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Influence of ethanol/water ratio in ultrasound and high-pressure/ high-temperature phenolic compound extraction from agri-food waste

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Summary

The valorisation and management of agri-food waste are currently hot investigation topics which probe the recovery of valuable compounds, such as polyphenols. In this study, high-pressure/high-temperature extraction (HPTE) and ultrasound-assisted extraction (UAE) have been used to study the recovery of phenolic compounds from grape marc and olive pomace in hydroalcoholic solutions. The main phenolic compounds in both extracts were identified by HPLC-DAD. Besides extraction yield (total polyphenol and flavonoid content) and the antiradical power, polyphenol degradation under HPTE and UAE has also been studied. HPTE with ethanol 75% gave higher phenolic extraction yields: 73.8 _ 1.4 mg of gallic acid equivalents per gram of dried matter and 60.0 mg of caffeic acid equivalents per gram of dried matter for grape marc and olive pomace, respectively. In this study, the efficient combination of ethanol/water mixture with HPTE or UAE has been used to enhance the recovery of phenolic compounds from grape marc and olive pomace. HPLC-DAD showed that UAE prevents phenolic species degradation damage because of its milder operative conditions.

Keywords: Grape marc, high-pressure/high-temperature extraction, olive pomace, polyphenols, ultrasound-assisted extraction.

Introduction

Recent decades have seen academic and industrial heads turned by the high variety of agri-food waste available as sources of bioactive compounds and fine chemicals. The literature highlights the pivotal role of enabling technologies for biowaste valorisation (Tabasso et al., 2015). High added value molecules can be recovered from these matrixes, such as fine chemicals (Tabasso et al., 2014), sugars (Hernoux-Villi_ere et al., 2013), bioplastics (Carnaroglio et al., 2015) and phenolic compounds (Moral & Mendez, 2006; Cioffi et al., 2010; Aliakbarian et al., 2011, 2012; Ramos et al., 2013). Polyphenols are a class of secondary metabolites produced by plant species and range from simple structures to complex molecules (Munin & Edwards-Lévy, 2011), providing a wide and interesting variety of biological effects (Tuck & Hayball, 2002), including antioxidant, anti-inflammatory, antibacterial and antiviral activity (Aliakbarian et al., 2009). These properties give polyphenols important roles to play in the nutraceutical and medical fields (Jang et al., 1997; Moure et al., 2001; Desco et al., 2002; Baur & Sinclair, 2006; Delmas et al., 2006; Loftsson & Duch^ene, 2007; Kumari et al., 2011; Aliakbarian et al., 2012; Palmieri et al., 2012).

Two of the most interesting agri-food wastes which contain phenolic species are grape marc (GM) and olive pomace (OP). GM production (skins and seeds) is approximately 20–25 kg for every 100 kg of grapevine produced (Passos et al., 2013), and OP is made up of the solid residue of olive pulp and seeds (*Olea europaea* L.), obtained during olive oil production. The extraction of high added value compounds from these two agri-food wastes can play an important role both in the economic sustainment of winemaking and olive oil production and in the reduction in this industrial waste's environmental impact. Several studies have described the extraction of polyphenols from these matrices both via conventional solid–liquid extraction and nonconventional techniques (Palma & Taylor, 1999; Buschmann & Schollmeyer, 2002; Astolfi-Filho et al., 2005; Bucić-Kojić et al., 2007; Vilkhu et al., 2008; Fiori et al., 2009; Aliakbarian et al., 2010, 2011; Casazza et al., 2010, 2012a). Recently, Antoniolli et al. (2015) reported the antioxidant capacity and the full phenolic composition

of Malbec grape pomace extract, highlighting the different quali–quantitative profile even within the same cultivar being influenced by locations, harvest time and the growth environment.

Of the techniques studied, high-pressure/high-temperature extraction (HPTE) and ultrasound-assisted extraction (UAE) are two of the most effective. The high temperatures in HPTE decrease solvent viscosity, leading to rapid solvent penetration (Palou, 1997; Oey et al., 2008). It can also enhance the disruption of the strong solute–matrix interactions caused by hydrogen bonds as well as dipole attractions and van der Waals interactions (Casazza et al., 2012b). The pressurisation of the steel reactor prevents the solvent boiling at the extraction temperature and enhances the intimate contact with the raw material over the entire extraction period (Sardar & Singhal, 2013), leading to increased target compound solubility and accelerated solvent desorption from the solid matrix (Richter et al., 1996). The high efficiency of UAE is mainly explained by the phenomenon of cavitation which is often used to obtain polyphenol-rich extracts from vegetal matrices (Ghafoor et al., 2009; De Souza Oliveira et al., 2011). The technique has been extensively studied on the laboratory scale and has also found some industrial applications (López-Córdoba et al., 2014). UAE is generally faster than traditional extraction techniques, because of the greater matrix–solvent contact area caused by a reduction in particle size (Casazza et al., 2010). The acoustic cavitation of power ultrasound (18–40 kHz) causes cell wall disruption, facilitates contact between the solvent and intracellular content and increases mass transfer (Astolfi-Filho et al., 2005).

Both of these nonconventional techniques have already been tested on grape by-products by our group (Casazza et al., 2010; Casazza et al., 2012), using pure methanol or ethanol, while only HPTE has been investigated on olive pomace (Aliakbarian et al., 2011, 2012). To the best of our knowledge, the effect of binary solvents on phenolic compound extraction from GM and OP is still unclear. The aim of this work was to study the effect of different ethanol/water ratios on the HPTE and UAE procedures. Extracts were compared in terms of total phenolic and total flavonoid contents and their antiradical power. High-performance liquid chromatography with a diode array detector (HPLC-DAD) was used to identify the main phenolic compounds. The degradation effects of HPTE and UAE

on phenolic species have been investigated using similar extraction protocols and were conducted on the standard polyphenol mixtures typically contained in grape and olive waste extracts.

Materials and method

Chemicals

Methanol, ethanol, acetic acid, n-hexane, acetonitrile, sodium carbonate, sodium hydroxide, aluminium chloride, sodium nitrite, the Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) and phenolic compound standards (gallic acid, caffeic acid, vanillic acid, syringic acid, tyrosol, epigallocatechin gallate, oleuropein and t-resveratrol) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard stock solutions were prepared with methanol (0.5 g L^{-1}) and stored at -20 °C in covered bottles.

Raw materials

Grape marc of Croatina cultivar (*Vitis vinifera* L.), typical of the northern part of Italy, was supplied by a local producer before the vinification process and stored at -20 °C. Following the methodology described by Casazza et al. (2011), GM was oven-dried at 50 °C until a constant moisture of about 4–5% and was obtained. The GM was then ground using a laboratory mixer to obtain a homogeneous powder, with a particle size of 0.8 mm obtained via sieve separation.

Olive pomace of Taggiasca cultivar (*Olea Europea* L.) from a three-phase oil extraction decanter was supplied by a north Italian olive oil industry and stored at -20 °C prior to analysis. As described by Aliakbarian et al. (2011), OP was washed with n-hexane to remove residual oil, oven-dried at 60 °C for about 48 h until constant moisture content was achieved (5–8%) and then ground using a laboratory mixer.

High-pressure/high-temperature extraction

Phenolic compounds were extracted from GM and OP with HPTE using an agitated reactor (model 4560; PARR Instrument Company, Moline, IL, USA). The effect of the ethanol/water ratio on phenolic compound recovery was studied using 100, 75, 50, 25 and 0% v/v in ethanol. GM extractions were performed at 150 °C for 270 min (Casazza et al., 2012b), while OP was extracted at 180 °C for 90 min (Aliakbarian et al., 2011). For both matrices, the extractions were performed in an inert atmosphere (N_2) and with a solid/liquid ratio of 1:10 (5.0 g of raw material and 50 mL of solution). After extraction, all samples were centrifuged on an ALC PK131 centrifuge (Alberta, Canada) at 6729 g for 10 min, filtered through a 0.22-μm filter (Sartorius Stedim Biotech GmbH, Göttingen, Germany) and stored at 4 °C before analysis.

Ultrasound-assisted extraction

Grape marc and OP polyphenols were subjected to UAE, with solvents at the same ethanol/water ratios used for HPTE. Briefly, 5 g of raw dried wastes was added to solvent solutions with a solid/liquid ratio of 1:10 and sonicated with an immersion titanium horn (tip O = 30 mm) for 15 min (frequency 19.9 kHz, input power 100 W, system provided by Danacamerini s.a. Turin), (Alexandru et al., 2013).

After treatment, the suspension was centrifuged at 1914 g for 20 min, filtered through a 0.22-μm filter and stored at 4 °C before analysis.

Total phenolic content

Grape marc and OP extracts obtained by HPTE and UAE were analysed in terms of total phenolic yield (TP) using the Folin–Ciocalteu colorimetric assay (Gutfinger, 1981): 0.2 mL of extract, 0.5 mL of Folin–Ciocalteu reagent and 1 mL of a 20% sodium carbonate solution were added to deionised water until a final volume of 10 mL was achieved by mixing the solution after each addition. Solutions were left at room temperature in the dark for 1 h. Measurements were carried out on a UV–Vis Lambda 25 spectrophotometer (Perkin Elmer, Wellesley, MA, USA) at a wavelength of 725 nm.

Calibration curves were obtained using methanolic standard solutions of gallic acid and caffeic acid, both ranging from 0.01 to 1.00 mg mL^{-1}. TP was expressed as milligrams of gallic acid equivalents (GAE) per gram of dried matter (mgGAE/gDM) for GM samples (Casazza et al., 2012a), and as milligrams of caffeic acid equivalents (CAE) per gram of dried matter (mgCAE/gDM) (Mulinacci et al., 2001) for OP samples. The method responses were described by linear eqns 1 (R^2 = 0.994) and 2 (R^2 = 0.996) for GM and OP, respectively

$$ABS_{725} = 0{:}0017 \text{ X TP} \quad (1)$$

$$ABS_{725} = 0{:}0023 \text{ X TP} \quad (2)$$

Total flavonoid content

Total sample flavonoid yield (TF), expressed as milligrams of catechin equivalent (CE) per gram of dried matter (mgCE/gDM), was calculated using the method described by Yang et al. (2009): 0.25 mL of diluted extract was mixed with 1.25 mL of deionised water and subsequently with 0.075 mL of 5% sodium nitrite solution. After 5 min of incubation, 0.15 mL of a 10% aluminium chloride solution was added and allowed to react for 6 min before 0.5 mL of 1 M sodium hydroxide was added. Distilled water was then added to bring the final volume of the mixture to 3 mL.

Measures were carried out at 510 nm using the same spectrophotometer as mentioned above, and the relationship between absorbance and flavonoid content was described by a linear curve (eqn 3) with R^2 = 0.991

$$ABS_{510} = 0{:}0021 \text{ X TF} \quad (3)$$

Antiradical activity determination

The antiradical activity of the extracts was measured in terms of hydrogen-donating or radical-scavenging ability by means of the radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$), according to the method described by Brand-Williams et al. (1995). The $DPPH^{\cdot}$ concentration in the reaction solution

($C_{DPPH^{\cdot}}$) was calculated from a calibration curve (eqn 4) using standard solutions of $DPPH^{\cdot}$ in the range 3–44 μg $DPPH^{\cdot}$ per mL:

$$ABS_{515} = 0{:}0023 \text{ X } C_{DPPH^{\cdot}} \quad (4)$$

with $R^2 = 0.999$.

The ratio, expressed in $\mu L_{extract}/\mu g_{DPPH}$, was plotted vs. the percentage of the residual $DPPH^{\cdot}$ concentration after 1 h vs. the initial value. The antiradical power value (ARP), expressed as mg$DPPH^{\cdot}$ per mLextract, is equal to the reciprocal of the effective concentration EC_{50} ($1/EC_{50}$), which is the concentration of the initial extract which is able to reduce 50% of the $DPPH^{\cdot}$ in solution. All analyses were performed in triplicate.

HPLC-DAD analysis

The quali–quantitative profile of the extracts was obtained by HPLC (Hewlett Packard, 1100 Series, Palo Alto, CA, USA) coupled with a DAD detector. This was equipped with a C18 reverse-phase column (Model 201TP54, Vydac, Hesperia, CA, USA), as described by De Faveri et al. (2008), with some modifications.

The mobile phase was made up of water/acetic acid 99:1% v/v (solvent A) and methanol/acetonitrile 50:50% v/v (solvent B), and the solvent gradient was changed according to the following settings: from 0% to 5% B in 5 min, from 5% to 30% B in 25 min, from 30% to 40% B in 10 min, from 40% to 48% B in 5 min, from 48% to 70% B in 5 min, from 70% to 100% B in 5 min, isocratic at 100% B for 5 min, followed by a return to initial conditions (10 min) and 12 min for column equilibration. Solvent flow rate, injection volume and column temperature were 1 mL min^{-1}, 20 μL and 30 °C, respectively. Before analyses, samples were filtered through a 0.22-μm membrane filter. Analyses were detected at 280 nm, and the concentration of each phenolic compound was calculated based on each standard solution.

Effect of extraction technique on single phenolic compound

To evaluate the effects of the extraction conditions on phenolic compound stability, standards of the most representative polyphenols in GM extracts (gallic acid, tyrosol, vanillic acid, epigallocatechin gallate, caffeic acid and trans-resveratrol) and OP (tyrosol, vanillic acid, syringic acid, caffeic acid and oleuropein) were mixed and solubilised in a 75:25 ethanol/water solution, with a concentration of 0.5 mg mL^{-1} for each single phenolic compound. The GM-like standard solution (GMss) and the OP-like standard solution (OPss) were treated with HPTE and UAE techniques, under the same conditions as described above, and analysed by HPLC-DAD to evaluate possible modifications in the phenolic profile due to the fact that it may be caused by high-temperature, high-pressure and strong acoustic cavitation.

Statistical analysis

The influence of the different ethanol/water ratios and extraction methods were assessed via an analysis of variance (ANOVA) and using Tukey's post *hoc test* ($P < 0.05$), using 'Statistica' software version 8.0 (Stat-Soft, Tulsa, OK, USA). The statistically significant differences are shown in tables and figures by different letters.

Results and discussion

Total phenolic yield and total flavonoid yield

Grape marc extracts obtained with HPTE and UAE techniques were characterised in terms of TP and TF, as shown in Fig. 1a,b.

In general, the addition of ethanol to the solvent mixture leads to increased TP: this is particularly evident in HPTE (Fig. 1a), where an increase in the ethanol fraction from 25% to 50% (v/v) resulted in a considerable increase in TP, from 27 ± 0.2 to 70.6 ± 0.4 mg_{GAE}/g_{DM}. This is probably because a higher ethanol concentration leads to better phenolic compound solubility and higher reactor chamber pressure, which can enhance GM matrix disruption. A further increase in ethanol fraction does not correspond to a statistically significant increase in TP ($P < 0.05$).

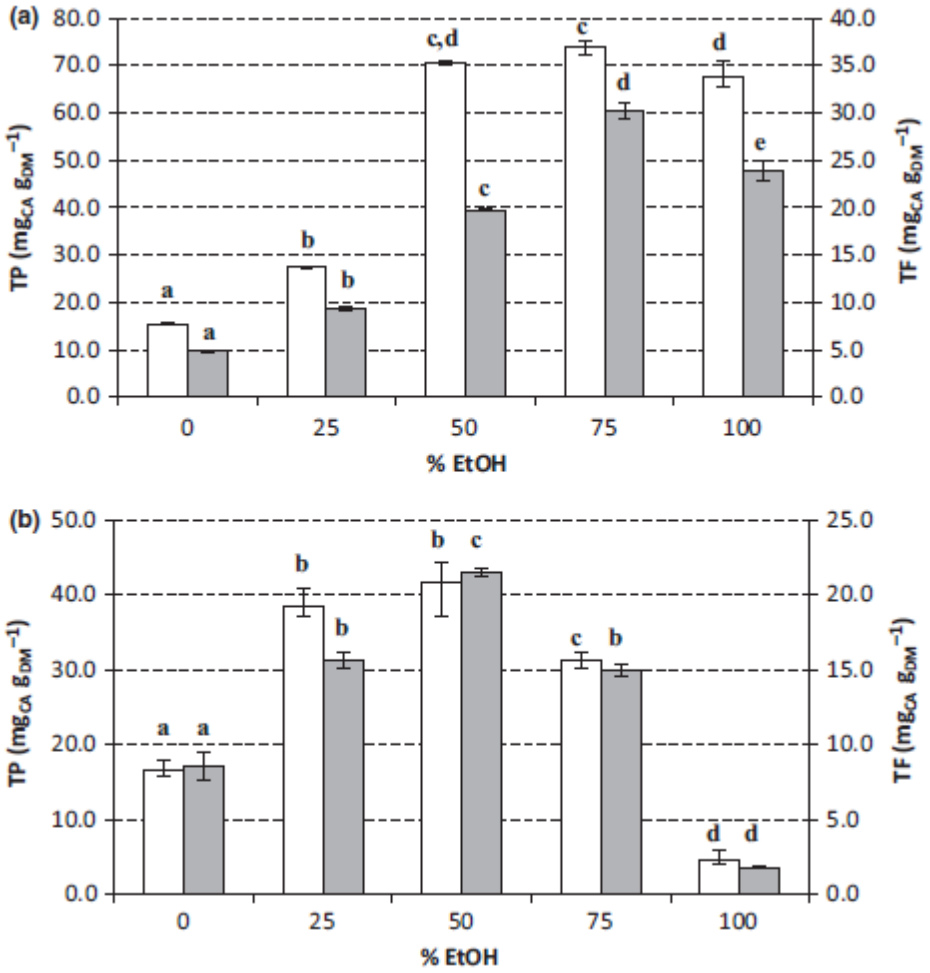

Fig. 1. TP yields (□) expressed as mg_{GAE}/g_{DM}, and TF yields (■), expressed as mg_{CE}/g_{DM}, of Croatina GM extracts using HPTE (a) and UAE (b) techniques with different ethanol/water ratio solvents. Different letters indicate significant differences in data at $p < 0.05$.

Ultrasound-assisted extraction samples show lower TP content than HPTE (Fig. 1b) when a high fraction of ethanol is present in the solvent mixture (from 50% to 100% v/v), while TP is 41% higher in the presence of 25% v/v of ethanol than in HPTE. In the absence of ethanol, the difference between the two extraction techniques is not statistically significant ($P < 0.05$), with TP values of 15.5 ± 0.3 mg_{GAE}/g_{DM} and 16.7 ± 1.2 mg_{GAE}/g_{DM} for HPTE and UAE, respectively. This can be explained by the fact that the higher vapour pressure and viscosity of ethanol, with respect to water, can decrease

the force of the implosion of the cavitation bubbles (Hemwimol et al., 2006), causing less effective solid material disruption.

TF is closely related to ethanol percentage in the mixture in both the techniques tested. In HPTE, TF increases from 4.9 ± 0.2 mg_{CE}/g_{DM}, in the absence of ethanol, to 30.2 ± 1.2 mg_{CE}/g_{DM} when a 75:25 ethanol/water solution was used (Fig. 1a).

Similar trend was reported by Spigno et al. (2007) using pressed marc of Barbera cultivar. They used two extraction cycles: first with absolute ethanol followed by a second extraction with different volumes of water added to ethanol (both steps at 60 °C for 5 h). The authors noticed that total phenolic yield improved when water was added to ethanol up to 30%. However, TP yield obtained in our study resulted to be much higher (maximum TP was 4.25 g_{GAE}/100 of freezed–dried extract when 60% of water was used). In UAE, the maximum yield was obtained with a 50:50 ethanol/water ratio, equal to 21.6 ± 0.4 mg_{CE}/ g_{DM}. However, the addition of a higher fraction of ethanol leads to less efficient UAE, while at lower

ethanol contents (0%, 25% v/v), this technique is more efficient in flavonoid extraction than HPTE.

Total phenolic and TF yields of OP extracts are shown in Fig. 2a,b.

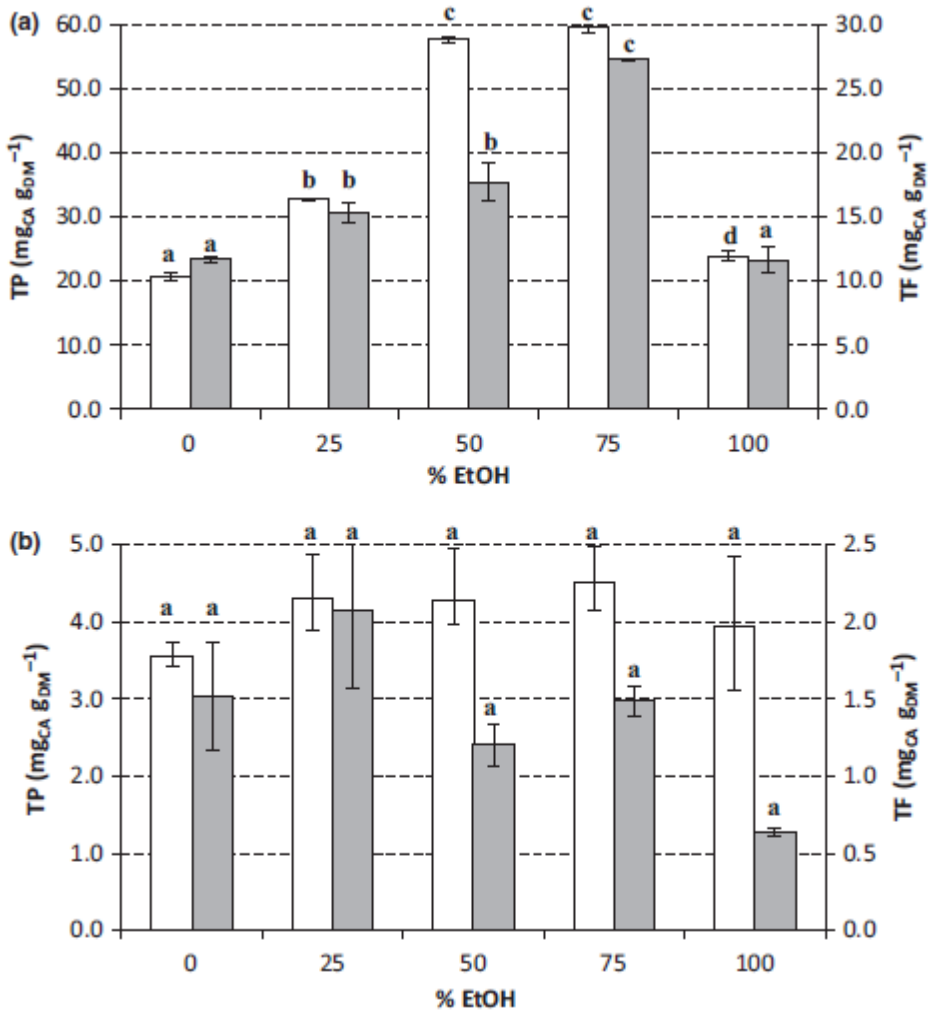

Fig. 2. TP yields (□), expressed as mg_{CAE}/g_{DM}, and TF yields (■), expressed as mg_{CE}/g_{DM}, of Taggiasca OP extracts using HPTE (a) and UAE (b) techniques with different ethanol/water ratio solvents. Different letters indicate significant differences in data at $p < 0.05$.

As regards OP, TP yield was higher than that reported by Aliakbarian et al. (2011) using HPTE in absolute methanol at 180 °C for 90 min (TP yield 45.2 mg_{CAE}/g_{DP}). In HPTE (Fig. 2a), TP yield enhances with increasing ethanol fraction, up to a value of 59.5 ± 0.9 mg_{CAE}/g_{DM} when a 75:25 ethanol/water ratio was used. As for GM, this behaviour can be explained if we consider the higher polyphenol solubility in the presence of polar organic solvents and the higher pressure in the reactor chamber, which can enhance OP matrix disruption. Extraction using pure ethanol leads to a statistically significant decrease in TP ($P < 0.05$), down to 23.8 ± 0.9 mg_{CAE}/g_{DM}, indicating that a

fraction of water is necessary for efficient phenolic extraction from OP when using the HPTE technique. This behaviour can also be noted in GM extraction where a lower but statistically significant decrease ($P < 0.05$) is present (-8.3%) when pure ethanol with respect to extraction with 75:25 ethanol/water.

As can be seen in Fig. 2b, different ethanol/water solvent ratios showed no statistically significant effects on TP ($P < 0.05$) when UAE is used and a TP yield of lower than 5 mg_{CAE}/g_{DM} was obtained in all the conditions tested. These results suggest that UAE is not suitable for the extraction of phenolic compounds from OP probably because of the composition of the raw material, which can compromise the effectiveness of ultrasound as extraction technology. Similar considerations can be made when we observe TF yields. In HPTE, TF increased upon increasing the quantity of ethanol in the solvent system, with the highest yield, equal to 27.3 ± 0.9 mg_{CE}/g_{DM}, recorded at a 75:25 ethanol/water ratio. According to TP results, a consistent decrease in TF content was detected (-57.3%) when pure ethanol is used. In UAE, TF yields were lower than those obtained with HPTE at all the conditions tested, and no significant differences ($P < 0.05$) can be noted between the samples.

Antiradical power of extracts

Antiradical power of the extracts is shown in Table 1.

For GM, the changes in ARP due to the addition of ethanol to the solvent system are similar to those observed in TP. In the absence of ethanol and at 25% v/v of ethanol, UAE has a higher ARP than HPTE. However, upon increasing the ethanol concentration, and in particular at 100% ethanol, HPTE samples show higher antiradical activity than UAE ones. In particular, HPTE gave the highest ARP value of 15.1 ± 2.6 $mg_{DPPH^{\cdot}}$ per $mL_{extract}$ at 50:50 ethanol/water.

For OP, ARP analyses confirmed that HPTE is more efficient than UAE at all ethanol/water ratios. The highest value, equal to 10.6 _ 1.0 $mg_{DPPH^{\cdot}}$ per $mL_{extract}$, was measured at an ethanol/water ratio of 50:50. This amount is lower than the value (15.80 ± 0.62 $mg_{DPPH^{\cdot}}$ per $mL_{extract}$) obtained by Aliakbarian et al. (2012) using the same technique with methanol as the solvent.

Table 1

Antiradical power of HPTE and UAE extracts from Croatina GM and Taggiasca OP, using solvents with different ethanol/water ratios, expressed as $mg_{DPPH}/mL_{extract}$.

	Croatina GM		Taggiasca OP	
Ethanol/water ratio	**HPTE**	**UAE**	**HPTE**	**UAE**
0:100	4.5 ± 0.1^{a}	7.1 ± 0.4^{a}	6.8 ± 1.5^{a}	1.5 ± 0.3^{a}
25:75	7.6 ± 0.1^{b}	10.2 ± 0.6^{b}	7.9 ± 0.1^{a}	2.9 ± 0.1^{b}
50:50	15.1 ± 2.6^{c}	11.3 ± 0.4^{c}	10.6 ± 1.0^{b}	2.0 ± 0.4^{a}
75:25	12.7 ± 0.5^{c}	8.8 ± 0.4^{d}	9.1 ± 0.8^{b}	1.2 ± 0.5^{a}
100:0	8.7 ± 2.0^{b}	2.0 ± 0.2^{e}	6.4 ± 0.1^{a}	0.2 ± 0.1^{c}

Data are shown as mean value ± standard deviation (n=3). Different letters within the column indicate significant differences in data at $p < 0.05$.

In HPTE, the addition of a higher amount of ethanol does not generate statistically significant differences in ARP ($P < 0.05$), while ARP decreases to 6.4 ± 0.1 $mg_{DPPH^{\cdot}}$ per $mL_{extract}$ at 100% ethanol, confirming that a fraction of water is necessary to obtain optimal phenolic compound extraction from this matrix. The same behaviour can also be observed in GM extracts. In general, the ARP profile is similar and clearly correlated with those observed in TP and TF yields.

HPLC-DAD analysis

All the GM and OP samples were analysed by HPLCDAD, to identify and quantify the main phenolic compounds in the extracts.

As shown in Table 2 and Fig. 3, using UAE, some phenolic compounds identified in GM extracts are not present in HPTE samples. These include tyrosol, catechin, epicatechin and trans-resveratrol. The most important detected flavonoid species, catechin, epicatechin and epigallocatechin gallate are present in high quantities only in UAE samples, suggesting that this technique is more suitable than HPTE for the preservation of these molecules after extraction. The differences in these results and the TF assays can be explained if we consider that other flavonoids, which are not detected by HPLC-DAD, can behave differently in different operative conditions, such as high temperature, and thus lead to higher yields in HPTE extracts.

Table 2

Content (mg/100g_{DM}) of main phenolic compounds in Croatina GM extracts with solvents at different ethanol/water ratios, analysed by HPLC-DAD.

	UAE ethanol/water ratio					HPTE ethanol/water ratio				
	0:100	**25:75**	**50:50**	**75:25**	**100:0**	**0:100**	**25:75**	**50:50**	**75:25**	**100:0**
GA[a]	14.78	23.41	9.92	8.02	6.49	69.57	12.17	60.51	98.40	105.24
Ty[b]	3.10	5.92	13.43	3.96	6.70	-	-	-	-	-
CA[c]	20.48	34.43	37.45	20.59	2.25	-	-	-	-	-
VA[d]	1.79	2.73	3.15	2.52	0.75	20.03	90.19	215.88	70.85	22.61
EGCG[e]	458.22	861.35	763.90	618.24	121.20	392.13	-	-	-	-
EC[f]	26.19	53.81	67.35	52.94	14.70	-	-	-	-	-
CAA[g]	-	-	-	-	-	7.66	5.07	31.08	28.06	8.38
***t*-Res**[h]	-	-	1.26	0.87	1.13	-	-	-	-	-

[a]Gallic acid; [b]Tyrosol; [c]Catechin; [d]Vanillic acid; [e]Epigallocatechin gallate; [f]Epicatechin; [g]Caffeic acid; [h]*trans*-Resveratrol.

trans-Resveratrol was only detected in UAE samples, suggesting that the harsher extraction conditions in HPTE (high pressure coupled with high temperature) can lead to the degradation of this compound after extraction from the GM matrix. On the other hand, the amount of gallic acid is higher in HPTE samples than in UAE ones, probably due to the degradation of epigallocatechin gallate. This suggests that HPTE extraction conditions can lead to greater modifications in the profiles of extracted polyphenols than UAE. These modifications may also be responsible for the discrepancy in TP and HPLCDAD results, as the large amount of the extracted polyphenols that are measured with the Folin–Ciocalteu assay cannot be identified with HPLC-DAD using the available standards (Casazza et al., 2011).

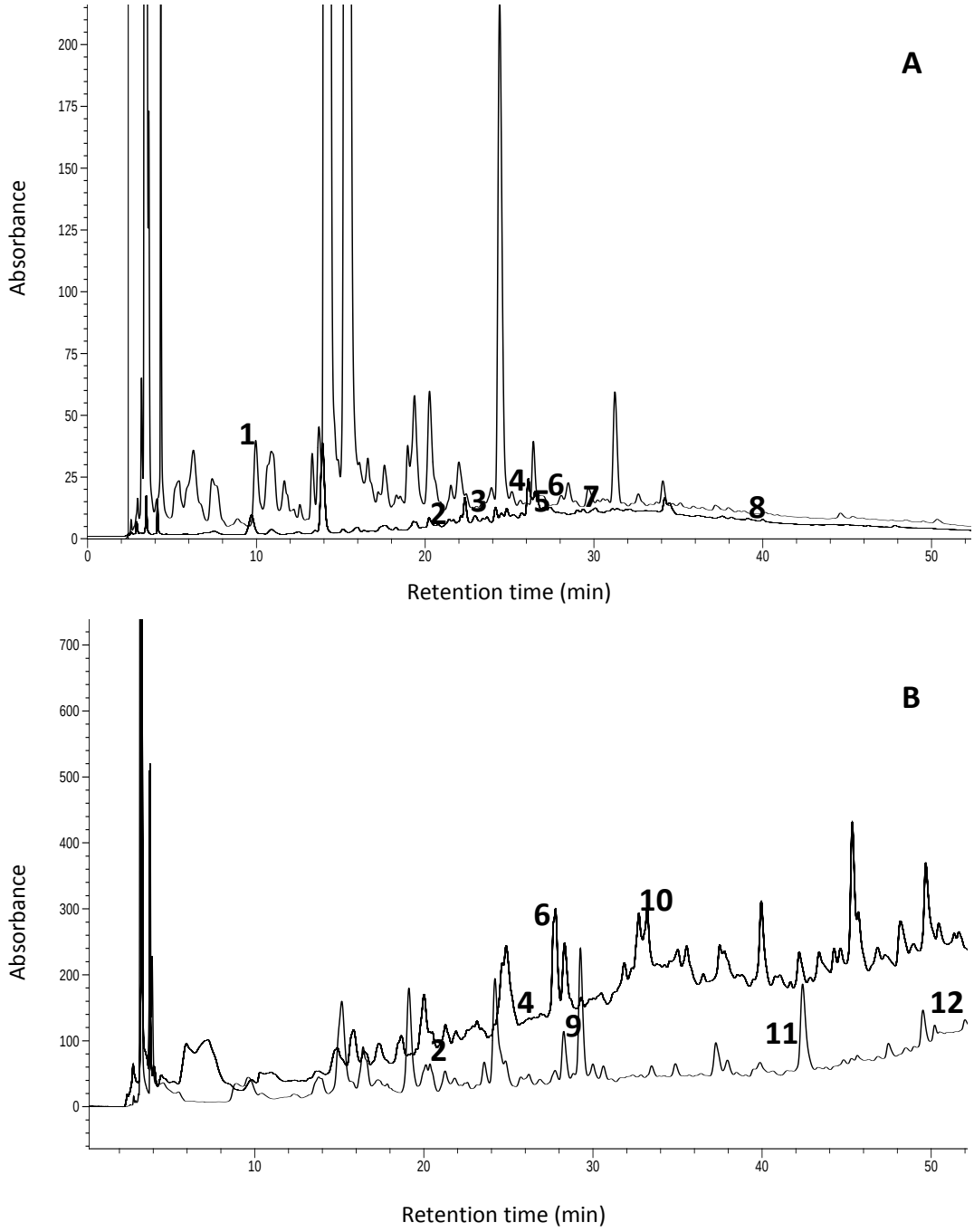

Fig. 3. HPLC chromatograms of grape marc (A) and olive pomace (B) extracts using UAE (dashed lines) and HPTE (continuous lines) with 75:25 ethanol/water.

1) Gallic acid; 2) Tyrosol; 3) Catechin; 4) Vanillic acid; 5) Epigallocatechingallate; 6) Epicatechin; 7) Caffeic acid; 8) *trans*-resveratrol; 9) Syringic acid; 10) Ferulic acid; 11) Oleuropein; 12) Apigenin.

Table 3 shows the polyphenol contents detected by HPLC-DAD in OP extracts.

According to TP and TF results, a comparison of the single phenolic compound in UAE and HPTE extracts shows that UAE is not the technique of choice for polyphenol extraction from OP: all the detected compounds except vanillic acid, which was not identified in HPTE extracts, showed higher contents in HPTE than in UAE at all the ethanol/water ratios used. This is particularly evident in oleuropein, the most abundant phenolic compound in olive oil and relative by-products, which reached a content of 542.03 mg per $100g_{DM}$ in the 75:25 ethanol/water HPTE extract. This value is 83 times higher than that in the respective UAE sample (6.51 mg per $100g_{DM}$).

Table 3
Content (mg/$100g_{DM}$) of main phenolic compounds in Taggiasca OP extracts with solvents at different ethanol/water ratios, analysed by HPLC-DAD.

	UAE ethanol/water ratio					HPTE ethanol/water ratio				
	0:100	**25:75**	**50:50**	**75:25**	**100:0**	**0:100**	**25:75**	**50:50**	**75:25**	**100:0**
Ty[a]	2.17	2.47	2.58	-	-	54.91	56.51	43.85	48.28	55.32
VA[b]	1.76	2.76	2.53	3.60	2.24	-	-	-	-	-
SA[c]	-	-	-	-	-	1.34	2.27	3.67	2.69	1.29
CAA[d]	2.09	2.12	2.18	1.54	-	3.22	8.88	3.66	3.57	3.22
FA[e]	0.94	0.84	0.90	0.47	-	5.61	6.04	3.19	1.85	4.55
Ol[f]	3.89	5.35	7.84	6.51	-	122.58	163.53	274.04	542.03	510.79
Ap[g]	-	-	0.88	1.36	0.44	2.18	1.49	7.34	3.89	3.09

[a]Tyrosol; [b]Vanillic acid; [c]Syringic acid; [d]Caffeic acid; [e]Ferulic acid; [f]Oleuropein; [g]Apigenin.

Effect of extraction techniques on single synthetic phenolic compounds

To evaluate the degradation effects on the extracted polyphenols which may be caused by the operative conditions, two mixtures of chemical standards (GMss and OPss) were analysed by HPLC-DAD before and after UAE and HPTE treatments.

As can be seen in Table 4, UAE operative conditions seem to have no degradation effect on single phenolic species, in both GMss and OPss; only caffeic acid decreased slightly in GMss (-4.2%) after UAE treatment.

Table 4
Percentage variation of polyphenol concentrations in GM-like standard solution (GMss) and OP-like standard solution (OPss) after UAE and HPTE treatments.

	GMss		OPss	
	UAE (%)	**HPTE (%)**	**UAE (%)**	**HPTE (%)**
GA[a]	2.3	-45.1	-	-
Ty[b]	2.4	8.1	1.6	6.4
CAA[c]	-4.2	-99.7	0.8	-99.2
VA[d]	2.8	-5.2	1.7	-9.5
EGCG[e]	0.2	-68.7	-	-
SA[f]	-	-	1.1	-11.3
***t*-Res**[g]	1.2	-6.4	-	-
Ol[h]	-	-	7.0	-98.8

[a]Gallic acid; [b]Tyrosol; [c]Caffeic acid; [d]Vanillic acid; [e]Epigallocatechin gallate; [f]Syringic acid; [g]*trans*-Resveratrol; [h]Oleuropein.

On the other hand, the harsh operative conditions in HPTE lead to a marked decrease in the phenolic species in solution, in both GMss and OPss. This is particularly evident in caffeic acid, which is fully degraded at high-temperature/high-pressure conditions, with percentage decreases of -99.7% and -99.2% in GMss and OPss, respectively. The same behaviour was noticed in oleuropein in OPss (-98.8%). For both standard solutions, new unidentified peaks appear in the chromatograms after HPTE treatment. These are due to the degradation products of the investigated phenolic compounds. Tyrosol is the only compound that appears not to be affected by HPTE treatment, as its content increased by 8.1% and 6.4% in GMss and OPss, respectively. This increase can be explained as tyrosol is known to be a degradation product of oleuropein.

Owing to the higher solubility of oleuropein in methanol than ethanol/water mixtures, Aliakbarian

et al. (2011) reported higher yield (2433 mg per 100 gDP). Despite degradation phenomena, HPTE gives higher TP than UAE at a 50:50 ethanol/water ratio as described in Figs 1 and 2. This means that the extraction rate of phenolics from solid material is higher than the degradation rate.

Conclusion

In this study, for the first time, the effect of ethanol/water ratio in UAE and HPTE of polyphenols from GM and OP was investigated. In GM extractions, HPTE gave higher total phenolic and flavonoid yields at higher ethanol percentages (75–100% v/v), while UAE was more efficient at low ethanol/water ratios (25–50% v/v). We can conclude that these techniques may play a fundamental role in the valorisation of winemaking and olive oil production wastes; meantime, ethanol-rich solutions arise safety concerns in pilot- and industrial-scale production.

Best results in terms of total polyphenol and flavonoid content, equal to 73.8 ± 1.4 mg_{GAE}/g_{DM} and 30.2 ± 1.2 mg_{CE}/g_{DM}, respectively, were obtained by HPTE at 75:25 ethanol/water. For OP, UAE gave lower TP, TF and ARP than HPTE under all the conditions tested. The HPLC-DAD of all the extracts and standard polyphenols solutions showed that UAE prevents phenolic species degradation damage because of its milder operative conditions. In particular, caffeic acid and oleuropein were found to be extremely sensitive to HPTE conditions. We can conclude that these techniques are able to play a fundamental role in the valorisation of winemaking and olive oil production wastes.

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