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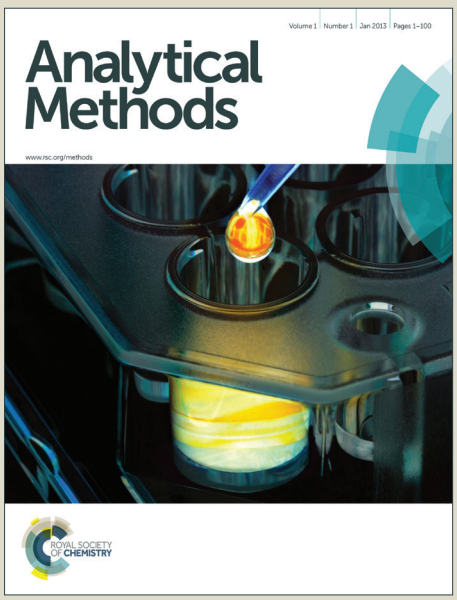

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ARTICLE

Fast analysis of phenolic terpenes by high-performance liquid chromatography using a fused-core column

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A fused-core column was used to develop a fast and efficient analytical method for separating phenolic terpenes by high-performance liquid chromatography. The main chromatographic parameters, such as composition of the mobile phase (mixtures of water and acetonitrile), flow rate of the mobile phase (1.0-2.5 mL/min), column temperature (30-55°C) and re-equilibration time after each injection (1-5 min) were studied and optimized during the development of the method. Using the current method, the major nonvolatile compounds from rosemary [rosmarinic acid (RA), rosmanol (RO), carnosol (CN), carnosic acid (CA) and methyl carnosate (MC)] could be separated in 4.7 min. The total time of analysis was 10 min, including the column cleanup and the re-equilibration period. The effect of the sample solvent was also studied. The combined influence of the injection volume and sample dilution on the performance of the chromatographic method was evaluated. The method was validated with several commercial samples, enabling the detection of low amounts (0.25 µg/mL) of CA and RA. The chromatographic profile showed excellent repeatability (intraday) and reproducibility (interday). Furthermore, the peak separation was good when using ethanol for sample dilution with respect to resolution (2.1, 3.7, 6.4, 10.6 and 21.7 for RO, MC, RA, CA and CN, respectively), selectivity (1.0 for MC; 1.1 for RO and CA; 1.3 for CN; and 1.5 for RA) and peak symmetry (1.0 for RA, CN and MC; 1.1 for CA; and 1.2 for RO).

1. Introduction

Phenolic terpenes are potentially useful in the pharmaceutical area for preventing diseases and are applied in the food and chemical industries as antioxidants. The rosemary extract is composed of two fractions. One of the fractions is the volatile oil composed mainly of terpenoids, while the other fraction consists of phenolic compounds, mainly the phenolic diterpenes carnosic acid, rosmarinic acid, carnosol and rosmanol (Fig. 1). The compounds presented in Fig. 1 are found mostly in rosemary and sage leaves (Fig. 2). The high antioxidant activity of carnosic acid (CA), for example, is leading researchers to study the potential of this bioactive compound as a natural antioxidant and as an agent against lipid oxidation in lamb meat,[1-3] chicken frankfurters,[4] buffalo meat patties and chicken patties.[5] Recent studies show that CA inhibits the oxidative damage in proteins caused by free radicals[6] and attenuates renal and liver injuries due to its antiapoptotic properties.[7, 8]

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Rosmarinic acid (RA) delays the motor dysfunction caused by progressive neurodegenerative diseases,[9] and rosmanol induces cellular apoptosis in human colon adenocarcinoma cells.[10]

Considering the wide range of applications of these bioactive substances, several methods for the separation and quantitation of nonvolatile rosemary compounds can be found in the scientific literature.[11-14] The main analytical methods use high-performance liquid chromatography (HPLC) with reverse phase columns packed with conventional totally porous particles (>5 µm). In this context, using columns with stationary phases containing small particles (<3 µm) can improve the performance of the column for separating the compounds and can reduce the time of analysis. However, using small particles implies a pressure increase, requiring systems able to support high pressures (>400 bar).

The constant need for upgrading the performance of stationary phases for liquid chromatography is promoting the development of technology in this area. One of the technologies showing great efficacy is related to the use of partially porous particles. Distinct from the totally porous particles used in conventional columns for liquid chromatography, the partially

porous particles (also known as fused-core, core-shell or porous-shell) are made from silica of a high purity containing a solid core covered with a porous thin layer.

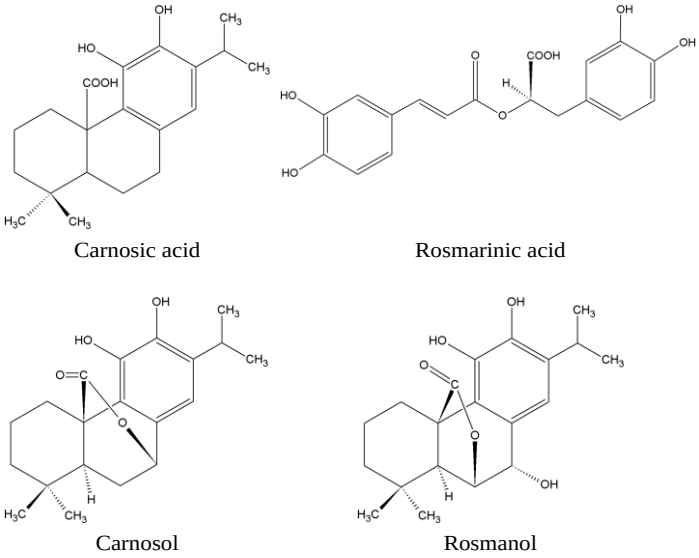

Fig. 1 Chemical structures of carnosic acid, rosmarinic acid, carnosol and rosmanol.

Fig. 2 Rosemary (left) and sage (right) leaves.

The stationary phases cited show lower eddy diffusion and more resistance to the mass transfer when compared to the stationary phases with smaller totally porous particles. Fused-core columns demonstrate higher performance due to the better distribution of particle size and the higher particle density. These aspects favor the production of efficiently packed beds. Due to these characteristics, fused-core columns exhibit higher efficiencies than columns packed with totally porous particles (comparison done at the same particle size).

This technology of stationary phases has already been used successfully for developing fast analytical methods (analysis times under 10 min) and for applying these methods to identify and quantify many compounds from several vegetal matrices, such as isoflavones from soybeans,[15, 16] phenolic compounds and alkaloids from tea, coffee, mate, energy and soft drinks,[17, 18] polyphenols from grape bagasse,[19] β-ecdysone from Brazilian ginseng (*Pfaffia glomerata*),[20] among others.

Despite the rather high importance of rosemary compounds and the advantages offered by these stationary phases (fused-core columns), no study of this application has been reported. Thus, the objective of this study is based on developing and validating a robust method for the fast analysis of nonvolatile compounds from rosemary (or other plants comprising the Lamiaceae botanical family) by high-performance liquid chromatography coupled to a photodiode array detector (HPLC-PDA) using a fused-core column.

2. Experimental

2.1. Chemicals and reagents

Reagents of HPLC grade used in this research were: acetic acid (Merck, Darmstadt, Germany), acetonitrile (Scharlab, Barcelona, Spain), methanol (Sigma Aldrich, São Paulo, Brazil) and ethanol (Chemco, Hortolândia, Brazil). Ultra-pure water was supplied by a Milli-Q Advantage water purifier system (Millipore, Bedford, USA). The reference standards used were carnosic acid ($C_{20}H_{28}O_4$ - CAS 3650-09-7 – Sigma Aldrich, St. Louis, USA) and rosmarinic acid ($C_{18}H_{16}O_8$ - CAS 20283-92-5 – Sigma Aldrich, St. Louis, USA).

2.2. Samples

Dried rosemary leaves were acquired in the Municipal Market of Campinas, Brazil. The leaves were comminuted in a knife mill (Marconi, MA-340, Piracicaba, Brazil) and stored in plastic bags at -18°C (HC-4, Metalfrio, São Paulo, Brazil) until production of the extract. The extracts from this raw material (process description in Section 2.2.1) were used for the development of the analytical method.

Five commercial samples of spices were analyzed for validation using the developed method. The samples consisted of (1) sage (*Salvia officinalis*) leaves, (2) rosemary (*Rosmarinus officinalis*) leaves, (3) a mixture of herbs (sage, oregano, thyme and rosemary), (4) a mixture for chimichurri sauce (onion, garlic, rosemary, sage and red pepper) and (5) oregano (*Origanum vulgare*) leaves.

2.2.1. Rosemary extracts. The rosemary extracts used to develop the analytical method were obtained using supercritical technology.

The extraction with supercritical CO_2 (SFE-CO_2) was performed in duplicate at 40°C and 30 MPa in a homemade multipurpose unit.[21] The extractors of 1 L each were filled with 475 g of comminuted rosemary, and the CO_2 (99% purity, Air Liquide, Campinas, Brazil) flow rate was maintained constant at 17.3 g/min for 6 h. The extract mass was determined using an analytical scale (Radwag, AS 200/C/2, Radom, Poland).

Another rosemary extract was obtained by low pressure solvent extraction (LPSE) in a soxhlet apparatus using ethanol as the solvent. Milled rosemary leaves (10 g) were wrapped in filter paper and placed in a soxhlet apparatus connected to a solvent flask with

0.3 L of ethanol. The system was refluxed for 6 h; then, the solvent was removed from the extracted mixture using a rotary vacuum evaporator at 40°C. The assays were performed in triplicate.

2.2.2. Extracts from commercial samples. The extracts were obtained by ultrasound-assisted solvent extraction in an ultrasonic bath (Unique, Indaiatuba Brazil) using ethanol as the solvent. The extraction conditions were: 50°C, ambient pressure (≈1 bar), ultrasound frequency of 40 kHz, and time of extraction equal to 6 h. The solvent mass to feed mass ratio (S/F) was equal to 20. The ethanolic extracts were filtered, and the solvent was removed from the extracted mixture using a rotary vacuum evaporator at 40°C. The assays were duplicated.

2.3. High-performance liquid chromatography (HPLC)

The chromatographic analyses were developed using the HPLC-PDA (Waters, Alliance E2695, Milford, USA) system, consisting of a separation module (2695) with integrated column heater and autosampler and a photodiode array (PDA) detector. Separation of compounds was carried out on a fused-core type column (Kinetex, C_{18}, 100 mm × 4.6 mm × 2.6 µm; Phenomenex, Torrance, USA). UV absorbance was monitored from 200 to 400 nm. The initial injection volume, the samples dilution and the solvents of the mobile phase are presented in the Table 1. Acetonitrile was selected as the solvent of mobile phase B because of its lower viscosity and lower backpressure generated compared to methanol.[20] Identification of CA and RA was achieved by comparing the retention times and UV spectra of the separated compounds with the authentic standards. Quantification was carried out by integrating the peak areas at 284 nm (carnosic acid) and 328 nm (rosmarinic acid) using the external standardization method. The standard curves of CA and RA were prepared by plotting each concentration (0.1, 0.25, 0.5, 1, 10, 50, 100, 125 and 380 mg/L) against the area of each relative peak. The assays were duplicated.

2.4. Ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS)

Rosmanol, carnosol and methyl carnosate were identified on a UPLC-MS (Waters, Milford, USA) system with a single quadrupole mass detector. A column composed of totally porous particles (Acquity, UPLC BEH C_{18}, 1.7 µm × 2.1 mm × 50 mm; Waters, Milford, USA) was used. Aiming to obtain the separation of the compounds, the mobile phase (eluents A and B) flow rate was 0.8 mL/min, and the column temperature was 55°C. The same gradient for peak separation applied in the HPLC-PDA system was used in the UPLC-MS system, but proportionally increasing the time of the gradient while decreasing the mobile phase flow rate. The compounds separated by negative ionization $[M-H]^-$ were evaluated by comparing the UV spectra with the mass to charge ratio (m/z) obtained through the Selected Ion Monitoring (SIM) technique. The mass spectrometric data were also monitored through the Total Ion Chromatograms (TICs).

3. Results and discussion

Some procedures were performed for developing and validating the analytical method. The method optimization integrated the execution of several tests with the goal of establishing the mobile phase (A and B) gradient, column temperature, mobile phase flow rate and re-equilibration time. The determination of the analytical properties comprised of injecting one sample 10 times during one day (intraday) and the same sample 10 times on another day (interday). The sample dilution was performed in three solvents: ethanol, methanol and acetonitrile. Each solvent was used at three different concentrations (70, 80 and 100%) in an aqueous mixture. The runs were performed in triplicate. Looking at the results, the robustness of the method was evaluated on combining five injection volumes (10, 20, 30, 40 and 50 µL) with five dilutions in ethanol: 1×, 2×, 3×, 4× and 5×, using one stock solution of 5 mg/mL; the runs were performed in triplicate. Afterwards, the standard curves were prepared by plotting the concentrations mentioned above against the areas of the peaks. Finally, the method validation comprised the injection of ethanolic solutions of 5 mg/mL obtained from five commercial samples. The responses obtained during the development and validation of the method were linked to the separation and quality of the target peaks by evaluating the parameters as follows: retention time, area, k prime, selectivity, resolution, symmetry factor and width @ baseline.

3.1. Selecting the conditions

The experimentation performed in this section comprised the selection of the conditions for chromatographic analysis. A sequence of trials using linear gradients of mobile phase B (acetonitrile with 0.1% (v/v) of acetic acid), requiring 20 min, was carried out. After the initial results were obtained and evaluated, another sequence of trials was performed to determine the method optimization. In this case, different non-linear gradients of the mobile phase were evaluated. Moreover, column temperature (30-55°C), mobile phase flow rate (1.0-2.5 mL/min) and re-equilibration period (1-5 min) were also studied. The maximum column operating temperature is 60°C because higher temperatures may significantly reduce the expected column life. The column temperature of 55°C was selected as the maximum working temperature.

Increasing the column temperature resulted in a mean reduction of the backpressure generated due to changes in the resistance of the mobile phase flow rate. There was therefore an increase in the mass transfer rates of the analytes between the mobile and stationary phases. Increasing temperature proportionated a mean reduction of the retention times of the analytes. For example, the retention time of CA was reduced in 6.9% when the temperature was increased from 30°C to 55°C, maintaining the same gradient of the mobile phase. Consequently, increasing the column temperature produced a narrowing of the peak widths, an increase in peak height and a better resolution for the compound separation.

The mobile phase flow rate was increased step-by-step from 1 mL/min to 2.5 mL/min. In this case, it was necessary to set the time of the separation gradient proportionally inverse to the increase of the mobile phase flow rate, aiming to accelerate the separation. For example, if the flow rate was doubled, the gradient time was reduced to half while maintaining the same percentage of solvents in the mobile phase (considering that the column maintained the same efficiency when the linear velocity of the mobile phase was larger). Maintaining the column at 30°C, the maximum system pressure was 181 bar and the retention time of CA was 11.29 min when using 1 mL/min. Maintaining the column at 55°C, the maximum system pressure was 118 bar and the retention time of CA was 10.51 min when using 1 mL/min. The retention time of CA was only 4.27 min when using 2.5 mL/min and 55°C. Consequently, the conditions described in the Table 1 were more suitable for this method, even though the maximum pressure reached in the system was 297 bar (below the pressure limitation of the system, which is 350 bar).

Increasing the mobile phase flow rate also allowed a reduction in the period necessary for re-equilibrating the initial conditions after each run because the mobile phase volume that passed through the column was increased. Re-equilibration time is necessary in gradient HPLC to ensure that the column environment has returned to the initial stable conditions. These conditions are particularly important when using gradient elution because the difference between the initial and final organic composition of the mobile phase is significant. Thus, we verified that 3 min was sufficient to reach the equilibrium (in pressure and gradient), which represented a solvent volume equivalent to 17.4 times the volume of the column. Doubling the mobile phase flow rate enabled the reduction of the time of the analysis by approximately 50%. Therefore, a shorter time of analysis (≈ 7 min) was obtained using the conditions described in Table 1. Such conditions provided better results relative to resolution in the separation of compounds.

Table 1 Conditions used in the HPLC-PDA system for the rosemary extract analyses

Parameter	Time (min)	Concentration of A (%, v/v)
Gradient	0	85
	1.5	75
	2.5	40
	3.5	40
Cleanup	5.0	10
Cleanup	6.0	10
Return to initial conditions	7.0	85
Equilibration period	10	85

Phase A	Ultra-pure water with 0.1% (v/v) of acetic acid
Phase B	Acetonitrile with 0.1% (v/v) of acetic acid
A + B flow rate	2.5 mL/min
Sample dilution	5 mg/mL
Injection volume	10 µL
Column temperature	55ºC

3.2. Characteristics of the method

Representative chromatograms of samples obtained using characteristic extractive methods (Soxhlet and SFE-CO_2) are shown in Fig. 3 and Fig. 4. The major compounds were identified through the retention times, maximum absorption wavelength and m/z ratio. Chromatograms in Fig. 3 and Fig. 4 were obtained from the wavelength of 328 nm and 284 nm, respectively.

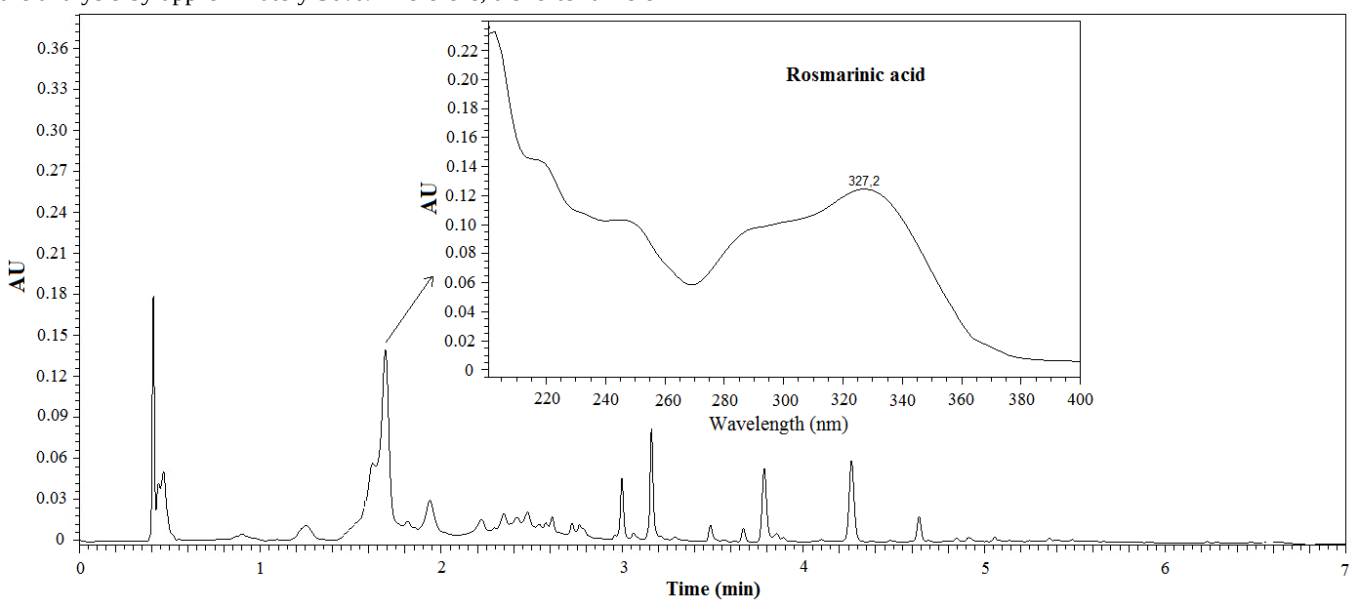

Fig. 3 Representative chromatogram of a sample (diluted to 5 mg/mL) obtained by conventional extraction (Soxhlet) using ethanol.

The method that had been developed was efficient for separating the major peaks of interest in this study. Many compounds were extracted from rosemary by SFE-CO_2, as seen in Fig. 4, of which the more pronounced were: RO, CN and its

isomer, CA and MC. The peaks were separated in less than 7 min of analysis. The values of selectivity were 1.04, 1.08, 1.14 and 1.31 for MC, RO, CA and CN, respectively. The values of symmetry factor were approximately 1.0 for CN and MC, 1.1 for CA and 1.2 for RO. The values of resolution were 2.1, 3.7, 10.6 and 21.7 for RO, MC, CA and CN, respectively.

RA, another phenolic diterpene found in rosemary and sage leaves, was not detected in samples obtained by SFE-CO_2. Nevertheless, this compound was identified and quantified in samples obtained with ethanol extraction in a Soxhlet apparatus, as shown in the chromatogram exhibited in Fig. 3. The explanation for this behavior is based on the hydrophobicity (polarity) of this compound because RA is composed of four –OH groups and one –COOH group (Fig. 1). Therefore, RA tends to be extracted by using a solvent with polar characteristics, instead of supercritical CO_2, which is a solvent with nonpolar characteristics. Because of this aspect, the retention time of RA is short (≈ 1.7 min), once RA tends to associate with the mobile phase composed mostly of A (ultra-pure water), that is, at the beginning of the analytical run.

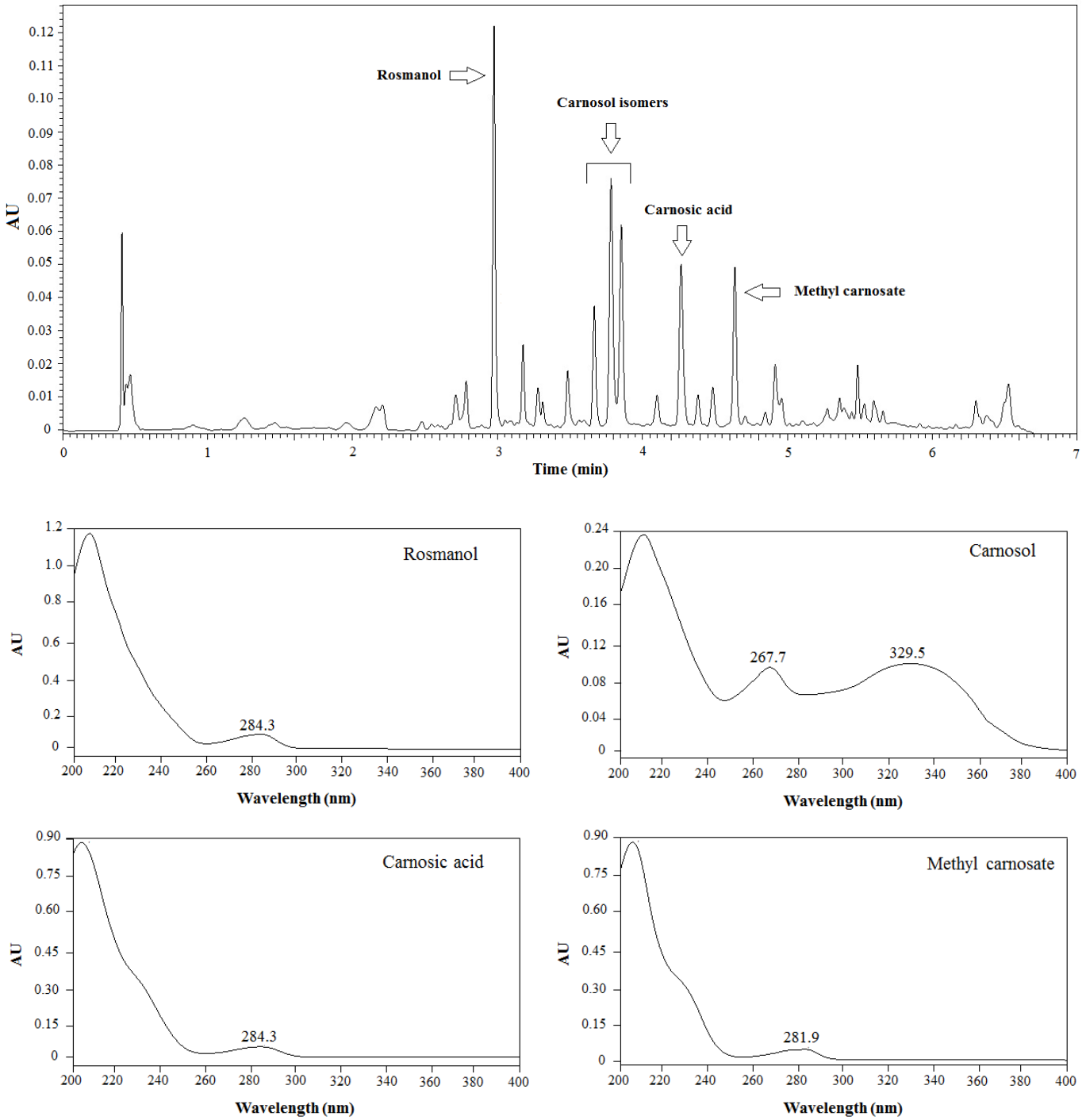

Fig. 4 Representative chromatogram of a sample (diluted to 5 mg/mL) obtained by SFE-CO_2.

3.3. Ultra-performance liquid chromatography coupled to mass spectrometry

The identity of the compounds found in the extract used for developing the method of fast analysis was initially proved based on the retention time and the absorption wavelength obtained in the UV spectra for the compounds for which the reference standards (RA and CA) were available, and also based on information found in the scientific literature. The identity of the compounds was confirmed by UPLC-MS. As previously mentioned, the main compounds identified in samples obtained by SFE-CO_2 were rosmanol, carnosol, CA and methyl carnosate. The relative intensities of the peaks were monitored by the scanning of the m/z ratio. The compounds cited and their spectra are displayed in Fig. 5 and Fig. 6.

Rosmanol, presenting a molar mass of 346.4 g/mol, was identified in m/z 345.41. Likewise, carnosol, presenting a molar mass of 330.4 g/mol, was identified in m/z 329.41. Such bioactive substances separated by negative ionization $[M-H]^-$ were evaluated through the UV spectra and the m/z ratio in mass spectrometry. Rosmanol and CA presented maximum absorption wavelength of 284 nm, while carnosol presented maximum absorption wavelength of 268/330 nm. These compounds detected in the rosemary extract were also identified by Borrás-Linares *et al*.[13]

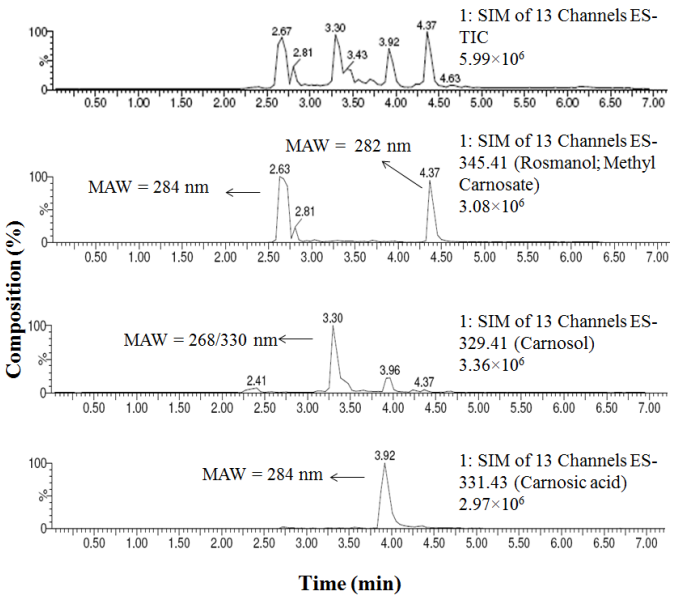

Fig. 5 Chromatograms obtained using UPLC-MS of a sample extracted by SFE-CO_2, SIM (Selected Ion Monitoring), TIC (Total Ion Chromatogram), ES^- (Negative Ionization), and MAW (Maximum Absorption Wavelength).

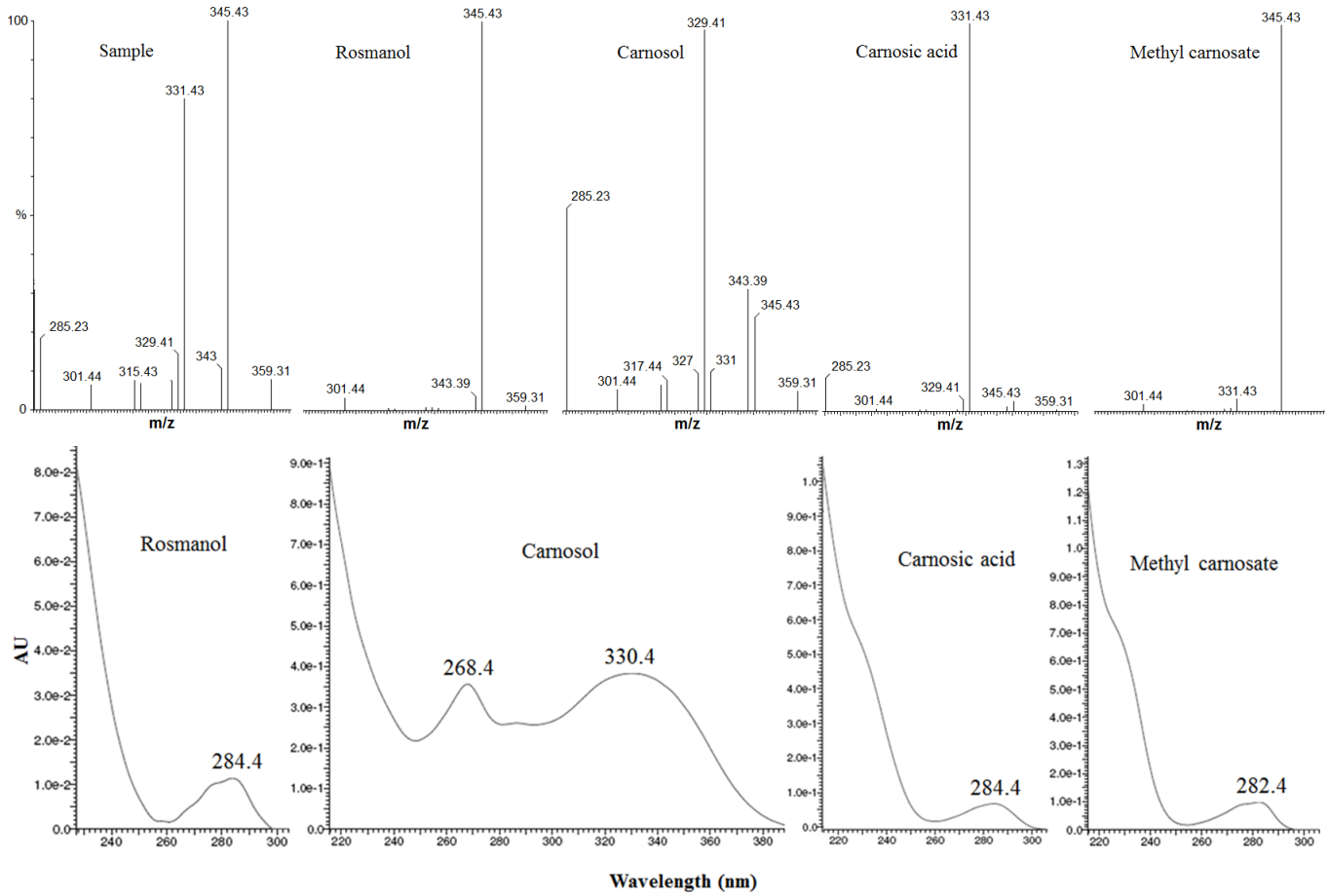

Fig. 6 Spectra and wavelength of rosemary compounds identified by UPLC-MS.

Methyl carnosate, presenting a molar mass of 346.2 g/mol, was also identified in m/z 345.41. Nevertheless, its differentiation from rosmanol is based on the differences in the retention time and spectra: 2.63 min/284 nm for rosmanol and 4.37 min/282 nm for methyl carnosate. These identifications were also obtained by evaluating the information available in the scientific literature, as well as the analyses of mass spectra performed by Señoráns *et al.*[12] and Mulinacci *et al.*.[14]

3.4. Repeatability and reproducibility

Repeatability and reproducibility of the chromatograms obtained using the developed method were studied with respect to the parameters that define the quality of the peaks, as expressed in Table 2. This step for validating the method was useful for showing its good applicability in identifying the five main compounds presented in this table.

In a general way, similar results were attained for all parameters of each identified phenolic diterpene, including the retention time (t_r) and area, for both intraday and interday injections. Low standard deviations (SDs) for most parameters were observed. In terms of coefficient of variation (SD/mean), values smaller than 0.6% and 0.2% were achieved for t_R and areas of the peaks, respectively. Likewise, the coefficients of variation for k prime and resolution were smaller than 0.9% and 0.4%, respectively. The coefficients of variation for the symmetry factor were also small for all compounds, reaching no more than 2.1%. Similar behavior was verified for the width @ baseline parameter.

Table 2 Repeatability and reproducibility of the responses of the developed method

Injections	Compound	t_R-RSD (%)	Area × 10^4 (µV×s)	*k* prime (-)	Selectivity (-)	Resolution (-)	Symmetry Factor (-)	Width @ Baseline × 10^2 (-)
Intraday (Repeatability)	Rosmarinic acid	0.2	22.8±0.4	3.46±0.01	1.50	6.4±0.1	0.97±0.02	9.5±0.4
	Rosmanol	0.3	14.9±0.1	6.88±0.02	1.08	2.1±0.1	1.17±0.01	8.3±0.5
	Carnosol	0.2	14.7±0.1	10.29±0.02	1.31	21.5±0.1	1.01±0.01	7.6±0.3
	Carnosic acid	0.2	7.5±0.1	3.46±0.03	1.14	10.6±0.1	1.09±0.01	9.5±0.4
	Methyl carnosate	0.3	6.5±0.1	11.24±0.04	1.04	3.6±0.1	1.00±0.01	8.0±0.3
Interday (Reproducibility)	Rosmarinic acid	0.3	23.2±0.2	3.45±0.01	1.50	6.3±0.1	0.98±0.01	10.2±0.9
	Rosmanol	0.2	14.5±0.3	6.84±0.02	1.08	2.06±0.02	1.20±0.01	8.6±0.2
	Carnosol	0.2	14.9±0.3	10.21±0.02	1.31	21.8±0.1	1.01±0.01	7.4±0.2
	Carnosic acid	0.1	7.7±0.1	3.45±0.03	1.14	10.57±0.03	1.11±0.01	8.8±0.5
	Methyl carnosate	0.2	6.3±0.1	11.22±0.03	1.04	3.7±0.1	1.01±0.01	7.3±0.4

± represents the standard deviation; t_R: retention time; RSD: relative standard deviation; (-), dimensionless.

3.5. Robustness: solvent for sample dilution

Fig. 7 and Table 3 show the results of the third step. Fig. 7 shows that different solvents and different concentrations of solvents (aqueous mixtures) influence the magnitude and quality of the peaks. In this figure, the same sample obtained by SFE-CO_2 was diluted to 5 mg/mL in each solvent.

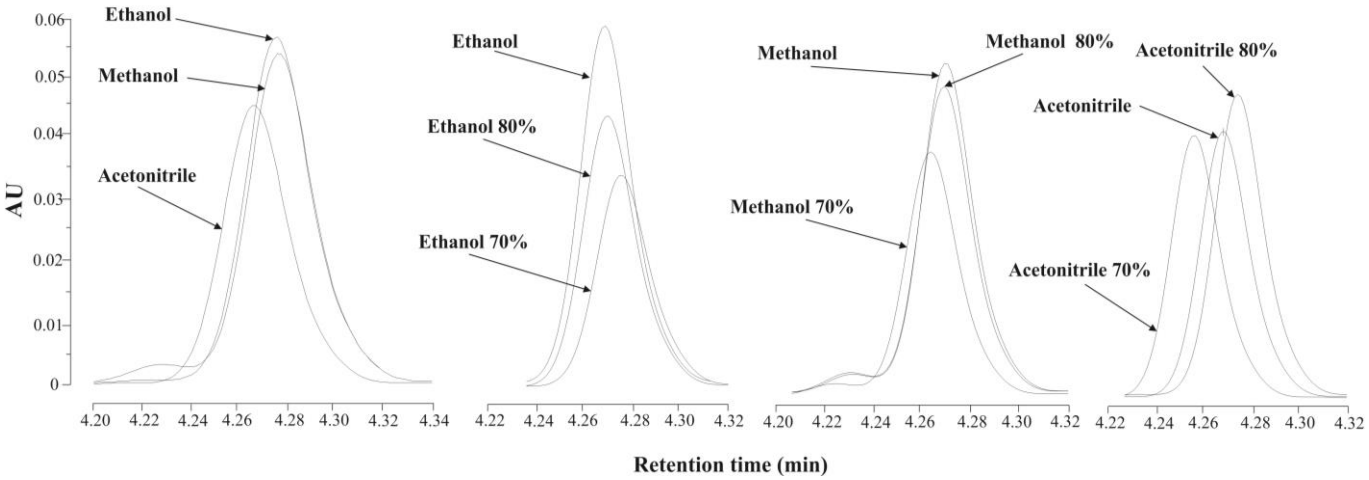

Fig. 7 Effect of the solvent on the intensity and resolution of the peak corresponding to carnosic acid.

Table 3 Influence of the sample solvent on the chromatographic performance of the current method

Solvent	Compound	t_R (min)	Area × 10^4 (μV×s)	*k* prime (-)	Selectivity (-)	Resolution (-)	Symmetry Factor (-)	Width @ Baseline × 10^2 (-)
EtOH	Rosmanol	2.98±0.01	16.0±0.1	6.84±0.02	1.08	5.71±0.04	1.17±0.01	9.0±0.3
	Carnosol	3.79±0.01	21.3±0.2	8.97±0.02	1.09	7.48±0.09	1.13±0.09	9.2±0.3
	Carnosic acid	4.27±0.01	9.1±0.1	10.24±0.02	1.14	3.52±0.03	1.14±0.01	8.8±0.6
	Methyl carnosate	4.64±0.01	7.2±0.1	11.20±0.01	1.04	3.58±0.02	1.00±0.01	7.3±0.2
80% EtOH	Rosmanol	2.99±0.01	16.1±0.1	6.86±0.02	1.08	5.95±0.03	1.18±0.01	9.1±0.5
	Carnosol	3.79±0.01	18.2±0.8	8.98±0.02	1.09	7.47±0.09	1.12±0.09	9.4±0.4
	Carnosic acid	4.28±0.01	6.3±0.7	10.26±0.03	1.14	3.49±0.01	1.15±0.01	8.8±0.4
	Methyl carnosate	4.64±0.01	6.6±0.2	11.21±0.03	1.04	3.59±0.03	1.00±0.01	7.8±0.3
70% EtOH	Rosmanol	2.99±0.01	14.4±0.1	6.86±0.02	1.08	5.98±0.04	1.18±0.01	9.2±0.2
	Carnosol	3.79±0.01	12.6±0.6	8.98±0.02	1.09	7.44±0.05	1.17±0.05	9.1±0.3
	Carnosic acid	4.28±0.01	5.9±0.5	10.27±0.02	1.14	3.50±0.02	1.15±0.01	9.0±0.1
	Methyl carnosate	4.65±0.01	5.9±0.2	11.25±0.01	1.04	3.57±0.03	0.99±0.01	7.2±0.4
MeOH	Rosmanol	2.99±0.01	16.0±0.1	6.84±0.01	1.08	6.01±0.02	1.19±0.01	9.1±0.2
	Carnosol	3.79±0.01	20.6±0.5	8.98±0.02	1.09	7.51±0.09	1.30±0.06	9.3±0.8
	Carnosic acid	4.28±0.01	9.1±0.2	10.26±0.02	1.14	3.53±0.02	0.84±0.01	12.2±0.3
	Methyl carnosate	4.64±0.01	7.1±0.1	11.21±0.04	1.04	3.56±0.03	1.00±0.02	7.3±0.5
80% MeOH	Rosmanol	2.99±0.01	15.2±0.1	6.86±0.02	1.08	6.04±0.02	1.19±0.01	8.9±0.3
	Carnosol	3.79±0.01	14.4±0.2	8.98±0.02	1.09	7.47±0.07	1.29±0.05	10.5±0.5
	Carnosic acid	4.28±0.01	8.6±0.2	10.26±0.03	1.14	3.55±0.02	0.82±0.01	12.5±0.5
	Methyl carnosate	4.64±0.01	6.5±0.3	11.21±0.04	1.04	3.59±0.02	0.99±0.03	7.5±0.6
70% MeOH	Rosmanol	2.98±0.01	15.4±0.1	6.85±0.02	1.08	6.06±0.02	1.18±0.01	9.3±0.4
	Carnosol	3.79±0.01	11.2±0.9	8.97±0.02	1.09	7.45±0.02	1.22±0.05	9.7±0.7
	Carnosic acid	4.27±0.01	6.6±0.1	10.24±0.03	1.14	3.56±0.01	0.93±0.01	11.6±0.2
	Methyl carnosate	4.63±0.01	5.2±0.4	11.19±0.02	1.04	3.57±0.01	1.00±0.02	7.5±0.6
ACN	Rosmanol	2.98±0.01	15.5±0.2	6.84±0.02	1.08	5.81±0.05	1.18±0.01	8.9±0.3
	Carnosol	3.79±0.01	25.2±0.8	8.96±0.03	1.09	7.59±0.07	1.21±0.03	9.0±0.5
	Carnosic acid	4.28±0.02	8.1±0.9	10.25±0.04	1.14	3.55±0.03	1.15±0.01	9.2±0.3
	Methyl carnosate	4.63±0.01	6.2±0.3	11.19±0.03	1.04	3.59±0.03	1.00±0.01	8.0±0.4
80% ACN	Rosmanol	2.99±0.01	15.4±0.1	6.86±0.01	1.08	5.96±0.04	1.19±0.01	9.6±0.3
	Carnosol	3.80±0.01	21.8±0.4	8.99±0.01	1.09	7.59±0.03	1.22±0.04	9.0±0.3
	Carnosic acid	4.28±0.01	7.5±1.6	10.27±0.01	1.14	3.52±0.02	1.15±0.01	8.9±0.3
	Methyl carnosate	4.65±0.01	6.6±0.2	11.24±0.02	1.04	3.57±0.01	0.98±0.01	7.2±0.4
70% ACN	Rosmanol	2.99±0.01	14.9±0.1	6.86±0.02	1.08	6.01±0.02	1.19±0.01	9.1±0.3
	Carnosol	3.79±0.01	18.3±0.3	8.98±0.03	1.09	7.55±0.04	1.24±0.03	9.3±0.3
	Carnosic acid	4.27±0.01	6.8±0.2	10.24±0.01	1.14	3.53±0.03	1.15±0.01	8.8±0.2
	Methyl carnosate	4.64±0.01	6.2±0.4	11.22±0.03	1.04	3.57±0.02	0.99±0.02	7.3±0.4

± represents the standard deviation; t_R: retention time; (-), dimensionless; EtOH: ethanol; MeOH: methanol; ACN: acetonitrile.

A small deformation at the beginning of the peaks, known as fronting, was identified when methanol was used, mostly pure methanol. Using different concentrations of acetonitrile, a reduction of the intensity of the peaks and a slightly variation in retention times (≈ 0.01 min) was observed. However, the solubility of CA in the solvent is decreased when the concentration of water is raised in mixtures of ethanol and water, resulting in a smaller area and lowered height of the peaks. Thus, pure ethanol is appropriate to be used as the solvent for analyzing CA in extracts. For example, the areas of the peaks for RO, MC, CA and CN were 15, 22, 54 and 69% higher than the areas obtained using ethanol 70%, respectively.

The extracts diluted in each solvent were analyzed using the current chromatographic method, aiming to understand whether the solvent for sample dilution influences the chromatographic separation and quality of the major peaks using the fused-core column. In addition to the evaluation of the results presented in Fig. 7, such an influence was also analyzed relative to the parameters presented in Table 3, through the quantitative description of the method efficiency.

According to the results shown in Table 3, no important differences (considering the standard deviation) were observed for the selectivity of the main compounds (rosmanol, carnosol, CA and methyl carnosate), using all the solvents tested in this study. Furthermore, no significant differences have been verified for the parameters retention time and k prime. The areas of the peaks were influenced by the solvent used for sample dilution. Larger signals from CA were achieved using pure ethanol or pure methanol. Higher areas of peaks corresponding to rosmanol were obtained by diluting the samples in ethanol, ethanol 80% or methanol. In the same way, higher areas of peaks corresponding to carnosol were obtained by diluting the samples in acetonitrile, acetonitrile 80%, ethanol, methanol or ethanol 80%. There were also differences for the symmetry factor of CA and width @ baseline of carnosol and CA, mainly when the solvent used for sample dilution is the methanol (Fig. 7). Nevertheless, both solvents are properly indicated when the resolution of the peaks is the parameter of most interest. Therefore, based on the results shown in Table 3 and Fig. 7, pure ethanol was selected as the solvent for sample dilution for the next steps of validating the analytical method.

3.6. Robustness of the method: sample concentration/dilution and injection volume

This section aims to present and to discuss the results linked to the step for determining the analytical properties of the current method, which consisted of combining five injection volumes (10, 20, 30, 40 and 50 µL) with five dilutions in ethanol: 1×, 2×, 3×, 4× and 5×, using the reference extract solution of 5 mg/mL. The robustness of the chromatographic method was evaluated by analyzing the results, which some of them are summarized in Table 4. The goal was to determine the constancy of the results when the conditions inherent to the method were deliberately varied.

Regarding the t_R of rosmanol, no significant differences were observed among all combinations tested in this step (p-value<0.05). Small differences in the t_R of CN (3.78±0.01 to 3.81±0.01 min) and CA (4.27±0.01 to 4.31±0.02 min) were observed, meaning a variation smaller than 2 s in the times corresponding to the maximum height of the peaks. A minimum tendency of increasing the t_R of these compounds was perceived when the samples were diluted to lower concentrations, it means the dilutions of 4× and 5× using the reference extract solution. However, the responses relative to the areas of the peaks were shown to be proportional to the dilutions and injected volumes. These results revealed a satisfactory application of the current method for several different conditions (except for the sample dilution of 5×, wherein poor peak shapes were seen, not enabling their integration).

In a general way, no pronounced differences were noted for k prime (capacity factor) and selectivity. Nevertheless, better resolution was observed when using lower injection volumes, independent of the dilution factor. For example, the resolution of the peak of CN was increased from 16.4 to 21.5 when reducing the injection volume from 50 µL to 10 µL. Likewise, the resolution of the peak of CA was increased from 8.3 to 10.5 when reducing the injection volume from 40 µL to 10 µL. The higher injection volumes (consequently higher solvent volumes) interfere with the quality of the peaks, causing band broadening. The higher amount of solvent plus analytes inside the small column might be diminishing its capacity of separating the peaks. In this case, different responses in the symmetry factor of each peak can also appear. Notwithstanding, all resolutions of the peaks were good.

Skoog *et al*.[22] suggest that the transference from one phase to the other requires energy, and the molecule must acquire this energy from its surroundings. Thus, the residence time in a given phase may be transitory after some transfers and relatively long after others. As a consequence, certain particles of a same substance flow rapidly due to their accidental inclusion in the mobile phase, whereas others flow slowly due to their association with the stationary phase.

Table 4 Parameters evaluated with relationship to the robustness of the method.

Compound	*k* prime (-)	Selectivity (-)	Resolution (-)	Symmetry Factor (-)	Width @ Baseline × 10^2 (-)
Rosmanol	6.84 – 6.90	1.08	1.7 – 2.0	0.6 – 1.2	7.1 – 20.6
Carnosol	8.96 – 9.04	1.31	16.4 – 21.5	0.7 – 1.0	7.5 – 12.7
Carnosic acid	10.22 – 10.33	1.14	8.3 – 10.5	0.7 – 1.1	8.2 – 15.1
Methyl carnosate	11.19 – 11.30	1.04	2.8 – 3.6	0.7 – 1.0	7.2 – 12.0

(-), dimensionless.

In this sense, these individual movements can lead to symmetric or asymmetric distribution of each peak, known as tailing or fronting. With respect to this study, increasing the injection volume from 10 µL to 50 µL caused a change in the symmetry of CN from 0.99 to 0.78, remembering that a symmetric peak presents symmetry of 1. Otherwise, the symmetries of peaks corresponding to CN and MC were seen to keep constant (0.99±0.01 to 1.01±0.01 and 0.99±0.01 to 1.00±0.03, respectively), for the same injection volume (10 µL).

The robustness of the proposed method was then accomplished. This study demonstrated that slight variations appeared when using different concentrations of the sample in the solvent for the same injection volume. Good robustness was shown by the nearly unchanged values of the parameters. It was found low relative standard deviation (RSD) values of the areas of the peaks along the triplicates. All of RSD values were lower than 5.3% for all compounds identified and presented in this paper. The method was considered robust because it was not much sensitive to variations in the experimental conditions.

In fact, the range of width @ baseline and symmetry factors was shown to be a function of the injection volume, as discussed before. Most of the values of theses parameters were near to 7.0 and to 1.0, respectively, when injecting 10 µL.

We therefore infer that several sample concentrations may be used by applying the developed method because the quantitative results were satisfactory. Aiming to avoid poor peak shapes, low injection volumes are indicated. These volumes need to be generally lower than 30 µL, because good resolution of the peaks was achieved using those conditions, mainly with 10 µL.

3.7. Comparison with other methods

There are numerous studies where nonvolatile compounds from rosemary are analyzed by HPLC using UV-Vis detection. Most of them use C_{18} columns and have a longer time of analysis.

One example is the study performed by Vicente *et al.*,[23] where the total time of analysis was 45 min using mixtures of acetonitrile and water as the mobile phase. Zhang *et al.*[24] applied another method for analyzing rosemary extract and identified the main compounds in times up to 20 min. Satisfactory separation of the major phenolic diterpenes found in rosemary was reached using mixtures of acetonitrile and water as the mobile phase, but in 58 min of total time of analysis.[14]

Couto *et al.*[25] developed a method for separating RA in 11.5±0.2 min. However, the chromatographic profile was regular, presenting a poor peak resolution. Thus, the greater advantage of this developed and validated method is its simplicity, robustness, reliability and shorter time of analysis. Combining fused-core columns with high temperatures and mobile phase flow rates is useful, because it affords higher sample processing capability compared to the previous cited methods. Furthermore, the fused-core technology represents a step forward in the available analytical methodology. The strategy employed resulted in a shortening of the time of analysis with high resolution of the chromatographic peaks corresponding to the phenolic terpenes.

3.8. Standard curves

The experiments performed in this section were focused on elaborating the standard curves, in duplicate, using the external standards of CA and RA. Nine concentrations were prepared in pure ethanol: 380, 125, 100, 50, 10, 1, 0.5, 0.25 and 0.1 mg/L.

An excellent correlation between the area of the peak and the concentration of the external standard was observed. The values of the coefficients of correlation (R^2) were 0.9999 and 0.9998 throughout the concentration range of CA and RA, respectively. The values of R^2 were also very good when we analyze only the three lower concentrations wherein the peaks were detected (0.25, 0.5 and 1 mg/L). These values of R^2 were 0.9959 and 0.9995 by fitting the area of each compound (CA and RA) with their corresponding concentration, respectively.

The order of magnitude of the areas equivalent to both compounds is different. For the same concentration, the area of the peak corresponding to RA is larger than the area of the peak corresponding to CA. The area of CA was 89×10^3 µV×s (Fig. 4), that is, the concentration of 60.4 mg/L. In the same context, the area of RA was 228×10^3 µV×s (Fig. 3), that is, a concentration of 48.7 mg/L. Thus, the method developed and validated in this study can be used with accuracy for detecting low concentrations of CA and RA and can be applied to different samples. As we have seen, it is possible to identify these compounds diluted to 0.25 mg/L.

The concentrations of the analytes showing signal-to-noise ratios 3:1 and 10:1 were considered as the limits of detection (LOD) and limits of quantification (LOQ). The values of LOD and LOQ were 0.25 µg/mL and 1 µg/mL, respectively, for both compounds: CA and RA.

3.9. Application the developed method to different samples

The current method was applied to the analysis of five different samples (rosemary, sage, the mixture for chimichurri sauce and a mixture of herbs). The extracts were obtained with ethanol, according to the procedure described in Section 2.2.1.

Fig. 8 presents the chromatograms of these samples. RA was identified in all samples, while CA, carnosol and rosmanol were identified in rosemary, sage, the mixture for chimichurri sauce and the mixture of herbs. The low intensity of the peak corresponding to RA is a consequence of the wavelength of chromatograms shown in Fig. 10. Both chromatograms were taken at 284 nm, while the maximum absorption wavelength of RA is 328 nm.

Table 5 exhibits the content of CA, RA, rosmanol (RO), carnosol (CN) and methyl carnosate (MC) in the five analyzed samples. The results note that the developed method is reliable and efficient for the analysis of phenolic diterpenes in vegetal matrices.

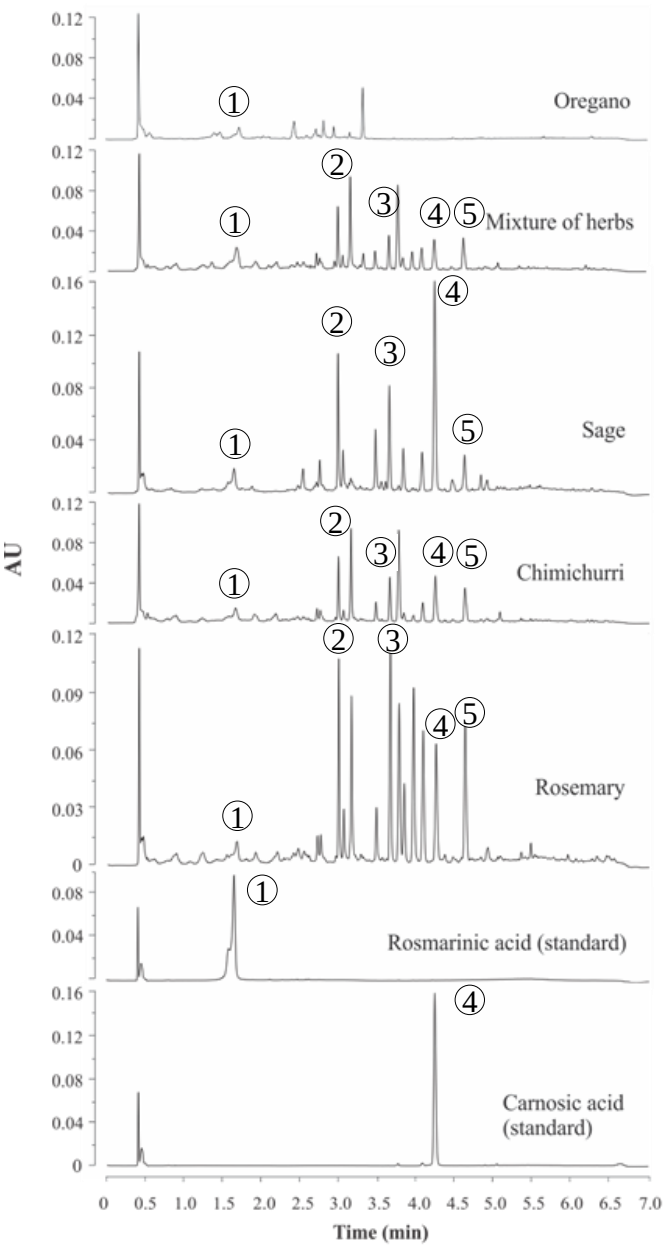

Fig. 9 Application of the developed method to different samples. 1: rosmarinic acid; 2: rosmanol; 3: carnosol; 4: carnosic acid; 5: methyl carnosate.

Table 5 Concentrations (mg/100 g of extract) of CA, RA, RO, CN and MC in different samples

Sample	CA	RA	RO**	CN**	MC**
Rosemary	14.9±0.4	3.3±0.1	15.3±0.5	17.5±0.6	19.0±0.3
Sage	33.4±0.1	2.0±0.1	10.8±0.2	9.8±0.3	5.7±0.1
*Chimichurri**	11.7±0.4	0.7±0.1	1.6±0.1	7.9±0.2	7.6±0.2
Mixture of herbs	7.9±0.4	1.4±0.1	1.9±0.2	7.0±0.2	6.8±0.1
Oregano	-	3.5±0.1	-	-	-

* commercial name; ** mg of CA equivalent/100 g of extract.

4. Conclusions

Separation of phenolic terpenes was attained in 4.7 min (time of each run equal to 7 min) using a fused-core column. It was possible by applying a systematic strategy of optimizing the chromatographic parameters (gradient and flow rate of the mobile phase, column temperature and re-equilibration time between the analytical runs) aiming to develop a simple, selective, reliable and robust method for fast analysis of phenolic terpenes. The total time of analysis was only 10 min, including a re-equilibration period. The method was validated and was found to be efficient with respect to its robustness, accuracy, repeatability, reproducibility, analyte detection levels and applicability to different samples. In the analysis of commercial samples, the results indicated different compositions and concentrations. The current method showed excellent chromatographic performance in terms of resolution, selectivity (separation factor), k prime (capacity factor), symmetry factor and width @ baseline. The precision of the method was confirmed by the low RSD of the replicated analytical runs. Good peak shapes were achieved using fused-core technology, indicating that this method has a great potential for determining some bioactive substances in natural products.

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