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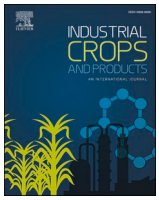

Utilizing green solvents in compressed fluids technologies for extracting bioactive compounds from *Ruta graveolens* L.

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ABSTRACT

Ruta graveolens L. is considered an important source of alkaloids and furanocoumarins, with attributed biological activity against phytopathogenic microorganisms. Pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) using bio-based solvents are green alternative extraction techniques to obtain specific type of compounds. From aerial parts, terpenes were favorably extracted by PLE, while by SFE using batch extraction, different fractions were obtained depending on the extraction pressure: at 200 bar, fatty acids enrichment was achieved from dry sample, while at 350 bar mainly alkaloids and furanocoumarins were obtained from moistened sample. Coumarins were extracted mainly from roots by the two extraction techniques tested. However, this family is always extracted together with alkaloids and furanocoumarins. Therefore, in this work a green methodology has been developed to obtain rue extracts enriched in different families of biologically active substances that could have antifungal or nematicidal activity for pathogen control in agricultural activities.

1. Introduction

Ruta graveolens L., commonly known as rue, is an aromatic, herbaceous, perennial plant that belongs to the Rutaceae family in the order of Sapindales. This plant in native to the Mediterranean region (Southern Europe and Northern Africa), where its use in traditional medicine became popular (San Miguel, 2003). Due to its used in folk medicine, over the years it has been introduced in various countries of America, China, and India and its cultivation is very abundant in Brazil and other tropical countries (Asgarpanah and Khoshkam, 2012). Several therapeutic properties described from *R. graveolens* includes the use of this plant in skin inflammation, earache, headache, cramps and menstrual disorders (França Orlanda and Nascimento, 2015).

The most valued constituents of *R. graveolens* are their essential oils. These rue essential oils are of interest because of their demonstrated nematicidal, antiprotozoal, antioxidant, antibacterial or anticancer activity (Al Qaisi et al., 2023; Al Qaisi et al., 2022; Al Qaisi et al., 2022; Al Qaisi et al., 2023; da Silva et al., 2014; Faria et al., 2013; Jianu et al., 2021). Rue biological activities are mainly attributed to the presence of secondary metabolites such as alkaloids and furanocoumarins (Oliva et al., 2003; Reis et al., 2015). There are other families of metabolites also identified in rue extracts, such as terpenes and fatty acids

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(Reyes-Vaquero et al., 2021). Some terpenes, above all L-carvone, pulegone and *trans*-anethole, have been reported to be more active to *Meloidogyne incognita* paralysis than as components in essential oils of some Greek aromatic plants (Ntalli et al., 2010). Myristic, palmitic, and oleic fatty acids were identified as the nematicidal principles in benzene extracts of roots of *Iris japonica* (Iridaceae) (Chitwood, 2002). As a result of this proven nematicidal activity of specific metabolites, there is an increasing demand for plant extracts that can be used as an alternative to substitute chemical pesticides for plant diseases control (Abubakar et al., 2020), many of them present in rue.

R. graveolens extracts are commonly obtained by maceration (Meepagala et al., 2005), percolation (Reis et al., 2015), and sonication (Reyes-Vaquero et al., 2021). Although these methods appear quite simple, they suffer from various drawbacks, including prolonged extraction time and/or a relative high solvent consumption, to highlight just a couple. Nowadays, more sustainable extraction processes with an enhanced extraction performance are required (Herrero et al., 2015). Pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) are innovative techniques that use pressurized solvents at medium-high temperatures and high pressures to extract target molecules. These compressed fluid techniques have several advantages compared to conventional extraction processes, such as faster extraction, use of lower volumes of extraction solvent, and improvement on mass transfer due to extraction temperatures and pressures applied (Sanchez-Camargo et al). Determining extraction parameters could affect not only the number of extractable compounds (Gallego et al., 2019) but also the nature of the compounds and, therefore, the bioactivity of the extracts.

Another critical point in extraction methodologies is solvent selection. Solvents derived from bio-based sources are environmentally friendly alternatives, as they are both biodegradable and non-toxic. These solvents are obtained from renewable sources and frequently demonstrate similar characteristics to traditional solvents (Li et al., 2016). Examples of these solvents include ethanol, ethyl acetate, ethyl lactate, D-limonene or CO_2. Such solvents can be derived from a range of biomass sources including energy crops, forestry resources, aquatic biomass, and waste materials through either fermentation or chemical conversion processes (Vovers et al., 2017). For these reasons, the mentioned bio-based solvents are popularly denoted as 'Green Solvents' (Calvo-Flores et al., 2018; Capello et al., 2007). In addition, the use of green solvents in combination with green techniques provide an added-value to obtain extracts from natural sources (Sanchez-Camargo et al., 2019). For a more comprehensive information of PLE and SFE applications using green solvents for bioactive extraction in plants, recent reviews are available (Gallego et al., 2019; Amador-Luna et al., 2023).

In order to choose theoretically the most suitable and safe alternative solvent for extraction purposes, Hansen solubility parameters (HSP) have been successfully employed as a real decision-making tool (Sanchez-Camargo et al., 2019). This predictive model determines the solubility of solutes in different solvents through their affinity and miscibility estimation (Hansen, 1969; Hansen and Hansen). As a result, the number of experiments and solvent consumption could be considerably reduced. The combined strategy HSP+PLE to extract target bioactive compounds from natural sources has been successfully employed previously (Sanchez-Camargo et al., 2019; Ballesteros-Vivas et al., 2019; dos Santos et al., 2021; Sanchez-Camargo et al., 2017). So far, there are few reports of *R. graveolens* metabolites obtained by SFE (Baldino et al., 2018; Sovová et al., 2017), but the use of PLE for this purpose has not been reported yet.

Within this framework, the aim of the present work was to explore the potential of PLE, involving HSP approach, and SFE using bio-based solvents to the selectively extraction of metabolite families from different parts of *R. graveolens* (aerial parts and roots), as well as to characterize the chemical composition of the obtained extracts, aiming to identify a diverse range of bioactive compounds potentially relevant for various applications.

2. Material and methods

2.1. Samples of Ruta graveolens L

Plants of *R. graveolens* were collected in the Campo experimental Emiliano Zapata, Yautepec, Morelos, México (18º 49" N to 99º 05" W, at 1064 masl), in January 2017. Taxonomic identification was performed, by cross-checking in the collection of the National Herbarium of Mexico at the UNAM (MEXU), a voucher specimen (No. 697262). Samples were separated in aerial parts and roots; all samples were indoor dried at 25 °C, grinded, sieved to a particle size of 500 μm and stored in the dark (Reyes-Vaquero et al., 2021).

2.2. Pressurized liquid extraction

Hansen solubility parameters (HSP) for alkaloids and furanocoumarins versus the green solvents ethanol, ethyl acetate, ethyl lactate and D-limonene were calculated using HSPiP® software v 5.0 at normal conditions. HSP were estimated following the method proposed by Sánchez-Camargo et al. (Sánchez-Camargo et al., 2021). Briefly, the Yamamoto-molecular break method from canonical SMILES (Simplified Molecular Input Line Entry Syntax, obtained from Pub-Chem website) was employed to estimate the dispersion (δ_D), dipole moment (δ_P) and hydrogen bond (δ_H) interaction parameters using "Do It Yourself" tool. The best solvents were chosen applying the R_a term as criteria, which concerns to the distance of a solute *i* and a solvent *j* in Hansen's three-dimensional space. This distance depends on the partial solubility parameters mentioned above, following Eq. (1):

$$Ra = \sqrt{4(\delta Di - \delta Dj)^2 + (\delta Pi - \delta Pj)^2 + (\delta Hi - \delta Hj)^2} \quad (1)$$

Therefore, the smaller R_a term, the greater the affinity between solute and solvent.

PLE parameters were optimized using a three-level factorial experimental design, considering relative abundance of the different families of compounds and yield (expressed as the percentage of extract weight per initial aerial part or root weight) as response variables, studying the effects of temperature (40, 105 and 170 °C) and percentage of ethyl acetate (0, 50, 100%) in D-limonene, as independent variables. A total of 12 extractions from both aerial parts and roots (nine points of the factorial design and three center points to consider the experimental error) were run in randomized order. The experimental design results and data analysis were performed using a response surface methodology. Furthermore, a quadratic model proposed for each response variable (Y_i) was proposed following Eq. (2):

$$Y_i = \beta_0 + \beta_1 S + \beta_2 T + \beta_{1,1} S^2 + \beta_{2,2} T^2 + \beta_{1,2} ST + \text{error} \quad (2)$$

where S stands for percentage of ethyl acetate in the solvent mixture and T for temperature, the independent variables, while β_0 is the intercept, β_1 and β_2 are the linear coefficients, $\beta_{1,1}$ and $\beta_{2,2}$ are the quadratic coefficients, $\beta_{1,2}$ is the interaction coefficient, and error is the error variable. Those parameters were estimated by multiple linear regression and their effect on the model and statistical significance, for each response variable, were illustrated in a Pareto chart. The goodness of fit of the model was evaluated by the coefficient of determination (R^2). The model also provided the optimum extraction conditions which maximized each response variable and results were graphically represented in a surface plot.

Extractions were carried out in an accelerated solvent extractor (ASE-200, Dionex Corp., Sunnyvale, CA, USA) equipped with a solvent controller unit. For each extraction, aliquots of 1 g of dried aerial parts or roots were mixed with sea sand (0.25–0.30 mm diameter, Panreac Química) in a proportion 1:1 (w/w). The mixture was placed into a 11 mL stainless-steel extraction cell. Extractions were performed by a

static cycle of 20 min at a pressure of 103 bar. Extracts were collected in previously weighted amber glass vials, evaporated by a gentle stream of nitrogen (TurboVap® LV Biotage, Uppsala, Sweden) and stored at 4 °C until chromatographic analyses.

2.3. Supercritical fluid extraction

Supercritical CO_2 ($scCO_2$) extractions were performed in a home-made compressed fluid extractor coupled to a supercritical peltier CO_2 pump PU–2080–CO_2 from Jasco (Pfungstadt, Germany) which introduces CO_2 into the extraction cell. Premier quality CO_2 was used (Carburos Metálicos, Grupo Air Products, España). Aerial parts or roots samples (1 g) were mixed with 1 g of sea sand and placed into an 8 mL stainless-steel extraction cell. Extractions were carried out at 40 °C using a flow rate, controlled using a needle valve as the variable restrictor, of 4 mL/min during 180 min. Pressure was held at 100 bar for 60 min, then raised to 200 bar next 60 min and finally up to 350 bar the latest 60 min. During the extraction process, two humidity conditions in the samples were evaluated: dried samples during the whole process and moistening samples (water content 35%, w/w) in the moment prior to raising pressure to 350 bar. SFE extracts were collected in ethanol or ethyl acetate, to avoid losses of volatile metabolites (Langenfeld et al., 1992). Obtained extracts were evaporated by a gentle nitrogen stream, weighed and stored in amber vials at 4 °C until chromatographic analyses.

2.4. Gas chromatography – mass spectrometry analysis (GC–q-TOF-MS)

Rue extracts obtained by PLE and SFE were chemically characterized by GC–q-TOF-MS. Dry extracts were dissolved in ethanol (HPLC grade) at a concentration of 1 mg/mL. Injection of 1 μL of sample was done in split mode (split ratio 4:1), in a 7890B Agilent gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to a 7200 quadrupole time-of-flight mass spectrometer (q-TOF-MS) (Agilent Technologies) equipped with an electronic ionization (EI) source. The temperature of the injector was kept at 250 °C and helium was the carrier gas at a constant flow rate of 1 mL/min. The separation was carried out using an Agilent Zorbax DB5-MS column (30 m × 0.20 mm id, with 0.25 μm film thickness) + 10 m DuraGuard capillary column. The oven temperature was programmed at 50 °C for 2 min then raised to 150 °C at 20 °C/min, followed by an increment of 3 °C/min until reached 225 °C, then increase by 15 °C/min until 280 °C, remaining at that temperature for 14 min and finally increased by 25 °C/min until it reached 300 °C for 5 min with a total run time of 55.5 min (Diwan and Malpathak, 2011).

The mass spectrometry parameters were: electron impact ionization at 70 eV, transfer line temperature of 320 °C, ion source temperature of 250 °C, quadrupole temperature of 150 °C, *m/z* scan range 50–600 Da (5 spectra per second). The tentative identification of compounds was achieved using the Agilent MassHunter Unknown Analysis tool that provides a systematic mass spectra deconvolution of chromatographic signals and the NIST Mass Spectral database (NIST MS Search 2.0). An alkane solution (C8-C34), 5 mg/L in dichloromethane was employed to calculate the linear retention index (LRI) of each analyte. Quantitative results for target compounds were expressed in terms of relative abundance.

2.5. Statistical analysis

A metabolite-relative abundance table, including extracts in columns and metabolites in rows, was submitted to cluster analysis and heat-mapping using freely available web server Heatmapper (www.heatmapper.ca, accessed on 18th October 2022). A data matrix was previously scaled using an auto-scaling approach; that is, the data were mean-centered and divided by the standard deviation of each variable. A hierarchical clustering was applied using an average linkage clustering method with Pearson distance measurement.

The relative abundance values of the main families found in the extracts from the aerial parts or roots of rue obtained by PLE were statistically analyzed using a response surface methodology. These analyses were performed using Statgraphics Centurion XVI.I software (StatPoint Technologies, Inc., Warrenton, VA, USA).

Relative abundance of compounds' families collected at different $scCO_2$ extraction conditions were compared using a t-test ($p < 0.05$) carried out with Excel 2013 (Microsoft, Washington, USA).

3. Results and discussion

3.1. Theoretical selection of green solvents for PLE

Hansen solubility parameters (HSP) analysis allowed theoretically identifying the green solvents with the highest solubility for characteristic compounds of *Ruta* genus such as furanocoumarins and alkaloids. In this study, D-limonene, ethyl acetate, ethyl lactate and ethanol were selected for the HSP study. These solvents are a real alternative to replace the petroleum-based solvents in order to implement the green approach of the PLE procedure.

Greater miscibility for the targeted furanocoumarins (isopimpinellin, xanthotoxin, bergapten and psoralen) was obtained with D-limonene (smaller R_a scores of the Hansen's three-dimensional space, Table 1). On the other hand, miscibility for alkaloids in different green solvents was more spread out; dictamnine and graveoline present lower R_a scores with D-limonene, while skimmianine and kokusaginine were more soluble in ethyl acetate, and cofusameline, rutacridone, arborinine and 1-hydroxy-10-methyl-9(10H)-acridinone with ethyl lactate (Table 1). These differences between alkaloids are due to its chemical structure that provide different contributions to dispersion, polarity and hydrogen bonding parameters. It must be taken into account that ethyl acetate showed very similar R_a scores than ethyl lactate for cofusameline, rutacridone and arborinine. Furthermore, both D-limonene and ethyl acetate showed low R_a value (<10) for 8 out of 12 compounds evaluated, and therefore they were selected as extraction solvents for PLE.

However, an experimental design was needed to verify the theoretical results taking into account the kinetics of the extraction process in the selection of the optimal composition of the green solvent to extract the target compounds from rue.

3.2. GC-q-TOF-MS analysis of compounds from aerial parts and roots extracts of R. graveolens

A complete characterization by GC-q-TOF was carried out,

Table 1
Distance of different green solvents from the center of the Hansen solubility sphere (R_a) for the different metabolites selected.

	R_a ($MPa^{1/2}$)			
	D-Limonene	Ethyl acetate	Ethyl lactate	Ethanol
Alkaloids				
Dictamnine	**7.39**	8.18	9.91	15.58
Skimmianine	7.55	**7.51**	8.88	14.58
Kokusaginine	7.55	**7.51**	8.88	14.58
Confusameline	11.07	9.22	**8.17**	12.63
Graveoline	**8.60**	10.04	11.40	16.50
Arborinine	14.16	12.98	**11.36**	14.29
1-Hydroxy-10-methyl-9 (10 H)-acridinone	14.83	13.48	**11.77**	14.59
Rutacridone	10.87	10.59	**10.50**	14.96
Furanocoumarins				
Psoralen	**8.67**	8.69	9.98	15.52
Xhantotoxin	**8.09**	8.79	10.18	15.61
Bergapten	**8.09**	8.79	10.18	15.61
Isopimpinellin	**6.25**	7.85	9.96	15.65

Table 2
Compounds tentatively identified in aerial parts and roots extracts of *Ruta graveolens* obtained by PLE and SFE.

RT (min)	Tentative identification	Acronym	LRI	Match factor	Monoisotopic mass	Main fragments (*m/z*)[a]	Ref.	PLE		SFE Collecting			
										EtOH		EtOAc	
								AP	R	AP	R	AP	R
	Alkaloids												
21.36	3-Methyl-2-nonyl-1H-quinolin-4-one	Al_01	1918	85	285.2093	**173.096**; 186.092; 200.107	36	-	-	-	+	-	+
22.16	Dictamnine	Al_02	1958	92	199.0633	156.060; 184.066; **199.104**	13, 36, 43	-	+	+	+	+	+
23.60	2-Methyl-3-undecyl-1H-quinolin-4-one	Al_03	1987	83	313.2406	172.091; **173.084**; 186.033		-	-	-	+	-	+
28.29	Fagarine	Al_04	2210	84	229.0739	186.092; 214.050; **229.073**	36, 43	+	+	+	-	+	-
28.94	Pteleine	Al_05	2234	88	229.0739	156.044; 200.012; **229.074**	43	-	+	+	+	+	+
32.75	Skimmianine	Al_06	2375	92	259.0845	230.081; **244.061**; 259.085	13, 36, 41, 43	+	+	+	+	+	+
34.34	Kokusaginine	Al_07	2478	89	259.0845	216.077; 244.087; **259.127**	13, 36, 41, 43	+	+	+	+	+	+
34.66	1-Hydroxy-10-methyl-9(10H)-acridinone	Al_08	2531	90	225.0790	154.065; 182.063; **225.101**	13, 41	-	+	-	+	-	+
34.80	3-Methyl-2-undecyl-1H-quinolin-4-one	Al_09	2550	86	313.2406	**173.122**; 186.101; 200.108	36	-	+	-	+	-	+
37.87	1-Hydroxy-3-methoxy-10-methyl-9(10H)-acridinone	Al_10	2825	92	255.0895	182.060; 226.086; **255.090**	13, 43	-	+	-	+	-	+
40.03	Furofoline I	Al_11	2996	80	265.0739	221.086; 250.048; **265.021**		-	+	-	-	-	-
40.17	Arborinine	Al_12	3007	90	285.1001	242.081; **270.076**; 285.099	13, 43	+	-	+	-	+	-
45.19	3-Methyl-2-pentyl-1H-quinolin-4-one	Al_13	3126	85	229.1467	130.065; **173.084**;186.091;		-	+	-	+	-	+
47.15	Graveoline	Al_14	3140	80	279.0895	220.176; 251.090; **279.123**	36, 43	-	+	-	+	-	+
	Furanocoumarins												
19.63	Psoralen	FC_01	1847	92	186.0317	102.046; 158.036; **186.032**	13, 36, 39–40, 43	+	+	+	+	+	+
22.19	4-(1,1-Dimethylallyl)-9-methoxy-7H-furo[3,2-g][1]benzopyran-7-one	FC_02	1959	85	284.1049	229.049; **269.081**;284.105;		-	-	-	+	-	+
24.06	Xanthotoxin	FC_03	2100	90	216.0423	173.023; 201.018; **216.042**	13, 43	-	+	-	+	-	+
25.67	Bergapten	FC_04	2120	90	216.0423	173.033; 145.031;216.0**59**	10, 13, 36, 41, 43	+	+	+	+	+	+
28.03	Chalepensin	FC_05	2205	80	254.0943	199.073; 239.106; **251.129**	43	+	+	+	+	+	+
29.39	Isopimpinellin	FC_06	2246	91	246.0528	160.016; **231.047**; 246.067	13, 36	-	+	+	+	+	+
35.82	Chalepin	FC_07	2688	82	314.1518	255.102; **299.129**; 314.152	43	+	+	+	+	+	+
37.80	Rutamarin	FC_08	2803	90	356.1624	**281.205**; 299.135; 356.178	43	+	+	+	+	+	+
	Terpenes												
6.34	Sabinene	T_01	961	81	136.1252	77.038; 91.054; **119.085**		-	+	-	-	-	-
6.40	*p*-Cymene	T_02	1021	91	134.1096	77.038; **91.054**; 93.069;	10	-	+	-	-	-	-
6.76	*m*-Cymenene	T_03	1084	97	132.0939	91.054; 121.101; **207.033**		+	+	-	-	-	-
6.88	*p*-Mentha-1,5,8-triene	T_04	1111	91	134.1096	**73.046**; 91.054; 133.012		-	+	-	-	-	-
7.22	*p*-Mentha-2,8-dien-1-ol	T_05	1122	93	152.1201	73.047; **91.054**;119.085		+	+	-	-	-	-
7.24	*trans*-Verbenol	T_06	1144	80	152.1201	41.341; 94.523; **109.065**		+	-	-	-	-	-
7.28	Citronellal	T_07	1158	86	154.1358	55.054; **69.070**; 83.085		-	+	-	-	-	-
7.50	*p*-Mentha-1(7),8-dien-2-ol	T_08	1186	84	152.1201	79.054; **109.065**;119.085		-	+	-	-	-	-
7.87	*trans*-3-Caren-2-ol	T_09	1188	80	152.1201	91.385; **119.040**;134.320		+	-	-	-	-	-
7.92	*trans*-3(10)-Caren-2-ol	T_10	1194	80	152.1201	41.459; 69.094; **109.053**		+	-	-	-	-	-
8.04	*trans*-Carveol	T_11	1217	93	152.1201	55.054; 91.054; **109.065**		+	+	-	-	-	-

(*continued on next page*)

Table 2 (*continued*)

RT (min)	Tentative identification	Acronym	LRI	Match factor	Monoisotopic mass	Main fragments (*m/z*)[a]	Ref.	PLE		SFE Collecting			
										EtOH		EtOAc	
								AP	R	AP	R	AP	R
8.06	*trans*-2-Caren-4-ol	T_12	1222	80	152.1201	67.054; **73.047**; 95.085		+	-	-	-	-	-
8.07	Carveol	T_13	1225	80	152.1201	91.102; **119.056**;135.059		+	-	-	-	-	-
8.15	Pulegone	T_14	1244	80	152.1201	67.943; 81.145; **152.539**		+	-	-	-	-	-
8.30	Carvenone	T_15	1258	87	152.1201	**54.046**; 91.054; 93.070		+	-	-	-	-	-
8.49	*trans*-Ascaridolglycol	T_16	1273	88	170.1307	79.054; 81.070; **109.065**		+	+	-	-	-	-
9.98	Isoascaridol	T_17	1318	80	168.1150	79.054; 95.085; **97.065**		+	+	-	-	-	-
11.68	α-Ionone	T_18	1422	80	192.1514	93.093; **121.187**; 136.235		-	-	-	+	-	+
12.28	Calarene	T_19	1440	81	204.1878	41.982; 105.056; **161.145**		-	-	+	-	+	-
12.34	*trans*-β-Ionone	T_20	1442	80	192.1514	43.090; 93.084; **121.537**		-	-	-	+	-	+
14.48	α-Eudesmol	T_21	1519	81	222.1984	**59.049**; 93.070; 149.132	10	-	-	+	+	+	+
22.48	Limonen-6-ol, pivalate	T_22	1966	80	236.1776	**57.070**; 85.100; 119.085		+	-	-	-	-	-
26.40	Phytol	T_23	2122	92	296.3079	**71.049**; 95.086; 123.117	13	+	-	+	-	+	-
43.13	Tocopherol	T_24	3112	85	430.3811	**165.090**; 341.019; 430.380	13	+	-	+	-	+	-
45.29	Campesterol	T_25	3131	88	400.3705	255.212; 315.305; **400.370**	13	+	-	+	+	+	+
47.56	γ-Sitosterol	T_26	3351	89	414.3862	145.101; 329.321; **414.650**	13	+	-	+	+	+	+
	Fatty acids												
9.37	Nonanoic acid	FA_01	1297	80	158.1307	**60.021**; 73.028; 115.074		-	-	+	+	+	+
10.76	*n*-Decanoic acid	FA_02	1387	85	172.1463	60.020; **73.028**; 29.090		-	-	+	+	+	+
11.82	2,5-Octadecadiynoic acid, methyl ester	FA_03	1426	82	290.2246	**91.054**; 105.070; 145.102	10	-	-	-	+	-	+
13.30	Fumaric acid, ethyl 2-methylallyl ester	FA_04	1476	82	198.0892	55.055; 99.043; **127.039**		-	-	+	-	+	-
15.31	Dodecanoic acid	FA_05	1555	84	200.1776	**73.028**; 129.091; 157.122	13	-	+	+	+	+	+
18.44	Myristic acid	FA_06	1765	88	228.2089	60.021; **73.029**; 129.091		-	-	+	-	+	-
21.94	Palmitic acid, methyl ester	FA_07	1928	80	270.2559	**74.036**; 87.044; 143.107		-	-	+	-	+	-
22.40	Palmitic acid	FA_08	1964	90	256.2402	**60.021**; 73.028; 129.091	13, 43	+	-	+	+	+	+
23.94	Palmitic acid, ethyl ester	FA_09	1997	80	284.2715	**88.051**; 101.059; 157.122		-	-	+	+	+	+
25.51	8,11-Octadecadienoic acid, methyl ester	FA_10	2112	83	294.2559	**67.054**; 81.069; 95.085		-	-	+	-	+	-
26.51	Linoleic acid	FA_11	2134	87	280.2402	**81.070**; 83.085; 95.085	13, 43	-	-	+	-	+	-
27.18	Oleic Acid	FA_12	2140	89	282.2559	**55.055**; 69.070; 83.085	13	-	-	+	-	+	-
27.25	Linolenic acid	FA_13	2143	88	280.2402	67.054; **79.054**; 93.070	13	-	-	+	-	+	-
27.87	Stearic acid	FA_14	2188	86	284.2715	60.021; **73.028**; 129.091	36	-	-	+	+	+	+
28.38	Linolenic acid, ethyl ester	FA_15	2215	80	306.2559	**79.054**; 121.101; 135.044		-	-	+	+	+	+
	Coumarins												
18.05	6,7,8-Trimethoxycoumarin	C_01	1727	85	236.0685	150.031; 193.049; **236.068**		-	-	-	+	-	+
18.17	7-Methoxycoumarin	C_02	1732	89	176.0473	**133.028**; 148.052; 176.046	11	-	-	+	-	+	-
23.89	5,7-Dimethoxycoumarin	C_03	1992	87	206.0579	163.073;178.048; **206.057**	36	-	-	-	+	-	+
25.32	Seselin	C_04	2101	80	228.0786	93.070; 123.116; **213.055**		-	+	-	-	-	-
27.05	Ostol	C_05	2138	87	244.1099	201.103; 229.105; **244.120**	36, 43	-	+	-	+	-	+

(*continued on next page*)

Table 2 (*continued*)

RT (min)	Tentative identification	Acronym	LRI	Match factor	Monoisotopic mass	Main fragments (*m/z*)[a]	Ref.	PLE		SFE Collecting			
										EtOH		EtOAc	
								AP	R	AP	R	AP	R
31.34	5-Hydroxy-7-methoxy-2-methyl-6-(3-methyl-2-butenyl)- chromone	C_06	2283	83	274.1205	219.066; **231.147**; 274.163	36	-	+	-	+	-	+
32.46	3-(1,1-dimethylallyl) scopoletin	C_07	2305	87	260.1049	217.088; 245.091; **260.111**	43	-	+	-	+	-	+
	Phenolic compounds												
8.18	1,3-bis(1,1-dimethylethyl)-benzene	PC_01	1249	80	190.1722	57.070; 91.054; **175.148**		-	-	+	+	+	+
11.46	4-Tert-butyl-2-(2-methylbutan-2-yl)phenol	PC_02	1419	83	220.1827	177.126; **205.159**; 220.183		-	+	-	-	-	-
14.55	2,4-Di-tert-butylphenol	PC_03	1521	83	206.1671	57.070; **191.144**; 206.166;		-	-	+	+	+	+
18.26	Turmeronol A	PC_04	1733	80	232.1463	**135.080**; 232.160; 217.121		+	-	+	+	+	+
18.33	(*E*)-Coniferyl alcohol	PC_05	1743	83	180.0786	124.098; **137.087**; 180.035		-	-	-	+	-	+
20.27	Isogentisin	PC_06	1884	80	258.0528	214.090; 242.118; **257.142**		-	-	+	+	+	+
	Amides												
21.47	Myristamide	Am_01	1921	84	227.2249	41.018; **59.037**; 72.045		-	+	-	-	-	-
33.04	Oleamide	Am_02	2397	90	281.2719	55.054; **59.099**;72.045		+	+	+	+	+	+
	Ketones												
9.79	2-Undecanone	K_01	1299	80	170.1671	43.049; **58.041**;59.064	3, 10, 13, 39–40, 43	-	-	+	+	+	+
13.42	2-Tridecanone	K_02	1481	85	198.1984	**58.042**; 71.049; 85.064	10, 13, 43	-	-	+	+	+	+
	Others												
7.40	Myrtenyl methyl ether	O_01	1160	80	166.1358	**77.038**; 91.054; 121.065		-	+	-	-	-	-
10.39	Piperonal	O_02	1329	80	150.0317	63.058; **149.135**; 150.768		-	-	-	+	-	+
10.55	Tricycloekasantalal	O_03	1343	85	178.1358	67.054; **93.070**; 105.069		-	-	+	+	+	+
10.61	7-Tetradecene	O_04	1370	83	196.2191	**55.054**; 69.070; 83.085		-	-	+	-	+	-
11.09	1,9-Nonanediol	O_05	1414	87	160.1463	**55.055**; 67.054; 81.070		-	-	+	-	+	-
11.78	Tricyclo[4.4.1.1(3,8)]dodeca-4,9-diene	O_06	1425	84	160.1252	79.054; **91.054**;105.070		-	-	+	-	+	-
11.97	2,6,10-Trimethyltetradecane	O_07	1431	81	240.2817	**57.070**; 71.085; 85.101		-	-	+	-	+	-
14.35	Heptacosane	O_08	1514	80	380.4382	43.101; **57.070**; 71.085	43	+	-	+	+	+	+
14.41	β-Acorenol	O_09	1516	80	222.1984	93.070; **119.086**; 161.134		-	-	+	-	+	-
14.99	5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-Benzofuranone	O_10	1538	80	180.1150	**111.049**; 137.096; 180.115		-	-	+	+	+	+
15.41	Syringaldehyde	O_11	1662	82	182.0579	111.145; 181.020; **182.350**		-	-	+	-	+	-
15.80	cis,α-Santalol	O_12	1681	81	234.1984	91.405; **93.045**; 121.230;		+	-	-	-	-	-
16.19	2,5,5,8a-Tetramethyl-6,7,8,8a-tetrahydro-5H-naphthalen-1-one	O_13	1686	83	204.1514	**135.044**; 148.051; 232.146		-	-	-	+	-	+
16.28	Illudol	O_14	1687	80	252.1725	**55.054**; 109.101; 135.044		-	-	+	-	+	-
17.41	2-Hexadecanol	O_15	1702	83	242.2610	**57.070**; 97.102; 111.116		-	-	+	-	+	-
18.57	Santalcamphor	O_16	1774	84	236.1776	95.086; 123.117; **207.033**		-	-	+	-	+	-
18.69	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydro2(4H) benzofuran	O_17	1784	83	196.1099	**111.044**; 140.047; 178.098		-	-	+	-	+	-
19.96	Heptadecane-2,4-dione	O_18	1874	82	268.2402	43.089; 85.115; **100.045**		-	-	-	+	-	+
21.29	7-Methyl-Z-tetradecen-1-ol acetate	O_19	1915	80	268.2402	**55.054**; 81.070; 67.055		-	-	+	-	+	-
22.05	11,13-Dimethyl-12-tetradecen-1-ol acetate	O_20	1955	81	282.2559	67.054; 69.071; **95.085**		-	-	+	-	+	-
23.76	Tetratetracontane	O_21	1990	85	618.7043	**57.070**; 71.086; 85.101	43	-	-	+	-	+	-

(*continued on next page*)

Table 2 (*continued*)

RT (min)	Tentative identification	Acronym	LRI	Match factor	Monoisotopic mass	Main fragments (*m/z*)[a]	Ref.	PLE		SFE Collecting			
										EtOH		EtOAc	
								AP	R	AP	R	AP	R
24.95	α-Tocospiro A	O_22	2071	82	462.3709	137.060; 237.113; **419.351**		-	-	+	-	+	-
41.95	3,4-bis(1,3-benzodioxol-5-ylmethyl)dihydro-(3R-trans)-2 (3H)-Furanone	O_23	3096	83	354.1103	77.038; **135.044**; 354.111		-	+	-	+	-	+

RT: retention time, LRI: linear retention index; AP: aerial parts; R: roots; - absence; + presence. a Quantitative *m/z* ion is written in bold. Ref. Ruta graveolens references.

considering the extracted metabolites in both, aerial parts and roots of *R. graveolens* obtained by PLE and SFE (Table 2). In order to facilitate the discussion, compounds were classified into families based on their chemical structure: alkaloids, terpenes, furanocoumarins, fatty acids, coumarins, phenolic compounds, amides, ketones, and "others" that does not correspond to the chemical structures previously mentioned. A total of 103 compounds were tentatively identified based on the positive match of the experimental mass spectra with theoretical MS data from databases, calculated mass accuracy for the $[M]^+$ molecular ion, linear retention index and data reported in literature. GC-HRMS parameters such as retention time, match factor values given by MS databases, monoisotopic mass, main MS/MS fragments and linear retention index are shown in Table 2. Identification reliability was considered satisfactory for all the compounds, showing a match factor value higher than 80%. In addition, to the best of our knowledge 63 new compounds were detected for the first time in rue extracts, mainly terpenes (sabinene, carveol, *m*-cymenene), amides (myristamide and oleamide), phenolic compounds (4-*tert*-butyl-2-(2-methylbutan-2-yl)phenol, turmeronol A, isogentisin) and coumarins (6,7,8-trimethoxycoumarin, and seselin).

All pressurized liquid or supercritical fluid extracts from rue were grouped according to their relative metabolites content after applying a clustering method to both rows and columns of the data matrix. The particular composition of rue extracts in terms of identified metabolites at different extraction conditions is depicted in the resulting heatmap displayed in Fig. 1, which shows a color code from lower (dark blue) to higher (dark yellow) concentration levels. Column (extracts) and row (metabolites) dendrograms can be found at supplementary material (Figures S1 and S2, respectively).

As expected, the column dendrogram clearly classified extracts into two big groups depending on the raw material: aerial parts on the left and roots on the right. Recently, it has been reported that the chemical profile of rue is different between the aerial parts and roots (Reyes-Vaquero et al., 2021). In addition, each group can be subdivided into three subgroups: PLE extracts, the extracts obtained by SFE at 350 bar with moistened sample and the rest of extracts from SFE for aerial parts, while for roots are PLE extracts, the extracts obtained by SFE at 350 bar with dry sample and the rest of extracts from SFE (Figure S1). These clusters were also expected since different green compressed fluids (neat CO_2 and diverse mixtures of limonene and ethyl acetate) and technologies were used to cover a wide range of polarities and thus trying to extract families of compounds selectively.

According to the row dendrogram (Figure S2), corresponding to identified metabolites which acronyms are in Table 2, five groups of metabolites are distinguished, and the relation of these groups and the extraction techniques are commented below.

The cluster A is related to rue roots extracts obtained by SFE. It is characterized mainly by the presence of coumarins and alkaloids (12 of 32 compounds). Within this cluster, coumarins such as ostol, 5-hydroxy-7-methoxy-2-methyl-6-(3-methyl-2-butenyl)-chromone, 3-(1,1-dimethylallyl) scopoletin, alkaloids such as 1-hydroxy-10-methyl-9(10 H)-acridinone, 1-hydroxy-3-methoxy-10-methyl-9(10 H)-acridinone and 3-methyl-2-pentyl-1 H-quinolin-4-one, and the furanocoumarin xanthotoxin and 3,4-bis(1,3-benzodioxol-5-ylmethyl)dihydro-(3R-*trans*)-2 (3 H)-furanone were present; these metabolites have been exclusively identified in rue roots extracts.

The cluster B is constituted mainly by terpenes (9 of 18 compounds) and is associated to root extracts obtained by PLE. In this cluster it is possible to observe also compounds exclusive from roots, such as alkaloids 3-methyl-2-undecyl-1H-quinolin-4-one and graveoline.

Clusters C, D and E are related to aerial parts. Terpenes can be found almost exclusively in cluster C (11 of a total of 14 terpenes extracted from aerial parts). This cluster is related to aerial parts extracts obtained by PLE. At this point, it can be noticed how the extraction of terpenes is favored by PLE regardless the part of the plant (aerial or root) and extraction conditions (solvent or temperature), as can be seen in clusters B and C.

The following cluster, cluster D, is defined by a mixture of furanocoumarins, alkaloids and terpenes obtained by PLE or SFE. Although some metabolites such as the furanocoumarins psoralen, bergapten, chalepensin, chalepin, rutamarin and the alkaloid skimmianine were identified in all type of extracts (Table 2), a significant increase in their relative abundance can be appreciated in the extracts from aerial parts independently of the extraction technique applied (PLE or SFE). Furthermore, in this cluster, metabolites exclusively from aerial parts can be found (arborinine, phytol and tocopherol). Regarding to furanocoumarins from rue, their presence in SFE extracts were expected (Baldino et al., 2018; Sovová et al., 2017) however, their extraction using PLE had not been reported yet.

Finally, the cluster E is associated to aerial parts extracts obtained by SFE and is defined by the presence of fatty acids and the compounds grouped as "others" (see Table 2). SFE using neat CO_2 as solvent has been reported previously for the extraction of fatty acids from passion fruit seeds (Vigano et al., 2016) or cocoa bean hulls (Mazzutti et al., 2018).

As has been reported, furanocoumarins and alkaloids are characteristic compounds of *R. graveolens* (Al Qaisi et al., 2022; Reyes-Vaquero et al., 2021; Al Qaisi et al., 2024; Husein et al., 2023; Kuzovkina et al., 2004; Oh et al., 2014; Stashenko et al., 2000). Stashenko et al. (Stashenko et al., 2000) identified the furanocoumarins bergapten, chalepensin, rutamarin, psoralen and chalepin, the alkaloids as dictamnina, and kokusaginina, and the ketone 2-undecanone as main metabolites. All these metabolites have been identified in this work. In addition, only three important metabolites described by Stashenko et al. (Stashenko et al., 2000) (geijenere, 6-(39,59-benzodioxyl)-3,3-dimethyl-1-hexene and 2-nonanone) were not found in our samples, which shows the good representativeness of the samples of both works.

Comparing with the previous work of Reyes-Vaquero et al. (Reyes--Vaquero et al., 2021), 68% of the compounds found in that work have been detected again; however only the major compounds in terms of relative abundance (>10%) isomaturnin and cirsimaritin have been missing. In that work, a relationship was established between antifungal activity and the presence of 12 compounds (skimmianine, linolenic acid, oleic acid, campesterol, phytol, palmitic acid, linoleic acid, dodecanoic acid, isopimpinelline, rutacridone, 1-hydroxy-10-methyl-9(10H)-acridinone and xanthotoxin), from which 11 of them have been identified and quantified in the present work.

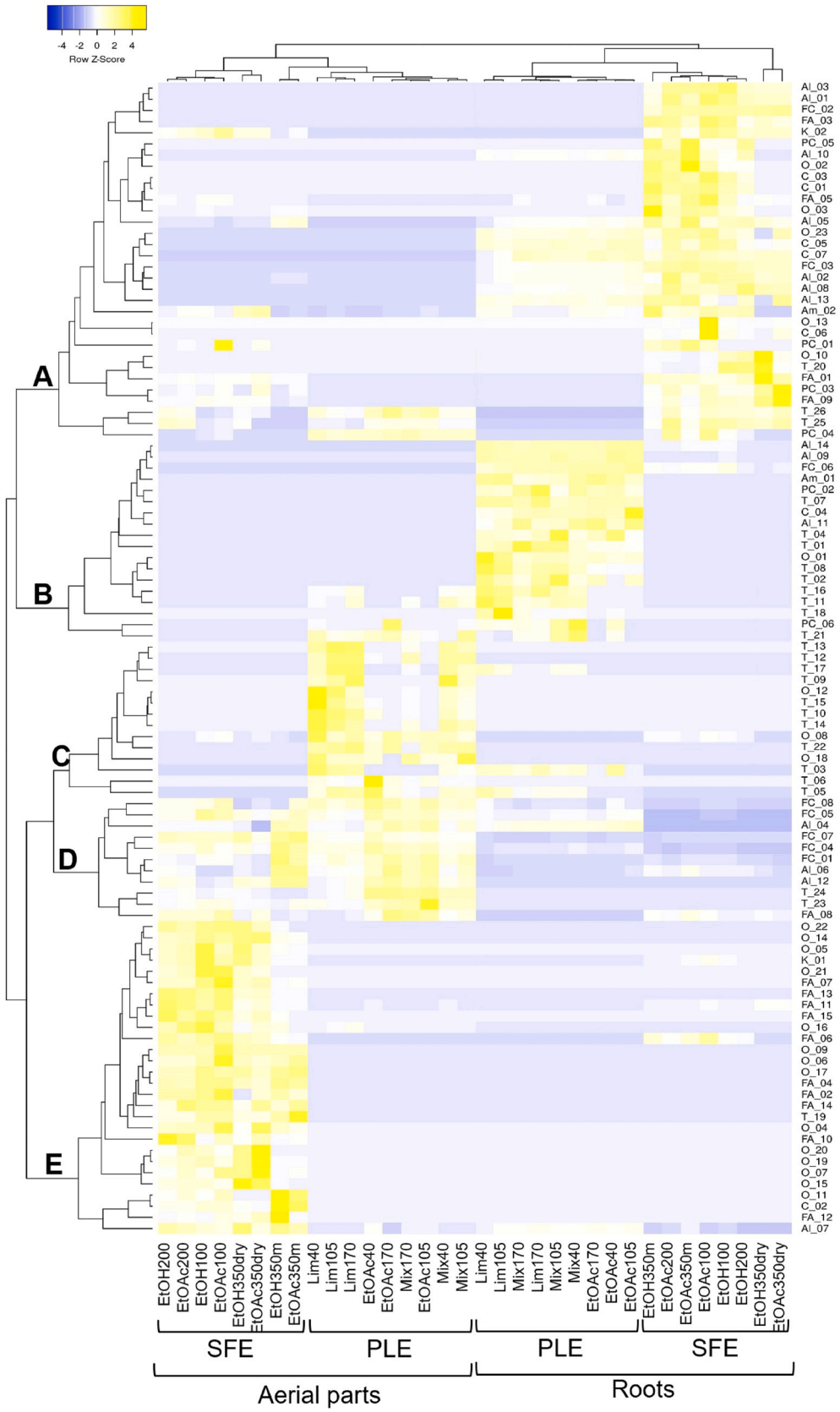

Fig. 1. Heatmap showing the differential relative abundance of identified compounds in aerial parts or roots rue extracts obtained by PLE or SFE. Color code: dark yellow (higher relative abundance); dark blue (lower relative abundance). Metabolites acronyms correspond to those identified in Table 2. Al: alkaloids; PC: phenolic compounds; FC: furanocoumarins; C: coumarins; K: ketones; FA: fatty acids, T: terpenes; Am: amides; O: others. EtOH: ethanol; EtOAc: ethyl acetate; Lim: limonene; M: mixture limonene/ethyl acetate 50/50 (v/v); m: moistened sample; dry: dry sample; Temperatures: 40, 105, 170 °C; Pressures: 100, 200, 350 bar.

This differences in terms of number and families of chemical compounds obtained could be attributed to environmental factors which impacts on the metabolites' biosynthetic pathways, moreover the use of more efficient extraction techniques such as PLE and SFE, in conjunction with the use of high resolution GC-q-TOF-MS, provides with higher sensitivity and resolution on a short time scale and high probability of identification of the compounds obtained in this work.

3.3. Pressurized liquid extraction of aerial parts and roots of R. graveolens

As it has been mentioned previously, *R. graveolens* is composed of several different families of compounds, some of them with different and known bioactive characteristics (Oliva et al., 2003; Reis et al., 2015; Meepagala et al., 2005). For this reason, optimizing extraction parameters trying to enrich the extract in one or more families of compounds is needed, because optimum extraction conditions can vary depending not only on the matrix (aerial parts or roots) but also on the target components. Moreover, to cover a wide range of conditions in the shortest possible time, an experimental design using response surface methodology (RSM) was applied trying to optimize the operating variables.

A three-level factorial experimental design was applied to optimize the extraction of alkaloids, furanocoumarins, terpenes and fatty acids from aerial parts and roots of *R. graveolens*. Two factors were selected: extraction temperature (40, 105 and 170 °C) and percentage of ethyl acetate (0, 50, and 100%) in D-limonene. Table 3 shows the yield and relative abundance of metabolites' families identified from aerial parts and roots extracts of *R. graveolens* using PLE.

As can be seen in Table 3, aerial parts and roots extracts showed similar extraction yield ranging from 2.15% to 18.11%. Relative abundance values indicate the relevant presence of terpenes, furanocoumarins and alkaloids regardless the part of the plant studied, the aerial part or root, and tested extraction conditions. Regarding the percentage of these families in each PLE extract (Table S1), on one hand, terpenes were the most abundant chemical group in aerial parts (30–63%), whereas alkaloids were mainly found in roots extracts (33–49%). Even when furanocoumarins were found at significant accumulation levels (from 22% to 40% in aerial part and, from 11% to 17% in roots), they were not the main compounds in the aerial parts nor roots. Moreover, fatty acids were detected in the aerial parts (from 2% to 8%), while in roots extracts were negligible (from 0.02% to 0.19%) as reported by Reyes-Vaquero et al. (Reyes-Vaquero et al., 2021). It is also observed that coumarins were found and identified only in roots extracts (from 6% to 10%). According to Stashenko et al. (Stashenko et al., 2000), and Reyes-Vaquero et al. (Reyes-Vaquero et al., 2021), coumarins and furoquinolines alkaloids were mainly identified in roots. Lastly, the percentage of amides and the rest of compounds was 10-fold higher in roots than in the aerial parts (in the former extract with an average incidence of 10%).

Figs. 2 and 3 show the standardized Pareto charts for each response variables and their corresponding response surface plot of PLE extracts from aerial parts and roots of rue. Standardized Pareto charts allows detecting the most important factors and interactions while displaying the absolute values of the effects and its significance at 95% confidence level; the different bar colors show the positive (grey) and negative (black) effects.

As can be seen, for the extraction of terpenes of aerial parts (Fig. 2a), results showed that the linear effect of solvent represents the only significant term in the model. In this sense, terpenes extraction was significantly favored by D-limonene (100%) while temperature had no effect. Aissou et al. (Aissou et al., 2017) reported that D-limonene is a suitable solvent for the extraction of terpenes, due to similar polarities. Another example of the solvent polarity effect using PLE has been reported by Péres et al. (Péres et al., 2006); showing that the extraction of terpenes from *Piper gaudichaudianum* leaves such as squalene, vitamin E, stigmasterol and β-sitosterol took place favorably with the less polar studied solvent.

The extraction of alkaloids, furanocoumarins and fatty acids from aerial parts followed a different pattern compared to terpenes. As illustrated in Fig. 2b-d, ethyl acetate proportion in the solvent and temperature are the most significant factors, both showing a positive effect. In general, these obtained PLE results are in accordance with other reports on the extraction of alkaloids from other matrices, where it has been observed that the solvent selection is more important than the

Table 3
Factor levels of the three-level two-factor experimental design (3^2) and results obtained for yield (%) and relative abundance of families of metabolites ($\times 10^3$) identified from aerial parts and roots extracts of *Ruta graveolens* using PLE.

	Factors		Response variables							
	Solvent (%)[a]	Temp. (°C)	Yield (%)	Al	FC	T	FA	C	Selectivity T/(Al+FC+C)	Differences to 33% for Al, FC, T
Aerial parts	50	40	4.69	7574	14,368	39,092	2720		1.78	59
	0	170	16.48	6545	12,424	35,677	1317		1.88	63
	50	105	5.57	8649	15,114	21,819	1754		0.92	29
	50	105	4.73	11,332	17,194	35,219	2788		1.23	43
	50	105	5.07	10,456	16,372	35,495	2705		1.32	46
	100	40	2.15	6755	12,026	10,104	1447		0.54	18
	0	105	9.95	6347	13,445	37,281	1275		1.88	62
	100	105	2.25	12,016	17,515	15,235	3265		0.52	14
	50	170	7.76	11,708	17,118	23,269	4054		0.81	23
	0	40	7.77	4779	11,830	30,772	1309		1.85	62
	50	105	4.40	11,763	17,650	36,443	3225		1.24	43
	100	170	5.31	11,459	18,699	16,995	4164		0.56	15
Roots	50	40	4.28	8540	2961	2333	17	1657	0.18	52
	0	170	18.11	5327	1815	3683	3	1008	0.45	49
	50	105	6.60	6978	2253	2562	10	1484	0.24	52
	50	105	6.59	8279	2924	1041	13	1767	0.08	52
	50	105	6.21	7490	2580	2288	18	1538	0.20	53
	100	40	3.21	7838	2822	3646	11	1690	0.30	49
	0	105	7.44	7491	2469	5004	19	1452	0.44	51
	100	105	12.06	9235	3152	659	6	1940	0.05	55
	50	170	10.75	7647	2621	2351	21	1517	0.20	52
	0	40	3.32	4775	1616	2065	16	1001	0.28	51
	50	105	6.46	7361	2392	751	31	1580	0.07	52
	100	170	9.89	5143	1758	3015	27	881	0.39	50

[a] Refers to % ethyl acetate in D-limonene. Temp.: temperature; Al: alkaloids; FC: furanocoumarins; T: terpenes; FA: fatty acids; C: coumarins.

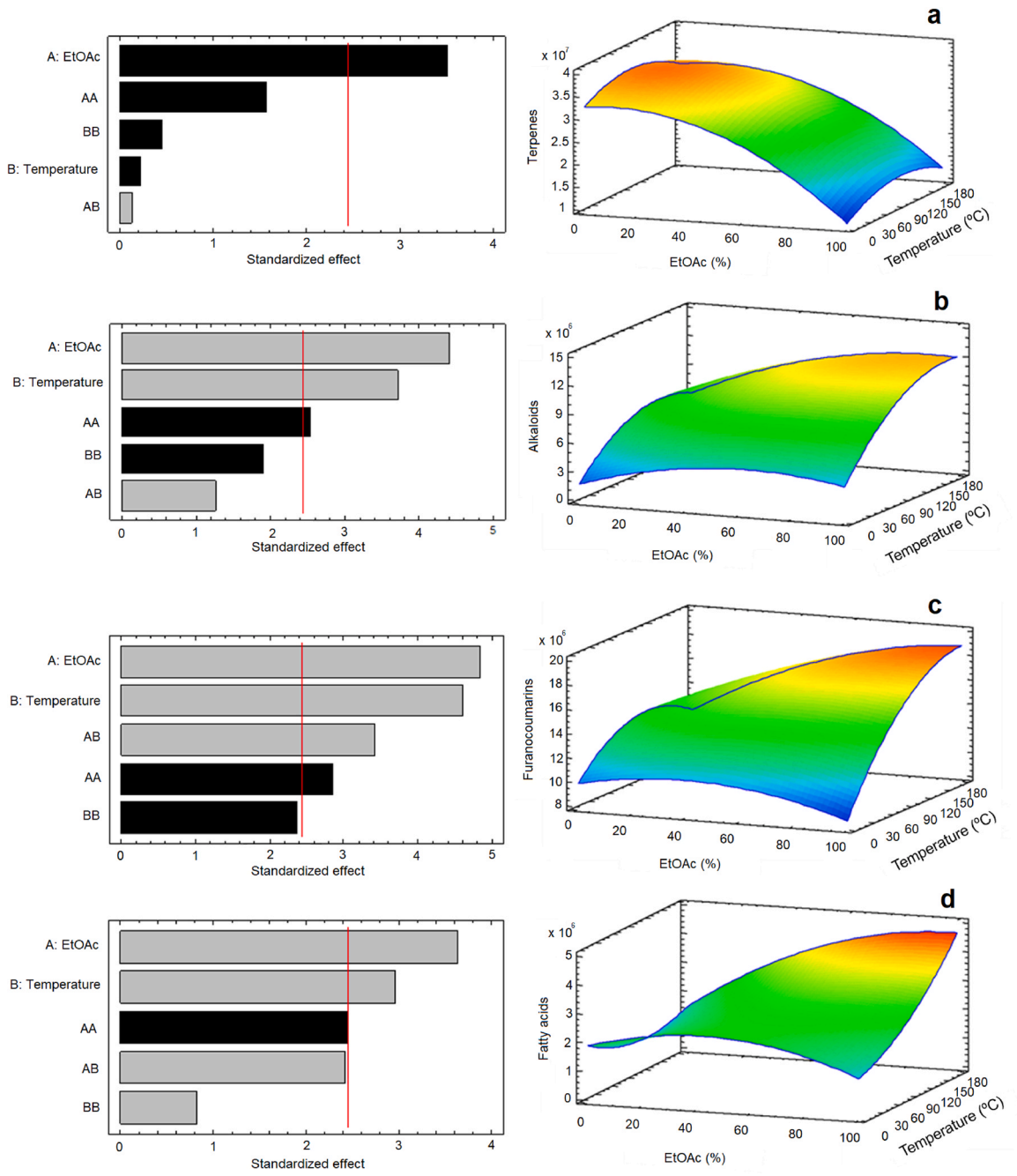

Fig. 2. Standardized Pareto charts (left) and their corresponding response surface plots (right) of the relative abundance of a) terpenes, b) alkaloids, c) furanocoumarins and d) fatty acids identified in extracts from aerial parts of *Ruta graveolens* obtained by PLE, according to temperature and percentage of ethyl acetate (0, 50, 100% in D-limonene). Bar color indicate the positive (grey) and negative (black) effect and red line 95% confidence level.

extraction temperature (Hossain et al., 2015); and, that the extraction of furanocoumarins improves at high temperatures (Skalicka-Wozniak and Glowniak, 2012).

The values of determination coefficients (R^2) of the studied families of metabolites from aerial parts extracts were between 0.726 and 0.927 (Table S2) and 0.977 for yield. The linear, quadratic and interaction equation coefficients for each response variable and studied extract are also provided in Table S2.

According to the individual mathematical models obtained in this study from aerial parts of rue, the optimum conditions to achieve enriched extracts of the different families of compounds are the following: 12.4% of ethyl acetate and 87.8 °C for terpenes; 87.6% ethyl acetate and 160.7 °C for alkaloids; 100% ethyl acetate and 170 °C for furanocoumarins, and 94.9% ethyl acetate and 170 °C for fatty acids. Regarding the extraction yield, the best results were achieved using PLE with 100% D-limonene at 170 °C. In this case, all the parameters of the model were significant, however the effect of solvent and temperature were more dominant ($p < 0.001$) (Table S2). A positive effect of temperature was expected to produce higher yields because high temperature increases compounds solubility, favoring the mass transfer rate and decreasing solvent viscosity.

Considering that terpenes were the main compounds of the aerial parts (Table 3, Table S1), a multiple response optimization was performed, maximizing the amount of terpenes and minimizing the amounts of alkaloids and furanocoumarins. In this way, the optimal conditions for terpenes extraction from aerial parts were 100% of D-

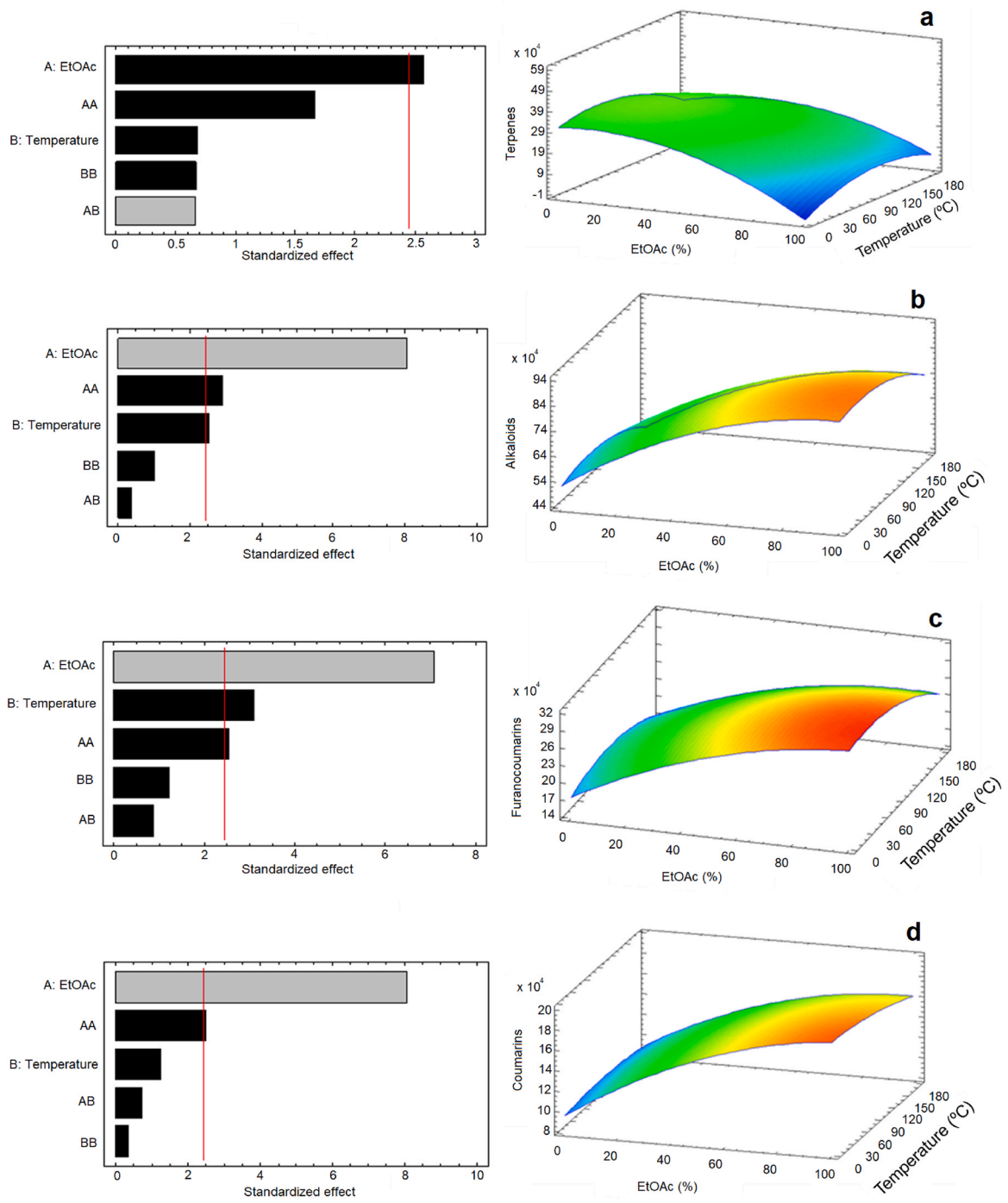

Fig. 3. Standardized Pareto charts (left) and their corresponding response surface plots (right) of the relative abundance of a) terpenes, b) alkaloids, c) furanocoumarins, d) coumarins identified in extracts from roots of *Ruta graveolens* obtained by PLE, according to temperature and percentage of ethyl acetate (0, 50, 100% in D-limonene). Bar color indicate the positive (grey) and negative (black) effect and red line 95% confidence level.

limonene and 40 °C.

For roots extracts, R^2 values of the studied families of metabolites ranged from 0.667 to 0.934 and 0.676 for extraction yield (Table S3). As it happened with the extracts of aerial parts, the extraction yield was favored by temperature, which in this case is the only significant factor of the model ($p < 0.005$) (Table S3). Moreover, the extraction of terpenes was positively favored by D-limonene (Fig. 3a) again, achieving the highest abundance with 100% of this solvent. The extraction of alkaloids, furanocoumarins and coumarins in roots was also favored by ethyl acetate, being the main effect in the models, followed by a minor negative contribution of the quadratic effect of solvent. However, as depicted in Fig. 3b-d, temperature showed a minor negative contribution for roots samples. This fact agrees with the observation done by Urbanová et al. (Urbanová et al., 2012), where temperatures above 80 °C produced a gradual decrease of alkaloids concentrations in their extraction from *Macleaya microcarpa* roots with pressurized methanol or ethanol. According to the results obtained in the present work, the effect of temperature on alkaloids and furanocoumarins extraction by PLE is diverse, mainly depending on the chemical characteristics and localization of metabolites; additionally, the structural composition of each kind of tissues analyzed should be considered.

Based on the individual mathematical models obtained in this study

from rue roots, the optimal conditions for the extraction of terpenes are 19.6% ethyl acetate and 67.1 °C; for alkaloids extraction, 98.4% ethyl acetate and 41.7 °C; while for furanocoumarins and coumarins, 100% ethyl acetate and 40 °C. For roots, the multiple response optimization made more sense for minimizing the amount of terpenes and maximizing the amounts of alkaloids, furanocoumarins and coumarins (Table 3); thus, their optimal extraction conditions were 100% of ethyl acetate and 40 °C.

Comparing the two multiple response optimizations, both optimal points are design points, opposites in terms of solvent for aerial parts and roots. For aerial part, the extract obtained with 100% of D-limonene at 40 °C presented a yield of 7.77% and a selectivity in terms of terpenes versus alkaloids, furanocoumarins and coumarins of 1.85, while the extraction of roots with 100% ethyl acetate at 40 °C presented a yield of 3.21% and a selectivity of 0.30 (Table 3).

In addition, it can be seen in the last column of Table 3 (differences to 33%), that extracts with a balance profile of terpenes, alkaloids and furanocumarins can also be obtained. The lower value of this column indicates the best-balanced extract in these three families.

As a conclusion of this part of the work, the extraction of alkaloids, furanocoumarins, coumarins, terpenes, and fatty acids can be carried out using green solvents by PLE, obtaining different selectivities just changing the solvent and/or the temperature.

3.4. *Supercritical fluid extraction ($scCO_2$) of aerial parts and roots of R. graveolens*

In order to use extracts of *R. graveolens* as an alternative to chemical

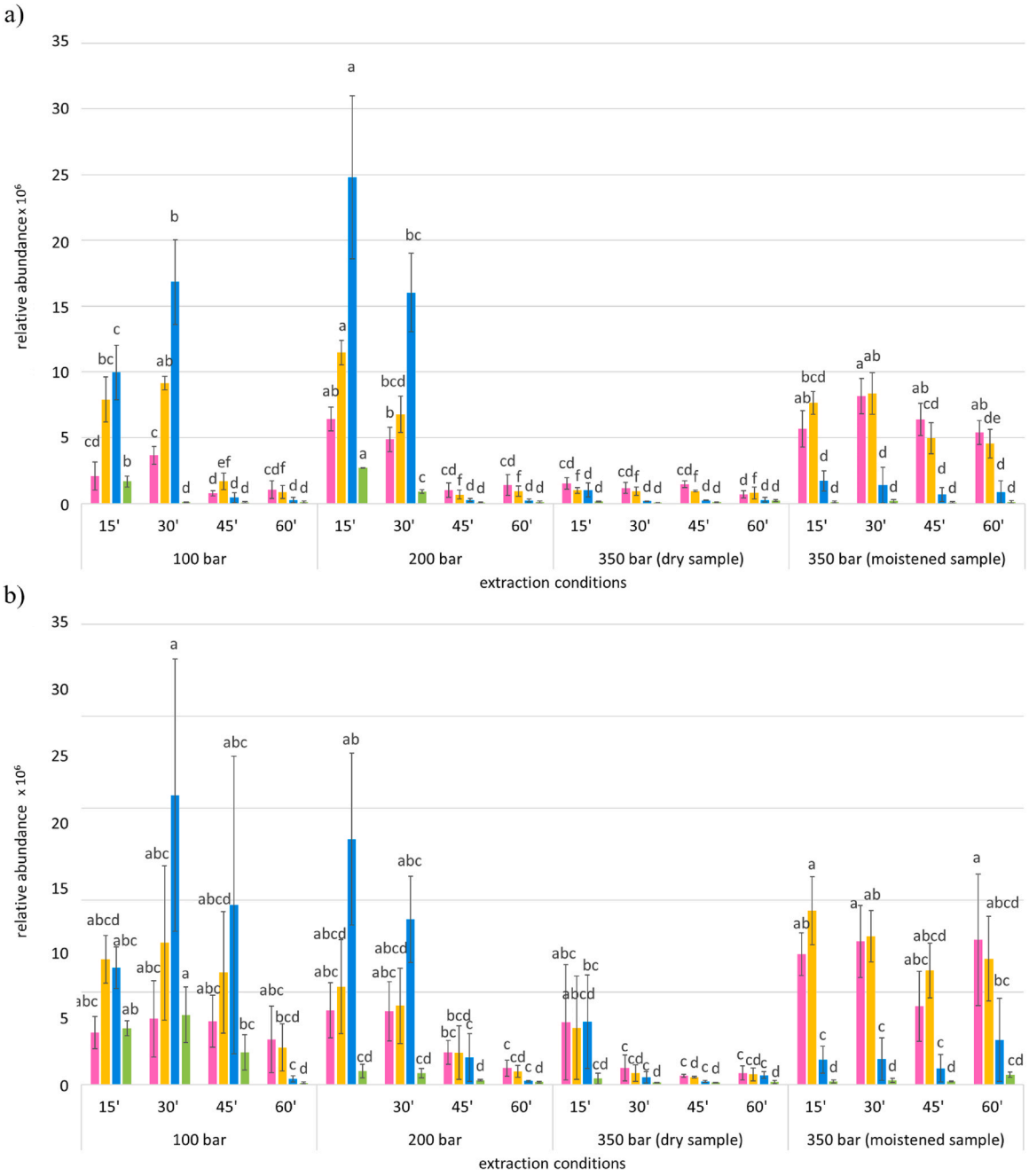

Fig. 4. Relative abundance of compounds identified in aerial parts from *Ruta graveolens* extracts obtained by SFE using different extraction conditions. Samples collected in: a) ethanol, b) ethyl acetate. Alkaloids (pink); furanocoumarins (yellow); fatty acids (blue); terpenes (green). Different letters within the same family show significant differences ($p < 0.05$).

pesticides in a future, neat CO_2 was selected as extraction solvent. CO_2 is considered a GRAS (generally recognized as safe) solvent because is inert, nontoxic, has a low cost, is abundant and easy to recover (Chemat et al., 2020), which makes it perfect to enhance the greenness of the extraction of rue metabolites.

Previous reports found in the literature on the use of $scCO_2$ for the extraction of furanocoumarins from *R. graveolens* suggested 40 °C at 200 bar as the most appropriate conditions (Baldino et al., 2018; Sovová et al., 2017). In our study, extraction temperature was kept constant at 40 °C, although different pressures were tested in a sequential mode (100, 200 and 350 bar), in order to extract, in addition to furanocoumarins, other groups of compounds as alkaloids, terpenes or fatty acids present in rue. This approach tried to follow the concept of biorefinery since is based on the continuous processing of the biomass (Bueno, 2020). Moreover, dry and moistened samples were tested in order to favor the extraction of alkaloids from rue, since it has been reported, for instance for caffeine, that to make alkaloids more readily available for $scCO_2$, samples should be wet (Mehr et al., 1996).

SFE obtained metabolites were grouped as alkaloids, coumarins, furanocoumarins, terpenes, fatty acids, ketones, amides, phenolic compounds and others (Table 2). The most abundant compounds families in aerial parts extracts were alkaloids, furanocoumarins and fatty acids (Fig. 4), while alkaloids and terpenes resulted the most important compounds families in root extracts (Fig. 5) regardless the collecting solvent used. This fact was expected since the differences between aerial parts and roots extracts regarding the chemical profile may be due to

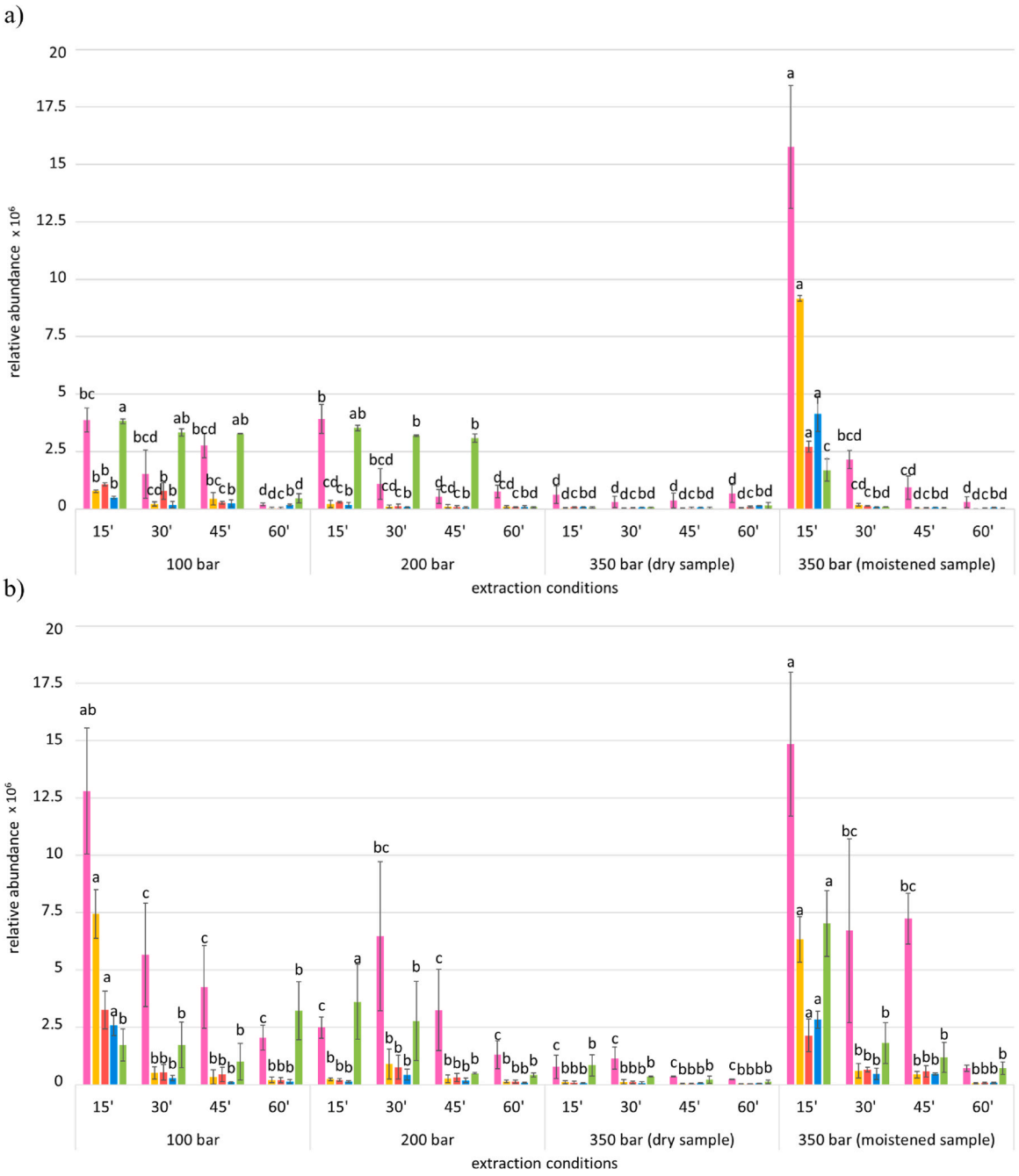

Fig. 5. Relative abundance of compounds identified in roots from *Ruta graveolens* extracts obtained by SFE using different extraction conditions. Samples collected in: a) ethanol, b) ethyl acetate. Alkaloids (pink); furanocoumarins (yellow); coumarins (red); fatty acids (blue); terpenes (green). Different letters within the same family show significant differences ($p < 0.05$).

facts such as physiological needs of the plant, physical structure of the organ used in the extraction, as well as to the site of biosynthesis and storage of metabolites within plant tissues (Verma and Shukla, 2015).

Regarding the collecting solvents, the global profiles recovered by ethanol or ethyl acetate were compared using a t-test for each type of matrix. Profiles were significantly different ($p < 0.05$) for aerial parts extracts in terms of phenolic compounds (Figure S3) while for root extracts differences appears for extracted alkaloids (Fig. 5). These differences are due to the different chemical structure and polarity of the identified compounds of rue, allowing a higher or lower interaction with the solvents (Thouri et al., 2017). In the case of phenolic compounds exclusive form aerial parts (1,3-bis(1,1-dimethylethyl)-benzene, 24-*ditert*-butylphenol, turmeronol A and isogentisin) and the alkaloids exclusive form roots (see Table 2), which are compounds with high octanol/water partition coefficient (log P > 3 and > 2, respectively), ethyl acetate was favored as collecting solvent because is a less polar solvent than ethanol. Increases of these families are 5-fold and 2-fold in ethyl acetate than in ethanol, respectively.

For extracts obtained from the same matrix (aerial parts or roots) and collecting solvent (ethanol or ethyl acetate), an ANOVA was performed on the relative abundance values of each family.

In the aerial parts, it could be inferred that for extracting alkaloids collected in ethanol, 30 min at 200 bar followed by 60 min at 350 bar after moistening the sample would be required. In the case of furanocoumarins, it would also be necessary to work first for 30 min at 100 bar (Fig. 4). In these conditions 71% and 89% of each family would be recovered, respectively. Focusing on the ANOVA evidences, when the extract is collected in ethyl acetate, alkaloids and furanocoumarins, appear more distributed in all the tested conditions. Therefore, a longer process would be required for recovery alkaloids and furanocoumarins in ethyl acetate (81% and 87%, respectively): 45 min at 100 bar followed by 30 min at 200 bar and the last 60 min at 350 bar after moistening the sample.

Most of the fatty acids (90%) would be removed after 30 min at 100 bar followed by 30 min at 200 bar (total of 60 min of extraction) using ethanol as collection solvent, while for reaching a 80% of recovery in ethyl acetate it would be needed 45 min at 100 bar follow by 30 min at 200 bar (total of 75 min of extraction). Favorable extraction conditions for the recovery of terpenes are 15 min at 100 bar followed by 30 min at 200 bar collecting the extract in ethanol or 45 min at 100 bar when the extract is collected in ethyl acetate (78% and 71% of the total of terpenes extracted). Nevertheless, the presence of terpenes in the global extract is 8-fold lower than fatty acids. It could be thought that this type of extraction with $scCO_2$ is not suitable for terpenes, however it should be noted that the terpenes extracted with $scCO_2$ are less polar (i.e. calarene, γ-stigmasterol with log P > 6) than those extracted in PLE (i.e. *m*-cymene, carvenone with log P < 4.50) (see Table 2).

In summary, for aerial parts, a batch extraction can be carried out to obtain enriched extracts in fatty acids while working until 200 bar, followed by the moistening of the sample and an increase in pressure to 350 bar to obtain a clean and enriched extract in alkaloids and furanocumarins, independently of the collecting solvent.

Regarding to roots (Fig. 5), alkaloids were obtained extracting 15 min at 100 bar followed by another 15 min at 200 bar and finally moistening the sample and extracting 15 min more at 350 bar using ethanol as collecting solvent. If ethyl acetate is used instead of ethanol, the optimal working conditions were 15 min at 100 bar followed by 30 min at 200 bar and 45 min at 350 bar after moistening the sample. At those conditions, a 66% of the total alkaloids extracted were recovered in 45 min using ethanol while 72% were recovered with ethyl acetate but doubling the time.

For terpenes, the other important family in roots, the optimal conditions were stablished at 45 min at 100 bar along with another 45 min at 200 bar with dry sample and ethanol as collecting solvent, whereas 15 min at 200 bar with subsequent extraction of 15 min at 350 bar with moistened sample are the conditions for the collection with ethyl acetate.

For a total extraction of compounds from root using $scCO_2$, the better conditions would be 15 min of extraction of the moistened sample at 350 bar using ethanol as collecting solvent, with a previous extraction of 15 min at 100 bar if ethyl acetate replace ethanol.

In general, as can be deduced from the results of this section, the presence of water is needed to improve the interactions of the solvent with the solute (Pereira and Meireles, 2010), not only for the alkaloids extraction as has been commented before, but also for other families of compounds, except for fatty acids.

In addition, our results are differing from the ones reported by Brandão et al. (Brandão et al., 2017) for alkaloids extraction from *Melocactus zehntneri*, where their optimal conditions included pressures of 300 bar, notwithstanding 300 bar was the highest pressure studied at that work. Therefore, an extra study comparing both pressures would be needed in order to work in the mildest possible conditions and thereby save energy.

As regards to furanocoumarins from rue, our results are consistent with those of Sovová et al. (Sovová et al., 2017), who reported that furanocoumarins were efficiently extracted from the aerial parts of rue at pressures of 120–280 bar, and at 300 bar the extraction was further improved. On the other hand, Baldino et al. (Baldino et al., 2018) developed a $scCO_2$ extraction method coupled with fractional separation to isolate furanocoumarins from rue leaves and fruits from waxes. This method lasts 6 h 40 min. In our case, we achieved a separation of lighter fatty acids (not included in either of the two previous articles) and a concomitant extraction of alkaloids and furanocumarins in just 120 min, collecting the extract in ethanol.

4. Conclusion

This work highlights the versatility of environmentally friendly extraction techniques (PLE and SFE) employing green solvents in selectively extracting specific metabolite families from different parts of rue. Terpenes were favorably extracted by PLE and fatty acids by SFE from aerial parts of rue. Coumarins were present and extracted exclusively from roots, and alkaloids enriched extracts were preferable obtained from roots, regardless the technique of extraction. A total of 103 compounds were tentatively identified in *R. graveolens* extracts, some of them with known biological activity against phytopathogenic microorganisms. Consequently, these enriched extracts hold promising potential as a green alternative to substitute chemical pesticides for plant diseases control, pending the confirmation of their nematicidal activity.

CRediT authorship contribution statement

Jose Mendiola: Writing – review & editing, Supervision, Methodology. **Alma Angélica Del Villar-Martínez:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Lorena Reyes-Vaquero:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Gerardo Álvarez-Rivera:** Writing – review & editing, Supervision, Methodology. **Elena Ibáñez:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Mónica Bueno:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, Conceptualization.

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

All relevant data are published as supplementary material.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2024.118717.

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