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Supercritical antisolvent precipitation as a green technology to fractionate an *Origanum majorana* extract: Relationship between fractions cellular antioxidant activity and phytochemical composition.

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A B S T R A C T

The aim of this work was to determine the optimal conditions to fractionate a marjoram extract using supercritical antisolvent precipitation (SAS) technology in order to improve its antioxidant activity. This activity was evaluated using a cellular antioxidant assay (CAA) and compared to a chemical method (ABTS). After extract fractionation, most of the phenolics were recovered in the precipitate, except for the less polar ones that were found in the separators, along with essential oil components, non-volatile terpenes and fatty acids. Precipitate fractions obtained at 40 °C and 20–25 MPa showed the best TEAC values (1.7 mmol Trolox/g extract). Nevertheless, no precipitate fraction exerted better CAA than the unfractionated extract. This activity was related to the presence of arbutin and rosmarinic acid in their composition. In contrast, separators showed a low TEAC value (0.3 mmol Trolox/g), but a higher cellular antioxidant activity, especially fractions obtained at 15–20 MPa and 40–50 °C. This enhanced antioxidant activity was mainly attributed to the presence of ursolic acid, sterubin, menthol, sabinene hydrate and terpineol, compounds with a less polar character that therefore could cross more easily the cell membrane and exert their antioxidant activity inside the cells. Thus, separator fractions obtained at 20 MPa and 40 °C increased a 30% the CAA value respect to UAE extract and could be used for designing nutraceuticals or functional foods with cellular antioxidant activity. Precipitate fractions, at these extraction conditions, also showed an increase in TEAC value (40%) and could be employed as natural antioxidants in food formulations.

1. Introduction

During decades, plant extracts have been considered sources of natural antioxidants, as their bioactive compounds, mainly phenolics, have been reported as potent antioxidants. *In vitro* chemical assays have been widely used to predict their antioxidant activity, as these methods are simple, rapid, and low-cost. However, these antioxidant assays (such as ABTS, DPPH and FRAP, among others) do not account for physiological conditions and their utility in predicting the *in vivo* antioxidant activity has been questioned (Amorati & Valgimigli, 2015). Conversely, cell-based assays offer a biological approach, including aspects like the absorption, bioavailability, distribution, and metabolism of the antioxidants in living systems (Apak, 2019). In this regard, the most employed cell-based assay is the Cellular Antioxidant Activity (CAA), originally developed by Wang and Joseph (1999), and popularized by Wolfe and Liu (2007) using HepG2 (human hepatocarcinoma) cells. The use of a different cell line, Caco-2 (human colorectal adenocarcinoma) cells, for CAA assays has been proposed as a more representative method as they can retain cell functions in cell culture and can reflect the intestinal absorption characteristics of antioxidants (Kellet et al., 2018; Wan et al., 2015) Thus, CAA assays based on the use of Caco-2 cells have been recently described to evaluate the antioxidant activity of plant extracts (Gutierrez-Grijalva et al., 2019; Liao et al., 2020).

Marjoram (*Origanum majorana* L.) leaf extracts have been reported to contain phenolic acids and flavonoids, in particularly rosmarinic acid, apigenin and luteolin (Dahchour, 2022), along with an essential oil fraction, in which terpinen-4-ol stands out (Napoli et al., 2022). Thus, significant antioxidant activity has been attributed to these marjoram

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extracts (Khayal et al., 2022). Nevertheless, the antioxidant activity in all these studies was assessed using chemical assays, mainly ABTS and DPPH. To the best of our knowledge, few cell-based assays have been previously reported.

Since plant extracts are complex matrices, it is challenging to attribute their antioxidant activity to one type of compounds. In this regard, supercritical antisolvent precipitation (SAS) is a technique recently used to fractionate plant extracts, in order to obtain fractions with a high concentration of bioactive compounds with different polarities (Mur et al., 2021; Quintana et al., 2020). During the SAS technique, supercritical CO_2 ($SCCO_2$) is used as an antisolvent, which is sprayed in co-current with the plant extract (dissolved in a polar solvent). The most polar compounds present in the extract precipitate due to their insolubility in the $SCCO_2$ (forming the precipitate fraction), meanwhile the less polar ones remain dissolved and are recovered downstream by pressure reduction (separator fraction) (Langa et al., 2019). In this process, CO_2 creates an inert environment that reduced the degradation of bioactive compounds due to its low critical temperature (Ozkan et al., 2019).

In this regard, Villalva et al. (2019) employed this technique to produce a selective fractionation of phenolic compounds from an *Achillea millefolium* L. extract to improve its antioxidant activity. Antioxidant phenolics were recovered in the precipitation fraction when a pressure of 10 MPa at 40 °C was applied. Similarly, Gimenez-Rota et al. (2019) successfully employed SAS to obtain fractions with a higher antioxidant activity from an ethanolic extract of *Lavandula luisieri* (Rozeira), using a pressure of 13 MPa. Also, Quintana et al. (2019) fractionated a rosemary extract, obtaining separator fractions with a higher antioxidant activity than precipitate ones. These separator fractions were enriched in carnosic acid and carnosol. However, all these authors only determined the antioxidant activity of the obtained fractions by chemical methods.

In this context, the objective of the present work was the optimization of SAS technology to fractionate an ethanolic marjoram extract to improve its antioxidant activity. The antioxidant activity of the fractions was determined using a cellular antioxidant assay and a chemical method to study the differences between the antioxidant values obtained by the two methods. Besides, the fractions were analysed by HPLC-PAD and GC-MS in order to relate the antioxidant activity to the presence of specific compounds. For this purpose, standards mixtures mimicking the chemical compositions of fractions were employed to show the relation between antioxidant activity and chemical composition. It is worth to mention that scarce studies demonstrate this relationship.

2. Materials and methods

2.1. Reagents and chemicals

CO_2 (99.98% purity) was obtained from Carburos Metálicos (Madrid, Spain). Ethanol was from Panreac (Barcelona, Spain). Formic acid (purity ≥99%) and acetonitrile (HPLC grade) were purchased from Acros Organics (Madrid, Spain) and Macron Fine Chemicals (Madrid, Spain), respectively. Reference standards (purity ≥95%) such as squalene, rosmarinic acid, sucrose, quercetin, thymol, valencene, phytol, carvacrol, eriodyctiol, sterubin, naringenin, menthol, orientin, sabinene hydrate, linalool, β-caryophyllene and linoleic acid were acquired from Sigma-Aldrich (Madrid, Spain). Spathulenol, sabinene, ursolic acid, linolenic acid and β-sitosterol were from Cymit quimica (Barcelona, Spain). 4-terpineol, taxifolin and oleanolic acid were from Fluka (Charlotte, North Carolina, USA) and Biosynth (Staad SG, Switzerland), respectively. Apigenin 7-*O*-glucuronide, apigenin, lithospermic acid, vicenin II, salvianolic acid and luteolin were obtained from Phytolab (Madrid, Spain). Ethyl gallate, luteolin 7-*O*-glucoside and caffeic acid were from Extrasynthese S.A. (Genay, France). Finally, arbutin and luteolin 7-*O*-glucuronide were from TCI (Zwijndrecht, Belgium) and HWI Analytic GmbH (Rülzheim, Germany), respectively.

2.2. Ultrasound assisted (UAE) marjoram ethanolic extract

Egyptian *Origanum majorana* L. (marjoram) dried leaves were acquired in a specialized herbalist's shop (Murciana herboristeria, Murcia, Spain). Marjoram leaves were ground (Grindomix GM 200, Retsch, Llanera, Asturias, Spain) and sieved (BA200N, CISA, Barcelona, Spain), to achieve a particle size ranging from 500 to 250 μm. Then, an ultrasonic device (Branson Digital Sonifier 250, Danbury, Connecticut, USA) was employed to perform the ultrasound assisted extraction (UAE) as described in Siles-Sanchez et al. (2022). Briefly, the extraction solvent used was ethanol (1:10 plant/solvent ratio) keeping the temperature at ≤ 50 °C for 20 min. Finally, the ethanolic extract was concentrated by vacuum rotary evaporation (RV 10 control VWR, IKA, Staufen, Germany) until a final concentration of 17.8 mg/mL and stored at −20 °C until used in the SAS process.

2.3. Supercritical antisolvent precipitation (SAS)

SAS was conducted using the supercritical technology equipment Thar SF2000 (Thar Technology, Pittsburgh, PA, USA), following the methodology described in Quintana et al. (2020) with some modifications. Briefly, marjoram ethanolic solution (17.8 mg/mL) was fractionated using different pressures (10, 15, 20, 25 MPa) and temperatures (40 and 50 °C). First, $SCCO_2$ was pumped with a flow rate of 60 g/min until pressure and temperature conditions were reached in the precipitation chamber. Then, the extract was sprayed through the nozzle at 1 mL/min for 45 min, while maintaining the $SCCO_2$ flow rate. After mixing, the marjoram extract components that were not soluble in $SCCO_2$+ethanol mixture precipitated and were collected in the precipitation vessel (precipitate fraction). The components soluble in $SCCO_2$+ethanol were recovered in the separator vessel.

These samples were rotary evaporated until an oleoresin product was obtained (separator fraction). The SAS fractions (precipitates and separators) were stored at −20 °C in darkness until further analysis.

2.4. Chemical composition analysis

2.4.1. HPLC-PAD-ESI-QTOF-MS/MS analysis

Identification of the phenolic composition of the ethanolic extract was performed in an Agilent 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled to a Photodiode array detector (PAD) (G4212A, Agilent Technologies, Santa Clara, CA, USA) and in line with an ultra-high-resolution QTOF instrument (6540 UHD, Agilent Technologies, Santa Clara, CA, USA). The mass spectrometer operated in the negative ion mode using an ESI source (Agilent Jet Stream, AJS, Santa Clara, CA, USA). The parameters for MS analysis were capillary voltage 3000 V and in-source Collision Induced Dissociation energy (isCID) 20 and 40 eV for MS/MS spectra. Nitrogen was the gas employed as nebulizer. An ACE Excell 3 Super C18 column (150 mm × 4.6 mm, 3 μm particle size) protected by a guard column (10 mm × 3 mm) operating at 35 °C was used for chromatographic separation purpose. Water with 0.1% formic acid (v/v) and acetonitrile with 0.1% formic acid (v/v) were respectively employed as solvent A and solvent B, following the gradient conditions described in Villalva et al. (2018). Samples were dissolved in methanol, and 2 μL of the filtered sample (0.45 μm PVDF filter) was injected.

Phenolic compounds were tentatively identified based on their accurate masses, and by comparing their fragmentation mass spectra with the NIST MS Data library, and UV–Vis spectra (Table 1S, supplementary material).

2.4.2. HPLC-PAD analysis

Quantification of the main phenolic compounds was performed in a 1260 HPLC system (1260 Infinity series, Agilent Technologies, Santa

Table 1
In vitro antioxidant activity (TEAC value) and cellular antioxidant activity (CAA) of UAE extract and SAS fractions (precipitates and separators) at different conditions employed.

SAS Conditions		Precipitate (P)		Separator (S)	
Pressure (MPa)	Temperature (°C)	TEAC value (mmol Trolox/g extract)	CAA value (μmol QE/g extract)	TEAC value (mmol Trolox/g extract)	CAA value (μmol QE/g extract)
10	40	1.59 ± 0.04^{b}	138.20 ± 3.86^{f}	0.22 ± 0.00^{d}	224.12 ± 7.61^{d}
15	40	1.61 ± 0.03^{b}	220.90 ± 13.94^{bc}	0.33 ± 0.01^{c}	319.60 ± 12.03^{ab}
20	40	1.71 ± 0.03^{a}	242.79 ± 15.97^{a}	0.34 ± 0.00^{c}	308.82 ± 3.21^{b}
25	40	1.75 ± 0.03^{a}	210.52 ± 14.89^{c}	0.37 ± 0.01^{b}	257.74 ± 21.18^{c}
15	50	1.52 ± 0.02^{c}	182.69 ± 5.62^{d}	0.34 ± 0.01^{c}	323.23 ± 10.5^{ab}
20	50	1.49 ± 0.03^{c}	164.01 ± 9.49^{e}	0.37 ± 0.01^{b}	340.51 ± 17.55^{a}
UAE extract		1.24 ± 0.02^{d}	232.49 ± 14.53^{ab}	1.24 ± 0.02^{a}	232.49 ± 14.53^{d}

^{a,b,c,d} Different letters denote statistical differences within the same column, according to one-way ANOVA followed by Tukey test ($p < 0.05$).

Clara, CA, USA) equipped with a PAD detector (G1315C Agilent Technologies, Santa Clara, CA, USA). The chromatographic method and conditions are described in the previous section. Analytical standards were used to develop individual calibration curves, except for luteolin-hexoside-pentoside and 6-hydroxyluteolin-7-*O*-glucoside (where vicenin II and luteolin-7-*O*-glucoside standards were used, respectively), lithospermic acid isomer (lithospermic acid standard) and salvianolic acid isomer (salvianolic acid standard). Moreover, according to their UV–Vis spectrum, the hydroxy methoxy flavones were quantified using apigenin curve. Ethyl gallate was added as internal standard in each analysed sample. Samples were dissolved in DMSO (1.5 mg/mL), filtered (0.45 PVDF filter), and 20 μL of sample was injected.

2.4.3. Gas chromatography-mass spectrometry (GC-MS) analysis

Volatile components of marjoram extract and SAS fractions were analysed by GC-MS-FID in splitless mode. For that purpose, an Agilent 7890A system (Agilent technologies, Santa Clara, CA, USA) equipped with a split/splitless injector, FID detector and a mass spectrometer detector (5975C triple-axis) was use. The samples were dissolved in chloroform:methanol (2:1) and filtered (0.45 μm PVDF filter) before injection (2 μL). Identification of the different compounds was carried out following the column, method and specifications described in Quintana et al. (2019). Quantification of each compound was performed using the calibration curve corresponding to its analytical standard, except for γ-terpinene, terpinen-4-ol, p-Menth-2-en-1-ol, and γ-terpineol acetate where α-terpinene, α-terpineol, menthol, and α-terpineol were employed.

The characterization and quantification of non-volatile compounds (saccharides, amino acids, fatty acids, and other non-volatile compounds) contained in the ethanolic extract and SAS fractions was performed using an Agilent 7890A GC-MS-FID (Agilent Technologies, Santa Clara, CA, USA). In this regard, samples were first derivatized at 10 mg/mL in bis(trimethylsilyl) trifluoroacetamide (BSTFA) at 75 °C for 1 h, and the derivatized samples were then analysed by gas chromatography–mass spectrometry as described in Herrera et al. (2019). The identification of the compounds was carried out by comparing the mass spectra with the NIST MS Data library. For quantification, calibration curves were generated using analytical standards prepared under the same derivatization procedure as described for the ethanolic extract and SAS fractions. Specifically, saccharides were quantified with sucrose, monosaccharides with D-glucose, amino acids with lysine, monoglycerides with 1-oleoyl-rac-glycerol, fatty acids with linolenic acid, and sterols with β-sitosterol. Oleanolic acid, ursolic acid, and squalene were quantified using their respective specific standards.

2.5. Determination of antioxidant activity

2.5.1. Antioxidant radical scavenging activity (ABTS assay)

$ABTS^{\bullet+}$ radical scavenging assay proposed by Re et al. (1999) was followed to measure the UAE extract and SAS fractions *in vitro* antioxidant activity. Briefly, samples were dissolved in ethanol and sample concentrations ranging from 2 to 0.5 mg/mL were tested to achieve 20%–80% radical inhibition. Results were expressed as TEAC value (mmol Trolox equivalent/g dry sample).

2.5.2. Cellular antioxidant activity (CAA)

The cellular antioxidant activity of the UAE extract and the SAS fractions were carried out following the method described in Kellet et al. (2018). For this purpose, human colorectal adenocarcinoma cell line Caco-2 (ATCC, Manassas, VA, USA) were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine (Gibco, Paisley, UK), in 95% humidified air containing 5% CO_2 at 37 °C. Cells were seeded in 96 wells plates, keeping a density of 2×10^5 cells/mL.

The toxic effect of the UAE extract and SAS fractions were tested on Caco-2 cells following the MTT assay described by Mosmann (1983).

Cells were incubated with subtoxic concentrations of the extracts dissolved in non-supplemented media with 25 μM DCFH-DA (2',7'-dichlorofluorescein diacetate). The media was then removed, and the cells were washed with PBS (x3). Then, 600 μM of AAPH (2,2'-Azobis(2-methylpropionamidine) dihydrochloride) dissolved in Hanks' balanced salt solution (HBSS) was added to each well. Then, fluorescence was measured (Cytation 5, Agilent Biotek, Santa Clara, CA, USA) every 5 min for 1 h (13 cycles) at excitation/emission wavelengths of 485/538 nm. For quantification, areas under the curve (AUC) were used to plot a curve. Then, the fluorescence reduction was calculated for each concentration against the blank as follows:

$$Inhibition\ (\%) = \left(1 - \frac{AUCsample}{AUCblank}\right) * 100$$

Once the percentage of inhibition was determined, the equation was calculated, and 50% inhibition was set as the EC_{50} value. Results were expressed as μmol quercetin equivalent (QE) per g extract, whereas for standards they were expressed as μmol QE per 100 μmol standard.

2.5.3. Antioxidant activity of standards mixtures

With the aim of finding a relationship between the compounds and their antioxidant activity, standards of the most abundant compounds presented in the UAE extract and SAS fractions (P20 40 and S20 40) were mixed keeping their ratio in the samples. SAS fractions obtained at 20 MPa and 40 °C were chosen because they presented the best antioxidant activity and UAE extract was used as a reference sample.

In that regard, mixture 1 (M1) contained the following authentic phenolic compounds standards: arbutin, vicenin II, taxifolin, rosmarinic acid, lithospermic acid, luteolin 7-*O*-glucuronide and sterubin, (dissolved in DMSO) in the ratio presented in UAE extract or fraction P20 40 °C. Meanwhile mixture 2 (M2) contained naringenin, sterubin, sabinene hydrate, linalool, menthol, terpineol, carvacrol, β-caryophyllene, spathulenol, α-linoleic acid, linolenic acid, β-sitosterol, ursolic acid and oleanolic acid, in the same proportion found in the S20 40 °C fraction. Finally, mixture 3 (M3) contained the phenolic compounds included in M1 (arbutin, vicenin II, taxifolin, rosmarinic acid, lithospermic acid, luteolin 7-*O*-glucuronide and sterubin), together with naringenin, sabinene hydrate, linalool, menthol, terpineol, carvacrol, β-caryophyllene, spathulenol, α-linoleic acid, linolenic acid, β-sitosterol,

ursolic acid and oleanolic acid, in the ratio presented in the UAE extract.

2.6. Statistical analysis

The statistical analysis of the data was performed using IBM SPSS Statistics 26 (SPSS Inc., Chicago, IL, USA). Results were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Tukey test were performed. All analyses were performance by triplicate.

3. Results and discussion

3.1. Antioxidant activity of UAE extract and SAS fractions

The antioxidant activity of the UAE extract and its SAS fractions, obtained under different pressure and temperature conditions, was evaluated employing an *in vitro* chemical assay (ABTS) and a cell-based assay (CAA). The results are shown in Table 1.

The UAE extract exerted a TEAC value of 1.24 ± 0.02 mmol Trolox/g extract. This value was close to the one reported by Garcia-Risco et al. (2017) for a UAE marjoram extract (1.0 mmol Trolox/g extract). Besides, this extract showed high cellular antioxidant activity with a CAA value of 235.5 μmol QE/g extract. This value was significantly higher than the result reported for a marjoram PLE (pressurized liquid extraction) ethanolic extract (149.16 μmol quercetin/g extract) and for a rosemary PLE extract (195.91 μmol quercetin/g extract) (Villanueva--Bermejo et al., 2024).

Regarding the SAS precipitate fractions, the ABTS method (results expressed as TEAC value) showed that all precipitates presented better antioxidant activity than the UAE extract. Thus, at 40 °C, the antioxidant activity increased with pressure, up to 20 MPa. At 50 °C, no differences were observed when pressure increased from 15 to 20 MPa. Consequently, the precipitate fractions obtained using 20 and 25 MPa at 40 °C exerted the highest antioxidant activity (1.7 mmol Trolox/g extract), increasing the TEAC value of the UAE extract by around 40%. In contrast, when the CAA assay was employed, the results showed that only the precipitate fraction obtained at 40 °C and 20 MPa presented the same CAA value as the UAE extract, meanwhile the other precipitates showed a lower cellular antioxidant activity than the unfractionated extract.

Concerning the antioxidant activity of the separator fractions measured by the ABTS method, all fractions showed similar, and significantly lower activity than the UAE extract (around 0.3 mmol Trolox/g extract). However, when this activity was determined using the CAA method, several fractions presented higher antioxidant activity than UAE extract, highlighting the fractions obtained using 15–20 MPa at 40 and 50 °C.

As can be observed, the antioxidant activity reported for the UAE extract and the SAS fractions, using both methods, was quite different. This difference has been attributed to the fact that in cell-based assays the antioxidants must be able to cross the cell membrane to exert their antioxidant activity inside the cells (Dienaitè et al., 2018). Thus, less polar compounds would pass through the membrane more easily (Wang et al., 2021). In this context, the higher antioxidant activity found in separator fractions could be related to this fact, since due to the characteristics of the SAS technique, the most polar compounds were collected in the precipitate fraction, while those with a less polar character were recovered in the separator (Langa et al., 2019).

3.2. Phenolic compounds analysis of UAE extract and SAS fractions

Considering that the antioxidant activity of marjoram extracts has been often related to the presence of phenolic compounds (Bieżanowska-Kopeć & Piątkowska, 2022), the analysis of phenolics presented in UAE extract and SAS fractions was carried out to establish a relationship between its phenolic composition and the antioxidant activity found.

The identification of the phenolic compounds revealed a large variety of phenolics (Table 1S). After that, the main phenolic compounds were quantified in the UAE extract (Table 2), highlighting the content of arbutin and rosmarinic acid, and to a less extent lithospermic acid and vicenin II. These compounds represented an 86% of all the identified phenolics. Concerning the SAS fractions, most of the phenolic compounds from the UAE extract were recovered in precipitate fractions (Table 2), mainly those with a polar character. However, the less polar ones were obtained in the separator vessels (Table 3). These compounds distribution between the two SAS fractions (precipitates and separators) according to their polarity was related to the solubility of the compounds in the $SCCO_2$-ethanol mixture (Langa et al., 2019).

All precipitate fractions showed an enrichment in polar phenolic compounds (those from arbutin to luteolin) with respect to the UAE extract, although the fractions obtained at 40 °C and 20–25 MPa presented the highest quantity. These fractions were enriched (around 1.6 times) in arbutin, rosmarinic acid, vicenin II and lithospermic acid, representing around 93% of their phenolic content. However, separator fractions presented a small quantity of phenolics, mainly less polar phenolic compounds, highlighting the enrichment in methoxyflavones and sterubin. These results indicated that SAS was an adequate technique to obtain fractions enriched in marjoram polar phenolic compounds, with 40 °C and 20–25 MPa being the optimal working conditions. Other authors also employed SAS technique to obtain fractions enriched in phenolic compounds. Thus, Villalva et al. (2019) fractionated a yarrow extract employing SAS under similar conditions (15 MPa at 40 °C) allowing a 3.2-fold enrichment in phenolics like chlorogenic acid, vicenin II, 3,5-DCQA and 4,5-DCQA. In the same way, Sanchez-Camargo et al. (2016) fractionated a rosemary extract obtaining a precipitate enriched around 2.7-fold in rosmarinic acid at 30 MPa at 40 °C.

3.3. Relationship between antioxidant activity and phenolic composition

In order to establish a relationship between the identified phenolic compounds and their antioxidant activity, mixtures containing authentic standards of the most abundant phenolic compounds in the UAE extract and SAS fractions were prepared. Standards proportion in the samples were maintained in the mixtures and their *in vitro* antioxidant activity was evaluated by ABTS and CAA methods. Thus, the mixture 1 (M1) contained a mix of authentic standards present in the UAE extract (arbutin, rosmarinic acid, vicenin II, lithospermic acid, taxifolin, luteolin 7-*O*-glucuronide and sterubin), representing 91% of the total identified phenolics. The antioxidant activity of this mixture was shown in Fig. 1. As can be observed, the TEAC value obtained with this mixture (M1) was slightly lower (1.08 mmol Trolox/g extract) than the value obtained for the UAE extract (1.24 mmol Trolox/g extract). These results indicated that the antioxidant activity of the UAE extract could be mainly attributed to the phenolics included in the mixture, although not entirely. The difference between the two antioxidant activity values, could be due either to the 9% of phenolic compounds that were not included in M1 or non-phenolic compounds presented in the UAE extract. With respect to the minority of phenolic compounds not included in the mixture, luteolin and apigenin have been reported to present antioxidant activity (Ahmadi et al., 2020; Zhao et al., 2022). In addition, other compounds different from phenolics could contribute to the antioxidant activity, including essential oil components, non-volatiles terpenes and fatty acids (Kazemi Pordanjani et al., 2022; Wang et al., 2019).

The CAA value obtained for M1 also indicated a lower antioxidant activity than UAE extract. As little data were found in literature about the antioxidant activity of arbutin, rosmarinic acid, vicenin II, lithospermic acid, taxifolin, luteolin 7-*O*-glucuronide and sterubin employing a cell-based assay, their antioxidant activity using the CAA method was performed. The results (Table 4) also included a quercetin

Table 2
Phenolic composition of UAE extract and SAS precipitates obtained at different conditions (mg/g dry fraction). P10 40 (precipitate obtained at 10 MPa and 40 °C), P15 40 (precipitate at 15 MPa and 40 °C), P20 40 (precipitate at 20 MPa and 40 °C), P25 40 (precipitate at 25 MPa and 40 °C), P15 50 (precipitate at 15 MPa and 50 °C), P20 50 (precipitate at 20 MPa and 50 °C).

Compound	UAE extract	P10 40	P15 40	P20 40	P25 40	P15 50	P20 50
Arbutin*	78.10 ± 1.18^{e}	110.54 ± 0.01^{c}	111.78 ± 2.55^{c}	125.15 ± 4.10^{a}	121.85 ± 0.83^{a}	116.40 ± 1.18^{b}	106.93 ± 1.87^{d}
Luteolin hexoside-pentoside	0.20 ± 0.01^{c}	0.29 ± 0.02^{b}	0.33 ± 0.02^{ab}	0.33 ± 0.03^{ab}	0.34 ± 0.01^{a}	0.28 ± 0.05^{ab}	0.31 ± 0.02^{ab}
Vicenin II*	4.56 ± 0.10^{c}	6.17 ± 0.53^{ab}	6.68 ± 0.16^{ab}	7.06 ± 0.35^{a}	6.85 ± 0.03^{a}	6.28 ± 0.75^{ab}	6.34 ± 0.16^{b}
Caffeic acid*	0.47 ± 0.01^{b}	0.55 ± 0.04^{a}	0.49 ± 0.01^{b}	0.43 ± 0.02^{c}	0.40 ± 0.01^{c}	0.49 ± 0.05^{ab}	0.42 ± 0.01^{c}
Orientin*	0.42 ± 0.01^{c}	0.63 ± 0.07^{ab}	0.68 ± 0.02^{a}	0.71 ± 0.04^{a}	0.72 ± 0.02^{a}	0.63 ± 0.09^{ab}	0.62 ± 0.01^{b}
6-hydroxyluteolin-7-*O*-glucoside	0.76 ± 0.01^{d}	0.99 ± 0.07^{c}	1.04 ± 0.02^{b}	1.04 ± 0.04^{b}	1.11 ± 0.01^{a}	0.97 ± 0.14^{abc}	0.96 ± 0.01^{c}
Luteolin 7-*O*-glucoside*	1.06 ± 0.08^{c}	1.41 ± 0.15^{ab}	1.51 ± 0.04^{a}	1.57 ± 0.08^{a}	1.58 ± 0.01^{a}	1.39 ± 0.19^{ab}	1.38 ± 0.02^{b}
Luteolin 7-*O*-glucuronide*	3.27 ± 0.09^{a}	2.07 ± 0.20^{bc}	2.28 ± 0.07^{b}	1.84 ± 0.09^{c}	2.20 ± 0.02^{b}	2.13 ± 0.25^{bc}	2.25 ± 0.05^{b}
Taxifolin*	2.33 ± 0.03^{c}	3.63 ± 0.23^{ab}	3.77 ± 0.08^{a}	3.96 ± 0.21^{a}	3.84 ± 0.02^{a}	3.47 ± 0.45^{ab}	3.43 ± 0.11^{b}
Apigenin 7-*O*-glucuronide*	2.24 ± 0.04^{a}	1.97 ± 0.17^{a}	2.16 ± 0.05^{a}	1.97 ± 0.11^{a}	2.15 ± 0.02^{a}	2.00 ± 0.25^{a}	2.08 ± 0.05^{a}
Rosmarinic acid*	53.07 ± 0.70^{e}	73.49 ± 0.17^{d}	76.62 ± 2.71^{c}	87.42 ± 0.05^{a}	85.05 ± 0.12^{b}	76.81 ± 1.09^{c}	75.21 ± 0.74^{c}
Lithospermic acid isomer*	8.48 ± 0.10^{e}	8.97 ± 0.27^{d}	9.79 ± 0.07^{b}	9.44 ± 0.29^{bc}	10.10 ± 0.04^{a}	9.43 ± 0.07^{c}	9.59 ± 0.40^{bc}
Salvianolic acid isomer*	2.09 ± 0.08^{a}	2.09 ± 0.15^{a}	1.86 ± 0.17^{a}	1.84 ± 0.26^{a}	2.06 ± 0.22^{a}	1.96 ± 0.37^{a}	1.77 ± 0.35^{a}
Eriodyctiol*	1.26 ± 0.01^{d}	1.74 ± 0.08^{a}	1.70 ± 0.05^{a}	1.65 ± 0.03^{a}	1.52 ± 0.01^{b}	1.61 ± 0.12^{ab}	1.47 ± 0.02^{c}
Luteolin*	0.21 ± 0.00^{c}	0.27 ± 0.05^{ab}	0.27 ± 0.02^{ab}	0.30 ± 0.01^{a}	0.30 ± 0.01^{a}	0.26 ± 0.02^{b}	0.27 ± 0.00^{b}
Trihydroxy-methoxy flavone	1.78 ± 0.01^{a}	0.95 ± 0.06^{b}	0.28 ± 0.01^{c}	0.22 ± 0.03^{de}	0.17 ± 0.02^{d}	0.33 ± 0.05^{c}	0.22 ± 0.07^{cd}
Trihydroxy-dimethoxy flavone I	1.34 ± 0.02^{b}	1.77 ± 0.16^{a}	1.16 ± 0.03^{c}	0.77 ± 0.04^{d}	0.63 ± 0.01^{e}	1.11 ± 0.14^{c}	0.77 ± 0.12^{de}
Trihydroxy-dimethoxy flavone II	1.02 ± 0.02^{a}	0.37 ± 0.02^{b}	0.19 ± 0.01^{c}	0.13 ± 0.01^{de}	0.12 ± 0.00^{d}	0.21 ± 0.02^{c}	0.15 ± 0.03^{cd}
Naringenin*	0.34 ± 0.00^{b}	0.42 ± 0.05^{a}	0.30 ± 0.04^{b}	0.21 ± 0.01^{d}	0.15 ± 0.01^{e}	0.35 ± 0.05^{ab}	0.24 ± 0.01^{c}
Apigenin*	0.21 ± 0.01^{c}	0.28 ± 0.02^{a}	0.25 ± 0.01^{ab}	0.22 ± 0.02^{b}	0.20 ± 0.00^{c}	0.24 ± 0.03^{ab}	0.20 ± 0.00^{c}
Trihydroxy-trimethoxy flavone	1.29 ± 0.03^{a}	0.93 ± 0.11^{b}	0.33 ± 0.01^{c}	0.14 ± 0.01^{e}	0.10 ± 0.00^{f}	0.37 ± 0.05^{c}	0.26 ± 0.02^{d}
Sterubin*	3.34 ± 0.05^{a}	2.19 ± 0.17^{b}	0.62 ± 0.01^{d}	0.31 ± 0.01^{e}	0.23 ± 0.00^{f}	0.78 ± 0.04^{c}	0.48 ± 0.22^{d}
Σ Phenolic compounds	167.84 ± 2.89^{d}	221.35 ± 3.57^{bc}	223.90 ± 3.73^{b}	246.58 ± 6.40^{a}	241.55 ± 1.88^{a}	227.29 ± 5.60^{b}	215.20 ± 3.00^{c}

^{a,b,c,d,e,f} Different letters denote statistical differences within the same line, according to one-way ANOVA followed by Tukey test ($p < 0.05$). *Identified and quantified via comparison with its authentic standard.

Table 3
Phenolic composition of UAE extract and SAS separator fractions (mg/g dry fraction). S10 40 (separator obtained at 10 MPa and 40 °C), S15 40 (separator at 15 MPa and 40 °C), S20 40 (separator at 20 MPa and 40 °C), S25 40 (separator at 25 MPa and 40 °C), S15 50 (separator at 15 MPa and 50 °C), S20 50 (separator at 20 MPa and 50 °C).

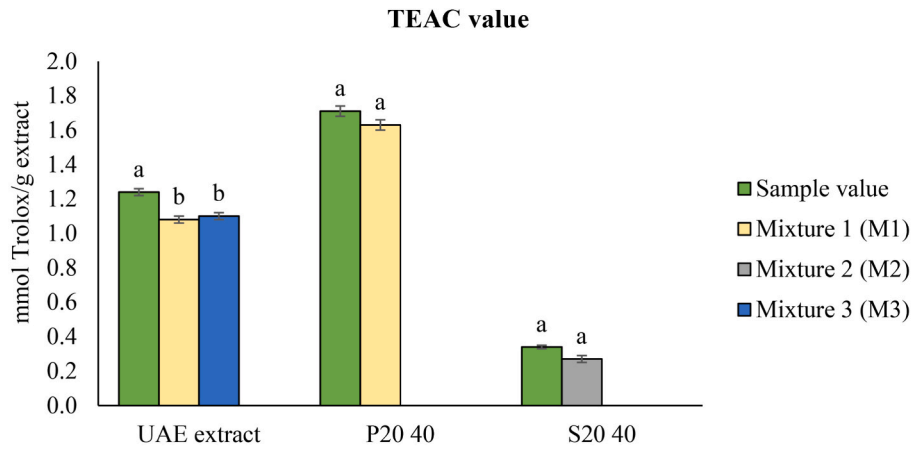
Compound	S10 40	S15 40	S20 40	S25 40	S15 50	S20 50
Arbutin*	Traces	Traces	0.06 ± 0.06^{c}	0.34 ± 0.18^{b}	Traces	0.67 ± 0.02^{a}
Caffeic acid*	0.30 ± 0.03^{d}	0.39 ± 0.03^{c}	0.48 ± 0.02^{b}	0.56 ± 0.00^{a}	0.33 ± 0.00^{d}	0.51 ± 0.02^{b}
Taxifolin*	0.33 ± 0.00^{e}	0.71 ± 0.03^{a}	0.43 ± 0.02^{d}	0.54 ± 0.01^{b}	0.33 ± 0.05^{f}	0.48 ± 0.02^{c}
Rosmarinic acid*	0.67 ± 0.03^{a}	0.66 ± 0.05^{a}	0.73 ± 0.03^{a}	0.75 ± 0.05^{a}	n.d.	0.70 ± 0.05^{a}
Eriodyctiol*	0.22 ± 0.02^{e}	0.33 ± 0.02^{d}	0.51 ± 0.02^{c}	0.74 ± 0.01^{a}	0.31 ± 0.00^{d}	0.61 ± 0.01^{b}
Trihydroxy-methoxy flavone	5.17 ± 0.13^{bcd}	5.08 ± 0.17^{bcd}	4.90 ± 0.21^{b}	5.25 ± 0.02^{b}	5.55 ± 0.01^{a}	5.14 ± 0.02^{c}
Trihydroxy-dimethoxy flavone I	0.70 ± 0.07^{e}	1.70 ± 0.08^{c}	2.38 ± 0.23^{d}	2.72 ± 0.01^{a}	1.48 ± 0.01^{d}	2.39 ± 0.01^{b}
Trihydroxy-dimethoxy flavone II	2.99 ± 0.32^{ab}	2.81 ± 0.07^{b}	2.64 ± 0.25^{b}	2.87 ± 0.02^{b}	3.03 ± 0.03^{a}	3.02 ± 0.03^{a}
Naringenin*	0.32 ± 0.03^{e}	0.51 ± 0.01^{d}	0.66 ± 0.04^{c}	0.81 ± 0.01^{a}	0.60 ± 0.02^{c}	0.75 ± 0.01^{b}
Apigenin*	0.14 ± 0.01^{d}	0.19 ± 0.05^{d}	0.33 ± 0.01^{c}	0.64 ± 0.02^{a}	0.52 ± 0.03^{b}	0.65 ± 0.05^{a}
Trihydroxy-trimethoxy flavone	4.20 ± 0.34^{c}	5.09 ± 0.13^{b}	5.05 ± 0.19^{ab}	5.29 ± 0.02^{a}	4.94 ± 0.02^{b}	5.33 ± 0.09^{a}
Sterubin*	7.55 ± 0.45^{d}	9.31 ± 0.15^{b}	9.63 ± 0.39^{ab}	10.00 ± 0.01^{a}	8.60 ± 0.00^{c}	9.92 ± 0.10^{a}
Σ Phenolic compounds	22.59 ± 2.15^{e}	26.78 ± 0.73^{c}	27.80 ± 0.53^{c}	30.51 ± 0.14^{a}	25.69 ± 0.22^{d}	30.17 ± 0.04^{b}

^{a,b,c,d,e} Different letters denote statistical differences within the same line, according to one-way ANOVA followed by Tukey test ($p < 0.05$). n.d.: non-detected. *Identified and quantified via comparison with its authentic standard.

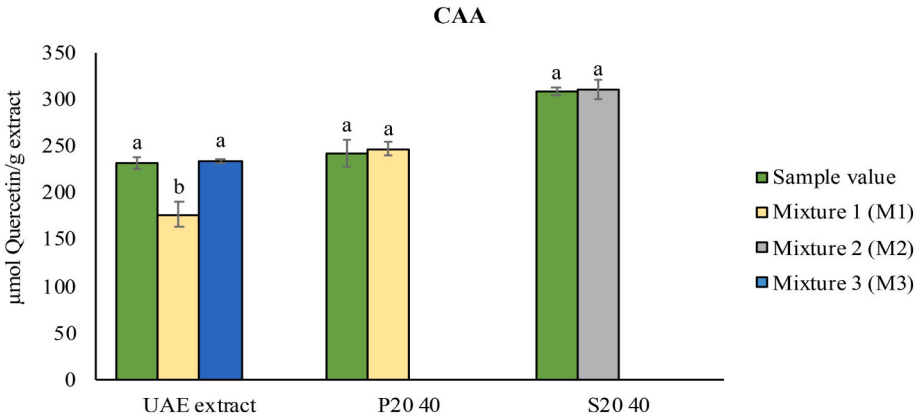
standard, since quercetin has been proposed as the reference compound to easily compare results (Martinelli et al., 2021). Rosmarinic acid (125.18 ± 7.64 μmol QE/100 μmol standard) showed a CAA values higher than the quercetin standard, while lithospermic acid (94.30 ± 7.25 μmol QE/100 μmol standard) did not present differences with quercetin. Thus, Gutierrez-Grijalva et al. (2019) also reported that 50 μg/mL of a rosmarinic acid standard presented a CAA value equal to quercetin. Luteolin 7-*O*-glucuronide, sterubin taxifolin, and, to a lesser extent arbutin also showed high CAA values, although lower than quercetin. Thus, the CAA of the UAE extract could be mainly attributed to rosmarinic acid and arbutin, since these compounds represented 78% of phenolics found in the extract, although the contribution of the other minority phenolic compounds (luteolin 7-*O*-glucuronide, sterubin and taxifolin) cannot be ruled out. In addition, other compounds in the UAE extract, different from phenolics, could also contribute to the CAA.

Regarding precipitate fractions, as the precipitate obtained at 20 MPa and 40 °C presented the highest antioxidant activity values employing both methods, it was chosen to simulate its phenolic composition. This mixture contained the same phenolics as the simulated UAE extract mixture (arbutin, rosmarinic acid, vicenin II, lithospermic acid, taxifolin, luteolin 7-*O*-glucuronide and sterubin), although in this case, the sum of these compounds represented around 95% of total phenolics. Data obtained (Fig. 1) showed that there were no significant differences in TEAC value measured from the standard mixture (1.63 ± 0.04 mmol Trolox/g extract) and precipitate fraction (1.71 ± 0.03 mmol Trolox/g extract). Thus, in this case the antioxidant activity of the precipitate fraction could be totally related to the phenolic compounds used in the mixture. Furthermore, applying the CAA method, no significant differences were found between the values of the mixture and the fraction either. As arbutin and rosmarinic acid were the main phenolics (86.2%) in the mixture employed, the cellular antioxidant activity of this extract could be mainly attributed to the presence of these compounds.

The separator fractions only presented a small quantity of phenolic compounds, mainly sterubin and a trihydroxy trimethoxy flavone. Although sterubin presented an important CAA value (Table 4), the antioxidant activity of these fractions could not be only attributed to



(A)



(B)

Fig. 1. UAE extract and SAS fractions (P20 40 and S20 40) *in vitro* antioxidant activity (TEAC value) (A) and cellular antioxidant activity (CAA) (B). Sample value corresponds to the UAE extract, P20 40 and S24 40, respectively. Mixture 1 (M1) includes arbutin, rosmarinic acid, vicenin II, lithospermic acid, taxifolin, luteolin 7-*O*-glucuronide and sterubin simulating the proportion in which they are found in the UAE extract and P20 40. Mixture 2 (M2) includes naringenin, sterubin, sabinene hydrate, linalool, menthol, terpineol, carvacrol, β-caryophyllene, spathulenol, α-linoleic acid, linolenic acid, β-sitosterol, ursolic acid and oleanolic acid simulating the proportion in which they are found S20 40. Mixture 3 (M3) contains the same amount of phenolic compounds as used in M1 (arbutin, rosmarinic acid, vicenin II, lithospermic acid, taxifolin, luteolin 7-*O*-glucuronide and sterubin), along to naringenin, sterubin, sabinene hydrate, linalool, menthol, terpineol, carvacrol, β-caryophyllene, spathulenol, α-linoleic acid, linolenic acid, β-sitosterol, ursolic acid and oleanolic acid simulating the proportion in which they are found in the UAE extract. Data shown represents mean ± S.D. (n = 3). $^{a\text{-}b}$ Different letters denote statistical differences at $p < 0.05$ between the UAE extract value and its mixtures (M1, 2 or 3).

these phenolics. Therefore, in order to establish a relationship between the composition of separator fractions and their antioxidant activity further analyses were performed.

3.4. GC-MS analysis of the UAE extract and separator fractions

As other compounds presented in the extract, different from phenolics, could contribute to the UAE extract and separator fractions' antioxidant activity, an exhaustive analysis of these samples was carried out by GC-MS. In a first step, the characterization of essential oil components was developed, followed by an analysis of non-volatile compounds as fatty acids, non-volatile terpenes and sterols.

3.4.1. Essential oil characterization

As can be observed in Table 5, a total of 17 compounds belonging to the volatile fraction of the essential oil were found in the UAE extract and the separator fractions. Regarding the UAE extract composition, the most abundant components were mainly monoterpenes and some sesquiterpenes, highlighting the content in terpinen-4-ol and sabinene hydrate, followed by α-terpineol. These essential oil components represented approximately a 74% of all the essential oil components identified. These results were in agreement with those obtained in a study carried out by Paudel et al. (2022), since they reported that terpineol and sabinene hydrate were the main constituents of a marjoram essential oil.

All separator fractions, regardless of the pressure and temperature

Table 4
Standards cellular antioxidant activity expressed as quercetin equivalents (μmol QE/100 μmol standard).

Type of compounds	Standard	μmol QE/100 μmol standard
Phenolic compounds	Quercetin	100.10 ± 1.00
	Arbutin	21.10 ± 7.27
	Vicenin II	6.27 ± 0.28
	Luteolin 7-*O*-glucuronide	70.10 ± 2.02
	Rosmarinic acid	125.18 ± 7.64
	Lithospermic acid	94.30 ± 7.25
	Taxifolin	42.39 ± 3.56
	Luteolin	185.54 ± 15.80
	Apigenin	12.06 ± 0.20
	Naringenin	14.41 ± 0.75
	Sterubin	62.09 ± 17.21
Essential oil components	Linalool	2.97 ± 0.01
	Spathulenol	4.49 ± 0.01
	α-terpineol	29.96 ± 1.52
	Sabinene hydrate	31.83 ± 3.03
	β-caryophyllene	8.84 ± 0.10
	Menthol	33.55 ± 0.65
Non-volatile terpenes	Ursolic acid	80.35 ± 2.30
	Oleanolic acid	24.29 ± 1.42
	Squalene	28.30 ± 2.74
Fatty acids	Linolenic acid	13.82 ± 1.00
	Linoleic acid	15.51 ± 0.80
Sterols	β-sitosterol	21.45 ± 0.20

conditions, showed an enrichment in essential oil components compared to the UAE extract. Among them, the one obtained at 40 °C and 10 MPa showed the highest quantity of essential oil components compared to other working conditions employed. However, regarding the main compounds (terpinen-4-ol, sabinene hydrate and α-terpineol) all the separator fractions contained a similar percentage of these compounds (around 63%).

3.4.2. Non-volatile fraction analysis

Table 6 shows the non-volatile fraction composition of the UAE extract and separator fractions. The UAE extract contained a large quantity of sugars (mainly monosaccharides and sucrose), fatty acids (α-linolenic acid), sterols (β-sitosterol), non-volatiles terpenes (oleanolic and ursolic acids), and a small amount of amino acids (pyroglutamic acid). Meanwhile, separators did not contain sugars, but the quantity of fatty acids, sterols and non-volatile terpenes was higher compared to the UAE extract. This composition was related to the higher solubility of these compounds in the $SCCO_2$+ethanol mixture. Regarding the fatty acids composition, the main one was α-linolenic acid, followed by linoleic and palmitic acids. The principal sterol was β-sitosterol and oleanolic and ursolic acids were the main non-volatile terpenes.

3.5. Relationship between antioxidant activity and separator fractions composition

As reported before, the relationship between the separator fraction components and their antioxidant activity was carried out using mixtures of authentic standards mimicking the composition of these fractions. The separator fraction obtained at 20 MPa and 40 °C, which presented one of the highest antioxidant activity values within the two methods, was chosen to carry out the standards mixture. Thus, the mixture 2 (M2) contained naringenin, sterubin, sabinene hydrate, linalool, menthol, terpineol, carvacrol, β-caryophyllene, spathulenol, α-linoleic acid, linolenic acid, β-sitosterol, ursolic acid and oleanolic acid, in the same proportion found in the fraction. Data obtained showed (Fig. 1) that the mixture presented the same TEAC value than the separator fraction. Regarding CAA, there were also no significant differences between the mixture (312.02 ± 10.60 μmol quercetin/g extract) and the fraction (308.82 ± 4.77 μmol quercetin/g extract). Thus, in this case the antioxidant activity of the separator fraction could be totally related to the compounds included in the mixture. Among all these components (Table 4), ursolic acid and sterubin presented the highest CAA, followed by menthol, sabinene hydrate and terpineol, although the rest of compounds included in the M2 also presented cellular antioxidant activity. The different antioxidant activity found in the separators fractions by the two methods employed highlights that the ABTS method could underestimate the antioxidant activity of plant extracts containing compounds with a less polar character.

As UAE extract also showed a small quantity of essential oil components, non-volatiles terpenes, sterols and fatty acids, a new mixture (M3) containing the same amount of phenolic compounds as M1 (arbutin, rosmarinic acid, vicenin II, lithospermic acid, taxifolin, luteolin 7-*O*-glucuronide and sterubin), along with naringenin, sterubin, sabinene hydrate, linalool, menthol, terpineol, carvacrol, β-caryophyllene, spathulenol, α-linoleic acid, linolenic acid, β-sitosterol, ursolic acid and oleanolic acid (in the ratio presented in UAE extract)

Table 5
Composition of identified volatile compounds by GC-MS in UAE extract and separator fractions (mg/g dry fraction). S10 40 (separator obtained at 10 MPa and 40 °C), S15 40 (separator at 15 MPa and 40 °C), S20 40 (separator at 20 MPa and 40 °C), S25 40 (separator at 25 MPa and 40 °C), S15 50 (separator at 15 MPa and 50 °C), S20 50 (separator at 20 MPa and 50 °C).

Compound	UAE extract	S10 40	S15 40	S20 40	S25 40	S15 50	S 20 50
Sabinene*	0.27 ± 0.05^{a}	0.16 ± 0.02^{b}	0.12 ± 0.01^{c}	0.09 ± 0.00^{d}	0.10 ± 0.01^{cd}	0.10 ± 0.01^{cd}	0.10 ± 0.01^{cd}
α -terpinene*	0.56 ± 0.01^{f}	2.19 ± 0.37^{a}	1.59 ± 0.03^{b}	1.27 ± 0.02^{de}	1.17 ± 0.10^{e}	1.48 ± 0.04^{c}	1.39 ± 0.10^{cd}
γ-terpinene	0.45 ± 0.02^{f}	1.07 ± 0.12^{a}	0.85 ± 0.01^{b}	0.70 ± 0.01^{c}	0.60 ± 0.01^{e}	0.71 ± 0.02^{c}	0.67 ± 0.01^{d}
Sabinene hydrate*	6.47 ± 0.08^{f}	30.68 ± 6.16^{a}	28.14 ± 1.78^{a}	16.12 ± 0.12^{e}	20.03 ± 0.36^{d}	22.95 ± 0.61^{b}	21.74 ± 0.54^{c}
Linalool*	1.36 ± 0.09^{d}	6.43 ± 0.10^{a}	3.00 ± 0.48^{b}	3.69 ± 0.20^{b}	1.50 ± 0.16^{c}	1.88 ± 0.23^{c}	1.62 ± 0.19^{c}
p-Menth-2-en-1-ol	1.77 ± 0.08^{e}	12.03 ± 2.48^{a}	9.66 ± 0.31^{a}	6.75 ± 0.10^{d}	6.64 ± 0.04^{d}	8.14 ± 0.28^{b}	7.51 ± 0.22^{c}
Terpinen-4-ol	11.91 ± 0.31^{f}	51.27 ± 6.18^{a}	32.74 ± 5.73^{b}	21.98 ± 0.14^{c}	14.55 ± 0.42^{e}	21.85 ± 1.44^{c}	18.48 ± 1.26^{d}
α -terpineol*	3.25 ± 0.19^{f}	8.95 ± 0.12^{a}	5.72 ± 0.11^{b}	8.74 ± 0.13^{a}	4.59 ± 0.01^{e}	5.39 ± 0.03^{c}	5.03 ± 0.15^{d}
γ-terpineol acetate	0.16 ± 0.00^{e}	1.46 ± 0.29^{a}	1.04 ± 0.04^{b}	0.26 ± 0.02^{d}	0.68 ± 0.06^{c}	0.81 ± 0.08^{c}	0.75 ± 0.05^{c}
Thymol*	0.15 ± 0.01^{e}	1.11 ± 0.27^{a}	0.75 ± 0.07^{b}	0.49 ± 0.02^{d}	0.51 ± 0.05^{d}	0.59 ± 0.00^{c}	0.54 ± 0.05^{cd}
Carvacrol*	0.91 ± 0.38^{e}	7.11 ± 1.33^{a}	4.51 ± 0.27^{b}	2.77 ± 0.01^{d}	2.76 ± 0.01^{d}	3.50 ± 0.20^{c}	2.81 ± 0.15^{d}
β -caryophyllene*	Traces	11.06 ± 2.76^{a}	5.93 ± 0.57^{b}	3.55 ± 0.01^{d}	3.35 ± 0.01^{e}	5.29 ± 0.17^{b}	4.29 ± 0.34^{c}
Valencene*	Traces	0.52 ± 0.00^{d}	0.90 ± 0.00^{a}	0.80 ± 0.01^{c}	0.85 ± 0.01^{b}	0.86 ± 0.03^{b}	0.84 ± 0.04^{bc}
Menthol*	0.26 ± 0.00^{c}	1.44 ± 0.21^{a}	1.01 ± 0.06^{b}	0.97 ± 0.04^{b}	0.91 ± 0.02^{b}	0.88 ± 0.05^{b}	0.88 ± 0.08^{b}
Spathulenol	1.67 ± 0.03^{e}	6.40 ± 1.15^{a}	4.73 ± 0.22^{b}	4.39 ± 0.05^{c}	4.09 ± 0.05^{d}	4.71 ± 0.13^{b}	4.32 ± 0.16^{c}
β-eudesmol*	Traces	2.20 ± 0.67^{a}	1.53 ± 0.08^{ab}	1.24 ± 0.22^{bc}	0.90 ± 0.00^{d}	1.27 ± 0.11^{c}	1.03 ± 0.20^{cd}
Phytol*	Traces	0.55 ± 0.24^{c}	0.41 ± 0.38^{c}	1.06 ± 0.10^{b}	1.65 ± 0.24^{a}	0.51 ± 0.00^{c}	1.48 ± 0.47^{ab}
Σ volatile compounds	29.17 ± 0.21^{f}	144.63 ± 13.68^{a}	102.63 ± 8.98^{b}	74.87 ± 0.11^{d}	64.08 ± 1.41^{e}	80.92 ± 3.15^{c}	73.48 ± 1.77^{d}

[a-f] Different letters denote statistical differences at $p < 0.05$. *Identified and quantified via comparison with its authentic standard.

Table 6
Characterization by GC-MS of non-volatile compounds in UAE and SAS separator fractions (mg/g sample). S10 40 (separator obtained at 10 MPa and 40 °C), S15 40 (separator at 15 MPa and 40 °C), S20 40 (separator at 20 MPa and 40 °C), S25 40 (separator at 25 MPa and 40 °C), S15 50 (separator at 15 MPa and 50 °C), S20 50 (separator at 20 MPa and 50 °C).

Compounds		UAE extract	S10 40	S15 40	S20 40	S25 40	S15 50	S20 50
Amino acid	Pyroglutamic acid	1.99 ± 0.20^{ab}	1.46 ± 0.10^{c}	1.87 ± 0.10^{bc}	2.14 ± 0.10^{a}	2.18 ± 0.10^{a}	1.56 ± 0.12^{c}	2.07 ± 0.10^{ab}
Sugars	Monosaccharides	121.83 ± 9.40	–	–	–	–	–	–
	Sucrose	98.36 ± 14.10	–	–	–	–	–	–
Fatty acids	Palmitic acid	6.75 ± 0.40^{c}	22.93 ± 0.30^{a}	19.14 ± 1.20^{b}	18.06 ± 0.70^{b}	17.65 ± 0.70^{b}	18.97 ± 1.30^{b}	17.40 ± 1.00^{b}
	Linoleic acid	8.52 ± 0.60^{d}	21.76 ± 2.00^{a}	18.79 ± 1.40^{abc}	18.71 ± 1.20^{abc}	18.14 ± 0.30^{b}	18.89 ± 1.50^{abc}	16.31 ± 0.90^{c}
	α-Linolenic acid	17.77 ± 1.00^{c}	45.69 ± 0.90^{a}	40.66 ± 2.80^{b}	42.98 ± 3.20^{ab}	41.68 ± 0.30^{b}	43.37 ± 3.30^{ab}	38.16 ± 3.40^{b}
	Stearic acid	2.90 ± 0.00^{c}	6.83 ± 0.20^{a}	5.79 ± 0.60^{b}	5.37 ± 0.30^{b}	5.46 ± 0.30^{b}	5.70 ± 0.40^{b}	5.43 ± 0.30^{b}
	Eicosanoic acid	–	3.10 ± 0.00^{a}	2.79 ± 0.20^{b}	2.53 ± 0.20^{b}	2.31 ± 0.00^{c}	2.66 ± 0.10^{b}	2.53 ± 0.10^{b}
	1-Monopalmitin	–	8.85 ± 0.10^{a}	8.31 ± 0.40^{ab}	8.32 ± 0.50^{ab}	7.59 ± 0.10^{c}	8.45 ± 0.50^{ab}	8.04 ± 0.30^{b}
	Monoglyceride n.i.	10.12 ± 1.20^{a}	7.21 ± 0.20^{b}	6.93 ± 0.10^{bc}	7.02 ± 0.30^{bc}	6.49 ± 0.10^{d}	7.10 ± 0.30^{bc}	6.74 ± 0.10^{c}
Sterols	Campesterol	–	7.01 ± 0.10^{a}	6.79 ± 0.23^{ab}	6.80 ± 0.20^{ab}	6.58 ± 0.10^{b}	7.01 ± 0.10^{a}	6.62 ± 0.10^{b}
	Stigmasterol	–	6.60 ± 0.10^{a}	6.42 ± 0.10^{ab}	6.23 ± 0.10^{bc}	6.22 ± 0.10^{bc}	6.44 ± 0.10^{ab}	6.24 ± 0.00^{c}
	β-Sitosterol	8.47 ± 0.10^{c}	14.47 ± 0.50^{a}	13.63 ± 1.50^{ab}	12.58 ± 1.70^{ab}	11.94 ± 1.44^{b}	13.87 ± 0.40^{ab}	12.11 ± 0.30^{b}
Non-volatil terpenes	Oleanolic acid	14.82 ± 0.10^{c}	12.18 ± 0.50^{d}	15.27 ± 0.50^{c}	16.93 ± 0.60^{ab}	16.07 ± 0.30^{b}	14.78 ± 0.10^{c}	18.38 ± 1.10^{a}
	Ursolic acid	17.73 ± 0.40^{a}	8.11 ± 0.40^{d}	9.25 ± 0.90^{cd}	13.54 ± 0.10^{b}	14.50 ± 1.40^{b}	9.40 ± 0.00^{c}	14.36 ± 2.00^{b}
	Squalene	–	8.53 ± 0.20^{c}	10.27 ± 0.90^{ab}	9.18 ± 0.90^{ab}	7.86 ± 0.20^{d}	9.76 ± 0.00^{a}	9.19 ± 0.30^{b}
	Pentacyclic triterpene n.i.	–	6.20 ± 0.00^{d}	6.94 ± 0.30^{b}	7.36 ± 0.20^{ab}	7.93 ± 0.70^{ab}	6.53 ± 0.10^{c}	8.00 ± 0.50^{a}

$^{a-d}$ Different letters denote statistical differences at $p < 0.05$ among the same line. n.i.: non-identified.

was also developed and its antioxidant activity was evaluated. As observed in Fig. 1, the TEAC value of the new mixture (M3) (1.14 ± 0.02 mmol Trolox/g extract) was very similar to that reported for the UAE extract (1.24 ± 0.02 mmol Trolox/g extract). Regarding CAA, there were no significant differences between the CAA value of the mixture M3 and UAE extract. Thus, the cellular antioxidant activity of the UAE extract was not only related to the phenolics content, but also to the other compounds presented in the extract, like essential oil components (linalool, menthol, terpineol and carvacrol), non-volatiles terpenes (ursolic and oleanolic acids), sterols (β-sitosterol) and fatty acids (α-linolenic and linolenic). Therefore, this CAA value could be mainly attributed to the presence of arbutin, rosmarinic acid, lithospermic acid, sterubin, sabinene hydrate, terpineol and ursolic acid, as they were the main compounds found in the UAE extract, and these pure standards exerted the highest CAA values.

4. Conclusions

The optimal working conditions to fractionate an UAE marjoram extract in order to obtain fractions with the highest antioxidant activity were 20 MPa and 40 °C. The precipitate fraction showed an increment in TEAC value (40%) meanwhile the CAA value did not presented differences with UAE extract. In contrast, the separator fraction showed a lower TEAC value but a higher CAA (30%) than UAE extract. The different results obtained by the two methods could be related to the presence of less polar compounds in the separator fraction. Thus, these compounds would pass through the cellular membrane more easily and exert the antioxidant activity inside the cells.

This work also allowed relating the antioxidant activity of the fractions to specific compounds. Thus, the chemical analysis of precipitate fraction allowed linking its antioxidant activity to polar phenolic compounds, mainly arbutin and rosmarinic acid. The separator activity was mostly related to the presence of ursolic acid and sterubin, and to a lesser extent to menthol, sabinene hydrate and terpineol. These results indicated that compounds, other than phenolics, could also contribute to the cellular antioxidant activity shown by plant extracts.

Consequently, the separator fraction would be used for designing nutraceuticals or functional foods with cellular antioxidant activity. Meanwhile, the precipitate fraction would be employed as a natural preservative with antioxidant function in food formulation.

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CRediT authorship contribution statement

María de las Nieves Siles-Sánchez: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Joaquin Navarro del Hierro:** Methodology. **Diana Martín:** Validation, Investigation. **Laura Jaime:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Susana Santoyo:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, 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

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.104103.

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