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**Title: 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 ºC to 120 ºC. 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.

**Keywords:** deep eutectic solvent; phenols; hydroxytyrosol; olive oil; by-product.

## 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. 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

49 the biorefinery application for the utilization of this by-product. Recently a new
50 alternative technology based on a thermal treatment with water steam at high pressure
51 and temperature has been developed, tested on a pilot plant scale and already
52 implemented at an industrial level, which achieves the separation of phases, making it
53 possible to use its components (cellulose, hemicellulose, pectic substances and lignin)
54 (Fernández-Bolaños et al., 1999, 2001). Bioactive compounds are also solubilized,
55 making them easy to isolate and recover (Rodríguez et al., 2017a, 2017b). Thus, the
56 recovery of high value-added products such as hydroxytyrosol, 3,4-
57 dihydroxyphenylglycol, mannitol, hemicellulosic polysaccharides, xylan type and
58 oligosaccharides with functional properties became possible (Fernández-Bolaños et al.,
59 2001, 2002, 2004). In addition, the thermal treatment produces a reduction in the solid
60 phase with a high content of oil which is rich in minor components (Lama-Muñoz et al.,
61 2011).

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

66 Recently the effect of DES has been tested on olive oil, where its capacity for the
67 extraction of phenolic compounds compared to the classical method of methanol / water
68 extraction has been demonstrated (García et al., 2016). The use of these new solvents
69 seems to be aimed at achieving not only an environmental improvement but
70 technological as well, since it can be used in conjunction with other treatments to
71 improve the results or even to reduce the energy requirements of the systems in which it
72 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 ºC 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

122 solubilization of the components and thus favor the interaction of hydrogen bond donors
123 and acceptors.
124
125 **2.4. Colorimetric method of determination of total phenols.**
126 Total phenolic content was determined by the Folin-Ciocalteu spectrophotometric
127 method with some modifications (Obied et al., 2005). For all samples 5 µL of sample
128 wereas placed in a test tube and mixed with 250 µL of Folin-Ciocalteu reagent and 200
129 µL of a 20% sodium carbonate solution in water (weight/volume (w/v)). The mixture
130 was stirred and allowed to react for 10 minutes and centrifuged to remove the pellet.
131 The supernatant was used to measure the absorbance at 655 nm. The results were
132 expressed as grams of gallic acid equivalents. A calibration curve was made with a set
133 of known concentrations of a gallic acid standard.
134
135 **2.5. Analysis of individual phenols**
136 In order to identify the phenolic compounds, a high-performance liquid chromatography
137 (Hewlett-Packard model 1100, Palo Alto, CA, USA) was used. This instrument was
138 equipped with an array detector monitoring at 254, 280 and 340 nm wavelength and a
139 C18 reverse-phase column (Spherisorb ODS-2; 250 x 4.6 mm i.d. and 5 µm particle
140 size) supplied by Teknokroma Tracer Extrasil OSD2 (Barcelona, Spain). The
141 temperature of the column was kept constant at 25 °C with a C18 guard column. All
142 samples treated with hydrothermal treatments were filtered through 0.45 µm pore size
143 filters and a volume of 20 µL and each sample was injected into the HPLC instrument at
144 a flow rate of 1 mL/min. The analytical method was carry out using a linear gradient of
145 two eluents: solvent A (Milli-Q water, pH 2.5 adjusted with 0.01% of trifluoroacetic
146 acid) and solvent B (acetonitrile), using the following gradient over a total run time of

147 55 min: 95% A and 5% B initially, 75% A and 25% B in 30 min, 50% A and 50% B in
148 45 min, 0% A and 100% B in 47 min, 75% A and 25% B in 95 min, and 95% A and 5%
149 B in 52 min until completion of the run. Quantification was carried out by integration of
150 the peaks at different wavelengths in function of the compounds, with reference to
151 calibrations made using external standards.
152
153 **2.6. Total Sugars**
154 The total content of neutral sugars was determined colorimetrically by the Antrona
155 method (Dische, 1962) using a spectrophotometer (BIO-RAD imark Microplate Reader,
156 USA). An aliquot of the 100 µL sample (in triplicate) was suitably diluted and 200 µL
157 of 0.2% (w/v) Antrone solution in concentrated sulfuric acid were added. The test tubes
158 were shaken in a Vortex and heated for 5 minutes at 100 °C in a water bath. When the
159 tubes were cooled in an ice bath, the absorbance at 630 nm was measured. Glucose was
160 used as an external standard to prepare a calibration with aqueous solutions in a
161 concentration range of 0.02-0.2 mg/mL.
162
163 **2.7. Total Uronic Acids**
164 The total content of uronic acids was quantified colorimetrically according to the
165 chromogen method phenylphenol or m-hydroxybiphenyl with some modification
166 (Blumenkrantz, and Asboe-Hansen, 1973). The concentration of uronic acid is
167 proportional to the pectin content with remarkable biological activities (Fernández-
168 Bolaños et al., 2004). 200 µL of sample were put in test tubes in triplicate and placed on
169 an ice bath. They were mixed with 1.2 mL of 0.0125 M sodium tetraborate in
170 concentrated sulfuric acid. Vortex tubes were shaken and heated at 100 °C for 5 minutes
171 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 ºC. 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 ºC, 50 ºC, 90 ºC, 120 ºC, 150 ºC and 180 ºC.

Time: two samples every 30 minutes for 2 hours for temperatures of 25 ºC to 90 ºC; 60 minutes for 120 ºC, 15 minutes for 150 ºC and 5 minutes for 180 ºC.

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

195 paper using a Buchner funnel for solid-liquid separation and the aqueous extract was
196 recovered and stored at – 20 ºC until the analysis.

197

198 2.8.1 Treatments by indirect heating in oil or water bath (25 ºC, 50 ºC, 90 ºC and 150
199 $^{o}$C).

200 Thermostatic baths with water or oil (PRECISBAT 6 rooms/Tanks 6001482) with a
201 temperature control heater were used. The DES mixture with alperujo was introduced
202 into glass vessels in the case of 25 ºC, 50 ºC and 90 ºC and stainless steel bottles for the
203 temperature of 150 ºC. The agitation was carried out with rotary propeller agitators. In
204 the case of treatments up to 90 ºC, kinetics was performed for up to two hours every 30
205 minutes for the two studied relationships. In the case of the treatment at 150 °C, a time
206 of 15 minutes was used for the previous experiment of the research group in the
207 application of thermal pre-treatments for alperujo. All treatments up to 150 ºC were
208 carried out by indirect heating.

209

210 2.8.2 Autoclave treatments to 120 $^{o}$C

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

214

215 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 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.

## 3. RESULTS AND DICUSSION

Different DES were test in order to evaluate the best extraction of phenols and sugars at 25 ºC, 50 ºC and 90 ºC (**Figures 1, 2** and **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 ºC-260 ºC and 119 ºC-260 ºC, respectively).

240 **3.1. Comparison of DES at lower temperatures.**

241 3.1.1. Total phenols.

242 The concentration of extracted total phenols is shown in **Figure 1** at different times (30,
243 60, 90, and 120 minutes) for the three chosen temperatures. In general, the
244 concentration of extracted phenols increased for all DES with the time and the
245 temperature. The quantification was not possible in some cases where the liquid-solid
246 separation was not efficient. The best extractions of phenols were made in the range of
247 20 mg/g of dry matter using DES5, DES6 and DES9, followed by DES7 and DES10
248 with 16 and 16.5 mg/g, and finally a group of DES with an average concentration of
249 phenols between 11 and 13,5 mg/g, specifically solvents DES3, DES4 and DES8. The
250 best extractions were made using the ratio of 1:1 for 90 ºC in all solvents. The quantity
251 of phenols extracted with DES4 and DES6 was high; although the difficult separation of
252 the liquid and solid phase makes their industrial management more difficult.

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

256 A hydro alcoholic extraction commonly used for phenols, such as metanol:water can
257 extract about 2.17 mg/g of total phenol from dry alperujo. This quantity is increased
258 when the alperujo is thermally treated up to 11.4 mg/g using the industrial condition of
259 160 ºC for 60 minutes (Rubio-Senent et al., 2012). The values obtained using DES5 and
260 DES9 at 90 ºC are higher than those reported by these authors, who found that their use
261 could help to diminish the industrial temperature for extracting a higher amount of total
262 phenols.

263

### 3.1.2. Total sugars.

The total sugar concentrations are shown in **Figure 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 ºC, for the ratio 1:1, or 87.84 and 93.41 mg/g of dry matter at 90 ºC 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 ºC for 90 and 120 minutes 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 ºC 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

287 extracted by Fernández-Bolaños et al. (2004) using an alcoholic extraction of alperujo
288 obtained at three different times during the season (70-130 mg/g of dry alperujo).

289

290 **3.1.3. Uronic acids**

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

295 The best solvent for extracting uronic acid was by far DES9 where the concentration of
296 14.4 and 6.67 mg/g for the alperujo:DES ratios 1:1 and 2:1 were found, respectively.
297 The acid sugar concentration increased with temperature and time, mainly at 90 ºC,
298 when there was a significant difference between the ratios of DES used.

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

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

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

312

313 **3.2. Study of selected DES at higher temperatures.**

314

315 3.2.1. Total phenols and sugars

316

317 Based on the comparison of the solubilization of phenols, total sugars and total uronic
318 acid, the solvents DES7 and DES9 were chosen to be combined with the thermal
319 treatments at higher temperatures. The conditions were 120 ºC, 150 ºC and 180 ºC for
320 60, 15 and 5 minutes, respectively, with the two last conditions being real alternatives
321 for the utilization of alperujo in the industry. DES9 is an acid solvent that showed the
322 best solubilization of sugars, and one of the best for phenol extraction. DES7 is an
323 alcoholic solvent which showed a promising solubilization of total sugars and phenols.
324 After the thermal treatments, each liquid phase was analyzed to determine the total and
325 individual phenols, the total sugars and the total uronic acid (**Figure 4**). The extraction
326 of total phenols at temperatures lower than 120 ºC was higher using DES7. At higher
327 temperatures, the use of DES9 led to extract values for total phenols of 28.69 mg/g at
328 120 ºC at a ratio of 1:1, and 21.67 mg/g at 180 ºC and a ratio 2:1. The maximum values
329 obtained with DES7 were 17.51 mg/g and 16.31 mg/g at 180 °C and ratios of 1:1 and
330 2:1, respectively. The use of higher temperatures helps DES extract phenols. The most
331 important finding was the high solubilization achieved at 120 ºC was higher than that
332 obtained at 180 ºC, meaning the use of DES helps to reduce the temperature to extract a
333 similar or even larger amount of these bioactive compounds. The industrial

334 solubilization of total phenols has been quantified at 11.40 mg/g as mentioned above,
335 which is less than half the values obtained in the present work for DES9 at 120 ºC.
336 With regard to the solubilization of total sugars the use of DES7 increased the
337 concentration up to 120 ºC, falling after 120 ºC and finally increasing at 180 ºC, when
338 the maximum was obtained (125.17 mg/g and 111.72 mg/g for the ratios 1:1 and 2:1,
339 respectively). The behavior of DES9 was different, although the maximum was also
340 found at 180 ºC (234.67 mg/g and 210.45 mg/g for the ratios 1:1 and 2:1, respectively).
341 In general, the extraction of sugars was higher using DES9. These values are higher
342 than those reported by other authors at the same thermal condition at 180 ºC without
343 DES, with 130 mg/g for untreated alperujo, and 141 mg/g for alperujo treated at 180 ºC
344 (Fernández-Bolaños et al., 2004). Besides the degradation of sugar increase with
345 temperature, the maximum was obtained at the highest temperature because the reaction
346 time was lower in comparison with the other treatments at lower temperatures. Thus, the
347 use of DES improved significantly the solubilization of total sugars from the
348 hemicellulosic composition present in the cell wall in combination with the thermal
349 treatment.
350 The uronic acid extraction was also significantly higher using the solvent DES9. In both
351 solvents the concentration of acid sugars increased with the temperature up to 120 ºC,
352 obtaining a maximum for DES7 of 4.42 and 4.06 mg/g, and for DES9 of 15.21 mg/g
353 and 19.52 mg/g for the ratios 1:1 and 2:1, respectively. These values are also higher
354 than the concentration of acid sugars reported by other authors, without both DES and
355 thermal treatment, of 5.4 mg/g (Rubio-Senent et al., 2015).
356 The best values at higher temperatures were obtained using DES9, and 120 ºC was the
357 temperature which produced the highest extraction of phenols and acid sugar with
358 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 ºC and 50 ºC, increasing at 90 ºC and at 180 ºC, obtaining similar values than those obtained with water. The best solubilization of DHPG was achieved using DES9 at 180 ºC, 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 ºC 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 ºC (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 ºC 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 ºC 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 ºC over the values found using water.

383 The use of DES9 decreased the concentration of vanillin with the temperature and the
384 time. The maximum concentration of acid vanillic was achieved using DES9, following
385 by DES7 and finally with water at 180 ºC. The extraction of luteonin and its glucoside
386 was higher using DES7.
387 In general, DES9 was the best for the extraction of total phenols from alperujo,
388 obtaining 28.69 mg/g at 120 ºC for the ratio of 1:1. This value was higher than the
389 concentration of phenol raising by water or with any solvent at higher temperatures. The
390 use of water solubilized 13.05 and 11.87 mg/g at 120 ºC and 60 minutes for the ratios
391 1:1 and 1:2, respectively, or 17.08 and 16.19 mg/g at 180 ºC and 60 minutes for the
392 ratios 1:1 and 1:2, respectively. In the bibliography values of 5.4 mg/g for 15 minutes or
393 11.43 mg/g for 75 minutes, both at the same thermal condition at 160 ºC without DES,
394 were found (Rubio-Senent et al., 2012). On the other hand, the sum of all the individual
395 phenols quantified using DES9 at 150 ºC (189.8 and 146.8 mg/g for the ratios 1:1 and
396 1:2, respectively) and 180 ºC (80.2 and 64.7mg/g for the ratios 1:1 and 1:2,
397 respectively) are higher and exceeded the values obtained calorimetrically as total
398 phenols. This fact could be due to the presence of interferences that do not allow for
399 accurate quantification by the Folin method (Rubio-Senent et al., 2012).

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

403 **3.3. Test for the individual components of the DES**

404 The possible changes in properties of DES at high temperatures was evaluated by other
405 authors, showing the DES formed by choline chloride with glycolic acid or oxalic acid a
406 degradation temperature of 226 ºC and 159 ºC, respectively (Florindo et al., 2014). It is

407 expected the use of oxalic and glycolic acids to form DES9 has a higher degradation
408 temperature that the lower one, the oxalic acid. Other authors also showed the use of
409 oxalic acid in DES should be at temperatures lower than the onset temperature of 134
410 ºC in which a degradation compounds start to be produced (Haz et al., 2016). To
411 evaluate the possible changes in properties of DES9 at 120 ºC using indirect heating and
412 to establish that the effects are due to the formation of the eutectic, but not its individual
413 constituents, the following test was carried out. A temperature of 120 ºC was chosen
414 and the concentrations of total sugars, phenols and uronic acid were measured. Each
415 component (glycolic acid, oxalic acid and choline chloride), and the mixture of the two
416 acids (glycolic and oxalic acids) were solved in water and added to the alperujo sample
417 in the ratio of 1:1 (w/v). The results are shown in **Figure 5**, where the effect of the
418 extraction of the constituents of DES9 (choline, glycolic acid and oxalic acid) was
419 compared with the eutectic and with the effect of the mixture of the two acids.

420 The extraction of total phenols showed that the maximum concentration was obtained
421 with the eutectic while there were no significant differences between each component
422 and the mixture of the two acids, with the smallest extraction being carried out with
423 water.
424 The extraction of total sugars also showed the best result for the eutectic, followed by
425 the mixture of the acids and the oxalic acid, obtaining the lowest concentration when the
426 rest of the components were used.
427 In the case of the uronic acid, the best result was obtained once again with the eutectic
428 followed by the mixture of the two acids, being no significant differences in the
429 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 ºC than the treatment at 180 ºC, 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 thermo-sensitive 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.

## DECLARATIONOFINTEREST

No conflict of interest exists in the submission of this manuscript.

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551 **Table 1.** Composition of the DES used in combination with thermal treatment to extract
552 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 |

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569 **Table 2**. Individual phenolic compounds and 5-hydroxymethylfurfural (HMF) concentration in mg/g of dry
570 alperujo (3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol 4-β-d-glucoside (Glu-HT), hydroxytyrosol
571 (HT), tyrosol (Ty), verbascoside (Ve), vanillic acid (VA), vanillin (Va) and luteonin (Lu)) by HPLC-UV in
572 the alperujo sample treated with water or with DES7 for the two relationship alperujo:DES. Different letters
573 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 | - | - | - | 0.001 a | 0.006 a | 0.002 a | Traces | 0.01 a | Traces | nd | Traces | 0.0008 |
| Water | 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 |
| DES7 | 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 |

574 **Table 3**. Individual phenolic compounds and 5-hydroxymethylfurfural (HMF) concentration in
575 mg/g of dry alperujo (3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol 4-β-d-glucoside
576 (Glu-HT), hydroxytyrosol (HT), tyrosol (Ty), verbascoside (Ve), vanillic acid (VA), vanillin
577 (Va) and luteonin (Lu)) by HPLC-UV in the alperujo sample treated with water or with DES9
578 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 | 25 | 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 |
| | | 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 |
| | 50 | 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 |
| | | 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 |
| | 90 | 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 |
| | | 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 |

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

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584 **Figures**

585 **Figure 1**. Colorimetric determination of total phenols by the Folin-Ciocalteu method of
586 ten different DES (DES1 to DES10) at three temperatures (25, 50 and 90 ºC) and four
587 times (30, 60, 90 and 120 minutes) for the two ratios of Alperujo/DES studied (1:1 in
588 blue and 2:1 in orange). *Significant differences ($p < 0.05$).

589 **Figure 2**. Colorimetric determination of total sugars by the Antrona method of eight
590 different DES (DES1 to DES10) at three temperatures (25, 50 and 90 ºC) and four times
591 (30, 60, 90 and 120 minutes) for the two ratios of Alperujo/DES studied (1:1 in blue and
592 2:1 in orange). *Significant differences ($p < 0.05$).

593 **Figure 3**. Colorimetric determination of the uronic acid solubilization of eight DES at
594 three temperatures (25, 50 and 90 ºC) at four different times (30, 60, 90 and 120
595 minutes) using two alperujo:DES ratios (1:1 in blue and 2:1 in orange). *Significant
596 differences ($p < 0.05$).

597 **Figure4.** Total phenols, total sugars and total acid sugars extracted by DES7 and DES9
598 in all treatments using two alperujo:DES ratios (1:1 in blue and 2:1 in red). Different
599 letters indicate significant differences ($p < 0.05$).

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

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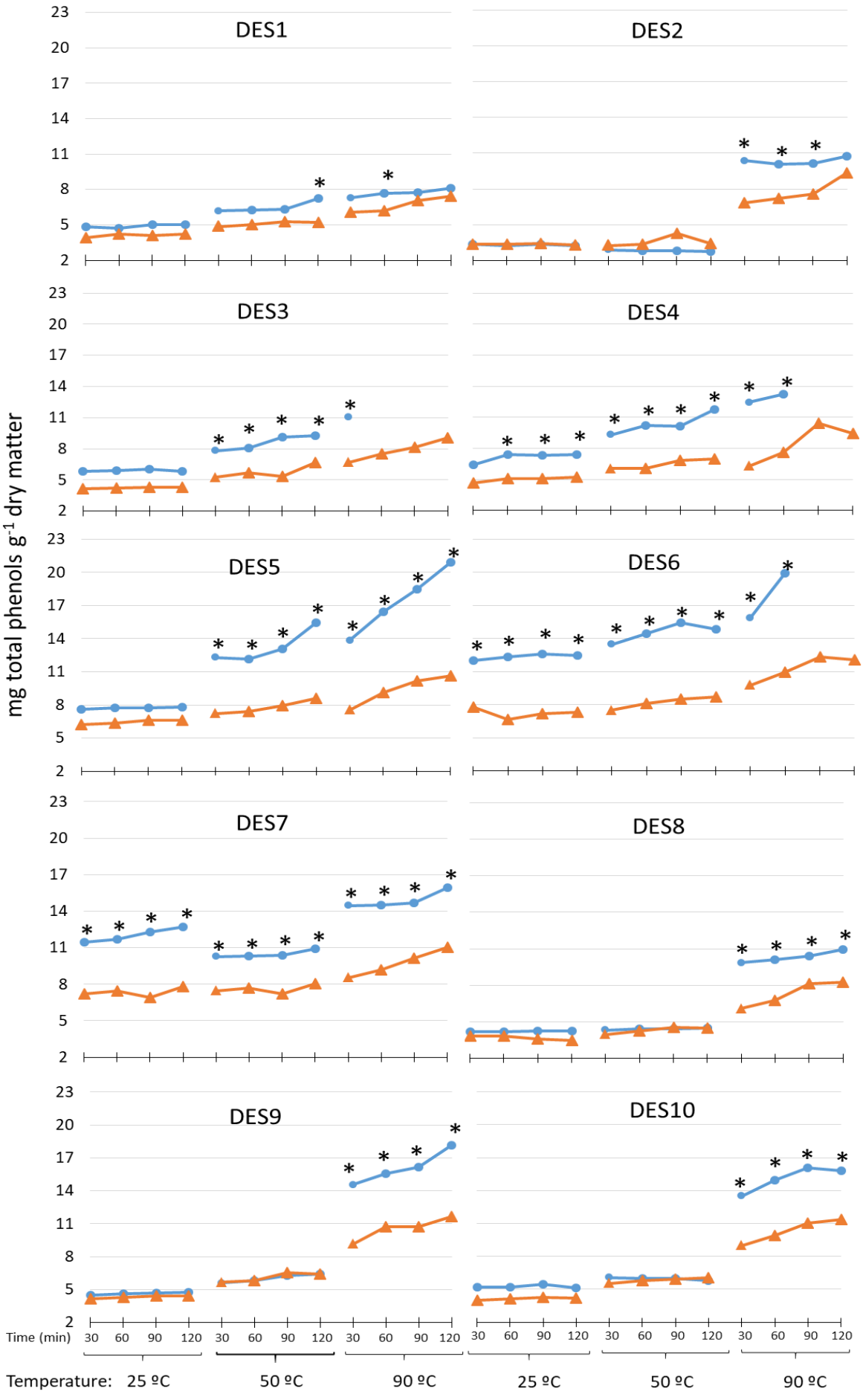
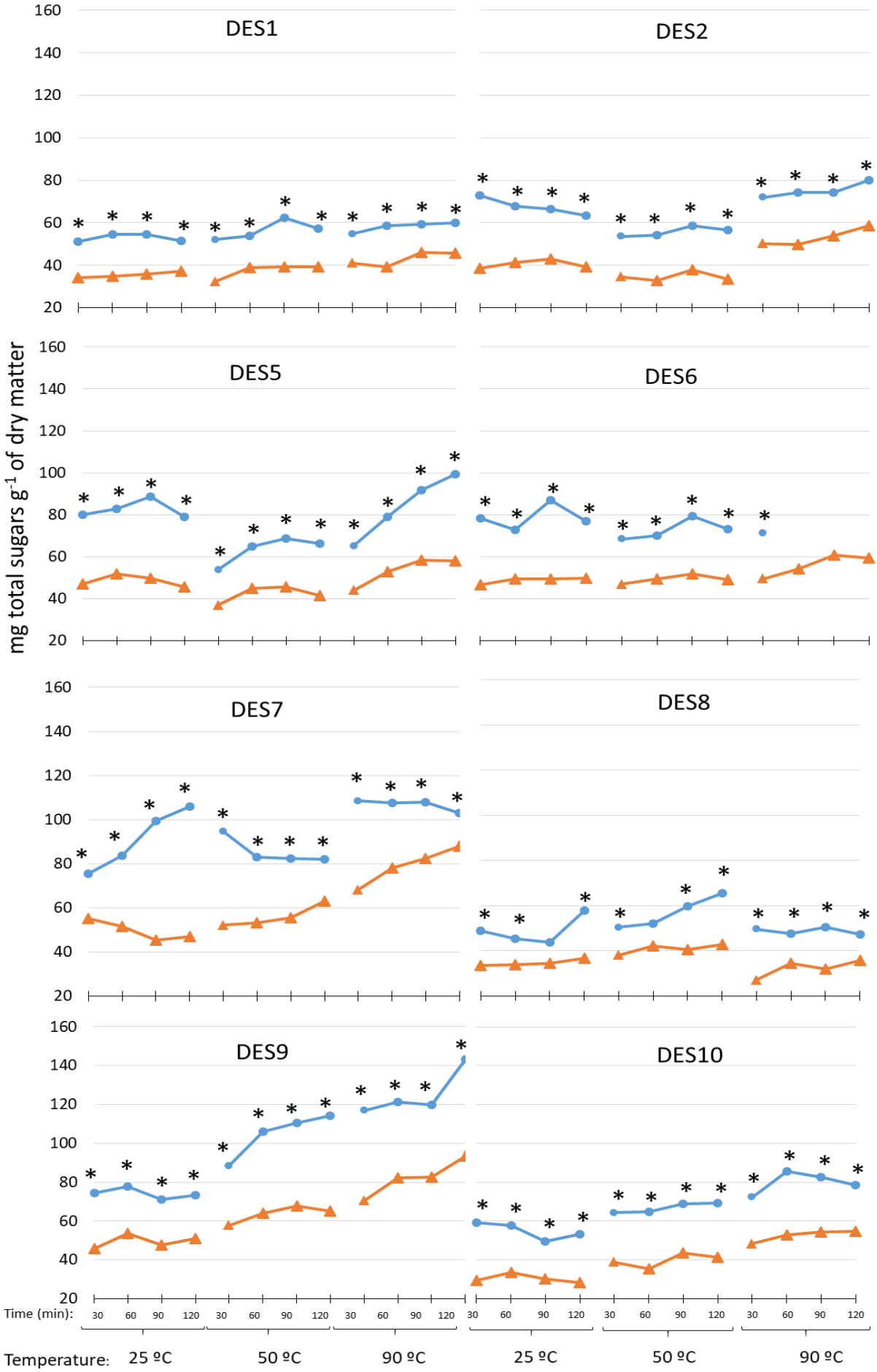
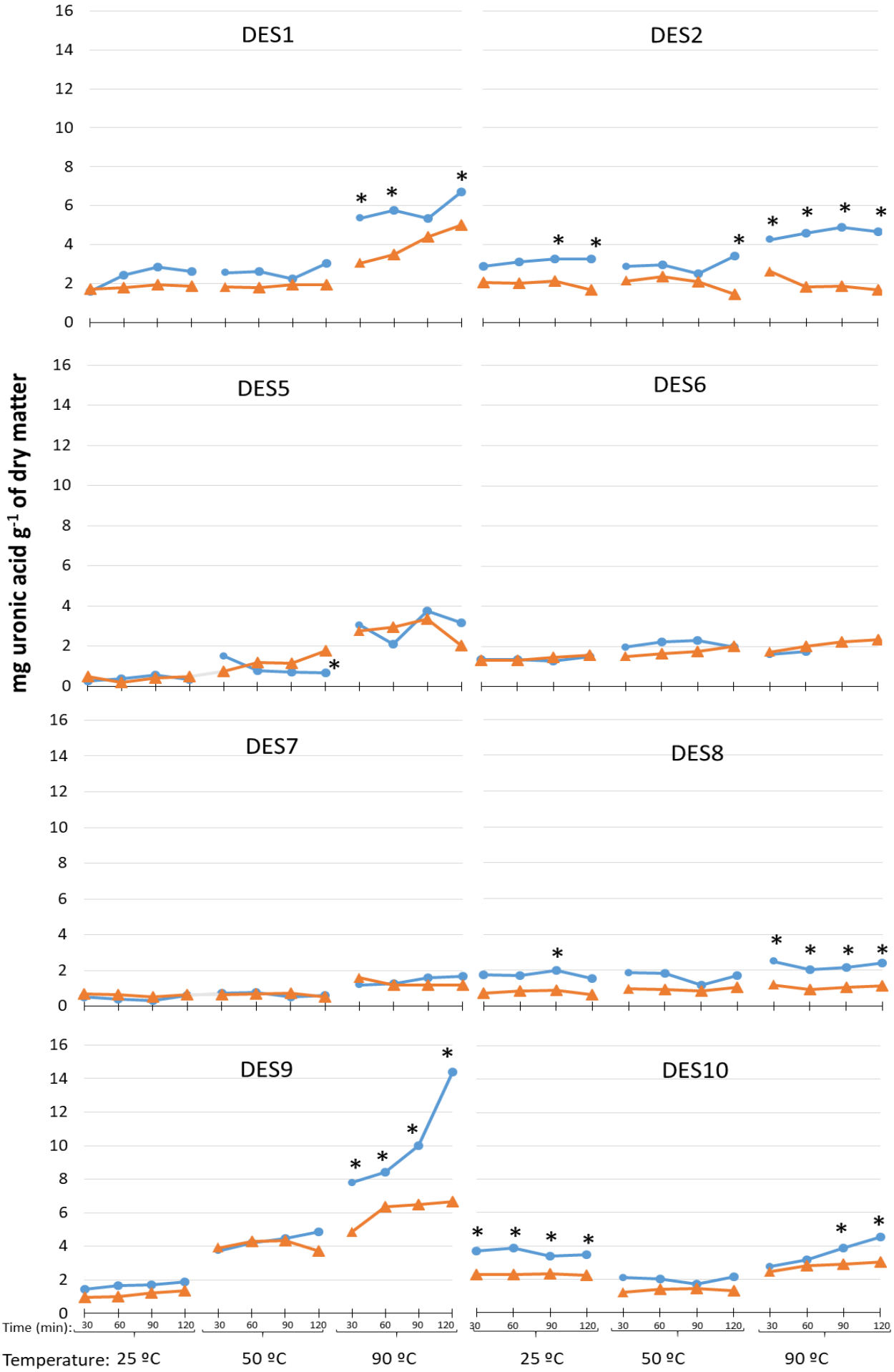

DES1
DES2
DES3
DES4
DES5
DES6
DES7
DES8
DES9
DES10
mg total phenols g-1 dry matter
Time (min)
30 60 90 120
Temperature: 25 ºC 50 ºC 90 ºC 25 ºC 50 ºC 90 ºC

605 Figure 1

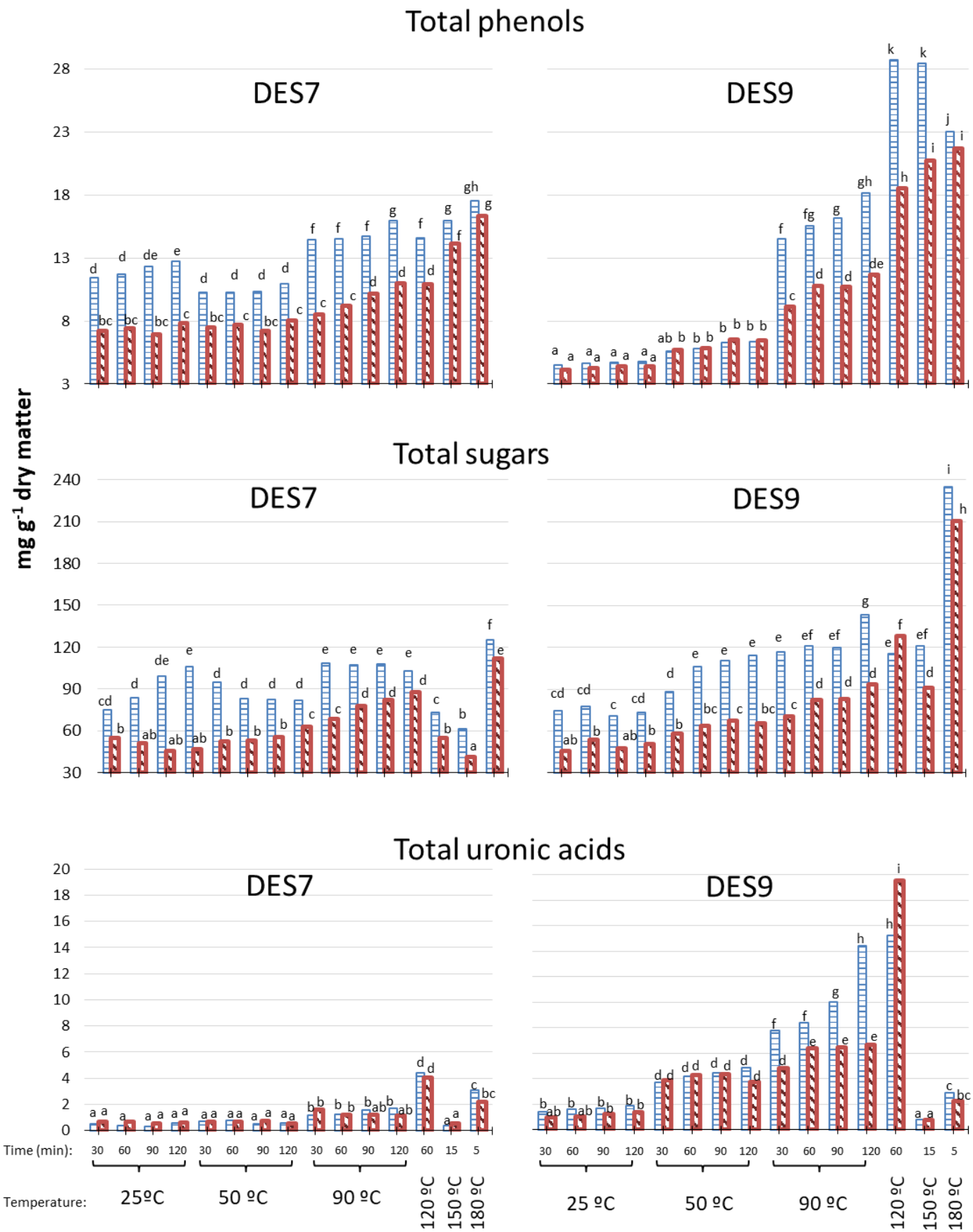

DES1
DES2
DES5
DES6
DES7
DES8
DES9
DES10
mg total sugars g-1 of dry matter
Time (min):
30 60 90 120
Temperature: 25 ºC 50 ºC 90 ºC 25 ºC 50 ºC 90 ºC

606 Figure 2


DES1
DES2
DES5
DES6
DES7
DES8
DES9
DES10
mg uronic acid g-1 of dry matter
Time (min):
Temperature: 25 ºC
50 ºC
90 ºC
25 ºC
50 ºC
90 ºC

607

Figure 3.




608 Figure 4

609

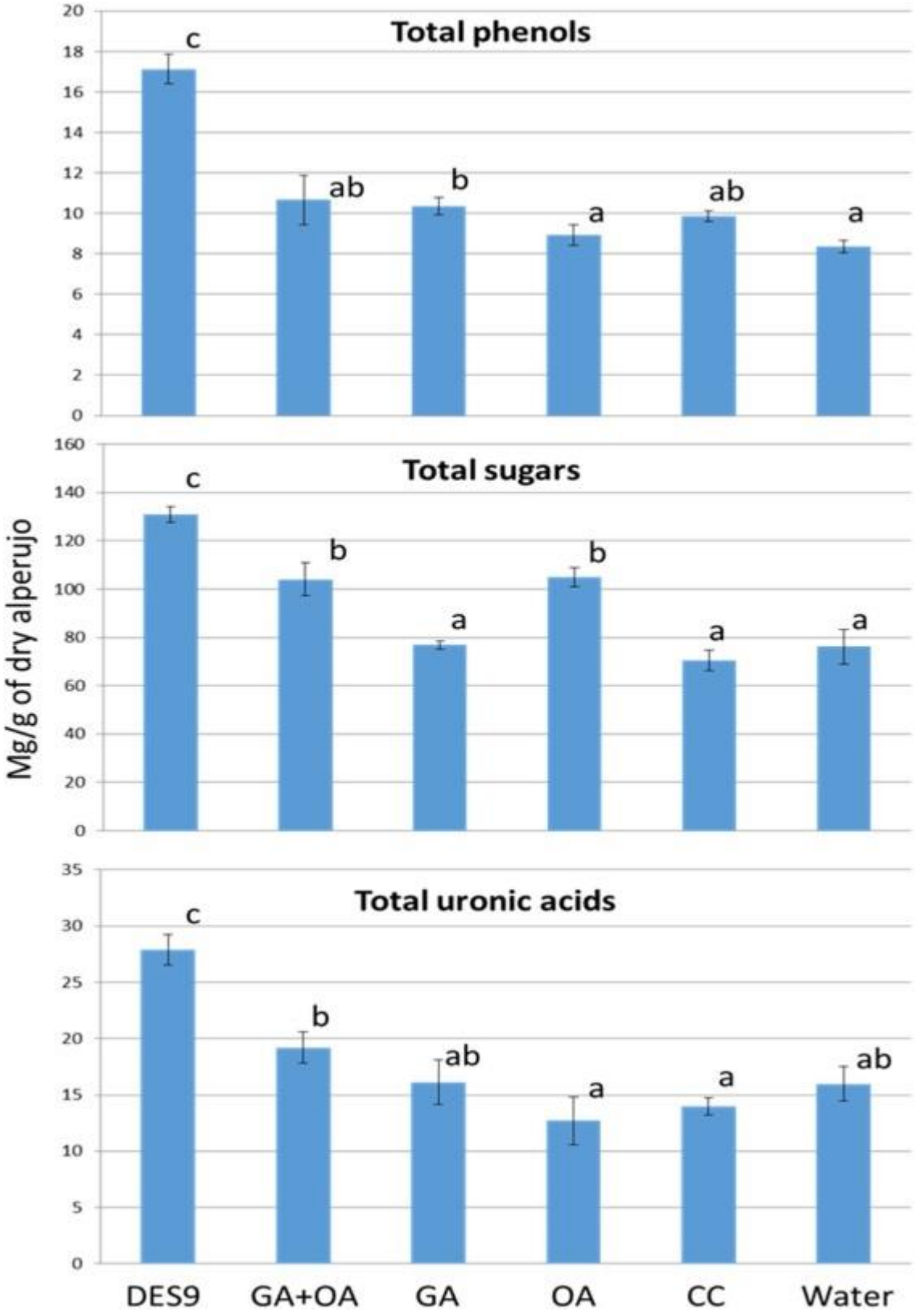

Total phenols
c
ab
b
a
ab
a
Total sugars
c
b
a
b
a
a
Total uronic acids
c
b
ab
a
a
ab
Mg/g of dry alperujo
DES9
GA+OA
GA
OA
CC
Water

Figure 5