



Influència de l'origen i del processat en el nivell de polifenols i antioxidants del tomàquet i dels seus derivats

Anna Vallverdú i Queralt

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Universitat de Barcelona
Facultat de Farmàcia
Departament de Nutrició i Bromatologia

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Anna Vallverdú i Queralt
2012

Universitat de Barcelona
Facultat de Farmàcia
Departament de Nutrició i Bromatologia
Programa de Doctorat
Alimentació i Nutrició
2008-2012

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de polifenols i antioxidants del tomàquet i dels seus
derivats.**

Memòria presentada per Anna Vallverdú i Queralt per optar al títol de doctor per
la Universitat de Barcelona, dirigida per:

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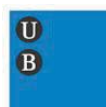


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ABREVIATURES

ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) = àcid 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfònic)

APCI (atmospheric pressure chemical ionization) = ionització química a pressió atmosfèrica

APPI (atmospheric pressure photoionization) = fotoionització a pressió atmosfèrica

CEE = Comunitat Econòmica Europea

CID (collision induced dissociation) = cel·la de col·lisió

DAD (diode array detector) = detector de feix de díodes

DART (direct analysis real time) = anàlisi directa a temps real

DESI (desorption electrospray ionization) = desorció ionització en electroesprai

DPPH (2,2-diphenyl-1-picrylhydrazyl) = 2,2-difenil-1-picrilhidrazil

EC = electroforesi capil·lar

ESI (electrospray ionization) = ionització en electroesprai

FAOSTAT (Food and Agriculture Organization of the United Nations) = Divisió d'Estadística de l'Organització per a l'Alimentació i l'Agricultura de les Nacions Unides

FC = Folin-Ciocalteu

FS = full scan

FTMS (fourier transform mass spectrometry) = transformada de fourier

GAE (gallic acid equivalent) = mg equivalents d'àcid gàl·lic

GC (gas chromatography) = cromatografia de gasos

HCD (high collision dissociation) = cel·la de col·lisió d'alta energia

HPLC (high-performance liquid chromatography) = cromatografia de líquids d'alta resolució

HPLC-MS (HPLC-mass spectrometry) = HPLC acoblada a espectrometria de masses

HPLC-MS/MS (HPLC-tandem mass spectrometry) = HPLC acoblada a espectrometria de masses en tàndem

HPLC-ESI-(Li⁺)-MS/MS = HPLC acoblada a MS/MS utilitzant ESI amb prèvia derivatització amb clorur de liti

HPLC-QToF-MS (HPLC coupled to quadrupole time of flight mass spectrometry) = HPLC acoblada a espectrometria de masses d'alta resolució amb un espectròmetre híbrid quadrupol-temps de volada

HPLC-LTQ-Orbitrap-MS (HPLC coupled to linear ion trap quadrupole Orbitrap mass spectrometry) = HPLC acoblada a espectrometria de masses d'alta resolució amb un espectròmetre híbrid de trampa d'ions-Orbitrap

IDA = information dependent acquisition

IT (*ion trap*) = trampa d'ions

MAGRAMA = Ministeri d'Agricultura, Alimentació i Medi Ambient

MALDI (*matrix assisted laser desorption ionization*) = mètode de desorció i ionització per làser

MAX (*mixed-mode anion exchange*) = mètode mixte aniònic

MoTo DB (*a metabolic database for tomato*) = base de dades dels metabòlits del tomàquet

MRM = *multiple reaction monitoring*

MS (*mass spectrometry*) = espectrometria de masses

MS/MS (*tandem mass spectrometry*) = espectrometria de masses en tàndem

MTBE (*methyl tert-butyl ether*) = terc-butil-metil-éter

m/z (*mass-to-charge*) = massa/càrrega

NL (*neutral loss*) = pèrdua neutra

NMR (*nuclear magnetic resonance*) = ressonància magnètica nuclear

OGM = organismes genèticament modificats

PCA (*principal component analysis*) = anàlisi de components principals

PEAIC = polsos elèctrics d'alta intensitat de camp

PEBIC = polsos elèctrics de baixa intensitat de camp

PIS = *product ion scan*

Prec = *precursor ion scan*

Q = quadrupol

QqQ = triple quadrupol

Q1 = primer quadrupol

Q3 = tercer quadrupol

QToF (*quadrupole time of flight*) = espectròmetre híbrid quadrupol-temps de volada

ROS (*reactive oxygen species*) = espècies reactives d'oxigen

RSD (*relative standard deviation*) = desviació estàndard relativa

SIM (*selected ion monitoring*) = monitoreig selectiu d'ions

SPE (*solid phase extraction*) = extracció en fase sòlida

ToF (*time of flight*) = temps de volada

Trolox = antioxidant de referència en els mètodes *ABTS⁺* i *DPPH*

UHT = *ultra high temperature*

USDA (*United States Department of Agriculture*) = Departament d'Agricultura dels Estats Units

UV (*ultraviolet*) = ultraviolat

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RESUM



I. RESUM

Actualment, s'estan invertint molts esforços en la detecció de components bioactius, com ara els polifenols, en fruites i hortalisses pel seu possible paper en la prevenció i el tractament de malalties cròniques. La quantitat de polifenols ingerits en la dieta és d'aproximadament 1 g/dia, quantitat més gran que la que prové de qualsevol altre antioxidant de la dieta. De fet, és aproximadament 10 vegades superior a la de vitamina C i 100 vegades superior a la de vitamina E i a la ingesta de carotens. D'altra banda, algunes dades epidemiològiques han mostrat una associació inversa entre el risc de mortalitat total o malalties cardiovasculars i el consum d'aliments rics en polifenols, com ara les fruites i hortalisses, el te, l'oli d'oliva verge i el vi.

El tomàquet és l'hortalissa més consumida a Espanya (14.5 kg/any per persona) amb una important repercussió econòmica. En els últims 5 anys, el consum del tomàquet ha crescut un 2.8% anual. En l'àmbit de la fruita/hortalissa transformada, el tomàquet també ocupa el primer lloc en consum amb una ingesta de tomàquet fregit envasat de 4.1 kg/any per persona i de tomàquet natural envasat de 3 kg/any per persona. Els tomàquets espanyols destaquen pel seu elevat contingut en flavonoides, encara que no hi ha gaires estudis que s'hagin centrat en com afecta el processat i l'elaboració industrial de les salses als polifenols, així com tampoc l'evolució d'aquests compostos durant la vida útil d'aquests productes.

El tomàquet i els seus derivats contenen altres fitoquímics valuosos com ara els carotens. El *trans*-licopè és el carotè més important dels tomàquets i el millor amortidor d'oxigen atòmic (captador de radicals lliures). A més del *trans*-licopè, els tomàquets també contenen altres carotens com els *cis*-isòmers del licopè i també α -, β -carotè i luteïna. La importància dels isòmers *cis* recau en la seva elevada biodisponibilitat en l'organisme humà, per la qual cosa són necessaris mètodes per a la identificació d'aquests carotens.

Alguns estudis han investigat l'efecte del tractament tèrmic en els carotens de salses de tomàquet, i s'hi ha observat un increment d'isòmers *cis* durant el processat. No obstant això, no hi ha estudis que valorin la influència de l'origen, el processat i l'emmagatzematge de tomàquets, salses, quètxups, gaspatxos i suc de tomàquet

Resum

quant als polifenols. Consegüentment, ens plantegem les hipòtesis i objectius que es detallen a continuació.

I. ABSTRACT

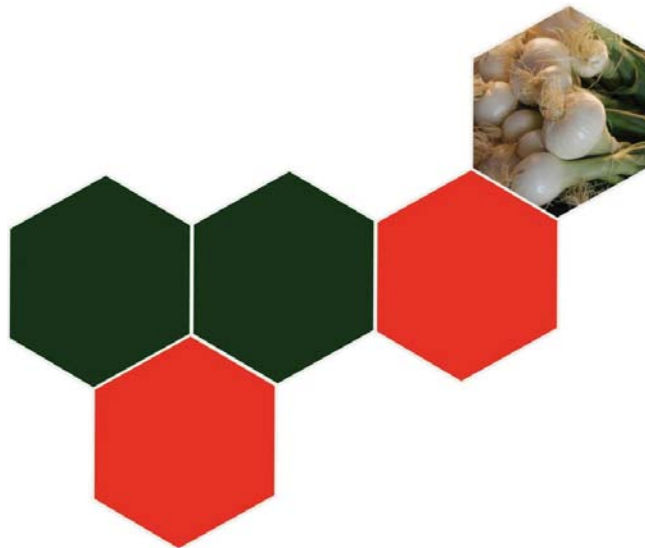
Currently many efforts are being invested in the detection of bioactive components of nutrients in fruits and vegetables, such as polyphenols, and their possible role in the prevention and treatment of chronic diseases. Total dietary polyphenol levels are approximately 1 g/day, a much larger amount than any other antioxidant that comes from the diet. In fact, it is about 10 times the dietary vitamin C and 100 times higher than vitamin E and carotene intake. Moreover, some epidemiological data have shown an inverse association between the risk of mortality or cardiovascular disease and consumption of foods rich in polyphenols, such as fruits and vegetables, tea, olive oil and wine.

The tomato is the most consumed vegetable in Spain (14.5 kg/year per person) and makes a significant economic impact. In the last 5 years, the consumption of tomatoes has grown by 2.8% annually. In the field of fruit/vegetables, tomatoes also rank first with the consumption of fried tomatoes at an intake of 4.1 kg/year and that of raw tomatoes at 3 kg/year per person. Spanish tomatoes are known for their high content of flavonoids, although there is a lack of studies focused on how the industrial processing of sauces affects the content of polyphenols, and also the evolution of these compounds during their shelf life.

The tomato and its derivatives contain other valuable phytochemicals such as carotenoids. *trans*-Lycopene is the most important carotene of tomato and it is an excellent free-radical scavenger; its capacity is more than twice that of β -carotene. In addition to *trans*-lycopene, tomatoes also contain other carotenoids such as *cis*-isomers of lycopene and α - and β -carotene and lutein. The importance of *cis*-isomers lies in their high bioavailability in the human body. Therefore, methods are needed to identify these carotenoids.

Some studies have studied the effect of heat treatment on carotenoids in tomato sauces, and an increase has been observed during processing. However, no studies have been focused on evaluating the influence of the origin, processing and storage of tomatoes, sauces, ketchups, tomato juices and gazpachos on the level of polyphenols. Consequently, we consider the assumptions and objectives listed below.

HIPÒTESIS I OBJECTIUS



II. HIPÒTESIS I OBJECTIUS

HIPÒTESIS

- ✓ Els compostos bioactius poden servir com a marcadors de diferents varietats de tomàquet.
- ✓ El processat afecta els compostos bioactius del tomàquet.
- ✓ Els tomàquets de cultius ecològics sintetitzen més polifenols a causa de l'estrès de la planta.
- ✓ Durant l'emmagatzematge de productes derivats del tomàquet es produeix una disminució dels compostos bioactius.
- ✓ Els polsos elèctrics poden incrementar els nivells de compostos bioactius del tomàquet.

OBJECTIU GENERAL

Estudiar els punts crítics des de la granja al consumidor “*from farm to fork*” que afecten la presència de compostos bioactius del tomàquet i dels seus productes transformats, per obtenir uns aliments amb més components funcionals.

OBJECTIUS ESPECÍFICS

1. Desenvolupar un mètode analític mitjançant *HPLC-LTQ-Orbitrap-MS*, *HPLC-QToF-MS* i *HPLC-MS/MS*, que permeti identificar i quantificar els polifenols del tomàquet i dels seus derivats.
2. Avaluar el perfil fenòlic i la capacitat antioxidant hidrofílica com a marcadors de diferents varietats de tomàquet.
3. Estudiar els canvis en el perfil fenòlic i l'activitat antioxidant durant la producció de salses i daus de tomàquet.
4. Estudiar les diferències entre tomàquets, suc de tomàquet i quètxups ecològics i no ecològics mitjançant anàlisis no dirigides (*HPLC-QToF-MS*) i dirigides (*HPLC-MS/MS*).
5. Avaluar les diferències en el contingut de carotens i capacitat antioxidant dels quètxups i gaspatxos mitjançant un mètode *HPLC-ESI-(Li⁺)-MS/MS*.
6. Estudiar els canvis en el perfil fenòlic i en el contingut de carotens durant l'emmagatzematge de quètxups, gaspatxos i suc de tomàquet.
7. Valorar l'aplicació de polsos elèctrics de baixa intensitat de camp per incrementar el contingut en compostos bioactius dels tomàquets.

II. HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

- ✓ The bioactive compounds may serve as markers of different varieties of tomato.
- ✓ The processing affects tomato bioactive compounds.
- ✓ Tomatoes from organic systems contain higher levels of polyphenols due to plant stress.
- ✓ During storage of tomato products, there is a reduction of bioactive compounds.
- ✓ Moderate-intensity pulsed electric fields may increase the levels of tomato bioactive compounds.

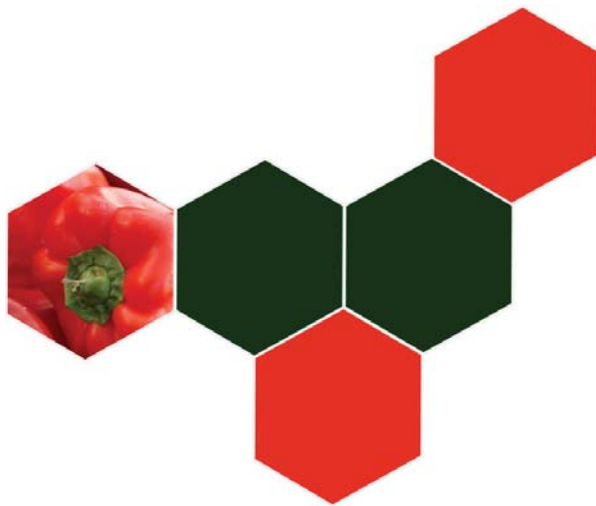
GENERAL OBJECTIVE

To study the critical steps from the farm to the consumer “from farm to fork” which affect the presence of bioactive compounds in tomatoes and tomato-based products in order to obtain food with more functional compounds.

SPECIFIC OBJECTIVES

1. To develop an analytical method using *HPLC-LTQ-Orbitrap-MS*, *HPLC-QToF-MS* and *HPLC-MS/MS* to identify and quantify the polyphenol compounds of tomatoes and tomato-based products.
2. To evaluate the phenolic profile and hydrophilic antioxidant capacity as markers of different varieties of tomato.
3. To study phenolic profile and antioxidant capacity changes during the production of sauces and diced tomatoes.
4. To study the differences between organic and conventional tomatoes, tomato juices and ketchups using non-focused (*HPLC-QToF-MS*) and focused (*HPLC-MS/MS*) analysis.
5. To assess the differences in carotenoid content and antioxidant capacity of ketchups and gazpachos using an *HPLC-ESI-(Li⁺)-MS/MS analysis*.
6. To study changes in the phenolic and carotenoid profile during storage of ketchups, tomato juices and gazpachos.
7. To assess the application of moderate-intensity pulsed electric fields to increase the content of bioactive compounds in tomatoes.

INTRODUCCIÓ



III. INTRODUCCIÓ

1. El tomàquet i els seus components

1.1. Història del tomàquet (*SOLANUM LYCOPERSICUM*)

El tomàquet té el seu origen a la zona compresa entre el nord de Xile, Colòmbia, el Perú i l'Equador, però es va cultivar a Mèxic. El nom del tomàquet deriva del mot *tomatl* de la llengua *Nahuac*, el significat del qual correspon a les plantes de fruits globosos o baies, amb moltes llavors i polpa aquosa. En els inicis, era semblant als *cherries* actuals. Va arribar a Europa a mitjan segle XVI, després del viatge que Hernán Cortés va realitzar a Mèxic, el 1519.

Després de la conquesta d'Amèrica del Sud, els espanyols van introduir el tomàquet a les seves colònies del Carib, a les Filipines i al continent asiàtic. A Espanya, Portugal i Itàlia es va utilitzar en l'alimentació humana; en canvi, a països com Alemanya i França el van destinar com a espècie ornamental, ja que ho van relacionar amb les solanàcies europees, considerades tòxiques per la presència d'alcaloides. L'alcaloide causant d'aquesta toxicitat és la tomatina, que es troba principalment en les fulles i en el fruit verd, però que es degrada en madurar. Aquesta creença es va mantenir en molts llocs fins a principis del segle XX (Jones *et al.*, 2001).

Botànicament pertany a la família de les *Solanàcies* (que tenen unes 1700 espècies), les quals s'acostumen a desenvolupar en climes càlids o temperats; les seves flors són de textura regular, s'agrupen en forma de rams i el calze i la corol·la constitueixen una peça dividida en cinc compartiments.

El tomàquet té un gust lleugerament àcid, la seva mida en les espècies silvestres és d'1 a 2 cm de diàmetre i té una grandària superior en les espècies cultivades.

Introducció

1.2. Producció i consum

El tomàquet és l'aliment d'origen vegetal més consumit arreu del món després de la patata, amb un consum de 12 kg/any per persona (Lugasi *et al.*, 2003). Als Estats Units és el quart aliment d'origen vegetal més consumit en fresc després de la patata, l'enciam i les cebes (Canene-Adams *et al.*, 2005). A Europa és la segona hortalissa més utilitzada en l'alimentació, excepte a Itàlia on ocupa el primer lloc, arran del consum en fresc i a la gran oferta de productes processats (Caris-Veyrat *et al.*, 2004).

La producció mundial de tomàquet fou, el 2010, de 146 milions de tones segons dades de la Divisió d'Estadística de l'Organització per a l'Alimentació i l'Agricultura de les Nacions Unides (FAOSTAT, 2010). Els principals països productors de tomàquet són la Xina, els Estats Units, l'Índia, Turquia, Egipte, Itàlia, Iran, Espanya, el Brasil i Mèxic, però la producció de tomàquet a Europa va disminuir, el 2010, en 1.65 milions de tones. El total de producció fou de 21.76 milions de tones, un 7% menys que el 2009.

A Europa, el tomàquet hi aporta un 30% del valor de la producció hortícola, i té un rendiment de 50 t/ha. Hi destaquen dues zones de producció: la zona mediterrània, integrada per Itàlia, Espanya, Grècia, Portugal i el sud de França, i que està orientada a subministrar matèria primera per a la indústria; i la zona nord, constituïda per Holanda, el nord de França, Bèlgica, el Regne Unit, Dinamarca i Alemanya, la producció principal dels quals és per al consum fresc. Itàlia fou, el 2010, el major productor de tomàquet a Europa amb una collita de 6.02 milions de tones, que representen aproximadament el 30% de la producció total europea. Espanya en va ser el segon productor amb 4.31 milions de tones i Grècia en va ser el tercer amb una producció de 1.41 milions de tones. Portugal i Holanda foren el quart i cinquè país (FAOSTAT, 2010).

A Espanya, la producció de tomàquet se situa principalment a Andalusia i Extremadura, ja que aquestes dues comunitats autònomes aporten més del 50% de la producció nacional. El seu consum a l'Estat espanyol es manté estable en els últims anys (2010-2011): aproximadament és de 15.3 kg/any per persona segons les dades del Ministeri d'Agricultura, Alimentació i Medi Ambient (MAGRAMA, 2011).

El tomàquet és una de les hortalisses de fruit amb més demanda a nivell mundial. Té qualitats sensorials molt apreciades i s'integra perfectament en l'alimentació de productes cuits i crus. El tomàquet és una font important d'antioxidants a causa del seu alt contingut en licopè, vitamina C i compostos fenòlics.

1.3. Composició i valor nutricional

La composició físicoquímica i nutricional del fruit del tomàquet es veu afectada per diversos factors: la varietat, l'estació de sembra, els nutrients disponibles en el sòl, la intensitat de llum, la temperatura d'exposició del cultiu, les tècniques d'agricultura, la disponibilitat d'aigua, els reguladors de creixement i el grau de maduració, entre d'altres (Abushita *et al.*, 2000). Un 1% del fruit del tomàquet el constitueixen la pell i les llavors. La matèria seca oscil·la entre un 5% i un 10%, un 50% de la qual la constitueixen els sucres (principalment glucosa 22% i fructosa 25%), un 10% àcids orgànics (especialment cítric i màlic), un 8% elements minerals i la resta està constituïda per sòlids insolubles (pectina, cel·lulosa i hemicel·lulosa), pigments, vitamines i lípids (Salles *et al.*, 2003). A la **Taula 1**, es mostra el valor nutricional del tomàquet (USDA, 2005).

Taula 1. Composició del tomàquet madur per cada 100 g.

Valors mitjans	Per 100 g
Valor energètic	21 kcal
Proteïnes	0.55 g
Greix total	0.55 g
Greixos saturats	0.05 g
Greixos monoinsaturats	0.05 g
Greixos poliinsaturats	0.11 g
Hidrats de carboni	2.9 g
Fibra	1.8 g
Aigua	93.80 g
Minerals	
Sodi	6 mg
Potassi	297 mg
Calci	13 mg
Fòsfor	27 mg
Magnesi	20 mg
Ferro	0.5 mg
Fluor	0.02 mg
Vitamines	
A (retinol)	133 µg
E (tocoferol)	0.80 mg
B1 (tiamina)	0.06 mg
B2 (riboflavina)	0.04 mg
B3 (niacina)	0.60 mg
B6 (piridoxina)	0.10 mg
C	24 mg

1.4. Compostos bioactius i capacitat antioxidant del tomàquet

Els compostos bioactius són substàncies funcionals que es troben en concentracions molt baixes en els aliments, intervenen en el metabolisme secundari dels vegetals i poden tenir un impacte important en la salut humana. Recentment alguns nutrients han estat inclosos com a compostos bioactius per presentar un efecte beneficiós per a la salut (Grieb *et al.*, 2009; Zhang *et al.*, 2009). Aquestes petites molècules que no poden ser sintetitzades en el cos i, per tant, s'han d'obtenir de la dieta, contribueixen al sistema de defensa antioxidant. Aquests compostos es poden agrupar segons la seva estructura química en vitamines, compostos fenòlics, fitoestrògens, pèptids i aminoàcids, minerals, àcids grassos poliinsaturats, fibra dietètica i carotens.

En els últims anys, s'han publicat gran nombre d'estudis epidemiològics que relacionen el consum d'una dieta rica en fruites i hortalisses amb una menor predisposició a patir malalties com ara el càncer i les malalties cardiovasculars, així com també, l'envelliment (Bagchi *et al.*, 2003; Blum *et al.*, 2005; Das *et al.*, 2005; Lambert & Yang, 2003; Nishino *et al.*, 2004).

1.4.1. Determinació de la capacitat antioxidant

Les reaccions d'oxidació desencadenades en l'organisme poden generar radicals lliures, els quals inicien reaccions en cadena que poden exercir danys cel·lulars. Els antioxidants són substàncies que retarden o tenen un efecte preventiu sobre l'oxidació mitjançant la seva pròpia oxidació, tot transferint electrons a una altra substància oxidant.

Determinació dels radicals lliures

Es mesura el temps necessari per consumir tots els antioxidants presents a la mostra. Aquest temps és equivalent al temps en què els antioxidants són capaços d'anul·lar una reacció d'oxidació. Es necessita una font de radicals, l'extracte que s'ha d'analitzar i un paràmetre que pugui determinar la modificació del procés oxidatiu per l'acció dels antioxidants. Una bona font de radicals és aquella que és constant i independent de la mostra que s'ha d'analitzar. Aquests tipus de test s'avaluen comparant l'activitat de l'extracte amb la d'un compost o extracte de referència. Quan

se sap el temps necessari per consumir tots els antioxidants presents a la mostra, es mesura la reacció en un temps determinat.

S'han desenvolupat diferents mètodes per mesurar l'activitat antioxidant basats en l'habilitat per atrapar radicals lliures en un medi aquós o lipofílic (Minoggio *et al.*, 2003; Odriozola-Serrano *et al.*, 2007; Vallverdú-Queralt *et al.*, 2011a). Les metodologies més senzilles són aquelles en què es genera un radical lliure i es mesura de manera directa la capacitat de la mostra o extracte per inhibir el radical. Alternativament, hi ha mètodes que combinen la generació de radicals amb l'oxidació d'un substrat o medi. En aquests casos, l'activitat antioxidant ve donada no només per la capacitat d'atrapar els radicals generadors de l'oxidació, sinó també per la capacitat de retardar l'oxidació i de reduir els productes que se'n deriven. A continuació es descriuran els mètodes que s'han utilitzat durant la part experimental d'aquesta tesi.

Determinació del radical 2,2-difenil-1-picrilhidrazil (DPPH')

Reconegut com a un radical estable, aquest radical sintètic ha estat un dels pioners en l'estudi de l'activitat antioxidant de compostos fenòlics. Es tracta d'un mètode de determinació de l'activitat antioxidant d'ús rutinari, l'èxit del qual es basa en la seva senzillesa, en el seu baix cost i, sobretot, a ser un mètode ràpid. És un mètode colorimètric basat en el compost cromogen *DPPH*, que presenta una coloració violeta en solució i groga en el producte final. En fer servir 515 nm com a longitud d'ona, aconseguim analitzar la quantitat romanent de radical *DPPH*, paràmetre que ens proporcionarà la dada necessària per al càlcul de la capacitat antioxidant de la mostra. En afegir els antioxidants una vegada format el radical, se'n determina l'absorbància a punt fix.

Determinació del radical 2,2'azino-bis(3-etilbenzotiazolina-6-sulfanat) (ABTS⁺)

És un mètode colorimètric basat en el compost cromogen blavós *ABTS*, amb un màxim d'absorció a 342 nm, soluble tant amb aigua com amb dissolvents orgànics i químicament estable. En reaccionar amb òxid de manganès, es genera el radical *ABTS⁺*, amb màxims a 414, 645, 734 i 815 nm. En afegir-hi els antioxidants una vegada format el radical, se'n determina l'absorbància a punt fix i se n'observa la decoloració en presència de compostos antioxidants.

1.4.2. Els compostos fenòlics

En el regne vegetal, els fenols constitueixen un grup d'estructures àmpliament distribuïdes. Els fenols funcionen com a antioxidants naturals en situacions de dany cel·lular com en el cas de l'estrès oxidatiu. Aquesta elevada activitat ve donada tant per la facilitat amb què els compostos fenòlics cedeixen el protó dels seus grups hidroxils, com per l'estabilitat del fenol oxidat producte de la cessió de l'electró. Aquest fenol oxidat conté un electró desaparellat i l'anell aromàtic facilita la deslocalització d'aquest electró.

Diversos estudis han constatat les relacions estructura-activitat d'aquests compostos. Lògicament, l'activitat dels fenols es deu principalment als hidroxils. Com més grups hidroxils, més activitat; però més important que la quantitat és la posició. Quan aquests hidroxils estan en posició *orto* la molècula resulta més activa. Perquè sigui així, hi ha dues raons bàsiques: la deslocalització electrònica resulta més efectiva, i a més s'incrementa l'activitat quelant de la molècula. Podem veure aquesta circumstància a l'anell B dels flavonoides, on les molècules més actives tenen els hidroxils en posició 3' i 4'. Els flavonoides tenen altres punts de quelació: la cetona en posició 4 de l'anell C pot interaccionar amb un hidroxil en posició 3 o amb l'hidroxil situat en posició 5 de l'anell A. Aquest hidroxil situat en posició 5, juntament amb el situat en 7, són dues propietats importants per incrementar l'activitat antioxidant dels flavonoides (Pietta, 2000; Silva *et al.*, 2002). Així mateix, un altre aspecte important en l'activitat antioxidant dels flavonoides és la presència d'un doble enllaç entre els carbonis 2 i 3, el grup hidroxil de la posició 3, i el grup cetona de la posició 4 en l'anell C, la qual cosa afavoreix la deslocalització electrònica. Tot aquest conjunt de factors també es poden veure en els àcids cinàmics i els seus èsters (**Figura 1**).

En el medi natural, els fenols es poden presentar units a sucres. Normalment, aquestes glicosilacions es produeixen en els grups hidroxils, la qual cosa redueix el potencial antioxidant de les molècules.

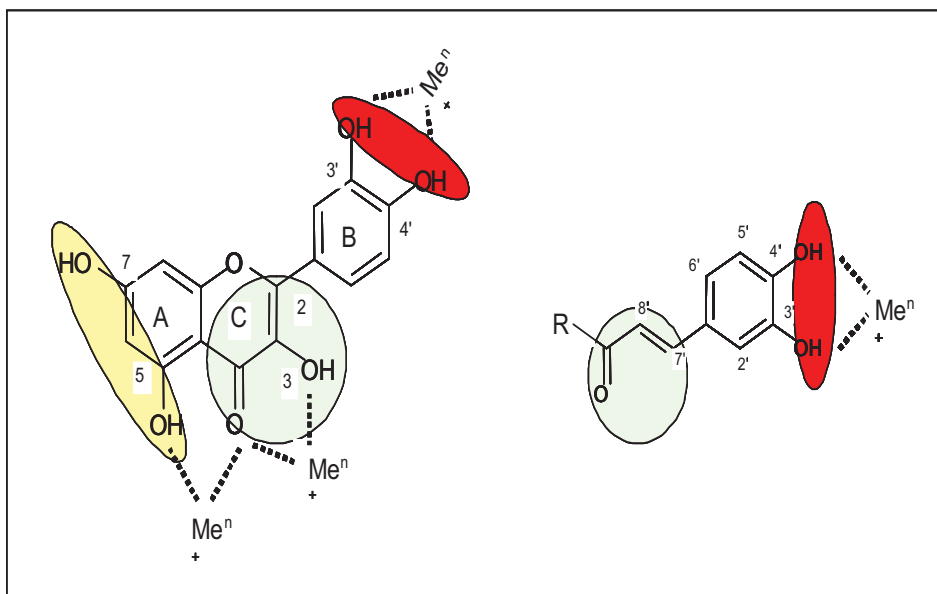


Figura 1. Relació estructura-activitat en els compostos fenòlics. En vermell, hidroxils en posició *orto*; en els flavonoides, en groc hidroxils en *meta*. En verd la relació doble enllaç-cetona, punt que es veu reforçat per un hidroxil en els flavonols. També podem veure els punts actius per la quelació de metalls.

Els compostos fenòlics són sintetitzats per la via de l'àcid mesònic o per la de l'àcid siquímic, o per les dues, com en el cas dels flavonoides (Taiz & Zeiger, 2006). Els compostos fenòlics es divideixen en dos grans grups, flavonoides i no flavonoides.

Classificació dels compostos fenòlics presents en el tomàquet

Flavonoides és el terme genèric amb el qual s'identifiquen els compostos polifenòlics caracteritzats per una estructura química basada en un esquelet C6-C3-C6, és a dir, un anell benzènic unit a una cadena propanoica i aquesta, a la vegada, unida a un altre anell benzènic. Els flavonoides comprenen diversos milers de compostos i es divideixen en flavonols, flavones, flavanones, antocians, isoflavonoides, flavanols, dihidroflavonols, calcones i dihidrocalcones. Quan aquests compostos es troben en la seva forma lliure, és a dir, no associats a cap altra estructura química, es parla d'aglicona.

A continuació es farà una petita descripció dels principals grups de flavonoides presents en el tomàquet i derivats.

Introducció

Flavonols

Distribuïts àmpliament en el regne vegetal, aquest grup d'estructures és d'una gran importància en la dieta. Es poden descriure unes 200 aglicones, de les quals destaquen la quercetina i el kaempferol com les estructures més àmpliament distribuïdes en tomàquets i derivats (Vallverdú-Queralt *et al.*, 2011b). En diversos casos, apareixen com a molècules glicosilades a l'hidroxil de la posició 3 a través d'un enllaç *O*-glicosídic (Tulipani *et al.*, 2011; Vallverdú-Queralt *et al.*, 2010). En el tomàquet i els seus derivats, el flavonol més abundant és la rutina (quercetina-3-*O*-rutinòsid), en concentracions que oscil·len entre 1 i 20 mg/100 g.

Flavanones

Són un grup de flavonoides poc distribuït dins del regne vegetal, amb una excepció en el grup dels cítrics, en què són la majoria. Es troben bàsicament en forma d'aglicones en aquests fruits. Les aglicones més comunes són l'hesperitina i la naringenina. Unes altres flavanones abundants són l'eriodictiol i la taxifolina. Les glicosilacions d'aquestes molècules tenen lloc preferentment en la posició 7, ja que no tenen la posició 3 hidroxilada (Vallverdú-Queralt *et al.*, 2011c). En el tomàquet i derivats, la naringenina és un dels flavonoides més abundants.

Flavones

Són uns dels flavonoides més comuns. Les aglicones més comunes són l'apigenina i la luteolina (Vallverdú-Queralt *et al.*, 2011c).

Dins del grup dels **no flavonoides** s'hi troben els àcids fenòlics i els estilbens. Els àcids fenòlics són els compostos no flavonoides més estudiats i es caracteritzen per tenir un àcid carboxílic funcional. Els àcids fenòlics formen un grup divers que inclou els derivats de l'àcid hidroxibenzoic i de l'àcid hidroxicinnàmic. Alguns exemples dels derivats de l'àcid hidroxibenzoic són l'àcid *p*-hidroxibenzoic, l'àcid gàl·lic i l'àcid el·làgic. En el tomàquet, s'hi troben presents l'àcid gàl·lic i el protocatèquic. Dins dels àcids hidroxicinnàmics hi podem citar els àcids *p*-cumàric, cafeic i ferúlic. Generalment, els àcids fenòlics estan presents en diverses formes conjugades i són més freqüents com a èsters que com a glucòsids. L'àcid clorogènic és el principal derivat dels àcids

hidroxicinnàmics. Aquest compost fenòlic es troba present en tomàquets en concentracions que oscil·len entre 0.14 i 8 mg/100 g.

1.4.3. Els carotens

El licopè

El licopè és un pigment vegetal que aporta al tomàquet el color vermell; pertany a la família dels carotens com el β -carotè (Vallverdú-Queralt *et al.*, 2011b). En el tomàquet, el carotè majoritari és el *trans*-licopè (80-90%), seguit del β -carotè (3-7%). A l'inici de la maduració, el contingut en *trans*-licopè és més alt. Durant el creixement del fruit, tant el nivell de llum com la temperatura afecten el contingut de licopè (Abushita *et al.*, 2000; Galicia *et al.*, 2008). La seva concentració depèn també de la varietat del tomàquet, les condicions de cultiu, el tipus de cultiu i les condicions climàtiques (Martínez-Valverde *et al.*, 2002). La concentració de licopè en el tomàquet és aproximadament de 50 mg/kg (Willcox *et al.*, 2003).

Pel que fa als isòmers del licopè, els *cis* isòmers del licopè són de gran interès nutricional, perquè són absorbits més fàcilment en l'intestí (Failla *et al.*, 2008; Unlu *et al.*, 2007). El *trans*-licopè representa el licopè més abundant i varia del 35% al 96% del licopè total; els 5-, 9-, 13- i 15-*cis*-licopè són els isòmers *cis* majoritaris (Frohlich *et al.*, 2007). Durant el tractament tèrmic, es duu a terme la *trans/cis* isomerització, per la qual cosa s'incrementa la proporció d'isòmers *cis* (Frohlich *et al.*, 2007; Lin & Chen, 2005). Els tractaments tèrmics trenquen les parets cel·lulars del fruit, que són les que dificulten l'absorció del licopè. No existeix evidència científica de quina seria la dosi òptima de licopè. A Espanya, el tomàquet i el puré de tomàquet són la principal font de licopè en la dieta i aporten el 42 i 71.6%, respectivament (O'Neill *et al.*, 2001).

El licopè protegeix les cèl·lules humanes de l'estrès oxidatiu produït per l'acció dels radicals lliures; actua com a potent neutralitzador de radicals lliures (òxid i peròxid) atenuant l'oxidació sobre els teixits (Das *et al.*, 2005; Djuric & Powell, 2001).

Introducció

β -carotè

El β -carotè és el precursor de la vitamina A, necessària per al bon desenvolupament de les funcions reproductores, per al bon desenvolupament del sistema ocular i també per a un bon funcionament de la pell. A més a més, intervé en la previsió de la degradació d'ossos, dents i nervis, en la protecció davant de diferents tipus de càncer i en l'activació del sistema immunitari (Das *et al.*, 2005; Djuric & Powell, 2001).

No obstant això, no tot el β -carotè emmagatzemat en l'organisme es transforma en vitamina A. Les activitats antioxidants dels carotens estan més relacionades amb aquest romanent no transformat que s'emmagatzema bàsicament en el fetge, que no pas amb la vitamina A. El β -carotè és capaç d'eliminar els radicals lliures, però és fàcilment oxidable (patint inclús processos d'autooxidació), la qual cosa fa que disminueixi el seu caràcter protector davant de la lipoperoxidació i d'altres processos oxidatius.

Altres carotens com l' α -carotè i la luteïna representen menys del 6.8% dels carotens totals (Raffo *et al.*, 2006). Per tant, la provitamina A dels tomàquets i derivats del tomàquet deriva principalment del β -carotè. Els tractaments tèrmics estimulen la transformació d'alguns carotens. Per exemple, el γ -carotè es cicla per formar anells de 6 carbonis al final de la molècula, la qual cosa pot portar a la formació del β -carotè (Quitão-Teixeira *et al.*, 2009).

1.4.4. Vitamina C

La vitamina C es troba present en el tomàquet en concentracions que poden oscil·lar entre 40-90 mg/100 g. La vitamina C inclou àcid ascòrbic, àcid deshidroascòrbic i sals d'ascorbat. En presència d'oxigen, l'àcid ascòrbic s'oxida a àcid deshidroascòrbic, que té la mateixa activitat vitamínica. Aquesta vitamina té propietats reductores i actua com a potent antioxidant que és capaç de reaccionar efectivament amb radicals superòxid i hidroxil.

La vitamina C és essencial per als humans. Les fonts més importants de vitamina C són els pebrots, els cítrics, les cols, els espinacs i altres fruites com el plàtan, la poma i la pinya, encara que la quantitat de vitamina C depèn de la varietat, les

condicions del sòl, el clima, la maduresa, les condicions d'emmagatzematge i de processat (Sahlin *et al.*, 2004)

1.5. Derivats del tomàquet

Les característiques ideals dels productes del tomàquet estan relacionades amb un color vermell intens, nivells alts de consistència i un gust i olor característic del fruit del tomàquet (Kalamaki *et al.*, 2003). El color vermell en el fruit és un important factor de qualitat en els derivats del tomàquet i és un dels atributs que més influeix en el moment d'adquirir aquests tipus de productes (Min & Zhang, 2003).

La consistència és un altre paràmetre de qualitat important en els productes derivats del tomàquet (Apaiah *et al.*, 2001). Els sòlids insolubles de la paret cel·lular (cel·lulosa, hemicel·lulosa, lignina i pectina) es troben estretament relacionats amb aquest paràmetre (Kalamaki *et al.*, 2003). Aquest atribut de qualitat es redueix durant els tractaments de calor i emmagatzematge (Apaiah *et al.*, 2001; Singh *et al.*, 2002). El processat tèrmic afecta l'estructura de la paret cel·lular dels teixits vegetals (Anthon & Barrett, 2006). La viscositat també té implicacions econòmiques, ja que, com més alta sigui la viscositat dels derivats del tomàquet, menys s'ha d'elaborar per arribar a la consistència desitjada.

El gust i l'olor són també paràmetres de reconeguda importància en aquest tipus de productes. Vénen determinats per la varietat utilitzada, la fase de maduració i les condicions de processat. El gust agredolç del tomàquet és degut al seu contingut de sucres, glucosa, fructosa i als àcids orgànics cítric i màlic (Roselló *et al.*, 2002; Salles *et al.*, 2003). L'olor dels tomàquets ve determinada per més de 400 substàncies volàtils que s'han identificat en aquest fruit (Aguiló-Aguayo *et al.*, 2010).

El pH dels tomàquets hauria de ser inferior a 4.7 perquè puguin ser processats com a aliments molt àcids. Com més madur és el tomàquet, més alt és el pH. El contingut d'àcids varia segons la seva maduresa, les condicions climàtiques i el mètode de cultiu. La concentració d'àcids és important, ja que afecta el gust i el pH. Els àcids cítric i màlic són els més abundants. L'acidesa mitjana de processament del tomàquet és aproximadament d'un 0.35%, expressada com a àcid cítric (Thakur *et al.*, 1996).

Introducció

1.5.1. Salsa de tomàquet

El procés de salses normalment es porta a terme quan els tomàquets frescos presenten la millor qualitat. S'utilitzen les varietats de tomàquet adequades per tal d'obtenir unes salses amb un alt contingut de cel·lulosa, pectina i polpa. Els únics ingredients que s'hi poden afegir són sal, alfàbrega i àcid cítric. El tractament inclou: rentat, trituració, addició d'una salsa rica en subproductes del processat (llavors i pells de tomàquet), preescalfament, tamisat, evaporació, esterilització i emmagatzematge. L'etapa més important és el preescalfament. En els processos en calent o *hot break*, els preescalfaments es realitzen entre 97-102 °C, mentre que en els processos en fred o *cold break*, es duen a terme entre 65-85 °C. La diferència entre aquests dos productes radica en la seva viscositat aparent. El producte *hot break* és més viscos i per tant més dens que el producte *cold break* (Scott Smith & Hui, 2004). Després del preescalfament, l'extracte es passa a través d'uns tamisos per eliminar les llavors i pells, i l'aigua s'evapora entre 75-80 °C fins a obtenir el contingut de sòlids solubles desitjat. Després d'això, les mostres s'esterilitzen a 112 °C durant 2 minuts i 20 segons i s'envasen asèpticament.

1.5.2. Daus de tomàquet

Des del punt de vista tecnològic, la principal dificultat en la producció de daus de tomàquet és la selecció de la varietat de tomàquet. Els daus de tomàquet estan sotmesos a la pèrdua d'integritat del teixit, ja que es sotmeten a altes temperatures i a un esforç tallant (Ma & Barrett, 2002). La paret cel·lular externa dels tomàquets consta de microfibrilles de cel·lulosa envoltades de pectines, hemicel·luloses i proteïnes. Durant el processat, la pectina es veu modificada pels enzims naturals de la fruita, i consegüentment, els tomàquets s'estoven. Aquestes alteracions poden afectar la ruptura de les cadenes de pectina per la qual cosa els tomàquets es processen amb sals de calci (CaCl_2). Els ions divalents de calci indueixen la formació de pactat de calci o pectinats de manera que s'aconsegueix un producte final més resistent (Gould, 2009).

En el procés tecnològic, primer els tomàquets es netegen i després es pelen i es trossegueixen en forma de daus. Posteriorment, els daus es barregen amb una salsa rica en subproductes del processat (llavors i pells de tomàquet) en proporció 1:1 a l'interior d'un tanc vertical especial per permetre l'intercanvi màxim de calor i el mínim dany

mecànic als cubs. La salsa utilitzada primer s'estabilitza durant 5 minuts a més de 90 °C. En aquesta etapa, s'hi afegeix CaCl_2 per millorar la textura del producte final (Gould, 2009). Després d'haver completat l'etapa de preparació, els daus s'esterilitzen gradualment fins a 115 °C durant 5 minuts i després es refreden ràpidament a uns 30-35 °C en un intercanviador tubular i s'envasen asèpticament. Els daus de tomàquet tenen una vida útil de 18 a 24 mesos.

1.5.3. Quètxup

El quètxup és un condiment dolç i agre típic de la dieta nord-americana. El procés tecnològic per obtenir el quètxup consisteix a barrejar la salsa de tomàquet (65%, 30-32 °Brix) amb sucre, vinagre, sal i traces de conservants. La barreja es pasteuritza a 96 °C durant 4-6 minuts i s'envasa. La temperatura ha de regular-se acuradament per assegurar l'absorció dels ingredients. Tot el procés de fabricació de quètxup es duu a terme durant un període de dues a tres hores (Galicia-Cabrera, 2010).

1.5.4. Suc de tomàquet

El suc és un producte intermedi de l'elaboració de pasta de tomàquet que es pot separar de la polpa mitjançant el filtrat, però més comunament tota la polpa s'utilitza com a suc. Els suc de tomàquet es formulen d'acord amb les característiques exigides pel mercat: el més comú és el suc amb oli verge d'oliva, sal i àcid cítric per ajustar-ne el pH. El producte s'embotella i es pasteuritza a 80 °C durant 20 minuts per allargar-ne la vida útil (Gahler *et al.*, 2003).

1.5.5. Gaspaxo

El gaspatxo és una sopa tradicional espanyola, que es pot definir com una sopa freda que conté principalment cinc ingredients: tomàquet, cogombre, pebrot verd, ceba i all, així com d'altres en menors proporcions com ara l'oli verge d'oliva, el vinagre, la sal, el sucre i l'aigua. La barreja es cuina i posteriorment es pasteuritza entre 92-95 °C durant 1 minut per allargar-ne la vida útil. Finalment, els gaspatxos són embotellats i empaquetats (Pinilla *et al.*, 2005).

1.6. L'agricultura ecològica

L'agricultura ecològica és el resultat d'una sèrie de reflexions i de diversos mètodes alternatius de producció que s'han anat desenvolupant, bàsicament al nord d'Europa, com a resposta a la revolució verda (Le Guillou & Sharpé, 2001). La revolució verda és coneguda avui com a agricultura convencional, model de producció intensiu que està basat en la hibridació, en la utilització de fertilitzants químics, plaguicides, herbicides i aplicacions de feines altament mecanitzades.

Entre els anys seixanta i setanta sorgeix la consciència de protegir el medi ambient; no obstant això, és als anys vuitanta quan es produeix la conscienciació cap a l'agricultura ecològica en la major part dels països europeus i en altres països com ara els Estats Units, el Canadà, Austràlia i el Japó (Le Guillou & Sharpé, 2001; Soto, 2003).

El contingut de polifenols en els tomàquets es veu influït per les condicions de cultiu, el grau de maduresa i la varietat (Barrett *et al.*, 2007; Connor *et al.*, 2002; Toor *et al.*, 2006). Les diferències fonamentals entre els sistemes de producció ecològics i convencionals es fonamenten sobretot en la manera de cultivar i adobar el sòl, la qual cosa pot afectar la composició nutritiva dels tomàquets i en particular els metabòlits secundaris (Brandt *et al.*, 2011).

Els sistemes convencionals i ecològics es diferencien en les quantitats de nutrients aplicats com a fertilitzants i en la matèria orgànica aplicada com ara el cultiu de cobertura lleguminosa. Els tomàquets convencionals poden rebre tractament amb herbicides i plaguicides, mentre que els cultius orgànics reben només plaguicides orgànics aprovats, com ara els compostos de sofre i benzotiofè. Les granges convencionals utilitzen fertilitzants que contenen nitrogen inorgànic soluble i altres nutrients, que són més directament absorbits per les plantes. La disponibilitat de nitrogen inorgànic, en particular, té potencial per influir en la síntesi dels metabòlits secundaris de les plantes, proteïnes i sòlids solubles. Els tomàquets convencionals solen rebre 50 kg/ha d'un fertilitzant que conté nitrogen, fòsfor i potassi i 118 kg/ha de nitrat d'amoni (Kong *et al.*, 2005).

A més a més, les plantes de producció ecològica tenen un període més llarg de maduració en comparació amb les plantes convencionals, a causa d'un alliberament més lent dels nutrients subministrats (Nielsen *et al.*, 1997), i com que els flavonoides

es formen en el període de maduració, es podria esperar un major contingut d'aquests compostos en les plantes de cultiu biològic (Brandt *et al.*, 2011).

1.6.1. Aspectes legislatius dels cultius ecològics

El Consell de les Comunitats Europees va aprovar el 24 de juny de 1991, el Reglament de la Comunitat Econòmica Europea (CEE) núm. 2092/91 sobre la producció agrícola ecològica i la seva indicació en els productes agraris i alimentaris. Posteriorment, el Consell de la Unió Europea i la Comissió de les Comunitats Europees han anat aprovant nous reglaments que modifiquen o completen el R. 2092/91.

Prohibició d'ús d'organismes modificats genèticament en l'agricultura ecològica

En el cultiu ecològic, no s'hi poden utilitzar organismes genèticament modificats (OGM) ni productes obtinguts a partir d'aquests organismes. Els productes de l'agricultura ecològica han d'estar lliures de contaminació d'OGM i dels seus derivats. Els operadors han d'adoptar, dins de l'àmbit de la seva responsabilitat, totes les mesures necessàries per evitar aquest tipus de contaminació en els seus productes durant la producció, l'elaboració, l'emmagatzematge i el transport.

Agricultura ecològica i medi ambient

És molt important que les parcel·les d'agricultura ecològica no alternin cultius ecològics i convencionals. S'ha de tenir en compte que, quan les parcel·les de conreus ecològics confinen amb parcel·les d'agricultura no ecològica, s'han d'establir mesures per tal d'evitar la contaminació. S'hi poden fer tanques vegetals, marges o altres mètodes que el Consell accepti.

La producció ecològica millora el medi ambient i fomenta la diversitat. La gestió dels fems del bestiar i dels residus vegetals produïts en l'explotació o provinents de l'exterior han de tenir com a objectiu assolir el màxim reciclat de nutrients amb el mínim de pèrdues. S'ha de prestar molta atenció a l'aplicació de fems per tal d'evitar la contaminació dels cursos d'aigua i de les aigües subterrànies.

El Consell pot denegar la qualificació ecològica a parcel·les amb risc d'acumulació excessiva de metalls pesants (mercuri, cadmi, plom, níquel, coure, crom i

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zinc) a causa de la contaminació de l'entorn, proximitat a carreteres de trànsit elevat o per culpa de les anteriors pràctiques agrícoles en la parcel·la.

Quant al reg de les parcel·les ecològiques, està permès utilitzar aigua regenerada provinent de depuradores d'indústries i/o explotacions exclusives certificades com a ecològiques. Està totalment prohibida la utilització d'aigües residuals per al reg i també la utilització d'aigua de reg contaminada amb aigües residuals o amb productes químics.

El maneig del sòl ha d'assegurar una aplicació regular de residus orgànics en forma de fem i/o restes vegetals i una activitat microbiana suficient per tal d'iniciar la descomposició dels materials orgànics i la meteorització dels minerals del sòl. D'aquesta manera, es formen nutrients en forma de sals solubles que poden ser utilitzats per les plantes. Els adobs minerals s'han de considerar com un suplement o esmena i no com a substituïdors d'adobs orgànics. S'han d'aplicar només quan no és possible assegurar la correcta nutrició del conreu amb les tècniques, els fems de bestiar i les matèries orgàniques autoritzades.

Es poden utilitzar fems de bestiar no ecològic sempre que es compleixin les següents condicions: fems procedents de sistemes de cria extensiva no ecològica en els quals no se superin les dues unitats de bestiar major per hectàrea de superfície farratgera, després de ser correctament compostats; fems d'aus (gallinassa) procedents dels sistemes de cria no ecològica d'aviram per a la producció de carn i producció d'ous i fems procedents de cria convencional de porcs, després de ser correctament compostats.

És important el disseny correcte de les rotacions, ja que ajuden al control de plagues i malalties, al manteniment de la fertilitat i minimitzen les pèrdues de nutrients. S'han d'incloure conreus amb diferents sistemes radiculars, que explotaran nivells diferents del sòl. És important una rotació de la família de les lleguminoses per enriquir el sòl amb nitrogen, per la qual cosa aquest sòl podrà ser utilitzat per conreus posteriors.

1.7. Tecnologia del processat d'hortalisses

El canvi en els hàbits de consum de la població ha tingut com a resultat una demanda més gran d'aliments naturals, d'aparença i valor nutricional semblants als productes frescos, sense additius sintètics, microbiològicament segurs i que, a la vegada, siguin fàcils de preparar i de consumir. La indústria conscient de la importància que significa satisfer les exigències dels clients per augmentar el consum, procura millorar contínuament els seus processos productius, buscant alternatives tecnològiques als tractaments tradicionals. Una de les respostes a la demanda d'aquests productes són les fruites i hortalisses mínimament processades, que obren nous mercats com a producte preparat i llest per al seu consum d'aquelles persones que, per comoditat o falta de temps, no tenen el costum de menjar fruites i hortalisses.

En aquests casos, s'hi aplica un processat mínim que consisteix a aplicar una sèrie de tecnologies que permeten mantenir les característiques dels aliments semblants als productes frescos i que augmenten la seva vida útil en termes microbiològics, sensorials i nutricionals. En aquest context, neixen les noves tecnologies de processat no tèrmic d'aliments com els polsos elèctrics d'alta intensitat de camp (PEAIC) que ajuden a contrarestar els efectes negatius produïts pel processat tèrmic. Els tractaments de polsos elèctrics s'han dut a terme com a alternativa a altres tècniques convencionals de producció d'aliments. En els últims anys, diversos estudis han demostrat la capacitat dels tractaments de PEAIC per obtenir aliments líquids amb alt valor nutricional mitjançant la inactivació de microorganismes i enzims (Espachs-Barroso *et al.*, 2003). En aquest cas, l'aplicació dels PEAIC pot causar dany letal a les cèl·lules a causa de la pèrdua irreversible de les propietats de permeabilitat de la membrana cel·lular (Aronsson *et al.*, 2001).

1.7.1. Tractament mitjançant polsos elèctrics d'alta intensitat de camp: PEAIC

L'aplicació del PEAIC és una tecnologia esperançadora en la conservació d'aliments. Aquesta tecnologia consisteix en l'aplicació de polsos d'alta intensitat de camp (kV/cm) durant un període de temps curt (μ s). Els principals avantatges d'aquest mètode de processat són que allarguen la vida del producte i mantenen les propietats nutricionals dels aliments. Els PEAIC només poden aplicar-se a aliments líquids, els

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quals han de ser homogenis i tenir una mida de partícula petita per no obstruir les conduccions ni la cambra de tractament. També és important evitar que l'aliment tingui bombolles d'aire, ja que pot causar arcs elèctrics en l'equip.

L'efectivitat d'aquest procés depèn de factors crítics entre els quals es troben els paràmetres que estan relacionats amb el tractament i d'altres que depenen del producte que s'ha de tractar. Dins dels factors de processat s'hi troben: la intensitat de camp, el temps de tractament, l'amplada del pols, la freqüència del pols, la forma del pols, la polaritat, l'energia i la temperatura aplicada.

1.7.2. Tractament mitjançant polsos elèctrics de baixa intensitat de camp: PEBIC

D'altra banda, els polsos elèctrics de baixa intensitat de camp (PEBIC) estan actualment en estudi, ja que les respostes fisiològiques a l'estrès induït pels polsos elèctrics no es coneixen bé. Recentment, els PEBIC s'apliquen com a tècnica per millorar l'extracció dels metabòlits intracel·lulars (Ade-Omowaye *et al.*, 2003; Soliva-Fortuny *et al.*, 2009) i per augmentar l'eficiència en processos de secat (Taiwo *et al.*, 2002). Algunes publicacions recents sobre polsos elèctrics han suggerit la possibilitat d'utilitzar aquesta tècnica per produir estrès oxidatiu a les cèl·lules i estimular el metabolisme secundari i, per tant, la biosíntesi de metabòlits beneficiosos per a la salut (Toepfl *et al.*, 2006). En els PEBIC, la distribució elèctrica és reversible i la viabilitat de la cèl·lula es manté a través de la biosíntesi de metabòlits secundaris addicionals com els polifenols o carotenoides, que són àmpliament coneguts per ser part de la resposta de defensa de les plantes a l'estrès oxidatiu, induït pel tractament dels PEBIC (Galindo *et al.*, 2009; Soliva-Fortuny *et al.*, 2009).

L'efectivitat d'aquest procés depèn de factors crítics entre els quals es troben els paràmetres que estan relacionats amb el tractament i d'altres que depenen del producte que s'ha de tractar. Els paràmetres crítics són la intensitat de camp, que normalment oscil·la entre 0.2 i 2 kV/cm, i el temps de tractament, que és el temps durant el qual el producte està sotmès al tractament de PEBIC.

1.7.3. Tractaments d'esterilització i pasteurització

La calor fins avui és la tècnica més eficaç per aconseguir la conservació adequada dels aliments, ja que és capaç de destruir els microorganismes i enzims que alteren els aliments. No obstant això, el tractament tèrmic fa malbé una gran quantitat de components bioactius presents en els aliments. Per aquest motiu la indústria alimentària necessita optimitzar els mètodes tèrmics per minimitzar els inconvenients com a conseqüència de la seva aplicació.

L'esterilització és un tractament tèrmic fort, i el seu objectiu és eliminar la majoria de microorganismes dels aliments, i obtenir com a resultat un aliment comercial estèril. Aquest tractament proporciona una major vida útil a l'aliment, inclús es pot emmagatzemar a temperatura ambient. No obstant això, l'esterilització té alguns desavantatges, ja que produeix canvis de textura, color, aroma, sabor i pèrdua de qualitat nutricional del producte. Els temps i temperatures d'escalfament varien, però el tractament ha de ser suficient per esterilitzar l'aliment. Normalment s'hi apliquen temperatures molt altes (entre 135 i 150 °C) que permeten temps d'escalfament curts (4-15 segons). Una manera d'eleva ràpidament la temperatura és utilitzant vapor en els sistemes de processat asèptic també anomenats *UHT (ultra high temperature)*. Aquest mètode de conservació per calor es fa servir molt en la indústria alimentària. La seva qualitat final es pot comparar amb els productes refrigerats, però amb una vida útil de com a mínim 6 mesos sense refrigeració.

La pasteurització és un tractament suau per calor, la finalitat del qual és eliminar selectivament els microorganismes patògens, reduir el recompte microbià i inactivar els enzims dels aliments, proporcionant les mínimes pèrdues d'aroma, sabor, textura i qualitat nutritiva. Aquest tractament també té inconvenients, ja que el producte resultant té una vida útil curta i ha de tenir un altre mitjà de conservació addicional, com ara la refrigeració o la congelació. Durant la pasteurització, els aliments se sotmeten a temperatures d'entre 90-95 °C entre 15-60 segons.

2. Anàlisi i identificació de compostos bioactius en tomàquets i derivats

2.1 Anàlisi i identificació de compostos fenòlics

La identificació de compostos fenòlics en aliments és una feina complexa, a causa de la gran varietat d'estructures presents en la natura i de la falta d'estàndards en el mercat. Algunes de les tècniques analítiques utilitzades per a la identificació de polifenols són: l'electroforesi capil·lar (EC) (Herrero-Martínez *et al.*, 2005), la cromatografia de gasos (GC) (Friedman, 2004), la ressonància magnètica nuclear (NMR) (Slimestad *et al.*, 2008), la cromatografia de líquids d'alta resolució (HPLC) amb detecció ultraviolat (UV) (Kerem *et al.*, 2004; Sun *et al.*, 2007) i l'HPLC acoblada a espectrometria de masses (MS) (Sánchez-Rabaneda *et al.*, 2003; Tsao & Deng, 2004; Vallverdú-Queralt *et al.*, 2010). Abans de l'ús d'aquestes tècniques modernes, però, s'havia fet ús de la cromatografia en paper i en capa fina per identificar un gran nombre d'aquests compostos. L'arribada posterior de noves tècniques cromatogràfiques ha servit per confirmar alguns dels productes coneguts i per augmentar considerablement el nombre de compostos identificats.

En aquesta tesi doctoral, la tècnica més utilitzada ha estat l'MS. Pel que fa a les anàlisis metabolòmiques, s'han realitzat amb l'HPLC acoblada a MS d'alta resolució amb un espectròmetre híbrid quadrupol-temps de volada (HPLC-QToF-MS). Aquesta tecnologia permet l'adquisició automàtica dels espectres de masses d'alta resolució en una sola injecció cromatogràfica. Les analítiques amb espectròmetre híbrid quadrupol-temps de volada (QToF) permeten obtenir la massa exacta dels ions, la qual cosa és essencial per assignar la composició elemental i, per tant, per a la caracterització de molècules petites.

Una altra eina que ha estat utilitzada és l'HPLC acoblada a MS d'alta resolució amb un espectròmetre híbrid de trampa d'ions-transformada de Fourier (HPLC-LTQ-Orbitrap-MS) (Vallverdú-Queralt *et al.*, 2010). Aquesta tècnica permet l'analítica d'MS i MSⁿ per facilitar l'obtenció d'informació estructural. A causa de l'especificitat de les mesures d'MSⁿ i la capacitat d'eliminar les interferències a través de l'etapa inicial de selecció de masses, l'HPLC-LTQ-Orbitrap-MS és un dels millors equips per a l'analítica qualitativa; ja que facilita l'elucidació de l'estructura de compostos desconeguts.

Per a la quantificació dels diferents compostos, s'ha fet ús de l'*HPLC* acoblada a espectrometria de masses en tàndem (*MS/MS*) amb un instrument de triple quadrupol (*HPLC-MS/MS*) per la seva gran sensibilitat en mode *multiple reaction monitoring* (*MRM*) (Vallverdú-Queralt *et al.*, 2011a; Vallverdú-Queralt *et al.*, 2011b). La sensibilitat de les tècniques en tàndem ha permès avançar en la quantificació de compostos fenòlics i reduir el temps d'anàlisi.

Espectrometria de masses: MS

L'*MS* és la tècnica més utilitzada per a l'anàlisi d'aliments i mostres biològiques a causa de la seva elevada sensibilitat, especificitat i la seva fàcil combinació amb tècniques cromatogràfiques, la qual cosa facilita la identificació, determinació i quantificació d'aquests compostos. Té alta sensibilitat, baix consum de mostra i la informació necessària s'obté fàcilment seleccionant tècniques d'ionització i analitzadors de masses adequats. Per a la majoria de compostos, l'espectròmetre de masses és més sensible i específic que cap altre detector que s'acobli al cromatògraf de líquids. A més, i a diferència dels detectors d'*UV*, l'*MS* pot analitzar substàncies que no tinguin cromòfors o actuïn com a tals, i és capaç de resoldre coelucions de compostos en un cromatograma, la qual cosa redueix la necessitat d'una separació cromatogràfica perfecta.

Instrumentació analítica

Els espectròmetres de masses consten de:

- A) Un **sistema d'introducció de la mostra**, que pot ser, entre altres, un cromatògraf de líquids o de gasos. En el nostre cas, un *HPLC*.
- B) Una **font d'ionització**, que és on es produeix la ionització dels analits de la mostra.
- C) Un **analitzador**, que separa els ions generats en funció de la seva relació massa/càrrega (m/z). Existeixen diferents tipus d'espectròmetres de masses en funció de l'analitzador que facin servir. Els analitzadors més comuns són: el quadrupol simple (Q), el triple quadrupol (QqQ), el sector magnètic, la trampa d'ions (*IT*), el temps de volada (*ToF*) i, més recentment, l'Orbitrap.

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D) Un **detector**, que recull i caracteritza els ions que surten de l'analitzador.

A més a més, tot el sistema es troba sotmès a alt buit (1×10^{-4} - 1×10^{-5} torr.) per evitar col·lisions entre els fragments iònics generats. A continuació, es mostra un esquema d'un sistema *HPLC-MS* en què s'especifiquen les diferents parts del sistema *MS* (**Figura 2**).

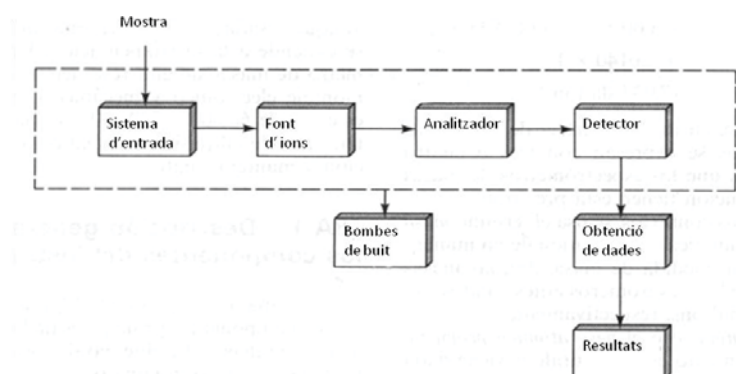


Figura 2. Esquema d'un sistema *HPLC-MS* en què s'especifiquen les diferents parts del sistema *MS*.

A) Sistema d'introducció de la mostra. Cromatografia de líquids d'alta resolució: *HPLC*

Les columnes de fase invertida són les més adequades per a l'anàlisi dels compostos fenòlics. Aquestes fases estacionàries de fase invertida acostumen a ser partícules de sílice que contenen molècules específiques lligades a la seva estructura. Les molècules de sílice milloren les separacions i n'augmenten l'especificitat. El material més utilitzat per a l'anàlisi de fenols és l'octadecilsilà (C_{18}) (Odriozola-Serrano *et al.*, 2009a; Torre-Carbot *et al.*, 2005; Vallverdú-Queralt *et al.*, 2010). No obstant això, també s'han emprat columnes C_8 o C_4 , però el seu ús no és tan comú.

Les dimensions de les columnes poden ser variables. Fa uns anys, les columnes més típiques tenien unes mides que oscil·laven entre els 100 i 300 mm de llargària x 4.6 mm de diàmetre, amb una mida de partícula inicialment de 10 μm . A mesura que han sorgit nous materials i que els empaquetaments de les columnes han estat millors, la mida i el diàmetre de partícula han disminuït. Actualment, s'utilitzen columnes de mida de partícula entre 3 i 5 μm , la qual cosa permet utilitzar columnes més curtes i

primes i, consegüentment, disminuir el temps d'anàlisi. L'últim avanç ha estat l'ús de columnes de nucli rígid de sílice tipus Kinetex o Ascentis i les columnes de sub-2 μm , que s'utilitzen per a cromatografia de líquids d'ultra alta resolució.

Els solvents més utilitzats en fase reversa són els sistemes binaris, els quals consten d'un dissolvent aquós amb modificadors de pH, i d'un altre solvent orgànic polar soluble en aigua com l'acetonitril o el metanol. Els modificadors més utilitzats acostumen a ser els àcids fòrmic, acètic, i també tampons volàtils (formiat d'amoni o acetat d'amoni). El pH és un dels factors més importants en la separació dels compostos fenòlics.

Els compostos fenòlics contenen enllaços dobles C-C que actuen com a cromòfors, la qual cosa fa possible l'anàlisi dels fenols mitjançant un UV. Els fenols simples es detecten amb facilitat a 280 nm, els àcids hidroxicinàmics a 320 nm, les flavanones i flavones a 280 nm i els flavonols a 365 nm. La sensibilitat dels detectors d'UV pot resultar adequada per a la determinació de compostos a altes concentracions, però en alguns casos, aquesta sensibilitat no és suficient i calen tècniques més potents com ara l'MS, la qual augmenta la confiança en els resultats qualitius i quantitatius que se n'obtenen.

B) Fonts d'ionització

A causa de la gran varietat estructural de compostos presents en les mostres d'aliments, no existeix una tècnica universal per a tots ells. En l'MS existeixen, principalment, 3 tècniques d'ionització depenent del pes molecular, de l'estructura i de la polaritat del compost que es vol analitzar: ionització en electroesprai (ESI), ionització química a pressió atmosfèrica (APCI) i fotoionització a pressió atmosfèrica (APPI). A continuació, es mostra el tipus de compostos que és possible analitzar per HPLC-MS segons les diferents fonts d'ionització (**Figura 3**).

Introducció

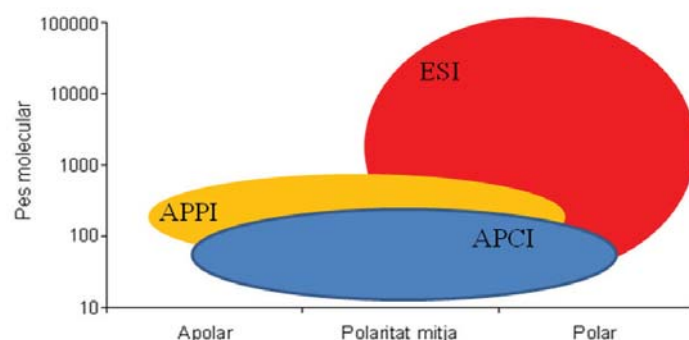


Figura 3. Tipus de compostos que és possible analitzar per *HPLC-MS* segons les diferents fonts d'ionització.

Ionització per electrosprai: ESI

L'*ESI* és la tècnica d'elecció per a compostos més polars i iònics. Aquesta font és molt útil per a l'anàlisi de compostos de pes molecular alt com ara: proteïnes, pèptids i oligonucleòtids, però també és una de les fonts més útils en l'anàlisi de compostos fenòlics.

Els ions es generen abans que la mostra arribi a l'espectròmetre de masses. La mostra entra junt amb la fase mòbil en una cambra que es troba a pressió atmosfèrica. Allà, la fase mòbil es nebulitza i es transforma en una mena d'esprai gràcies a un camp elèctric i a la presència d'un gas a alta temperatura. El camp elèctric dissocia les molècules de l'analit, mentre el gas evapora el solvent en forma de petites gotes. A mesura que la mida de les gotes disminueix, n'augmenta la càrrega, i s'afavoreix la dissociació dels diferents ions a la nova fase gasosa. Aquests ions passen posteriorment a l'interior de l'analitzador.

Ionització química a pressió atmosfèrica: APCI

Per a l'anàlisi d'analits de baixa polaritat, l'*APCI* proporciona millor eficàcia i selectivitat. Una font de calor (250-400 °C) evapora la fase mòbil junt amb la mostra que entra en forma d'esprai a la cambra d'ionització. Per acció d'una agulla que descarrega electrons, s'inicia el procés d'ionització. És aquí quan els ions de la fase mòbil transfereixen la càrrega a les molècules de la mostra a través de reaccions químiques (ionització química). Els ions passen llavors a l'analitzador.

Fotoionització a pressió atmosfèrica: APPI

L'APPI és una tècnica recent en l'HPLC acoblada a MS. El fonament és el mateix que la ionització per APCI, però la ionització la provoca una làmpada que genera fotons en un estret rang d'energies d'ionització. Aquest rang d'energia ionitza el màxim de molècules dels analits i minimitza la ionització de les molècules de la fase mòbil. Els ions resultants passen a l'analitzador.

L'APPI i l'APCI tenen més tolerància a les sals i a l'efecte matriu si es compara amb l'ESI (Schlüsener & Bester, 2005). L'APCI presenta un rang d'aplicació similar a l'APPI, però està més estesa a compostos no polars.

Recentment, s'ha descobert la desorció ESI (DESI) com una tècnica d'ionització per a la identificació de metabòlits (Williams *et al.*, 2006). No necessita preparació prèvia de la mostra i es pot utilitzar directament en teixits i organismes vius (Wiseman *et al.*, 2000). Una altra nova font d'ionització és l'anàlisi directa a temps real (DART) en la qual la mostra s'introdueix directament (sòlida, líquida o gasosa). Serveix tant per a l'anàlisi qualitativa com quantitativa.

La tècnica d'ionització i desorció per làser (MALDI) no té gaires aplicacions per l'anàlisi de polifenols en mostres d'aliments.

C) Analitzadors de masses

En principi, qualsevol tipus d'analitzador es pot utilitzar en l'anàlisi per HPLC-MS. Els avantatges i desavantatges d'aquests analitzadors depenen dels requeriments de cada anàlisi. Els analitzadors de masses es classifiquen en dos grans grups segons la seva resolució. En general els analitzadors de baixa resolució, com ara els quadrupols i trampes d'ions, tenen una resolució en un rang menor de 1000, mentre que els analitzadors d'alta resolució tenen una resolució mínima de 15000 per a analitzadors de temps de volada i per sobre de 50000 per a analitzadors de transformada de Fourier (Peterman *et al.*, 2006). En aquesta tesi, es fa una breu descripció del Q, QqQ, ToF, QToF, IT, transformada de Fourier (FTMS) i LTQ-Orbitrap.

Introducció

C.1. Quadrupol simple: Q

El Q està format per quatre cilindres paral·lels que formen un quadrat (**Figura 4**). Els voltatges que s'apliquen als cilindres generen camps electromagnètics que determinen quins ions, segons la seva relació m/z , poden arribar al detector a un temps determinat. La resta d'ions no segueixen una trajectòria estable i mai no arribaran al detector.

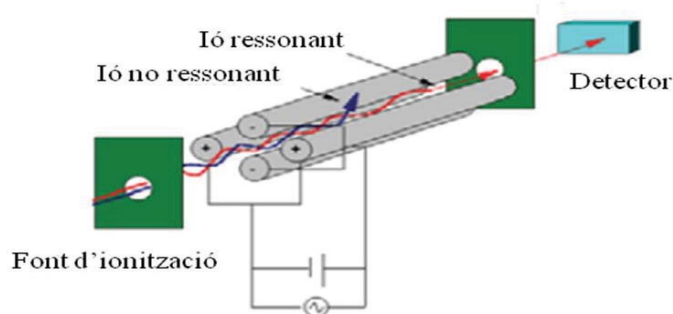


Figura 4. Esquema d'un Q. Els ions que tenen la càrrega adequada (ressonants) arriben al detector.

Hi ha dues maneres d'operar amb aquest analitzador: en mode d'escombratge complet, *full scan* (FS), i seleccionant i monitoritzant un ió concret, *selected ion monitoring* (SIM). En mode FS, l'analitzador monitoritza un rang predeterminat de relacions m/z . És el mètode utilitzat per dur a terme anàlisis qualitatives. En mode SIM, l'analitzador monitoritza només unes relacions de m/z determinades. És molt més sensible que el mode d'escanejat, la qual cosa el fa molt recomanable per realitzar anàlisis quantitatives, però es perd la informació espectral.

C.2. Triple quadrupol: QqQ

L'analitzador QqQ consisteix en tres quadrupols connectats en sèrie. El quadrupol 1(Q1) i el 3(Q3) funcionen com a analitzadors. El quadrupol 2(Q2) o cel·la de col·lisió (CID) se situa entremig del Q1 i Q3. A continuació, es mostra un esquema d'un QqQ i les seves parts (**Figura 5**).

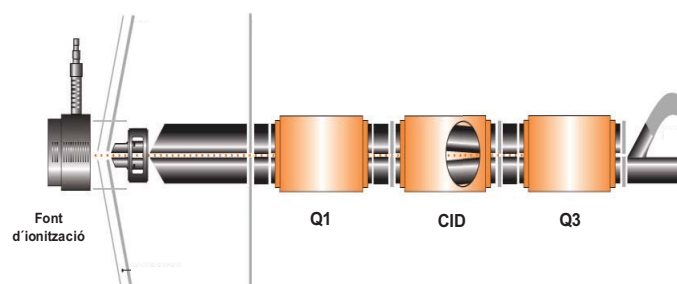


Figura 5. Esquema d'un QqQ.

En el cas del QqQ, es poden realitzar quatre tipus d'experiments, que es mostren en la **figura 6**, en què Q1 i Q3 estan controlades segons l'experiment i tenen una funció específica. Per exemple, si es busca un compost o família específica de compostos s'utilitzaran experiments de *precursor ion scan* (*Prec*) o de *neutral loss* (*NL*), mentre que si el que es vol és confirmar la identitat d'un compost determinat es duran a terme experiments de *product ion scan* (*PIS*). Finalment, l'*MRM* es fa servir bàsicament per obtenir millors límits de detecció i per quantificar els diferents analits. Aquest últim és el mètode més sensible per a l'anàlisi quantitativa, però també presenta una manca d'informació espectral.

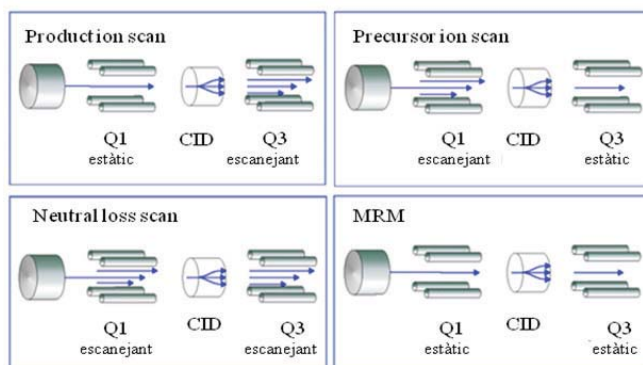


Figura 6. Esquema dels diferents experiments *MS/MS*.

Introducció

C.3. Temps de volada: ToF

En aquest tipus d'analitzador, s'hi aplica una força electromagnètica uniforme a tots els ions al mateix temps, la qual cosa accelera el pas dels ions a través d'un tub anomenat *time of flight*. Els ions més lleugers viatgen més ràpid i arriben primer al detector. Així, les relacions de m/z dels ions estan condicionades i es determinen segons el seu temps d'arribada. No obstant això, amb aquest tipus d'analitzador no es poden realitzar experiments d' MS/MS .

C.4. Quadrupol-temps de volada: QToF

Aquest analitzador combina l'estabilitat d'un QqQ amb l'alta eficàcia, sensibilitat i exactitud d'un ToF. És capaç de seleccionar un ió en particular i aconseguir mesures simultànies i precises d'ions de tot el ventall de masses. Ofereixen una gran sensibilitat i exactitud en front el QqQ quan es treballa en mode FS. Aquest equip s'utilitza per a les anàlisis metabolòmiques per fer una exhaustiva identificació dels metabòlits presents en mostres d'aliments i biològiques. Les analítiques amb QToF permeten obtenir la massa exacta dels ions i dels seus productes, la qual cosa és essencial per assignar la composició elemental i, per tant, per a la caracterització de molècules petites. A diferència del ToF, amb aquest equip es poden fer mesures d' MS/MS .

C.5. Trampa d'ions: IT

Està formada per una cambra amb dos elèctrodes i dues peces de finalització que atrapen els ions gràcies a una sèrie de camps electromagnètics. Una vegada dins de la cambra, pot aplicar-se un altre camp electromagnètic, romanen en la cambra només els ions seleccionats. Aquest analitzador permet fer múltiples anàlisis o fragmentacions (MS^n) d'una mateixa molècula sense requerir un acoblament amb altres analitzadors de masses addicionals (Anari *et al.*, 2003). Aquest procés es pot seqüenciar automàticament fent, per exemple, que els ions més abundants de cada etapa es fragmentin de nou. Aquestes tècniques de múltiples processos de trencament permeten descartar en una primera etapa els ions que no ens interessin.

C.6. Transformada de Fourier: FTMS

Aquest analitzador està basat en l'acció que un camp magnètic exerceix sobre un ió girant en un camp de radiofreqüència. Mitjançant el camp magnètic, els ions són dirigits a una cambra on giren descrivint òrbites de diàmetre reduït i mínima freqüència. Per l'aplicació d'un senyal de radiofreqüència, els ions són excitats a descriure òrbites espirals d'amplitud creixent. Arriba un moment en què l'òrbita descrita és de diàmetre igual a la distància de separació entre dos elèctrodes detectors, moment en el qual són detectats generant una imatge de corrent, que és la funció directa de la seva relació m/z . Aquesta imatge de corrent és integrada mitjançant una transformada de Fourier i convertida a un senyal proporcional a la seva intensitat. L'espectre complet s'obté mitjançant un escombrat del camp de radiofreqüència que varia entre 8 KHz i 100 MHz. El principal avantatge d'aquest tipus d'analitzador és l'altíssima precisió en les mesures de masses (0.001% i superiors) i un poder de resolució quasi il·limitat.

C.7. Trampa d'ions-transformada de Fourier: LTQ-Orbitrap

És un dels millors analitzadors per a l'anàlisi de polifenols en aliments i mostres biològiques (Peterman *et al.*, 2006). Combina els avantatges dels analitzadors *IT* i *FTMS*. Aquesta metodologia permet l'analítica d' MS i MS^n per facilitar l'obtenció d'informació estructural amb una mesura acurada de les masses amb un error inferior a 2 ppm i sense necessitat de calibrar contínuament. A causa de l'especificitat de les mesures d' MS^n i la capacitat d'eliminar les interferències a través de l'etapa inicial de selecció de masses, l'*LTQ-Orbitrap-MS* és un equip amb grans possibilitats per a l'analítica qualitativa, ja que facilita l'elucidació de l'estructura de compostos desconeguts. A continuació es presenta un esquema d'un analitzador *LTQ-Orbitrap*.

Introducció

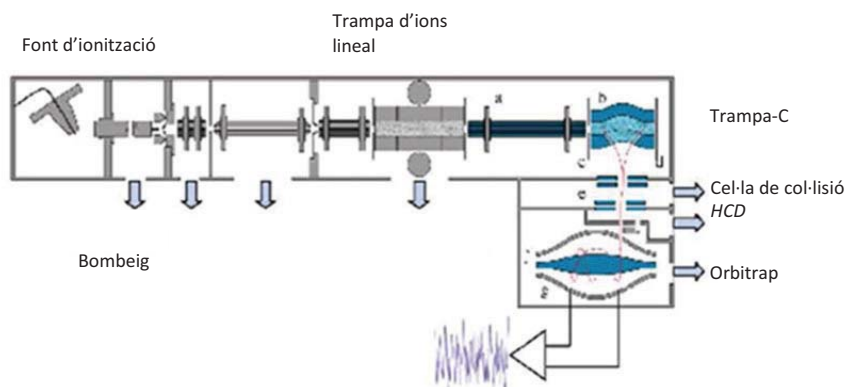


Figura 7. Esquema d'un *LTQ-Orbitrap*.

A continuació es descriuen les diferents parts d'aquest equip.

C.7.1. Font d'ionització

Els ions es generen tant en la fase de solució mitjançant *ESI* com en la fase gas mitjançant *APCI*. Després, els analits passen de la font d'ionització a la regió de l'analitzador mitjançant una sèrie de lents que combinen corrent continu, voltatge de radiofreqüència i un gradient de buit.

C.7.2. LTQ-trampa d'ions lineal

És la primera trampa lineal en dues dimensions i ofereix diversos avantatges respecte els instruments en 3-Dimensions. Aquesta trampa lineal té una gran sensibilitat, més velocitat d'exploració i més eficiència.

C.7.3. Trampa-C

La trampa-C és un Q de voltatge de radiofreqüència en forma de C, la funció del qual és assegurar que els ions s'injectin a l'*Orbitrap*.

C.7.4. Cel·la de col·lisió d'alta energia: HCD

És un nou disseny de cel·la de col·lisió que es connecta directament amb la trampa-C. Això permet que es duguin a terme col·lisions d'alta energia per generar espectres d'*MS/MS*. Una vegada els ions s'han fragmentat, es transfereixen a la trampa-C amb un gradient de potencial i des d'allí a l'*Orbitrap*.

C.7.5. Orbitrap

L'*Orbitrap* consta de dos elèctrodes un de central, al voltant del qual els ions es mouen descrivint una espiral, i un d'exterior. De les tres freqüències característiques que poden descriure els ions en l'*Orbitrap*, només la component axial és independent de l'energia inicial, dels angles i de les posicions i pot ser detectada a l'*Orbitrap*. Després s'emptra una transformada de Fourier per obtenir freqüències d'oscil·lació dels ions amb masses diferents. Finalment, s'obté una lectura precisa de la seva m/z i masses d'alta resolució.

D) Detectors

Comercialment, són assequibles diferents tipus de detectors per a espectròmetres de masses. En els primers anys, predominaven els fotomultiplicadors d'electrons. Després, van aparèixer d'altres detectors per a deteccions puntuals com els Channeltron de baix cost i ús general, i els sistemes de conversió fotònica més sofisticats i sensibles. També, han aparegut sistemes de detecció simultània com ara les bateries de micromultiplicadors multicanal combinades amb posterior conversió fotònica, l'aportació de les quals als nous desenvolupaments instrumentals en MS ha estat important.

Eines per a la identificació de polifenols mitjançant HPLC-MS/MS

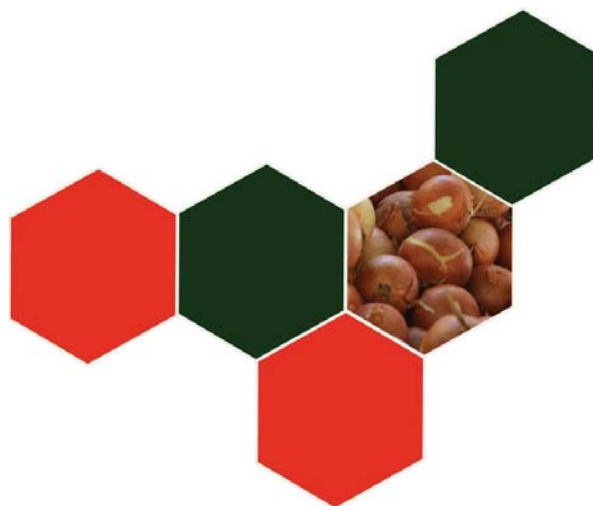
Hi ha diferents maneres de confirmar la identitat dels compostos presents en mostres d'aliments. En aquesta tesi, primer es van fer servir experiments d'*MS/MS* en el QqQ. No obstant això, per la confirmació definitiva es van fer servir equips d'alta resolució com el *QToF* i l'*LTQ-Orbitrap*, els quals permeten obtenir la massa exacta. La composició elemental es va seleccionar en funció de la massa exacta i del patró isotòpic amb l'ajuda del Diccionari de Productes Naturals i la base de dades dels metabòlits del tomàquet (*MoTo DB*) (Grennan, 2009). La interpretació dels espectres de masses (*MS/MS* i *MSⁿ*) en comparació amb els trobats a la literatura ha estat l'eina principal per a la identificació dels polifenols dels tomàquets i derivats.

2.2. Anàlisi i identificació de carotens

Fins fa poc, la identificació de carotens es portava a terme mitjançant fases estacionàries C_{18} (Azevedo-Meleiro & Rodríguez-Amaya, 2009; Liu *et al.*, 2009). Més recentment, s'estan fent servir fases estacionàries C_{30} , la qual cosa proporciona més resolució i selectivitat (García-Alonso *et al.*, 2009). Per a la identificació de carotens, l'*HPLC-UV* és la tècnica més utilitzada (Rentel *et al.*, 1998). No obstant això, l'*MS* s'ha començat a fer servir per l'anàlisi de carotens. La tècnica d'ionització més utilitzada és l'*APCI*, però amb aquesta tècnica és necessària molta quantitat de mostra. Per això, una bona alternativa és l'*ESI* amb una prèvia derivatització amb sals de sodi o calci (D'Alexandri *et al.*, 2010) per aconseguir una bona ionització de compostos apolars com els carotens.

En aquesta tesi doctoral, s'han utilitzat dues tècniques per a la identificació dels carotens: l'*HPLC-UV* i l'*HPLC* acoblada a *MS/MS* amb un instrument de QqQ, utilitzant *ESI* amb prèvia derivatització amb clorur de liti (*HPLC-ESI-(Li⁺)-MS/MS*). Pel que fa a les característiques i als equips emprats per a la seva identificació són els mateixos que els descrits per a l'anàlisi de compostos fenòlics.

MATERIALS I MÈTODES



IV. MATERIALS I MÈTODES

1. Recepció i preparació de la mostra

Les varietats de tomàquet procedents de l'empresa CONESA de Vegas Bajas del Guadiana, Badajoz, Espanya, escollides per a la realització d'aquesta tesi van ser: *malva*, H-9661, H-9776, H-9997, *albastro*, *guadiva* i *elegy*. Les mostres de salses (*passata* 10/12, *hot break* 22/24, *hot break* 28/30 i *cold break* 28/30) i les mostres de daus de tomàquet (*cold process*, *cold process* amb CaCl_2 i *hot process*) també procedien de la mateixa zona.



Tots els suc de tomàquet, quètxups i gaspatxos van ser adquirits a diferents mercats de Barcelona. Per a la realització dels estudis de PEBIC, es van escollir mostres del mercat de Lleida.

Tots els productes es van emmagatzemar en refrigeració o congelació, depenent dels requeriments de la mostra, fins a la seva anàlisi.



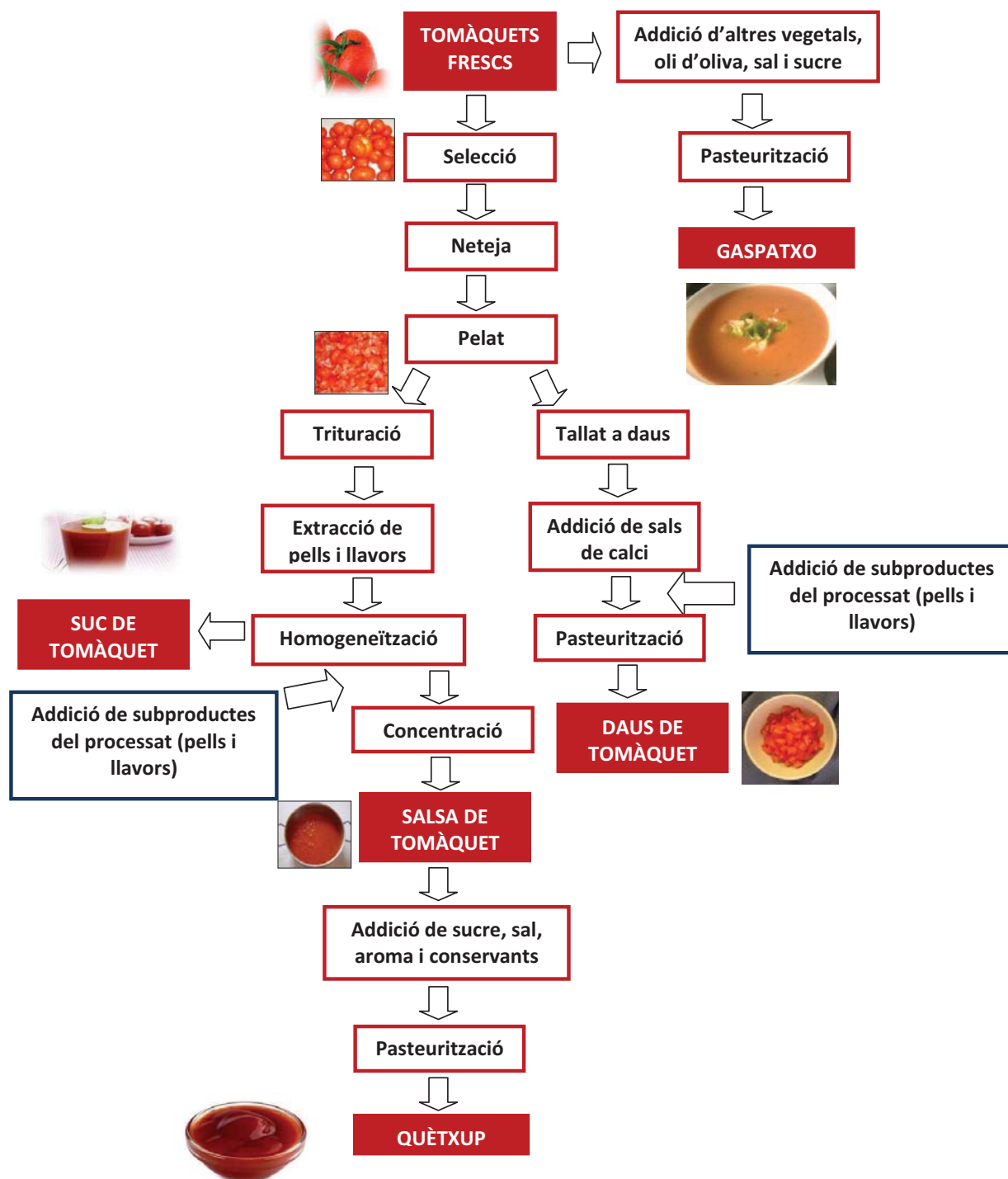


Figura 8. Diagrama de flux pels productes derivats del tomàquet.

2. Caracterització

Per a la caracterització d'algunes de les mostres, es va dur a terme la determinació de diferents propietats físicoquímiques com ara: color, pH, textura i sòlids solubles.

Color: Per mesurar el color de tomàquets sencers, es va fer servir un colorímetre Minolta CR-400 (Konica, Minolta, Sensing, Inc., Osaka, Japó). Aquests equips, a partir dels valors de refractància, proporcionen les coordenades del sistema cromàtic uniforme CIElab (a^* , b^* i L^*).

pH: el pH es va mesurar potenciomètricament amb un pHmetre Crisol 2001 (Crisol Instruments SA, Alella, Barcelona, Espanya).

Sòlids solubles: Els sòlids solubles ($^{\circ}$ Brix) de les mostres de tomàquet es van determinar mitjançant un refractòmetre digital RX-100 (Atago Comapny, Ltd., Japó).

Textura: La textura dels tomàquets es va determinar mitjançant un texturòmetre TA-XT2 (Stable Micro Systems Ltd., Surrey, Anglaterra). La resistència a la penetració es va mesurar com la força necessària perquè una sonda de 4 mm de diàmetre i de base plana penetrés en el tomàquet 5 mm. Els paràmetres van ser els següents: velocitat preassaig, 2 mm/seg; velocitat assaig, 3 mm/seg i velocitat postassaig, 10 mm/seg.

3. Extraccions dels compostos bioactius en mostres de tomàquets i els seus derivats

3.1. Extracció dels polifenols

Per tal d'evitar l'exposició a la llum, a l'oxigen i a les altes temperatures, l'extracció dels polifenols es va dur a terme en la foscor i en fred. En primer lloc, 0.5 grams de mostra fresca es van homogeneïtzar amb 5 mL d'una solució d'un 80% etanol. La mescla es va sotmetre a sonicació durant 5 minuts per afavorir l'extracció dels polifenols i es va centrifugar (4000 rpm a 4 °C) durant 20 minuts. El sobrenedant es va transferir a un tub de recollida de mostra i l'extracció es va repetir. Ambdós sobrenedants es van combinar i es van evaporar sota corrent de N_2 i, finalment, el residu es va reconstituir amb aigua Milli-Q (0.1% d'àcid fòrmic) fins a 4 mL o 1.5 mL depenent del tipus de derivat de tomàquet (Vallverdú-Queralt *et al.*, 2010).

A continuació, es va procedir a fer una extracció en fase sòlida (SPE) dels extractes obtinguts per tal d'eliminar les substàncies interferents, com ara la vitamina

Materials i mètodes

C, sucres reductors o ferro (II), en la determinació de polifenols totals. Per a l'SPE, es van seleccionar els cartutxos Oasis® MAX 30 mg (*mixed-mode anion-exchange and reversed-phase solvent*) per obtenir amb ells millor recuperació que amb altres cartutxos d'SPE. Aquests cartutxos permeten una alta selectivitat i sensibilitat amb els compostos fenòlics estudiats a causa de la mescla del mode d'intercanvi aniònic i el solvent de fase reversa. El procediment es detalla a continuació (**Figura 9**):



Acondicionament 1: Metanol al 100% (1 mL)

Acondicionament 2: Acetat sòdic 50 mM pH 7 (1 mL)



Mostra: Extractes diluïts i acidificats (2 mL)



Neteja: Acetat sòdic 50 mM pH 7 al 5% de metanol (1 mL)



Elució: Metanol al 2% d'àcid fòrmic (1.8 mL)



Evaporació a sequedat sota corrent de N₂
Reconstitució amb aigua Milli-Q al 0.1% d'àcid fòrmic (4 mL)

Figura 9. SPE pels extractes de tomàquet.

3.2. Extracció dels carotens

Per tal d'evitar l'exposició a la llum, a l'oxigen i a les altes temperatures, l'extracció dels carotenoides es va dur a terme en la foscor i en fred. En primer lloc, 0.5 grams de mostra fresca es van homogeneïtzar amb 5 mL d'una solució d'etanol: hexà (4:3 v/v). La mescla es va sotmetre a sonicació durant 5 minuts per afavorir l'extracció dels carotens i es va centrifugar (4000 rpm a 4 °C) durant 15 minuts. El sobrenedant es va transferir a un tub de recollida de mostra i l'extracció es va repetir. Ambdós sobrenedants es van combinar i es van evaporar sota corrent de N₂ i, finalment, el residu es va reconstituir amb terc-butil-metil-èter (MTBE) fins a 1 mL (Rodríguez-Bernaldo de Quirós & Costa, 2003).

4. Determinacions analítiques

4.1. Polifenols totals: Folin-Ciocalteu

Aquest mètode es fa servir per a la mesura dels polifenols totals. És un mètode colorimètric basat en una reacció redox, en què els grups hidroxils dels diferents fenols presents a la mostra reaccionen amb el reactiu de *Folin-Ciocalteu* (FC). Aquesta mescla, en medi bàsic, pren un color blavós proporcional a la concentració de substàncies reductores presents a la mostra, coloració que es mesura amb un espectrofotòmetre a 765 nm. Per dur a terme la quantificació, es necessita una recta patró preparada amb una substància pura de referència, com per exemple l'àcid gàl·lic, i el resultat s'expressa com a mil·ligrams d'àcid gàl·lic per gram d'extracte (Medina-Remón *et al.*, 2010; Vallverdú-Queralt *et al.*, 2011d).

4.2. Licopè total

El contingut de licopè es va determinar espectrofotomètricament seguint el mètode proposat per Odrizola-Serrano *et al.* (2007). Aproximadament uns 0.6 g de mostra fresca es van mesclar amb 5 mL de butilat hidroxitoluè en acetona al 0.05% (p/v), 5 mL d'etanol al 95% i 10 mL d'hexà. La barreja es va centrifugar (4000 rpm a 4 °C) durant 15 minuts. Després es van afegir 3 mL d'aigua Milli-Q, i es va fer la lectura de l'absorbància de la part orgànica a 503 nm.

4.3. Vitamina C

El mètode utilitzat per l'extracció i determinació de la vitamina C de les mostres va ser validat per Odrizola-Serrano *et al.* (2007). Es van pesar 5 g de mostra fresca i s'hi van afegir 5 mL d'una solució que contenia 9 g/L d'àcid metafosfòric i 1.5 g/L de DL-ditiotreitòl com a agent reductor. La barreja es va centrifugar (4000 rpm a 4 °C) durant 15 minuts. Passat aquest temps, el sobrenedant es va filtrar amb un filtre de tefló de 0.45 µm i 13 mm de diàmetre. Una alíquota de 20 µL es va injectar en el sistema cromatogràfic Agilent 1100 (Agilent, Waldbronn, Alemanya) equipat amb una bomba quaternària, un termòstat automàtic de mostres, un forn, un injector automàtic i un detector de feix de díodes (DAD). La separació es va realitzar en una columna de fase reversa C₁₈ Luna de 50 mm de llarg, 2.0 mm de diàmetre i 5 µm de mida de partícula interna. Es va fer servir una fase mòbil que constava d'una solució d'àcid sulfúric al 0.01% a pH 2.6. La columna es va mantenir a temperatura ambient i el flux de la fase mòbil va ser d'1 mL/min. La detecció es va realitzar a longitud d'ona de 245 nm. Per a la quantificació, es va construir la corba de calibratge a cinc nivells de concentració de l'àcid ascòrbic (5, 10, 15, 30 i 50 mg/kg) amb valors de R² entre 0.9896 i 0.9990.

4.4. Radicals lliures: capacitat antioxidant

L'activitat antioxidant es va determinar pel mètode dels radicals estables ABTS⁺ i DPPH segons els mètodes descrits per Minoggio *et al.* (2003) amb algunes modificacions. Es duen a terme les extraccions lipofíliques o hidrofíliques segons si es vol determinar la capacitat antioxidant lipofílica o hidrofílica. 5 µL de sobrenedant hidrofílic o lipofílic es van mesclar amb 245 µL d'una solució metanòlica de DPPH o ABTS⁺. Després de 60 min a la foscor pel mètode d'ABTS⁺ i 30 min pel mètode DPPH, es va mesurar l'absorbància a 515 nm en el cas del DPPH i a 734 nm en el cas d'ABTS⁺. El percentatge d'inhibició respecte l'absorbància inicial obtinguda per la solució metanòlica de DPPH o ABTS⁺ es va expressar com a mmol equivalents d'antioxidant de referència (Trolox) /100 g mostra.

5. Cromatografia de líquids d'alta resolució acoblada a espectrometria de masses en tàndem amb un instrument de triple quadrupol: *HPLC-MS/MS*

Es va fer servir la cromatografia de líquids d'alta resolució acoblada a l'espectròmetre de masses de QqQ API 3000 (ABSciex, Concord, Ontario, Canadà) per a la identificació i quantificació de polifenols i carotens en mostres de tomàquets i de derivats. Aquest espectròmetre està equipat amb una font d'*ESI* i opera amb nitrogen d'alta puresa com a gas de nebulització, gas auxiliar i gas cortina. Per als compostos polifenòlics, normalment, es treballa en mode negatiu, i per als carotens en mode positiu. Per a l'anàlisi dels carotens es va fer servir una addició postcolumna d'una solució de LiCl (500 mg/L) per tal d'afavorir la ionització d'aquests compostos.



Figura 10. Espectròmetre de masses QqQ amb font d'*ESI*.

Es van dur a terme diferents tipus d'experiments. Primer, les mostres es van injectar en mode *FS* de m/z 100 a 800 per tal de fer una primera identificació dels compostos. Seguidament, es van realitzar experiments d'*MS/MS* com ara: l'*NL* en què els dos quadrupols analitzadors duen a terme escombratges complets amb una diferència d'unitats de massa que correspon a la pèrdua d'una molècula neutra; el *Prec*, el qual escaneja els possibles ions precursors d'un determinat ió i el *PIS*, el qual aïlla un ió en el Q1, el fragmenta a la cel·la de col·lisió i escaneja els seus fragments al Q3. Aquesta última és la tècnica més utilitzada per identificar i/o confirmar compostos comparant l'espectre de masses del patró amb el compost a la mostra.

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Finalment, es van injectar les mostres en mode *MRM* que és l'experiment d'anàlisi quantitativa per excel·lència a causa de la seva elevada sensibilitat, encara que no dóna informació espectral.

El cromatògraf de líquids és un Agilent 1100 (Agilent, Waldbronn, Alemanya) equipat amb una bomba quaternària, un injector automàtic amb termòstat i un forn. Les determinacions analítiques dels compostos polifenòlics es van dur a terme utilitzant la columna C_{18} Luna de 50 mm de llarg, 2.0 mm de diàmetre i 5 μ m de mida de partícula interna (Phenomenex, Torrance, CA, EUA). Les fases mòbils van ser aigua Milli-Q (0.1% d'àcid fòrmic) (solvent A) i acetonitril (0.1% d'àcid fòrmic) (solvent B). Es va utilitzar un gradient d'augment lineal de dissolvent B. La separació es va dur a terme en 15 minuts en les següents condicions: 0 min, 5% B; 10 min, 18% B; 13 min, 100% B; 14 min, 100% B; 15 min, 5% B. Es va treballar a un flux de 0.4 mL/min i el volum d'injecció va ser de 20 μ L. Aquest gradient pot variar lleugerament, depenent dels tipus de productes que s'hagin d'analitzar, ja siguin tomàquets, salses, quètxups, suc o gaspatxos (Vallverdú-Queralt *et al.*, 2011a; Vallverdú-Queralt *et al.*, 2011b).

Les determinacions analítiques dels carotens es van dur a terme utilitzant una columna C_{30} de 250 mm de llarg, 4.6 mm de diàmetre i 5 μ m de mida de partícula interna (YMCTM, Waters Co, Milford, MA, EUA). El volum d'injecció va ser de 20 μ L i el flux d'1 mL/min. La fase mòbil constava de dues mescles de dissolvents: A-aigua:metanol: *MTBE* (4:26:70 v/v) i B aigua:metanol: *MTBE* (4:90:6, v/v). El gradient va ser lineal de 26% B a 90% B en 23 minuts. La columna es va equilibrar durant 10 minuts abans de cada anàlisi. L'*MTBE* s'utilitza com a modificador per facilitar l'elució de licopè, que és fortament retinut en un ambient metanòlic.

6. Cromatografia de líquids d'alta resolució acoblada a espectrometria de masses d'alta resolució amb un espectròmetre híbrid de trampa d'ions-transformada de Fourier: *HPLC-LTQ-Orbitrap-MS*

Es va fer servir la cromatografia de líquids d'alta resolució acoblada a l'espectròmetre LTQ Orbitrap Velos (Thermo Scientific, Hemel Hempstead, Regne Unit), que és un espectròmetre híbrid d'*IT* i *FTMS*, per a l'anàlisi de polifenols en mostres de tomàquet i derivats amb una mesura acurada de les masses i errors inferior a 2 ppm. És un instrument amb un gran potencial en l'anàlisi qualitativa amb un poder de resolució de 30000. Combina els avantatges de l'analitzador d'*IT* amb l'analitzador d'*FTMS*.

Aquest espectròmetre està equipat amb una font d'*ESI* i opera amb nitrogen d'alta puresa com a gas de nebulització, gas auxiliar i gas cortina i a una temperatura capil·lar de 275 °C. Es va treballar en mode negatiu.

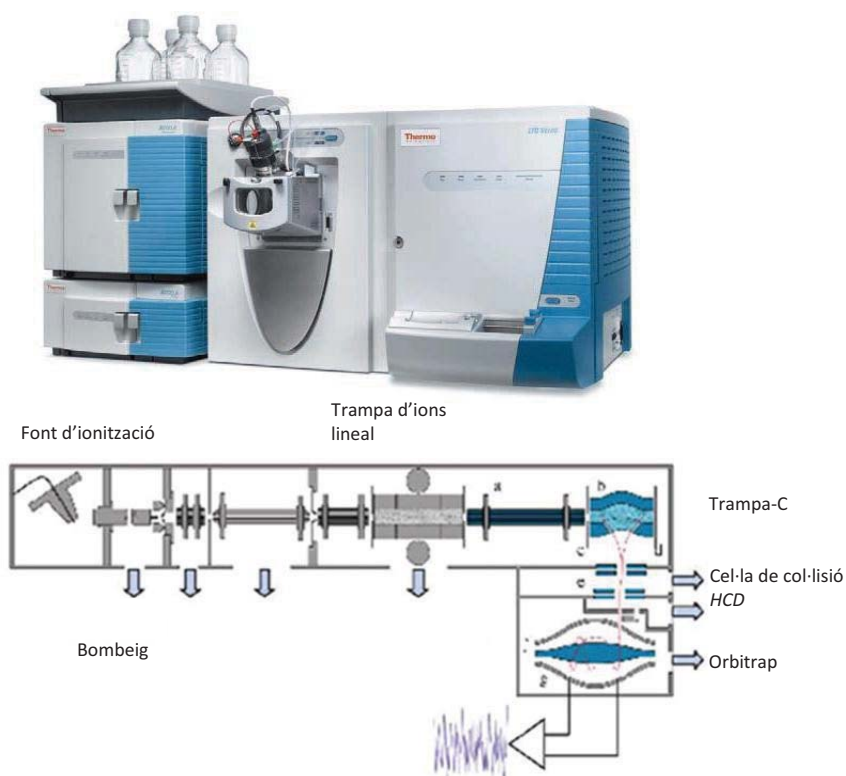


Figura 11. Espectròmetre de masses *LTQ-Orbitrap* amb font d'*ESI*.

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Es van dur a terme diferents tipus d'experiments. En primer lloc, l'*FTMS*, que és l'escombratge complet a l'analitzador de transformada de Fourier de m/z 100 a 1000, per tal de fer una primera identificació dels compostos. Seguidament, es van realitzar experiments d'*MSⁿ* per facilitar l'obtenció d'informació estructural dels polifenols. Per a l'anàlisi de les dades es va utilitzar el programari Xcalibur.

El cromatògraf de líquids és un Accela (Thermo Scientific, Hemel Hempstead, Regne Unit) equipat amb una bomba quaternària, un termòstat automàtic de mostres, un injector automàtic i un *DAD* (Vallverdú-Queralt *et al.*, 2010). Es va fer servir una columna C_{18} Luna de 50 mm de llarg, 2.0 mm de diàmetre i 5 μ m de mida de partícula interna (Phenomenex, Torrance, CA, EUA). El gradient d'elució es va realitzar amb aigua Milli-Q (0.1% d'àcid fòrmic) (solvent A) i acetonitril (0.1% d'àcid fòrmic) (dissolvent B) amb un flux de 0.4 mL/min i el volum d'injecció va ser de 5 μ L. Es va utilitzar un gradient d'augment lineal de dissolvent B. La separació es va dur a terme en 15 minuts en les següents condicions: 0 min, 5% B; 10 min, 18% B; 13 min, 100% B; 14 min, 100% B; 15 min, 5% B. Aquest gradient pot variar lleugerament, depenent dels tipus de productes que s'analitzen, ja siguin tomàquets, salses, quètxups, sucros o gaspatxos.

7. Cromatografia de líquids d'alta resolució acoblada a espectrometria de masses d'alta resolució amb un espectròmetre híbrid quadrupol-temps de volada: *HPLC-Q-ToF-MS*

Es va fer servir la cromatografia de líquids d'alta resolució acoblada a l'espectròmetre QSTAR Elite (ABSciex) per a les anàlisis metabolòmiques de tomàquets i els seus derivats. Aquest equip presenta l'avantatge dels analitzadors d'alta resolució de *ToF* amb un rang il·limitat de masses i la potencialitat de dur a terme experiments d'*MS/MS* i analitzar els ions fragment en alta resolució. Aquest espectròmetre està equipat amb una font d'*ESI* i opera amb nitrogen d'alta puresa com a gas de nebulització, gas auxiliar i gas cortina i a una temperatura de 400 °C. Es va treballar en mode negatiu.

Es van dur a terme diferents tipus d'experiments. En primer lloc, el *ToF-MS* de m/z 90 a 1100. Seguidament, es van dur a terme experiments d'*MS/MS*. Es va fer ús de

l'IDA (Information Dependent Acquisition), que permet l'adquisició automàtica dels espectres d'*MS* i *MS/MS* en una sola injecció cromatogràfica.

El cromatògraf de líquids és un Agilent 1200 RRLC (Agilent, Waldbronn, Alemanya) equipat amb una bomba quaternària, un termòstat automàtic de mostres, un forn, un injector automàtic i un *UV*. Es va fer servir una columna *C₁₈ Luna* de 50 mm de llarg, 2.0 mm de diàmetre i 5 µm de mida de partícula interna (Phenomenex, Torrance, CA, EUA). El gradient d'elució es va realitzar amb aigua Milli-Q (0.1% d'àcid fòrmic) (solvent A) i acetonitril (0.1% d'àcid fòrmic) (dissolvent B) amb un flux de 0.4 mL/min i el volum d'injecció va ser de 5 µL. Es va utilitzar un gradient d'augment lineal de dissolvent B. La separació es va dur a terme en 15 minuts en les següents condicions: 0 min, 5% B; 10 min, 18% B; 13 min, 100% B; 14 min, 100% B; 15 min, 5% B (Vallverdú-Queralt *et al.*, 2011e).



Figura 12. Espectròmetre de masses *QToF-MS* amb font d'*ESI*.

8. Anàlisis Estadístiques

Les anàlisis de variància permeten avaluar si existeixen diferències significatives ($P \leq 0.05$) entre mostres i tractaments aplicats. En alguns casos, es van determinar correlacions entre les variables estudiades mitjançant el test de Pearson. En d'altres casos, es va realitzar una anàlisi de components principals (*PCA*) per establir possibles relacions entre variables.

A través del programa informàtic Design Expert 7.0.1 (Stat Ease Inc., Minneapolis, USA) es van realitzar les superfícies de resposta i es van obtenir les

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equacions polinòmiques de segon ordre. Les variables numèriques independents van ser el nombre de polsos i la intensitat de camp. La funció de segon ordre es va predir mitjançant l'equació 1

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j \quad (1)$$

on Y és la variable independent β_0 és el punt central dels sistema β_i , β_{ii} i β_{ij} els coeficients de la regressió corresponent als efectes lineals, quadràtics i de les interaccions, respectivament; X_i , X_i^2 i $X_i X_j$, els efectes lineals quadràtics i de les interaccions de les variables independents.

Per a les anàlisis metabolòmiques, es van fer servir els programes Marker viewTM 1.2 (Applied Biosystems, MDS SCIEX, Toronto, Ontario, Canadà), Unscrambler 9.0 (CAMO A/S, Trondheim, Noruega) i l'Statgraphics Plus 5.1 (Statistical Graphics Co., Rockville, EUA).

RESULTATS



V. RESULTATS

En la següent secció, s'hi exposen els resultats obtinguts dels treballs experimentals realitzats en aquesta tesi doctoral. Aquests resultats s'han concretat en 12 publicacions, totes elles com a primera signant, en 10 revistes del primer quartil i 2 del segon, del *Science Citation Index*.

Davant de cada publicació, hi ha un resum en què es detallen els objectius, els resultats i les principals conclusions de cada estudi.

1. Capítol I: Caracterització dels polifenols del tomàquet mitjançant *HPLC-LTQ-Orbitrap-MS* i *HPLC-MS/MS*

Anna Vallverdú-Queralt, Olga Jáuregui, Alexander Medina-Remón, Cristina Andrés-Lacueva & Rosa M. Lamuela-Raventós. *Rapid Communications in Mass Spectrometry*. 2010, 24: 2986-2992.

Resum: Aquesta publicació presenta una identificació exhaustiva dels polifenols presents en el tomàquet mitjançant la combinació de dos equips: *HPLC-MS/MS* i *HPLC-LTQ-Orbitrap-MS*. Primer, es va fer una identificació amb *HPLC-MS/MS* i després es van injectar les mostres en l'equip d'alta resolució (*HPLC-LTQ-Orbitrap-MS*). Aquesta metodologia permet l'anàlítica d'*MS*, *MS/MS* i *MSⁿ*, la qual cosa facilita l'obtenció d'informació estructural. A causa de l'especificitat de les mesures d'*MSⁿ* i la capacitat d'eliminar les interferències a través de l'etapa inicial de selecció de masses, l'*LTQ-Orbitrap-MS* és un dels millors equips per a l'anàlítica qualitativa; facilita l'elucidació de l'estructura de compostos desconeguts. Consegüentment, es van identificar 38 compostos en mostres de tomàquets amb errors inferiors a 2 mDa, tres dels quals no havien estat descrits en tomàquets.

Improved characterization of tomato polyphenols using liquid chromatography/electrospray ionization linear ion trap quadrupole Orbitrap mass spectrometry and liquid chromatography/electrospray ionization tandem mass spectrometry

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Tomato (*Lycopersicon esculentum* Mill.) is the second most important fruit crop worldwide. Tomatoes are a key component in the Mediterranean diet, which is strongly associated with a reduced risk of chronic degenerative diseases. In this work, we use a combination of mass spectrometry (MS) techniques with negative ion detection, liquid chromatography/electrospray ionization linear ion trap quadrupole-Orbitrap-mass spectrometry (LC/ESI-LTQ-Orbitrap-MS) and liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) on a triple quadrupole, for the identification of the constituents of tomato samples. First, we tested for the presence of polyphenolic compounds through generic MS/MS experiments such as neutral loss and precursor ion scans on the triple quadrupole system. Confirmation of the compounds previously identified was accomplished by injection into the high-resolution system (LTQ-Orbitrap) using accurate mass measurements in MS, MS² and MS³ modes. In this way, 38 compounds were identified in tomato samples with very good mass accuracy (<2 mDa), three of them, as far as we know, not previously reported in tomato samples. Copyright © 2010 John Wiley & Sons, Ltd.

Tomatoes are the most frequently consumed vegetables in Spain (16.7 kg/year per person) and their consumption has increased rapidly in recent years (2.8% annually), with important economical consequences and potential health-promoting implications.¹

Tomato polyphenols are bioactive molecules distributed in plant species, influencing their morphology, growth and reproduction, as well as their resistance to parasites and environmental stress.² Phenolic compounds contribute to the antioxidant activities of tomatoes and tomato products, and play an important role in the benefits attributed to the consumption of these vegetables.

Identification of phenolic compounds is a complex task in food matrices compared to enriched supplements or drugs, due to the wide variety of structures present in nature and the lack of standard polyphenols commercially available. Several methods have been used for the determination of

polyphenols, such as capillary electrophoresis (CE),³ gas chromatography (GC),^{4,5} nuclear magnetic resonance (NMR),⁶ and, most commonly, high-performance liquid chromatography (HPLC) coupled to ultraviolet (UV)^{7,8} and mass spectrometry (MS).^{9–11}

Linear ion trap quadrupole-Orbitrap-mass spectrometry (LTQ-Orbitrap-MS) delivers single-stage mass analysis providing molecular mass information, two-stage mass analysis (MS/MS) and multi-stage mass analysis (MSⁿ) delivering structural information. Because of the specificity of MS/MS measurements and the ability to eliminate interferences through the initial mass selection stage, qualitative analysis of target compounds is accomplished using LTQ-Orbitrap-MS. Exact mass measurements and elemental composition assignment are essential for the characterization of small molecules. Accurate mass measurement of the product ions, formed in MSⁿ experiments, facilitates the elucidation of the structures of unknown compounds.

The objective of this work was to identify the maximum number of polyphenols in raw tomatoes. To identify the phenolic compounds, samples were analyzed after a suitable

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work-up extraction procedure using two complementary instruments: an LTQ-Orbitrap and a triple quadrupole mass spectrometer, to determine structures based on fragmentation patterns using several modalities (product ion scan, neutral loss scan and precursor ion scan). Thus, the combination of LTQ-Orbitrap and triple quadrupole enabled us to identify 38 polyphenols in tomato samples.

EXPERIMENTAL

Reagents

All samples and standards were handled without exposure to light. Gallic, caffeic, protocatechuic, ferulic and chlorogenic acids, quercetin and rutin were purchased from Sigma[®] (Madrid, Spain); naringenin, kaempferol-3-O-glucoside and kaempferol-3-O-rutinoside from Extrasynthèse (Genay, France); hydrochloric acid 35% from Panreac (Barcelona, Spain); and anhydrous sodium acetate (2 mol/L) from Merck (Darmstadt, Germany). Methanol, formic acid and glacial acetic acid HPLC grade were obtained from Scharlau (Barcelona, Spain) and water from a Milli-Q ultrapure water system (Millipore, Bedford, USA).

Extraction of polyphenols

Sample treatment was performed in a darkened room with a red safety light to avoid the oxidation of the analytes during the process following the procedure of Ami *et al.* with minor modifications.¹² Tomato fruits (0.5 g) were homogenized with a blender over an ice bed with 5 mL of 80% ethanol in Milli-Q water (0.1% formic acid). Then they were sonicated for 5 min and centrifugated at 4000 rpm for 20 min at 4°C. The supernatant was collected and the extraction procedure was repeated. Both supernatants were combined and evaporated under a nitrogen flow; finally, the residue was reconstituted with Milli-Q water (0.1% of formic acid) up to 4 mL.

Solid-phase extraction (SPE) of these extracts was carried out following the procedure of Medina-Remón *et al.*¹³ Firstly, 1 mL of methanol and subsequently 1 mL of sodium acetate 50 mmol/L pH 7 were loaded into Oasis[®] MAX cartridges from Waters to equilibrate it; then, 1 mL of each extract was diluted with 1 mL of Milli-Q water and acidified with 34 µL of 35% hydrochloric acid before being loaded into the cartridges separately. These were rinsed with sodium acetate 50 mmol/L pH 7 (5% methanol). The polyphenols were eluted with 1800 µL of methanol (2% formic acid). The eluted fractions were evaporated under a nitrogen flow, and the residue was reconstituted with water (0.1% formic acid) up to 250 µL and filtered through a 13 mm, 0.45 µm PTFE filter (Waters) into an insert-amber vial for HPLC analysis. Samples were stored at -20°C until analysis.

Apparatus and experimental conditions

For accurate mass measurements, an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source in negative mode was used to acquire mass spectra in profile mode with a setting of 30000 resolution at m/z 400. Operation parameters were as follows: source voltage, 4 kV; sheath gas, 20 (arbitrary units);

auxiliary gas, 10 (arbitrary units); sweep gas, 2 (arbitrary units); and capillary temperature, 275°C. Default values were used for most other acquisition parameters (Fourier transform (FT) Automatic gain control (AGC) target $5 \cdot 10^5$ for MS mode and $5 \cdot 10^4$ for MSⁿ mode). Tomato samples were first analyzed in full MS mode with the Orbitrap resolution set at 30 000 at m/z 400. The successive analyses were done in MSⁿ mode with the Orbitrap resolution also set at 30 000 at m/z 400. An isolation width of 100 amu was used and precursors were fragmented by high-energy C-trap dissociation (HCD) with normalized collision energy of 2% and an activation time of 10 ms. The maximum injection time was set to 100 ms with two micro scans for MS mode and to 1000 ms with one micro scan for MSⁿ mode. The mass range was from m/z 100 to 1000. The data analyses were performed using XCalibur software. The liquid chromatograph was an Accela (Thermo Scientific, Hemel Hempstead, UK) equipped with a quaternary pump, a photodiode array detector (PDA) and a thermostated autosampler. A Luna C₁₈ column (50 × 2.0 mm i.d., 5 µm; Phenomenex, Torrance, CA, USA) was used. Gradient elution was performed with water/0.1% formic acid (solvent A) and acetonitrile/0.1% formic acid (solvent B) at a constant flow rate of 0.4 mL/min, and injection volume was 5 µL. An increasing linear gradient of solvent B was used. Separation was carried out in 15 min under the following conditions: 0 min, 5% B; 10 min, 18% B; 13 min, 100% B; 14 min, 100% B; 15 min, 5% B. The column was equilibrated for 5 min prior to each analysis. These conditions were adapted from a previous study with some modifications.¹⁴

An API 3000 triple quadrupole mass spectrometer (ABSciex, Concord, Ontario, Canada) equipped with a Turbo Ionspray source in negative-ion mode was used to obtain MS/MS data. Turbo Ionspray source settings were as follows: capillary voltage, -3500 V; nebulizer gas (N₂), 10 (arbitrary units); curtain gas (N₂), 12 (arbitrary units); collision gas (N₂), 4 (arbitrary units); focusing potential, -200 V; entrance potential, 10 V; drying gas (N₂), heated to 400°C and introduced at a flow rate of 8000 mL/min. The declustering potential and collision energy were optimized for each compound in infusion experiments: individual standard solutions (10 µg/µL) dissolved in 50:50 (v/v) mobile phase were infused at a constant flow rate of 5 µL/min using a syringe pump (Harvard Apparatus, Holliston, MA, USA). Full-scan data acquisition was performed scanning from m/z 100 to 800 in profile mode and using a cycle time of 2 s with a step size of 0.1 u and a pause between each scan of 2 ms. To confirm the identity of some compounds, neutral loss scan and precursor ion scan experiments were carried out. MS/MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer and mass analyzed using the instrument's second analyzer. Additional experimental conditions for MS/MS included collision energy (depending on the compound), CAD gas (nitrogen) at 6 (arbitrary units), and scan range, as necessary for the precursor selected. Neutral loss scan of 162 u was done by scanning within the m/z range from 200 to 800 u, and precursor ion scan experiments were carried out by scanning Q1 between 300

and 800 u. In all the experiments, both quadrupoles (Q1 and Q3) were operated at unit resolution. The liquid chromatograph was an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, a thermostated autosampler and a column oven set to 30°C. The separations were conducted using the column and the gradient elution described above. The injection volume was 20 µL and the flow rate was 0.4 mL/min.

The MS/MS conditions used in this work were adapted from a previous work dealing with the identification of phenolic compounds in cocoa (*Theobroma cacao*).¹⁰

RESULTS AND DISCUSSION

In this work, first we tested for the presence of polyphenolic compounds through MS/MS experiments of neutral loss and precursor ion scan. The final confirmation was accomplished by injection into the high-resolution system (LTQ-Orbitrap) using accurate mass measurements. The elemental composition was selected according to the accurate masses and the isotopic pattern. Then, the elemental composition obtained was identified in the Dictionary of Natural Products¹⁵ and the MoTo database.¹⁶ The interpretation of the observed MS/MS spectra in comparison with those found in the literature was the main tool for putative identification of polyphenols. Table 1 shows the list of 38 compounds identified through triple quadrupole MS/MS and Orbitrap experiments, along with retention times (rt), accurate mass (acc. mass), molecular formula (MF), mDa of error between the mass found and the accurate mass of each polyphenol, the MS/MS ions used for the identification, and neutral loss (NL) and precursor ion (PI) experiment settings.

Hydroxycinnamic and benzoic acid derivatives

Examination of the chromatograms in full-scan mode in the triple quadrupole system revealed the presence of several polyphenols, some of them positively identified by comparison with available standards. The spectra generated for cinnamic and benzoic acids showed the deprotonated molecule $[M-H]^-$ and some fragments. The typical loss of CO₂ was observed for gallic, protocatechuic, caffeic and ferulic acids, giving $[M-H-44]^-$ as a characteristic ion; loss of a methyl group $[M-H-15]^-$ was observed for ferulic acid. These results were confirmed by injection into the high-resolution system (LTQ-Orbitrap) using accurate mass measurements with errors between 0.11 and 0.41 mDa.

Three caffeoylquinic acid derivatives (m/z 353) were first detected in the triple quadrupole system through neutral loss scan of 162 u. This experiment for caffeoylquinic acids confirmed the loss of a caffeic acid unit from neochlorogenic, chlorogenic and cryptochlorogenic acids. It was possible to differentiate the three isomers on relative intensities in MS² spectra using the triple quadrupole system according to the method cited by Clifford *et al.*¹⁷ Chlorogenic and neochlorogenic acids gave m/z 191 $[M-H-162]^-$ corresponding to quinic acid in the product ion spectra, whereas cryptochlorogenic acid showed m/z 173 $[quinic-H-H_2O]^-$ as a base peak in the product ion scan spectra. Chlorogenic and neochlorogenic acids could be differentiated by different

intensities of the fragment ion m/z 179, which was of 80% for neochlorogenic and less than 5% for chlorogenic acid. Only chlorogenic acid (5-caffeoylquinic acid) could be confirmed by comparing its spectrum and retention time with those of a standard. These results were confirmed by LTQ-Orbitrap experiments. The chromatograms in the FTMS mode confirmed the presence of the three caffeoylquinic acids with an error below 0.03 mDa showing the deprotonated molecule $[M-H]^-$ (m/z 353) and the ion $[M-H-162]^-$ (m/z 191) corresponding to the loss of a caffeic acid unit.

In addition, two dicaffeoylquinic acid isomers (m/z 515) were also identified at different retention times in the triple quadrupole system through neutral loss scan of 162 u. This experiment for dicaffeoylquinic acids showed peaks at m/z 353 and 515 which confirmed the losses of two caffeic acid units. The analysis in the LTQ-Orbitrap confirmed their presence with an error below 0.03 mDa showing the deprotonated molecule $[M-H]^-$ (m/z 515), the ion $[M-H-162]^-$ (m/z 353, corresponding to the loss of a caffeic acid unit) and the deprotonated quinic acid (m/z 191).

Moreover, four caffeic acid-O-hexosides (m/z 341) were first detected in the triple quadrupole system through neutral loss scan of 162 u and precursor scan experiments of m/z 179. The neutral loss scan gave the deprotonated molecule at m/z 341 and the precursor ion scan gave an extracted ion chromatogram showing four caffeic acid-O-hexosides. The precursor ion spectra for these compounds showed the $[M-H]^-$ ion at m/z 341. The analysis in the LTQ-Orbitrap confirmed the presence of these caffeic acid-O-hexosides with an error below 0.12 mDa. The FT mass spectrum showed ions corresponding to the deprotonated molecule $[M-H]^-$, the loss of a hexose $[M-H-162]^-$ and the loss of the acid group as characteristic fragment ions.

Homovanillic acid-O-hexoside (m/z 343) and ferulic acid-O-hexoside (m/z 355) were also identified on the triple quadrupole system as revealed by neutral loss scan of 162 u. This experiment gave the deprotonated molecule at m/z 343 for homovanillic acid-O-hexoside and at m/z 355 for ferulic acid-O-hexoside. The analysis in the LTQ-Orbitrap confirmed the presence of these compounds with errors below 0.07 and 0.04 mDa, respectively.

The higher sensitivity of the LTQ-Orbitrap system compared to the triple quadrupole system enabled the identification of low-intensity signals obtained in the triple quadrupole corresponding to coumaroylquinic acid (m/z 337) and coumaric acid-O-hexoside (m/z 325) with errors below 0.95 mDa. These compounds could not be detected using the triple quadrupole system due to the lack of sensitivity in full-scan mode. MS² of m/z 337 revealed peaks at m/z 191 and 163 corresponding to the deprotonated quinic acid unit $[M-H-146]^-$ and coumaric acid $[M-H-174]^-$, respectively, with errors below 0.04 mDa. MS² of m/z 325 showed a peak at m/z 163 corresponding to the cleavage of a sugar moiety with an error of 0.22 mDa. Moreover, another peak was identified in the FT mass spectrum corresponding to feruoylquinic acid-O-hexoside (m/z 529) confirmed by the exact mass measurements with an error of 0.21 mDa. MS² of this ion showed peaks at m/z 367 and 191 corresponding to the loss of a hexose $[M-H-162]^-$ and the deprotonated quinic acid, with errors of 1.49 and 0.47 mDa, respectively.

Table 1. List of compounds identified in tomato samples

Compound	rt (min)	[M-H] ⁻	MS/MS ions (% intensities)	NL	PI	Acc. mass	mDa	MF
Gallic acid ^a	0.97	169	169 (55), 125 (100)			169.0142	0.28	C ₇ H ₅ O ₅
Protocatechuic acid ^a	2.08	153	153 (30), 109 (100)			153.0193	0.11	C ₇ H ₅ O ₄
Caffeic acid-O-hexoside 1	2.98	341	341 (25), 179 (100), 135 (10)	162	179	341.0877	0.12	C ₁₈ H ₁₉ O ₆
Caffeic acid-O-hexoside 2	3.29	341	341 (25), 179 (100), 135 (10)	162	179	341.0877	0.10	C ₁₈ H ₁₉ O ₆
Neochlorogenic acid (3-caffeoylquinic acid)	3.36	353	191 (100), 179 (80), 135 (43)	162		353.0877	0.02	C ₁₉ H ₁₉ O ₇
Caffeic acid-O-hexoside 3	4.39	341	341 (25), 179 (100), 135 (10)	162	179	341.0877	0.09	C ₁₈ H ₁₉ O ₆
Homovanillic acid-O-hexoside	4.97	343	181 (100), 137 (20), 121 (5)	162		343.1034	0.07	C ₁₉ H ₁₉ O ₆
Feruloylquinic acid-O-hexoside	5.56	529	529 (100), 367 (25), 191 (10)			529.1561	0.21	C ₂₃ H ₂₅ O ₈
Ferulic acid-O-hexoside	5.78	355	355 (40), 193 (100), 178 (30)	162		355.1034	0.04	C ₁₉ H ₁₉ O ₆
Caffeic acid-O-hexoside 4	5.80	341	341 (25), 179 (100), 135 (10)	162	179	341.0877	0.12	C ₁₈ H ₁₉ O ₆
Coumaric acid-O-hexoside	6.41	325	325 (70), 187 (40), 163 (100)			325.0928	0.95	C ₁₈ H ₁₉ O ₆
Caffeic acid ^a	6.85	179	179 (40), 135 (100)			179.0349	0.34	C ₇ H ₅ O ₄
Chlorogenic acid (5-caffeoylquinic acid) ^a	7.15	353	191 (100), 179 (<5)	162		353.0877	0.03	C ₁₉ H ₁₉ O ₇
Cryptochlorogenic acid (4-caffeoylquinic acid)	8.28	353	191 (40), 179 (70), 173 (100), 135 (25)	162		353.0877	0.02	C ₁₉ H ₁₉ O ₇
Naringenin-O-dihexoside 1	8.53	595	595 (20), 433 (100), 271 (30)	162		595.1665	0.33	C ₂₇ H ₃₁ O ₁₅
Rutin-O-hexoside-pentoxide	8.64	903 ^b	741 (100), 609 (70), 300 (100) ^a			903.2411	0.58	C ₃₄ H ₄₁ O ₁₅
Naringenin-O-dihexoside 2	8.89	595	595 (20), 433 (100), 271 (30)	162		595.1665	0.30	C ₂₇ H ₃₁ O ₁₅
Rutin-O-hexoside	8.97	771	771 (100), 609 (50)		301	771.1989	0.42	C ₃₁ H ₃₅ O ₁₅
Naringenin-C-diglycoside	9.01	595	595 (100), 475 (30), 385 (45), 355 (30)			595.1667	0.13	C ₂₇ H ₃₁ O ₁₅
Coumaroylquinic acid	9.28	337	191 (100), 163 (10)			337.0930	0.13	C ₁₆ H ₁₇ O ₅
Ferulic acid ^a	9.56	193	193 (20), 178 (70), 149 (20), 134 (100)			193.0506	0.41	C ₁₆ H ₁₇ O ₅
Kaempferol-O-rutinoside-hexoside	9.72	755 ^b	593 (100), 285 (5) ^b			755.2039	0.40	C ₃₁ H ₃₅ O ₁₅
Naringenin-O-hexoside 1	10.11	433	433 (45), 271 (100)	162		433.1140	0.10	C ₁₈ H ₁₉ O ₆
Floridizin-C-diglycoside	10.37	759	741 (20), 609 (30), 639 (80), 621 (20), 579 (60), 549 (100), 519 (65)			759.2346	0.70	C ₃₁ H ₃₅ O ₁₅
Naringenin-O-hexoside 2	10.57	433	433 (45), 271 (100)	162		433.1140	0.08	C ₁₈ H ₁₉ O ₆
Rutin-O-pentoxide	10.93	741	609 (60), 300 (100)	132		741.1883	0.85	C ₃₁ H ₃₅ O ₁₅
Rutin/queretin 3-O-rutinosyl-glucoside ^a	11.16	609	609 (100), 300 (50)		301	609.1460	0.49	C ₂₇ H ₃₁ O ₁₅
Phloretin-C-diglycoside	11.25	597	597 (30), 477 (50), 417 (40), 387 (90), 357 (100)			597.1819	0.10	C ₂₇ H ₃₁ O ₁₅
Kaempferol-3-O-rutinoside ^a	11.35	593	593 (100), 285 (80)			593.1511	0.58	C ₂₇ H ₃₁ O ₁₅
Prunin (Naringenin-7-O-glucoside) ^a	11.45	433	433 (45), 271 (100)	162		433.1140	0.10	C ₁₈ H ₁₉ O ₆
Eriodictyol-O-hexoside	11.50	449	449 (20), 287 (100), 151 (30)	162		449.1089	0.12	C ₂₁ H ₂₃ O ₇
Dicaffeoylquinic acid 1	11.52	515	515 (65), 353 (50), 191 (100)	162		515.1194	0.03	C ₂₃ H ₂₅ O ₈
Dicaffeoylquinic acid 2	11.62	515	515 (65), 353 (50), 191 (100)	162		515.1194	0.02	C ₂₃ H ₂₅ O ₈
Naringenin-O-hexoside 3	11.64	433	433 (45), 271 (100)	162		433.1140	0.09	C ₁₈ H ₁₉ O ₆
O-acetylprunin	11.68	475	433 (15), 271 (100)			475.1245	0.07	C ₂₃ H ₂₅ O ₈
Kaempferol-3-O-glucoside ^a	11.76	447	447 (100), 285 (20)	162		447.0932	0.14	C ₂₁ H ₂₃ O ₇
Naringenin ^a	12.07	271	271 (100), 177 (20), 151 (100), 119 (30)			271.0611	0.03	C ₁₅ H ₁₅ O ₃
Quercetin ^a	13.63	301	301 (60), 151 (100)			301.0353	0.15	C ₁₅ H ₁₁ O ₇

^a Comparison with standard.

rt, retention time; NL, neutral loss; PI, precursor ion scan; Acc. mass, accurate mass; MF, molecular formula; mDa, millidaltons of error between the mass found and the accurate mass of each polyphenol.

^b MS² of 903 and MS³ of 741.^c MS² of 755 and MS³ of 593.

Flavonol derivatives

Examination of the chromatograms obtained in full-scan mode in the triple quadrupole system revealed the presence of some peaks at m/z 609, 593, 447 and 301 corresponding to rutin, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside and quercetin, respectively. These results were confirmed by injection into the high-resolution system (LTQ-Orbitrap) using accurate mass measurements with errors below 0.58 mDa. Flavonol-*O*-dihexosides such as rutin and kaempferol-3-*O*-rutinoside were identified by comparing their retention times with those of reference substances. The FT mass spectra of these compounds showed ions corresponding to the deprotonated molecule $[M-H]^-$ and the loss of the rutinoside unit, $[M-H-308]^-$. Flavonol aglycones such as quercetin gave as a characteristic ion the deprotonated molecule $[M-H]^-$ and ions corresponding to retro-Diels-Alder (RDA) fragmentation in the C-ring involving 1,3-scission as described by other authors.^{18,19}

In addition, rutin-*O*-hexoside (m/z 771) and rutin-*O*-pentoside (m/z 741) were identified in the triple quadrupole system through experiments of neutral loss scans of 162 and 132 u and a precursor ion scan of m/z 301. Neutral loss scan experiments of 162 u gave the deprotonated molecule at m/z 771 corresponding to rutin-*O*-hexoside, and neutral loss scan of 132 u gave the deprotonated molecule at m/z 741, corresponding to rutin-*O*-pentoside. A precursor ion scan experiment of m/z 301 was carried out in order to investigate the presence of three quercetin derivatives. The results of this experiment showed the presence of three compounds with m/z 609, 741 and 771. The analysis in the LTQ-Orbitrap confirmed the presence of rutin-*O*-hexoside and rutin-*O*-pentoside with errors below 0.85 mDa. The MS² mass spectrum of m/z 771 showed peaks at m/z 609 and 300 corresponding to the loss of a hexoside $[M-H-162]^-$ and to the radical anion of the aglycone (m/z 300). This high difference (>0.1 Da) in respect of the m/z of the deprotonated quercetin (m/z 301) could be explained by the formation of a radical anion $[M-H]^-$, as described previously by Hvattum and Ekeberg²⁰ using a triple quadrupole mass spectrometer. Lastly, the MS² mass spectrum of m/z 741 was assessed, showing peaks at m/z 609 and 300 corresponding to the loss of a pentoside $[M-H-132]^-$ and to the radical anion of the aglycone (m/z 300) with errors below 2 mDa.

As mentioned above, the use of the LTQ-Orbitrap was crucial for the structural determination of unknown compounds that could not be identified in the triple quadrupole system, probably due to their low concentration. Ions at m/z 755 and 903 have been identified in the FTMS experiment with errors below 0.58 mDa. The MS² mass spectrum of m/z 755 showed a fragment ion at m/z 593 with an error of 2.24 mDa corresponding to the loss of a hexose unit $[M-H-162]^-$. Thus, MS³ of m/z 593 revealed the loss of a rutinoside unit with an error of 0.87 mDa. Therefore, this compound was tentatively identified as kaempferol-*O*-rutinoside-hexoside. The MS² mass spectrum of m/z 903 showed a fragment ion at m/z 741 with an error below 2 mDa corresponding to the loss of a hexose unit $[M-H-162]^-$. Thus, MS³ of m/z 741 showed peaks at m/z 609 and 300 corresponding to the loss of a pentoside moiety $[M-H-132]^-$ and to the radical anion of the aglycone (m/z 300) with errors

below 2 mDa. No characteristic ions of the sugar part were observed in the negative-ion mode. Thus, this compound was tentatively identified as rutin-*O*-hexoside-pentoside.

As far as we know, rutin-*O*-hexoside and rutin-*O*-pentoside have been found in tomato samples,²¹ whereas this is the first time that rutin-*O*-hexoside-pentoside has been reported in tomato fruit. Rutin and rutin glycosylated derivatives are valuable phytochemicals consistently associated with anti-oxidant and anticarcinogenic activities, and a relatively longer circulation time *in vivo*.^{22,23}

Flavanone derivatives

The chromatograms in full-scan mode obtained in the triple quadrupole system revealed the presence of naringenin (m/z 271). Naringenin has been identified as one of the major phenolics in tomatoes and tomato by-products. Naringenin gave as a characteristic ion the deprotonated molecule $[M-H]^-$ and the ions corresponding to RDA fragmentation in the C-ring involving 1,3-scission (m/z 151), as mentioned above for quercetin (m/z 301). Naringenin was identified by comparing its retention time with that of a reference substance. The analysis in the LTQ-Orbitrap confirmed the presence of naringenin with an error of 0.03 mDa.

Four naringenin-*O*-hexosides (m/z 433) and two naringenin-*O*-dihexosides (m/z 595) were also identified in the triple quadrupole system as revealed by a neutral loss scan of 162 u. This scan gave ions corresponding to m/z 433 for monohexosides and m/z 433 and 595 for dihexosides. These results were confirmed by injection into the high-resolution system (LTQ-Orbitrap) using accurate mass measurements with errors below 0.33 mDa. The MS² mass spectrum of m/z 433 showed an ion at m/z 271 corresponding to the loss of a hexoside moiety $[M-H-162]^-$ and the MS² mass spectrum of m/z 595 showed ions at m/z 433 and 271, corresponding to the loss of one hexoside unit $[M-H-162]^-$ and two hexoside units $[M-H-162-162]^-$, respectively.

Naringenin-*C*-diglycoside was also present in tomato fruit. Naringenin-*C*-diglycoside (m/z 595) was detected in the triple quadrupole system and confirmed by injection into the high-resolution system (LTQ-Orbitrap) using accurate mass measurements with an error of 0.13 mDa. As pointed out by several authors, *C*-glycosides show a fragmentation pattern different from those of *O*-glycosides. The spectra of *C*-glycosides displayed losses of 120 and 90 u corresponding to cross-ring cleavages in the sugar unit as reported previously in fennel (*Foeniculum vulgare*) and cocoa (*Theobroma cacao*) using liquid chromatography/tandem mass spectrometry.^{10,24} The MS² spectrum of m/z 595 showed the characteristic losses of 90 and 120 u from m/z 595 and 475, respectively, which confirmed the presence of two hexose units.

LTQ-Orbitrap-MS enabled us to identify low-intensity signals corresponding to eriodictiol-*O*-hexoside (m/z 449) and *O*-acetyl-prunin (m/z 475) with errors below 0.12 mDa. These compounds have not been detected using the triple quadrupole system. The MS² mass spectrum of m/z 449 gave a product ion corresponding to the loss of a hexoside moiety and that characteristic for RDA fragmentation of aglycones.

Resultats

RCM

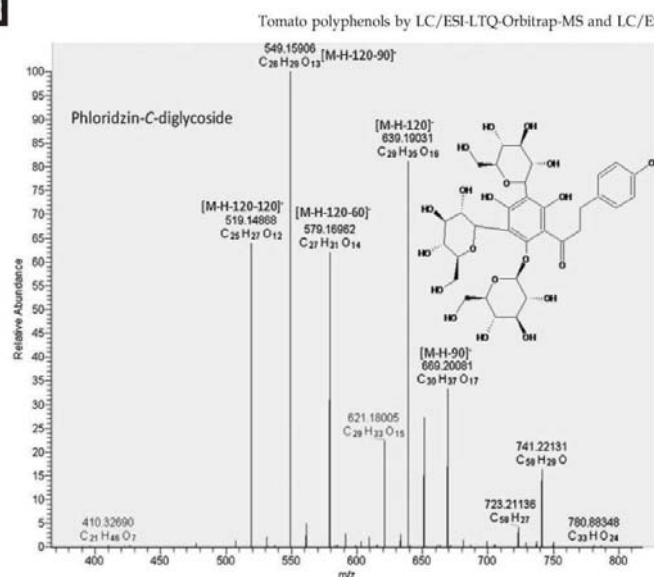


Figure 1. Identification of phloridzin-C-diglycoside in tomato samples. The MS² product ion scan of m/z 759 shows the characteristic fragment ions of a C-diglycoside polyphenol.

The MS² mass spectrum of m/z 475 exhibited fragments at m/z 433 and 271, corresponding to the loss of an acetyl group $[M-H-42]^-$ and the naringenin aglycone, respectively. Thus, this polyphenol was assigned as *O*-acetyl-prunin and, to the best of our knowledge, has never been reported in tomato samples until now.

Dihydrochalcone derivatives

Phloretin-C-diglycoside (m/z 597) was detected in the triple quadrupole system and confirmed by injection into the high-resolution system (LTQ-Orbitrap) using accurate mass measurements with an error of 0.10 mDa. Phloridzin-C-diglycoside (m/z 759) could only be identified in the LTQ-Orbitrap due to the lack of sensitivity of the triple quadrupole, probably due to its low concentration, with an error of 0.70 mDa. Figure 1 shows the MS² spectrum of m/z 759 of phloridzin-C-diglycoside displaying the losses of 90 and 120 u from m/z 759 and 639, respectively, which confirmed the presence of two hexose units. Moreover, in the product ion spectra of m/z 759, loss of H₂O was observed showing an ion at m/z 741, which displayed a loss of 120 u (m/z 621). Phloretin-C-diglycoside followed the same pattern, showing losses of 90 and 120 u as characteristic fragment ions in the MS/MS mode. Using the LTQ-Orbitrap in MS and MS² modes, the comprehensive fragmentation pathways of dihydrochalcone-C-glycosides were unambiguously determined with errors below 0.70 mDa. As far as we know, phloridzin-C-diglycoside is reported here in tomato samples

for the first time, whereas phloretin-C-diglycoside has been previously described in tomato fruit by Slimestad *et al.*⁶

CONCLUSIONS

In conclusion, the experiments led to new results in the field of tomato fruit. We applied a combination of techniques, LC-ESI-LTQ-Orbitrap-MS and LC-ESI-MS-MS, to accurately identify 38 phenolics of tomato samples, three of them, *O*-acetylprunin, phloridzin-C-diglycoside and rutin-*O*-hexoside-pentoside, as far as we know, reported for the first time. LC-ESI-LTQ-Orbitrap-MS allowed unambiguous assignment of all fragment ions with fewer experiments and easier interpretation than previous methods. Compared to the experiments with the triple quadrupole, which consumed a lot of instrument time and also a high volume of samples, LC-ESI-LTQ-Orbitrap-MS gave more sensitive and better-resolved FT mass spectra, which facilitated the identification of tomato constituents. The use of the LTQ-Orbitrap was crucial for the structural determination of coumaric acid-*O*-hexoside, feruloylquinic acid-*O*-hexoside, coumaroylquinic acid, rutin-*O*-hexoside-pentoside, *O*-acetylprunin and phloridzin-C-diglycoside, which could not be identified in the triple quadrupole system probably due to their low concentration. Using LC/ESI-LTQ-Orbitrap-MS very good mass accuracies were obtained for all molecular ions with the LTQ-Orbitrap instrument. In general, the differences between calculated and measured m/z values were below 2 mDa.

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2. Capítol II: Perfil fenòlic i capacitat antioxidant hidrofílica com a marcadors de diferents varietats de tomàquet

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Resum: Aquesta publicació pretén presentar el perfil fenòlic i la capacitat antioxidant hidrofílica com a marcadors de diferents varietats de tomàquet que, fins a l'actualitat, no havien estat estudiats. Les varietats procedien de l'empresa CONESA de Vegas Bajas del Guadiana, Badajoz, Espanya. Les anàlisis es van dur a terme durant dues collites consecutives, 2008 i 2009. Totes les varietats presentaven el mateix grau de maduresa, mida i no diferien en el sistema de regadiu emprat. Es va utilitzar el mètode d'anàlisi de polifenols totals, amb prèvia neteja mitjançant *SPE*, l'assaig de capacitat antioxidant hidrofílica i l'anàlisi de polifenols individuals mitjançant *HPLC-MS/MS*. Es pot concloure que el perfil fenòlic i la capacitat antioxidant hidrofílica són els responsables de la diferenciació entre varietats.

Phenolic Profile and Hydrophilic Antioxidant Capacity
as Chemotaxonomic Markers of Tomato VarietiesAnna Vallverdú-Queralt,^{†,‡} Alexander Medina-Remón,^{†,‡} Miriam Martínez-Huélamo,[†] Olga Jáuregui,[§]
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ABSTRACT: Tomatoes (*Solanum lycopersicum* L.), the second most important vegetable crop worldwide, are a key component in the so-called "Mediterranean diet", which is strongly associated with a reduced risk of chronic degenerative diseases. In this work, we evaluate the differences in the total and individual polyphenol content and hydrophilic antioxidant capacity of seven varieties of tomato cultivated in Vegas Bajas del Guadiana, Badajoz (Spain), which were collected from two consecutive harvests (2008–2009). Hydrophilic antioxidant capacity was evaluated using the TEAC assay, while the Folin–Ciocalteu assay with a previous cleanup was used to establish total polyphenol content. The method was optimized and validated. Individual polyphenols were quantified using liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) on a triple quadrupole. All compounds were found to be significantly different when analysis of variance was performed. Results from the principal component analysis show that phenolic compounds and hydrophilic antioxidant capacity were responsible for the differences among tomato samples according to variety.

KEYWORDS: Tomato varieties, chemotaxonomic markers, hydrophilic antioxidant capacity, total polyphenols, LC-MS-MS, principal component analysis

■ INTRODUCTION

Epidemiological studies have shown that consumption of raw tomato and its tomato-based products (ketchup, tomato sauce, and tomato juice) is associated with a decrease in chronic degenerative diseases.¹ In addition to its micronutrient content, such as minerals (potassium), vitamin C, vitamin E, and folate,² tomato contains some valuable bioactive components, including antioxidants. The main tomato antioxidants are carotenoids, principally lycopene, which is largely responsible for the red color of the fruit. A number of epidemiologic studies have associated lycopene with a lower risk of prostate cancer.³ In addition to carotenoids, other functional compounds such as phenolics also contribute to the beneficial effects of tomato products. Recently, the high content of phenolic compounds such as flavonoids and hydroxycinnamic acids in tomato has been gaining interest because of their apparent multiple biological effects, including free-radical scavenging, metal chelation, inhibition of cellular proliferation, and modulation of enzymatic activity and signal transduction pathways.⁴ The presence of antioxidants in tomatoes has been the object of several studies^{5–7} on the positive physiological properties attributed to these compounds. However, to our knowledge, no study has used the phenolic profile and hydrophilic antioxidant capacity as a varietal marker of tomatoes.

One of the important features of phenolic compounds is their usage as taxonomical markers.^{8–12} Singleton et al.⁸ showed that the patterns of phenolic substances in Chenin blanc, French Colombard, Semillon, and Thompson wines are influenced by the genotypes of the grapevine. The polyphenolic profile determined

by HPLC revealed a similarity within a grape variety and differences between varieties. Similarly, varieties of white musts,¹¹ wines,^{10,13} and sparkling wines¹² have been shown to have different phenolic profiles.

Moreover, Russo et al.⁹ studied the relationship between chemical composition and biotypes of 24 steam-distilled samples of essential oils from inflorescences of *Origanum vulgare* ssp. *hirtum*. Four chemotypes were identified on the basis of the phenolic content, i.e., thymol, carvacrol, thymol/carvacrol, and carvacrol/thymol chemotypes. Another investigation was carried out into the composition and variability of the essential oils contained in 63 individual plants taken from 21 populations of *Thymus zygis* L. in southern Spain.¹⁴ A chemometric investigation of the infraspecific variability of the essential oils of these populations led to the distinction of seven main chemotypes: thymol, carvacrol, linalool, α -terpinyl acetate, thymol/*p*-cymene/*y*-terpinene, 1,8-cineole/myrcene/spathulenol, and 1,8-cineole/ α -terpineol. The phenolic compound thymol was the most common constituent in the majority of the samples studied.

An attempt was made, by means of principal component analysis (PCA) and linear discriminant analysis (LDA), to identify those parameters that could be useful to classify clones. Phenolic compounds such as anthocyanins, flavonols, and hydroxycinnamates

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were used as taxonomical markers to discriminate *Vitis vinifera* L. cv 'Barbera' clones.¹⁵

The distribution and expression of phenolic constituents and hydrophilic antioxidant capacity can differ significantly in tomato varieties. Our initial purpose was to evaluate total polyphenols (TP), hydrophilic antioxidant capacity, and the levels of flavonols (kaempferol-3-O-glucoside, rutin, and quercetin), flavanones (naringenin), hydroxycinnamic acids (chlorogenic, caffeic, caffeic-O-hexoside, and ferulic acids) and phenolic acids (protocatechuic and gallic acids) in seven varieties of tomato by liquid chromatography coupled to mass spectrometry in tandem mode (LC-ESI-MS/MS). We observed a different characteristic phenolic profile for each variety and, surprisingly, found that tomatoes could be grouped within varieties by their polyphenol content and hydrophilic antioxidant activity. In this article, 7 different varieties of tomato (H-9661, H-9997, H-9776, Albastro, Guadiva, Elegy, and Malva) harvested over two consecutive years, 2008 and 2009, were identified and classified by their phenolic profile and hydrophilic antioxidant activity. The analytical variables were subjected to analysis of variance (ANOVA) and principal component analysis (PCA).

MATERIALS AND METHODS

Standards and Reagents. All samples and standards were handled without exposure to light. Quercetin, rutin, caffeic, gallic, ferulic, protocatechuic and chlorogenic acids, and Folin–Ciocalteu (F–C) reagent, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), PBS (phosphate-buffered saline pH 7.4), Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid 97%), and manganese dioxide were purchased from Sigma (Madrid, Spain); naringenin and kaempferol-3-O-glucoside from Extrasintese (Genay, France); hydrochloric acid 35% and acetic acid 99.8% from Panreac (Barcelona, Spain); and anhydrous sodium acetate (2M) from Merck (Darmstadt, Germany). Methanol and formic acid were obtained from Scharlau (Barcelona, Spain) and ultrapure water (Milli-Q) from Millipore System (Bedford, USA).

Sampling and Processing Conditions. Tomato varieties (Malva, H-9661, H-9776, H-9997, Albastro, Guadiva, and Elegy) were cultivated in Vegas Bajas del Guadiana, Badajoz (Spain) over two consecutive years, 2008 and 2009. Each variety was independently sampled three times. All varieties were harvested at the same degree of ripeness (4.3–5°Brix) and were of normal size (75–80 g) to accurately compare polyphenol levels and hydrophilic antioxidant capacity.

The technique of crop management was transplantation with a root ball, with small plants grown in a greenhouse for a period of approximately 40–45 days. Planting was done on beds 1.5 m wide. The period for transplantation was between the first days of April and late May, and the cycles of these varieties ranged between 100 and 125 days. The mean maximum temperature ranged from 25 to 30 °C, and the variations in mean minimum temperature were 15–20 °C for the two years.

As a method of fertilization, fertigation (application of fertilizers, soil amendments, or other water-soluble products through an irrigation system) was applied.

Extraction of Hydrophilic Compounds. First, tomatoes were washed, sorted, and collected in a sterilized recipient. Tomato fruits were then beaten and homogenized over an ice bed; 0.5 g was weighed and homogenized with 4 mL of 80% ethanol in Milli-Q water; and they were sonicated for 5 min and centrifuged (4000 rpm at 4 °C) for 20 min. The supernatant was transferred into a flask, and extraction was repeated. Both supernatants were combined and evaporated under nitrogen flow; finally, the residue was reconstituted with Milli-Q water (0.1% of formic acid) up to 4 mL. Samples were frozen at –20 °C until analysis.

For the solid phase extraction (SPE) cleanup, Oasis MAX cartridges with 30 mg of mixed-mode anion-exchange and reversed-phase solvent from Waters (Milford, USA) were equilibrated with 1 mL of methanol 100% and 1 mL of 50 mM sodium acetate at pH 7, 1 mL of Milli-Q water, and 34 µL of hydrochloric acid at 35% were added to 1 mL of nitrogen evaporation extract before being loaded into the cartridges separately. These were rinsed with 50 mM sodium acetate at pH 7 (5% methanol), and finally, the polyphenols were eluted with 1800 µL of methanol (2% formic acid). The eluted fractions were evaporated under nitrogen, and the residue was reconstituted with water (0.1% formic acid) up to 250 µL and filtered through a 13 mm, 0.45 µm PTFE filter (Waters).

Analysis of Total Polyphenols. For the TP assay, each sample was analyzed in triplicate; 20 µL of the eluted fractions was mixed with 188 µL of Milli-Q water in a thermo microtiter 96-well plate (nunc, Roskilde, Denmark), 12 µL of F–C reagent and 30 µL of sodium carbonate (200 g/L) were added following the procedure described by Medina-Remón et al.¹⁶ The mixtures were incubated for 1 h at room temperature in the dark. After the reaction period, 50 µL of Milli-Q water was added, and the absorbance was measured at 765 nm in a UV/vis Thermo Multiskan Spectrum spectrophotometer (Vantaa, Finland). This spectrophotometer allowed the absorbance of a 96-well plate to be read in 10 s. Results were expressed as mg of gallic acid equivalents (GAE)/100 g fresh weight (FW).

Folin–Ciocalteu Assay validation. The method was validated with gallic acid. To evaluate the linearity, standards between 0.25 and 10 mg/L were studied. The sensitivity was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ). These were calculated by measuring the analytical background response by reading 10 blanks at the maximum sensitivity, and the standard deviation (SD) was calculated. The LOD was defined as 3 times the SD of the 10 blanks, whereas the LOQ was 10 times the SD. The intra- and interday accuracy and precision were determined by spiking the matrix with known levels of gallic acid at seven different concentrations. The precision was calculated as the relative standard deviation (RSD). The assay was validated according to the recommendations of AOAC International.¹⁷

The short-term temperature stability was evaluated by freezing three aliquots of each of the seven concentrations between 0.25 and 10 mg/L of gallic acid in Milli-Q water at –20 and –80 °C. The aliquots were thawed at room temperature for 6 h (the mean sample preparation time) and then analyzed. To evaluate the stability after successive freeze–thaw cycles, three aliquots of each concentration were stored at –20 and –80 °C for 24 h and then thawed at room temperature up to four times over a one-week period. Gallic acid was assessed in each aliquot in a single run at the end of the last freeze–thaw cycle. Aliquots of each concentration for long-term stability were prepared and immediately frozen at –20 and –80 °C until analysis (within 2 years). The concentrations of all the stability samples were compared with the mean back-calculated values for the standards at each concentration from the first day of long-term stability testing. The same procedures were followed for sample stability. We used HPLC-UV to calculate the recovery of seven representative polyphenols with different polarities after spiking the tomato samples with 5, 10, 25, and 50 mg/L of each standard.

We tested the possible interferences from vitamin C (ascorbic acid at 50 mg/L and 200 mg/L), sugar (glucose at 2 mg/L), iron (Fe(II) at 1 mg/L), and amino acids (phenylalanine, tyrosine, glutamine, and arginine at 1 mg/L) according to Roura et al.¹⁸

Hydrophilic Antioxidant Capacity. The antioxidant activity in the tomato samples was measured using ABTS^{•+} radical decolorization assay.^{19,20} The ABTS^{•+} radical cation was prepared by passing a 5 mM aqueous stock solution of ABTS (in PBS) through manganese dioxide powder. The manganese dioxide excess was removed by filtering the solution through a 13 mm, 0.45 µm PTFE filter (Waters). Prior to the assay, the solution was diluted in PBS at pH 7.4 to give an absorbance at

Table 1. Intra- and Interday Accuracy and Precision of the Assay

calibrator concentration (mg L ⁻¹)		means measured concentration (mg L ⁻¹)		precision, RSD ^a (%)		recovery, error (%)	
intraday	interday	intraday	interday	intraday	interday	intraday	interday
1.12	1.07	1.01	0.98	0.10	0.12	90.57	91.97
2.24	2.14	2.24	2.06	0.10	0.11	100.08	96.25
4.48	4.27	4.38	4.25	0.09	0.09	97.77	99.60
6.73	6.41	6.69	6.40	0.09	0.10	99.38	99.87
8.97	8.55	8.93	8.21	0.06	0.08	99.58	96.00
11.21	10.69	11.62	11.03	0.05	0.07	103.67	103.21
13.40	13.81	13.89	14.51	0.04	0.05	103.66	105.10

^aRSD: relative standard deviation.

734 nm of 1.0 ± 0.01 and preincubated in ice. The ABTS⁺ radical cation solution was prepared daily.

One millimolar Trolox in PBS at pH 7.4 was used as an antioxidant standard for the ABTS assay. Working standards were prepared each day by diluting 1 mM Trolox with PBS at pH 7.4.

Then, 245 μ L of ABTS⁺ solution was added to 5 μ L of Trolox or to the tomato samples, and the solutions were stirred for 30 s. The absorbance was recorded continuously every 30 s with a UV/vis Thermo Multiskan Spectrum spectrophotometer for 1 h. PBS blanks were run in each assay.

The working range for Trolox (final concentration 0–750 μ M) was based on triplicate determinations and consisted of plotting the absorbance as a percentage of the absorbance of the uninhibited radical cation (blank). The activities of the tomato samples were assessed at four different concentrations, which were within the range of the dose–response curve. Each sample was analyzed in triplicate at these four concentrations. Results of Trolox equivalent antioxidant capacity (TEAC) were expressed as (mmol Trolox equivalent (TE)/100 g FW).

LC-MS/MS Analysis. Tomato fruit polyphenols were identified in a previous study using LC-ESI-LTQ-Orbitrap-MS.²¹ We selected the most predominant polyphenols of these tomatoes to evaluate the differences between the seven varieties of tomato. These polyphenols were quantified using LC-ESI-MS/MS. An API 3000 (PE Sciex, Concord, Ontario, Canada) triple quadrupole mass spectrometer equipped with a Turbo IonSpray source in negative-ion mode was used to obtain MS/MS data. Turbo IonSpray source settings were as follows: capillary voltage, –3500 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 4 (arbitrary units); focusing potential, –200 V; entrance potential, –10 V; and drying gas (N_2), heated to 400 °C and introduced to a flow rate of 8000 cm³/min. The declustering potential and collision energy were optimized for each compound in infusion experiments: the individual standard solution (10 μ g/mL) dissolved in 50:50 (v/v) mobile phase was infused at a constant flow rate of 5 μ L/min using a model syringe pump (Harvard Apparatus, Holliston, MA, USA).

For quantification purposes, data was collected in the multiple reaction monitoring (MRM) mode, tracking the transition of the specific parent and product ions for each compound. In particular, we selected 10 transitions corresponding to the most abundant polyphenols in tomato fruit: ferulic acid m/z 193→134 (CE: –20 V); chlorogenic acid m/z 353→191 (CE: –20 V); caffeic acid m/z 179→135 (CE: –20 V); caffeic acid-O-hexoside m/z 341→179 (CE: –20 V); quercetin m/z 301→151 (CE: –30 V); rutin m/z 609→300 (CE: –50 V); protocatechuic acid m/z 153→109 (CE: –20 V); gallic acid m/z 169→125 (CE: –20 V); naringenin m/z 271→151 (CE: –30 V) and kaempferol-3-O-glucoside m/z 477→285 (–30 V); ethyl gallate was used as an internal standard m/z 197→169 (CE: –25 V). The standards of these polyphenols confirmed the previously reported fragmentation patterns. The compounds were deduced from the different transition signals and from comparing the observed accurate transitions and retention times with those of the standards.

Quantification of polyphenols was performed by the internal standard method. Polyphenols were quantified with respect to their corresponding standard. When standards were not available, as in the case of caffeic acid-O-hexoside, it was quantified with respect to the corresponding hydroxycinnamic acid (caffeic acid).

The liquid chromatograph was an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, an autosampler, and a column oven set to 30 °C. A Luna C₁₈ column 50 × 2.0 mm i.d., 5 μ m (Phenomenex, Torrance, CA, USA) was used. The injection volume was 20 μ L, and the flow rate was 0.4 mL/min. Mobile phases consisted of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B). Separation was carried out in 15 min under the following conditions: 0 min, 5% B; 10 min, 18% B; 13 min, 100% B; 14 min, 100% B; 15 min, 5% B. The column was equilibrated for 5 min prior to each analysis.

Statistical Analysis. The significance of the results was analyzed using the Statgraphics Plus v.5.1 Windows Package (Statistical Graphics Co., Rockville, MD). Analysis of variance (ANOVA) was used to compare the means of groups of measurement data, and principal component analysis (PCA) was carried out to obtain correlations among variables.

RESULTS

Validation of the Method for the Analysis of Total Polyphenols in Tomato Samples. The F–C method was linear over 0.25 to 10 mg/L for gallic acid. Least squares regression analysis gave the following result for the calibration curve: mean (SD); slope, 0.100 (0.001); y intercept, –0.003 (0.001); $r^2 = 0.9993$ (0.0004); and a standard deviation of residuals, 0.053. The LOD and LOQ were 0.006 mg/L and 0.019 mg/L, respectively. The intra- and interday precision and accuracy were evaluated at seven different concentrations (Table 1); for the values analyzed, recovery and precision were between the accepted values for AOAC International.¹⁷

Freeze and thaw cycles did not significantly modify the gallic acid concentration at either temperature tested; they were between 91.7% and 106.5% at –80 °C and between 94.9% and 106.2% at –20 °C. For short-term stability, the recoveries of gallic acid were between 90.1% and 104.0% at –20 °C and between 85.2% and 102.6% when the temperature was –80 °C. Long-term storage at –20 and –80 °C did not affect the gallic acid concentration. It decreased to 95% after 2 years, at both temperatures. We observed no statistically significant differences ($P > 0.05$) in TPs in the tomato samples after testing freeze and thaw cycles, and short-term and long-term stability. The storage and sample handling conditions used for the assays allowed the phenols to remain stable.

Resultats

Table 2. Total Polyphenols (mg GAE/100g FW) and Hydrophilic Antioxidant Capacity (mmol TE/100g FW) of the Seven Varieties (Mean $\pm$ SD), Malva, H-9661, H-9776, H-9997, Albastro, Guadiva, and Elegy, Investigated in this Study over 2008 and 2009^a

	total polyphenols (mg GAE/100 g FW)	hydrophilic antioxidant capacity (mmol TE/100 g FW)
Malva	10.04 $\pm$ 0.30 e	1.79 $\pm$ 0.10 e
H-9661	13.32 $\pm$ 0.70 g	2.80 $\pm$ 0.20 g
H-9776	8.64 $\pm$ 0.20 b	1.29 $\pm$ 0.10 b
H-9997	9.01 $\pm$ 0.40 c	1.40 $\pm$ 0.10 c
Albastro	8.60 $\pm$ 0.30 a	1.25 $\pm$ 0.09 a
Guadiva	9.06 $\pm$ 0.20 d	1.63 $\pm$ 0.08 d
Elegy	12.69 $\pm$ 0.40 f	1.98 $\pm$ 0.10 f

^a Different letters in the columns represent statistically significant differences ($P < 0.05$). SD, standard deviation; GAE, gallic acid equivalent; FW, fresh weight; TE, Trolox equivalent

Table 3. Quantification of Individual Polyphenols (Mean $\pm$ SD) Described in the Literature^{21,37} for Seven Varieties, Malva, H-9661, H-9776, H-9997, Albastro, Guadiva, and Elegy, Expressed as μ g/g FW over 2008 and 2009^a

	ferulic acid	chlorogenic acid	caffeic acid	caffeic acid-O-hexoside	quercetin	rutin	protocatechuic acid	gallic acid	naringenin	kaempferol-3-O-glucoside
Malva	BQL	0.36 $\pm$ 0.01 a	0.68 $\pm$ 0.01 c	0.26 $\pm$ 0.01 a	0.73 $\pm$ 0.02 f	19.79 $\pm$ 0.77 e	0.20 $\pm$ 0.02 a	BQL	3.03 $\pm$ 0.21 d	BQL
H-9661	0.35 $\pm$ 0.03 c	0.46 $\pm$ 0.01 c	1.02 $\pm$ 0.05 d	0.94 $\pm$ 0.04 f	0.76 $\pm$ 0.04 f	20.44 $\pm$ 0.47 e	0.46 $\pm$ 0.02 b	BQL	6.90 $\pm$ 0.19 e	BQL
H-9776	0.10 $\pm$ 0.05 a	0.40 $\pm$ 0.01 b	0.53 $\pm$ 0.01 a	0.32 $\pm$ 0.01 b	0.42 $\pm$ 0.01 a	2.68 $\pm$ 0.05 b	BQL	BQL	0.75 $\pm$ 0.04 b	BQL
H-9997	BQL	0.38 $\pm$ 0.03 b	0.53 $\pm$ 0.04 a	0.47 $\pm$ 0.08 d	0.47 $\pm$ 0.04 b	4.65 $\pm$ 0.04 c	BQL	BQL	0.50 $\pm$ 0.01 a	0.27 $\pm$ 0.03 a
Albastro	0.20 $\pm$ 0.01 b	0.39 $\pm$ 0.02 b	0.55 $\pm$ 0.02 a	0.38 $\pm$ 0.02 c	0.63 $\pm$ 0.04 c	0.79 $\pm$ 0.06 a	BQL	0.96 $\pm$ 0.04 a	1.59 $\pm$ 0.25 c	BQL
Guadiva	BQL	0.38 $\pm$ 0.03 b	1.25 $\pm$ 0.01 e	0.44 $\pm$ 0.06 d	0.69 $\pm$ 0.06 d	6.07 $\pm$ 0.51 d	BQL	BQL	1.71 $\pm$ 0.06 c	BQL
Elegy	0.21 $\pm$ 0.01 b	0.40 $\pm$ 0.02 b	0.63 $\pm$ 0.01 b	0.83 $\pm$ 0.02 e	0.69 $\pm$ 0.05 d	21.80 $\pm$ 0.68 f	BQL	0.93 $\pm$ 0.01 a	0.70 $\pm$ 0.01 b	0.59 $\pm$ 0.01 b

^a Different letters in the columns represent statistically significant differences ($P < 0.05$). SD, standard deviation; FW, fresh weight; BQL, below quantification limit.

Absolute recoveries for the 7 polyphenol standards in the tomato samples using SPE were as follows: polyphenol, mean (SD); gallic acid, 92.5 (4.5); naringenin, 98.2 (3.4); caffeic acid, 96.7 (10.7); rutin, 87.3 (4.8); quercetin, 77.5 (5.4); protocatechuic acid, 108.8 (3.2); and chlorogenic acid, 101.1 (5.2). These recoveries are between the accepted values established by AOAC International.¹⁷

Total Polyphenols and Hydrophilic Antioxidant Capacity in Tomato Samples. SPE has been introduced to eliminate interfering nonphenolic reductants before the F-C assay. We tested for interferences from vitamin C (ascorbic acid at 50 mg/L and 200 mg/L), sugar (glucose at 2 mg/L), iron (Fe(II) at 1 mg/L), and amino acids (phenylalanine, tyrosine, glutamine, and arginine at 1 mg/L), according to Roura et al.¹⁸ None of these substances reacted with the F-C reagent after SPE. After the SPE procedure, the phenolic levels were between 56.54% and 64.70% lower than the values obtained before the extraction procedure.

The content of TP evaluated by the F-C assay after SPE and the relative contribution of individual compounds to the hydrophilic antioxidant capacity of tomato samples from both seasons (2008 and 2009) were analyzed and expressed as the mean $\pm$ SD as shown in Table 2. The F-C assay showed that Albastro and H-9776 contained the lowest concentrations of phenolics, followed by H-9997 and Guadiva. The varieties that possessed the highest concentrations were H-9661, Elegy, and Malva for the F-C assay.

A similar trend was observed in the TEAC results. The lowest hydrophilic antioxidant capacities were determined for Albastro and H-9776, followed by H-9997 and Guadiva. H-9661 contained the highest antioxidant capacity with Elegy ranked second, followed by Malva. No significant changes ($P > 0.05$) in TP content and hydrophilic antioxidant capacity were observed over

the two consecutive years, 2008 and 2009. This data indicates that the TP and hydrophilic antioxidant content of tomatoes was mainly influenced by varietal factors rather than environmental conditions or year changes.

Quantification of Individual Polyphenols in Tomato Samples. The most abundant polyphenols in tomato varieties are quantified in Table 3. The statistically significant differences found between the varieties for each compound analyzed were highlighted with different superindexes. An HPLC chromatogram of the crude extract of the H-9661 variety, including the identification of each peak, is shown in Figure 1.

The main polyphenol in all the varieties was rutin (m/z 609 $\rightarrow$ 300), present at levels ranging between 0.79 and 21.80 μ g/g FW, followed by naringenin (m/z 271 $\rightarrow$ 151), which was found at levels between 0.50 and 6.90 μ g/g FW. The variety with the highest levels of individual polyphenols was H-9661, followed by Elegy and Malva, and this was reflected in the analysis of total phenolics and hydrophilic antioxidant capacity as well.

Hydroxycinnamic acids were mainly represented by ferulic acid (m/z 193 $\rightarrow$ 134) in H-9661, H-9776, Albastro, and Elegy, with chlorogenic acid (m/z 353 $\rightarrow$ 191), caffeic acid (m/z 179 $\rightarrow$ 135), and caffeic acid-O-hexoside (m/z 341 $\rightarrow$ 179) present in all the varieties. Chlorogenic acid levels were similar in all the varieties, whereas the amounts of caffeic and caffeic acid-O-hexoside were higher in H-9661, Elegy, and Guadiva. Ferulic acid was particularly high in H-9661, whereas in other varieties, this hydroxycinnamic acid was below the limit of quantification.

Regarding the family of phenolic acids, the main compound was gallic acid (m/z 169 $\rightarrow$ 125) in Albastro and Elegy, followed by protocatechuic acid (m/z 153 $\rightarrow$ 109) in Malva and H-9661. In some varieties, gallic and protocatechuic acids were below the detection level.

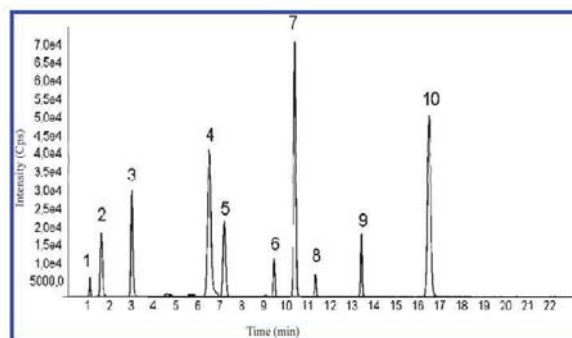


Figure 1. HPLC chromatogram of the crude extract of the H-9661 variety including the identification of each peak: 1: gallic acid; 2: protocatechuic acid; 3: caffeic-O-hexoside; 4: caffeic acid; 5: chlorogenic acid; 6: ferulic acid; 7: rutin; 8: kaempferol-3-O-glucoside; 9: naringenin; 10: quercetin.

Flavonol concentration varied from one variety to another. Flavonols were mainly characterized by quercetin (m/z 301→151) and rutin (m/z 609→300) in all the varieties, and kaempferol-3-O-glucoside (m/z 477→285) in H-9997 and Elegy. Quercetin levels were similar in all the varieties, whereas the amounts of rutin were higher in Malva, H-9661, and Elegy. Kaempferol-3-O-glucoside reached its maximum concentration in Elegy and H-9997.

Flavonones were mainly represented by naringenin (m/z 271→151) in all the varieties. As can be observed, the amounts of naringenin present in H-9661 and Malva were higher than those in other varieties.

Correlation between Tomato Compounds and Seven Different Varieties. The ANOVA of the polyphenolic data and hydrophilic antioxidant activity showed that there were significant differences ($P > 0.05$) among the varieties (Table 3). PCA was performed on all the samples and variables (total phenolics, hydrophilic antioxidant activity, ferulic, chlorogenic, caffeic, caffeic-O-hexoside, protocatechuic, and gallic acids, and quercetin, rutin, naringenin, and kaempferol 3-O-glucoside) and was able to separate the tomato samples according to variety.

Two principal components (PC1 and PC2) were obtained and accounted for 74.59% of the variability of the original data (Figure 2). The statistical analysis for the data showed a strong positive correlation between TP content, rutin, and ferulic and caffeic-O-hexoside acids. Moreover, a positive correlation was observed between protocatechuic acid and naringenin. In contrast, ferulic and caffeic-O-hexoside acids, rutin and TP content were weakly correlated with naringenin and caffeic and protocatechuic acids.

The score plot of PC1 versus PC2 from the full-data PCA model plotted in Figure 2 describes differences between the seven varieties of tomato. Tomato samples are gathered in seven different groups (Figure 3). Guadiva and Malva are situated close together in the bottom part of the plot; H-9997, Albastros and H-9776 are situated in the left part of the score plot; whereas the variety H-9661 appears on the right-hand side and Elegy in the upper part of the plot. The H-9661 variety correlated well with naringenin and protocatechuic acids, whereas the H-9997 and H-9776 varieties scored lower values of these parameters as they were located on the left-hand side. However, the Elegy variety correlated well with kaempferol-3-O-glucoside and gallic acid,

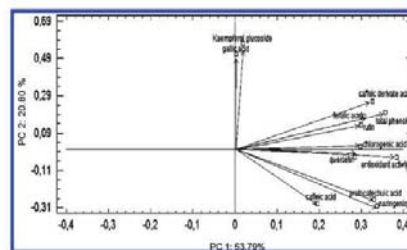


Figure 2. Principal components plot of the seven varieties of tomato.

although this sample scored lower values of naringenin and caffeic and protocatechuic acids. Moreover, H-9661 and Elegy correlated well with rutin, TP content, hydrophilic antioxidant capacity, and caffeic-O-hexoside and ferulic acids as these varieties scored the highest levels of these parameters.

DISCUSSION

The antioxidant capacity and polyphenol content of tomatoes are greatly affected by both the ripening stage and part of the fruit;^{22,23} therefore, in our experiment tomato fruits were harvested at the same degree of ripeness (4.3–5°Brix). Since 98% of total flavonols occur in the skin, tomato types with different fruit size and thus different skin–volume ratios or different skin color are expected to have varying flavonol contents.²⁴ However, in our experiment, since all the varieties were normal-sized (75–80 g), the skin–volume ratio and fruit size were not expected to be a determining factor for the polyphenol content.

In addition, all the studied varieties were cultivated in Vegas Bajas del Guadiana, Badajoz (Spain), in order to attenuate the variability due to the country of origin. Analysis of cherry tomato cv. Favorita obtained from Spain, South Africa, England, and Scotland has shown widely varying flavonol contents of 21.5, 16.0, 3.4, and 6.6 $\mu\text{g/g}$ FW, respectively, based on whole fruit samples.²⁵

Stewart et al.²⁵ suggested that choice of cultivar is a major factor contributing to the total phenol content in tomatoes when grown under similar environmental conditions. Two normal-sized field

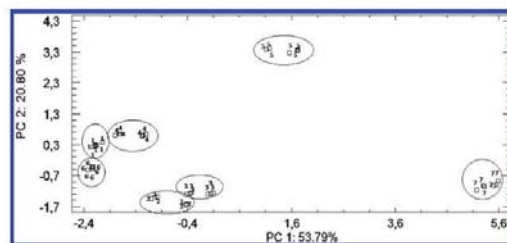


Figure 3. Score plot of PC1 vs PC2 of all the varieties analyzed. Varieties: 1, H-9997; 2, Guadiva; 3, Malva; 4, Albastros; 5, Elegy; 6, H-9776; 7, H-9661.

grown tomatoes, cv.'s Bond and Havanera, grown alongside each other in Spain, contained 10.9 and 6.6 $\mu\text{g/g}$ FW of flavonols, respectively. Therefore, to evaluate the effects of the cultivar, we chose seven normal-sized varieties of tomato harvested at the same degree of ripeness (4.3–5°Brix) from Vegas Bajas del Guadiana, Badajoz (Spain): Malva, H-9661, H-9776, H-9997, Albastros, Guadiva, and Elegy.

Total phenolic content is indicative of the amount of polyphenols in vegetables. The F–C method is the most commonly used assay to analyze TP in tomatoes.^{26,27} However, TP content is sometimes overvalued, as tomatoes contain reducing substances (ascorbic acid, sugars, and amino acids) that interfere with the assay.^{16,28} In this work, we took into account the hydrophilic antioxidant capacity due to polyphenols and vitamin C.

A prerequisite for the evaluation of the total antioxidant contents and antioxidative capacities of tomato products is the separation of hydrophilic and lipophilic fractions, which contain vitamin C and polyphenols or vitamin E and carotenoids, respectively. In this work, we took into account the hydrophilic antioxidant capacity due to polyphenols and vitamin C. Curiously, our TP content results were comparable to the existing data. However, total polyphenol levels in tomatoes have been overestimated until now because previously cleanup SPE was not assessed. Gahler et al.²⁹ reported TPs in tomatoes, and their results varied from 9.86 to 22.32 mg of GAE/100 g FW. Minogio et al.¹⁹ analyzed different polyphenol fractions of tomato cultivars and found that the content of total phenolics varied from 4.43 to 25.84 mg GAE/100 g FW. Our data are in agreement with the results shown by these authors. Shen et al.³⁰ also analyzed tomato cultivars and determined a TP content from 1.8 to 2.3 mg GAE/100 g FW. Therefore, the tomatoes analyzed in this work from Vegas Bajas del Guadiana had a slightly higher total phenolic content than tomatoes analyzed in the other works.

When the hydrophilic antioxidant capacity was evaluated, our results were within the range described by other authors. Gahler et al.²⁹ determined hydrophilic antioxidant capacity in tomato fruit, and their results were 0.045 mmol TE/100 g FW, whereas Minogio et al.¹⁹ reported hydrophilic antioxidant capacities from 60 to 230 mmol TE/100 g FW in tomato lines and cultivars. These results show that the hydrophilic antioxidant capacity differs greatly between studies, probably due to the variety, stage of ripeness, light, temperature, climatic growing conditions, or soil characteristics of tomato fruits.³¹ The antioxidative effects of the examined tomatoes correlated with the TP content, which suggests that these bioactive components contribute significantly to the hydrophilic antioxidant capacity of tomato fruits. Surprisingly, Minogio et al.¹⁹ found that almost all the tomato lines

they tested with low carotenoid content produced high levels of phenolics and consequently had the strongest antioxidant capacity.

The qualitative and quantitative determination of individual polyphenols in foods is becoming extremely interesting in the field of nutrition and food technology. The highest concentrations of tomato polyphenols have been found in epidermal and placental tissues.²⁴ Stewart et al.²⁵ measured the distribution of flavonols in Spanish cherry tomatoes and found a flavonol content of 25.3 $\mu\text{g/g}$ FW in the whole fruits, 143.3 $\mu\text{g/g}$ FW in the peel or epidermis, and 0.12 $\mu\text{g/g}$ FW in the flesh, whereas the seed had 1.5 $\mu\text{g/g}$ FW.

According to the USDA flavonoid database, red tomatoes contain on a year average basis 15 $\mu\text{g/g}$ FW of flavonoids determined as aglycones. Naringenin (45%) is reported to be the main flavonoid, followed by quercetin (39%), myricetin (10%), and kaempferol (5%).²⁴ Other studies report rutin to be the major flavonoid in several tomato cultivars.^{32,33} In our investigation, rutin (quercetin-3-O-rutinoside) was the dominant flavonol in all the samples, followed by naringenin.

The naringenin content in these varieties fell within the range (4.50–9.50 $\mu\text{g/g}$ FW) reported by Martínez-Valverde et al.³⁴ The rutin content of Malva, H-9661, and Elegy was very similar to the value (19.4 $\mu\text{g/g}$ FW) found by Proteggente et al.³⁵ Minogio et al.¹⁹ also reported levels of rutin between 0.7 and 23.5 $\mu\text{g/g}$ FW, and our values are in accordance with these results.

Some studies have reported quercetin and kaempferol in tomato fruits.^{36,37} In our study, quercetin achieved a content of 0.76 $\mu\text{g/g}$ FW in the H-9661 variety, and kaempferol-3-O-glucoside was only detected in H-9997 and Elegy. The differing flavonol levels in tomato fruits found in different studies are probably due to the variety, state of ripeness, light, temperature, climatic growing conditions, or soil.

Tomato fruits also contain hydroxycinnamic acids, which are found as simple esters, chlorogenic acid being the most predominant.³⁸ The concentration of this hydroxycinnamic acid in pulp is nearly twice that in pericarp tissues.³⁹ The chlorogenic acid content in the varieties we studied fell within the range (0.3–5.8 $\mu\text{g/g}$ FW) reported by Minogio et al.¹⁹ All the varieties contained caffeic acid, and our results are in accordance with the value (<1 $\mu\text{g/g}$ FW) found by Minogio et al.¹⁹ Ferulic acid was also found in this work, but only in H-9661, H-9776, Albastros, and Elegy. The variations in the content of hydroxycinnamic acids depend on the degree of maturity of the tomato fruits concerned and on agricultural practices. Chlorogenic acid appears in young fruits and decreases during fruit maturity.³⁸

Some of the varieties we analyzed also contained phenolic acids, the most predominant being gallic acid, as described in

other studies,³⁰ with a value lower than 1 µg/g FW in Albastro and Elegy. We also found protocatechuic acid, but only in Malva and H-9661, and at a level close to the limit of detection.

The variables studied enabled the differentiation of H-9661, H-9997, H-9661, Malva, Guadiva, Albastro, and Elegy. Significant differences among the varieties were observed after applying analysis of variance, and these were then confirmed by principal component analysis. H-9661 and Elegy were clearly distinct from all other varieties, while this distinction was not so clear for Guadiva and Malva.

In conclusion, phenolic and hydroxycinnamic acids and flavonoids, total polyphenols, and hydrophilic antioxidant capacity can be used as chemotaxonomic tomato markers to distinguish between tomatoes according to variety.

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ABBREVIATIONS USED

F—C, Folin—Ciocalteu; SPE, solid phase extraction; TP, total polyphenols; LC-MS/MS, liquid chromatography coupled to mass spectrometry in tandem mode; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); PBS, phosphate-buffered saline 5 mM; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; GAE, mg of gallic acid equivalents; FW, fresh weight; LOD, limit of detection; LOQ, limit of quantification; SD, standard deviation; HPLC, high pressure liquid chromatography; TEAC, Trolox equivalent antioxidant capacity; TE, Trolox equivalent; FS, full scan; CE, collision energy; PIS, product ion scan; Prec, precursor ion scan; NL₀, Neutral loss; MRM, multiple reaction monitoring; ANOVA, analysis of variance; PCA, principal component analysis; PC, principal component.

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3. Capítol III: Efecte del processat industrial de salses de tomàquet en el perfil fenòlic i en la capacitat antioxidant hidrofílica

Anna Vallverdú-Queralt, Alexander Medina-Remón, Isidre Casals-Ribes, Andrew L. Waterhouse, Cristina Andrés-Lacueva & Rosa M. Lamuela-Raventós. LWT-Food Science and Technology. 2012, 47: 154-160.

Resum: Aquesta publicació presenta el primer estudi que tracta d'avaluar diferents tractaments industrials (*hot* 22/24, *hot* 28/30, *cold* 28/30 i *passata* 10/12) per a la producció de salses de tomàquet. L'estudi es va dur a terme durant els anys 2008 i 2009. Es va fer l'anàlisi de polifenols totals, amb prèvia neteja mitjançant *SPE*, l'assaig de capacitat antioxidant mitjançant els mètodes: *DPPH* i *ABTS*⁺ i finalment l'anàlisi de fenols individuals mitjançant *HPLC-MS/MS*. En aquest estudi, el tractament *passata* 10/12 és el que millor conserva els polifenols i antioxidants durant el processat de les salses, ja que necessita temps d'evaporació més curts (productes finals d'entre 10 i 12 °Brix); en canvi el *hot* 28/30 presenta pèrdues més elevades de compostos bioactius, ja que es caracteritza per temps llargs d'evaporació (productes finals d'entre 28 i 30 °Brix). Per tant, el temps d'evaporació és un element crític que cal controlar durant la producció de salses. D'altra banda, l'addició d'una salsa rica en pells i llavors durant el processat és important per enriquir el producte final amb més contingut de components bioactius.



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Effect of tomato industrial processing on phenolic profile and hydrophilic antioxidant capacity

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ABSTRACT

Industrial processing of tomatoes involves juice addition and heat treatment that may affect (increase or decrease) the levels of phenolic compounds. In this work, we evaluated the effect of each processing step in the paste-making process. Four technological processes were investigated on an industrial scale: Hot Break 28/30, Hot Break 22/24, Cold Break 28/30 and Passata 10/12; and four stages were monitored in each process: (1) fresh tomatoes; (2) juice after scalders and cream addition; (3) tomato paste from evaporators and (4) final paste. The effect of each processing stage was evaluated by different analyses: first, hydrophilic antioxidant capacity was evaluated using ABTS^{•+} and DPPH assays and total phenolics using Folin–Ciocalteu method; then, individual polyphenols were quantified using HPLC-ESI-MS/MS. This multifaceted approach has revealed that each processing stage induces different changes in the antioxidant and phenolic profile. The results of this study indicated that cream addition increases the hydrophilic antioxidant capacity and the amount of phenolic compounds positively, while heat treatment and, to a lesser extent, the sterilization stage during the paste-making process affect these compounds negatively.

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1. Introduction

Tomatoes and tomato-based products are consistently associated with a lower risk of several types of cancer (Grieb et al., 2009; Zhang et al., 2009) and also, to a lesser extent, to a lower incidence of coronary heart disease (Stahl & Sies, 2005). All tomato-based products contain micronutrients, such as potassium, antioxidant vitamin C, vitamin E and folate (Agarwal, Shen, Agarwal, & Rao, 2001). In addition to their micronutrient content, tomatoes, and therefore their tomato-based products, also contain valuable phytochemicals or bioactive components: mainly phenolic compounds and carotenoids, such as lycopene. Phenolic compounds in tomatoes are mainly represented by flavanones (naringenin glycosylated derivatives) and flavonols (quercetin, rutin and kaempferol glycosylated derivatives) (Baharun, Luximon-Ramma, Crozier,

& Aruoma, 2004; Le Gall et al., 2003; Slimestad, Fossen, & Verheul, 2008; Stewart et al., 2000).

The industrial processing of tomatoes into tomato-based products includes several thermal treatment steps such as drying, heating and pasteurizing. These treatments inactivate microorganisms or enzymes, decrease the moisture content and concentrate the product. During thermal treatments, several additional changes can occur to affect the appearance, composition, nutritional value and sensory properties in terms of color, texture and flavor of the product (Capanoglu, Beekwilder, Boyacioglu, Hall, & de Vos, 2008). However, processed tomatoes may have a lower content of health-benefiting compounds than fresh ones (Abushita, Daoud, & Biacs, 2000; Takeoka et al., 2001). So, the evaluation of food processing on health-benefiting compounds is necessary to preserve the activity and bioaccessibility of these compounds.

The technological effects on the lycopene content of tomatoes have been fully examined (Goula, Adamopoulos, Chatzidakis, & Nikas, 2006; Sharma & Le Maguer, 2004). However, little is known about the effects of these treatments on polyphenolic antioxidants. Some studies indicate that a considerable loss of

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hydrophilic antioxidants is caused by the processing approach. Crozier et al. studied the effect of cooking on the quercetin content of tomatoes. Boiling, microwaving and frying reduced the quercetin content (Crozier, Lean, McDonald, & Black, 1997). In contrast, the total phenolic content was found to be increased as a result of processing in other studies. In experiments carried out by Chang et al., two tomato varieties were air dried at 80 °C for 2 h and then at 60 °C for 6 h. Analyses showed that the total flavonoid and total phenolic content were increased when compared to the corresponding levels in fresh tomatoes (Chang & Liu, 2007).

The aim of this work was to examine extensively the effects of different kinds of tomato processing, in the production of paste on an industrial scale, on the hydrophilic antioxidant capacity, total polyphenols (TP), the content of flavonols (kaempferol-3-O-rutinoside, rutin and quercetin), flavanones (naringenin and naringenin-7-O-glucoside), hydroxycinnamic acids (chlorogenic, caffeic, caffeic-O-hexoside, ferulic and ferulic-O-hexoside acids) and benzoic acid (protocatechuic acid) in four different tomato processes: Passata 10/12, Hot Break 22/24, Hot Break 28/30 and Cold Break 28/30. Sampling and processing were conducted in commercial processing facilities over the autumn of 2008 and 2009.

2. Materials and methods

2.1. Standards and reagents

All samples and standards were handled without exposure to light; all the standards were HPLC-grade. Caffeic, ferulic, protocatechuic and chlorogenic acids, quercetin and rutin, Folin–Ciocalteu (F–C) reagent, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), PBS: phosphate-buffered saline pH 7.4, Trolox: (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid 97%, manganese dioxide and DPPH: 2,2-diphenyl-1-picrylhydrazyl were purchased from Sigma® (Madrid, Spain); naringenin, naringenin-7-O-glucoside and kaempferol-3-O-rutinoside from Extrasynthèse (Genay, France); hydrochloric acid 35% and acetic acid 99.8% from Panreac (Barcelona, Spain); and anhydrous sodium acetate (2 M) from Merck (Darmstadt, Germany). Methanol and formic acid were obtained from Scharlau (Barcelona, Spain) and ultrapure water (Milli-Q) from Millipore System (Bedford, MA, USA).

2.2. Sampling and processing conditions

Tomatoes in this study were represented by a mixture of varieties – H-9661, H-9997, H-9776, Albastros, Malva, Guadiva and Elegy – harvested in Vegas Bajas del Guadiana, Badajoz (Spain). All samples of this study were obtained from an industry in Badajoz (Spain). In order to estimate differences in original fruit sources and years, each step in the process was independently sampled six times from different fruit batches processed over two consecutive years, 2008 and 2009.

Four different processes were evaluated: Passata 10/12, Hot Break 22/24, Hot Break 28/30 and Cold Break 28/30. The Passata process normally takes place during the peak of the tomato season, when fresh tomatoes are at their best quality; a suitable variety of tomatoes is used in order to obtain high cellulose, pectin and pulp content; the only added ingredients allowed are salt, basil and citric acid (100 g for 1500 kg of tomato). The Hot Break process involves fresh tomato being chopped when heated, at higher temperatures than the Cold Break process. The difference between these two products lies in their apparent viscosity. The Hot Break product is more viscous and therefore denser than the Cold Break product. The Hot Break product is usually used for ketchup and different sauces, while the Cold Break product is most commonly used for triple-concentrated paste for domestic use (Scott Smith & Hui,

2004). Citric acid (100 g citric acid for 1500 kg of tomato) was also added in Hot and Cold breaks to adjust the pH.

The processing included: washing, triturating, addition of a cream rich in peels and seeds in Passata 10/12, Hot Break 22/24 and Hot Break 28/30 scalders, preheating (Hot Break, Cold Break or Passata), sieving, evaporation, sterilization, filling and storage. The Hot Break and Passata preheatings were performed between 97 °C and 102 °C, whereas the Cold Break preheating was performed between 65 °C and 85 °C. Then the extract was transferred through the sieves to remove the seeds and peels, and water was evaporated under vacuum between 75 °C and 80 °C to reach a total soluble solid content between 10 and 12 Brix, 22 and 24 Brix or 28 and 30 Brix. After this, samples were sterilized at 112 °C for 2 min and 20 s, aseptically filled and packaged. The flow diagram is shown in Fig. 1.

Four sample stages were monitored for each process: (1) fresh tomatoes; (2) juice after scalders; (3) tomato paste from evaporators and (4) final paste. After sampling, all products were kept on ice and taken to the laboratory and stored at –20 °C until analysis.

The processing of the samples was performed following the procedure of Vallverdú-Queralt et al. First, samples (0.5 g) were weighed and homogenized with 4 mL (80 mL ethanol/100 mL Milli-Q water); the homogenates were sonicated for 5 min and centrifugated (2140×g at 4 °C) for 20 min. The supernatant was transferred into a flask and extraction was repeated. Both supernatants were combined and evaporated under nitrogen flow; finally, the residue was reconstituted with Milli-Q water (0.1 mL formic acid/100 mL Milli-Q water) up to 4 mL. Samples were frozen

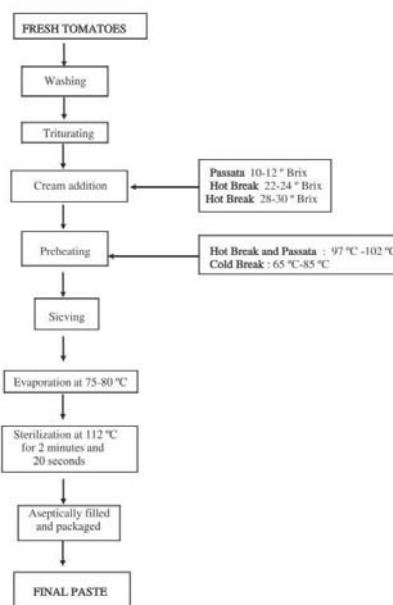


Fig. 1. Flow diagram of tomato paste production.

at -20°C until analysis (Vallverdú-Queralt, Jáuregui, Medina-Remón, Andrés-Lacueva, & Lamuela-Raventós, 2010).

2.3. Analysis of total polyphenols

For TP analysis, solid phase extraction (SPE) was carried out to eliminate interferences such as ascorbic acid, amino acids and reductant sugars, which are proved to overestimate values of TP (Singleton, Orthofer, & Lamuela-Raventós, 1999). For this procedure, firstly, 1 mL of methanol and subsequently 1 mL of sodium acetate 50 mmol/L pH 7 were loaded into Oasis[®] MAX cartridges from Waters (Milford, USA) to equilibrate the sample; then, 1 mL of each extract was diluted with 1 mL of Milli-Q water and acidified with 34 μL of hydrochloric acid before being loaded into the cartridges separately. These were rinsed with sodium acetate 50 mmol/L pH 7 (5 mL/100 mL methanol). The polyphenols were eluted with 1800 μL of methanol (2 mL formic acid/100 mL methanol).

The content of TP was analyzed spectrophotometrically using the F-C method following the procedure of (Medina-Remón et al., 2009). Gallic acid was used as calibrator standard, and the TP content was expressed as mg of gallic acid equivalents (GAE)/100 g dry material (DM).

2.4. Hydrophilic antioxidant capacity

The hydrophilic antioxidant capacity was measured using an ABTS⁺ radical decolorization assay and DPPH assay, following the procedure described by Vallverdú-Queralt, Medina-Remón, Andrés-Lacueva, and Lamuela-Raventós (2011).

2.5. HPLC-ESI-MS/MS analysis

The polyphenols of tomato fruits were identified in a previous study using HPLC-ESI-LTQ-Orbitrap-MS (Vallverdú-Queralt et al., 2010). To evaluate the effects of processing on the phenolic compounds, we selected the most predominant polyphenols in tomato fruits found in this study. These polyphenols were quantified using HPLC-ESI-MS/MS. An API 3000 (PE Sciex, Concord, Ontario, Canada) triple quadrupole mass spectrometer equipped with a Turbo IonSpray source in negative-ion mode was used to obtain MS/MS data. Turbo IonSpray source settings were as follows: capillary voltage, -3500 V ; nebulizer gas (N_2), 10 a.u. (arbitrary units); curtain gas (N_2), 12 a.u.; collision gas (N_2), 4 a.u.; focusing potential, -200 V ; entrance potential, -10 V ; drying gas (N_2), heated to 400°C and introduced to a flow rate of $8000\text{ cm}^3/\text{min}$. The declustering potential and collision energy were optimized for each compound in infusion experiments: individual standard solutions (10 $\mu\text{g}/\text{mL}$) dissolved in 50:50 (v/v) mobile phase were infused at a constant flow rate of 5 $\mu\text{L}/\text{min}$ using a model syringe pump (Harvard Apparatus, Holliston, MA, USA).

For quantification purposes, data was collected in the multiple reaction monitoring (MRM) mode, tracking the transition of specific parent and product ions for each compound. In particular, we selected 11 transitions corresponding to the most abundant polyphenols in tomato fruits: ferulic acid m/z 193 $\rightarrow$ 134 (CE: -20 V); ferulic acid-O-hexoside m/z 355 $\rightarrow$ 193 (CE: -20 V); chlorogenic acid m/z 353 $\rightarrow$ 191 (CE: -20 V); caffeic acid m/z 179 $\rightarrow$ 135 (CE: -20 V); caffeic acid-O-hexoside m/z 341 $\rightarrow$ 179 (CE: -20 V); protocatechuic acid m/z 153 $\rightarrow$ 109 (CE: -20 V); quercetin m/z 301 $\rightarrow$ 151 (CE: -30 V); rutin m/z 609 $\rightarrow$ 300 (CE: -50 V); naringenin m/z 271 $\rightarrow$ 151 (CE: -30 V); naringenin-7-O-glucoside m/z 433 $\rightarrow$ 271 (CE: -20 V); kaempferol-3-O-rutinoside m/z 593 $\rightarrow$ 285 (CE: -30 V); ethyl gallate (internal standard) m/z 197 $\rightarrow$ 169 (CE: -25 V).

The standards of these polyphenols confirmed the previously reported fragmentation patterns (Sanchez-Rabaneda et al., 2003).

The compounds were deduced from the different transition signals and by comparing the observed accurate transitions and retention times with those of the standards. Quantification of polyphenols was performed by the internal standard method. Polyphenols were quantified with respect to their corresponding standard. When standards were not available, as in the case of caffeic-O-hexoside and ferulic-O-hexoside acids, they were quantified with respect to the corresponding hydroxycinnamic acid (caffeic and ferulic acids).

The liquid chromatograph was an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, an autosampler and a column oven set to 30°C . A Luna C₁₈ column $50 \times 2.0\text{ mm i.d.}$, $5\text{ }\mu\text{m}$ (Phenomenex, Torrance, CA, USA) was used. The injection volume was 20 μL and the flow rate was 0.4 mL/min. Mobile phases consisted of 0.1 mL/100 mL in Milli-Q water (A) and 0.1 mL/100 mL formic acid in acetonitrile (B). Separation was carried out in 15 min under the following conditions: 0 min, 5% B; 10 min, 18% B; 13 min, 100% B; 14 min, 100% B; 15 min, 5% B. The column was equilibrated for 5 min prior to each analysis.

2.6. Statistical analysis

Data was processed using the Statgraphics Plus v.5.1 Windows Package (Statistical Graphics Co., Rockville, Md). Analysis of variance (ANOVA) was used to compare the means of groups of measurement data, and principal components analysis (PCA) to visualize the original arrangement of the tomato samples in an n -dimensional space, by identifying the directions in which most of the information is retained. The scores plot was carried out to define different groups, and the loadings plot to highlight differences among variables.

3. Results

3.1. Analysis of TP content and hydrophilic antioxidant activity during production of tomato paste

The relative contributions of individual compounds to TPs and hydrophilic antioxidant activity of the four technological processes are shown in Table 1. No significant changes ($P > 0.05$) were observed between the harvests (data not shown). To avoid the effect of water evaporation and concentration of solids taking place on the quantification during thermal processing, the estimated values were expressed as dry weight [per g of dry matter (DM)]. The ANOVA of the TPs and hydrophilic antioxidant capacity showed that statistically significant differences were detected between the different processing steps and treatments.

Statistically significant increases ($P < 0.05$) in TPs and hydrophilic antioxidant capacity occurred when fresh tomatoes were processed into juice in Hot Break 22/24, Hot Break 28/30 and Passata 10/12, due to the addition into the juices of cream rich in peels and seeds with high levels of antioxidants and TPs (Table 1). Within the three tomato paste processes, the maximum change was observed in Passata 10/12, with an increase in TPs of 37.94% and one of 72.30% and 72.76% for ABTS⁺ and DPPH assays, respectively. It should be noted that in Cold Break 28/30 no increase was observed because of the lack of cream addition.

As tomato juices passed through evaporators to obtain the desired final paste, consistent decreases ($P < 0.05$) in TPs and hydrophilic antioxidant capacity were observed. Passata 10/12 caused the smallest changes in the TPs and hydrophilic antioxidant levels, by 20.32% for TPs, 25.69% and 22.76% for ABTS⁺ and DPPH assays.

Resultats

Table 1

Total phenolic content and hydrophilic antioxidant capacity expressed as mean $\pm$ SD of the four technological processes ($n = 6$); different letters in the columns represent statistically significant differences ($P < 0.05$).

Sample	TP (mg GAE/100 g DM)	ABTS ^a (mmol TE/100 g DM)	DPPH (mmol TE/100 g DM)
<i>Passata 10/12</i>			
Fresh tomatoes	169.5 $\pm$ 6.3 ^a	29.6 $\pm$ 1.8 ^a	32.3 $\pm$ 1.4 ^a
Juice after scalders	233.8 $\pm$ 10.1 ^b	51.0 $\pm$ 2.4 ^b	55.8 $\pm$ 1.9 ^b
Evaporator	200.3 $\pm$ 9.6 ^c	39.4 $\pm$ 1.4 ^c	45.6 $\pm$ 1.3 ^c
Final paste	186.3 $\pm$ 7.5 ^d	37.9 $\pm$ 2.1 ^d	43.1 $\pm$ 1.4 ^d
Cream 10/12	64.2 $\pm$ 1.2	21.9 $\pm$ 1.1	26.5 $\pm$ 1.9
<i>Hot Break 28/30</i>			
Fresh tomatoes	198.3 $\pm$ 8.9 ^a	31.2 $\pm$ 1.0 ^a	34.5 $\pm$ 1.9 ^a
Juice after scalders	251.4 $\pm$ 12.3 ^b	51.4 $\pm$ 3.2 ^b	52.2 $\pm$ 2.6 ^b
Evaporator	199.6 $\pm$ 1.5 ^c	24.0 $\pm$ 1.4 ^c	28.9 $\pm$ 1.8 ^c
Final paste	162.8 $\pm$ 7.1 ^d	19.4 $\pm$ 1.8 ^d	25.6 $\pm$ 1.3 ^d
Cream 28/30	53.9 $\pm$ 1.6	20.3 $\pm$ 1.2	24.8 $\pm$ 1.2
<i>Hot Break 22/24</i>			
Fresh tomatoes	196.5 $\pm$ 7.5 ^a	30.4 $\pm$ 1.8 ^a	32.6 $\pm$ 1.1 ^a
Juice after scalders	246.0 $\pm$ 9.1 ^b	44.3 $\pm$ 2.1 ^b	44.6 $\pm$ 1.8 ^b
Evaporator	171.2 $\pm$ 8.4 ^c	28.7 $\pm$ 1.6 ^c	30.5 $\pm$ 1.1 ^c
Final paste	157.2 $\pm$ 4.4 ^d	27.6 $\pm$ 1.2 ^d	28.4 $\pm$ 1.7 ^d
Cream 22/24	49.6 $\pm$ 1.9	13.5 $\pm$ 1.0	19.8 $\pm$ 1.1
<i>Cold Break 28/30</i>			
Fresh tomatoes	226.5 $\pm$ 9.6 ^a	32.6 $\pm$ 1.2 ^a	34.9 $\pm$ 1.4 ^a
Juice after scalders	203.4 $\pm$ 12.4 ^b	25.4 $\pm$ 1.4 ^b	27.8 $\pm$ 1.9 ^b
Evaporator	148.5 $\pm$ 9.5 ^c	23.6 $\pm$ 1.5 ^c	26.1 $\pm$ 1.3 ^c
Final paste	146.3 $\pm$ 3.0 ^m	18.2 $\pm$ 1.7 ^d	21.2 $\pm$ 1.2 ^m

SD: standard deviation; TP: total polyphenols; GAE: gallic acid equivalent; TE: Trolox equivalent; DM: dry matter.

3.2. Quantization of individual polyphenols during production of tomato paste

The changes in specific polyphenol compounds were monitored during the four technological processes (Tables 2 and 3). No significant changes ($P > 0.05$) were observed during the two consecutive years, 2008 and 2009 (data not shown). The ANOVA for polyphenol data showed statistically significant differences between processing steps and treatments.

Hydroxycinnamic acids were mainly represented by ferulic (m/z 193 $\rightarrow$ 134), ferulic-O-hexoside (m/z 355 $\rightarrow$ 193), chlorogenic (m/z 353 $\rightarrow$ 191), caffeic (m/z 179 $\rightarrow$ 135) and caffeic-O-hexoside (m/z 341 $\rightarrow$ 179) acids. Although there was considerable variation between processes in the content of hydroxycinnamic acids, the trends reflecting the effects of processing were similar in Hot Break 22/24, Hot Break 28/30 and Passata 10/12, as a result of the cream addition (Table 2). The largest increases ($P < 0.05$) occurred for chlorogenic acid, whereas for caffeic acid they were nearly undetectable.

In the latter stages of the paste-making process, further vacuum evaporation in processes requiring more extensive processing at higher temperatures (Hot Break 28/30) reduced chlorogenic, ferulic-O-hexoside and caffeic-O-hexoside acids by 42.81%, 33.46% and 36.27%, respectively, in comparison to Passata 10/12 which produced slight reductions. The subsequent sterilization stage prior to the final paste did not further affect ($P > 0.05$) the content of chlorogenic, ferulic-O-hexoside and caffeic-O-hexoside acids.

In contrast to other hydroxycinnamic acids, further processing steps to produce paste by vacuum evaporation caused statistically significant increases ($P < 0.05$) in caffeic and ferulic acid levels. This increase in the final stages was highly reproducible between the different technological processes, probably due to a hydrolysis from the ester or glycosidic forms.

As regards the family of phenolic acids, the main phenolic acid was protocatechuic acid (m/z 153 $\rightarrow$ 109), which showed statistically significant increases ($P < 0.05$) when fresh tomatoes were processed into juice in Passata 10/12 and Hot Break 28/30. Subsequently, levels of protocatechuic acid significantly decreased as a consequence of the evaporation stage ($P < 0.05$). Passata 10/12 caused the smallest reductions in the protocatechuic acid levels. The subsequent sterilization stages did not further modify ($P > 0.05$) the levels of protocatechuic acid.

Flavonols in these products are characterized by rutin (m/z 609 $\rightarrow$ 300), kaempferol-3-O-rutinoside (m/z 593 $\rightarrow$ 285) and quercetin (m/z 301 $\rightarrow$ 151). Statistically significant increases ($P < 0.05$) were observed in the first stage of the process as a result of the cream addition in Passata 10/12, Hot Break 22/24 and Hot Break 28/30 (Table 3). The concentration of rutin was relatively high

Table 2

Content of hydroxycinnamic and phenolic acids ($\mu\text{g/g DM}$) expressed as mean $\pm$ SD of the four technological processes ($n = 6$); different letters in the columns represent statistically significant differences ($P < 0.05$).

	Ferulic acid	Ferulic-O-hexoside acid	Chlorogenic acid	Caffeic acid	Caffeic-O-hexoside acid	Protocatechuic acid	Total hydroxycinnamic and phenolic acids
<i>Passata 10/12</i>							
Fresh tomatoes	1.73 $\pm$ 0.07 ^a	33.45 $\pm$ 0.87 ^a	17.18 $\pm$ 0.12 ^a	3.47 $\pm$ 0.15 ^a	22.27 $\pm$ 0.93 ^a	2.80 $\pm$ 0.05 ^a	80.90
Juice after scalders	18.37 $\pm$ 0.15 ^b	117.69 $\pm$ 4.48 ^b	150.31 $\pm$ 0.09 ^b	4.44 $\pm$ 0.13 ^b	26.91 $\pm$ 0.88 ^b	4.41 $\pm$ 0.39 ^b	322.13
Evaporator	31.42 $\pm$ 1.95 ^c	87.76 $\pm$ 3.28 ^c	142.99 $\pm$ 2.92 ^c	10.71 $\pm$ 0.05 ^c	20.70 $\pm$ 0.01 ^c	4.17 $\pm$ 0.14 ^c	297.75
Final paste	44.09 $\pm$ 1.19 ^d	85.33 $\pm$ 0.13 ^c	138.77 $\pm$ 1.46 ^d	15.58 $\pm$ 0.52 ^d	20.17 $\pm$ 0.14 ^c	3.96 $\pm$ 0.09 ^d	307.90
Cream 10/12	15.61 $\pm$ 0.29	112.89 $\pm$ 0.20	129.78 $\pm$ 2.59	1.78 $\pm$ 0.08	4.54 $\pm$ 0.11	2.16 $\pm$ 0.04	266.76
<i>Hot Break 28/30</i>							
Fresh tomatoes	2.80 $\pm$ 0.09 ^a	37.69 $\pm$ 2.17 ^a	17.38 $\pm$ 0.61 ^a	3.00 $\pm$ 0.10 ^a	21.01 $\pm$ 0.11 ^d	2.85 $\pm$ 0.06 ^a	84.73
Juice after scalders	16.41 $\pm$ 0.97 ^b	139.70 $\pm$ 5.86 ^b	212.32 $\pm$ 7.18 ^b	4.19 $\pm$ 0.18 ^b	28.01 $\pm$ 2.79 ^b	6.45 $\pm$ 0.12 ^b	407.08
Evaporator	21.27 $\pm$ 0.55 ^c	92.95 $\pm$ 6.34 ^c	121.42 $\pm$ 1.98 ^c	9.76 $\pm$ 0.11 ^c	17.85 $\pm$ 0.53 ^c	4.83 $\pm$ 0.27 ^c	268.08
Final paste	24.39 $\pm$ 0.99 ^d	85.86 $\pm$ 5.62 ^c	118.57 $\pm$ 3.12 ^c	11.41 $\pm$ 0.39 ^c	17.42 $\pm$ 0.78 ^c	4.58 $\pm$ 0.13 ^d	262.23
Cream 28/30	12.75 $\pm$ 0.49	109.96 $\pm$ 2.63	235.69 $\pm$ 2.74	2.89 $\pm$ 0.14	7.15 $\pm$ 0.02	3.69 $\pm$ 0.06	372.13
<i>Hot Break 22/24</i>							
Fresh tomatoes	1.79 $\pm$ 0.14 ^a	37.42 $\pm$ 2.84 ^a	50.17 $\pm$ 0.36 ^a	3.33 $\pm$ 0.13 ^a	27.63 $\pm$ 0.16 ^d	5.38 $\pm$ 0.29 ^b	125.72
Juice after scalders	13.93 $\pm$ 0.98 ^b	118.17 $\pm$ 8.12 ^b	144.80 $\pm$ 11.14 ^b	3.84 $\pm$ 0.23 ^b	34.68 $\pm$ 1.50 ^b	3.52 $\pm$ 0.14 ^c	318.94
Evaporator	20.46 $\pm$ 0.73 ^c	81.91 $\pm$ 2.94 ^c	100.04 $\pm$ 3.59 ^c	12.71 $\pm$ 0.73 ^b	24.30 $\pm$ 1.32 ^c	3.08 $\pm$ 0.01 ^d	242.50
Final paste	23.17 $\pm$ 0.68 ^d	79.54 $\pm$ 5.16 ^c	94.21 $\pm$ 1.50 ^c	13.05 $\pm$ 0.20 ^c	23.01 $\pm$ 0.03 ^d	2.76 $\pm$ 0.19 ^b	235.74
Cream 22/24	10.58 $\pm$ 0.64	106.74 $\pm$ 2.65	116.60 $\pm$ 3.68	2.13 $\pm$ 0.03	6.13 $\pm$ 0.26	3.01 $\pm$ 0.06	245.19
<i>Cold Break 28/30</i>							
Fresh tomatoes	1.79 $\pm$ 0.14 ^a	37.42 $\pm$ 2.84 ^a	50.17 $\pm$ 0.36 ^a	3.33 $\pm$ 0.13 ^a	27.63 $\pm$ 0.16 ^d	5.39 $\pm$ 0.29 ^b	125.73
Juice after scalders	13.29 $\pm$ 0.81 ^b	34.07 $\pm$ 0.94 ^b	43.03 $\pm$ 1.74 ^b	5.71 $\pm$ 0.46 ^b	26.86 $\pm$ 0.48 ^b	3.76 $\pm$ 0.14 ^c	126.72
Evaporator	17.94 $\pm$ 0.45 ^c	23.20 $\pm$ 0.13 ^b	35.95 $\pm$ 0.10 ^c	6.87 $\pm$ 0.43 ^b	20.69 $\pm$ 0.45 ^c	3.08 $\pm$ 0.02 ^d	107.73
Final paste	20.65 $\pm$ 0.28 ^m	22.92 $\pm$ 0.05 ^b	33.97 $\pm$ 0.66 ^c	9.90 $\pm$ 0.10 ^c	19.57 $\pm$ 0.61 ^c	2.91 $\pm$ 0.07 ^b	109.92

SD: standard deviation; DM: dry matter.

Table 3

Content of flavonols and flavanones ($\mu\text{g/g DM}$) expressed as mean $\pm$ SD of the four technological processes ($n = 6$); different letters in the columns represent statistically significant differences ($P < 0.05$).

	Rutin	Quercetin	Naringenin	Kaempferol-3-O-rutinoside	Naringenin-7-O-glucoside	Total flavonols and flavanones
Passata 10/12						
Fresh tomatoes	279.00 $\pm$ 13.09 ^a	6.12 $\pm$ 0.45 ^a	108.29 $\pm$ 1.08 ^a	1.06 $\pm$ 0.05 ^a	33.24 $\pm$ 2.04 ^a	427.71
Juice after scalders	456.60 $\pm$ 8.94 ^b	11.12 $\pm$ 0.15 ^b	305.99 $\pm$ 24.08 ^b	2.51 $\pm$ 0.08 ^b	41.52 $\pm$ 0.01 ^b	817.74
Evaporator	379.72 $\pm$ 1.09 ^c	6.57 $\pm$ 0.12 ^c	259.96 $\pm$ 8.83 ^c	2.46 $\pm$ 0.06 ^b	39.04 $\pm$ 1.04 ^c	687.75
Final paste	376.54 $\pm$ 2.04 ^c	6.25 $\pm$ 0.32 ^c	241.66 $\pm$ 15.33 ^d	2.43 $\pm$ 0.04 ^b	37.67 $\pm$ 1.74 ^d	664.55
Cream 10/12	197.38 $\pm$ 8.68	4.10 $\pm$ 0.26	195.39 $\pm$ 4.90	2.31 $\pm$ 0.14	9.23 $\pm$ 0.19	408.41
Hot Break 28/30						
Fresh tomatoes	326.56 $\pm$ 0.16 ^d	9.04 $\pm$ 0.06 ^d	115.50 $\pm$ 6.87 ^a	1.15 $\pm$ 0.10 ^a	34.34 $\pm$ 0.99 ^e	486.59
Juice after scalders	557.98 $\pm$ 34.26 ^e	11.77 $\pm$ 0.33 ^e	304.59 $\pm$ 10.64 ^b	1.66 $\pm$ 0.08 ^c	39.00 $\pm$ 2.00 ^f	915.00
Evaporator	462.15 $\pm$ 16.77 ^f	4.27 $\pm$ 0.04 ^f	242.08 $\pm$ 10.93 ^c	1.34 $\pm$ 0.08 ^d	35.58 $\pm$ 0.09 ^g	745.42
Final paste	455.48 $\pm$ 5.09 ^f	4.24 $\pm$ 0.11 ^f	208.97 $\pm$ 10.31 ^f	1.28 $\pm$ 0.01 ^d	34.73 $\pm$ 2.22 ^{g,h}	704.70
Cream 28/30	296.59 $\pm$ 14.70	4.71 $\pm$ 0.25	219.21 $\pm$ 1.48	2.17 $\pm$ 0.03	7.54 $\pm$ 0.12	530.22
Hot Break 22/24						
Fresh tomatoes	298.79 $\pm$ 0.01 ^e	15.33 $\pm$ 0.54 ^e	298.14 $\pm$ 10.3 ^a	3.25 $\pm$ 0.25 ^e	30.23 $\pm$ 0.19 ^h	645.74
Juice after scalders	592.36 $\pm$ 7.01 ^h	19.82 $\pm$ 0.23 ^h	461.09 $\pm$ 6.36 ^b	3.49 $\pm$ 0.10 ^f	32.51 $\pm$ 0.01 ⁱ	1109.27
Evaporator	503.58 $\pm$ 18.26 ^g	8.03 $\pm$ 0.14 ^g	373.67 $\pm$ 13.96 ^c	3.35 $\pm$ 0.18 ^e	31.51 $\pm$ 1.13 ^j	920.14
Final paste	486.10 $\pm$ 9.00 ^g	8.01 $\pm$ 0.07 ^g	341.09 $\pm$ 4.17 ^c	3.32 $\pm$ 0.09 ^e	30.77 $\pm$ 0.23 ^{j,k}	869.29
Cream 22/24	322.32 $\pm$ 3.59	5.03 $\pm$ 0.03	278.89 $\pm$ 1.76	2.20 $\pm$ 0.08	2.68 $\pm$ 0.12	611.12
Cold Break 28/30						
Fresh tomatoes	298.79 $\pm$ 0.01 ^h	15.33 $\pm$ 0.54 ^e	298.14 $\pm$ 10.03 ^a	3.25 $\pm$ 0.25 ^e	30.23 $\pm$ 0.19 ^h	645.74
Juice after scalders	205.05 $\pm$ 2.74 ^k	14.80 $\pm$ 0.59 ^f	140.46 $\pm$ 1.56 ^k	2.85 $\pm$ 0.19 ^f	29.09 $\pm$ 0.06 ^h	392.25
Evaporator	172.26 $\pm$ 1.97 ⁱ	5.78 $\pm$ 0.39 ^f	105.56 $\pm$ 1.96 ⁱ	2.59 $\pm$ 0.13 ^h	27.15 $\pm$ 0.35 ^j	313.34
Final paste	169.02 $\pm$ 3.32 ^j	5.46 $\pm$ 0.49 ^f	95.34 $\pm$ 3.01 ^m	2.43 $\pm$ 0.02 ^h	26.27 $\pm$ 0.57 ^j	298.52

SD: standard deviation; DM: dry matter.

in the cream, which explains the high content in the juice after scalders. Rutin was mostly affected by the evaporation stage, while the sterilization stage caused an insignificant reduction ($P > 0.05$) in rutin content. The content of rutin remained stable between 81.64% and 82.47% for the Hot Break 28/30 and Passata 10/12 processes, respectively. For quercetin, significant changes ($P > 0.05$) occurred as the tomato fruit was processed. Quercetin decreased more than one-fold as a result of processing, since it is a phenolic compound with potent antioxidant properties, and therefore more easily oxidized. Taking into account, kaempferol-3-O-rutinoside, evaporation and sterilization steps did not significantly modify ($P < 0.05$) the levels of kaempferol-3-O-rutinoside for Passata 10/12 and Hot Break 22/24.

Flavanones were mainly represented by naringenin (m/z 271 $\rightarrow$ 151) and naringenin-7-O-glucoside (m/z 433 $\rightarrow$ 271). We checked the UV spectroscopic properties of naringenin and chalconaringenin. The polyphenol found in our varieties is naringenin, as this polyphenol gave its maximum UV absorption at 280 nm, since chalconaringenin has its maximum UV absorption at 370 nm and was not present in our samples.

Statistically significant increases ($P < 0.05$) occurred for naringenin as the tomato fruit was processed into juice in Passata 10/12, Hot Break 22/24 and Hot Break 28/30 (Table 3). The concentration of naringenin was relatively high in the cream, which explained the high content in the juice after scalders in comparison to naringenin-7-O-glucoside. As a result of further processing steps, naringenin remained stable from 67.87% for Cold Break 28/30 to 78.98% for Passata 10/12. For naringenin-7-O-glucoside, significant changes ($P < 0.05$) were observed in the evaporation stage in the Hot Break 28/30 process. Subsequent sterilization steps did not further modify ($P > 0.05$) the levels of flavanones.

3.3. Correlation between phenolic compounds and the four technological processes

A PCA was performed to visualize the effect of each stage of industrial tomato processing. The phenolic and antioxidant profile were influenced by different processing steps and treatments. Two

principal components (PC1 and PC2) accounted for 70.14% of the variability of the original data (Fig. 2). PC1 allowed differentiation between samples in which cream had been added, which are located on the right-hand side of the plot, and the remaining steps of the technological processes, which are situated more on the left-hand side. PC2 corresponded to the stepwise processing of fresh tomatoes, situated in the bottom part of the plot (step 1), to final paste (steps 3 and 4). Samples from stage 2 of the Hot Break processes are well related to TP, hydrophilic antioxidant capacity, ferulic-O-hexoside, chlorogenic, caffeic-O-hexoside and protocatechuic acids and quercetin, rutin, naringenin, naringenin-7-O-glucoside and kaempferol-3-O-rutinoside, whereas samples from stage 2 of the Cold processes scored the lowest values of these parameters, as they were located more on the left-hand side (Fig. 3). Moreover, stages 3 and 4 were better related to caffeic and ferulic acids, as both hydroxycinnamic acids increased during thermal processing.

4. Discussion

In this study we focused on the effects of processing on tomato polyphenols subjected to different processes: Passata 10/12, Cold

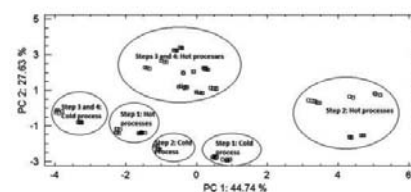


Fig. 2. Score plot of PC1 vs PC2 of the different processing stages and treatments. Stage 1: fresh tomatoes; stage 2: tomato paste from scalders; stages 3 and 4: tomato paste from evaporators and final stages. PC: principal component.

Resultats

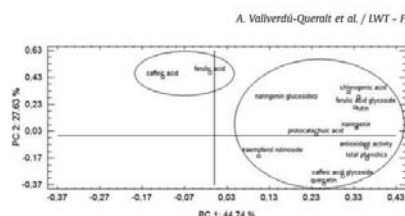


Fig. 3. Principal components plot of all the technological processes studied. PC: principal component.

Break 28/30, Hot Break 28/30 and Hot Break 22/24. Thermal processing of tomatoes into paste involved a number of heating stages which could be expected to have an effect on heat-labile and oxidizable compounds. The intactness of the cellular matrix of tomatoes determines the bioavailability of TPs and hydrophilic antioxidant capacity. Processing can usually disrupt this cellular matrix. Dewanto et al. reported that total polyphenols did not change during the processing of tomatoes (Dewanto, Wu, Adom, & Liu, 2002). In contrast, our results first showed an increase in TPs when fresh tomatoes were processed into Passata 10/12, Hot Break 22/24 and Hot Break 28/30 juices as a consequence of cream addition. It should be noted that in Cold Break 28/30, antioxidant levels and TPs did not increase, as this process was not enriched with cream. Due to the supposed negative effects of the industrial processing of tomato-based products, the main strategy for minimizing eventual processing damage was "reconstitution", achieved by adding a cream rich in seeds and peels with high levels of antioxidants and polyphenols to tomato-based products. It is a source of functional ingredients and is a common practice in food industries, as compounds present in the seed and skin fragments are separately extracted and then returned to paste during this stage. As a result, TP and antioxidant activity experienced significant increases. Moreover, cream addition increases viscosity of the product. Later, after the addition of the cream, significant losses in TPs and antioxidant capacity were observed when the conditions required more extensive processing. At the last stage, after sterilization and bottling, a slight decrease was detected in TP content and antioxidant activity.

The changes in specific polyphenols were monitored during the paste-production process. Phenolic antioxidants, including rutin and naringenin, were identified as major antioxidants in these extracts, as was reported in other studies (Le Gall et al., 2003; Slimestad et al., 2008). The abundance of these flavonoids increased significantly in the second step for Passata 10/12, Hot Break 22/24 and Hot Break 28/30, as these processes were subjected to cream addition, whereas for Cold Break 28/30 the increase was not observed. The concentrations of rutin and naringenin were relatively high in the cream, which explained their high content in the final paste. For subsequent stages of the processes, the content of naringenin decreased. Our results are in accordance with Re et al., who analyzed the effects of three different methods of processing tomatoes into tomato sauce (Re, Bramley, & Rice-Evans, 2002). The methods used were: Hot Break processing at 90 °C, Cold Break processing at 65 °C and super Cold Break processing. Naringenin was strongly affected by processing. Naringenin-7-O-glucoside followed the same pattern during the paste-making process. The decreases in naringenin and naringenin-7-O-glucoside could be attributed to the formation of reactive-O-quinones and the extensive oxidation process created during thermal processing (Singleton, 1987).

Capanoglu et al. found that rutin decreased after treating the samples in a three-effect evaporator unit which included heat treatment up to 80 °C (Capanoglu et al., 2008). However, it could not be hypothesized that the disappearance of rutin is due to hydrolysis, as quercetin (its hydrolysis product) did not increase during heat thermal processing. These results are in agreement with those described previously (Lavelli, Hippeli, Peri, & Elstner, 1999). It is obvious that the presence of oxygen accelerates the degradation of quercetin and rutin. The decay is caused by the so-called reactive oxygen species. These radicals may be captured by flavonols, and during the thermal processing the flavonols are able to transfer one or two hydrogen atoms, forming quinonoid structures, which leads to the formation of quinones (Hvattum, Stenstrom, & Ekeberg, 2004). In the case of rutin, the 3-hydroxy-function at the C-ring of the flavonoid is blocked by a sugar moiety, whereas for quercetin it remains unoccupied. Thus, the blockage of the 3-hydroxyl group is the essential reason for the higher stability of rutin toward oxidation (Makris & Rossiter, 2000).

Similarly, for kaempferol-3-O-rutinoside, the 3-hydroxy-function is blocked. This explains the thermal stability of kaempferol-3-O-rutinoside toward quercetin. Kaempferol conjugates are commonly identified in food plants (Andrade, Mendes, Falco, Valentao, & Seabra, 2001; Le Gall et al., 2003) such as tomato fruit and wine grape varieties. Monagas et al. found that vinification and maturation stimulated the hydrolysis of glycosides in *Vitis vinifera* grapes and, as a consequence, kaempferol conjugates decreased at a rate consistent with temperature (Monagas, Bartolome, & Gomez-Cordoves, 2005). These results are in accordance with our study. Kaempferol-3-O-rutinoside decreased when the conditions required more extensive processing at higher temperatures.

Tomato varieties also contain hydroxycinnamic acids such as chlorogenic acid, which increased after the second stage (juice after scalders), especially for Passata 10/12 and Hot Break 28/30; and then slightly decreased for all the technological processes studied. Chlorogenic acid may be oxidized to reactive O-quinones through the catalytic oxidation process. O-quinones could interact with NH₂ groups of amino acids, causing nutritional damage (Friedman, 1992). The same effect was observed for caffeic acid-O-hexoside and ferulic acid-O-hexoside. It has been found that after air-drying at 80 °C, the number of free hydroxyl phenol groups increases owing to the hydrolysis of flavonoid glycosides and/or the release of cell wall polyphenols (Lavelli et al., 1999). As a result of thermal processing, ferulic and caffeic acids increased between one- and three-fold in all the processes. Our results are in accordance with those reported by Re et al., in which caffeic, ferulic and p-coumaric acids increased during heat treatment (Re et al., 2002).

Phenolic acids are also present in tomato fruit (Shen, Chen, & Wang, 2007); protocatechuic acid was found to increase after the addition of cream in Passata 10/12 and Hot Break 28/30; then slightly decreased for the Passata 10/12 process, and the greatest decreases were observed for Hot Break 28/30. This can be related to oxygen exposure during processing and may result in the formation of oxidation products.

5. Conclusions

The most critical events in tomato processing are the cream addition process and the evaporation stage, as is reflected in the ANOVA and PCA analysis. Firstly, the importance of the cream addition is seen in its balancing effect on the technological process. Secondly, the evaporation stage and, to a lesser extent, the sterilization stage during the paste-making process have a negative effect on the number of dominant polyphenols in tomatoes. A gradual and significant decrease was observed during the industrial processing of tomatoes. The greatest losses in antioxidant and phenolic

compounds were associated with conditions requiring more extensive processing. The polyphenol changes in tomato paste during processing may result from two different reactions: (a) the oxidative degradation of polyphenols which lead to the formation of reactive-O-quinone and the autocatalytic phenolic oxidation products; (b) the hydrolysis of conjugate structures. So as to minimize losses in bioactive compounds, recommendations focus on shortening processing time and adding a cream rich in functional ingredients present in the seed and skin fragments which are separately extracted and then returned to paste so as to balance possible losses in phenolic and antioxidants compounds.

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4. Capítol IV: Canvis en el perfil fenòlic i en l'activitat antioxidant durant la producció de daus de tomàquet

Anna Vallverdú-Queralt, Alexander Medina-Remón, Cristina Andrés-Lacueva & Rosa M. Lamuela-Raventós. *Food Chemistry*. 2011, 126: 1700-1707.

Resum: Aquesta publicació tracta d'avaluar diferents tractaments industrials [*hot*, *cold* i *cold* amb sals de calci (CaCl_2)] per a la producció de daus de tomàquet. L'estudi es va dur a terme durant els anys 2008 i 2009. Es va fer l'anàlisi de polifenols totals, amb prèvia neteja mitjançant *SPE*, l'assaig de capacitat antioxidant mitjançant els mètodes: *DPPH* i *ABTS*⁺ i, finalment, l'anàlisi de fenols individuals mitjançant *HPLC-MS/MS*. En aquest estudi, el tractament *cold* sense l'addició de CaCl_2 és el que millor conserva els polifenols i antioxidants durant el processat dels daus; en canvi, el tractament *hot* produeix les pèrdues més importants de compostos fenòlics. Per tant, el tractament tèrmic és un factor clau a tenir en compte en la producció de daus de tomàquet. D'altra banda, l'addició d'una salsa rica en pells i llavors durant el processat permet enriquir el producte final amb més contingut de components bioactius.



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Changes in phenolic profile and antioxidant activity during production of diced tomatoes

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ABSTRACT

Tomatoes and tomato-based products are rich in antioxidants such as carotenoids, vitamin C and polyphenols. The industrial processing of diced tomatoes involves heat treatments in which these antioxidant compounds may be potentially affected. In this study, we evaluate the effect of each separate step in the dice-making process. Three technological processes were investigated: Hot, Cold and Cold treated with calcium salt (CaCl_2). Four stages were monitored in each process: (1) fresh tomatoes; (2) peeled tomatoes; (3) diced tomatoes; and (4) final product after sauce addition. The main tool for minimising or counteracting the eventual processing damage was the strategy of 'reconstitution', achieved by adding a sauce rich in seeds and peels with high levels of antioxidants and phenolics to the diced tomatoes. Different analyses were carried out in order to evaluate the effect of each processing step. First, total polyphenols (TP) were evaluated using Folin–Ciocalteu (F–C) assay and antioxidant activity using ABTS[•] and DPPH assays. Flavonols, flavanones, hydroxycinnamic and phenolic acids were then quantified using liquid chromatography/electrospray ionisation tandem mass spectrometry (HPLC–ESI–MS/MS). The combination of principal component analysis (PCA) and analysis of variance (ANOVA) revealed that each processing step induces alterations in the antioxidant and phenolic profile, and in particular sauce addition and calcium treatment significantly affected the levels of antioxidants and phenolics during the dice-making process.

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1. Introduction

The cultivated tomato (*Lycopersicon esculentum*) originated in the New World from wild species that are native to the Andean region of South America. In the 16th century, the tomato was taken to Europe. The tomato fruit was accepted as a food remarkably slowly at first, but then became very popular (Beecher, 1998). It has been suggested that consuming tomatoes may reduce the risk of several chronic diseases such as cancer, cardiovascular and cerebrovascular diseases, diabetes and hypertension (Bagchi et al., 2003; Blum, Monir, Wirsansky, & Ben-Arzi, 2005; Das, Otani, Maulik, & Das, 2005; Lambert & Yang, 2003; Ness & Powles, 1997; Nishino et al., 2004; Yochum, Kushi, Meyer, & Folsom, 1999).

Diced tomatoes represent a reservoir of potentially healthy micronutrients such as ascorbic acid, vitamin E and minerals (potassium). In addition to their micronutrient content, diced

tomatoes also contain valuable phytochemicals or antioxidants. The antioxidant activity of tomato end products often changes when more invasive processing steps are used (Powell & Benet, 2002). Greater attention should be given to minimising the detrimental effects of the processing methods. Although the effect of processing on the lycopene levels of tomatoes has previously been reviewed (Shi & Le Maguer, 2000), the exact levels of gains or losses reported during processing differ widely depending on the type and conditions of the process applied (Dewanto, Wu, Adom, & Liu, 2002; Giovanelli, Zanoni, Lavelli, & Nani, 2002; Lavelli, Peri, & Rizzolo, 2000; Re, Bramley, & Rice-Evans, 2002). Understanding the mechanisms taking place during processing will lead to several innovations in the food industry, which is required to monitor optimal technical and environmental parameters.

Diced tomatoes are processed in a similar way to peeled tomatoes up to the peeling stage, after which the product is cut into cubes and preheated to 50 °C. Then it is sent for the final sauce addition, at 90 °C, and packaged. Diced tomatoes are subjected to losses of tissue integrity as they are submitted to high temperatures and shear stress through pumps, strainers, fillers and tanks (Ma & Barrett, 2002). The outer cell wall of tomatoes is made of

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cellulose microfibrils embedded in a matrix of pectins, hemicelluloses and proteins. During processing, pectin is altered by naturally occurring enzymes in the fruit, causing the fruit to soften. These alterations may involve the breakdown of the pectin chains. Therefore, tomatoes are processed with calcium salt (CaCl_2) to improve the texture of the final product (Gould, 2009). Divalent calcium ions induce the formation of calcium pectate or pectinates by binding pectin methylsterase, which results in a firmer and more resistant product.

The objective of this work was to extensively examine the effects of thermal processing and calcium treatment on diced tomatoes, taking into account their antioxidant activity, total polyphenols (TP), and content of flavonols (kaempferol-3-O-rutinoside, rutin and quercetin), flavanones (naringenin and naringenin-7-O-glucoside), hydroxycinnamic acids (chlorogenic, caffeic, caffeic-O-hexoside, ferulic and ferulic-O-hexoside), and phenolic acids (protocatechuic and gallic) in three different processes: Hot treatment, Cold treatment and Cold treatment processed with calcium salt (CaCl_2).

2. Materials and methods

2.1. Standards and reagents

All samples and standards were handled without exposure to light. Quercetin, rutin, caffeic, ferulic, protocatechuic and chlorogenic acids, Folin-Ciocalteu (F-C) reagent, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), PBS: phosphate-buffered saline pH 7.4, Trolox: ($\pm$)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid 97% and manganese dioxide were purchased from Sigma® (Madrid, Spain); DDPH: 2,2-diphenyl-1-picrylhydrazyl, naringenin, naringenin-7-O-glucoside and kaempferol-3-O-rutinoside from Extrasynthèse (Genay, France); hydrochloric acid 35% and acetic acid 99.8% from Panreac (Barcelona, Spain); and anhydrous sodium acetate (2 M) from Merck (Darmstadt, Germany). Methanol and formic acid were obtained from Scharlau (Barcelona, Spain). Ultrapure water (Milli-Q) from Millipore Corporation (Bedford, MA, USA).

2.2. Sampling and processing conditions

The tomatoes in these samples were a mixture of varieties H-9661, H-9997, H-9776, Albastros, Malva, Guadiva and Elegy, harvested in the surrounding areas of Extremadura, Spain. In order to estimate differences in the original fruit sources or harvest years, each step in the process was independently sampled six times from different fruit batches processed over two consecutive years, 2008 and 2009. From a technological point of view, the main difficulty in the production of diced-tomato products with a good consistency is choosing the right variety of fruit with suitable characteristics.

In the technological process, tomatoes were first cleaned, then peeled and diced. After that, the dices were sent to mixing tanks. Dices were mixed with a tomato sauce in a ratio of 50/50 inside a special upright tank to allow maximum heat exchange, as well as minimal mechanical damage to the cubes. The sauce for the diced mixture was stabilised at over 90 °C for at least 5 min. In this stage, CaCl_2 was added in one of the processes to cause the diced product to harden, thereby making it firmer and consequently easier to pump. Calcification can occur by direct addition of calcium to the container or by conveying the dices through a calcium bath. The amount of calcium cannot exceed 0.08% by weight (Code of Federal Regulations, 2000). Having completed the preparation stage, the dices were sent to the aseptic sterilising-cooling system. Inside the tubular steriliser, the product was gradually sterilised up

to a temperature of 115 °C, kept at this temperature for at least 5 min and then quickly cooled to about 30–35 °C in a tubular exchanger, using cold and/or refrigerated water. The aseptic diced products were put into special bags. Dices have an 18- to 24-month shelf life.

In this study, tomatoes were subjected to three different treatments: Hot, Cold and Cold processed with CaCl_2 . During Hot treatment, fresh tomatoes were processed at higher temperatures than in the two Cold treatments; in one of the Cold treatments, CaCl_2 was added to dices in order to keep the consistency of the product. Four sample stages were monitored: (1) fresh tomatoes; (2) peeled tomatoes (3) diced tomatoes; and (4) final product.

2.3. Preparation of the samples

Sample treatments were performed following the procedure of Vallverdú-Queralt, Jáuregui, Medina-Remón, Andrés-Lacueva, & Lamuela-Raventós (2010), after the extraction procedure, solid phase extraction (SPE) was carried out. Firstly, 1 ml of methanol and subsequently 1 ml of sodium acetate 50 mmol/l pH 7 were loaded into Oasis® MAX cartridges from Waters to equilibrate the sample; then, 1 ml of each extract was diluted with 1 ml of Milli-Q water and acidified with 34 μ l of hydrochloric acid before being loaded into the cartridges separately. These were rinsed with 50 mmol/l pH 7 (5% methanol) sodium acetate. The polyphenols were eluted with 1800 μ l of methanol (2% formic acid). The eluted fractions were evaporated under nitrogen flow, and the residues were reconstituted with water (0.1% formic acid) up to 250 μ l and filtered through a 13 mm, 0.45 μ m PTFE filter (Waters) into an insert-amber vial for HPLC analysis. Samples were stored at –20 °C until analysis.

2.4. Analysis of total polyphenols

For the TP assay, each sample was analysed in triplicate: 20 μ l of the eluted fractions were mixed with 188 μ l of Milli-Q water in a thermo microtiter 96-well plate (nunc™, Roskilde, Denmark); afterwards, 12 μ l of F-C reagent and 30 μ l of sodium carbonate (200 g/l) were added (Medina-Remón et al., 2009). The mixtures were incubated for 1 h at room temperature in the dark. After the reaction period, 50 μ l of Milli-Q water were added and the absorbance was measured at 765 nm in a UV/VIS Thermo Multiskan Spectrum spectrophotometer (Vantaa, Finland). This spectrophotometer allowed the absorbance of a 96-well plate to be read in 10 s. Results were expressed as mg of gallic acid equivalents (GAE)/100 g dry material (DM).

2.5. Antioxidant activity

The antioxidant activity in diced tomatoes was measured using an ABTS® radical decolorization assay (Minoggio et al., 2003) and DDPH assay (Odziozola-Serrano, Soliva-Fortuny, & Martín-Belloso, 2008), with minor modifications.

2.5.1. ABTS® assay

1 mM Trolox (antioxidant standard) was prepared in PBS once a week. Working standards were prepared daily by diluting 1 mM Trolox with PBS.

An ABTS® radical cation was prepared by passing a 5 mM aqueous stock solution of ABTS (in PBS) through manganese dioxide powder. Excess manganese dioxide was filtered through a 13 mm 0.45 μ m filter PTFE (Waters). Before analysis, the solution was diluted in PBS pH 7.4 to give an absorbance at 734 nm of 1.0 ± 0.1 , and pre-incubated in ice. Afterwards, 245 μ l of ABTS® solution was added to 5 μ l of Trolox or to tomato samples and the solutions were stirred for 30 s. The absorbance was recorded continuously

every 30 s with a UV/VIS Thermo Multiskan Spectrum spectrophotometer for 1 h and PBS blanks were run in each assay.

The working range for Trolox (final concentration 0–750 μM) was based on triplicate determinations and consisted of plotting the absorbance as a percentage of the absorbance of the uninhibited radical cation (blank). The activities of the tomato samples were assessed at four different concentrations, which were within the range of the dose–response curve. Each sample was analysed in triplicate at each concentration. Results were expressed as mmol Trolox equivalent (TE)/100 g DM.

2.5.2. DPPH assay

The antioxidant activity was also studied through the evaluation of free radical-scavenging effect on DPPH radical. Solutions of known Trolox were used for calibration. 5 μL of tomato samples or Trolox were mixed with 250 μL of methanolic DPPH (0.025 g/L). The homogenate was shaken vigorously and kept in darkness for 30 min. Absorption of the samples was measured on a UV/VIS Thermo Multiskan Spectrum spectrophotometer at 515 nm. The percentage of inhibition of the DPPH was calculated and plotted as a function of the concentration of Trolox for the standard reference data. The final DPPH values were calculated using a regression equation between the Trolox concentration and the percentage of DPPH inhibition and results were expressed as mmol TE/100 g DM.

2.6. HPLC–ESI–MS/MS analysis

The polyphenols of tomato fruits were identified in a previous study using HPLC/ESI-LTQ-Orbitrap-MS (Vallverdú-Queralt et al., 2010). To evaluate the effect of the dicing process on polyphenols, we selected the most predominant polyphenols in tomato fruit. Those polyphenols were quantified using HPLC–ESI–MS/MS. An API 3000 (PE Sciex, Concord, Ontario, Canada) triple quadrupole mass spectrometer equipped with a Turbo Ionspray source in negative-ion mode was used to obtain MS/MS data. Turbo Ionspray source settings were as follows: capillary voltage, –3500 V; nebuliser gas (N_2), 10 arbitrary units (a.u.); curtain gas (N_2), 12 a.u.; collision gas (N_2), 10 a.u.; focusing potential, –200 V; entrance potential, 10 V; drying gas (N_2), heated to 400 °C and introduced at a flow rate of 8000 m^3/min . The declustering potential and collision energy were optimised for each compound in infusion experiments: individual standard solutions (10 $\mu\text{g}/\text{mL}$) dissolved in 50:50 (v/v) mobile phase were infused at a constant flow rate of 5 $\mu\text{L}/\text{min}$ using a model syringe pump (Harvard Apparatus, Holliston, MA, USA).

For quantification purposes, data was collected in the multiple reaction monitoring (MRM) mode, tracking the transition of specific parent and product ions for each compound. In particular, we selected 12 transitions corresponding to the most abundant polyphenols in tomato fruits: ferulic acid m/z 193 $\rightarrow$ 134 (CE: –20 V); ferulic acid-O-hexoside m/z 355 $\rightarrow$ 193 (CE: –20 V); chlorogenic acid m/z 353 $\rightarrow$ 191 (CE: –20 V); caffeic acid m/z 179 $\rightarrow$ 135 (CE: –20 V); caffeic acid-O-hexoside m/z 341 $\rightarrow$ 162 (CE: –20 V); protocatechuic acid m/z 153 $\rightarrow$ 109 (CE: –20 V); gallic acid m/z 169 $\rightarrow$ 125 (CE: –20 V); quercetin m/z 301 $\rightarrow$ 151 (CE: –30 V); rutin m/z 609 $\rightarrow$ 300 (CE: –50 V); naringenin m/z 271 $\rightarrow$ 151 (CE: –30 V); naringenin-7-O-glucoside m/z 433 $\rightarrow$ 271 (CE: –20 V); kaempferol-3-O-rutinoside m/z 593 $\rightarrow$ 285 (CE: –30 V); ethyl galate (internal standard) m/z 197 $\rightarrow$ 169 (CE: –25 V). The standards of these polyphenols confirmed the previously reported fragmentation patterns. The compounds were deduced from the different transition signals and by comparing the observed accurate transition and retention times with those of the standards.

Quantification of polyphenols was carried out using the internal standard method. Polyphenols were quantified with respect to their corresponding standard. When standards were not available,

as in the case of caffeic acid-O-hexoside and ferulic-O-hexoside, they were quantified with respect to the corresponding hydroxycinnamic acid (caffeic and ferulic acids).

The liquid chromatograph was an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, an autosampler and a column oven set to 30 °C. A Luna C₁₈ column 50 $\times$ 2.0 mm i.d., 5 μm (Phenomenex, Torrance, CA, USA) was used. The injection volume was 20 μL and the flow rate was 0.4 mL/min. Mobile phases consisted of water/0.1% formic acid (A) and acetonitrile/0.1% formic acid (B). Separation was carried out in 15 min under the following conditions: 0 min, 5% B; 10 min, 18% B; 13 min, 100% B; 14 min, 100% B; 15 min, 5% B. The column was equilibrated for 5 min prior to each analysis.

2.7. Statistical analysis

The significance of the results was analysed using the Statgraphics Plus v.5.1 Windows Package (Statistical Graphics Co., Rockville, Md). Analysis of variance (ANOVA) was used to compare the means of groups of measurement data, and principal component analysis (PCA) was carried out to obtain correlations amongst variables. PCA is a multivariate statistical technique that allows us to visualise the original arrangement of tomato samples in an n -dimensional space, by identifying the directions in which most of the information is retained. The scores plot was created to define different groups and the loadings plot to highlight differences among variables.

3. Results

3.1. Analysis of TP content and antioxidant activity during production of diced tomatoes

The relative contributions of individual compounds to TPs and the antioxidant activity of the three technological processes are shown in Table 1. No significant changes ($P > 0.05$) were observed over the two consecutive years, 2008 and 2009. To avoid water evaporation and concentration of solids affecting the quantification during thermal processing, the estimated values were dry weight based, per gram of dry matter (DM).

The content of TPs in diced tomatoes varied from 320.8 ± 7.1 mg GAE/100 g DM for the final product after Cold treatment to 138.2 ± 4.2 mg GAE/100 g DM for diced tomatoes subjected to Hot treatment. The antioxidant activity measured by ABTS⁺ assay was between 20.8 ± 1.0 mmol TE/100 g DM for the final product after Cold treatment and 9.5 ± 0.6 mmol TE/100 g DM for diced tomatoes subjected to Hot treatment. The values obtained by DPPH assay were between 22.1 ± 0.9 mmol TE/100 g DM for the final product after Cold treatment and 13.3 ± 0.4 mmol TE/100 g DM for diced tomatoes subjected to Hot treatment. The content of TPs and antioxidant activity decreased significantly ($P < 0.05$) from fresh to peeled tomatoes. The greatest decrease appeared to be associated with tomatoes subjected to Hot treatment. In the second step, when peeled tomatoes were cut into cubes, TP decreased by 3.02% and antioxidant activity by 12.84% and 15.82% for ABTS⁺ and DPPH assays, respectively.

Consistent changes ($P < 0.05$) in TPs and antioxidant activity were observed as diced tomatoes were mixed with a sauce to obtain the final product. This sauce contains seeds and peels with high levels of antioxidants and polyphenols (Table 1). The greatest increase in total phenolics and antioxidant activity occurred when dices were subjected to Cold treatment without adding CaCl_2 .

Resultats

Table 1

TPs and antioxidant activity (ABTS[•], DPPH) expressed as mean ± SD of the three technological processes; different letters in the columns represent statistically significant differences ($P < 0.05$).

Sample	Total polyphenols (mg GAE/100 g sample DM)	ABTS [•] (mmol TE/100 g DM)	DPPH (mmol TE/100 g DM)
Process 1: Hot treatment			
1 Fresh tomatoes	150.3 ± 7.4 ^a	17.5 ± 0.5 ^{ab}	20.8 ± 0.3 ^a
2 Peeled tomatoes	142.5 ± 5.1 ^b	10.9 ± 0.2 ^c	15.8 ± 0.2 ^b
3 Diced tomatoes	138.2 ± 4.2 ^c	9.5 ± 0.6 ^d	13.3 ± 0.4 ^c
4 Final product	190.7 ± 4.5 ^d	17.0 ± 0.4 ^a	20.2 ± 0.3 ^a
Hot sauce	179.5 ± 6.1	12.4 ± 0.7	14.6 ± 0.4
Process 2: Cold treatment			
1 Fresh tomatoes	190.5 ± 7.9 ^e	18.2 ± 0.6 ^b	20.5 ± 0.3 ^a
2 Peeled tomatoes	181.5 ± 6.3 ^f	12.6 ± 0.3 ^c	18.8 ± 0.4 ^d
3 Diced tomatoes	178.4 ± 4.3 ^g	11.4 ± 0.4 ^d	16.5 ± 0.5 ^e
4 Final product	320.8 ± 7.1 ^h	20.8 ± 1.0 ^f	22.1 ± 0.9 ^f
Cold sauce	180.4 ± 5.6	14.4 ± 0.8	18.0 ± 0.6
Process 3: Cold treatment with CaCl₂			
1 Fresh tomatoes	190.5 ± 7.9 ^e	18.2 ± 0.6 ^b	20.5 ± 0.3 ^a
2 Peeled tomatoes	181.5 ± 6.3 ^f	12.6 ± 0.3 ^c	18.8 ± 0.4 ^d
3 Diced tomatoes	178.4 ± 4.3 ^g	11.4 ± 0.4 ^d	16.5 ± 0.5 ^e
4 Final product	220.2 ± 4.9 ^h	16.6 ± 0.3 ^h	19.4 ± 0.3 ^g
Cold sauce	180.4 ± 5.6	14.4 ± 0.8	18.0 ± 0.6

SD, standard deviation; GAE, gallic acid equivalent; DM, dry matter; TE, Trolox equivalent.

Table 2

Content of flavonols and flavanones expressed as mean ± SD of the three technological processes; different letters in the columns represent statistically significant differences ($P < 0.05$).

	Rutin (μg/g DM)	Quercetin (μg/g DM)	Kaempferol-3-O-rutinoside (μg/g DM)	Naringenin (μg/g DM)	Naringenin-7-O-glucoside (μg/g DM)
Process 1: Hot treatment					
1 Fresh tomatoes	141.89 ± 4.11 ^a	11.66 ± 0.89 ^a	4.71 ± 0.44 ^a	24.50 ± 0.28 ^a	13.41 ± 0.62 ^a
2 Peeled tomatoes	116.10 ± 5.19 ^b	8.99 ± 0.08 ^b	3.83 ± 0.12 ^b	19.29 ± 0.07 ^b	9.57 ± 0.35 ^b
3 Diced tomatoes	114.64 ± 0.97 ^b	7.68 ± 0.25 ^c	3.62 ± 0.03 ^c	18.34 ± 0.42 ^b	8.94 ± 0.10 ^b
4 Final product	154.36 ± 1.87 ^c	10.91 ± 0.11 ^d	4.22 ± 0.00 ^d	142.65 ± 3.42 ^c	17.47 ± 0.56 ^c
Hot sauce	310.60 ± 0.63	4.93 ± 0.11	1.16 ± 0.04	107.56 ± 4.19	10.20 ± 0.46
Process 2: Cold treatment					
1 Fresh tomatoes	289.73 ± 4.85 ^a	14.78 ± 0.17 ^a	7.19 ± 0.25 ^a	54.47 ± 0.03 ^a	17.61 ± 0.44 ^a
2 Peeled tomatoes	260.28 ± 0.26 ^b	10.96 ± 0.08 ^b	6.34 ± 0.13 ^b	43.70 ± 1.28 ^a	14.54 ± 0.10 ^b
3 Diced tomatoes	255.84 ± 0.92 ^c	9.83 ± 0.06 ^b	6.24 ± 0.25 ^c	42.50 ± 2.54 ^a	13.56 ± 0.07 ^b
4 Final product	417.43 ± 2.80 ^d	14.20 ± 0.23 ^b	7.30 ± 0.11 ^d	208.56 ± 3.47 ^d	40.18 ± 1.79 ^d
Cold sauce	288.64 ± 3.01	5.04 ± 0.28	1.81 ± 0.03	205.00 ± 7.07	15.07 ± 1.03
Process 3: Cold treatment with CaCl₂					
1 Fresh tomatoes	289.73 ± 23.24 ^a	14.78 ± 0.17 ^a	7.19 ± 0.25 ^a	54.47 ± 0.03 ^a	17.61 ± 0.44 ^a
2 Peeled tomatoes	260.28 ± 0.26 ^b	10.96 ± 0.02 ^b	6.34 ± 0.13 ^b	43.70 ± 1.28 ^a	14.54 ± .10 ^b
3 Diced tomatoes	255.84 ± 9.40 ^c	9.83 ± 0.00 ^b	6.24 ± 0.46 ^b	42.50 ± 2.54 ^a	13.56 ± 0.07 ^b
4 Final product	281.81 ± 5.47 ^d	10.99 ± 0.13 ^c	6.82 ± 0.08 ^d	158.96 ± 9.21 ^d	17.59 ± 1.30 ^c
Cold sauce	288.64 ± 5.83	5.04 ± 0.28	1.81 ± 0.03	205.00 ± 7.07	15.07 ± 1.03

DM, dry matter; SD, standard deviation.

3.2. Quantisation of individual polyphenols in tomato samples during processing

The changes in specific polyphenol compounds were monitored during three technological processes (Tables 2 and 3). The ANOVA of the polyphenol data showed statistically significant differences between different processing steps and treatments. In all the samples, rutin was identified as the main polyphenol at levels ranging between 114.64 and 417.43 μg/g DM, followed by naringenin and chlorogenic acid.

Hydroxycinnamic acids were mainly represented by ferulic (m/z 193 → 134), ferulic-*O*-hexoside (m/z 355 → 193), chlorogenic (m/z 353 → 191), caffeic (m/z 179 → 135) and caffeic-*O*-hexoside (m/z 341 → 179) acids. Although there was a considerable variation between processes in the content of hydroxycinnamic acids, the trends reflecting the effects of processing were similar. The content of hydroxycinnamic acids decreased from tomato fruits to dices. The greatest decrease ($P < 0.05$) occurred for ferulic and caffeic acids. Within the three tomato-dicing stages, the decrease

in ferulic acid varied between 33.93% for dices subjected to Cold treatment and 39.38% using Hot treatment. Similarly, caffeic acid decreased by between 39.41% and 40.14% during the dice-making process in dices subjected to Cold and Hot treatments, respectively. The smallest changes were shown for ferulic-*O*-hexoside, which decreased by 3.43% and 2.12% in dices subjected to Hot treatment and Cold treatment, respectively.

In the latter stages of the dice-making process, further addition of a sauce rich in peels and seeds caused the levels of hydroxycinnamic acids in the product to increase (Table 3). This increase in the final stages was highly reproducible between the different technological treatments. Statistically significant increases ($P < 0.05$) were shown for ferulic and ferulic acid-*O*-hexoside when dices were subjected to Cold treatment without CaCl₂. Both hydroxycinnamic acids increased more than 1-fold when sauce was added to obtain the final product. With regard to the other hydroxycinnamic acids, increases were not so pronounced.

As regards the family of phenolic acids, it was observed that the main phenolic acid was gallic (m/z 169 → 125), followed by

Table 3

Content of hydroxycinnamic and phenolic acids expressed as mean $\pm$ SD of the three technological processes; different letters in the columns represent statistically significant differences ($P < 0.05$).

	Ferulic ac. ($\mu\text{g/g DM}$)	Ferulic ac.-O-hexoside ($\mu\text{g/g DM}$)	Chlorogenic ac. ($\mu\text{g/g DM}$)	Caffeic ac. ($\mu\text{g/g DM}$)	Caffeic ac.-O-hexoside ($\mu\text{g/g DM}$)	Protocatechuic ac. ($\mu\text{g/g DM}$)	Gallic ac. ($\mu\text{g/g DM}$)
Process 1: Hot treatment							
1 Fresh tomatoes	9.60 $\pm$ 0.38 ^a	47.21 $\pm$ 1.81 ^a	102.71 $\pm$ 4.00 ^a	21.75 $\pm$ 0.76 ^a	18.10 $\pm$ 0.53 ^a	2.90 $\pm$ 0.06 ^a	3.48 $\pm$ 0.07 ^a
2 Peeled tomatoes	6.25 $\pm$ 0.10 ^b	47.18 $\pm$ 0.36 ^a	93.64 $\pm$ 1.92 ^b	13.56 $\pm$ 0.51 ^b	14.68 $\pm$ 1.19 ^b	2.75 $\pm$ 0.16 ^b	2.98 $\pm$ 0.11 ^b
3 Diced tomatoes	5.82 $\pm$ 0.03 ^c	45.59 $\pm$ 0.16 ^a	84.94 $\pm$ 2.99 ^c	13.02 $\pm$ 0.50 ^b	13.83 $\pm$ 0.08 ^c	1.93 $\pm$ 0.06 ^c	2.61 $\pm$ 0.09 ^c
4 Final product	18.14 $\pm$ 0.62 ^d	94.94 $\pm$ 2.28 ^b	100.34 $\pm$ 0.84 ^a	14.90 $\pm$ 0.36 ^c	18.94 $\pm$ 0.99 ^a	3.86 $\pm$ 0.04 ^d	6.68 $\pm$ 0.08 ^d
Hot sauce	15.47 $\pm$ 0.25	77.42 $\pm$ 1.36	87.58 $\pm$ 5.71	1.63 $\pm$ 0.02	5.57 $\pm$ 0.07	4.52 $\pm$ 0.22	4.57 $\pm$ 0.00
Process 2: Cold treatment							
1 Fresh tomatoes	8.34 $\pm$ 0.27 ^a	69.96 $\pm$ 5.30 ^c	113.93 $\pm$ 4.08 ^a	22.48 $\pm$ 0.47 ^d	23.63 $\pm$ 1.63 ^d	3.78 $\pm$ 0.08 ^e	3.88 $\pm$ 0.05 ^e
2 Peeled tomatoes	6.53 $\pm$ 0.03 ^d	68.56 $\pm$ 1.20 ^c	112.00 $\pm$ 3.54 ^d	15.55 $\pm$ 0.21 ^a	20.18 $\pm$ 0.42 ^c	3.32 $\pm$ 0.08 ^d	3.26 $\pm$ 0.16 ^d
3 Diced tomatoes	5.51 $\pm$ 0.12 ^d	68.48 $\pm$ 0.30 ^c	105.72 $\pm$ 6.97 ^e	13.62 $\pm$ 0.71 ^b	19.74 $\pm$ 1.09 ^c	2.94 $\pm$ 0.18 ^d	3.23 $\pm$ 0.07 ^d
4 Final product	32.06 $\pm$ 1.05 ^b	196.05 $\pm$ 5.68 ^d	170.30 $\pm$ 8.62 ^f	19.70 $\pm$ 0.14 ^d	42.05 $\pm$ 2.36 ^f	10.63 $\pm$ 0.60 ^b	18.06 $\pm$ 1.05 ^f
Cold sauce	11.77 $\pm$ 0.21	90.67 $\pm$ 0.83	123.09 $\pm$ 4.70	4.16 $\pm$ 0.06	26.85 $\pm$ 0.40	6.80 $\pm$ 0.28	11.77 $\pm$ 0.21
Process 3: Cold treatment with CaCl₂							
1 Fresh tomatoes	8.34 $\pm$ 0.27 ^a	69.96 $\pm$ 5.30 ^c	113.93 $\pm$ 4.08 ^a	22.48 $\pm$ 0.47 ^d	23.63 $\pm$ 1.63 ^d	3.78 $\pm$ 0.08 ^e	3.88 $\pm$ 0.05 ^e
2 Peeled tomatoes	6.53 $\pm$ 0.03 ^d	68.56 $\pm$ 1.20 ^c	112.00 $\pm$ 3.54 ^d	15.55 $\pm$ 0.21 ^a	20.18 $\pm$ 0.42 ^c	3.32 $\pm$ 0.08 ^d	3.26 $\pm$ 0.16 ^d
3 Diced tomatoes	5.51 $\pm$ 0.12 ^d	68.48 $\pm$ 0.30 ^c	105.72 $\pm$ 6.97 ^e	13.62 $\pm$ 0.71 ^b	19.74 $\pm$ 1.09 ^c	2.94 $\pm$ 0.18 ^d	3.23 $\pm$ 0.07 ^d
4 Final product	18.61 $\pm$ 1.18 ^b	130.21 $\pm$ 8.61 ^d	154.48 $\pm$ 5.10 ^f	16.23 $\pm$ 0.58 ^d	23.13 $\pm$ 1.37 ^d	9.96 $\pm$ 1.16 ^c	11.88 $\pm$ 0.45 ^b
Cold sauce	11.77 $\pm$ 0.21	90.67 $\pm$ 0.83	123.09 $\pm$ 4.70	4.16 $\pm$ 0.06	26.85 $\pm$ 0.40	6.80 $\pm$ 0.28	11.77 $\pm$ 0.21

DM, dry matter; SD, standard deviation.

protocatechuic (m/z 153 $\rightarrow$ 109). Both phenolic acids, as observed above for hydroxycinnamic acids, experienced a decrease ($P < 0.05$) when fresh tomatoes were processed. Gallic acid remained stable between 75.0% and 83.25% when subjected to Hot and Cold treatments, respectively. Similarly, using these treatments, protocatechuic acid remained stable between 77.78% and 66.55%, respectively. Notably, the abundance of both phenolic acids increased as a result of sauce addition. The greatest increase was observed when diced tomatoes were subjected to Cold treatment without adding CaCl₂.

Flavonols were characterised by rutin (m/z 609 $\rightarrow$ 300), kaempferol-3-O-rutinoside (m/z 593 $\rightarrow$ 285) and quercetin (m/z 301 $\rightarrow$ 151). When dices were subjected to Hot treatment, levels of rutin, quercetin and kaempferol-3-O-rutinoside remained stable at 80.79%, 65.87% and 76.86%, whereas when they were processed using Cold treatment, they remained stable at 88.30%, 66.51% and 86.79%, respectively. As shown for hydroxycinnamic and phenolic acids, further addition of the sauce caused the level of flavonols in the product to increase. The concentration of rutin was relatively high in the sauce, which explained the high content in the final paste. Rutin increased by 63.16% when dices were subjected to Cold treatment without CaCl₂, whereas when dices were processed with CaCl₂, rutin increased by 10.15%. The same pattern was observed for kaempferol-3-O-rutinoside and quercetin.

Finally, flavanones were mainly represented by naringenin (m/z 271 $\rightarrow$ 151) and naringenin-7-O-glucoside (m/z 433 $\rightarrow$ 271). Naringenin remained stable at 78.02% and 74.86% when dices were subjected to Cold and Hot treatments, respectively. The relative levels of these compounds increased after the sauce addition treatment, compared to intact fruits. The concentration of naringenin was relatively high in the sauce, which explained the high content in the final paste. Levels of naringenin increased more than 5-fold

when dices were subjected to Cold treatment, but relatively low increases were detected using other treatments. The same pattern was observed for naringenin-7-O-glucoside.

3.3. Correlation between the health-related compounds of diced tomatoes and the three technological processes studied

To visualise the effect of each industrial tomato processing step, a PCA was performed on all the samples and variables (total phenolics, ABTS⁺, DPPH, ferulic, ferulic-O-hexoside, chlorogenic, caffeic, caffeic-O-hexoside, gallic and protocatechuic acids and quercetin, rutin, naringenin, naringenin-7-O-glucoside and kaempferol-3-O-rutinoside). As shown in the score plot (Fig. 1), the phenolic and antioxidant profiles were mainly influenced by different processing steps and treatments. Two principal components (PC1 and PC2) were obtained and accounted for 85.25% of the variability of the original data. PC1 allowed for differentiation between samples subjected to Hot treatment, which are located more on the left-hand side, whereas samples produced when dices were subjected to Cold treatment without adding CaCl₂, samples were located more on the right-hand side. PC2 corresponded to the stepwise processing from fresh tomatoes, situated in the bottom part of the plot, to final paste, located more in the central part of the plot.

Final products subjected to Cold treatment without adding CaCl₂ are well linked to total phenolics, antioxidant activity (ABTS⁺ and DPPH), ferulic-O-hexoside, caffeic-O-hexoside, chlorogenic, gallic and protocatechuic acids and rutin, naringenin and naringenin-7-O-glucoside, as these samples scored the highest levels of these parameters, whereas samples subjected to Hot treatment or Cold treatment with CaCl₂ scored lower values of these parameters, as they were located more on the left-hand side (Fig. 2). Moreover, fresh tomatoes were better linked to caffeic acid,

Resultats

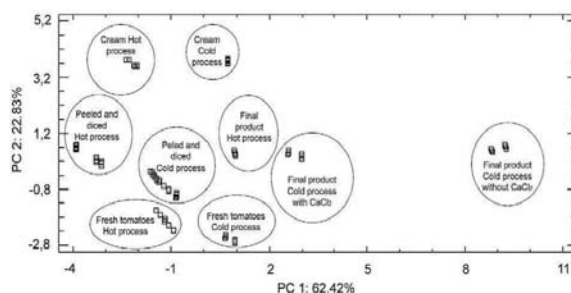


Fig. 1. Score plot of PC1 vs. PC2 of different processing steps and treatments. Fresh tomatoes; peeled and diced tomatoes; final product and sauces Hot and Cold.

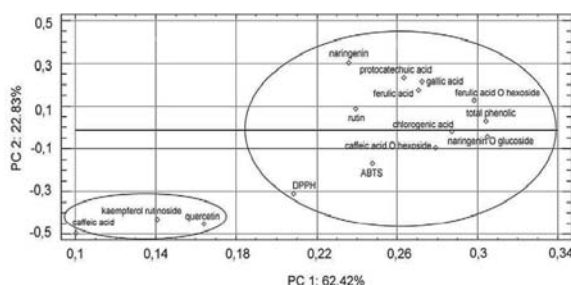


Fig. 2. Principal components plot of all the technological processes studied.

kaempferol-3-O-rutinoside and quercetin, while final products scored the lowest values of these parameters.

4. Discussion

The effects of paste-making processes on tomato fruit compounds, such as the antioxidant potential, lycopene, β -carotene and vitamin C, have been widely studied in the literature (Abushita, Daoud, & Biacs, 2000; Re et al., 2002; Takeoka et al., 2001); however, the effects of processing on the antioxidant activity and polyphenols of diced tomatoes have not been investigated. Diced tomatoes are widely consumed worldwide and contain remarkably high concentrations of micronutrients, such as minerals (potassium), antioxidant vitamin C, vitamin E and antioxidants. Therefore, it is of relevant importance to evaluate the effect of the dice-making process on health-related compounds. In this study, tomatoes were subjected to three different treatments: Hot, Cold and Cold with CaCl_2 .

Some studies have reported that industrial processing of tomatoes involves heat treatments which may help to increase the levels of some bound antioxidants, but other studies have reported that some labile antioxidants may be damaged as a result of industrial-scale processes (Abushita et al., 2000; Dewanto et al., 2002). In our study, a gradual and significant decrease in TP and antioxidant activity was observed in tomato dices compared to fresh tomatoes, especially for tomatoes subjected to Hot treatment. The higher temperatures required to produce the desired diced tomatoes may be associated with these increased losses. The de-

crease after the second step could be attributed to oxidation during factory-scale tomato processing.

Due to the supposed negative effects of the industrial processing of diced tomatoes, the main strategy for minimising eventual processing damage was "reconstitution", achieved by adding a sauce rich in seeds and peels with high levels of antioxidants and polyphenols to the diced tomatoes. As a result, TP and antioxidant activity experienced significant increases. The greatest increases occurred in Cold treatment without CaCl_2 . Calcium salts may inhibit the accumulation of phenolic compounds which play a part in subsequent reactions that cause tissue browning. There is controversy regarding the effects of calcium salts on the level of polyphenols. Floros et al. reported that calcium salts make high thermal treatments on the product unnecessary, as calcium salts inhibit peroxidase and polyphenol oxidase enzyme activity (Floros, Ekanayake, Abide, & Nelson, 1992). However, Rensburg et al. found that calcium treatments suppressed both respiration and polyphenol oxidation, and also reduced the content of total phenolic and other antioxidants (Rensburg & Engelbrecht, 1986).

In our study, diced tomatoes were found to be a rich source of rutin and naringenin, as has been reported in other studies (Galensa & Herrmann, 1980; Le Gall et al., 2003; Slimstad, Fossen, & Verheul, 2008). The abundance of these flavonoids decreased as tomato fruits were processed to obtain dices. The reduction could be attributed to the presence of oxygen, which accelerates the degradation. The decay is caused by the so-called reactive oxygen species (ROS) (Hvattum, Stenstrom, & Ekeberg, 2004). In the later processing stages, the content of rutin, naringenin and naringenin-7-O-glucoside increased as a result of sauce addition. The

concentrations of rutin and naringenin were relatively high in the cream, which explained their high content in the final product. As mentioned above, the highest increase was observed for dices subjected to Cold treatment without CaCl_2 . Polyphenols have a strong tendency to form complexes with mono-, di- and tri-valent cations, which hinders their bioaccessibility (Sharma, Jood, & Sehgal, 1996).

In contrast to rutin, results obtained for quercetin indicate a highly significant decrease following industrial processing from fresh to diced tomatoes. In the case of rutin, the 3-hydroxy-function at the C-ring of the flavonoid is blocked by a sugar moiety, whereas for quercetin it remains unoccupied. Thus, the blockage of the 3-hydroxyl group is the essential reason for the higher stability of rutin towards oxidation (Makris & Rossiter, 2000).

Similarly, for kaempferol-3-O-rutinoside, the 3-hydroxy-function is blocked. This explains the thermal stability of kaempferol-3-O-rutinoside towards quercetin. Further sauce addition increased levels of quercetin and kaempferol-3-O-rutinoside. Odriozola et al. found levels of quercetin between 9 and 9.6 $\mu\text{g/g}$ FM in tomato slices (Odriozola-Serrano, Oms-Oliu, Soliva-Fortuny, & Martín-Belloso, 2009), but in our study it only achieved a content of 14.20 $\mu\text{g/g}$ DM (2.93 $\mu\text{g/g}$ FM) in dices subjected to Cold treatment without CaCl_2 . This could be attributed to the differences between the state of ripeness, light, temperature, climatic growing conditions or soil of tomato fruits.

Diced tomatoes also contain hydroxycinnamic acids, which are found as simple esters in tomatoes. Hydroxycinnamic acids decreased as fresh tomatoes were processed. This may be attributed to the oxidation of hydroxycinnamic acids to reactive O-quinones; O-quinones may interact with NH_2 groups of amino acids leading to nutritional damage (Friedman, 1992). Notably, a significant change in hydroxycinnamic acids was observed after sauce addition. It appears that this step is especially important in changing the polyphenol profile during the dice-making process. The concentration of chlorogenic acid was relatively high in the sauce, which explained the high content in the final products. Chlorogenic acid was found at concentrations of 100.34–170.30 $\mu\text{g/g}$ DM (19.57–35.08 $\mu\text{g/g}$ FM). These results fell within the range (21.2–22 $\mu\text{g/g}$ FM) reported by Odriozola et al. in tomato slices (Odriozola-Serrano et al., 2009). With regard to other hydroxycinnamic acids, caffeic and ferulic acids were found in minor quantities. Caffeic acid was obtained in concentrations of 14.90–19.70 $\mu\text{g/g}$ DM (2.91–4.06 $\mu\text{g/g}$ FM), and ferulic of 18.14–32.06 $\mu\text{g/g}$ DM (3.54–6.60 $\mu\text{g/g}$ FM). Our results are in accordance with those found by Martínez-Valverde et al. in which levels of caffeic and ferulic acids were between 1.39 and 13.00 $\mu\text{g/g}$ FM, and between 1.60 and 5.38 $\mu\text{g/g}$ FM, respectively (Martínez-Valverde, Periago, Provan, & Chesson, 2002). Odriozola et al. also found values of caffeic acid ranging between 2.5 and 2.7 $\mu\text{g/g}$ FM, and ferulic acid between 3.9 and 4.2 $\mu\text{g/g}$ FM. Our results are in agreement with this study (Odriozola-Serrano et al., 2009).

Besides hydroxycinnamic acids, phenolic acids are also present in tomato fruit and tomato-based products (Shen, Chen, & Wang, 2007). The analysis pointed to significant differences in specific processing stages. Protocatechuic acid was found to decrease as tomato was processed into dices. This could be due to exposure to oxygen during processing and may result in the formation of O-quinones. The later processing stages increased levels of protocatechuic and gallic acids due to sauce addition. Results on the content of gallic acid, 6.68–18.06 $\mu\text{g/g}$ DM (1.30–3.72 $\mu\text{g/g}$ FM), were higher in comparison with the existing data, which reported values of <1 $\mu\text{g/g}$ FM in tomato varieties (Minogio et al., 2003). This could be explained by the sauce addition, used as a strategy to balance the effects of the dice-making process.

The study led to new results in the study of tomato fruit. Clearly, a gradual and significant decrease in antioxidant and polyphenol levels was observed during the industrial processing of

diced tomatoes; the extent of the decrease was influenced by both the temperature and the medium in which the diced tomatoes were treated. As is reflected in the ANOVA and PCA analysis, the most critical event in the industrial processing of diced tomatoes is the sauce addition process without CaCl_2 . However, sauce addition is seen as being an important way of balancing or enriching diced tomatoes. It is a source of functional ingredients and is a common practice in food industries, as compounds present in the seed and skin fragments are separately extracted and then returned to paste during this stage.

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5. Capítol V: Perfil fenòlic de derivats de tomàquet mitjançant tecnologies d'alta resolució (HPLC-ESI-QToF)

Anna Vallverdú-Queralt, Olga Jáuregui, Giuseppe Di Lecce, Cristina Andrés-Lacueva & Rosa M. Lamuela-Raventós. *Food Chemistry*. 2011, 129: 877-883.

Resum: Aquesta publicació presenta el primer estudi que realitza una identificació acurada dels polifenols en productes derivats del tomàquet: quètxups, gaspatxos i suc de tomàquet mitjançant una tecnologia d'alta resolució (HPLC-QToF-MS) i l'aplicació d'IDA. Es van identificar 45 polifenols amb errors inferiors a 2.8 mDa, cinc dels quals no havien estat descrits abans en tomàquets; l'addició d'altres ingredients en la formulació d'aquests aliments com ara l'oli d'oliva, alls i cebes permet augmentar el nombre de polifenols respecte els presents en el tomàquet fresc.



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Screening of the polyphenol content of tomato-based products through accurate-mass spectrometry (HPLC–ESI–QTOF)

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ABSTRACT

Tomatoes, the second most important vegetable crop worldwide, are a key component in the so-called “Mediterranean diet” and its consumption has greatly increased worldwide over the past 2 decades, mostly due to a growing demand for tomato-based products such as ketchups, gazpachos and tomato juices.

In this work, tomato-based products were analysed after a suitable work-up extraction procedure using liquid chromatography/electrospray ionisation-time of flight-mass spectrometry (HPLC–ESI–QTOF) with negative ion detection using information-dependent acquisition (IDA) to determine their phenolic composition. The compounds were confirmed by accurate mass measurements in MS and MS² modes. The elemental composition was selected according to the accurate masses and isotopic pattern. In this way, 47 compounds (simple phenolic and hydroxycinnamoylquinic acids and flavone, flavanol, flavanone and dihydrochalcone derivatives) were identified in tomato-based products, five of them, as far as was known, were previously unreported in tomatoes. The phenolic fingerprint showed that tomato-based products differ in phenolic composition, principally in protocatechuic acid-*O*-hexoside, apigenin and its glycosylated forms, quercetin-*O*-dihexoside, kaempferol-*C*-hexoside and eriodictyol-*O*-dihexoside. Gazpacho showed the highest number of phenolic compounds due to the vegetables added for its production.

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1. Introduction

Tomato and tomato products are considered to be healthy food since they are low in fat and calories, free of cholesterol and rich in vitamins A and C, β -carotene, lycopene and potassium, as well as octadecadienoic acid (Kim et al., 2011). Moreover, the tomato and its products are an important source of phenolic compounds for the human diet. It has recently been proposed that the phenolic constituents of tomato such as flavanones, flavonols and hydroxycinnamates and conjugated structures (caffeoylquinic acids) are bioactive compounds involved in the prevention of cancer (Grieb et al., 2009; Zhang et al., 2009) and cardiovascular diseases (Silaste, Alfthan, Aro, Kesaniemi, & Horkko, 2007).

Tomato consumption worldwide has greatly increased over the past 2 decades, mostly due to a growing demand for tomato-based

products such as ketchups, gazpachos and tomato juices. In general, tomato-based products undergo the following processing steps: preliminary processes such as size selection, washing and tray placing; dehydration, hydration or cooking and post treatments such as inspections, cleaning and packaging. Olive oil added to tomato-based products such as tomato juice and gazpachos could be an important factor governing the *in vivo* bioavailability of phenolic compounds, playing a crucial role in their accessibility and extractability from food (Bugianesi et al., 2004; Simonetti et al., 2005).

Although tomatoes have been widely studied for their phenolic content (Slimestad & Verheul, 2009), little information is available in the literature on the nonvolatile constituents of ketchups and tomato juice (Stewart et al., 2000) and there is no data on the phenolic profile of gazpachos.

Some previous work on polyphenol analysis has been carried out by NMR (Slimestad, Fossen, & Verheul, 2008), capillary electrophoresis (CE) (Peng, Zhang, & Ye, 2008), gas chromatography (GC) (Friedman, 2004), and most commonly high-performance liquid chromatography (HPLC) coupled to ultraviolet (UV) (Ozturk,

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Tuncel, & Tuncel, 2007) and mass spectrometry (MS) (Clifford, Johnston, Knight, & Kuhnert, 2003; Justesen, 2000).

For this kind of application, liquid chromatography/electrospray ionisation-time of flight-mass spectrometry (HPLC-ESI-QTOF) enables automated acquisition of both TOF-MS (survey) and MS/MS (dependent) spectra during a single chromatographic injection or infusion analysis. The high resolution and accurate mass MS/MS data acquired make IDA (information dependent acquisition) very informative. In IDA experiments, a TOF-MS survey scan is used to generate a peak list of ions present in the spectrum at a particular analysis time. This peak list is subjected to a series of user-defined criteria to select precursor ions of interest based on filters such as intensity threshold, charge state, isotope pattern and others. Once precursor ions are selected, they are submitted for MS/MS analysis. This cycle is repeated throughout the duration of the acquisition, generating large amounts of informative data. The use of QTOF technologies allows the exact mass measurements of both MS and MS/MS ions to be achieved, which is essential for elemental composition assignment and thus for the characterisation of small molecules.

The aim of this work was to describe the phenolic composition of gazpachos, unreported until now, and to accurately examine the differences between phenolic profiles of ketchups, gazpachos and tomato juices. Thus, samples were analysed using HPLC-ESI-QTOF to determine structures based on fragmentation patterns using accurate mass measurements of the deprotonated molecules and product ions in MS/MS experiments. Consequently, HPLC-ESI-QTOF enabled us to identify 47 polyphenols in tomato-based products.

2. Materials and methods

2.1. Standards and reagents

All samples and standards were handled without exposure to light. Caffeic, p-coumaric, protocatechuic, ferulic and chlorogenic acids, rutin, eriodictyol and quercetin were purchased from Sigma® (Madrid, Spain); naringenin, apigenin-7-O-glucoside, kaempferol-3-O-glucoside and kaempferol-3-O-rutinoside from Extrasynthèse (Genay, France). Ethanol and formic acid HPLC grade were obtained from Scharlau (Barcelona, Spain) and ultrapure water (Milli-Q) from Millipore System (Bedford, USA).

2.2. Processing conditions and ingredients of commercial samples

2.2.1. General

Six different commercial brands of each tomato-based product (ketchup, tomato juice and gazpacho) were analysed. All the studied brands contained the same ingredients but in some cases the amount of each ingredient was not known.

2.2.2. Ketchup

Ketchup is a sweet-and-sour condiment typical of the American diet. The technological process to obtain ketchup consists of mixing the tomato paste (65%, 30–32°Bx) with sugar, wine vinegar, salt, aroma and preservatives in traces. The mixture is pasteurised at 96 °C for 4–6 min and packaged in bottles. The temperature must be carefully regulated to ensure absorption of the ingredients without overcooking. The entire process of ketchup manufacturing generally takes two to three hours (Galicia-Cabrera, 2007).

2.2.3. Tomato juice

Juice is an intermediate product in the processing of tomato paste, obtained in the juice extraction stage of a process that eventually results in tomato concentrate. Tomato juice can be sepa-

rated from the pulp by filtering, but more commonly the entire pulp is used as juice. The juice is formulated according to the characteristics demanded by the market: the most common is juice with extra virgin olive oil, salt and citric juice to adjust the pH. The product is then bottled and usually pasteurised (80 °C, 20 min) to extend its shelf life (Gahler, Otto, & Bohm, 2003; Galicia-Cabrera, 2010).

2.2.4. Gazpacho

Gazpacho is a traditional Spanish soup, which can be defined as a cold soup containing mainly five vegetables, tomato, cucumber, green pepper, onion and garlic, as well as other minor components such as extra virgin olive oil, wine vinegar, salt, sugar and water. The vegetable mixture is cooked and usually pasteurised (92–95 °C, 1 min) to extend its shelf life. The samples in this study were of canned, bottled and packed gazpachos (Pinilla, Plaza, Sanchez-Moreno, de Ancos, & Cano, 2005).

2.3. Extraction and isolation of phenolic compounds

Samples were treated in a darkened room with a red safety light to avoid oxidation of the analytes, following the procedure of Valverdú-Queralt et al. with some modifications (Valverdú-Queralt, Jáuregui, Medina-Remón, Andrés-Lacueva, & Lamuela-Raventós, 2010).

Ketchups (1.0 g) were weighed and homogenised with 80% ethanol in Milli-Q water (4 ml); they were then sonicated for 5 min and centrifugated (4000 rpm at 4 °C) for 20 min. The supernatant was transferred into a flask and extraction was repeated. Both supernatants were combined and evaporated under nitrogen flow; finally, the residue was reconstituted with Milli-Q water (0.1% of formic acid) up to 1.5 ml.

Gazpachos and tomato juices (10 ml) were centrifuged (4000 rpm at 4 °C) for 10 min. The supernatant was rejected and 4 ml of 80% ethanol in Milli-Q water was added to the pellet. They were sonicated for 5 min and centrifugated (4000 rpm at 4 °C) for 20 min following the aforementioned procedure for the extraction of ketchups.

Solid-phase extraction (SPE) of these extracts was carried out following the procedure of Medina-Remón et al. (2009). Firstly, 1 ml of methanol and subsequently 1 ml of sodium acetate 50 mmol/l pH 7 were loaded into Oasis® MAX cartridges from Waters to equilibrate it; then 1 ml of each extract was diluted with 1 ml of Milli-Q water and acidified with 34 µl of hydrochloric acid at 35% before being loaded into the cartridges separately. These were rinsed with 50 mmol/l pH 7 sodium acetate (5% methanol). The polyphenols were eluted with 1800 µl of methanol (2% formic acid). The eluted fractions were evaporated under nitrogen flow, and the residue was reconstituted with water (0.1% formic acid) up to 250 µl and filtered through a 13 mm, 0.45 µm PTFE filter (Waters) into an insert-amber vial for HPLC analysis. Samples were stored at –20 °C until analysis.

2.4. HPLC-ESI-QTOF analysis

The chromatography was performed on an Agilent 1200 RRIC (Agilent, Waldbronn, Germany) using a Luna C₁₈ column 50 × 2.0 mm internal diameter, 5 µm (Phenomenex, Torrance, CA, USA). Flow rate was 0.4 ml/min and injection volume was 5 µl. Mobile phases consisted of 0.1% formic acid in Milli-Q-water (A) and 0.1% formic acid in acetonitrile (B). Separation was carried out in 20 min under the following conditions: 0 min, 5% B; 16 min, 40% B; 17 min, 95% B; 19 min, 95% B; 19.5 min, 5% B. The column was equilibrated for 5 min prior to each analysis. The HPLC system was coupled to a hybrid quadrupole time-of-flight QSTAR Elite (ABSciex). The MS acquisition was performed in negative

ionisation using information-dependent acquisition (IDA) between m/z 90–1100. MS parameters were: ion spray voltage, –4200 V; declustering potential, –60 V; focusing potential, –190 V; declustering potential 2, –15 V; ion release delay, 6 V; ion release width, 5 V; temperature, 400 °C with curtain gas (N_2), 50 a.u. (arbitrary units); auxiliary gas, 50 a.u. and nebulizer gas (N_2), 50 a.u. IDA was performed using the following criteria: ions that exceeded 5 counts, ion tolerance 50 mDa, collision energy fixed at –30 V, dynamic background subtract activated. The Q-TOF was calibrated as recommended by the manufacturer.

The elemental composition of each polyphenol was selected according to the accurate masses and the isotopic pattern (through the Formula Finder feature in Analyst QS 2.0) and searched for in the Dictionary of Natural Products (Chapman & Hall/CRC) and the MOTO database (<http://appliedbioinformatics.wur.nl/moto>). The interpretation of the observed MS/MS spectra in comparison with those found in the literature was the main tool for putative identification of polyphenols.

3. Results and discussion

3.1. Characterisation of phenolic compounds and its derivatives

3.1.1. General

Table 1 shows the list of 47 compounds identified through HPLC-ESI-QTOF experiments along with their retention times (rt), fraction (ketchup, gazpacho or tomato juice), accurate mass (acc. mass), molecular formula (MF), mDa of error between the mass found and the accurate mass of each polyphenol and the MS-MS ions used for identification. No differences were observed in the phenolic profiles among the six brands analysed for each tomato-based product.

3.1.2. Simple phenolic acid derivatives

Phenolic acids and their derivatives are widely distributed in plants. They are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but also because of

Table 1
List of compounds identified in tomato by-products.

Compound	Fraction	tr	[M–H]	m/z ions	Acc. mass	mDa	M.F.
Protocatechuic acid-O-hexoside	A,C	1.10	315	153 (100), 109 (40)	315.0721	0.90	$C_{13}H_{16}O_9$
Protocatechuic acid ^a	A,B,C	1.25	153	153 (100), 109 (100)	153.0193	0.50	$C_7H_6O_4$
Caffeic acid-O-dihexoside	A	1.35	503	341 (80), 179 (100)	503.1406	1.10	$C_{21}H_{30}O_{14}$
Caffeic acid-O-hexoside 1	A,B	1.43	341	179 (100), 135 (20)	341.0877	1.60	$C_{15}H_{18}O_9$
Caffeic acid-O-hexoside 2	A,B,C	1.54	341	179 (100), 135 (20)	341.0877	0.70	$C_{15}H_{18}O_9$
Neochlorogenic acid	A,B,C	1.65	353	191 (100), 179 (80), 135 (30)	353.0877	0.70	$C_{16}H_{20}O_9$
Caffeic acid-O-hexoside 3	A,B,C	2.16	341	179 (100)	341.0877	2.30	$C_{15}H_{18}O_9$
Homovanillic acid-O-hexoside 1	A,B,C	2.22	343	343 (100), 137 (70), 109 (40)	343.1034	1.10	$C_{15}H_{20}O_9$
Ferulic acid-O-hexoside	A,B,C	2.49	355	193 (65), 178 (30), 149 (100)	355.1034	2.50	$C_{16}H_{20}O_9$
Homovanillic acid-O-hexoside 2	A,B,C	2.82	343	343 (100), 137 (70), 109 (40)	343.1034	2.10	$C_{15}H_{20}O_9$
Caffeic acid-O-hexoside 4	A,B,C	2.90	341	179 (100), 135 (35)	341.0877	0.70	$C_{15}H_{18}O_9$
Coumaric acid-O-hexoside 1	A,C	2.99	325	163 (75), 119 (100)	325.0928	1.40	$C_{15}H_{18}O_8$
Vanillic acid-O-hexoside	A,B	3.01	329	167 (100)	329.0877	0.69	$C_{14}H_{18}O_8$
Coumaric acid-O-hexoside 2	A,C	3.26	325	163 (80), 119 (100)	325.0928	0.20	$C_{15}H_{18}O_8$
Chlorogenic acid ^a	A,B,C	3.43	353	191 (100)	353.0877	1.50	$C_{16}H_{20}O_9$
Caffeic acid ^a	A,B,C	3.80	179	135 (100), 107 (20)	179.0349	1.40	$C_9H_8O_4$
Cryptochlorogenic acid	A,B,C	4.14	353	191 (50), 173 (100), 135 (20)	353.0877	0.80	$C_{16}H_{20}O_9$
Naringenin-C-diglycoside	A,B,C	4.96	595	595 (100), 505 (25), 475 (50), 385 (50), 355 (45)	595.1667	0.70	$C_{27}H_{34}O_{15}$
Chlorogenic acid isomer	A,B,C	5.15	353	191 (100)	353.0877	1.00	$C_{16}H_{20}O_9$
Rutin-O-hexoside	A,B,C	5.20	771	771 (100), 609 (70), 300 (20)	771.1989	1.60	$C_{33}H_{40}O_{21}$
Coumaric acid ^a	A,B,C	5.82	163	163 (20), 119 (100)	163.0400	0.86	$C_9H_8O_3$
Coumaroylquinic acid	A,B,C	6.01	337	191 (100), 163 (10)	337.0930	1.50	$C_{16}H_{20}O_8$
Apigenin-C-hexoside-hexoside	C	6.26	593	503 (10), 473 (20), 353 (10)	593.1511	0.30	$C_{27}H_{30}O_{15}$
Feruloylquinic acid	A,B,C	6.58	367	193 (15), 191 (100)	367.1034	0.10	$C_{17}H_{20}O_9$
Dihydroxy-dimethoxychalcone-C-diglycoside	A,B	7.01	623	623 (100), 503 (20), 383 (20)	623.1981	0.60	$C_{28}H_{36}O_{15}$
Rutin-O-pentoside	A,B,C	7.66	741	741 (100), 609 (80), 300 (20)	741.1883	0.70	$C_{32}H_{38}O_{20}$
Apigenin-C-hexoside-pentoside	C	7.78	563	503 (10), 473 (25), 383 (15), 353 (25)	563.1406	0.90	$C_{26}H_{32}O_{14}$
Quercetin-O-dihexoside	C	7.90	625	463 (100), 300 (30)	625.1410	1.60	$C_{27}H_{30}O_{17}$
Rutin ^a	A,B,C	8.21	609	609 (100), 300 (30)	609.1460	0.50	$C_{27}H_{30}O_{16}$
Quercetin-O-hexoside	A,B,C	8.44	463	463 (100), 300 (20)	463.0881	2.80	$C_{21}H_{24}O_{12}$
Phloretin-C-diglycoside	A,B,C	8.62	597	477 (30), 387 (100), 357 (85), 417 (15)	597.1819	0.30	$C_{27}H_{34}O_{15}$
Naringenin-C-hexoside	A,C	9.03	433	433 (40), 343 (20), 313 (40)	433.1140	0.60	$C_{21}H_{22}O_{10}$
Naringenin-O-hexoside	A,B,C	9.12	433	433 (10), 271 (80)	433.1140	0.10	$C_{21}H_{22}O_{10}$
Kaempferol-3-O-rutinoside ^a	A,B,C	9.18	593	593 (100), 285 (60)	593.1511	0.70	$C_{27}H_{30}O_{15}$
Kaempferol-C-hexoside	C	9.39	447	357 (15), 327 (100), 285 (<5)	447.0932	0.60	$C_{21}H_{22}O_{11}$
Dicafeoylquinic acid 1	A,B,C	9.50	515	515 (20), 353 (100), 335 (20), 191 (35), 173 (60)	515.1194	0.10	$C_{29}H_{36}O_{12}$
Apigenin-7-O-glucoside ^a	C	9.66	431	431 (25), 269 (50), 161 (75)	431.0983	0.80	$C_{21}H_{22}O_{10}$
Dicafeoylquinic acid 2	A,B,C	10.25	515	515 (25), 353 (50), 191 (100), 179 (75)	515.1194	1.60	$C_{29}H_{36}O_{12}$
Kaempferol-3-O-glucoside ^a	A,B,C	10.31	447	447 (50), 285 (100)	447.0932	1.80	$C_{21}H_{22}O_{11}$
Eriodictyol-O-dihexoside	B	10.50	611	611 (20), 449 (100), 287 (30)	611.1606	1.10	$C_{27}H_{32}O_{16}$
Naringenin-7-O-glucoside (prunin) ^a	A,B,C	10.70	433	433 (10), 271 (100)	433.1140	1.10	$C_{21}H_{22}O_{10}$
Eriodictyol-O-hexoside	A,B,C	10.81	449	449 (10), 287 (100)	449.1089	2.50	$C_{21}H_{22}O_{11}$
Tricafeoylquinic acid 1	A,B	11.31	677	677 (45), 515 (100), 353 (40), 191 (15)	677.1511	1.40	$C_{34}H_{40}O_{15}$
Eriodictyol ^a	A,B,C	11.41	287	151 (70), 135 (100)	287.0560	1.20	$C_{14}H_{14}O_6$
Quercetin ^a	A,B,C	11.93	301	301 (10), 151 (100)	301.0353	0.40	$C_{15}H_{12}O_7$
Tricafeoylquinic acid 2	A,B,C	12.98	677	677 (75), 515 (100), 353 (20), 173 (15)	677.1511	1.00	$C_{34}H_{40}O_{15}$
Naringenin ^a	A,B,C	13.03	271	151 (80), 119 (100)	271.0611	1.50	$C_{15}H_{12}O_5$

^a Comparison with standard. Subfraction: A, ketchup; B, tomato juices and C, gazpachos. rt, retention time; Acc. mass, accurate mass; MF, molecular formula; mDa, millidaltons of error between the mass found and the accurate mass of each polyphenol.

their stable radical intermediates, which prevent the oxidation of various food ingredients (Cuvelier, Richard, & Berset, 1992). Phenolic acids have also been shown to have antimutagenic and antiglycemic properties (Friedman, 1997). In addition, caffeic, coumaric, ferulic, and protocatechuic acids can exert an antifungal effect.

The examination of the chromatograms in TOF-MS mode of ketchups, gazpachos and tomato juices revealed the presence of protocatechuic (m/z 153), caffeic (m/z 179) and coumaric (m/z 163) acids. MS^2 of these ions showed the deprotonated molecule $[M-H]^-$ and the typical loss of CO_2 giving $[M-H-44]^-$ as a characteristic ion. These compounds were confirmed by comparison with the retention time of the standard and the MS^2 fragmentation patterns.

Eight phenolic acid-*O*-hexosides were identified in TOF-MS mode using accurate mass measurements and MS^2 fragmentations. Caffeic acid-*O*-hexosides (m/z 341) were detected in all tomato-based products. The MS^2 of m/z 341 showed a characteristic fragmentation involving cleavage of the intact sugar $[M-H-162]^-$ (m/z 179) and the ion corresponding to the loss of CO_2 (m/z 135).

In addition, the analysis in TOF-MS mode revealed the presence of two homovanillic acid-*O*-hexosides (m/z 343), two coumaric acid-*O*-hexosides (m/z 325) and one vanillic acid-*O*-hexoside (m/z 329). Homovanillic acid-*O*-hexosides were present in all tomato-based products, whereas coumaric acid-*O*-hexosides were only detected in ketchups and gazpachos and vanillic acid-*O*-hexoside in ketchups and tomato juices. The MS^2 of m/z 343, m/z 325 and m/z 329 showed ions corresponding to the deprotonated molecule $[M-H]^-$, the loss of a hexose $[M-H-162]^-$ and the loss of the carboxylic group.

The analysis in TOF-MS mode also showed the presence of protocatechuic acid-*O*-hexoside (m/z 315) in ketchups and gazpachos and ferulic acid-*O*-hexoside (m/z 355) in all tomato-based products. The MS^2 of m/z 355 showed ions at m/z 193, 178 and 149 corresponding to the loss of a hexose moiety $[M-H-162]^-$ and the loss of a methyl and acid group from the aglycone. The MS^2 of m/z 315 showed ions at m/z 153 and m/z 109, corresponding to the loss of a hexose and loss of CO_2 .

In ketchups, a peak showing m/z 503 could be attributed to caffeic acid-*O*-dihexoside. The fragmentation of *O*-dihexosides in the product ion scan mode involves the cleavage of the glycosidic bond and the loss of the sugar moieties, thus showing the deprotonated aglycon $[A-H]^-$ and the intermediate *O*-hexoside $[M-H-162]^-$ ion fragment as described by other authors (Cuyckens & Claeys, 2004; Parejo et al., 2004; Valverdú-Queralt et al., 2010). Assignment of the position of the hexose (glucose or galactose) unit was not possible due to the lack of both standards and reported data.

As far as we know, glycosylated caffeic, vanillic, coumaric, homovanillic and ferulic acids have been found in tomato (Slimestad & Verheul, 2009), whereas protocatechuic acid-*O*-hexoside and caffeic acid-*O*-dihexoside have not.

As described by Ruiz-Rodríguez, Marín, Ocaña, and Soler-Rivas (2008), the occurrence of protocatechuic acid-*O*-hexoside in gazpachos and some ketchups could be a consequence of thermal processing, which leads to intense degradation of quercetin to form protocatechuic acid or its glycosylated derivatives (Ruiz-Rodríguez et al., 2008). As mentioned above for protocatechuic acid, the presence of caffeic acid-*O*-dihexoside was tentatively attributed in ketchups to the technological process applied in the manufacture of this sauce, which could lead to the formation of caffeic acid or its glycosylated forms from more complex structures.

3.1.3. Hydroxycinnamoylquinic acid derivatives

Preliminary structure-activity relationship studies of cinnamic acids and derivatives have pointed out the importance of the catechol group in the antiradical efficacy (Silva et al., 2000). Peaks showing m/z 353 revealed the presence of neochlorogenic, chloro-

genic, cryptochlorogenic and caffeoylquinic isomer acids in all the tomato-based products. Peak identification was accomplished using accurate mass measurements. It was possible to differentiate the four isomers of chlorogenic acid by their relative intensities in MS^2 spectra according to the method cited by other authors using liquid-chromatography tandem mass spectrometry (Clifford et al., 2003; Parejo et al., 2004). The caffeoylquinic isomer might be a stereoisomer of the neochlorogenic acid, chlorogenic acid or cryptochlorogenic acid as described by Fang, Yu, and Prior (2002).

Coumaroylquinic acid (m/z 337) and feruloylquinic acid (m/z 367) were also present in all the tomato-based products. MS^2 of m/z 367 revealed peaks at m/z 191 corresponding to the deprotonated quinic acid unit and m/z 193 (ferulic acid) and m/z 337 showed peaks at m/z 191 and m/z 163 (coumaric acid).

In addition, two dicaffeoylquinic acid isomers (m/z 515) were also detected in all the tomato-based products at different retention times. The MS^2 of m/z 515 showed the ion $[M-H-162]^-$ (m/z 353, corresponding to the loss of a caffeic acid unit) and the deprotonated quinic acid (m/z 191).

Lastly, two tricaffeoylquinic acids (m/z 677) were identified in ketchups, gazpachos and tomato juices. MS^2 experiments of m/z 677 of both compounds revealed characteristic fragmentations involving cleavage of three caffeoyl moieties for tricaffeoylquinic acid isomers.

3.1.4. Flavone derivatives

The flavone apigenin is present in fruit and vegetables and is known to have a number of biological functions such as anti-inflammatory, anticancer and free radical scavenging properties. Studies of human malignant cancer cell lines have shown that apigenin inhibits cancer cell growth via apoptosis (Choi & Kim, 2009). Three flavone derivatives were found to occur in gazpachos. Apigenin-7-*O*-glucoside (m/z 431), apigenin-C-hexoside-pentoside (m/z 563) and apigenin-C-hexoside-hexoside (m/z 593) were tentatively identified in TOF-MS mode. C-glycosides and O-glycosides exhibited different fragmentation patterns in MS^2 mode as described previously in *Cocoa Theobroma* (Sanchez-Rabaneda et al., 2003) and *Artemisia annua* (Han et al., 2008) using liquid-chromatography tandem mass spectrometry with a triple quadrupole instrument. The product ion scan of C-diglycosides revealed the characteristic losses of 60, 90 and 120 u corresponding to cross-ring cleavages in the sugar unit whereas O-hexosides involved the cleavage of the glycosidic bond and the loss of the sugar moieties, thus showing the $[M-H-162]^-$ ion fragment.

The product ion scan of m/z 431 for apigenin-*O*-glucoside showed peaks at m/z 269 and 151, which were attributed to the loss of a glucose moiety $[M-H-162]^-$ and to the ion corresponding to retro-Diels Alder (RDA) fragmentation, as described by other authors (Fabre, Rustan, de, & Quetin-Leclercq, 2001; Justesen, 2000; Sanchez-Rabaneda et al., 2003).

Apigenin-C-hexoside-hexoside and apigenin-C-hexoside-pentoside could be distinguished by the presence of the ion $[M-H-60]^-$ according to the method cited by Han et al. using liquid chromatography coupled to electrospray ionisation mass spectrometry (Han et al., 2008). MS^2 of m/z 593 produced ions at m/z 503 $[M-H-90]^-$, 473 $[M-H-120]^-$ and 353 $[M-H-120-120]^-$ while the ion of $[M-H-60]^-$ was absent, whereas MS^2 of m/z 563 yielded the ions at m/z 503 $[M-H-60]^-$, 473 $[M-H-90]^-$, 383 $[M-H-90-90]^-$ and 353 $[M-H-90-120]^-$ suggesting the presence of a pentose substitution. Considering all of the above information, these compounds were tentatively identified as apigenin-C-hexoside-hexoside and apigenin-C-hexoside-pentoside (Fig. 1A and B).

Although apigenin has been previously described in bell pepper and garlic (Miean & Mohamed, 2001), apigenin-7-*O*-glucoside, apigenin-C-hexoside-hexoside and apigenin-C-hexoside-pentoside have been reported here for the first time. The presence of these

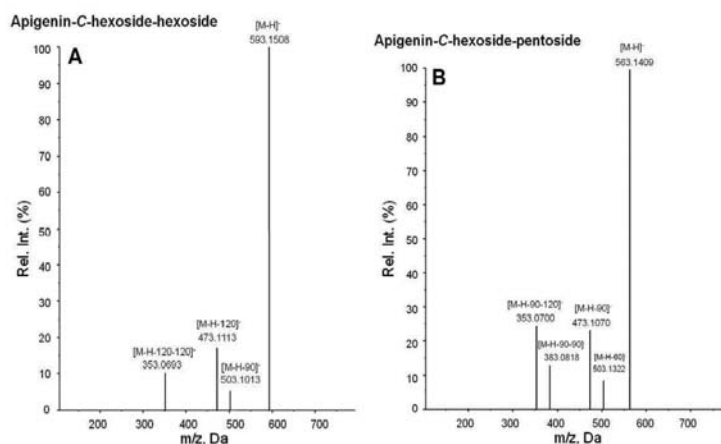


Fig. 1. (A) Mass spectrum of Apigenin-C-hexoside-hexoside and (B) mass spectrum of Apigenin-C-hexoside-pentoside.

new compounds found in tomato-based products could be attributed to bell pepper and garlic. Moreover, the technological parameters or the percentage of vegetable used to manufacture gazpachos could have an influence on the phenolic profile.

3.1.5. Flavanone derivatives

The chromatograms in TOF-MS mode revealed the presence of naringenin (m/z 271) and eriodictyol (m/z 287) in ketchups, gazpachos and tomato juices. Naringenin and eriodictyol were identified by comparing their retention times with that of a reference substance using accurate mass measurements. Naringenin has been identified as one of the major polyphenols in tomatoes. A recent study demonstrated that sub-chronic administration of flavanones significantly attenuated the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the decrease in striatal dopamine (DA) concentrations. This suggests that these compounds can be considered as potential candidates for a dietary supplement in the treatment of Parkinson's disease (Zbarsky et al., 2005).

Two naringenin-O-hexosides (m/z 433) and one naringenin-C-hexoside (m/z 433) were tentatively identified in the TOF-MS mode. Both naringenin-O-hexosides were present in all the tomato-based products whereas naringenin-C-hexoside was only detected in ketchups and gazpachos. The MS^2 mass spectrum of m/z 433, which has been assigned to O-substitution, showed an ion at m/z 271 corresponding to the loss of a hexoside moiety $[M-H-162]^-$, whereas the product ion scan of naringenin-C-hexoside revealed characteristic losses of 90 and 120 u from m/z 433 corresponding to cross-ring cleavages in the sugar unit.

Moreover, in all the tomato-based products, a peak showing m/z 595 could be attributed to naringenin-C-diglycoside. The MS^2 of this ion displayed losses of 90 and 120 u from m/z 595 and 475, respectively, which confirmed the presence of two hexose (glucose or galactose) units.

Eriodictyol-O-hexoside (m/z 449) and eriodictyol-O-dihexoside (m/z 611) were also detected in TOF-MS mode. Eriodictyol-O-hexoside was present in all the tomato-based products whereas eriodictyol-O-dihexoside was only detected in tomato juices. MS^2 of m/z 449 revealed the deprotonated molecule $[M-H]^-$ and the ion corresponding to the loss of a hexoside moiety $[M-H-162]^-$,

whereas MS^2 of m/z 611 showed ions at m/z 449 and 287 corresponding to the loss of one hexoside unit and two hexoside units, respectively.

Eriodictyol and eriodictyol derivatives have been previously reported in tomatoes (Slimestad & Verheul, 2009). However, in this study, the presence of eriodictyol-O-dihexoside only in tomato juices could be attributed to the addition of citric juice. The main aglycones in citrus juices are naringenin, hesperetin and glycosylated eriodictyol (Gardana, Nalin, & Simonetti, 2008).

3.1.6. Flavonol derivatives

Flavonol derivatives are valuable phytochemicals consistently associated with antioxidant and anticarcinogenic activities, and a relatively longer circulation time *in vivo* (Kurisawa, Chung, Uyama, & Kobayashi, 2003; Nagasawa et al., 2003). The examination of the chromatograms in TOF-MS mode of ketchups, gazpachos and tomato juices revealed the presence of some peaks at m/z 609, 593 and 301 corresponding to rutin, kaempferol-3-O-rutinoside and quercetin, respectively.

Rutin and kaempferol-3-O-rutinoside were identified by comparing their retention times with those of reference substances. The MS^2 of these ions showed peaks corresponding to the deprotonated molecule $[M-H]^-$ and the loss of the rutinoside unit, $[M-H-308]^-$. Flavonol aglycons such as quercetin gave as a characteristic ion the deprotonated molecule $[M-H]^-$ and ions corresponding to RDA fragmentation.

The analysis in the TOF-MS confirmed the presence of rutin-O-hexoside (m/z 771) and rutin-O-pentoside (m/z 741). The MS^2 mass spectrum of m/z 771 showed peaks at m/z 609 and 300 corresponding to the loss of a hexoside $[M-H-162]^-$ and to the radical anion of the aglycone (m/z 300) as described in other studies using a triple quadrupole mass spectrometer (Hvattum & Ekeberg, 2003; Valverdú-Queralt et al., 2010). The MS^2 mass spectrum of m/z 741 was assessed, showing peaks at m/z 609 and 300 corresponding to the loss of a pentoside $[M-H-132]^-$ and to the radical anion of the aglycone (m/z 300). Rutin-O-hexoside and rutin-O-pentoside have been previously found in tomato (Moco et al., 2006).

In addition, hyperoside (m/z 463) was identified by comparing its retention time with that of a reference substance using accurate mass measurements. Quercetin-O-dihexoside (m/z 625) was also

identified in the TOF-MS. Hyperoside was present in all the tomato-based products analysed whereas quercetin-*O*-dihexoside was only detected in gazpachos. The differences observed in the phenolic profiles of these tomato-based products could be explained by the addition of onions to gazpacho (Ruiz-Rodríguez et al., 2008). The MS² of *m/z* 463 showed an ion at *m/z* 300 corresponding to the loss of a hexoside moiety [M–H–162][–] and MS² of *m/z* 625 showed ions at *m/z* 463 and 300 corresponding to the loss of one hexoside unit and two hexoside units, respectively.

Lastly, in gazpachos a peak showing *m/z* 477 could be attributed to kaempferol-3-*C*-hexoside, whereas kaempferol-3-*O*-glucoside (*m/z* 477) was detected in all three tomato products. The MS² of kaempferol-*O*-glucoside revealed the deprotonated molecule [M–H][–] and the ion corresponding to the loss of a hexoside moiety [M–H–162][–], whereas the MS² of kaempferol-3-*C*-hexoside did not reveal the deprotonated aglycon [M–H][–], although characteristic losses of 90 and 120 u from *m/z* 477, corresponding to cross-ring cleavages in the sugar unit, were observed.

As far as we know, this is the first time kaempferol-*C*-hexoside has been reported in gazpachos, whereas kaempferol-3-*O*-glucoside has been previously described in tomatoes (Slimestad & Verheul, 2009). The presence of kaempferol-*C*-hexoside in gazpachos was tentatively attributed to the vegetables added in its production and to the technological process in its manufacture, which could lead to the formation of kaempferol-3-*C*-glucoside from more complex forms, as mentioned above for protocatechuic acid.

3.1.7. Dihydrochalcone derivatives

Dihydrochalcones are a family of bicyclic flavonoids defined by the presence of two benzenoid rings joined by a three-carbon bridge. Dihydrochalcones show significant radical scavenging activity, suppressing lipid peroxidation, and have various advantages for application as food additives, being safe, stable, colourless and easy to prepare by the usual hydrogenation of citrus flavanones (Nakamura, Watanabe, Miyake, Kohno, & Osawa, 2003).

In ketchups and tomato juices, a peak showing *m/z* 623 could be attributed to dihydroxy-dimethoxychalcone-*C*-diglycoside, whereas phloretin-*C*-diglycoside (*m/z* 597) was tentatively identified in all tomato-derived products. Both chalcones showed losses of 90 and 120 u as characteristic fragment ions in the MS/MS mode. As far as we know, this is the first time dihydroxy-dimethoxychalcone-*C*-diglycoside is reported in ketchups and tomato juices, whereas phloretin-*C*-diglycoside has been previously described in tomato by Slimestad et al. (2008). The presence of dihydroxy-dimethoxychalcone-*C*-diglycoside only in ketchups and tomato juices is probably due to technological practices such as dehydrations and stabilization procedures (Gahler et al., 2003; Galicia-Cabrera, 2010).

3.2. Technological conditions and cultural practices may affect the phenolic profile of tomato-based products

From a technological point of view, the main difficulty in the production of ketchups, tomato juices and gazpachos with a good consistency is choosing the right variety of tomatoes with suitable characteristics. The products are obtained in accordance with good commercial practices to maintain the physicochemical parameters of the tomatoes and other ingredients and provide products with the best characteristics. Moreover, tomato varieties and agricultural conditions such as cultivar selection, temperature, light, fertilisation and irrigation, temperature and season may have an influence on phenolic accumulation (Slimestad & Verheul, 2009).

Among the six brands analysed for each tomato-based product, no differences were observed in the phenolic profiles. The typical olive oil phenols such as oleuropein and ligstroside (Torre-Carbot et al., 2005) were not found in the tomato-based products. Phenolic

composition varied among the ketchups, gazpachos and tomato juices, with gazpacho generally showing the highest number of phenolic compounds. These results could be explained by the content of several vegetables in gazpacho that are a rich source of bioactive compounds.

4. Conclusions

Ketchups, tomato juices and gazpachos, three tomato-based products, were studied with high-resolution HPLC-ESI-QTOF. This technique is able to distinguish between different phenolic compounds of several chemical classes. Using HPLC-ESI-QTOF, good mass accuracies were obtained for all molecular ions both in MS and MS/MS experiments: the differences between calculated and measured *m/z* values were below 2.8 mDa in MS mode. In the IDA experiment, the data itself directs the kind of mass analysis function used. Its main advantage is that, within a single run, product ion spectra can be generated for precursor ions that are unknown beforehand. Its application in tomato-based products has allowed the identification of 47 phenolic compounds including simple phenolic and hydroxycinnamoylquinic acids, and flavone, flavanol, flavanone and dihydrochalcone derivatives, five of which, as far as it is known, have never been reported in tomatoes before.

The phenolic fingerprint unambiguously identified with HPLC-QTOF showed that tomato-based products differ in phenolic composition, principally in protocatechuic acid-*O*-hexoside, apigenin and its glycosylated forms, quercetin-*O*-dihexoside, kaempferol-*C*-hexoside and eriodictyol-*O*-dihexoside. As described in the literature, protocatechuic acid and its derivatives can be formed from more complex structures as a consequence of thermal processing. Quercetin-*O*-dihexoside, kaempferol-*C*-hexoside and apigenin were present in gazpachos due to the other vegetables added in its production, such as onion, garlic and bell pepper. Finally, eriodictyol-*O*-dihexoside was detected only in tomato juices, probably due to the addition of citric juice.

These three marketed tomato-based products are interesting for their content of bioactive phenolic compounds that have beneficial effects on human health. Moreover, the transformation of tomatoes is a useful economic strategy to extend their shelf life.

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6. Capítol VI: Avaluació d'un mètode per caracteritzar el perfil fenòlic de tomàquets orgànics i convencionals

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Resum: Aquest estudi pretén comparar el perfil fenòlic de tomàquets ecològics i convencionals mitjançant l'anàlisi *HPLC-MS/MS*. La metodologia es va optimitzar i validar. Per a la identificació dels compostos, es van fer servir experiments com: el *PIS*, l'*NL* i el *Prec*. L'*MRM* es va utilitzar per quantificar els flavonols, flavones, flavanones i àcids cinnàmics presents en les mostres de tomàquet. La confirmació dels compostos, prèviament identificats amb el *QqQ*, es va dur a terme amb un equip d'alta resolució (*QToF-MS*). D'aquesta manera, es van identificar 34 compostos. Les recuperacions dels compostos seleccionats eren superiors al 78% per als tomàquets convencionals i ecològics. Els valors de desviació estàndard relativa (*RSD%*), per a la precisió *intraday* i *interday*, van ser inferiors al 12% en tots els casos. Es van trobar nivells significativament ($P < 0.05$) superiors de polifenols en els tomàquets ecològics. Aquesta metodologia permet trobar diferències en el contingut de polifenols en tomàquets ecològics i convencionals que no havien estat descrites anteriorment.

Evaluation of a Method To Characterize the Phenolic Profile of Organic and Conventional Tomatoes

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ABSTRACT: The present study aims to compare the phenolic profiles of organic and conventional tomatoes bought in the market. For the quantification and identification of individual polyphenols, liquid chromatography coupled to mass spectrometry in tandem mode (LC-MS/MS) was carried out. Confirmation of the compounds previously identified on the triple-quadrupole was accomplished by injection in the high-resolution system (QToF-MS). In this way, 34 compounds were identified in tomato fruits. Recoveries of targeted polyphenols exceed 78% for conventional and organic tomatoes, respectively. The method intraday precision ranged between 3 and 5%, whereas the interday one was below 12%. Comparing the analyses of tomatoes from conventional and organic production systems demonstrated statistically higher levels ($P < 0.05$) of phenolic compounds in organic tomatoes. This methodology allowed finding differences in the bioactive components of organic and conventional tomatoes not previously reported.

KEYWORDS: organic tomatoes, conventional tomatoes, polyphenols, LC-ESI-MS/MS, QToF-MS

INTRODUCTION

The consumption of raw tomato is associated with a decrease in chronic degenerative diseases.¹ Tomatoes contain some valuable bioactive components, including polyphenols.^{2–4} The polyphenol content of plants is affected by cultivation, growing conditions, degree of ripeness, and plant variety.^{5–9} The nutrient composition of plants, including secondary plant metabolites, may be affected by different production systems, such as organic and conventional.^{10,11} These systems differ in the amounts of nutrients applied as fertilizers and in organic matter applied to the soil. The synthesis of secondary plant metabolites, proteins, and soluble solids is influenced by inorganic nitrogen availability. Moreover, organically produced plants have a longer ripening period because of a slower release of the supplied nutrients compared to conventional plants,¹² and as polyphenols are synthesized during the ripening period and the plant synthesizes them as phytoalexins under stress conditions, one may expect a higher content of these compounds in organically grown plants. In previous works made with market tomato-based products, organic ketchups and tomato juices had a significantly higher phenolic content than conventional alternatives.^{13,14} However, for these products, there is a lack of information about which tomato varieties have been used and if there are some differences in the technological treatments applied, so it is necessary to perform a study with the same varieties and grown under the same weather conditions, in which the only difference is organic or conventional growth.

Grinder-Pedersen et al.¹⁵ compared conventionally produced diets (CPD) and organically produced diets (OPD) in a human crossover intervention study ($n = 16$) in terms of the intake and excretion of five selected flavonoids and the effect on markers

of oxidative defense. The urinary excretion of quercetin and kaempferol was higher ($P < 0.05$) after 22 days of intake of the OPD than of the CPD. Therefore, the food production method not only affected the content of the major flavonoids in foods but also affected urinary flavonoids and markers of oxidation in humans.

Liquid chromatography (LC) coupled to mass spectrometry (MS) with electrospray ionization (ESI) is one of the most powerful tools to analyze phenolic compounds. For this kind of application, liquid chromatography electrospray ionization time-of-flight mass spectrometry (LC-ESI-QToF) allows the exact mass measurements of both MS and MS/MS ions to be achieved, which is essential for the characterization of small molecules.

We carried out a study to find phenolic profile differences between conventional and organic tomatoes in the content of flavonols (kaempferol-3-O-rutinoside, rutin, and quercetin), flavanones (naringenin and naringenin-7-O-glucoside), flavones (apigenin-7-O-glucoside), and hydroxycinnamic acids (ferulic, *p*-coumaric, caffeic, and chlorogenic acids). Therefore, we propose an analytical approach to obtain the phenolic profile of organic and conventional tomatoes. The methodology was optimized and validated. Confirmation of the compounds was accomplished by injection in the high-resolution system (LC-QToF-MS) using accurate mass measurements in MS and MS² modes. Moreover, the combination of LC-QToF-MS and

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triple-quadrupole enabled us to identify 34 polyphenols in tomato fruits.

MATERIALS AND METHODS

Standards and Reagents. All samples and standards were handled without exposure to light. Caffeic, *p*-coumaric, and chlorogenic acids, rutin, and quercetin were purchased from Sigma (Madrid, Spain) and naringenin, naringenin-7-*O*-glucoside, apigenin-7-*O*-glucoside, and kaempferol-3-*O*-rutinoside from Extrasynthèse (Genay, France). Hydrochloric acid 35% was from Panreac (Barcelona, Spain) and anhydrous sodium acetate (2 mol/L) from Merck (Darmstadt, Germany). Ethanol and formic acid of HPLC grade were obtained from Scharlau (Barcelona, Spain), and ultrapure water (Milli-Q) was from a Millipore system (Bedford, MA, USA).

Samples: Organic and Conventional Tomatoes. Organic and conventional tomatoes (cv. Daniella) were bought in Barcelona markets over two consecutive years, 2010 and 2011. All conventional and organic tomatoes selected had the same degree of ripeness (4.3–5 °Brix) and were normal-sized (75–80 g). Conventional farms utilize fertilizers containing soluble inorganic nitrogen and other nutrients, which are more directly available to plants. Organic systems emphasize the accumulation of soil organic matter and fertility over time through the use of cover crops, manures, and composts and rely on the activity of a diverse soil ecosystem to make nitrogen (N) and other nutrients available to plants. The amount of N present in cover crops varies from year to year, but, typically, organic plots currently receive between 240 and 260 kg of N ha⁻¹ per year in addition to the N fixed by the legume cover crop. Conventional tomatoes usually receive 50 kg ha⁻¹ of an N–P–K starter fertilizer and 118 kg ha⁻¹ of ammonium nitrate as side dressing.¹⁶ Tomatoes were bought and frozen until the analyses were carried out.

Extraction and Isolation of Phenolic Compounds. Sample treatment was performed by duplicate, in a darkened room with a red safety light to avoid the oxidation of the analytes during the process, following the procedure of Vallverdú-Queralt et al.⁵ with some modifications. Tomatoes (skin, flesh, and pips) were blended to obtain a tomato paste containing an average particle size of about 1 mm. Then, 0.5 g of the paste was weighed and homogenized with 4 mL of 80% ethanol in Milli-Q water and centrifuged (4000 rpm at 4 °C) for 20 min. The supernatant was transferred into a flask, and the extraction was repeated on the pellet with the same conditions. Both supernatants were combined and evaporated under nitrogen flow. Finally, the residue was reconstituted with up to 1.5 mL of Milli-Q water containing 0.1% formic acid.

Solid-phase extraction (SPE) of these extracts was carried out following the procedure previously reported^{17,18} using Oasis MAX cartridges (30 mg) from Waters. These cartridges were selected because they provided the highest recoveries. First, 1 mL of methanol and subsequently 1 mL of sodium acetate (50 mmol/L, pH 7) were loaded into the cartridges to equilibrate the cartridges; then, 1 mL of each extract was diluted with 1 mL of Milli-Q water and acidified with 34 µL of hydrochloric acid at 35% before being loaded into the cartridges. These were rinsed with 1 mL of 50 mmol/L sodium acetate (pH 7) at 5% of methanol. The polyphenols were eluted with 1800 µL of methanol containing 2% formic acid. The eluted fractions were evaporated under nitrogen flow, and the residue was reconstituted with up to 250 µL of water containing 0.1% formic acid and filtered with 13 mm polytetrafluoroethylene (PTFE) 0.45 µm filters from Waters (Milford, MA, USA) into an insert-amber vial for HPLC analysis. Samples were stored at –20 °C until analysis.

HPLC-ESI-MS/MS Analysis. To evaluate the differences between organic and conventional production systems, target analytes were identified and quantified using HPLC-ESI-MS/MS. An API 3000 (PE Sciex, Concord, ON, Canada) triple-quadrupole mass spectrometer equipped with a Turbo IonSpray source in negative ion mode was used to obtain MS/MS data. Turbo IonSpray source settings were as follows: capillary voltage, –3500 V; nebulizer gas (N₂), 10 arbitrary units (au); curtain gas (N₂), 12 au; collision gas (N₂), 4 au; focusing potential, –200 V; entrance potential, –10 V; drying gas (N₂), heated

to 400 °C and introduced to a flow rate of 6000 cm³/min. The declustering potential and collision energy were optimized for each compound in infusion experiments: individual standard solutions (10 µg/mL) dissolved in 50:50 (v/v) mobile phase were infused at a constant flow rate of 5 µL/min using a model syringe pump (Harvard Apparatus, Holliston, MA, USA). Full-scan data acquisition was performed scanning from *m/z* 100 to 800 in profile mode and using a cycle time of 2 s with a step size of 0.1 u and a pause between each scan of 2 ms. To confirm the identity of some compounds, neutral loss scan and precursor ion scan experiments were carried out as described by Vallverdú-Queralt et al.¹⁹

For quantitative purposes, two MRM transitions were selected for each of them, after having observed their product ion scan spectra. Table 1 shows the two MRM transitions together with the collision

Table 1. HPLC-MS/MS Parameters for Polyphenols and Internal Standard

analyte	rt ^a	MRM transition ^b	collision energy
chlorogenic acid	3.43	353→191 353→179	–20.00
caffeic acid	3.80	179→135 179→107	–20.00
<i>p</i> -coumaric acid	5.82	163→119 163→145	25.00
ferulic acid	6.00	193→134 193→149	–20.00
rutin	8.21	609→300 609→151	–50.00
kaempferol-3- <i>O</i> -rutinoside	9.18	593→285 593→255	–30.00
apigenin-7- <i>O</i> -glucoside	9.66	431→269 431→161	–20.00
naringenin-7- <i>O</i> -glucoside	10.70	433→271 433→151	25.00
quercetin	11.93	301→151 301→121	–30.00
naringenin	13.03	271→151 271→119	–30.00
internal standard: ethyl gallate	8.65	197→169	–20.00

^art, retention time. ^bThe first line reports the quantifier MRM transition and the second line the qualifier transition.

energy for polyphenols and internal standard (ethyl gallate). Each polyphenol was identified on the basis of its retention time, two selected MRM transitions, and their relative abundance. Quantitative analysis was performed by means of standard addition method because blank samples were not available.

The liquid chromatograph was an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, an autosampler, and a column oven set to 30 °C. A Luna C₁₈ column 50 × 2.0 mm i.d., 5 µm (Phenomenex, Torrance, CA, USA), was used. Mobile phases consisted of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B). The injection volume was

20 μL , and the flow rate was 0.4 mL/min. Separation was carried out in 20 min under the following conditions: 0 min, 5% B; 16 min, 40% B; 17 min, 95% B; 19 min, 95% B; 19.5 min, 5% B. The column was equilibrated for 5 min prior to each analysis.

HPLC-ESI-QToF Analysis. For accurate mass measurements, a HPLC-ESI-QToF was used following the procedure of Vallverdú-Queralt et al.²⁰ The chromatography was performed on an Agilent 1200 RRLC using the column and the gradient elution described above. Flow rate was 0.4 mL/min, and injection volume was 5 μL . The column was equilibrated for 5 min prior to each analysis. The HPLC system was coupled to a hybrid quadrupole ToF QSTAR Elite (ABSciex). The MS acquisition was performed in negative ionization using IDA between m/z 90 and 1100. MS parameters were as follows: ion spray voltage, -4200 V ; declustering potential, -60 V ; focusing potential, -190 V ; declustering potential 2, -15 V ; ion release delay, 6 V; ion release width, 5 V; temperature, 400°C with curtain gas (N_2), 50 au; auxiliary gas, 50 au; and nebulizer gas (N_2), 50 au. IDA was performed using the following criteria: ions that exceed 5 counts; ion tolerance, 50 mDa; collision energy, fixed at -30 V ; dynamic background subtract activated. The QToF was calibrated as recommended by the manufacturer.

The elemental composition of each polyphenol was selected according to the accurate masses and the isotopic pattern (through the Formula Finder feature in Analyst QS 2.0) and searched for in the Dictionary of Natural Products (Chapman & Hall/CRC) and the MOTO database (<http://appliedbioinformatics.wur.nl/moto>). The interpretation of the observed MS/MS spectra in comparison with those found in the literature was the main tool for putative identification of polyphenols.

Validation Method. Validation assays were performed by means of the standard addition method. Therefore, we estimated the unknown amount of the analytes in organic and conventional tomatoes, and we evaluated the linear dynamic range and sensitivity in the analysis in both organic and conventional tomatoes. Recoveries, precisions, limits of detection (LODs), limits of quantitation (LOQs), and stabilities were calculated after determination of the natural levels of each polyphenol in organic and conventional tomatoes.

Standard Addition Method. Analyte quantification was performed by the standard addition method (five points). For this purpose, five aliquots (0.5 g each) of organic tomato and five aliquots (0.5 g each) of conventional tomato were spiked with the same amount of the internal standard (ethyl gallate); four aliquots of each group were then spiked with different concentrations of phenols (between 11.70 and 465.80 $\mu\text{g/g}$) and submitted to the extraction process. The spiked level was chosen to increase the original content of polyphenols in organic and conventional tomatoes by a factor between 2 and 3.

Recovery and Precision. After a preliminary screening of the phenolic profile in organic and conventional tomatoes, analyte recoveries were assessed by analyzing the percentage of recovery of each polyphenol in conventional and organic tomatoes. The samples were spiked with four different concentrations (50, 100, 150, and 200% of expected value) of each polyphenol before extraction. The spiked samples were extracted by triplicate and analyzed under the previously established optimal conditions. The percentage of recovery was calculated for each polyphenol, whereas the corresponding relative standard deviation (RSD) was representative for intraday precision. Interday precision was estimated as the RSD performed within three different days.

Limits of Detection and Limits of Quantitation. The sensitivity of the method was evaluated determining the LODs and LOQs. With regard to the two MRM transitions, the quantifier transition was used for quantitative purposes, whereas the confirmation transition was used for qualitative analysis and for method limits. The LOD was calculated as the quantity of analyte able to produce a chromatographic peak 3 times higher than the noise of the baseline in a chromatogram ($S/N = 3$) of a nonfortified sample, after having estimated the endogenous amount. The LOQ was set at 10 times higher than the noise of the baseline in a chromatogram ($S/N = 10$).²¹

Stability. Three aliquots with four different quantities (50, 100, 150, and 200% of expected value) were used to evaluate the short-term temperature stability at -20 and -80°C . The aliquots were thawed at room temperature for 6 h (the mean sample preparation time) and then analyzed. To evaluate the stability after successive freeze–thaw cycles, three aliquots of each concentration (50, 100, 150, and 200% of expected value) were stored at -20 and -80°C for 24 h and then thawed at room temperature up to four times over a 1 week period. Analytes were assessed in each aliquot in a single run at the end of the last freeze–thaw cycle. Aliquots of each concentration for long-term stability were prepared and immediately frozen at -20 and -80°C until analysis (within 1 year). The concentrations of all the stability samples were compared with the mean of back-calculated values for the standards at each concentration from the first day of long-term stability testing.

RESULTS AND DISCUSSION

It is well-known that the biosynthesis of phenolic compounds in plants is strongly influenced by the cultivar⁵ and mode of fertilization.²² The level of carbon-based secondary metabolites such as phenolic compounds is usually higher in organic plants^{23,24} due to their defensive role in plants under stress conditions.²⁵ Data on the phenolic composition of fruits and vegetables grown either organically or conventionally remain scarce in the literature as these compounds have only recently been considered to be interesting functional microconstituents due to their potential role in the prevention of cardiovascular diseases, degenerative diseases, and cancer.²⁶ Among papers mentioning total phenolic content, the majority describe a higher phenolic concentration in organically grown fruits or vegetables.²⁷ Our results are in accordance with these studies because organic tomatoes showed a higher content of polyphenols than conventional tomatoes.

Validation Results. Standard Addition Method. Linear regression parameters are reported in Table 2. A good linearity was verified, with all correlation coefficients exceeding 0.9907. Slopes were very similar for curves representing organic and conventional tomatoes, suggesting a comparable matrix effect. LODs and LOQs are also shown in Table 2. The noise level depended on the matrix and, therefore, the same analyte was characterized by different LODs and LOQs in organic and conventional tomatoes. The LODs varied between 0.13 and 0.66 $\mu\text{g/g}$ fresh weight (FW) for conventional tomatoes and between 0.18 and 0.61 $\mu\text{g/g}$ FW for organic tomatoes. The LOQs ranged between 0.43 and 2.20 $\mu\text{g/g}$ FW for conventional tomatoes and between 0.60 and 2.04 $\mu\text{g/g}$ FW for organic tomatoes.

Recovery and Precision. After a preliminary determination of the polyphenol content in organic and conventional tomatoes, Table 3 reports the definitive values of the estimated quantities by the standard addition, recoveries, and precision data in organically and conventionally grown tomatoes. Recoveries ranged between 78 and 98%, and intraday and interday precisions were less than 5 and 11%, respectively, for all of the analytes. For the values analyzed, recovery and precision were between the accepted values of AOAC International.²¹

Stability. Freeze and thaw cycles did not significantly modify the polyphenol concentration at either temperature tested; polyphenol recoveries were between 85 and 98% at -80°C and between 81 and 97% at -20°C . For short-term stability, the recoveries of polyphenols were between 80 and 98% at -20°C and between 78 and 94% when the temperature was -80°C . Long-term storage at -20 and -80°C did not affect the polyphenol recoveries. They were between 75 and 91% after

Table 2. Method Validation Parameters: Slopes (m), Correlation Coefficients (R^2) and Limits of Detection (LOD) and Quantification (LOQ)

analyte	conventional tomatoes				organic tomatoes			
	m (10^3)	R^2	LOD ($\mu\text{g/g FW}$)	LOQ ($\mu\text{g/g FW}$)	m (10^3)	R^2	LOD ($\mu\text{g/g FW}$)	LOQ ($\mu\text{g/g FW}$)
caffeic acid	3.50	0.9933	0.30	0.99	3.30	0.9911	0.22	0.73
chlorogenic acid	4.23	0.9943	0.26	0.88	3.60	0.9907	0.29	0.97
ferulic acid	0.78	0.9958	0.42	1.39	0.43	0.9926	0.53	1.77
<i>p</i> -coumaric acid	4.40	0.9934	0.28	0.94	4.08	0.9934	0.22	0.73
naringenin	2.10	0.9932	0.19	0.63	2.60	0.9918	0.20	0.67
naringenin-7- <i>O</i> -glucoside	2.10	0.9934	0.13	0.43	2.35	0.9958	0.18	0.60
rutin	7.30	0.9927	0.21	0.70	7.62	0.9990	0.24	0.80
quercetin	2.90	0.9919	0.66	2.20	2.35	0.9957	0.61	2.04
kaempferol-3- <i>O</i> -rutinoside	22.35	0.9957	0.36	1.20	25.30	0.9908	0.33	1.10
apigenin-7- <i>O</i> -glucoside	0.82	0.9985	0.23	0.77	0.80	0.9950	0.26	0.86

1 year, at both temperatures. Therefore, the storage conditions used for the assays allowed the phenols to remain stable.

Application of the Method for the Analysis of Conventional and Organic Tomatoes. The results concerning the quantitative determination of the target polyphenols are summarized in Table 3, whereas those related to the screening of polyphenols (hydroxycinnamic acids, flavonols, flavanones, flavones, and their derivatives) are given in Table 4. To avoid the effect of water evaporation and concentration of solids taking place on the quantification, the estimated values were expressed as FW and dry weight (DW).

The main polyphenol in all tomatoes was rutin, followed by naringenin, as reported in other studies.^{5,28} Rutin and naringenin concentrations were significantly higher in organically grown tomatoes. Chassy et al.²⁹ found significantly higher mean levels of soluble solids, flavonoids, total phenolics, and ascorbic acid in organic tomatoes than in their conventional counterparts grown in model plots over a 3 year period. However, in their study, a complete phenolic profile identification was not performed because only flavonoids were identified.

Simple Phenolic Acid Derivatives. Phenolic acids and their derivatives are widely distributed in plants. They are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but also because of their stable radical intermediates, which prevent the oxidation of various food ingredients.³⁰

The examination of the chromatograms in full scan mode on the triple-quadrupole system revealed the presence of ferulic (m/z 193), caffeic (m/z 179), and *p*-coumaric (m/z 163) acids (Table 4). MS^2 of these ions showed the deprotonated molecule $[M - H]^-$ and the typical loss of CO_2 , giving $[M - H - 44]^-$ as a characteristic ion. These results were confirmed by injection in the high-resolution system (ToF-MS) using accurate mass measurements and the MS^3 fragmentation patterns.

Absolute differences in the levels of ferulic, caffeic, and *p*-coumaric acid were found in organic and conventional tomatoes (Table 3). Phenolic acids were higher in organic tomatoes, having contents of 34.25 $\mu\text{g/g FW}$ (380.55 $\mu\text{g/g DW}$) for *p*-coumaric acid and 41.70 $\mu\text{g/g FW}$ (463.33 $\mu\text{g/g DW}$) for caffeic acid, whereas for conventional tomatoes phenolic acids ranged between 20.59 $\mu\text{g/g FW}$ (257.38 $\mu\text{g/g DW}$) for *p*-coumaric acid and 22.88 $\mu\text{g/g FW}$ (286.00 $\mu\text{g/g DW}$) for caffeic acid. We tentatively attributed the higher concentration of phenolic acids in organic tomatoes to mode of fertilization.²³

Phenolic acid-*O*-hexosides were identified in ToF-MS mode using accurate mass measurements and MS^2 fragmentations

(Table 4). Caffeic acid-*O*-hexosides (m/z 341) were detected in all conventional and organic tomatoes. The MS^2 of m/z 341 showed a characteristic fragmentation involving cleavage of the intact sugar $[M - H - 162]^-$ (m/z 179) and the ion corresponding to the loss of CO_2 (m/z 135).

In addition, the analysis in ToF-MS mode revealed the presence of two homovanillic acid-*O*-hexosides (m/z 343) and two coumaric acid-*O*-hexosides (m/z 325). The MS^2 of m/z 343 and 325 showed ions corresponding to the deprotonated molecule $[M - H]^-$, the loss of a hexose $[M - H - 162]^-$, and the loss of the carboxylic group (Table 4).

The analysis in ToF-MS mode also showed the presence of ferulic acid-*O*-hexoside (m/z 355). The MS^2 of m/z 355 showed ions at m/z 193, 178, and 149 corresponding to the loss of a hexose moiety $[M - H - 162]^-$ and the loss of a methyl and acid group from the aglycone (Table 4).

Hydroxycinnamoylquinic Acid Derivatives. Preliminary structure-activity relationship studies of cinnamic acids and derivatives demonstrated the importance of the catechol group in the antiradical efficacy.³¹ The examination of chromatograms in full scan mode in the triple-quadrupole system of organic and conventional tomatoes revealed a peak at m/z 353 corresponding to chlorogenic acid (Table 4). Chlorogenic acid was the most abundant hydroxycinnamic acid, ranging from 36.87 $\mu\text{g/g FW}$ (460.88 $\mu\text{g/g DW}$) in conventional tomatoes to 56.99 $\mu\text{g/g FW}$ (633.22 $\mu\text{g/g DW}$) in organic tomatoes (Table 3). These results are in line with those reported by Caris-Veyrat et al.,³² who retrieved significantly higher concentrations of chlorogenic acid from organic tomatoes ($P < 0.05$) in comparison to the conventional variant. These results were confirmed by injection in the ToF-MS using accurate mass measurements and the MS^2 fragmentation patterns.

Neochlorogenic and cryptochlorogenic acid were also present (Table 4). It was possible to differentiate the isomers of chlorogenic acid by their relative intensities in MS^2 spectra according to the method cited by other authors using liquid chromatography-tandem mass spectrometry.³³ Another caffeoylquinic isomer acid was also detected, which might be a stereoisomer of the neochlorogenic acid, chlorogenic acid, or cryptochlorogenic acid as described by other authors.^{20,34}

The analysis in ToF-MS mode also showed the presence of two dicaffeoylquinic acid isomers (m/z 515). The MS^2 of m/z 515 showed the ion $[M - H - 162]^-$ (m/z 353, corresponding to the loss of a caffeic acid unit) and the deprotonated quinic acid (m/z 191). Two tricaffeoylquinic acids (m/z 677) were also identified in both varieties of tomatoes. MS^2 experiments of m/z 677 of both compounds revealed characteristic

Table 3. Estimated Quantities and Recoveries (Relative Standard Deviations) of Polyphenols from Organic and Conventional Tomatoes

analyte	conventional tomatoes				organic tomatoes			
	estimated quantity ($\mu\text{g/g FW} \pm \text{SD}^a$)	lowest spiked level ^b	recovery ^c $\pm$ SD	RSD ^d (%)	estimated quantity ($\mu\text{g/g FW} \pm \text{SD}$)	lowest spiked level	recovery $\pm$ SD	RSD (%)
caffeic acid	22.88 $\pm$ 0.45	46.60	98.40 $\pm$ 3.22	3.27	41.70 $\pm$ 0.81	93.16	97.04 $\pm$ 2.95	3.05
chlorogenic acid	36.87 $\pm$ 0.61	69.90	96.23 $\pm$ 2.89	3.00	56.99 $\pm$ 0.90	116.50	96.12 $\pm$ 3.10	3.23
ferulic acid	21.69 $\pm$ 0.40	69.90	94.15 $\pm$ 4.49	4.77	35.11 $\pm$ 0.63	69.90	96.88 $\pm$ 4.01	4.15
<i>p</i> -coumaric acid	20.59 $\pm$ 0.36	46.60	94.89 $\pm$ 2.87	3.02	34.25 $\pm$ 0.55	69.90	97.65 $\pm$ 2.90	2.97
naringenin	36.46 $\pm$ 0.69	116.50	85.11 $\pm$ 3.97	4.69	87.38 $\pm$ 0.98	163.00	88.10 $\pm$ 3.51	3.98
naringenin-7-O-glucoside	7.68 $\pm$ 0.15	23.30	87.02 $\pm$ 3.14	3.58	13.91 $\pm$ 0.28	23.30	88.63 $\pm$ 3.02	3.41
rutin	119.82 $\pm$ 1.49	233.00	93.44 $\pm$ 4.72	5.05	272.75 $\pm$ 2.98	465.80	95.11 $\pm$ 4.51	4.74
quercetin	5.69 $\pm$ 0.12	11.70	77.81 $\pm$ 3.88	5.00	11.42 $\pm$ 0.18	23.30	78.20 $\pm$ 3.41	4.36
kaempferol-3-O-rutinoside	6.03 $\pm$ 0.11	11.70	89.15 $\pm$ 3.27	3.67	12.70 $\pm$ 0.24	23.30	91.34 $\pm$ 3.02	3.31
apigenin-7-O-glucoside	28.28 $\pm$ 0.63	46.60	91.05 $\pm$ 2.65	2.90	31.63 $\pm$ 0.52	46.60	93.54 $\pm$ 2.87	3.07

^aSD, standard deviation. ^bThe lowest spiked level (50%) was applied for doubling or tripling the endogenous concentration of each compound. ^cMean recovery of all spiked levels (50, 100, 150, and 200%). ^dRSD, relative standard deviation.

Table 4. Analytes Tentatively Identified in Organic and Conventional Tomatoes

analyte ^a	rt ^b	[M - H] ⁻	m/z ions	accurate mass	mDa ^c	MF ^d
caffeic acid-O-hexoside 1	1.43	341	179 (100), 135 (30)	341.0877	1.60	C ₁₃ H ₁₈ O ₆
caffeic acid-O-hexoside 2	1.54	341	179 (100), 135 (30)	341.0877	0.70	C ₁₃ H ₁₈ O ₆
neochlorogenic acid	1.65	353	191 (100), 179 (70), 135 (30)	353.0877	0.70	C ₁₈ H ₁₆ O ₆
caffeic acid-O-hexoside 3	2.16	341	179 (100)	341.0877	2.30	C ₁₃ H ₁₈ O ₆
homovanillic acid-O-hexoside 1	2.22	343	343 (100), 137 (70), 109 (40)	343.1034	1.10	C ₁₃ H ₂₀ O ₆
ferulic acid-O-hexoside	2.49	355	193 (60), 178 (30), 149 (100)	355.1034	2.50	C ₁₈ H ₂₀ O ₆
homovanillic acid-O-hexoside 2	2.82	343	343 (100), 137 (70), 109 (40)	343.1034	2.10	C ₁₃ H ₂₀ O ₆
caffeic acid-O-hexoside 4	2.90	341	179 (100), 135 (50)	341.0877	0.70	C ₁₃ H ₁₈ O ₆
coumaric acid-O-hexoside 1	2.99	325	163 (85), 119 (100)	325.0928	1.40	C ₁₃ H ₁₆ O ₆
coumaric acid-O-hexoside 2	3.26	325	163 (90), 119 (100)	325.0928	0.20	C ₁₃ H ₁₆ O ₆
chlorogenic acid*	3.43	353	191 (100), 179 (5)	353.0877	1.50	C ₁₈ H ₁₈ O ₆
caffeic acid*	3.80	179	135 (100), 107 (40)	179.0349	1.40	C ₉ H ₈ O ₄
cryptochlorogenic acid	4.14	353	191 (70), 173 (100), 135 (20)	353.0877	0.80	C ₁₈ H ₁₆ O ₆
naringenin-C-diglycoside	4.96	595	595 (100), 505 (35), 475 (60), 385 (50), 355 (45)	595.1667	0.70	C ₂₇ H ₃₂ O ₁₃
chlorogenic acid isomer	5.15	353	191 (100)	353.0877	1.00	C ₁₈ H ₁₈ O ₆
rutin-O-hexoside	5.20	771	771 (100), 609 (80), 300 (20)	771.1989	1.60	C ₂₃ H ₃₀ O ₂₁
<i>p</i> -coumaric acid*	5.82	163	163 (40), 119 (100)	163.0400	0.86	C ₉ H ₈ O ₃
ferulic acid*	6.00	193	193 (20), 178 (70), 149 (20), 134 (100)	193.0506	1.19	C ₁₈ H ₁₆ O ₄
apigenin-C-hexoside-hexoside	6.26	593	503 (10), 473 (40), 353 (10)	593.1511	0.30	C ₂₇ H ₃₀ O ₁₃
rutin-O-pentoside	7.66	741	741 (100), 609 (90), 300 (20)	741.1883	0.70	C ₂₃ H ₂₈ O ₂₀
apigenin-C-hexoside-pentoside	7.78	563	503 (10), 473 (35), 383 (15), 353 (25)	563.1406	0.90	C ₂₆ H ₂₈ O ₁₄
quercetin-O-dihexoside	7.90	625	463 (100), 300 (40)	625.1410	1.60	C ₂₇ H ₃₀ O ₁₇
rutin*	8.21	609	609 (100), 300 (40)	609.1460	0.50	C ₂₇ H ₃₀ O ₁₆
naringenin-C-hexoside	9.03	433	433 (30), 343 (20), 313 (40)	433.1140	0.60	C ₂₁ H ₂₂ O ₁₀
naringenin-O-hexoside	9.12	433	433 (10), 271 (70)	433.1140	0.10	C ₂₁ H ₂₂ O ₁₀
kaempferol-3-O-rutinoside*	9.18	593	593 (100), 285 (70), 255 (20)	593.1511	0.70	C ₂₇ H ₃₀ O ₁₅
dicaffeoylquinic acid 1	9.50	515	515 (20), 353 (100), 335 (20), 191 (45), 173 (60)	515.1194	0.10	C ₂₇ H ₃₂ O ₁₂
apigenin-7-O-glucoside*	9.66	431	431 (25), 269 (60), 161 (75)	431.0983	0.80	C ₂₁ H ₂₀ O ₁₀
dicaffeoylquinic acid 2	10.25	515	515 (25), 353 (70), 191 (100), 179 (75)	515.1194	1.60	C ₂₇ H ₃₂ O ₁₂
naringenin-7-O-glucoside (prunin)*	10.70	433	433 (30), 271 (100), 151 (50)	433.1140	1.10	C ₂₁ H ₂₂ O ₁₀
tricaffeoylquinic acid 1	11.31	677	677 (25), 515 (100), 353 (40), 191 (15)	677.1511	1.40	C ₃₄ H ₄₀ O ₁₅
quercetin*	11.93	301	301 (10), 151 (100)	301.0353	0.40	C ₁₅ H ₁₀ O ₇
tricaffeoylquinic acid 2	12.98	677	677 (55), 515 (100), 353 (20), 173 (15)	677.1511	1.00	C ₃₄ H ₄₀ O ₁₅
naringenin*	13.03	271	151 (100), 119 (70)	271.0611	1.50	C ₁₃ H ₁₂ O ₅

^aThe asterisk (*) indicates comparison with standard. ^brt, retention time. ^cmDa, millidaltons of error between the mass found and the of each polyphenol. ^dMF, molecular formula.

fragmentations involving cleavage of three caffeoyl moieties for tricaffeoylquinic acid isomers (Table 4).

Flavone Derivatives. The flavone apigenin is known to have a number of biological functions such as possible anti-inflammatory,

cytotoxic, and free radical scavenging properties. Studies of human malignant cancer cell lines have shown that apigenin inhibits cancer cell growth via apoptosis.³⁵ The analysis in full scan mode in the triple-quadrupole system showed the presence of apigenin 7-O-glucoside (m/z 431). The product ion scan of m/z 431 showed peaks at m/z 269 and 151, which were attributed to the loss of a glucose moiety $[M - H - 162]^-$ and to the ion corresponding to retro-Diels-Alder (RDA) fragmentation, as described by other authors.^{36,37} These results were confirmed by injection in the ToF-MS using accurate mass measurements and the MS² fragmentation patterns (Table 4). The smallest differences between organic and conventional tomatoes were shown for apigenin-7-O-glucoside. Levels of apigenin-7-O-glucoside were higher in organic tomatoes than in conventional ones. However, the differences were not so pronounced as in the case of phenolic and hydroxycinnamoylquinic acid derivatives. Organic tomatoes contained 31.63 $\mu\text{g/g}$ FW (351.44 $\mu\text{g/g}$ DW) apigenin-7-O-glucoside, whereas conventional tomatoes contained 28.28 $\mu\text{g/g}$ FW (353.50 $\mu\text{g/g}$ DW) (Table 3).

Apigenin-C-hexoside-pentoside (m/z 563) and apigenin-C-hexoside-hexoside (m/z 593) were tentatively identified in ToF-MS mode in conventional and organic tomatoes. The product ion scan of C-diglycosides revealed characteristic losses of 60, 90, and 120 u corresponding to cross-ring cleavages in the sugar unit (Table 4). Apigenin-C-hexoside-hexoside and apigenin-C-hexoside-pentoside could be distinguished by the presence of the ion $[M - H - 60]^-$ according to the method cited by other authors.^{30,38}

Flavonone Derivatives. The chromatograms in the triple-quadrupole revealed the presence of naringenin (m/z 271). It was identified by comparing their retention times with their reference substance. These results were confirmed by injection in the high-resolution system (ToF-MS) using accurate mass measurements and the MS² fragmentation patterns (Table 4). Naringenin has been identified as one of the major polyphenols in tomatoes. A recent study demonstrated that subchronic administration of flavanones significantly attenuated the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the decrease in striatal dopamine (DA) concentrations. This suggests that these compounds may be considered as potential candidates for a dietary supplement in the treatment of Parkinson's disease.³⁹

Two naringenin-O-hexosides (m/z 433) and one naringenin-C-hexoside (m/z 433) were tentatively identified in the ToF-MS mode (Table 4). The MS² mass spectrum of m/z 433, which has been assigned to O-substitution, showed an ion at m/z 271 corresponding to the loss of a hexoside moiety $[M - H - 162]^-$ whereas the product ion scan of naringenin-C-hexoside revealed characteristic losses of 90 and 120 u from m/z 433 corresponding to cross-ring cleavages in the sugar unit.

Both naringenin and naringenin-7-O-glucoside (prunin) were higher in organic tomatoes (Table 3). Naringenin levels were 36.46 $\mu\text{g/g}$ FW (455.75 $\mu\text{g/g}$ DW) in conventional tomatoes and 87.38 $\mu\text{g/g}$ FW (970.88 $\mu\text{g/g}$ DW) in organic tomatoes, whereas levels of naringenin-7-O-glucoside varied from 7.68 $\mu\text{g/g}$ FW (96.00 $\mu\text{g/g}$ DW) in conventional to 13.91 $\mu\text{g/g}$ FW (154.56 $\mu\text{g/g}$ DW) in organic tomatoes.

Moreover, a peak showing m/z 595 could be attributed to naringenin-C-diglycoside (Table 4). The MS² of this ion displayed losses of 90 and 120 u from m/z 595 and 475, respectively, which confirmed the presence of two hexose (glucose or galactose) units.

Flavonol Derivatives. The examination of the chromatograms in the triple-quadrupole in MS mode of organic and conventional tomatoes revealed the presence of some peaks at m/z 609, 593, and 301 corresponding to rutin, kaempferol-3-O-rutinoside, and quercetin, respectively. These results were confirmed by injection in the high-resolution system (ToF-MS) (Table 4). The MS² of m/z 609 and 593 showed peaks corresponding to the deprotonated molecule $[M - H]^-$ and the loss of the rutinoside unit, $[M - H - 308]^-$. Flavonol aglycons such as quercetin gave as a characteristic ion the deprotonated molecule $[M - H]^-$ and ions corresponding to RDA fragmentation.

The organic tomatoes had higher levels of rutin, 272.75 $\mu\text{g/g}$ FW (3030.56 $\mu\text{g/g}$ DW), than the conventional ones, 119.82 $\mu\text{g/g}$ FW (1497.75 $\mu\text{g/g}$ DW). Similarly, the rates of quercetin and kaempferol-3-O-rutinoside were much lower in the conventionally produced tomatoes (Table 3). Mitchell et al.⁴⁰ described results in line with our study, reporting that the mean levels of the flavonoids quercetin and kaempferol were significantly higher ($P < 0.001$) in tomato samples from the organic cropping system than in those produced conventionally. They suggested that there is a significant difference between the two systems in the amount of flavonoids occurring in ripe fruit at harvest. Therefore, different food cultivation methods may result in differences in the content of secondary metabolites such as polyphenol compounds, and such an increase may have health-related effects because polyphenols increase significantly after organic food consumption.

The analysis in the ToF-MS confirmed the presence of rutin-O-hexoside (m/z 771) and rutin-O-pentoside (m/z 741) in both conventional and organic tomatoes (Table 4). The MS² mass spectrum of m/z 771 showed peaks at m/z 609 and 300 corresponding to the loss of a hexoside $[M - H - 162]^-$ and to the radical anion of the aglycone (m/z 300) as described in other studies using a triple-quadrupole mass spectrometer.^{19,41} The MS² mass spectrum of m/z 741 was assessed, showing peaks at m/z 609 and 300 corresponding to the loss of a pentoside $[M - H - 132]^-$ and to the radical anion of the aglycone (m/z 300).

In addition, quercetin-O-dihexoside (m/z 625) was also identified in the ToF-MS (Table 4). The MS² of m/z 625 showed ions at m/z 463 and 300 corresponding to the loss of one hexoside unit and two hexoside units, respectively.

A number of studies have addressed the question of whether agricultural chemicals and other agricultural methods including organic farming affect nutrient content. The question is still unresolved. When plants are grown with artificial nutrients, they are supposed to lose their natural defense mechanisms. This may result in reduced disease resistance and diluted contents of minerals, vitamins, and defense-related secondary metabolites, which are considered beneficial for human health. In the present study the growing conditions of tomatoes (conventional versus organic) affected the content of phenolic compounds of these vegetables. The organically produced tomatoes displayed a higher phytochemical concentration than conventionally produced tomatoes. Thus, vegetable and fruit products grown in organic agriculture would be expected to be more health-promoting than those produced conventionally. The LC-MS-MS method was completely validated, providing a sensitive analysis for polyphenol detection and showing satisfactory data for all parameters tested. Good results were obtained with respect to linearity and recovery as well as an excellent level of precision.

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7. Capítol VII: Hi ha alguna diferència en el perfil fenòlic de suc de tomàquet orgànics i convencionals?

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Resum: El contingut de polifenols en els tomàquets es veu influït per les condicions de cultiu, el grau de maduresa i la varietat. Les diferències fonamentals entre els sistemes de producció ecològics i convencionals es fonamenten sobretot en la manera de cultivar i adobar el sòl, la qual cosa pot afectar la composició nutritiva dels tomàquets i en particular els metabòlits secundaris. Els tomàquets convencionals reben herbicides i plaguicides, mentre que els cultius orgànics reben només plaguicides orgànics aprovats, com els compostos de sofre i benzotiofè. Les granges convencionals utilitzen fertilitzants que contenen nitrogen inorgànic soluble i altres nutrients, que són més directament absorbits per les plantes. La disponibilitat de nitrogen inorgànic, en particular, té el potencial per influir en la síntesi dels metabòlits secundaris de les plantes, de les proteïnes i dels sòlids solubles. Per tant, en aquest capítol, es pretén estudiar les diferències entre suc de tomàquet orgànics i convencionals en relació al nivell de polifenols totals i individuals i capacitat antioxidant. L'estudi es va dur a terme durant l'any 2010. Els suc de tomàquet orgànics van presentar nivells més elevats de polifenols i antioxidants que els convencionals. Aquests resultats es podrien explicar pels mecanismes de defensa de les plantes segons el tipus d'adob emprat en el cultiu orgànic o en el convencional. En tots els cultius, el factor més important per al creixement de la planta és el nitrogen. Ara bé, en el cas de l'agricultura convencional, aquest nitrogen es pot afegir a la planta com a nitrogen soluble, com a fertilitzant, etc., mentre que en els cultius orgànics la planta no rep nutrients artificials, i reacciona activant mecanismes de defensa que augmenten els nivells de polifenols. En conseqüència, les plantes d'agricultura tradicional es poden tornar menys resistents a malalties i presentar un contingut més baix en nutrients, minerals i metabòlits secundaris.



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Analytical Methods

Is there any difference between the phenolic content of organic and conventional tomato juices?

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ABSTRACT

The present study aims to compare the phenolic and hydrophilic antioxidant profiles of organically and conventionally produced tomato juices. Comparisons of analyses of archived samples from conventional and organic production systems demonstrated statistically higher levels ($P < 0.05$) of phenolic compounds in organic tomato juices. This increase corresponds not only with increasing amounts of soil organic matter accumulating in organic plots but also with reduced manure application rates once soils in the organic systems had reached equilibrium levels of organic matter. Using principal component analysis, results show that phenolic compounds and hydrophilic antioxidant capacity were responsible for the differentiation between organic and conventional tomato juices. Thus, there appear to be genuine differences in the bioactive components of organic and conventional tomato juices not previously reported.

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1. Introduction

Polyphenols are a widespread family of phytochemicals with diverse biological functions in plants. Interest in their analysis has increased due to their recognised physiological actions. Numerous studies relate the ingestion of polyphenols in diet to a lower risk of cardiovascular diseases (Silaste, Alfthan, Aro, Kesaniemi, & Horkko, 2007) and development of cancers (Grieb et al., 2009; Zhang et al., 2009).

Due to the increasing demand for processed tomato products, it is extremely important to determine the phenolic compounds and antioxidants of tomato juices and their possible beneficial impact on human health. Juice is an intermediate product in the processing of tomato paste, obtained in the juice extraction stage of a process that eventually results in tomato concentrate. Tomato juice can be separated from the pulp by filtering, but more commonly the entire pulp is used as juice. The juice is formulated according to the characteristics demanded by the market: the most common is with extra virgin olive oil (1.8%), salt and citric juice to adjust the pH. The product is then bottled and pasteurised to extend its shelf life (Galicia-Cabrera, 2007).

It is known that many of the tomato nutrients may function individually, or in concert, to protect lipoproteins and vascular cells from oxidation. Several of these substances have been shown to exhibit other cardioprotective functions, such as reduction of homocysteine, platelet aggregation, and blood pressure levels. Evidence of a preventive effect of tomato product consumption on cardiovascular disease and atherosclerosis is, to date, circumstantial but rapidly accumulating (Libby, Ridker, & Maseri, 2002).

In addition, consumption of tomato juice in type 2 diabetic subjects caused a significant elevation of plasma lycopene as well as increased resistance of low-density lipoprotein (LDL) to oxidation (Gianetti et al., 2002).

Polyphenol content in plants is influenced by cultivation and harvesting conditions such as growing conditions, degree of ripeness and plant variety (Barrett, Weakley, Diaz, & Watnik, 2007; Connor, Luby, Tong, Finn, & Hancock, 2002; Herrmann, 1976; Toor, Savage, & Heeb, 2006). Fundamental differences between organic and conventional production systems, particularly in soil fertility management, may affect the nutritive composition of plants, including secondary plant metabolites (Daniel, Meier, Schlatter, & Frischknecht, 1999; Lydon & Duke, 1989; Williams, 2002). Conventional and organic systems differ in the amount of irrigation received (rain fed or irrigated), in the amounts of nutrients applied as fertilizers and in organic matter applied to the soil as crop residues, winter legume cover crops or composted manure. The availability of inorganic nitrogen in particular, has the potential to influence the synthesis of secondary plant metabolites, proteins

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and soluble solids. In addition, organically produced plants have a longer ripening period compared to conventional plants, because of a slower release of the supplied nutrients (Nielsen, Mølgaard, & Lærke, 1997), and as flavonoids are formed in the ripening period, one could expect a higher content of these compounds in organically grown plants.

The aim of this work was to find phenolic profile differences between conventional and organic tomato juices. Therefore, total polyphenol content (TP), hydrophilic antioxidant capacity and the content of flavonols (kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside, rutin and quercetin), flavanones (naringenin and naringenin-7-O-glucoside) and hydroxycinnamic acids (ferulic-O-hexoside, caffeic-O-hexoside, caffeic, chlorogenic, cryptochlorogenic and dicaffeoylquinic acids) were analyzed in conventional and organic tomato juices.

2. Materials and methods

2.1. Standards and reagents

All samples and standards were handled without exposure to light. Caffeic and chlorogenic acids, rutin and quercetin, Folin-Ciocalteu (F–C) reagent, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), PBS: phosphate-buffered saline pH 7.4, Trolox: (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid 97%, manganese dioxide and DPPH: 2,2-diphenyl-1-picrylhydrazyl were purchased from Sigma® (Madrid, Spain); naringenin, naringenin-7-O-glucoside, kaempferol-3-O-rutinoside and kaempferol-3-O-glucoside from Extrasynthèse (Genay, France). Ethanol and formic acid HPLC grade were obtained from Scharlau (Barcelona, Spain) and ultrapure water (Milli-Q) from Millipore System (Bedford, USA).

2.2. Processing conditions of organic and conventional tomato juices

Processing of both organic and conventional tomato juice includes multiple steps such as washing, fruit selection, grinding and sieving. Juice is an intermediate product in the processing of tomato paste since it is obtained in the juice extraction stage; when the process continues it results in tomato concentrate. Tomato juice can be separated from the pulp by filtering, but more commonly the entire pulp is used as juice. The juice is formulated according to the characteristics demanded by the market: the most common is the juice with extra virgin olive oil (1.8%), salt, and citric juice, which is added to adjust the pH. Then the product is bottled and pasteurised to extend its shelf life (Galicia-Cabrera, 2007). In this study, three independent production events, spread over 2010, were collected. In each event, a total of 11 tomato juices were analyzed, six of which were conventional and five were organic. The tomato juices analyzed were commercial juices that were available in Barcelona markets.

2.3. Extraction and isolation of phenolic compounds

Sample treatment was performed in duplicate in a darkened room with a red safety light to avoid the oxidation of the analytes during the process following the procedure of Vallverdú-Queralt et al. with some modifications (Vallverdú-Queralt, Jáuregui, Medina-Remón, Andrés-Lacueva, & Lamuela-Raventós, 2010). All juices (10 ml) were centrifuged (4000 rpm at 4 °C) for 10 min. The supernatant was discarded and 4 ml of 80% ethanol in Milli-Q water was added to the pellet; this was sonicated for 5 min and centrifuged (4000 rpm at 4 °C) for 20 min. The supernatant was transferred into a flask and the extraction was repeated with 4 ml of 80% ethanol in Milli-Q water and centrifuged (4000 rpm at 4 °C) for

20 min. Both supernatants were combined and evaporated under nitrogen flow and finally the residue was reconstituted with Milli-Q water (0.1% of formic acid) up to 1.5 ml.

Solid-phase extraction (SPE) of these extracts was carried out following the procedure of another work (Medina-Remón et al., 2009). Firstly, 1 ml of methanol and subsequently 1 ml of sodium acetate 50 mmol/l pH 7 were loaded into Oasis® MAX cartridges from Waters to equilibrate the samples; then, 1 ml of each extract was diluted with 1 ml of Milli-Q water and acidified with 34 µl of hydrochloric acid at 35% before being loaded into the cartridges separately. These were rinsed with 50 mmol/l of sodium acetate pH 7 (5% methanol). The polyphenols were eluted with 1800 µl of methanol (2% formic acid). The eluted fractions were evaporated under nitrogen flow, and the residue was reconstituted with water (0.1% formic acid) up to 250 µl and filtered through a 13 mm, 0.45 µm PTFE filter (Waters) into an insert-amber vial for HPLC analysis. Samples were stored at –20 °C until analysis.

2.4. Analysis of total polyphenols

For the TP assay, each sample was analyzed in triplicate: 20 µl of the eluted fractions were mixed with 188 µl of Milli-Q water in a thermo microtiter 96-well plate (nunc™, Roskilde, Denmark); afterwards, 12 µl of F–C reagent and 30 µl of sodium carbonate (200 g/l) were added. The mixtures were incubated for 1 h at room temperature in the dark. After the reaction period, 50 µl of Milli-Q water were added and the absorbance was measured at 765 nm in a UV/VIS Thermo Multiskan Spectrum spectrophotometer (Vantaa, Finland). This spectrophotometer allowed the absorbance of a 96-well plate to be read in 10 s. Results were expressed as mg of gallic acid equivalents (GAE)/100 g fresh weight (FW) (Singleton & Rossi, 1965).

2.5. Antioxidant activity

The antioxidant activity in tomato juices was measured using an ABTS® radical decolorization assay and DPPH® assay with minor modifications (Vallverdú-Queralt, Medina-Remón, Andrés-Lacueva & Lamuela-Raventós, 2011).

2.5.1. ABTS® assay

One millimole Trolox (antioxidant standard) was prepared in PBS once a week. Working standards were prepared daily by diluting 1 mM Trolox with PBS.

An ABTS® radical cation was prepared by passing a 5 mM aqueous stock solution of ABTS (in PBS) through manganese dioxide powder. Excess manganese dioxide was filtered through a 13 mm 0.45 µm filter PTFE (Waters). Before analysis, the solution was diluted in PBS pH 7.4 to give an absorbance at 734 nm of 1.0 ± 0.1 , and pre-incubated in ice. Then, 245 µl of ABTS® solution were added to 5 µl of Trolox or to tomato samples and the solutions were stirred for 30 s. The absorbance was recorded continuously every 30 s with a UV/VIS Thermo Multiskan Spectrum spectrophotometer for 1 h and PBS blanks were run in each assay.

The working range for Trolox (final concentration 0–750 µM) was based on triplicate determinations and consisted of plotting the absorbance as a percentage of the absorbance of the uninhibited radical cation (blank). The activities of the tomato samples were assessed at four different concentrations that were within the range of the dose–response curve. Each sample was analyzed in triplicate at each concentration. Results were expressed as mmol Trolox equivalent (TE)/100 g FW.

2.5.2. DPPH® assay

The antioxidant activity was also studied through the evaluation of the free radical-scavenging effect on DPPH radicals.

Solutions of known Trolox were used for calibration. Five microlitres of tomato samples or Trolox were mixed with 250 μ l of methanolic DPPH (0.025 g/l). The homogenate was shaken vigorously and kept in darkness for 30 min. Absorption of the samples was measured on a UV/VIS Thermo Multiskan Spectrum spectrophotometer at 515 nm. The percentage of inhibition of the DPPH was calculated and plotted as a function of the concentration of Trolox for the standard reference data. The final DPPH• values were calculated using a regression equation between the Trolox concentration and the percentage of DPPH• inhibition and results were expressed as mmol TE/100 g FW.

2.6. HPLC-ESI-MS/MS analysis

To evaluate the differences between organic and conventional production systems, phenolic compounds were quantified using HPLC-ESI-MS/MS (Vallverdú-Queralt et al., 2011). An API 3000 (PE Sciex, Concord, Ontario, Canada) triple quadrupole mass spectrometer equipped with a Turbo Ionspray source in negative-ion mode was used to obtain MS/MS data. Turbo Ionspray source settings were as follows: capillary voltage, -3500 V; nebulizer gas (N_2), 10 a.u. (arbitrary units); curtain gas (N_2), 12 a.u.; collision gas (N_2), 4 a.u.; focusing potential, -200 V; entrance potential, -10 V; drying gas (N_2), heated to 400 °C and introduced to a flow rate of 8000 cm³/min. The declustering potential and collision energy were optimised for each compound in infusion experiments: individual standard solutions (10 μ g/ml) dissolved in 50:50 (v/v) mobile phase were infused at a constant flow rate of 5 μ l/min using a model syringe pump (Harvard Apparatus, Holliston, MA, USA). Full-scan data acquisition was performed scanning from m/z 100 to 800 in profile mode and using a cycle time of 2 s with a step size of 0.1 u and a pause between each scan of 2 ms. To confirm the identity of some compounds, neutral loss scan and precursor ion scan experiments were carried out. MS/MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer and mass analyzed using the instrument's second analyzer. Additional experimental conditions for MS/MS included collision energy (depending on the compound), CAD gas (nitrogen) at 6 (arbitrary units), and scan range, as necessary for the precursor selected. Neutral loss scan of 162 u was done by scanning within the m/z range from 200 to 800 u, and precursor ion scan experiments were carried out by scanning Q1 between 300 and 800 u. In all the experiments, both quadrupoles (Q1 and Q3) were operated at unit resolution.

For quantification purposes, data was collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound. In particular 12 transitions were selected which corresponded to ferulic acid-*O*-hexoside m/z 355 $\rightarrow$ 193 (CE: -20 V); chlorogenic acid m/z 353 $\rightarrow$ 191 (CE: -20 V); cryptochlorogenic acid m/z 353 $\rightarrow$ 191 (CE: -20 V); dicaffeoylquinic acid m/z 515 $\rightarrow$ 353 (CE: -20 V); caffeic acid m/z 179 $\rightarrow$ 135 (CE: -20 V); caffeic acid-*O*-hexoside m/z 341 $\rightarrow$ 179 (CE: -20 V); quercetin m/z 301 $\rightarrow$ 151 (CE: -30 V); rutin m/z 609 $\rightarrow$ 300 (CE: -50 V); naringenin m/z 271 $\rightarrow$ 151 (CE: -30 V); naringenin-7-*O*-glucoside m/z 433 $\rightarrow$ 271 (CE: -20 V); kaempferol-3-*O*-glucoside m/z 477 $\rightarrow$ 285 (CE: -30 V); kaempferol-3-*O*-rutinoside m/z 593 $\rightarrow$ 285 (CE: -30 V) and ethyl galate (internal standard) m/z 197 $\rightarrow$ 169 (CE: -25 V).

Quantification of phenolic compounds was determined by an internal standard. Polyphenols were quantified with respect to their corresponding standard. When standards were not available, as in the case of caffeic-*O*-hexoside and ferulic-*O*-hexoside acids, they were quantified with respect to the corresponding hydroxycinnamic acid (caffeic and ferulic acids). The isomers of chlorogenic

acid (caffeoylquinic and dicaffeoylquinic acids) were quantified with respect to the chlorogenic acid.

The liquid chromatograph was an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, an autosampler and a column oven set to 30 °C. Mobile phases consisted of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B). The injection volume was 20 μ l and the flow rate was 0.4 ml/min. Separation was carried out in 20 min under the following conditions: 0 min, 5% B; 16 min, 40% B; 17 min, 95% B; 19 min, 95% B; 19.5 min, 5% B. The column was equilibrated for 5 min prior to each analysis.

2.7. Statistical analysis

The significance of the results was analyzed using the Statgraphics Plus v.5.1 Windows Package (Statistical Graphics Co., Rockville, MD). Analysis of variance (ANOVA) was used to compare the means of groups of measurement data, and principal component analysis (PCA) was carried out to obtain correlations amongst variables. PCA visualises the original arrangement of tomato juice samples in an n -dimensional space, by identifying the directions in which most of the information is retained.

3. Results

3.1. Analysis of TP content and hydrophilic antioxidant capacity of tomato juices

The content of TP evaluated by F-C assay after SPE and the relative contribution of individual compounds to the hydrophilic antioxidant capacity of tomato juices were analyzed. Results are shown in Table 1. No significant changes ($P > 0.05$) in TP content and hydrophilic antioxidant capacity were observed in the different production events.

The F-C assay showed that organic tomato juices contained significantly ($P < 0.05$) higher concentrations of TP than conventionally produced tomato juices. TP content ranged from (9.28–10.55) mg GAE/100 g FW in conventional tomato juices to (11.04–12.89) mg GAE/100 g FW in organic tomato juices.

A similar trend was observed for hydrophilic antioxidant capacities. Tomato juices contained significantly ($P < 0.05$) higher concentrations of hydrophilic antioxidants than conventionally produced tomato juices. The lowest were determined for conventional tomato juices. For the ABTS[•] assay, results ranged from

Table 1
Total phenolic content (mg GAE/100 g FW) and ABTS[•] and DPPH• (mmol TE/100 g FW) assays expressed as mean $\pm$ SD of organic and conventional tomato juices; different letters in the columns represent statistically significant differences ($P < 0.05$).

Tomato juices	TPy	ABTS [•]	DPPH
<i>Organic</i>			
O _A	12.89 $\pm$ 0.32 ^a	3.26 $\pm$ 0.08 ^c	5.78 $\pm$ 0.12 ^{b,c}
O _B	11.50 $\pm$ 0.25 ^d	3.71 $\pm$ 0.08 ^{a,b}	5.90 $\pm$ 0.11 ^b
O _C	11.34 $\pm$ 0.47 ^d	3.59 $\pm$ 0.06 ^c	5.68 $\pm$ 0.12 ^c
O _D	12.23 $\pm$ 0.34 ^c	3.82 $\pm$ 0.13 ^a	6.11 $\pm$ 0.10 ^a
O _E	12.69 $\pm$ 0.30 ^b	3.25 $\pm$ 0.07 ^c	5.70 $\pm$ 0.11 ^d
<i>Conventional</i>			
C _A	10.55 $\pm$ 0.25 ^e	2.38 $\pm$ 0.09 ^d	4.11 $\pm$ 0.10 ^e
C _B	9.34 $\pm$ 0.23 ^e	1.50 $\pm$ 0.05 ^b	2.99 $\pm$ 0.08 ^d
C _C	10.15 $\pm$ 0.20 ^f	2.90 $\pm$ 0.10 ^d	3.25 $\pm$ 0.12 ^d
C _D	9.28 $\pm$ 0.23 ^e	2.38 $\pm$ 0.08 ^d	3.15 $\pm$ 0.07 ^b
C _E	9.54 $\pm$ 0.28 ^e	1.95 $\pm$ 0.08 ^d	2.67 $\pm$ 0.09 ^d
C _F	10.37 $\pm$ 0.26 ^d	2.77 $\pm$ 0.09 ^d	3.61 $\pm$ 0.10 ^d

GAE, gallic acid equivalents; FW, fresh weight; TE, Trolox equivalents; SD, standard deviation.

(1.50–2.90) mmol TE/100 g FW in conventional tomato juices to (3.25–3.82) mmol TE/100 g FW in organic tomato juices. For the DPPH• assay, antioxidant capacities were higher than those determined by the ABTS• assay. Values were between (2.67–4.11) mmol TE/100 g FW in conventional tomato juices and (5.68–6.11) mmol TE/100 g FW in organic tomato juices.

3.2. Quantization of individual polyphenols in conventional and organic tomato juices

Specific phenolic compounds were monitored for conventional and organic tomato juices. Results are quantified in Table 2 and Table 3. The main phenolic compound in all the tomato juices was rutin, present at levels ranging between 42.50 and 89.07 µg/g FW. Organic tomato juices contained significantly ($P < 0.05$) higher levels of phenolic compounds than conventional tomato juices, as reflected in the analysis of total phenolics and hydrophilic antioxidant capacity.

Hydroxycinnamic acids were mainly represented by ferulic acid-*O*-hexoside (m/z 355 → 193), which ranged from (19.23–29.91) µg/g FW in conventional tomato juices to (31.92–45.17) µg/g FW in organic tomato juices. Caffeic acid-*O*-hexosides (m/z 341 → 179) and caffeic acid (m/z 179 → 135) followed a similar trend, with higher levels in organic tomato juices.

Chlorogenic acid isomers (m/z 353 → 191) and dicaffeoylquinic acid (m/z 515 → 353) were also present in tomato juices. Chlorogenic acid was the most abundant caffeoylquinic acid, ranging from 8.41–10.35 µg/g FW in conventional tomato juices to 12.48–15.63 µg/g FW in organic tomato juices. Dicaffeoylquinic acid was found in lower concentrations than caffeoylquinic acids, with higher levels in organic tomato juices (2.12–2.71) µg/g FW compared to conventional juices (1.38–1.84) µg/g FW.

Flavonols were characterised by rutin followed by kaempferol-3-*O*-rutoside, kaempferol-3-*O*-glucoside and quercetin. The organic tomato juices had higher levels of rutin (75.06–89.07) µg/g FW compared with the conventional juices (42.50–63.33) µg/g FW. The same pattern was followed by quercetin, with levels ranging from (1.05–2.63) µg/g FW in conventional tomato juices to (2.75–5.47) µg/g FW in those produced organically. Organic tomato juices also showed the highest content of kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutoside.

Flavanones were represented by naringenin and naringenin-7-*O*-glucoside, whose maximum concentrations were determined in organic tomato juices, like the above-mentioned flavonols and hydroxycinnamic acids. Naringenin levels ranged from (35.97–52.96) µg/g FW in conventional tomato juices to (57.54–70.43) µg/g FW in organic ones and levels of naringenin-7-*O*-glucoside varied from

(1.25–3.27) µg/g FW in conventional tomato juices to (3.40–5.79) µg/g FW in organic ones.

3.3. Principal component analysis

In order to establish the variation between the different organic and conventional agronomic techniques and to determine which phenolic compound mostly affects the overall composition of tomato juices, a PCA of the data set was performed. PCA allows us to visualise the original arrangement of tomato juices in an n -dimensional space, by identifying the directions in which most of the information is retained. The scores plot was created to define different groups as shown in Fig. 1. Samples are clustered together according to the different agronomic techniques (organic and conventional). Two principal components (PC1 and PC2) accounted for 85.30% of the variability of the original data. The first component (X-axis) explained 77.43% of the total variation of the data set. This component points to the differentiation between organic and conventional tomato juices. The group of organic juices is situated in the right-hand side of the plot, which is divided into five different subgroups: O_A , O_B , O_C , O_D and O_E . In contrast, the group of conventional juices is situated on the left-hand side of the plot, which is divided into six different subgroups: C_A , C_B , C_C , C_D , C_E and C_F . Among the compounds that were significantly higher ($P < 0.05$) in organic juices versus conventional ones were total polyphenols, caffeoylquinic and dicaffeoylquinic acids, caffeic and caffeic acid-*O*-hexoside, ferulic acid-*O*-hexoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutoside, naringenin-*O*-hexoside, naringenin, rutin and quercetin, as well as hydrophilic antioxidant capacity.

4. Discussion

It is known that organic foods have superior sensory attributes, contain lower levels of pesticides and synthetic fertilisers, have higher levels of nutrients and protective phytochemicals and lower levels of nitrate than conventionally produced foods. Worthington et al. found more iron, magnesium, phosphorus, and vitamin C and less nitrates in organic crops when compared to conventional crops. This phenomenon is possibly due to a higher water content in conventional crops, which causes nutrient dilution (Worthington, 1998). Conversely, it has also been suggested that application of manure and reduced use of fungicides and antibiotics in organic farming could result in a greater contamination of organic foods by microorganisms or microbial products (Hoefkens et al., 2009).

Few data exist regarding the influence of the production method (organic versus conventional) on the phytochemical concentration in tomato juices, because these compounds have only been recently

Table 2

Content of hydroxycinnamic (µg/g FW) expressed as mean ± SD of organic and conventional tomato juices, different letters in the columns represent statistically significant differences ($P < 0.05$).

Tomato juices	Ferulic acid- <i>O</i> -hexoside	Caffeic acid- <i>O</i> -hexoside	Caffeic acid	Cryptochlorogenic acid	Chlorogenic acid	Dicaffeoylquinic acid
<i>Organic</i>						
O_A	35.26 ± 1.06 ^d	4.20 ± 0.11 ^d	4.74 ± 0.14 ^d	7.86 ± 0.18 ^c	12.78 ± 0.37 ^c	2.43 ± 0.06 ^b
O_B	31.92 ± 0.95 ^d	6.80 ± 0.12 ^d	3.24 ± 0.08 ^d	9.55 ± 0.25 ^a	12.48 ± 0.31 ^c	2.33 ± 0.09 ^b
O_C	36.66 ± 1.28 ^c	5.48 ± 0.09 ^b	3.67 ± 0.14 ^c	9.61 ± 0.17 ^a	13.24 ± 0.35 ^b	2.12 ± 0.07 ^c
O_D	39.14 ± 0.94 ^b	4.35 ± 0.10 ^c	3.54 ± 0.13 ^c	7.71 ± 0.20 ^c	15.63 ± 0.40 ^a	2.30 ± 0.09 ^b
O_E	45.17 ± 0.97 ^a	4.15 ± 0.13 ^d	4.16 ± 0.10 ^b	8.57 ± 0.26 ^b	13.28 ± 0.22 ^b	2.71 ± 0.06 ^a
<i>Conventional</i>						
C_A	29.91 ± 0.96 ^d	3.75 ± 0.12 ^a	2.38 ± 0.10 ^d	5.80 ± 0.27 ^a	9.36 ± 0.14 ^a	1.48 ± 0.04 ^f
C_B	20.76 ± 0.70 ^f	2.16 ± 0.10 ^f	2.29 ± 0.07 ^e	4.36 ± 0.16 ^f	8.41 ± 0.29 ^a	1.64 ± 0.06 ^e
C_C	26.66 ± 1.08 ^b	3.27 ± 0.11 ^f	1.46 ± 0.06 ^f	4.79 ± 0.11 ^a	9.43 ± 0.25 ^a	1.52 ± 0.04 ^f
C_D	25.24 ± 0.79 ^b	2.56 ± 0.05 ^b	1.81 ± 0.07 ^d	5.65 ± 0.21 ^a	9.26 ± 0.18 ^a	1.50 ± 0.07 ^f
C_E	19.23 ± 0.64 ^d	2.83 ± 0.11 ^b	2.24 ± 0.05 ^d	4.71 ± 0.22 ^a	8.79 ± 0.35 ^a	1.84 ± 0.07 ^d
C_F	28.75 ± 0.87 ^a	2.73 ± 0.12 ^b	2.30 ± 0.09 ^d	5.67 ± 0.25 ^a	10.35 ± 0.12 ^a	1.51 ± 0.04 ^f

FW, fresh weight; SD, standard deviation.

Table 3

Content of flavonols and flavanones ($\mu\text{g/g}$ FW) expressed as mean $\pm$ SD of conventional and organic tomato juices, different letters in the columns represent statistically significant differences ($P < 0.05$).

Tomato juices	Rutin	Quercetin	Naringenin	Naringenin-7-O-glucoside	Kaempferol-3-O-rutinoside	Kaempferol-3-O-glucoside
Organic						
O _A	89.07 $\pm$ 1.36 ^a	2.99 $\pm$ 0.06 ^d	58.89 $\pm$ 1.40 ^b	5.79 $\pm$ 0.10 ^a	28.31 $\pm$ 1.19 ^c	10.47 $\pm$ 0.36 ^c
O _B	80.72 $\pm$ 2.89 ^b	5.47 $\pm$ 0.11 ^a	57.54 $\pm$ 1.75 ^b	3.40 $\pm$ 0.13 ^d	26.47 $\pm$ 0.86 ^d	10.47 $\pm$ 0.40 ^c
O _C	81.96 $\pm$ 1.61 ^b	4.53 $\pm$ 0.06 ^b	59.79 $\pm$ 1.24 ^b	4.33 $\pm$ 0.08 ^b	29.64 $\pm$ 1.02 ^b	9.58 $\pm$ 0.41 ^d
O _D	75.06 $\pm$ 3.40 ^c	3.42 $\pm$ 0.05 ^c	59.61 $\pm$ 2.20 ^b	4.02 $\pm$ 0.07 ^c	35.67 $\pm$ 1.16 ^a	11.24 $\pm$ 0.23 ^b
O _E	77.00 $\pm$ 3.20 ^c	2.75 $\pm$ 0.05 ^e	70.43 $\pm$ 0.80 ^a	5.72 $\pm$ 0.07 ^a	27.91 $\pm$ 1.19 ^c	11.67 $\pm$ 0.46 ^a
Conventional						
C _A	62.84 $\pm$ 2.13 ^d	1.58 $\pm$ 0.04 ^h	45.47 $\pm$ 1.66 ^f	1.34 $\pm$ 0.05 ^h	18.54 $\pm$ 0.69 ^g	9.37 $\pm$ 0.26 ^f
C _B	42.50 $\pm$ 1.85 ^e	1.29 $\pm$ 0.05 ⁱ	36.14 $\pm$ 0.99 ^g	1.25 $\pm$ 0.04 ^h	18.51 $\pm$ 0.82 ^g	7.51 $\pm$ 0.29 ^f
C _C	61.74 $\pm$ 1.93 ^d	1.67 $\pm$ 0.01 ^g	35.97 $\pm$ 1.26 ^g	2.41 $\pm$ 0.07 ^f	15.92 $\pm$ 0.41 ^h	6.25 $\pm$ 0.19 ^h
C _D	57.58 $\pm$ 2.68 ^e	2.63 $\pm$ 0.04 ^f	48.83 $\pm$ 1.52 ^d	3.27 $\pm$ 0.11 ^f	16.47 $\pm$ 0.40 ^h	7.24 $\pm$ 0.22 ^h
C _E	50.28 $\pm$ 2.39 ^f	1.05 $\pm$ 0.03 ⁱ	38.24 $\pm$ 1.04 ^f	2.26 $\pm$ 0.06 ^f	20.97 $\pm$ 0.85 ^f	6.31 $\pm$ 0.36 ^h
C _F	63.33 $\pm$ 2.43 ^d	1.59 $\pm$ 0.04 ^h	52.96 $\pm$ 2.23 ^c	2.34 $\pm$ 0.08 ^f	24.72 $\pm$ 0.26 ^e	5.72 $\pm$ 0.26 ^f

FW, fresh weight; SD, standard deviation.

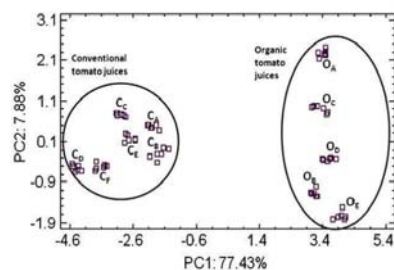


Fig. 1. Score plot of PC1 vs PC2 of different organic and conventional tomato juices. Conventional: C_A, C_B, C_C, C_D, C_E, C_F; Organic: O_A, O_B, O_C, O_D, O_E.

considered as interesting bioactive microconstituents, due to their intervention in human health (Grieb et al., 2009; Zhang et al., 2009), especially cardiovascular diseases (Silaste et al., 2007).

From a technological point of view, the main difficulty in the production of tomato juices is choosing the right variety of tomatoes with suitable characteristics. Tomatoes destined to produce juices are harvested at an appropriate degree of ripeness through the use of selected crops with adequate irrigation systems and suitable physical and chemical parameters.

Among published studies mentioning total phenolic contents, the majority describe a higher phenolic concentration in organically grown fruits or vegetables (Agence Française de Sécurité Sanitaire des Aliments, 2003). Our results are in agreement with these studies. Organic tomato juices were found to have a higher content of phenolic compounds and hydrophilic antioxidants than conventional juices. It is well-known that the biosynthesis of phenolic compounds in plants is strongly influenced by cultivar (Hakkinen & Torronen, 2000), mode of fertilisation (Macheix, Feuriet, & Billot, 1990), temperature, light and seasonal variations (Slimestad & Verheul, 2009). The tendency of higher polyphenol concentrations in organically produced fruits and vegetables could be explained by a higher phosphorus uptake and limited nitrogen availability (Awad, de Jager, & van Westing, 2000; Weibel, Bickel, Leuthold, & Alfoldi, 2000). An increased phosphorus uptake can provide the necessary energy for the synthesis of phytochemicals (Weibel et al., 2000). Furthermore, it has been shown that plants synthesise more flavonoids when nitrogen is limited (Mitchell et al., 2007; Weibel et al., 2000). These differences are reflected in the principal component analysis. Twelve phenolic compounds were detected as chemotaxonomic tomato juice markers able to

distinguish between different agronomic treatments. One of the important features of phenolic compounds is their usage as chemotaxonomic markers (Andres-Lacueva, Ibern-Gomez, Lamuela-Raventos, Buxaderas, & de la Torre-Boronat, 2002; De la Presa-Owens, Lamuela-Raventos, Buxaderas, & De la Torre-Boronat, 1995; Romero-Perez, Lamuela-Raventos, Buxaderas, & De la Torre-Boronat, 1996; Russo, Galletti, Bocchini, & Carnacini, 1998; Singleton & Trousdale, 1983).

From the investigation, rutin (quercetin-3-O-rutinoside) was found to be the predominant flavonol in all tomato juices, followed by naringenin, which was also reported in other studies (Andrade, Mendes, Falco, Valentao, & Seabra, 2001; Hermann, 1976; Le Gall et al., 2003). Rutin and naringenin levels were significantly higher in juices made from organically grown tomatoes. Chassy et al. found that the flavonoid concentration in organically produced tomatoes and bell peppers was higher than in those produced conventionally (Chassy, Bui, Renaud, Van Horn, & Mitchell, 2006). Hakkinen et al. compared strawberries cultivated by organic and conventional methods and found high amounts of phenolic acids and flavonols in organic strawberries (Hakkinen & Torronen, 2000).

Similarly, the levels of quercetin and kaempferol were much lower in the conventionally grown produce. Flavonols such as quercetin and kaempferol are antimicrobial compounds synthesized by plants in response to pathogen attack (Dixon & Paiva, 1995). Since organically grown products are produced by cultural methods using very few pesticides, pathogenic pressures may explain the higher TP levels found in the organically grown samples.

The chlorogenic acid was found to be the most abundant caffeoylquinic acid in both conventional and organic tomato juices, which agrees with the results reported by Caris-Veyrat et al. Significantly higher concentrations of chlorogenic acid and glycoalkaloids were retrieved in organic tomatoes and derived purees ($P < 0.05$) in comparison to the conventional ones (Caris-Veyrat et al., 2004).

Another study performed to evaluate the phenolic compounds of apples (Golden Delicious) grown under defined organic and conventional conditions showed significant differences in levels of chlorogenic acid, flavanols and flavonols with the organic fruit showing higher concentrations (Stracke, Rufer, Weibel, Bub, & Watzl, 2009). Absolute differences are also apparent in both systems regarding levels of dicaffeoylquinic acids.

A number of studies have addressed the question of whether agricultural chemicals and other agricultural methods including organic farming affect nutrient content. The question is still unresolved, in part due to the large amount of variability in agricultural data resulting from uncontrollable factors such as rainfall and sunlight, which also influence nutrient content. When plants are

grown with artificial nutrients, they are supposed to lose their natural defence mechanisms. This may result in reduced disease resistance and diluted contents of minerals, vitamins and defence-related secondary metabolites, which are considered beneficial for human health. In the present study the growing conditions of tomatoes (conventional versus organic) affected the content of twelve phenolic compounds and the antioxidant capacity in tomato juices. The organically produced tomato juices displayed a higher phytochemical concentration and a higher hydrophilic antioxidant capacity than conventionally produced juices. Thus, vegetable and fruit products grown in organic agriculture would be expected to be more health-promoting than those produced conventionally.

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8. Capítol VIII: Aproximacions metabolòmiques per establir diferències entre quètxups orgànics i convencionals

Anna Vallverdú-Queralt, Alexander Medina-Remón, Isidre Casals-Ribes, Mercedes Amat & Rosa M. Lamuela-Raventós. *Journal of Agricultural and Food Chemistry*. 2011, 59: 11703-11710.

Resum: Les tècniques modernes d'anàlisi global, denominades "omics", permeten estudiar la biocomplexitat dels efectes dels nutrients. Concretament, la metabolòmica ha estat proposada com a eina de gran valor per explorar les complexes relacions entre nutrició i metabolisme, investigant el paper que juguen els components de la dieta en el manteniment de la salut o en el desenvolupament de malalties. Actualment, un repte important dels estudis de metabolòmica és identificar nous marcadors que permetin establir relacions entre marcadors i diferents tipus d'aliments. En aquest estudi elaborat durant l'any 2010, es va dur a terme una anàlisi metabolòmica en quètxups orgànics i convencionals. Es van trobar 14 marcadors que permeten clarament distingir entre els dos tipus de quètxups. Els quètxups orgànics presenten més contingut de polifenols que els quètxups convencionals. D'altra banda, en els quètxups convencionals s'hi van poder identificar dos dipèptids, un derivat del triptòfan i un de la fenilalanina, que fins llavors no havien estat descrits en productes derivats del tomàquet.

A Metabolomic Approach Differentiates between Conventional and Organic Ketchups

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ABSTRACT: The agronomic environments in which tomatoes are cultivated potentially affect the levels of antioxidants and other metabolites in commercial products. In this study, biochemical and metabolomic techniques were used to assess the differences between ketchups produced by organic and conventional systems. An untargeted metabolomic approach using QToF-MS was used to identify those nutrients that have the greatest impact on the overall metabolomic profile of organic ketchups as compared to conventional ones. Individual polyphenols were quantified using LC-ESI-QqQ. This multifaceted approach revealed that the agronomic environment in which tomatoes are grown induces alterations in the content of antioxidant capacity, phenolics, and other metabolites in ketchups. Organic cultivation was found to provide tomatoes and tomato-derived products with a significantly higher content of antioxidant microconstituents, whereas glutamylphenylalanine and *N*-malonyltryptophan were detected only in conventional ketchups.

KEYWORDS: ketchups, organic agriculture, conventional agriculture, metabolomics, HPLC-ESI-QToF, LC-ESI-QqQ

INTRODUCTION

The increasing demand for organic foods can be explained mainly by consumer concerns about the quality and safety of foods and the perception that organically produced foods are healthier and safer than conventional foods. A wide range of processed organic tomato products are available including pasta sauces, tomato sauces, juices, ketchups, tinned tomatoes (whole and diced), dried tomatoes, and pastes.¹

Fundamental differences between organic and conventional production systems, particularly in soil fertility management, may affect the nutritive composition of plants, including secondary plant metabolites.² Organic agriculture is an ecological production management system that promotes and enhances biodiversity, biological cycles, and soil biological activity. Organic systems rely on the activity of a diverse soil ecosystem to make nitrogen and other nutrients available to plants, whereas conventional farms utilize fertilizers containing soluble inorganic nitrogen and other nutrients, which are more directly available to plants.³ The availability of inorganic nitrogen in particular has the potential to influence the synthesis of secondary plant metabolites, proteins, and soluble solids. It is well-known that the biosynthesis of phenolics in plants is strongly influenced by cultivar, environmental conditions, especially light, and the mode of fertilization.^{4,5} Vallverdú-Queralt et al.⁶ suggested that choice of cultivar is a major factor contributing to the total phenol content of tomatoes when grown under similar environmental conditions. Seven normal-sized field-grown tomatoes, grown alongside one another in Spain, showed different content of flavonols, flavanones, and phenolic and hydroxycinnamic acids.

Moreover, the phenolic content of plants may be influenced by manipulating the agronomic environment in which they are grown.^{7,8} The level of nitrogen influences the level of phenol.^{9,10} Even if the levels of nitrogen, as well as potassium and phosphorus, were higher in organic conditions, the bioavailability of nitrogen from organic fertilization would remain lower than that achieved by synthetic fertilization; the levels of carbon-based secondary metabolites such as phenolic compounds are higher in organic plants.^{9,10}

The development of tools and metabolomic markers to monitor the quality of these products is necessary. With the recent developments in plant metabolomic techniques,¹¹ it is possible to detect several hundred metabolites simultaneously and to compare samples reliably for differences and similarities in a semiautomated and untargeted manner.

Until now, metabolomic approaches in tomatoes have only been used to define alterations to fruit induced by mutations¹² and to investigate metabolomic changes taking place during fruit development.^{13,14} To our knowledge, comprehensive biochemical studies on the effect of the agronomic environment on the phenolic content of tomato-based products have not been reported. Many studies have shown that phenol intake improves health and prevents chronic diseases.¹⁵ We carried out a pilot study to identify the differences between organic and conventional

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ketchups on the market through a combination of MS techniques, liquid chromatography electrospray ionization time-of-flight mass spectrometry (LC-ESI-QTOF-MS) and liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-QqQ), and two additional analyses, total polyphenol (TP) and hydrophilic antioxidant capacity assays.

MATERIALS AND METHODS

Standards and Reagents. All samples and standards were handled without exposure to light. Caffeic and chlorogenic acids, rutin and quercetin, Folin-Ciocalteu (F-C) reagent, 2,2'-azobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), phosphate-buffered saline (PBS), pH 7.4, ($\pm$)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) 97%, manganese dioxide, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma (St. Louis, MO) and naringenin, naringenin-7-O-glucoside, and kaempferol-3-O-rutinoside from Extrasynthèse (Genay, France). Ethanol and formic acid of HPLC grade were obtained from Scharlau (Barcelona, Spain), and ultrapure water (Milli-Q) was from Millipore (Bedford, MA).

Processing Conditions of Organic and Conventional Tomatoes. Conventional tomatoes receive herbicides and other pesticides as needed, whereas organic crops receive only organically approved pesticides such as sulfur and benzothiazophene compounds. Conventional farms utilize fertilizers containing soluble inorganic nitrogen and other nutrients, which are more directly available to plants. Organic systems emphasize the accumulation of soil organic matter and fertility over time through the use of cover crops, manures, and composts and rely on the activity of a diverse soil ecosystem to make nitrogen (N) and other nutrients available to plants. The amount of N present in cover crops varies from year to year, but, typically, organic plots currently receive between 240 and 260 kg of N ha⁻¹ per year in addition to the N fixed by the legume cover crop. Conventional tomatoes usually receive 50 kg ha⁻¹ of an N-P-K starter fertilizer and 118 kg ha⁻¹ of ammonium nitrate as side dressing.¹⁶

Processing Conditions and Ingredients of Commercial Ketchups. From a technological point of view, the main difficulty in the production of ketchups is choosing the most appropriate variety of organic or conventional tomatoes with good consistency and suitable degrees Brix to produce tomato paste between 30 and 32 °Brix. Moreover, the use of resistant and tolerant varieties can be advantageous in the management of pest control. For example, varieties such as Supreme, Gold, Fresh, and Belle are more resistant to diseases and are the most commonly used for tomato-based organic products such as ketchups.

Organic and conventional tomatoes are used to produce organic and conventional ketchups. Tomatoes are sorted, washed, and chopped, and then the tomato paste (65%, 30 and 32 °Brix) is mixed with sugar (organic ketchups) or high-fructose corn syrup (conventional ketchups), wine vinegar, salt, aroma, and trace preservatives. The mixture is pasteurized at 96 °C for 4–6 min. The entire process of ketchup manufacturing generally takes between 2 and 3 h. Finally, the ketchup is bottled.^{18,17}

The manufacturing date controls are January 2010, April 2010, and September 2010. A total of 15 ketchups commercially available in Barcelona markets were analyzed on each manufacturing date: 10 conventional and 5 organic.

Extraction and Isolation of Phenolic Compounds. All ketchups (1.0 g) were weighed and homogenized with 4 mL of 80% ethanol in Milli-Q water. They were then sonicated for 5 min and centrifuged (4000 rpm at 4 °C) for 20 min. The supernatant was transferred into a flask, and the extraction was repeated. Both supernatants were combined and evaporated under nitrogen flow. Finally, the residue was reconstituted with up to 1.5 mL of Milli-Q water containing 0.1% formic acid. Samples were stored at -20 °C until analysis.

Analysis of Total Polyphenols. Solid-phase extraction (SPE) was carried out to eliminate interferences such as ascorbic acid, amino acids, and reducing sugars, which could contribute to an overestimation of the values of TP. For this procedure, Oasis MAX cartridges with 30 mg of mixed-mode anion-exchange and reversed-phase sorbent from Waters (Milford, MA) following the procedure of Valverdú-Queralt et al.¹⁸ were used. The eluted fractions were evaporated under nitrogen flow, and the residue was reconstituted with up to 250 μ L of Milli-Q water containing 0.1% formic acid and filtered through a 13 mm, 0.45 μ m PTFE filter (Waters) into an insert-amber vial for HPLC analysis.

For the TP assay, each sample was analyzed in triplicate: 20 μ L of the eluted fractions was mixed with 188 μ L of Milli-Q water in a thermo microtiter 96-well plate (Nunc, Roskilde, Denmark); then, 12 μ L of F-C reagent and 30 μ L of sodium carbonate (200 g/L) were added. The mixtures were incubated for 1 h at room temperature in the dark. After the reaction period, 50 μ L of Milli-Q water was added, and the absorbance was measured at 765 nm in a UV-vis Thermo Multiskan Spectrum spectrophotometer (Vantaa, Finland). This spectrophotometer allowed the absorbance of a 96-well plate to be read in 10 s. Results were expressed as milligrams of gallic acid equivalents (GAE) per 100 g of fresh weight (FW).¹⁹

Antioxidant Capacity. The antioxidant capacity in tomato juices was measured using an ABTS⁺ radical decolorization assay and a DPPH assay following the procedure of Valverdú-Queralt et al.¹⁸ with minor modifications.

ABTS⁺ Assay. One millimolar Trolox (antioxidant standard) was prepared in PBS once a week. Working standards were prepared daily by diluting 1 mM Trolox with PBS.

An ABTS⁺ radical cation was prepared by passing a 5 mM aqueous stock solution of ABTS (in PBS) through manganese dioxide powder. Excess manganese dioxide was filtered through a 13 mm 0.45 μ m filter PTFE (Waters). Before analysis, the solution was diluted in PBS, pH 7.4, to give an absorbance at 734 nm of 1.0 ± 0.1 and preincubated in ice. Then, 245 μ L of ABTS⁺ solution was added to 5 μ L of Trolox or to tomato samples, and the solutions were stirred for 30 s. The absorbance was recorded continuously every 30 s with a UV-vis Thermo Multiskan Spectrum spectrophotometer for 1 h, and PBS blanks were run in each assay.

The working range for Trolox (final concentration = 0–750 μ M) was based on triplicate determinations and consisted of plotting the absorbance as a percentage of the absorbance of the uninhibited radical cation (blank). The activities of the tomato samples were assessed at four different concentrations that were within the range of the dose-response curve. Each sample was analyzed in triplicate at each concentration. Results were expressed as millimoles of Trolox equivalent (TE) per 100 g of FW.

DPPH Assay. The antioxidant capacity was also studied through the evaluation of the free radical-scavenging effect on DPPH radicals. Solutions of known Trolox were used for calibration. Five microliters of tomato sample or Trolox was mixed with 250 μ L of methanolic DPPH (0.025 g/L). The homogenate was shaken vigorously and kept in darkness for 30 min. Absorption of the samples was measured on a UV-vis Thermo Multiskan Spectrum spectrophotometer at 515 nm. The percentage of inhibition of the DPPH was calculated and plotted as a function of the concentration of Trolox for the standard reference data. The final DPPH values were calculated using a regression equation between the Trolox concentration and the percentage of DPPH inhibition, and results were expressed as millimoles of TE per 100 g of FW.

HPLC-ESI-QTOF Analysis. The chromatography was performed on an Agilent 1200 RRLLC (Agilent, Waldbronn, Germany) using a Luna C₁₈ column 50 $\times$ 2.0 mm internal diameter, 5 μ m (Phenomenex, Torrance, CA). The flow rate was 0.4 mL/min, and the injection volume was 5 μ L. Mobile phases consisted of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B). Separation was carried out

over 20 min under the following conditions: 0 min, 5% B; 16 min, 40% B; 17 min, 95% B; 19 min, 95% B; 19.5 min, 5% B. The column was equilibrated for 5 min prior to each analysis. The HPLC system was coupled to a hybrid quadrupole time-of-flight QSTAR Elite (AB Sciex). The MS acquisition was performed in negative ionization using information-dependent acquisition (IDA) between m/z 90 and 1100. MS parameters were as follows: ion spray voltage, -4200 V; declustering potential, -60 V; focusing potential, -190 V; declustering potential 2, -15 V; ion release delay, 6 V; ion release width, 5 V; temperature, 400 °C with curtain gas (N_2), 50 au (arbitrary units); auxiliary gas, 50 au; and nebulizer gas (N_2), 50 au. IDA was performed using the following criteria: ions that exceed 5 counts; ion tolerance, 50 mDa; collision energy fixed at -30 V; dynamic background subtraction activated. The QToF was calibrated as recommended by the manufacturer. The sequences of injections were randomized.

The significance of the results was analyzed using MarkerView 1.2 software (Applied Biosystems, MSD SCIEX, Toronto, ON, Canada), which performs feature extraction by peak-finding for each sample and alignment using a minimum peak width of 1 mg/L with a noise threshold of 5 and a subtraction multiple factor of 1.5. Alignment used 0.04 mDa mass tolerance and 0.06 min retention time tolerance. Additionally, variables were required to be present in at least five samples. With these parameters a data set containing 1200 mass features was obtained.

LC-ESI-QQQ Analysis. To evaluate the differences between organic and conventional production systems, phenolic compounds were quantified using LC-ESI-QQQ. An API 3000 (PE Sciex, Concord, ON, Canada) triple quadrupole mass spectrometer equipped with a Turbo IonSpray source in negative-ion mode was used to obtain MS/MS data. Turbo IonSpray source settings were as follows: capillary voltage, -3500 V; nebulizer gas (N_2), 10 au (arbitrary units); curtain gas (N_2), 12 au; collision gas (N_2), 4 au; focusing potential, -200 V; entrance potential, -10 V; drying gas (N_2), heated to 400 °C and introduced at a flow rate of 8000 cm³/min. The declustering potential and collision energy were optimized for each compound in infusion experiments: individual standard solutions (10 µg/mL) dissolved in 50:50 (v/v) mobile phase were infused at a constant flow rate of 5 µL/min using a model syringe pump (Harvard Apparatus, Holliston, MA). Full-scan data acquisition was performed scanning from m/z 100 to 800 in profile mode and using a cycle time of 2 s with a step size of 0.1 u and a pause between each scan of 2 ms. To confirm the identity of some compounds, neutral loss scan and precursor ion scan experiments were carried out. MS/MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple-quadrupole mass spectrometer and mass analyzed using the instrument's second analyzer. Additional experimental conditions for MS/MS included collision energy (depending on the compound), CAD gas (nitrogen) at 6 (arbitrary units), and scan range, as necessary for the precursor selected. Neutral loss scan of 162 u was carried out by scanning within the m/z range from 200 to 800 u, and precursor ion scan experiments were carried out by scanning Q1 between 300 and 800 u. In all of the experiments, both quadrupoles (Q1 and Q3) were operated at unit resolution.

For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound. In particular, we selected 15 transitions corresponding to ferulic acid-O-hexoside m/z 355 $\rightarrow$ 193 (CE, -20 V); ferulic acid m/z 193 $\rightarrow$ 134 (CE, -20 V); chlorogenic acid m/z 353 $\rightarrow$ 191 (CE, -20 V); cryptochlorogenic acid m/z 353 $\rightarrow$ 191 (CE, -20 V); neochlorogenic acid m/z 353 $\rightarrow$ 191 (CE, -20 V); dicaffeoylquinic acids m/z 515 $\rightarrow$ 353 (CE, -20 V); caffeic acid m/z 179 $\rightarrow$ 135 (CE, -20 V); caffeic acid-O-hexoside m/z 341 $\rightarrow$ 179 (CE, -20 V); quercetin m/z 301 $\rightarrow$ 151 (CE, -30 V); rutin m/z 609 $\rightarrow$ 300 (CE, -50 V); naringenin m/z 271 $\rightarrow$ 151 (CE, -30 V); naringenin-7-O-glucoside m/z 433 $\rightarrow$ 271 (CE, -20 V); kaempferol-3-O-rutinoside m/z

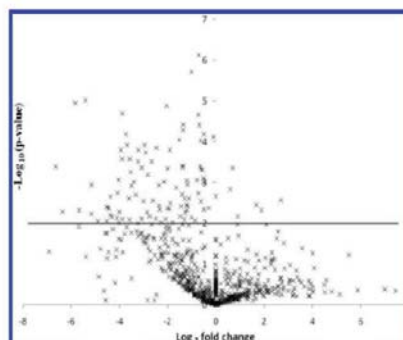


Figure 1. Volcano plot ($\log_2$ fold change vs $-\log_{10}$ p value) representing the entire set of data (1200 features).

593 $\rightarrow$ 285 (CE, -30 V); and ethyl gallate (internal standard) m/z 197 $\rightarrow$ 169 (CE, -25 V).

Quantification of phenolic compounds was performed by the internal standard method. Polyphenols were quantified with respect to their corresponding standard. When standards were not available, as in the case of caffeic-O-hexoside and ferulic-O-hexoside acids, they were quantified with respect to the corresponding hydroxycinnamic acid (caffeic and ferulic acids). The isomers of chlorogenic acid (caffeoylquinic and dicaffeoylquinic acids) were quantified with respect to the chlorogenic acid.

The liquid chromatograph was an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, an autosampler, and a column oven set to 30 °C. Mobile phases consisted of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B). The injection volume was 20 µL, and the flow rate was 0.4 mL/min. Separation was carried out for 20 min under the following conditions: 0 min, 5% B; 16 min, 40% B; 17 min, 95% B; 19 min, 95% B; 19.5 min, 5% B. The column was equilibrated for 5 min prior to each analysis.

Statistical Analysis. Mass profiles were obtained using Marker View 1.2 software and Statgraphics Plus 5.1. Analysis of variance (ANOVA) was used to remove possible mass features shared by all ketchups that could lead to a common pattern of noninformative signals. Therefore, one-way ANOVA was used to compare the effects of the different types of ketchups on metabolome modifications. Prior to each analysis, the data set was mean-centered and Pareto-scaled. We selected this scaling because it effectively increases the importance of low-concentration metabolites, albeit not to an extent that noise contributes to the model.

RESULTS AND DISCUSSION

The view that organic foods are "healthier" than those produced conventionally appears to be based on the perception that organic foods have superior sensory attributes, contain lower levels of pesticides and synthetic fertilizers, and have higher levels of nutrients and protective phytochemicals. Conversely, it has been suggested that the application of manure and reduced use of fungicides and antibiotics in organic farming could result in a greater contamination of organic foods by micro-organisms or microbial products.²⁰

Untargeted Metabolomic Analysis. To establish the variations between the different organic and conventional agronomic techniques and to determine which metabolite has the greatest

Table 1. Significantly Different Compounds ($P < 0.05$) between Organic and Conventional Ketchups

compound	type	[M - H] ⁻	m/z ions	acc mass	mDa	MF	ratio mean conventional/mean organic
glutamylphenylalanine	conventional	293	164 (70), 147 (100), 103 (40)	293.1143	0.4	C ₁₄ H ₁₈ N ₂ O ₅	
<i>N</i> -malonyl tryptophan	conventional	289	203 (40), 159 (20), 142 (40)	289.083	0.9	C ₁₄ H ₁₆ N ₂ O ₅	
caffeic acid- <i>O</i> -hexoside 1	organic	341	179 (100), 135 (20)	341.0877	1.6	C ₁₃ H ₁₆ O ₉	0.7033
caffeic acid- <i>O</i> -hexoside 2	organic	341	179 (100), 135 (20)	341.0877	0.7	C ₁₃ H ₁₆ O ₉	0.6139
caffeic acid ^a	organic	179	135(100), 107(20)	179.0349	1.4	C ₉ H ₈ O ₄	0.6366
neochlorogenic acid	organic	353	191 (100), 179 (80), 135 (30)	353.0877	0.7	C ₁₆ H ₁₈ O ₉	0.3912
cryptochlorogenic acid	organic	353	191 (50) 173 (100), 135 (20)	353.0877	0.8	C ₁₆ H ₁₈ O ₉	0.3894
chlorogenic acid ^a	organic	353	191 (100)	353.0877	1.5	C ₁₆ H ₁₈ O ₉	0.6468
ferulic acid- <i>O</i> -hexoside	organic	355	193 (65), 178 (30), 149 (100)	355.1034	2.5	C ₁₈ H ₂₀ O ₉	0.6240
rutin ^a	organic	609	609 (100), 300 (30)	609.1460	0.5	C ₂₇ H ₃₀ O ₁₆	0.7101
quercetin ^a	organic	301	301 (10), 151 (100)	301.0353	0.4	C ₁₅ H ₁₀ O ₇	0.4419
kaempferol-3- <i>O</i> -rutinoside ^a	organic	593	593 (100), 285 (60)	593.1511	0.7	C ₂₇ H ₃₀ O ₁₅	0.6540
naringenin ^a	organic	271	151 (80), 119 (100)	271.0611	1.5	C ₁₅ H ₁₂ O ₅	0.6790
naringenin-7- <i>O</i> -glucoside ^a	organic	433	433 (10), 271 (100)	433.1140	1.1	C ₂₁ H ₂₂ O ₁₀	0.6503
dicafeoylquinic acid 1	organic	515	515 (20), 353 (100), 191 (50)	515.1194	0.1	C ₂₃ H ₂₄ O ₁₂	0.7028
dicafeoylquinic acid 2	organic	515	515 (20), 353 (100), 191 (50)	515.1194	0.1	C ₂₃ H ₂₄ O ₁₂	0.6065

^a Comparison with standard.

effect on the overall metabolomic composition of ketchups, mass profiles were obtained using Marker View 1.2 software and Statistics Plus 5.1.

A volcano plot ($\log_2$ fold change vs $-\log_{10}$ p value) representing the entire set of data (1200 features) is shown in Figure 1. Metabolites with statistically significant differences between organic and conventional ketchups lie above the horizontal threshold line. Then, the data set was mean-centered and Pareto-scaled after removal of all mass features showing nonsignificant p values or other mass features that were due to interferences between organic and conventional ketchup samples. A list of significantly different mass chromatographic features between organic and conventional ketchups is shown in Table 1. The elemental composition was selected according to the accurate masses and isotopic pattern (through the Formula Finder feature in Analyst QS 2.0). Then, the elemental composition obtained was sought in the *Dictionary of Natural Products* (Chapman and Hall/CRC), the MOTO database (<http://appliedbioinformatics.wur.nl/moto>), and CAS SciFinder. Interpretation of the observed MS/MS spectra in comparison with results found in the literature was the main tool for putative identification of metabolites.

The compounds found in significantly higher ($P < 0.05$) amounts in organic rather than conventional ketchups were caffeoylquinic and dicafeoylquinic acids, caffeic and caffeic acid hexosides, kaempferol-3-*O*-rutinoside, ferulic-*O*-hexoside, naringenin-7-*O*-glucoside, naringenin, rutin, and quercetin, whereas glutamylphenylalanine and *N*-malonyltryptophan were detected only in conventional brands. It was possible to differentiate the isomers of caffeoylquinic, dicafeoylquinic, and caffeic-*O*-hexoside acid in relative intensities in MS² spectra using LC-ESI-QToF. Table 1 shows the list of 16 compounds identified through HPLC-QToF-MS having levels that were significantly different ($P < 0.05$) between organic and conventional brands, along with retention times (rt), accurate mass (acc mass), molecular formula (MF), and millidaltons (mDa) of error between the mass found and the accurate mass of each metabolite and the MS-MS ions used for the identification.

Mass Characterization of Glutamylphenylalanine and *N*-Malonyltryptophan in Conventional Ketchups. Although

mass characterization of phenolic compounds has been widely described in the literature for tomatoes and tomato-based products,^{21–23} this is the first time that glutamylphenylalanine and *N*-malonyltryptophan have been found in conventional tomato ketchups.

Examination of the chromatograms in the ToF-MS mode suggested the presence of glutamylphenylalanine (m/z 293) and *N*-malonyltryptophan (m/z 289) with errors of 0.39 and 0.90 mDa, respectively. The MS² of m/z 293 showed ions at m/z 164, 147, and 103, which may correspond to losses of C₅H₇NO₃, NH₃, and COO, respectively. The MS² of m/z 289 showed ions at m/z 203, 159, and 142, which could be attributed to losses of C₅H₇O₃, COO, and NH₃, respectively. Glutamylphenylalanine is reported here for the first time, whereas *N*-malonyltryptophan is a predominant indole compound widespread in higher plants and found in leaves, fruits, and seeds of wheat and tomatoes.^{22,24,25}

It is well-known that the biosynthesis of phenolic compounds in plants is strongly influenced by the cultivar⁶ and mode of fertilization.²⁶ The level of carbon-based secondary metabolites such as phenolic compounds is usually higher in organic plants^{9,10} due to their defensive role in plants under stress conditions.²⁷ Our results for nitrogen compounds are in line with a German review reporting a lower nitrate content in organic vegetables in nearly all cases and less protein in organic cereal grains.²⁸ These differences are reflected in our metabolomic analysis, which detected 14 metabolites as chemotaxonomic ketchup markers able to distinguish between varieties from different agronomic treatments. The metabolomic approach makes it possible to detect several hundred metabolites simultaneously and to reliably compare samples for differences and similarities in a semi-automated and, above all, untargeted manner.

Analysis of Total Phenolic Content and Hydrophilic Antioxidant Capacity. The F-C assay showed that organic ketchups contained significantly ($P < 0.05$) higher concentrations of TP than conventionally produced ketchups (results shown in Table 2). A similar trend was observed for hydrophilic antioxidant capacity, with the lowest being determined in conventional ketchups.

Table 2. Total Phenolic Content and Hydrophilic Antioxidant Capacity Using ABTS^a and DPPH Assays Expressed as Mean $\pm$ SD of Organic and Conventional Ketchups^a

ketchup	total phenolic content (mg GAE/100 g FW)	ABTS ^a (mmol TE/100 g FW)	DPPH (mmol TE/100 g FW)
conventional			
C ₁	9.43 $\pm$ 0.10 ab	3.78 $\pm$ 0.09 a	4.95 $\pm$ 0.11 a
C ₂	9.61 $\pm$ 0.12 b	3.81 $\pm$ 0.07 a	4.67 $\pm$ 0.08 b
C ₃	9.03 $\pm$ 0.08 c	3.89 $\pm$ 0.10 b	4.93 $\pm$ 0.08 a
C ₄	8.14 $\pm$ 0.11 d	3.45 $\pm$ 0.06 c	4.20 $\pm$ 0.09 c
C ₅	9.40 $\pm$ 0.17 a	3.37 $\pm$ 0.08 c	3.94 $\pm$ 0.08 d
C ₆	9.61 $\pm$ 0.13 b	3.65 $\pm$ 0.08 d	4.21 $\pm$ 0.07 c
C ₇	8.62 $\pm$ 0.16 e	2.71 $\pm$ 0.08 e	3.21 $\pm$ 0.09 e
C ₈	8.46 $\pm$ 0.19 c	3.24 $\pm$ 0.08 f	4.15 $\pm$ 0.10 c
C ₉	7.62 $\pm$ 0.13 f	2.59 $\pm$ 0.06 g	3.46 $\pm$ 0.09 f
C ₁₀	8.07 $\pm$ 0.10 d	2.65 $\pm$ 0.09 eg	3.25 $\pm$ 0.11 e
organic			
O ₁	11.56 $\pm$ 0.18 g	4.25 $\pm$ 0.07 h	5.38 $\pm$ 0.12 g
O ₂	12.61 $\pm$ 0.11 h	4.41 $\pm$ 0.06 i	5.50 $\pm$ 0.10 h
O ₃	12.22 $\pm$ 0.15 i	4.27 $\pm$ 0.06 h	5.21 $\pm$ 0.09 i
O ₄	12.37 $\pm$ 0.16 i	4.19 $\pm$ 0.07 h	5.09 $\pm$ 0.10 j
O ₅	12.30 $\pm$ 0.12 i	4.53 $\pm$ 0.07 j	5.45 $\pm$ 0.09 gh

^a Letters in the columns represent significant statistical differences ($P < 0.05$). GAE, gallic acid equivalents; TE, Trolox equivalent; FW, fresh weight; SD, standard deviation.

Data on the phenolic composition of fruits and vegetables grown either organically or conventionally remain scarce in the literature, because these compounds have only recently been considered as interesting functional microconstituents due to their role in the prevention of cancer, cardiovascular diseases, and degenerative diseases. Among papers mentioning total phenolic content, the majority describe a higher phenolic concentration in organically grown fruits or vegetables.²⁹ Our results are in accordance with these studies because organic ketchups showed a higher content of polyphenols and antioxidant capacity than conventional ketchups.

Quantitation of Individual Polyphenols in Conventional and Organic Ketchups. Specific polyphenol compounds were monitored in conventional and organic ketchups, with the results shown in Table 3. The main polyphenol in all ketchups was rutin, present at levels ranging between 40.88 and 77.65 $\mu\text{g/g}$ FW, followed by naringenin, as reported in other studies.^{6,30} Rutin and naringenin concentrations were significantly higher in the ketchups made from organically grown tomatoes. Chassy et al.³¹ found significantly higher mean levels of soluble solids, flavonoids, total phenolics, and ascorbic acid in organic tomatoes than in their conventional counterparts grown in model plots over a 3-year period.

Hydroxycinnamic acids were mainly represented by ferulic acid-*O*-hexoside, the content of which ranged from 15.59–29.24 $\mu\text{g/g}$ FW in conventional ketchups to 34.50–40.85 $\mu\text{g/g}$ FW in organic ketchups. Caffeic acid-*O*-hexosides and caffeic acid followed a similar trend, with higher levels found in organic ketchups.

Table 3. Content of Polyphenols Expressed as Mean $\pm$ SD of Organic and Conventional Ketchups; superscript ^a

ketchup	$\mu\text{g/g}$ FW				
	ferulic acid-O-hexoside	caffeic acid-O-hexoside 1	caffeic acid-O-hexoside 2	caffeic acid	
conventional					
C ₁	29.24 ± 0.72 a	3.87 ± 0.11 a	1.23 ± 0.06 a	1.41 ± 0.04 a	
C ₂	28.08 ± 0.40 b	3.79 ± 0.14 a	1.52 ± 0.04 b	1.34 ± 0.05 b	
C ₃	28.32 ± 0.87 b	3.52 ± 0.11 b	1.48 ± 0.07 b	1.33 ± 0.05 b	
C ₄	15.59 ± 0.36 c	2.20 ± 0.07 c	1.36 ± 0.05 c	1.18 ± 0.04 c	
C ₅	25.91 ± 0.58 d	3.92 ± 0.05 a	1.36 ± 0.07 c	1.90 ± 0.05 d	
C ₆	19.23 ± 0.64 e	2.93 ± 0.08 d	1.37 ± 0.07 c	1.24 ± 0.05 c	
C ₇	19.58 ± 0.72 ef	2.84 ± 0.08 d	1.03 ± 0.04 d	1.19 ± 0.05 c	
C ₈	22.76 ± 0.75 g	3.20 ± 0.11 e	1.36 ± 0.05 c	1.74 ± 0.05 e	
C ₉	26.83 ± 0.75 h	2.50 ± 0.09 f	1.15 ± 0.04 e	1.76 ± 0.06 e	
C ₁₀	20.14 ± 0.53 f	3.33 ± 0.10 g	1.40 ± 0.04 c	1.40 ± 0.04 a	
organic					
O ₁	34.84 ± 0.38 h	4.02 ± 0.09 h	2.00 ± 0.06 f	2.08 ± 0.03 f	
O ₂	40.85 ± 0.91 i	4.51 ± 0.13 i	2.39 ± 0.04 g	2.89 ± 0.04 g	
O ₃	39.62 ± 0.72 j	4.99 ± 0.11 j	2.20 ± 0.06 h	2.40 ± 0.04 h	
O ₄	39.04 ± 0.94 j	4.00 ± 0.10 h	1.88 ± 0.06 i	1.73 ± 0.03 i	
O ₅	34.50 ± 0.69 h	5.30 ± 0.06 k	2.33 ± 0.05 g	2.28 ± 0.05 j	
ketchup	$\mu\text{g/g}$ FW				
	neochlorogenic acid	cryptochlorogenic acid	chlorogenic acid	dicafeoylquinic acid 1	dicafeoylquinic acid 2
conventional					
C ₁	0.56 ± 0.02 a	0.18 ± 0.01 a	10.24 ± 0.14 a	0.36 ± 0.01 a	0.24 ± 0.01 a
C ₂	0.57 ± 0.01 a	0.22 ± 0.01 b	9.38 ± 0.09 b	0.50 ± 0.01 b	0.35 ± 0.01 b
C ₃	0.76 ± 0.03 ^{bc}	0.26 ± 0.01 c	9.47 ± 0.15 bc	0.50 ± 0.02 b	0.42 ± 0.02 c

Table 3. Continued

ketchup	$\mu\text{g/g FW}$				
	neochlorogenic acid	cryptochlorogenic acid	chlorogenic acid	dicafeoylquinic acid 1	dicafeoylquinic acid 2
C_4	$0.43 \pm 0.02 \text{ d}$	$0.14 \pm 0.01 \text{ d}$	$5.58 \pm 0.10 \text{ d}$	$0.47 \pm 0.02 \text{ c}$	$0.31 \pm 0.01 \text{ d}$
C_5	$0.73 \pm 0.02 \text{ b}$	$0.32 \pm 0.01 \text{ e}$	$9.54 \pm 0.12 \text{ c}$	$0.55 \pm 0.02 \text{ d}$	$0.44 \pm 0.01 \text{ e}$
C_6	$0.79 \pm 0.03 \text{ c}$	$0.35 \pm 0.01 \text{ f}$	$11.04 \pm 0.12 \text{ e}$	$0.48 \pm 0.01 \text{ c}$	$0.45 \pm 0.01 \text{ e}$
C_7	$0.65 \pm 0.02 \text{ c}$	$0.36 \pm 0.01 \text{ f}$	$6.49 \pm 0.06 \text{ f}$	$0.43 \pm 0.01 \text{ c}$	$0.41 \pm 0.01 \text{ c}$
C_8	$0.91 \pm 0.02 \text{ f}$	$0.29 \pm 0.01 \text{ g}$	$8.85 \pm 0.12 \text{ g}$	$0.39 \pm 0.02 \text{ f}$	$0.44 \pm 0.01 \text{ e}$
C_9	$0.88 \pm 0.01 \text{ g}$	$0.27 \pm 0.01 \text{ c}$	$7.09 \pm 0.07 \text{ h}$	$0.46 \pm 0.02 \text{ c}$	$0.34 \pm 0.01 \text{ b}$
C_{10}	$0.77 \pm 0.02 \text{ c}$	$0.32 \pm 0.01 \text{ e}$	$6.93 \pm 0.08 \text{ i}$	$0.33 \pm 0.01 \text{ g}$	$0.36 \pm 0.01 \text{ b}$
organic					
O_1	$1.57 \pm 0.02 \text{ h}$	$0.66 \pm 0.01 \text{ h}$	$13.28 \pm 0.15 \text{ j}$	$0.71 \pm 0.01 \text{ h}$	$0.71 \pm 0.01 \text{ f}$
O_2	$1.20 \pm 0.02 \text{ i}$	$0.44 \pm 0.01 \text{ i}$	$12.49 \pm 0.13 \text{ k}$	$0.74 \pm 0.01 \text{ i}$	$0.61 \pm 0.02 \text{ g}$
O_3	$2.44 \pm 0.02 \text{ j}$	$0.96 \pm 0.01 \text{ j}$	$13.53 \pm 0.17 \text{ l}$	$0.66 \pm 0.01 \text{ j}$	$0.67 \pm 0.01 \text{ h}$
O_4	$2.32 \pm 0.07 \text{ k}$	$0.57 \pm 0.02 \text{ k}$	$12.81 \pm 0.13 \text{ m}$	$0.56 \pm 0.01 \text{ k}$	$0.58 \pm 0.02 \text{ i}$
O_5	$1.48 \pm 0.06 \text{ l}$	$0.85 \pm 0.02 \text{ l}$	$13.30 \pm 0.15 \text{ j}$	$0.51 \pm 0.01 \text{ b}$	$0.53 \pm 0.01 \text{ j}$
ketchup	$\mu\text{g/g FW}$				
	rutin	quercetin	naringenin	naringenin-7-O-glucoside	kaempferol-3-O-rutinoside
conventional					
C_1	$64.00 \pm 1.04 \text{ a}$	$0.56 \pm 0.02 \text{ a}$	$44.30 \pm 1.10 \text{ a}$	$3.41 \pm 0.08 \text{ a}$	$29.37 \pm 0.82 \text{ a}$
C_2	$54.50 \pm 1.59 \text{ b}$	$0.61 \pm 0.01 \text{ b}$	$54.96 \pm 1.24 \text{ b}$	$3.34 \pm 0.13 \text{ ab}$	$29.88 \pm 1.28 \text{ a}$
C_3	$60.24 \pm 1.33 \text{ c}$	$0.67 \pm 0.02 \text{ c}$	$40.13 \pm 1.25 \text{ c}$	$3.23 \pm 0.13 \text{ b}$	$29.25 \pm 0.76 \text{ ab}$
C_4	$55.84 \pm 1.24 \text{ b}$	$0.26 \pm 0.01 \text{ d}$	$34.97 \pm 1.43 \text{ d}$	$2.22 \pm 0.07 \text{ c}$	$16.34 \pm 0.52 \text{ c}$
C_5	$49.58 \pm 1.03 \text{ d}$	$0.61 \pm 0.01 \text{ b}$	$45.66 \pm 1.48 \text{ a}$	$2.39 \pm 0.09 \text{ d}$	$29.08 \pm 0.99 \text{ ab}$
C_6	$45.28 \pm 1.21 \text{ e}$	$0.63 \pm 0.02 \text{ b}$	$37.74 \pm 3.23 \text{ e}$	$2.70 \pm 0.08 \text{ e}$	$21.31 \pm 0.78 \text{ d}$
C_7	$53.88 \pm 0.98 \text{ b}$	$0.50 \pm 0.02 \text{ e}$	$27.87 \pm 1.14 \text{ f}$	$2.25 \pm 0.08 \text{ c}$	$16.13 \pm 0.64 \text{ c}$
C_8	$49.07 \pm 1.36 \text{ f}$	$0.49 \pm 0.01 \text{ e}$	$38.55 \pm 1.00 \text{ e}$	$1.77 \pm 0.07 \text{ f}$	$28.97 \pm 0.68 \text{ ab}$
C_9	$41.96 \pm 1.61 \text{ g}$	$0.53 \pm 0.01 \text{ f}$	$29.79 \pm 1.24 \text{ g}$	$1.27 \pm 0.06 \text{ g}$	$27.48 \pm 0.94 \text{ c}$
C_{10}	$40.88 \pm 1.78 \text{ g}$	$0.39 \pm 0.02 \text{ g}$	$40.28 \pm 1.58 \text{ h}$	$1.99 \pm 0.08 \text{ h}$	$28.24 \pm 0.75 \text{ bc}$
organic					
O_1	$69.50 \pm 1.26 \text{ h}$	$0.73 \pm 0.01 \text{ h}$	$52.76 \pm 1.60 \text{ i}$	$3.69 \pm 0.07 \text{ i}$	$34.17 \pm 1.02 \text{ e}$
O_2	$75.15 \pm 1.27 \text{ i}$	$1.20 \pm 0.03 \text{ i}$	$60.81 \pm 1.17 \text{ j}$	$3.64 \pm 0.07 \text{ i}$	$35.98 \pm 1.42 \text{ f}$
O_3	$68.57 \pm 1.55 \text{ h}$	$0.80 \pm 0.02 \text{ j}$	$57.33 \pm 1.88 \text{ k}$	$4.01 \pm 0.05 \text{ j}$	$40.11 \pm 1.02 \text{ g}$
O_4	$71.90 \pm 1.66 \text{ j}$	$1.96 \pm 0.05 \text{ k}$	$67.16 \pm 1.37 \text{ l}$	$3.06 \pm 0.03 \text{ k}$	$38.65 \pm 1.03 \text{ h}$
O_5	$77.65 \pm 0.88 \text{ k}$	$1.25 \pm 0.03 \text{ l}$	$52.25 \pm 1.42 \text{ m}$	$4.49 \pm 0.02 \text{ l}$	$46.85 \pm 1.28 \text{ i}$

^a Letters in the columns represent significant statistical differences ($P < 0.05$). FW, fresh weight.

Chlorogenic acid isomers were also present in ketchups. Chlorogenic acid was the most abundant caffeoylquinic acid, ranging from 5.58 – $11.04 \mu\text{g/g FW}$ in conventional ketchups to 12.49 – $13.53 \mu\text{g/g FW}$ in organic ketchups. These results are in line with those reported by Caris-Veyrat et al.,³² who retrieved significantly higher concentrations of chlorogenic acid and glycoalkaloids from organic tomatoes ($P < 0.05$) in comparison to the conventional variant. Absolute differences in the levels of dicafeoylquinic acids were also found in the two types of ketchup, dicafeoylquinic acid 1 being the most abundant among the majority, ranging from 0.33 – $0.55 \mu\text{g/g FW}$ in conventional ketchups to 0.51 – $0.74 \mu\text{g/g FW}$ in organic ketchups.

Flavanones were represented by naringenin and naringenin-7-O-glucoside, with maximum concentrations being determined in organic ketchups. Naringenin levels ranged from 27.87 – $54.96 \mu\text{g/g FW}$ in conventional ketchups to 52.25 – $67.16 \mu\text{g/g FW}$ in organic ketchups, and levels of naringenin-7-O-glucoside varied

from 1.27 – $3.41 \mu\text{g/g FW}$ in conventional ketchups to 3.06 – $4.49 \mu\text{g/g FW}$ in organic ketchups.

Flavonols were represented by rutin followed by kaempferol-3-O-rutinoside and quercetin. The organic ketchups had higher levels of rutin (68.57 – $77.65 \mu\text{g/g FW}$) than the conventional ones (40.88 – $64.00 \mu\text{g/g FW}$). The same pattern was observed for quercetin and kaempferol-3-O-rutinoside.

Similarly, the concentrations of quercetin and kaempferol were much lower in the conventionally produced ketchups. Mitchell et al.³³ described results in line with our study, reporting that the mean level of the flavonoids quercetin, naringenin, and kaempferol was significantly higher ($P < 0.001$) in tomato samples from the organic cropping system than in those produced conventionally. They suggested that there is a significant difference between the two systems in the amount of flavonoids occurring in ripe fruit at harvest. Therefore, different food cultivation methods may result in differences in the content of secondary metabolites such as polyphenolic compounds. Grindler-Pedersen et al.

compared conventionally produced diets (CPD) and organically produced diets (OPD) in a human crossover intervention study ($n = 16$) in terms of the intake and excretion of five selected flavonoids and the effect on markers of oxidative defense. The urinary excretion of quercetin and kaempferol was higher after 22 days of intake of the OPD than of the CPD ($P < 0.05$). Therefore, the food production method affected the content of the major flavonoids, quercetin and kaempferol, in foods and also affected urinary flavonoids and markers of oxidation in humans.³⁴

In another study performed to evaluate the polyphenol content of apples (Golden Delicious) grown under defined organic and conventional conditions, it was found that organically produced apples showed significantly higher concentrations of chlorogenic acid, flavonols, flavanols, and dihydrochalcones than the conventionally produced fruits.³⁵

The results obtained in this study of metabolite profiling led to the distinction of features that differentiate between organic and conventional ketchups. Following chromatographic alignment and peak detection, statistical analysis was performed to identify metabolites that may serve as markers for organic and conventional ketchups. It was observed that organic cultivation can provide tomatoes and tomato-derived products with a significantly higher content of phenolic compounds, whereas glutamylphenylalanine and *N*-malonyltryptophan were detected only in conventional brands. Plant defense-related secondary metabolites are generally considered to be the most important determinant of the nutritional value of fruits and vegetables, and thus organically grown products are more health-promoting than conventional products.

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ABBREVIATIONS USED

F-C, Folin–Ciocalteu; SPE, solid-phase extraction; TP, total polyphenols; HPLC-QToF-MS, liquid chromatography coupled to a hybrid quadrupole time-of-flight mass spectrometer; LC-ESI-Qq, liquid chromatography coupled to mass spectrometry in tandem mode; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; PBS, phosphate-buffered saline, 5 mM; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; GAE, mg of gallic acid equivalents; FW, fresh weight; SD, standard deviation; TE, Trolox equivalent; MRM, multiple reaction monitoring; ANOVA, analysis of variance.

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9. Capítol IX: Diferències en el contingut de carotens dels quètxups i gaspatxos a través d'un mètode *HPLC-ESI-(Li⁺)-MS/MS* correlacionat amb la seva capacitat antioxidant

Anna Vallverdú-Queralt, Míriam Martínez-Huélamo, Sara Arranz-Martínez, Esther Miralles & Rosa M. Lamuela-Raventós. *Journal of the Science of Food and Agriculture*. 2012, 92: 2043-2049.

Resum: Els carotens són compostos antioxidants molt importants en la dieta humana. Poden existir en forma *cis* o *trans*, els quals són difícils de diferenciar en mostres naturals. En quètxups i gaspatxos, les formes isomèriques *trans* són les predominants. No obstant això, durant el processat tèrmic es poden formar isòmers en forma *cis*. Per tant, la determinació de carotens en mostres d'aliments és necessària per a l'avaluació del seu valor nutricional.

El carotè majoritari dels quètxups és el *trans*-licopè, el qual és el principal responsable de la capacitat antioxidant dels quètxups, mentre que, en els gaspatxos, el 5-, 9- i 13-*cis*-licopèns, la luteïna, el β - i α -carotens contribueixen de manera significativa en la capacitat antioxidant. Els quètxups presenten una capacitat antioxidant més elevada a causa del seu elevat contingut en *trans*-licopè, el principal carotè dels tomàquets.

L'anàlisi directa de carotens per *MS* sovint proporciona baixes sensibilitats, per la qual cosa són necessàries tècniques de derivatització. Consegüentment, es presenta un mètode per *MS* fent servir l'*ESI* com a font d'ionització per analitzar els carotens dels quètxups i gaspatxos mitjançant una addició postcolumna de clorur de liti per afavorir la cationització dels carotens. Aquest és el primer estudi que presenta la identificació del 5-, 9- i 13-*cis*-licopèns en quètxups i gaspatxos.

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Differences in the carotenoid content of ketchups and gazpachos through HPLC/ESI(Li⁺)-MS/MS correlated with their antioxidant capacity

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Abstract

BACKGROUND: Carotenoids are important antioxidant compounds in the human diet. Owing to their constrained polyene structure, carotenoids can exist in *cis* and *trans* isomeric forms that can be difficult to differentiate in natural samples. In ketchups and gazpachos, all-*trans* isomeric forms are predominant. However, during thermal processing, *cis* isomers are formed. Therefore the determination of carotenoid isomers is required for the assessment of the nutritional value of foods.

RESULTS: The main carotenoid found in ketchups was *trans*-lycopene, whereas in gazpachos a higher contribution to the total carotenoid content was made by other carotenoids such as 5-, 9- and 13-*cis*-lycopene, lutein, β -carotene and α -carotene. Ketchups exhibited the highest lipophilic antioxidant content owing to their higher content of *trans*-lycopene, the main carotenoid in tomatoes.

CONCLUSION: Direct analysis of carotenoids by electrospray ionisation mass spectrometry (ESI-MS) often produces poor results requiring offline time- and sample-consuming derivatisation techniques. Therefore in this work a simple ESI-MS approach is described for the direct analysis of carotenoids in ketchups and gazpachos using the post-column addition of lithium chloride to promote the cationisation of carotenoids. To the best of the authors' knowledge, this paper presents for the first time the identification of 5-, 9- and 13-*cis*-lycopene in ketchups and gazpachos.

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Keywords: ketchup; gazpacho; carotenoids; HPLC/ESI(Li⁺)-MS/MS; antioxidant capacity

INTRODUCTION

Tomatoes and tomato-based products provide an optimal mix of dietary antioxidants such as phenolic compounds, ascorbic acid, vitamin E and carotenoids. Carotenoids are naturally occurring pigments that are found in a wide variety of plant and animal sources, including various fruits and vegetables and certain animal tissues and biological fluids. Studies have proven that consumption of β -carotene, lycopene and other carotenoids decreases the risk of degenerative diseases such as certain kinds of cancer and cardiovascular diseases.^{1,2} They act as antioxidants and as free radical scavengers *in vivo*.

Owing to their constrained polyene structure, carotenoids can exist in *cis* and *trans* isomeric forms. The identification of either different carotenoids or *cis/trans* isomers of a particular carotenoid is of special interest.^{3,4} In tomatoes and tomato-based products, all-*trans* isomeric forms are predominant. However, during thermal processing, *cis* isomers are formed. Research indicates that more than 50% of the carotenoids found in the human body are present in the *cis* isomeric form, leading to the hypothesis that the *cis* isomeric form is the most bioavailable form in the human body.⁵

Typically, separations of carotenoids have been carried out through the use of C₁₈ stationary phases.^{6,7} More recently, a C₃₀

stationary phase has been developed that gives dramatically improved resolution and selectivity of carotenoids and their isomers.^{8,9}

For the identification and quantification of carotenoids, high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection is the preferred technique.¹⁰ More recently, mass spectrometry (MS) has been introduced. The common ionisation technique used is atmospheric pressure chemical ionisation (APCI).

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However, a large number of samples is necessary and sometimes this cannot be achieved.¹¹ A good alternative is the use of electrospray ionisation (ESI) where only a small number of samples is necessary and there are no problems with thermal instability of some carotenoids. However, this technique is more susceptible to mobile phase interference, and low detection limits are usually achieved when compared with APCI for carotenoid analysis. More recently, efficient ionisation of low-polar compounds such as carotenoids using ESI has been achieved with prior derivatisation via the addition of sodium or lithium salts.¹¹

The aim of this work was to describe the carotenoid composition of gazpachos and ketchups through the use of HPLC-UV and HPLC/ESI(Li⁺)-MS/MS with a non-polar C₃₀ stationary phase. In this way we identified seven carotenoids: lutein, α -carotene, β -carotene, *trans*-lycopene and 5-, 9- and 13-*cis*-lycopene. It is important to evaluate bioactive compounds together owing to possible synergistic and/or antagonistic effects on their contribution to antioxidant capacity.

MATERIALS AND METHODS

Standards and reagents

All samples and standards were handled without exposure to light. β -Carotene, α -carotene, lycopene, lutein, methyl *tert*-butyl ether (MTBE) and hexane were purchased from Sigma (St Louis, MO, USA), ethanol and methanol of HPLC grade from Scharlau (Barcelona, Spain), and ultrapure water (Milli-Q) from Millipore (Bedford, MA, USA).

Processing conditions and ingredients of commercial samples

Six different commercial brands of each of the tomato-based products (ketchup and gazpacho) were analysed. All studied brands contained the same ingredients, but in some cases the amount of each ingredient was not known.

Ketchup

Ketchup is a sweet-and-sour condiment typical of the American diet. The technological process used to obtain ketchup consists of mixing the tomato paste (65%, 30–32° Brix) with sugar, wine vinegar, salt, aroma and preservatives in traces. The mixture is pasteurised at 96 °C for 4–6 min and packaged in bottles. The temperature must be carefully regulated to ensure absorption of the ingredients without overcooking. The entire process of ketchup manufacturing generally takes 2–3 h.¹²

Gazpacho

Gazpacho is a traditional Spanish soup that can be defined as a cold soup containing mainly five vegetables – tomato, cucumber, green pepper, onion and garlic – as well as other minor components such as extra virgin olive oil, wine vinegar, salt, sugar and water. The vegetable mixture is cooked and usually pasteurised (92–95 °C, 1 min) to extend its shelf life.¹³ The samples in this study were of canned, bottled packed, commercial gazpachos.

Extraction and isolation of carotenoid compounds from tomato-based products

The extraction of carotenoids must be carried out very quickly, avoiding exposure to light, oxygen, high temperatures and also pro-oxidant metals such as iron or copper in order to minimise autooxidation and *cis/trans* isomerisation.

Ketchup and gazpacho samples (0.5 g) were homogenised with 5 mL of ethanol/hexane (4:3 v/v) following a procedure described in the literature.¹⁴ The homogenate was sonicated for 5 min and centrifuged at 2140 $\times g$ for 15 min at 4 °C. The supernatant was transferred into a flask and the extraction was repeated. The two supernatants were combined and evaporated under nitrogen flow. Finally, the residue was reconstituted with MTBE up to 1 mL and filtered through a 13 mm, 0.45 μ m polytetrafluoroethylene (PTFE) filter (Waters, Milford, MA, USA) into an insert-amber vial for HPLC analysis. Samples were stored at –20 °C until analysis.

HPLC separation of carotenoids in ketchups and gazpachos

Chromatographic analysis was performed using an HP 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany) equipped with a quaternary pump, an autosampler and a column oven. The analytes were separated on a 250 mm $\times$ 4.6 mm i.d., 5 μ m C₃₀ column (YMCTM, Waters) maintained at 20 °C. The injection volume was 20 μ L and the flow rate was 1 mL min^{–1}. The mobile phases were water (A), methanol (B) and MTBE (C). Separation was carried out in 23 min under the following conditions: 0 min, 70% B; 10 min, 20% B; 20 min, 6% B; 21 min, 6% B; 23 min, 70% B. Water was kept constant at 4% throughout the analysis. The column was equilibrated for 10 min prior to each analysis. The modification procedure followed the protocol previously published for *Ocimum basilicum*.⁹ MTBE was used as a modifier to facilitate the elution of lycopene, which is strongly retained in a methanol environment.

Diode array detector

Commercially available carotenoid standards (lutein, α -carotene, β -carotene and *trans*-lycopene) were used to identify analytes by retention times and UV–visible spectra. The HPLC–UV chromatograms were acquired at 450 nm wavelength. Afterwards the UV–visible spectra were recorded in the range of 350–550 nm for the tentative identification of carotenoids and their geometrical isomers (*cis*-lycopene isomers) on the basis of the retention times and absorption spectrum characteristics described in the literature.^{9,15}

Mass spectrometry

An API 3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, ON, Canada) equipped with a Turbo Ionspray source in positive ion mode was used to obtain MS/MS data. The Turbo Ionspray source settings were as follows: capillary voltage, 5500 V; nebuliser gas (N₂), 10 (arbitrary units); curtain gas (N₂), 12 (arbitrary units); collision gas (N₂), 4 (arbitrary units); focusing potential, 200 V; entrance potential, 10 V; drying gas (N₂), heated to 400 °C and introduced at a flow rate of 6000 cm³ min^{–1}. The declustering potential and collision energy were optimised for each compound in infusion experiments: individual standard solution (10 μ g mL^{–1}) dissolved in 50:50 (v/v) mobile phase was infused at a constant flow rate of 5 μ L min^{–1} using a syringe pump (Harvard Apparatus, Holliston, MA, USA).

Full-scan data acquisition was performed by scanning from *m/z* 100 to 800 in profile mode and using a cycle time of 2 s with a step size of 0.1 unit and a pause between each scan of 2 ms. MS/MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple-quadrupole mass spectrometer and mass analysed using the instrument's second analyser. In all experiments, both quadrupoles Q1 and Q3 were operated at unit resolution. Additional experimental conditions for MS/MS

included collision energy (depending on the compound), CAD gas (N_2) at 6 (arbitrary units) and scan range, as necessary for the precursor selected.

A solvent delivery system connected post-column was used for the delivery of $100 \mu L \text{ min}^{-1}$ of lithium chloride solution at a concentration of 500 mg L^{-1} .

The combination of DAD and MS was indispensable for the screening of carotenoids. MS can provide additional information such as molecular weight and characteristic fragmentation patterns when used in tandem MS mode. Carotenoids were quantified with respect to their corresponding standards according to the internal standard method using *trans*- β -apo-8'-carotenal. When standards were not available, as in the case of *cis* isomers of lycopene, they were quantified on the basis of the peak area of the *trans*-lycopene standard. Results were expressed as mg kg^{-1} dry weight (DW).

Lipophilic antioxidant capacity

The lipophilic antioxidant capacity of ketchup and gazpacho extracts was measured using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid cation (ABTS⁺) radical decolorisation assay and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay following the procedure of Vallverdú-Queralt *et al.*¹⁶ with minor modifications.

ABTS⁺ assay

Antioxidant standard (1 mmol L^{-1} Trolox) was prepared in methanol once a week. Working standards were prepared daily by diluting 1 mmol L^{-1} Trolox with methanol.

An ABTS⁺ radical solution was prepared by passing a 5 mmol L^{-1} aqueous stock solution of ABTS (in methanol) through manganese dioxide powder. Excess manganese dioxide was removed by means of a 25 mm , $0.45 \mu\text{m}$ PTFE filter (Waters). Before analysis the solution was diluted in methanol to give an absorbance at 734 nm of 1.0 ± 0.1 and pre-incubated in ice. Then $250 \mu\text{L}$ of ABTS⁺ solution was added to $5 \mu\text{L}$ of Trolox or gazpacho/ketchup extract and the solutions were stirred for 60 s . The absorbance was recorded every 30 s for 1 h with a Thermo Multiskan Spectrum UV-visible spectrophotometer (Vantaa, Finland), and methanol blanks were run in each assay.

The working range for Trolox (final concentration $0\text{--}1000 \mu\text{mol L}^{-1}$) was based on triplicate determinations and consisted of plotting the absorbance as a percentage of the absorbance of the uninhibited radical cation (blank). The activities of ketchup and gazpacho extracts were assessed at four different concentrations within the range of the dose-response curve. Each sample was analysed in triplicate at each concentration. Results were expressed as $\text{mmol Trolox equivalent (TE) kg}^{-1}$ DW.

DPPH assay

The antioxidant capacity was also studied through the evaluation of free radical-scavenging effects on the DPPH radical. Trolox solutions of known concentration were used for calibration. First, $5 \mu\text{L}$ of Trolox or ketchup/gazpacho extract was mixed with $250 \mu\text{L}$ of methanolic DPPH (0.025 g L^{-1}). The homogenates were shaken vigorously and kept in darkness for 30 min . Absorption of the samples was measured at 515 nm . The percentage of DPPH inhibition was calculated and plotted as a function of the concentration of Trolox for the standard reference data. The final DPPH values were calculated using a regression equation between the Trolox concentration and the percentage of DPPH inhibition. Results were expressed as mmol TE kg^{-1} DW.

Statistical analysis

The significance of the results was analysed using Statgraphics Plus Version 5.1 for Windows (Statistical Graphics Co., Rockville, MD, USA). Analysis of variance was used to compare the means of groups of measurement data. Relationships between variables were examined using Pearson correlation coefficients. *P* values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

It is well known that tomatoes are the main dietary source of lycopene and are one of the most frequently consumed vegetables in the Mediterranean area. Their consumption has greatly increased worldwide over the past two decades, mostly owing to a growing demand for tomato-based products such as ketchups and gazpachos.^{16,17}

Lithium adducts $[M + Li]^+$ appear to be very important in the ionisation process of carotenoid compounds. Without the addition of lithium chloride the molecular ions $[M]^+$ and $[M + H - H_2O]^+$ could be detected with a high limit of detection, even when other additives such as formic acid and acetic acid were used. Table 1 shows the HPLC and MS characteristics of identified carotenoids. The results found in this study are in line with those reported by other authors.¹⁵

In the case of both β -carotene (m/z 536.4) and α -carotene (m/z 536.4) the fragment ion spectrum of the $[M]^+$ ion showed that the most abundant fragment ion was $[M - 92]^+$ at m/z 444.4 corresponding to the loss of toluene, indicating the presence of extensive conjugation within the molecule. This ion is abundant in the tandem mass spectra of α -carotene, β -carotene, lutein and lycopene. The product ion spectra of β -carotene and α -carotene also highlighted ions at m/z 177.4 and 137.4 probably originating from breaking double bonds. The most intense ion at m/z 177.4 might be stabilised by the formation of a ring structure. Similar results were described by Gentili and Caretti,¹⁸ who reported that the product ion spectrum of β -carotene presented two series of fragment ions. The first series originated from breaking double bonds and the second series was due to the cleavage of single bonds, showing fragment ions at m/z 123 and 413. Moreover, the UV-visible absorption spectrum of β -carotene displayed λ_{max} at 450 and 475 nm, which is consistent with conjugated double bonds in the polyene chain and two double bonds in the β rings. These results are in line with those reported by Mercadante *et al.*¹⁹ The UV-visible absorption spectrum of α -carotene displayed λ_{max} at 450 and 473 nm.

For lutein (m/z 568.5) the most abundant fragment ion was at m/z 550.5 corresponding to the loss of water, followed by the ions at m/z 476.5 and 429.5 corresponding to the loss of toluene and water plus the terminal ring respectively. The UV-visible absorption spectrum of lutein displayed λ_{max} at 425, 448 and 472 nm.

For both *trans*-lycopene and *cis*-lycopene isomers the most abundant fragment ions were at m/z 467.4 and 444.4, which could be attributed to the loss of isoprene and toluene groups respectively. Moreover, in the UV-visible absorption spectra, *trans*-lycopene and 5-*cis*-lycopene displayed λ_{max} at 444, 472 and 500 nm and at 445, 472 and 503 nm respectively, whereas 9- and 13-*cis*-lycopene displayed λ_{max} at 440, 462 and 495 nm and at 440, 462 and 490 nm respectively.

Resultats

Table 1. List of carotenoids identified in ketchups and gazpachos

Compound	RT (min)	[M - H] ⁺	m/z ions	Absorption maxima (nm)	MF
Lutein ^a	6.20	568.5	568.5 (10), 550.5 (90), 476.5 (50), 429.5 (50)	425, 448, 472	C ₄₀ H ₅₆ O ₂
α -Carotene ^a	10.25	536.4	536.4 (20), 444.4 (80), 177.4 (50), 137.4 (80)	450, 473	C ₄₀ H ₅₆
β -Carotene ^a	11.40	536.4	536.4 (20), 444.4 (80), 177.4 (60), 137.4 (80)	450, 475	C ₄₀ H ₅₆
9- <i>cis</i> -Lycopene	14.20	536.4	536.4 (20), 467.4 (80), 444.4 (50)	440, 462, 495	C ₄₀ H ₅₆
13- <i>cis</i> -Lycopene	16.50	536.4	536.4 (20), 467.4 (70), 444.4 (70)	440, 462, 490	C ₄₀ H ₅₆
<i>trans</i> -Lycopene ^a	20.10	536.4	536.4 (20), 467.4 (80), 444.4 (50)	444, 472, 500	C ₄₀ H ₅₆
5- <i>cis</i> -Lycopene	21.10	536.4	536.4 (20), 467.4 (70), 444.4 (70)	445, 472, 503	C ₄₀ H ₅₆

RT, retention time; MF, molecular formula.
^a Comparison with standard.

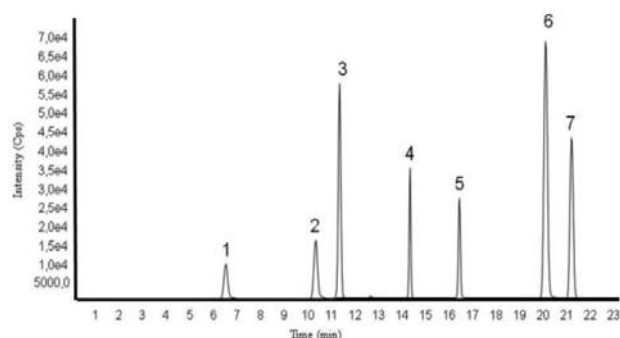


Figure 1. HPLC chromatogram of carotenoid profile, including identification of each peak: 1, lutein; 2, α -carotene; 3, β -carotene; 4, 9-*cis*-lycopene; 5, 13-*cis*-lycopene; 6, *trans*-lycopene; 7, 5-*cis*-lycopene.

Antioxidant capacity and carotenoid content of ketchups and gazpachos

It was observed in data from a population-based case-control study that there was an inverse correlation between total antioxidant capacity intake and the risk of gastric cancer.¹ An HPLC chromatogram of the carotenoid profile, including identification of each peak, is shown in Fig. 1.

Ketchups exhibited the highest lipophilic antioxidant capacity, as shown in Table 2. For ketchups the antioxidant capacity varied between 27.01 and 29.70 mmol TE kg⁻¹ DW and between 28.60 and 30.10 mmol TE kg⁻¹ DW using the ABTS⁺ and DPPH assays respectively. For gazpachos it was slightly lower, ranging from 20.22 to 25.76 mmol TE kg⁻¹ DW and from 23.83 to 27.14 mmol TE kg⁻¹ DW using the ABTS⁺ and DPPH assays respectively. These values were slightly higher than those reported by other authors for tomato products,^{20,21} probably owing to the onion, garlic and olive oil added in the production of ketchups and gazpachos. However, the lipophilic antioxidant capacity per serving is much higher in gazpacho (200 mL) than in ketchup (10 mL).

A Pearson correlation was calculated to establish the relationship between the individual parameters and to ascertain their relative significance in determining the lipophilic antioxidant capacity of ketchups and gazpachos. For ketchups the highest correlation coefficient was found for *trans*-lycopene and DPPH ($R^2 = 0.8908$, $P < 0.05$), indicating a relatively strong and sig-

nificant relationship between the variables. A significant and moderately strong relationship was found between ABTS⁺ and *trans*-lycopene ($R^2 = 0.6791$, $P < 0.05$). No significant correlations were found between other carotenoids and lipophilic antioxidant capacity.

For gazpachos the highest correlation coefficient was observed for ABTS⁺ and β -carotene ($R^2 = 0.9240$, $P < 0.05$) followed by ABTS⁺ and lutein ($R^2 = 0.8580$, $P < 0.05$), ABTS⁺ and 5-*cis*-lycopene ($R^2 = 0.8186$, $P < 0.05$) and ABTS⁺ and *trans*-lycopene ($R^2 = 0.7896$, $P < 0.05$). Significant relationships were also found between DPPH and 5-*cis*-lycopene ($R^2 = 0.9249$, $P < 0.05$) and DPPH and *trans*-lycopene ($R^2 = 0.6816$, $P < 0.05$).

These results are in line with the well-recognised fact that the lipophilic antioxidant capacity of tomato fruits is mainly attributable to the presence of lycopene and β -carotene.^{20,21} Although for ketchups the lipophilic antioxidant capacity essentially resulted from *trans*-lycopene, in the case of gazpachos, 5-*cis*-lycopene, β -carotene, *trans*-lycopene and lutein contributed significantly to their lipophilic antioxidant capacity. This fact could well be attributed to the different composition of ketchups and gazpachos. A lower proportion of tomato and an increase in the content of cucumber, green pepper and onion in gazpachos may be the reason for the different carotenoid proportion between gazpachos and ketchups. Lutein and β -carotene are characteristic carotenoids in green peppers, onions and cucumbers.²²

Table 2. Lipophilic antioxidant capacity (mmol Trolox equivalent kg⁻¹ dry weight) of gazpachos and ketchups through ABTS⁺ and DPPH assays

Tomato-based product	ABTS ⁺	DPPH
<i>Ketchups</i>		
A	29.59 ± 1.73a	29.35 ± 1.68a
B	27.01 ± 1.37b	28.60 ± 1.51b
C	29.70 ± 2.32a	30.10 ± 1.64c
D	28.45 ± 1.15c	29.14 ± 1.55a
E	28.11 ± 1.56c	29.01 ± 1.55a
F	27.23 ± 1.44b	28.97 ± 1.48b
<i>Gazpachos</i>		
A	20.22 ± 1.95d	23.83 ± 1.98d
B	25.76 ± 1.90e	27.14 ± 2.53e
C	21.36 ± 1.74f	26.00 ± 1.84f
D	23.18 ± 1.55g	24.47 ± 1.69g
E	20.30 ± 1.94d	24.83 ± 2.01g
F	24.95 ± 2.95h	26.84 ± 2.24h

Values are expressed as mean ± standard deviation. Different letters in a column denote statistically significant differences ($P < 0.05$).

trans-Lycopene content

Quantitative data on lycopene composition are presented in Table 3. Lycopene shows strong antioxidant capacity both *in vitro* and *in vivo*.²³ Moreover, thermal processing, such as cooking, required for the preparation of ketchups and gazpachos is recommended because it increases the bioavailability of lycopene in the human body.²⁴ Bearing in mind that most lycopene is associated with the lipophilic fraction in tomato fruits,²¹ a real estimation of the antioxidant capacity of the lipophilic fraction of ketchups depends on a real estimation of lycopene content. These results are also in line with those reported by Lenucci *et al.*,^{25,26} who found that tomatoes are characterised by a high lycopene content (>200 mg kg⁻¹ fresh weight).

Lycopene contents were significantly different among the studied gazpachos and ketchups ($P < 0.05$). The differences

between different tomato-based products could be mainly due to genotypic factors and to enhanced enzymatic activity of phytoene synthase I, which causes high production of lycopene precursors in ripening tomatoes.²⁷ Lycopene was the most abundant carotenoid, ranging from 661.81 to 2477.91 mg kg⁻¹ DW in ketchups and from 59.73 to 86.30 mg kg⁻¹ DW in gazpachos. This variation in the content of lycopene depends on the amount of tomato added. As mentioned above for lipophilic antioxidant capacity, a lower proportion of tomato and an increase in the content of cucumber, green pepper and onion in gazpachos may be the reason for the differences in lycopene content.

cis-Lycopene content

We also analysed *cis*-lycopene isomers (Table 3), because human studies have focused on the nutritional benefits of consuming tomato products with an increased proportion of *cis*-lycopene isomers. *cis*-Lycopene isomers are better absorbed in the intestine.^{28,29} This lycopene isomer profile is in line with that described by other authors in different tomato products. They reported that *trans*-lycopene was the most abundant lycopene isomer but that it varied from 35 to 96% of the total lycopene content, with 5-, 9-, 13- and 15-*cis*-lycopene being the main *cis* isomers detected.¹⁵

It has been shown that, during the preparation of meals, lycopene undergoes *trans/cis* isomerisation, increasing the proportion of *cis* isomers.³⁰ In the present study, 15-*cis*-lycopene was not detected in either gazpachos or ketchups. However, we found 5-*cis*-lycopene in all tomato-based products analysed. Garcia-Alonso *et al.*⁸ measured the lycopene content in a number of tomato-based foodstuffs and meals and found that the proportion of 5-*cis*-lycopene fluctuated between 4 and 27%, 9-*cis*-lycopene ranged from <1 to 14% and 13- and 15-*cis*-lycopene ranged (together) from <1 to 7%, while the sum of the other *cis* isomers varied between <1 and 22% of the total lycopene content. These results are in line with those obtained in our study on ketchups. For ketchups, *cis* isomers varied between 9 and 23% of the total lycopene content, whereas *cis* isomers detected in gazpachos accounted for between 85 and 90% of the total lycopene content. As mentioned above, *cis*-lycopene isomers

Table 3. Carotenoid contents (mg kg⁻¹ dry weight) of ketchups and gazpachos

Tomato-based Product	Lutein	α -Carotene	β -Carotene	<i>trans</i> -Lycopene	5- <i>cis</i> -Lycopene	9- <i>cis</i> -Lycopene	13- <i>cis</i> -Lycopene	Total
<i>Ketchups</i>								
A	1.43 ± 0.03a	10.01 ± 0.61a	257.89 ± 23.37a	1342.04 ± 116.71a	115.69 ± 7.60a	74.65 ± 1.67a	32.69 ± 1.88a	1784.39
B	1.41 ± 0.05a	28.28 ± 2.33b	485.74 ± 29.72b	810.49 ± 53.88b	56.29 ± 4.43b	85.26 ± 8.05b	51.80 ± 3.39b	1519.26
C	1.40 ± 0.08a	18.49 ± 1.06c	315.73 ± 34.75c	2477.91 ± 266.69c	99.24 ± 5.17c	136.54 ± 4.60c	71.82 ± 3.47c	3121.12
D	1.42 ± 0.07a	10.61 ± 1.28d	237.30 ± 19.20d	1107.32 ± 75.89d	180.35 ± 9.92d	66.58 ± 3.67d	20.37 ± 0.91d	1623.95
E	1.68 ± 0.06b	17.08 ± 1.16e	382.59 ± 23.11e	1156.71 ± 64.32e	211.14 ± 15.67e	105.24 ± 9.79e	26.47 ± 2.48e	1900.92
F	1.13 ± 0.06c	5.00 ± 0.58f	231.89 ± 13.68d	661.81 ± 11.37f	181.22 ± 3.87d	63.58 ± 4.03f	17.07 ± 1.96f	1168.67
<i>Gazpachos</i>								
A	0.89 ± 0.04d	5.06 ± 0.37f	181.98 ± 3.78f	64.71 ± 1.44g	47.48 ± 1.25f	23.69 ± 0.86g	32.75 ± 0.46a	356.54
B	1.58 ± 0.04e	6.32 ± 0.64g	313.83 ± 10.11c	86.30 ± 1.66h	68.38 ± 0.35g	14.76 ± 0.40b	28.34 ± 1.07g	519.52
C	1.26 ± 0.11f	5.39 ± 0.48h	228.61 ± 9.57g	65.08 ± 3.42g	59.27 ± 8.43h	20.81 ± 0.68i	27.52 ± 0.63g	407.95
D	1.52 ± 0.17g	7.35 ± 0.92i	291.21 ± 31.54h	63.67 ± 6.68g	56.62 ± 1.58i	17.26 ± 0.78j	26.96 ± 0.87h	464.59
E	1.02 ± 0.10h	6.25 ± 0.38g	214.31 ± 12.75i	59.73 ± 3.65i	54.19 ± 1.06i	27.16 ± 2.19k	27.16 ± 0.84gh	389.81
F	1.78 ± 0.13i	10.52 ± 0.29d	315.59 ± 13.19c	77.21 ± 7.45j	67.24 ± 2.22g	34.16 ± 1.18l	32.95 ± 2.62a	539.46

Values are expressed as mean ± standard deviation. Different letters in a column denote statistically significant differences ($P < 0.05$).

are more bioavailable than *trans*-lycopene *in vitro* and *in vivo*, probably because *cis* isomers are more soluble in bile acid micelles and may be preferentially incorporated into chylomicrons.⁸ Therefore consuming gazpachos would be expected to be more health-promoting owing to their high proportion of *cis* isomers.

β -Carotene, α -carotene and lutein contents

While lycopene is the main carotenoid in tomatoes (~90–96% of total carotenoids), we also measured the β -carotene, α -carotene and lutein contents (Table 3). The β -carotene content ranged between 231.89 and 485.74 mg kg⁻¹ DW in ketchups and between 181.98 and 315.59 mg kg⁻¹ DW in gazpachos. For ketchups, β -carotene accounted for between 10 and 32% of the total carotenoid content. For gazpachos it varied between 50 and 62% and was the major carotenoid. This difference may be due to the addition of onion and garlic to gazpachos. It has been reported that onion and garlic generally improve the retention of β -carotene during cooking.³¹

Other carotenoids such as α -carotene and lutein account for less than 6.8% of total carotenoids.²⁰ The α -carotene content varied from 5.00 to 28.28 mg kg⁻¹ DW in ketchups and from 5.06 to 10.52 mg kg⁻¹ DW in gazpachos. The lutein content was in the range 1.13–1.68 mg kg⁻¹ DW for ketchups and in the range 0.89–1.78 mg kg⁻¹ DW for gazpachos. Therefore provitamin A of ketchups and gazpachos is derived essentially from β -carotene. Thermal treatments might stimulate the transformation of some carotenoids into β -carotene. γ -Carotene may undergo cyclisation to form six-membered rings at one end of the molecule, giving β -carotene as a product.³²

CONCLUSIONS

In this work, ketchups and gazpachos were studied for their carotenoid content. The main carotenoid found in ketchups was *trans*-lycopene, whereas for gazpachos a higher contribution to the total carotenoid content from other carotenoids, mainly β -carotene, 5-, 9- and 13-*cis*-lycopene, lutein and α -carotene, was observed. To the best of our knowledge, this paper presents for the first time the identification of 5-, 9- and 13-*cis*-lycopene isomers in ketchups and gazpachos using a C₃₀ stationary phase column through the combination of two techniques, HPLC/ESI(LI⁺) -MS/MS and HPLC-UV, enabling better ionisation of carotenoids and consequently better identification. The methodology described in this work is currently under investigation to establish whether it could be used to assess the carotenoid status in human blood and tissue samples.

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10. Capítol X: Canvis en el perfil fenòlic dels quètxups i suc de tomàquet durant l'emmagatzematge

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Resum: En aquesta publicació es pretén estudiar els efectes de l'emmagatzematge de suc de tomàquet i quètxups en el contingut de polifenols totals, capacitat antioxidant i polifenols individuals (àcids hidroxicinnàmics, flavonols i flavanones). Aquest estudi es va dur a terme durant l'any 2010. Fins ara, no existia cap estudi que, durant nou mesos, avalués els efectes de l'emmagatzematge en aquests tipus de productes, que actualment estan en demanda creixent gràcies a l'augment del consum de productes processats de tomàquet. S'observa que durant 9 mesos, el nivell de polifenols i antioxidants disminueix, sobretot les aglicones, en comparació amb els polifenols glicosilats, però, en cap cas, pot comportar un perjudici per al consumidor.

Changes in Phenolic Content of Tomato Products during Storage

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ABSTRACT: The effect of storage on the total polyphenol content and individual phenolic compounds as well as on the hydrophilic antioxidant capacity of ketchups and tomato juices was studied. The total polyphenol content was determined using the Folin–Ciocalteu assay, and the antioxidant capacity of the hydrophilic fraction was determined using DPPH and ABTS^{•+} assays. Individual polyphenols were identified and quantified using liquid chromatography/electrospray ionization tandem mass spectrometry on a triple quadrupole. All analyses were carried out for ketchups and tomato juices after storage for 3, 6, and 9 months. The total polyphenol content and antioxidant capacity of the hydrophilic fraction decreased during storage of ketchups and tomato juices. Ketchups, in general, showed a slightly greater stability during storage than tomato juices. The most significant decrease was observed for quercetin followed by caffeic and ferulic acids, whereas glycosylated polyphenols showed greater stability during storage.

KEYWORDS: Polyphenols, HPLC-MS/MS, antioxidant capacity, storage, stability

■ INTRODUCTION

Tomato consumption has greatly increased worldwide over the past 2 decades, mostly due to a growing demand for tomato-based products such as ketchups and tomato juices. Ketchups and tomato juices, as well as raw tomatoes, possess antioxidative, anti-inflammatory, antimutagenic, and anticarcinogenic properties and the capacity to modulate some key cellular enzyme functions, this being ascribed in part to their many phenolic compounds.^{1,2} When considering the importance of the role of polyphenols as health-protecting factors, the consumption of tomato and tomato-based products could be seen as a nutritional indicator of good dietary habits and a healthy lifestyle.

Tomato-based products may potentially contain the same range of phenolic compounds as the tomatoes from which they are derived; however, because of differences between varieties of tomatoes and their origin,^{3,4} the total polyphenol (TP) content can vary considerably. The nutritional quality of tomato products is affected by storage conditions as well as by thermal treatments during processing. In particular, thermal treatments are generally believed to be the main cause of the depletion in natural antioxidants.^{5,6}

Studies investigating the impact of storage conditions on the stability of tomato antioxidants have shown marked lycopene and ascorbic acid losses during the first months of storage.^{7,8} During the storage of tomato-based products, various changes may occur, for example, as a result of browning and degradation reactions. It is well-known that naturally occurring antioxidants are significantly lost as a consequence of processing and storage.⁹

The literature data for individual and/or TP content, as well as the antioxidant activity of commercial juices, are limited. Although some data on the content of hydroxycinnamic acids and flavanone derivatives in fresh and stored juices are available,^{10–12} storage studies lasting longer than 6 months are scarce. Therefore, further

studies are necessary to determine the nutritional quality of their bioactive compounds during storage. Moreover, there is no information concerning changes in flavonols, flavanones, and hydroxycinnamic acids in ketchups and tomato juices during storage.

The aim of this study was to evaluate the stability of the hydrophilic antioxidant compounds present in six commercially available tomato juices and ketchups over a storage period of 9 months. Both products were kept at storage temperature. The hydrophilic antioxidant capacity, TP, and content of flavonols (kaempferol-3-O-rutinoside, rutin, and quercetin), flavanones (naringenin and naringenin-7-O-glucoside), and hydroxycinnamic acids (chlorogenic, caffeic, caffeic-O-hexoside, ferulic, and ferulic-O-hexoside acids) were analyzed.

■ MATERIALS AND METHODS

Standards and Reagents. All samples and standards were handled without exposure to light. Caffeic and chlorogenic acids, rutin, quercetin, Folin–Ciocalteu (F-C) reagent, ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)], PBS (phosphate-buffered saline, pH 7.4), Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid 97%], manganese dioxide, and DPPH (2,2-diphenyl-1-picrylhydrazyl) were purchased from Sigma Chemical Co (St. Louis, MO); naringenin, naringenin-7-O-glucoside, and kaempferol-3-O-rutinoside were purchased from Extrasynthèse (Genay, France). Ethanol and formic acid, high-performance liquid chromatography (HPLC) grade, were obtained from Scharlau Chemie, S.A. (Barcelona, Spain); and ultrapure water (Milli-Q) was from Millipore System (Bedford, MA).

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Processing Conditions and Ingredients of Commercial Samples. In this study, a 9 month storage trial was designed to investigate the stability of the main bioactive antioxidant compounds of six commercially available tomato juices and six commercial ketchups. All of the studied brands contained the same ingredients, but in some cases, the amount of each ingredient was not known. Each brand of ketchup and tomato juice was analyzed at the start of the trial (month 0). Three cartons of each brand of ketchup and tomato juice were stored for 3, 6, and 9 months. Ketchups and tomato juices were kept in Tetra Paks at a storage temperature of 4.0 ± 0.1 . Both products analyzed were available in Barcelona markets.

Extraction and Isolation of Phenolic Compounds. Samples were treated in triplicate in a darkened room with a red safety light to avoid oxidation of the analytes, following the procedure of Vallverdú-Queralt et al. with some modifications.¹³

Ketchup samples (1.0 g) were weighed and homogenized with 80% ethanol in Milli-Q water (4 mL); they were then sonicated for 5 min and centrifuged (4000 rpm at 4 °C) for 20 min. The supernatant was transferred into a flask, and the extraction was repeated. Both supernatants were combined and evaporated under nitrogen flow. Finally, the residue was reconstituted with Milli-Q water (0.1% formic acid) up to 1.5 mL.

Tomato juices (10 mL) were centrifuged (4000 rpm at 4 °C) for 10 min. The supernatant was discarded, and 4 mL of 80% ethanol in Milli-Q water was added to 1 g pellets. They were sonicated for 5 min and centrifuged (4000 rpm at 4 °C) for 20 min following the procedure described above for the extraction of ketchups.

Solid-phase extraction (SPE) of these extracts was carried out following the procedure of other authors.^{14,15} First, 1 mL of methanol and subsequently 1 mL of sodium acetate 50 mmol/L pH 7 were loaded into Oasis MAX cartridges from Waters to equilibrate it; then, 1 mL of each extract was diluted with 1 mL of Milli-Q water and acidified with 34 μ L of 35% hydrochloric acid before being loaded into the cartridges separately. These were rinsed with sodium acetate 50 mmol/L pH 7 (5% methanol). The polyphenols were eluted with 1800 μ L of methanol (2% formic acid). The eluted fractions were evaporated under nitrogen flow, and the residue was reconstituted with water (0.1% formic acid) up to 250 μ L and filtered through a 13 mm, 0.45 μ m PTFE filter (Waters) into an insert-amber vial for HPLC analysis. Samples were stored at -20 °C until analysis.

Analysis of TPs. The content of TP was analyzed using the F-C method. All ketchup and tomato juice extracts (20 μ L of the eluted fractions) were mixed with 188 μ L of Milli-Q water in a thermo microtiter 96-well plate (Nunc, Roskilde, Denmark). Then, 12 μ L of F-C reagent and 30 μ L of sodium carbonate (200 g/L) were added following procedures described previously.^{16,17} The mixtures were incubated for 1 h at room temperature in darkness. After the reaction period, 50 μ L of Milli-Q water was added, and the absorbance was measured at 765 nm in a UV/vis Thermo Multiskan Spectrum spectrophotometer (Vantaa, Finland). Gallic acid was used as a calibration standard, and the TP content was expressed as mg of gallic acid equivalents (GAE)/100 mg fresh weight (FW).

Antioxidant Capacity of the Hydrophilic Fraction. The antioxidant capacity of the hydrophilic fraction in tomato juices and ketchups was measured by ABTS[•] and DPPH assays following the procedure described previously.^{18–20}

ABTS[•] Assay. A 1 mM concentration of Trolox (antioxidant standard) was prepared in methanol once a week. Working standards were prepared daily by diluting 1 mM Trolox with methanol. Solutions of known Trolox were used for calibration.

An ABTS[•] radical cation was prepared by passing a 5 mM aqueous stock solution of ABTS (in methanol) through manganese dioxide powder. Excess manganese dioxide was filtered through a 13 mm 0.45 μ m filter PTFE (Waters). Then, 245 μ L of ABTS[•] solution was added to 5 μ L of Trolox or to tomato samples, and the solutions were stirred for 30 s. The homogenate was shaken vigorously and kept in

darkness for 1 h. Absorption of the samples was measured on a UV/vis Thermo Multiskan Spectrum spectrophotometer at 734 nm, and methanol blanks were run in each assay. Results were expressed as mmol Trolox equivalent (TE)/100 g DM.

DPPH Assay. The antioxidant activity was also studied through the evaluation of the free radical-scavenging effect on DPPH radical. Solutions of known Trolox were used for calibration. Five microliters of tomato samples or Trolox was mixed with 250 μ L of methanolic DPPH (0.025 g/L). The homogenate was shaken vigorously and kept in darkness for 30 min. The absorption of the samples was measured on the spectrophotometer at 515 nm. Results were expressed as mmol TE/100 g DM.

HPLC-ESI-MS/MS Analysis. To evaluate the effect of storage on the content of flavonols, flavanones and hydroxycinnamic acids, polyphenols were quantified using HPLC-electrospray ionization tandem mass spectrometry (ESI-MS/MS) following the procedure of Vallverdú-Queralt et al.¹⁶ An API 3000 (PE Sciex, Concord, Ontario, Canada) triple quadrupole mass spectrometer equipped with a Turbo Ionspray source in negative-ion mode was used to obtain MS and MS/MS data.

For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound. In particular, we selected 12 transitions corresponding to ferulic acid-O-hexoside m/z 355 $\rightarrow$ 193; ferulic acid m/z 193 $\rightarrow$ 134; chlorogenic acid m/z 353 $\rightarrow$ 191; caffeic acid m/z 179 $\rightarrow$ 135; caffeic acid-O-hexoside m/z 341 $\rightarrow$ 179; quercetin m/z 301 $\rightarrow$ 151; rutin m/z 609 $\rightarrow$ 300; naringenin m/z 271 $\rightarrow$ 151; naringenin-7-O-glucoside m/z 433 $\rightarrow$ 271; kaempferol-3-O-rutinoside m/z 593 $\rightarrow$ 285; and ethyl gallate (internal standard) m/z 197 $\rightarrow$ 169.

Quantification of polyphenols was performed by the internal standard method. Polyphenols were quantified with respect to their corresponding standard. When standards were not available, as in the case of caffeic-O-hexoside and ferulic-O-hexoside acids, they were quantified with respect to the corresponding hydroxycinnamic acid (caffeic and ferulic acids).

The liquid chromatograph was an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, an autosampler, and a column oven set to 30 °C. Mobile phases consisted of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B). A Luna C₁₈ column 50 mm $\times$ 2.0 mm i.d., 5 μ m (Phenomenex, Torrance, CA), was used. The injection volume was 20 μ L, and the flow rate was 0.4 mL/min. Separation was carried out in 20 min under the following conditions: 0 min, 5% B; 16 min, 40% B; 17 min, 95% B; 19 min, 95% B; and 19.5 min, 5% B. The column was equilibrated for 5 min prior to each analysis.

Statistical Analysis. The significance of the results was analyzed using the Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, MD). Analysis of variance (ANOVA) was used to compare the means of groups of measurement data.

RESULTS AND DISCUSSION

Effect of Storage on the Total Phenolic Content and Antioxidant Capacity of the Hydrophilic Fraction. Generally, thermal processing of tomato-based products may lead to gains or losses in total phenol content.^{6,20} Thermal processing such as traditional pasteurization applied to ketchups and tomato juices might release more bound phenolic compounds in the breakdown of cellular constituents. However, cellular disruption can release oxidative enzymes that could destroy phenolic compounds. The total phenolic content observed in these samples is in the range of those reported in tomato and tomato products (between 100 and 500 mg/kg).^{9,21,22} As shown in Figure 1A,B, time of storage slightly affected the TP content as determined by F-C assay. Although there was a slight variation in the TP content

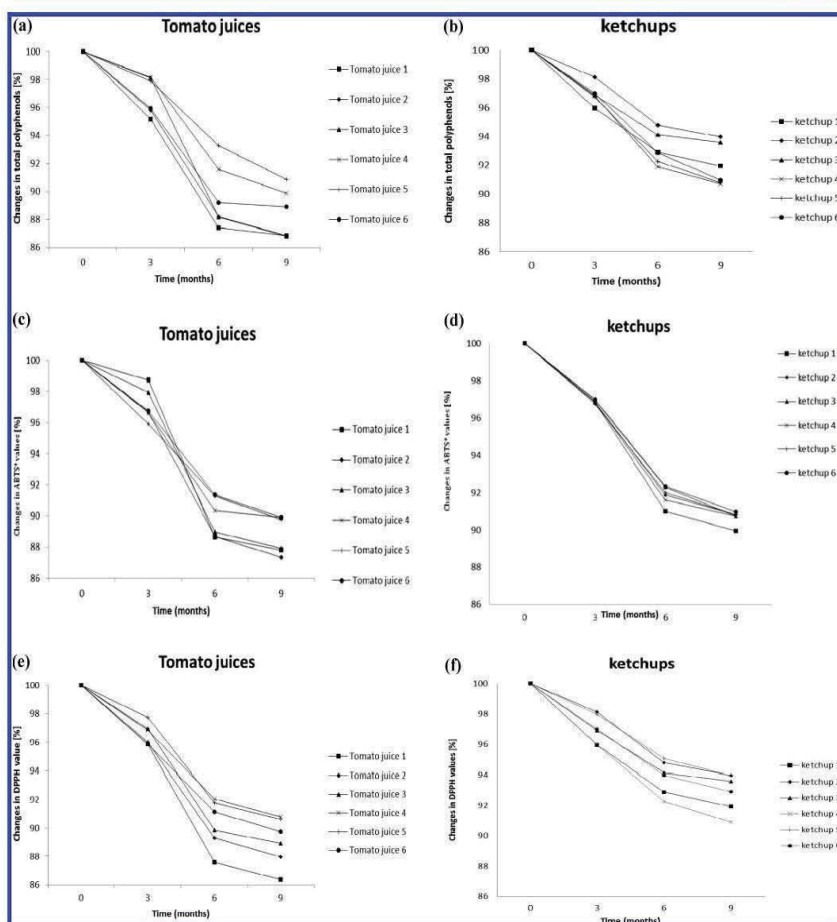


Figure 1. Changes in TPs in tomato juices (A) and ketchups (B). Changes in ABTS⁺ [%] in tomato juices (C) and ketchups (D). Changes in DPPH [%] in tomato juices (E) and ketchups (F).

between different brands of ketchups and tomato juices, the trends reflecting the effects of storage were similar. The polyphenol content decreased by about 12% for tomato juices and 7% for ketchups over the whole of the storage period. The most significant decrease was observed between 0 and 6 months of storage. These results are in line with those reported by Klimczak et al.²³ They found that after 6 months of storage of orange juices, there was approximately a 10–22% decline in TPs and vitamin C content. Other studies reported that phenolic compounds were stable during storage of tomato juice at 4 °C for 3 months⁹ and storage of tomato pulp at 4 and 20 °C for 3–5 months.²⁴

The antioxidant capacity of the hydrophilic fraction of tomato-based products was evaluated using the DPPH and ABTS⁺ assays.

Both methods are easy and accurate assays for measuring the antioxidant capacity of food. The relative contributions of individual compounds to the antioxidant capacity of the hydrophilic fraction (ABTS⁺ and DPPH) of tomato-based products are shown in Figure 1C–F. The ABTS⁺ and DPPH values decreased during storage. Results were similar to those reported for the TP content. It is widely known that ascorbic acid and phenolic compounds are the major contributors to the antioxidant capacity of the hydrophilic fraction. Therefore, polyphenol losses were paralleled by the decrease in hydrophilic antioxidant capacity. The most significant decrease was observed between 0 and 6 months of storage. For tomato juices, the antioxidant capacity of the hydrophilic fraction decreased between 10 and

Resultats

Table 1. Changes in the Content of Hydroxycinnamic Acids ($\mu\text{g/g}$ FW) in Ketchups and Tomato Juices ($n = 3$) during 9 Months of Storage^a

months of storage	caffeic acid	caffeic acid-O-hexoside	ferulic acid	ferulic acid-O-hexoside	chlorogenic acid
ketchup 1					
0	1.41 $\pm$ 0.04 a	3.87 $\pm$ 0.11 a	25.15 $\pm$ 1.21 a	29.24 $\pm$ 0.72 a	10.24 $\pm$ 0.14 a
3	1.33 $\pm$ 0.06 b	3.72 $\pm$ 0.23 b	24.97 $\pm$ 1.01 b	28.34 $\pm$ 1.34 b	9.98 $\pm$ 0.20 b
6	1.26 $\pm$ 0.05 c	3.61 $\pm$ 0.19 c	22.50 $\pm$ 1.44 c	27.50 $\pm$ 1.19 c	9.71 $\pm$ 0.39 c
9	1.22 $\pm$ 0.05 d	3.49 $\pm$ 0.22 d	22.30 $\pm$ 1.38 d	27.10 $\pm$ 1.04 d	9.32 $\pm$ 0.30 d
ketchup 2					
0	1.34 $\pm$ 0.05 a	3.79 $\pm$ 0.14 a	21.31 $\pm$ 1.50 a	28.08 $\pm$ 0.40 a	9.38 $\pm$ 0.09 a
3	1.30 $\pm$ 0.07 b	3.72 $\pm$ 0.26 b	20.85 $\pm$ 0.89 b	27.69 $\pm$ 1.33 b	9.15 $\pm$ 0.33 b
6	1.25 $\pm$ 0.06 c	3.58 $\pm$ 0.21 c	20.22 $\pm$ 1.25 c	26.81 $\pm$ 1.21 c	8.90 $\pm$ 0.34 c
9	1.19 $\pm$ 0.05 d	3.45 $\pm$ 0.18 d	19.20 $\pm$ 1.10 d	26.54 $\pm$ 1.09 d	8.50 $\pm$ 0.31 d
ketchup 3					
0	1.33 $\pm$ 0.05 a	3.52 $\pm$ 0.11 a	22.45 $\pm$ 1.02 a	28.32 $\pm$ 0.87 a	9.47 $\pm$ 0.15 a
3	1.29 $\pm$ 0.07 b	3.47 $\pm$ 0.17 b	21.71 $\pm$ 0.98 b	27.40 $\pm$ 0.99 b	9.10 $\pm$ 0.31 b
6	1.25 $\pm$ 0.06 c	3.38 $\pm$ 0.20 c	20.38 $\pm$ 1.05 c	26.30 $\pm$ 1.01 c	8.81 $\pm$ 0.25 c
9	1.15 $\pm$ 0.04 d	3.25 $\pm$ 0.18 d	20.11 $\pm$ 1.19 d	25.49 $\pm$ 1.22 d	8.60 $\pm$ 0.36 d
ketchup 4					
0	1.18 $\pm$ 0.04 a	2.20 $\pm$ 0.07 a	24.11 $\pm$ 1.15 a	15.59 $\pm$ 0.36 a	5.58 $\pm$ 0.10 a
3	1.11 $\pm$ 0.03 b	2.09 $\pm$ 0.12 b	23.90 $\pm$ 1.40 b	14.92 $\pm$ 0.45 b	5.41 $\pm$ 0.19 b
6	1.08 $\pm$ 0.03 c	2.06 $\pm$ 0.16 c	22.51 $\pm$ 1.03 c	14.54 $\pm$ 0.49 c	5.20 $\pm$ 0.24 c
9	1.02 $\pm$ 0.05 d	2.03 $\pm$ 0.18 d	22.01 $\pm$ 1.22 d	14.22 $\pm$ 0.56 d	5.09 $\pm$ 0.17 d
ketchup 5					
0	1.90 $\pm$ 0.05 a	3.92 $\pm$ 0.05 a	22.30 $\pm$ 1.09 a	25.91 $\pm$ 0.58 a	9.54 $\pm$ 0.12 a
3	1.81 $\pm$ 0.08 b	3.85 $\pm$ 0.19 b	21.15 $\pm$ 1.08 b	25.41 $\pm$ 1.15 b	9.15 $\pm$ 0.15 b
6	1.74 $\pm$ 0.09 c	3.77 $\pm$ 0.21 c	20.88 $\pm$ 1.01 c	24.41 $\pm$ 1.15 c	8.90 $\pm$ 0.19 c
9	1.65 $\pm$ 0.07 d	3.64 $\pm$ 0.13 d	20.50 $\pm$ 1.23 d	24.12 $\pm$ 1.10 d	8.69 $\pm$ 0.21 d
ketchup 6					
0	1.24 $\pm$ 0.05 a	2.93 $\pm$ 0.08 a	21.22 $\pm$ 1.22 a	19.23 $\pm$ 0.64 a	11.04 $\pm$ 0.12 a
3	1.18 $\pm$ 0.06 b	2.86 $\pm$ 0.14 b	20.91 $\pm$ 1.09 b	19.01 $\pm$ 0.99 b	10.76 $\pm$ 0.10 b
6	1.14 $\pm$ 0.07 c	2.74 $\pm$ 0.16 c	20.09 $\pm$ 1.33 c	18.25 $\pm$ 1.11 c	10.51 $\pm$ 0.33 c
9	1.09 $\pm$ 0.06 d	2.70 $\pm$ 0.10 d	19.41 $\pm$ 1.13 d	18.02 $\pm$ 1.28 d	10.01 $\pm$ 0.39 d
tomato juice 1					
0	2.38 $\pm$ 0.10 a	3.75 $\pm$ 0.12 a	20.65 $\pm$ 1.14 a	29.91 $\pm$ 0.96 a	9.36 $\pm$ 0.14 a
3	2.31 $\pm$ 0.13 b	3.69 $\pm$ 0.22 b	20.05 $\pm$ 1.21 b	28.49 $\pm$ 1.45 b	9.15 $\pm$ 0.41 b
6	2.20 $\pm$ 0.11 c	3.51 $\pm$ 0.19 c	19.21 $\pm$ 1.01 c	27.91 $\pm$ 1.34 c	8.50 $\pm$ 0.51 c
9	2.01 $\pm$ 0.09 d	3.44 $\pm$ 0.20 d	18.01 $\pm$ 1.10 d	27.21 $\pm$ 1.41 d	8.30 $\pm$ 0.47 d
tomato juice 2					
0	2.29 $\pm$ 0.07 a	2.16 $\pm$ 0.10 a	21.11 $\pm$ 1.20 a	20.76 $\pm$ 0.70 a	8.41 $\pm$ 0.29 a
3	2.23 $\pm$ 0.14 b	2.11 $\pm$ 0.15 b	20.65 $\pm$ 1.33 b	20.32 $\pm$ 1.21 b	8.22 $\pm$ 0.44 b
6	2.06 $\pm$ 0.16 c	2.07 $\pm$ 0.16 c	18.97 $\pm$ 1.26 c	19.71 $\pm$ 1.15 c	7.65 $\pm$ 0.51 c
9	1.91 $\pm$ 0.10 d	2.00 $\pm$ 0.13 d	18.51 $\pm$ 1.09 d	18.89 $\pm$ 1.26 d	7.40 $\pm$ 0.36 d
tomato juice 3					
0	1.46 $\pm$ 0.06 a	3.27 $\pm$ 0.11 a	23.46 $\pm$ 1.24 a	26.66 $\pm$ 1.08 a	9.43 $\pm$ 0.25 a
3	1.39 $\pm$ 0.13 b	3.11 $\pm$ 0.11 b	22.68 $\pm$ 1.13 b	25.80 $\pm$ 1.23 b	8.66 $\pm$ 0.39 b
6	1.28 $\pm$ 0.11 c	3.03 $\pm$ 0.21 c	21.11 $\pm$ 1.19 c	24.31 $\pm$ 1.16 c	8.37 $\pm$ 0.46 c
9	1.22 $\pm$ 0.09 d	2.96 $\pm$ 0.17 d	20.40 $\pm$ 1.25 d	24.10 $\pm$ 1.10 d	8.31 $\pm$ 0.41 d
tomato juice 4					
0	1.81 $\pm$ 0.07 a	2.56 $\pm$ 0.05 a	19.14 $\pm$ 1.22 a	25.24 $\pm$ 0.79 a	9.26 $\pm$ 0.18 a
3	1.77 $\pm$ 0.11 b	2.49 $\pm$ 0.14 b	18.05 $\pm$ 1.12 b	24.49 $\pm$ 1.01 b	9.15 $\pm$ 0.34 b
6	1.61 $\pm$ 0.14 c	2.35 $\pm$ 0.16 c	17.38 $\pm$ 1.10 c	23.91 $\pm$ 1.12 c	8.49 $\pm$ 0.46 c

Table 1. Continued

months of storage	caffeic acid	caffeic acid- <i>O</i> -hexoside	ferulic acid	ferulic acid- <i>O</i> -hexoside	chlorogenic acid
9	1.55 ± 0.11 d	2.31 ± 0.19 d	17.16 ± 1.09 d	23.11 ± 1.41 d	8.11 ± 0.36 d
tomato juice 5					
0	2.24 ± 0.05 a	2.83 ± 0.11 a	21.03 ± 1.36 a	19.23 ± 0.64 a	8.79 ± 0.35 a
3	2.19 ± 0.12 b	2.72 ± 0.09 b	20.34 ± 1.24 b	18.32 ± 0.99 b	8.22 ± 0.39 b
6	2.03 ± 0.09 c	2.68 ± 0.23 c	19.07 ± 1.09 c	17.99 ± 1.13 c	7.91 ± 0.44 c
9	1.92 ± 0.13 d	2.59 ± 0.25 d	18.87 ± 1.02 d	17.27 ± 1.08 d	7.65 ± 0.40 d
tomato juice 6					
0	2.30 ± 0.09 a	2.73 ± 0.12 a	22.11 ± 1.41 a	28.75 ± 0.87 a	10.35 ± 0.12 a
3	2.17 ± 0.14 b	2.53 ± 0.11 b	21.31 ± 1.29 b	27.80 ± 1.08 b	9.86 ± 0.19 b
6	2.01 ± 0.11 c	2.68 ± 0.15 c	19.66 ± 1.38 c	26.31 ± 1.12 c	9.15 ± 0.26 c
9	1.99 ± 0.08 c	2.52 ± 0.19 d	19.14 ± 1.12 d	26.10 ± 1.23 d	8.97 ± 0.33 d

^a Different letters in the columns represent statistically significant differences ($P < 0.05$).

12% after 9 months of storage. For ketchups, the changes were between 7 and 9%. These results are in line with those reported by Gliszczynska-Swiglo and Tyrakowska.²⁵ They found that storage of apple juice at room temperature results in a decrease in the TEAC value by about 6–14%.

Effect of Storage on Individual Polyphenols in Ketchups and Tomato Juices. Tables 1 and 2 show polyphenol compounds identified and quantified by HPLC-ESI-MS/MS. Identification was aided by making comparisons with reference standards and the MS² fragmentation patterns.¹³

Hydroxycinnamic acids were represented by ferulic and caffeic acids, ferulic acid-*O*-hexoside, and caffeic acid-*O*-hexoside. Hydroxycinnamic acids have become a subject of much interest because they make up a significant proportion of the total phenolics ingested in a normal diet and are readily absorbed in the digestive tract.²¹ Although there was a variation between tomato-based products in the content of hydroxycinnamic acids, the trends reflecting the effects of storage were similar.

The content of hydroxycinnamic acids decreased after 9 months of storage at 4 °C. The greatest decrease ($P < 0.05$) occurred in ferulic and caffeic acids in comparison to their glycosidic forms. In ketchups, ferulic and caffeic acids decreased by 9.49 and 12.84%, respectively; in tomato juices, they decreased by 12.03 and 15.18%. In the case of caffeic-*O*-hexoside and ferulic-*O*-hexoside, the 3-hydroxy-function at the C ring of the flavonoid is blocked by a sugar moiety, whereas in the case of caffeic and ferulic acids, it remains unoccupied. Thus, the blockage of the 3-hydroxyl group is perhaps one of the reasons for the greater stability of glycosidic forms toward their aglycones.²⁶

Chlorogenic acid was also present in tomato-based products. Chlorogenic acid decreased by 9.10 and 12.32% for ketchups and tomato juices, respectively. Again, ketchups had a greater stability during storage than tomato juices. Chlorogenic acid may be oxidized to reactive *O*-quinones through the catalytic oxidation process. As mentioned by Odriozola et al.,²⁷ tomato-based products underwent a substantial loss of chlorogenic acid from 4.4 to 3.5–3.8 mg/100 mL FW after 56 days of storage at 4 °C. Peroxidase may be involved in the oxidative degradation of phenolic compounds. Therefore, the degradation of phenolic compounds during storage might be associated with the residual activity of peroxidase.

Tomato and tomato-based products have been found to be rich sources of flavonoids, with rutin and kaempferol as the main flavonols.¹⁶ As Table 2 shows, the initial concentrations of rutin

in the studied ketchups were 45.28–64.00 and 42.50–63.33 μg/g FW for tomato juices, whereas kaempferol-3-*O*-rutinoside was found at concentrations of 15.92–24.72 μg/g FW for tomato juices and 16.34–29.88 μg/g FW for ketchups. Rutin decreased by 7.20–10.61% in ketchups, whereas in tomato juices it decreased by 10.28–12.19%. These results are in line with those described previously.²⁸ During storage, peroxidase may be involved in the oxidative degradation of phenolic compounds that are able to interact with reactive oxygen species, transferring one or two hydrogen atoms, forming quinonoid structures, which leads to the formation of quinones.²⁹ In the case of rutin, the 3-hydroxy-function at the C ring of the flavonoid is blocked by a sugar moiety, whereas for quercetin it remains unoccupied. Quercetin decreased significantly throughout the storage of tomato-based products, thus reaching values of 0.21–0.51 μg/g FW for ketchups and 0.82–2.15 μg/g FW for tomato juices. Quercetin is a phenolic compound with potent antioxidant properties and, therefore, more easily oxidized. The blockage of the 3-hydroxyl group is perhaps one of the reasons for the greater stability of rutin toward oxidation.³⁶ Similarly, for kaempferol-3-*O*-rutinoside, the 3-hydroxy function is blocked. This explains the thermal stability of kaempferol-3-*O*-rutinoside toward quercetin. In contrast, other studies reported that glycosylation of the hydroxy substituent on C-3 decreases antioxidant activity.³⁰ The content of kaempferol in vegetables has not been extensively studied and there are few data about its occurrence in tomatoes. We found higher levels (between 15.92 and 29.88 μg/g FW) than those reported by Martínez-Valverde et al.²¹ who reported values of kaempferol between 1.16 and 2.07 μg/g FW.

A slight decrease in naringenin and naringenin-7-*O*-glucoside was observed. Table 2 shows the changes in flavanone concentrations during storage. The small changes in individual and total flavanone content could be explained by the high stability of these compounds. The naringenin content found in ketchups and tomato juices was higher than that reported by Martínez-Valverde et al.,²¹ which was 4.50 μg/g FW for raw tomatoes cv. Remate and 12.55 μg/g FW for cv. Daniella, whereas Wu M and Burrell RC³¹ found naringenin levels of 40, 95, and 142.5 μg/g FW for the Haubners Vollendung, Ponderosa, and Sunny Ray varieties, respectively. The initial concentrations of naringenin in the studied ketchups were between 34.97 and 54.96 μg/g FW and between 35.97 and 52.96 μg/g FW for tomato juices, whereas naringenin-7-*O*-glucoside was found at concentrations between 1.25 and 3.27 μg/g FW for tomato juices and between 2.22 and 3.41 μg/g FW for ketchups.

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Table 2. Changes in the Content of Flavonols and Flavanones ($\mu\text{g/g FW}$) in Ketchups and Tomato Juices ($n = 3$) during 9 Months of Storage^a

months of storage	rutin	quercetin	kaempferol-3-O-rutinoside	naringenin	naringenin-7-O-glucoside
ketchup 1					
0	64.00 $\pm$ 1.04 a	0.56 $\pm$ 0.02 a	29.37 $\pm$ 0.82 a	44.30 $\pm$ 1.10 a	3.41 $\pm$ 0.08 a
3	62.31 $\pm$ 3.22 b	0.51 $\pm$ 0.04 b	28.91 $\pm$ 1.99 b	42.81 $\pm$ 2.50 b	3.35 $\pm$ 0.20 b
6	59.97 $\pm$ 2.56 c	0.47 $\pm$ 0.03 c	27.99 $\pm$ 1.54 c	41.10 $\pm$ 2.23 c	3.21 $\pm$ 0.11 c
9	57.21 $\pm$ 2.71 d	0.43 $\pm$ 0.01 d	26.71 $\pm$ 1.63 d	39.50 $\pm$ 2.25 d	3.10 $\pm$ 0.09 d
ketchup 2					
0	54.50 $\pm$ 1.59 a	0.61 $\pm$ 0.01 a	29.88 $\pm$ 1.28 a	54.96 $\pm$ 1.24 a	3.34 $\pm$ 0.13 a
3	53.09 $\pm$ 3.21 b	0.57 $\pm$ 0.02 b	29.21 $\pm$ 1.22 b	53.40 $\pm$ 1.99 b	3.29 $\pm$ 0.09 b
6	51.45 $\pm$ 2.44 c	0.52 $\pm$ 0.02 c	28.51 $\pm$ 1.43 c	51.50 $\pm$ 2.40 c	3.22 $\pm$ 0.08 c
9	49.01 $\pm$ 2.88 d	0.47 $\pm$ 0.03 d	27.11 $\pm$ 1.31 d	49.81 $\pm$ 2.33 d	3.10 $\pm$ 0.12 d
ketchup 3					
0	60.24 $\pm$ 1.33 a	0.67 $\pm$ 0.02 a	29.25 $\pm$ 0.76 a	40.13 $\pm$ 1.25 a	3.23 $\pm$ 0.13 a
3	58.01 $\pm$ 1.41 b	0.63 $\pm$ 0.04 b	28.88 $\pm$ 1.99 b	38.94 $\pm$ 1.56 b	3.17 $\pm$ 0.14 b
6	56.40 $\pm$ 3.00 c	0.57 $\pm$ 0.03 c	27.91 $\pm$ 1.23 c	37.33 $\pm$ 1.99 c	3.08 $\pm$ 0.16 c
9	54.23 $\pm$ 2.41 d	0.51 $\pm$ 0.01 d	26.60 $\pm$ 1.55 d	36.31 $\pm$ 2.31 d	2.97 $\pm$ 0.10 d
ketchup 4					
0	55.84 $\pm$ 1.24 a	0.26 $\pm$ 0.01 a	16.34 $\pm$ 0.52 a	34.97 $\pm$ 1.43 a	2.22 $\pm$ 0.07 a
3	54.61 $\pm$ 2.20 b	0.24 $\pm$ 0.02 b	15.49 $\pm$ 0.33 b	33.55 $\pm$ 1.94 b	2.20 $\pm$ 0.09 b
6	52.20 $\pm$ 2.12 c	0.22 $\pm$ 0.01 c	15.09 $\pm$ 0.44 c	32.95 $\pm$ 1.67 c	2.11 $\pm$ 0.12 c
9	51.00 $\pm$ 1.29 d	0.21 $\pm$ 0.01 d	14.98 $\pm$ 0.31 d	32.01 $\pm$ 1.88 d	2.07 $\pm$ 0.14 d
ketchup 5					
0	49.58 $\pm$ 1.03 a	0.61 $\pm$ 0.01 a	29.08 $\pm$ 0.99 a	45.66 $\pm$ 1.48 a	2.39 $\pm$ 0.09 a
3	49.01 $\pm$ 1.89 b	0.57 $\pm$ 0.03 b	28.50 $\pm$ 1.33 b	45.00 $\pm$ 1.99 b	2.32 $\pm$ 0.12 b
6	47.11 $\pm$ 2.15 c	0.52 $\pm$ 0.04 c	27.88 $\pm$ 1.12 c	44.01 $\pm$ 1.85 c	2.30 $\pm$ 0.08 c
9	46.01 $\pm$ 2.99 d	0.49 $\pm$ 0.03 d	27.09 $\pm$ 1.09 d	43.21 $\pm$ 1.88 d	2.27 $\pm$ 0.09 d
ketchup 6					
0	45.28 $\pm$ 1.21 a	0.63 $\pm$ 0.02 a	21.31 $\pm$ 0.78 a	37.74 $\pm$ 3.23 a	2.70 $\pm$ 0.08 a
3	44.99 $\pm$ 1.99 b	0.60 $\pm$ 0.03 b	21.01 $\pm$ 1.96 b	37.01 $\pm$ 2.33 b	2.61 $\pm$ 0.14 b
6	43.20 $\pm$ 2.01 c	0.56 $\pm$ 0.02 c	20.35 $\pm$ 1.81 c	36.11 $\pm$ 2.41 c	2.56 $\pm$ 0.13 c
9	41.51 $\pm$ 2.67 d	0.51 $\pm$ 0.03 d	19.67 $\pm$ 1.72 d	35.01 $\pm$ 2.20 d	2.52 $\pm$ 0.16 d
tomato juice 1					
0	62.84 $\pm$ 2.13 a	1.58 $\pm$ 0.04 a	18.54 $\pm$ 0.69 a	45.47 $\pm$ 1.66 a	1.34 $\pm$ 0.05 a
3	60.41 $\pm$ 3.01 b	1.48 $\pm$ 0.09 b	18.15 $\pm$ 0.88 b	43.00 $\pm$ 2.33 b	1.29 $\pm$ 0.09 b
6	58.97 $\pm$ 3.15 c	1.39 $\pm$ 0.07 c	17.54 $\pm$ 0.76 c	41.51 $\pm$ 2.11 c	1.26 $\pm$ 0.08 c
9	56.02 $\pm$ 2.19 d	1.26 $\pm$ 0.06 d	16.99 $\pm$ 0.59 d	40.46 $\pm$ 2.99 d	1.22 $\pm$ 0.05 d
tomato juice 2					
0	42.50 $\pm$ 1.85 a	1.29 $\pm$ 0.05 a	18.51 $\pm$ 0.82 a	36.14 $\pm$ 0.99 a	1.25 $\pm$ 0.04 a
3	40.98 $\pm$ 1.30 b	1.21 $\pm$ 0.04 b	18.14 $\pm$ 0.97 b	33.20 $\pm$ 1.60 b	1.22 $\pm$ 0.07 b
6	39.51 $\pm$ 2.09 c	1.15 $\pm$ 0.07 c	17.50 $\pm$ 0.91 c	32.40 $\pm$ 1.78 c	1.18 $\pm$ 0.06 c
9	38.11 $\pm$ 2.21 d	1.07 $\pm$ 0.04 d	17.40 $\pm$ 0.85 d	32.10 $\pm$ 2.34 d	1.13 $\pm$ 0.05 d
tomato juice 3					
0	61.74 $\pm$ 1.93 a	1.67 $\pm$ 0.01 a	15.92 $\pm$ 0.41 a	35.97 $\pm$ 1.26 a	2.41 $\pm$ 0.07 a
3	59.87 $\pm$ 3.01 b	1.59 $\pm$ 0.03 b	15.31 $\pm$ 1.01 b	34.45 $\pm$ 1.99 b	2.31 $\pm$ 0.08 b
6	56.32 $\pm$ 2.56 c	1.43 $\pm$ 0.02 c	14.98 $\pm$ 0.99 c	33.90 $\pm$ 2.20 c	2.29 $\pm$ 0.06 c
9	55.20 $\pm$ 2.99 d	1.28 $\pm$ 0.05 d	14.52 $\pm$ 0.73 d	32.42 $\pm$ 2.00 d	2.23 $\pm$ 0.08 c
tomato juice 4					
0	57.58 $\pm$ 2.68 a	2.63 $\pm$ 0.04 a	16.47 $\pm$ 0.40 a	48.83 $\pm$ 1.52 a	3.27 $\pm$ 0.11 a
3	55.65 $\pm$ 2.09 b	2.46 $\pm$ 0.03 b	16.11 $\pm$ 0.55 b	46.00 $\pm$ 1.60 b	3.12 $\pm$ 0.10 b
6	53.07 $\pm$ 2.87 c	2.27 $\pm$ 0.06 c	15.37 $\pm$ 0.60 c	45.51 $\pm$ 2.01 c	3.09 $\pm$ 0.09 c

Table 2. Continued

months of storage	rutin	quercetin	kaempferol-3-O-rutinoside	naringenin	naringenin-7-O-glucoside
9	51.03 ± 2.99 d	2.15 ± 0.06 d	15.09 ± 0.72 d	43.56 ± 1.50 d	2.95 ± 0.08 d
tomato juice 5					
0	50.28 ± 2.39 a	1.05 ± 0.03 a	20.97 ± 0.85 a	38.24 ± 1.04 a	2.26 ± 0.06 a
3	48.78 ± 2.12 b	0.97 ± 0.04 b	20.01 ± 0.80 b	37.11 ± 1.23 b	2.19 ± 0.05 b
6	46.20 ± 2.09 c	0.86 ± 0.02 c	19.33 ± 0.76 c	36.89 ± 1.26 c	2.11 ± 0.07 c
9	45.11 ± 1.85 d	0.81 ± 0.01 d	19.15 ± 0.82 d	34.68 ± 1.94 d	2.06 ± 0.06 d
tomato juice 6					
0	63.33 ± 2.43 a	1.59 ± 0.04 a	24.72 ± 0.26 a	52.96 ± 2.23 a	2.34 ± 0.08 a
3	59.66 ± 2.01 b	1.33 ± 0.03 b	23.30 ± 0.85 b	49.55 ± 1.65 b	2.19 ± 0.09 b
6	57.21 ± 2.48 c	1.29 ± 0.02 c	22.50 ± 0.96 c	48.12 ± 1.87 c	2.15 ± 0.11 c
9	55.61 ± 2.66 d	1.22 ± 0.05 d	22.31 ± 0.68 d	47.11 ± 2.00 d	2.11 ± 0.15 d

^a Different letters in the columns represent statistically significant differences ($P < 0.05$).

Naringenin slightly decreased throughout the storage of tomato-based products. The naringenin content in ketchups decreased by 5.37–10.84% and in tomato juices by 9.31–12.18%. For naringenin-7-O-glucoside, the trends reflecting the effects of storage were similar to naringenin.

To our knowledge, this is the first time a study has been carried out to evaluate the stability of flavonols, flavanones, and hydroxycinnamic acids in ketchups and tomato juices during storage. Storage of tomato juices and ketchups results in a slight decrease in their polyphenol content and also in the hydrophilic antioxidant capacity, but the levels achieved would not represent a nutritional drawback. This information is of interest to consumers and nutritionists: They can expect a beneficial effect from the consumption of tomato juices and ketchups throughout storage.

The main degradation was in quercetin oxidation, whereas in the case of rutin, the 3-hydroxy function at the C ring of the flavonoid is blocked by a sugar moiety. An important fact to note is that during the storage of individual polyphenols, the glycosides of flavonols and hydroxycinnamic acids are more stable to oxidation than aglycones, probably due to the blockage of the 3-hydroxyl group at the C ring.

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11. Capítol XI: Estabilitat del compostos fenòlics i carotens dels gaspatxos durant l'emmagatzematge

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Resum: En aquesta publicació es pretén estudiar els efectes de l'emmagatzematge de gaspatxos en la capacitat antioxidant lipofílica i hidrofílica i en el nivell de carotens i polifenols individuals. Aquest estudi es va dur a terme durant l'any 2010. Fins ara, no existia cap estudi que, durant 3 mesos, avalués els efectes de l'emmagatzematge en gaspatxos. S'hi observa que, durant aquest període, el nivell de polifenols disminueix, sobretot les aglicones en comparació amb els polifenols glicosilats. Pel que fa als carotens els isòmers *trans* disminueixen més acusadament que els *cis*. El 5-*cis*-licopè, fins i tot, augmenta lleugerament durant l'emmagatzematge, la qual cosa es pot atribuir a la *cis/trans* isomerització que té lloc durant els tractaments tèrmics per a la producció de derivats de tomàquet. Les capacitats antioxidants hidrofíliques i lipofíliques també disminueixen lleugerament durant els 3 mesos d'emmagatzematge. No obstant això, aquestes disminucions no comporten un perjudici per al consumidor.

Stability of the Phenolic and Carotenoid Profile of Gazpachos during Storage

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ABSTRACT: Gazpacho is a ready-to-use vegetable soup containing tomato, cucumber, pepper, olive oil, and other minor constituents such as onion, garlic, wine vinegar, sea salt, and water. In this work, changes in individual phenolic and carotenoid compounds of commercial gazpachos, as well as in their hydrophilic and lipophilic antioxidant capacities (measured through ABTS[•] and DPPH radicals), were assessed for 3 months at 4 °C. The storage of gazpachos at 4 °C for 3 months results in a slight decrease in their polyphenol and carotenoid content and also in the hydrophilic and lipophilic antioxidant capacities, but the levels achieved could not be construed as a nutritional drawback. The main degradation was quercetin oxidation because the hydroxy function at the C-ring of the flavonoid is not blocked by a sugar moiety as it is in the case of rutin and kaempferol-3-O-rutinoside and glycosylated caffeic and ferulic acids. Lycopene underwent significant losses throughout storage as 11 conjugated double bonds are present in its structure and should be more reactive than *trans*-lutein and *trans*- β -carotene. *cis*-Lycopene isomers slightly decreased. However, *5-cis*-lycopene underwent a slight increase. This phenomenon could be explained by *cis*-isomerization increasing the proportion of *cis*-isomers.

KEYWORDS: gazpachos, stability, carotenoids, polyphenols, antioxidant capacity, storage

INTRODUCTION

The popularity of gazpachos, a ready-to-use vegetable soup, is rapidly becoming more widespread partly due to the fact that a high consumption of fruits and vegetables has been associated with the prevention of cancer, cardiovascular illnesses, and several degenerative chronic diseases.^{1,2} These beneficial properties of fruits and vegetables have been attributed partially to their content of functional compounds, such as carotenoids and phenolic compounds.³ Tomatoes and tomato-based products such as gazpachos are of great interest because of their high content of carotenoids. Some of these compounds, such as β -carotene and *trans*- α -carotene, may exhibit provitamin A activity.⁴ Studies have proven that the consumption of lycopene decreases the risk of degenerative diseases, such as certain kinds of cancers and cardiovascular diseases.⁵

In addition, tomato-based products are a good source of phenolic compounds, which contribute substantially to their antioxidant potential.⁶ Several studies have demonstrated that a diet rich in phenolic compounds correlates with a reduced risk of coronary heart disease. Tomatoes possess antioxidative, anti-inflammatory, antimutagenic, and anticarcinogenic properties and the capacity to modulate some key cellular enzyme functions, thus being ascribed to many phenolic compounds.^{7,8}

Tomato-based products may potentially contain the same range of phenolic compounds and carotenoids as the tomatoes from which they are derived; however, due to differences among varieties of tomatoes and their origin,^{9,10} polyphenol and carotenoid contents could vary considerably.

Storage conditions and thermal treatments affect the quality of tomato products. In particular, thermal treatments are known to be the main cause of the depletion of natural antioxidants. Browning and oxidation reactions are the main causes of degradation of naturally occurring antioxidants during the storage of tomato products.^{11,12}

Because of the important health-protecting role of polyphenols and carotenoids, the consumption of tomatoes and tomato-based products could be seen as a nutritional indicator of good dietary habits and a healthy lifestyle. As gazpachos typically have a commercial shelf life of 3 months, as provided by commercial labels, studies are necessary to determine their nutritional quality during storage. However, as far as we know, no information is currently available on how the phenolic and carotenoid compounds of gazpachos are influenced by storage time. Moreover, there is no information available concerning changes in flavonols, flavanones, hydroxycinnamic acids, and individual carotenoids of gazpachos during storage.

We carried out a pilot study to evaluate the stability of antioxidant compounds present in commercially available gazpachos during 3 months of storage. Therefore, hydrophilic and lipophilic antioxidant capacities and the content of

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flavonols, flavanones, hydroxycinnamic acids, and carotenoids were analyzed.

MATERIALS AND METHODS

Standards and Reagents. All samples and standards were handled without exposure to light. Caffeic and chlorogenic acids, rutin and quercetin, *trans*- β -carotene, β -apo-8'-carotenal, *trans*- α -carotene, *trans*-lycopene, and *trans*-lutein, methyl *tert*-butyl ether (MTBE), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ($\pm$)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%), manganese dioxide, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma (Madrid, Spain); naringenin, naringenin-7-O-glucoside, kaempferol-3-O-rutinoside, and hexane were from Extrasynthèse (Genay, France). Ethanol, methanol, *p*-phenylenediamine, hydrogen peroxidase, monobasic and dibasic sodium phosphate, and formic acid of HPLC grade were obtained from Scharlau (Barcelona, Spain), and ultrapure water (Milli-Q) was from Millipore (Bedford, MA).

Commercial Samples. In this study, a 3 month storage trial was designed to investigate the stability of the main antioxidant compounds of six commercially available gazpachos. All of the studied brands contained the same ingredients (tomato, cucumber, pepper, olive oil, and other minor constituents such as onion, garlic, wine vinegar, sea salt, and water), but in some cases the amount of each ingredient was not known. The processing date was March 2010. Each brand of gazpacho was analyzed immediately after purchase (month 0). Three cartons of each brand of gazpacho were stored for 4, 8, and 12 weeks. Gazpachos were kept in Tetra Paks at a storage temperature of 4.0 ± 0.1 °C. All products analyzed were available in Barcelona markets.

Extraction of Phenolic Compounds from Gazpachos. Samples were treated in triplicate in a darkened room with a red safety light to avoid oxidation of the analytes, following a procedure found in the literature¹³ with some modifications.

Lyophilized gazpachos (0.5 g) were weighed and homogenized with 80% ethanol in Milli-Q water (4 mL); they were then sonicated for 5 min and centrifuged (4000 rpm at 4 °C) for 20 min. The supernatant was transferred into a flask, and extraction was repeated. The supernatants were combined and evaporated under nitrogen flow; finally, the residue was reconstituted with Milli-Q water (0.1% of formic acid) up to 1.5 mL and filtered through a 13 mm, 0.45 μ m PTFE filter (Waters).

Extraction of Carotenoid Compounds from Gazpachos. There is a wide range of carotenoids present in vegetables and fruits. Therefore, the identification of these compounds in the food matrix is a difficult task. To avoid exposure to light, oxygen, and high temperatures and to pro-oxidant metals such as iron or copper, the extractions of carotenoids were carried out quickly in darkness and using dry ice to minimize autooxidation and *cis-trans* isomerization.

Lyophilized gazpachos (0.5 g) were weighed and homogenized with 5 mL of EtOH/hexane (4:3, v/v) following a procedure described in the literature;¹⁴ they were then sonicated for 5 min and centrifuged (4000 rpm at 4 °C) for 15 min. The supernatant was transferred into a flask, and extraction was repeated. The supernatants were combined and evaporated under nitrogen flow; finally, the residue was reconstituted with MTBE to 1 mL and filtered through a 25 mm, 0.45 μ m PTFE filter (Waters).

Antioxidant Capacity. The hydrophilic and lipophilic antioxidant capacities in gazpachos were measured by ABTS¹⁵ and DPPH assays following the procedures described in the literature.^{15,16}

Peroxidase (POD) Activity Measurement. POD activity of gazpachos was measured using the method described by Aguiló-Aguayo et al.¹² Enzyme extracts were obtained by homogenization of 10 mL of gazpacho with 20 mL of 0.2 mol L⁻¹ sodium phosphate buffer (pH 6.5). Then, the homogenate was centrifuged (4000 rpm, 15 min) at 4 °C. The supernatant was filtered through a Whatman no. 1 paper and the resulting liquid constituted the enzymatic extract, which was immediately used for the POD activity determination. POD activity was assayed spectrophotometrically by placing 260 μ L of 0.050

mol L⁻¹ sodium phosphate buffer (pH 6.5), 20 μ L of *p*-phenylenediamine (10 g kg⁻¹) as H-donor, 10 μ L of hydrogen peroxidase (15 g kg⁻¹) as oxidant, and 10 μ L of enzymatic extract in a 96-well plate. The oxidation of *p*-phenylenediamine was measured at 509 nm and 25 °C using a UV-vis Thermo Multiskan Spectrum spectrophotometer (Vantaa, Finland). POD activity was determined by measuring the initial rate of the reaction, which was computed from the linear portion of the plotted curve. One unit of POD activity was defined as a change in absorbance at 509 nm min⁻¹ mL⁻¹ of enzymatic extract. The percentage of residual POD activity (RA) was defined as indicated by eq 1

$$RA = 100 \times \frac{A_t}{A_0} \quad (1)$$

where A_t is the enzyme activity of gazpachos from week 0, 4, 8, or 12 and A_0 is the enzyme activity of gazpachos from week 0.

Analysis of Polyphenols in Gazpachos. HPLC-ESI-MS/MS was used to evaluate the effect of time on the content of flavonols, flavanones, and hydroxycinnamic acids.¹³ An API 3000 (PE Sciex, Concord, ON, Canada) triple-quadrupole mass spectrometer equipped with a Turbo Ionspray source in negative-ion mode was used to obtain MS/MS data. Turbo Ionspray source settings were as follows: capillary voltage, -3500 V; nebulizer gas (N₂), 10 au (arbitrary units); curtain gas (N₂), 12 au; collision gas (N₂), 4 au; focusing potential, -200 V; entrance potential, -10 V; drying gas (N₂), heated to 400 °C and introduced at a flow rate of 8000 cm³ min⁻¹. The declustering potential and collision energy were optimized for each compound in infusion experiments: individual standard solutions (10 μ g mL⁻¹) dissolved in 50:50 (v/v) mobile phase were infused at a constant flow rate of 5 μ L min⁻¹ using a model syringe pump (Harvard Apparatus, Holliston, MA). Full-scan data acquisition was performed scanning from m/z 100 to 800 using a cycle time of 2 s with a step size of 0.1 unit and a pause between each scan of 2 ms. To confirm the identity of some compounds, neutral loss scan and precursor ion scan experiments were carried out.¹⁷

For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific to each compound. Quantification of polyphenols was performed by the internal standard method. Polyphenols were quantified with respect to their corresponding standard. When standards were not available, as in the case of caffeoyl-hexoside and feruloyl-hexoside acids, they were quantified with respect to the corresponding hydroxycinnamic acid (caffeic and ferulic acids).

The liquid chromatograph was an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, an autosampler, and a column oven set to 30 °C. Mobile phases consisted of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B). The injection volume was 20 μ L, and the flow rate was 0.4 mL min⁻¹. Separation was carried out in 20 min under the following conditions: 0 min, 5% B; 16 min, 40% B; 17 min, 95% B; 19 min, 95% B; 19.5 min, 5% B. The column was equilibrated for 5 min prior to each analysis.

Analysis of Carotenoids in Gazpachos. Chromatographic analysis was performed using the HPLC system described for polyphenol analysis. The analytes were separated on a C₃₀ column, 250 $\times$ 4.6 mm i.d., 5 μ m (YMC, Waters Co., Milford, MA), and kept at 20 °C. The injection volume was 20 μ L and the flow rate, 1 mL min⁻¹. The mobile phase consisted of two different solvent mixtures: A, water/methanol/MTBE (4:26:70, v/v), and B, the same solvents but in the range 4:90:6 (v/v). The linear gradient was from 26 to 90% B in 23 min. The column was equilibrated for 10 min prior to each analysis. The modification procedure followed the protocol previously published.¹⁸ MTBE was used as a modifier to facilitate the elution of lycopene, which is strongly retained in a methanol environment.

Diode Array Detector. Commercially available carotenoid standards (*trans*-lutein, *trans*- α -carotene, *trans*- β -carotene, and *trans*-lycopene) were used to identify analytes by retention times and UV-vis spectra. The LC-DAD chromatograms were acquired by selecting the 450 nm

Table 1. Changes in the Hydrophilic and Lipophilic Antioxidant Capacities and Relative Residual POD Activity in Gazpachos ($n = 3$) during 12 Weeks of Storage^a

mmol TE 100 g ⁻¹ FW					
weeks of storage	ABTS [•] hydrophilic	DPPH hydrophilic	ABTS [•] lipophilic	DPPH lipophilic	relative residual POD activity (%)
Gazpacho 1					
0	3.38 ± 0.04 a	4.68 ± 0.11 a	1.01 ± 0.01 a	1.19 ± 0.10 a	100 a
4	3.27 ± 0.07 b	4.55 ± 0.26 b	0.97 ± 0.08 b	1.12 ± 0.04 b	90.25 ± 4.08 b
8	3.13 ± 0.04 c	4.36 ± 0.23 c	0.93 ± 0.05 c	1.08 ± 0.02 c	85.11 ± 4.05 c
12	3.09 ± 0.02 d	4.29 ± 0.28 d	0.92 ± 0.03 c	1.05 ± 0.04 d	82.15 ± 3.97 d
Gazpacho 2					
0	3.20 ± 0.05 a	4.23 ± 0.14 a	1.29 ± 0.03 a	1.36 ± 0.04 a	100 a
4	3.13 ± 0.05 b	4.12 ± 0.32 b	1.23 ± 0.06 b	1.32 ± 0.05 b	90.51 ± 3.18 b
8	3.05 ± 0.08 c	4.02 ± 0.28 c	1.19 ± 0.03 c	1.28 ± 0.05 c	88.16 ± 4.07 c
12	3.02 ± 0.03 d	3.93 ± 0.16 d	1.17 ± 0.09 c	1.26 ± 0.09 c	84.46 ± 4.12 d
Gazpacho 3					
0	3.95 ± 0.05 a	4.82 ± 0.11 a	1.07 ± 0.02 a	1.30 ± 0.07 a	100 a
4	3.84 ± 0.05 b	4.69 ± 0.22 b	1.02 ± 0.02 b	1.27 ± 0.08 b	93.51 ± 3.11 b
8	3.70 ± 0.04 c	4.50 ± 0.28 c	0.99 ± 0.05 c	1.25 ± 0.06 c	86.10 ± 4.18 c
12	3.63 ± 0.06 d	4.41 ± 0.10 d	0.97 ± 0.09 c	1.24 ± 0.03 c	83.00 ± 4.03 d
Gazpacho 4					
0	3.41 ± 0.04 a	4.69 ± 0.07 a	1.16 ± 0.05 a	1.22 ± 0.06 a	100 a
4	3.30 ± 0.05 b	4.54 ± 0.18 b	1.12 ± 0.03 b	1.20 ± 0.05 b	91.10 ± 4.09 b
8	3.15 ± 0.07 c	4.41 ± 0.20 c	1.09 ± 0.02 c	1.16 ± 0.04 c	87.43 ± 3.91 c
12	3.09 ± 0.03 d	4.36 ± 0.25 d	1.08 ± 0.03 c	1.14 ± 0.06 c	83.00 ± 3.87 d
Gazpacho 5					
0	4.11 ± 0.05 a	5.00 ± 0.05 a	1.02 ± 0.09 a	1.24 ± 0.05 a	100 a
4	4.03 ± 0.06 b	4.85 ± 0.25 b	0.99 ± 0.08 b	1.22 ± 0.08 b	90.10 ± 4.15 b
8	3.95 ± 0.07 c	4.76 ± 0.30 c	0.97 ± 0.07 c	1.15 ± 0.07 c	83.56 ± 4.02 c
12	3.91 ± 0.09 d	4.65 ± 0.19 d	0.95 ± 0.05 c	1.14 ± 0.03 c	80.01 ± 4.06 d
Gazpacho 6					
0	3.07 ± 0.05 a	4.29 ± 0.08 a	1.25 ± 0.02 a	1.34 ± 0.06 a	100 a
4	2.98 ± 0.08 b	4.16 ± 0.18 b	1.21 ± 0.05 b	1.30 ± 0.04 b	91.70 ± 4.09 b
8	2.89 ± 0.05 c	4.09 ± 0.20 c	1.18 ± 0.04 c	1.25 ± 0.07 c	87.50 ± 3.89 c
12	2.84 ± 0.09 d	4.01 ± 0.06 d	1.17 ± 0.03 c	1.23 ± 0.02 c	84.18 ± 3.73 d

^aLetters in the columns represent statistically significant differences ($P < 0.05$).

wavelength; afterward, the UV-vis spectra were recorded in the range of 350–550 nm for the tentative identification of carotenoids and their geometrical isomers (*cis*-lycopene isomers), on the basis of the retention times and absorption spectrum characteristics described in the literature.^{18,19}

Mass Spectrometry. The API 3000 (PE Sciex) triple-quadrupole mass spectrometer in positive-ion mode was used to obtain MS/MS data for carotenoid analysis. Turbo IonSpray source settings were the same as previously described for polyphenol analysis. A solvent delivery system connected postcolumn (Applied Biosystems, Foster City, CA) was used for the delivery of 100 $\mu\text{L min}^{-1}$ of a LiCl solution at a concentration of 500 mg L⁻¹.

Statistical Analysis. The significance of the results was analyzed using the Statgraphics Plus v.5.1 Windows Package (Statistical Graphics Co., Rockville, MD). Analysis of variance (ANOVA) was used to compare the means of groups of measurement data. Relationships between variables were examined using Pearson correlation coefficients. P values of <0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of Time of Storage on Hydrophilic and Lipophilic Antioxidants in Gazpachos. The contribution of carotenoids to total antioxidant capacity is considered to be small in comparison with hydrophilic antioxidant capacity. In tomatoes, the hydrophilic fraction displays greater antioxidant capacity than the lipophilic fraction. Wu et al.²⁰ reported that

hydrophilic antioxidant capacity represents >90% of the total antioxidant capacity of fruits and vegetables. These results concur with our study. Although hydrophilic antioxidant capacity does not account for >90% of the total antioxidant capacity, hydrophilic antioxidant content is greater than lipophilic content (Table 1).

The ABTS[•] and DPPH values decreased over the 12 weeks of storage for both lipophilic and hydrophilic antioxidants. Although there was a slight variation between different brands of gazpachos in the content of antioxidants, the trends reflecting the effects of storage were similar. The content of hydrophilic antioxidants decreased by about 7.34% for the ABTS[•] assay and by 7.42% for the DPPH assay. For lipophilic antioxidant capacity, the contents decreased by 7.95 and 7.76% for the ABTS[•] and DPPH assays, respectively. The most significant decrease was observed after 4 weeks of storage. Del Caro et al.²¹ described a decrease of about 5% in the TEAC value for orange juice stored for 12 days at 4 °C. Controversially, Piga et al.²² reported an increase in the DPPH content over 15 days of storage of mandarin juices at 4 °C. They attributed this increase in the antioxidant capacity to the formation of Maillard reaction products.

Effect of Time of Storage on Individual Phenolic Compounds in Gazpachos. POD is involved in the oxidative degradation of phenolic compounds.²³ Therefore, the residual

Table 2. Changes in the Content of Hydroxycinnamic Acids in Gazpachos ($n = 3$) during 12 Weeks of Storage^a

weeks of storage	$\mu\text{g g}^{-1}$ FW				
	caffeic acid	caffeic acid-O-hexoside	ferulic acid	ferulic acid-O-hexoside	chlorogenic acid
Gazpacho 1					
0	4.23 $\pm$ 0.04 a	10.69 $\pm$ 0.11 a	31.10 $\pm$ 1.21 a	34.47 $\pm$ 0.72 a	10.93 $\pm$ 0.14 a
4	4.09 $\pm$ 0.09 b	10.49 $\pm$ 0.27 b	30.51 $\pm$ 1.15 b	33.48 $\pm$ 1.44 b	10.63 $\pm$ 0.25 b
8	3.99 $\pm$ 0.07 c	10.20 $\pm$ 0.15 c	29.43 $\pm$ 1.54 c	32.91 $\pm$ 1.29 c	10.31 $\pm$ 0.29 c
12	3.84 $\pm$ 0.08 d	10.00 $\pm$ 0.26 c	29.00 $\pm$ 1.45 c	32.31 $\pm$ 1.14 c	10.02 $\pm$ 0.40 c
Gazpacho 2					
0	3.33 $\pm$ 0.05 a	13.44 $\pm$ 0.14 a	24.11 $\pm$ 1.50 a	39.62 $\pm$ 0.40 a	9.32 $\pm$ 0.09 a
4	3.27 $\pm$ 0.10 b	13.09 $\pm$ 0.29 b	23.54 $\pm$ 0.92 b	38.29 $\pm$ 1.43 b	8.99 $\pm$ 0.43 b
8	2.98 $\pm$ 0.12 c	12.67 $\pm$ 0.18 c	22.87 $\pm$ 1.30 c	37.80 $\pm$ 1.31 c	8.52 $\pm$ 0.40 c
12	2.91 $\pm$ 0.09 d	12.31 $\pm$ 0.22 c	22.09 $\pm$ 1.20 d	36.60 $\pm$ 1.29 d	8.43 $\pm$ 0.41 d
Gazpacho 3					
0	3.67 $\pm$ 0.05 a	9.68 $\pm$ 0.11 a	26.51 $\pm$ 1.02 a	35.12 $\pm$ 0.87 a	12.11 $\pm$ 0.15 a
4	3.51 $\pm$ 0.03 b	9.41 $\pm$ 0.27 b	25.60 $\pm$ 0.95 b	34.07 $\pm$ 0.89 b	11.91 $\pm$ 0.21 b
8	3.42 $\pm$ 0.09 c	9.05 $\pm$ 0.22 c	23.99 $\pm$ 1.10 c	33.31 $\pm$ 1.15 c	11.22 $\pm$ 0.35 c
12	3.30 $\pm$ 0.08 d	8.82 $\pm$ 0.15 d	23.02 $\pm$ 1.22 d	32.97 $\pm$ 1.32 d	11.08 $\pm$ 0.26 c
Gazpacho 4					
0	4.05 $\pm$ 0.04 a	15.35 $\pm$ 0.07 a	21.46 $\pm$ 1.15 a	28.98 $\pm$ 0.36 a	11.19 $\pm$ 0.10 a
4	3.97 $\pm$ 0.07 b	15.09 $\pm$ 0.15 b	20.87 $\pm$ 1.53 b	27.50 $\pm$ 0.55 b	11.01 $\pm$ 0.29 b
8	3.78 $\pm$ 0.08 c	14.55 $\pm$ 0.19 c	20.09 $\pm$ 1.13 c	26.78 $\pm$ 0.59 c	10.79 $\pm$ 0.34 c
12	3.72 $\pm$ 0.08 d	14.38 $\pm$ 0.21 c	19.70 $\pm$ 1.32 c	26.68 $\pm$ 0.65 c	10.54 $\pm$ 0.27 d
Gazpacho 5					
0	4.47 $\pm$ 0.05 a	17.34 $\pm$ 0.05 a	30.41 $\pm$ 1.09 a	25.70 $\pm$ 0.58 a	9.55 $\pm$ 0.12 a
4	4.29 $\pm$ 0.10 b	16.98 $\pm$ 0.22 b	28.60 $\pm$ 1.15 b	25.01 $\pm$ 1.25 b	9.21 $\pm$ 0.25 b
8	4.17 $\pm$ 0.13 c	16.51 $\pm$ 0.25 c	28.01 $\pm$ 1.11 c	24.86 $\pm$ 1.23 c	9.10 $\pm$ 0.29 c
12	4.13 $\pm$ 0.05 d	16.39 $\pm$ 0.16 c	27.94 $\pm$ 1.33 c	24.23 $\pm$ 1.30 c	8.92 $\pm$ 0.31 d
Gazpacho 6					
0	3.85 $\pm$ 0.05 a	11.87 $\pm$ 0.08 a	20.57 $\pm$ 1.22 a	33.64 $\pm$ 0.64 a	10.09 $\pm$ 0.12 a
4	3.71 $\pm$ 0.10 b	11.61 $\pm$ 0.18 b	20.21 $\pm$ 1.19 b	33.01 $\pm$ 0.89 b	9.59 $\pm$ 0.15 b
8	3.53 $\pm$ 0.04 c	11.44 $\pm$ 0.20 c	19.82 $\pm$ 1.40 c	32.28 $\pm$ 1.21 c	9.34 $\pm$ 0.43 c
12	3.45 $\pm$ 0.09 d	11.31 $\pm$ 0.15 d	19.47 $\pm$ 1.23 d	32.12 $\pm$ 1.38 c	9.20 $\pm$ 0.29 c

^aLetters in the columns represent statistically significant differences ($P < 0.05$).

activity of POD might be associated with the degradation of phenolic compounds during storage. Table 1 shows the relative POD activity after 4, 8, and 12 weeks of storage. Ferulic, caffeic, and chlorogenic acid, ferulic acid-O-hexoside, and caffeic acid-O-hexoside were identified in gazpachos (Table 2). Levels of caffeic acid in gazpachos were in line with those reported by Martínez-Valverde et al.²⁴ for tomato products, which ranged between 1.39 and 13 $\mu\text{g g}^{-1}$ FW. The content of ferulic acid was higher than that found by Luthria et al.²⁵ for tomato products, who reported values between 9 and 15 $\mu\text{g g}^{-1}$ FW, whereas in our study the results were between 20.57 and 31.10 $\mu\text{g g}^{-1}$ for ferulic acid and between 25.70 and 39.62 $\mu\text{g g}^{-1}$ for its glycosidic form. The trends reflecting the effects of storage were similar for all of the gazpachos analyzed.

The content of hydroxycinnamic acids decreased after 12 weeks of storage at 4 °C. The greatest decrease ($P < 0.05$) occurred for ferulic (8.33%) and caffeic (9.68%) acids during the 12 weeks of storage, whereas their glycosidic forms decreased by 6.37 and 6.71%, respectively. The higher stability of the glycosidic forms toward their aglycones could be attributed to the blockage of the 3-hydroxy function at the C-ring by a sugar moiety.²⁶ Similarly, chlorogenic acid decreased by 7.94%.

As described by Gliszczynska-Swiglo and Tyrakowska,²⁷ caffeic and chlorogenic acids decreased during the storage of apple juices. They found that the initial concentrations of

caffeic acid were 3.31–8.66 mg L^{-1} and underwent losses reaching values of 1.51 to 3.49 mg L^{-1} . The same pattern was observed for chlorogenic acids. Hydroxycinnamic acids are formed in plant products via the action of phenylalanine ammonia-lyase activity (PAL) due to the phenylpropanoid metabolism.

Biosynthesis of flavonols consists of the sequential addition of three molecules of malonyl-CoA to hydroxycinnamic acid CoA esters to form chalcones. These chalcones are then isomerized into (2S)-flavanones, which, through hydroxylation and desaturation, are converted into flavonols.²⁸ In our study, it could not be hypothesized that the disappearance of hydroxycinnamic acids is due to their conversion to flavonols, as quercetin, rutin, and kaempferol-3-O-rutinoside did not increase during storage and other flavonols were not detected. Therefore, decreases may be due to the oxidation of chlorogenic to reactive O-quinones through the catalytic oxidation process.²⁹

A slight decrease in naringenin and naringenin-7-O-glucoside was observed (Table 3). The naringenin content found in gazpachos was in the range reported by other authors in tomato products.^{24,30} The initial concentrations of naringenin in the studied gazpachos were 44.08–46.83 $\mu\text{g g}^{-1}$ FW, whereas naringenin-7-O-glucoside was found at concentrations of 2.16–2.54 $\mu\text{g g}^{-1}$ FW. The naringenin content in gazpachos

Table 3. Changes in the Content of Flavonols and Flavanones in Gazpachos ($n = 3$) during 12 Weeks of Storage^a

weeks of storage	$\mu\text{g g}^{-1}$ FW				
	rutin	quercetin	kaempferol-3-O-rutinoside	naringenin	naringenin-7-O-glucoside
Gazpacho 1					
0	59.98 $\pm$ 1.04 a	2.79 $\pm$ 0.02 a	20.47 $\pm$ 0.82 a	44.08 $\pm$ 1.10 a	2.48 $\pm$ 0.08 a
4	57.93 $\pm$ 3.30 b	2.63 $\pm$ 0.07 b	19.80 $\pm$ 1.79 b	42.90 $\pm$ 2.60 b	2.39 $\pm$ 0.18 b
8	55.81 $\pm$ 2.66 c	2.48 $\pm$ 0.05 c	19.19 $\pm$ 1.44 c	41.49 $\pm$ 2.30 c	2.32 $\pm$ 0.16 c
12	54.00 $\pm$ 2.90 d	2.29 $\pm$ 0.03 d	18.53 $\pm$ 1.53 d	41.00 $\pm$ 2.35 c	2.28 $\pm$ 0.11 d
Gazpacho 2					
0	56.99 $\pm$ 1.59 a	2.47 $\pm$ 0.01 a	20.77 $\pm$ 1.28 a	46.83 $\pm$ 1.24 a	2.26 $\pm$ 0.13 a
4	54.91 $\pm$ 3.31 b	2.32 $\pm$ 0.03 b	22.35 $\pm$ 1.12 b	45.21 $\pm$ 1.87 b	2.22 $\pm$ 0.12 b
8	52.81 $\pm$ 2.54 c	2.15 $\pm$ 0.04 c	19.81 $\pm$ 1.33 c	44.29 $\pm$ 2.25 c	2.19 $\pm$ 0.05 c
12	51.93 $\pm$ 2.98 c	2.00 $\pm$ 0.05 d	19.50 $\pm$ 1.21 c	43.02 $\pm$ 2.13 d	2.17 $\pm$ 0.18 c
Gazpacho 3					
0	54.40 $\pm$ 1.33 a	2.61 $\pm$ 0.02 a	19.57 $\pm$ 0.76 a	44.46 $\pm$ 1.25 a	2.19 $\pm$ 0.13 a
4	52.67 $\pm$ 1.50 b	2.44 $\pm$ 0.06 b	18.90 $\pm$ 1.59 b	42.21 $\pm$ 1.76 b	2.14 $\pm$ 0.19 b
8	50.54 $\pm$ 2.85 c	2.33 $\pm$ 0.05 c	18.31 $\pm$ 1.13 c	41.51 $\pm$ 2.09 c	2.10 $\pm$ 0.26 c
12	49.31 $\pm$ 2.60 d	2.16 $\pm$ 0.03 d	17.92 $\pm$ 1.56 d	41.33 $\pm$ 2.15 c	2.07 $\pm$ 0.15 d
Gazpacho 4					
0	60.68 $\pm$ 1.24 a	2.48 $\pm$ 0.01 a	20.59 $\pm$ 0.52 a	46.50 $\pm$ 1.43 a	2.53 $\pm$ 0.07 a
4	58.03 $\pm$ 2.31 b	2.32 $\pm$ 0.05 b	20.01 $\pm$ 0.23 b	45.65 $\pm$ 1.87 b	2.49 $\pm$ 0.13 b
8	57.37 $\pm$ 2.23 c	2.21 $\pm$ 0.02 c	19.70 $\pm$ 0.34 c	44.80 $\pm$ 1.75 c	2.45 $\pm$ 0.15 c
12	57.00 $\pm$ 1.25 c	2.15 $\pm$ 0.04 d	19.25 $\pm$ 0.40 c	44.01 $\pm$ 1.99 c	2.41 $\pm$ 0.24 d
Gazpacho 5					
0	51.04 $\pm$ 1.03 a	2.08 $\pm$ 0.01 a	19.05 $\pm$ 0.99 a	44.90 $\pm$ 1.48 a	2.16 $\pm$ 0.09 a
4	50.00 $\pm$ 1.95 b	2.00 $\pm$ 0.05 b	18.70 $\pm$ 1.43 b	43.01 $\pm$ 1.87 b	2.10 $\pm$ 0.22 b
8	48.77 $\pm$ 2.25 c	1.83 $\pm$ 0.06 c	18.00 $\pm$ 1.22 c	42.90 $\pm$ 1.75 c	2.09 $\pm$ 0.05 c
12	48.00 $\pm$ 2.89 c	1.75 $\pm$ 0.05 d	17.88 $\pm$ 1.12 d	42.50 $\pm$ 1.98 c	2.01 $\pm$ 0.06 d
Gazpacho 6					
0	61.83 $\pm$ 1.21 a	2.74 $\pm$ 0.02 a	20.41 $\pm$ 0.78 a	45.20 $\pm$ 3.23 a	2.54 $\pm$ 0.08 a
4	58.00 $\pm$ 1.79 b	2.55 $\pm$ 0.06 b	19.87 $\pm$ 1.01 b	43.18 $\pm$ 2.43 b	2.41 $\pm$ 0.14 b
8	57.43 $\pm$ 2.31 c	2.49 $\pm$ 0.04 c	19.37 $\pm$ 1.91 c	42.61 $\pm$ 2.50 c	2.35 $\pm$ 0.15 c
12	57.03 $\pm$ 2.77 c	2.25 $\pm$ 0.05 d	19.05 $\pm$ 1.82 c	42.40 $\pm$ 2.30 c	2.30 $\pm$ 0.18 d

^aLetters in the columns represent statistically significant differences ($P < 0.05$).

decreased by 6.53% and that of naringenin-7-O-glucoside by about 6.44%.

Gazpachos have been found to be a rich source of flavonols such as quercetin, kaempferol, and rutin. Naringenin (45%) is reported to be the main flavonoid, followed by quercetin (39%), myricetin (10%), and kaempferol (5%).⁹ Other studies report rutin to be the major flavonoid in several tomato cultivars.¹⁷ In this study, rutin was the dominant flavonol in all of the gazpachos, followed by kaempferol-3-O-rutinoside and quercetin.

The initial concentrations of quercetin in the studied gazpachos were 2.08–2.79 $\mu\text{g g}^{-1}$ FW, whereas kaempferol-3-O-rutinoside was found at concentrations of 19.05–20.77 $\mu\text{g g}^{-1}$ FW. Quercetin underwent significant losses throughout the gazpacho storage period, reaching values of 1.75–2.29 $\mu\text{g g}^{-1}$ FW. Our results were in line with those found by Vallverdú-Queralt et al.³¹ during the storage of ketchups and tomato juices.

Changes in rutin content are also shown in Table 3; rutin decreased by 8.00%. It could be hypothesized that the oxidative degradation of phenolic compounds is due to the residual peroxidase activity and the autocatalytic oxidative reactions. Flavonols may capture reactive oxygen species and, during the thermal processing, flavonols could form quinonoid structures, which leads to the formation of quinones. In the case of rutin and kaempferol-3-O-rutinoside, in comparison with quercetin,

the 3-hydroxy function at the C-ring of the flavonoid is blocked by a sugar moiety. Thus, the blockage of the 3-hydroxyl group might explain the higher stability of rutin and kaempferol toward oxidation.

Effect of Time of Storage on Individual Carotenoids in Gazpachos. Table 4 shows the evolution of the carotenoid content in gazpachos over 12 weeks of storage at 4 °C. The initial concentrations of *all-trans*-lycopene in the studied gazpachos were 2.99–4.31 $\mu\text{g g}^{-1}$ FW. *all-trans*-Lycopene underwent significant losses throughout the storage of gazpachos, reaching values of 2.45–3.51 $\mu\text{g g}^{-1}$ FW. *trans*-Lycopene decreased by 16.91% in comparison to *trans*- β -carotene, *trans*- α -carotene, and *trans*-lutein, which decreased by 8.54, 10.24, and 5.44%, respectively.

Our results are in line with those reported by Lin and Chen,³² who attributed the higher stability of *trans*-lutein and *trans*- β -carotene to the coplanar structure of *trans*-lycopene, in which 11 conjugated double bonds are present and which should be more reactive than *trans*-lutein and *trans*- β -carotene.

In another study, Zaroni et al.³³ reported that *trans*-lycopene underwent a high loss of 50% in dried tomatoes after a 30 day storage period and a loss of 70% after 90 days. Similarly, Odriozola-Serrano et al.¹¹ reported final *trans*-lycopene losses of approximately 70% in tomato juice stored at 4 °C for 3 months. Moreover, similar lycopene degradation patterns (35–75%) have been described by Giovanelli and Paradiso³⁴ during

Table 4. Changes in the Content of Carotenoids in Gazpachos ($n = 3$) during 12 Weeks of Storage^a

weeks of storage	$\mu\text{g g}^{-1}$ FW						
	<i>trans</i> - α -carotene	<i>trans</i> - β -carotene	<i>trans</i> -lutein	<i>trans</i> -lycopene	5- <i>cis</i> -lycopene	9- <i>cis</i> -lycopene	13- <i>cis</i> -lycopene
Gazpacho 1							
0	0.25 $\pm$ 0.01 a	9.10 $\pm$ 0.04 a	2.04 $\pm$ 0.02 a	3.24 $\pm$ 0.16 a	2.37 $\pm$ 0.05 a	1.18 $\pm$ 0.02 a	1.64 $\pm$ 0.16 a
4	0.21 $\pm$ 0.01 b	8.75 $\pm$ 0.12 b	2.00 $\pm$ 0.09 b	3.10 $\pm$ 0.13 b	2.35 $\pm$ 0.10 a	1.15 $\pm$ 0.04 b	1.55 $\pm$ 0.15 b
8	0.20 $\pm$ 0.01 c	8.45 $\pm$ 0.11 c	1.98 $\pm$ 0.08 c	2.85 $\pm$ 0.15 c	2.40 $\pm$ 0.07 b	1.15 $\pm$ 0.06 b	1.54 $\pm$ 0.12 b
12	0.19 $\pm$ 0.01 c	8.34 $\pm$ 0.03 c	1.95 $\pm$ 0.06 d	2.74 $\pm$ 0.17 d	2.45 $\pm$ 0.09 c	1.14 $\pm$ 0.05 b	1.52 $\pm$ 0.10 c
Gazpacho 2							
0	0.32 $\pm$ 0.03 a	15.69 $\pm$ 0.05 a	1.98 $\pm$ 0.08 a	4.31 $\pm$ 0.19 a	3.42 $\pm$ 0.04 a	0.74 $\pm$ 0.01 a	1.42 $\pm$ 0.12 a
4	0.30 $\pm$ 0.02 b	15.35 $\pm$ 0.02 b	1.92 $\pm$ 0.09 b	4.06 $\pm$ 0.15 b	3.45 $\pm$ 0.08 b	0.71 $\pm$ 0.02 b	1.36 $\pm$ 0.011 b
8	0.30 $\pm$ 0.01 b	14.75 $\pm$ 0.09 c	1.89 $\pm$ 0.07 c	3.78 $\pm$ 0.17 c	3.46 $\pm$ 0.03 c	0.70 $\pm$ 0.03 b	1.35 $\pm$ 0.10 b
12	0.29 $\pm$ 0.01 b	14.50 $\pm$ 0.07 c	1.85 $\pm$ 0.06 d	3.51 $\pm$ 0.14 d	3.50 $\pm$ 0.02 d	0.70 $\pm$ 0.01 b	1.32 $\pm$ 0.09 c
Gazpacho 3							
0	0.27 $\pm$ 0.02 a	11.43 $\pm$ 0.01 a	1.90 $\pm$ 0.04 a	3.25 $\pm$ 0.16 a	2.96 $\pm$ 0.07 a	1.04 $\pm$ 0.04 a	1.38 $\pm$ 0.11 a
4	0.26 $\pm$ 0.02 b	11.13 $\pm$ 0.05 b	1.84 $\pm$ 0.05 b	3.05 $\pm$ 0.19 b	2.98 $\pm$ 0.06 b	1.02 $\pm$ 0.05 b	1.32 $\pm$ 0.10 b
8	0.25 $\pm$ 0.01 b	10.69 $\pm$ 0.04 c	1.80 $\pm$ 0.04 c	2.82 $\pm$ 0.10 c	3.00 $\pm$ 0.05 b	1.00 $\pm$ 0.03 c	1.30 $\pm$ 0.09 c
12	0.25 $\pm$ 0.01 b	10.42 $\pm$ 0.08 c	1.79 $\pm$ 0.03 c	2.71 $\pm$ 0.15 d	3.05 $\pm$ 0.07 c	0.98 $\pm$ 0.01 c	1.29 $\pm$ 0.08 c
Gazpacho 4							
0	0.37 $\pm$ 0.03 a	14.56 $\pm$ 0.04 a	1.88 $\pm$ 0.04 a	3.18 $\pm$ 0.12 a	2.83 $\pm$ 0.11 a	0.86 $\pm$ 0.02 a	1.35 $\pm$ 0.11 a
4	0.35 $\pm$ 0.02 b	14.20 $\pm$ 0.05 b	1.81 $\pm$ 0.05 b	2.99 $\pm$ 0.19 b	2.86 $\pm$ 0.15 b	0.85 $\pm$ 0.06 a	1.28 $\pm$ 0.06 b
8	0.35 $\pm$ 0.03 b	13.65 $\pm$ 0.09 c	1.76 $\pm$ 0.07 c	2.71 $\pm$ 0.11 c	2.90 $\pm$ 0.12 c	0.83 $\pm$ 0.07 b	1.27 $\pm$ 0.05 b
12	0.34 $\pm$ 0.03 b	13.34 $\pm$ 0.08 c	1.73 $\pm$ 0.08 d	2.67 $\pm$ 0.15 d	2.89 $\pm$ 0.06 d	0.82 $\pm$ 0.05 b	1.25 $\pm$ 0.07 b
Gazpacho 5							
0	0.31 $\pm$ 0.03 a	10.72 $\pm$ 0.03 a	1.85 $\pm$ 0.05 a	2.99 $\pm$ 0.14 a	2.71 $\pm$ 0.06 a	1.36 $\pm$ 0.09 a	1.36 $\pm$ 0.11 a
4	0.30 $\pm$ 0.02 b	10.33 $\pm$ 0.06 b	1.81 $\pm$ 0.07 b	2.80 $\pm$ 0.15 b	2.73 $\pm$ 0.06 b	1.32 $\pm$ 0.12 b	1.30 $\pm$ 0.07 b
8	0.29 $\pm$ 0.02 b	9.95 $\pm$ 0.04 c	1.80 $\pm$ 0.06 c	2.61 $\pm$ 0.13 c	2.76 $\pm$ 0.03 c	1.30 $\pm$ 0.10 c	1.27 $\pm$ 0.05 c
12	0.28 $\pm$ 0.02 b	9.75 $\pm$ 0.03 d	1.78 $\pm$ 0.09 d	2.45 $\pm$ 0.19 d	2.79 $\pm$ 0.08 d	1.25 $\pm$ 0.08 d	1.25 $\pm$ 0.09 c
Gazpacho 6							
0	0.53 $\pm$ 0.05 a	15.78 $\pm$ 0.04 a	1.95 $\pm$ 0.06 a	3.86 $\pm$ 0.15 a	3.36 $\pm$ 0.08 a	1.71 $\pm$ 0.15 a	1.65 $\pm$ 0.15 a
4	0.51 $\pm$ 0.05 b	15.40 $\pm$ 0.05 b	1.90 $\pm$ 0.06 b	3.61 $\pm$ 0.16 b	3.39 $\pm$ 0.10 b	1.69 $\pm$ 0.16 b	1.57 $\pm$ 0.13 b
8	0.50 $\pm$ 0.04 b	14.73 $\pm$ 0.06 c	1.88 $\pm$ 0.05 c	3.30 $\pm$ 0.14 c	3.41 $\pm$ 0.15 c	1.65 $\pm$ 0.14 c	1.55 $\pm$ 0.12 c
12	0.49 $\pm$ 0.03 b	14.33 $\pm$ 0.08 c	1.87 $\pm$ 0.07 c	3.21 $\pm$ 0.15 d	3.45 $\pm$ 0.19 d	1.62 $\pm$ 0.13 d	1.53 $\pm$ 0.14 c

^aLetters in the columns represent statistically significant differences ($P < 0.05$).

the storage of tomato paste. Controversially, García Alonso et al.³⁵ found that the total lycopene content remained fairly stable throughout the storage trial and varied from 99 to 120 mg kg^{-1} in tomato juice packaged in Tetra Paks and from 96 to 115 mg kg^{-1} in samples stored in glass bottles. For both types of samples, no clear temperature dependency was revealed regarding the rate of total lycopene loss, which was affected only by storage time. At the end of the storage trial, the final losses in the total lycopene content varied from 7 to 17%. The differences in lycopene stability between these studies could be attributed to the presence and stability of other antioxidants such as polyphenols or to the thermal inactivation of oxidizing enzymes during tomato processing.³⁶

We also looked at *cis*-isomers of lycopene. Human health studies have demonstrated the nutritional benefits of *cis*-isomers in tomato products, because these compounds seem to be better absorbed in the intestine.³⁶ We tentatively identified three isomers in gazpachos: 5-, 9-, and 13-*cis*-lycopene (Table 4). This lycopene isomer profile is in line with that described by other authors in different tomato products, in which *trans*-lycopene represents the most abundant lycopene isomer, varying from 35 to 96% of total lycopene, with 5-, 9-, 13-, and 15-*cis*-lycopene being the main *cis*-isomers detected.^{19,37} It was shown that during meal preparation, lycopene undergoes *trans/cis*-isomerization, increasing the proportion of *cis*-isomers.³² As mentioned above, *all-trans*-lycopene underwent

a 16.91% loss in gazpachos in comparison to *cis*-lycopene isomers, which slightly decreased, 5.52 and 7.27% for 9-*cis*-lycopene and 13-*cis*-lycopene, respectively. An increase of 2.72% for the 5-*cis*-lycopene isomer was observed in gazpacho products. These results are in line with those reported by Lin and Chen.³² They observed the formation of *cis*-lycopene isomers during the 3 month storage period of tomato juice. This could be explained by the isomerization phenomenon. *all-trans*-Lycopene can be converted to 13-*cis*-lycopene, which can then be converted into other *cis*-isomers.

To our knowledge, this is the first time a study has been carried out to evaluate the stability of flavonols, flavanones, hydroxycinnamic acids, and individual carotenoids in gazpachos during storage.

The storage of gazpachos at 4 °C results in a slight decrease in their polyphenol and carotenoid contents and also in the hydrophilic and lipophilic antioxidant capacities, but the levels achieved do not signify a nutritional drawback. Therefore, we can expect a beneficial effect from the consumption of gazpachos at any point during their shelf life.

The main degradation was observed for quercetin oxidation because the hydroxy function at the C-ring of the flavonoid is not blocked by a sugar moiety as it is in the case of rutin, kaempferol-3-O-rutinoside, and glycosylated caffeic and ferulic acids.

Lycopene underwent significant losses throughout storage, due mainly to the 11 conjugated double bonds that are present in its structure, and should be more reactive than *trans*-lutein and *trans*- β -carotene. *cis*-Lycopene isomers slightly decreased. However, 5-*cis*-lycopene underwent a slight increase; this phenomenon could be explained by *cis*-isomerization increasing the portion of *cis*-isomers.

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12. Capítol XII: Efectes dels polsos elèctrics en el contingut de compostos bioactius i capacitat antioxidant dels tomàquets

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Resum: L'aplicació dels polsos elèctrics en el processat d'aliments ha atret gran interès com una alternativa als tractaments tèrmics per mantenir la qualitat nutricional dels productes. No obstant això, els polsos elèctrics també es poden fer servir per estimular la producció de metabòlits secundaris com els polifenols i els carotens. Es va fer servir un disseny de superfície de resposta per avaluar l'efecte dels tractaments de polsos elèctrics en la producció de polifenols, carotens i vitamina C. Les variables independents van ser la intensitat de camp (de 0.4 a 2.0 kV/cm) i el nombre de polsos (de 5 a 30). Els tomàquets tractats amb polsos elèctrics es van guardar a 4 °C durant 24 hores. Després es van dur a terme les anàlisis de capacitat antioxidant, vitamina C, polifenols totals i licopè total. S'observa un increment en el contingut de polifenols, licopè total i capacitat antioxidant a diferents combinacions de polsos elèctrics i intensitat de camp. La vitamina C disminueix lleugerament. Consegüentment, els polsos elèctrics es poden fer servir per augmentar el contingut de compostos bioactius en tomàquets i derivats de tomàquet, com ara suc de tomàquet, quètxups i gaspatxos.

Effects of Pulsed Electric Fields on the Bioactive Compound Content and Antioxidant Capacity of Tomato Fruit

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ABSTRACT: The effect of moderate intensity pulsed electric fields (MIPEF) on the bioactive compounds (total polyphenol, lycopene, and vitamin C content) as well as on the antioxidant capacity of tomato fruit was studied. The MIPEF treatment conditions were optimized to obtain tomato fruit with a high content of bioactive compounds. Tomato fruits were subjected to different electric field strengths (from 0.4 to 2.0 kV/cm) and number of pulses (from 5 to 30) and then immediately refrigerated at 4 °C for 24 h. A concentration of bioactive compounds higher than that of untreated tomatoes was obtained in MIPEF-treated tomatoes. A 44% increase in total polyphenol content was achieved under 30 pulses at 1.2 kV/cm. The hydrophilic antioxidant capacity was also enhanced by 44% applying 18 pulses at 1.2 kV/cm, and the lipophilic antioxidant capacity was increased by 37% under 5 pulses at 1.2 kV/cm. The maximum overall level of bioactive compounds and antioxidant capacity in the treated tomatoes was obtained under 16 pulses at 1 kV/cm. Therefore, MIPEF treatments could be considered an effective method to enhance the bioactive compound content and antioxidant potential of tomatoes.

KEYWORDS: MIPEF treatments, lycopene, total polyphenols, antioxidant capacity, vitamin C, stress reactions

■ INTRODUCTION

Diet-related chronic diseases have become a major public health concern due to their increasing prevalence in recent years. In this context, the consumption of tomatoes could be considered as a nutritional indicator of good dietary habits and healthy lifestyle due to the important health protecting role of their bioactive compounds, principally polyphenols, carotenoids, and vitamin C.^{1,2} In particular, lycopene has been associated in epidemiological studies with a lower risk of prostate cancer.³ The high content of polyphenols in tomatoes has also been gaining interest because of their multiple biological effects, including free-radical scavenging, inhibition of cellular proliferation, and modulation of enzymatic activity and signal transduction pathways.⁴

High intensity pulsed electric fields have been proposed as an alternative to conventional techniques of food preservation. Several studies have demonstrated the ability of high intensity pulsed electric fields to obtain shelf-stable liquid foods with high nutritional value by inactivating microorganisms and enzymes.⁵ Other applications of pulsed electric fields technology are currently being developed. Moderate intensity pulsed electric fields (MIPEF) may cause lethal damage to cells or induce sublethal stress by permeabilizing tissue structures, thus improving intracellular metabolite extraction^{5,6} and enhancing drying efficiency.⁷ Recent studies have also suggested the possibility of using MIPEF to stress cells and thus stimulate the biosynthesis of secondary metabolites.⁸ MIPEF affect the metabolism of vegetables with the consequent generation of reactive oxygen species (ROS),^{9,10}

ROS are endogenous signal components required for the synthesis of secondary metabolites such as polyphenols or carotenoids, which are known to be part of the defense response of plants to stress.¹¹

Metabolic responses to MIPEF treatments have been studied in potato tissues,^{10,12} but as far as we know, no data is available on the effects of MIPEF on other fruit and vegetables. Therefore, the aim of this research was to study the effects of processing parameters of MIPEF, namely, electric field strength and number of pulses, on the bioproduction of polyphenols, lycopene and vitamin C as well as on the antioxidant capacity of tomato fruits. In addition, MIPEF processing parameters were optimized to obtain tomato fruit with enhanced levels of bioactive compounds.

■ MATERIALS AND METHODS

Reagents. All samples and standards were handled without exposure to light. Folin–Ciocalteu (F–C) reagent, L-ascorbic acid, lycopene, 2,2'-azino-bis(3)-ethylbenzothiazoline-6-sulfonic acid (ABTS), Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) 97%, and manganese dioxide were purchased from Sigma (Madrid, Spain); metaphosphoric acid and DL-1,4-dithiothreitol (DTT) were purchased from Acros Organics (New Jersey, U.S.A.); hydrochloric acid 35% and acetic acid 99.8% were from Panreac (Barcelona, Spain); and anhydrous sodium acetate (2 M) was from

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Merck (Darmstadt, Germany). Butylated hydroxytoluene (BHT), methanol, hexane, and formic acid were obtained from Scharlau (Barcelona, Spain). Ultrapure water (Milli-Q) was from Millipore Corporation (Bedford, MA, USA).

Tomatoes. Commercially mature tomato fruit (*Lycopersicon esculentum* Mill. cv. Daniella) were purchased from a local supermarket (Lleida, Spain). The pH (Crison 2001 pH-meter; Crison Instruments SA, Alella, Barcelona, Spain), soluble solids content (Atago RX-1000 refractometer; Atago Company Ltd., Japan), firmness (Texturometer-XT2 Stable Micro Systems Ltd., Surrey, England), and color (Minolta CR-400, Konica Minolta Sensing, Inc., Osaka, Japan) of the tomato fruit were determined (Table 1).

Table 1. Analytical Characteristics of Tomato Fruits

parameters ^a	tomato fruits
pH	4.45 ± 0.01
firmness (N·s) ^b	20.4 ± 2.51
soluble solids (°Brix)	4.42 ± 0.02
L*	38.5 ± 0.4
a*	18.1 ± 1.9
b*	24.6 ± 1.8

^aResults are the mean ± SD of three measurements. ^bN·s: Newtons-second.

MIPEF Processing. MIPEF treatments were conducted in bath equipment manufactured by Physics International (San Leandro, CA, USA), which can deliver pulses from a capacitor of 0.1 μF with an exponential decaying waveform. A stainless steel parallel plate treatment chamber was used. A batch of tomato fruit was placed in the treatment chamber filled with tap water. Tomato fruits were treated at 0.4–2 kV/cm using 5–30 monopolar pulses of 4 μs at a frequency of 0.1 Hz. Each treatment was repeated twice.

MIPEF-treated tomato fruits were collected and immediately refrigerated at 4 °C for 24 h as previously described by Galindo et al.¹⁰ Untreated tomatoes were stored separately at 4 °C for 24 h. Both untreated and MIPEF-treated tomatoes were lyophilized after 24 h and frozen at –20 °C until analysis.

Extraction and Analyses of Total Polyphenol Content. Samples were treated in triplicate following the procedure of Vallverdú-Queralt et al.¹³ with some modifications. Lyophilized tomato fruits (0.2 g) were weighed and homogenized with 80% ethanol in Milli-Q water (4 mL); the homogenate was then sonicated for 5 min and centrifuged (4000 rpm at 4 °C) for 15 min. The supernatant was transferred into a flask, and the extraction was repeated. Both supernatants were combined and evaporated under nitrogen flow. Finally, the residue was reconstituted with Milli-Q water (0.1% of formic acid) up to 2 mL and filtered through a 13 mm, 0.45 μm polytetrafluoroethylene (PTFE) filter from Waters (Milford, USA) into an insert-amber vial.

Solid phase extraction (SPE) was carried out to eliminate interferences such as ascorbic acid, amino acids, and reducing sugars, which are proved to overestimate values of total polyphenol (TP) content. For this procedure, Oasis MAX cartridges with 30 mg of mixed-mode anion-exchange and reversed-phase solvent from Waters (Milford, USA) were used following the procedure of Vallverdú-Queralt et al.¹⁴ The eluted fractions were evaporated under nitrogen flow, and the residue was reconstituted with up to 500 μL of Milli-Q water containing 0.1% formic acid.

For the TP content assay, each extract was analyzed as follows: 20 μL of the eluted fractions was mixed with 188 μL of Milli-Q water in a thermo microtiter 96-well plate (Nunc, Roskilde, Denmark), and 12 μL of Folin–Ciocalteu (F–C) reagent and 30 μL of sodium carbonate (200 g/L) were added. The mixtures were incubated for 1 h at room temperature in the dark. After the reaction period, 50 μL of Milli-Q water was added, and the absorbance was measured at 765 nm in a UV/vis Thermo Multiskan Spectrum spectrophotometer (Vantaa, Finland). This spectrophotometer allowed the absorbance of a 96-well

plate to be read in 10 s. TP content was expressed as mg of gallic acid equivalents (GAE)/100 g dry weight.

Extraction and Analyses of Vitamin C Content. The extraction procedure was carried out in triplicate following a method described by Odriozola-Serrano et al.¹⁵ Five grams of lyophilized tomato fruits were weighed and homogenized with 5 mL of a solution containing 45 g of metaphosphoric acid and 7.5 g of DTT per liter. The mixture was centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was transferred into a flask, and the extraction was repeated. Both supernatants were combined and filtered through a 0.45 μm PTFE filter into an insert-amber vial for HPLC analysis.

For chromatographic separations an HP 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany) equipped with a diode-array detector and an automatic sample injector was used. The mobile phase was a 0.01% solution of sulphuric acid adjusted to pH 2.6. The separation of ascorbic acid was performed with a Luna C₁₈ column 50 × 2.0 mm i.d., 5 μm (Phenomenex, Torrance, CA, USA). The injection volume was 20 μL, and the flow rate was 1 mL/min. All UV spectra were recorded at 245 nm. Identification of the ascorbic acid was carried out comparing the retention time and UV–visible absorption spectrum with those of the standard. Vitamin C content was expressed as mg vitamin C/100 g dry weight.

Extraction and Analyses of Lycopene Content. The extraction of lycopene was carried out in triplicate following the procedure of Odriozola-Serrano et al.¹⁶ This method determines the content of lycopene and other derivatives such as hydroxy lycopene and lycopene epoxides. Approximately 0.2 g of lyophilized tomatoes were weighed and added to 2.5 mL of 0.05% (w/v) BHT in acetone, 2.5 mL of 95% USP-grade ethanol, and 5 mL of hexane. The homogenate was centrifuged at 4000 rpm for 20 min at 4 °C. After shaking, 1.5 mL of distilled water was added. The vials were then agitated for 5 min and left at room temperature to allow phase separation. The lycopene content of each sample was measured using the absorbance at 503 nm. Lycopene content was expressed as mg lycopene/100 g dry weight.

Antioxidant Capacity Determination. The tomato extracts prepared to determine the TP and lycopene contents were also used to analyze the hydrophilic antioxidant capacity (HAC) and lipophilic antioxidant capacity (LAC), respectively. The HAC and LAC were measured using an ABTS[•] radical decolorization assay and DPPH assay following the procedure of Vallverdú-Queralt et al.¹⁷ with minor modifications.

ABTS[•] Assay. An ABTS[•] radical cation was prepared by passing a 5 mM aqueous stock solution of ABTS (in methanol) through manganese dioxide powder. Excess manganese dioxide was filtered through a 13 mm 0.45 μm filter PTFE. Before analysis, the solution was diluted in methanol pH 7.4 to give an absorbance at 734 nm of 0.9 ± 0.1 and preincubated in ice. Two hundred forty-five microliters of ABTS[•] solution was added to 5 μL of tomato extracts, and solutions were stirred for 30 s. The homogenates were kept in darkness for 1 h, and the absorbance was recorded with a UV/vis Thermo Multiskan Spectrum spectrophotometer at 734 nm against a blank of methanol without ABTS[•]. The results were expressed as mmol Trolox equivalent (TE)/100 g dry weight.

DPPH Assay. HAC and LAC were also measured by the DPPH assay. Five microliters of tomato extracts or Trolox were mixed with 250 μL of methanolic DPPH (0.025 g/L). The homogenate was shaken vigorously and kept in darkness for 30 min. Absorption of the samples was measured on the spectrophotometer at 515 nm against a blank of methanol without DPPH. The results were expressed as mmol Trolox equivalent (TE)/100 g dry weight.

TP, lycopene, and vitamin C relative contents were defined as the percentage of compound content of MIPEF-treated tomatoes compared to that of the untreated tomatoes. Relative HAC and LAC were defined as the percentage of HAC or LAC of MIPEF-treated tomatoes compared to that of the untreated tomatoes.

Experimental Design. A face-centered central composite response surface design was used to determine the effect of electric field strength and number of pulses on TP, vitamin C, and lycopene content as well as on HAC and LAC. The independent variables were electric field strength (from 0.4 to 2 kV/cm) and number of pulses

Table 2. Central Composite Response Surface Design for Relative TP, Vitamin C, and Lycopene Content and Relative Antioxidant Capacity of Tomato Fruits under Different MIPEF Treatments^a

E (kV/cm)	n (number of pulses)	relative TP content (%) ^b	relative vitamin C content (%) ^b	relative lycopene content (%) ^b	relative HAC ^c (DPPH) (%) ^b	relative HAC ^c (ABTS) (%) ^b	relative LAC ^c (DPPH) (%) ^b	relative LAC ^c (ABTS) (%) ^b
1.2	30	144.61 ± 2.29 a	98.97 ± 1.11 a	110.13 ± 2.74 a	134.67 ± 1.64 a	129.76 ± 1.30 a	123.34 ± 2.77 a	115.45 ± 2.96 a
1.2	5	121.89 ± 2.92 b	86.05 ± 1.45 b	131.75 ± 2.96 b	120.80 ± 2.38 b	112.69 ± 1.77 b	137.35 ± 2.02 b	130.72 ± 2.23 b
1.2	18	137.19 ± 2.73 c	99.36 ± 0.99 a	117.77 ± 1.37 c	144.20 ± 2.08 c	141.36 ± 1.76 c	126.47 ± 1.33 c	120.42 ± 1.50 c
2	18	90.59 ± 2.88 d	94.51 ± 1.96 c	100.56 ± 2.40 d	122.48 ± 1.87 d	114.69 ± 1.38 b	113.93 ± 1.64 d	105.51 ± 3.57 d
0.4	18	121.21 ± 2.69 b	95.30 ± 1.88 d	112.98 ± 2.41 a	133.80 ± 1.99 a	130.20 ± 2.38 a	122.10 ± 2.31 a	118.76 ± 2.12 c
2	30	98.96 ± 4.65 e	94.94 ± 1.50 c	93.01 ± 1.39 e	109.85 ± 1.67 e	103.06 ± 1.53 d	110.42 ± 2.43 e	99.93 ± 2.38 e
0.4	30	134.49 ± 3.00 c	94.31 ± 0.97 c	103.83 ± 3.00 f	125.81 ± 2.15 f	117.30 ± 1.37 e	116.75 ± 3.08 f	111.16 ± 2.26 f
2	5	81.37 ± 1.95 f	81.00 ± 1.85 e	110.97 ± 3.09 a	101.60 ± 2.45 g	92.61 ± 0.95 f	120.74 ± 2.27 g	114.62 ± 1.84 a
0.4	5	106.64 ± 1.68 g	83.22 ± 1.60 f	126.02 ± 2.82 g	114.15 ± 2.57 h	106.57 ± 1.51 g	130.86 ± 1.99 h	123.47 ± 3.37 g

^aDifferent letters in the columns represent statistically significant differences ($P < 0.05$). ^bData shown are the mean ± SD of two PEF treatment repetitions; each assay was performed in triplicate. ^cHAC, hydrophilic antioxidant capacity; LAC, lipophilic antioxidant capacity.

Table 3. Analysis of Variance of the Second-Order Models for Relative TP, Vitamin C, and Lycopene Content and Relative Antioxidant Capacity

source ^a	relative TP content	relative vitamin C content	relative lycopene content	relative HAC ^b (DPPH)	relative HAC ^b (ABTS)	relative LAC ^b (DPPH)	relative LAC ^b (ABTS)
quadratic model	200.13 ^c	6463.34 ^c	178.83 ^c	285.53 ^c	176.78 ^c	118.43 ^c	62.54 ^c
E	267.08 ^c	94.23 ^c	171.15 ^c	142.18 ^c	79.41 ^c	99.17 ^c	77.34 ^c
n	148.49 ^c	14777.62 ^c	445.27 ^c	102.25 ^c	60.76 ^c	241.80 ^c	124.43 ^c
E ²	465.53 ^c	3279.01 ^c	265.51 ^c	356.29 ^c	215.01 ^c	245.60 ^c	103.99 ^c
n ²	2.80	7656.7 ^c	11.69 ^d	375.28 ^c	245.68 ^c	21.43 ^d	2.43
E·n	5.06	187.89 ^c	3.14	1.56	0.01	3.50	0.59
lack of fit	0.38	2.61	0.42	2.89	2.90	2.13	1.47
std. dev.	2.28	1.36	1.20	1.36	2.00	1.01	1.55
mean	121.98	129.55	113.70	129.55	124.13	123.68	117.06
coefficient of variation	1.87	1.05	1.05	1.05	1.61	0.82	1.32
R ²	0.9931	0.9898	0.9922	0.9951	0.9921	0.9883	0.9781
adj R ²	0.9881	0.9896	0.9867	0.9916	0.9865	0.9799	0.9624

^aE) electric field strength, n) number of pulses. ^bHAC, hydrophilic antioxidant capacity; LAC, lipophilic antioxidant capacity. ^cSignificant at $p < 0.001$. ^dSignificant at $p < 0.05$.

(from 5 to 30). The levels for each independent parameter were chosen considering sample and equipment limitations. MIPEF experimental design was performed twice, and the order of assays was randomized. Experimental data were fitted to a polynomial response surface. The second-order response function was predicted by eq 1:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j \quad (1)$$

where Y is the dependent variable, β_0 is the center point of the system, β_i , β_{ii} , and β_{ij} represent the coefficients of the linear, quadratic, and interactive effect, respectively, and X_i , X_i^2 , and $X_i X_j$ represent the linear, quadratic, and interactive effect of the independent variables, respectively. The nonsignificant terms were deleted from the second-order polynomial model after an ANOVA test, and a new ANOVA was performed to obtain the coefficients of the final equation for improved accuracy. Design Expert 7.0.1 software (Stat Ease Inc., Minneapolis, MN) was used to generate quadratic models that fit the experimental data and to draw the response surface plots.

The optimization was done following the method proposed by Derringer et al.¹⁸ All the individual desirability functions obtained for each response were combined into an overall expression, which is defined as the geometrical mean of the individual functions. The closer the desirability value is to the unit, the more suitable is the system. In the present study, desirability functions were developed to obtain tomato fruits with optimum levels of health-related compounds.

A set of 88 experiments, choosing intensities between 0.4 and 2 kV/cm and number of pulses between 5 and 30, was carried out to validate the predictive models on TP, vitamin C, and lycopene content as well as on HAC and LAC. Correlations between predicted and observed retentions were evaluated by Pearson's test.

RESULTS AND DISCUSSION

Effect of MIPEF on TP Content. The TP content of untreated tomatoes was 138.2 mg/100 g dry weight, which was consistent with values reported in the literature.^{18,19} Results for relative TP content obtained under the different experimental conditions are shown in Table 2. After 24 h of MIPEF treatments, a higher TP content was observed in MIPEF-treated tomatoes than in untreated samples except at 2 kV/cm. The increases in relative TP content of tomatoes ranged from 6.6% (5 pulses at 0.4 kV/cm) to 44.6% (30 pulses at 1.2 kV/cm). These results are in accordance with Galindo et al.,¹⁰ who reported that 24 h after MIPEF treatments, potato tissue metabolism showed plant stress responses characterized by changes in polyphenols, amino acids, and the hexose pool. Similar responses in plants to other types of stress have also been observed. Matsuda et al.²⁰ reported a 50% increase of chlorogenic acid in wound-healing potato tuber tissue, which is known to boost tissue protection against oxidative stress.

Increases of TP content are difficult to explain due to the complexity of chemical reactions occurring in natural systems.

Table 4. Significant Regression Coefficients of the Quadratic Model for Relative TP, Vitamin C, and Lycopene Content and Relative Antioxidant Capacity of MIPEF-Treated Tomatoes

source ^a	relative TP content	relative vitamin C content	relative lycopene content	relative HAC ^b (DPPH)	relative HAC ^b (ABTS [•])	relative LAC ^b (DPPH)	relative LAC ^b (ABTS [•])
intercept value	74.338	69.237	120.413	80.107	66.220	125.996	119.264
E	95.417	14.778	35.962	49.782	57.167	30.562	26.589
n	0.909	1.967	-1.374	4.011	4.742	-1.142	-0.564
E ²	-47.692	-7.087	-18.308	-24.200	-27.615	-14.871	-13.973
n ²		-0.044	0.016	-0.102	-0.121	-0.018	
E-n		0.090					

^aE, electric field strength; n, number of pulses. ^bHAC, hydrophilic antioxidant capacity; LAC, lipophilic antioxidant capacity.

Polyphenols are formed in plant products via the action of phenylalanine ammonia-lyase (PAL) in the phenylpropanoid metabolism.²¹ It could be hypothesized that MIPEF induced stress and increased PAL activity, thus enhancing TP content. This stress response is initiated when the plant recognizes a stimulus at the cellular level, which is initiated by the activity of specific ion channels.²² Voltage-gated ion channels are a specific type of transmembrane ion channel embedded in a cell membrane, which are activated by changes in the membrane electrical potential. Therefore, MIPEF may influence the voltage-gated ion channels and increase the membrane permeability for Ca²⁺ at the cellular level, followed by a rapid influx of Ca²⁺ through cation channels. Through this process, Ca²⁺-dependent protein kinase (CDPK) phosphorylates PAL,²³ which regulates the phenylpropanoid metabolism. The CDPK can also increase the ROS,²⁴ which are endogenous signal components required for the synthesis of secondary metabolites, such as polyphenols, known to be part of the plant defense response to stress.¹¹

The effect of the number of pulses and electric field strength on relative TP content was evaluated by a design surface methodology. The statistical analyses showed that the quadratic model proposed for relative TP content was adequate ($P < 0.001$), with a satisfactory determination coefficient ($R^2 = 0.9931$) (Table 3). The model showed no significant ($P > 0.05$) lack-of-fit, indicating a good fit for prediction within the range of assayed conditions. The linear term of electric field strength ($P < 0.001$), the linear term of number of pulses ($P < 0.001$), and the quadratic term of electric field strength ($P < 0.001$) significantly affected the relative TP content of tomato fruits (Table 3). Coefficients of the fitted equations are shown in Table 4. An overall increase in relative TP content was observed as the number of pulses rose (Figure 1). These results provide evidence that a fast metabolic response took place under 25–30 pulses at 0.7–1.1 kV/cm. The application of MIPEF results in an opening of pores in the cell membrane and consequently an efflux and influx of molecules. The influence of different stress factors such as wounding and light on the increased expression of phenol-biosynthetic genes has been reported,²⁵ and MIPEF may elicit a similar response. As can be seen in Figure 1, tomato fruit treated at the highest intensity field strength (2 kV/cm) and minimum number of pulses (5 pulses) showed the lowest relative TP content. It seems that treatments at 2 kV/cm may cause lethal damage to cells due to irreversible loss of cell membrane permeability properties.²⁶

Effect of MIPEF on Vitamin C Content. The vitamin C content of untreated tomato fruits was 98 mg/100 g dry weight, which is consistent with values reported in the literature.¹⁶ The relative vitamin C content of tomatoes 24 h after MIPEF

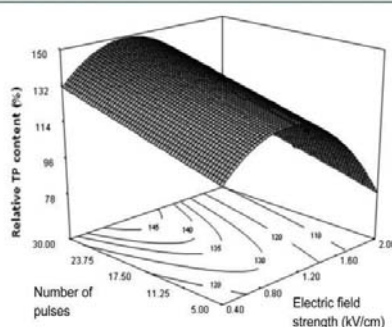


Figure 1. Effect of electric field strength and number of pulses on the relative TP content of tomatoes.

treatments ranged between 81.0% (5 pulses at 2 kV/cm) and 99.4% (18 pulses at 1.2 kV/cm) (Table 2). Ade-Omowaye et al.⁶ reported a relative vitamin C content ranging from 89.6% to 96.5% in red bell peppers immediately after the application of MIPEF treatments (50 pulses at 2 kV/cm, pulse duration 400 ms). Therefore, the application of MIPEF does not produce synthesis of vitamin C.

In order to evaluate the effects of number of pulses and electric field strength on relative vitamin C content, a surface design methodology was used. A second-order model showed a good fit with the relative vitamin C content results ($P < 0.001$). The determination coefficient, R^2 , was 0.9898, and the lack of fit was not significant ($P > 0.05$). The linear terms of electric field strength ($P < 0.001$) and number of pulses ($P < 0.001$), the quadratic terms of electric field strength ($P < 0.001$) and number of pulses ($P < 0.001$), and the interaction between both parameters ($P < 0.001$) had significant effects on the vitamin C content of the tomato fruits (Table 3). Coefficients of the fitted equations are presented in Table 4. As shown in Figure 2, a lower relative vitamin C content was observed in MIPEF-treated tomatoes than in untreated samples. The highest relative vitamin C content (99.4%) was obtained under 18–25 pulses at 0.8–1.2 kV/cm. Vitamin C is a typically labile nutrient and is also vulnerable to enzyme-catalyzed oxidation (specifically by ascorbate oxidase and peroxidase).²⁷ Our results demonstrate that 24 h after the application of MIPEF, tomato metabolism did not show an increase in vitamin C. Even more a slight decrease was observed in vitamin C in treated fruits.

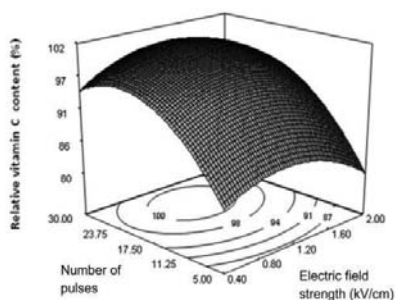


Figure 2. Effect of electric field strength and number of pulses on the relative vitamin C content of tomatoes.

Effect of MIEPEF on Lycopene Content. In untreated tomatoes, the lycopene content was 65 mg/100 g dry weight, which is consistent with values reported in the literature.¹⁰ A higher relative lycopene content was achieved in tomato fruits 24 h after the application of MIEPEF, except under 30 pulses at 2 kV/cm (Table 2). The increase in the relative lycopene content of tomato fruit ranged from 0.6% (18 pulses at 2 kV/cm) to 31.8% (5 pulses at 1.2 kV/cm), indicating an influence of MIEPEF on the carotenoid pattern. The maturation of tomato fruit is characteristically accompanied by a burst of ethylene.²⁸ Ethylene production, which leads to lycopene biosynthesis in tomatoes, is also known to be rapidly increased by stress, e.g., a pathogenic attack or elevated salinity.^{28,29} Thus, it is possible that MIEPEF promoted ethylene production and activated enzymes involved in lycopene biosynthesis, such as carotenoid isomerase or ζ -carotene desaturase.³⁰

A second-order model showed an adequate fit with the relative lycopene content ($P < 0.001$) with a satisfactory determination coefficient ($R^2 = 0.9922$) and insignificant lack of fit ($P > 0.05$) (Table 3). Coefficients of the fitted equations are shown in Table 4. The relative lycopene content was significantly affected by the linear terms of electric field strength ($P < 0.001$) and number of pulses ($P < 0.001$), the quadratic terms of electric field strength ($P < 0.001$), and number of pulses ($P < 0.05$). A fast metabolic response was observed when electric field strength increased between 0.4 and 1.2 kV/cm, showing a maximum bioproduction between 0.8 and 1.2 kV/cm, although the relative lycopene content decreases when electric field strength increases above 1.2 kV/cm. Moreover, treatments carried out at 5 pulses resulted in the highest lycopene content of tomato fruits. At 0.4 kV/cm, an enhancement of about 22% was observed with 5 pulses compared to 30 pulses (Figure 3). Similarly, increases of 22% and 18% were obtained with 5 pulses at 1.2 kV/cm and 30 pulses at 2 kV/cm, respectively. The metabolic response is initiated when the plant recognizes a stimulus at the cellular level, including changes in membrane electric potential. Therefore, MIEPEF may influence the voltage-gated ion channels and increase the membrane permeability for Ca^{2+} at the cellular level, followed by a rapid influx of Ca^{2+} through cation channels²³ and an acceleration of the biosynthesis of carotenoids in tomatoes.

Effect of MIEPEF on Antioxidant Capacity. *Hydrophilic Antioxidant Capacity (HAC).* Table 2 shows the relative HAC

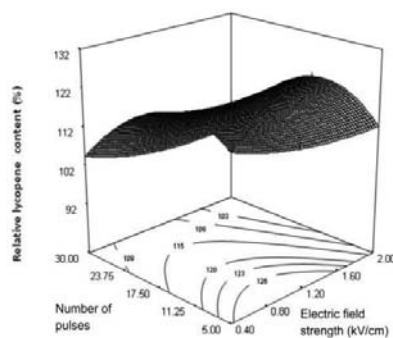


Figure 3. Effect of electric field strength and number of pulses on the relative lycopene content of tomatoes.

of MIEPEF-processed tomato fruits treated under the studied experimental conditions using DPPH and ABTS[•] methods. In untreated tomatoes, the HAC (DPPH) was 5.30 mmol TE/100 g dry weight, and HAC (ABTS[•]) was 4.85 mmol TE/100 g dry weight, being consistent with values reported in the literature.¹⁴ The increases in relative HAC of tomatoes 24 h after MIEPEF treatments ranged from 1.6% (5 pulses at 2 kV/cm) to 44.2% (18 pulses at 1.2 kV/cm) for the DPPH assay, and from 3.1% (30 pulses at 2 kV/cm) to 41.4% (18 pulses at 1.2 kV/cm) for the ABTS[•] assay. Thus, MIEPEF treatment significantly affected the metabolome after 24 h in promoting an increase in hydrophilic compounds. These results are in agreement with those reported by Galindo et al.,¹⁰ who strongly suggested that MIEPEF treatments significantly affect the tissue metabolome only in the long term (time scale of hours).

The effects of electric field strength and number of pulses on the relative HAC were studied. A second-order model fitted well ($P < 0.001$) with the relative HAC results in both assays (ABTS[•] and DPPH). The determination coefficients, R^2 , were 0.9951 (DPPH) and 0.9921 (ABTS[•]), and the lack of fit was not significant ($P > 0.05$). The linear terms of electric field strength ($P < 0.001$) and number of pulses ($P < 0.001$), and the quadratic terms of electric field strength ($P < 0.001$) and number of pulses ($P < 0.001$) had significant effects on the relative HAC of tomato fruits in both DPPH and ABTS[•] assays (Table 3). Thus, the HAC was represented by polynomial quadratic equations in terms of the studied PEF parameters (Table 4).

As can be seen in Figure 4a and b, the analyzed tomato fruits showed the same tendencies in both DPPH and ABTS[•] assays. A rise in relative HAC was observed when the electric field strength and number of pulses were increased between 0.4 and 1.2 kV/cm and 5–18, respectively, although it seemed to decrease when these parameters were increased further (Figure 4a and b). The maximum relative HAC was achieved with 0.6–1 kV/cm and 15–18 pulses (Figure 4a and b).

Antioxidant capacity is related to the amount and composition of bioactive compounds present in food.³¹ It is widely known that ascorbic acid together with polyphenols may be important in conferring antioxidative-related health benefits in tomatoes.¹⁴ A moderate relationship was found between relative HAC and TP content, $R^2 = 0.6794$ (DPPH) and $R^2 =$

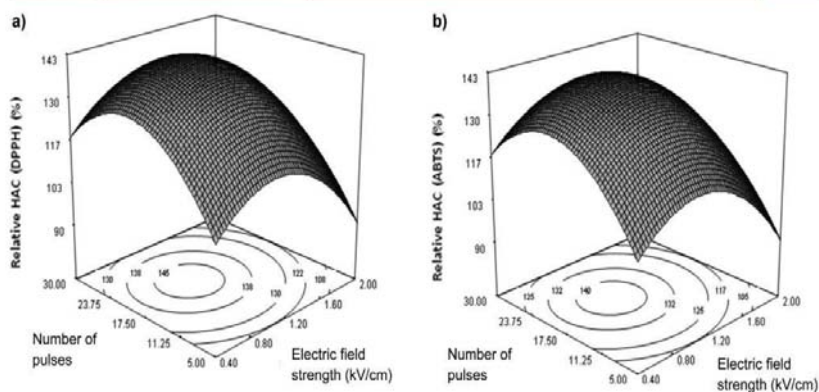


Figure 4. Effect of electric field strength and number of pulses on the relative hydrophilic antioxidant capacity of tomatoes. (a) DPPH. (b) ABTS[•].

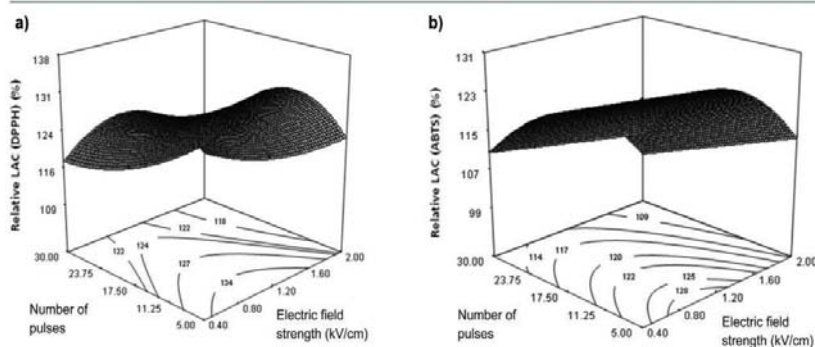


Figure 5. Effect of electric field strength and number of pulses on the relative lipophilic antioxidant capacity of tomatoes. (a) DPPH. (b) ABTS[•].

0.6515 (ABTS[•]), whereas a stronger relationship was observed between relative HAC and vitamin C content, $R^2 = 0.7272$ (DPPH) and $R^2 = 0.7319$ (ABTS[•]). As can be seen by comparing Figure 1 with Figure 4a and b, relative HAC and relative TP content did not follow the same pattern, the former reaching a peak at 15–22 pulses and the latter at 30 pulses. Thus, the levels of vitamin C and other antioxidant compounds in the tomato fruits seem to have exerted an important effect on the HAC, which increased 24 h after the MIEF treatments.

Lipophilic Antioxidant Capacity (LAC). The LAC of tomato fruits was measured on the basis of the DPPH and ABTS[•] assays. The LAC (DPPH) of untreated tomatoes was 4.73 mmol TE/100 g dry weight, and LAC (ABTS[•]) was 4.51 mmol TE/100 g dry weight. These results are in the range of those reported by Cano et al.³² MIEF-processed tomato fruits showed an increase in relative LAC between 10.4% (30 pulses at 2 kV/cm) and 37.4% (5 pulses at 1.2 kV/cm) in the DPPH assay and between 5.5% (18 pulses at 2 kV/cm) and 30.7% (5 pulses at 1.2 kV/cm) in the ABTS[•] assay 24 h after MIEF treatments (Table 2).

The effects of the number of pulses and electric field strength on relative LAC were also evaluated. A second-order model

showed a good fit with the relative LAC data (Table 3). The determination coefficient, R^2 , was 0.9883 (DPPH) and 0.9781 (ABTS[•]), and the lack-of-fit was not significant ($P > 0.05$). The linear terms of electric field strength ($P < 0.001$) and number of pulses ($P < 0.001$), and the quadratic term of electric field strength ($P < 0.001$) had significant effects on the relative LAC of tomato fruits in both DPPH and ABTS[•] assays (Table 3), while the quadratic term of number of pulses ($P < 0.05$) significantly affected the relative LAC of tomato fruits in the DPPH assay (Table 3). Coefficients of the fitted equations are shown in Table 4.

A rise in relative LAC was observed when electric field strength was increased between 0.4 and 1 kV/cm, beyond which it decreased (Figure 5a and b). The number of pulses also had a significant effect ($P < 0.001$) on the relative LAC of tomatoes. A maximum content of tomato lipophilic antioxidants was achieved by combining an electric field strength of 0.8–1.3 kV/cm with 5–8 pulses (Figure 5a and b), when a fast metabolic response was observed.

Lipophilic compounds such as carotenoids are responsible for the LAC of tomato fruits.¹⁴ A close relationship was found between the relative LAC and lycopene content: $R^2 = 0.9708$

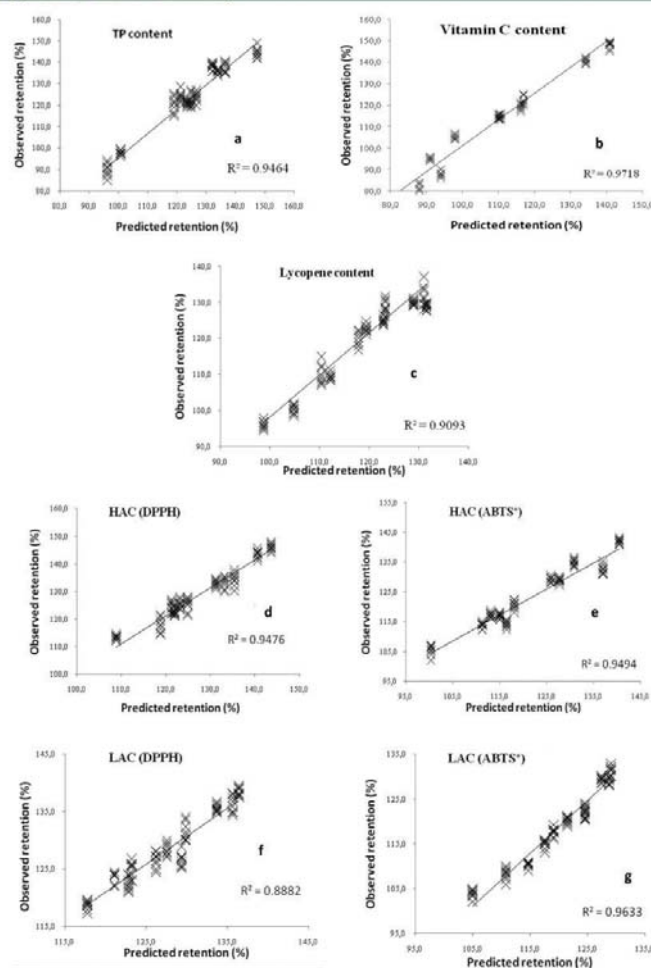


Figure 6. Scatter plots of the observed and predicted data of tomato fruit TP content (a), vitamin C content (b), lycopene content (c), HAC (ABTS^{•+}) (d), HAC (DPPH) (e), LAC (ABTS^{•+}) (f), and LAC (DPPH) (g). Retention of the validated trials. The straight line indicates the correlation between both groups of data.

(DPPH) and $R^2 = 0.9592$ (ABTS^{•+}). As can be seen when comparing Figure 3 with Figure 5a and b, the relative LAC and lycopene content show a similar tendency, indicating that the increases in LAC observed 24 h after MIPEF treatment could be due to the higher total lycopene content. These results are in

agreement with those of Cano et al.³² who described lycopene as the most important carotenoid in the lipophilic fraction of tomatoes.

Optimization and Validation of the MIPEF Processing Conditions. The MIPEF critical parameters that provided

tomato fruits with the highest nutritional quality were determined. The same priority was assigned to each dependent variable in order to obtain tomatoes with maximal content of TP, vitamin C, lycopene, and antioxidant capacity. All the individual desirability functions obtained for each response were combined into an overall expression, which is defined as the geometrical mean of the individual functions. The closer the desirability value to the unit, the more adequate the system. A desirability value of 0.822 was obtained when MIPEF treatment was conducted with 16 pulses at 1 kV/cm. Under these conditions, the predicted relative contents were TP (136.58%), vitamin C (98.33%), lycopene (120.10%), HAC-DPPH (143.83%), HAC-ABTS* (140.70%), LAC-DPPH (128.03%), and LAC-ABTS* (122.84%).

To validate the predictive models, a set of 88 experiments was carried out. The data comparison (Figure 6) showed that the proposed predicted expressions (Table 4) were accurate enough to fit experimental results. There were strong correlations between the observed and predicted retention data: TP (0.9464), vitamin C (0.9718), lycopene (0.9093), HAC-DPPH (0.9476), HAC-ABTS* (0.9494), LAC-DPPH (0.8882), and LAC-ABTS* (0.9633).

Conclusions. Increases in TP and lycopene contents as well as in the antioxidant capacity of MIPEF-treated tomato fruit were observed 24 h after treatments, depending on the electric field strength (0.4–2 kV/cm) and number of pulses (5–30). Maximum increases in TP (36.58%) and lycopene (20.10%) contents were obtained by combining 1 kV/cm and 16 pulses, contributing to an increase in the antioxidant capacity of tomato fruit by more than 20%.

Our results confirm an increase in bioactive compounds, which could be attributed to a MIPEF-induced stress response but also to an increased permeability of the cellular membrane due to MIPEF processing, which could potentially make the extraction of bioactive constituent more efficient. MIPEF treatments may induce stress reactions in tomato fruits after 24 h of refrigeration by stimulating metabolic activity and accumulating secondary metabolites. However, further investigations should be carried out to study in depth the MIPEF-induced stresses in plants.

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Notes

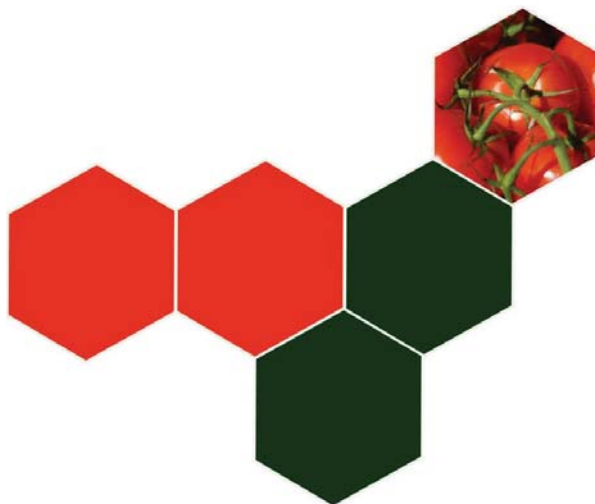
The authors declare no competing financial interest.

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DISCUSSIÓ GLOBAL



VI. DISCUSSIÓ GLOBAL

La discussió general dels resultats derivats d'aquesta tesi es presenta a continuació estructurada a partir dels articles anteriorment descrits al llarg del treball d'investigació. La primera part del treball d'investigació de la tesi doctoral comprèn la posada a punt de mètodes d'extracció optimitzats i específics de compostos polifenòlics en mostres de tomàquet i els seus derivats. Es va posar a punt i validar un mètode per a la determinació de polifenols totals en aquestes mostres fent servir l'anàlisi d'*FC*. Per aplicar aquesta metodologia va ser necessari fer una neteja prèvia de les mostres mitjançant *SPE* per eliminar les possibles interferències de substàncies reductores que reaccionen amb el reactiu d'*FC*. Es van utilitzar plaques de 96 pous, la qual cosa permet analitzar 96 mostres en 3 hores a diferència d'altres mètodes que necessitaven un temps d'aproximadament 12.5 hores (Saura-Calixto & Goñi, 2006; Singleton VL *et al.*, 1999). Les plaques de 96 pous fan la metodologia més pràctica en l'anàlisi d'un gran nombre de mostres d'aliments. Es van provar diferents tipus de cartutxos per obtenir la major sensibilitat i menor efecte matriu en la posterior detecció per *MS*. Els cartutxos de bescanvi aniònic amb fase reversa (Oasis® *MAX*) van donar els millors resultats de recuperació i precisió en els flavonols, flavanones i àcids fenòlics.

La metodologia va ser validada amb 10 patrons de polifenols de diferents polaritats i a diferents concentracions. Les recuperacions es van trobar en el rang 77-109% i els valors d'*RSD%*, per a la precisió *intraday* i *interday*, van ser inferiors al 15% en tots els casos, per la qual cosa compleix els criteris *AOAC International* (Horwitz, 2003). A més a més, es va estudiar l'estabilitat dels polifenols a curt i llarg termini i, també, després de diversos cicles de congelació i descongelació a -20 °C i a -80 °C. Els compostos fenòlics van ser estables en les condicions de manipulació i emmagatzematge d'aquesta metodologia.

Aquesta metodologia es va aplicar per fer l'anàlisi de polifenols en diferents varietats de tomàquet (publicació 1). Posteriorment a la fase de preparació, es van fer servir dues tècniques (*HPLC-LTQ-Orbitrap-MS* i *HPLC-MS/MS*) per a la identificació de 38 polifenols, 3 dels quals, l'*O*-acetilprunina, un *C*-glicòsid de la ploridzina i un diglicòsid de la rutina no havien estat identificats anteriorment en aquest tipus de

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productes. Els *O*-glicòsids mostren una fragmentació diferent dels *C*-glicòsids. Els *O*-glicòsids presenten pèrdues de 162 o 132 unitats, segons si són hexoses o pentoses, mentre que els *C*-glicòsids mostren pèrdues de 120 i 90 unitats, la qual cosa ja havia estat descrita anteriorment en fonoll (*Foeniculum vulgare*) i cacau (*Theobroma cacao*) fent servir *HPLC-MS/MS* (Parejo *et al.*, 2004; Sánchez-Rabaneda *et al.*, 2003).

L'*HPLC-LTQ-Orbitrap-MS* permet l'anàlisi d'*MS*, *MS/MS* i *MSⁿ* i facilita l'obtenció d'informació estructural. A causa de l'especificitat de les mesures d'*MSⁿ* i la capacitat d'eliminar les interferències a través de l'etapa inicial de selecció de masses, l'*HPLC-LTQ-Orbitrap-MS* és un dels millors equips per l'anàlisi qualitativa; facilita l'elucidació de l'estructura de compostos desconeguts. Per exemple, l'*HPLC-LTQ-Orbitrap-MS* és crucial per a la determinació estructural de glicòsids dels àcids *p*-coumàric, feruloilquínic i coumaroilquínic, així com, també, de diglicòsids de la rutina, *C*-glicòsids de la ploridzina i l'*O*-acetilprunina. L'*HPLC-LTQ-Orbitrap-MS* permet obtenir molt bones exactituds per a tots els ions moleculars. En general, les diferències entre la *m/z* calculada i mesurada va ser inferior a 2 mDa.

Una de les característiques més importants dels polifenols és el seu ús com a marcadors entre varietats (Andrés-Lacueva *et al.*, 2002; De La Presa Owens *et al.*, 1995; Romero Pérez *et al.*, 1996; Russo *et al.*, 1998; Singleton & Trousdale, 1983). En la publicació 2, es va estudiar el perfil fenòlic de diferents varietats de tomàquet (H-9661, H-9997, H-9661, *malva*, *guadiva*, *albastro* i *elegy*) procedents de l'empresa CONESA de Vegas Bajas del Guadiana, Badajoz, Espanya, durant dos anys consecutius, 2008 i 2009. Es va portar a terme l'anàlisi de polifenols totals, pel mètode de l'*FC*, amb prèvia *SPE*; l'anàlisi de la capacitat antioxidant, mitjançant el mètode *ABTS⁺*; i l'anàlisi individual de polifenols, mitjançant *HPLC-MS/MS*. S'hi van trobar 10 marcadors responsables de la diferenciació entre aquestes varietats: la quercetina, la rutina, la naringenina, el 3-*O*-glucòsid del kaempferol, els àcids cafeic, un glicòsid del cafeic, el ferúlic, el clorogènic, el gàl·lic i el protocatèquic. A més a més, els polifenols totals i la capacitat antioxidant van facilitar la diferenciació entre les diferents varietats de tomàquet analitzades.

Els resultats obtinguts van ser coherents amb els que prèviament havien observat Singleton *et al.* (1983) en diferents vins produïts a partir de les varietats de raïm: *chenin blanc*, *french colombard*, *semillon* i *thompson*. En aquest estudi, es va descriure que les diferents varietats de vins es veuen influïdes pel perfil fenòlic. De la

mateixa manera, diferents varietats de mosts (De La Presa Owens *et al.*, 1995), vins (Darias-Martín *et al.*, 2008; Romero Pérez *et al.*, 1996) i caves (Andrés-Lacueva *et al.*, 2002) presenten diferents perfils fenòlics. Russo *et al.* (1998) van estudiar la relació entre la composició química de 24 mostres d'olis essencials. Van trobar 4 marcadors en base al perfil fenòlic: timol, carvacrol, timol/carvacrol i carvacrol/timol. Consegüentment, el perfil fenòlic i la capacitat antioxidant són unes eines essencials tant per diferenciar entre varietats de vins i olis essencials com també diferents tipus de tomàquets.

En les publicacions 3 i 4, es van analitzar els efectes del processat industrial en el perfil fenòlic de salses i daus de tomàquet. Les mostres es van obtenir de l'empresa CONESA de Vegas Bajas del Guadiana, Badajoz, Espanya, durant dos anys consecutius 2008 i 2009. Es va portar a terme l'anàlisi de polifenols totals, pel mètode de l'FC, amb prèvia SPE; l'anàlisi de la capacitat antioxidant, mitjançant els mètodes ABTS⁺ i DPPH; i l'anàlisi individual de polifenols, mitjançant HPLC-MS/MS. Els marcadors estudiats van ser: la rutina, la quercetina, la naringenina, el 7-O-glucòsid de la naringenina, el 3-O-glucòsid del kaempferol i els àcids ferúlic, cafeic, clorogènic, protocatèquic, un glicòsid del cafeic i un glicòsid del ferúlic. En les salses i daus de tomàquet, el compost fenòlic majoritari va ser la rutina, seguida de la naringenina. Aquests resultats ja s'havien obtingut prèviament en altres estudis (Le Gall *et al.*, 2003; Slimestad *et al.*, 2008).

Per a l'obtenció de salses de tomàquet, publicació 3, els processos estudiats van ser: *passata* 10/12, *cold break* 28/30, *hot break* 28/30 i *hot break* 22/24. En cada un dels processos, s'hi van monitoritzar 4 punts: (1) tomàquets frescs, (2) salsa després de l'addició de pells i llavors riques en antioxidants, (3) salsa després de l'evaporador i (4) producte final.

El processat tèrmic dels tomàquets per obtenir el producte final inclou diverses etapes que necessiten l'aplicació de calor, la qual cosa pot tenir un efecte negatiu en els compostos termolàbils, com ara els polifenols. La integritat de la matriu cel·lular dels tomàquets determina la biodisponibilitat dels polifenols i antioxidants. El processat pot trencar aquesta matriu cel·lular. Dewanto *et al.* (2002) van estudiar que el contingut de polifenols totals no es veu modificat durant el processat dels tomàquets. En canvi, els resultats obtinguts en aquesta tesi doctoral mostren un increment en el contingut de polifenols i la capacitat antioxidant quan els tomàquets

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frescs són processats per obtenir salses en els processos *passata* 10/12, *hot break* 22/24 i *hot break* 28/30. Aquest fet és degut a l'addició de pells i llavors riques en antioxidants, les quals s'extreuen per separat durant el processat dels tomàquets i després es retornen a la salsa en aquest punt (punt 2). L'addició de pells i llavors s'utilitza com a tècnica de reconstitució en indústries per augmentar la viscositat dels productes. Cal tenir en compte que en el procés *cold break* 28/30, no s'hi van observar aquests augments, ja que no es va dur a terme l'addició de pells i llavors durant el processat industrial.

En l'etapa d'evaporació (punt 3), quan els temps d'evaporació eren més llargs (procés *hot break* 28/30), es van observar pèrdues significatives ($P < 0.05$) en el nivell de polifenols i capacitat antioxidant, la qual cosa és comparable amb els resultats obtinguts per Capanoglu *et al.* (2008) que van observar una disminució en el contingut d'alguns polifenols després de tractar les mostres tèrmicament a 80 °C en un evaporador.

A l'últim pas, després de l'etapa d'esterilització i envasat (punt 4), les pèrdues en el nivell de polifenols i capacitat antioxidant no van ser tan acusades com després de l'etapa d'evaporació (punt 3). Aquests resultats són similars als obtinguts per Re *et al.* (2002) Ells van estudiar l'efecte de 3 mètodes de processats per obtenir salses de tomàquet: *hot break* a 90 °C, *cold break* a 65 °C i *super cold break* i van concloure que durant el processat de salses de tomàquet disminueix el nivell de flavonoides.

Per a l'obtenció de daus de tomàquet, publicació 4, els processos estudiats van ser: *hot break*, *cold break* i *cold break* amb sals de calci (CaCl_2). L'addició de CaCl_2 es porta a terme a les etapes finals de processat, la funció de les quals és aconseguir un producte amb elevada fermesa. En cada un dels processats, s'hi van monitoritzar 4 punts: (1) tomàquets frescs, (2) tomàquets pelats, (3) daus de tomàquet i (4) producte final. Aquest ha estat el primer estudi que ha analitzat els canvis durant el processat de daus de tomàquet en l'àmbit industrial.

Durant el processat, s'observa una disminució gradual significativa ($P < 0.05$) en el nivell de polifenols i antioxidants des del punt 1 al punt 3, com ja s'ha comentat anteriorment, en les salses de tomàquet (publicació 3). El procés que presenta la disminució més important és el *hot break*. Les elevades temperatures que són necessàries per produir els productes desitjats han estat associades amb les altes

pèrdues en el contingut de compostos bioactius. En aquest cas, també es fa servir l'estratègia de reconstitució per augmentar la viscositat del producte mitjançant l'addició de pells i llavors riques en antioxidants. L'increment més elevat s'obté en el procés *cold break* sense sals de calci, les quals poden inhibir l'acumulació de compostos fenòlics. Existeix certa controvèrsia respecte els efectes que les sals de calci poden causar en el nivell de polifenols. Floros *et al.* (1992) van estudiar que les sals de calci fan innecessaris els tractaments tèrmics a altes temperatures, ja que aquestes sals inhibeixen l'activitat de la peroxidasa i la fenoloxidasa. No obstant això, Rensburg & Engelbrecht (1986) van descriure que els tractaments amb sals de calci disminueixen el contingut de polifenols i altres antioxidants.

La disminució en el contingut de polifenols i la capacitat antioxidant durant el processat de salses i daus de tomàquet pot ser a causa de la degradació oxidativa dels polifenols. Aquests resultats són similars als obtinguts per l'estudi de Lavelli *et al.* (1999). És obvi que la presència d'oxigen accelera la degradació dels polifenols. La degradació és ocasionada per les ROS. Aquests radicals poden ser capturats pels flavonols, la qual cosa pot portar a la formació de quinones. A més a més, els àcids fenòlics, també, es poden oxidar i formar quinones. Les O-quinones poden interaccionar amb el NH₂ dels aminoàcids i causar pèrdues nutricionals (Friedman, 1992). Durant els tractaments tèrmics de salses i daus de tomàquet, la quercetina és el polifenol que presenta una disminució més elevada a diferència de la rutina o el 3-O-glucòsid del kaempferol. En el cas de la rutina i del 3-O-glucòsid del kaempferol, el 3-OH de l'anell d'aquests flavonoides està bloquejat per una hexosa, mentre que per a la quercetina roman desocupat. Per tant, el bloqueig del 3-OH és la raó fonamental per a l'elevada estabilitat de la rutina i del 3-O-glucòsid del kaempferol davant l'oxidació (Makris & Rossiter, 2000).

El següent treball de la tesi doctoral, publicació 5, va consistir en la identificació exhaustiva dels polifenols de tres derivats del tomàquet: quètxups, gaspatxos i suc de tomàquet. Per això es va fer servir un *HPLC-QToF-MS*. El perfil fenòlic mostra clarament que aquests tres derivats del tomàquet es diferencien en la seva composició fenòlica, principalment en 7 tipus de marcadors: un glicòsid de l'àcid protocatèquic, l'apigenina i les seves formes glicosilades, un dihexòsid de la quercetina, un C-hexòsid del kaempferol i un dihexòsid de l'eriodictiol.

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El glicòsid de l'àcid protocatèquic es va detectar en gaspatxos i quètxups. Com s'ha descrit en la literatura, l'àcid protocatèquic i els seus derivats poden provenir d'altres estructures més complexes com a conseqüència dels tractaments tèrmics (Ruíz-Rodríguez *et al.*, 2008). L'O-dihexòsid de l'eriodictiol només es va detectar en suc de tomàquet i el dihexòsid de la quercetina, el C-hexòsid del kaempferol i l'apigenina i els seus derivats només es van trobar en els gaspatxos, probablement causat per la presència d'altres hortalisses per a la seva producció com ara les cebes i pebrots. És possible distingir entre els diferents derivats de l'apigenina (un C-dihexòsid i un C-hexòsid-pentòsid) per la presència de l'ió $[M-H-60]^-$. Aquests resultants ja s'havien obtingut prèviament en un estudi realitzat per Han *et al.* (2008) mitjançant *HPLC-MS/MS*. L' MS^2 del C-dihexòsid del kaempferol produeix pèrdues de 120 i 90 unitats, però no s'observa la pèrdua de 60 unitats; en canvi, per al C-hexòsid-pentòsid s'hi observen pèrdues de 180, 120, 90 i 60 unitats, la qual cosa suggereix la presència d'una substitució de 5 carbonis.

Tant els quètxups, com els gaspatxos i els suc de tomàquet són productes d'un elevat contingut en polifenols, els quals són beneficiosos per a la salut. A més a més, la transformació del tomàquet a derivats de tomàquet és una estratègia econòmica per preservar la vida útil dels tomàquets frescs que inevitablement es deterioren amb el temps. Els compostos fenòlics dels derivats del tomàquet no es veuen afectats pels tractaments tèrmics aplicats, i no s'hi observen reduccions en comparació als tomàquets frescs (Vallverdú-Queralt *et al.*, 2010).

Els pròxims estudis de la tesi doctoral, publicacions 6, 7 i 8, es van centrar en les diferències entre tomàquets, suc de tomàquet i quètxups convencionals i ecològics. En els tres estudis, s'hi ha observat un major contingut de polifenols en els cultius ecològics que en els tradicionals. En canvi, els quètxups de cultiu tradicional presentaven un contingut més elevat de dos dipèptids, un derivat del triptòfan i un de la fenilalanina. Els nostres resultants són similars als que ja s'havien trobat anteriorment per Woese *et al.* (1997) que van descriure que en cereals de cultiu ecològic s'hi troba menys quantitat de proteïnes que en els de cultiu convencional. Aquestes diferències estan clarament reflectides en l'anàlisi metabòlica, la qual va permetre establir clares diferències entre el cultiu ecològic i tradicional. L'anàlisi

metabolòmica permet detectar molts metabòlits simultàniament i comparar mostres per trobar similituds i diferències d'una manera no dirigida.

Les diferències fonamentals entre els sistemes de producció ecològics i convencionals es fonamenten sobretot en la manera de cultivar i adobar el sòl, la qual cosa pot afectar la composició nutritiva dels tomàquets i en particular els metabòlits secundaris (Daniel *et al.*, 1999; Williams, 2002). En el cultiu convencional, està permès utilitzar fertilitzants que contenen nitrogen inorgànic soluble i altres nutrients, que són més directament absorbits per les plantes. La disponibilitat de nitrogen inorgànic condiona la síntesi dels metabòlits secundaris de les plantes, de les proteïnes i dels sòlids solubles. A més a més, les plantes de producció ecològica tenen un període més llarg de maduració en comparació amb les plantes convencionals, a causa d'un alliberament més lent dels nutrients subministrats (Nielsen *et al.*, 1997), i com que els flavonoides es formen en el període de maduració, es podria esperar un contingut més elevat d'aquests compostos en les plantes de cultiu ecològic (Vallverdú-Queralt *et al.*, 2012).

Els estudis de composició fenòlica en hortalisses cultivades de manera ecològica i tradicional són escassos. En general, es descriu un contingut més elevat de compostos fenòlics en fruites i hortalisses ecològiques (Brandt *et al.*, 2011). El nostre estudi presenta els mateixos resultats, ja que els tomàquets i derivats del tomàquet ecològics presenten més contingut de polifenols i capacitat antioxidant que els convencionals. Chassy *et al.* (2006) van trobar continguts més elevats de sòlids solubles, flavonoides, polifenols totals i àcid ascòrbic en tomàquets ecològics durant un període de tres anys consecutius. Pel que fa a polifenols individuals, Caris-Veyrat *et al.* (2004) van atribuir concentracions superiors d'àcid clorogènic en tomàquets de cultiu ecològic. Pel que fa als nivells de quercetina, naringenina i kaempferol, els nivells eren també superiors en cultius ecològics. Els nostres resultats estan en línia amb els que van descriure Mitchell *et al.* (2007), els quals van detectar nivells de quercetina, naringenina i kaempferol significativament superiors ($P < 0.001$) en tomàquets ecològics. En un altre estudi dissenyat per avaluar el contingut fenòlic en pomes (Golden Delicious) cultivades mitjançant agricultura ecològica i convencional, es va trobar que les pomes ecològiques presentaven concentracions significativament més

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elevades d'àcid clorogènic, flavonols, flavanols i dihidrocalcones que les produïdes mitjançant agricultura convencional (Stracke *et al.*, 2009).

Pel que fa a estudis en humans, Grønder-Pedersen *et al.* (2003) van comparar dietes que provenien de cultiu ecològic amb altres de cultiu convencional en estudis d'intervenció creuats en humans (n=16) en termes d'excreció de 5 flavonoides. L'excreció urinària de la quercetina i el kaempferol va ser més elevada després de 22 dies de consum de dietes que provenien de cultiu ecològic ($P < 0.05$). Per tant, el mètode de producció dels aliments no només afecta el contingut de flavonoides en aliments, sinó també el contingut de flavonoides en orina. No obstant això, Stracke *et al.* (2010) van comparar dietes que provenien de pomes de cultiu ecològic amb pomes de cultiu tradicional en estudis a curt i llarg termini i no es van observar augments en la concentració de polifenols en plasma i orina.

El següent estudi d'aquesta tesi doctoral, publicació 9, es va centrar en la identificació i quantificació de carotens en mostres de tomàquets i derivats de tomàquet. Es va utilitzar la combinació de dues tècniques: *HPLC-UV* i *HPLC-ESI-(Li⁺)-MS/MS*. S'hi van identificar 7 carotens: luteïna, α -carotè, β -carotè, *trans*-licopè, 5-, 9- i 13-*cis*-licopè. A més a més, es va estudiar la capacitat antioxidant lipofílica mitjançant els mètodes *DPPH* i *ABTS⁺*. Els resultats mostren que l'activitat antioxidant lipofílica ve determinada principalment per la presència de *trans*-licopè i β -carotè (Cano *et al.*, 2003; Raffo *et al.*, 2006). El perfil de carotens varia en funció del derivat de tomàquet a estudiar a causa de les diferents proporcions d'ingredients com el tomàquet, l'oli, les cebes o els alls utilitzats per a la preparació del producte. El *trans*-licopè és el carotè més abundant en tomàquets (Abushita *et al.*, 2000; Leonardi *et al.*, 2000). El processat tèrmic, que és necessari per a la preparació dels quètxups i gaspatxos, incrementa la biodisponibilitat del licopè (Boehm & Bitsch, 1999). Les diferències entre els derivats de tomàquet podrien ser degudes principalment als factors genètics i a l'augment de l'activitat enzimàtica de la fitoè sintasa I, que causa una elevada producció de precursors de licopè en tomàquets d'elevada maduresa (Fraser *et al.*, 2009).

Pel que fa als isòmers del *cis*-licopè, són de gran interès nutricional, perquè són absorbits més fàcilment en l'intestí (Failla *et al.*, 2008; Unlu *et al.*, 2007). El perfil d'isòmers derivats del *cis*- i *trans*-licopè en els diferents derivats de tomàquet estudiats és el mateix que ja havia estat descrit anteriorment. El *trans*-licopè representa el licopè

més abundant i varia del 35% al 96% del licopè total; els 5-, 9-, 13- i 15-*cis*-licopè són els isòmers *cis* majoritaris (Frohlich *et al.*, 2007). Durant el tractament tèrmic, es porta a terme la *trans/cis* isomerització, per la qual cosa s'incrementa la proporció d'isòmers *cis* (Lin & Chen, 2005).

Pel que fa a la luteïna, α -carotè i β -carotè també mostren clares diferències depenent del tipus de derivat de tomàquet. Per als quètxups, el β -carotè representa entre el 10% i el 32% del contingut total de carotens, mentre que, per als gaspatxos, varia entre el 50% i el 62% i és el carotè majoritari. Aquesta diferència pot ser deguda a l'addició d'all i cebes utilitzada per a la preparació dels gaspatxos. S'ha descrit que l'all i la ceba milloren la retenció de β -carotè en el processat tèrmic (El-Sherefa., 2006). Altres carotens com l' α -carotè i la luteïna representen menys del 6.8% dels carotens totals (Raffo *et al.*, 2006). La provitamina A dels tomàquets, quètxups i gaspatxos deriva principalment del β -carotè. Els tractaments tèrmics estimulen la transformació d'alguns carotens. Per exemple, el γ -carotè es cicla per formar anells de 6 carbonis al final de la molècula, la qual cosa pot portar a la formació del β -carotè (Quitao-Teixeira *et al.*, 2009).

Els següents estudis, publicacions 10-11, es van centrar a avaluar els efectes de l'emmagatzematge de suc de tomàquet, quètxups i gaspatxos a 4 °C en el contingut de polifenols i carotens mitjançant l'anàlisi de polifenols totals, pel mètode de l'FC, amb prèvia SPE; l'anàlisi de la capacitat antioxidant mitjançant els mètodes ABTS⁺ i DPPH; i l'anàlisi individual de polifenols i carotens, mitjançant HPLC-MS/MS i HPLC-UV.

El contingut de polifenols i la capacitat antioxidant hidrofílica van disminuir durant l'emmagatzematge a 4 °C dels diferents derivats del tomàquet. Els polifenols que van presentar més disminució van ser el ferúlic, el cafeic i la quercetina; en canvi, els polifenols glicosilats van disminuir només lleugerament. L'elevada estabilitat de les formes glicosilades respecte les aglicones pot ser atribuïda al bloqueig de la posició 3-OH de l'anell dels polifenols per un glicòsid (Makris & Rossiter, 2000). La degradació és ocasionada per les ROS. Aquests radicals poden ser capturats pels flavonoides, la qual cosa pot portar a la formació de quinones. A més a més, els àcids fenòlics també es poden oxidar i formar quinones. Les O-quinones poden interaccionar amb el NH₂ dels aminoàcids i causar pèrdues nutricionals (Friedman, 1992).

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Pel que fa als carotens i capacitat antioxidant lipofílica també van disminuir durant l'emmagatzematge de derivats del tomàquet a 4 °C. El *trans*-licopè, el qual conté 11 dobles enllaços conjugats, és més reactiu que la luteïna, l' α -carotè o el β -carotè i va presentar una disminució d'un 17% després de 3 mesos d'emmagatzematge de gaspatxos a 4 °C. En un altre estudi, Zandoni *et al.* (1998) també van descriure que el *trans*-licopè presentava pèrdues del 50% en tomàquets després de l'emmagatzematge durant 30 dies i del 70% després de 90 dies. Odriozola-Serrano *et al.* (2008) van descriure pèrdues del 70% en *trans*-licopè en suc de tomàquet emmagatzemat durant 3 mesos a 4 °C. Pel que fa als *cis*-isòmers del lycopè, van disminuir lleugerament i el 5-*cis*-licopè va augmentar durant l'emmagatzematge de gaspatxos. Resultats similars ja havien estat descrits en suc de tomàquet (Lin & Chen, 2005) emmagatzemats durant 3 mesos, ja que s'hi observava com augmentava la proporció d'isòmers *cis* del lycopè, la qual cosa es pot atribuir al fenomen d'isomerització que es porta a terme durant el tractament tèrmic (Lin & Chen, 2005).

Per tant, l'emmagatzematge de derivats de tomàquet a 4 °C provoca una disminució en el contingut de polifenols i carotens com també en les capacitats antioxidants hidrofíliques i lipofíliques. No obstant això, aquestes disminucions no presenten cap desavantatge per al consumidor. Es pot esperar un efecte beneficiós de la consumició d'aquests derivats en qualsevol punt del seu emmagatzematge.

Un dels pròxims reptes en l'anàlisi d'aliments és utilitzar tècniques per incrementar el nivell de polifenols i carotens en aliments. En la publicació 12, s'hi han utilitzat els PEBIC per augmentar el nivell de compostos bioactius i capacitat antioxidant en els tomàquets. Els polsos elèctrics provoquen un estrès al fruit amb la consegüent generació de metabòlits secundaris al cap de 24 h de refrigeració a 4 °C.

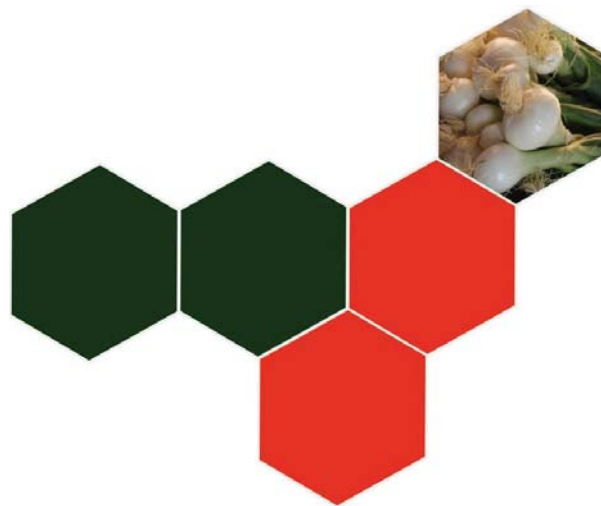
En aquests estudis, s'hi va treballar a intensitats de camp entre 0.4 i 2 kV/cm i entre 5 i 30 polsos. A intensitats de camp de 1.2 kV/cm, s'aconsegueix el màxim increment de polifenols i carotens; en canvi a 2 kV/cm, disminueix el nivell de compostos bioactius i capacitat antioxidant. Per tant, en aquestes condicions els PEBIC poden causar danys irreversibles a les cèl·lules del fruit amb la consegüent pèrdua de la permeabilitat de les membranes (Aronsson *et al.*, 2001). Pel que fa al nombre de polsos, a 5 polsos s'hi observa un increment en la biosíntesi dels carotens; en canvi, en

els polifenols la màxima producció és a 30 polsos. Pel que fa a la vitamina C, disminueix lleugerament després de l'aplicació dels PEBIC.

És difícil explicar els increments dels polifenols a causa de la complexitat de reaccions químiques que es duen a terme en productes naturals. Els flavonoides es formen per l'acció de la fenilalanina amoni-liasa mitjançant el metabolisme dels fenilpropanoids (Odriozola-Serrano *et al.*, 2009b). Els tractaments de PEBIC podrien induir estrès i incrementar l'activitat de la fenilalanina amoni-liasa i, per tant, augmentar el nivell de polifenols. Aquesta resposta a l'estrès és iniciada quan la planta reconeix un estímul a nivell cel·lular, que és iniciat per l'activitat específica de diferents canals iònics (Gaspar *et al.*, 2002). Els canals iònics són proteïnes o un agregat de proteïnes que obren porus en la membrana cel·lular i es poden obrir responenent a un estímul. Aquest estímul pot ser un canvi de voltatge. Per tant, els PEBIC poden influir en els canals iònics i incrementar la permeabilitat del Ca^{2+} a nivell cel·lular, seguit per un influx ràpid de Ca^{2+} a través dels canals. A través d'aquest procés, les quinases depenents del transport de Ca^{2+} fosforilen la fenilalanina amoni-liasa, la qual regula el metabolisme dels fenilpropanoids (Sanders *et al.*, 2002).

Els mecanismes implicats en la síntesi de carotens en plantes són relativament poc coneguts. Els enzims biosintètics són codificats per gens nuclears i les proteïnes precursors són importades cap als plastidis, en els quals té lloc la síntesi de carotens. La ruptura dels teixits provocada per l'estrès pot promoure la transcripció dels gens o el transport de l'àcid ribonucleic missatger relacionat amb la síntesi de carotens (Bartley & Scolnik, 1995). La maduració dels tomàquets va acompanyada d'una producció d'etilè i una consegüent producció de licopè (Dannehl *et al.*, 2011). La producció d'etilè s'incrementa amb l'estrès com, per exemple, en casos d'elevada salinitat o atac de patògens (Dannehl *et al.*, 2011; Shibli *et al.*, 2007). Per tant, els polsos elèctrics podrien accelerar la producció d'etilè i activar els enzims implicats en la síntesi de licopè i els seus derivats (DellaPenna & Pogson, 2006).

CONCLUSIONS



VII. CONCLUSIONS

Conclusions generals

- ✓ La varietat, el tipus de cultiu, el processat i l'emmagatzematge afecten el nivell de compostos bioactius.
- ✓ El coneixement dels punts crítics pot permetre obtenir aliments amb més compostos bioactius.

Conclusions específiques

- ✓ S'ha validat un mètode sensible, específic, ràpid i reproducible per analitzar els polifenols totals en mostres de tomàquet, amb prèvia neteja mitjançant *SPE*. Aquest mètode permet obtenir més sensibilitat i menor efecte matriu en la posterior detecció per *MS*.
- ✓ S'ha utilitzat la combinació de dues tècniques (*HPLC-LTQ-Orbitrap-MS* i *HPLC-MS/MS*) per a la identificació de 38 polifenols en tomàquets i derivats de tomàquet, 3 dels quals, l'*O*-acetilprunina, un *C*-diglicòsid de la ploridzina i un diglicòsid de la rutina, no havien estat identificats anteriorment.
- ✓ La varietat condiciona el perfil fenòlic i la capacitat antioxidant del tomàquet.
- ✓ Durant el processat, s'observa una disminució gradual significativa en el nivell de polifenols i antioxidants. Les temperatures elevades són el principal responsable de les pèrdues en el contingut d'aquests compostos bioactius. No obstant això, l'addició de pells i polpa de tomàquet permet obtenir un producte final amb un contingut més elevat que la matèria primera.
- ✓ S'ha efectuat una identificació exhaustiva dels polifenols de tres derivats del tomàquet: quètxups, gaspatxos i suc de tomàquet mitjançant *HPLC-QToF-MS*. El perfil fenòlic mostra que aquests tres derivats del tomàquet es diferencien principalment en 7 tipus de marcadors: un glicòsid de l'àcid protocatèquic, l'apigenina i les seves formes glicosilades, un dihexòsid de la quercetina, un *C*-hexòsid del kaempferol i un dihexòsid de l'eriodictiol.

Conclusions

- ✓ S'ha observat un major contingut de polifenols en els cultius ecològics que en els tradicionals. En canvi, en els quètxups de cultiu tradicional s'hi va detectar la presència de dos dipèptids, un derivat del triptòfan i un de la fenilalanina. Aquest comportament està clarament reflectit en l'anàlisi metabolòmica, la qual va permetre establir clares diferències entre el cultiu ecològic i tradicional.
- ✓ S'han identificat i quantificat els carotens en quètxups i gaspatxos mitjançant la combinació de dues tècniques (*HPLC-UV* i *HPLC-ESI-(Li⁺)-MS/MS*). S'hi van identificar 7 carotens: luteïna, α -carotè, β -carotè, *trans*-licopè, 5-, 9- i 13-*cis*-licopè i es va estudiar la capacitat antioxidant lipofílica. Els resultats mostren que l'activitat antioxidant lipofílica ve determinada principalment per la presència de *trans*-licopè i β -carotè.
- ✓ L'emmagatzematge de derivats de tomàquet a 4 °C provoca una disminució en el contingut de polifenols i carotens com també en les capacitats antioxidants. No obstant això, aquestes disminucions no comporten un perjudici per al consumidor quant als compostos bioactius. Es pot esperar un efecte beneficiós del consum d'aquests derivats en qualsevol punt del seu emmagatzematge.
- ✓ S'han utilitzat els polsos elèctrics de baixa intensitat de camp per augmentar el nivell de compostos bioactius i la capacitat antioxidant dels tomàquets. A intensitats de camp d'1.2 kV/cm, s'aconsegueix el màxim increment de polifenols, carotens i vitamina C; en canvi a 2 kV/cm, disminueix el nivell de compostos bioactius i la capacitat antioxidant. Pel que fa al nombre de polsos, a 5 polsos s'observa un increment en la biosíntesi dels carotens; en canvi, per als polifenols, la màxima producció és a 30 polsos.

VII. CONCLUSIONS

General conclusions

- ✓ The variety, type of cultivation, processing and storage affect the level of bioactive compounds.
- ✓ Knowledge of the critical points may provide food with a higher level of bioactive compounds.

Specific conclusions

- ✓ A sensitive, specific, rapid and reproducible method using *SPE* has been validated to analyse polyphenols from tomato samples. This method provides greater sensitivity and less matrix effect on the subsequent detection by mass spectrometry.
- ✓ A combination of two techniques (*HPLC-LTQ-Orbitrap-MS* and *HPLC-MS/MS*) has been used to identify 38 polyphenols in tomatoes and tomato-based products, 3 of which, *O*-acetylprunine, a *C*-diglycoside of phloridzin and a diglycoside of rutin, have not been previously identified.
- ✓ The variety determines the phenolic profile and antioxidant activity of tomatoes.
- ✓ During processing, there was a significant gradual decrease in the level of polyphenols and antioxidants. The high temperatures are responsible for the decreases in these compounds. However, the addition of tomato seeds and skin fragments provide a final product with a higher content of bioactive compounds than raw tomatoes.
- ✓ An extensive identification of the phenolic profile of ketchups, tomato juices and gazpachos has been carried out by *HPLC-QToF-MS*. The phenolic profile shows that these three tomato-based products mainly differ in 7 types of markers: a glycoside of protocatechuic acid, apigenin and its glycosylated forms, a dihexoside of quercetin, a *C*-hexoside of kaempferol and a dihexoside of eriodictyol.

Conclusions

- ✓ Organic cultivation was found to provide tomatoes and tomato-based products with a significantly higher content of antioxidants. However, in conventional ketchups the presence of two dipeptides, a derivative of tryptophan and a derivative of phenylalanine, was detected. This behaviour is clearly reflected in the metabolomic analysis, which allowed clear differences between traditional and organic systems to be established.
- ✓ Carotenoids of ketchups and gazpachos have been identified and quantified through a combination of two techniques (*HPLC-UV* and *HPLC-ESI-(Li⁺)-MS/MS*). Seven carotenoids were identified: lutein, α -carotene, β -carotene, *trans*-lycopene, and 5-, 9-, and 13-*cis*-lycopene. Moreover, lipophilic antioxidant capacity was also studied. The results show that lipophilic antioxidant capacity is mainly attributed to the presence of *trans*-lycopene and β -carotene.
- ✓ Storage of tomato-based products at 4 °C results in a slight decrease in their polyphenol and carotenoid content, and also in the antioxidant capacities. However, the levels achieved do not represent a drawback. Therefore, we can expect a beneficial effect from the consumption of tomato-based products at any point during their shelf life.
- ✓ Moderate-intensity pulsed electric fields have been used to increase the level of bioactive compounds and antioxidant capacity of tomatoes. An electric field strength of 1.2 kV/cm achieved the highest increase of polyphenols, carotenoids and vitamin C, in contrast to 2 kV/cm which decreased the level of bioactive compounds and antioxidant capacities. Regarding the number of pulses, 5 pulses provided an increase in the biosynthesis of carotenoids, whereas polyphenols obtained their maximum increase at 30 pulses.

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ANNEX



IX. ANNEX

1. Altres publicacions en revistes

En aquest annex s'inclouen altres publicacions, no incloses com a treball de tesis doctoral, en les quals també s'ha fet ús de l'MS, caracterització dels metabòlits i aplicació de polsos elèctrics de baixa i alta intensitat.

Publicació 13: Metabolite profiling of phenolic and carotenoid content in tomatoes after moderate-intensity pulsed electric field treatments

Anna Vallverdú-Queralt, Gemma Oms-Oliu, Isabel Odriozola-Serrano, Rosa M. Lamuela-Raventós, Olga Martín-Belloso & Pedro Elez-Martínez. *Food Chemistry*. 2013, 136: 199-205.



Metabolite profiling of phenolic and carotenoid contents in tomatoes after moderate-intensity pulsed electric field treatments

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ABSTRACT

A metabolite profiling approach was used to study the effect of moderate-intensity pulsed electric field (MIPEF) treatments on the individual polyphenol and carotenoid contents of tomato fruit after refrigeration at 4 °C for 24 h. The MIPEF processing variables studied were electric field strength (from 0.4 to 2.0 kV/cm) and number of pulses (from 5 to 30).

Twenty four hours after MIPEF treatments, an increase was observed in hydroxycinnamic acids and flavanones, whereas flavonols, coumaric and ferulic acid-O-glucoside were not affected. Major changes were also observed for carotenoids, except for the 5-*cis*-lycopene isomer, which remain unchanged after 24 h of MIPEF treatments. MIPEF treatments, conducted at 1.2 kV/cm and 30 pulses, led to the greatest increases in chlorogenic (152%), caffeic acid-O-glucoside (170%) and caffeic (140%) acids. On the other hand, treatments at 1.2 kV/cm and 5 pulses led to maximum increases of α -carotene, 9- and 13-*cis*-lycopene, which increased by 93%, 94% and 140%, respectively. Therefore, MIPEF could stimulate synthesis of secondary metabolites and contribute to production of tomatoes with high individual polyphenol and carotenoid contents.

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1. Introduction

Tomatoes (*Solanum lycopersicum* L.) contain some valuable bioactive components, including antioxidants such as carotenoids and phenolic compounds. Recently, the high content of phenolic compounds, such as flavonoids and hydroxycinnamic acids in tomatoes has been attracting interest because of their apparent multiple biological effects (Crozier, Jaganath, & Clifford, 2009). Moreover, tomatoes contain carotenoids such as β -carotene and α -carotene, which possess provitamin A activity (Olson, 1989). The consumption of β -carotene, lycopene and other carotenoids decreases the risk of certain cancers and cardiovascular diseases (Das, Otani, Maulik, & Das, 2005; Djuric & Powell, 2001; Giovannucci, 1999; Giovannucci et al., 1995). *Trans*-lycopenes represents the most abundant lycopene isomer in tomato, with 5-, 9-, 13-, and 15-*cis*-lycopene being the main *cis*-isomers detected (Frohlich, Conrad, Schmid, Breithaupt, & Bohm, 2007). Human studies have demonstrated the nutritional benefits of *cis* isomers in tomato products, because these compounds seem to be better absorbed in the intestine than are *trans* isomers (Takeoka et al., 2001).

The application of pulsed electric fields at high field strength has been proposed as an alternative to conventional food preservation techniques. Several studies have demonstrated the ability of high intensity pulsed electric fields to produce shelf-stable liquid foods with high nutritional value by inactivating microorganisms and enzymes (Soliva-Fortuny, Balasa, Knorr, & Martín-Belloso, 2009). Other applications of pulsed electric fields, at moderate field strength, are currently under development. Moderate-intensity pulsed electric fields (MIPEF) permeabilise tissue structures, thus improving intracellular metabolite extraction (Soliva-Fortuny et al., 2009) and enhancing drying efficiency (De Vito, Ferrari, Lebovka, Shynkaryk, & Vorobiev, 2008). Metabolic responses of plant cells (Cai et al., 2011) and tissues (Galindo et al., 2009; Gomez Galindo, Wadso, Vicente, & Dejmek, 2008) upon the application of MIPEF have been studied. The analysis of potato demonstrated that, 24 h after the application of MIPEF, tissue metabolism showed MIPEF-specific stress responses, characterised by changes in the hexose pool that may involve starch and ascorbic acid degradation (Galindo et al., 2009). MIPEF-induced stresses could include a burst of ROS, which are endogenous signal components required for synthesis of secondary metabolites, such as polyphenols or carotenoids, which are known to be part of the defence response of plants to stress (Shohael et al., 2006).

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Metabolic analysis was used to characterise stress responses of vegetable tissues subjected to MIEPF, providing insights of how potato tissue responds to treatment (Galindo et al., 2009; Gomez Galindo et al., 2008). However, as far as we know, this is the first time that a targeted metabolite profiling of tomato polyphenols and carotenoids has been used to study the effects of MIEPF on the biosynthesis of secondary metabolites in whole fruits. The objective of this research was to characterise the biosynthesis of individual polyphenols and carotenoids in tomato 24 h after MIEPF treatments at different electric field strengths and numbers of pulses.

2. Materials and methods

2.1. Standards and reagents

All samples and standards were handled without exposure to light. Caffeic, ferulic, *p*-coumaric and chlorogenic acids, rutin and quercetin, β -carotene, β -apo-8'-carotenal, α -carotene, *trans*-lycopene, lutein and methyl tert-butyl ether (MTBE) were purchased from Sigma® (Madrid, Spain); naringenin, naringenin-7-O-glucoside, eriodictyol and hexane were from Extrasynthèse (Genay, France). Ethanol, methanol and formic acid (HPLC grade) were obtained from Scharlau (Barcelona, Spain) and ultrapure water (Milli-Q) from Millipore (Bedford, USA).

2.2. Tomatoes

Commercially mature tomato fruit (c.v. Daniella) were purchased from a local supermarket (Lleida, Spain). The pH (Crison 2001 pH-metre; Crison Instruments SA, Alella, Barcelona, Spain), soluble solids content (Atago RX-1000 refractometer; Atago Company Ltd., Japan), firmness (TA-XT2 Texture Analyzer; Stable Micro Systems Ltd, Surrey, England) and colour (Minolta CR-400, Konica Minolta Sensing, Inc., Osaka, Japan) of the tomato fruit were determined. The physicochemical characteristics of tomatoes were: pH = 4.45 ± 0.01 , firmness = 20.4 ± 2.51 N s, soluble solids = 3.8 ± 0.1 Brix, colour: $L^* = 38.5 \pm 0.4$, $a^* = 18.1 \pm 1.9$, $b^* = 24.6 \pm 1.8$.

2.3. MIEPF processing

MIEPF treatments were conducted in a bath apparatus manufactured by Physics International (San Leandro, CA, USA), which can deliver pulses from a capacitor of 0.1 μ F with an exponential decaying waveform. A stainless steel parallel plate treatment chamber was used. A batch of tomato fruit was placed in the treatment chamber filled with tap water. Tomato fruit were treated at 0.4–2 kV/cm, using 5–30 monopolar pulses of 4 μ s at a frequency of 0.1 Hz. Two replicates of each treatment were carried out.

MIEPF-treated tomato fruit were collected and immediately refrigerated at 4 °C during 24 h, as previously described by Galindo et al. (2009). Untreated tomatoes were stored separately, also at 4 °C during 24 h. Then, both untreated and MIEPF-treated tomatoes were lyophilised and frozen at –20 °C prior to analysis.

2.4. Extraction and analysis of polyphenols

2.4.1. Extraction of polyphenols

Each replicate of the treatment was extracted and analysed three times in a darkened room with a red safety light to avoid oxidation of the analytes, following the procedure of Vallverdú-Queralt, Jáuregui, Medina-Remón, Andrés-Lacueva, and Lamuela-Raventós (2010) with some modifications.

Lyophilised tomato fruits (0.2 g) were weighed and homogenised with 80% ethanol in Milli-Q water (4 ml); the homogenate was sonicated for 5 min and centrifuged (4000 rpm at 4 °C) for

15 min. The supernatant was transferred into a flask and extraction was repeated. Both supernatants were combined and evaporated under nitrogen flow; finally, the residue was reconstituted with up to 2 ml of Milli-Q water containing 0.1% formic acid and filtered through a 13 mm, 0.45 μ m Polytetrafluoroethylene (PTFE) filter from Waters (Milford, USA) into an insert-amber vial.

Solid phase extraction (SPE) was carried out to eliminate interferents, such as ascorbic acid, amino acids and reducing sugars. For this procedure, Oasis® MAX cartridges, with 30 mg of mixed-mode anion-exchange and reversed-phase sorbent from Waters (Milford, USA), following the procedure of Vallverdú-Queralt et al. (2011). The eluted fractions were evaporated under nitrogen flow and the residue was reconstituted with up to 500 μ l of Milli-Q water containing 0.1% formic acid and filtered through a 13 mm, 0.45 μ m PTFE filter into an insert-amber vial for HPLC analysis.

2.4.2. Analysis of polyphenols

HPLC-ESI-MS/MS was used to evaluate the content of flavonols, flavanones and hydroxycinnamic acids, following the procedures of other studies. (Vallverdú-Queralt, Jáuregui, Medina-Remón, & Lamuela-Raventós, 2012; Vallverdú-Queralt et al., 2011). Method parameters appear in these studies of the same authors.

Liquid chromatography was performed with an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, an autosampler and a column oven set to 30 °C. Mobile phase consisted of Milli-Q water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The injection volume was 20 μ l and the flow rate was 0.4 ml/min. Separation was carried out in 20 min under the following conditions: 0 min, 5% B; 16 min, 40% B; 17 min, 95% B; 19 min, 95% B; 19.5 min, 5% B. The column was equilibrated for 5 min prior to each analysis.

An API 3000 (PE Sciex, Concord, Ontario, Canada) triple quadrupole mass spectrometer, equipped with a Turbo IonSpray source in negative-ion mode, was used to obtain MS/MS data. Turbo IonSpray source settings were as follows: capillary voltage, –3500 V; nebuliser gas (N_2), 10 a.u. (arbitrary units); curtain gas (N_2), 12 a.u.; collision gas (N_2), 4 a.u.; focusing potential, –200 V; entrance potential, –10 V; drying gas (N_2), heated to 400 °C and introduced to a flow rate of 6000 cm³/min. The declustering potential and collision energy were optimised for each compound in infusion experiments: individual standard solutions (10 μ g/ml) dissolved in 1:1 (v/v) mobile phase were infused at a constant flow rate of 5 μ l/min, using a model syringe pump (Harvard Apparatus, Holliston, MA, USA). Full-scan data acquisition was performed scanning from *m/z* 100 to 800 in profile mode and using a cycle time of 2 s with a step size of 0.1 u and a pause between scans of 2 ms. Identification of some compounds was carried out through neutral loss scan and precursor ion scan experiments. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound.

Quantification of polyphenols was performed by the internal standard method. Polyphenols were quantified, related to their corresponding standard, and results were expressed as μ g/g dry weight. When standards were not available, as in the case of caffeic acid-O-glucoside, *p*-coumaric acid-O-glucoside and ferulic acid-O-glucoside, polyphenols were quantified related to the corresponding hydroxycinnamic acid (caffeic, *p*-coumaric and ferulic acids). Cryptochlorogenic and dicaffeoylquinic acids were quantified with respect to the chlorogenic acid.

The total polyphenol (TP) content was obtained by the sum of all individual polyphenols.

2.6. Statistical analysis

Using the Unscrambler (v.9, CAMO A/S, Trondheim, Norway), a PLS-DA analysis was performed to cluster tomato samples according to similar metabolite profiling. PLS-DA is a partial least squares regression of a set of binary variables, describing the categories of the categorical variables (pulsed electric field treatments) on a set of predictor variables (bioactive compounds of tomatoes). Further details about building the PLS-DA can be found in Pedreschi et al. (2009). For the PLS-DA, pulse treatment conditions (number of pulses and electric field strength) were considered as X-variables, while individual polyphenol and carotenoid contents of tomatoes, were Y-variables. The data were mean-centred and variables were weighted by their standard deviation to give them equal variance. Cross-validation was applied to test the performance of the models; since the number of observations is relatively small it is impossible to validate the models on an independent test set. Multifactor analysis of variance (ANOVA) was carried out at $P < 0.05$ in order to assess for absolute changes in polyphenol and carotenoid contents among the different pulse electric field treatment conditions (number of pulses and electric field strength), using Statgraphics plus v. 5.1 software (Manugistics, Inc., Rockville, MA, USA). A Duncan's multiple range test was applied to determine differences among means, with a significance level of $P = 0.05$.

3. Results and discussion

3.1. Plant metabolite profile of MIPEF-treated tomatoes

3.1.1. General

A PLS-DA analysis was carried out to discriminate among the pulse electric field treatments according to similar metabolite profiles in tomatoes. Score vectors and loading vectors are formed in PLS-DA biplot, as can be shown in the Fig. 1. PLS accounts for 11% and 11% of the total X-variation and 40% and 30% of the total Y-variation by the first two latent variables (LV1 and LV2). This analysis maximises the covariance between the explanatory variables X (pulse electric field treatments) and the response Y (phenolic compounds and carotenoids). The nearer the variable Y is

Fig. 1. PLS-DA biplot of samples representing tomato fruits treated with different electric field strengths and numbers of pulses. (X,Y): X: Electric field strength (0.4, 1.2 and 2.2 kV/cm); Y: number of pulses (5, 18 and 30); FAG, ferulic acid- α -glucoside; FA, ferulic acid; COUMAG, coumaric acid- α -glucoside; CAG, caffeic acid- α -glucoside; CA, caffeic acid; CHA, chlorogenic acid; CRYA, cryptochlorogenic acid; NG, naringenin-7- α -glucoside; N, naringenin; E, eriodictyol; Q, quercetin; R, rutin; DICA, dicaffeoylquinic acid; TP, total polyphenols; Lic T, trans-lutein; Lic SC, 5-cis-lycopene; Lic 9, 9-cis-lycopene; Lic 13, 13-cis-lycopene; Lic 15, 15-cis-lycopene; BCaR, β -carotene; CBaR, 9-cis- β -carotene; ACaR, α -carotene; LuT, lutein; LuT 9C, 9-cis-lutein; TC, total carotenoids.

The total carotenoid (TC) content was obtained by the sum of all individual carotenoids.

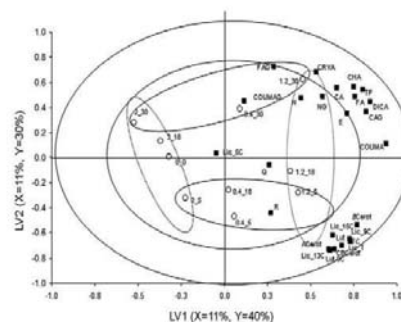


Fig. 1. PLS-DA biplot of samples representing tomato fruits treated with different electric field strengths and numbers of pulses. (X,Y): X: Electric field strength (0.4, 1.2 and 2.2 kV/cm); Y: number of pulses (5, 18 and 30); FAG, ferulic acid- α -glucoside; FA, ferulic acid; COUMAG, coumaric acid- α -glucoside; CAG, caffeic acid- α -glucoside; CA, caffeic acid; CHA, chlorogenic acid; CRYA, cryptochlorogenic acid; NG, naringenin-7- α -glucoside; N, naringenin; E, eriodictyol; Q, quercetin; R, rutin; DICA, dicaffeoylquinic acid; TP, total polyphenols; Lic T, trans-lutein; Lic SC, 5-cis-lycopene; Lic 9, 9-cis-lycopene; Lic 13, 13-cis-lycopene; Lic 15, 15-cis-lycopene; BCaR, β -carotene; CBaR, 9-cis- β -carotene; ACaR, α -carotene; LuT, lutein; LuT 9C, 9-cis-lutein; TC, total carotenoids.

located to the axis origin (=loading), the smaller is the contribution to the class distinction among treatments. Thus, plant metabolites, such as 5-*cis*-lycopene, quercetin, rutin and coumaric acid-*O*-glucoside may not contribute to such discrimination among MIPF treatments (Fig. 1). On the other hand, large loadings for variables, such as caffeic, ferulic, chlorogenic, dicaffeoylquinic and coumaric acids, caffeic acid-*O*-glucoside, β -carotene, *trans*-lycopene and 9-*cis*-lycopene, had a high discriminating power. Changes in total polyphenol and carotenoid content of tomatoes could be mainly attributed to changes in some individual compounds. It can be observed that the total polyphenol content is highly correlated with individual phenolic compounds, such as caffeic, ferulic, and chlorogenic acid. On the other hand, the total carotenoid content is highly correlated with compounds such as β -carotene, 9-*cis*-lycopene, 15-*cis*-lycopene and *trans*-lycopene. Use of the supervised PLS-DA technique sharpened the discrimination among the treatments. The biplot (Fig. 1) shows the marked discrimination among the pulse electric field treatments, as evidenced by pattern clusters formed among the different pulsed electric field treatments and their separation along the first two latent variables, LV1 and LV2, respectively. The latent variable 1 (LV1) of the PLS-DA model is able to explain the variation among treatments at different electric field strengths, while the latent variable 2 (LV2) explains the variation among treatments at different number of pulses. On the basis of the marked discrimination of the treatments, it can be clearly observed that certain individual polyphenols and carotenoids are clearly characteristic or highly correlated with a specific treatment. For instance, tomatoes, treated at 1.2 kV/cm, located to the positive side of the LV1 axis, are characterised by high levels of polyphenols (TP, chlorogenic, caffeic and ferulic acid) and carotenoids (TC, β -carotene, *trans*-lycopene and 9-*cis*-lycopene), whereas tomatoes treated with 2 kV/cm, located towards the negative side of the LV1 axis, are related to low levels of these metabolites. Tomatoes treated with 5 pulses, located to the negative side of LV2 axis, are characterised by high levels of carotenoids, whereas tomatoes treated with 30 pulses, located towards the positive side of the LV2 axis, are related to high contents of polyphenols. According to the PLS-DA plot (Fig. 1), a high content of phenolic compounds and carotenoids is strongly correlated with MIPF treatments at 1.2 kV/cm, which can be related to a bioproduction of these metabolites. At this field strength, some polyphenols were markedly characteristic of MIPF-treated tomatoes treated with 30 pulses (hydroxycinnamic, phenolic acids and flavanones), whereas some carotenoids were characteristic of MIPF treatments of 5 pulses (β -carotene, α -carotene and *cis*-lycopene isomers). Therefore, the increased number of pulses at 1.2 kV/cm seems to promote the synthesis of polyphenols rather than that of carotenoids in tomatoes. Such a metabolite production is induced as a stress response, initiated when the plant recognises a stimulus at the cellular level, such as changes in electrical potential differences of the membranes. MIPF may influence the voltage-gated ion channels and increase the membrane permeability for Ca^{2+} at the cellular level, followed by a rapid influx of Ca^{2+} through cation channels. Through this process, Ca^{2+} -dependent protein kinase (CDPK) phosphorylates PAL (Sanders, Pelloux, Brownlee, & Harper, 2002), which regulates the phenylpropanoid metabolism. The CDPK can also increase the ROS which are endogenous signal components required for the synthesis of secondary metabolites, such as polyphenols, known to be part of the plant defence response to stress (Shohael et al., 2006).

3.1.2. Effect of MIPF on individual polyphenol content

The content of individual polyphenols of untreated samples were within the range described in the literature for tomato products (Martínez-Valverde, Periago, Provan, & Chesson, 2002; Vallverdú-Queralt, Medina-Remon, Martínez-Huelamo, Jauregui,

Andrés-Lacueva & Lamuela-Raventós, 2011). Hydroxycinnamic acid and flavanone concentration were enhanced 24 h after MIPF treatments ($P < 0.05$), whereas flavonols were not affected ($P > 0.05$).

TP content was between 1969 $\mu\text{g/g}$ DW (2 kV/cm-5 pulses) and 3622 $\mu\text{g/g}$ DW (1.2 kV/cm-30 pulses). The maximum TP content was obtained at 1.2 kV/cm and 30 pulses (Table 1). Concentrations of hydroxycinnamic acids in untreated tomatoes ranged from 37.6 $\mu\text{g/g}$ DW for *p*-coumaric acid to 561 $\mu\text{g/g}$ DW for ferulic acid-*O*-glucoside (Table 2). Concentrations of ferulic, *p*-coumaric, caffeic and caffeic acid-*O*-glucoside higher than that of untreated tomatoes were observed in MIPF-treated tomatoes with different combinations of electric field strength (0.4 and 1.2 kV/cm) and numbers of pulses (5–30 pulses), but not for treatments carried out at 2 kV/cm (Table 2). It seems that treatments at 2 kV/cm may cause lethal damage to cells, due to irreversible loss of cell membrane permeability properties (Aronsson, Lindgren, Johansson, & Rönner, 2001). The concentration of hydroxycinnamic acids in MIPF-treated tomatoes was enhanced from 40.9 $\mu\text{g/g}$ DW for *p*-coumaric acid to 517 $\mu\text{g/g}$ DW for ferulic acid (Table 2). The maximum enhancement was attained at 1.2 kV/cm and 30 pulses ($P < 0.05$); caffeic acid increased by 140%, ferulic acid by 110%, *p*-coumaric acid by 39% and caffeic acid-*O*-glucoside by 170 %, followed by treatments carried out at 1.2 kV/cm-18 pulses and 0.4 kV/cm-30 pulses. Bioproduction of hydroxycinnamic acids seems to be enhanced when electric field strength increases, with treatments at 1.2 kV/cm leading to the highest content of hydroxycinnamic acids. Glycosylated ferulic and *p*-coumaric acids were not affected by MIPF treatments ($P > 0.05$), whereas caffeic acid-*O*-glucoside concentration was enhanced by 170% at 1.2 kV/cm and 30 pulses. This fact could be attributed to the presence of an easily oxidisable *ortho*-diphenol group in caffeic acid which is not present in ferulic and *p*-coumaric acids (Kilmartin, Zou, & Waterhouse, 2001).

It is difficult to explain changes of individual phenolic compounds by one single factor due to the complexity of chemical reactions occurring in natural systems. Polyphenols are formed in plant products via the action of phenylalanine ammonia-lyase (PAL) activity due to the phenylpropanoid metabolism. Activation of PAL activity has been observed in response to several causes of physiological stress (Assis, Maldonado, Muñoz, Escobedo, & Merodio, 2001). MIPF may stimulate the activity of PAL and, therefore, hydroxycinnamic acids increase. Some authors have demonstrated that wounding stimulates PAL activity, with further production of the main phenolic compounds and synthesis of new polyphenolic substances (Assis et al., 2001).

Chlorogenic acid was the most abundant hydroxycinnamic acid (Table 2). The content of chlorogenic acid in untreated tomatoes was 335 $\mu\text{g/g}$ DW. The levels of chlorogenic acid of MIPF-treated tomatoes ranged from 376 $\mu\text{g/g}$ DW (0.4 kV/cm-5 pulses) to 843 $\mu\text{g/g}$ DW (1.2 kV/cm-30 pulses). This increased by 152% when tomatoes were processed at 1.2 kV/cm-30 pulses, followed by treatments carried out at 1.2 kV/cm-18 pulses and 0.4 kV/cm-30 pulses. It seems that electric field strength substantially affected the chlorogenic acid content of MIPF-treated tomatoes. Tomato fruit treated at the highest electric field strength (2 kV/cm) showed a decrease in the levels of chlorogenic acid. This means that treatments over 1.2 kV/cm may cause lethal damage to cells, due to irreversible loss of cell membrane permeability properties and, thus, chlorogenic content is not enhanced (Aronsson et al., 2001). The trends reflecting the effects of MIPF were similar for cryptochlorogenic and dicaffeoylquinic acids. Hydroxycinnamic acids in tomatoes have been gaining interest because of their apparent multiple biological effects, including free radical-scavenging, metal chelation, inhibition of cellular proliferation, and modulation of enzymatic activity and signal transduction pathways (Crozier

Table 1
Total polyphenol (TP) content and total carotenoid (TC) content of MIPEF-treated tomatoes.

E(kV/cm)	n (Number of pulses)	Total polyphenol (TP) content (μg/g)	Total carotenoid (TC) content (μg/g)
0	0	2306 ± 35.7 ^a	57.0 ± 1.53 ^a
0.4	5	2313 ± 40.1 ^a	75.1 ± 2.04 ^b
0.4	18	2555 ± 57.9 ^d	68.5 ± 2.11 ^d
0.4	30	3226 ± 72.1 ^b	58.2 ± 1.60 ^c
1.2	5	2863 ± 49.3 ^c	78.6 ± 2.24 ^a
1.2	18	3288 ± 76.6 ^b	71.9 ± 2.86 ^c
1.2	30	3622 ± 81.4 ^a	61.6 ± 1.89 ^e
2	5	1969 ± 23.8 ^e	66.8 ± 1.90 ^d
2	18	2135 ± 31.0 ^f	50.6 ± 1.22 ^f
2	30	2364 ± 37.9 ^c	44.1 ± 1.33 ^f

The TP and TC contents were obtained by the sum of all individual polyphenols and carotenoids, respectively. Different letters in the columns represent statistically significant differences ($P < 0.05$).

Table 2
Contents of hydroxycinnamic acids (μg/g DW) of MIPEF-treated tomatoes.

E(kV/cm)	n (number of pulses)	Ferulic acid	Ferulic acid-O-glucoside	p-Coumaric acid	p-Coumaric acid-O-glucoside	Caffeic acid	Caffeic acid-O-glucoside	Chlorogenic acid	Cryptochlorogenic acid	Dicaffeoylquinic acid
0	0	246 ± 6.95 ^d	561 ± 2.55 ^d	37.6 ± 3.00 ^d	68.5 ± 1.92 ^d	43.9 ± 0.51 ^f	180 ± 5.89 ^c	335 ± 1.45 ^f	17.4 ± 0.13 ^e	29.9 ± 0.56 ^b
0.4	5	138 ± 6.14 ^b	559 ± 1.19 ^d	42.5 ± 3.26 ^{d,e}	68.3 ± 1.03 ^d	49.9 ± 0.09 ^e	236 ± 3.26 ^d	376 ± 5.61 ^e	20.4 ± 0.59 ^e	32.8 ± 0.32 ^d
0.4	18	356 ± 8.37 ^a	556 ± 3.10 ^d	40.9 ± 1.00 ^{d,e}	68.0 ± 1.66 ^d	50.6 ± 0.10 ^e	279 ± 6.62 ^d	387 ± 10.13 ^e	14.1 ± 0.10 ^f	3937 ± 2.12 ^f
0.4	30	468 ± 8.60 ^b	562 ± 3.72 ^d	42.5 ± 2.65 ^{d,e}	69.0 ± 1.53 ^d	75.5 ± 0.12 ^c	279 ± 6.32 ^d	805 ± 14.36 ^b	23.5 ± 0.05 ^b	43.5 ± 0.26 ^b
1.2	5	402 ± 9.21 ^d	563 ± 1.50 ^d	47.3 ± 1.53 ^{b,c}	68.4 ± 1.63 ^d	87.4 ± 0.54 ^b	272 ± 8.75 ^d	489 ± 4.52 ^d	18.9 ± 0.36 ^d	40.7 ± 0.49 ^{d,e}
1.2	18	423 ± 10.1 ^c	555 ± 2.29 ^d	47.9 ± 3.65 ^{b,c}	68.9 ± 1.02 ^d	48.5 ± 0.38 ^e	457 ± 4.11 ^b	753 ± 4.49 ^c	20.1 ± 0.12 ^c	41.2 ± 0.29 ^c
1.2	30	517 ± 5.58 ^a	557 ± 1.55 ^d	52.1 ± 0.25 ^d	68.9 ± 1.88 ^d	105 ± 0.55 ^d	485 ± 5.18 ^a	843 ± 7.43 ^b	28.6 ± 0.24 ^d	49.5 ± 1.35 ^d
2	5	187 ± 7.56 ^d	558 ± 2.78 ^d	37.6 ± 1.13 ^e	68.9 ± 1.50 ^d	17.3 ± 0.34 ^f	98.5 ± 3.96 ^d	198 ± 5.39 ^b	12.3 ± 0.34 ^e	28.7 ± 0.88 ^b
2	18	147 ± 7.42 ^b	563 ± 2.05 ^d	37.2 ± 2.64 ^e	67.5 ± 1.25 ^d	60.2 ± 0.99 ^d	148 ± 9.31 ^f	294 ± 10.75 ^d	20.1 ± 0.15 ^c	33.6 ± 1.23 ^d
2	30	227 ± 7.47 ^c	564 ± 3.38 ^d	31.7 ± 2.23 ^f	67.7 ± 1.58 ^d	43.7 ± 0.31 ^f	175 ± 9.78 ^e	327 ± 5.23 ^d	16.9 ± 0.52 ^e	28.5 ± 0.58 ^b

Data shown are the means ± SD of two MIPEF treatment repetitions; each assay was performed in triplicate. Different letters in the columns represent statistically significant differences ($P < 0.05$).

Table 3
Contents of flavonol and flavanones (μg/g DW) of MIPEF-treated tomatoes.

E(kV/cm)	n (Number of pulses)	Flavonones			Flavonols		
		Naringenin	Naringenin-7-O-glucoside	Eriodictyol	Quercetin	Rutin	
0	0	503 ± 4.32 ^c	25.7 ± 0.63 ^d	11.4 ± 0.60 ^c	61.1 ± 0.81 ^a	186 ± 0.59 ^a	
0.4	5	509 ± 4.36 ^c	24.4 ± 3.71 ^d	10.4 ± 0.01 ^d	62.2 ± 0.09 ^a	187 ± 0.12 ^d	
0.4	18	472 ± 3.92 ^c	31.8 ± 0.43 ^c	13.7 ± 0.20 ^c	62.1 ± 0.15 ^a	188 ± 0.15 ^a	
0.4	30	560 ± 2.52 ^b	38.3 ± 0.94 ^{a,b}	12.8 ± 0.19 ^d	62.2 ± 0.05 ^a	185 ± 0.58 ^a	
1.2	5	576 ± 3.79 ^a	36.2 ± 0.75 ^b	12.7 ± 0.14 ^d	62.3 ± 0.20 ^a	186 ± 2.01 ^d	
1.2	18	577 ± 13.2 ^a	30.5 ± 1.41 ^c	16.2 ± 0.26 ^b	62.23 ± 0.23 ^a	188 ± 1.80 ^a	
1.2	30	578 ± 0.68 ^a	42.9 ± 2.36 ^a	17.1 ± 0.01 ^d	62.3 ± 0.73 ^a	185 ± 0.68 ^a	
2	5	468 ± 15.4 ^c	32.4 ± 0.25 ^c	13.0 ± 0.27 ^c	62.7 ± 0.75 ^a	187 ± 0.63 ^a	
2	18	471 ± 3.66 ^c	34.8 ± 0.01 ^b	11.4 ± 0.58 ^e	61.9 ± 0.27 ^a	187 ± 1.25 ^a	
2	30	491 ± 16.8 ^d	24.0 ± 0.22 ^d	11.0 ± 0.04 ^f	62.2 ± 0.13 ^a	185 ± 0.51 ^a	

Data shown are the means ± SD of two MIPEF treatment repetitions; each assay was performed in triplicate. Different letters in the columns represent statistically significant differences ($P < 0.05$).

et al., 2009). MIPEF may activate the synthesis of these hydroxycinnamic acids in tomatoes.

Flavanones (naringenin, naringenin-7-O-glucoside, eriodictyol) increased 24 h after the application of MIPEF treatments (Table 3). The concentration of flavanones in untreated tomatoes varied from 11.4 μg/g DW for eriodictyol to 503 μg/g DW for naringenin. Concentrations of flavanones in MIPEF-treated tomatoes were enhanced from 11.4 μg/g DW for eriodictyol to 578 μg/g DW for naringenin. The highest increase in the content of flavonols was obtained with treatments carried out at 1.2 kV/cm and 30 pulses. Naringenin-7-O-glucoside, eriodictyol and naringenin content, increased by 66%, 50% and 15%, respectively. Such a bioproduction of eriodictyol, naringenin and its glycosylated form in MIPEF-treated tomatoes could be attributed to the induction of flavanone

synthase from 4-coumaroyl-CoA and malonyl-CoA (Schröder, Heller, & Hahlbrock, 1979) when MIPEF-treatments were applied.

3.1.3. Effect of MIPEF on individual carotenoid contents

The contents of individual carotenoids of untreated samples were in the range described in the literature for tomato products (Martínez-Valverde et al., 2002). In this study, *trans*-lycopene, 9-, 13-, 15-*cis*-lycopene and TC content were enhanced ($P < 0.05$) when MIPEF-treatments were applied. TC content was in the range 44.1 μg/g DW (2 kV/cm-30 pulses) to 78.6 μg/g DW (1.2 kV/cm-5 pulses) (Table 1). The maximum TC content was obtained at 1.2 kV/cm and 5 pulses. The concentrations of *trans*-lycopene and *cis*-lycopene isomers in untreated tomatoes ranged from 2.27 μg/g DW for 13-*cis*-lycopene to 17.6 μg/g DW for *trans*-lycopene (Ta-

Table 4
Content of carotenoids ($\mu\text{g/g DW}$) of MPEF-treated tomatoes.

E(kV/cm)	n (number of pulses)	13- <i>cis</i> -Lycopene	15- <i>cis</i> -Lycopene	9- <i>cis</i> -Lycopene	<i>trans</i> -Lycopene	5- <i>cis</i> -Lycopene
0	0	2.27 $\pm$ 0.07 ^a	5.96 $\pm$ 0.14 ^a	2.62 $\pm$ 0.13 ^a	17.6 $\pm$ 0.10 ^f	2.81 $\pm$ 0.04 ^a
0.4	5	5.14 $\pm$ 0.06 ^b	7.14 $\pm$ 0.04 ^b	4.94 $\pm$ 0.04 ^b	21.7 $\pm$ 0.06 ^b	2.80 $\pm$ 0.05 ^a
0.4	18	4.10 $\pm$ 0.03 ^d	6.10 $\pm$ 0.18 ^d	4.13 $\pm$ 0.04 ^d	20.8 $\pm$ 0.09 ^c	2.82 $\pm$ 0.04 ^a
0.4	30	2.89 $\pm$ 0.10 ^e	5.85 $\pm$ 0.04 ^d	3.27 $\pm$ 0.06 ^e	17.2 $\pm$ 0.09 ^f	2.82 $\pm$ 0.04 ^a
1.2	5	5.47 $\pm$ 0.06 ^a	7.91 $\pm$ 0.62 ^a	5.08 $\pm$ 0.04 ^a	22.3 $\pm$ 0.06 ^a	2.81 $\pm$ 0.04 ^a
1.2	18	4.65 $\pm$ 0.05 ^c	6.83 $\pm$ 0.20 ^c	4.67 $\pm$ 0.06 ^c	21.1 $\pm$ 0.28 ^c	2.83 $\pm$ 0.09 ^a
1.2	30	3.35 $\pm$ 0.02 ^f	5.81 $\pm$ 0.05 ^f	3.48 $\pm$ 0.05 ^f	18.3 $\pm$ 0.12 ^c	2.82 $\pm$ 0.05 ^a
2	5	3.81 $\pm$ 0.05 ^e	6.85 $\pm$ 0.04 ^c	3.80 $\pm$ 0.05 ^e	20.2 $\pm$ 0.08 ^d	2.81 $\pm$ 0.05 ^a
2	18	2.74 $\pm$ 0.08 ^e	5.81 $\pm$ 0.04 ^f	2.80 $\pm$ 0.12 ^b	15.8 $\pm$ 0.15 ^b	2.83 $\pm$ 0.05 ^a
2	30	1.97 $\pm$ 0.08 ^f	3.26 $\pm$ 0.08 ^g	2.37 $\pm$ 0.22 ^g	16.5 $\pm$ 0.42 ^e	2.80 $\pm$ 0.08 ^a
		Lutein	9- <i>cis</i> -Lutein	α -Carotene	β -Carotene	9- <i>cis</i> - β -Carotene
0	0	1.64 $\pm$ 0.11 ^f	1.20 $\pm$ 0.11 ^e	0.71 $\pm$ 0.01 ^e	19.8 $\pm$ 0.41 ^e	3.43 $\pm$ 0.04 ^f
0.4	5	2.63 $\pm$ 0.07 ^b	2.23 $\pm$ 0.07 ^b	1.22 $\pm$ 0.03 ^b	24.9 $\pm$ 0.14 ^b	4.61 $\pm$ 0.05 ^b
0.4	18	2.14 $\pm$ 0.12 ^d	2.05 $\pm$ 0.09 ^d	1.00 $\pm$ 0.03 ^c	23.5 $\pm$ 0.09 ^d	3.84 $\pm$ 0.06 ^d
0.4	30	1.44 $\pm$ 0.08 ^e	1.30 $\pm$ 0.06 ^f	0.67 $\pm$ 0.02 ^e	21.0 $\pm$ 0.18 ^f	3.13 $\pm$ 0.06 ^e
1.2	5	2.94 $\pm$ 0.04 ^a	2.40 $\pm$ 0.03 ^a	1.37 $\pm$ 0.04 ^a	25.3 $\pm$ 0.13 ^a	4.95 $\pm$ 0.04 ^a
1.2	18	2.46 $\pm$ 0.05 ^c	2.11 $\pm$ 0.03 ^c	1.06 $\pm$ 0.04 ^c	24.3 $\pm$ 0.30 ^c	4.40 $\pm$ 0.14 ^c
1.2	30	1.92 $\pm$ 0.12 ^e	1.63 $\pm$ 0.08 ^e	0.73 $\pm$ 0.04 ^e	21.4 $\pm$ 0.58 ^f	3.31 $\pm$ 0.07 ^f
2	5	2.07 $\pm$ 0.05 ^d	2.04 $\pm$ 0.04 ^d	0.84 $\pm$ 0.02 ^d	23.1 $\pm$ 0.69 ^e	3.64 $\pm$ 0.06 ^e
2	18	1.37 $\pm$ 0.04 ^g	1.15 $\pm$ 0.02 ^g	0.61 $\pm$ 0.02 ^f	15.9 $\pm$ 0.10 ^h	2.85 $\pm$ 0.05 ^h
2	30	1.11 $\pm$ 0.04 ^h	1.02 $\pm$ 0.02 ^h	0.59 $\pm$ 0.01 ^f	12.8 $\pm$ 0.12 ^h	2.64 $\pm$ 0.08 ^h

Data shown are the means $\pm$ SD of two treatment repetitions, each assay was performed in triplicate. Different letters in the columns represent statistically significant differences ($P < 0.05$).

ble 5). Enhanced concentrations of lycopene isomers in MPEF-treated tomatoes between 2.74 $\mu\text{g/g DW}$ for 13-*cis*-lycopene and 22.3 $\mu\text{g/g DW}$ for *trans*-lycopene were observed. The maximum enhancement was attained with 1.2 kV/cm-5 pulses; *trans*-lycopene increased by 27% whereas 9-, 13- and 15-*cis*-lycopene were enhanced by 94%, 140% and 33%. However, these MPEF conditions did not affect 5-*cis*-lycopene content ($P > 0.05$). Moreover, treatments carried out at 2 kV/cm and 30 pulses resulted in a decrease of *trans*-lycopene and 9-, 13- and 15-*cis*-lycopene. Therefore, treatments at the highest electric field strength may cause lethal damage to cells due to irreversible loss of cell membrane permeability properties (Aronsson et al., 2001). Little is known about the mechanisms involved in carotenoid biosynthesis in plants. The biosynthetic enzymes are encoded by nuclear genes, and precursor proteins are post-translationally imported into plastids, where carotenoid biosynthesis occurs. Disruption of tissue by wounding could promote the transcription of the genes or the transport of the mRNA related to the synthesis of carotenoids (Bartley & Scolnik, 1995). The production of *cis*-lycopene isomers due to MPEF treatments could be explained by the activation of enzymes such as phytoene synthase and ζ -carotene desaturase (ZDS). Phytoene undergoes a set of desaturation reactions, each of which creates a new double bond and extends the chromophore by two conjugated double bonds; the end-product is lycopene (DellaPenna & Pogson, 2006). The increases in *cis*-lycopene isomers at 0.4 and 2 kV/cm and low number of pulses could be due to the activation of these specific enzymes. On the other hand, the production of *trans*-lycopene (Table 4) could be explained through the enzyme action of desaturases. These enzymes introduce a series of four double bonds in *cis* configuration forms, which are isomerised to the all-*trans* configurations by the carotenoid isomerase enzyme (CRTISO). MPEF treatments may also produce the activation of CRTISO and enhance *trans*-lycopene bioproduction. There is evidence that the CRTISO enzyme may act in concert with the ZDS enzyme (DellaPenna & Pogson, 2006). The results found in our study are in line with those described by other authors in different tomato products, in which *trans*-lycopene represents the most abundant lycopene isomer which varied from 35% to 96% of total lycopene, with 5-, 9-, 13-, and 15-*cis*-lycopene being the main *cis* isomers detected (Frohlich et al., 2007; Schierle et al., 1997). Human studies

have demonstrated the nutritional benefits of *cis* isomers in tomato products, because these compounds seem to be better absorbed in the intestine than are *trans* isomers (Failla, Chittumoonchokchai, & Ishida, 2008; Takeoka et al., 2001). In our study, *cis*-isomers are enhanced after MPEF treatments, reflecting the importance of applying this methodology to increasing health-related compounds.

Moreover, α -, β - and 9-*cis*- β -carotene concentrations increased significantly ($P < 0.05$) 24 h after MPEF treatments were applied (Table 4). The concentration of α -, β - and 9-*cis*- β -carotene in untreated tomatoes ranged from 0.71 $\mu\text{g/g DW}$ for α -carotene to 19.8 $\mu\text{g/g DW}$ for β -carotene. Concentrations of α -, β - and 9-*cis*- β -carotene in MPEF-treated tomatoes were enhanced from 0.73 $\mu\text{g/g DW}$ for α -carotene to 25.3 $\mu\text{g/g DW}$ for β -carotene. Maximum enhancement was attained at 1.2 kV/cm-5 pulses; α -, β - and 9-*cis*- β -carotene increased by 93%, 28% and 44%, respectively, followed by treatments carried out at 0.4 kV/cm-5 pulses. As shown in Table 4, α -, β - and 9-*cis*- β -carotene decreased as the number of pulses increased, whereas treatments carried out at 1.2 kV/cm led to the highest content of these three carotenoids. Therefore, the number of pulses seems to be the determining factor for enhancing α -, β - and 9-*cis*- β -carotene bioproduction. The carotenoid biosynthetic pathway in plants has two main branches after lycopene, distinguished by different cyclic end-groups. Two beta rings lead to the β , β branch (β -carotene and its derivatives) through β -carotene cyclase, whereas one beta and one epsilon ring define the β , ϵ branch (α -carotene and its derivatives) through ϵ - and β -carotene cyclase. MPEF-processing may activate ϵ - and β -carotene cyclase and, thus stimulate α -, β - and 9-*cis*- β -carotene production. Both the β LCY and ϵ -cyclase (ϵ LCY) enzymes are required to form α -carotene and lutein (Cunningham & Gantt, 1998). Increasing or decreasing ϵ LCY expression resulted in lutein levels ranging from 10% to 180% (Pogson & Rissler, 2000). In our study, the content of lutein and 9-*cis*-lutein in untreated tomatoes were 1.64 $\mu\text{g/g DW}$ and 1.20 $\mu\text{g/g DW}$, respectively (Table 4). Both lutein and 9-*cis*-lutein were enhanced ($P < 0.05$) from 1.92 $\mu\text{g/g DW}$ to 2.40 $\mu\text{g/g DW}$, while they were maintained or slightly decreased when treatments were carried out at the highest electric field strength (2 kV/cm) or over long treatment times (30 pulses). The maximum increase was attained at 1.2 kV/cm and 5 pulses.

Lutein was enhanced by 79% and 9-cis-lutein by 44%. This could be attributed to the physiological responses to MPEF-induced stress.

4. Conclusions

This study characterised the bioproduction of individual polyphenols and carotenoids in MPEF-treated tomato fruit after 24 h of refrigeration at 4 °C. Most compounds could be clustered in groups, showing either increasing polyphenol content (e.g., caffeic, ferulic, chlorogenic, dicaffeoylquinic, coumaric acids and caffeic acid-O-glucoside) and/or increasing carotenoid content (e.g., β -carotene, α -carotene and cis-lycopene isomers) of MPEF-treated tomato fruit. At 1.2 kV/cm, some polyphenols were markedly characteristic of MPEF-treated tomatoes treated at 30 pulses (hydroxycinnamic, phenolic acids and flavanones), whereas some carotenoids were characteristic of MPEF treatments at 5 pulses (β -carotene, α -carotene and cis-lycopene isomers).

MPEF treatments could be used to induce stress reactions in tomato fruit after 24 h of refrigeration at 4 °C by enhancing metabolic activity and accumulating secondary metabolites. Therefore, the metabolic profiling approach used in this study allowed identification of changes in the metabolome induced by MPEF treatments, helping in developing new strategies to improve phytochemical content of tomatoes.

Acknowledgments

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Publicació 14. Changes in polyphenol profile of tomato juices processed by pulsed electric fields

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Changes in the Polyphenol Profile of Tomato Juices Processed by Pulsed Electric Fields

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ABSTRACT: The effect of pulsed electric fields on the polyphenol profile of tomato juices was studied. First, tomatoes were subjected to moderate-intensity pulsed electric fields (MIPEFs) and then were immediately refrigerated at 4 °C for 24 h. Treated and untreated juices were then subjected to high-intensity pulsed electric fields (HIPEFs) or thermal treatment (90 °C for 60 s). In comparison to references, tomatoes subjected to MIPEF treatments led to juices with a higher content of polyphenol compounds. A slight decrease in polyphenol compounds was observed over time in thermal- and HIPEF-treated juices, with the exception of caffeic acid. However, HIPEF-processed tomato juices had a higher content of polyphenol compounds (ferulic acid, caffeic-O-glucoside acid, *p*-coumaric acid, chlorogenic acid, rutin, and naringenin) just after processing and through storage than those thermally treated. Therefore, the combination of MIPEFs and HIPEFs could be proposed as a strategy for producing tomato juices with a higher content of phenolic compounds.

KEYWORDS: Tomato juices, HIPEF, MIPEF, thermal treatments, polyphenols, tomato fruit

■ INTRODUCTION

The consumption of raw tomato and tomato-based products, such as tomato juices, is associated with a decrease in chronic degenerative diseases.¹ These beneficial effects could be attributed in part to their content in polyphenols, which have been gaining interest because of their multiple biological effects, such as free-radical scavenging, inhibition of cellular proliferation, and modulation of enzymatic activity and signal transduction pathways.² Polyphenols in tomatoes are mainly represented by flavanones (naringenin-glycosylated derivatives) and flavonols (quercetin-, rutin-, and kaempferol-glycosylated derivatives).^{3,4}

The application of pulsed electric fields in food processing has been studied as a possible treatment to induce stress reactions in plant systems to enhance or stimulate the production of secondary plant metabolites, such as polyphenols.⁵ Vallverdú-Queralt et al.⁶ reported that moderate-intensity pulsed electric field (MIPEF) treatments induce stress reactions in tomato fruits after 24 h of refrigeration by stimulating metabolic activity and accumulating secondary metabolites. MIPEFs affect metabolism as a result of stress, with the consequent generation of reactive oxygen species (ROS). ROS are required for the synthesis of polyphenols, which are widely known to be part of the defense response of plants to stress.^{6,7}

Thermal processing is the most common method to extend the shelf life of juices, by inactivating microorganisms and enzymes. However, heat treatments reduce the sensory and nutritional qualities of these products. Up to now, studies have suggested that high-intensity pulsed electric field (HIPEF)

treatment is efficient enough to destroy microorganisms in fruit juices at levels equivalent to those achieved by heat pasteurization without greatly affecting their nutritional and sensory properties.^{8,9} On the other hand, some studies suggested that HIPEF processing may enhance the antioxidant properties of juices compared to untreated juices.^{10,11} However, knowledge about the effects of this emerging technology on the antioxidant potential of juices prepared with fruits treated by MIPEFs is not currently available. Therefore, the aim of this work was to evaluate the influence of the consecutive application of MIPEF and HIPEF treatments on the polyphenol profile of tomato juices.

■ MATERIALS AND METHODS

Standards and Reagents. All samples and standards were handled without exposure to light. Caffeic, ferulic, *p*-coumaric, and chlorogenic acids, rutin, quercetin, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%), and manganese dioxide were purchased from Sigma (Madrid, Spain). Naringenin, naringenin-7-O-glucoside, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Extrasynthèse (Genay, France). Hydrochloric acid (35%) and acetic acid (99.8%) were purchased from Panreac (Barcelona, Spain). Anhydrous sodium acetate (2 M) was purchased from Merck (Darmstadt, Germany). Ethanol, methanol, and formic acid [high-performance liquid chromatography (HPLC) grade] were obtained

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from Scharlau (Barcelona, Spain). Ultrapure water (Milli-Q) was obtained from Millipore (Bedford, MA).

Tomato Juice. Tomatoes. Tomato fruits (*Lycopersicon esculentum* Mill., cv. Daniela) at commercial maturity were purchased from a local supermarket (Lleida, Spain). pH (Crison pH-meter; Crison Instruments SA, Alella, Barcelona, Spain), soluble solids content (Atago RX-1000 refractometer; Atago Company, Ltd., Japan), firmness (Texturimeter-XT2 Stable Micro Systems, Ltd., Surrey, U.K.), and color (Minolta CR-400, Konica Minolta Sensing, Inc., Osaka, Japan) of the tomatoes were determined. The physicochemical characteristics of tomatoes were as follows: pH, 4.45 ± 0.0 ; soluble solids, $3.8 \pm 0.1^\circ$ Brix; firmness, 20.4 ± 2.51 N s; and color, with L^* , 38.5 ± 0.4 ; a^* , 18.1 ± 1.9 ; and b^* , 24.6 ± 1.8 .

MIPEF Processing of Tomatoes. MIPEF treatments were conducted in a batch equipment manufactured by Physics International (San Leandro, CA), which deliver pulses from a capacitor of 0.1 μ F. A stainless-steel parallel-plate treatment chamber was used. A batch of tomato fruit was placed in the treatment chamber filled with tap water. Tomato fruit were treated at 1 kV cm^{-1} using 16 monopolar pulses of 4 μ s at a frequency of 0.1 Hz according to a previous study.⁶ MIPEF-treated tomato fruit were collected and immediately refrigerated at 4°C for 24 h, as previously described by Galindo et al.¹² Untreated tomatoes were stored separately at 4°C for 24 h.

Preparation of Tomato Juice. Both untreated and MIPEF-treated tomatoes were ground and then filtered through 2 mm steel sieves. The physicochemical characteristics of untreated tomato juices were as follows: electrical conductivity, $0.73 \pm 0.02 \text{ S m}^{-1}$; pH, 4.24 ± 0.19 ; soluble solids, $3.8 \pm 0.1^\circ$ Brix; and color, with L^* , 22.8 ± 0.8 ; and b^* , 35.6 ± 1.8 . The parameters of MIPEF-treated tomato juices were as follows: electrical conductivity, $0.76 \pm 0.01 \text{ S m}^{-1}$; pH, 4.41 ± 0.17 ; soluble solids, $3.9 \pm 0.1^\circ$ Brix; and color, with L^* , 24.8 ± 0.9 ; and b^* , 38.6 ± 1.4 .

HIPEF Processing of Tomato Juices. Pulse treatments were carried out using a continuous flow bench-scale system (OSU-4F, The Ohio State University, Columbus, OH) that provides square-wave pulses within eight co-field flow chambers in series. Each chamber had a treatment volume of 0.012 cm^3 , delimited by two stainless-steel electrodes and separated by a gap of 0.29 cm. The flow rate of the process was adjusted to 60 mL min^{-1} and controlled by a variable-speed pump (model 752210-25, Cole Palmer Instrument Company, Vernon Hills, IL). The treatment temperature was kept below 40°C using a cooling coil, which was connected before and after each pair of chambers and submerged in an ice–water shaking bath. HIPEF treatment was set up at 35 kV cm^{-1} for 1500 μ s using bipolar square-wave pulses of 4 μ s and a frequency of 100 Hz.⁸

Thermal Treatment of Tomato Juice. To compare the effect of HIPEF treatment to that of conventional thermal treatment, tomato juice was subjected to heat processes at 90°C for 60 s. Tomato juice was thermally processed in a tubular stainless-steel heat-exchange coil immersed in a hot-water shaking bath (University of Lleida, Lleida, Spain). A gear pump was used to maintain the desirable juice flow rate. After thermal processing, the juice was immediately cooled in a heat-exchange coil immersed in an ice–water bath.

Packaging and Storage Conditions. Polypropylene, sterile, 100 mL bottles were filled directly from the outlet of the treatment systems, leaving as little headspace as possible. Afterward, the container was tightly closed and stored at $4 \pm 1^\circ\text{C}$ for 56 days.

Extraction and Analysis of Polyphenols. Extraction of Polyphenols. Samples were treated in a dark room with a red safety light to avoid oxidation of the analytes. Tomato juices (0.5 g) were weighed and homogenized with 80% ethanol in Milli-Q water (4 mL); the homogenates were sonicated for 5 min and centrifugated (4000 rpm at 4°C) for 15 min. The supernatant was transferred into a flask, and extraction was repeated. The supernatants were combined and evaporated under nitrogen flow. Finally, the residue was redissolved with up to 2 mL of Milli-Q water containing 0.1% formic acid and filtered through a 13 mm, 0.45 μ m polytetrafluoroethylene (PTFE) filter from Waters (Milford, MA) into an insert-amber vial.¹³

Solid-phase extraction (SPE) was carried out to eliminate interferences, such as ascorbic acid, amino acids, and reducing sugars.

For this procedure, Oasis MAX cartridges with 30 mg of mixed-mode anion-exchange and reversed-phase sorbent from Waters were used.¹⁴ The eluted fractions were evaporated under nitrogen flow, and the residue was redissolved with up to 500 μ L of Milli-Q water containing 0.1% formic acid and filtered through a 13 mm, 0.45 μ m PTFE filter into an insert-amber vial for HPLC analysis.

Analysis of Polyphenols. HPLC–electrospray ionization (ESI)–tandem mass spectrometry (MS/MS) was used to evaluate the content of flavonols, flavanones, and hydroxycinnamic acids.¹⁵ The liquid chromatography equipment was an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, an autosampler, and a column oven set to 30°C . The mobile phase consisted of Milli-Q water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The separation of phenolic compounds was performed with a Luna C₁₈ column, 50×2.0 mm inner diameter, 5 μ m (Phenomenex, Torrance, CA). The injection volume was 20 μ L, and the flow rate was 0.4 mL min^{-1} . Separation was carried out in 20 min under the following conditions: 0 min, 5% B; 16 min, 40% B; 17 min, 95% B; 19 min, 95% B; and 19.5 min, 5% B. The column was equilibrated for 5 min prior to each analysis.

An API 3000 (PE Sciex, Concord, Ontario, Canada) triple quadrupole mass spectrometer equipped with a Turbo Ionspray source in negative-ion mode was used to obtain MS/MS data.¹⁵ Quantification of polyphenols was performed by the internal standard method. Polyphenols were quantified in relation to their corresponding standard, and results were expressed as $\mu\text{g g}^{-1}$ of dry weight. When standards were not available, as in the case of caffeic-O-glucoside and ferulic-O-glucoside acids, they were quantified with respect to the corresponding hydroxycinnamic acid (caffeic and ferulic acids).

The total polyphenol (TP) content was obtained by the sum of each individual polyphenol.

Antioxidant Capacity of the Hydrophilic Fraction. The tomato juice extracts, which were prepared for the analysis of the polyphenols, were used to analyze the hydrophilic antioxidant capacity (HAC). The HAC was measured using an ABTS[•] radical decolorization assay and a DPPH assay.¹⁶

ABTS[•] Assay. A total of 1 mM Trolox (antioxidant standard) was prepared in methanol. Working standards were carried out by diluting 1 mM Trolox with methanol. Solutions of known Trolox were used for calibration.

An ABTS[•] radical cation was prepared by passing a 5 mM aqueous stock solution of ABTS (in methanol) through manganese dioxide powder. Excess manganese dioxide was filtered through a 13 mm, 0.45 μ m PTFE filter (Waters). Then, 245 μ L of ABTS[•] solution was added to 5 μ L of Trolox or tomato juice extracts, and the solutions were stirred for 30 s. The homogenate was shaken vigorously and kept in the dark for 1 h. Absorption of the samples was measured on a UV–vis Thermo Multiskan Spectrum spectrophotometer at 734 nm, and methanol blanks were run in each assay. Results were expressed as millimoles of Trolox equivalent (TE) 100 g^{-1} of fresh weight (FW).

DPPH Assay. The antioxidant capacity was also studied through the evaluation of the free-radical-scavenging effect on the DPPH radical. Solutions of known Trolox were used for calibration. A total of 5 μ L of tomato juice extracts or Trolox were mixed with 250 μ L of methanolic DPPH (0.025 g L^{-1}). The homogenate was shaken vigorously and kept in the dark for 30 min. Absorption of the samples was measured on the spectrophotometer at 515 nm. Results were expressed as millimoles of TE 100 g^{-1} of FW.

Statistical Analysis. Treatments of tomato juice were carried out in duplicate, and each replicate was analyzed 3 times. Significance of the results and statistical differences were analyzed using Statgraphics Plus, version 5.1, software (Manugistics, Inc., Rockville, MA). Data were analyzed by multifactor analysis of variance, and a Duncan multiple range test was applied to determine differences among means, with a significance level of $p = 0.05$.

RESULTS AND DISCUSSION

Effect of Processing and Storage on Polyphenol Compounds. The initial TP content of tomato juices, obtained by the sum of each individual polyphenol, ranged from 148 to 151 $\mu\text{g g}^{-1}$ of FW for those prepared with untreated tomatoes and from 175 to 180 $\mu\text{g g}^{-1}$ of FW for juices made of MIPEF-treated tomatoes (Figure 1). The results

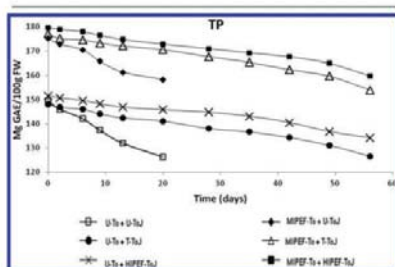


Figure 1. Effects of HIPEFs and heat treatments on TP of tomato juices made of untreated and MIPEF-treated tomatoes through storage at 4 °C. U-To + U-ToJ, untreated tomato + untreated tomato juice; U-To + HIPEF-ToJ, untreated tomato + HIPEF-treated tomato juice; U-To + T-ToJ, untreated tomato + thermal-treated tomato juice; MIPEF-To + U-ToJ, MIPEF-treated tomato + untreated tomato juice; MIPEF-To + HIPEF-ToJ, MIPEF-treated tomato + HIPEF-treated tomato juice; and MIPEF-To + T-ToJ, MIPEF-treated tomato + thermal-treated tomato juice. Data shown are the mean $\pm$ standard deviation.

were significantly higher ($p < 0.05$) in juices made of MIPEF-treated tomatoes in comparison to those made of untreated tomatoes. This fact could be attributed to the defense response of plants to MIPEFs.⁶ MIPEF treatment provides potential to induce stress reactions in tomato fruits after 24 h of refrigeration by enhancing metabolic activity and accumulating secondary metabolites but also an increased permeability of the cellular membrane because of MIPEF processing, which could potentially make the extraction of the bioactive constituent more efficient.

HIPEF processing better maintained the content of polyphenols in tomato juices than thermal treatments. Lower processing temperatures reached through HIPEF processing ($T < 40$ °C) would explain the higher retention of polyphenols in HIPEF-treated tomato juices compared to the thermally processed samples.

HIPEF-processed tomato juices from untreated and MIPEF-treated tomatoes presented significantly ($p < 0.05$) higher TP content (134.2–160.0 $\mu\text{g g}^{-1}$ of FW) throughout the storage period than those treated at 90 °C for 60 s. Juices treated by heat showed levels of TP content ranging from 127.5 to 155.2 $\mu\text{g g}^{-1}$ of FW (Figure 1) at 56 days of cold storage. The TP concentration was kept stable during the first 10 days of storage in fresh and treated juices. Afterward, a significant decrease ($p > 0.05$) was observed in fresh juices, whereas for HIPEF- and thermal-treated juices, the TP content significantly depleted ($p > 0.05$) after day 30 of cold storage. This trend is in accordance with other studies that determined the effects of heat and HIPEF treatments over storage on tomato juices made of untreated tomatoes.¹⁷ Peroxidase and oxidase are involved in the oxidative degradation of phenolic compounds. Thus, the degradation of phenolic compounds during storage might be

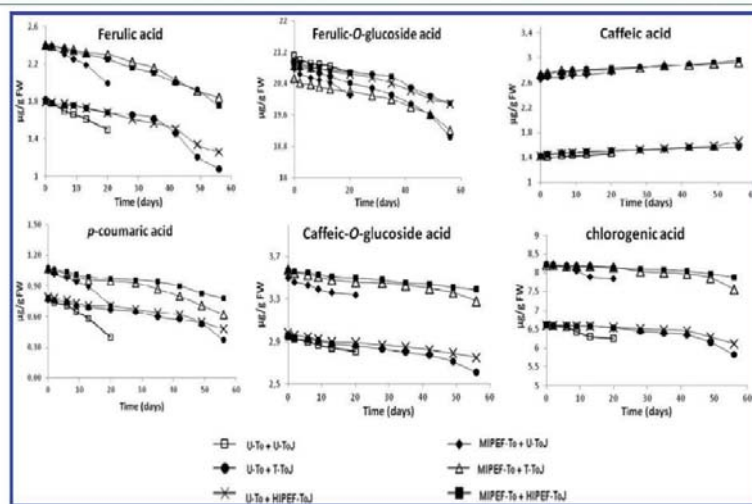


Figure 2. Effects of HIPEF and heat treatments on hydroxycinnamic acids of tomato juices made of untreated and MIPEF-treated tomatoes through storage at 4 °C. U-To + U-ToJ, untreated tomato + untreated tomato juice; U-To + HIPEF-ToJ, untreated tomato + HIPEF-treated tomato juice; U-To + T-ToJ, untreated tomato + thermal-treated tomato juice; MIPEF-To + U-ToJ, MIPEF-treated tomato + untreated tomato juice; MIPEF-To + HIPEF-ToJ, MIPEF-treated tomato + HIPEF-treated tomato juice; and MIPEF-To + T-ToJ, MIPEF-treated tomato + thermal-treated tomato juice. Data shown are the mean $\pm$ standard deviation.

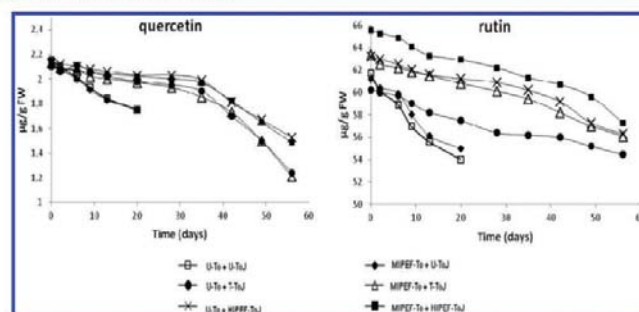


Figure 3. Effects of HIPEF and heat treatments on flavonols of tomato juices made of untreated and MIPEF-treated tomatoes through storage at 4 °C. U-To + U-ToJ, untreated tomato + untreated tomato juice; U-To + HIPEF-ToJ, untreated tomato + HIPEF-treated tomato juice; U-To + T-ToJ, untreated tomato + thermal-treated tomato juice; MIPEF-To + U-ToJ, MIPEF-treated tomato + untreated tomato juice; MIPEF-To + HIPEF-ToJ, MIPEF-treated tomato + HIPEF-treated tomato juice; and MIPEF-To + T-ToJ, MIPEF-treated tomato + thermal-treated tomato juice. Data shown are the mean $\pm$ standard deviation.

associated with the residual activity of phenol oxidase or phenol peroxidase. It has been demonstrated that both thermal and HIPEF treatments could partially inhibit peroxidase in tomato juices.¹⁸ However, a residual peroxidase activity of 10 and 5% was obtained after applying thermal and HIPEF treatments, respectively. Therefore, it could be the reason for the high content of polyphenols in juices treated with HIPEF treatments.

Phenolic Acids. The main hydroxycinnamic acid derivative in tomato juices was chlorogenic acid. The content of chlorogenic acid was 25% higher in juices prepared with MIPEF-treated tomatoes than in juices made of untreated tomatoes (Figure 2).

Chlorogenic acid was also found to be significantly ($p < 0.05$) higher concentrations just after processing and over time in HIPEF-treated tomato juices than in juices processed by heat, irrespective of the tomatoes used. Tomato juices underwent a substantial loss ($p < 0.05$) of chlorogenic acid from 6.6 to 5.8–6.1 $\mu\text{g g}^{-1}$ of FW in juices made of untreated tomatoes and from 8.2 to 7.7–7.9 $\mu\text{g g}^{-1}$ of FW in juices prepared with MIPEF-treated tomatoes at 56 days of storage at 4 °C (Figure 2). These results were similar to those reported by Odriozola-Serrano et al.,⁸ in which chlorogenic acid was also found to be in greater concentrations over the time in juices processed by HIPEFs than in those thermally treated, when tomato juice was prepared with untreated tomatoes. Tomato juices have been found to be a rich source of caffeic acid (1.4–2.7 $\mu\text{g g}^{-1}$ of FW), ferulic acid (1.8–2.4 $\mu\text{g g}^{-1}$ of FW), and *p*-coumaric acid (0.8–1.0 $\mu\text{g g}^{-1}$ of FW) and their glycosylated forms. Significantly ($p < 0.05$) lower concentrations of caffeic acid (87%), *p*-coumaric acid (34%), and ferulic acid (33%) were obtained in juices prepared with untreated tomatoes compared to those made of MIPEF-processed tomatoes (Figure 2). Ferulic-*O*-glucoside acid was not affected by MIPEF treatments ($p > 0.05$), whereas caffeic-*O*-glucoside acid was enhanced from 3 to 3.5 $\mu\text{g g}^{-1}$ of FW in juices made of MIPEF-treated tomatoes.

Higher concentrations in ferulic, *p*-coumaric, and caffeic acids were obtained just after processing in HIPEF-treated tomato juices than in heat-treated juices, regardless of the tomatoes used, related to the higher processing temperatures reached in thermally processed samples. Changes in ferulic, *p*-coumaric,

and caffeic acids during storage are shown in Figure 2. The caffeic acid concentration was slightly enhanced ($p < 0.05$) during the storage time, reaching maximal values of 1.6–1.7 $\mu\text{g g}^{-1}$ of FW at 56 days in juices prepared with untreated tomatoes and 2.9–3.0 $\mu\text{g g}^{-1}$ of FW in juices made of MIPEF-treated tomatoes. Tomato juices underwent a substantial depletion ($p < 0.05$) of *p*-coumaric acid over time, leading to values of 0.4–0.8 $\mu\text{g g}^{-1}$ of FW at 56 days of cold storage, which may be a consequence of its conversion to caffeic acid.¹⁹ Therefore, the increase of caffeic acid in tomato juices during 56 days of storage may be directly associated with residual hydroxylase activities, which convert *p*-coumaric acid in caffeic acid. Ferulic-acid- and caffeic-acid-glycosylated forms decreased ($p < 0.05$) during storage. The content of ferulic-*O*-glucoside acid was maintained during the first 35 days, whereas the caffeic-*O*-glucoside acid concentration was kept during 49 days of cold storage, irrespective of the processing treatment applied. In caffeic-*O*-glucoside and ferulic-*O*-glucoside acids, the 3-hydroxy function at the C ring of the flavonoid is blocked by a sugar moiety. Thus, the blockage of the 3-hydroxyl group is perhaps one of the reasons for the greater stability of glycosidic forms toward their aglycones.²⁰

Flavonols. The content of quercetin and rutin was not enhanced in juices prepared with tomatoes processed by MIPEFs. The initial content of rutin in tomato juices was 60.3–63.2 $\mu\text{g g}^{-1}$ of FW in juices made of untreated tomatoes and 61.2–65.6 $\mu\text{g g}^{-1}$ of FW in juices prepared with MIPEF-treated tomatoes. However, quercetin was found at concentrations of 2.1 $\mu\text{g g}^{-1}$ of FW in just processed juices, irrespective of the tomatoes used. Stewart et al.³ reported levels of quercetin in tomato juices ranging from 2.8 to 3.7 mg 100 mL⁻¹ of FW. Valverdú-Queralt et al.¹⁵ measured concentrations of quercetin between 0.4 and 0.8 $\mu\text{g g}^{-1}$ of FW for different commercial tomato cultivars. The quercetin concentration of tomatoes varies according to fruit cultivar, country of origin, harvesting seasons, and growing condition.

The quercetin content depleted significantly ($p < 0.05$) throughout the storage of tomato juices, reaching values of 1.2–1.5 $\mu\text{g g}^{-1}$ of FW (Figure 3) at 56 days, whereas rutin slightly decreased during storage. This fact could be attributed to the bonded 3-hydroxyl group in the case of rutin, which may be one of the reasons for the greater stability of rutin forms

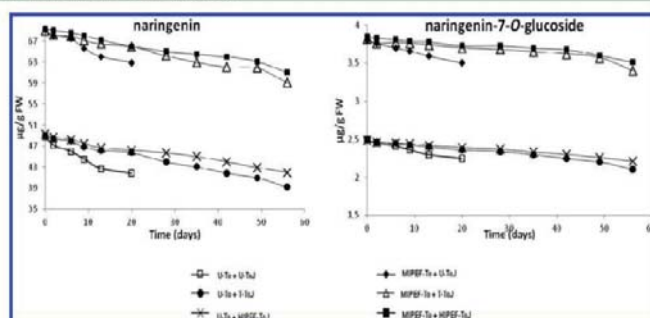


Figure 4. Effects of HIPEF and heat treatments on flavanones of tomato juices made of untreated and MIPEF-treated tomatoes through storage at 4 °C. U-To + U-ToJ, untreated tomato + untreated tomato juice; U-To + HIPEF-ToJ, untreated tomato + HIPEF-treated tomato juice; U-To + T-ToJ, untreated tomato + thermal-treated tomato juice; MIPEF-To + U-ToJ, MIPEF-treated tomato + untreated tomato juice; MIPEF-To + HIPEF-ToJ, MIPEF-treated tomato + HIPEF-treated tomato juice; and MIPEF-To + T-ToJ, MIPEF-treated tomato + thermal-treated tomato juice. Data shown are the mean $\pm$ standard deviation.

toward quercetin.²⁰ However, HIPEF-treated juices showed significantly ($p < 0.05$) greater quercetin and rutin contents than juices processed by heat just after processing and during storage for 56 days at 4 °C, irrespective of the tomatoes used. These results are in agreement with those reported by Odriozola-Serrano et al.,⁶ who studied the effects of thermal and HIPEF treatments on tomato juices made with untreated tomatoes.

Flavanones. The content of naringenin was 40% higher in juices made of MIPEF-treated tomatoes in comparison to juices prepared with untreated tomatoes. The same tendency was observed for the naringenin glycosidic form. The content of naringenin-7-O-glucoside in juices prepared with MIPEF-treated tomatoes was 52% higher than in juices made of untreated tomatoes. After MIPEF treatments, the resealing process takes place in a time scale of seconds or minutes. The higher content of naringenin and its glycosylated form in juices made of MIPEF-treated tomatoes could be attributed to the induction of flavanone synthase when MIPEF treatments are applied.⁶

Thermal and HIPEF processing did not modify the initial content of naringenin and naringenin-7-O-glucoside in tomato juices. The naringenin concentration depleted significantly ($p < 0.05$) throughout the storage, leading to values of 39.2–42.0 $\mu\text{g g}^{-1}$ of FW for juices made of untreated tomatoes and 59.1–62.9 $\mu\text{g g}^{-1}$ of FW for juices prepared with MIPEF-treated tomatoes (Figure 4), whereas glycosylated naringenin slightly changed during storage.²¹ HIPEF-processed tomato juices better maintained the flavanones content during the storage period than thermally treated and untreated juices (Figure 4).

Effect of Processing and Storage on HAC. Figure 5 shows changes in the antioxidant capacity of tomato juices measured through the DPPH and ABTS assays. According to both methods, tomato juices made of MIPEF-treated tomatoes had significantly ($p < 0.05$) higher HAC (4.28–6.24 mmol of TE 100 g^{-1} of FW) than tomato juices prepared with untreated tomatoes (3.07–5.47 mmol of TE 100 g^{-1} of FW). HIPEF-processed tomato juices obtained from untreated and MIPEF-treated tomatoes showed significantly ($p < 0.05$) higher HAC (3.3–5.6 mmol of TE 100 g^{-1} of FW) just after processing and throughout the storage period than those treated at 90 °C for 60 s. Juices treated by heat showed levels of HAC ranging from

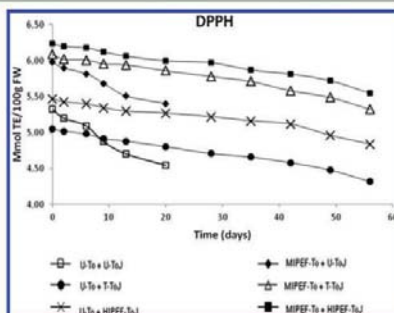


Figure 5. Effects of HIPEF and heat treatments on antioxidant capacities of tomato juices made of untreated and MIPEF-treated tomatoes through storage at 4 °C. U-To + U-ToJ, untreated tomato + untreated tomato juice; U-To + HIPEF-ToJ, untreated tomato + HIPEF-treated tomato juice; U-To + T-ToJ, untreated tomato + thermal-treated tomato juice; MIPEF-To + U-ToJ, MIPEF-treated tomato + untreated tomato juice; MIPEF-To + HIPEF-ToJ, MIPEF-treated tomato + HIPEF-treated tomato juice; and MIPEF-To + T-ToJ, MIPEF-treated tomato + thermal-treated tomato juice. Data shown are the mean $\pm$ standard deviation.

2.6 to 5.3 mmol of TE 100 g^{-1} of FW at 56 days of storage (Figure 5). The changes in the antioxidant capacity over time might be associated with the variations of the individual phenolics. The antioxidant capacity of fruits and vegetables is known to depend upon a wide number of compounds. A high relationship was found between relative HAC and TP contents, with $R^2 = 0.9972$ (DPPH) and $R^2 = 0.9834$ (ABTS[•]). The magnitude of changes in values of DPPH and ABTS inhibition were highly correlated to the presence of flavanones ($R^2 = 0.9749$ –0.9864) and phenolic acids ($R^2 = 0.9571$ –0.9986) rather than flavonol content ($R^2 = 0.2398$ –0.3554) in tomato juices.

An enhancement of polyphenolic compounds (phenolic acids and flavanones) and HAC were observed in juices made of tomatoes processed by MIPEFs. Increases of chlorogenic acid (25%) and naringenin-7-O-glucoside (52%) were obtained

in juices made of MIPF-treated tomatoes compared to those prepared with untreated tomatoes. However, the content of flavonols was not enhanced after MIPF treatments. The amounts of individual polyphenol compounds underwent a substantial loss during storage in both juices prepared with MIPF-treated and untreated tomatoes, with the exception of the caffeic acid content. However, HIPEF-processed tomato juices better maintained the individual polyphenols just after processing and during the storage period than thermally treated and fresh juices. Consecutive application of MIPF and HIPEF treatments could be proposed as a strategy for producing tomato juices with high antioxidant properties.

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Notes

The authors declare no competing financial interest.

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Publicació 15: Gazpacho consumption is associated with lower blood pressure and reduced hypertension in a high cardiovascular risk cohort. Cross-sectional study within the PREDIMED trial

Alexander Medina-Remón, Anna Vallverdú-Queralt, Sara Arranz-Martínez, Emilo Ros, Miguel Ángel Martínez-González, Emilo Sacanella, Maribel Covas, Dolores Corella, Jordi Salas-Salvadó, Enrique Gómez-Gracia, Valentina Ruíz-Gutiérrez, José Lapetra, Miquel Fiol, Fernando Arós, Guillermo Saez, Lluís Serra-Majem, Xavier Pinto; Miguel Ángel Muñoz, Ramón Estruch & Rosa M. Lamuela-Raventós. *Nutrition Metabolism and Cardiovascular Diseases*. 2012 (in press).

Publicació 16: The Effect of Polyphenol Consumption on Blood Pressure

Alexander Medina-Remón, Ramón Estruch, Anna Tresserra-Rimbau, Anna Vallverdú-Queralt & Rosa M. Lamuela-Raventós. *Mini Reviews in Medicinal Chemistry*. 2012 (in press).

Publicació 17: Light Gazpachos Contain Higher Phytochemical Levels than Conventional Gazpachos

Anna Vallverdú-Queralt, Alexander Medina-Remón, Ramón Estruch & Rosa M. Lamuela-Raventós. *Food Science and Research International*. 2012 (in press).

Annex

Publicació 18: Chemical and Sensory Analysis of Organic and Conventional Tomato Juices

Anna Vallverdú-Queralt, Federica Tesini, Enrico Valli, Rosa M. Lamuela-Raventós, Tullia Gallina Toschi & Alessandra Bendini. *Food Chemistry*. 2012 (in revision).

Publicació 19: Chemical and sensory analysis of HIPEF and thermal treated juices prepared with MIPEF and untreated tomatoes

Anna Vallverdú-Queralt; Sara Barbieri, Giuseppe Di Lecce, Olga Martín-Belloso, Tullia Gallina Toschi & Alessandra Bendini. *Food Research International*. 2012 (in revision).

Publicació 20: Changes in carotenoid profile of tomato juices after PEF treatments

Anna Vallverdú-Queralt, Isabel Odriozola-Serrano, Gemma Oms-Oliu, Rosa M. Lamuela-Raventós, Pedro Elez-Martínez & Olga Martín-Belloso. *Journal of Agricultural and Food Chemistry*. 2012 (in revision).

2. Comunicacions en congressos

Comunicació 1: Presentació oral

1er Congreso tecnológico del tomate para industria. Badajoz, España 2009.

“Folin-Ciocalteu assay for total polyphenols analysis in tomato samples needs a previous clean up procedure.”

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& Rosa M. Lamuela-Raventós^{1,2}.

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Tomatoes are one of the most widely consumed vegetables worldwide, not only in fresh form, but also in a wide range of processed products. The Folin Ciocalteu (FC) assay has been used for many years to determine the total polyphenol (TPs) content in tomatoes (1,2). However, it is affected by several interfering substances (3). The aim of this study was to improve the determination of TPs in tomato samples using solid phase extraction (SPE) with Oasis[®] MAX 96-well plate in order to eliminate these substances before the F-C assay.

The F-C assay was optimised, to eliminate interferences by reducing substances, and validated. The sensitivity, linearity, precision and accuracy were evaluated by spiking the tomato matrix with known levels of gallic acid standard at six different concentrations. The stability after successive freeze and thaw cycles, and short- and long-term temperature stability were also evaluated. We used high-performance liquid chromatography to calculate the recovery of nine representative polyphenols with different polarity, after spiking the tomato samples with four different concentrations of each standard. Seven tomato samples were analysed and TPs were expressed as mg of gallic acid equivalents (GAE)/100g dry material (DM).

The results of the F-C assay were linear over the working range 1 to 75 mgL⁻¹. Assay precision and accuracy were between the accepted values according to AOAC INTERNATIONAL(4). The samples and standards were stable under the storage and sample handling condition used and after successive freeze and thaw cycles. The recoveries for nine polyphenol standards after SPE were between 79.12% and 108.75%. The concentrations of TPs for tomato samples after SPE were between 149.99 and 247.12 mg of GAE/100g DM.

We have optimized and validated a method for analysis of TPs in tomato samples, eliminating the interferences with the F-C assay using 96-well plate cartridges to carry out SPE. This methodology is fast and simple, and it allows the analysis of a large number of samples at the same time.

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Comunicació 2: Pòster

1er Congreso tecnológico del tomate para industria. Badajoz, España 2009.

“The extraction of the solid phase of tomato samples before the Folin-Ciocalteu method to reduce interferences.”

Anna Vallverdú-Queralt, Vaila Mallace, Alexander Medina-Remón, Cristina Andrés-Lacueva & Rosa M. Lamuela-Raventós.



UNIVERSITAT DE BARCELONA

Folin-Ciocalteu assay for total polyphenols analysis in tomato samples needs a previous clean up procedure

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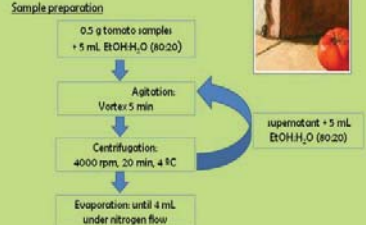
1. Introduction

Tomatoes are one of the most widely consumed vegetables worldwide, not only in fresh form, but also in a wide range of processed products. Foods rich in polyphenols such as tomatoes may have an important effect in preventing diseases, specifically they have been related with a lower risk of cancer and cardiovascular diseases. The Folin-Ciocalteu (F-C) assay has been used for many years to determine the total polyphenol (TPs) content in tomatoes. However, TPs content is sometimes overestimated for some reducing substances such as ascorbic acid, sugars and aminoacids which interfere in the assay.

Aim: The aim of this study was to eliminate non-phenolics interfering substances before the F-C assay using solid phase extraction (SPE) in order to improve the determination of TPs in tomato samples. This is the first study that has assessed TPs content in tomato samples after SPE, allowing the analysis of a large number of samples at the same time, using Oasis[®] MAX 96-well so as to eliminate these interfering substances.

2. Method

Sample preparation



Sample cleaning-up: Oasis MAX 96 well plate

- 1st Condition: 1 mL of methanol 98-100%.
- 2nd Condition: 1 mL of sodium acetate 50mM pH 7.
- Load: 1mL samples + 1mL Milli-Q water + 34 µL HCl.
- Cleaning: 1 mL sodium acetate 50mM pH 7/ 5% methanol
- Elution: 1.8 mL methanol at 2% formic acid.

Analysis of total polyphenols in tomatoes, in thermo microtiter 96 well plate.

- 20 µL of each methanolic fraction eluted after SPE.
- 188 µL of Milli-Q water.
- 12 µL of F-C reagent.
- 30 µL of sodium carbonate (200 g/L).
- Incubation 60 min. in the darkroom.
- After the reaction period: 50 µL of Milli-Q.
- Measure absorbance at 765 nm at the Spectrophotometers UV/VIS Thermo Multiskan Spectrum.

Validation:

- Linearity: 0.25-10 mg/L (gallic acid)
- Sensitivity: LoD= 3*SD, LoQ= 10*SD (10 blanks at 765 nm)
- Accuracy: % recoveries
- Precision: RSD=SD/X*100
- Stability: - Short-term - Long-term - After successive freeze-thaw cycles

- Recoveries of gallic, caffeic, p-coumaric, protocatechuic, p-hydroxybenzoic and chlorogenic acids, naringenin, rutin and quercetin, at 5, 10, 25 and 50 mg/L.

- Seven samples were analysed and results were expressed as mg of gallic acid equivalents/100g dry material.

3. Results

Linearity: 0.25-10mg/L → 0.1 x - 0.003

Sensitivity: LOD: 0.006 mg/L, LOQ: 0.019 mg/L



The intra and inter-day precision and accuracy are between the accepted values for the AOAC International (Table 1).

Table 1: Intra- and inter-day accuracy and precision of the assay

Calibrator concentration (mg/L)		Means measured concentration (mg/L)		Precision, R.S.D. (%)		Recovery, error (%)	
Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
1.12	1.07	1.01	0.98	0.10	0.12	90.57	91.97
2.24	2.14	2.24	2.06	0.10	0.11	100.08	96.25
4.48	4.27	4.38	4.25	0.09	0.09	97.77	99.60
6.73	6.41	6.69	6.40	0.09	0.10	99.38	99.87
8.97	8.55	8.93	8.21	0.06	0.08	99.58	96.00
11.21	10.69	11.62	11.03	0.05	0.07	103.67	103.21
13.40	13.81	13.89	14.51	0.04	0.05	103.66	105.10

RSD: relative standard deviation

Stability

Table 2: Freeze-thaw cycle, short and long-term stability

Temperature	Freeze-thaw cycles stability	Short-term stability	Long-term stability
-20 °C	94.9-106.2 %	90.1-104.0 %	95 %
-80 °C	91.7-106.5 %	85.2-102.6 %	95 %

Absolute recoveries

Table 3: Absolute recoveries of the nine polyphenols

Polyphenol	Mean recoveries %	Standard deviation (S.D.)
Gallic acid	92.47	4.45
Naringenin	98.20	3.39
Caffeic acid	98.74	10.72
Rutin	87.32	4.81
Quercetin	77.52	5.43
p-Coumaric acid	96.14	3.36
Protocatechuic acid	108.75	3.15
p-Hydroxybenzoic acid	98.53	2.00
Chlorogenic acid	101.11	5.17

The concentrations of TPs for tomato samples vary between 260.80 and 472.23 mg of GAE/100 g DM when SPE was not used and between 113.32 and 174.85 mg of GAE/100g DM after the SPE. So this means that the results have been overestimated until now.

4. Conclusions

- It is the first time that SPE has been used to eliminate interfering nonphenolics reductants before the F-C assay.
- The method described may be useful as a routine control assay for total polyphenols determination in food, especially food such as fruit and vegetables that are rich in non phenolic reductant substances that react with FC reagent and interfere in the analysis.
- Samples require minimal sample preparation before TPs analysis. It is ideal to analyse large batches of samples, as the Oasis[®] MAX 96-well plate accommodates much larger sample volumes.

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I International conference about industrial tomato, Extremadura España

Comunicació 3: Pòster

The 4th International Conference on Polyphenols and Health. Harrogate International Centre, Yorkshire, England, 2009.

“An improved method to determine total polyphenols in tomato samples eliminating interferences with Folin-Ciocalteu assay.”

Anna Vallverdú-Queralt, Alexander Medina-Remón, Vaila Mallace, Cristina Andrés-Lacueva & Rosa M. Lamuela-Raventós.



UNIVERSITAT DE BARCELONA

An improved method to determine total polyphenols in tomato samples eliminating interferences with Folin-Ciocalteu assay.

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1. Introduction

Tomatoes are one of the most widely consumed vegetables worldwide, not only in fresh form, but also in a wide range of processed products. Foods rich in polyphenols such as tomatoes may have an important effect in preventing diseases, they have specifically been related with a lower risk of cancer and cardiovascular diseases. The Folin-Ciocalteu (F-C) assay has been used for many years to determine the total polyphenol (TP) content in tomatoes. However, TP content is sometimes overestimated for some reducing substances such as ascorbic acid, sugar and aminoacids which interfere in the assay.

Aim: The aim of this study was to eliminate non-phenolic interfering substances before the F-C assay using solid phase extraction (SPE) in order to improve the determination of TP in tomato samples. This is the first study that has analysed TP content in tomato samples after SPE, allowing the analysis of a large number of samples at the same time, using Oasis[®] MAX 96-well so as to eliminate these interfering substances.

2. Method

Sample preparation

- 0.5 g tomato samples + 5 mL EtOH:H₂O (80:20)
- Agitation: Vortex 5 min
- Centrifugation: 4000 rpm, 20 min, 4 °C
- Evaporation: until 4 mL under nitrogen flow

Sample clean-up: Oasis MAX[®] 96 well plate

- 1st Condition: 1 mL of methanol 98-100%.
- 2nd Condition: 1 mL of sodium acetate 50 mM pH 7.
- Load: 1 mL samples + 1 mL Milli-Q water + 34 µL HCl.
- Cleaning: 1 mL sodium acetate 50 mM pH 7/ 5% methanol
- Elution: 1.8 mL methanol at 2 % formic acid.

Analysis of total polyphenols in tomatoes, in thermo microtiter 96 well plate.

- 20 µL of each methanolic fraction eluted after SPE.
- 188 µL of Milli-Q water.
- 12 µL of F-C reagent.
- 30 µL of sodium carbonate (200 g/L).
- Incubation 60 min. in the darkroom.
- After the reaction period: 50 µL of Milli-Q
- Measure absorbance at 765 nm at the Spectrophotometers UV/VIS Thermo Multiskan Spectrum.

Validation:

- Linearity: 0.25-10 mg/L (gallic acid)
- Sensitivity: LeD= 3*SD, LeQ= 10*SD (10 blanks at 765 nm)
- Accuracy: % recoveries
- Precision: RSD=SD/X*100
- Stability:
 - Short-term
 - Long-term
 - After successive freeze-thaw cycles

- Recoveries of gallic, caffeic, p-coumaric, protocatechuic, p-hydroxybenzoic and chlorogenic acids, naringenin, rutin and quercetin, at 5, 10, 25 and 50 mg/L.

- Seven samples were analysed and results were expressed as mg of gallic acid equivalents/100g dry material.

3. Results

Linearity: 0.25-10 mg/L $y = 0.1x - 0.003$

Sensitivity: LOD: 0.006 mg/L, LOQ: 0.019 mg/L

The intra and inter-day precision and accuracy are between the accepted values for AOAC International (Table 1).

Table 1: Intra- and inter-day accuracy and precision of the assay

Calibrator concentration (mg/L)	Means measured concentration (mg/L)		Precision, R.S.D. (%)		Recovery, error (%)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
1.12	1.07	1.01	0.98	0.10	0.12	90.57
2.24	2.14	2.24	2.06	0.10	0.11	100.08
4.48	4.27	4.38	4.25	0.09	0.09	97.77
6.73	6.41	6.69	6.40	0.09	0.10	99.38
8.97	8.55	8.93	8.21	0.06	0.08	99.58
11.21	10.69	11.62	11.03	0.05	0.07	103.67
13.40	13.81	13.89	14.51	0.04	0.05	103.66

RSD, relative standard deviation

Stability

Table 2: Freeze-thaw cycle, short and long-term stability

Temperature	Freeze-thaw cycles stability	Short-term stability	Long-term stability
-20 °C	94.9-106.2 %	90.1-104.0 %	95 %
-80 °C	91.7-106.5 %	85.2-102.6 %	95 %

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Table 3: Absolute recoveries of the nine polyphenols

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The concentrations of TP_i for tomato samples vary between 260.80 and 472.23 mg of CAE/100 g DM when SPE was not used and between 118.32 and 174.85 mg of CAE/100g DM after the SPE. So this means that the results have been overestimated until now.

4. Conclusions

- It is the first time that SPE has been used to eliminate interfering non-phenolic reductants before the F-C assay.
- The method described may be useful as a routine control assay for total polyphenol determination in food, especially food such as fruit and vegetables that are rich in non phenolic reductant substances that react with FC reagent and interfere in the analysis.
- Samples require minimal sample preparation before TP analysis. It is ideal to analyse large batches of samples, as the Oasis[®] MAX 96-well plate accommodates much larger sample volumes.

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6. Supported by

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4th International Conference on Polyphenols and Health, Yorkshire England

Comunicació 4: Pòster

II Congreso FESNAD. Barcelona, España, 2010.

“Polifenoles totales en muestras de tomate eliminando sustancias interferentes por el método de FOLIN-CIOCALTEU.”

Anna Vallverdú-Queralt, Alexander Medina-Remón, Cristina Andrés-Lacueva & Rosa M. Lamuela-Raventós.

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Nutr Hosp Vol. 25. Suplemento 1. Marzo 2010

nivel de significancia del 5% ($p < 0,05$), hay una diferencia estadísticamente significativa para la cantidad total de fósforo, siendo los valores más altos en yogur de cabra, mientras que calcio y magnesio no muestran diferencias estadísticamente significativas entre las distintas especies.

P154 Polifenoles totales en muestras de tomate eliminando sustancias interferentes por el método de Folin-Ciocalteu

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Introducción: El método de Folin-Ciocalteu (F-C) se ha utilizado durante años para determinar el contenido de polifenoles totales (PTs) en muestras de tomate; no obstante otras sustancias presentes en el tomate con poder reductor (como ácido ascórbico, dehidráscorbico, azúcares...) interfieren en la cuantificación de los PTs.

Objetivo: El objetivo de nuestro estudio es mejorar la determinación de polifenoles totales en muestras de tomate utilizando la extracción en fase sólida (SPE) con placas Oasis[®] MAX 96 con el objetivo de eliminar las sustancias interferentes antes de la determinación de PTs con el método de F-C.

Método: El método de F-C ha sido optimizado, para reducir las interferencias causadas por sustancias reductoras, y validado. Se ha determinado la sensibilidad, precisión, exactitud y linealidad del método añadiendo 6 concentraciones de ácido gálico a la matriz de tomate. La estabilidad a corto y largo plazo y después de sucesivos ciclos de congelación-descongelación también se ha evaluado. Mediante cromatografía líquida de alta resolución se han determinado las recuperaciones de nueve polifenoles con diferente polaridad, después de adicionar a la matriz de tomate 4 concentraciones distintas de cada patrón estudiado. Se han analizado siete muestras de tomate y los resultados se expresan en mg equivalentes de ácido gálico/100 g de materia seca.

Resultados: El intervalo de linealidad del método F-C oscila entre 1 y 75 mg/L. Los valores de precisión y exactitud están comprendidos entre los niveles que acepta la AOAC INTERNATIONAL. Las muestras y patrones son estables tanto a corto como a largo plazo, así como después de los sucesivos ciclos de conge-

lación descongelación. Las recuperaciones de los nueve polifenoles después de la SPE están comprendidos entre 79,12% y 108,75%. Las concentraciones de PTs en las muestras de tomate después de SPE oscilan entre 149,99 Y 247,12 mg equivalentes de ácido gálico/100 g materia seca.

Conclusión: Se ha optimizado y validado un método de análisis de PTs en muestras de tomate eliminando las interferencias con el método F-C utilizando SPE con placas Oasis[®] MAX 96 obteniendo unos resultados entre 149,99 Y 247,12 mg de GAE/100 g materia seca.

P155 Valor nutritivo de tomate maduro en base a su perfil metabólico

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Introducción: Las modernas técnicas de análisis global, denominadas 'omics' permiten estudiar la complejidad nutritiva de los alimentos. La Metabolómica se ha aplicado en este trabajo como herramienta para explorar el valor nutritivo de tomate maduro en base a su perfil metabólico.

Objetivo: obtener un mapa global de los metabolitos polares que componen el tomate tales como azúcares, ácidos orgánicos y aminoácidos.

Métodos: La cromatografía de gases-espectrometría de masas (GC/MS) permitió el análisis simultáneo de los metabolitos polares presentes en tomate. No obstante, la inyección de la muestra en un GC requiere una conversión previa de los compuestos a derivados volátiles mediante un proceso de derivatización. La identificación de los metabolitos se realizó a través de la librería "Fiehn GC/MS metabolomic library". Las concentraciones relativas de los metabolitos se calcularon a partir de los factores respuesta de cada uno de los compuestos, relacionando el área de cada pico con la del estándar interno (ribitol) presente en cada cromatograma.

Resultados: En los extractos de tomate maduro se identificaron 41 metabolitos. Los compuestos mayoritarios en tomate (glucosa, fructosa y ácido cítrico) se detectaron en concentraciones relativas de 1.344,72, 1.739,46 y 352,16, respectivamente. No obstante, se detectaron otros azúcares como la galactosa, sacarosa, inositol, ácido galacturónico, manosa, maltosa, glucosa 6-fosfato, rafinosa, manitol, galactitol, sorbitol, y ácidos tales como el isocítrico, gulónico, glucónico, tartárico, sacárico, succínico, maleico, láctico, citramálico, fumárico,

Annex

Comunicació 5: Pòster

25th International Conference on Polyphenols. Montpellier, France, 2010.

“Phenolic profile and antioxidant capacity in tomato varieties from markers.”

Anna Vallverdú-Queralt, Alexander Medina-Remón, Miriam Martínez-Huélamo, Olga Jáuregui, Cristina Andrés-Lacueva & Rosa M. Lamuela-Raventós.



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Phenolic profile and antioxidant capacity in tomato varieties from markers.

Anna Vallverdú-Queralt^{1,2}, Alexander Medina-Ramón^{1,3}, Miriam Martínez-Huélamo⁴, Olga Jauregui-Pallares⁵, Cristina Andrés-Lacueva^{1,6} and Rosa M. Lamuela-Raventós^{1,2*}

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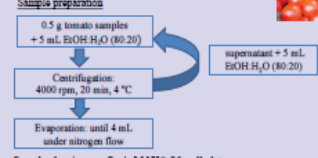
1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is the second most important vegetable crop worldwide. Epidemiological studies have pointed out that consumption of fruits and vegetables imparts health benefits, e.g. reduced risk of coronary heart disease and stroke, as well as certain types of cancer. Tomatoes are a key component in the so-called "Mediterranean diet" which is strongly associated with a reduced risk of chronic degenerative diseases.

Aims: Total polyphenols, individual polyphenols and antioxidant activity of seven commercial varieties of tomato produced in Spain were studied. The Folin-Ciocalteu (F-C) assay was used to analyse total polyphenol (TP) after solid phase extraction (SPE). Liquid chromatography coupled to mass spectrometry in tandem mode (LC/MS/MS) with negative ion detection was carried out to identify and quantify flavonols, flavanones, cinnamic acid derivatives and phenolic acids. Antioxidant capacity was analyzed by the ABTS assay.

2. Method

Sample preparation



Analysis of total polyphenols in tomatoes, in 96-well microtiter plates.

- 20 µL of each methanolic fraction diluted after SPE.
- 188 µL of Milli-Q water
- 12 µL of F-C reagent
- 30 µL of sodium carbonate (200 g/L).
- Incubation: 60 min. in the darkroom.
- After the reaction period: 50 µL of Milli-Q
- Measure absorbance at 765 nm in the Spectrophotometer UVVIS. Results were expressed as mg of gallic acid equivalents (GAE)/100 g fresh material (FM).

Analysis of total antioxidants in tomatoes.

The antioxidant activity of the compounds is determined by the decolorization of the ABTS^{•+}, through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm in the Spectrophotometer UVVIS. Results of Trolox equivalent antioxidant capacity (TEAC) were expressed as (mmol Trolox equivalent (TE)/100 g FM).

calibration

Trolox mM	Trolox mL	ABTS ^{•+} mM	ABTS ^{•+} mL
0	5	0	245
5	5	250	245
8	5	400	245
10	5	500	245
15	5	750	245

sample

Dilution	Sample mL	FB mL
0	0	200
40	5	195
20	10	190
10	20	180

Calculation

$$INH\% = \frac{(A_{\text{solvent}} - A_{\text{compound}})}{(A_{\text{solvent}}) \times 100}$$

$$TEAC = \frac{INH\% \text{ of sample}}{INH\% \text{ of Trolox}} \times 250$$

Analysis of flavonols, flavanones, cinnamic acid derivatives and phenolic acids

Time (min)	H ₂ O ACN 0.1%FA/0.1%
0	95
10	82
13	0
14	0
15	95

Column: 50 x 2.0 mm i.d., 5 µm Luna C18
Injection volume: 20 µL
Flow: 4 mL/min
Column temperature: 30 °C
Modes: Full scan, Product ion scan, neutral loss, multiple reaction monitoring

3. Results

Table 1. Total polyphenols and antioxidant activity expressed as mean ± SD of the seven varieties.

Varieties	Total polyphenols (mg GAE/100g FM)	Antioxidant activity (mmol TE/100 g FM)
malva	10.64 ± 0.30	1.79 ± 0.10
H-9661	13.32 ± 0.70	2.80 ± 0.20
H-9776	8.64 ± 0.20	1.29 ± 0.10
H-9997	9.01 ± 0.40	1.40 ± 0.10
albatro	8.69 ± 0.30	1.25 ± 0.09
guadiva	9.06 ± 0.20	1.63 ± 0.08
elegy	12.69 ± 0.40	1.98 ± 0.10

Albatro variety contained the lowest concentrations of phenolics, followed by H-9997 and Guadiva. The varieties with highest TP concentrations were H-9661, Elegy and Malva. A similar trend was observed for antioxidant activity.

Table 2. Quantification of cinnamic and phenolic acids (mean ± SD) expressed as µg/g fresh weight of seven varieties described in the literature and investigated in this study.

Varieties	Ferulic acid (µg/kg FM)	Chlorogenic acid (µg/kg FM)	Caffeic acid (µg/kg FM)	Protocatechuic acid (µg/kg FM)
malva	N.Q.	0.36 ± 0.01	0.68 ± 0.01	0.20 ± 0.02
H-9661	0.35 ± 0.03	0.46 ± 0.01	1.02 ± 0.05	0.46 ± 0.02
H-9776	0.10 ± 0.05	0.40 ± 0.01	0.53 ± 0.01	N.Q.
H-9997	N.Q.	0.38 ± 0.03	0.53 ± 0.04	N.Q.
albatro	0.39 ± 0.01	0.39 ± 0.02	0.55 ± 0.02	N.Q.
guadiva	N.Q.	0.38 ± 0.03	1.25 ± 0.01	N.Q.
elegy	0.21 ± 0.01	0.40 ± 0.02	0.63 ± 0.01	N.Q.

N.Q.: Not quantified

Table 3. Quantification of flavonols and flavanones (mean ± SD) expressed as µg/g fresh weight of seven varieties described in the literature and investigated in this study.

Varieties	Quercetin (µg/kg FM)	Rutin (µg/kg FM)	Naringenin (µg/kg FM)	Kaempferol-3-O-Glucoside (µg/kg FM)
malva	0.73 ± 0.02	19.79 ± 0.77	3.03 ± 0.21	N.Q.
H-9661	0.76 ± 0.04	20.44 ± 0.47	6.90 ± 0.19	N.Q.
H-9776	0.42 ± 0.01	2.68 ± 0.05	0.75 ± 0.04	N.Q.
H-9997	0.47 ± 0.04	4.65 ± 0.04	0.50 ± 0.01	0.27 ± 0.03
albatro	0.63 ± 0.04	0.79 ± 0.06	1.59 ± 0.25	N.Q.
guadiva	0.69 ± 0.06	6.07 ± 0.51	1.71 ± 0.06	N.Q.
elegy	0.69 ± 0.05	21.80 ± 1.68	0.70 ± 0.01	0.59 ± 0.01

N.Q.: Not quantified

The main polyphenol in all the varieties is rutin, which is present at levels ranging between 0.79 and 21.80 µg/g FM, followed by naringenin, which is found at levels between 0.50 and 6.90 µg/g FM. The variety with higher levels of individual polyphenols is H-9661 followed by Malva and Elegy, as it is reflected in the analysis of total polyphenols and antioxidant activity, as well.

4. Conclusions

- It is the first time that SPE has been used to eliminate interfering non-phenolic reductants before the analysis of tomato varieties.
- The variables studied allowed the differentiation of H-9661, H-9997, H-9661, malva, guadiva, albatro and elegy. Significant differences ($P < 0.05$) among varieties were obtained.
- H-9661 possessed the highest concentrations of total polyphenols and antioxidants, followed by elegy and malva.
- Rutin (quercetin-3-O-rutinoside) was the dominant flavonol amongst all the samples, followed by naringenin.

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The authors express their gratitude to CONESA for the samples and to CICTA (AGL2006-14228-C03-02, AGL2007-66638-C02 and AGL2010-22119-C03), RETICS RD06/0604/0003 from the Spanish Ministry of Science and Innovation (MICINN) and grant PI070240 from Instituto de Salud Carlos III, Spain for financial support.

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25th International Conference on Polyphenols, Montpellier France

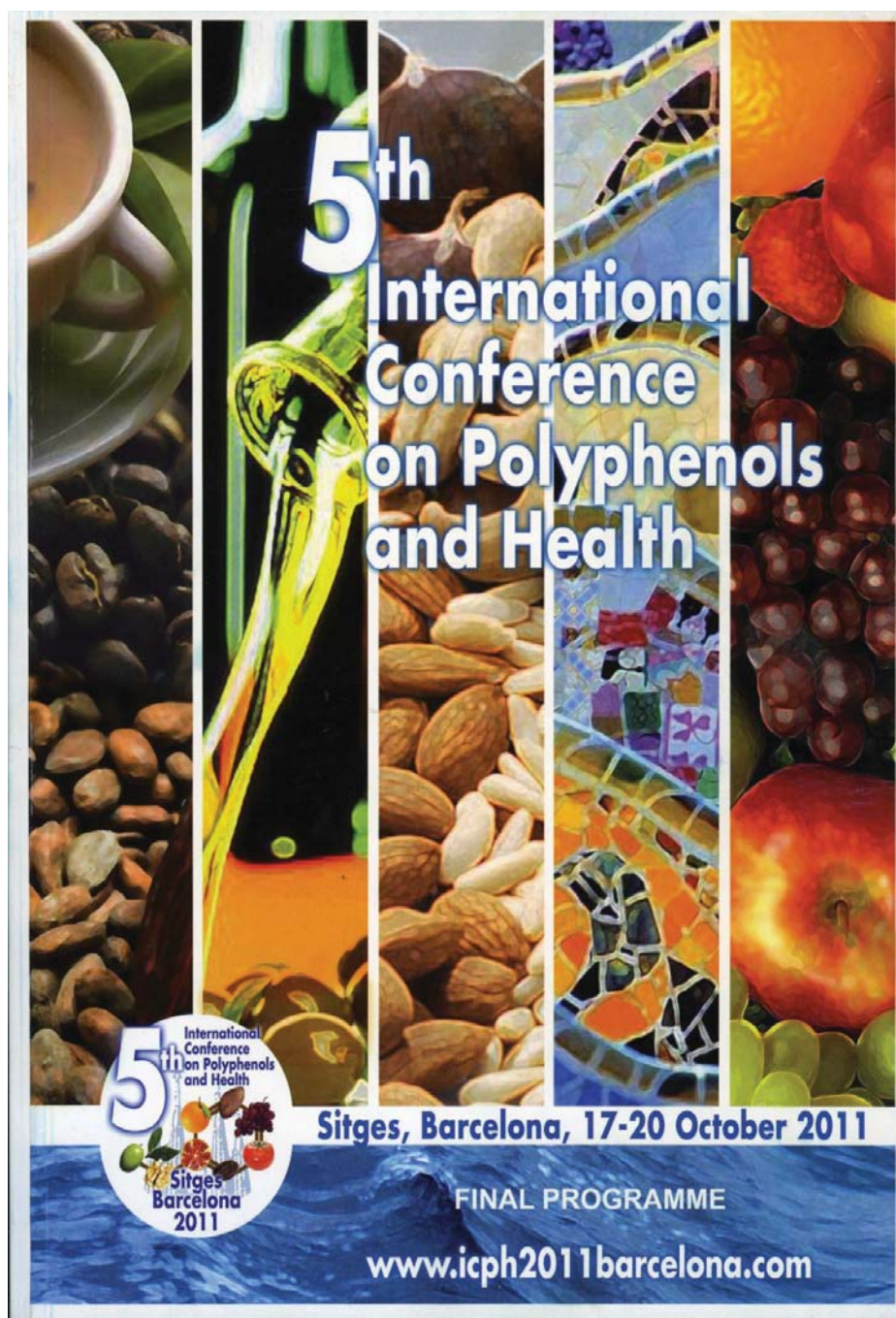
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Comunicació 6: Presentació Oral

The 5th International Conference on Polyphenols and Health. Sitges, Barcelona, Spain 2011.

“Oil addition increase anti-inflammatory effects of tomato.”

Miriam Martínez-Huélamo, Sara Tulipani, Palmira Valderas-Martínez, Giuseppe Di Lecce, Anna Vallverdú-Queralt, Ramón Estruch, Xavier Torrado, Elvira Escribano Ferrer & Rosa M. Lamuela-Raventós.



O52 - Co-sponsored Lecture by IOOC

NUTRIGENOMIC EFFECTS OF MEDITERRANEAN DIET AND OLIVE OIL CONSUMPTION ON CARDIOVASCULAR RISK

M. Fitó^{1,2}, V. Konstantinidou³, O. Khymenets⁴, M. Pujadas⁵, D. Muñoz-Aguayo¹, O. Castañer¹, M. Farras¹, MC. Tormos⁶, G. Blanchart¹, V. Ruiz-Gutiérrez⁷, G. Sáez⁸, R. De la Torre R^{1,4}, M.I. Covas^{1,2}, Cardiovascular Risk and Nutrition and e Human Pharmacology and Clinical Neurosciences Researchs Groups, IMIM-Research Institute Hospital del Mar, and b CIBER de Fisiopatología de la Obesidad y Nutrición, (CIBEROBN), Barcelona Spain¹, CIBER de Fisiopatología de la Obesidad y Nutrición, (CIBEROBN), Barcelona Spain², Hellenic Health Foundation, Athens-Greece³, Bromatology Department, Facultat de Farmàcia de Barcelona Spain⁴, Human Pharmacology and Clinical Neurosciences Researchs Groups IMIM-Research Institute Hospital del Mar⁵, Department of Biochemistry and Molecular Biology, Valencia University, Valencia-Spain⁶, Nutrition and Lipids Metabolism, Instituto de La Grasa de Sevilla-Spain⁷

Introduction Adherence to traditional Mediterranean diet, in which olive oil is the main source of fat, has been associated with reductions in cardiovascular morbidity and mortality. The objective of the present study was to assess the in vivo gene expression changes, protective for cardiovascular disease, associated with the consumption of traditional Mediterranean diet, virgin olive oil, and its phenolic compounds, in healthy volunteers.

Materials and methods: A randomized, parallel, controlled, double-blind trial (ISRCTN53283428) with three dietary interventions was performed. Ninety Volunteers (20-50 years) were recruited. Whole blood and urine samples were collected at 0 hours (baseline) and after 3 months of intervention. Volunteers were grouped into three groups: a) Group A: Traditional Mediterranean diet with virgin olive oil high in phenolic content; b) Group B: Traditional Mediterranean diet with olive oil with very low phenolic content; Group C: habitual diet. Changes in the expression of several cardiovascular disease-related genes were assessed. Plasma glucose, lipid profile, oxidative damage, tyrosol/ hydroxytyrosol, and in inflammation markers were also measured.

Results: Traditional Mediterranean diet consumption, decreased: 1) plasma oxidative and inflammatory status and; 2) the expression of genes related with both inflammation (interferon-gamma; IFNgamma, Rho-GTPase activating protein15; ARHGAP15, interleukin-7 receptor; IL7R) and oxidative stress (adrenergic-beta-2 receptor; ADRB2, and polymerase (DNA directed)-kappa; POLK) in mononuclear cells. All effects, with the exception of the decrease in POLK expression, were particularly observed when virgin olive oil was present in the TMD dietary pattern.

Conclusions: Olive oil phenolic compounds, within the frame of the traditional Mediterranean diet, enhance the health protective nutrigenomic effect. Our results support the idea that the benefits associated with a Mediterranean diet, virgin olive oil, and olive oil phenolic compounds consumption on cardiovascular risk could be mediated through changes in the expression of cardiovascular risk-related genes.

O53 - Co-sponsored Lecture by IOOC

OL ADDITION INCREASE ANTI-INFLAMMATORY EFFECTS OF TOMATO

Miriam Martínez-Huélamo^{1,2}, Sara Tulipani^{1,3}, Palmira Valderas-Martínez^{2,4}, Giuseppe Di Lecce¹, Anna Vallverdú-Queralt^{1,4}, Ramón Estruch^{2,4}, Xavier Torrado¹, Elvira Escribano Ferrer⁶, Rosa M. Lamuela-Raventós^{1,2}, Nutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Barcelona-Spain¹, CIBER 06/003 Physiopathology of obesity and nutrition (CIBEROBN) and RETICS RD06/0045/0003, Institute of Health Carlos III, Madrid-Spain², Ingenio Consolider Program, FUN-C-FOOD (CSD2007-063), Barcelona-Spain³, Department of Internal Medicine, Hospital Clinic, Institute of Biomedical Investigation August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona-Spain⁴, Department of Pharmacy and Pharmaceutical Technology, Biopharmaceutics and Pharmacokinetics Unit, Faculty of Pharmacy-Spain⁵

Introduction: Food matrix seems to be an important factor modulating absorption and further biological activity of dietary polyphenols. In order to verify whether the oral absorption and metabolism of the phenolic compounds contained in tomato and tomato sauces is influenced by the processing and the addition of a lipid matrix during sauce processing, we performed a prospective randomized, cross-over study with 40 healthy volunteers and three interventions: raw tomatoes, oil-free tomato sauce and refined olive oil-enriched tomato sauce.

Methods and materials: The phenolic content of the raw tomatoes and tomato sauces was characterized by UPLC-MS/MS analysis by Triple Quadrupole mass spectrometer, with negative ion detection mode. The corresponding biological metabolites were identified in plasma and urine by UPLC-MS/MS after solid-phase extraction of the biological samples with Oasis HLB 96-well plates. The identification of the compounds was carried out on the basis of their mass spectra in full scan mode and confirmed by MS/MS experiments (PIS, NL). Multiple



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reaction monitoring experiments were used for quantification purposes. Peripheral blood mononuclear cells were separated by Fycoll-Hypaque gradient. Adhesion molecule expression on T-lymphocytes and monocytes surface was determined by using a double-direct immunofluorescence test. Determinations were performed using a FACSCalibur flow cytometer. Bio-Plex cytokines assays were performed in soluble inflammatory biomarkers, using Luminex-100 equipment.

Results and conclusions: The processing of tomato to sauce and the addition of a lipid matrix during sauce preparation changes the phenolic composition of the product, probably due to changes in the bioaccessibility of phenolics from the food matrix. The use of oil in tomato sauce increases the anti-inflammatory effect since it reduces the expression of certain adhesion molecules associated with atherosclerosis than after raw tomatoes, oil-free tomato sauce. The plasma kinetics and urinary excretion of the flavanone naringenin and naringenin glucuronides after the interventions confirmed the highest bioaccessibility of this flavanone after tomato processing to sauce. The plasma pharmacokinetic of naringenin suggests a two-phase absorption only following the ingestion of oil-enriched tomato sauce. These findings suggest the occurrence of re-absorption events by enterohepatic circulation.

Polyphenols as preventive ingredients: in vitro experiment, animal models and human intervention studies

O54 - Invited Speaker

POLYPHENOLS IN THE PREVENTION OF CHRONIC DISEASES AND RELATED COMPLICATIONS: IN VITRO STUDIES WITH THE RIGHT MOLECULES

Daniele Del Rio, Luca Calani, Margherita Dall'Asta

The Laboratory of Phytochemicals in Physiology, Department of Public Health, University of Parma, Parma-Italy
On the basis of prospective, cross-sectional and intervention studies linking polyphenols to human health, several experimental papers in the literature have tried to evaluate the molecular mechanisms involved in their bioactivity. Polyphenols are reported to in vitro inhibit cancer cell proliferation, reduce vascularisation, protect neurons, stimulate vasodilation and improve insulin secretion, but are often studied as aglycones or as sugar conjugates and at non-physiological concentration. However, it is now well established that polyphenols undergo substantial metabolism after being ingested by humans in dietary relevant amount and that concentrations of plasma metabolites after a normal dietary intake rarely exceed nmol/L. This lecture intends to highlight that uncritical judgments made on the basis of the published literature about polyphenol bioactivity, may sometimes have been misled and misleading and to conclude that it is phenolic metabolites, formed in the small intestine and hepatic cells, and low molecular weight metabolic products of the colonic microbiota to travel around the human body in the circulatory system or reach body tissues to elicit bioactive effects. Therefore, this lecture will bring to the audience attention the most recent advancements in the field of polyphenol physiological activities, with particular attention to their antiinflammatory, estrogenic and antiglycative effects investigated with the most innovative and conservative experimental techniques.

O55 - Invited Speaker

PREVENTION OF AGING-RELATED ENDOTHELIAL DYSFUNCTION BY POLYPHENOLS: EVIDENCE FROM ANIMAL STUDIES

Cyril Auger, Noureddine Idris-Khodja, Valérie B. Schini-Kerth

Cardiovascular Pharmacology and Pathophysiology group, UMR CNRS 7213-Laboratory of Biophotonics and Pharmacology, Faculty of Pharmacy, University of Strasbourg Illkirch-Graffenstaden-France.

Several cardiovascular diseases, such as hypertension, diabetes, and aging are associated with an endothelial dysfunction. Since in vitro and ex-vivo studies have demonstrated that polyphenols can induce the formation of vasoprotective factors such as NO and EDHF-mediated responses, they may also exert beneficial effects on the endothelial function in vivo. Indeed, experimental studies indicate that chronic intake of polyphenol-rich products can prevent or improve the endothelial dysfunction in various animal models of cardiovascular diseases such as hypertension, and vascular aging. This presentation will also discuss the possible mechanistic aspects underlying the beneficial effects of polyphenols on the endothelial function.

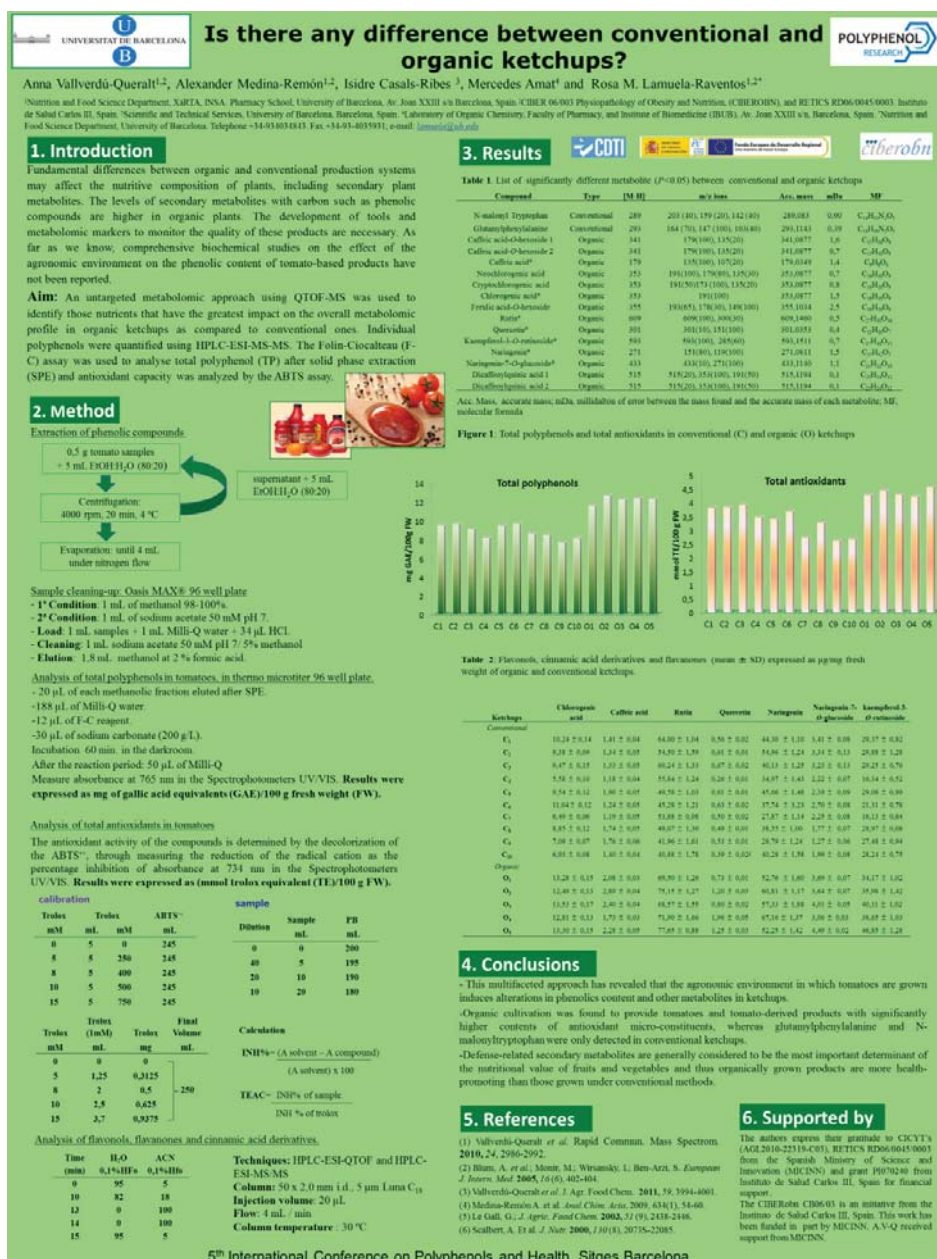
Annex

Comunicació 7: Pòster

The 5th International Conference on Polyphenols and Health. Sitges, Barcelona, Spain 2011.

“Is there any difference between conventional and organic ketchups?”

Anna Vallverdú-Queralt, Alexander Medina-Remón, Isidre Casals-Ribes, Mercedes Amat
& Rosa M. Lamuela-Raventós.



Annex

Comunicació 8: Pòster

The 5th International Conference on Polyphenols and Health. Sitges, Barcelona, Spain 2011.

“Effects of pulsed electric fields on total polyphenol content in tomato fruits: an emerging perspective.”

Anna Vallverdú-Queralt, Gemma Oms-Oliu, Isabel Odriozola-Serrano, Pedro Elez-Martínez, Rosa M^a Lamuela Raventós & Olga Martín-Belloso*.

POLYPHENOL RESEARCH

Effects of pulsed electric fields on total polyphenol content in tomato fruits: an emerging perspective

Anna Vallverdú-Queralt^{1,2}, Gemma Oms-Oliu¹, Isabel Odrizola-Serrano³, Pedro Elez-Martínez², Rosa M. Lamuela-Raventós^{1,2} and Olga Martín-Belloso³

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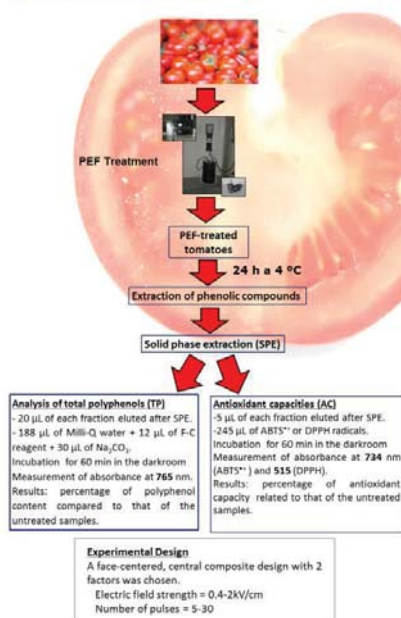
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1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is the second most important fruit crop worldwide. Epidemiological studies have pointed out that consumption of fruit and vegetables imparts health benefits, e.g. reduced risk of coronary heart disease and stroke, as well as certain types of cancer.

The application of Pulsed Electric Fields (PEF) in food processing has attracted large interest as a non-thermal preservation technology to maintain the nutritional quality and freshness of plant products. However, little research has been carried out to evaluate the feasibility of applying low-intensity PEF for producing secondary plant metabolites, such as polyphenol compounds, by inducing stress reactions. Therefore, the effects of PEF treatments on polyphenol content and antioxidant capacities of tomato fruit have been investigated.

2. Materials and Methods



Acknowledgments

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5th International Conference on Polyphenols and Health, 17th-20th October 2011, Sitges Barcelona

3. Results

Table 1 Central composite response surface design for TP and AC relative content (%) of tomato fruits treated under different PEF treatments.

E (kV/cm)	n (number of pulses)	TP relative content (%) ^a	AC (DPPH) relative content (%) ^a	AC (ABTS) relative content (%) ^a
1.2	30	144.61 ± 2.29	134.67 ± 1.64	129.76 ± 1.30
1.2	5	121.89 ± 2.92	120.80 ± 2.38	112.69 ± 1.77
1.2	18	137.19 ± 2.73	144.20 ± 2.08	141.36 ± 1.76
2	18	90.59 ± 2.88	122.40 ± 1.87	114.69 ± 1.38
0.4	18	121.21 ± 2.69	133.80 ± 1.99	130.20 ± 2.38
2	30	98.98 ± 4.65	109.85 ± 1.67	103.08 ± 1.53
0.4	30	134.49 ± 2.00	125.81 ± 2.15	117.30 ± 1.37
2	5	81.27 ± 1.95	101.60 ± 2.45	92.61 ± 0.95
0.4	5	106.84 ± 1.68	114.15 ± 2.57	106.57 ± 1.51

^a Data shows are the mean ± SD of two treatment repetitions, each assay was performed in quadruplicate.

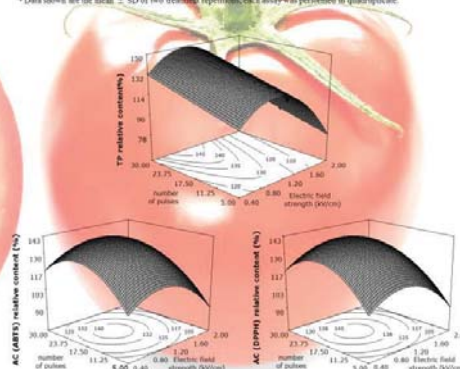


Figure 1 Effect of electric field strength and number of pulses on TP relative content (%) and AC relative content (%) of tomatoes.

The statistical analysis indicates that the quadratic model proposed was adequate ($P < 0.001$) to determine the effect of PEF treatments on TP and AC in tomato fruits with satisfactory coefficients of determination ($R^2 \geq 0.9865$).

PEF treatments affect TP and AC relative content of tomatoes depending on the electric field strength and number of pulses. An enhance in TP and AC was observed in PEF-treated tomatoes, except for treatments carried out at 2 kV/cm. A maximum increase of 40% in TP was attained with PEF treatments of 30 pulses and 1.2 kV/cm, whereas the maximum increase on AC was obtained with PEF treatments of 18 pulses and 1.2 kV/cm. Wounds stimulate phenylalanine ammonia-lyase activity and, thus, induce the enhancement of the main phenolic compounds and synthesis of new polyphenolic substances. Thus, it could be hypothesized that PEF increase the activity of phenylalanine ammonia-lyase and, therefore, some polyphenol compounds increase.

4. Conclusions

PEF treatments induced an increase on the TP and AC of tomato fruits depending on the electric field strength and number of pulses. PEF can contribute to obtain tomato-based products with high content of polyphenols and antioxidants.

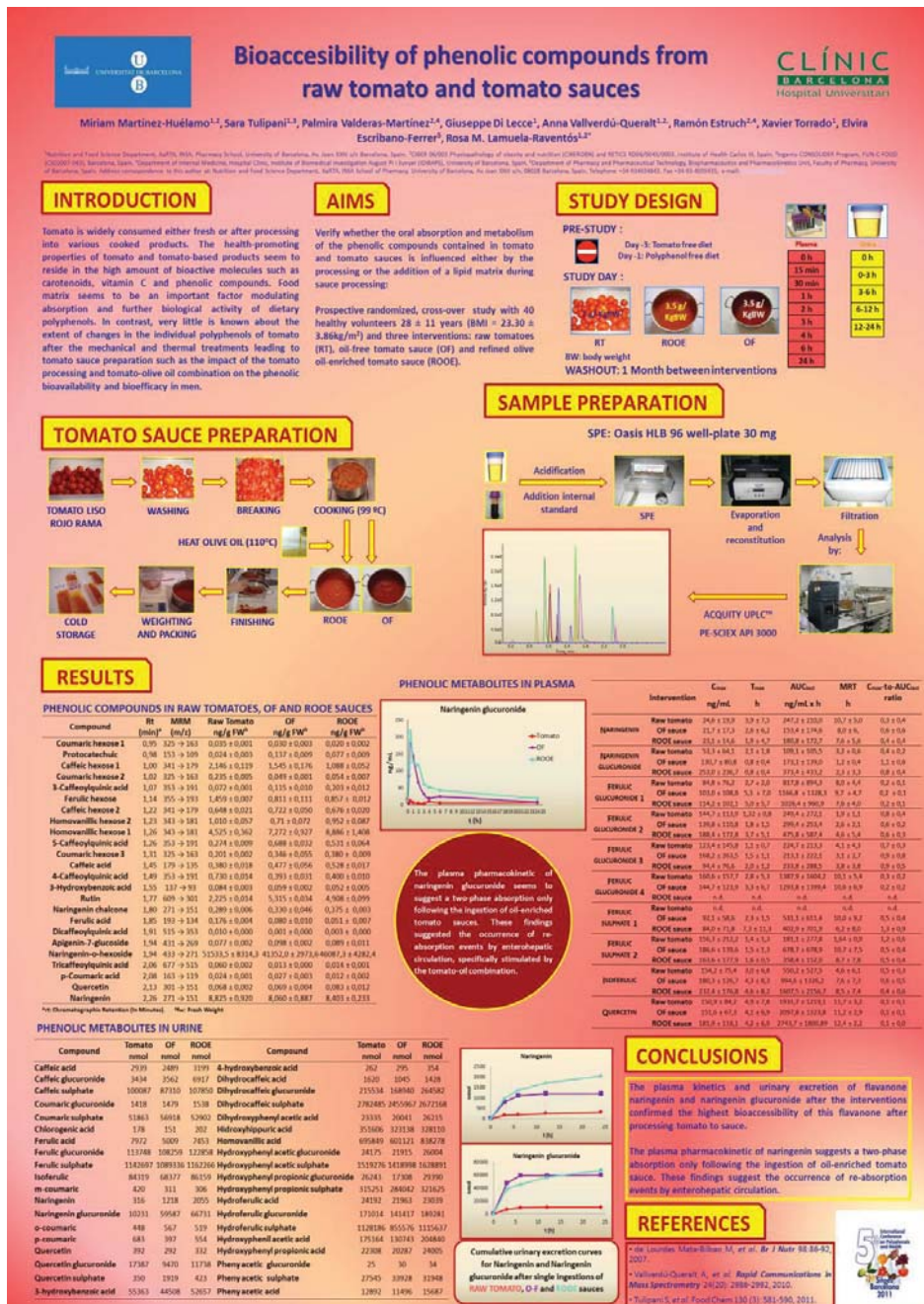
Annex

Comunicació 9: Pòster

The 5th International Conference on Polyphenols and Health. Sitges, Barcelona, Spain 2011.

“Bioaccessibility of phenolic compounds from raw tomato and tomato sauces.”

Miriam Martínez-Huélamo, Sara Tulipani, Palmira Valderas-Martínez, Giuseppe Di Lecce, Anna Vallverdú-Queralt, Ramón Estruch, Xavier Torrado, Elvira Escribano Ferrer & Rosa M. Lamuela-Raventós.



Annex

Comunicació 10: Pòster

The 5th International Conference on Polyphenols and Health. Sitges, Barcelona, Spain 2011.

“Gazpacho Consumption in high-risk Mediterranean Population and Prevalence of Hypertension: the PREDIMED trial.”

Alexander Medina-Remón, Anna Vallverdú-Queralt, Sara Arranz, Miguel Angel Martínez-González, Maribel Covas, Ramón Estruch & Rosa M. Lamuela-Raventós.



Gazpacho consumption in high-risk Mediterranean population and prevalence of hypertension. The PREDIMED trial.

Alexander Medina-Remón^{1,2,3}, Anna Valverde-Queralt^{1,2}, Sara Arranz-Martínez⁴, Miguel Ángel Martínez-González^{2,5}, Maribel Covas^{2,6}, Ramon Estruch^{2,3,4}, and Rosa M. Lamuela-Raventós^{1,2,3}, on behalf of the PREDIMED Study Investigators

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1. INTRODUCTION

Hypertension is a major public health problem and a leading cause of death and disability in both developed and developing countries, affecting one-quarter of the world's adult population and causing 7.6 million premature deaths and 6% of all cases of disability-adjusted life years worldwide [1]. The principal environmental factors influencing BP (blood pressure) are diet, physical activity and psychosocial factors [2]. Changing dietary habits is considered the principal lifestyle measure in BP control [3]. More recently, increasing epidemiological and clinical evidence points to the TMD (traditional Mediterranean diet) as an alternative dietary pattern for BP control [4-6]. Of note, the healthy diets recommended to subjects with or at risk of hypertension should be low in salt [7], and rich in fruits and vegetables [8]. Gazpacho is presumably beneficial to human health due to its high content in bioactive compounds such as carotenoids (mainly lycopene), vitamin C and polyphenols [9].

Aims: We undertook a substudy of a large nutritional intervention study, the PREDIMED (prevention with Mediterranean diet) trial, in order to assess the cross-sectional association between consumption of gazpacho and, systolic and diastolic BP, and/or with the prevalence of hypertension, in individuals at high cardiovascular risk.

2. SUBJECTS and METHODS

3955 potential participants recruited in primary health centres affiliate to five different hospitals in Spain from the PREDIMED trial (www.predimed.org) (ISCN135736439).

Men: 55-80 years old
Women: 60-80 years old
High CV risk without CVD
type 2 diabetes
3+ risk factors

1. Smoking
2. Hypertension
3. LDL
4. HDL
5. Overweight/obesity
6. Family history CVD

At baseline:

- Validated semiquantitative FFQ (food frequency questionnaire) with 137 items.
- Validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire.
- Validated 14-point Mediterranean diet score.
- 47-item questionnaire about education, lifestyle, history of diseases and medication use.

Statistical analyses were performed using SPSS software v17.0 (Chicago, USA).

3. RESULTS

Table 1: Baseline characteristics of study subjects by categories of gazpacho consumption.

	No consumption (0 g/d)	Moderate consumption (1-19 g/d)	High consumption (>20 g/d)	P for trend
No. of subjects	1878	994	1080	
Age (y) mean (SD)	68 (8)	67 (8)	67 (8)	0.501
Women, n (%)	1039 (55)	609 (61)	628 (58)	0.21
BMI (kg/m ²) mean (SD)	30 (4)	30 (4)	30 (4)	0.022
Systolic BP (mmHg) mean (SD)	130 (20)	148 (20)	147 (18)	<0.001
Diastolic BP (mmHg) mean (SD)	84 (11)	82 (11)	82 (11)	<0.001
Hypertension, n (%)	1546 (82)	819 (82)	814 (80)	0.29
Current smoker, n (%)	261 (14)	146 (15)	179 (16)	0.17
Medication, n (%)				
ACE-inhibitors	1344 (72)	714 (72)	802 (75)	0.40
Diuretics	146 (8)	72 (8)	99 (9)	0.29
Statins or other hypolipemic drugs	872 (47)	479 (48)	514 (47)	0.66
Insulin	109 (6)	76 (8)	83 (8)	0.012
Oral hypoglycemic drugs	551 (29)	306 (31)	358 (33)	<0.001
Energy expenditure in physical activity (kcal/d) mean (SD)	231 (218)	197 (200)	202 (238)	<0.001

Figure 1: Multivariate linear regression analyses (difference of means, 95% confidence intervals) of systolic and diastolic blood pressure according to categories of gazpacho consumption.

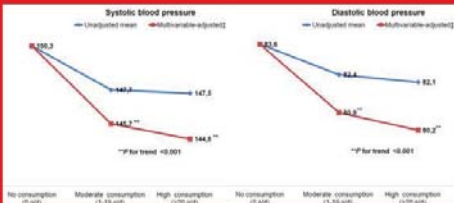
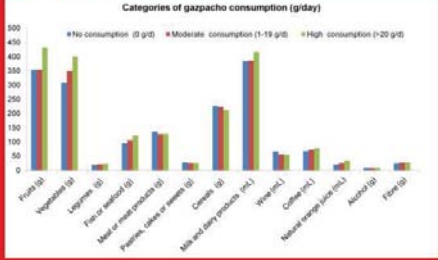


Table 2: Multivariate adjusted odds ratios (95% CI) for hypertension by gazpacho consumption expressed as 250 g/d.

Model	OR for 250 g/d	95% CI for OR (95% CI)	P
Model 1	0.93	0.84 - 1.02	0.099
Model 2	0.92	0.84 - 1.01	0.090
Model 3	0.84	0.73 - 0.98	0.023

Figure 2: Daily intake of selected foods and nutrients according to gazpacho consumption category.



4. CONCLUSIONS

In this cross-sectional study within the framework of the PREDIMED trial in a high-risk Mediterranean population, gazpacho consumption was inversely associated with systolic and diastolic BP and prevalence of hypertension. The beneficial effect of gazpacho on BP is probably due to synergy among several bioactive compounds present in the vegetable ingredients used to make the recipe.

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6. SUPPORTED BY

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5th International Conference on Polyphenols and Health, Sitges, Barcelona.

Annex

Comunicació 11: Pòster

5th International Symposium on Recent Advances in FOOD ANALYSIS. Prague, Czech Republic, 2011.

“Evaluation of a method based on *LC-ESI-MS* for the characterization of the polyphenol profile of organic and conventional tomatoes.”

Anna Vallverdú-Queralt, Olga Jáuregui, Alexander Medina-Remón & Rosa M. Lamuela-Raventós.



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Política de Cohesión

Evaluation of a method based on LC-ESI-MS/MS for the characterization of polyphenol profile of organic and conventional tomatoes



CIBEROBN

Anna Vallverdú-Queralt^{1,2}, Olga Jáuregui³, Alexander Medina-Remón^{1,2} and Rosa M. Lamuela-Raventós^{1,2*}

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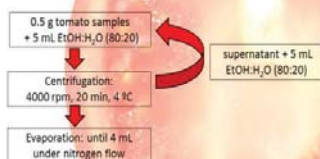
1. Introduction

Fundamental differences between organic and conventional production systems may affect the nutritive composition of plants, including secondary plant metabolites. Conventional and organic systems differ in the amount of nutrients applied as fertilizers and in organic matter applied to the soil as crop residues, winter legume cover crops or composted manure. The availability of inorganic nitrogen in particular has the potential to influence the synthesis of secondary plant metabolites, proteins and soluble solids.

Aim: The aim of this work was to find phenolic profile differences between conventional and organic tomatoes in the content of flavonols (kaempferol-3-O-rutinoside, rutin and quercetin), flavanones (naringenin and naringenin-7-O-glucoside), flavones (apigenin-7-O-glucoside) and hydroxycinnamic acids (ferulic, p-coumaric, caffeic and chlorogenic acids). Therefore, we propose an analytical approach to obtain the phenolic profile of organic and conventional tomatoes. The methodology was optimised and validated.

2. Material and Methods

Extraction of phenolic compounds



Sample cleaning-up: Oasis MAX[®] 96 well plate

- Condition: 1 mL of methanol 99-100% + 1 mL of sodium acetate 50 mM.
- Load: 1 mL samples + 1 mL Milli-Q water + 34 µL HCl.
- Cleaning: 1 mL sodium acetate 50 mM pH 7/ 5% methanol
- Elution: 1.8 mL methanol at 2 % formic acid.

Analysis of flavonols, flavanones and cinnamic acid derivatives

Time (min)	H ₂ O	ACN	0.1% H ₂ O	0.1% H ₂ O
0	95	5		
10	82	18		
13	0	100		
14	0	100		
15	95	5		

Column: 50 x 2.0 mm i.d., 5 µm Luna C₁₈
Injection volume: 20 µL
Flow: 4 mL/min
Column temperature: 30 °C
Modes: Full scan, Product ion scan, neutral loss, multiple reaction monitoring

Validation Method parameters

-Linearity:
Standard addition method.

-Sensitivity:

LoD= 3*5/N
LoQ= 10*5/N

-Accuracy: % recoveries

-Precision: RSD=SD/X*100

-Stability

- Short-term
- Long-term
- After successive freeze-thaw cycles

5th International Symposium on Recent Advances in Food Analysis, November 1-4 2011, Prague, Czech Republic

3. Results

The LC-MS/MS method was completely validated, providing a sensitive analysis for polyphenols detection and showing satisfactory data for all the parameters tested. Good results were obtained with respect to linearity and recovery as well as an excellent level of precision.

Table 1. Method validation parameters: slopes (m), correlation coefficients (R²) and limits of detection (LOD) and quantification (LOQ).

Analytes	Conventional tomatoes				Organic tomatoes			
	m (10 ³)	R ²	LOD (µg/g FW)	LOQ (µg/g FW)	m (10 ³)	R ²	LOD (µg/g FW)	LOQ (µg/g FW)
Caffeic acid	3.50	0.9933	0.30	0.99	3.30	0.9911	0.22	0.73
Chlorogenic acid	4.23	0.9943	0.28	0.88	3.80	0.9907	0.29	0.97
Ferulic acid	0.78	0.9958	0.42	1.38	0.43	0.9936	0.53	1.77
p-Coumaric acid	4.40	0.9934	0.28	0.94	4.08	0.9934	0.22	0.73
Naringenin	2.10	0.9832	0.18	0.63	2.60	0.9918	0.20	0.67
Naringenin-7-O-glucoside	2.10	0.9934	0.13	0.43	2.88	0.9958	0.18	0.60
Rutin	7.30	0.9927	0.21	0.70	7.62	0.9950	0.24	0.80
Quercetin	2.90	0.9919	0.66	2.20	2.36	0.9957	0.61	2.04
Kaempferol-3-O-rutinoside	22.38	0.9957	0.38	1.20	25.30	0.9928	0.33	1.10
Apigenin-7-O-glucoside	0.82	0.9990	0.23	0.77	0.80	0.9950	0.28	0.88

FW: fresh weight

Table 2. Estimated quantities, recoveries (%) and precision (RSD %) of polyphenols from organic and conventional tomatoes

Analytes	Conventional tomatoes				Organic tomatoes			
	Estimated quantity (µg/g FW ± SD) ^a	Lowest Spiked level ^b	Recovery ± SD	RSD (%) ^c	Estimated quantity (µg/g FW ± SD) ^a	Lowest Spiked level	Recovery ± SD	RSD (%)
Caffeic acid	22.88 ± 0.65	46.60	98.80 ± 3.22	3.27	41.70 ± 0.81	93.16	97.04 ± 2.95	3.05
Chlorogenic acid	36.87 ± 0.61	69.90	96.21 ± 2.89	3.00	56.99 ± 0.90	116.50	96.12 ± 3.10	3.23
Ferulic acid	21.69 ± 0.40	69.90	94.25 ± 4.49	4.77	35.11 ± 0.63	69.90	96.88 ± 4.01	4.33
p-Coumaric acid	20.59 ± 0.36	46.60	94.89 ± 2.87	3.02	34.25 ± 0.55	69.90	97.65 ± 2.90	2.97
Naringenin	36.46 ± 0.69	116.50	85.11 ± 3.97	4.69	87.38 ± 0.98	163.00	88.10 ± 3.51	3.98
Naringenin-7-O-glucoside	7.68 ± 0.15	23.30	87.02 ± 3.34	3.58	13.91 ± 0.28	23.30	88.63 ± 3.02	3.43
Rutin	119.62 ± 1.49	233.00	93.44 ± 4.72	5.05	272.25 ± 2.98	465.80	95.11 ± 4.51	4.74
Quercetin	5.69 ± 0.12	11.70	77.81 ± 3.88	5.00	11.42 ± 0.18	23.30	78.20 ± 3.41	4.36
Kaempferol-3-O-rutinoside	6.03 ± 0.11	11.70	89.35 ± 3.27	3.67	12.70 ± 0.24	23.30	91.34 ± 3.02	3.31
Apigenin-7-O-glucoside	28.28 ± 0.63	46.60	93.05 ± 2.85	2.90	31.63 ± 0.52	46.60	93.94 ± 2.87	3.07

^aSD: standard deviation

^bThe lowest spiked level (50%) was applied for doubling or tripling the endogenous concentration of each compound.

^cMean recovery of all spiked levels (50%, 100%, 150%, 200%).

^dRSD: relative standard deviation.

4. Conclusions

In the present study the growing conditions of tomatoes (conventional versus organic) affected the content of phenolic compounds of these vegetables. The organically produced tomatoes displayed a higher phytochemical concentration than conventionally produced tomatoes.

The LC-MS/MS method was completely validated, providing a sensitive analysis for polyphenols detection and showing satisfactory data for all the parameters tested. Good results were obtained with respect to linearity and recovery as well as a good level of precision.

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