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Deep eutectic solvents improve the biorefinery of alperujo by extraction of bioactive molecules in combination with industrial thermal treatments

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ABSTRACT

Thermal treatments are the latest developments in the olive oil biorefinery industry to extract bioactive compounds from its by-products, mainly the alperujo. To reach these goals and reduce energy consumption the utilization of deep eutectic solvents has been studied. An initial screening led to define a eutectic mix of choline chloride, glycolic and oxalic acid (DES9) as one of the adequate solvents to increase the solubilization of phenols and sugars, with a temperature reduction from 180 °C to 120 °C. DES9 increased the concentration of acid sugars by six times and the concentration of hydroxytyrosol by 30 times, to up to 85.81 mg/g of dry matter. The activity of DES9 is not due to the activity of each component separately or to the mixture of the two acids, but to the eutectic mixture of all of them. In the future, these solvents could improve the extraction, stability and bioavailability of bioactive compounds and the biorefinery of alperujo.

1. Introduction

The olive oil production industry generates huge amounts of alperujo or two olive oil extraction waste which has a great environmental impact because of its high phytoxicity. In addition, the remarkable increase in production and the sensitivity of the population to environmental problems during recent years (Wiesman, 2009) have led to the adaptation and transformation of the traditional strategies of valorization. Currently, after drying and extracting the oil from the pomace, alperujo is destined to the cogeneration of electrical and thermal energy by means of combustion. Research has seen little success in finding a more environmentally and economically viable solution for general adoption so far. The potential uses of by-products from olive oil are related to health (cosmetics, pharmaceuticals, food additives, etc.), biofertilizers and/or compost, animal feed and with the production of alternative energy as a real biorefinery (biodiesel, gasification, methane production, etc.) (Serrano et al., 2017; Fernández-Bolaños et al., 1999).

The olive oil industry is changing in line with the new concept of quality, in which the most important focus is not only on the final product, but also its implication to both human and environmental health. In this way the by-products generated from the olive oil industry are being managed in order to obtain bioactive compounds and the total utilization of the rest of the components. The bioactive compounds, like phenols, and the monounsaturated fatty acids of the olive are responsible for the well-known beneficial effects of olive oil (Pérez-Jiménez et al., 2007). Approximately 98–99% of phenols remain in the alperujo after the extraction of olive oil (Sacchi et al., 2014). They are being widely studied for their biological activities such as antioxidants, antiplatelet, anti-inflammatory, antimicrobial, antiviral, anti-cancer, antihypertensive and cardioprotective (Benavente-García et al., 2000; Cicerale et al., 2012; Rozzi and Malpei, 1996).

Some of the most important commercial antioxidants found in olives and olive oil are hydroxytyrosol (HT), tyrosol (Ty), and oleuropein.

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Other important organic compounds that can be selectively recovered are lipids, water-soluble carbohydrates and proteins.

Therefore, waste treatment technologies which focus on the recovery of bioactive compounds from olive oil waste represent an interesting alternative that makes possible the biorefinery application for the utilization of this by-product. Recently a new alternative technology based on a thermal treatment with water steam at high pressure and temperature has been developed, tested on a pilot plant scale and already implemented at an industrial level, which achieves the separation of phases, making it possible to use its components (cellulose, hemicellulose, pectic substances and lignin) (Fernández-Bolaños et al., 1999, 2001). Bioactive compounds are also solubilized, making them easy to isolate and recover (Rodríguez et al., 2007a, b). Thus, the recovery of high value-added products such as hydroxytyrosol, 3,4-dihydroxyphenylglycol, mannitol, hemicellulosic polysaccharides, xylan type and oligosaccharides with functional properties became possible (Fernández-Bolaños et al., 2001, 2002, 2004). In addition, the thermal treatment produces a reduction in the solid phase with a high content of oil which is rich in minor components (Lama et al., 2011).

Another alternative to the extraction of components of interest is the use of conventional organic solvents. However, the use of large quantities has a negative effect on the environment, so during recent years other types of solvents have been sought, such as ionic solvents (IL) and deep eutectic solvents (DES).

Recently the effect of DES has been tested on olive oil, where its capacity for the extraction of phenolic compounds compared to the classical method of methanol/water extraction has been demonstrated (García et al., 2016). The use of these new solvents seems to be aimed at achieving not only an environmental improvement but technological as well, since it can be used in conjunction with other treatments to improve the results or even to reduce the energy requirements of the systems in which it is combined. This could be the case of the use of DES with treatments which are beginning to be used for a better management of by-products, such as thermal treatments.

Once it had been determined that the use of the DES increases the extraction of bioactive compounds in olive oil, and that the application of thermal treatments makes the industrial production of these compounds from by-products (alperujo) possible, the combination of the use of DES with thermal treatments should improve the extraction of bioactive components and make it possible to reduce the temperatures used, save energy and decrease the degradation of the components of interest.

For the first time, this work focuses on studying the combination of the use of eutectic solvents with thermal treatments, with the aim of lowering the temperature for the extraction of bioactive molecules from alperujo, making easier the further application of bioprocess for the total utilization of this by-product. A study was carried out on the behavior of different solvents at low temperatures with alperujo through solid-liquid separation, the extraction of total phenols, total sugars and uronic acids. After that, the most suitable solvents were chosen and studied at higher temperatures, through the same parameters plus the profile of individual phenols. Finally, it was verified that the positive effects were due to DES and not to its separate components.

2. Materials and methods

2.1. Samples

The samples of fresh alperujo were taken at the middle of the 2016–2017 harvesting season, from the olive oil experimental mill at the Instituto de la Grasa (CSIC) (Seville, Spain). The alperujo (olive oil) came from olives of the Arbequina variety.

The samples were taken directly without pitting (45–50% of pit referred to as dry matter) at the end of the horizontal centrifuge of the two-phase extraction process. The samples were stored at −20 °C before extraction.

2.2. Chemicals

Trifluoroacetic acid (TFA) (CAS 76-05-1, purity 99%), anthrone (CAS 90-44-8, purity 97%) and Folin–Ciocalteu phenol were purchased from Sigma-Aldrich Química (Madrid, Spain). Na_2CO_3 (CAS 497-19-8, purity ≥99.5%) and methanol (CAS 67-56-1, purity ≥99.9%) were from Panreac Química S.A. (Barcelona, Spain). A standard of gallic acid (GA) (CAS 149-91-7, purity ≥97.5%) was purchased from Sigma-Aldrich Química. Acetonitrile (CAS 75-05-08, purity ≥99.9%) was of HPLC-grade purity (Romyl, Teknokroma, Barcelona, Spain). The acetone (CAS 67-64-1, purity ≥99.8%) was from Sharlau (Barcelo, Spain).

2.3. Preparation of eutectic solvents

To synthesize eutectic solvents, the starting components were mixed according to the indications in Table 1. The mixtures corresponding to DES1, DES2, DES5, DES7, DES8, DES9 and DES10 were placed in round-bottomed flacks and heated in a water bath set at 60 °C with stirring until the formation of a viscous, colorless and stable liquid. However, for those that contained sucrose in their compositions, DES3 and DES4, all the components were previously dissolved with water. To synthesize these solvents, the excess of water was eliminated in a rotatory evaporator under vacuum. Mixtures of choline chloride with sugars (sucrose and xylitol) and with 1,2-propanediol did not form a clear liquid when subjected to the same treatment as the rest of the DESs. In the sugar-based DES and 1,2-propanediol, distilled water was added to allow the solubilization of the components and thus favor the interaction of hydrogen bond donors and acceptors.

2.4. Colorimetric method of determination of total phenols

Total phenolic content was determined by the Folin–Ciocalteu spectrophotometric method with some modifications (Obied et al., 2005). For all samples 5 μL of sample wereas placed in a test tube and mixed with 250 μL of Folin-Ciocalteu reagent and 200 μL of a 20% sodium carbonate solution in water (weight/volume (w/v)). The mixture was stirred and allowed to react for 10 min and centrifuged to remove the pellet. The supernatant was used to measure the absorbance at 655 nm. The results were expressed as grams of gallic acid equivalents. A calibration curve was made with a set of known concentrations of a gallic acid standard.

2.5. Analysis of individual phenols

In order to identify the phenolic compounds, a high-performance liquid chromatography (Hewlett-Packard model 1100, Palo Alto, CA, USA) was used. This instrument was equipped with an array detector monitoring at 254, 280 and 340 nm wavelength and a C18 reverse-phase column (Spherisorb ODS-2; 250 × 4.6 mm i.d. and 5 μm particle size) supplied by Teknokroma Tracer Extrasil OSD2 (Barcelona, Spain). The temperature of the column was kept constant at 25 °C with a C18 guard column. All samples treated with hydrothermal treatments were filtered through 0.45 μm pore size filters and a volume of 20 μL and each sample was injected into the HPLC instrument at a flow rate of 1 mL/min. The analytical method was carry out using a linear gradient of two eluents: solvent A (Milli-Q water, pH 2.5 adjusted with 0.01% of trifluoroacetic acid) and solvent B (acetonitrile), using the following gradient

Table 1 – Composition of the DES used in combination with thermal treatment to extract the alperujo sample (% of water is referred to the volume of all constituents).

Component 1	Component 2	Component 3	% Water	Relation (w/v)	pH	Name
Choline chloride	Glycerol	–	–	1:2	6.3	DES1
Choline chloride	Xylitol	–	11	2:1:3	4.8	DES2
Choline chloride	Sucrose	–	25	1:1:9	4.6	DES3
Betaine	Sucrose	–	12.8	2:1:5		DES4
Choline chloride	Malonic acid	–	–	1:1	0.42	DES5
Betaine	Levulinic acid		–	1:2	0.40	DES6
Choline chloride	1,4-butanediol	–	–	1:5	6.1	DES7
Choline chloride	1,2-propanediol	–	7.5	1:1:1	5.1	DES8
Choline chloride	Glycolic acid	Oxalic acid	–	1:1.7:0.3		DES9
Choline chloride	Ethylene glycol	–	–	1:2		DES10

over a total run time of 55 m in. 95% A and 5% B initially, 75% A and 25% B in 30 min, 50% A and 50% B in 45 min, 0% A and 100% B in 47 min, 75% A and 25% B in 95 min, and 95% A and 5% B in 52 min until completion of the run. Quantification was carried out by integration of the peaks at different wavelengths in function of the compounds, with reference to calibrations made using external standards.

2.6. *Total sugars*

The total content of neutral sugars was determined colorimetrically by the Antrona method (Dische, 1962) using a spectrophotometer (BIO-RAD imark Microplate Reader, USA). An aliquot of the 100 μL sample (in triplicate) was suitably diluted and 200 μL of 0.2% (w/v) Antrone solution in concentrated sulfuric acid were added. The test tubes were shaken in a Vortex and heated for 5 min at 100 °C in a water bath. When the tubes were cooled in an ice bath, the absorbance at 630 nm was measured. Glucose was used as an external standard to prepare a calibration with aqueous solutions in a concentration range of 0.02–0.2 mg/mL.

2.7. *Total uronic acids*

The total content of uronic acids was quantified colorimetrically according to the chromogen method phenylphenol or *m*-hydroxybiphenyl with some modification (Blumenkrantz and Asboe-Hansen, 1973). The concentration of uronic acid is proportional to the pectin content with remarkable biological activities (Fernández-Bolaños et al., 2004). 200 μL of sample were put in test tubes in triplicate and placed on an ice bath. They were mixed with 1.2 mL of 0.0125 M sodium tetraborate in concentrated sulfuric acid. Vortex tubes were shaken and heated at 100 °C for 5 min in a water bath. Once the tubes were cooled, 20 μL of 0.15% *m*-hydroxybiphenyl in 0.5 % NaOH. A sensitive and specific reagent of the anhydrides of the uronic acids, were added. The color developed by vigorous agitation was measured at 520 nm in the microplate reader. Galacturonic acid was used as the external calibration standard. A calibration curve was prepared with the absorbance values of solutions of known concentration (from 0.02 to 0.2 mg/ mL).

2.8. *Thermal treatments*

All the experiments in this work have been done combining the use of DEs with heat treatments. Three types of tests were planned. In the first test, different DES were tested at low temperatures, up to 90 °C. A second test was carried out in which, two of the best DES, according to the results obtained, were combined with thermal treatments up to 180 °C. Finally, a test was done at the optimum temperature to determine the effectiveness of DES against its components separately. The variables to be studied, apart from the DES were chosen according to previous studies carried out to extract bioactive compounds from olive oil by-products based on maximum solubilization of hydroxytyrosol and soluble sugars using the industrial thermal technologies (Fernández Bolaños et al., 2002, 2004, 2010):

Temperatures: 25 °C, 50 °C, 90 °C, 120 °C, 150 °C and 180 °C.

Time: two samples every 30 min for 2 h for temperatures of 25 °C–90 °C; 60 min for 120 °C, 15 min for 150 °C and 5 min for 180 °C.

Solvent ratio: sample/solvent ratios (w/v): 1: 1 and 2: 1.

100 g of weed alperujo were used in duplicate plus the addition of water of DES for all the thermal treatments. After the treatments the samples were filtered through filter paper using a Buchner funnel for solid–liquid separation and the aqueous extract was recovered and stored at −20 °C until the analysis.

2.8.1. *Treatments by indirect heating in oil or water bath (25 °C, 50 °C, 90 °C and 150 °C)*

Thermostatic baths with water or oil PRECISBAT 6 rooms/Tanks 6,001,482 with a temperature control heater were used. The DES mixture with alperujo was introduced into glass vessels in the case of 25 °C, 50 °C and 90 °C and stainless steel bottles for the temperature of 150 °C. The agitation was carried out with rotary propeller agitators. In the case of treatments up to 90 °C, kinetics was performed for up to two hours every 30 min for the two studied relationships. In the case of the treatment at 150 °C, a time of 15 min was used for the previous experiment of the research group in the application of thermal pre-treatments for alperujo. All treatments up to 150 °C were carried out by indirect heating.

2.8.2. *Autoclave treatments to 120 °C*

DES mixtures and alperujo, together with the control were introduced into glass bottles numbered with the ratios 1:1 and 2:1 (w/v). They were placed in the autoclave for one hour at 120 °C. The samples were cooled and the phases were separated.

2.8.3. *Thermal pre-treatment with steam at 180 °C*

The hydrothermal treatment has been patented (Fernández-Bolaños et al., 2010) and was performed using a steam treatment reactor prototype that can operate at temperatures up to 190 °C and at a maximum pressure of 1.2 MPa. The heating of the alperujo was performed by the direct and indirect injection of steam. Only in this treatment an extra water was

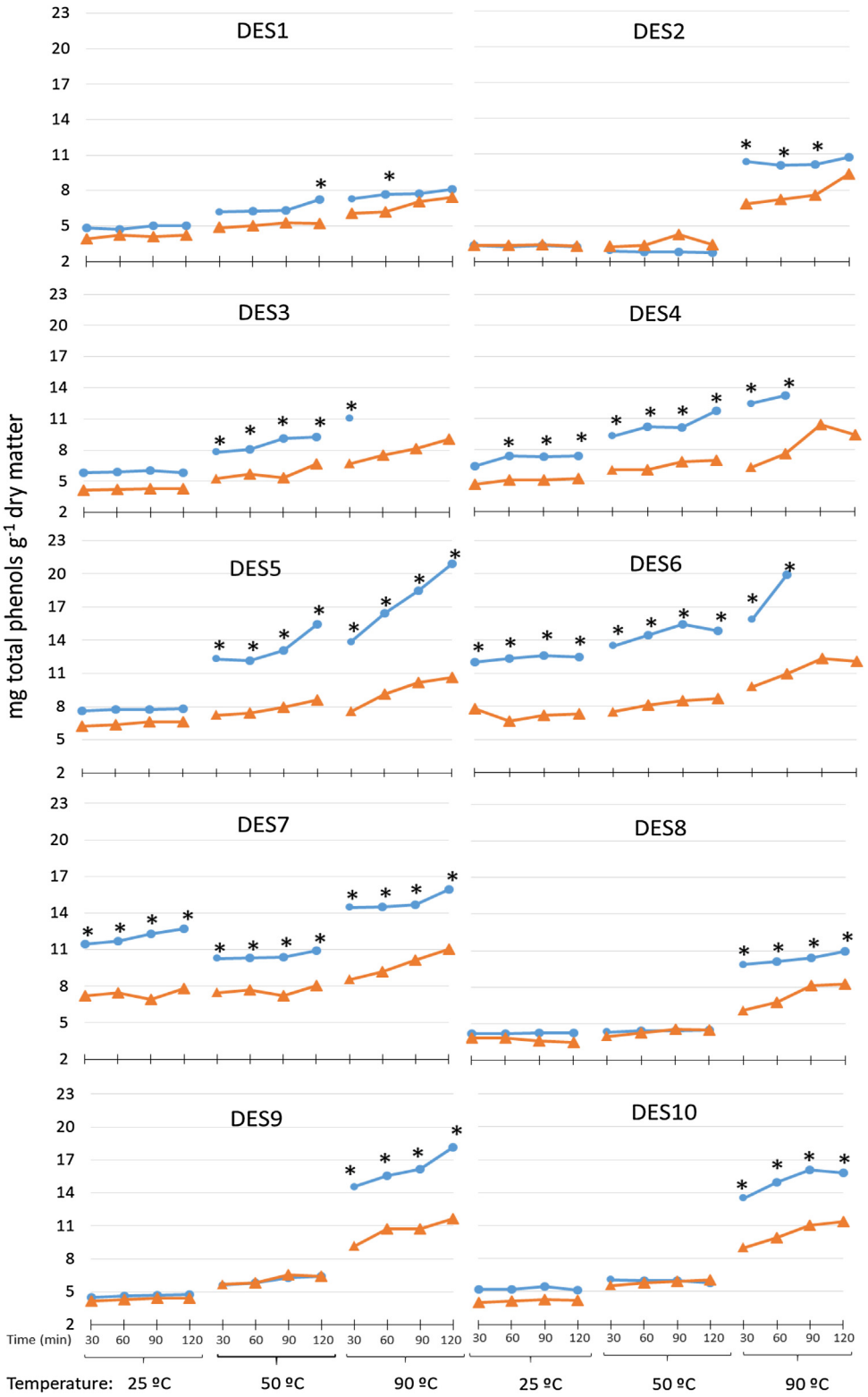

Fig. 1 – Colorimetric determination of total phenols by the Folin-Ciocalteu method of ten different DES (DES1 to DES10) at three temperatures (25, 50 and 90 °C) and four times (30, 60, 90 and 120 min) for the two ratios of Alperujo/DES studied (1:1 in circle and 2:1 in triangle). *Significant differences ($p < 0.05$).

added to the sample by the steam condensation in a relation sample/water of 1:5 (w/v) approximately. The use of direct steam in the steam explosion treatment can break the hydrogen bonds in the DES by the addition of extra water, unlike treatments with indirect heating. Therefore the effect of the addition of DES seems not to be due to the eutectic but to the components separately when the water addition is produced during the thermal treatment.

2.9. *Statistical analysis*

The results were expressed as mean values ± standard deviations. STATGRAPHICS® plus software was used for statistical analyses. Comparisons amongst samples were made using one-way analysis of variance (ANOVA) with Student's t test and the LSD method at the same confidence level. A p value of < 0.05 indicated statistically significant differences.

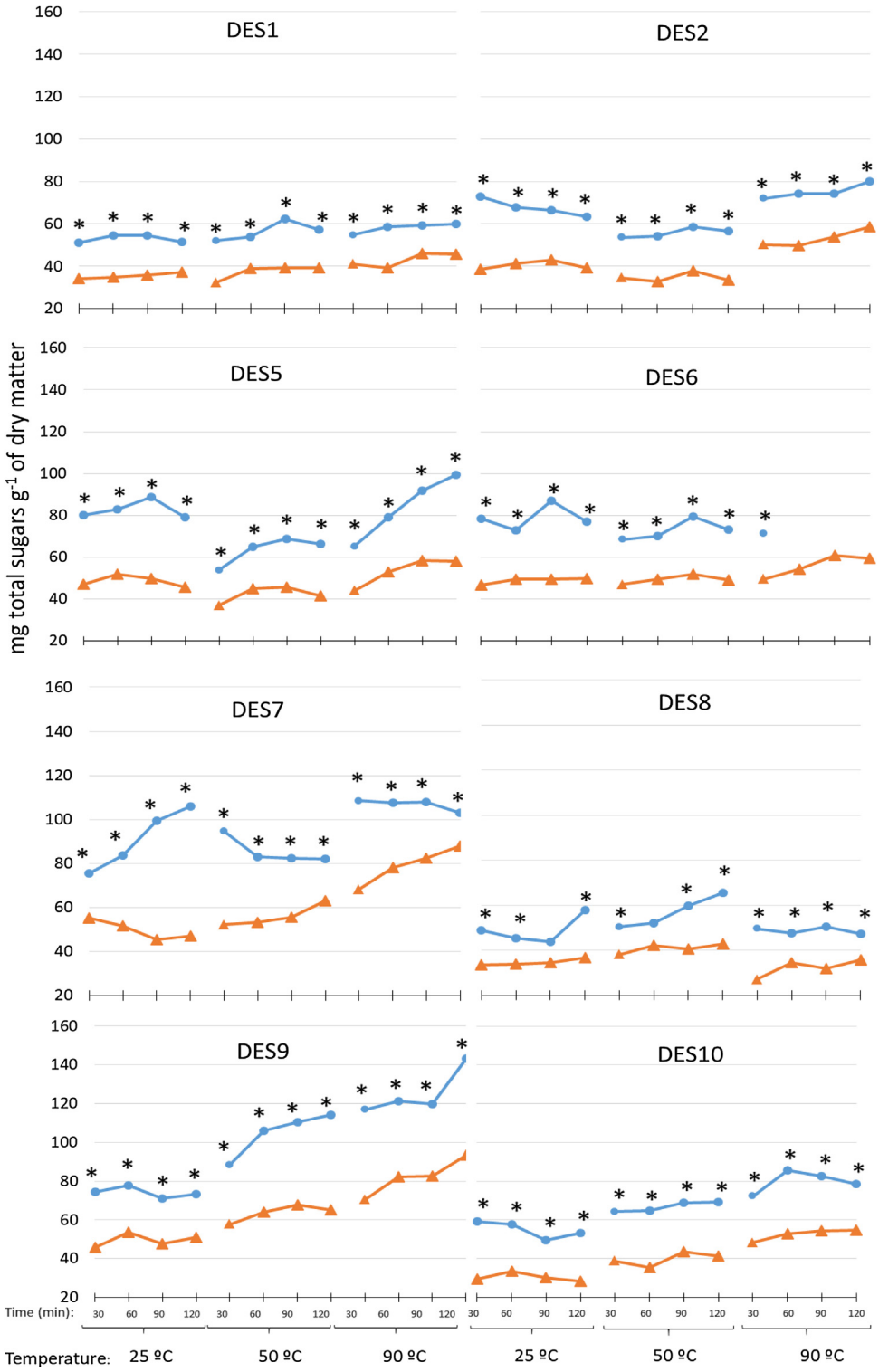

Fig. 2 – Colorimetric determination of total sugars by the Antrona method of eight different DES (DES1 to DES10) at three temperatures (25, 50 and 90 °C) and four times (30, 60, 90 and 120 min) for the two ratios of Alperujo/DES studied (1:1 in circle and 2:1 in triangle). *Significant differences ($p < 0.05$).

3. Results and discussion

Different DES were test in order to evaluate the best extraction of phenols and sugars at 25 °C, 50 °C and 90 °C (Figs. 1–3), using two ratios of alperujo:DES (1:1 and 2:1 (w/v)). The thermal stability of DES has been studied for different combinations, showing some authors (Wenjun et al., 2018) high range of degradation temperatures for the eutectic formed by choline chloride and sugars or alcohols (160 °C–260 °C and 119 °C–260 °C, respectively).

3.1. *Comparison of DES at lower temperatures*

3.1.1. *Total phenols*

The concentration of extracted total phenols is shown in Fig. 1 at different times (30, 60, 90, and 120 min) for the three chosen temperatures. In general, the concentration of extracted phenols increased for all DES with the time and the temperature. The quantification was not possible in some cases where the liquid-solid separation was not efficient. The best extractions of phenols were made in the range of 20 mg/g of dry mat-

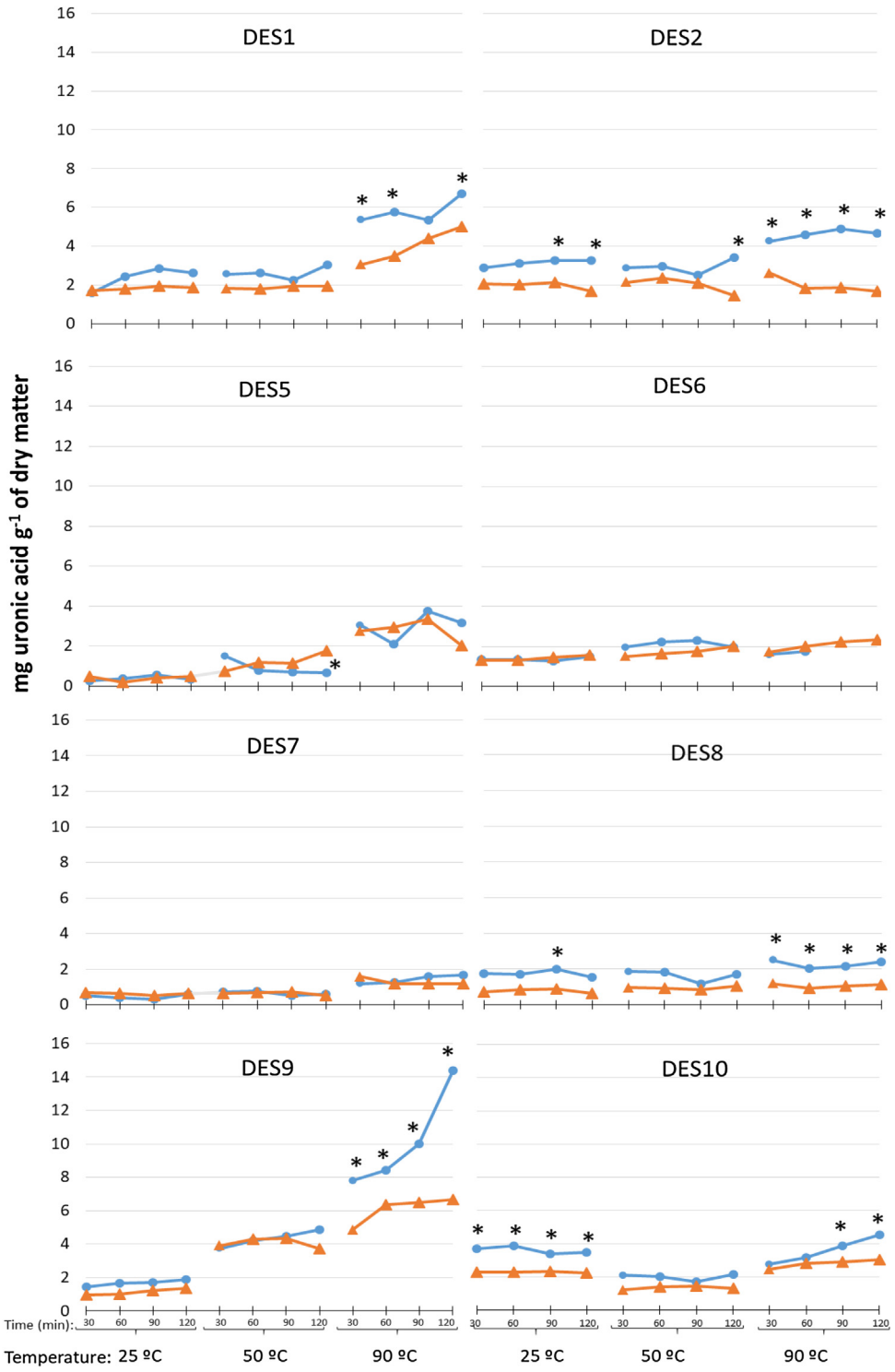

Fig. 3 – Colorimetric determination of the uronic acid solubilization of eight DES at three temperatures (25, 50 and 90 °C) at four different times (30, 60, 90 and 120 min) using two alperujo:DES ratios (1:1 in circle and 2:1 in triangle). *Significant differences ($p < 0.05$).

ter using DES5, DES6 and DES9, followed by DES7 and DES10 with 16 and 16.5 mg/g, and finally a group of DES with an average concentration of phenols between 11 and 13,5 mg/g, specifically solvents DES3, DES4 and DES8. The best extractions were made using the ratio of 1:1 for 90 °C in all solvents. The quantity of phenols extracted with DES4 and DES6 was high; although the difficult separation of the liquid and solid phase makes their industrial management more difficult.

The fact that great differences were seen between the results obtained from the eutectic solvents underlines the importance of the composition of DES, making the appropriate design of this kind of green solvents crucial.

A hydro alcoholic extraction commonly used for phenols, such as metanol:water can extract about 2.17 mg/g of total phenol from dry alperujo. This quantity is increased when the alperujo is thermally treated up to 11.4 mg/g using the industrial condition of 160 °C for 60 min (Rubio-Senent et al., 2012). The values obtained using DES5 and DES9 at 90 °C are higher than those reported by these authors, who found that

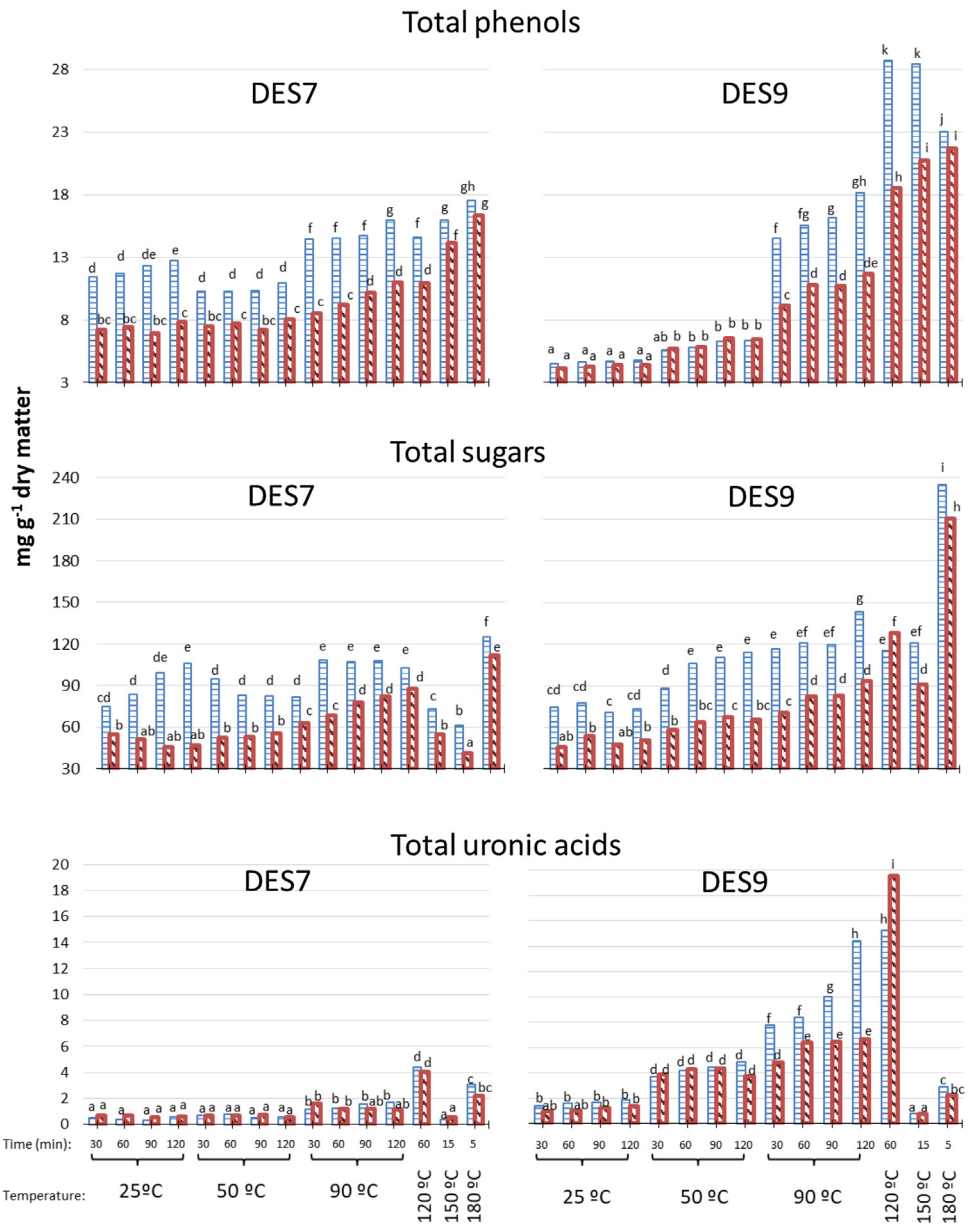

Fig. 4 – Total phenols, total sugars and total acid sugars extracted by DES7 and DES9 in all treatments using two alperujo:DES ratios (1:1 horizontal lines and 2:1 oblique lines). Different letters indicate significant differences ($p < 0.05$).

their use could help to diminish the industrial temperature for extracting a higher amount of total phenols.

3.1.2. *Total sugars*

The total sugar concentrations are shown in Fig. 2 for all the DES used, except the solvents whose compositions included sugars in high proportions (DES3 and DES4), a fact that makes it impossible to determine the solubilized sugars.

The best results for the solubilization of total sugars were achieved using DES7 and DES9 with values of 108.52 and 143.26 mg/g of dry matter at 90 °C, for the ratio 1:1, or 87.84 and 93.41 mg/g of dry matter at 90 °C for the ratio of 2:1, respectively. The best ratio found was 1/1, where the values for sugar were in some cases two times or even three times higher than the value obtained from the ratio 2/1.

The use of other solvents like DES1, DES6, DES8, DES5 and DES2 did not result in significant differences among the three temperatures; while the significant difference was only based on the ratio alperujo:DES. The use of DES6 at 90 °C for 90 and 120 min did not allow for solid/liquid separation, making its utilization in combination with higher temperatures difficult.

The solubilization of sugars increased slightly using DES5 and DES10, and values close to 100 mg/g of dry matter at 90 °C were obtained.

The final values for sugars are the balance between solubilization and degradation. Thermal treatment enhanced the solubilization of the hemicellulose from the cell wall material, but also increased the degradation of sugars, thus obtaining hydroxymethylfurfural or furfural, in addition to other compounds. This balance depends on the severity of the treatment and the use or not of acid or bases (Rodríguez et al., 2007a). For the temperatures used, the degradation is very low, but the solubilization of sugar seems to be in the same range or even higher than the sugars extracted by Fernández-Bolaños et al. (2004) using an alcoholic extraction of alperujo obtained at three different times during the season (70–130 mg/g of dry alperujo).

Table 2 – Individual phenolic compounds and 5-hydroxymethylfurfural (HMF) concentration in mg/g of dry alperujo (3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol 4-β-D-glucoside (Glu-HT), hydroxytyrosol (HT), tyrosol (Ty), verbascoside (Ve), vanillic acid (VA), vanillin (Va) and luteonin (Lu)) by HPLC-UV in the alperujo sample treated with water or with DES7 for the two relationship alperujo:DES. Different letters indicate significant differences ($p < 0.05$). Traces: concetration <0.1 mg/L, nd: non detected.

DES	Temp(C)	Time (min)	Relation Alp:DES	DHPG	Glu-HT	HT	Ty	Va	Ve	HMF	VA	Lu
Control Water	–	–	–	0.001 a	0.006 a	0.002 a	Traces	0.01 a	Traces	nd	Traces	0.0008
DES7	120	60	1:1	0.62 e	0.50 d	1.33 h	0.18 b	0.38 c	0.15 b	nd	0.06 a	0.02 a
			2:1	0.56 de	0.36 c	1.30 h	0.21 b	0.28 c	0.16 bc	nd	0.06 a	0.01 a
	150	15	1:1	1.01 g	0.35 c	0.33 c	0.10 b	2.06 ij	nd	nd	0.05 a	0.03 a
			2:1	0.35 c	0.43 cd	1.96 i	0.39 cd	1.53 h	nd	3.75 c	0.01 a	0.02 a
	180	5	1:1	1.19 f	3.38 k	2.74 jk	0.28 c	6.73 l	0.27 e	nd	0.12 b	0.50 i
			2:1	1.23 gh	2.54 j	2.66 j	0.30 c	5.36 l	0.23 d	nd	0.11 b	0.32 fg
	25	30	1:1	0.20 bc	0.27 c	0.14 b	0.10 b	Traces	0.12 b	nd	0.04 a	0.22 d
		60	1:1	0.21 c	0.42 d	0.17 b	0.07 ab	Traces	0.10 ab	nd	0.05 a	0.34 g
		90	1:1	0.22 c	0.50 d	0.17 bc	0.09 ab	Traces	0.10 ab	nd	0.06 a	0.40 h
		120	1:1	0.21 c	0.38 c	0.12 b	0.08 ab	Traces	0.08 a	nd	0.05 a	0.36 gh
		30	2:1	0.16 bc	0.30 c	0.17 b	0.03 a	Traces	0.06 a	nd	0.03 a	0.13 b
		60	2:1	0.19 bc	0.40 cd	0.23 c	0.03 a	Traces	0.07 a	nd	0.03 a	0.18 c
		90	2:1	0.20 c	0.41 d	0.20 b	0.05 a	Traces	0.06 a	nd	0.03 a	0.19 cd
		120	2:1	0.20 bc	0.39 c	0.13 b	0.05 a	Traces	0.04 a	nd	0.03 a	0.19 cd
	50	30	1:1	0.26 c	0.43 d	0.35 c	0.13 b	Traces	0.15 bc	nd	0.06 a	0.55 ij
		60	1:1	0.26 c	0.41 d	0.30 c	0.14 b	Traces	0.13 b	nd	0.05 a	0.58 j
		90	1:1	0.25 c	0.41 d	0.35 c	0.19 b	Traces	0.18 c	nd	0.05 a	0.65 k
		120	1:1	0.24 c	0.39 c	0.29 c	0.15 b	Traces	0.13 b	nd	0.05 a	0.47 i
		30	2:1	0.22 bc	0.36 c	0.41 d	0.04 a	Traces	0.17 c	nd	0.04 a	0.33 g
		60	2:1	0.23 c	0.38 c	0.44 d	0.03 a	Traces	0.18 c	nd	0.04 a	0.35 gh
		90	2:1	0.22 c	0.36 c	0.38 cd	0.05 a	Traces	0.16 bc	nd	0.04 a	0.33 g
		120	2:1	0.26 c	0.47 d	0.59 de	0.01 a	Traces	0.21 d	nd	0.04 a	0.45 i
	90	30	1:1	0.53 d	0.43 d	0.37 c	0.26 c	Traces	0.14 b	nd	0.07 a	0.63 jk
		60	1:1	0.29 c	0.36 c	0.32 c	0.26 c	Traces	0.10 ab	nd	0.04 a	0.41 hi
		90	1:1	0.41 cd	0.40 c	0.51 d	0.47 d	Traces	0.16 bc	nd	0.06 a	0.69 k
		120	1:1	0.26 c	0.29 bc	0.32 c	0.27 c	Traces	0.09 ab	nd	0.03 a	0.32 fg
		30	2:1	0.31 c	0.33 c	0.33 c	0.05 a	Traces	0.11 b	nd	0.01 a	0.31 f
		60	2:1	0.40 cd	0.34 c	0.45 d	0.10 ab	Traces	0.16 bc	nd	0.01 a	0.42 hi
		90	2:1	0.33 c	0.34 c	0.37 c	0.08 a	Traces	0.11 b	nd	0.01 a	0.34 g
		120	2:1	0.42 d	0.34 c	0.50 d	0.15 b	Traces	0.15 bc	nd	0.01 a	0.35 g
	120	60	1:1	0.30 c	0.57 de	1.05 g	0.46 d	0.30 c	0.32 fg	nd	0.04 a	0.02 a
			2:1	0.37 c	0.51 d	0.88 f	0.34 c	0.28 c	0.24 de	nd	0.01 a	0.01 a
	150	15	1:1	0.30 c	0.41 d	1.51 hi	0.59 de	3.48 k	nd	2.13 b	0.01 a	0.02 a
			2:1	0.29 c	0.30 c	2.48 j	0.56 d	2.16 j	nd	4.65 d	Traces	0.01 a
	180	5	1:1	1.32 h	3.80 k	2.81 k	0.40 c	7.33 l	0.33 g	nd	0.13 b	0.53 j
			2:1	1.25 gh	2.62 j	2.70 k	0.44 d	6.07 l	0.23 de	nd	0.12 b	0.34 gh

3.1.3. *Uronic acids*

The analysis of uronic acid showed that the acid sugars were solubilized by the DES. The total uronic acid is shown in Fig. 3 for all samples except the DES that contain sugars (DES3 and DES4) because of the difficulty of the determination of initial sugars under these concentrations.

The best solvent for extracting uronic acid was by far DES9 where the concentration of 14.4 and 6.67 mg/g for the alperujo:DES ratios 1:1 and 2:1 were found, respectively. The acid sugar concentration increased with temperature and time, mainly at 90 °C, when there was a significant difference between the ratios of DES used.

The values obtained for DES1 did not change for 25 °C or 50 °C, increasing significantly at 90 °C, when the values found for the two ratios were also significantly different, reaching maximums of 6.69 and 4.99 mg/g for the ratios 2:1 and 1:1, respectively. DES2 showed a similar behavior, where the highest solubilization of uronic acids was at 90 °C using the 1:1 ratio (4.86 mg/g). For DES5 the concentration of acid sugars increased with the temperature and the ratio alperujo:DES, obtaining the highest values at 90 °C for the ratios 1:1 and 2:1 of 3.75 and 3.32 mg/g, respectively.

There were no differences in the uronic concentration among the three temperatures using DES6, DES7, DES8 and DES10, neither for the two alperujo:DES ratios in the cases of (DES6 and DES7).

Remarkable values were found using DES9, which were even three times higher than those reported by other authors using conditions like 5 mg/g at 160 °C for 30 min without DES (Rubio-Senent et al., 2015).

3.2. *Study of selected DES at higher temperatures*

3.2.1. *Total phenols and sugars*

Based on the comparison of the solubilization of phenols, total sugars and total uronic acid, the solvents DES7 and DES9 were chosen to be combined with the thermal treatments at higher temperatures. The conditions were 120 °C, 150 °C and 180 °C for 60, 15 and 5 min, respectively, with the two last conditions being real alternatives for the utilization of alperujo in the industry. DES9 is an acid solvent that showed the best solubilization of sugars, and one of the best for phenol extraction. DES7 is an alcoholic solvent which showed a promising solubilization of total sugars and phenols.

Table 3 – Individual phenolic compounds and 5-hydroxymethylfurfural (HMF) concentration in mg/g of dry alperujo (3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol 4-β-D-glucoside (Glu-HT), hydroxytyrosol (HT), tyrosol (Ty), verbascoside (Ve), vanillic acid (VA), vanillin (Va) and luteonin (Lu)) by HPLC-UV in the alperujo sample treated with water or with DES9 for the two relationship alperujo:DES. Different letters indicate significant differences ($p < 0.05$).

DES	Temp(C)	Time (min)	Relation Alp:DES	DHPG	Glu-HT	HT	Ty	Va	Ve	HMF	VA	Lu
DES9		30	1:1	0.20 c	0.58 d	0.77 e	nd	1.68 i	0.27 ef	nd	0.07 a	0.48 i
		60	1:1	0.51 d	0.73 e	1.37h	nd	0.84 f	0.20 dc	nd	0.10 b	0.29 f
		90	1:1	0.54 d	0.74 e	1.48 hi	0.08 a	0.86 f	0.26 e	nd	0.11 b	0.31 fg
		120	1:1	0.56 d	0.74 e	1.50 i	0.10 ab	0.91 fg	0.21 d	nd	0.11 b	0.32 fg
	25	30	2:1	0.31 c	0.15 b	1.74 i	0.17 b	0.91 fg	0.23 de	nd	0.07 a	0.22 d
		60	2:1	0.33 c	0.45 d	0.97 g	0.08 a	0.55 d	0.02 a	nd	0.07 a	0.13 b
		90	2:1	0.38 c	0.46 d	1.01 g	0.09 a	0.56 d	0.14 b	nd	0.07 a	0.14 b
		120	2:1	0.39 c	0.45 d	0.98 g	0.08 a	0.56 d	0.14 b	nd	0.06 a	0.13 b
		30	1:1	0.71 e	0.69 e	0.70 e	0.02 a	0.74 e	0.08 a	nd	0.08 a	0.35 gh
		60	1:1	0.54 d	0.64 e	0.59 d	0.01 a	0.61 de	0.08 a	nd	0.07 a	0.28 ef
		90	1:1	0.69 e	0.65 e	0.73 e	0.08 a	0.63 de	0.10 ab	nd	0.08 ab	0.32 fg
		120	1:1	0.84 ef	0.73 e	0.98 g	0.21 bc	0.71 e	0.12 b	0.33 a	0.10 b	0.44 i
	50	30	2:1	0.45 d	0.40 cd	0.95 g	0.07 a	0.65 e	0.13 b	nd	0.06 a	0.18 c
		60	2:1	0.58 d	0.42 d	1.19 gh	0.12 ab	0.75 e	0.12 b	nd	0.06 a	0.23 de
		90	2:1	0.41 cd	0.40 cd	0.91 g	0.07 a	0.57 de	0.11 ab	nd	0.05 a	0.17 c
		120	2:1	0.45 d	0.39 c	1.01 g	0.09 a	0.57 d	0.14 b	nd	0.05 a	0.16 bc
		30	1:1	0.53 d	0.84 f	1.95 ij	0.37 c	nd	0.31 fg	2.1 b	0.11 b	0.42 hi
		60	1:1	0.37 c	0.86 f	2.17 j	0.60 e	nd	0.23 de	10.50 e	0.12 b	0.42 hi
		90	1:1	0.42 cd	0.90 f	2.95 k	0.93 fg	nd	0.17 c	27.34 g	0.16 c	0.47 i
		120	1:1	0.36 c	0.58 de	2.30 j	0.77 e	nd	0.07 a	26.41 fg	0.12 b	0.31 fg
	90	30	2:1	0.47 d	0.53 d	1.28 h	0.14 b	0.62 e	0.20 cd	nd	0.06 a	0.21 d
		60	2:1	0.49 d	0.53 d	1.43 h	0.25 c	0.50 d	0.26 e	nd	0.06 a	0.22 d
		90	2:1	0.49 d	0.58 d	1.80 i	0.42 d	0.51 d	0.29 f	1.50 ab	0.08 a	0.27 ef
		120	2:1	0.36 c	0.47 d	1.43 h	0.36 c	0.41 cd	0.20 d	1.96 ab	0.06 a	0.20 cd
	120	60	1:1	0.52 d	0.30 c	26.61 n	2.01 i	0.52 d	0.23 d	29.74 g	0.16 c	0.17 c
			2:1	0.80 ef	0.24 bc	10.24 l	1.46 h	0.75 e	0.24 de	23.86 fg	0.09 a	0.13 b
	150	15	1:1	0.45 d	0.49 d	85.81 q	2.28 j	6.65 kl	nd	93.90 j	0.02 a	0.10 ab
			2:1	0.41 cd	0.34 c	61.93 p	1.60 hi	4.85 k	nd	77.53 ij	0.01 a	0.08 a
	180	5	1:1	1.42 h	nd	28.26 n	2.27 ij	nd	nd	47.26 h	0.17 c	0.56 j
			2:1	1.36 h	nd	18.49 m	2.37 j	nd	nd	41.71 h	0.17 c	0.43 i

Traces: concetration <0.1 mg/L, nd: non detected.

After the thermal treatments, each liquid phase was analyzed to determine the total and individual phenols, the total sugars and the total uronic acid (Fig. 4). The extraction of total phenols at temperatures lower than 120 °C was higher using DES7. At higher temperatures, the use of DES9 led to extract values for total phenols of 28.69 mg/g at 120 °C at a ratio of 1:1, and 21.67 mg/g at 180 °C and a ratio 2:1. The maximum values obtained with DES7 were 17.51 mg/g and 16.31 mg/g at 180 °C and ratios of 1:1 and 2:1, respectively. The use of higher temperatures helps DES extract phenols. The most important finding was the high solubilization achieved at 120 °C was higher than that obtained at 180 °C, meaning the use of DES helps to reduce the temperature to extract a similar or even larger amount of these bioactive compounds. The industrial solubilization of total phenols has been quantified at 11.40 mg/g as mentioned above, which is less than half the values obtained in the present work for DES9 at 120 °C.

With regard to the solubilization of total sugars the use of DES7 increased the concentration up to 120 °C, falling after 120 °C and finally increasing at 180 °C, when the maximum was obtained (125.17 mg/g and 111.72 mg/g for the ratios 1:1 and 2:1, respectively). The behavior of DES9 was different, although the maximum was also found at 180 °C (234.67 mg/g and 210.45 mg/g for the ratios 1:1 and 2:1, respectively). In general, the extraction of sugars was higher using DES9. These values are higher than those reported by other authors at the same thermal condition at 180 °C without DES, with 130 mg/g for untreated alperujo, and 141 mg/g for alperujo treated at 180 °C (Fernández-Bolaños et al., 2004). Besides the degradation of sugar increase with temperature, the maximum was obtained at the highest temperature because the reaction time was lower in comparison with the other treatments at lower temperatures. Thus, the use of DES improved significantly the solubilization of total sugars from the hemicellulosic composition present in the cell wall in combination with the thermal treatment.

The uronic acid extraction was also significantly higher using the solvent DES9. In both solvents the concentration of acid sugars increased with the temperature up to 120 °C, obtaining a maximum for DES7 of 4.42 and 4.06 mg/g, and for DES9 of 15.21 mg/g and 19.52 mg/g for the ratios 1:1 and 2:1, respectively. These values are also higher than the concentration of acid sugars reported by other authors, without both DES and thermal treatment, of 5.4 mg/g (Rubio-Senent et al., 2015).

The best values at higher temperatures were obtained using DES9, and 120 °C was the temperature which produced the highest extraction of phenols and acid sugar with minimal differences between ratios 1:1 or 1:2.

3.2.2. *Individual phenols*

The composition of individual phenols was shown and the use of water with DES7 (Table 2) and DES9 (Table 3) as extracting agents in combination with thermal treatments was compared. The control was measured as a methanolic extraction of the alperujo without thermal treatment. The DHPG concentration increased with temperature. In the case of DES7, the DHPG remained constant between 25 °C and 50 °C, increasing at 90

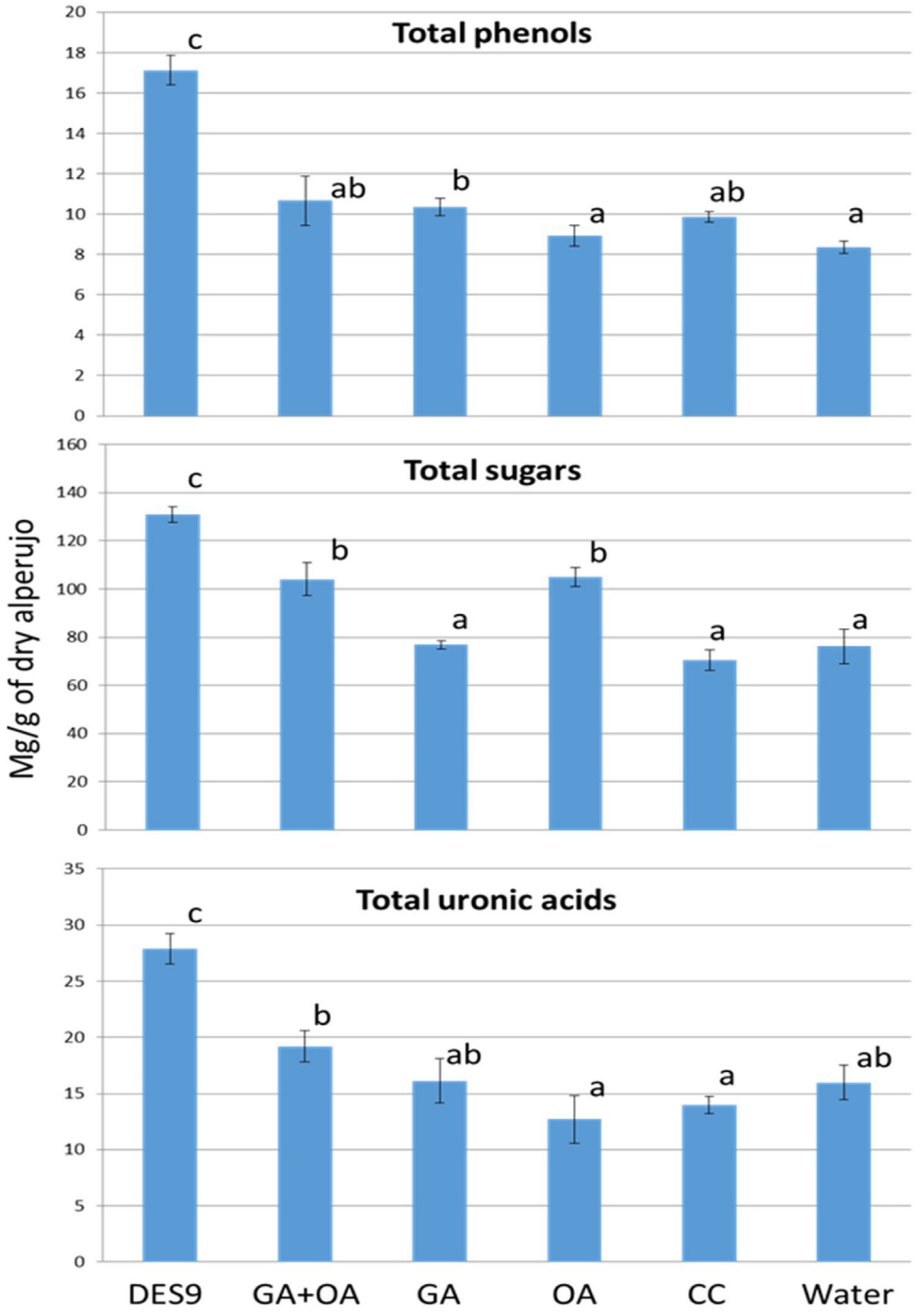

Fig. 5 – Total phenols, total sugars and total acid sugars extracted by DES9, its components (glycolic acid (GA), oxalic acid (OA) and choline chloride (CC)), the mixture of the two acids (GA + OA) and water at 120 °C in the ratio of 1:1 (DES or component:alperujo). Different letters indicate significant differences ($p < 0.05$).

°C and at 180 °C, obtaining similar values than those obtained with water. The best solubilization of DHPG was achieved using DES9 at 180 °C, to obtain 1.42 mg/g, which is higher than the control or the value obtained with DES7 (1.23 and 1.31 mg/g, respectively). The use of DES9 led to an increase in the concentration of DHPG (1.42 mg/g) at 180 °C over the values obtained with water and DES7. The concentration of HT was similar when water and DES7 were used; and the extraction with DES9 was remarkable, where the maximum values were achieved at 150 °C (85.81and 61.93 mg/g for 1:1 and 1:2 relationships, respectively). The acidic nature of DES9 enhanced the hydrolysis of HT precursors, thus increasing the final concentration of this phenol significantly. The high concentration of HMF indicated the degradation of sugars at higher temperatures. The increment in vanillin and vanillic acid at 180 °C with water as a degradation product of ligning (Brebu and Vasile, 2010) also indicated the severe increase when the DES was used because this increment was higher at 150 °C with DES than with water.

The extraction of tyrosol was very similar to the HT extraction; while the rest of the quantified phenols presented other kinds of behavior. In this way, the vanillin increased with the temperature in the case of DES7 at 120 °C over the values found using water. The use of DES9 decreased the concentration of vanillin with the temperature and the time. The maximum concentration of acid vanillic was achieved using DES9, fol-

lowing by DES7 and finally with water at 180 °C. The extraction of luteonin and its glucoside was higher using DES7.

In general, DES9 was the best for the extraction of total phenols from alperujo, obtaining 28.69 mg/g at 120 °C for the ratio of 1:1. This value was higher than the concentration of phenol raising by water or with any solvent at higher temperatures. The use of water solubilized 13.05 and 11.87 mg/g at 120 °C and 60 min for the ratios 1:1 and 1:2, respectively, or 17.08 and 16.19 mg/g at 180 °C and 60 min for the ratios 1:1 and 1:2, respectively. In the bibliography values of 5.4 mg/g for 15 min or 11.43 mg/g for 75 min, both at the same thermal condition at 160 °C without DES, were found (Rubio-Senent et al., 2012). On the other hand, the sum of all the individual phenols quantified using DES9 at 150 °C (189.8 and 146.8 mg/g for the ratios 1:1 and 1:2, respectively) and 180 °C (80.2 and 64.7 mg/g for the ratios 1:1 and 1:2, respectively) are higher and exceeded the values obtained calorimetrically as total phenols. This fact could be due to the presence of interferences that do not allow for accurate quantification by the Folin method (Rubio-Senent et al., 2012).

It is important to mention that the addition of water during the treatment at 180 °C should broke the hydrogen bonds of DES. Thus, it seems the effect must be due to individual components or their mixture but not due to the formation of DES.

3.3. *Test for the individual components of the DES*

The possible changes in properties of DES at high temperatures was evaluated by other authors, showing the DES formed by choline chloride with glycolic acid or oxalic acid a degradation temperature of 226 °C and 159 °C, respectively (Florindo et al., 2014). It is expected the use of oxalic and glycolic acids to form DES9 has a higher degradation temperature that the lower one, the oxalic acid. Other authors also showed the use of oxalic acid in DES should be at temperatures lower than the onset temperature of 134 °C in which a degradation compounds start to be produced (Haz et al., 2016). To evaluate the possible changes in properties of DES9 at 120 °C using indirect heating and to establish that the effects are due to the formation of the eutectic, but not its individual constituents, the following test was carried out. A temperature of 120 °C was chosen and the concentrations of total sugars, phenols and uronic acid were measured. Each component (glycolic acid, oxalic acid and choline chloride), and the mixture of the two acids (glycolic and oxalic acids) were solved in water and added to the alperujo sample in the ratio of 1:1 (w/v). The results are shown in Fig. 5, where the effect of the extraction of the constituents of DES9 (choline, glycolic acid and oxalic acid) was compared with the eutectic and with the effect of the mixture of the two acids.

The extraction of total phenols showed that the maximum concentration was obtained with the eutectic while there were no significant differences between each component and the mixture of the two acids, with the smallest extraction being carried out with water.

The extraction of total sugars also showed the best result for the eutectic, followed by the mixture of the acids and the oxalic acid, obtaining the lowest concentration when the rest of the components were used.

In the case of the uronic acid, the best result was obtained once again with the eutectic followed by the mixture of the two acids, being no significant differences in the concentration obtained using the rest of the components.

Thus, the effectiveness of the eutectic is due to the formation of these solvents but not for the individual components when the thermal treatments were carried out by indirect heating.

4. Conclusion

The best results for the extraction of phenols and sugars were achieved by DES9 formed by choline, glycolic acid and oxalic acid. The use of DES9 allows for the same or even a better solubilization of total sugars and total phenols at 120 °C than the treatment at 180 °C, increasing the concentration of acid sugars by six times and the concentration of hydroxytyrosol by 30 times. In this way the use of this kind of green solvent allows to reduce the temperature in the thermal treatments that are used industrially nowadays for the valorization of alperujo. The activity of DES9 is not due to the activity of each component separately, nor to the mixture of the two acids, but to the eutectic mixture of all of them for the indirect heating treatments. The future of these solvent should be focused on the use of components obtained from by-products to also treat by-products. Further studies will be necessary to assess the application of DES to improve the extraction of other thermosensitive phenols, or to be used directly as a carrier of the bioactive compounds solubilized, improving, besides others, their own bioavailability. In the same way, further analysis to determine the possible thermal degradation and the final structure of the DES will be necessary.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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