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Un model d'estrès oxidatiu en embrió de peix zebra. Aplicació per a l'avaluació de l'activitat protectora de substàncies antioxidants.

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Identification of phenolic compounds in red wine extract samples and zebrafish embryos by HPLC-ESI-LTQ-Orbitrap-MS

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ABSTRACT

The zebrafish embryo is a highly interesting biological model with applications in different scientific fields, such as biomedicine, pharmacology and toxicology. In this study, we used liquid chromatography/electrospray ionisation-linear ion trap quadrupole-Orbitrap-mass spectrometry (HPLC/ESI-LTQ-Orbitrap-MS) to identify the polyphenol compounds in a red wine extract and zebrafish embryos. Phenolic compounds and anthocyanin metabolites were determined in zebrafish embryos previously exposed to the red wine extract. Compounds were identified by injection in a high-resolution system (LTQ-Orbitrap) using accurate mass measurements in MS, MS^2 and MS^3 modes. To our knowledge, this research constitutes the first comprehensive identification of phenolic compounds in zebrafish by HPLC coupled to high-resolution mass spectrometry.

1. Introduction

The Mediterranean diet is rich in natural antioxidants such as polyphenols, whose main dietary sources are fruits, vegetables, red wine, coffee, olives and virgin olive oil (Tresserra-Rimbau et al., 2013; Vallverdú-Queralt, Regueiro, Rinaldi De Alvarenga, Torrado & Lamuela-Raventos, 2014). Considerable interest has been focused on the bioactive phenolic compounds in wine and wine extracts, notably anthocyanins, flavanols, flavonols and resveratrol, since they possess many biological activities, such as antioxidant, cardioprotective, anticancer, anti-inflammation, anti-aging and antimicrobial properties (Xia, Deng, Guo, & Li, 2010). Although positive effects of polyphenols have been demonstrated in humans and animals, the chemical and *in vitro* biological techniques regularly used to evaluate their antioxidant capacity are not useful for determining their underlying mechanisms of action (Frankel & Finley, 2008).

The zebrafish (ZF), *Danio rerio*, is a tropical fish of the Cyprinidae family, which was first used as a model in the 1970s to study vertebrate development. Since then, the ZF embryo has become a biological model with wide-ranging applications in different scientific fields, such as biomedicine, pharmacology, toxicology, etc. The ZF has numerous advantages: easy maintenance, a large number of offspring, and transparent embryos that develop outside the mother and are easily accessible for experimental manipulation (Scholz et al., 2008). The ZF is characterised by a very rapid embryonic development: the main organs are present at 48 h post-fertilisation (hpf), embryos hatch at about 2–3 days post-fertilisation (dpf), organogenesis is complete by 5–6 dpf and sexual

Abbreviations: ZF, zebrafish; hpf, hours post-fertilisation; dpf, days post-fertilisation; HRMS, high resolution mass spectrometry; LTQ, linear ion trap; MS/MS, two-stage mass analysis; MS^n, multi-stage mass analysis; HPLC, high performance liquid chromatography; MTC, maximum tolerable concentration; AGC, automatic gain control; HCD, high-energy C-trap dissociation; PDA, photodiode array detector; TP, total polyphenols; GAE, gallic acid equivalents; AC, antioxidant capacity; TE, Trolox equivalent; rt, retention time; acc mass, accurate mass.

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maturity is reached in 3 months (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). The ZF presents genes and enzymes which are implicated in defence processes against oxidative stress. These systems are analogous to mammalian antioxidant systems (Timme-Laragy et al., 2012). Thus, we selected the ZF embryo model as a potential method to study the effects of natural bioactive compounds from dietary sources.

One of the limitations of ZF embryos is that they need to take up substances through the chorion and other membranes, because the gills of the adult fish are not yet developed. The determination of a target compound in the ZF embryo is very important to confirm its bioavailability and also its capacity to produce an effect (Scholz et al., 2008).

High-resolution mass spectrometry (HRMS) can provide abundant information for the structural elucidation of a wide range of polyphenol compounds in red wine extracts and ZF embryos. Recently, the combination of Orbitrap technology with a linear ion trap (LTQ) has been shown to enable fast, sensitive and reliable detection and identification of small molecules, regardless of relative ion abundance. LTQ-Orbitrap-MS delivers single-stage mass analysis, providing molecular mass information, two-stage mass analysis (MS/MS) and multi-stage mass analysis (MS^n) delivering structural information. Exact mass measurements and elemental composition assignment are essential for the characterisation of small molecules (Vallverdú-Queralt et al., 2014).

The aim of the present work was to provide accurate and comprehensive identification of the phenolic constituents found in wine extracts and ZF embryos using high performance liquid chromatography (HPLC) coupled with LTQ-Orbitrap analysis. *In vivo* studies using ZF allow the observation of pathological responses that integrate physiological functions, as well as providing the advantages of an *in vitro* model. Thus, we selected the ZF embryo model to study the effects of natural bioactive compounds from dietary sources. To our knowledge, this research constitutes the first comprehensive identification of phenolic compounds in ZF by HPLC coupled to HRMS.

2. Materials and methods

2.1. Standards and samples

All samples and standards were handled without exposure to light. Caffeic, ferulic, *p*-coumaric, protocatechuic and gallic acid, quercetin, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, catechin, epicatechin, peonidin and malvidin-3-*O*-glucoside, ABTS (2,2′azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97%), anhydrous sodium acetate, Folin–Ciocalteu (F–C) 2 N reagent, sodium carbonate, and manganese dioxide were purchased from Sigma–Aldrich (Madrid, Spain), and DPPH (2,2-diphenyl-1-picrylhydrazyl) from Extrasynthèse (Genay, France). Acetonitrile, ethanol and methanol, all of HPLC grade, and formic acid (⩾98%) were acquired from Scharlau (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q Gradient water purification system (Millipore Bedford, MA). Samples were stored at 4 °C and protected from light before analysis.

To obtain the red wine extract, 300 mL of wine sample, protected from light, were concentrated under vacuum below 30 °C to a volume of 250 mL, to remove ethanol.

2.2. Zebrafish maintenance and embryo production

Adult ZF were obtained from a commercial supplier (Pisciber, Barcelona) and housed separately in a closed flow-through system in standardised dilution water according to ISO 7346 (ISO, 1996: 2 mM $CaCl_2{\cdot}2H_2O$, 0.5 mM $MgSO_4{\cdot}7H_2O$, 0.75 mM $NaHCO_3$, 0.07 mM KCl). Fish were maintained at 26 ± 1 °C on a 14/10 h light/dark cycle, and were fed with commercial dry flake food (API® Tropical) and live brine shrimp. Males and females were placed in a breeding tank with an 8:10 male:female ratio. Spawning and fertilisation took place the next morning, 30 min after the lights had been turned on. The embryos were collected, successively cleaned and staged according to Kimmel et al. (1995). Fertilisation success was checked under a dissection stereomicroscope (Motic SMZ168, Motic China group, Ltd., China).

2.3. Embryo exposure

Range-finding experiments with different dilutions of the red wine extract with ultrapure water were performed to select the maximum tolerable concentration (MTC), that is, the highest concentration of red wine extract at which the embryos did not show any adverse effects. A 1–800 dilution of the red wine extract was selected as the working concentration and the embryos were exposed to it from 24 to 48 h post-fertilisation (hpf). The red wine extract was diluted in Danieau's buffer (17.4 mM NaCl, 0.2 mM KCl, 0.1 mM $MgSO_4{\cdot}7H_2O$, 0.2 mM $Ca(NO_3)_2{\cdot}4H_2O$, 1.5 mM HEPES; pH 6.5) and filtered through a 0.22-μm PTFE membrane filter. The pH of the dilution was adjusted to 6.5. Sixty randomly selected embryos were distributed in glass dishes. Until the period of exposure, embryos were maintained in 25 mL of Danieau's buffer at 27 ± 1 °C and protected from light. At 24 hpf the embryos were exposed to the red wine extract dilution and maintained under the conditions previously detailed. After 24 h of exposure, the embryos were manually dechorionated and transferred to a new glass dish where they were washed 3 × 5 min with Danieau's buffer. The pool of 60 ZF embryos was homogenised in 250 μL of 0.1% aqueous formic acid. Homogenisation was performed for 3 × 10 s using a Kontes® Pellet Pestle®. The homogenates were stored at −20 °C until they were analysed.

2.4. HPLC-LTQ-Orbitrap-MS

The red wine extract was centrifuged at 3000*g* for 10 min at 4 °C, filtered through a 0.22-μm PTFE membrane filter and directly injected into the HPLC. The ZF homogenate was also centrifuged at 3000*g* for 10 min at 4 °C, filtered through a 0.22-μm PTFE membrane filter and directly injected into the HPLC.

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 resolution of 30,000 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 (FT Automatic gain control (AGC) target, 5×10^5 for MS mode and 5×10^4 for MS^n mode). The LTQ delivers single-stage mass analysis providing molecular mass information, two-stage mass analysis (MS/MS) and multi-stage mass analysis (MS^n) which gives structural information. Exact mass measurements are accomplished using Orbitrap-MS. Red wine extract and ZF were first analysed in full MS mode with the resolution set at 30,000 at *m*/*z* 400. The successive analyses were done in MS^n mode with the resolution set at 15,000 at *m*/*z* 400. An isolation width of 100 amu was used and precursors were fragmented by high-energy C-trap dissociation (HCD) with normalised 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 MS^n 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 C_{18} column (50 × 2.0 mm i.d., 5 μm; Phenomenex, Torrance, CA) 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**; 12 min, 100% **B**; 13 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 (Vallverdú-Queralt et al., 2012).

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

2.5. Analysis of total polyphenols

For the total polyphenol (TP) assay, each sample was analysed in triplicate; 20 μL of the eluted fractions were mixed with 188 μL of Milli-Q water in a microtitre 96-well plate (Nunc, Thermo Fisher Scientific, Roskilde, Denmark), 12 μL of F–C reagent and 30 μL of sodium carbonate (200 g/L) were added following the procedure described by Vallverdú-Queralt, de Alvarenga, Estruch, and Lamuela-Raventós (2013). 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)/L of sample.

2.6. Antioxidant capacity

The red wine extract and the ZF embryos were also analysed for their antioxidant capacity (AC) using a DPPH assay (Vallverdú-Queralt et al., 2013).

The AC was studied through the evaluation of the free radical-scavenging effect on the DPPH radical. Solutions of known Trolox concentration were used for calibration. A 5-μL aliquot of red wine extract or ZF embryos homogenate (0.1% formic acid in water) 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 at 515 nm. Results were expressed as mmol Trolox equivalents (TE)/L of sample.

3. Results and discussion

The TP content in the red wine extract samples, measured by the TP assay, was 2180 ± 145 mg GAE/L of sample. The AC of the red wine extract samples, measured by the DPPH assay, was 153 ± 0.9 mmol TE/L of sample. The TP and AC capacity were also measured in the homogenate of ZF embryos that had been exposed to red wine extract. In the ZF embryo homogenate, the TP content was 10.8 ± 0.20 mg GAE/L of sample and AC was 1.53 ± 0.08 mmol TE/L of sample.

The red wine extract and ZF embryo homogenate were analysed by HPLC-ESI-LTQ-Orbitrap-MS to identify the polyphenols in the samples. Table 1 shows the 43 compounds identified in the red wine extract along with their retention times (rt), accurate mass (acc. mass), molecular formula, mDa of error between the mass found and the accurate mass of each polyphenol and the MS–MS ions used for identification.

The spectra generated by cinnamic and benzoic acids showed the deprotonated molecule $[M-H]^-$ and some fragments. The typical loss of CO_2 was observed for gallic, protocatechuic, caffeic, *p*-coumaric, genistic, caftaric, *p*-hydroxybenzoic and ferulic acids, giving $[M-H-44]^-$ as a characteristic ion. All these phenolic acids were positively identified by comparison with available standards, and, with the exception of gentisic and ferulic acids, they were also found in the ZF embryo homogenate (Table 2).

Phenolic acid-*O*-glucosides, such as coumaric acid-*O*-hexoside and protocatechuic acid-*O*-hexoside, were identified in the red wine extract samples, giving as corresponding fragments the loss of a hexose $[M-H-162]^-$. Protocatechuic acid-*O*-hexoside and coumaric acid-*O*-hexoside were also found in ZF embryos (Table 2). It has been demonstrated that polyphenols are mainly metabolised in plasma, and that free forms represent only 5–24% of the total polyphenol metabolites (Zhao, Egashira, & Sanada, 2003).

C-glycosides of caffeic acid were also present, showing a different fragmentation pattern from *O*-glycosides. The spectra of *C*-glycosides displayed losses of 120 and 90 amu corresponding to cross-ring cleavages in the sugar unit, as reported previously in tomatoes using liquid chromatography/tandem mass spectrometry (Vallverdú-Queralt et al., 2012).

Four trimeric proanthocyanidins (*m*/*z* 865) and two dimers (*m*/*z* 577) were also detected in the red wine extract. The most common classes of proanthocyanidins consist of subunits of catechin, epicatechin, and their gallic acid esters (B-type oligomers). Various studies of oligomeric and polymeric procyanidins in wine seed extracts have proposed a fragmentation scheme of ions derived from B-type procyanidins (Gu et al., 2003; Sun & Miller, 2003). However, we hypothesised that proanthocyanidins were not able to enter the ZF embryo without metabolisation, as they were not found in the ZF embryo homogenate (Table 2).

Catechin, epicatechin and some other typical polyphenols from wine products, such as resveratrol and piceid derivatives, were also detected in red wine extract samples (Table 1) and their identity was confirmed by comparison with available standards. Moreover, another peak present in the red wine extract at *m*/*z* 441 was identified as epicatechin-3-*O*-gallate, its retention time and mass spectra matching the standard. The mass spectra of epicatechin-3-*O*-gallate showed two fragment ions arising from the cleavage of the ester bond, at *m*/*z* 289 for deprotonated epicatechin and *m*/*z* 169 for a deprotonated gallic acid moiety. Resveratrol, catechin and piceid were the main compounds of the red wine extract and were also detected in the ZF embryo homogenate.

Analysis of chromatograms in the positive mode detected the presence of the anthocyanins malvidin and peonidin-3-*O*-glucoside in the red wine extract, which was confirmed by comparison with commercial standards. The mass spectra showed the loss of $[M-H-162]^-$, corresponding to the loss of a hexoside. Other anthocyanins, such as delphinidin, cyanidin and petunidin-*O*-hexosides, were also detected in the red wine extract. The mass spectra of these compounds showed fragments corresponding to the loss of one hexoside unit $[M-H-162]^-$ and two hexoside units $[M-H-162-162]^-$, respectively, corresponding to those reported in the literature (Da Costa, Horton, & Margolis, 2000). In contrast, the only anthocyanins detected in ZF embryos were peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, delphinidin-*O*-hexoside and petunidin-*O*-hexoside.

Naringenin and quercetin were positively identified in the red wine extract by comparison with available standards and the mass spectra gave as a characteristic ion the deprotonated molecule

Table 1
Compounds identified in red wine extracts.

	Compound	rt (min)	$[M-H]^-/[M+H]^+$	MS/MS ions	Acc Mass	mDa	Molecular formula
1	Procyanidin trimer type B isomer 1	1.50	865	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	865.1985	4	C45H38O18
2	Gallic acid*	1.57	169	125 (100)	169.0142	1.1	C7H6O5
3	Protocatechuic acid-*O*-hexoside	1.86	315	153 (100), 109 (20)	315.0721	2.2	C13H16O9
4	Gentisic acid*	2.70	153	123 (100), 109 (10)	153.0188	0.4	C7H6O4
5	Protocatechuic acid*	2.79	153	153 (40), 109 (90)	153.0193	0.9	C7H6O4
6	Caftaric acid*	3.22	311	149 (100)	311.0408	2	C13H12O9
7	Procyanidin dimer type B isomer 1	3.40	577	425 (100), 451 (60), 407 (50), 559(30), 289 (30)	577.1346	2.9	C30H26O12
8	Caffeic acid-*C*-hexoside 1	3.54	341	281 (90), 251 (100), 221 (40), 179 (60), 135 (10)	341.0877	2.1	C15H18O9
9	Procyanidin dimer type B isomer 2	4.09	577	425 (100), 451 (60), 407 (50), 559(30), 289 (30)	577.1346	2.9	C30H26O12
10	Caffeic acid-*C*-hexoside 2	4.19	341	281 (90), 251 (100), 221 (40), 179 (60), 135 (10)	341.0877	2	C16H18O8
11	Coumaric-*O*-hexoside	4.36	325	163 (100), 119 (12)	325.0928	1.2	C15H18O8
12	Catechin*	4.43	289	245 (100)	289.0718	1.7	C15H14O6
13	*p*-hydroxybenzoic acid*	4.49	137	93 (100)	137.0244	0.7	C7H6O3
14	Procyanidin trimer type B isomer 2	4.75	865	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	865.1985	4	C45H38O18
15	Procyanidin trimer type B isomer 3	5.50	865	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	865.1985	4	C45H38O18
16	Caffeic acid*	5.72	179	135 (100)	179.0349	1.1	C9H8O4
17	Peonidin-3-*O*-glucoside*	6.25	463 $[M+H]^+$	301(100)	463.1233	0.2	$C22H23O11^+$
18	Malvidin-3-*O*-glucoside*	6.30	493 $[M+H]^+$	331 (100)	493.1341	0.1	$C23H25O12^+$
19	Epicatechin*	6.47	289	245 (100)	289.0718	1.9	C15H14O6
20	Eriodictyol-*O*-hexoside	6.64	449	287 (100), 259 (40), 269 (38)	449.1089	2.6	C16H18O8
21	Procyanidin trimer type B isomer 4	7.29	865	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	865.1985	4	C45H38O18
22	Delphinidin-*O*-dihexoside	7.76	627 $[M+H]^+$	465 (100), 303 (70)	627.1556	0.1	$C27H31O17^+$
23	Coumaric acid*	8.24	163	119 (100)	163.0400	0.9	C9H8O3
24	Myricetin-*O*-hexoside	8.49	479	316 (100), 317 (50)	479.0826	2.2	C21H20O13
25	Kaempferol-3-*O*-rutinoside*	8.79	593	285 (100)	593.1511	1.6	C27H30O15
26	Ferulic acid*	9.17	193	178 (70), 149 (100), 134 (50)	193.0506	1.2	C10H10O4
27	Piceid acid isomer 1	9.50	389	227 (100), 193 (80), 341 (70)	389.1241	2	C20H22O8
28	Cyanidin-*O*-dihexoside	9.65	611 $[M+H]^+$	303 (100), 465 (40)	611.1607	1.6	$C27H31O16^+$
29	Epicatechin-*O*-gallate	9.8	441	289 (100), 169 (30)	441.0822	1.8	C22H18O10
30	Kaempferol-3-*O*-glucoside*	10.20	447	285 (100)	447.0932	2.4	C21H20O11
31	Quercetin-*O*-hexoside*	10.20	463	301 (100), 293 (40), 271 (15), 169 (10)	463.0881	2.2	C21H20O12
32	Delphinidin-*O*-hexoside	10.2	465 $[M+H]^+$	303 (100), 447 (20), 369 (8)	465.1028	0.2	$C21H21O12^+$
33	Cyanidin-*O*-hexoside	11.66	449 $[M+H]^+$	287 (100), 431 (30), 263 (5)	449.1078	0.4	$C21H21O11^+$
34	Quercetin-*O*-rhamanoside	11.67	447	301 (100), 284 (20), 285 (20)	447.0932	1.9	C21H20O11
35	Petunidin-*O*-hexoside	11.94	479 $[M+H]^+$	317 (100), 359 (5), 461 (12)	479.1184	0.1	$C22H23O12^+$
36	Piceid acid isomer 2	12.23	389	227 (100)	389.1241	2.2	C20H22O8
37	Delphinidin acetyl hexoside	13.5	507 $[M+H]^+$	303(100), 489 (10), 187 (5)	507.1138	1.7	$C23H23O13^+$
38	Delphinidin	16.28	303 $[M+H]^+$	257 (100), 229 (70), 285 (60), 165 (50), 137 (20)	303.0499	0.9	$C15H11O7^+$
39	Quercetin*	16.30	301	301 (30), 179 (100), 151 (90), 107 (10)	301.0353	1.5	C15H10O7
40	Resveratrol	16.58	227	185 (100), 143 (30)	227.0713	2.5	C14H12O3
41	Naringenin*	17.88	271	271 (20), 151 (100), 177 (20), 119 (10)	271.0611	1.7	C15H12O5
42	Cyanidin	18.29	287 $[M+H]^+$	177 (70), 165 (40), 241 (45), 213 (30)	287.0550	0.1	$C15H11O6^+$
43	Petunidin	18.43	317 $[M+H]^+$	302 (100), 285 (40), 165 (10), 139 (10)	317.0656	0.2	$C16H13O7^+$

* Comparison with standard: rt, retention time; Acc. mass, accurate mass; mDa, millidaltons of error between the mass found and the accurate mass of each polyphenol.

Table 2
Compounds identified in zebrafish embryos.

	Compound	rt (min)	$[M-H]^-/[M-H]^+$	MS/MS ions	Acc Mass	mDa	Molecular formula
1	Gallic acid	1.57	169	125 (100)	169.0142	1.8	C7H6O5
2	Protocatechuic acid-*O*-hexoside	1.86	315	153 (100), 109 (20)	315.0721	0.8	C13H16O9
3	Protocatechuic acid	2.79	153	153 (40), 109 (90)	153.0193	0.5	C7H6O4
4	Caftaric acid	3.22	311	149 (100)	311.0408	1	C13H12O9
5	Coumaric-*O*-hexoside	4.36	325	163 (100), 119 (12)	325.0928	1.2	C15H18O8
6	Catechin	4.43	289	245 (100)	289.0718	1.2	C15H14O6
7	*p*-Hydroxybenzoic acid	4.49	137	93 (100)	137.0244	0.6	C7H6O3
8	Caffeic acid	5.72	179	135 (100)	179.0349	0.5	C9H8O4
9	Peonidin-3-glucoside	6.25	463 $[M+H]^+$	301(100)	463.1233	0.6	$C22H23O11^+$
10	Malvidin-3-glucoside	6.30	493 $[M+H]^+$	331 (100)	493.1341	0.2	$C23H25O12^+$
11	Ferulic acid*	9.17	193	178 (70), 149 (100), 134 (50)	193.0506	1.2	C10H10O4
12	Piceid acid isomer 1	9.50	389	227 (100), 193 (80), 341 (70)	389.1241	0.9	C20H22O8
13	Quercetin-*O*-hexoside	10.20	463	301 (100), 293 (40), 271 (15), 169 (10)	463.0881	1	C21H20O12
14	Delphinidin-*O*-hexoside	10.2	465 [M+H]+	303 (100), 447 (20), 369 (8)	465.1028	0.2	$C21H21O12^+$
15	Kaempferol-3-*O*-glucoside	10.20	447	285 (100)	447.0932	0.8	C21H20O11
16	Petunidin-*O*-hexoside	11.94	479 [M+H]+	317 (100), 359 (5), 461 (12)	479.1184	0.2	$C22H23O12^+$
17	Piceid acid isomer 2	12.23	389	227 (100)	389.1241	1.1	C20H22O8
18	Quercetin-*O*-glucuronide	14.80	477	301 (100)	477.0674	0.6	C21H18O13
19	Resveratrol	16.58	227	185 (100), 143 (30)	227.0713	0.9	C14H12O3

* Comparison with standard: rt, retention time; Acc. mass, accurate mass; mDa, millidaltons of error between the mass found and the accurate mass of each polyphenol.

$[M-H]^-$ and the ions corresponding to retro-Diels Alder fragmentation in the *C*-ring involving 1,3 scission (*m*/*z* 151). Some quercetin derivatives were also identified in the red wine extract samples (Table 1). In the homogenate of ZF embryos, quercetin-*O*-glucoronide was detected (Table 2). As this compound was not observed in red wine extracts (Table 1), it could be hypothesised that the ZF embryos metabolised quercetin-*O*-glucuronide from quercetin. The metabolism of quercetin has been evaluated in rats and humans, as this flavone has a putative ability to prevent cancer and cardiovascular diseases. After intravenous administration to rats, quercetin was found in both plasma and urine, whereas quercetin metabolites (sulfate and glucuronide conjugates and methylated forms) were isolated from the plasma, urine and bile (Jones et al., 2004). Derivatives of the flavonol kaempferol were also present in the red wine extract, while only kaempferol-3-*O*-glucoside was detected in the ZF embryo samples (Table 2).

The ZF embryo model has been used to study the effects of polyphenols (Pardal, Caro, Tueros, Barranco, & Navarro, 2014; Park et al., 2014; Richetti et al., 2011). However, as far as we know, the present study is the first time that polyphenols such as phenolic and hydroxycinnamic acids, proanthocyanidins and anthocyanins have been detected in ZF embryos. The ZF embryo has been described as an extremely valuable vertebrate model organism that can replace other juvenile or adult animal models (Kimmel et al., 1995). It has been proposed as a model organism to study oxidative stress effects in basic biology and pathology (Fang & Miller, 2012), as it shares oxidative pathways with humans (Timme-Laragy et al., 2012). During embryonic stages, ZF are covered by a chorion membrane and perivitelline space, so embryos can only take up the target compounds through their membranes (Wiegand, Pflugmacher, Giese, Frank, & Steinberg, 2000). As far as we know, there is no other research available in the literature referring to the absorption of polyphenols by ZF embryos. Based on the results of our study, it could be hypothesised that caffeic acids, protocatechuic and coumaric acid-*O*-hexosides and some proanthocyanidins and anthocyanins are able to cross the chorion membrane and be absorbed by the ZF embryo (Table 2) to develop antioxidant functions.

Caco-2 cells have been incubated with gallic acid to explore the mechanism of absorption, revealing non-polarised, dose-dependent and pH-independent permeability, and the same results were obtained for protocatechuic and hydroxybenzoic acids (Konishi, Kobayashi, & Shimizu, 2003). Other authors found that caffeic acid, caftaric acid, and esters of caffeic acid can also be absorbed in rat models (Lafay, Morand, Manach, Besson, & Scalbert, 2006).

The rate of uptake, metabolism and excretion are important parameters for determining the bioavailability and internal effective concentrations of an environmental chemical in an organism (Scholz et al., 2008). The membranes surrounding the ZF embryo may be a restriction factor for compound uptake and absorption. The chorion membrane of the ZF embryos contains pores which might result in size-dependent limitations in the uptake of large compounds but low molecular weight compounds are easily absorbed (Langheinrich, 2003). As most natural polyphenol compounds have a relatively low molecular weight, ranging from 500 to 3000 Da (Haslam, 1996), it should be possible for them to cross the chorion membrane, be absorbed by the ZF embryos and therefore produce an effect. Moreover, exposure to compounds in early life stages of ZF is through the culture medium, and most polyphenols are soluble, according to their polarity and chemical structure (Bravo, 1998).

In this study, we determined anthocyanin metabolites in ZF embryos previously exposed to red wine extracts. As the exposure of the ZF embryo model occurred through the ambient medium, maternal metabolism of the test substance can be discounted (Mattson, Ullerås, Patring, & Oskarsson, 2012). From the early development of the ZF, the presence of basic enzymes that facilitate biotransformation and elimination of chemicals can be presumed (Otte, Schmidt, Hollert, & Braunbeck, 2010; Tseng, Hseu, Buhlerb, Wang, & Hu, 2005). The detection of quercetin-*O*-glucuronide in the samples of ZF embryo homogenate indicates some metabolic activity in ZF embryos, a very important consideration for the assessment of anthocyanin effects in an *in vivo* model. There is evidence pointing to a positive association between anthocyanin intake and healthy biological effects displayed *in vivo* (Kong, Chia, Goh, Chia, & Brouillard, 2003; Prior & Wu, 2006). In this sense, some glycosylated anthocyanins may be more efficiently absorbed than aglycone forms. Some studies have reported that anthocyanins are readily detected in plasma in their parent forms, possibly as a result of their absorption through the gastric wall (Cao, Muccitelli, Sanchez-Moreno, & Prior, 2001; Mulleder, Murkovic, & Pfannhauser, 2002). Talavera et al. (2004) and Talavera et al. (2005) reported only native anthocyanins in the stomach, while in other organs (jejunum, liver, and kidney) native and methylated as well as conjugated anthocyanidins (monoglucuronides) were observed. Anthocyanin bioavailability is generally presumed, but whether their effect is due to the native compounds or other forms, or which mechanisms are involved is still unknown. In a recent study, rats were fed with a blackberry-enriched diet for 12 days and anthocyanin metabolites (methylated anthocyanins and glucurono-conjugated derivatives) were detected in bladder, prostate, testes, heart and adipose tissue (Felgines et al., 2009). Dietary anthocyanins from grapes and berries are also reported to reach the brain (Andres-Lacueva et al., 2005; Passamonti, Vrhovsek, Vanzo, & Mattivi, 2005; Talavera et al., 2005). Domitrovic (2011) stated that anthocyanidins reach higher concentrations in the brain than in plasma due to their capacity to cross the blood–brain barrier and distribute in the central nervous system. It could also be hypothesised that non-absorbed anthocyanins may modify the microbiota environment, thereby affecting human metabolism, or could act at the membrane border, inducing signal transduction pathways (Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014).

In conclusion, the HPLC-LTQ-Orbitrap-MS has permitted the identification of the polyphenols present in red wine extracts. This is the first time that polyphenols have been identified in ZF embryos previously exposed to red wine extract, and the metabolic capacity of ZF embryos has been observed.

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