New vacuum cooking techniques with extra-virgin olive oil show a better phytochemical profile than traditional cooking methods: A foodomics study

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Abstract

In this work, the major changes in extra-virgin olive oil (EVOO) composition during cooking were assessed. A foodomics approach based on both metabolomics and lipidomics was used to evaluate the impact of six different cooking techniques, three traditional and three more innovative (Crock-pot®, Roner® and Gastrovac®), and the effect of temperature and cooking time. The lipophilic and hydrophilic fractions of EVOO that underwent different cooking processes were characterized by untargeted high-resolution mass spectrometry approaches. Multivariate statistics were used to unravel the differences in chemical signatures. The different cooking methods resulted in broadly different phytochemical profiles, arising from thermally driven reactions accounting for hydrolysis, synthesis, and oxidation processes. The innovative cooking techniques marginally altered the phytochemical profile of EVOO, whereas sauteing was the cooking method determining the most distinctive profile. Conventional cooking methods (oven, pan-frying, and deep-frying) produced more oxidation products (epoxy- and hydroxy-derivatives of lipids) and markedly induced degradation processes.

Keywords: healthy cooking; thermal processing; Mediterranean diet; fat oxidation; polyphenol; cuisinomics.

1.Introduction

Extra Virgin Olive Oil (EVOO) is the main source of fat in the Mediterranean diet, well appreciated due to its flavor and known health effects. EVOO is consumed both raw and cooked; however, during thermal processes, its components can undergo transformations, oxidations, and/or degradations (Casal, Malheiro, Sendas, Oliveira, & Pereira, 2010). EVOO is mainly composed of triglycerides, with a high content of monounsaturated fatty acid (MUFA) and relatively low polyunsaturated fatty acid

(PUFA) amounts, which makes it more stable than other edible oils richer in PUFAs (Aşkın & Kaya, 2020; Cui, Hao, Liu, & Meng, 2017).

Despite having higher stability than other edible oils, the cooking process can transform some EVOO compounds, thus producing (among others) peroxides, polar compounds, and aldehydes. Also, triglycerides can be hydrolyzed and polymerized (ben Hammouda, Freitas, Ammar, Da Silva, & Bouaziz, 2017; Santos, Cruz, Cunha, & Casal, 2013). In this regard, the products of hydrolysis, monoglycerides, and fatty acids, have been proposed as a marker of cooked oil (Cao et al., 2019). However, unrefined oils like EVOO also contain some other compounds, such as terpenoids, vitamins, carotenoids, and polyphenols, among others which provide a protective effect during cooking (Blasi et al., 2018; Jiménez, García, Bustamante, Barriga, & Robert, 2017). As a consequence of their protective properties these compounds tend to degrade during cooking (Kalantzakis, Blekas, Pegklidou, & Boskou, 2006). In the case of phenolic compounds, previous studies found that despite the degradation, cooking at moderate temperature (i.e., 120 ºC), the EVOO preserve enough phenolics to comply with the European Health claim (European Commission Regulation EC No. 432/2012, 2012; Lozano-Castellón et al., 2020). Those bioactive compounds can migrate from the oil to the food enriching it (J. D. P. Ramírez-Anaya, Samaniego-Sánchez, Castañeda-Saucedo, Villalón-Mir, & De La Serrana, 2015; Vallverdú-Queralt, Regueiro, Rinaldi de Alvarenga, Torrado, & Lamuela-Raventos, 2014). Furthermore, the food can protect those phenols from degradation during cooking (J. del P. Ramírez-Anaya et al., 2019). Besides, carotenoids, normally present as *all-E* isomers, can form *Z*-isomers during heating (Vallverdú-Queralt, Regueiro, de Alvarenga, Torrado, & Lamuela-Raventos, 2015) which are more bioavailable (Unlu et al., 2007). Carotenoids during heating are also transformed to apo-

63 carotenoids (Rinaldi de Alvarenga et al., 2019), which still present antioxidant activity
64 (Müller, Reynaud, Goupy, Caris-Veyrat, & Böhm, 2012).

65 Depending on the type of cooking, the potential reactions may change according to the
66 contact with the air, the temperature of cooking, and some other factors able to alter the
67 oxidation and degradation processes (Goulas, Orphanides, Pelava, & Gekas, 2015; Santos
68 et al., 2013). For example, Saleem *et al.* (2017) reported that cooking at 140 ºC does not
69 deteriorate the EVOO (Saleem et al., 2017).

70 Previous studies have assessed the effect of cooking on fatty acids and/or some minor
71 compounds, mainly by using targeted analytical approaches (Chiou & Kalogeropoulos,
72 2017). These latter are useful to determine the degradation rate of selected compounds,
73 but all the new products formed remain still unclear. Also, few studies exploited an
74 untargeted analytical approach; in this regard, Blasi et al (2018) reported the differences
75 in terms of phenolics between EVOO and EVOO enriched with a carotenoid extract
76 during frying, but with no reference about the effect of cooking methods on the entire
77 EVOO metabolome (Blasi et al., 2018). Furthermore, most of the previous studies dealing
78 with EVOO and cooking have been done in the lab and not real conditions, i.e., heating
79 the oil for extended periods (36h) (Brenes, García, Dobarganes, Velasco, & Romero,
80 2002) or in a non-normally gadget, such as a beaker (Brkić Bubola, Klisović, Lukić, &
81 Novoselić, 2020).

82 Therefore, starting from the previous considerations, the aim of this study was to
83 comparatively assess the effect of different cooking methods on the phytochemical profile
84 of EVOO, considering both its hydrophilic and lipophilic fractions. To determine which
85 changes occur and how EVOO functional compounds could be modified. To this aim, we
86 used untargeted metabolomics and lipidomics approaches based on ultra-high-pressure
87 liquid chromatography (UHPLC) coupled with high-resolution mass spectrometry

(HRMS) detectors (such as QTOF and Orbitrap) to provide a wide perspective on EVOO chemical profile. Overall, six different cooking techniques have been assessed, including three conventional and three more innovative, to evaluate each technique's impact on the degradation of EVOO. These different techniques inherently require different temperatures and present different oxygen contact; cooking time was an additional processing factor under investigation. To the best of our knowledge, this is the first work using both metabolomics and lipidomics on EVOO to find possible marker compounds of each cooking methods under investigation. The final aim was to hierarchically assess each factor's weight to affect the EVOO quality, thus considering the cooking method itself and different combinations of temperature/time.

2. Materials and methods

2.1. Reagents

Methanol, water, tert-butyl methyl ether, and isopropanol were LC-MS grade supplied by Merck (Darmstadt, Germany). Formic acid and ammonium formate (LC-MS grade) were supplied by sigma Aldrich (St. Louis, USA).

2.2. EVOO samples and cooking processes

The EVOO sample was kindly provided by "Organización Interprofesional del Aceite de oliva Español". The sample was from "Picual" cultivar and labeled as extra-virgin olive oil, it was harvested in autumn 2019 in Spain. To standardize the experimental conditions, all EVOO samples used for the cooking processes came from the same batch. In this work, we used three conventional techniques, namely sauteing, deep-frying, and oven. Moreover, three innovative cooking methods consisting of a low-temperature vacuum cooking carried out with a Roner® apparatus, a slow-cooker pot carried out with a Crockpot® apparatus, and the vacuum pot cooking process, carried out with a Gastrovac® apparatus were also chosen. The cooking processes were performed at the

Food Torribera Campus, University of Barcelona (Santa Coloma de Gramenet, Spain), except for the Gastrovac® experiments, which were carried out at CETT, School of Tourism, Hospitality and Gastronomy, University of Barcelona (Barcelona, Spain). The EVOO was cooked using the six different techniques, using for every technique 2 temperatures (one high and one low) within the common interval of use. These temperatures were selected according to the chef's criteria, based on those temperatures normally applied at domestic conditions for each traditional technique and at restaurant conditions for the more innovative ones, except for the slow-cooker experiments, which was determined by the apparatus as explained below. Furthermore, two cooking times were assessed for each temperature, one in which the food was with "al dente" texture and one with the food well cooked. In order to find the optimal time for each technique and temperature, a regular portion of potatoes and chicken (200 and 100 g, respectively) was cooked and the time was determined. **Table 1** reports the times and temperatures chosen for each cooking technique. The detailed and compared cooking processes are explained below. All the cooking process were done in triplicate. After each cooking cycle, the oil was cooled down and stored in vacuum bags at -20 ºC for further analysis.

2.2.1. Sauteing

For the sauteing process, the pan (20 cm diameter, 0.8 mm thickness, stainless steel 18/10, Excalibur, Pujadas, Girona, Spain) was heated in on an electrical cooking plate (180 mm diameter, 1500 W, model Encimera EM/30 2P, Teka®, Madrid, Spain) until the required temperature was reached. Then 200 g of EVOO were added, and the oil was heated for the desired time. The temperature was monitored with a laser thermometer (error: ±1 °C, ScanTemp 410, TFA Dostmann GmbH & Co. KG, Wertheim, Germany) during all the process and the heat was reduced or increased when needed.

2.2.2. Deep-frying

For the deep-frying process, 200 g of EVOO were heated in a domestic fryer (model Electro 1L, Electrodomésticos JATA, S.A., Tudela, Spain) at the convenient temperature for the appropriate time. The temperature was monitored and controlled with its own thermometer.

2.2.3. Oven

For the oven, a professional restauration oven was used (model Welbilt WED 106, Cleveland Range Ltd. Concord, Canada). The oven was heated to the desired temperature, then the oil placed in a steel tray was introduced inside the oven for the required cooking time.

2.2.4. Crock-pot apparatus (slow cooking)

For the slow-cooker process, a Crock-pot® apparatus was used (Model SCCPRC507B-050, Oster Electrodomésticos Ibérica S.L., Aravaca, Spain), first it was needed to determine the temperatures, as the apparatus just has "low" and "high" program, but the temperature cannot be controlled. For the low temperature, the "low" program was selected, and the temperature was monitored to be 70 ºC. In the case of the "high" program, the temperature was 85 ºC. For the cooking process 200 g of EVOO were added to the pot, the desired program was selected, and the oil was cooked for the required time.

2.2.5. Roner apparatus (low temperatures)

For the low-temperature process, a Roner® apparatus was used (model 99999951, J.P.Selecta S.A., Abrera, Spain). The water bath was set at the desired temperature and when it was reached 200 g of EVOO inside a plastic vacuum bag, were placed in the bath and cooked.

2.2.6. Gastrovac apparatus (vacuum cooking)

Finally, for the vacuum pot cooking process a Gastrovac® apparatus was used (model 20000, International Cooking Concepts S.A., Barcelona, Spain). The apparatus consists of an electric stove, a pot connected to a vacuum machine and a thermometer. The pot was heated and when the corresponding temperature was achieved 200 g of EVOO was added and the vacuum was set at maximum; the oil was cooked for each time.

2.3. Extraction of hydrophilic compounds

For the extraction of the hydrophilic compounds, 200 µL of EVOO sample were weighed and suspended in 1 mL of a hydroalcoholic solution of methanol 80%, acidified with 0.1% formic acid (Blasi et al., 2018). The samples were stirred in a conical stirrer, centrifuged for 3 min at 1574 g and 4 ºC, and then 500 µL of the methanol extract was directly filtered with a 0.22 µm syringe filter in a 2-mL vial before further analysis. Analyses were done immediately after the extraction step.

2.4. Extraction of lipophilic compounds

For the extraction of the lipophilic compounds, 200 µL of EVOO sample were weighted and solved with 800 µL of tert-butyl methyl ether. The samples were frozen at -20 ºC for the precipitation of the triglyceride fraction. Thereafter, 500 µL were taken and overnight evaporated. Samples were then resuspended in 500 µL of a solution consisting of 65% isopropanol, 30% methanol, and 5% water, then stirred in a conical stirrer and centrifuged at 2460 x g for 5 min at 4 ºC. Finally, 300 µL of the supernatant was transferred to a 2 mL vial. Analyses were done immediately after the extraction.

2.5. Phenolic profiling by UHPLC-QTOF mass spectrometry

A metabolomics-based analysis was performed using ultra-high-pressure liquid chromatography (Agilent 1200 series) coupled to a quadrupole time-of-flight mass spectrometer (Agilent 6550 iFunnel) as previously reported by (Rocchetti et al., 2017). Briefly, the extracts were acquired in positive polarity (ESI+), using a full scan mode with

187 an *m/z* range of 100-1200 (0.8 spectra/s). The mass spectrometer worked in extended
188 dynamic range mode (nominal mass resolution = 30,000 FWHM). The separation was
189 achieved using an Agilent Zorbax Eclipse Plus C18 column (50 × 2.1 mm, 1.8 μm) with
190 water-acetonitrile gradient elution and a linear gradient from 6 to 94% of acetonitrile in
191 35 min, using 0.1% formic acid in both phases. The electrospray conditions were
192 previously optimized by the group (Rocchetti et al., 2018). The injection volume was 6
193 μL, the sequence was randomized and pooled Quality Control samples (QCs) were
194 injected every ten samples and at the beginning of the sequence. QCs were analyzed in
195 data-dependent MS/MS mode using 12 precursors per cycle (1 Hz, 50–1200 m/z, positive
196 polarity, active exclusion after 2 spectra), with collision energies of 10, 20 and 40 eV for
197 collision-induced decomposition.

198 The data was processed using Agilent Profinder B.06 (Agilent Technologies) software
199 according to the targeted "find-by-formula" algorithm. Combining monoisotopic mass
200 information and isotope profile allowed us to achieve the highest confidence in
201 annotation, adopting a 5-ppm tolerance for mass accuracy. For compound annotation, we
202 used the comprehensive database Phenol-Explorer 3.6 (http://phenol-explorer.eu/). Also,
203 mass and retention time alignment and compounds filtering were performed in Agilent
204 Profinder B.06 software. Those compounds with mass accuracy higher than 5 ppm, with
205 a frequency of detection within 100% of replicates in at least one kind of sample, and
206 having a plausible chromatogram peak feature, were retained and finally used for
207 multivariate statistical data analysis. In those QTOF experiments, a Level 2 of annotation
208 was achieved (i.e., putatively annotated compounds), as reported by COSMOS
209 Metabolomics Standards Initiative (Salek, Steinbeck, Viant, Goodacre, & Dunn, 2013).
210 The post-acquisition data analysis for phenolic compounds (by UHPLC/QTOF) was done
211 using the software Mass Profiler Professional (version: B.12.06; from Agilent

Technologies) as previously reported (Giuberti et al., 2018). Therein, compounds were filtered by abundance (area > 10000 counts), Log2 transformed, normalized at 75th percentile, and baselined for the median. Besides, some compounds were structurally confirmed by using a dedicated MS/MS workflow based on QC samples and MS-Dial software.

2.6. Lipidomic profiling by UHPLC-Q-Orbitrap mass spectrometry

The untargeted UHPLC-MS lipidomics analysis was done on a Q Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) coupled to a Vanquish ultra-high-pressure liquid chromatography (UHPLC) pump and equipped with a HESI-II probe (Thermo Scientific, USA). The chromatographic separation was achieved by using a BEH C18 (2.1x100 mm, 1.7 µm) analytical column maintained at 40 °C. The mobile phases consisted of (A) 5 mM ammonium formate and 0.1% formic acid in water/methanol (95/5, v/v), and (B) 5 mM ammonium formate and 0.1% formic acid in 2-propanol/methanol/water (65/30/5, v/v/v). The linear gradient and flow rate increased linearly as follows, considering time (min), %B, flow rate (µL/min): (0,10,200), (5, 50, 200), (15, 80, 250), (28, 100, 250), (30, 100, 250), (30.9, 10, 250), and (35,10, 250). For the full scan MS analysis, the acquisition was performed using both positive and negative ionization with a mass resolution of 70,000 at *m/z* 200. The automatic gain control target (AGC target) and the maximum injection time (IT) were 1e^6 and 100 ms, respectively. Also, separate and randomized injections of pooled quality control (QC) samples were performed in a data-dependent (Top N = 3) MS/MS mode with full scan mass resolution reduced to 17,500 at *m/z* 200, with an AGC target value of 1e^5, maximum IT of 100 ms, and isolation window of 1.0 *m/z*, respectively. For the stage of data-dependent MS/MS, the Top N ions were selected for further fragmentation under stepped normalized collisional energy (i.e. 10, 20, 40 eV). The injection volume was 5

μL and the *m/z* range for the full scan analyses was 100-1500. Heated electrospray ionization (HESI) parameters were as follows: sheath gas flow 30 arb (arbitrary units) auxiliary gas flow 10 arb, spray voltage 3.5 kV for ESI+ and 2.8kV for ESI-, capillary temperature 320 °C. Prior to data collection, the mass spectrometer was calibrated using Pierce™ positive and negative ion calibration solutions (Thermo Fisher Scientific, San Jose CA, USA). To avoid possible bias, the sequence of injections for EVOO samples was randomized.

The collected UHPLC-HRMS data (.RAW file) were converted into .abf file using the Reifycs Abf Converter and then further processed using the software MS-DIAL (version 4.24) (Tsugawa et al., 2015). In this regard, automatic peak finding, LOWESS normalization and annotation via spectral matching (against the database LipidBlast) were performed. The mass range 100-1500 *m/z* was searched for peaks with a minimum peak height of 10000 cps for ESI + and ESI - polarities. The MS and MS/MS tolerance for peak centroiding was set to 0.01 and 0.05 Da, respectively. Retention time information was excluded from the calculation of the total score. Accurate mass tolerance for identification was 0.01 Da for MS and 0.05 Da for MS/MS. The identification step was based on mass accuracy, isotopic pattern, and spectral matching. In MS-DIAL, these criteria were used to calculate a total identification score. The total identification score cut off was 75%, considering the most common ion adducts for lipidomics. Gap filling using peak finder algorithm was performed to fill in missing peaks, considering 5 ppm tolerance for *m/z* values. Finally, the software MS-Finder (Tsugawa et al., 2016) was used to provide *in-silico* fragmentation of the not annotated mass features, considering both Lipid Maps and FoodDB libraries available in the same software. To this aim, those compounds presenting an *in-silico* prediction score > 5 were retained.

2.7. Multivariate data analysis

The multivariate data analysis was done separately for the lipidomics and phenolic profile data. Unsupervised hierarchical clustering analysis (HCA) was made using the online software MetaboAnalyst (Chong, Wishart, & Xia, 2019). The HCA grouped the different samples with no class information, thus allowing to hierarchically assess the weight of each variable (i.e., method, time, temperature) of cooking. Next, the datasets containing the raw annotations were exported into the SIMCA software (Umetrics), and supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) was performed using three different class discrimination criteria (i.e., cooking technique, temperature, and time). Hotelling's T2 was carried out to investigate the presence of outliers by using 95% and 99% confidence limits for suspicious and strong outliers, respectively. Model validation parameters were also produced, goodness-of-fit (R^2Y) and goodness-of-prediction (Q^2Y), adopting a Q^2Y prediction ability of >0.5 as acceptability threshold. Finally, cross-validation of the model was performed using ANOVA on cross-validated residuals ($p < 0.01$), whereas permutation testing (200 permutations) was done to exclude overfitting. The variables' importance in projection (VIP) was used to extrapolate marker compounds, i.e., those possessing a VIP score > 1. Finally, a fold change (FC) analysis of the discriminant VIP markers (ANOVA, $p < 0.05$) was done for each cooking method against the raw EVOO, retaining those significant compounds with a FC value > 2.

3. Results

3.1. Phenolic compounds

The UHPLC-QTOF data obtained by using the Phenol-Explorer database for the annotation step allowed us to putatively annotate 143 phenolic compounds in the EVOO samples, mainly flavonoids, tyrosols, phenolic acids, and lignans. A comprehensive list containing phenolic annotations is available in the supplementary material, where

compounds are reported together with raw abundance values and composite mass spectra. Also, the dedicated MS/MS experiment on MS-Dial allowed us to structurally confirm some of the most abundant compounds (supplementary material).

The dataset containing the significant annotations was then used for unsupervised HCA, made from the fold-change heat map, to group the different cooking techniques and visualize the changes in phenolic profile considering the different temperatures and cooking time. The resulting grouping from the technique, the cooking temperature, and the interaction between those two factors is showed in **Figure 1**, the complete HCA are in supplementary material. This clustering approach was used for evaluating the combination between temperatures and cooking techniques to hierarchically determine possible interactions between the factors considered. The samples clustering by time were very similar to the grouping by temperature, thus indicating an interaction between time and temperature. Thereafter, in order to monitor the changes in phenolic profiles between the raw EVOO sample and the different cooking methods, ANOVA ($p < 0.05$) and Fold Change (FC) analyses (cut-off > 2) were combined in Volcano Plot, and the differential compounds grouped in classes and listed either as degradation products or created compounds. A comprehensive list of these compounds and their $\log_2$FC, together with their VIP scores, can be found in supplementary material and a list of a representative compound of each phenolic group is shown in **Table 2**.

Later, a supervised analysis based on OPLS-DA was used to find the discriminant compounds, mostly describing the changes observed as a function of the cooking method, temperatures and times. The cooking technique and temperature OPLS-DA score plots are reported in **Figures 2A** and **2C**, and the other is in the supplementary material. As it could be seen, the temperature and the cooking method presented a marked effect on the EVOO samples, whilst time had no significant effect. Also, the VIP selection method

312 listed 72 marker compounds for the different cooking techniques, 68 for the cooking
313 temperature, with 48 of those compounds being shared between the two comparisons.
314 These compounds, together with their VIP score for temperature and/or cooking
315 technique, standard error and LogFC values, are shown in supplementary material and a
316 representative compound of each phenolic group is listed in **Table 2**.

317 *3.2. Lipidomic profile*

318 The raw data obtained from Q-Orbitrap detection allowed identifying 1163
319 compounds, reported in supplementary material together with their nominal exact mass,
320 exact mass, fragmentation pattern, retention time, and identification score. The
321 unsupervised HCA produced from the fold-change heat map was built considering the
322 cooking technique, time and temperature. A further HCA considering only the
323 temperature and cooking technique was built to assess the effect of the interaction
324 between those parameters and to identify which has a more marked effect. The resulting
325 grouping from the technique, the cooking temperature, and the interaction between those
326 two factors is showed in **Figure 1**, the complete HCA are in supplementary material.
327 Thereafter, ANOVA ($p < 0.05$) and Fold Change values against raw EVOO (cut-off > 2)
328 were combined in a Volcano Plot and the discriminant compounds were retained. Finally,
329 the supervised OPLS-DA models were built for cooking techniques, temperature, and
330 time, and the graphs are shown in **Figures 2B and 2D** and in the supplementary material
331 (time). Multivariate elaboration provided outputs that corroborated the outcome of
332 elaborations from phenolic profiling, with cooking time showing a hierarchically less
333 relevant effect. Also, oven and deep-frying cooking methods presented similar lipidomic
334 profiles, whilst sauteing was differentiated from the other cooking methods, with the
335 innovative cooking techniques showing a comparatively less relevant impact on the
336 phytochemical profile of raw EVOO.

The differential compounds were again grouped in compounds that increase or degrade or both, as a function of the specific cooking method. In the case of the generated compounds, they were sorted by class and according to the type of generation, i.e., oxidation, synthesis, and hydrolyzation processes. Finally, the LogFC values, together with the VIP scores of the cooking technique and/or temperature, and the standard error of these marker compounds, are shown in supplementary material, while a representative marker of each group and the amount of marker compounds per group is listed in **Table 3**.

4. Discussion

4.1. Multivariate data hierarchical modelling of cooking, temperature and time

In this work, multivariate outputs from both lipidomic and phenolic profiles allowed us to observe very similar sample groupings, meaning that thermal stability of phenolics and lipids during cooking are related and tend to degrade and/or transform correlatively during the different cooking processes.

Going into details, HCA analysis was built for both phenolic and lipids, and considering cooking technique, temperature and time. Moreover, to determine the effect of the interaction and to assess which factor had a higher effect, an HCA of cooking technique and temperature was also developed (**Figure 1** and supplementary material). In the case of the stronger cooking processes, sauteing was totally differentiated from deep-fry and oven. The multivariate elaborations show that the innovative cooking techniques preserve EVOO, as presented profiles more similar to the raw EVOO than conventional cooking. Specifically, the HCA of the cooking temperature showed that those clusters, including 170 and 200 ºC samples, were characterized by a completely different profile. Also, intermediate temperatures (140 and 150 ºC) were found to cluster together, whilst the mildest conditions (70, 80 and 85 ºC) produced similar profiles.

Finally, samples cooked at 120 ºC cluster by themselves. This particularity was also observed in the HCA built with both cooking technique and temperature, in which sauteing at 120 ºC clusters on its own. Through this analysis, it was possible to observe some different outcomes between the phenolic and lipidomic fractions. In the case of the lipidomics, there was a major effect of the high temperatures, as the first group division was between those samples heated at 170 ºC or more and the others, independently of the cooking method. Furthermore, the raw EVOO sample was separated from the mildly cooked samples, and the sauteing at 120 ºC creates its own distinct group. This suggests that the temperature plays a pivotal role in the EVOO transformation during cooking, even though the cooking technique is also important, as the sauteing at 120 ºC was well differentiated from those samples heated even more, but with an oven or with a vacuum pot apparatus. On the other hand, the phenolic profile observed in HCA allowed us to observe that the specific combination of temperature and cooking method determined the actual phenolic profile of EVOO. This might be related to the differences in oxygen availability during cooking. For example, considering the cluster composed of Gastrovac samples cooked at 140 ºC and roner at 85 ºC, the absence of oxygen during the cooking process was more important in the modification of the phenolic compounds than the temperature at which the EVOO was heated. Besides, both deep-fried samples clustered together and created a grouping with the oven at 200 ºC, whilst the sauteing at 170 ºC clustered with the oven at 150 ºC. Interestingly, those two last techniques have a similar surface-to-volume ratio and, then, a similar oxygen availability, which makes it possible to justify the grouping observed. Indeed, polyphenols are compounds typically associated with redox imbalance processes, both in vitro and in vivo, and are well-recognized antioxidant and radical scavenging compounds (Choe & Min, 2009). On the contrary, phenolics are reported to be relatively stable when mild-intermediate temperatures (in the

range 60-100 °C) are applied (Volf, Ignat, Neamtu, & Popa, 2014). However, the fatty chain of lipids is known to undergo autoxidation and thermal oxidation via the removal of allylic hydrogen (especially in polyunsaturated chains, where a bis-allylic methylene position is present). Then, the radical form of these lipids can eventually react with triplet oxygen very quickly (Choe & Min, 2009). Even if the process leads to combinatory and not fully elucidated steps, this indicates that the removal of allylic hydrogen is a rate-limiting step for thermal stability of fatty chains, thus supporting our postulations. Within this context, the free radical scavenging activity provided by phenolics in contrast to lipid oxidation processes is well-known and has been related to their ability to provide low bond dissociation energies for the O-H group in nonpolar solvents (i.e., where hydrogen bonding is not present) (Choe & Min, 2009). Consequently, phenolic compounds react not only when the temperature is high but also when the oxygen availability is also high and, on the contrary, fatty acids need a high temperature to remove the allylic hydrogen. For this reason, lipidomic results were more affected by the temperature while the phenolics suffer greater transformations or degradations with the interaction between temperature and cooking technique, which determines the oxygen availability.

In order to confirm these results from a supervised point of view, the OPLS-DA score plots were built. As in the case of the hierarchical analysis, the orthogonal analysis results of the untargeted profiles were found to be very similar. With this regard, it is important to consider that this supervised modelling approach allows to stress out the predictive variability, i.e., the variability that correlated to the factor(s) under investigation. This resulted in emphasizing that the effect on the phenolic compounds and lipidic compounds was somehow correlated. The OPLS-DA considering the cooking method as a discrimination parameter showed that innovative techniques tend to cluster with the raw EVOO, whilst the oven and deep-frying aggregate together. Interestingly, as showed in

412 the HCA, the sauteing process produced a more specific profile when considering both
413 phenolics and lipid compounds. Besides, the OPLS-DA models built according to the
414 temperature showed that the higher the temperature, the more extensive the
415 metabolome/lipidome changes occurring. This is suggested by the occurrence of quite
416 close groups in the hyperspace at high temperatures, indicating a flattening of cooking-
417 specific effects. However, cooking at 120 ºC via sauteing, demonstrated to have a totally
418 different impact on the EVOO oil, likely because of the high surface-to-volume ratio and
419 the consequent exposure to air, compared with the other techniques. Therefore, even by
420 this supervised modelling, we could support the previously mentioned cooking-related
421 involvement of oxygen mediated processes. Interestingly, we also found that the cooking
422 time did not play a significant role in determining the major changes during EVOO
423 cooking. A similar conclusion was achieved in previous experiments, in which the change
424 of EVOO phenolic profile during a domestic sauteing process was not altered by cooking
425 time (Lozano-Castellón et al., 2020).

426 *4.2. Marker compounds*

427 Combining the VIP scores and the FC analysis, it was possible to create a table with
428 the most discriminant compounds (VIP score > 1) for the cooking technique or for the
429 temperature, also presenting a *p*-value < 0.05 (ANOVA, Bonferroni multiple testing
430 correction) and a FC value >2. The changes of these compounds were compared for all
431 cooking-temperature combinations vs. the raw EVOO sample. Therefore, two tables were
432 created with these values, one for the phenolic (**Table 2**) and another for the lipidomic
433 profile (**Table 3**).

434 *4.2.1. Phenolic compounds*

435 In the case of the phenolic profile, the most degraded compounds were flavonoids; for
436 example, luteolin-*O*-hexoside had a VIP score of 1.2 for the temperature and a LogFC of

approximately -17 for the oven, deep-frying, and sauteing cooking. Lignans were also important marker compounds, secoisolariciresinol-sesquilignan presents a VIP score of 1.4 for the cooking technique and a LogFC value lower than -17. Some phenolic acids were also degraded, as *m*- and *p*-coumaric acids, which despite not having high VIP scores, were found to be markers of both technique and temperature. In this case, it is important to highlight that they were mostly degraded during a roner cooking, meaning those compounds are really labile. Finally, some tyrosols were also marker compounds, likely affected during the cooking process. For example, tyrosol acetate (*p*-HPEA-AC) was degraded during the crockpot, but not during the other techniques; this trend could be explained as it is degraded, but could also be formed during cooking, which compensates for its degradation during the other cooking process. This compound is formed during the ester breakdown of ligstroside aglycone or one of its derivatives (Lozano-Castellón et al., 2020).

On the contrary, among the marker compounds generated during the cooking process, we found some lignan derivatives, such as 7-hydroxysecoisolariciresinol, an oxidized form of secoisolariciresinol. Also, some synthesis products were observed, including *p*-coumaroyl tyrosine. In addition, 3,4-dihydroxyphenylglycol was another marker compound of a new synthesis, which was recently reported as a marker of cooking time in a work dealing with how ingredients and time affect the metabolome of sofrito following the addition of garlic or onion (Rinaldi de Alvarenga et al., 2020). Finally, some other degradation products (including two hydroxybenzoic acids) were detected and reported in **Table 2**.

In addition to the phenolic compounds tentatively identified by UHPLC-QTOF analysis, the UHPLC-Q-Orbitrap also revealed some more lipophilic phenolics, found to be also markers of the cooking process, showing either an increasing or decreasing trend

462 compared with the raw EVOO. This was the case of 2-(3,4-dihydroxyphenylethyl)-6-epi-
463 elenaiate, which is a tyrosol derivative, whose concentration was increased in the
464 innovative cooking process and in sauteing. In fact, it is classified as a degradation
465 product, but as the process gets more aggressive (in the case of deep-frying and oven),
466 this compound undergoes degradation, and its concentration tends to decrease. Similarly,
467 during EVOO cooking, some phenolic compounds can interact with other compounds, as
468 is the case of (3*β*,22*E*)-26,27-dinorergosta-5,22-dien-3-ol, which results from conjugation
469 between a ferulic acid and a terpenoid.

470 *4.2.2. Fatty acids and derivatives*

471 Triacylglycerols (TG) are the main components of EVOO from a quantitative point of
472 view. During cooking, these compounds react due to temperature, light, and oxygen to
473 form new compounds. In this regard, the main reactions widely reported are
474 hydrolyzation and polymerization (Santos et al., 2013). Also, the fatty acids (FA) can
475 undergo some transformations as oxidation or reaction with other EVOO components
476 (Santos et al., 2013). Overall, all of these reactions could be reflected in the markers we
477 found. In our experimental conditions, we found several degradation products of TGs; in
478 particular, following the traditional cooking processes (oven, deep-frying and sauteing).
479 The same could be observed for some other fatty compounds, that are less degraded by
480 modern cooking techniques.

481 Also, FA and diglycerides (DG) underwent synthesis reactions with other compounds
482 in EVOO, thus forming esters, ethers, and amides. They reacted with amino acids,
483 forming, for example, N-palmitoyl isoleucine, i.e., the amide between palmitic acid with
484 isoleucine. Overall, Napolitano *et al*. (2018) showed similar compounds derived from the
485 roasting of hazelnuts (Napolitano, Cerulli, Pizza, & Piacente, 2018).

Another marker found to increase during cooking was 16:2-Glc-Stigmasterol, which results from the conjugation between a FA, a carbohydrate and a sterol. Furthermore, some hydrolysis products were proposed as marker compounds, mainly monoglycerides and diglycerides. One of these monoglycerides is the stearic acid, previously proposed as a marker of cooked oil (Cao et al., 2019).

In addition, PUFAs underwent oxidation through the attack of reactive oxygen species to form oxylipins. These latter are bioactive compounds, being modulators of inflammation, blood pressure, and immune action (Gabbs, Leng, Devassy, Monirujjaman, & Aukema, 2015). In this work, the oxylipins formed were mainly derived from linoleic acid, in which one or both unsaturations are oxidized to epoxy, peroxide, acid or alcohol group (Brühl, 2014). We also found possible intramolecular esterification of fatty acids to form lactones, as in the case of muricatacin. Oxylipins, namely 9,12,12-TriHODE (trihydroxy- octadecadienoic acid) and 9,12,12-TriHOME (trihydroxy-octadecenoic acid) were also found as markers of the addition of garlic and onion in a sofrito cooking process (Rinaldi de Alvarenga et al., 2020).

Finally, we found a group of FA and derivatives compounds that were markers of the cooking process, resulting in increased or degraded depending on the process. One example is the case of phosphatidylethanolamines or some diacylglycerols (DG), which are formed from the hydrolysis of one ester linkage of the TG.

4.2.3. Carotenoids

Carotenoids are EVOO antioxidant compounds, two main are lutein and *β*-carotene (Martakos, Kostakis, Dasenaki, Pentogennis, & Thomaidis, 2019). In our experimental conditions, two degraded carotenoids were found to be markers of the cooking process, namely violaxanthin and 3-hydroxy-*β, E*-caroten-3'-one, i.e., two xanthophylls. On the

other hand, we found some generated marker compounds, as tangeraxanthin, which is a lutein derivative.

4.2.4. Other compounds

Other health-related compounds present in EVOO are triterpenic acids. In particular, the predominant ones are maslinic, oleanolic, and ursolic acids (Giménez et al., 2015). In this work, oleanolic and ursolic acids were markers of the cooking process. In fact, these latter were degraded to produce discriminant degradation products. One example is queretaroic acid, which is an oleanolic acid with an alcohol group. This compound was mainly generated during conventional cooking processes.

EVOO is also an important source of lipophilic vitamins, mainly vitamin E (Martakos et al., 2019). During the cooking process, α-tocopherol is degraded, and some derived compounds are formed, as 13'-hydroxy-α-tocopherol. We also identified some vitamin D derivatives in the oil, both degraded and generated. Finally, one vitamin K derivative, namely demethylphylloquinone, was found to be a marker of the process, and it was a generated compound.

Finally, we found other marker compounds likely degraded or generated during the cooking process. These latter are part of the unsaponifiable fraction of EVOO, which is complex due to this oil is unrefined. Between the generated ones, the majority were oxidation products including epoxy, alcohols, lactones, and acids.

5. Conclusions

In this work, the specific impact of different cooking techniques, cooking time, and temperature on EVOO composition was comprehensively investigated by targeting both hydrophilic and lipophilic compounds. The results have suggested that innovative (and milder) cooking techniques preserved EVOO phytochemical profile and highlighted as the cooking time has a rather limited effect. Interestingly, the temperature presented a

hierarchically higher weight in determining the observed differences compared to cooking time. Nonetheless, the clustering of treatments unraveled some distinctive effects of cooking-temperature combinations, considering the more hydrophilic (polyphenols) and nonpolar fractions. In particular, it may be postulated that phenolics were more sensitive to cooking conditions (likely because of the related oxygen availability conditions), while nonpolar components were more affected by cooking temperature (thus suggesting thermal degradation processes were predominant).

The untargeted profiling allowed to holistically shed light on a broad diversity of changes occurring in EVOO during cooking. Indeed, among the marker compounds, we found chemically diverse compounds, including FAs, oxylipins, phenolic compounds, and vitamins.

Overall, this work provides new insights into the appropriate selection of specific cooking methods (both traditional and innovative) in order to preserve the EVOO components that relate to health-promoting aspects, while preserving a good palatable product. Further investigations in how food matrix affect EVOO should be done, as they can interact during cooking.

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Conflict of interest

Rosa María Lamuela-Raventos reports receiving lecture fees from Cerveceros de España and receiving lecture fees and travel support from Adventia. The other authors declare no conflict of interest.

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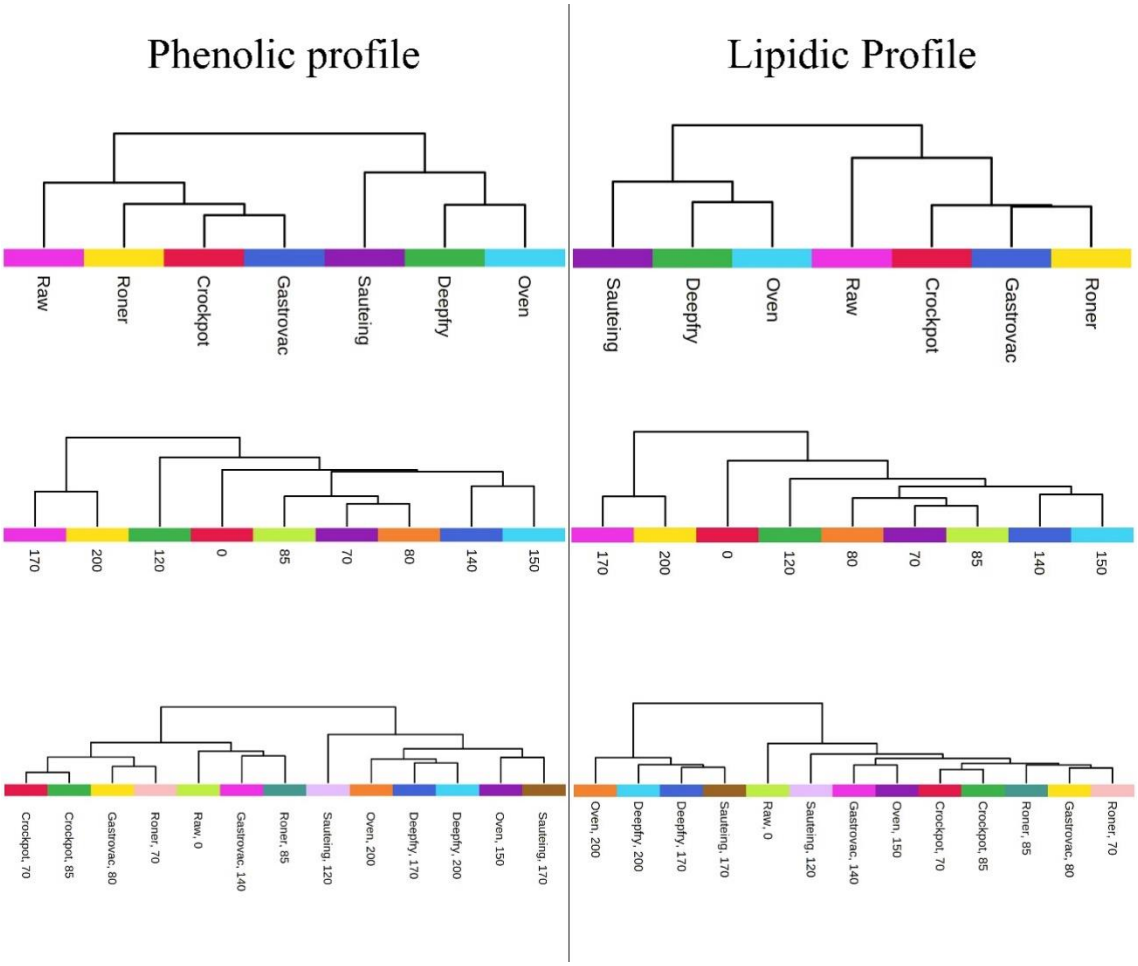
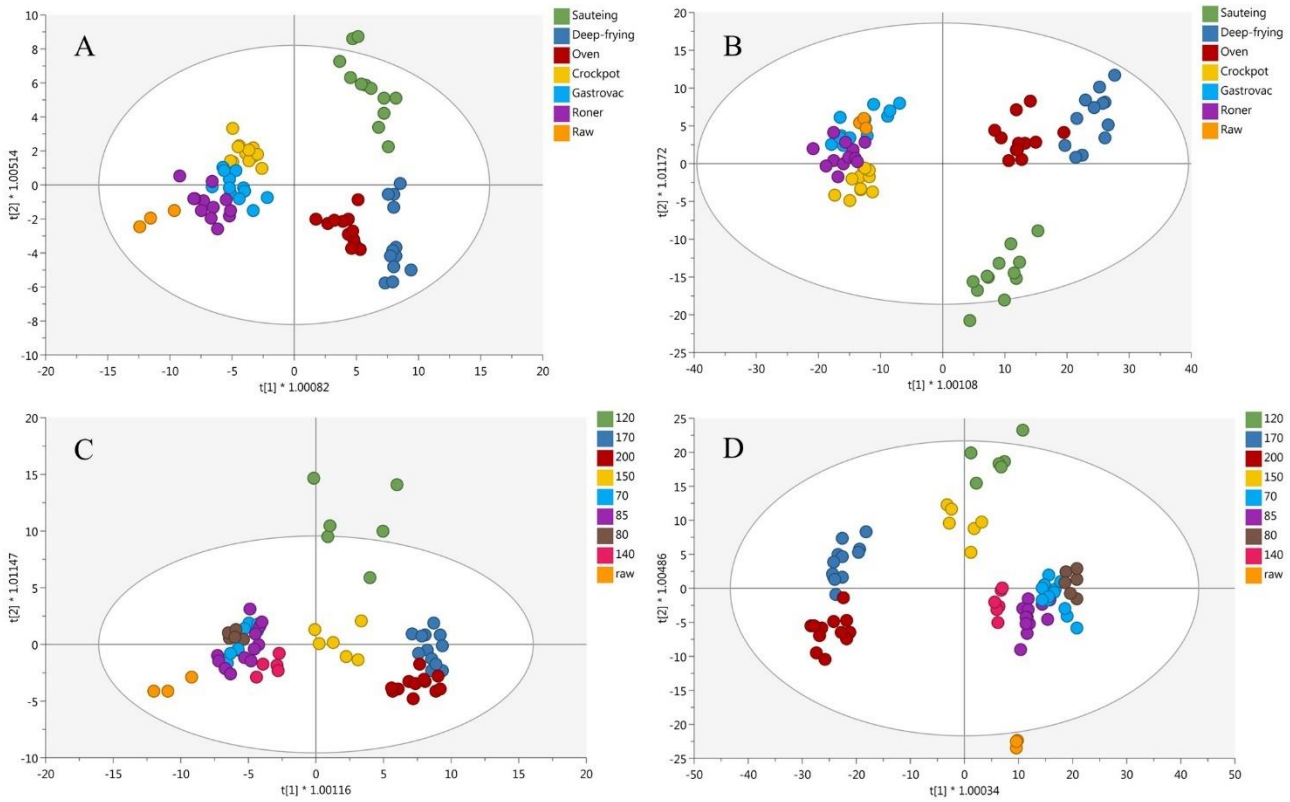
Figures

Figure 1:



Grouping obtained from the HCA made with the QTOF data (phenolic profile) or Orbitrap data (lipidic profile) built with the factors cooking technique, cooking temperature (in ºC) and the interaction.

Figure 2:



A: OPLS-DA of QTOF data using cooking technique as discriminant factor. B: OPLS-DA of Orbitrap data using cooking technique as discriminant factor. C: OPLS-DA of QTOF data using cooking temperature as discriminant factor. D: OPLS-DA of Orbitrap data using cooking temperature as discriminant factor.

Tables

Table 1: Summary of EVOO cooking treatment. ↓: low, ↑: high.

Technique	↓Temperature ↓Time	↓Temperature ↑Time	↑Temperature ↓Time	↑Temperature ↑Time
Sauteing	120ºC, 8 min	120ºC, 12 min	170ºC, 5 min	170ºC, 8 min
Deeep-frying	170ºC, 5 min	170ºC, 10 min	200ºC, 4 min	200ºC, 8 min
Oven	150ºC, 20 min	150ºC, 30 min	200ºC, 15 min	200ºC, 25 min
Crockpot	70ºC, 150 min	70ºC, 285 min	85ºC, 150 min	85ºC, 270 min
Gastrovac	80ºC, 30 min	80ºC, 45 min	140ºC, 15 min	140ºC, 25 min
Roner	70ºC, 165 min	70ºC, 300 min	85ºC, 165 min	85ºC, 300 min

Table 2: Marker phenolic compounds of each class found through QTOF analyses, separated by if they are increase or degraded. VIP: variables' importance in projection of the OPLS-DA built with Temperature or cooking technique as discriminant factor. Log FC: Log(2) of the fold-change comparing each cooking technique with the raw sample. *p*-HPEA-AC: Tyrosol acetate.

Generated	**discriminant compounds**	**most discriminant compound**	**VIP score Temp**	**VIP score technique**	**LogFC Crockpot**	**LogFC Gastrovac**	**LogFC Roner**	**LogFC deep-frying**	**LogFC oven**	**Log FC sauteing**
Flavonoids										
Flavones	1	Sinensetin	1.15	1.07	17.09	19.35	18.82	21.18	20.97	20.80
Flavonols	1	Kaempferol-*O*-trihexoside	>1	1.11	0.38	0.23	0.35	0.70	0.42	1.01
Lignans	5	Dimethylmatairesinol	1.04	1.20	16.34	8.97	8.36	0.27	1.69	4.36
Phenolic acids										
Hydroxybenzoic acids	2	2-Hydroxybenzoic acid	1.07	1.00	0.12	-0.01	1.83	19.51	21.24	10.56
Hydroxycinnamic acids		Ferulic acid-*O*-hexoside	>1	1.09	0.12	2.92	0.07	14.12	9.01	4.45
Other polyphenols										
Alkylmethoxyphenols	1	4-Vinylsyringol	1.15	1.17	1.86	1.19	0.74	3.21	2.49	3.89
Furanocoumarins	1	Isopimpinellin	1.15	1.12	0.29	0.29	0.06	19.59	19.17	20.14
Other polyphenols	1	3,4-Dihydroxyphenylglycol	1.15	1.07	-0.11	-0.56	-0.26	0.70	0.24	1.04
Phenolic terpenes	1	Carnosol	1.02	1.03	-0.22	7.19	-0.29	17.00	13.87	9.63
Degraded										
Flavonoids										
Anthocyans	6	Delphinidin-*O*-pentoside	1.31	1.10	-0.08	-0.42	-0.38	-11.20	-5.43	-2.26
Dihydrochalcones	1	Phloridzin	>1	1.05	-3.20	-3.15	-1.55	-3.99	-1.78	-2.11
Flavanones	3	8-Prenylnaringenin	1.10	1.02	-0.19	-9.69	-0.24	-19.10	-19.10	-17.44
Flavones	2	Luteolin-*O*-hexoside	1.24	1.07	-14.15	-13.12	-13.12	-17.14	-17.16	-17.28
Flavonols	2	Quercetin-*O*-pentoside	1.31	1.12	-0.16	-0.38	-0.36	-11.05	-5.34	-2.21

Isoflavonoids	2	Glycitin	1.01	1.08	-18.50	-13.42	-0.45	-18.35	-18.37	-18.50
Lignans										
Lignans	3	Lariciresinol-sesquilignan	>1	1.40	-20.01	-20.01	-20.01	-19.84	-19.79	-19.96
Phenolic acids										
Hydroxybenzoic acids	2	4-Hydroxybenzoic acid -*O*-hexoside	1.08	1.12	-1.30	-1.03	-1.09	-7.43	-13.97	-1.58
Hydroxycinnamic acids	3	*m*-Coumaric acid	1.06	1.16	-2.43	-2.37	-16.11	-7.32	-4.71	-7.08
Other polyphenols										
Alkylphenols	1	5-Heptadecylresorcinol	>1	1.01	-0.59	-0.55	-0.05	-15.39	-0.89	-22.89
Hydroxybenzaldehydes	1	Vanillin	>1	1.03	-17.25	-0.49	-0.03	-17.25	-11.45	1.04
Tyrosols	3	*p*-HPEA-AC	1.15	1.21	0.31	-1.28	0.48	0.60	0.55	0.53

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742 Table 3: Marker compounds of each class found through Orbitrap analyses, separated by if they are increased, degraded or both. VIP: variables' importance in
743 projection of the OPLS-DA built with Temperature or cooking technique as discriminant factor. Log FC: Log(2) of the fold-change comparing each cooking
744 technique with the raw sample.

Generated	Discriminant compounds	Most discriminant compound	VIP score Temp	VIP score technique	LogFC Crockpot	LogFC Gastrovac	LogFC Roner	Log FCdeep-frying	LogFC oven	LogFC sauteing
New Formed										
FA and derivatives	32	PI(18:1/15:0)	1.74649	1.41877	4.391944	8.2872715	3.9429774	8.524108	8.915601	9.683893
Phenolic compounds and derivatives	14	*O*-Caffeoylshikimic acid	1.62856	1.41505	5.3970065	5.6248736	6.3705034	7.3163686	8.557368	7.966763
Others	12	Geranyl acetate	1.41995	1.58213	2.622311	2.151964	2.964643	2.5024965	2.571333	2.353322
Oxydation products										
Oxylipins	18	5-Butyltetrahydro-2-oxo-3-furancarboxylic acid	1.51034	1.66126	1.071096	0.39695692	1.0114352	0.9875431	0.82941115	1.0001428
Other FA and derivatives	8	3-Hydroxy-hexadecanoic acid	1.60505	1.61002	0.87744284	0.45202762	1.0277257	0.8459569	0.90590745	0.65938294
Phenolic compounds and derivatives	20	13-Hydroxy-5'-O-methylmelledonal	1.45667	1.2772	1.9838253	2.0072188	2.103234	1.9235957	2.1474628	2.3930402
Carotenoids and derivatives	5	4,4'-Diapophytofluene	1.4534	1.31368	1.3135228	-0.18357341	0.17269167	-0.9017002	-0.7525671	1.8951955
Terpenic acid and derivatives	4	Methyl 3*β*-hydroxy-13(18)-oleanen-28-oate	1.38992	1.28585	2.0006738	1.5461024	0.84843785	0.92612	0.04273605	2.7423096
Vitamins and derivatives	11	13'-Hydroxy-*α*-tocopherol	1.41301	1.35148	1.5694126	1.5583464	1.7986315	1.849115	2.0009272	1.8696846
Others	46	Pterin	1.43307	1.58319	1.705614	1.300201	2.019796	1.5977775	1.673532	1.3951392
Hydrolization										
FA	12	*α*-Linolenic acid	1.54097	1.65921	4.6568446	2.2539718	4.4372306	3.169782	2.8576748	2.9702747
Phenolic compounds and derivatives	1	Hydroxydaidzein/Apigenin	1.29803	1.29585	1.0344255	0.1897362	0.44370523	-0.9023163	-0.8047352	0.5877536

Degraded										
FA and derivatives	67	10-Hydroxy-8-decenoic acid	1.30323	1.30866	-1.0943787	0.23167211	-0.43355703	-2.2567995	-1.161508	-3.1817083
Phenolic compounds and derivatives	33	Butyl *O*-caffeoylquinate	1.57418	1.35638	-1.3318475	0.6814499	-2.3654459	0.04668316	-0.6469526	0.9593978
Carotenoids and derivatives	2	3-Hydroxy-*β*, *E*-caroten-3'-one	1.08632	1.03855	-0.26328963	-1.6357712	-0.2701796	0.15731113	-0.5132378	-1.3777789
Terpenic acid and derivatives	3	Katonic acid/ Ursolic acid	1.13848	1.20005	-0.18030563	-0.4533887	0.25251913	-1.7398878	-1.4326813	-1.4248419
Vitamins and derivatives	14	1*α*,24,25-trihydroxy-22-oxavitamin D3	1.13527	1.06238	0.14750274	0.20444027	0.16522105	-1.5150608	-1.2004497	-0.5781701
Others	45	4*α*-Formyl-4*β*-methyl-5*α*-cholesta-8-en-3*β*-ol	1.65249	1.40777	-2.316343	-0.09582913	-3.181325	-0.8616666	-1.2215797	-0.10636723
Generated and degraded										
FA and derivatives	14	*O*-α- Glucopyranosyl - mannitol	1.30383	1.32783	1.0625315	0.07058764	0.3712975	-1.3880334	-1.1108049	0.59258974
Phenolic compounds and derivatives	7	Artoindonesianin R	1.28768	1.26356	1.0390304	-0.6923979	0.4073812	-3.398105	-2.3019292	-0.5265567
Others	11	Integerrimine N-oxide	1.33708	1.36664	1.5224164	0.04542001	0.5260237	-2.0447052	-1.8462828	0.9733645

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