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# Antioxidant activity, cytotoxic activity and metabolic profiling of juices obtained from saffron (*Crocus sativus* L.) floral by-products

Running title: Juices from saffron floral by-products as bioactive compounds source

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**ABSTRACT**

Juices obtained from cold-pressed saffron (*Crocus sativus* L.) floral by-products were evaluated as a potential source of compounds with antioxidant and cytotoxic activities. Floral by-products were split in two batches for extraction 24 and 48 hours after flower harvesting, respectively. The *in vitro* anti-oxidant activity of these extracts was tested using the FRAP and DPPH chemical tests, and two biological models of lipid oxidation (activity in preventing cholesterol degradation and protection against $Cu^{2+}$-mediated degradation of the liposomal unsaturated fatty acids). The cytotoxic activity was evaluated using the MTT assay. The results show that extracts obtained 48h post-harvest contained higher levels of total polyphenols and had the highest antioxidant activity in all of the performed assays. The LC-DAD and LC-ESI-(HR)$MS^n$ metabolic profiles showed high levels of kaempferol derivatives and anthocyanins. This study suggests that juices from saffron floral by-products could potentially be used to develop new products for the food and health industry.

**Keywords:** floral by-products; *Crocus sativus* L.; LDL oxidation; cholesterol degradation; LC-DAD; LC-ESI-(HR)$MS^n$

## 1. Introduction

Harvesting and processing of vegetables and fruit generate large amounts of by-products and bio-waste that can be used as an inexpensive source of important bio-molecules such as polyphenols and antioxidants. Saffron (*Crocus sativus* L.) is a plant widely used for the production of the saffron spice from the stigmas. This is the most valuable part of the flower due to its numerous uses both in the food and health sector. Traditionally, only stigmas are collected, dried and used as a spice while the remaining parts of the flower are discarded. Approximately 90 % of harvested saffron flowers is discarded. However, the phytochemical composition of these parts of the flower has recently gained interest from the scientific community because of its potential use in the health and food industries. So far a variety of carbohydrates (glucose, fructose, sucrose, maltose, inositol, sorbitol, mannitol) (Serrano-Díaz, Sánchez, Martínez-Tomé, Winterhalter, & Alonso, 2013), proteins, lipids, dietary fiber, minerals (P, Mg, Ca, Fe, K, Na) (Serrano-Díaz et al.,2013), volatiles (butyrolactone and 2,3-butanediol) (Argento, Branca, Siracusa, Strano, Napoli, & Ruberto, 2010; Zheng, Li, Ma, Han, & Qin, 2011), carotenoid derivates (crocetins, crocins, and lutein diesters) (Montoro, Maldini, Luciani, Tuberoso, Congiu, & Pizza, 2012; Goupy, Vian, Chemat, & Caris-Veyrat, 2013; Vignolini, Heimler, Pinelli, Ieri, Sciullo, & Romani, 2008), monoterpenoids (picrocrocin and crocusatins) (Li, Lee, & Wu, 2004), and phenolic compounds (benzoic acids, hydroxycinnamic acids (Li et al., 2004), anthocyanins, and flavonoids (Serrano-Díaz, Sánchez, Martínez-Tomé, Winterhalter, & Alonso, 2014a)) have been detected. Among the anthocyanins, delphinidin 3,5-di-*O*-glucoside is the most concentrated one, followed by petunidin 3,7-di-*O*-glucoside, petunidin 3-*O*-glucoside and malvidin 3-*O*-glucoside (Goupy et al., 2013; Serrano-Díaz et al., 2014a; Serrano-Díaz, Estevan, Sogorb, Carmona, Alonso, & Vilanova, 2014b). Flavonoids are mainly represented by kaempferol, quercetin and isorhamnetin glycosides with kaempferol 3-*O*-sophoroside being the most abundant (Goupy et al., 2013; Montoro, Tuberoso, Maldini, Cabras, & Pizza, 2008; Serrano-Díaz et al., 2014a; Serrano-Díaz et al., 2014b;

Termentzi, & Kokkalou, 2008; Vignolini et al., 2008). Saffron flower extracts have been studied for their biological activities, showing antityrosinase activity (Li et al., 2004; Sariri, Sabbaghzadeh, & Poumohamad, 2011), antioxidant (Sánchez-Vioque, Rodríguez-Conde, Reina-Ureña, Escolano-Tercero, Herraiz-Peñalver, & Santana-Méridas, 2012; Sariri et al., 2011; Serrano-Díaz, Sánchez, Maggi, Martínez-Tomé, García-Diz, Murcia, & Alonso, 2012; Termentzi et al., 2008), and antiradical activities (Montoro et al., 2012; Sánchez-Vioque et al., 2012; Serrano-Díaz et al., 2012; Zheng et al., 2011), metal chelating properties (Sánchez-Vioque et al., 2012), cytotoxic effect (Serrano-Díaz et al., 2014b; Zheng et al., 2011), antifungal activity (Zheng et al., 2011), and have been shown to have some benefits in the treatment of mild-to-moderate depression (Moshiri, Akhondzadeh, Noorbala, Jamshidi, Abbasi, & Akhondzadeh, 2006). These extracts are usually obtained with hydro-alcoholic solution for hydrophilic compounds or with *n*-hexane for lipophilic compounds, and often several purification steps are required. Plant material can be dried (Montoro et al., 2012; Moshiri et al., 2006; Sánchez-Vioque et al., 2012; Zheng et al., 2011), vacuum freeze-dried (Goupy et al., 2013; Serrano-Díaz et al., 2012; Serrano-Díaz et al., 2013; Serrano-Díaz et al., 2014a), grounded or powdered (Goupy et al., 2013; Moshiri et al., 2006; Sánchez-Vioque et al., 2012; Serrano-Díaz et al., 2014b), pre-extracted (Argento et al., 2010; Goupy et al., 2013; Termentzi at al., 2008; Zheng et al., 2011), filtered and/or centrifuged in order to obtain purified fractions. However, most of these methods are not very practical for large-scale extraction of these valuable compounds from saffron floral waste. Therefore, currently there is a lot of interest in developing low-cost environmentally friendly production methods. Green extraction techniques have become increasingly appealing for the industries (Chemat, Vian, & Cravotto, 2012), and cold-press seems to currently be one of the most desirable technique (Cravotto, Binello, & Orio, 2011; Heng, Tan, Yong, & Ong, 2013), thanks to the lack of use of auxiliary substances, such as solvents.

The aim of this work was to evaluate the antioxidant activity of juices obtained from cold-pressing of saffron (*Crocus sativus* L.) floral by-products with different *in-vitro* systems, and to investigate the polar fraction of these extracts using high resolution mass spectrometry (LC-ESI-$MS^n$). Additionally, the cytotoxic activity of these extracts was assessed using the MTT assay, and also the content of total polyphenols (Folin-Ciocalteu's assay) and CIE $L^*a^*b^*C^*_{ab}h°_{ab}$ chromaticity coordinates of the juices were determined. To study the potential decline of the quality of the floral by-products, juices obtained 24 and 48 hours after saffron flower harvesting were analyzed.

## 2. Materials and methods

### *2.1. Reagents*

All the solvents and chemicals used in this study were of analytical grade. Methanol, acetonitrile, and 85% phosphoric acid were purchased from Merck (Darmstadt, Germany). Acetonitrile and formic acid, both LC/MS grade, were obtained from Merck (Darmstadt, Germany). Ultrapure water (18 MΩ·cm) was obtained with a Milli-Q Advantage A10 System apparatus (Millipore, Milan, Italy). Fatty acids, triolein, trilinolein, cholesterol, 5-cholesten-3*β*-ol-7-one (7-keto), 5-cholestene-3*β*,7*β*-diol (7*β*-OH), desferal (deferoxamine mesylate salt), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the mixture of phospholipids (bovine brain extract, Type VII, purity > 99%), 1,1,3,3,-tetraethoxypropane (TEP), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), phenylalanine, tyrosine, gallic acid, ferrous sulphate, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), Folin-Ciocalteu's reactive, sodium carbonate, ferric chloride and $CuSO_4•5H_2O$ were purchased from Sigma-Aldrich, Fluka

(Milan, Italy). Standards of anthocyanins and flavonoids were purchased from Extrasynthese (Genay, France). Cell culture materials were purchased from Invitrogen (Milan, Italy).

### *2.2. Saffron samples and extraction method*

Saffron floral by-products (n = 3) were obtained from saffron spice producers in Sardinia (Italy) in November 2014. Stigma separation was performed using the traditional procedures required for the Protected Designation of Origin "Zafferano di Sardegna" (Gazzetta Ufficiale Serie Generale n. 47, 2009). The floral by-products were split in two batches for extraction following 24 and 48 h from harvesting. The juice extraction procedure was done squeezing the floral by-products using a Polsinelli Enologia mod. 30 stain-less steel manual press (Isola del Liri, FR, Italy). For the analytical assays, the juices were centrifuged at 6000 rpm, filtered through cellulose acetate GD/X septa (0.45 $\mu$m, 25 mm Ø, Whatman, Milan, Italy) and stored at -20 °C in dark glass bottles until analysis, which was performed within 1 month from sample collection.

### *2.3. Water content of saffron floral by-products and dry matter of juices*

The water content of the fresh flowers was evaluated drying 5 g of floral by-products at 105 ± 1 °C for 90 min. The floral juices' dry matter was assessed by drying 1000 $\mu$L of solution for 2 h in a thermostatic oven at 105 ± 1 °C, and weighing until a constant weight value was achieved.

### *2.4. CIE $L^*a^*b^*C^*_{ab}h°_{ab}$ coordinates and pH measurement*

The chromaticity coordinates were measured with a UV–visible spectrophotometer Varian series Cary 50 Scan (Varian, Leini, TO, Italy), and data were processed analyzed using Cary Win UV Color Application V. 2.00 software. The transmittance of the whole visible spectrum (380-780 nm) was measured at a wavelength interval of 5 nm, using D65 illuminat and a 10° observation angle. Floral juices were used without any dilution, and transmittance was measured in a 5 mm

quartz cuvette subtracting a blank (air). The pH measurements were performed with a CyberScan pH 510 Meter (Eutech Instruments, Landsmeer, The Netherlands) which was calibrated with standard buffer solutions of pH 7 and pH 4, prior to measuring the pH.

*2.5. Determination of total phenolic content (Folin-Ciocalteu's assay***)**

The total phenols (TP) content was determined by spectrophotometric assay with a modified Folin-Ciocalteu's method (Tuberoso, Boban, Bifulco, Budimir, & Pirisi, 2013). One hundred $\mu$L of floral juice diluted with $H_2O$ 1:50 (v/v) were added to 500 $\mu$L of Folin-Ciocalteu's reactive. After 5 min, 3 mL of 10% $Na_2CO_3$ (w/v) were added, the mixture was shaken, and diluted with $H_2O$ to a final volume of 10 mL. After a 90 min incubation period at room temperature, the absorbance was read at 725 nm on a 10 mm optical polystyrene cuvette (Kartell® 01937) using a Varian Cary 50 spectrophotometer (Varian, Leinì, TO, Italy), subtracting a blank. The TP content results, expressed as mg/kg of gallic acid equivalent (GAE), were obtained using a calibration curve of a freshly prepared gallic acid standard solution (10–200 mg/L, r = 0.9999).

*2.6. Total antioxidant activity (FRAP test)*

The FRAP assay was performed preparing a ferric complex of 2,4,6-tris(pyridin-2-yl)-1,3,5-triazine (TPTZ) and $Fe^{3+}$ (0.3123 g TPTZ, 0.5406 g $FeCl_3 \cdot 6H_2O$ in 100 mL acetate buffer pH 3.6) (Tuberoso et al., 2013). Twenty $\mu$L of floral juice (1:50, v/v, with water) were dissolved in 2 mL of ferric complex and, after an incubation period of 4 min in the dark, absorbance at 593 nm was measured with a Cary 50 Scan spectrophotometer using a 10 mm optical polystyrene cuvette. Quantitative analysis was performed according to the external standard method ($FeSO_4$, 0.1-2 mmol/L), correlating the absorbance with the concentration and results were expressed as mmol/L of $Fe^{2+}$. FRAP assay for each sample was performed in triplicate.

*2.7. Free radical scavenging activity (DPPH Assay)*

A spectrophotometric analysis using DPPH, and a comparison with the Trolox calibration curve was performed (Tuberoso et al., 2013). Fifty microliters of diluted floral juice (same dilution as for FRAP assay) were dissolved in 2 mL of 0.04 mmol/L DPPH in methanol. A calibration curve in the range 0.05–1.0 mmol/L (r = 0.9997) was used for the Trolox, and data were expressed as Trolox equivalent antioxidant capacity (TEAC, mmol/L). Spectrophotometric readings were carried out with a Cary 50 Scan spectrophotometer at 517 nm, using a 10 mm optical polystyrene cuvette. DPPH assay for each sample was performed in triplicate.

*2.8. Cholesterol assay*

The cholesterol oxidation assay was conducted in dry state as previously described (Kim, & Nawar, 1993; Rosa, Melis, Deiana, Atzeri, Appendino, Corona, et al., 2008). Aliquots of 0.5 mL (2586 nmol) of cholesterol solution (2 mg/mL of MeOH) were dried in a round-bottom test tube under vacuum, and then incubated in a bath at 140 °C for 1 h (oxidized controls, 0) under artificial light exposure. Controls (non-oxidized cholesterol, Ctrl) were kept at 0 °C in the dark. In a different set of experiments, aliquots (0.1–30 µL) of the saffron flower juices were added to 0.5 mL of cholesterol solution (2 mg/mL MeOH), the mixtures cholesterol/juice were dried under vacuum, and then incubated in dry state in a bath at 140 °C for 1 h. The oxidation process was stopped by immersing the test tubes in an ice bath for 10 minutes and adding 1 mL of MeOH. Quantification of cholesterol, 7-ketocholesterol (7-keto), and 7$\beta$-hydroxycholesterol (7$\beta$-OH) was carried out with an Agilent Technologies 1100 liquid chromatograph equipped with a diode array detector (HPLC-DAD). Cholesterol and oxysterols were analyzed using a Chrompack Inertsil 5 ODS-3 (150 mm × 3 mm, 5 $\mu$m particle size) column, with a mobile phase in the isocratic mode of 100% MeOH at a flow rate of 0.4 mL/min. Cholesterol and 7$\beta$-OH were detected at 203 nm and 7-keto at 245 nm (Rosa, Tuberoso, Atzeri, Bifulco, Melis, & Dessì, 2011). The data were collected and analyzed using the Agilent Chemstation A.10.02. software.

182 *2.9. Liposomes oxidation assay*

183 Liposomes were prepared according to the Bangham method with slight modifications
184 (Bangham, Standish, & Watkins, 1965; Rosa et al., 2011). Phospholipids were dissolved in
185 chloroform (1 mg/mL) in a round-bottom flask. After chloroform evaporation under vacuum, the
186 thin lipid film was hydrated for 20 min with iced 0.9% NaCl solution. The resulting suspension (1
187 mg lipids/mL) was sonicated in ice for 15 min in a Bandelin Sonorex Super RK 510H sonicator
188 water bath (Bandelin Electronic, Berlin, Germany). Aliquots (300 $\mu$g) of liposomes in 1 mL of
189 saline solution were incubated for 24 h in presence of 5 μM $CuSO_4$ at 37 °C in a thermostatic water
190 bath (Falc Instruments, Treviglio, Italy), exposed to air and artificial light. Controls were kept at
191 0 °C in the dark. Different amounts of saffron flower juices (1, 5, 10 $\mu$L) were added to 300 $\mu$g
192 of liposomes in 1 mL of saline solution and the mixtures were incubated for 24 h at 37 °C. The
193 oxidation was stopped by cooling samples in a mixture of ice and water for 10 minutes. Aliquots
194 (100 $\mu$L) of liposome samples were used for malondialdehyde (MDA) quantification. Liposome
195 fatty acids were obtained by mild saponification as previously described (Rosa et al., 2011) and
196 analyzed with an Agilent Technologies 1100 HPLC-DAD system. Quantification of unsaturated
197 fatty acids (detected at 200 nm) were carried out using a XDB-C18 Eclipse (150 mm × 4.6 mm,
198 3.5 $\mu$m particle size) column equipped with a Zorbax XDB-C18 Eclipse (12.5 mm × 4.6 mm, 5
199 $\mu$m particle size) guard column (Agilent Technologies), with a mobile phase of
200 acetonitrile/$H_2O$/$CH_3COOH$ (75/25/0.12, v/v/v) at a flow rate of 2.3 mL/min (Rosa et al., 2011).
201 The temperature of the column was kept at 37 °C.

202 MDA levels in the liposome samples were measured with the method described by Templar
203 et al. with few modifications (Templar, Kon, Milligan, Newman, & Raftery, 1999; Rosa et al.,
204 2011). Briefly, 100 $\mu$L of 10% TCA and 200 $\mu$L of 0.6% TBA were added to the liposome

dispersions (100 $\mu$L). The mixtures were incubated in a water bath at 90 °C for 45 min, cooled and centrifuged at 12000 g for 10 min. MDA quantification was conducted by HPLC analysis. A standard curve was prepared using a TEP solution (0.05-10 $\mu$M). The MDA-TBA adduct (revealed at 532 nm) was eluted using a reverse phase column Varian Inertsil ODS-2, 150 × 4.6 mm; the mobile phase was a mixture of 50 mM $KH_2PO_4$ pH 7/MeOH (65/35 v/v) at a flow rate of 1 mL/min.

*2.10. Cell cultures*

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC) (Salisbury, Wiltshire U.K.). Caco-2 cells have been obtained from a human colon adenocarcinoma (Travelin, Gråsjö, Taipalensuu, Ocklind, & Artursson, 2002). Subcultures of the Caco-2 cells were grown in T-75 culture flasks and passaged with a trypsin-EDTA solution. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and penicillin (100 units/mL)–streptomycin (100 $\mu$g/mL), at 37 °C in 5% $CO_2$.

*2.11. Cytotoxic activity of saffron juices in cancer Caco-2 Cells: MTT assay*

The cytotoxic effect of saffron extracts was evaluated in cancer Caco-2 cells by the MTT assay (Rosa, Scano, Atzeri, Deiana, & Falchi, 2013; Schiller, Klainz, Mynett, & Gescher, 1992). Caco-2 cells were seeded in 96-well plates at a density of $5 \times 10^4$ cells/mL in 100 $\mu$L of medium and cultured overnight. Prior to the addition of saffron flower juices, the cell culture medium was removed, Caco-2 cells were washed with PBS containing $Ca^{2+}$ and $Mg^{2+}$, and then fresh medium was added. Cells were subsequently exposed to various aliquots of the floral juices (0.1-50 $\mu$L/mL) in complete culture medium and incubated for 24 h. The cell culture medium was then removed from each well of the 96-well plates; an 8 $\mu$L portion of MTT solution (5 mg/mL of $H_2O$) was added to cells in fresh medium and left for 4 h at 37 °C. The medium was aspirated, 100 $\mu$L of DMSO was added to the wells, and color development was measured at 570 nm with an Infinite

200 auto microplate reader (Infinite 200, Tecan, Austria). The absorbance was proportional to the number of viable cells.

*2.12. LC-DAD analysis*

Detection and quantitative analyses of polar compounds were carried out using an LC-DAD method. An HPLC Varian system ProStar was employed, fitted with a pump module 230, an autosampler module 410, and a ThermoSeparation diode array detector SpectroSystem UV 6000lp (ThermoSeparation, San Jose, CA). Separation was obtained with a Gemini C18 column (150 × 4.60 mm, 3 $\mu$m, Phenomenex, Casalecchio di Reno, BO, Italy) using 0.2 M phosphoric acid (solvent A), and acetonitrile (solvent B) at a constant flow rate of 1.0 mL/min. The gradient (v/v) was generated decreasing from 100% of solvent A to 85% in 20 min; to 65% in 40 min; to 10% in 50 min. Before each injection, the system was stabilized for 10 min with the initial A/B ratio (100:0, v/v). The injection volume was 10 $\mu$L and the column temperature was set at 30 °C. Chromatograms and spectra were elaborated with a ChromQuest V. 2.51 data system (ThermoQuest, Rodano, Milan, Italy) and data were acquired at 520 nm for anthocyanins, 360 nm for flavonols and 210 for amino acids. Juices were diluted with ultrapure water (1:50, v/v), filtered through Econofilter RC membrane (0.45 $\mu$m, Ø 25mm, Agilent Technologies, Milan, Italy) and then injected in HPLC without any further purification.

The established method was validated in agreement with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidance note which describes validation of analytical methods (ICH Topic Q2 (R1), 2005) by determining linearity, limits of detection (LOD), limits of quantification (LOQ), precision and accuracy. Stock standard solutions were prepared in methanol for phenolic compounds (kaempferol, kaempferol 3-*O*-glucoside, isorhamnetin, isorhamnetin 3-*O*-rutinoside, quercetin,

quercetin 3-*O*-glucoside, delphinidin 3,5-di-*O*-glucoside, petunidin 3,5-di-*O*-glucoside, delphinidin 3-*O*-glucoside, and petunidin 3-*O*-glucoside), and in methanol: 0.1 M HCl 1:1 (v/v) for amino acids. The working standard solutions were prepared in ultrapure water. The linearity was evaluated by preparing standard mixtures at six different concentrations and analyzing them by HPLC-DAD. The calibration curves for commercial standards were plotted with the method of external standard, correlating the peak area with the concentration by means of the least-squares method, with coefficient of determination ($r^2$) > 0.997 for all compound. The LODs and LOQs were calculated according to the equation LOD = 3.3r/S and LOQ = 10r/S, respectively (where r = standard deviation of the blank, and S = slope of the calibration curve). LODs ranged from a minimum of 0.4 mg/L (kaempferol, isorhamnetin, and isorhamnetin 3-*O*-rutinoside) to a maximum of 1. 9 mg/L (tyrosine), and the limits of quantification (LOQ) were below 6 mg/L for all compounds (Table 2). The precision of this method was evaluated testing intra- and inter-day repeatability. Six injections of the same standard containing all the phenolic compounds within one day and over three consecutive days, were performed. The relative standard deviation (RSD) for the area under the peak was determined as a measure of precision, and all RSDs were lower than 5 %. The accuracy of the method was evaluated using recovery rates. Saffron floral juices (24h) were spiked with two concentrations of kaempferol 3-*O*-glucoside (50 and 200 mg/L), delphinidin 3-*O*-glucoside (20 and 100 mg/L) and phenylalanine (20 and 100 mg/L), and each spiked sample was analyzed in triplicate. Recovery rates were between 95.3 and 103.2 % (data not shown). The matrix effect was evaluated comparing the response of a standards mix prepared with kaempferol 3-*O*-glucoside (200 mg/L), delphinidin 3-*O*-glucoside (200 mg/L) and phenylalanine (200 mg/L), both in 24h saffron floral by-products juice and in water. No statistical differences were observed ($p < 0.05$). The specificity, intended as the lack of interference with other substances detected in the region of interest, was assessed by the ChromQuest purity calculation software

index (total peak purity ≥ 0.99), and resulted to be specific with no any other peak interfering at the retention times of the dosed compounds in the HPLC-DAD detection mode.

*2.13. LC/ESI/FT (Orbitrap)MS and LC/ESI/FT (Orbitrap)MS/MS analysis*

The confirmation of the identity of the molecules detected and quantified by HPLC-DAD was performed by LC–MS/MS according to Mari, Montoro, D'Urso, Macchia, Pizza and Piacente (2015) with slight modifications. The electrospray ionization (ESI) source of a Thermo Scientific LTQ-Orbitrap XL (Thermo Scientific, Germany) mass spectrometer was tuned to positive ion mode with a standard solution of kaempferol 3-*O*-glucoside (l $\mu$g/mL) infused at a flow rate of 5 $\mu$L/min with a syringe pump. Negative ionization mode for the MS analysis was used with data-dependent automatic switching between MS and MS/MS and $MS^n$ acquisition modes. The instrument was calibrated using the manufacturer's calibration standards. The scan was collected in the Orbitrap at a resolution of 30000 in a *m*/*z* range of 50–1500 amu. The source voltage was 5 kV and capillary voltage -12 kV was, the tube lens offset -121.47 V and the capillary temperature was set at 280 °C, auxiliary gas was set at 5 (arbitrary units) and no sheath gas was used. Tuning optimization was performed on the specific value of kaempferol 3-*O*-glucoside at *m/z* 447.0369 amu. In positive ion mode tuning experiment were performed by infusing a solution of delphinidin 3-*O*-glucoside (l $\mu$g/mL) infused at a flow rate of 5 $\mu$L/min with a syringe pump. Positive ionization mode for the MS analysis was used with data-dependent automatic switching between MS and MS/MS and $MS^n$ acquisition modes. The instrument was calibrated using the manufacturer's calibration standards. The scan was collected in the Orbitrap at a resolution of 30000 in a *m*/*z* range of 50–1500 amu. The source voltage was 3 kV and capillary voltage 49 kV was, the tube lens offset 120 V and the capillary temperature was set at 280 °C, auxiliary gas was set at 5 (arbitrary units) and no sheath gas was used. The tuning optimization was performed on the specific value of delphinidin at *m/z* 303.0504 amu.

Qualitative LC/ESI/MS in negative ion mode was performed using a Thermo Scientific Accela HPLC system (Thermo Scientific, Germany) equipped with a Waters (Milford, MA, USA) X-Select RP C18 column (150 mm × 2.1 mm, 3.5 $\mu$m) and coupled to a LTQ-Orbitrap XL mass spectrometer. Linear gradient elution with a mobile phase comprising water acidified with 0.1% formic acid (solvent A) and acetonitrile acidified with 0.1% formic acid (solvent B) starting from 100% A, was converted in 85% A in 20 min, from 85% to 65% (A) in following 20 min and from 65% to 10% (A) in 10 min followed by 10 min of maintenance. The mobile phase was supplied at a flow rate of 200 $\mu$L/min keeping the column at room temperature, and the effluent was injected directly into the ESI source. The mass spectrometer was operated in the negative ion mode. The auxiliary gas was set at 5 (arbitrary units) and no sheath gas was used. In LC/ESI/MS experiments Total Ion Current (TIC) profile was produced by monitoring the intensity of all the ions produced and acquired in every scan during the chromatographic run.

Qualitative LC/MS in positive ion mode was performed using a Thermo Scientific Accela HPLC system (Thermo Scientific, Germany) equipped with a Waters (Milford, MA, USA) X-Select RP C18 column (150 mm × 2.1 mm, 3.5 $\mu$m) and coupled to a LTQ-Orbitrap XL mass spectrometer. The auxiliary gas that was set at 20 (arbitrary units) and the sheath gas at 10 (arbitrary units). The gradient programme was the same described above.

In LC/ESI/MS experiments Total Ion Current (TIC) profile was produced by monitoring the intensity of all the ions produced and acquired in every scan during the chromatographic run. Additional LC/ESI/(Orbitrap)MS/MS experiments were carried out in order to select and submit these ions to fragmentation experiments using the parameters previously chosen by ESI/MS direct infusion experiments, and a collision energy of 30%. Compounds were identified by matching experimental MS/MS spectra with those reported in a public repository of mass spectral data called Mass Bank (Chang, & Wong, 2004).

### *2.14. Statistical analyses*

Graph Pad INSTAT software (GraphPad software, San Diego, CA, USA) was used to calculate the means and standard deviations (SD) of two or three independent experiments involving duplicate or triplicate analyses for each sample/condition. The evaluation of statistical significance of observed differences was performed by one-way analysis of variance (One-way ANOVA), using Bonferroni Multiple Comparisons Test.

## 3. Results and discussion

### *3.1. Technological properties of saffron floral by-products and juices*

The present paper represents the first study of saffron juices since previous reports were focused on extracts obtained with different solvents (Argento et al., 2010; Cravotto et al., 2011; Goupy et al., 2013; Li et al., 2004; Montoro et al., 2012; Sariri et al., 2011; Serrano-Díaz et al., 2013; Serrano-Díaz et al., 2014b; Vignolini et al., 2008; Zheng et al., 2011). Significant differences between floral by-products pressed after 24 and 48 hours were noticed (Table 1). Particularly, the juice yield increase after 48 hours (16.8 ± 1.3 mL/100 g). This could be due at least partially to the loss of water (ca. 6 %, w/w) which was observed after 48h and to the possible breakdown of the vegetable structures, which can facilitate the juice extraction process. The use of industrial presses may allow to squeeze bio-waste more efficiently, which may lead to obtain similar amounts of juice from the 24h and 48h floral by-products. Values of the physical-chemical parameters are quite similar, although they are significantly different at $p < 0.05$ (Table 1). The dry matter obtained from the juices was 6.1 ± 0.0 and 6.3 ± 0.0 %, respectively for the 24h and 48h juices. The chromatic coordinates showed similar values of luminosity (L*) (72.9 ± 0.3 and 73.7 ± 0.2, respectively for 24h and 48h juices), but the color turn to reddish after 48 h ($C^*_{ab}$ = 79.9 ± 0.3). It

346 is possible that the color change was caused by anthocyanins release from intra-cellular vacuoles
347 and/or by a decrease of the pH (pH = 5.73 ± 0.01 at 24h, and pH = 5.00 ± 0.01 at 48h). Similar
348 phenomenon was indicated as being responsible for color changing in saffron flowers subjected to
349 different dehydration treatments (Serrano-Díaz, Sánchez, Alvarruiz, & Alonso, 2013b).

350 *3.2. In-vitro antioxidant properties of the juices obtained from saffron floral by-products*

351 The *in-vitro* antioxidant assays that were used indicated high values for total phenols (TP),
352 antioxidant (FRAP) and antiradical (DPPH) properties in the two juices (Table 1). Juices at 24h
353 had TP, FRAP, DPPH values of 4616.1 ± 133.9 mg GAE/L, 35.5 ± 1.4 mmol $Fe^{2+}$/L, and 7.0 ±
354 0.3 mmol TEAC/L, respectively. Juices at 48h had an increased + 61, + 56, and + 76% for TP,
355 FRAP and DPPH, respectively. These findings are of particular interest since these values are at
356 least 2-3 times higher than the ones reported for red wines or berries extracts (Tuberoso et al.,
357 2013). The juices were also tested for their antioxidant activity during cholesterol oxidation in dry
358 state at 1 h. The consumption of cholesterol and the formation of its mayor oxidation products, the
359 7-keto and 7$\beta$-OH derivatives, were measured as markers of oxidative process. Figure 1 shows the
360 values of cholesterol, 7$\beta$-OH, and 7-keto (expressed as $\mu$g) measured during cholesterol oxidation
361 in the controls (Ctrl) and in oxidized samples in the absence (0) or in the presence of different
362 amounts (0.1-30 $\mu$L) of 24h (Fig. 1A) and 48h-juices (Fig. 1B). The 48h-juice exerted a complete
363 inhibition of cholesterol degradation from 0.25 $\mu$L showing a 50% protection at 0.1 $\mu$L. In this
364 system the 24h-juice, although less effective at low amounts, showed a complete inhibition of the
365 oxidative process from 0.5 $\mu$L, with a 50% protection at 0.25 $\mu$L. The 24h juice could significantly
366 inhibit the formation of both oxysterols from 0.25 μL, while the 48h juice exerted a significant
367 decrease in 7-keto and 7$\beta$-OH formation from 0.5 $\mu$L. The oxidative degradation of cholesterol,
368 which is an important component of biological membranes/lipoproteins, is known to play a role in

the development of tissue damage and in a wide range of other pathological events. Furthermore, oxysterols also have a variety of biological activities of interest to the biomedical research field (e.g. cytotoxicity, angiotoxicity, and mutagenicity) (Garenc, Julien, & Levy, 2010). This model of lipid oxidation has been widely used to assess the antioxidant properties of extracts and pure phenolic compounds (Rosa et al, 2008; Rosa et al, 2011). Both saffron juices protected sterol against free radical attack and inhibited oxysterol formation, showing scavenging ability against peroxyl radicals LOO• (Rosa et al, 2008; Rosa et al, 2011).

To study lipid peroxidation, a model based on liposomes was used. Liposomes are considered an important membrane model and much research has been carried out to understand the effects of lipid oxidation in phospholipid membranes to develop strategies to prevent, modulate and treat oxidative damage (Mosca, Ceglie, & Ambrosone, 2011). Therefore, the protective effect of saffron juices for the lipid target of liposome oxidative injury was evaluated. Liposomes were treated with 5 $\mu$M $Cu^{2+}$ at 37 °C for 24 h and the changes in unsaturated fatty acids concentration, together with the increase of the MDA secondary oxidative products, were analyzed as an index of the lipid peroxidation process (Rosa et al., 2011). Control liposome (0 °C) values were: 18:1 n-9, 95.97 ± 3.76 $\mu$g; 20:4, 17.40 ± 0.85 $\mu$g; 22:4, 18.20 ± 0.90 $\mu$g; 22:6, 25.10±1.43 $\mu$g/mg liposomes. Figure 2 shows the total values of the main unsaturated fatty acids 20:4 n-6, 22:4 n-6 and 22:6 n-3 (PUFA) (expressed as μg/mg liposomes) (Fig. 2A) and MDA (% control) (Fig. 2B), measured in the control (Ctrl) and during liposome oxidation at 37 °C for 24 h with 5 $\mu$M $CuSO_4$ in the absence (0) and in the presence of different concentrations (1, 5, 10 μL) of 24h and 48h juices. A strong significant decrease of the PUFA level was observed at 24 h oxidation. The 48h juice significantly protected against $Cu^{2+}$-mediated unsaturated fatty acid degradation starting at a volume of 5 $\mu$L, significantly reducing MDA production in the liposome system at 10 $\mu$L. In this test, the 24h juice was not very effective at protecting PUFA against oxidative degradation, and a

significant reduction in MDA generation was only observed when a 10 $\mu$L volume was used. Conceivably, in this system the 24h-juice protected the liposome particles from copper-induced oxidative damage by scavenging peroxyl radicals (acting as chain breaking antioxidant) and chelating copper ions at the aqueous phase or at the liposome particle surface and/or core (Rosa et al, 2008; Rosa et al, 2011).

The saffron juices were also tested and compared for cytotoxicity (MTT assay) in colon cancer Caco-2 cell cultures. Figure 3 shows the viability, expressed as % of the control, of the cancer Caco-2 cells after a 24 h incubation in the presence of different concentrations of extracts (0.1-50 $\mu$L/mL). The treatment with the 48h-juice caused a significant reduction in cell viability (30%), in comparison with control, from the concentration of 10 $\mu$L/mL. A significant 32% decrease in Caco-2 cell viability was also observed with the 24h-juice, but only at a concentration of 50 $\mu$L/mL. Saffron stigma extracts were extensively studied for their chemopreventive potential against cancer. Previous studies have also demonstrated the cytotoxic activity of liposoluble extracts obtained from saffron floral by-products against cancer cell lines (Zheng et al., 2011). Interestingly, aqueous extracts obtained from saffron floral bio-residues did not show cytotoxicity against non-tumorigenic 3T3 fibroblast cells (Serrano-Díaz et al., 2014b).

### *3.3. Chemical composition of the juices obtained from saffron floral by-products*

Recently, several studies have reported the use of LC-MS techniques to study *C. sativus* floral by-products. However, this is is the first description of the use of high resolution mass spectrometry approach to do a similar study. There is evidence for the use of metabolomics approaches based on High Resolution Mass Spectrometry (MS) as a tool for the selection of appropriate markers (Mathon, Duret, Kohler, Edder, Bieri, & Christen, 2013). The correct determination of the elemental composition is the starting point for the identification of biomarkers using untargeted metabolomics. Therefore, high mass accuracy, in combination with high spectral

accuracy is a prerequisite and can only be achieved by using high resolution mass spectrometers, such as Orbitrap, ToF and QToF technologies. In plant metabolomic, MS/MS or $MS^n$ analyzers are considered a useful tool for the identification of secondary metabolites. In particular, LTQ FT (Orbitrap)MS analyzers have $MS^n$ capabilities for enhanced levels of structural analysis. Secondary metabolites belonging to the phenolic class have been frequently reported in *C. sativus* petals. Anthocyanins and flavonoids are the typical metabolites of saffron petals. In LC-MS, when positive ionization was performed, the flavonoids and flavonoids glycosides displayed the pseudo molecular ion $[M+H]^+$. On the other hand, ionization was obtained in negative ion mode too, obtaining a deprotonated pseudomolecular ion $[M-H]^-$ for each of the compounds of interest. Thus, the MS spectra in ESI positive and negative mode were both useful for the determination of the molecular weight. The aim of identifying the flavonoids, anthocyanins and flavonoids glycosides contained in *C. sativus* extracts was to compare the metabolic profiles of different waste products obtained during production of the very expensive saffron spice. This study also aimed at describing a system which possibly confirms the identity of these compounds without the use of external standards, based on high resolution mass spectrometry data. The identification of the flavonoids was in fact based on mass spectral data in combination with published data. Crude extracts obtained from flowers were analyzed by an analytical method developed in LC/ESI/(Orbitrap)MS and LC/ESI/(Orbitrap)MS/MS, in negative ion mode and positive ion mode. The positive LC-MS profile indicated the presence of anthocyanins, and flavonoids. The negative LC-MS profile indicated the presence of a larger group of compounds (Figure 4) corresponding to the deprotonated molecular ions of different flavonoids and anthocyanins. The individual components were identified by comparison of their m/z values in the Total Ion Courrent (TIC) profile with those of the selected compounds described in the literature (Table 2). Additional LC/ESI/(Orbitrap)MS/MS experiments were carried out in order to select and submit these ions to fragmentation experiments using the parameters previously chosen by ESI/MS direct infusion

experiments. The compounds were identified by matching experimental MS/MS spectra with those reported in a public repository of mass spectral data (Mass Bank).

The major compounds identified and quantified by HPLC-DAD and LC-ESI-$MS^n$ were phenolic compounds such as flavonols and anthocyanins. The total flavonoid levels in the 24h- and 48h-juices were 4259.8 ± 105.0 and 3813.0 ± 82.1 mg/L, respectively. Among the flavonoids, kaempferol 3-*O*-sophoroside was the most abundant (2.790.7 ± 52.4 and 2.854.5 ± 36.0 mg/L in 24h and 48h juice, respectively) followed by other kaempferol, quercetin and isorhamnetin glycosides. Kaempferol derivatives accounted for more than 80 % of total flavonoids in both 24h- and 48h juices. It is interesting to observe that the kaempferol 3,7-di-*O*-glucoside amount decreased from 368.1 ± 4.1 to 21.9 ± 0.5 mg/L while kaempferol 7-*O*-glucoside increased from 96.3 ± 13.5 to 232.4 ± 39.0 mg/L, in the 24h- and 48h- juices, respectively. Contrary to what saw with the flavonoids, total anthocyanin levels increased from 1075.9 ± 20.2 in the 24h-juices to 1316.7 ± 109.8 mg/L in the 48h-juices. Delphinidin 3,5-di-*O*-glucoside was the most abundant anthocyanin, reaching 1003.6 ± 81.7 mg/L in the 48h juices and accounting for 76% of the total anthocyanins, followed by delphinidin 3-*O*-glucoside, malvidin 3,5-di-*O*-glucoside, petunidin 3-*O*-glucoside and petunidin 3,7-di-*O*-glucoside. Qualitative and quantitative profile of the compounds detected in the juices matches what has been previously reported (Goupy et al., 2013; Serrano-Díaz et al., 2014a; Serrano-Díaz et al., 2014b). The high levels of anthocyanins in saffron juices suggest that, similar to what was proposed for saffron flower ethanolic extracts (Kakhki, 2010), they could also be used as beverage supplements. Some of the compounds found in saffron juices are well-known for their biological activities. In particular, delphinidin 3,5-di-*O*-glucoside was proven to have a dose dependent activity on the AhR–CYP1A1 signaling pathway, which is involved in drug metabolism and carcinogenesis (Kamenickova, Anzenbacherova, Pavek, Soshilov, Denison, Zapletalova, et al., 2013). Moreover, delphinidin 3,5-*O*-diglucoside has been

466 shown to play a role in preventing dry eye by managing tear secretion activity in the lacrimal gland
467 (Nakamura, Tanaka, Imada, Shimoda, & Tsubota, 2014). Kaempferol 3-*O*-sophoroside has
468 antiradical (Delazar, Lasheni, Fathi-Azad, Nahar, Rahman, Asnaashari, et al., 2010) and anti-
469 inflammatory activities (Kim, Ku, Lee, & Bae, 2012), and increases collagen production in a dose-
470 dependent manner (Yim, Jang, Moon, Lee, Kim, & Lee, 2015).

471 HPLC-DAD and LC-ESI-$MS^n$ chromatogram showed also the presence of some unknown
472 peaks (Figure 4) and the amino acids tyrosine (Tyr) and phenylalanine (Phe). The differences in
473 the 24h and 48h juices were not statistically significant, with concentrations of 679.8 ± 146.9 and
474 1085.6 ± 18.8 mg/L for Tyr and Phe, respectively, in 48h juices.

475

476 **4. Conclusions**

477 The overall data suggest that juices obtained from *C. sativus* floral by-products are an
478 interesting source of bioactive molecules. Moreover, floral wastes do not need excessive
479 precautions to preserve the integrity of their bioactive compounds and can be processed up to two
480 days after harvesting. The juices showed remarkable antioxidant activity in several *in vitro* systems
481 of oxidative stress. Additionally, they showed a mild effect on cell viability in colon cancer cell.
482 Future studies are needed to further investigate the mechanism underlying some of these bioactive
483 properties and to promote the use of extracts derived from saffron floral by-product.

484

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**Figure captions**

**Figure 1.** Cholesterol and oxysterols 7$\beta$-OH and 7-keto (expressed as μg) measured in controls (Ctrl) and oxidized samples in the absence (0) or in the presence of different amounts (0.1-30 $\mu$L) of 24h and 48h saffron flower juices, during the oxidation of cholesterol at 140 °C for 1 h. Three independent experiments were performed and data are presented as mean ± SD (n = 6); [a] = $p< 0.001$; [b] = $p< 0.01$; [c] = $p< 0.05$ versus Ctrl; [e] = $p< 0.01$ versus 0. All data are highly significant ($p< 0.001$) from the amount of 0.5 $\mu$L.

**Figure 2.** Levels of total polyunsaturated fatty acids (PUFA) ($\mu$g/mg liposomes) (A) and malonildyaldeide (MDA) (% control) (B) in control (Ctrl) samples and during liposome oxidation at 37 °C for 24 h with 5 $\mu$M $CuSO_4$ in the absence (oxidized control, 0) or in the presence of different amounts of 24h and 48h saffron flower juices. Two independent experiments were performed and data are presented as mean ± SD (n = 6); [a] = $p< 0.001$; [b] = $p< 0.01$; [c] = $p< 0.05$ versus Ctrl; [d] = $p< 0.001$; [f] = $p< 0.05$ versus 0; [g] = $p< 0.001$ versus 1 μL; [l] = $p< 0.001$ versus 5 $\mu$L;

**Figure 3.** Viability MTT analysis, expressed as % of the control, following incubation in human cancer of Caco-2 cell culture for 24 h with different concentrations of 24h and 48h saffron flower juices (0.1-50 $\mu$L/mL). Three independent experiments were performed and data are presented as mean ± SD ($n$ = 18); [b] = $p< 0.01$; [c] = $p< 0.05$ versus Ctrl.

**Figure 4.** LC-ESI-FT (Orbitrap) MS profile of 24h saffron flower juices in (+) ESI positive mode and (-) ESI negative mode. Chromatographic conditions are described in the text. List of compounds in Table 2; U = unknowns compounds.

**Table 1**

Humidity of saffron floral by-products. Yield, humidity, CIE chromatic coordinates, total phenols and antioxidant and antiradical properties of the saffron floral by-products juices

| Parameter | Sample[a] | | | |
|---|---|---|---|---|
| | 28h floral by-products | | 48h floral by-products | |
| Humidity (%, w/w) | 88.6a | ± 0.5 | 82.4b | ± 1.3 |
| Dry residue (%, w/w) | 11.4a | ± 0.5 | 17.6b | ± 1.3 |
| | 24h juice | | 48h juice | |
| Yield (mL/100 g floral by-product) | 5.4a | ± 0.3 | 16.8 | ± 1.3 |
| Humidity (%, w/v) | 93.9a | ± 0.0 | 93.7b | ± 0.0 |
| Dry matter (%, w/v) | 6.1a | ± 0.0 | 6.3b | ± 0.0 |
| pH | 5.73a | ± 0.01 | 5.00b | ± 0.01 |
| CIE chromatic coordinates | | | | |
| L* | 72.9a | ± 0.3 | 73.7b | ± 0.2 |
| a* | -5.2a | ± 0.2 | 6.1b | ± 0.2 |
| b* | 22.5a | ± 0.2 | 22.8a | ± 0.3 |
| $C^*_{ab}$ | 23.1a | ± 0.2 | 23.6b | ± 0.2 |
| $h°_{ab}$ | 103.0a | ± 0.3 | 79.9b | ± 0.3 |
| Total phenols[b] (mg GAE/L) | 4616.1a | ± 133.9 | 7421.8b | ± 380.7 |
| FRAP[c] (mmol $Fe^{2+}$/L) | 35.5a | ± 1.4 | 55.4b | ± 4.2 |
| DPPH[d] (mmol TEAC/L) | 7.0a | ± 0.3 | 12.3b | ± 1.9 |

[a] Means ± standard deviation ($n$ = 3) in each row followed by different letters are significantly different at $p < 0.05$. [b] GAE: gallic acid equivalent. [c] FRAP value is expressed as $Fe^{2+}$ millimolar concentration, obtained from a $FeSO_4$ solution having an antioxidant capacity equivalent to that of the dilution of the juice. [d] DPPH value is expressed as TEAC millimolar concentration, obtained from a Trolox solution having an antiradical capacity equivalent to that of the dilution of the juice.

**Table 2**
Phenolic compounds and amino acids[a] in saffron floral by-products juices

| | Compound | tR | LOD | LOQ | Floral juices | | | | | | Typical ions | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | min | mg/L | mg/L | 24h | | 48h | | | | *m/z* | | | |
| | | | | | mg/L | ± SD | mg/L | ± SD | MW | Formula | $[M]^+/[M+H]^+$ | $MS^2$ | $[M-H]^-$ | $MS^2$ |
| | Flavonoids | | | | | | | | | | | | | |
| F1 | Kaempferol 3-*O*-sophoroside-7-*O*-glucoside[b] | 18.41 | 0.5 | 1.5 | 25.0a | 0.8 | 27.7b | 0.8 | 772.2066 | $C_{33}H_{40}O_{21}$ | 773.2140 | 611.1612 | 771.6502 | 609.5096 |
| F2 | Kaempferol 3,7-di-*O*-glucoside[b] | 22.64 | 0.5 | 1.5 | 368.1a | 4.1 | 21.9b | 0.5 | 610.1533 | $C_{27}H_{30}O_{16}$ | 611.1621 | 449.1083 | 609.1459 | 447.3690 |
| F3 | Kaempferol tri-*O*-glucoside[b] Isomer 1 | 24.56 | 0.5 | 1.5 | 22.6a | 3.0 | 3.3b | 0.6 | 772.6581 | $C_{33}H_{40}O_{21}$ | 773.2140 | 611.1622 | 771.6502 | 609.1453<br>285.0397 |
| F4 | Kaempferol tri-*O*-glucoside[b] Isomer 2 | 26.85 | 0.5 | 1.5 | 9.7a | 1.2 | 8.6a | 2.6 | 772.6581 | $C_{33}H_{40}O_{21}$ | 773.2140 | 611.1622 | 771.6502 | 609.1453<br>285.0397 |
| F5 | Isorhamnetin 3,4'-di-*O*-glucoside[c] | 27.38 | 0.4 | 1.1 | 3.1a | 0.2 | 2.9a | 0.2 | 640.1639 | $C_{28}H_{32}O_{17}$ | 641.1717 | 479.1189 | 639.5355 | 477.4525 |
| F6 | Kaempferol tri-*O*-glucoside[b] Isomer 3 | 28.10 | 0.5 | 1.5 | 172.5a | 7.6 | 87.3b | 4.2 | 772.6581 | $C_{33}H_{40}O_{21}$ | 773.2140 | 611.1622 | 771.6502 | 609.1453<br>285.0397 |
| F7 | Quercetin 3-*O*-sophoroside[d] | 28.43 | 0.6 | 1.7 | 194.1a | 5.2 | 207.4a | 8.9 | 626.1483 | $C_{27}H_{30}O_{17}$ | 627.1561 | 465.1030 | 625.1490 | 301.0346 |
| F8 | Quercetin 3-*O*-glucoside sophoroside[d] | 28.88 | 0.6 | 1.7 | 2.6a | 0.1 | 2.5a | 0.1 | 788.2011 | $C_{33}H_{40}O_{22}$ | 789.2089 | 627.1561 | 787.1965 | 625.1856 |
| F9 | Kaempferol 3-*O*-sophoroside[b] | 29.23 | 0.5 | 1.5 | 2.790.7a | 52.4 | 2.854.5a | 36.0 | 610.5175 | $C_{27}H_{30}O_{16}$ | 611.1612 | 449.1083 | 609.5096 | 285.0314 |
| F10 | Kaempferol 3- *O*-glucoside | 29.92 | 0.5 | 1.5 | 2.0a | 0.2 | 3.6b | 0.6 | 448.3769 | $C_{21}H_{20}O_{11}$ | 449.1083 | 287.0585 | 447.0369 | 285.0012 |
| F11 | Isorhamnetin 3-*O*-rutinoside | 30.48 | 0.4 | 1.1 | 268.4a | 4.1 | 128.9a | 0.8 | 624.5440 | $C_{28}H_{32}O_{16}$ | 625.1768 | 479.1189 | 623.5361 | 315.0426 |
| F12 | Quercetin 3- *O*-glucoside | 30.93 | 0.6 | 1.7 | 184.1a | 14.8 | 154.6b | 13.5 | 464.3763 | $C_{21}H_{20}O_{12}$ | 465.1933 | 303.0512 | 463.3684 | 301.0343 |
| F13 | Kaempferol 7- *O*-glucoside[b] | 33.08 | 0.5 | 1.5 | 96.3a | 13.5 | 232.4b | 39.0 | 448.3789 | $C_{21}H_{20}O_{11}$ | 449.1083 | 303.0553 | 447.3690 | 301.0325 |
| F14 | Quercetin | 41.61 | 0.5 | 1.6 | 1.6a | 0.0 | 1.9b | 0.2 | 302.0426 | $C_{15}H_{10}O_7$ | 303.0553 | - | 301.0345 | - |
| F15 | Kaempferol | 45.68 | 0.4 | 1.3 | 50.0a | 4.3 | 58.0a | 5.4 | 286.0477 | $C_{15}H_{10}O_6$ | 287.0445 | - | 285.0397 | - |
| F16 | Isorhamnetin | 50.27 | 0.4 | 1.4 | 1.2a | 0.2 | 17.5b | 0.1 | 316.0583 | $C_{16}H_{12}O_7$ | 317.0681 | - | 315.0374 | - |
| | Anthocyanins | | | | | | | | | | | | | |
| A1 | Delphinidin 3,5-di-*O*-glucoside | 19.89 | 0.7 | 2.2 | 822.7a | 20.4 | 1.003.6b | 81.7 | 627.1561 | $C_{27}H_{31}O_{17}$ | 627.1561 | 465.1045 | - | - |
| A2 | Petunidin 3,5-di-*O*-glucoside | 21.00 | 0.6 | 1.7 | 84.6a | 2.9 | 137.7b | 3.8 | 641.1717 | $C_{28}H_{33}O_{17}$ | 641.0014 | 465.1022 | - | - |
| A3 | Delphinidin 3-*O*-glucoside | 25.38 | 0.7 | 2.0 | 111.3a | 2.3 | 111.7a | 28.3 | 465.1033 | $C_{21}H_{21}O_{12}$ | 465.1033 | 303.0098 | - | |
| A4 | Petunidin 3-*O*-glucoside | 27.51 | 0.5 | 1.67 | 28.7a | 1.0 | 30.3a | 1.6 | 479.1189 | $C_{22}H_{23}O_{12}$ | 479.1258 | 317.2702 | - | - |
| | Amino acids | | | | | | | | | | | | | |
| Tyr | Tyrosine | 2.21 | 1.9 | 5.7 | 618.7a | 89.0 | 679.8a | 146.9 | 181.0738 | $C_9H_{11}NO_3$ | 182.0817 | 165.0543 | 180.1806 | 162.0127 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phe | Phenilalanine | 8.03 | 1.6 | 5.0 | 1.072.9 | 140.9 | 1.085.6 | 18.8 | 165.0789 | $C_9H_{11}NO_2$ | 166.0868 | 120.0805 | - | - |

[a] Means ± SD (n = 3) in each row followed by different letters are significantly different at $p < 0.05$. [b] Dosed with the calibration curve of kaempferol 3-*O*-glucoside. [c] Dosed with the calibration curve of isorhamnetin 3-*O*-rutinoside. [d] Dosed with the calibration curve of quercetin 3-*O*-glucoside. nd = < LOD. tr = < LOQ

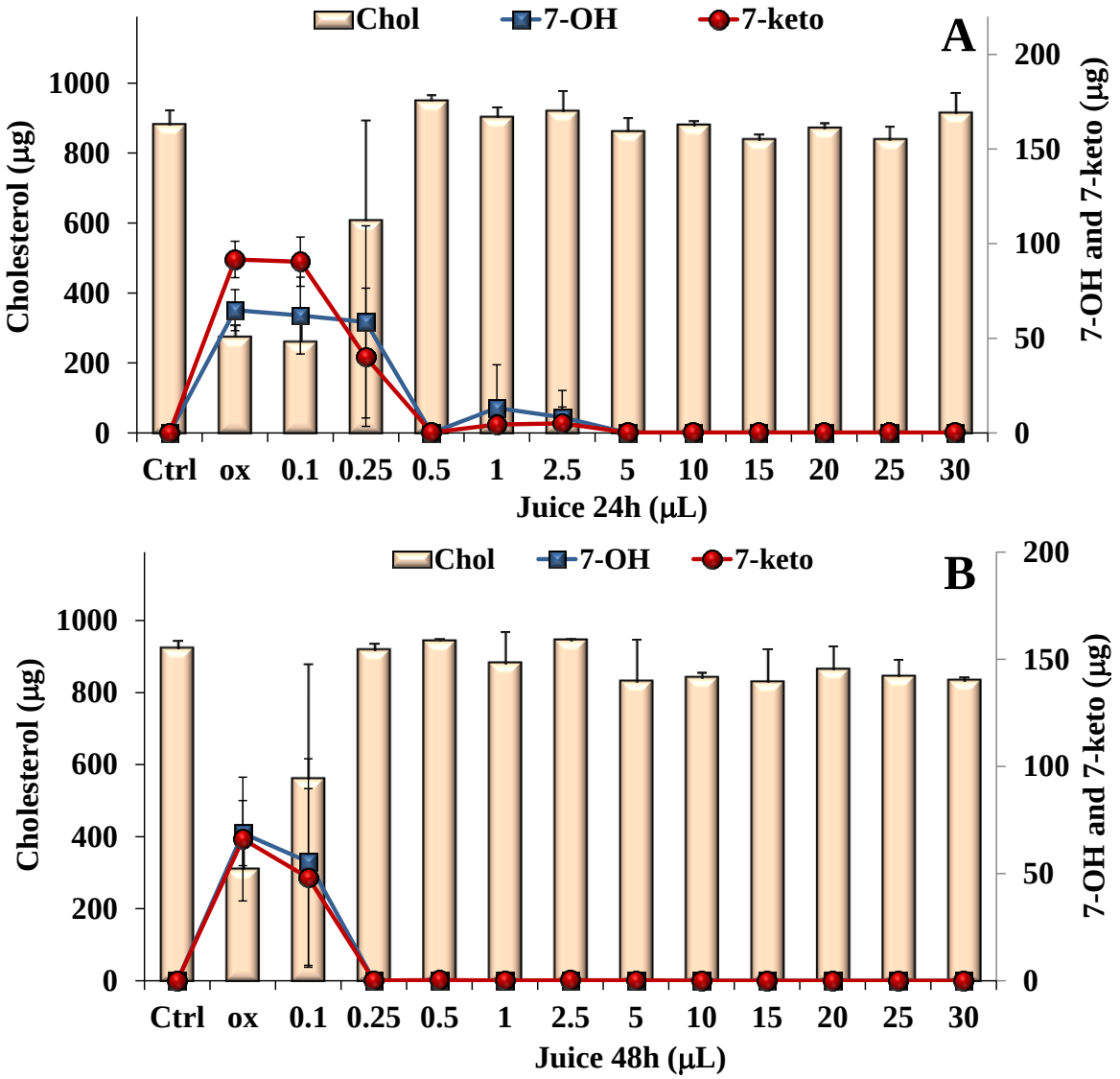
Chol
7-OH
7-keto
A
Cholesterol (µg)
7-OH and 7-keto (µg)
0
200
400
600
800
1000
0
50
100
150
200
Ctrl
ox
0.1
0.25
0.5
1
2.5
5
10
15
20
25
30
Juice 24h (µL)
Chol
7-OH
7-keto
B
Cholesterol (µg)
7-OH and 7-keto (µg)
0
200
400
600
800
1000
0
50
100
150
200
Ctrl
ox
0.1
0.25
0.5
1
2.5
5
10
15
20
25
30
Juice 48h (µL)

**Fig 1.**

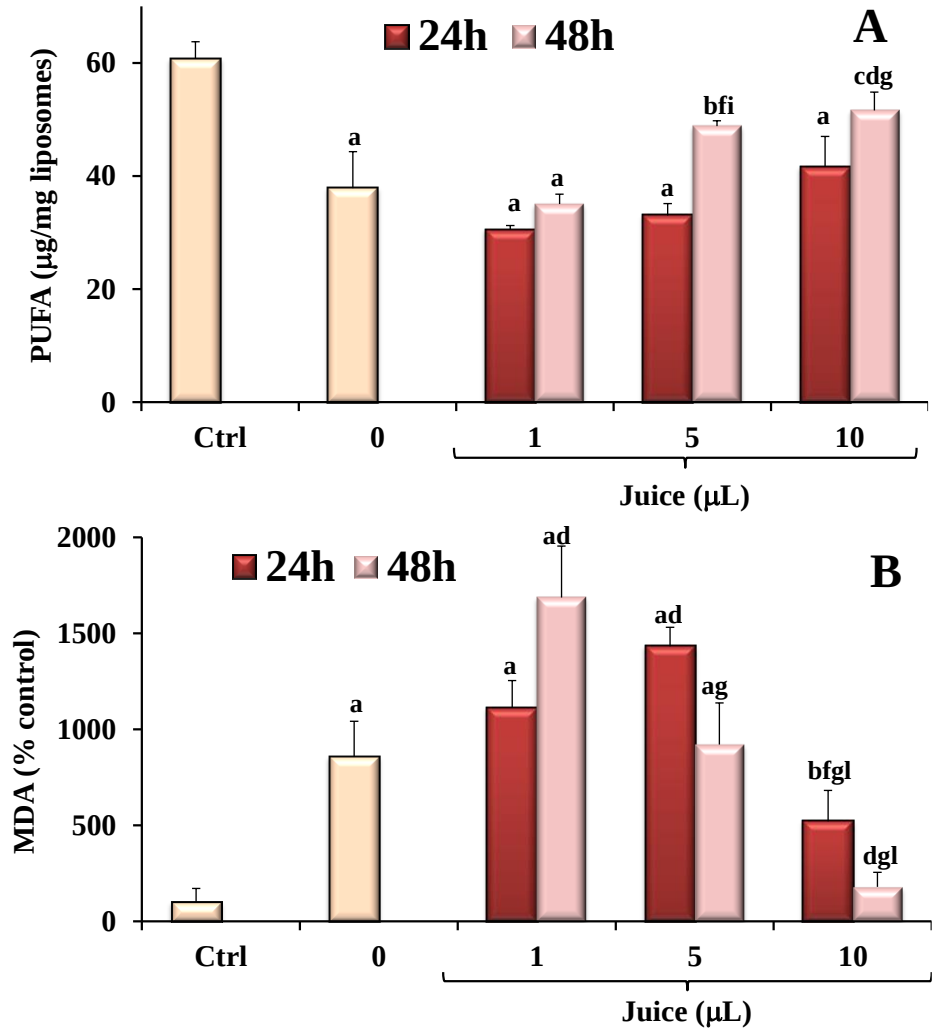
24h
48h
A
PUFA (µg/mg liposomes)
60
40
20
0
a
a
a
bfi
a
cdg
Ctrl
0
1
5
10
Juice (µL)
2000
1500
1000
500
0
MDA (% control)
24h
48h
B
a
a
ad
ad
ag
bfgl
dgl
Ctrl
0
1
5
10
Juice (µL)

**Fig 2.**

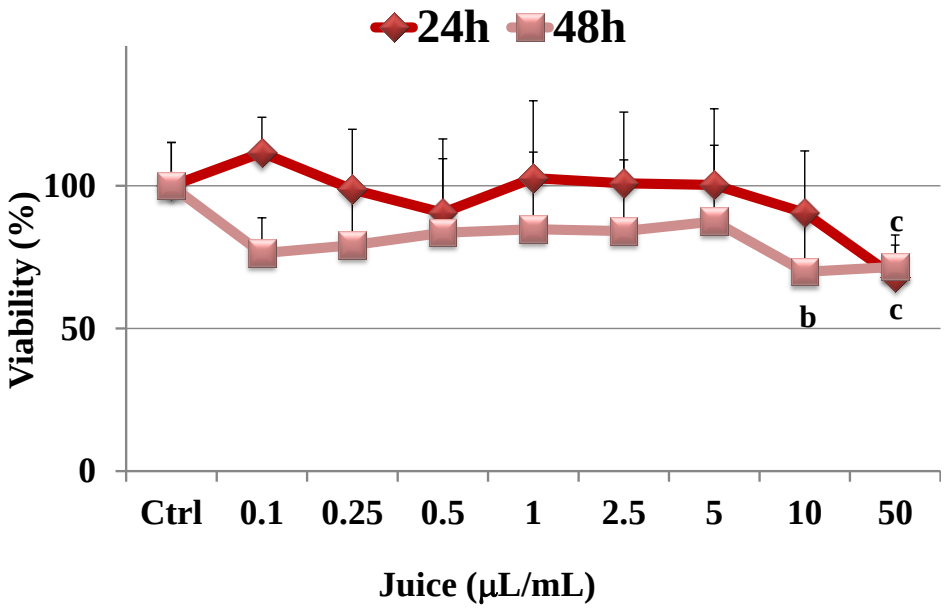
24h
48h
Viability (%)
100
50
0
Ctrl
0.1
0.25
0.5
1
2.5
5
10
50
b
c
c
Juice (µL/mL)

**Fig 3.**

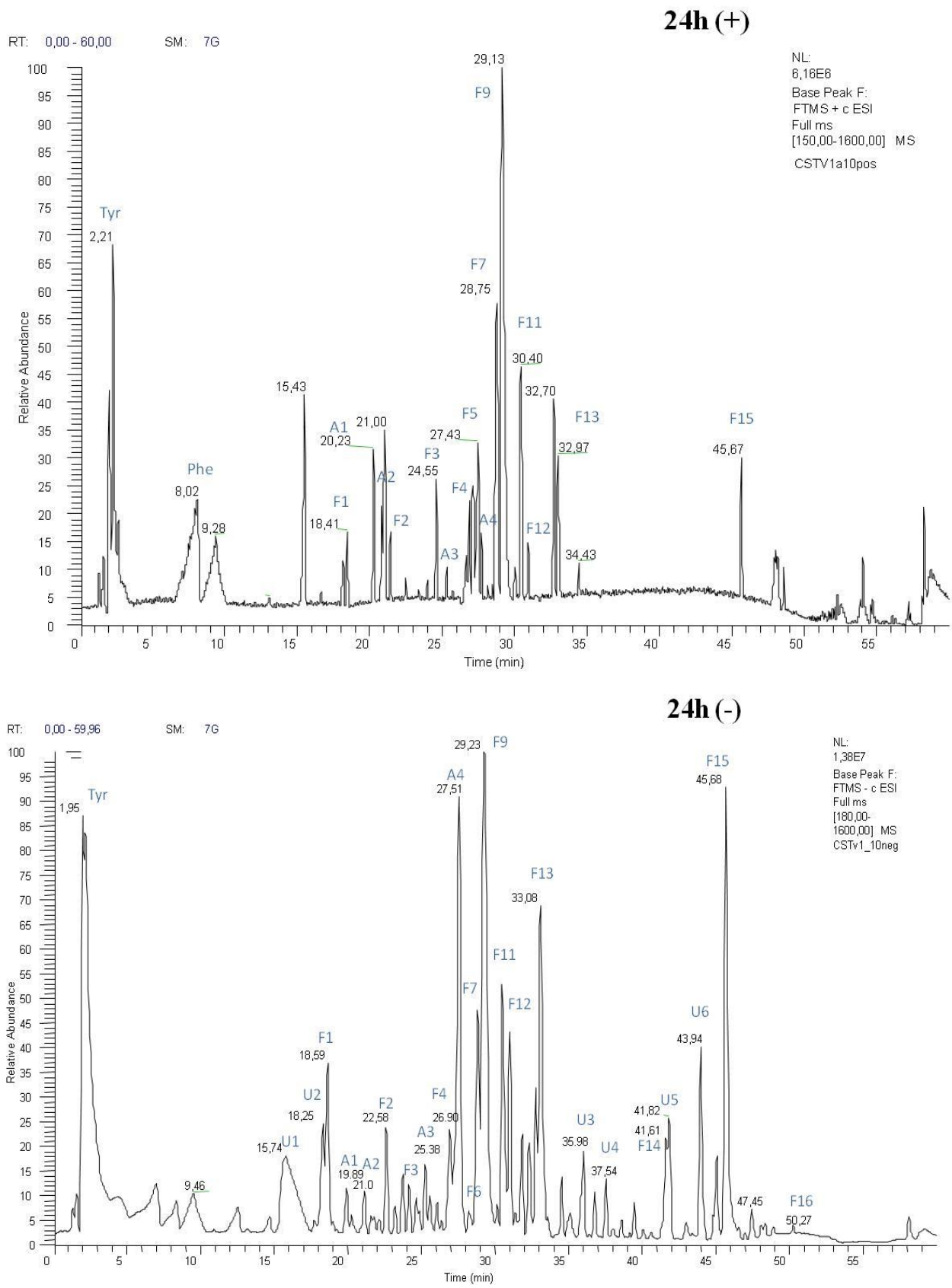
24h (+)
RT: 0,00 - 60,00
SM: 7G
NL:
6,16E6
Base Peak F:
FTMS + c ESI
Full ms
[150,00-1600,00] MS
CSTV1a10pos
Relative Abundance
Time (min)
Tyr
2,21
Phe
8,02
9,28
15,43
A1
20,23
21,00
A2
F1
18,41
F2
F3
24,55
A3
F4
27,43
F5
29,13
F9
A4
F7
28,75
F11
30,40
F12
32,70
F13
32,97
34,43
F15
45,67
24h (-)
RT: 0,00 - 59,96
SM: 7G
NL:
1,38E7
Base Peak F:
FTMS - c ESI
Full ms
[180,00-
1600,00] MS
CSTv1_10neg
Relative Abundance
Time (min)
Tyr
1,95
U1
15,74
9,46
F1
18,59
U2
18,25
A1
19,89
A2
21,0
F2
22,58
F3
25,38
A3
F4
26,90
A4
27,51
F9
29,23
F6
F7
F11
F12
F13
33,08
U3
35,98
U4
37,54
U5
41,82
41,61
F14
U6
43,94
F15
45,68
47,45
F16
50,27

**Fig 4.**