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Optimization of bioactive compounds by ultrasound extraction and gas chromatography - mass spectrometry in fast-growing leaves

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ABSTRACT

Fast-growing crops are of great economic importance in the production of paper pulp and energy. The commercialization of the bioactive components from underutilized parts could provide additional value to these crops. In the present study, chromatography gas coupled to mass spectrometry triple quadrupole (GC–MS/MS) and ultrasound extraction technique was used in the bioactive compounds determination in fast-growing crops leaves. Twenty-one terpenes and eight polyphenolic bioactive compounds were studied. Prior to GC–MS/MS, polydimethylsiloxane (PDMS) - stir bars preconcentration and trimethylsilane (TMS) derivatization steps were used for terpenes and polyphenolic compounds, respectively. Several parameters (pH, temperature, ethanol–water mixtures, ultrasound power, and ultrasound time) of the extraction step were optimized by central composite experimental design. Ethanol content, ultrasound power, and ultrasound time were the parameters that most influence the extraction efficiency of terpenes in biomass residues, while for polyphenolic compounds it was temperature and ultrasound time.

The optimal ultrasound extraction conditions for terpenes were 60% ethanol, pH 4, 80 W, 40 °C, and 15 min, whereas for polyphenolic compounds they were 60% ethanol, pH 4, 120 W, 50 °C, and 15 min. The detection limits (LOD) were in the range of 0.200–3.02 μg kg^{-1} and 7.9–540 μg kg^{-1} for terpenes and polyphenolic compounds, respectively.

The developed analytical method was applied to twelve fast-growing leaves (*Leucaene (diversifolia and leucocephala)*, *Eucalyptus (globulus and urograndis)*, *Populus (I214 and AF2)*, *Prosopis (alba and juliflora)*, *Robinia*, *Tagasaste*, *Ulmus pumila,* and *Paulownia*). Eucalyptol was the mayor terpene present in all the plants studied, while chlorogenic and quinic acid were the mayor phenolic compounds.

1. Introduction

Fast-growing plants are used to in the production of paper pulp [1,2] and bioenergy crops [2–4] generate a significant amount of biomass residues, such as branches and leaves. Furthermore, these biomass residues present a high content of bioactive compounds with high added value, such as phenolic compounds [5–12], terpenes [13–18], and flavonoids [7]. Many of these compounds have antioxidant [7,12,17,19], antibacterial [7,12,14,15,17–19], or antitumour [7,20] properties.

In recent decades, the need to replace synthetic additives with natural ones has become apparent. Therefore, it is interesting to extract high levels of these bioactive compounds from biomass residues of fast-growing plants to be valorized, as high value-added products, for the agricultural, food, health or pharmaceutical industries [6,21–23]. Also, the isolation, identification, and characterization of potentially bioactive compounds play a decisive role.

The extraction efficiency of bioactive compounds depends on several parameters, as solvent type and concentration, extraction time, temperature, and extraction method. The classical Soxhlet extraction method requires long extraction times, a large amount of solvent, and may also cause degradation of some compounds [6,7,10,11,24–26]. For this reason, this conventional extraction method is being replaced by

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green extraction methods, which reduce extraction times and solvent amounts. These green extraction methods such as supercritical fluid extraction, microwave assisted extraction [24,27], and ultrasound extraction [6,28,29] methods have been used for extraction of bioactive compounds from biomass residues. Ultrasound extraction methods present a good efficiency and low cost. The energy (frequency) ultrasound waves generates cavitation force, which increases mass transfer causing tissue disruption and a good solvent penetration into the tissue matrix. The properties of the solvent such as vapour pressure, viscosity, surface tension influence the intensity of the ultrasonic cavitation.

On the other hand, the separation and determination of bioactive compounds have been based on the gas chromatography coupled to mass spectrometry (GC–MS) [30–32] and high performance liquid chromatography with UV (HPLC-UV) [6,11,30,32] technique. Gas chromatography provides a high resolution, but due to the polarity, hydrophilicity and low volatility of some bioactive compounds, they need to be converted into volatile ones by a derivatization step. The silytation reaction is a single-step derivatization method that has been most commonly used in the analysis of polyphenolic compound in plants, i.e., trimethylsilane (TMS) derivatives [9,25,33,34].

In the present study, the efficiency of the ultrasound extraction step was optimized for bioactive compounds (terpenes and polyphenolic compounds) in fast-growing plants biomass residues (*E. globulus* leaves). GC–MS was used to quantify the bioactive compounds. Temperature, pH, ethanol–water mixtures, ultrasound power, and ultrasound time were optimized by central composite experimental design. The optimized method was applied to several fast-growing plant biomass residues.

2. Materials and methods

2.1. Standards and chemicals reagents

Individual terpenes standards (α-pinene, sabinene, β-pinene, α-phellandrene, (Z)-3-hexenyl acetate, 3-carene, 1,4-cineole, 2-carene, o-cymene, limonene, eucalyptol, β-ocimene, γ-terpinene, linalool, fenchol, menthone, α-terpineol, myrtenal, geraniol, epiglobulol, and viridiflorol) and polyphenol standards (ellagic acid, mandelic acid, quinic acid, gallic acid, caffeic acid, epicatechin, catechin hydrate, and chlorogenic acid) were obtained from Sigma Aldrich.

Bromochlorobenzene (BCB) and 1-naphthol from Sigma Aldrich was used as internal standards (IS) for terpenes and polyphenol compounds, respectively. N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS), and pyridine were purchased from sigma Aldrich. Ethanol, methanol, and chloroform were obtained from Romil (grade HPLC). Individual terpenes and polyphenols stock solutions were prepared by weighing at a concentration of 2000 mg L^{-1} in ethanol and stored at –20 °C. The working standard solutions were prepared daily.

2.2. Fast-growing leaves

Fresh leaves of *Eucalyptus* (*globulus* and *urograndis*), *Leucaene* (*diversifolia* and *leucocephala*), *Populus* (*I214* and *AF2*), *Prosopis* (*alba* and *juliflora*), *Robinia*, *Tagasaste*, *Ulmus pumila,* and *Paulownia* from Campus "La Rábida" (University of Huelva) were collected and taken to the laboratory for washing and hand cutting into 5 cm fractions. Each test was carried out on 100 g of fresh and clean leaves with an average moisture content of 75% (±4%).

2.3. Ultrasound extraction process

An ultrasonic homogenizer (Cobos, Ultrasonic Baths Power Sonic 510, made by Hawing Technology, Seoul, Korea) with a maximum frequency of 40 kHz was used for extraction. To maintain the selected initial temperature, a Julabo Corio C Heating Immersion Circulators has been used. Aliquots of 2.0 g of *Eucalyptus globulus* leaves and 20 mL of solvent at a fixed ratio of 1:10 (w/v, dry weight) [29] were used for the extraction optimization. A central composite experimental design was used to evaluate the significance of the variables affecting the extraction, as well as the interactions between them.

According to previous research by the authors [29], the variables studied were pH (2–6), adjusted with HCl or NaOH, temperature (T) (20–60 °C), ethanol–water mixture solvent (EW) (0–60% (v/v)), ultrasound power (P) (0–160 W), and ultrasound time (t) (0–20 min). All variables were evaluated on five levels (Table 1). This design involved 29 experiments which were performed in random order.

After extraction, the solids were discarded and in the supernatant was analyzed for bioactive compounds. Terpenic compounds were analyzed immediately, while an aliquot of 0.5 mL of supernatant was freeze-dried for polyphenolic compounds analysis.

2.4. Terpenic compounds analysis by SBSE-TD-GC–MS

Terpenic compounds in the extract were pre-concentrated on PDMS stir bar sorptive extraction (SBSE) using a previously optimized procedure [29]. 1 mL of fast-growing leaves extract was placed in a 40 mL glass vial containing 10 mL of 20% NaCl. Then, a stir bar (20 mm length × 0.5 mm film thickness) coated with 47 μL of PDMS from Gerstel (Mulheim an der Ruhr, Germany) was placed in the solution, and stirred at 900 rpm for 90 min at room temperature using a Gerstel magnetic stirrer. Next, stir bar was removed from the vial, rinsed with Milli-Q water, dried with a paper tissue, and placed in a glass tube of the thermal desorption system unit (TD-20, Shimadzu, Japan) coupled to the GC–MS/MS (GCMSQP6030 Ultra, Shimadzu, Japan). Terpenic compounds were desorbed at 280 °C for 15 min, pre-concentrated on the cold trap at −20 °C, and then thermally desorbed at 300 °C for 8 min. Separation was carried out using a HP-5 MS column (60 m, 0.25 mm ID, 0.25 μm film thickness, J&W Scientific, Agilent Technologies, USA). The column was held at 60 °C for 7 min, ramped at 8 °C min^{-1} to 280 °C, and held for 6 min. Helium, at constant flow rate of 1.3 mL min^{-1}, was used as the carrier gas. The temperatures of the transfer line and ion source were maintained at 280 and 230 °C, respectively. The acquisition mode was performed in total ion current (TIC) (*m/z* 42 to 450) (Table 2).

Quantitative analysis was carried out adding 10 ppb of BCB (as instrumental internal standard) to the extract. Response factors were obtained for each standard in a six-point calibration curve and compared with the response factors of the samples.

2.5. Polyphenolic compounds analysis by GC–MS/MS

The polyphenolic compounds were derivatizated using a procedure originally developed by Zuo et al. (2002) [35]. 50 μL of pyridine and BSTFA/TMCS (90:10) were added to an aliquot freeze-dried supernatant and incubated for 30 min at 70 °C. Next, 1 μL of trimethylsilyl (TMS) derived extract was injected into GC–MS/MS (GCMSQP8030 Ultra System, Shimadzu, Japan). Separation was carried out on a HP-5 MS column (60 m, 0.25 mm ID, 0.25 μm film thickness, J&W Scientific, Agilent Technologies, USA). The GC oven was programmed as follows: 60 °C for 7 min, ramped at 8 °C min^{-1} to 280 °C, and held for 4 min. Helium, at constant flow rate of 1.3 mL min^{-1}, was used as the carrier

Table 1
Variables and levels investigated for the ultrasonic extraction step using a central composite experiment design.

	pH [a]	T (°C) [b]	EW (% v/v) [c]	P (W) [d]	t (min) [e]
level (–2)	2	20	0	0	0
level (–1)	3	30	15	40	5
level (+1)	5	50	45	120	15
Level (+2)	6	60	60	160	20
Center (0)	4	40	30	80	10

[a] pH, [b] Temperature (T), [c] Ethanol in water percentage (EW), [d] Ultrasound power (P), and [e] Ultrasound time (t).

Table 2
Retention times, quantifier ion and characteristic mass (intensity relative in %) of terpenic compounds by SBSE-TD-GC–MS.

	$t_{retention}$ (min)	Molecular Weight	Quantifier ion (*m/z*)	Characteristic mass
α-Pinene	11.22	136	93	93(100), 91(54), 77(31), 136(7)
Sabinene	12.45	136	93	93(100), 91(54), 77(42), 136(15)
β-Pinene	12.56	136	93	93(100), 91(36), 69(34), 136(7)
α-Phellandrene	13.35	136	93	93(76), 91(97), 77 (42), 136(25)
(Z)-3-Hexenyl acetate	13.38	142	43	43(100), 67(97), 82(45), 142(<1)
3-Carene	13.51	136	93	93(100), 79 (38),121(20),136 (14)
1,4-Cineole	13.63	154	43	43(100), 71(69), 111(75), 154(18)
2-Carene	13.69	136	93	93(100), 121(90), 136(55), 77(34)
o-Cymene	13.92	134	119	119(100), 91(36), 134(32), 77(8)
Limonene	14.03	136	68	68(100), 93(77), 121(21), 136(16)
Eucalyptol	14.09	154	154	43(100), 81(55), 108(37), 154(23)
β-Ocimene	14.22	136	93	93(100), 91(61), 79(41), 136(1)
γ-Terpinene	14.82	136	93	93(100), 136(34), 77(38),121(26)
Linalool	15.79	154	71	71(100), 93(76), 55(66), 154(<1)
Fenchol	16.21	154	81	81(100), 80(64), 69(27), 111(14), 154(<1)
BCB (IS)	16.49	192	192	
Menthone	17.15	154	112	112(100), 69(91), 139(51), 154(36)
α-Terpineol	17.96	154	59	59(100), 93(64), 121(49), 136(39), 154(<1)
Myrtenal	18.11	150	79	79(100), 107(62), 91(39), 150(<1)
Geraniol	19.20	154	69	69(100), 93(11), 123(10), 154(<1)
Epiglobulol	24.70	222	43	43(100), 82(64), 109(62), 161(55), 222(<1)
Viridiflorol	25.26	222	43	43(100), 69(58), 109(64), 161(54), 204 (<1)

gas. The temperatures of the injection port (split mode 10:1), transfer line, and ion source were maintained at 250, 280, and 230 °C, respectively. GC–MS/MS was operated in multiple reaction monitoring (MRM) mode. The MRM transitions of each compound were optimized by performing collision-induced dissociation (CID) experiments. First, the full scan positive ion electron ionization mass spectrometry (EI-MS) data for each of the compounds were acquired, and precursor ions were chosen. Next, these precursor ions were subjected to further fragmentation to produce product ions, and quantitative and qualitative MRM transitions were selected for each compound. For each terpenic compound two transitions were monitored for quantification and confirmation purposes. For labelled internal standard (1-naphthol) one transition was used. The most abundant transition was chosen for quantification and the other transition was selected for confirmation (Table 3). The collision energy (CE) for each mass transition was optimized using individual standard solutions of the terpenic compounds.

Quantitative analysis was carried out adding 10 ppm of 1-naphthol (as instrumental internal standard) to the extract. Response factors were determined for each of the standards in a six-point calibration curve and compared with the response factors of the samples.

Table 3
Quantification and confirmation transition and collision energies (CE) for polyphenol TMS derivatives compounds using GC–MS/MS by MRM mode acquisition.

	$t_{retention}$ (min)	Quantification transition		Confirmation transition	
		m/z	CE (mV)	*m/z*	CE (mV)
Mandelic acid	6.61	179 > 73	12	147 > 73	24
1-Naphthol (IS)	7.03	216 > 185	24		
Quinic acid	8.34	345 > 255	18	255 > 73	22
Gallic acid	8.78	458 > 281	13	433 > 399	16
Caffeic acid	9.58	396 > 219	16	381 > 307	20
Epicatechin	14.9	368 > 249	26	355 > 267	34
Catechin hydrate	15.1	368 > 249	26	355 > 267	34
Chlorogenic acid	18.2	345 > 255	19	307 > 73	20
Ellagic acid	21.3	590 > 575	14	575 > 487	30

2.6. Statistical analysis

The influence of the variables pH, temperature (T), ethanol–water mixture solvent (EW), ultrasound power, and ultrasound time (t) on the extraction of terpenic and polyphenolic compounds from *E. globulus* was investigated through a central composite experimental design.

Statistical significance of the variables was determined at the 5% probability level ($p < 0.05$). The data obtained from the 29 experimental design were fitted to second order polynomial equations and where possible the models were simplified by elimination of statistically insignificant terms. The significance of the coefficients was investigated through one-way analysis of variance (ANOVA) using the Statistica version 10.0 software (Stat soft Inc., Tulsa, OK, USA). Assays to validate the optimal extraction conditions and analysis of each point of the experimental design were performed in triplicate.

3. Results and discussion

3.1. Ultrasound extraction optimization of terpene from E. Globulus leaves

A preliminary experiment was performed in order to select the best terpenes pre-concentration conditions using a 47 μL of PDMS coated stir bar sorptive extraction. The effect of the extraction time (30, 60, 90 and 120 min) and ionic strength (0, 5, 10, 20 and 30% NaCl) were tested. 2% ethanol aqueous solutions spiked with 50 μL terpene solution at levels ranging from 1 to 3 mg L^{-1} were used in the experiments. The results showed that the highest response for all terpenic compounds were obtained with an extraction time higher than 90 min (Fig. 1a) and NaCl concentration of 20% (Fig. 1b). Therefore, 20% of NaCl and 90 min duration were used for further experiments.

Ultrasound conditions for terpenes extraction from biomass residues were optimized using a central composite experimental design with *E. globulus* leaves. The results are presented in Table S1 (supplementary material). Eucalyptol was the most abundant followed by α-pinene, α-terpineol, viridiflorol, limonene, epiglobulol, and geraniol. Other compounds, such as β-pinene and O-cymene, were the least abundant. Table 4 shows the coefficients obtained from a polynomial regression between the analytical response and the independent variables for the most abundant terpenes, as well as the variations in the dependent

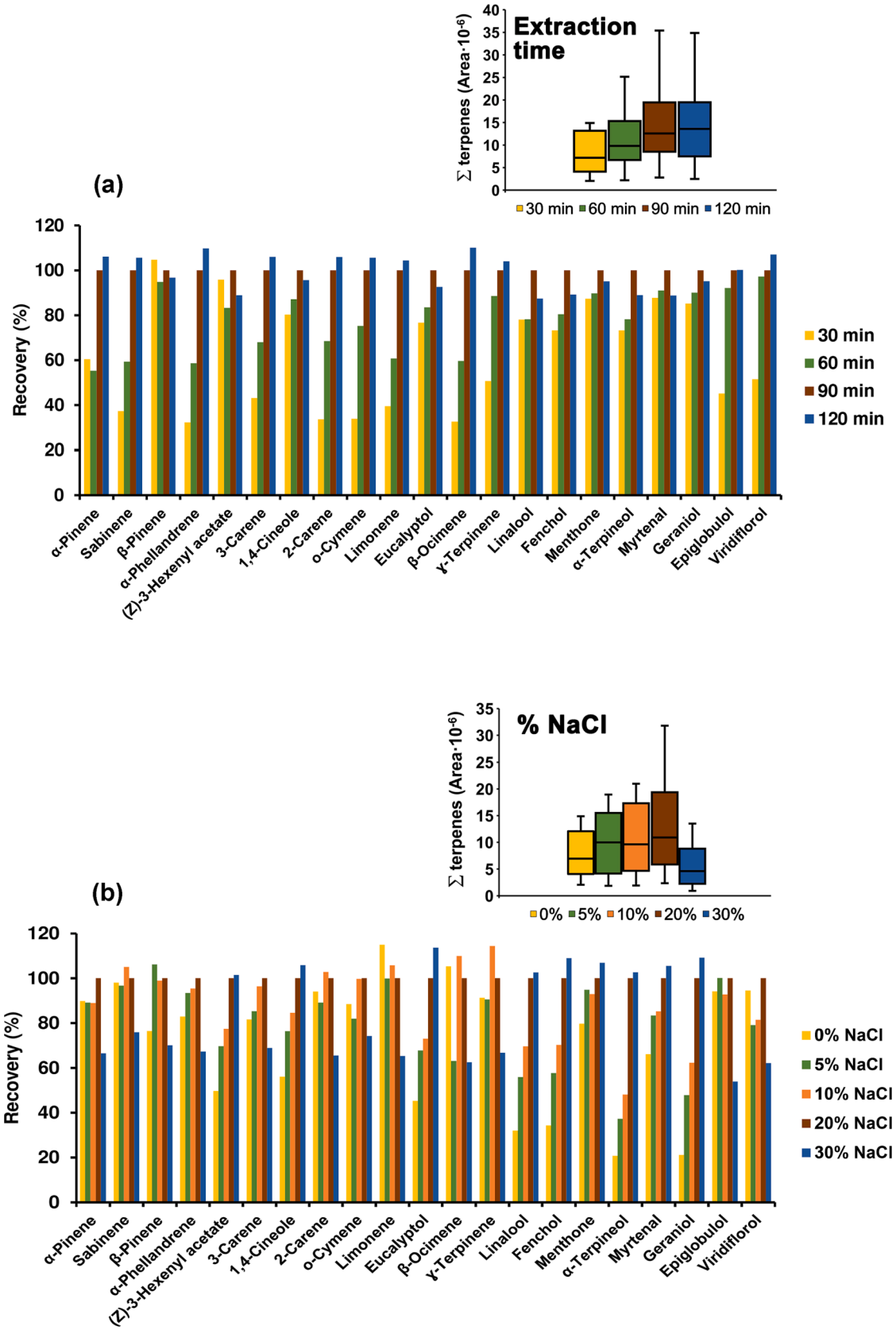

Fig. 1. Effect of (a) extraction time and (b) ionic strength on pre-concentration terpenic compounds using 47 μL of PDMS coated stir bar sorptive extraction.

Table 4
Coefficients for terpenes (mg kg^{-1} dry leaves) model as a function of the independent variables (normalized values).

	α-Pinene	Eucalyptol	Epiglobulol	Viridiflorol	α-Therpineol	Geraniol	∑ Terpenes
Intercept	7.95*	421*	0.953*	3.28*	4.83*	0.696	441*
pH	–0.207	–4.350	0.096	–0.015	–1.188	–0.443	–6.40
pH^2	–0.557	–40.97	–0.058	–0.301	0.776	0.258	–40.9
T	0.526	92.25	0.060	0.529	2.87*	0.806*	97.4
T^2	–1.70*	–152*	–0.078	–0.675	–1.80	–0.332	–158*
EW	1.08	163*	0.633*	2.01*	0.261	0.237	169*
EW^2	–2.36*	36.82	0.046	0.152	0.443	0.377	37.780
P	3.73*	90.93	0.178	0.903	2.69*	0.697*	99.346
P^2	–1.91*	–137*	–0.403	–0.940	–0.870	0.045	–142*
t	–0.483	129*	0.035	0.602	2.52*	0.558	133*
T^2	–0.536	–55.89	–0.127	–0.455	–0.956	–0.144	–58.6
pH·T	0.093	–60.80	–0.079	–0.812	–1.58	–0.600	–64.1
pH·EW	–0.525	–62.30	–0.088	–0.940	–0.624	–0.103	–64.6
T·EW	–0.090	50.91	0.033	0.105	1.32	0.242	53.0
pH·P	1.10	–4.28	0.086	0.243	0.281	0.105	–2.483
T·P	2.24*	73.24	0.041	0.481	1.67	0.686	79.2
EW·P	–0.822	47.05	–0.011	0.124	1.87	0.615	49.3
pH·t	0.511	11.74	–0.045	–0.332	1.31	0.474	13.5
T·t	–2.49*	–40.39	–0.055	–0.419	–1.16	–0.175	–44.6
EW·t	1.27	14.07	–0.035	–0.106	0.402	–0.014	15.4
P·t	–0.702	10.73	–0.013	0.000	0.891	0.154	10.6
r^2	0.921*	0.895	0.822	0.843	0.857	0.850	0.899
r	0.960*	0.946	0.907	0.918	0.926	0.922	0.948

Ultrasound power (P), Temperature (T), pH, ethanol in water percentage (EW), and ultrasound time (t).
*Values: statistically significant.

variables with respect to the changes in the independent variables. The determination coefficient (r^2) of the terpenes was >0.822. In addition, Fig. 2 shows t-value effects of the significant independent variables ($p < 0.05$) on the response dependent variable studied. The results show that each compound has a different response to the selected variables. pH has no significant influence on the response of the terpenes. However, the other variables have a significant effect on the efficiency of terpene extraction.

The ethanol–water solvent affects the extraction of eucalyptol, epiglobulol and viridiflorol extraction. Solvent properties such as vapor pressure, viscosity, surface tension or dielectric constant influence the intensity of cavitation. Increasing the ethanol content of the solvent increases the terpene concentration. This may be due to an increase in the solubility of terpene compounds as the ethanol concentration increases [36]. However, for α-pinene, the quadratic ethanol–water solvent coefficient had a negative effect on extraction efficiency, the optimum conditions being with an intermediate ethanol–water solvent. This trend may be due to the fact that the increase in ethanol concentration increases the extraction efficiency of α-pinene, with an increase in cavitation intensity, up to a maximum ethanol concentration and then has a negative effect on the extraction efficiency due to an increase in cavitation bubbles. The linear coefficient power ultrasound has positive significant effect on the extraction efficiency of α-pinene, α-terpineol, and geraniol. This was due to the cavitation effect causing a tissue disruption and solvent penetration into the tissue matrix [36]. In addition, in the quadratic coefficient models of α-pinene and eucalyptol (Table 4) present negative significant contribution due to high power ultrasund, the cavitation effect decreased at higher bubble volume [36], and decreased the extraction efficiency of terpenic compounds. Temperature extraction has significant effect on the extraction of α-terpineol and geraniol, whereas the quadratic coefficient models (Table 4) present a negative insignificant contribution. For α-pinene and eucalyptol, the quadratic temperature had negative significant effect. The increase in temperature increase the solubility of the analytes and decrease the viscosity of the solvent increased the mass transfer [36]. However, at high temperature increase vapour pressure and reduce the surface

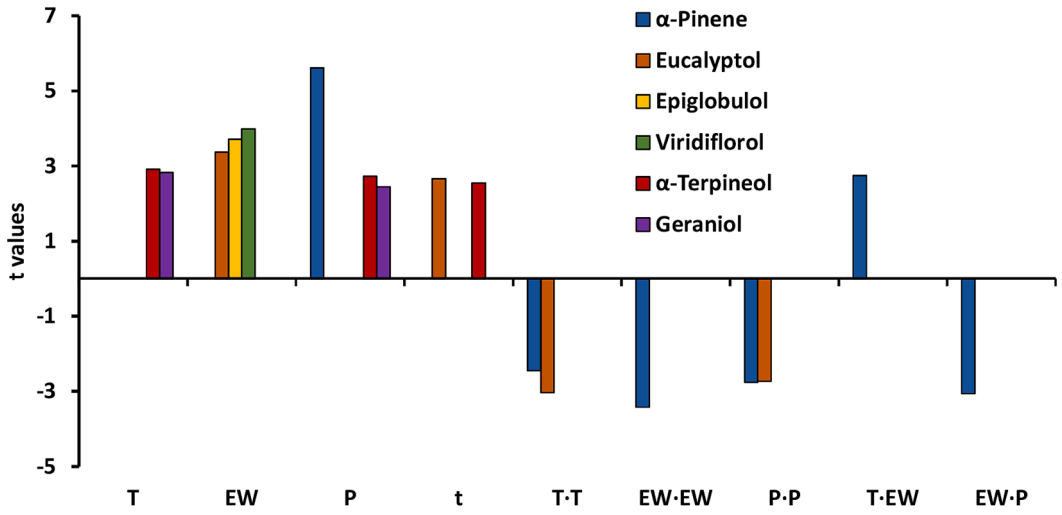

Fig. 2. Pareto diagram obtained from the central composite experimental design for the optimization of the ultrasound parameters for the terpenes extraction in leaves of *E. globulus*.

tension of the solvent decreased the number of cavitation bubbles is large causing a decrease on extraction efficiency [36]. Extraction time had positive significant influence on extraction of eucalyptol and α-terpineol, while the quadratic coefficient had negative insignificant effect. The interaction temperature and solvent ethanol content had a positive effect on α-pinene extraction, while interaction of ultrasonic power and solvent ethanol content had a negative effect.

In addition, the optimal extraction conditions for each terpene were quite different due to the different characteristics and degradability of each compound, however, for industrial extraction purposes, suitable conditions to obtain an optimal amount of total terpenes must be selected. Therefore, to select the optimal conditions, the sum of the extracted compounds at each point of the experimental design was carried out. Temperature, ethanol–water solvent, ultrasonic power, and extraction time had highest significant influence on the efficiency of terpene extraction from *E. globulus* leaves. Fig. 3 shows the three-dimensional surface plot of the model for total terpenes from *E. globulus*. The terpenes extraction efficiency decreased with lower ethanol concentration, probably due to the increase in polarity of the solvent. The maximum total terpene extraction efficiency extraction was obtained using high ethanol percentage (60%), medium ultrasonic power (80 W), temperature (40 °C), and pH 4 for 15 min.

3.2. Ultrasound extraction optimization of polyphenols from E. Globulus leaves

In this study, BSTFA was used as derivatization reagent for the determination of the polyphenolic compounds in *E. gloublus* leaves by GC–MS/MS. The BSTFA silylation method requires anhydrous conditions. Therefore, a preliminary step to remove the water was studied. Liquid-liquid extraction and freeze-dried were evaluated. Aliquots of 0.5 mL of aqueous solutions spiked with 50 μL polyphenols solution at a level ranging from 1 to 3 mg L^{-1} were used in the experiments. The solvents chloroform and ethyl acetate [34] were evaluated for liquid–liquid extraction. The higher percentage recoveries were obtained when the samples were freeze-dried. Therefore, this method was selected for further experiments.

Table S2 (supplementary material) shows the polyphenols concentration extracted from *E. globulus* leaves under different conditions using a central composite experimental design. Chorogenic and quinic acids were the major polyphenolic compounds present in leaves (92 ± 10% of total polyphenols), followed by ellagic acid (6 ± 8%) and gallic acid (1.4 ± 2.3%). Catechin hydrate, epicatechin, caffeic acid, and mandelic acid accounted for <1% of the total polyphenolic compounds.

The coefficients obtained by means of a multiple polynomial regression of the variables for each polyphenolic compound are shown in Table 5. The regression model was significant for all polyphenolic compounds with $r^2 \geq 0.74$. To visualize the relative significant influence of each independent variable ($p > 0.05$), t-values effect diagram was presented in Fig. 4. The variable does not have a significant influence on mandelic and ellagic acids extraction; therefore, they were not included in the diagram. The results show that temperature had a positive effect on all polyphenolic compounds, except for caffeic acid. Similar results were obtained for total phenolic content [37] or chlorogenic acid [37]. This may be due to the fact that increasing the temperature increases the solubility of the compounds in the solvent and improves the extraction efficiency as has been reported by for total phenolic content or chlorogenic acid [37]. Furthermore, cavitation and vibration, induced by ultrasound, may contribute to the improved extraction efficiency by facilitating better solvent penetration [36].

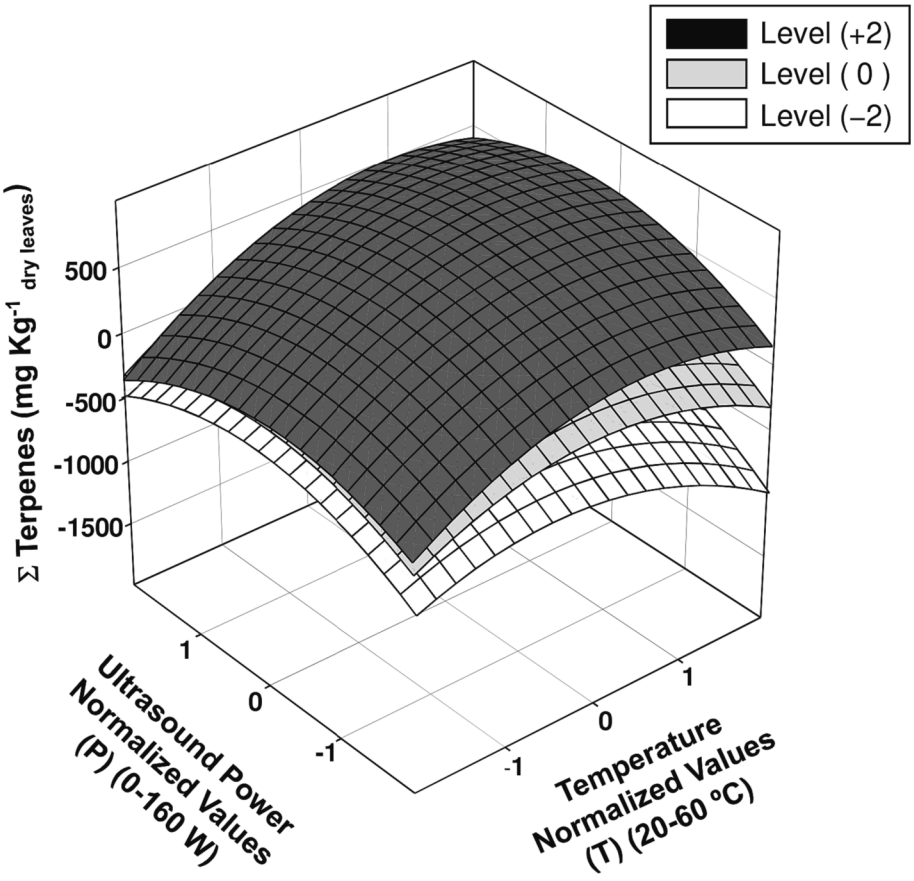

Fig. 3. Three-dimensional surface plot of the model for optimization of the ultrasound parameters to total terpenes extraction in l*eaves of E. globulus* at pH 2, 0% Ethanol in water, 1 min (Level –2); pH 4, 30% Ethanol in water, 10 min (Level 0); pH 6, 60% Ethanol in water, 20 min (Level +2).

Table 5
Coefficients for the polyphenols (mg kg^{-1} dry leaves) model as a function of the independent variables (normalized values).

	Mandelic Ac.	Quinic Ac.	Gallic Ac.	Caffeic Ac.	Epicatechin	Catechin hydrate	Chlorogenic Ac.	Ellagic Ac.	∑ polyphenols
Intercept	0.195	3381*	40.9	0.309	3.86	24.1*	7815*	799	12065*
pH	0.069	263	56.1	–0.300	1.37	3.76	803	110	1238
pH^2	0.093	–322*	15.4	0.163	–0.277	–2.70	–782	–142	–1233
T	0.024	1202*	61.8	–0.068	2.11	9.08*	3474*	1326	4882*
T^2	–0.023	48.8	13.3	0.129	0.651	0.145	–137	–76.6	–151
EW	0.018	148	–44.2	–0.150	–0.484	3.60	1929	163	2199
EW^2	–0.034	–132	11.4	0.247	1.17	2.06	1058	81.6	1022
P	0.019	615*	–36.8	–0.002	–1.17	2.48	1401	11. 5	1992
P^2	–0.040	–257	7.81	0.459*	0.250	–2.55	–1359	–283	–1893
T	0.023	1231*	46.71	0.290	2.44*	8.38*	3159*	95.9	4544*
t^2	–0.052	–130	11.3	0.113	0.015	–1.68	–543	–2.50	–665
pH·T	–0.027	–327*	80.3	0.199	3.25*	2.76	98.4	45.0	–96.8
pH·EW	–0.018	–40.4	–68.0	–0.050	–1.14	–0.748	465	–10.0	345
T·EW	0.028	–41.3	–62.8	0.092	–0.469	–0.006	299	2.64	198
pH·P	0.005	–11.8	–61.7	–0.194	0.212	0.151	334	–1.63	259
T·P	0.018	289	–58.7	–0.092	–0.311	1.59	1324	3.99	1560
EW·P	–0.005	–497*	58.5	0.096	1.60	0.349	–169	41.9	–563
pH·t	0.000	–205	59.7	0.049	0.041	0.453	389	54.2	298
T·t	0.004	709*	63.1	0.170	0.721	5.38	2326	45.1	3150
EW·t	–0.004	9.157	–61.3	–0.201	–1.43	–0.368	309	–10.4	245
P·t	–0.007	503*	–67.3	–0.299	–2.56	0.789	1115	–21.5	1527
r^2	0.630	0.977	0.861	0.740	0.798	0.876	0.849	0.555	0.872
r	0.746	0.988*	0.928	0.860	0.893	0.936	0.921	0.745	0.934

Ultrasound power (P), Temperature (T), pH, ethanol in water percentage (EW), and ultrasound time (t).
* Values: statistically significant.

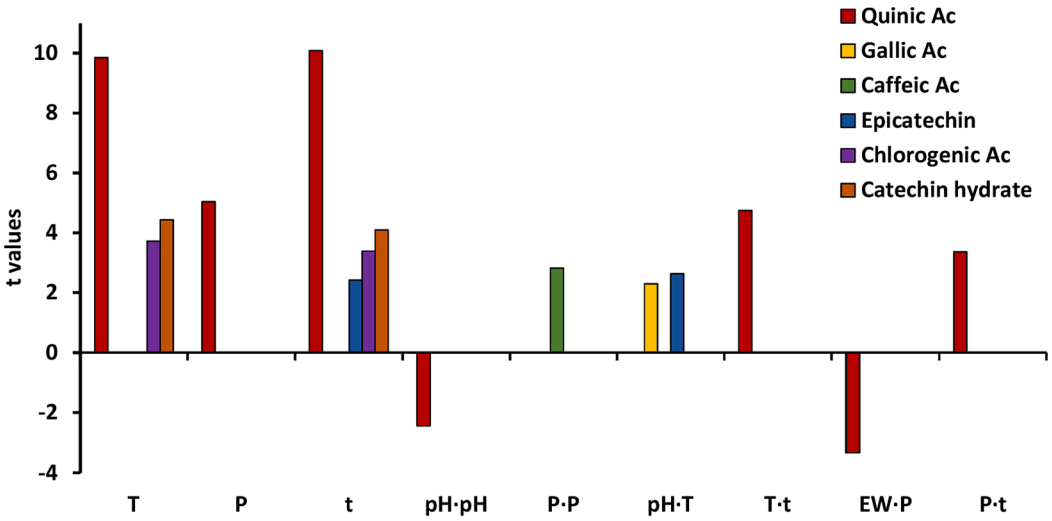

Fig. 4. Pareto diagram obtained from central composite experimental design to optimize the ultrasonic parameters for extracting terpenes from *E. globulus* leaves.

Extraction time was another important parameter with significant effect on the extraction efficiency of the polyphenolic compounds studied, except for gallic and caffeic acid. The linear effect was positive, meaning an increase in extraction efficiency. However, the quadratic effect of time was not statistically significant and varied among the different compounds. Epicatechin, gallic and caffeic acids showed a positive effect, whereas catechin hydrate, quinic, chlorogenic, and ellagic acids showed a negative effect. This may indicate that long extraction times may degrade these last compounds. Similar results were obtained by other authors for total phenolic content and flavanols [37,38]. Ultrasonic power had a statistical influence on the extraction of quinic and caffeic acids. For quinic acid, as can be observed in the Table 5, the significant linear and no significant quadratic effect are positive and negative respectively, indicating that higher ultrasonic power can degrade it. However, the extraction efficiency of caffeic acid shows a significant positive effect of the ultrasonic power quadratic term, indicating an increase with amplitude. Similar results have been obtained by other authors [39,40]. Finally, the pH only statistically influences quinic acid, a significant but negative effect of the quadratic term of pH, which caused a decrease in the extraction of quinic acid.

The interaction of time with temperature and ultrasound power had a positive effect in quinic acid, while the interaction with ethanol content and power had a negative effect. In the quinic acid model (Table 5), the ethanol content and its quadratic coefficient have a positive and negative contribution, respectively. These negative effects may be due to the polarity of the solvents and the polyphenol compound on the extraction efficiency. For the other polyphenolic compounds models, the ethanol content was statistically insignificant, however the comportment is different for each one. Catechin hydrate, chlorogenic, and ellagic acids models present a positive contribution of both coefficients, linear and quadratic, increasing the extraction efficiency with ethanol content. However, gallic acid, caffeic acid, and epicatechin models show a negative effect with ethanol content, increasing the extraction efficiency as the ethanol content decreased. Several authors [36,41,42] have indicated that a mixture of alcohol and water is more effective for phenolic compounds extraction than alcohol alone. Alcohol reduces the

dielectric constant of the extraction solvent, allowing the analytes to attach to the solvent molecules, while water creates a more polar medium and the breaking of hydrogen bonds facilitates the extraction of the most and least polar polyphenolic compounds [42]. The efficiency of gallic acid and epicatechin extraction was statistically influenced by the interaction between pH and temperature.

This leads to the conclusion that the optimum extraction conditions are slightly different depending on the selected compound. However, for industrial use, suitable conditions should be selected for most of the polyphenols. To estimate the optimal conditions for the extraction of polyphenols, the total sum was considered. Temperature and extraction time had most significant influence on the extraction efficiency of polyphenols from *E. globulus* leaves. The three-dimensional graphical representation of the total sum of the extracted polyphenols is shown in Fig. 5. The efficiency of polyphenol extraction increased with temperature and time, indicating that these parameters have a positive effect on the extraction of these compounds. The best conditions were obtained using medium ultrasound power (120 W), high temperature (50 °C), pH 4, and ethanol in water (60%) for 15 min.

3.3. Analytical characteristics of terpenes and polyphenols method

Linear range, detection, and quantification limits of the method were performed based on TIC and MRM for terpenes and polyphenol derivatives, respectively. A six-point matrix-matched calibration and internal standards were used (Table 6).

The detection and quantification limits (LOD and LOQ, respectively) were calculated with a signal to noise ratio of 3 (LOD) and 10 (LOQ). LOD and LOQ were in the range of 0.200 to 3.02 μg kg^{-1} and 0.665 to 10.1 μg kg^{-1}, respectively, for terpenes in leaves. For polyphenolic compounds, LOD and LOQ were between 7.9–540 μg kg^{-1} and 26–1800 μg kg^{-1}, respectively.

The response was linear between LOD and two orders of magnitude of ppb and ppm for polyphenols and terpenes, respectively, with determination coefficients (r^2) above 0.999.

LODs obtained by the proposed GC–MS/MS method were much lower than those obtained by HPLC-UV [11,43] or LC-MS/MS [30] for

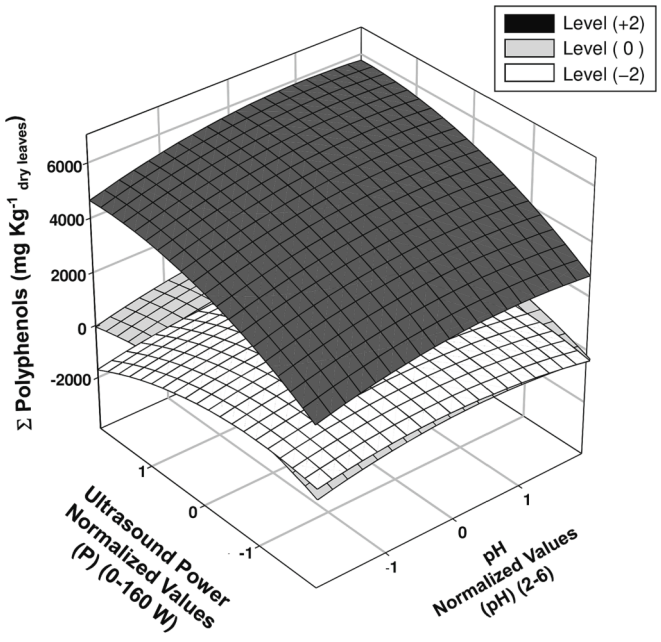

Fig. 5. Three-dimensional surface plot of the model to total polyphenols extraction in leaves of *E. globulus* at 0% Ethanol in water, 1 min and 20 °C (Level –2); 30% Ethanol in water, 10 min, 40 °C (Level 0); 60% Ethanol in water, 20 min and 60 °C (Level +2).

Table 6
Analytical performance for the determination of terpenes and polyphenols in leaves *E. globulus* by ultrasonic extraction and SBSE-TD-GC–MS (scan mode) and TMS derivatives-GC–MS/MS (MRM mode), respectively.

	Linear range (mg L^{-1})	r^2	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
Terpenes				
α-Pinene	$(0.01–27)\cdot10^{-3}$	0.999	0.51	1.7
Sabinene	$(0.01–10)\cdot10^{-3}$	1.000	0.61	2.0
β-Pinene	$(0.01–30)\cdot10^{-3}$	0.999	0.35	1.2
α-Phellandrene	$(0.01–10)\cdot10^{-3}$	1.000	0.43	1.5
3-Carene	$(0.01–27)\cdot10^{-3}$	1.000	0.41	1.4
1,4-Cineole	$(0.03–29)\cdot10^{-3}$	1.000	1.4	4.5
o-Cymene	$(0.01–14)\cdot10^{-3}$	0.999	0.40	1.3
Limonene	$(0.01–10)\cdot10^{-3}$	0.999	0.53	1.8
Eucalyptol	$(0.004–26)\cdot10^{-3}$	0.999	0.20	0.67
γ-Terpinene	$(0.01–29)\cdot10^{-3}$	0.999	0.45	1.5
Linalool	$(0.02–28)\cdot10^{-3}$	0.999	0.81	2.7
Fenchol	$(0.006–25)\cdot10^{-3}$	0.999	0.29	0.97
Menthone	$(0.003–26)\cdot10^{-3}$	0.999	0.13	0.44
α-Terpineol	$(0.04–21)\cdot10^{-3}$	0.999	1.9	6.2
Myrtenal	$(0.02–19)\cdot10^{-3}$	0.999	0.78	2.6
Geraniol	$(0.23–25)\cdot10^{-3}$	0.999	13	44
Epiglobulol	$(0.03–20)\cdot10^{-3}$	0.999	1.4	4.7
Viridiflorol	$(0.06–10)\cdot10^{-3}$	1.000	3.0	10
Polyphenols				
Mandelic acid	0.010–10	0.999	21	71
Quinic acid	0.013–22	0.999	26	85
Gallic acid	0.006–19	0.999	13	42
Caffeic acid	0.017–19	0.999	33	$1.1\cdot10^2$
Epicatechin	0.016–161	0.999	31	$1.0\cdot10^2$
Catechin hydrate	0.004–24	0.999	7.9	26
Chlorogenic acid	0.157–143	0.999	$3.1\cdot10^2$	$1.0\cdot10^3$
Ellagic acid	0.270–224	0.999	$5.4\cdot10^2$	$1.8\cdot10^3$

all polyphenolic compounds of this study.

To study the precision of the overall process, five samples of *Eucalyptus* leaves, from different trees, were subjected to ultrasonic extraction, and analysed under optimum conditions for terpenes and polyphenolic compounds. The average relative standard derivation (RSD) of the overall process for the twenty-one terpenes and eight polyphenolic compounds was <13.8% and 12.3%, respectively.

3.4. Applications of the analytical method for the study of terpenes and polyphenols in fast-growing plants leaves

The developed analytical method was applied to the determination of terpenes and polypenols in leaves of fast-growing plants, such as *Leucaene*, *Eucalyptus*, *Populus*, *Proposis*, *Robinia*, *Tagasaste*, and *Ulmus Pumila* using the previously determined optimal ultrasound extraction conditions: 60% ethanol, pH 4, 80 W, 40 °C, and 15 min for terpenes, and 60% ethanol, pH 4, 120 W, 50 °C, and 15 min for polyphenolic compounds. The results showed that polyhenols and terpenes concentrations were in the order of mg kg^{-1} and μg kg^{-1}, respectively (Table 7). *Eucalyptus* leaves showed the highest terpene concentrations.

Eucalyptol was the mayor terpene in all leaves with values between 65 and 100% of the total terpernes. Similar results were obtained by other authors [16,19,31,44] for different *Eucalyptus* species, such as *parvula*, *cinerea*, *pulverulenta*, *globulus*, *radiata*, *maculata* [16,19,31,44], and *hydrosols* [31]. However, other authors [7,14,15,32,45–47] found lower percentages (<48%) in *E. globulus*, *E. camaldulensis*, *P. Alba*, *P. balsamifera*, and *P. nigra* by HS-SPME-GC–MS [32], hydrodistilled [14,15,46], Soxhlet [7], or shaking extraction with methanol [7,45] or hexane [47].

Variable percentages were found for the remaining of terpenes: α-pinene (0.8–26%), β-pinene (0.3–4.2%), o-cymene (0.04–16%), limonene (0.1–5%), and α- terpineol (1–3%). Also, some authors [7,15,16,19,31,32,44] have also reported these compounds in

Table 7
Concentration of terpenes and polyphenols (mg kg^{-1}) in leaves of fast-growing plants under optimal extraction conditions.

	L. diversifolia	*L. leucocephala*	*P. juliflora*	*P. alba*	*E. urograndis*	*E. globulus*	*P. I214*	*P. AF2*	*Tagasaste*	*U. pumila*	*Robinia*	*Paulownia*
α-pinene	0.164 ± 0.015	0.192 ± 0.016	0.154 ± 0.012	<LOD	0.301 ± 0.023	4.99 ± 0.37	<LOD	0.129 ± 0.010	0.657 ± 0.098	0.079 ± 0.006	0.079 ± 0.007	0.492 ± 0.036
β-Pinene	0.086 ± 0.007	0.072 ± 0.007	<LOD	<LOD	<LOD	2.42 ± 0.15	<LOD	<LOD	0.061 ± 0.005	<LOD	<LOD	0.176 ± 0.012
3-Carene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.180 ± 0.016
o-Cymene	<LOD	<LOD	<LOD	<LOD	0.996 ± 0.076	<LOD	<LOD	<LOD	1.04 ± 0.09	0.014 ± 0.001	0.16 ± 0.013	0.689 ± 0.055
Limonene	<LOD	<LOD	<LOD	<LOD	0.802 ± 0.069	0.706 ± 0.052	0.340 ± 0.032	<LOD	0.355 ± 0.030	0.124 ± 0.011	<LOD	<LOD
Eucalyptol	2.37 ± 0.19	1.44 ± 0.12	1.28 ± 0.10	1.97 ± 0.16	35.0 ± 3.0	688 ± 52	3.02 ± 0.20	0.367 ± 0.030	5.00 ± 0.40	3.51 ± 0.33	1.00 ± 0.08	2.89 ± 0.24
α-Terpineol	<LOD	<LOD	<LOD	<LOD	1.01 ± 0.096	7.03 ± 0.62	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
$\Sigma_{Terpenes}$	2.62 ± 0.18	1.70 ± 0.13	1.44 ± 0.12	1.97 ± 0.16	38.1 ± 3.1	703 ± 52	3.36 ± 0.23	0.50 ± 0.02	7.11 ± 0.40	3.72 ± 0.33	1.24 ± 0.07	4.43 ± 0.25
Mandelic acid	0.109 ± 0.010	0.157 ± 0.011	0.470 ± 0.039	0.123 ± 0.009	0.11 ± 0.01	0.115 ± 0.012	0.685 ± 0.063	0.200 ± 0.019	0.038 ± 0.004	0.313 ± 0.028	0.007 ± 0.001	0.622 ± 0.045
Quinic acid	0.71 ± 0.07	(2.13 ± 0.19)·10^3	716 ± 51	28.4 ± 2.5	333 ± 27	(7.35 ± 0.45)·10^3	0.347 ± 0.028	(21.59 ± 1.18)·10^3	0.944 ± 0.088	16.3 ± 1.3	2.95 ± 0.27	119 ± 9
Gallic acid	1.55 ± 0.11	5.21 ± 0.44	3.55 ± 0.29	6.63 ± 0.51	38.0 ± 3.0	73.7 ± 4.8	6.81 ± 0.56	4.26 ± 0.35	0.451 ± 0.036	2.79 ± 0.22	0.091 ± 0.007	6.97 ± 0.57
Caffeic acid	4.06 ± 0.39	17.9 ± 1.49	4.44 ± 0.40	4.50 ± 0.40	8.64 ± 0.74	1.83 ± 0.16	2.38 ± 0.23	60.2 ± 3.2	1.02 ± 0.09	39.9 ± 2.6	0.067 ± 0.006	92.3 ± 4.8
Epicatechin	0.067 ± 0.006	25.7 ± 2.0	5.26 ± 0.51	3.75 ± 0.36	0.081 ± 0.008	10.1 ± 0.8	0.217 ± 0.021	24.71 ± 2.2	4.85 ± 0.47	320 ± 18	0.041 ± 0.004	5.89 ± 0.54
Catechin hydrate	5.22 ± 0.45	1.69 ± 0.09	17.0 ± 1.6	14.1 ± 1.3	3.13 ± 0.32	68.5 ± 6.5	0.568 ± 0.060	215 ± 9	0.250 ± 0.027	9.64 ± 0.60	0.835 ± 0.066	0.033 ± 0.003
Chlorogenic acid	<LOD	(5.60 ± 0.53)·10^3	(6.03 ± 0.32)·10^3	363 ± 35	45.7 ± 4.0	(29.99 ± 1.67)·10^3	<LOD	(695.9 ± 38.5)·10^3	133 ± 9	(783.5 ± 44.6)·10^3	<LOD	(1.70 ± 0.16)·10^3
Ellagic acid	<LOD	2.17 ± 0.22	<LOD	<LOD	<LOD	(1.43 ± 0.11)·10^3	<LOD	3.17 ± 0.29	<LOD	<LOD	<LOD	<LOD
$\Sigma_{Polyphenols}$	11.7 ± 0.57	(7.78 ± 0.66)·10^3	(6.78 ± 0.28)·10^3	420 ± 36	428 ± 33	(38.61 ± 2.17)·10^3	11.0 ± 0.9	(717.8 ± 39.7)·10^3	141 ± 10	(783.9 ± 44.6)·10^3	3.99 ± 0.31	(1.92 ± 0.18)·10^3

*Each value is the average of three samples (Mean ± SD, standard deviation <5%).

Eucalyputs leaves. No results have been published on the terpene content of *Leucaena*, *Proposis*, *Robinia*, *Tagasaste*, *Ulmus*, and *Paulownia* leaves to the best of the authors' knowledge.

P. AF2 and *U. Pumila* leaves showed the highest concentrations of polyphenols with values of 718 and 784 g kg^{-1}, respectively, followed by *E. globulus* (38.6 g kg^{-1}), *L. leucocephala* (7.8 g kg^{-1}), and *P. Juliflora* (6.8 g kg^{-1}). The percentage of each polyphenol varied between plant species. Chlorogenic acid was the mayor polyphenol in *U. pumila, P. AF2*, *Tagasaste, P. juliflora,* and *Paulownia* (>88%), but it was not detected in *L. diversifolia*, *P. I214,* and *Robina*. This polyphenol has several important therapeutic roles, such as antioxidant activity, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, antiviral, anti-microbial, anti-hypertension, free radical scavenger, and a central nervous system (CNS) stimulator [48]. Catechin (45%), caffeic acid (35%), and gallic acid (13%) were the main compounds present in *diversifolia* species, and gallic acid (62%) and caffeic acid (22%) in *P. I214*. These results are in ageement with those obtained by other authors such as González-Burgos et al. (2018) [49]. However, some authors [5,7,32] reported that gallic and ellagic acids were the main polyphenolic compounds in *E. globulus* leaves. Also, Ashraf et al. (2015) [45] found that gallic acid was the main phenolic acid in *E. camalducence* leaves.

4. Conclusions

Ultrasound extraction is an economical and efficient technique for the extraction of bioactive compounds (terpenes and polyphenolic compounds) from the leaves extracts of fast-growing crops. The variables temperature, pH, ethanol–water mixtures, ultrasound power, and ultrasound time were optimized using a central composite experimental design. The structural differences between the terpenic and phenolic compounds influenced the optimal extraction conditions: 60% ethanol, pH 4, 80 W, 40 °C, and 15 min for terpenes, and 60% ethanol, pH 4, 120 W, 50 °C, and 15 min for polyphenolic compounds.

21 terpenes and 8 polyphenolic compounds were identified and quantified in leaves extracts of 12 fast-growing plants (*Eucalyptus* (*globulus* and *urograndis*) *Leucaene* (*diversifolia* and *leucocephala*), *Populus* (*I214* and *AF2*), *Prosopis* (*alba* and *juliflora*), *Robinia*, *Tagasaste*, *Ulmus pumila*, and *Paulownia*) by GC–MS. Prior to GC–MS, terpenes were preconcentrated by PDMS stir bar sorption and polyphenols were

preconcentrated by TMS derivatization. Eucalyptol was the most abundant terpene in *Leucaene*, *Eucalyptus*, *Populus*, *P. I214*, and *U. Pumila*. Chlorogenic acid was the most abundant polyphenol in *Populus*, *Tagasaste*, *U. Pumila,* and *Paulwnia*.

The bioactive compounds studied have antioxidant, antibacterial, and antitumor properties. For this reason, this work aims to optimize a methodology for the extraction and quantification of these bioactive compounds from a large amount of biomass residues from fast-growing plants to be valued as high value-added products useful for the agricultural, food, and pharmaceutical industries. The growing economic importance of bioactive compounds may lead to the search for new compounds in the leaves of different biomass crops and attempts to optimize their extraction individually in the future.

CRediT authorship contribution statement

Alberto Palma: Investigation, Methodology, Formal analysis, Writing - original draft. **Mercedes Ruiz-Montoya:** Investigation, Methodology, Supervision. **Manuel Jesús Díaz:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Inmaculada Giráldez:** Investigation, Data curation, Writing – review & editing, Project administration. **Emilio Morales:** Conceptualization, Methodology, Validation.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2023.109231.

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