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Green subcritical water extraction of *Mentha x rotundifolia* leaves collected in different annuities

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Abstract

Aim: In this work, the development for the first time of a green and efficient method to obtain bioactive extracts from *Mentha x rotundifolia* leaves has been investigated.

Methods: The efficiency of three techniques [microwave-assisted extraction (MAE), subcritical water extraction (SWE), and ultrasound-assisted extraction (UAE)] was compared in terms of total phenolic content (TPC) and antioxidant activity [1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzthioazoline-6-sulfonic acid) (ABTS) assays].

Results: Under similar operating conditions, SWE outperformed MAE and UAE for providing *M. rotundifolia* extracts with improved antioxidant activity. Further in-depth optimization of the SWE method by means of a Box-Behnken experimental design showed 120°C, 5 min, 0.08 g dry sample: 1 mL water and 2 extraction cycles as optimal experimental parameters to provide the maximum yield of phenolics and the highest bioactivity. The application of the developed SWE method to *M. rotundifolia* leaves collected in different annuities (2014–2017) showed, in general, no significant differences regarding both composition and antioxidant capacity, as expected from plant samples grown in field under drip irrigation conditions.

Conclusions: The SWE method here optimized is shown as a sustainable and efficient alternative for providing bioactive *M. rotundifolia* extracts with application as functional ingredients, natural preservatives, etc. in the food industry, among others.

Keywords

Mentha x rotundifolia, microwave-assisted extraction, subcritical water extraction, ultrasound-assisted extraction, phenolic compounds, antioxidant activity, liquid chromatography-mass spectrometry

Introduction

Bioactive compounds obtained from natural sources are the basis for the development of new supplements or functional foods. Plants are probably the most studied natural source of bioactives, mainly due to their availability, diversity, and wide variability regarding composition. Among them, *Mentha* is a genus consisting of around 20 species and several hybrids that can occur naturally or in cultivation. Several species of *Mentha* are used in the food industry [1, 2], not only as food additives and taste enhancers [3], but also as functional ingredients, considering their bioactive constituents (terpenes, polyphenols, etc.) which are responsible for their numerous beneficial properties (anti-inflammatory, anesthetics, antioxidant and anti-microbial, among others) [4].

Mentha x rotundifolia is a hybrid between *Mentha longifolia* and *Mentha suaveolens*. It generally grows wild on the banks of irrigated areas, although it is also cultivated for culinary and medicinal purposes. Most studies regarding *Mentha x rotundifolia* have been focused on the chemical profiling and bioactive properties (antimicrobial, antioxidant, etc.) of its essential oils, mainly obtained by distillation or hydrodistillation [5–7]. However, studies regarding the composition and bioactivity of extracts from this *Mentha* species obtained using polar solvents are scarce [8, 9]. Moreover, no previous study has addressed the changes with harvesting year for extracts from *M. rotundifolia* samples collected at the same harvesting stage.

Although solid-liquid extraction (SLE) is the most widely used procedure to obtain bioactive extracts because of their undeniable advantages in terms of simplicity and affordability, advanced extraction techniques such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) and subcritical water extraction (SWE), are gaining importance as efficient alternatives for bioactive extraction [10]. While UAE [11–14] and MAE [8, 11, 15] have been previously described to provide improved performance for the extraction of *Mentha* bioactives, SWE has been scarcely applied for this purpose.

SWE is an environmentally friendly technique in which the application of high pressures to water throughout the extraction process usually gives rise to better extraction yields and shorter extraction times than traditional extraction procedures [16]. This technique has been successfully used for the selective extraction of oxygenated flavour compounds [17] and phenolics [18] from *Mentha piperita* and for the extraction of carbohydrates, proteins, and phenolic compounds from a Japanese mint (*Mentha arvensis*) [19]. However, to the best of our knowledge, no previous application of this technique to other *Mentha* species and, in particular, to *M. rotundifolia* has been carried out.

Therefore, in this manuscript, a new method by SWE has been optimized to obtain bioactive extracts of *Mentha x rotundifolia*, prior comparison of the performance of this technique with MAE and UAE. The optimized method was subsequently applied to obtain bioactive extracts from *M. rotundifolia* samples collected in different annuities.

Materials and methods

Samples and standards

Mentha x rotundifolia plants from different annuities [2014 (MR14), 2015 (MR15), 2016 (MR16) and 2017 (MR17)] were kindly provided by Dr. J. Navarro Rocha from Centro de Investigación y Tecnología Agroalimentaria de Aragón (Spain). These plants were experimentally grown in field in Ejea de los Caballeros (Zaragoza, Spain; 42°8'8.73" N, 1°12'31.50" W/346 m a.s.l.) and were provided with drip irrigation from June to August every annuity due to the low rainfall in this area. Leaves of *M. rotundifolia* plants, harvested at their flowering stage, were air-dried at ambient temperature and in the absence of light, stored in closed amber vials for preservation of their original composition, ground in a domestic mill (Moulinex, Barcelona, Spain) and sieved (< 500 μm) before extraction.

Caffeic acid and chlorogenic acid were purchased from Sigma-Aldrich® (St. Louis, MO, USA), luteolin 7-*O*-glucoside and salvianolic acid B were acquired from Extrasynthese (Genay, France) and rosmarinic acid from Cayman Chemical Company (Michigan, USA). All standards used were of analytical grade (purity ≥ 95%).

Selection of extraction technique

A preliminary study was carried out to select the optimal extraction technique (UAE, MAE, or SWE). In all cases, 1 g of sample (MR16) and 12 mL of ultrapure water (Milli-Q® System, Millipore, Burlington, MA, USA) were used as sample to solvent volume ratio (*s*/*v*). All experiments were carried out in triplicate at 50°C (otherwise below specified) for 5 min and the extracts thus obtained were frozen at –20°C until analysis. For the selection of the optimal technique, total phenolic content (TPC, section 2.5) and the antioxidant activity [1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzthioazoline-6-sulfonic acid) (ABTS) methods, sections 2.6] of the extracts were used as response variables to be maximized.

UAE

UAE of bioactive compounds was carried out using both an ultrasonic bath and two sonoprobes of different dimensions. The US bath (SONICA sweep system EP 2200 SOLTEC®), provided with a metallic rack to hold in place 50 mL Falcon® tubes, was set at 45 kHz. The process was carried out at 45°C ± 5°C.

A Branson 450 Digital Sonifier (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a sonoprobe of either 12 mm or 3 mm of diameter (Biogen Científica, SL) was used for UAE. The system consisted of an integrated temperature sensor and a digital unit for control of wave amplitude and time. To provide the mild conditions required for extraction of phenolics, both sonoprobes operated in pulsed mode (1 s on/1 s off) at a frequency of 20 kHz and an amplitude of 30%. In both cases, the probe was immersed in the sample at a constant depth of 2 cm and overheating of the sample above the set temperature was also avoided by placing the Falcon tubes in a water/ice bath.

MAE

MAEs were carried out in a MARS 6 (CEM, NC, USA) equipment provided with 100 mL X-Press 1500 vessels (CEM) and an optical fiber probe for temperature control. Microwave power was set at 900 W.

SWE

Extractions were carried out using a PSE ONE instrument (Applied Separations, PA, USA). *Mentha x rotundifolia* leaves were mixed with 7 g of washed sea sand (Panreac, Barcelona, Spain) and introduced into the stainless steel extraction cell between two layers of sea sand at the top and bottom of the cell. A single static extraction cycle was performed (unless indicated) at a constant pressure of 100 bar. A nitrogen (99.999% purity) purge was used for 1 min at the end of the extraction cycle to ensure complete recovery of the extract.

Optimization of SWE operating conditions

First, the *s*/*v* ratio was optimized by evaluating three sample amounts (s), i.e., 0.5 g, 1.0 g, and 1.5 g, and measuring the resulting volume of extract recovered after each experiment. The selection of the optimal sample amount was based on the extraction yield of three phenolic compounds of different nature (luteolin 7-*O*-glucoside, rosmarinic acid, and salvianolic acid B), previously described in the literature as the most abundant of *Mentha* extracts [20–22].

Subsequent optimization of the extraction temperature (*T*, °C) and time (*t*, min) was carried out using a 3-level factorial design (Table S1) with the aim of maximizing the extraction efficiency and of providing a fast SWE extraction method. The quadratic model proposed was:

$$R = \beta_0 + \beta_1 T + \beta_2 t + \beta_{1,1} T^2 + \beta_{2,2} t^2 + \beta_{1,2} Tt + \varepsilon \text{ (equation 1)}$$

In equation 1, β_0 is the intercept, β_i are the first-order coefficients, $\beta_{i,i}$ the quadratic coefficients for ith factors, $\beta_{i,j}$ the coefficients for the interaction of factors i and j, and ε is the error. The ranges of the experimental factors evaluated were *T* = 50°–150°C and *t* = 5–30 min. Temperature values above the boiling point of water were considered to evaluate the benefits associated to the extraction of *M. rotundifolia* phenolics under the pressurized conditions provided by SWE. Potential adverse effects such as the possible degradation of thermolabile phenolics were also considered in the selection of the temperature range.

As response variables (R) in equation 1, the individual content (R_x, in mg g^{-1} of dry sample) of selected bioactives (luteolin 7-*O*-glucoside, rosmarinic acid, and salvianolic acid B), the TPC (R_{TPC}) and the antioxidant activity, as determined by the DPPH method (R_{DPPH}) and the ABTS (R_{ABTS}) assay, were considered. The parameters of the model that individually maximized R_x, R_{TPC}, R_{DPPH} and R_{ABTS}, were estimated by multiple linear regression (MLR) using Statgraphics Centurion XV software (Statistical Graphics Corporation, Rockville, MD, USA).

A desirability function (D) that simultaneously maximized all previously mentioned individual responses was also considered to provide optimal SWE extraction. This D function takes values between 0 (completely undesirable value) and 1 (completely desirable or ideal response). Finally, the number of extraction cycles (C1–C3) that maximized the extraction yield of target compounds was also evaluated.

Liquid chromatography-mass spectrometry analysis

A high performance liquid chromatography (LC; HPLC)-ultraviolet (UV)/mass spectrometry (MS) apparatus consisting of a 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) was used in this study for characterization of SWE extracts. This equipment was provided with an in-line degasser, a binary pump, a Rheodyne® 7125 injection valve, and a column oven (Kariba Instruments, UK), coupled via an electrospray ionization (ESI) interface to a single quadrupole MS detector (MSD) 1100 (Agilent Technologies). The operating parameters of the electrospray source were as follows: spray voltage, 4 kV; drying gas (N_2, 99.5% purity) temperature, 300°C; drying gas flow, 12 L min^{-1}; nebulizer (N_2, 99.5% purity) pressure, 276 kPa; and fragmentor voltage, 80–100 V. Quasimolecular ions for target compounds were recorded in the selected ion monitoring (SIM) mode under negative polarity [$(M-H)^-$ 179, 353, 359, 447, and 717 for caffeic acid, chlorogenic acid, rosmarinic acid, luteolin 7-*O*-glucoside, and salvianolic acid B, respectively]. Data acquisition and processing were performed using HP ChemStation Rev. A.07.01 software.

Chromatographic separations were carried out on a reverse-phase Luna C18 analytical column (Phenomenex, Cheshire, UK; 100 mm × 2.0 mm i.d., 3 µm) operating at a flow rate of 0.4 mL min^{-1}. The mobile phase was a binary mixture of solvent A (water with 0.1% acetic acid) and B (acetonitrile with 0.1% acetic acid), according to the following gradient: 0 min 5% B; 20–25 min 36% B; 35 min 90% B; 36–50 min 5% B. Injection volume was 5 µL and the column temperature was set at 25°C.

Quantitation of target phenolics was performed in triplicate using external standard calibration curves within the range 0.01–100 µg mL^{-1}. Goodness of fitting for these calibration curves and reproducibility of the method were previously assured. Results were expressed in milligrams per gram of dry sample.

TPC

TPC of *M. rotundifolia* extracts was determined using the Folin-Ciocalteu reagent (2N) and gallic acid as standard (both from Sigma-Aldrich®), according to the method of Soria et al. [23] with slight modifications. An aliquot (100 µL) of extracts previously diluted [1:5–1:175, *v*/*v*], 100 µL of MeOH (Sigma-Aldrich®) and 100 µL of Folin-Ciocalteu reagent were vortexed in a 1.5 mL eppendorf tube. After 5 min, 700 µL of 75 g L^{-1} Na_2CO_3 (Panreac, Barcelona, Spain) were added and the samples were vortexed briefly. The eppendorfs were then allowed to stand in the dark for 20 min at room temperature. Following this, the samples were centrifuged at 4,400 g for 3 min and their absorbance was measured [number of replicates (n) = 3] at 750 nm using a BioTek ELx800™ microplate reader (BioTek Instruments, Inc., USA). The same procedure was repeated with aqueous solutions of gallic acid in the 10–100 mg L^{-1} concentration range to build up a calibration curve. Results were expressed as gallic acid equivalents (GAE; mg mL^{-1} or mg g^{-1} dry sample).

Antioxidant activity

DPPH method

The antioxidant activity of *M. rotundifolia* extracts was measured (n = 3) in terms of hydrogen donating or free radical scavenging ability using the stable DPPH radical method [24]. Aliquots (50 µL) of different dilutions (1:20–1:70, *v*/*v*) of SWE extracts were mixed with 45 µL of a 0.001 mol/L methanolic solution

of DPPH (Sigma-Aldrich®). After 30 min of incubation in the darkness at 40°C, the decrease in absorbance was measured at 540 nm by using the absorbance microplate reader described in section 2.5. Antioxidant reagent Trolox (Sigma-Aldrich®) was used as positive control and results were expressed as Trolox equivalents (TE; mg TE mL^{-1}).

ABTS assay

An aqueous solution of 7 mmol/L ABTS (Sigma-Aldrich®) and 2.45 mmol/L $K_2S_2O_8$ (Sigma-Aldrich®) was prepared and allowed to stand at 4°C in the absence of light for 16 h. After this time, the $ABTS^+$ was equilibrated at 30°C and diluted (1:30, *v*/*v*) with ethanol. For colorimetric measurements by using a BioTek ELx800™ microplate reader, 50 µL of diluted (1:500, *v*/*v*) *Mentha* extracts and 150 µL of the diluted $ABTS^+$ solution were added to each well. The same procedure was carried out for the antioxidant agent Trolox (0.005–1 mmol/L) in ethanol (positive control). After stirring the mixtures, absorbance ($n = 3$) at 750 nm was read. The results of this assay were expressed as TE (mg TE mL^{-1}).

Statistical analysis

Data were subjected to statistical analysis by using Statistica 7.0 software (StatSoft, Inc., Tulsa, OK, USA). Significance ($P < 0.05$) of differences was assessed by the analysis of variance (ANOVA, Tukey test).

Results

Selection of the optimal technique

The efficiency of UAE, MAE and SWE in terms of TPC and antioxidant activity of *Mentha x rotundifolia* MR16 extracts obtained under similar extraction conditions (50°C and 5 min) was evaluated (Table 1).

Table 1. Antioxidant activity (DPPH and ABTS methods, expressed as TE, and TPC expressed as GAE) of *Mentha x rotundifolia* MR16 extracts obtained by UAE, MAE, and SWE at 50°C for 5 min

Technique	TPC (GAE, mg mL^{-1})	ABTS (TE, mg mL^{-1})	DPPH (TE, mg mL^{-1})
UAE bath	5.7 (0.1)*,[a]	3.3 (0.2)[a,b]	4.4 (0.5)[c]
UAE 12 mm probe	3.38 (0.06)[c]	2.4 (0.5)[b]	5.0 (0.9)[c]
UAE 3 mm probe	1.85 (0.04)[d]	1.7 (0.2)[b]	2.6 (0.8)[d]
MAE	3.03 (0.06)[c]	4.3 (0.5)[a]	9.9 (0.9)[b]
SWE	4.5 (0.2)[b]	4.3 (0.1)[a]	11.2 (0.5)[a]

* Mean value and standard deviation in parentheses ($n = 3$); [a,b,c,d] different letters in the same column indicate significant ($P < 0.05$) differences among the techniques evaluated

For UAE, a US bath and two sonoprobes with different internal diameter (3 mm and 12 mm) were used. Although no significant differences were observed in the antioxidant activity of the extracts obtained using the 12 mm probe and the bath, a higher content of phenolic compounds was found for the last treatment. As regards the different sonoprobes, significantly lower TPC and antioxidant activity values were achieved using the 3 mm probe.

As compared with MAE and SWE treatments, UAE bath provided the highest TPC. However, the antioxidant activity measured by the DPPH method of UAE extracts was significantly lower than those provided by MAE and SWE, ruling out UAE for further investigation.

Although no significant differences were found in the antioxidant activity (ABTS method) of extracts obtained by MAE and SWE, this last technique was selected for subsequent studies and for in-depth optimization on the basis of its higher TPC yield and greater antioxidant activity (DPPH assay).

Selection of the optimal SWE conditions to obtain bioactive extracts of *Mentha* sp.

SWE parameters (*s*, *t*, *T* and the number of cycles) were optimized to enhance the extraction of *M. rotundifolia* bioactives. As previously mentioned, the volume of solvent is not a fixed parameter in SWE, since it depends on the capacity of the extraction cell and on the amount of sample and sea sand used. Thus, a number

of experiments using different sample amounts (*s*: 0.5 g, 1 g, and 1.5 g of MR16 leaves) were carried out under the same operating conditions (100°C for 18 min).

It shows in Table 2 the concentrations of selected bioactives (luteolin 7-*O*-glucoside, rosmarinic acid, and salvianolic acid B) from *M. rotundofolia* extracted using the different sample amounts (*s*) evaluated. Higher concentrations of rosmarinic and salvianolic acid B were recovered with 0.5 g and 1 g, whereas this last amount provided the most efficient recovery of luteolin 7-*O*-glucoside. Therefore, 1 g of sample (corresponding to an s/v of 0.08 g mL^{-1}) was selected for further experiments.

Table 2. Concentration of selected phenolics present in SWE (100°C, 18 min) extracts obtained from different amounts (*s*) of *Mentha x rotundifolia* (MR16)

s (g)	Concentration (mg g^{-1} dry sample)		
	Luteolin 7-*O*-glucoside	Rosmarinic acid	Salvianolic acid B
0.5	0.032 (0.003)*,b	2.21 (0.06)^{a}	0.29 (0.03)^{a}
1.0	0.051 (0.005)^{a}	3.8 (0.2)^{a}	0.33 (0.02)^{a}
1.5	-	0.79 (0.06)^{b}	0.135 (0.004)^{b}

* Mean value and standard deviation in parentheses (n = 3); ^{a,b} different letters indicate significant (P < 0.05) differences for each compound among the experiments carried out with different sample amounts; -: none

Optimization of SWE temperature and time was carried out following a Box-Behnken experimental design, considering as response variables the TPC (R_{TPC}), the antioxidant activity determined by the DPPH (R_{DPPH}) and ABTS (R_{ABTS}) methods and the concentrations (mg g^{-1} of dry sample) of previously selected phenolic compounds (R_x, Table S1).

In general, the highest TPC and antioxidant activity were obtained at high temperatures and intermediate times (e.g., R_{TPC} 6.0 mg GAE mL^{-1}, R_{ABTS} 14.02 mg TE mL^{-1}, R_{DPPH} 12.2 mg TE mL^{-1} at 150°C, 18 min). However, the highest concentrations of rosmarinic acid (3.8 mg g^{-1} dry sample), salvianolic acid B (0.72 mg g^{-1} dry sample) and luteolin 7-*O*-glucoside (0.068 mg g^{-1} dry sample) were extracted at lower temperatures (100°C, 18 min).

Response surface methodology was used to calculate the coefficients of the quadratic models proposed and to estimate the statistical significance of the regression coefficients. The equations of each model and the optimal conditions for the different responses considered (R_x, R_{TPC}, R_{DPPH}, and R_{ABTS}) are shown in Table 3. In general, extractions at temperatures above 100°C maximized all responses, with T and T^2 being the most significant (P < 0.05) variables. Optimal value of TPC was achieved at the highest temperature and the longest time (142°C, 20 min), while the extraction of rosmarinic acid, salvianolic acid B, and luteolin 7-*O*-glucoside, which shared a similar trend, was maximal between 98°C and 109°C, and 15 min and 18 min. The optimal conditions that provided the highest antioxidant activity, measured by both the DPPH and ABTS methods, were intermediate between the previous cases, requiring 120°C, 5 min, and 128°C, 18 min, respectively.

Table 3. Equations of the models and optimal conditions for maximization of every individual response in the development of a SWE method to obtain bioactive extracts from *M. rotundifolia* (MR16) leaves

Response	Equation of the model (R^2)	Optimal conditions
$R_{luteolin\ 7\text{-}O\text{-}glucoside}$	$y = -0.111 + 0.003T - 0.165 \cdot 10^{-4}T^2$ (R^2 = 84%)	98ºC, 18 min
$R_{rosmarinic\ acid}$	$y = -7.55 + 0.20T - 9.09 \cdot 10^{-4}T^2$ (R^2 = 75%)	109ºC, 15 min
$R_{salvianolic\ acid\ B}$	$y = -1.23 + 0.03T - 1.60 \cdot 10^{-4}T^2$ (R^2 = 74%)	103ºC, 16 min
R_{TPC}	$y = 2.35 + 0.05T - 1.7 \cdot 10^{-4}T^2$ (R^2 = 73%)	142ºC, 20 min
R_{DPPH}	$y = 697.38 + 10.15T + 4.23t - 0.04T^2 - 0.04Tt - 0.11t^2$ (R^2 = 71%)	120ºC, 5 min
R_{ABTS}	$y = -33.808 + 1.366T - 0.005T^2$ (R^2 = 89%)	128ºC, 18 min

R^2: *R*-squared statistic indicates the percentage of variability explained by the model

Finally, when a multiple response was considered to simultaneously maximize all the response variables above mentioned, the optimal extraction parameters were found to be 120°C and 5 min (D = 0.67).

Evaluation of the number of extraction cycles required to achieve an exhaustive extraction of the phenolic compounds present in this matrix was also considered. Whereas salvianolic acid B was completely recovered after the first extraction cycle, 67% of rosmarinic acid and 39% of luteolin 7-*O*-glucoside were extracted. Therefore, a second extraction cycle was required to maximize the recovery of all target phenolic compounds (84% luteolin 7-*O*-glucoside and 90% rosmarinic acid). In summary, the optimal SWE conditions were 120°C, 5 min, 0.08 g dry sample per 1 mL of water and 2 extraction cycles.

Application of the optimized SWE method to *Mentha x rotundifolia* samples collected in different annuities

Concentration of bioactives in the SWE extracts obtained from MR14–MR17 leaf samples was determined by LC-UV/MS. Apart from the phenolics considered for the optimization of the method, caffeic and chlorogenic acids were detected and quantified in these extracts. As it is shown in Table 4, no significant differences in the concentration of bioactives were found among the samples in study, except for rosmarinic acid, whose concentration decreased with the collection annuity down to half of its initial value (MR14: 4.16 mg g^{-1} of dry sample, MR17: 2.5 mg g^{-1} of dry sample). TPC and antioxidant activity of MR14–MR17 extracts was also stable irrespective of the harvesting year, with only sample collected in 2015 (MR15) providing an unexpectedly higher TPC.

Table 4. Concentration (mg g^{-1} of dry sample) of main phenolic compounds and antioxidant activity (DPPH and ABTS methods, expressed as TE, and TPC expressed as GAE) in SWE extracts of *Mentha x rotundifolia* from different annuities obtained with a *s*/*v* of 0.08 g mL^{-1} at 120ºC for 5 min and with 2 extraction cycles

Samples	Concentration (mg g^{-1} of dry sample)					TPC (GAE, mg mL^{-1})	ABTS (TE, mg mL^{-1})	DPPH (TE, mg mL^{-1})
	Luteolin 7-*O*-glucoside	Rosmarinic acid	Salvianolic acid B	Caffeic acid	Chlorogenic acid			
MR14	0.047 (0.003)*,a	4.16 (0.02)a	0.49 (0.01)a	0.059 (0.006)a	0.004 (0.001)a	6.8 (0.5)b	11.6 (1.4)a	11.0 (0.3)a
MR15	0.051 (0.001)a	3.62 (0.02)a,b	0.32 (0.02)a	0.06 (0.01)a	0.0044 (0.0003)a	8.2 (0.8)a	16.2 (0.6)a	11.0 (1.2)a
MR16	0.050 (0.006)a	3.36 (0.04)b	0.345 (0.001)a	0.039 (0.005)a	0.0032 (0.0003)a	5.7 (0.1)b	13.41 (1.69)a	12.4 (0.1)a
MR17	0.06 (0.03)a	2.5 (0.1)c	0.5 (0.1)a	0.043 (0.008)a	0.0033 (0.0007)a	6.5 (0.2)b	15.75 (1.17)a	10.4 (0.4)a

* Mean value and standard deviation in parentheses (n = 3); a,b,c different letters in the same column indicate significant (P < 0.05) differences among extracts of *M. rotundifolia* collected in different annuities

Discussion

Among the techniques used in this study to obtain bioactive extracts from *Mentha x rotundifolia*, UAE has advantages such as its simplicity and the use of low temperatures that can prevent compound degradation. Moreover, the use of a sonoprobe could favor the cell wall breakdown, allowing the penetration of the solvent into the matrix and assisting the extraction of the compounds with antioxidant activity from the plant. However, the more energetic conditions provided by the 3 mm probe could cause the degradation of the antioxidant compounds during extraction, justifying the obtained results (Table 1). This behaviour agrees with that previously described by Šic Žlaburo et al. [25] for the extraction of phenolic compounds from *Stevia rebaudiana*, using a UAE probe of either 22 mm or 7 mm. On the contrary, Silva et al. [26] reported that the probe diameter (10 mm or 20 mm) did not exert an influence on the extraction yield of phenolic compounds from *Rhodiola rosea*, evidencing the need to evaluate this operating parameter for every intended application.

In this study a better performance was obtained for UAE bath compared to sonoprobes. These results are in agreement with Roshanpour et al. [27] who obtained promising results for the extraction of antioxidants from *M. piperita* leaves by using an ultrasound bath [optimal conditions were: 65°C, 50 min and 59% (*v*/*v*) ethanol/water as extractant]. However, when the three techniques here evaluated were compared, SWE outperformed MAE and UAE bath for the intended extraction of bioactives from *Mentha x rotundifolia*.

After optimization of SWE conditions and quantitation of major phenolics, TPC and antioxidant activity were evaluated in *Mentha x rotundifolia* extracts. TPC has been reported to be dependent on the extraction technique and the solvent used as extractant [28, 29]. The *Mentha* sp. considered [28–30], the development stage of the plant, and the environmental conditions during cultivation [31, 32] are also key factors affecting

recovery of bioactives. Values here reported on TPC (68–98 mg g–1 dry weight, Table 4) have been found to be similar to those previously reported by Ranjbar et al. [29] for ethanolic extracts of *M. spicata*, *M. aquatica*, and *M. longifolia* (61–87 mg g^{-1} dry weight) obtained by maceration of samples collected at different seasonal stages (April, June, and August). In a study by Benabdallah et al. [28] on bioactivity of methanolic extracts of different Algerian mints species, *Mentha x rotundifolia* and *Mentha pulegium* extracts showed no differences in their TPC content (15 mg g^{-1} and 17 mg g^{-1} dry weight, respectively), whereas extracts from *Mentha aquatica*, *Mentha arvensis*, and *Mentha piperita* showed a significantly higher TPC value (in the range 31–43 mg GAE g^{-1} dry weight). The lower yield of *M. rotundifolia* phenolics reported in this last study, as compared with the results described in the present paper, could be explained as the result of the improved extraction performance of SWE over conventional SLE, even when water is used as extractant.

As reported in the literature [22, 33, 34], essential oils and extracts of some of the major genera belonging to Lamiaceae family (e.g., *Mentha*, *Salvia*, etc.) have been described to possess high antioxidant activity. However, whereas the number of papers dealing with the antioxidant activity of *Mentha* extracts obtained by conventional SLE is high [28, 29], there are fewer reports regarding the bioactivity of extracts obtained by advanced extraction techniques [18, 19], and none of them refers to *M. rotundifolia*. This makes difficult the comparison of experimental data in this study with previous literature.

As previously mentioned, whereas a number of papers have aimed the effect of harvesting stage on the composition and bioactivity of different *Mentha* species [29], very few studies have reported the variability in phenolic composition and antioxidant activity of *Mentha* extracts obtained from plant samples collected at different annuities [30]. In the present study, *M. rotundifolia* plants have been cultivated in field under no control of environmental conditions other than water irrigation during summer time, and have been harvested in the same period of the year (flowering season). Therefore, the minimal expected variation, both in composition and bioactivity, of SWE extracts from samples MR14–MR17, has been confirmed by the experimental results here obtained. Although further research would be necessary to stablish a reliable composition-bioactivity relationship for *M. rotundifolia* subcritical water extracts, the fact that rosmarinic acid has been found to significantly vary with the harvesting year seems to justify that the antioxidant activity of these extracts is not only due to this major phenolic, but to the synergic effect of different *M. rotundifolia* constituents present in these extracts.

Finally, Zeljkovic et al. [35] found that different *Mentha* species such as *M. longifolia*, *M. microphylla* and *M. x villosa*, which are cultivated for ornamental purposes, have beneficial properties for humans and could therefore be used in the food industry as *M. spicata*, *M. x piperita* and *M. arvensis*, already established for such uses. In this sense, and taking into account the results obtained in this work, *M. rotundifolia* could be considered a promising and scarcely investigated source of bioactives.

In conclusion, an efficient and environmentally friendly SWE method based on the use of subcritical water as extractant has been fully optimized for the first time to obtain extracts rich in bioactives from *M. rotundifolia* leaves. As supported by the results here obtained, the exploitation of these extracts as natural food ingredients, preservatives, etc. in the food field, among others, would be an application of this methodology worthy of exploration. Moreover, the conclusions here drawn on SWE extracts from samples collected in different annuities could also contribute to promote the benefits of cultivation of this endemic *Mentha* species as a new and rather stable source of bioactives.

Abbreviations

ABTS: 2,2'-azino-bis (3-ethylbenzthioazoline-6-sulfonic acid)

DPPH: 1,1-diphenyl-2-picrylhydrazyl

GAE: gallic acid equivalents

MAE: microwave-assisted extraction

MS: mass spectrometry

D: desirability function

s/*v*: sample to volume ratio

SLE: solid-liquid extraction

SWE: subcritical water extraction

TE: Trolox equivalents

TPC: total phenolic content

UAE: ultrasound-assisted extraction

Supplementary materials

The supplementary material for this article is available at: https://www.explorationpub.com/uploads/Article/file/10104_sup_1.pdf.

Declarations

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Author contributions

MJGS and PGI: Methodology, Formal analysis. ACS: Conceptualization, Supervision, Writing—review & editing, Funding acquisition. MLS: Conceptualization, Supervision, Writing—original draft.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent to publication

Not applicable.

Availability of data and materials

Not applicable.

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