Application of pressurized liquid extraction to grape by-products as a circular economy model to provide phenolic compounds enriched ingredient.

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Abstract

The aim of this study was to valorise wine-making by-products optimizing the recovery of phenolic compounds from Cannonau grape pomaces using Pressurized Liquid Extraction (PLE). To achieve these goals, PLE experiments were performed by a Response Surface Methodology (RSM) based on a Central Composite Design 2^3 model to address the polar compound extraction. The selected independent variables were: temperature, composition of extractant solvent (% ethanol) and extraction time. The response variables were extraction yield and recovery of phenolic compounds. Phytochemical profile of PLE extracts was analysed by high-performance liquid chromatography coupled to a time-of-flight mass analyser (HPLC-ESI-TOF-MS) in order to address the individual phenolic extraction effectiveness. Hence, the optimum values to maximize phenolics extraction were 130°C, 55% ethanol and 22 min. Extraction results obtained by using PLE system was compared with that obtained with conventional solid-liquid extraction. The proposed experimental model has proven to be a valuable alternative method to optimize extraction of bioactive compounds from wine-making by-products.

Keywords: grape pomace; HPLC-ESI-TOF-MS; Pressurized liquid extraction; Solid liquid extraction; Green Production; Circular economy

1. Introduction

Circular economy concept is a win-win and self-sustaining system that promotes sustainability, recovery and valorisation of wastes with the purpose to better manage and to prevent their production (Camana et al., 2021; Chebbi et al., 2021; Tacchini et al., 2019). In this sense, the European Union has made the circular economy one of its cornerstones, approving in 2020 "A new Circular Economy Action Plan for a cleaner and more competitive Europe" (European Commission, 2020). In recent years, also according to its principles, market is ever more interested into waste-to-value products. Waste-to-value products are those value-added products obtained through the valorisation of wastes or by-products generated in the food-supply chain (Coderoni and Perito, 2020). In this context, grape pomace represents an agro-industrial by-product that offers a wide range of valorisation and recycle possibilities. Grape pomace is the principal solid waste generated during wine-making process. It is mainly composed by seeds, stalks and skins and represent the 20% of the total harvested grapes (Ferri et al., 2020). The ever-growing interest for this by-product is due to their high content into high value bioactive compounds, such as fibres, proteins, lipids, minerals and phenolics (Ferri et al., 2020). It has been estimated that around 70% of phenolic compounds are still present into the pomace (Dwyer et al., 2014).

The high value of these compounds is due to their beneficial effects for human health. It has been proved that grape pomace derived phenolics are able to exercise a wide range of beneficial effects, such as antioxidant, antimicrobial, anti-glycation, cytotoxic effect against tumoral cells (Olszewska et al., 2020; Peixoto et al., 2018; Sri Harsha et al., 2014).For example, Peixoto et Al. evaluated the antioxidant, cytotoxic and antibacterial activities of grape pomaces (skins, seeds and their mixture) extracts. They found that seeds have the highest concentrations of phenolic compounds and the highest antioxidant, cytotoxic and antibacterial activities. So, they pointed out that this by-product is a valuable source whose use in the extractions of phenolic compounds must be increased to be applied

in various fields (Peixoto et al., 2018). In our previous study, we obtain an optimal extract from Cannonau pomace (Manca et al., 2020).

It is well known that the extraction process represents the critical step in the isolation and identification of phenolics (Castellanos-Gallo et al., 2022). The most common method is solid-liquid extraction (SLE) coupled with mechanical stirring, which often implies the use of a high quantity solvents, in some cases of organic nature (Wasilewski et al., 2022). In recent years, new non-conventional methods, such as supercritical fluid extraction (SFE), PLE, pulsed electric fields, are arousing an ever-growing interest due to their higher efficiency, lower extraction time and costs, being also environmentally-friendly (Castellanos-Gallo et al., 2022).

Several authors successfully performed PLE from grape by-products obtaining high-value extracts that may increase their value with potential applications in different industrial sectors like cosmetics or nutraceutics (Cejudo-Bastante et al., 2021; Ferri et al., 2020; Otero-Pareja et al., 2015).For instance, Nieto et al. performed PLE with ethanol:water mixtures as extractant solvent to obtain phenolic antioxidants from grape stems. The optimal extraction conditions were determined by using a central composite rotatable design. RSM determined 30% ethanol, 120 °C and 10 min as the optimal extraction conditions, that lead to obtaining an extract with a high phenolic content and a remarkable antioxidant activity from Merlot grape stems (Nieto et al., 2020).

Besides, Otero-Pareja et al. performed both SFE with CO_2 + 20% ethanol and PLE with either ethanol, water or an ethanol/water (50:50) mixture as the extraction solvents on different varieties of grape pomaces. The comparison of the two techniques showed that PLE using hydro-alcoholic mixture as solvent was more efficient than SFE using CO_2 + 20% ethanol in terms of both phenolic content and antioxidant activity. The global yield and the yield of anthocyanins and phenolic compounds was higher for PLE than SFE. Finally, they demonstrated that PLE is a successful extraction method to obtain antioxidant phenolic compounds from winemaking by-products (Otero-Pareja et al., 2015).

The main objective of the present research was to optimize a PLE process for Cannonau grape pomace to increase the extraction efficiency of polar compounds compared to conventional methods, and the comprehensive characterization of the obtained extracts with an advanced analytical technique, concretely HPLC-ESI-TOF-MS. RSM based on Central Composite Design 2^3 model was applied for the optimization considering %EtOH in aqueous mixtures, extraction time and temperature as independent variables at two levels. Furthermore, the response variables consist of extraction yield and detailed composition of the obtained extracts by HPLC-ESI-TOF-MS.

2. Material and methods

2.1. Materials

All chemicals used in this study were of analytical reagent grade and used as received. For extraction procedure, purified water was obtained by a Milli-Q system from Millipore (Bedford, MA, USA) and ethanol purchased from VWR chemicals (Radnor, PA, USA). For the mobile phase preparation used for analysis, formic acid was ordered from Sigma-Aldrich (Steinheim, Germany) and Acetonitrile of LC-MS grade was acquired from Fisher chemicals (Waltham, MA, USA). The pure standards used for the preparation of the calibration curves, were purchased from Sigma–Aldrich (St. Louis, MO, USA), Arbo Nova (Turku, Finland) and Extrasynthese (Lyon, France).

2.2. Sample preparation

Grape pomaces from Cannonau red wine were provided by Cantine Argiolas (Sardinia, Italy). Samples were dried at 42°C for about 48 h, to reduce the moisture content from 60.7± 0.7 % up to 7.4± 1.1 %, and ground using an ultra-centrifugal mill ZM200 (Retsch GmbH, Haan, Germany) at room temperature. Then, they were stored under vacuum, in darkness, at room temperature, until extraction experiments.

2.3. Extraction method

119 Phytocomplex was obtained by conventional solid-liquid extraction and PLE. To select the best
120 solvent to extract the polar fraction, grape pomace (3.5 g) was dispersed in five different blends
121 (50 mL each) of ethanol:water with 0, 30, 50, 70, and 100 % EtOH for SLE 1, SLE 2, SLE 3, SLE 4
122 and SLE 5, respectively. The mixtures were shaken for 60 min, at room temperature to allow the
123 extraction. The samples were then centrifuged at 13000 rpm for 10 min using with Sorvall ST 16 R,
124 Thermo Scientific instrument (Leicestershire, UK), and the supernatant was collected and filtered
125 through a 0.45 μm filter. The solvent was evaporated under vacuum in a Savant™ SpeedVac
126 Concentrator SC250 EXP (Thermo Scientific, Sunnyvale, CA, USA) and kept at -20 °C until HPLC-
127 MS analysis.
128 PLE was performed in a ASE™ 350 system (Dionex, Sunnyvale, CA, USA) equipped with a solvent
129 controller. The experimental designconsist of a total of 15 experiments, including center and star
130 point, using different solvent composition (ethanol and water), temperatures and static cycle
131 extraction times. The pre-set default condition was the extraction pressure, fixed at 11 MPa. The
132 solvents were previously degassed for 15 min to remove the dissolved oxygen for avoiding any
133 possible degradation of the target compounds by oxidation reactions. Pomace (3.5 g) was mixed with
134 sea sand (10 g) to improve the extraction process increasing the contact surface between sample and
135 solvent. The mixture was loaded onto stainless-steel extraction cells (33 mL) putting in the up and
136 bottom of the cell cellulose filters and a portion of sand (5 g). This disposition was optimized to avoid
137 the clogging of the metal frits and the interior conducts. The obtained extracts were dried under
138 vacuum in a Savant™ SpeedVac Concentrator SC250 EXP (Thermo Scientific, Sunnyvale, CA,
139 USA) at room temperature and stored at −20 °C.
140 *2.4. Experimental design*
141 RSM was applied to enhance the recovery of polar phytochemicals from grape pomace using a central
142 composite design 2^3 model with axial points. Percentage of ethanol (15, 50, 85 %) in aqueous
143 mixtures, static extraction time (5, 12.5, 20 min) and temperature (40, 110, 180 °C), were chosen as

144 independent variables and their effect was evaluated in 15 experiments conducted in a randomized
145 order (Table 1). The response variables were extraction yield and chemical composition of the
146 extracts determined by HPLC-ESI-TOF-MS. The extraction yield was calculated following the
147 equation Eq. 1:

148 Eq. 1 $$Yield\ (\%) = \frac{Weight\ of\ dried\ extract\ (g)}{Weight\ of\ dried\ grape\ pomaces\ extracted\ (g)} \times 100$$

149 **Table 1.** Central composite design 2^3 model and values of selected independent variables.

Experiment	**Ethanol (%)**	**Extraction time (min)**	**Temperature (C°)**	**Dielectric Constant**
PLE 1	15	20	40	66.11
PLE 2	85	5	40	32.26
PLE 3	5	12.5	110	53.19
PLE 4	50	22	110	36.98
PLE 5	15	5	40	66.11
PLE 6	50	12.5	20	51.83
PLE 7	50	3	110	36.98
PLE 8	50	12.5	110	36.98
PLE 9	15	5	180	33.93
PLE 10	50	12.5	110	36.98
PLE 11	85	20	40	32.26
PLE 12	85	5	180	21.63
PLE 13	85	20	180	21.63
PLE 14	95	12.5	110	20.78
PLE 15	50	12.5	200	25.14

150 In order to explain the response behaviour of variables, the experimental data were fitted to a
151 quadratic polynomial model following the general equation 2:

152 Eq. 2 $$Y = \alpha_0 + \sum_{i=1}^{k} \alpha_i x_i + \sum_{i=1}^{k} \alpha_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \alpha_{ij} x_i x_j$$

where Y represents the predicted response; α_0 is a constant coefficient that fixed the response at the central point of the experiments, k is the number of variables and α_i, α_{ii} and α_{ij} are the regression coefficients of the linear, quadratic and interaction terms, respectively; x_i and x_j represent the value of independent variables. The parameters of the models, determination of the optimum conditions and plot of response surface were estimated by using Statgraphics Centurion software XVI provided by Statpoint Technologies (Warrenton, VA, USA). The adequacy of the model obtained for PLE, were checked by the quadratic coefficient of determination (R^2), the lack of fit value and the coefficient of variation. Values were considered significantly different when p was < 0.05. The relationship between independent variables and responses were analysed by 3D response surface plots reporting the dependent variables as a function of the two most influent independent variables. Optimum conditions were calculated considering the maximization of individual response variables. Therefore, independent variables were kept in ranges while response was optimized.

2.5. HPLC-ESI-TOF-MS analysis of grape pomace extracts

The obtained extracts were dissolved in DMSO (10 mg/ml) and analysed by HPLS-ESI-TOF-MS. The HPLC instrument was a RRLC 1200 series (Agilent Technologies, Palo Alto, CA, USA), equipped with a vacuum degasser, autosampler, a binary pump, and a DAD detector. The stationary phase for reverse mode was composed of C18, concretely a Zorbax Eclipse Plus C18 (Agilent Technologies, Palo Alto, CA, USA) whose dimensions were a 150 mm x 4.6 mm, 1.8 μm of particle size. The mobile phases composition were composed of 0.1 % aqueous formic acid (mobile phase A) and acetonitrile (mobile phase B). The gradient elution followed a multistep linear profile: 5 % B as initial condition; increasing until 95 % B at 55 min; and decreasing until initial condition in 5 min and maintain them another 5 min to equilibrate the system before the next injection. The flow rate was set at 0.5 mL/min, injection volume was 10 μL at room temperature.

The detection of the compounds was also monitorized by a TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) by an electrospray interface (model G1607 from Agilent Technologies, Palo Alto,

178 CA, USA). The detection was performed in negative and in positive ionization mode with a mass
179 range of 50-1000 m/z. The flux from the HPLC column was reduced at 125 µl min^{-1} using a "T" type
180 splitter before being introduced into the mass spectrometer (split ratio 1:3).
181 The optimum ionization and transfer parameters were for negative mode: capillary voltage of +3.5
182 kV; drying gas temperature, 210 ºC; drying gas flow, 9 L min^{-1}; nebulizing gas pressure, 2.3 bar;
183 capillary exit, -120 V; skimmer 1, -40 V; hexapole 1, -23 V; RF hexapole, 80 Vpp; and skimmer 2, -
184 22.5 V. In contrast, for positive ionization mode the optimum values were: capillary voltage of +4
185 kV; drying gas temperature, 190 ºC; drying gas flow, 9 L min-1; and nebulizing gas pressure, 2.0 bar;
186 whereas the values of transfer parameters were: capillary exit, +120 V; skimmer 1, +40 V; hexapole
187 1, +23 V; RF hexapole, 100 Vpp; and skimmer 2, +22.5 V.
188 The instrument and the acquired chromatograms were calibrated externally with a 74900-00-05 Cole
189 Palmer syringe pump (Vernon Hills, IL, USA) using as standard a 10 mM sodium formate cluster
190 solution. The mixture was injected at the beginning of each run and all the spectra were calibrated
191 prior to compound identification. Data were processed through the software Data Analysis 4.0
192 (Bruker Daltonics). For each chromatogram a list of possible elemental formulas by Generate-
193 Molecular Formula Editor from each peak was obtained thank to the CHNO algorithm and its
194 standard functionalities, mainly minimum and maximum elemental range, electron configuration and
195 ring-plus double bonds equivalents. This combination provides a sigma value resulting from the
196 comparison of the theoretical and measured isotope pattern, which help to increase the confidence in
197 the proposed molecular formula. For quantitation purposes, gallic acid, catechin, epigallocatechin
198 gallate and quercetin were used as standards to quantify phenolics in samples. Calibration curves
199 were obtained with nine calibration levels at different concentrations (from 0.5 to 100 µg/mL). The
200 linearity of all calibration curves was demonstrated with regression coefficients higher than 0.98. The
201 total phenolic content in Cannonau grape pomaces extracts were calculated as the sum of the
202 individual compound concentrations obtained by HPLC-MS.

3. Results and discussion

3.1. Identification of phytochemical compounds

The components of grape pomace extracts obtained by solid or pressurized liquid extraction were identified by HPLC–ESI–TOF–MS from the analysis of chromatograms (Figure 1A, B C and D). The analysis allowed the detection of forty-five compounds, thirty-five of them were presented in both extracts and only ten in that obtained by solid liquid extraction. Among these, thirty-nine compounds were identified while six of them could not be identified with the analytical platform used (Table 2).

Table 2. Chemicals found in the extracts obtained from grape pomace by solid liquid extraction (SLE) or pressurised liquid extraction (PLE), retention time, molecular formula, experimental and theoretic m/z, error, mSigma, and bibliographic references that supported their identification.

Peak	RT (min)	Formula	m/z exp.	m/z theoric	Error (ppm)	mSigma	Name	Extraction	Reference
[M−H]⁻									
Organic acids									
1	3.1	$C_6H_{12}O_7$	195.0508	195.0510	1.2	16.0	Gluconic acid	SLE, PLE	(Gika et al., 2012; Perra et al., 2021)
2	3.4	$C_4H_6O_6$	149.0100	149.0092	5.5	50.4	Tartaric acid	SLE, PLE	(Perra et al., 2021)
3	3.9	$C_4H_6O_5$	133.0157	133.0142	10.7	13.7	Malic acid	SLE, PLE	(Perra et al., 2021)
4	4.0	$C_6H_8O_7$	191.0201	191.0197	2.0	20.0	Citric acid	SLE, PLE	(Perra et al., 2021)

10	17.9	$C_7H_{12}O_5$	175.0612	175.0612	0.2	27.3	2-Isopropylmalicacid	SLE, PLE	(Perra et al., 2021)
Amino acids									
7	16.3	$C_{13}H_{16}O_{10}$	203.0835	203.0826	-4.5	6.6	Tryptophan	SLE	(Sica et al., 2018)
Phenols									
5	10.5	$C_7H_6O_5$	169.0153	169.0142	3.7	5.8	Gallic acid	PLE	(Gika et al., 2012)
Tannins									
6	13.3	$C_{13}H_{16}O_{10}$	331.0657	331.0671	4.2	24.1	Galloyl-glucoside	SLE, PLE	(Romani et al., 2012; Yan et al., 2016)
Flavonoids									
11	18.7	$C_{15}H_{14}O_6$	289.0716	289.0718	0.7	7.0	Catechin	SLE, PLE	(Hashim et al., 2020; Jiménez-Sánchez et al., 2016; Nastić et al., 2019)
13	20.4	$C_{15}H_{14}O_6$	289.0716	289.0718	0.5	5.5	Catechin isomer	SLE, PLE	(Hashim et al., 2020; Jiménez-Sánchez et al., 2016; Nastić et al., 2019)

17	30.2	$C_{15}H_{10}O_7$	301.0353	301.0354	0.4	21.3	Quercetin	SLE, PLE	(Hashim et al., 2020)
20	33.0	$C_{15}H_{10}O_6$	285.0395	285.0405	3.4	7.1	Fisetin	SLE, PLE	(de Araújo Rodrigues et al., 2019)
21	33.3	$C_{16}H_{12}O_7$	315.0502	315.0510	2.6	13.7	Quercetin-methyl ether	SLE, PLE	(Ji et al., 2015)
Proanthocyanidins									
9	17.0	$C_{30}H_{26}O_{12}$	577.1336	577.1351	2.7	17.3	Proanthocyanidin B2 isomer	SLE, PLE	(Jia et al., 2019; Nastić et al., 2019)
12	19.5	$C_{30}H_{26}O_{12}$	577.1329	577.1351	4.0	24.1	Proanthocyanidin B2 isomer	SLE, PLE	(Jia et al., 2019; Nastić et al., 2019)
Iridoids									
8	16.6	$C_{21}H_{32}O_{10}$	443.1927	443.1923	0.9	68.0	Penstemide or *epi*-DPA-G	SLE	(Cretin et al., 2019; Fayad et al., 2020; Noui et al., 2018)
Fatty acids									
18	31.0	$C_{18}H_{32}O_5$	327.2173	327.2177	1.3	51.3	prostaglandin F1alpha isomer	SLE	(Fu et al., 2010; García-

									Villalba et al., 2008)
19	32.4	$C_{18}H_{34}O_5$	329.2324	329.2333	3.0	27.6	trihydroxy-octadecenoic acid	SLE, PLE	(Tao et al., 2016)
24	40.9	$C_{18}H_{32}O_4$	311.2227	311.2228	0.4	71.8	13-hydroperoxy-octadecadienoic acid	SLE	(Jiménez-Sánchez et al., 2016)
26	41.7	$C_{18}H_{32}O_4$	311.2220	311.2228	2.4	14.2	13-hydroperoxy-octadecadienoic acid	SLE	(Jiménez-Sánchez et al., 2016)
27	43.7	$C_{18}H_{30}O_3$	293.2121	293.2122	0.4	15.3	Hydroxy-octadecatrienoic acid isomer	SLE, PLE	(Jiménez-Sánchez et al., 2016; Nastić et al., 2019)
28	46.0	$C_{18}H_{32}O_3$	295.2273	295.2279	2.1	6.6	Hydroxy-octadecatrienoic acid	SLE, PLE	(Jiménez-Sánchez et al., 2016; Nastić et al., 2019)
29	47.6	$C_{18}H_{30}O_3$	293.2125	293.2122	0.9	28.7	Hydroxy-octadecatrienoic acid isomer	SLE	(Jiménez-Sánchez et al., 2016;

									Nastić et al., 2019)
30	55.2	$C_{18}H_{30}O_2$	277.2163	277.2173	3.6	45.9	Linolenic acid	SLE	(Crews et al., 2006; Della Corte et al., 2015; Jiménez-Sánchez et al., 2016)
Unknown compounds									
14	23.1	$C_{18}H_{12}N_6O_8$	441.0804	441.0800	-0.8	22.4	UK1	SLE	
15	24.9	$C_{32}H_{30}O_{14}$	637.1560	637.1563	0.4	35.5	UK2	SLE, PLE	
16	25.6	$C_{32}H_{32}O_{15}$	655.1662	655.1668	1.0	21.3	UK3	SLE, PLE	
22	38.4	$C_{24}H_{48}O_6$	431.3370	431.3378	2.0	30.3	UK4	SLE, PLE	
23	39.5	$C_7H_4N_8O_3$	247.0326	247.0334	3.3	16.7	UK5	SLE	
25	41.3	$C_{10}H_{13}NO_3$	194.0825	194.0823	-1.2	23.3	UK6	SLE, PLE	
[M−H]$^+$									
Phenols									
a	12.1	$C_7H_6O_5$	171.0282	171.0288	3.7	5.8	Gallic acid	SLE, PLE	(Gika et al., 2012)
Flavonoids									
d	17.0	$C_{15}H_{14}O_6$	291.0857	291.0863	2.0	5.1	Catechin	SLE, PLE	(Hashim et al., 2020; Nastić et al., 2019)

g	18.8	$C_{15}H_{14}O_6$	291.0859	291.0863	1.4	2.7	Catechin isomer	SLE, PLE	(Hashim et al., 2020; Nastić et al., 2019)
h	21.5	$C_{30}H_{26}O_{14}$	611.1377	611.1395	3.0	12.9	Delphinidin glucoside	SLE, PLE	(De Villiers et al., 2011)
i	22.0	$C_{32}H_{30}O_{15}$	655.1639	655.1657	2.8	8.5	Malvidin caffeoyl-glucoside	SLE, PLE	(Pérez-Navarro et al., 2019)
j	22.5	$C_{31}H_{28}O_{14}$	625.1548	625.1552	0.6	9.3	Isorhamnetin glucoside	SLE, PLE	(Panighel et al., 2015)
k	25.4	$C_{15}H_{10}O_8$	319.0430	319.0448	5.9	13.0	Myricetin	SLE, PLE	(Bevilacqua et al., 2004)
l	28.8	$C_{15}H_{10}O_7$	303.0484	303.0499	4.9	8.7	Quercetin	SLE, PLE	(Hashim et al., 2020)
n	31.7	$C_{15}H_{10}O_6$	287.0538	287.0550	4.3	7.4	Fisetin	SLE, PLE	(de Araújo Rodrigues et al., 2019)
o	32.0	$C_{16}H_{12}O_7$	317.0647	317.0656	2.7	9.0	Quercetin-methyl ether	SLE, PLE	(Ji et al., 2015)
Stilbenes									
m	30.3	$C_{28}H_{22}O_6$	455.1470	455.1489	4.1	11.3	ε-viniferin	SLE, PLE	(Flamini et al., 2015; Pugajeva et al., 2018)

Anthocyanins									
e	17.6	$C_{23}H_{24}O_{12}$	493.1330	493.1341	2.1	5.4	Malvidin glucoside	SLE, PLE	(Pérez-Navarro et al., 2019)
Proanthocyanidins									
b	15.3	$C_{30}H_{26}O_{12}$	579.1464	579.1497	5.7	6.7	Proanthocyanidin B2 isomer	SLE, PLE	(Jia et al., 2019; Nastić et al., 2019)
c	16.3	$C_{30}H_{26}O_{12}$	579.1464	579.1497	5.7	6.3	Proanthocyanidin B2 isomer	SLE, PLE	(Jia et al., 2019; Nastić et al., 2019)
f	17.8	$C_{30}H_{26}O_{12}$	579.1464	579.1497	4.9	41.1	Proanthocyanidin B2 isomer	SLE, PLE	(Jia et al., 2019; Nastić et al., 2019)

Most of the compounds identified were previously found in grape or in its by-products (Flamini et al., 2015; Perra et al., 2021) and belonged to different chemical classes including organic acids, amino acids, phenols (tannins, flavonoids, stilbenes, anthocyanins, proanthocyanidins), iridoids and fatty acids.

3.1.1. Phenolic compounds

Twenty-four different phenols with different chemical structure were identified by the used analyses, in particular, one tannin, eight flavonoids, two stilbenes, one anthocyanin, five proanthocyanidins and two simple phenols were found.

3.1.1.1. Tannins

223 Retention time and mass spectra allowed to recognize that peak 6 corresponded to galloyl-glucoside,
224 which was detected for the first time in this study in grape or its by-products and only using the
225 negative ionization mode (Romani et al., 2012; Yan et al., 2016).
226 *3.1.1.2. Flavonoids*
227 Mass spectra and elution profile of extracts disclosed the presence of fourteen different flavonoids,
228 including four aglycons and four derivatives. All the found flavonoids, except delphinidin, malvidin
229 and isorhamnetin, were detected using positive and negative ionization mode and all of them have
230 been previously described in grape or its by-products (de Araújo Rodrigues et al., 2019; De Villiers
231 et al., 2011; Flamini et al., 2015; Hashim et al., 2020; Ji et al., 2015; Panighel et al., 2015; Pérez-
232 Navarro et al., 2019; Perra et al., 2021). Aglycons and derivatives were detected in the extracts
233 obtained by solid liquid extraction and those obtained by pressurized liquid extraction and they were
234 catechin and catechin isomers (peaks 11,13, d and g); myricetin (peak k); quercetin (peaks 17 and l);
235 fisetin (peaks 20 and n); quercetin-methyl ether (peaks 21 and o); delphinidin glucoside (peak h);
236 malvidin caffeoyl-glucoside (peak i); isorhamnetin glucoside (peak j).
237 *3.1.1.3. Stilbenes*
238 The only stilbene detected was ε-viniferin, which was found in all the extracts only using the positive
239 ionization mode. It was previously described in grape by Flamini et al. (Flamini et al., 2015).
240 *3.1.1.4. Anthocyanin and proanthocyanidin*
241 Malvidin glucoside (peak e) and proanthocyanidin B2 isomers (peaks 9,12, b, e and f), were the only
242 anthocyanin and proanthocyanidin respectively, detected in all the extracts, irrespective to the
243 extraction method used. Proanthocyanidin B2 isomers were detected by using both ionization modes,
244 while malvidin glucoside was detected only using the positive mode. These compounds have been
245 largely described in bibliography in grape or its by-products (Jia et al., 2019; Pérez-Navarro et al.,
246 2019; Perra et al., 2021).
247 *3.1.1.5. Phenols*

248 Gallic acid (peaks 5 and a) was the only simple phenol detected by using both positive and negative
249 ionization mode in the extracts obtained by solid liquid extraction and in those obtained by
250 pressurized liquid extraction. This compound has been widely described in grape or its by-products
251 (Gika et al., 2012).
252 *3.1.2. Other polar compounds*
253 According to mass spectroscopy data and the HPLC elution profile, six compounds were identified
254 to be organic acids. According to this result, gluconic, tartaric, malic, citric acid and 2-isopropimalic
255 acid (peaks 1,2,3,4 and 10) have been commonly found in grape or its by-products (Gika et al., 2012;
256 Perra et al., 2021). The elution time at 16.3 and *m/z* 203.0835 allowed to identify peak 7 as
257 tryptophan, which was the unique amino acid detected solely in the extract obtained by pressurized
258 liquid extraction. The elution time at 16.6 and *m/z* 443.1927 allowed to recognise peak 8 as that of
259 penstemide (or *epi*-DPA-G). Gallic acid has been detected using the two ionization modes, the other
260 were detected only using the negative mode.
261 *3.1.3. Fatty Acids*
262 According to mass spectra and elution profile, eight fatty acids were found, among these, 3
263 trihydroxy-octadecenoic acid (peak 19), hydroxy-octadecatrienoic isomer (peaks 27 and 29) and
264 hydroxy-octadecatrienoic acid (peak 28), were detected in extracts obtained by solid liquid extraction
265 and in those obtained by pressurized liquid extraction. Prostaglandin F1alpha isomer (peak 18), 13-
266 hydroperoxy-octadecadienoic acid (peaks 24 and 26) and linolenic acid (peak 30) were recognised
267 only in the extracts obtained by solid liquid extraction. Among these compounds, only linolenic acid
268 has been previously described in grape (Della Corte et al., 2015).
269 *3.2 Quantification of polar compounds by HPLC-ESI-TOF-MS*
270 In order to quantify the number of polar compounds present in grape pomaces, four commercial
271 standard molecules (gallic acid, catechin, epigallocatechin gallate and quercetin) were used to obtain
272 calibration curves using the positive and negative ionization mode (Supporting information Table 1).

The other molecules were quantified by using the curve of gallic acid to quantify galloyl-glucoside, that of catechin for catechin isomers, that of epigallocatechin gallate for proanthocyanidin b2 isomer, malvidin-glucoside, delphinidin glucoside and **ε**-viniferin, that of quercetin for malvidin caffeoyl-glucoside, isorhamnetin glucoside, myricetin, fisetin and quercetin-methyl ether. The analysis of malvidin-glucoside, delphinidin glucoside, **ε**-viniferin, malvidin caffeoyl-glucoside, isorhamnetin glucoside, myricetin, fisetin and quercetin-methyl ether were carried out by using the positive ionization mode.

Considering that the regression curve of the used standards could differ from that of the compounds actual present in the grape pomace extracts, their quantification/amount should be considered only an estimation of their real concentration. Using these values, the extraction yield and the total content of all polar compounds, as the sum of the concentrations of all the components, were calculated (Table 3).

Table 3. Predicted, experimental yield and concentration of total polar compounds (TPC), and relative coefficient of variation (CV) calculated for the extracts obtained by pressurized liquid extraction (PLE) or solid liquid extraction (SLE) at different experimental conditions (reported in Table 1). Results were expressed in μg of analyte/g of grape pomace. Values ± standard deviations were reported.

	Yield (%)			**TPC (μg/g of grape pomace)**		
	Predicted	Experimental	CV	Predicted	Experimental	CV (%)
PLE 1	7.88	7.27	5.7	601.772	460 ± 13	18.8
PLE 2	5.86	6.02	1.8	582.447	612± 16	3.5
PLE 3	14.75	11.82	15.6	833.079	1059 ± 10	16.9
PLE 4	13.45	14.86	7.0	2841.61	2809 ± 30	0.8
PLE 5	7.88	8.15	2.4	404.746	330 ± 3	14.4
PLE 6	5.77	5.41	4.5	525.173	610 ± 14	10.5
PLE 7	13.45	12.62	4.5	2166	2125 ± 8	14.5
PLE 8	13.45	15.11	8.2	2214.54	2076 ± 41	14.3

PLE 9	25.26	27.05	4.8	587.551	526 ± 20	7.8
PLE 10	13.45	14.71	6.3	2214.54	2451 ± 39	7.2
PLE 11	5.86	6.48	7.0	932.447	1023 ± 16	6.6
PLE 12	23.25	20.67	8.3	1078.58	1249 ± 16	10.4
PLE 13	23.25	23.33	0.3	1948.3	2052 ± 39	9.8
PLE 14	12.16	11.70	2.7	1361.31	1064 ± 27	17.3
PLE 15	28.12	28.65	1.3	1295.74	1140 ± 14	9.0
SLE 1		11.35			235 ± 60	
SLE 2		4.79			67 ± 1	
SLE 3		7.12			929 ± 73	
SLE 4		6.40			1080 ± 58	
SLE 5		7.06			645 ± 2	

The total phenols for each family and individual compound concentrations in the two extracts were also calculated (Supporting Information Table 2 and 3). Results disclosed that the extracts obtained by pressurized liquid extractions contained a higher amount (330-2809 μg of analyte/g of grape pomace) and type of polar compounds ensuring a higher extraction yield than the extract obtained by conventional solid liquid extractions. This increase may be related to higher temperature and pressure used in this method, that facilitated the cell breakdown and consequently the release of phytochemicals from the grape tissues. The highest concentrations of compounds were obtained pleasing the conditions PLE 4, PLE 7, PLE 8, PLE 10 and PLE 13 (see Table 1 and Table 3), which permit to obtain 2809, 2125, 2076, 2452 and 2052 μg analyte/g grape pomace, respectively. In these experimental conditions temperatures above 110 °C and ethanol concentrations higher than 50 % were used (Table 1 and Table 3). When the solid liquid extraction was used, the highest recovery of polar compounds (1080 μg analyte/g of grape pomace) was reached using the extraction condition SLE 4 (blend of water 30 % and ethanol 70 %), while the lowest yield (4.79 %) and amount of polar compounds (67.18 μg of analyte/g of grape pomace) were achieved by using the condition SLE 2 (blend of water 70 % and ethanol 30 %) (Table 3).

306 Considering the different phytochemicals groups, in general, pressurised liquid extraction achieved
307 better results in terms of phytochemicals concentration. For flavonoids, the most abundant group,
308 PLE 4, with ~ 1470 μg flavonoids/g grape pomace, represented the best experimental condition to
309 obtain high quantities of flavonoids. Regarding solid-liquid extraction the best condition for
310 flavonoids extraction was SLE 4, with ~ 300 μg analyte/g grape pomace. When SLE was performed
311 these phytochemicals were extracted efficiently, but always in lower concentration that achieved by
312 PLE. Despite being thermosensitive compounds, flavonoids required an increment of temperature for
313 improving their extraction (around 110°C), for this reason lower and higher extraction temperatures
314 achieved a lesser concentration of flavonoids. These results may indicate that temperature and
315 pressure are needed to enhance the release of phytochemical from plant cell, and consequently, the
316 extraction condition provided by PLE are suitable for flavonoid extraction.
317 Regarding anthocyanins recovery, PLE extracts were ranged from 0 to 933 μg anthocyanins/g grape
318 pomace being PLE 7, the best extraction condition to recover these phytochemicals, whereas SLE 3
319 and SLE 4, which were the best SLE conditions for these phytochemicals, revealed lower
320 anthocyanins recovery, ~ 440 and ~ 540 μg anthocyanins/g grape pomace respectively. As with the
321 extraction of flavonoids, anthocyanins are temperature-sensitive compounds, but looking at the
322 results obtained, it can be seen, at the same ethanol concentration (50%) an increase in temperature
323 (110 °C) is necessary to promote the extraction of these phytochemicals. For this reason, comparing
324 SLE 3 (50% ethanol) and PLE 7 (110°C, 50% ethanol and 3minutes) it can be observed that more
325 than twice as much anthocyanin is extracted when PLE is applied.
326 In addition, proanthocyanidins were another group detected in the extracts at high concentrations
327 (Figure 2). These phytochemicals were found in higher concentrations in PLE extracts which were
328 ranged from 0 to 1014 μg proanthocyanidins/g grape pomace, being PLE 13 the best condition to
329 recover these phytochemicals. On the other hand, the concentration of proanthocyanidins achieved
330 after performing SLE extraction were ranged from 0 to 90 proanthocyanidins/g grape pomace. This

large difference in concentration may be due to the need to apply high temperatures (around 180) to cause the separation of these compounds from the matrix (Cádiz-Gurrea et al., 2019)**,** for this reason, SLE was not able to recover large amounts of these compounds as it was carried out at room temperature.

Regarding the minority groups in the extracts, PLE 12 ensured the highest tannins extraction (163µg of tannins/g of pomace), while the lowest results were obtained when PLE 9 were performed (Supporting information Table 2 and 3). Figure 2 shows how solid-liquid extraction is not a good technique for tannins recovery since their recovery is very low compared to PLE extracts (ranged from 0 to 77µg of tannins/ g of pomace). This may be due to the structure of these phytochemicals and their strong binding to the matrix, requiring the application of energy in the form of heat or pressure to achieve their separation from the matrix.

In addition, phenolic acids were found in range from 0 to 110 µg of phenolic acids/ g of pomace when PLE was applied being PLE 13 the best condition and 0 to 60 when SLE extraction were performed, being SLE 4 the best of them. As can be seen in Figure 2, the extraction of these phytochemicals was similar independently of extraction methodology applied.

Finally, stilbenes group was the least recovered phytochemicals. They were ranged from 0 to 32 µg of stilbenes/ g of pomace when PLE extraction were performed and from 0 to 13µg of stilbenes/ g of pomace during SLE extractions.

Considering these results, PLE reached higher extraction yield and higher TPC content, and consequently higher quality extracts, consuming less solvents and expending less time for extractions when it is compared with SLE. In this sense, PLE revealed to be a better technique to obtain extracts enriched with several phytochemicals from Cannonau grape pomace allowing for extraction processes that facilitate the achievement of a circular economy.

3.3 RSM analysis of pressurized liquid extraction

3.3.1 Model fitting

As previously mentioned, the proposed experimental design was applied to maximize the individual response variables. An individual analysis of variance (ANOVA) for each response was performed to fit and optimize the statistical model (Table 4). The obtained fitting results followed a quadratic polynomial model (Eq. 1) and regression coefficients were the result of the method of least squares (Supporting information Table 4).

Table 4. Yield and total polar compounds found in the extracts obtained by PLE and quantified by the regression models as a function of A (temperature), B (ethanol), C (time). Sum of squares (SS), degrees of freedom (Df), mean square (MS), F-ratio, p value, quadratic coefficient of determination (R^2) and percentage of coefficient of variation (CV) were reported.

Source	**YIELD**					**TOTAL POLAR COMPOUNDS**				
	SS	Df	MS	F-Ratio	P value	SS	Df	MS	F-Ratio	P value
A	637.167	1	637.167	8010.16	0.0071*	749188	1	749188	10.61	0.1897
B	9.25312	1	9.25312	116.22	0.0322*	352067	1	352067	4.98	0.2681
C	0.95474	1	0.95474	12.00	0.1789	586932	1	586932	8.31	0.2126
AA	23.6417	1	23.6417	297.21	0.0369*	3211290	1	3211290	45.47	0.0937
AB	2.89587	1	2.89587	36.41	0.1046	32681.3	1	32681.3	0.46	0.6197
AC	0.277577	1	0.277577	3.49	0.3129	89921.4	1	89921.4	1.27	0.4617
BB	5.63789	1	5.63789	70.88	0.0753	2357440	1	2357440	33.38	0.1091
BC	2.30368	1	2.30368	28.96	0.1170	7790.13	1	7790.13	0.11	0.7959
CC	0.141294	1	0.141294	1.78	0.4098	161688	1	161688	2.29	0.3718
Lack-of-fit	11.3353	4	2.83383	35.63	0.1232	257296	4	64323.9	0.91	0.6462
Pure error	0.0795448	1	0.0795448			70631.6	1	70631.6		
Total (corr.)	803.457	14				8864700	14			
R^2	0.986					0.963				
CV	≤ 3.37					≤ 7.02				

365 The first parameter to be considered for the study of the data variability was the coefficient of
366 determination (R^2). This indicator can be explained by the proposed mathematical model and
367 consequently, it possesses the ability to predict the behaviour of the response variables. The obtained
368 R^2 for total polar compounds was 0.963, indicating a good correlation, as well as those of flavonoids
369 and anthocyanins, both very similar ~ 0.950, while that of tannins was the lowest (0.694), indicating
370 a lower correlation (Supporting information Table 4).
371 Moreover, to verify the fitting quality of the used model the lack-of-fit test was also calculated. In
372 this sense, most of the results of this test were non-significant ($p > 0.05$), pointing out a good fit out
373 of the proposed mathematical model (Table 4 and Supporting information Table 4). Moreover, the
374 coefficient of variation, which explain the reproducibility of the experimental data compared to the
375 ones predicted by the model, was always lower than 16, suggesting a small variability of the data and
376 a great reproducibility (Table 4 and Supporting information Table 4) (Liyana-Pathirana and Shahidi,
377 2005).
378 Additionally, 3D response surface plots were depicted to evaluate graphically the relationship
379 between experimental parameters and response variables (Figure 3).
380 *3.3.2 Extraction yield*
381 The coefficient of determination of extraction yield was very high (0.986) due to the considerable
382 part of the variance within data. In addition, the lack of fit value was not significant, and the
383 coefficient of variation indicated a good reproducibility of the data (≤ 3.37 %). All these statistical
384 values permitted to confirm a great fit toward the proposed model which could be used to predict and
385 optimize the extraction yield of grape pomace. The results from ANOVA underlined that temperature
386 and ethanol concentration significantly affected the extraction yield ($p < 0.05$), being temperature the
387 most influent factor. In particular, individual and quadratic effects of temperature and individual
388 effect of ethanol were the most relevant variables, which permit to optimize the extraction yield.

389 Considering the importance of these parameters, a model equation (Eq. 3) was obtained fitting
390 experimental data and keeping only the significant parameters in the quadratic model:
391 Eq. 3 $$Yield = 6.4467 + 0.0293\,A - 0.0288\,B + 0.0004\,A^2$$
392 The extraction yields obtained by the Eq. 3 were ranged from 5.4 to 28.6 %. According to the results
393 reported in Table 4, the highest yields were reached when temperature increased regardless the
394 ethanol concentration used, these is also in accordance with results calculated by the Eq. 3, where the
395 positive effect of temperature (linear and quadratic) allowed an increase of yield extraction, including
396 using the extreme temperatures around 200 °C. The high extraction yield can be related to the better
397 ability of high temperatures to facilitate the breakdown of cells, enhancing the release of
398 phytochemicals toward the solvent. Moreover, in order to evaluate the prediction ability of the
399 proposed experimental model, the values of yield obtained by the Eq. 3 and the experimental yield
400 values (Table 3) were compared. The results revealed a slight variance (coefficient of variation ≤ 3.37
401 %) confirming that the proposed equation permits to predict valuable values comparable to
402 experimental ones. The most promising optimized conditions foresee an extraction at 200 °C, using
403 18 % ethanol for 22 minutes, the theoretical yield that should be achieved is 30 %.
404 *3.3.3 Total polar compounds*
405 The coefficient of determination of total polar compounds was very high, 0.963, indicating a huge
406 variety of the results provided by the method. In addition, the lack of fit value was not significant,
407 and the coefficient of variation results revealed a good reproducibility of the data (≤ 7.02 %). The
408 results revealed a great adjust of the proposed model which could be used to predict and optimize the
409 extraction of polar compounds from grape pomace. After evaluating the ANOVA results, any factor
410 had a significant effect on this response variable ($p > 0.05$).
411 The amount of total polar compounds ranged from 330 to 2809 µg/g of grape pomace. Higher
412 recovery of phytochemicals were achieved when middle proportions of ethanol and temperature were
413 used during large extraction cycles (Figure 2). This behaviour may be due to the simultaneous effect

414 of the dielectric constant of the blend of ethanol and water and the temperature applied, which
415 synergically enabled the recovery of a high variety of phytochemicals. In spite of the ANOVA results
416 for factor effects, the experimental results displayed that middle temperature and ethanol
417 concentrations (at 110 °C and 50 % of ethanol respectively), regardless of the extraction time,
418 achieved the highest contents of total polar compounds. Similarly, the prediction ability of the
419 proposed experimental model was confirmed by the low variance (coefficient of variation ≤ 7.02%)
420 and the selected optimized conditions were 129 ºC, 55 % of ethanol and 22 minutes, which theoretical
421 permits to extract 2731 µg of total polar compounds in each g of grape pomace.
422 With the purpose of understand the behaviour of TPC response and the non-significant effect of the
423 factors, since it was considered as a joint variable of different families of compounds, it was
424 performed an individual statistical analysis of each chemical group detected in the evaluated extracts.
425 In this sense, tannins, flavonoids, and stilbenes, which represent an important part of phenolic content,
426 were not affected significatively by any factor (Supporting information Table 4). As mentioned
427 above, despite revealing a good fitting (lack of fit >0.05), the R^2 presented a low value (0.694), and
428 hence, the model cannot be used to predict its behaviour reliably. Although, flavonoids response
429 variable was not affected significatively by any factor, the high R^2 (0.929) and the results of lack of
430 fit test ($p>0.05$) revealed a good fitting of this response, allowing a reliable optimal conditions at
431 139°C, 54% ethanol and 22 minutes, which were similar to optimal condition for TPC (129°C, 55%
432 and 22 minutes). This result may be associated to the important contribution of this phenolic
433 compound to the total phenolic content (Figure 2). Finally, stilbenes revealed an acceptable fitting to
434 the model since R^2 was 0.830 and lack of fit test was non-significant, pointing out that the optimal
435 conditions proposed by the model (116°C, 95% and 6 minutes) would allow a predicted value of 33µg
436 od stilbenes/ g of pomace.
437 On the other hand, anthocyanins, proanthocyanins and phenolic acids presented a good fitting of the
438 model, being anthocyanins the chemical group with similar optimal conditions that TPC (105°C, 54%

439 and 12 minutes). In the same way that flavonoids, its relevant contribution (2nd chemical group most
440 abundant in the extracts) promoted these results. Moreover, it was negatively influences by
441 temperature, since at higher temperature, the recovery of anthocyanins was lower. For these reasons
442 the optimum conditions for reaching the highest anthocyanins concentration should be performed at
443 105°C, 54% and 3 minutes. However, proanthocyanins and phenolic acids revealed optimal
444 conditions that are far from those at TPC (temperatures above 180 and more than 89% EtOH).
445 Additionally, the factors presented different effect on these responses. For instance,
446 proanthocyanidins were affected by temperature and time whereas phenolic acids were affected by
447 temperature and ethanol. These results may be due to two reasons. Proanthocyanins are tightly bound
448 to cellular structures and therefore require high temperatures to enhance their recovery (Cádiz-Gurrea
449 et al., 2019). On the other hand, phenolic acids, which may be the result of thermal degradation of
450 certain more complex phenolic compounds, such as tannins and flavonoids (Chaaban et al., 2017;
451 Sebestyén et al., 2019). For these reasons, the optimum recovery temperature for these compounds
452 may be so high (Supporting information Table 4).
453
454 **4. Conclusions**
455 Circular economy promotes the shift of nowadays linear path production and consumption to circular
456 ones, where wastes are no longer a problem but a resource, reducing the use of raw materials (Camana
457 et al., 2021). According to the "new Circular Economy Action Plan" of European Commission "for
458 citizens, the circular economy will provide high-quality, functional and safe products, which
459 are efficient and affordable, last longer and are designed for reuse, repair, and high-quality
460 recycling" (European Commission, 2020). The high efficiency of pressurized liquid extraction has
461 been well established (Álvarez-Casas et al., 2014; Castellanos-Gallo et al., 2022; Ju and Howard,
462 2003; Pereira et al., 2019). Compared to conventional solid-liquid extraction, pressurised liquid
463 extraction allows to reduce solvent volume, ethanol concentration and extraction time (Nieto et al.,

2020). In this study, phytocomplexes from Cannonau grape pomace were obtained by solid liquid extraction and pressurized liquid extraction, and the methods were optimized by central composite design 2^3. The proposed model allowed an accurate prediction of the two response variables since slight differences were found between predicted and experimental data. Temperature has proven to be the most relevant parameter in increasing the extraction yield, probably due to the cell break down, which enhance the phytochemicals releasing and the extraction yield. The content of total polar compounds was influenced by both, temperature and percentage of ethanol, as higher results were obtained with high temperature (≥ 110 °C) and high concentration of ethanol (≥ 50 %). Any significant difference in the composition of the extracts was detected as a function of the used extraction technique, however, as expected, pressurized liquid extraction has proven to be a more efficient process than solid liquid extraction and it can be successfully applied to wine-making by-products to obtain a phytocomplex enriched in phenolic compounds that can be successfully implemented in different health promoting sectors, such as cosmeceutics or nutraceutics, closing the loop of the winemaking chain.

CRediT authorship contribution statement

Matteo Perra: Investigation, Formal analysis, Data curation, Writing- Original draft preparation. **Francisco-Javier Leyva-Jimenez**: Investigation, formal analysis, writing. **Maria Letizia Manca**: Investigation, Data curation, Writing- Original draft preparation. **Maria Manconi:** Supervision, Project administration, Writing - Review & Editing. **Hiba N. Rajha:** Review & Editing. **Isabel Borrás-Linares:** Methodology, Investigation, Writing. **Antonio Segura-Carretero:** Methodology, Investigation. **Jesús Lozano-Sánchez**: Methodology, Validation, Writing - Review & Editing.

Declaration of Competing Interest

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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Figure 1. Representative base peak chromatograms and extracted ions chromatograms of grape extracts obtained by solid liquid extraction (A and B) and pressurized liquid extraction (C and D) and analysed by HPLC-ESI-TOF-MS in negative ionization (A and C) or positive ionization mode (B and D).

Figure 2. Quantity of compounds (μg analyte/g of Cannonau grape pomace) found in the extracts obtained by pressurized liquid extraction (PLE) and solid-liquid extraction (SLE) using different experimental conditions. Mean values ± standard deviations are reported in Supporting information Table 2 and 3.

Figure 3. Effect of pressurized liquid extraction factors on yield (A), total polar compounds (B), tannins (C), flavonoids (D), stilbenes (E), anthocyanins (F), proanthocyanidins (G) and phenols (H).