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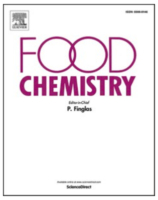

New selective and sustainable ultrasound-assisted extraction procedure to recover carnosic and rosmarinic acids from *Rosmarinus officinalis* by sequential use of bio-based solvents

Rodrigo S. Pizani [a], Juliane Viganó [a,b], Letícia S. Contieri [a], Monique M. Strieder [a], Renan K. Kamikawashi [c], Wagner Vilegas [c], Leonardo M. de Souza Mesquita [a], Maurício A. Rostagno [a,*]

[a] *Multidisciplinary Laboratory of Food and Health (LabMAS), School of Applied Sciences (FCA), University of Campinas, Rua Pedro Zaccaria 1300, 13484-350 Limeira, São Paulo, Brazil*
[b] *Centro de Ciências da Natureza, Universidade Federal de São Carlos, Rod. Lauri Simões de Barros, km 12 – SP 189, Buri, SP 18290-000, Brazil*
[c] *UNESP – São Paulo State University, Institute of Biosciences, São Vicente, Brazil*

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ABSTRACT

Carnosic (CA) and rosmarinic (RA) acids are the primary phenolic acids in hydrophilic rosemary extracts. Their combination exhibits high antioxidant activity and can be explored in several applications. This study aimed to develop an extraction procedure using bio-based solvents to recover two rosemary extracts, one rich in CA and the other in RA. By using ultrasound-assisted extraction (UAE) and a pool of 34 solvents, we evaluated nominal power (W), extraction time (min), and solvent water percentage (% H_2O) regarding yield and selectivity. The authors propose a sequential UAE procedure validated by applying ethanol 99.5 % (v/v), 240 W, and 5 min to recover a rich fraction of 24.0 $mg_{CA}.g^{-1}_{biomass}$; followed by a second step using AmAc:LA (1:2 M ratio), 20 % H_2O (m/m), 320 W, and 5 min that resulted in 8.4 $mg_{RA}.g^{-1}_{biomass}$. Our results indicate that modulating the solvent composition and process temperature is critical to increasing extraction yields and selectivity.

1. Introduction

Rosemary (*Rosmarinus officinalis*) is a Mediterranean plant widely used as a condiment in food preparations due to its flavor and health benefits. Its biomass has a complex composition, including hydrophilic phenolic compounds and hydrophobic volatile oils responsible for the characteristic aroma, which is much appreciated in culinary (Gonçalves, Panzarin, Bernardo Gonçalves, & da Costa Rodrigues, 2020). In this sense, food industries use rosemary leaves as a flavoring and antioxidant agent since it is also a potent preservative ingredient, increasing the shelf life of food products (Nieto, Ros, & Castillo, 2018).

The isolation of rosemary compounds through extractive processes allows the use of phenolic-rich fractions as food and cosmetic ingredients. However, existing studies that involve the extraction of these compounds still struggle to address their separation and selectivity, even when emerging techniques are employed, such as microwaves and ultrasound (Pizani et al., 2022). Rosemary extract can be used as a preservative in meat-based products, improving flavor and product shelf-life (Kaur, Gupta, Bronlund, & Kaur, 2021). Besides presenting flavoring and antioxidant properties, rosemary extracts also exhibit UV light protection and natural colorant characteristics that can potentially produce cosmetic skin products (González-Minero, Bravo-Díaz, & Ayala-Gómez, 2020). Specifically, carnosic diterpenes and triterpene phenolic acids are the main components of the non-volatile fraction of rosemary extract (Kontogianni et al., 2013). In the hydrophilic fraction, the most abundant phenolic compounds are carnosic acid (CA) and rosmarinic acid (RA), which are primarily responsible for their benefits to human health (Ali, Chua, & Chow, 2019). Studies assign RA the property to improve skin health by being a photoprotective agent in sunscreens and improving symptoms of human dermatitis (de Oliveira Bispo et al., 2023; Lee, Jung, Koh, Kim, & Park, 2008). CA, in turn, is attributed to anti-inflammatory activity, especially in liver diseases (Donmez, Kacar, Bagci, & Sahinturk, 2020; Song et al., 2018).

Conventional processes extract several compounds from rosemary

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* Corresponding author.
E-mail address: mauricio.rostagno@fca.unicamp.br (M.A. Rostagno).

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together, resulting in extracts with a complex composition (Hirondart, Rombaut, Fabiano-Tixier, Bily, & Chemat, 2020). However, not all are essential for a target industrial application. Therefore, selective extraction with higher yields is still challenging, mainly for solid–liquid extraction. Thus, to fully benefit from the bioactive properties of rosemary phenolic compounds, the extraction process must be selective and efficient, i.e., obtaining high extraction yields in a specific rich fraction. Conventionally, the most used techniques to fractionate liquid extracts are liquid–liquid extraction, solid-phase extraction, or other physicochemical alternatives that enable an efficient separation/purification of compounds (Chemat et al., 2020). However, these techniques represent additional costs and high operational time (de Souza Mesquita et al., 2023). A straightforward alternative is to find solvents that modulate the extraction of a specific compound without co-extracting non-desired ones, mitigating time and costs. Moreover, from another perspective, isolating phenolic compounds allows individual investigation of their bioactive properties, which enables studies for specific purposes (food, cosmetics, pharmaceuticals, and health). Separated compounds are also attractive for obtaining purer and more concentrated fractions, increasing their commercial value, and facilitating the production of chemical standards.

Furthermore, extraction techniques such as Soxhlet, infusions, and macerations are the primary choices for obtaining phenolic compounds from rosemary (Pizani et al., 2022). However, these techniques are very time-consuming and often utilize toxic organic solvents such as methanol, acetone, ether, and hexane (Osorio-Tobón, 2020), which researchers must avoid when the food segment is the intended application. In this sense, ultrasound-assisted extraction (UAE), an emergent and promising technique, provides environmental advantages. It shortens the extraction time and reduces the solvent volume required by the process (Chemat et al., 2020).

UAE revolves around acoustic cavitation that promotes mechanical and thermal effects on the extractive medium. The acoustic waves from ultrasound equipment produce microbubbles in the liquid medium that collapse, promoting cell wall ruptures and a temperature increase. This phenomenon enhances mass transfer and facilitates the extraction of compounds bound within raw materials (Jambrak, Nutrizio, Pingret, Fabiano-Tixier, & Chemat, 2022). It is worth noting that many plant compounds are thermosensitive, so the equipment must monitor the temperature rise by ultrasound cavitation to prevent eventual losses. Regarding rosemary phenolic compounds, high temperatures (>80.0 °C) can degrade CA into carnosol and rosmanol (Erkan, Ayranci, & Ayranci, 2009). Therefore, extraction techniques should increase extraction yield and avoid thermal losses.

The solvent choice is crucial for achieving higher yields and selectivity, regardless of the extraction process. The solvent polarity and viscosity are essential parameters for the extraction performance, mainly regarding the solvency power by promoting the solubilization of specific compounds from biomass (Ventura et al., 2017). Additionally, considering the updated sustainability credentials of the 2030 agenda (United Nations (2015), 2015), solvents should be nontoxic and biodegradable. Therefore, eutectic mixtures, also known as deep eutectic solvents - (D)ES, are alternatives that meet these requirements. They can be produced with non-expensive compounds and are relatively simple to prepare (Chemat et al., 2019). Eutectic solvents combine organic compounds that produce a steady fluid replete with hydrogen bonds when mixed at a specific stoichiometric proportion and with limited water content (Abbott, Capper, Davies, Rasheed, & Tambyrajah, 2003). Thus, due to the countless hydrogen bond acceptors and donors available to formulate an eutectic solvent, these mixtures are highlighted as design solvents with customizable physicochemical properties that can be modulated to a specific extraction procedure (Hansen et al., 2021).

Therefore, this work aimed to develop a new, fast, sustainable extraction procedure to recover the main bioactive compounds from rosemary selectively. In this sense, we studied eutectic solvents and UAE parameters to obtain CA- and RA-rich fractions without further purification post-extraction approaches. Thus, to our knowledge, this is the first study to focus on the selective extraction of phenolic acids from rosemary, eliminating the necessity for additional purification steps. This approach aligns with the latest principles of sustainability engineering.

2. Material and methods

2.1. Chemicals and raw material

The authors purchased dried rosemary (*Rosmarinus officinalis*) from a local market (Limeira, São Paulo, Brazil). The raw material was grounded, sieved from 300 to 1000 μm (WS Tyler, Wheeling, USA), and stored at −20 °C until further experiments. This study used the particles retained in the 500 and 300 μm sieves. Analytical standards, methanol, and acetonitrile were purchased from Sigma Aldrich (São Paulo, Brazil) and were used only in the analytical apparatus. Cholinium chloride, betaine, L-proline, ammonium acetate, 1,4-butanediol, sorbitol, citric acid, acetic acid, malic acid, and lactic acid, were used to prepare the eutectic solvents, all purchased from Dinâmica (São Paulo, Brazil).

2.2. Solvent preparation & screening assay

The authors prepared twenty-two eutectic solvents by combining the hydrogen bond acceptors (HBA) cholinium chloride, betaine, L-proline, and ammonium acetate with the hydrogen bond donors (HBD) 1,4-butanediol, sorbitol, citric acid, acetic acid, malic acid, and lactic acid (**Table SM1**). Initially, we prepared all eutectic mixtures at a molar ratio of 1:2 (HBA:HBD) with the addition of 20 % water (% H_2O m/m) alongside HBA and HBD (taking into account the initial moisture content) in an oily bath at 60 °C under magnetic stirring (180 rpm) until a transparent liquid is obtained. The authors also tested aqueous solutions of HBA and HBD used to produce the eutectic solvents (10 substances) as a solvent (20 % H_2O m/m). Distilled water and ethanol 99.5 % (v/v) were used for comparison purposes, totaling 34 different solvents. **Table SM1** presents abbreviations and additional reagent data.

2.3. Ultrasound-assisted extraction (UAE)

2.3.1. Ultrasonic bath scanning (UAE$_{bath}$)

The extraction of phenolic compounds from rosemary using the 34 different solvents was adapted from (de Souza Mesquita et al., 2023) and performed at a solvent-to-feed ratio of 20 (w/v) in a 37 kHz bath ultrasound (Elmasonic Schmidbauer GmbH, Singen, Germany), at 150 W, and 40 °C for 60 min. We used this extraction condition to select the solvents that produced the highest phenolic compounds extraction yields ($mg_{phenolic\ acids}\cdot g_{biomass}^{-1}$) from rosemary. After the extraction, we diluted, centrifuged, and filtered the samples through a nylon syringe filter (0.22 μm) for further analysis. The authors evaluated the solvent's selectivity regarding the yield of RA and CA, i.e., whether the solvent could extract RA, CA, or both.

2.3.2. Ultrasonic probe evaluation (UAE$_{probe}$)

After scanning and selecting the solvents, we evaluated the low-frequency ultrasound-assisted extraction (UAE) using a 20 kHz and 13 mm probe-type ultrasound (Ultronique, São Paulo, Brazil). We evaluated the effects of water percentage in the solvent (20, 30, 40, and 50 %, H_2O, m/m), nominal ultrasound power (0, 160, 240, 320, and 400 W), and extraction time (1, 3, 5, 7, and 9 min) on the extraction of phenolic acids ($mg_{phenolic\ acids}\cdot g_{biomass}^{-1}$). Extractions occurred using a 20 (w/v) solvent-to-feed ratio, starting at room temperature. Moreover, the authors recorded the UAE temperature in intervals of 10 s as a process control. All extractions were performed in triplicate. After the extraction, we diluted, centrifuged, and filtered the samples through a nylon syringe filter (0.22 μm) for further analysis.

Calorimetric assays determined the acoustic power supplied to the samples according to the methodology described by (Mason, Lorimer, Bates, & Zhao, 1994). In brief, we subjected 50 g of milli-Q water to ultrasound at nominal power values of 160, 240, 320, and 400 W. Temperature rise was measured every 10 s for 2 min. The instrument dissipates about 402 W/L and 612 W/L in the extractive medium at respective nominal powers of 240 W and 320 W.

2.3.3. Ultrasonic sequential extraction (UAE$_{probe}$)

The authors performed the sequential extraction study in two steps to obtain different fractions of phenolic compounds (CA and RA) from rosemary. The selected UAE and solvent conditions identified by the steps performed in section 2.3.2 were employed. Thus, we evaluated the order of the sequential extraction conditions in terms of RA and CA extraction yields. All extractions were performed in triplicate. After the extraction, we diluted, centrifuged, and filtered the samples through a nylon syringe filter (0.22 μm) for further analysis.

2.4. Conventional extraction

The authors conducted sequential conventional extractions to compare the extraction yields with section 2.3.3. We selected the solvents according to the results obtained in section 2.3.2. Maceration was conducted at room temperature for 24 h with a solvent-to-feed ratio of 20 (w/v) in the absence of light. Agitation extraction used a magnetic stirrer (IKA C-MAG HS 7, Guangdong, China) at 180 rpm and the same conditions used in maceration. Adding solvent corrected eventual volume losses by evaporation. Extractions were performed in triplicate; we diluted, centrifuged, and filtered the samples through a nylon syringe filter (0.22 μm) for further analysis.

2.5. Phenolic compounds identification and quantification

The authors identified and quantified the phenolic compounds extracted from rosemary using mass spectrometry and liquid chromatography.

2.5.1. Identification by flow injection analysis electrospray ion trap mass spectrometry (FIA-ESI-IT-MS/MS)

Phenolic compound identification was adapted from (de Souza Mesquita et al., 2023), using a flow-injection ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray ionization source. The authors calibrated the equipment by infusing standard rosmarinic and carnosic acid (1 $\mu g_{standard}.mL^{-1}_{methanol}$) at a flow rate of 5 $\mu L.min^{-1}$. The analysis conditions were as follows: capillary voltage −35 V, spray voltage 5–10 kV, tube lens offset 75 V, capillary temperature 300 °C, sheath gas (N_2) flow rate 8 (arbitrary units). Negative ion mass spectra were recorded in *m/z* 100–2000 Da. After a first full scan to acquire *m/z* range data, a second scan MS/MS experiment was performed using a data-dependent scan on deprotonated molecules from the compounds at a collision energy of 25–30 % and activation time of 30 ms. Xcalibur software (version 2.2 SP1.48) processed the acquired data.

2.5.2. Quantification by ultra-high performance liquid chromatography (UHPLC)

UHPLC analyses were modified from the method described by (Zabot, Moraes, Rostagno, & Meireles, 2014) and performed in an Acquity UPLC H-Class system (Waters, Miliford, MA, USA) using as mobile phase a gradient of water (solvent A) and acetonitrile (solvent B), both containing 0.1 % (v/v) acetic acid. The gradient used was as follows: 0 min (88 % A); 0.5 min (85 % A); 1.0 min (80 % A); 1.2 min (77 % A); 1.5 min (75 % A); 1.7 min (73 % A); 2.0 min (70 % A); 2.3 min (67 % A); 2.5 min (65 % A); 3.0 min (60 % A); 6.0 min (30 % A); 7.0 min (88 % A). A Waters Acquity C_{18} column (2.1 × 5.0 mm, 1.7 μm) performed the separation at 55 °C, with a flow rate of 0.6 $mL.min^{-1}$ and an injection volume of 1 μL. Absorbance was monitored between 210 and 400 nm, and the quantifications were performed at 284 nm for CA and 330 nm for RA. The authors identified the compounds by comparing retention times, UV spectra, and co-elution with authentic RA and CA standards. Expressed results regard rosemary dry weight (mean ± standard deviation of the mean).

2.6. Thermal stability of carnosic and rosmarinic acids

A degradation essay evaluated the thermal stability of CA and RA by heating its extracts in an oven (Memmert GmbH, Schwabach, Germany). Vials containing 1.0 mL of rosemary extracts were kept in the oven at 55 °C, 80 °C, and 105 °C for 12 h. Thus, the vials were removed at different times, cooled using an ice bath, and analyzed according to section 2.5.2. The authors integrated chromatographic peak areas over time for CA, carnosol, and rosmanol using the ethanolic extract and for RA using the extract produced with the eutectic solvent selected in section 2.3.1.

2.7. Statistical analysis

Graphical and statistical analysis of the data employed Microsoft Excel 2013 and Minitab 17.3.1.0 software, respectively. Shapiro-Wilk and Levene's test evaluated the variables for normal distribution and equality of variances. We applied two-sided one-way ANOVA with Tukey's post-hoc test to check for significant differences in parameter evaluation, considering a 95 % confidence level ($p < 0.05$).

3. Results and discussion

3.1. Identification of compounds from rosemary

Fig. 1 shows a chromatogram obtained at 284 nm for the extract obtained by UAE$_{bath}$ with 99.5 % (v/v) ethanol, as described in section 2.3.1. Authentic standards confirmed the extract's presence of RA (peak 2) and CA (peak 12). According to their UV–vis spectra and mass spectrometry, peaks 7 and 11 are rosmanol and carnosol, respectively (Zhang et al., 2012). Moreover, mass spectrometry detection allowed the identification of flavonoids such as isorhamnetin, luteolin, and apigenin in glycosylated form. **Table SM2** presents a complete list of the identified compounds and their retention times.

3.2. Eutectic solvent screening and selectivity

The authors evaluated each solvent's performance considering RA and CA extraction yields (Fig. 2). Two combinations of L-proline solvents are missing because they resulted in inhomogeneous mixtures, probably due to the non-stabilization of hydrogen bonds. Most eutectic solvents showed higher extraction yields than their individual component solutions, except for the cholinium chloride (ChCl) aqueous solution. Moreover, with some exceptions, eutectic solvents extracted a higher RA yield than ethanol and pure water. That might be explained considering the RA chemical structure since it is a polar molecule, presenting four peripheral phenolic hydroxyls and a central carboxyl group. Thus, we expect RA to have higher solubilization in eutectic solvents than in ethanol or water. Our results are in line with the study of (Barbieri et al., 2020), who obtained higher RA content using an eutectic mixture composed of cholinium chloride:1,2-propanediol (1:2, 10 % H_2O m/m) than in pure ethanol. Despite the fact that 1,4-butanediol and 1,2-propanediol present similar molecular structures (number and type of functional groups), the authors reported that ChCl:1,4-b extracted 13 ± 2 $mg_{RA}.g^{-1}_{biomass}$ and 6 ± 1 $mg_{CA}.g^{-1}_{biomass}$, while Beta:Sorb extracted 13 ± 1 $mg_{RA}.g^{-1}_{biomass}$ and AmAc:LA extracted 15 ± 1 $mg_{RA}.g^{-1}_{biomass}$, both with a negligible amount of CA.

CA is a less polar compound than RA. Therefore, ethanol performs better in extracting CA, recovering 36 ± 1 $mg_{CA}.g^{-1}_{biomass}$ (Fig. 2). Fig. 2 also indicates that the extraction of CA using eutectic solvents still needs

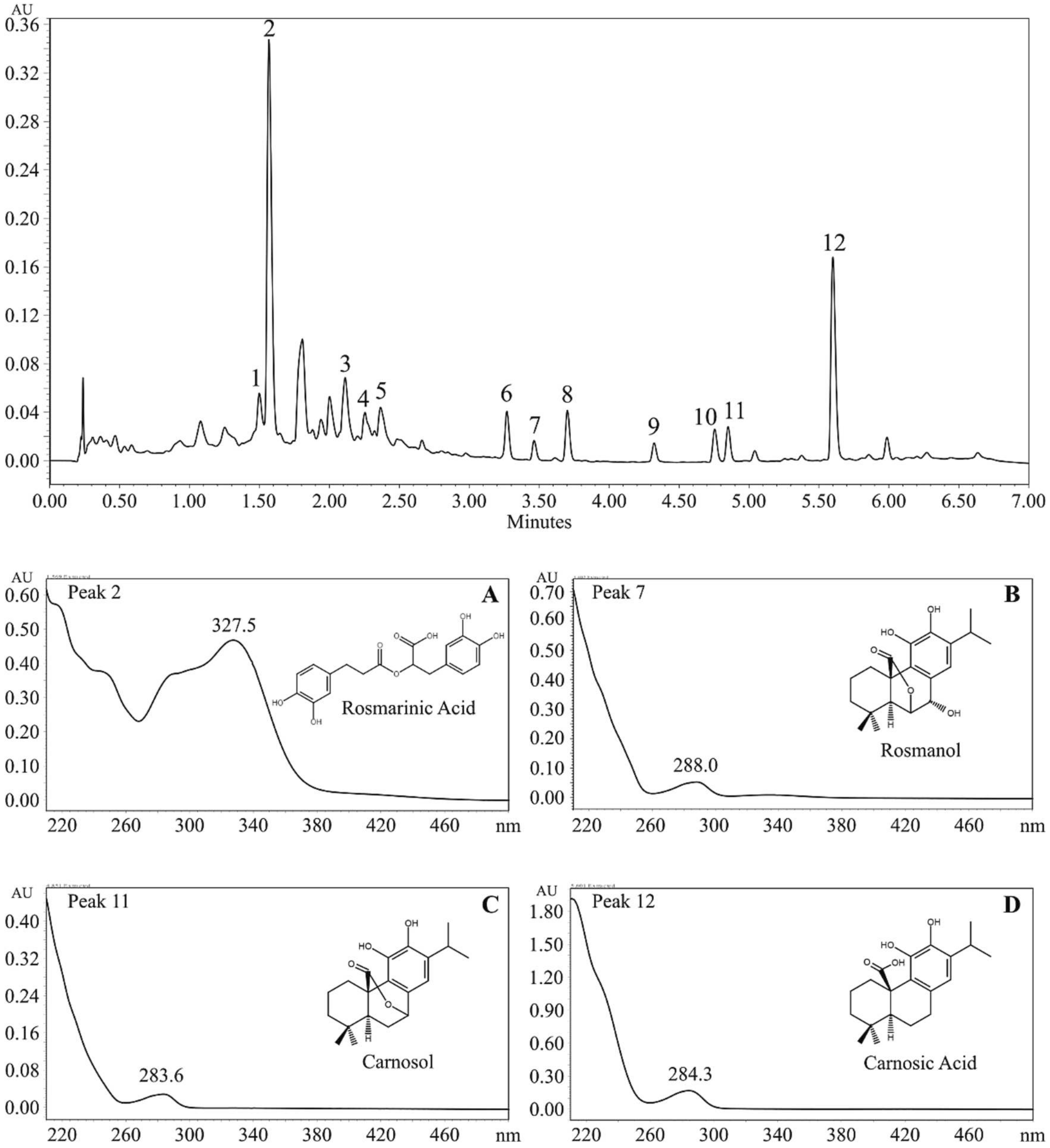

Fig. 1. Chromatogram obtained at 284 nm from ethanolic (99.5 %, v/v) rosemary extract in by UAE$_{bath}$. Identified peaks: (1) Rosmarinic acid-3-*O*-glucoside; (2) Rosmarinic acid; (3 and 5): Luteolin-3′-*O*-(*O*-acetyl)-β-D-glucuronide isomers; (4) Isorhamnetin-3-*O*-rutinoside; (6) Luteolin-7-*O*-glucuronide; (7) Rosmanol; (8) Genkwanin; (9) Rosmadial; (10) Apigenin-7-*O*-glucoside; (11) Carnosol; (12) Carnosic acid. (A) UV–vis spectrum of Rosmarinic acid. (B) UV–vis spectrum of Rosmanol. (C) UV–vis spectrum of Carnosol. (D) UV–vis spectrum of Carnosic acid.

to be improved since ethanol produced a larger CA extraction yield. On the other hand, the selective characteristic of eutectic solvents to obtain extracts with distinct phenolic acid composition from rosemary can be an advantage. The AmAc:LA showed the highest RA yield but was inefficient for extracting CA. Because of that characteristic, we selected AmAc:LA and ethanol to obtain different extract fractions from rosemary, one rich in RA and the other rich in CA.

UAE can achieve high yields with short extraction times; however, the process is generally not selective when using conventional solvents (Vinatoru, Mason, & Calinescu, 2017). In this sense, target compounds are concentrated in the extract but mixed with other compounds from the plant material. However, specific applications require target compounds in both pure and concentrated forms, and extraction selectivity is highly desirable. Thus, Fig. 2 data represents a breakthrough in using eutectic mixtures since it demonstrates the selective characteristics of this class of solvents for extracting target compounds from rosemary.

3.3. *Extraction parameters evaluation*

3.3.1. *Effect of eutectic solvent molar ratio and water percentage on RA extraction*

The authors evaluated UAE variables through a univariate experimental design using eutectic solvent and ethanol as solvents (performed by UAE$_{probe}$). Fig. 3A shows the RA extraction yields using AmAc:LA at different molar ratios, evidencing that the molar ratios of 1:2 and 3:1 AmAc:LA were significantly more efficient for RA extraction than the others tested (ANOVA $p < 0.05$). A plausible explanation for this phenomenon might be that, under such conditions, the solvent exhibits maximum affinity for RA's chemical structure. Moreover, the extraction yield decreased by increasing the proportion of lactic acid (HBD). Altering the HBA to HBD ratio leads to viscosity variations in the solvent. Lactic acid is a viscous fluid that contributes to the eutectic mixture's denseness. As a result, we expect that elevating the lactic acid proportion

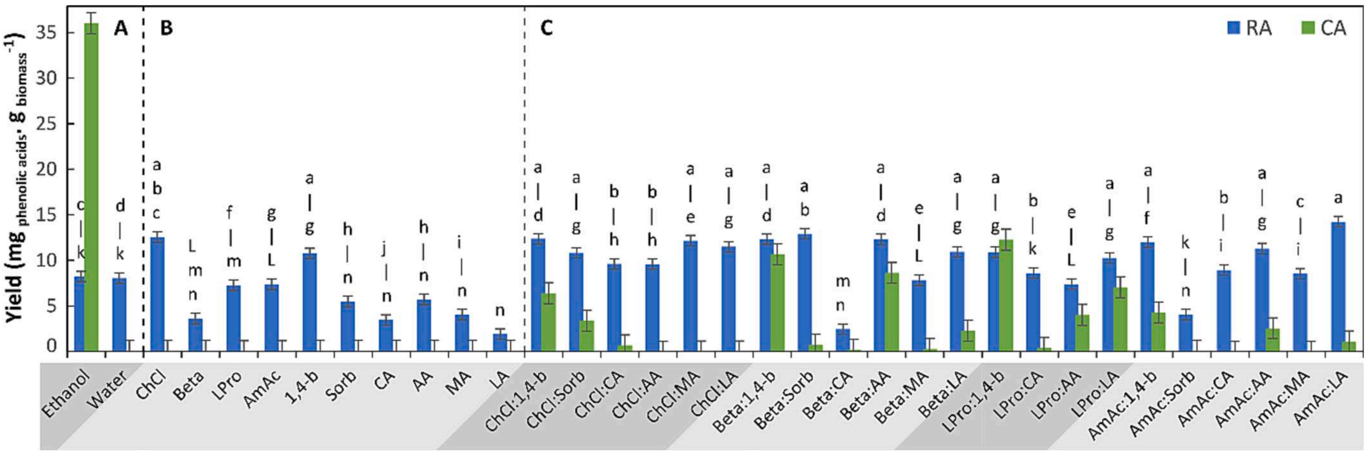

Fig. 2. Rosmarinic acid (RA) and Carnosic acid (CA) yield ($mg_{phenolic\ acids}{\cdot}g^{-1}_{biomass}$) in UAE_{bath} for (A) ethanol 99.5 % (v/v) and pure water, (B) aqueous solutions of the starting materials used to formulate the eutectic solvents (20 % H_2O m/m), and (C) eutectic solvents HBA:HBD (1:2), 20 % H_2O (m/m). Distinct letters above the columns represent different significant groups in one-way ANOVA ($p < 0.05$) Tukey's posthoc test.

will result in heightened viscosity, hindering the propagation of ultrasonic waves and thereby diminishing the efficacy of cavitation. As shown in solvent screening, CA is poorly soluble in this eutectic solvent (Fig. 2). The AmAc:LA 1:2 ratio was adopted considering the acquisition cost and ease of handling since ammonium acetate is a hygroscopic solid at room temperature.

The next variable evaluated was the water percentage in the eutectic solvent. Fig. 3B presents the effects of the water percentage in the eutectic solvent on the RA extraction yield. RA extraction yield decreased by increasing the water percentage from 20 % to 50 % H_2O (m/m). RA has more affinity for the eutectic components than water (Fig. 2). Thus, the increase in water percentage decreased the extraction yield. These results corroborated those found by (Bakirtzi, Triantafyllidou, & Makris, 2016), who obtained a higher total phenolic content from sage by using AmAc:LA (1:3) with 20 % H_2O (v/v) than pure water. Sage (*Salvia officinalis*) belongs to the Lamiaceae family, the same family as rosemary. The main phenolic compounds of sage are also RA and CA (Jakovljević et al., 2019).

On the other hand, utilizing less than 20 % H_2O (m/m) was challenging since the eutectic solvent has a high viscosity. Under this solvent condition, the rosemary sample floated, and there was a low contact surface area between the solvent and the rosemary particles. In addition to the low surface contact, the high viscosity can hinder the propagation of the ultrasonic waves in the extractive medium. As mechanical wave propagation is fundamental for acoustic cavitation, much more energy is required to promote the phenomenon in a viscous solvent (Jambrak et al., 2022).

3.3.2. Ethanol-water percentage on CA extraction

After evaluating RA extraction using AmAc:LA, we selected ethanol to obtain a CA-rich extract. Firstly, we evaluated the extraction performance using different percentages of water in ethanol (Fig. 4A). Ethanol 99.5 % and 85.0 % (v/v) showed similar efficiency in extracting CA but recovered different RA content. The extraction of RA increases by increasing the water content of ethanol. (Bernatoniene et al., 2016) also achieved a higher RA yield using 70 % ethanol than 90 % ethanol in an UAE_{bath}. The addition of water to ethanol increases the polarity of the solvent, which facilitates the extraction/solubilization of RA (more polar) and hinders the extraction of CA (less polar). (Psarrou, Oreopoulou, Tsimogiannis, & Oreopoulou, 2020) also observed similar behavior in extracting RA and CA from a rosemary residue. Therefore, since it was more selective for CA, the authors chose ethanol 99.5 % (v/v) for this step.

3.3.3. Ultrasound power, extraction time, and temperature on RA and CA extraction

Fig. 3C, 3D, and 4B, 4C, 4D showcase ultrasound power, extraction time, and temperature behavior on the extraction of RA and CA. The nominal power affected the RA and CA yields for both eutectic solvent and ethanol (Fig. 3C and 4B). RA reached maximum yield at 320 W using AmAc:LA, and CA reached maximum yield at 240 W applying ethanol. Ethanol requires less ultrasound energy to obtain the same intensity of shear forces than the eutectic solvent because it has a lower viscosity and surface tension. It is known that increasing ultrasound power causes an increase in shear forces, which facilitate the diffusion of metabolites from the plant matrix into the solvent. However, high powers can promote the compound's thermal degradation by increasing the medium temperature (Kumar, Srivastav, & Sharanagat, 2021).

Furthermore, even at a fixed and low temperature, high power values can promote the mechanical degradation of compounds by mechanical friction of the plant particles. Intense cavitation can break hydrogen bonds and polymeric structures (Wang et al., 2018), but a power value that degrades one compound may not affect another. Consequently, the selectivity of some molecules can be described as a function of the applied power (Chemat et al., 2017). In this sense, Fig. 3C and 4B show a drop in RA and CA yield when the nominal power increases to 400 W. Therefore, the acoustic energy sourced at this power probably promoted thermal and mechanical degradation of the compounds.

The equipment registered the extractive medium's temperature during extraction (Fig. 4D). As previously discussed, the increase in the medium's temperature during UAE extractions depends on the solvents' characteristics, such as viscosity, boiling point, and surface tension (Jambrak et al., 2022). In this sense, we observed that the eutectic solvent process reached the highest temperature, which may favor or disfavor the extraction of some components.

RA was more stable than CA at higher temperatures. The extraction of RA in AmAc:LA reached a temperature of 103.7 °C at 5 min and stabilized at about 115.7 °C from 7 to 9 min without yield loss. In other words, RA yield remained steady from 5 to 9 min in the low-frequency UAE, even with increasing temperature (Fig. 3D). On the other hand, CA showed yield loss after reaching 78.2 °C. When changing the solvent to ethanol, extraction reached its boiling point of 78.2 °C at 5 min (Haynes, 2017), sustained for further times. CA yield reached maximum yield at 5 min of extraction but dropped noticeably for the longer extraction times (Fig. 4C).

Indeed, the temperature increase and the presence of free radicals convert CA into its derivatives, carnosol and rosmanol (Masuda et al., 2002). It is believed that the combination of these three compounds (carnosic acid, carnosol, and rosmanol) is the main reason for the

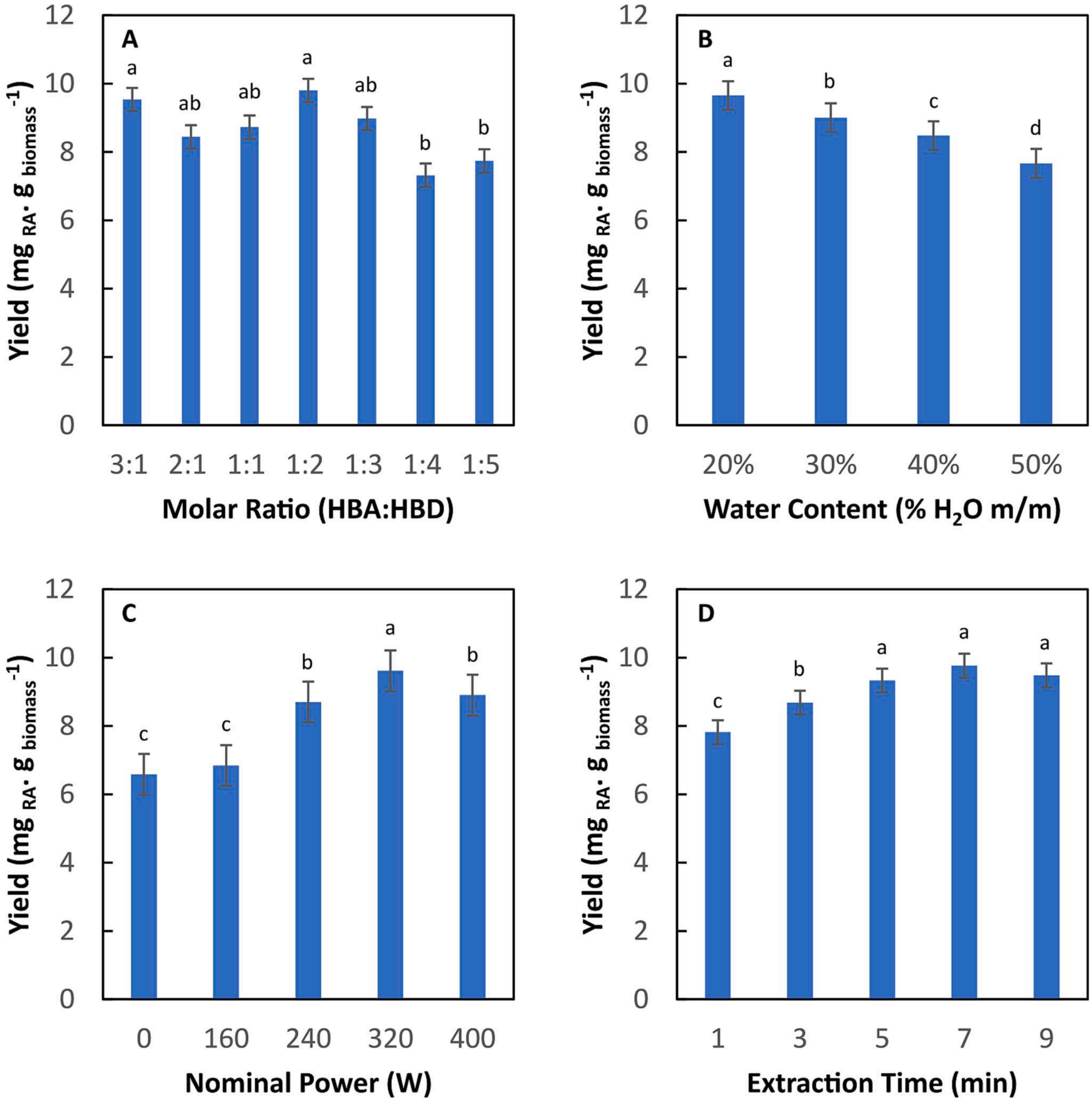

Fig. 3. Rosmarinic acid (RA) yield ($mg_{RA}.g^{-1}_{biomass}$) for rosemary extracts obtained from UAE_{probe} using AmAc:LA. (A) Different AmAc:LA molar ratios at 20 % H_2O (m/m), 240 W, and 5 min; (B) Different water percentages in AmAc:LA (1:2), at 240 W, and 5 min; (C) Different UAE nominal powers using 20 % H_2O (m/m) in AmAc:LA (1:2), and 5 min; (D): Different UAE extraction times using 20 % H_2O (m/m) in AmAc:LA (1:2), and 320 W. Distinct lowercase letters in the same graph represent different significant groups in one-way ANOVA ($p < 0.05$) Tukey's posthoc test.

excellent antioxidant power of rosemary extracts (Lešnik, Furlan, & Bren, 2021). The authors detail discuss the degradation of CA in section 3.5.

3.4. Sequential extraction process and conventional extraction

Considering the findings reported in section 3.3, we developed the sequential extraction of CA and RA from rosemary in two steps (using the same biomass). The extraction sequence to obtain these acids changed depending on which solvent was used first: eutectic solvent (AmAc:LA) or ethanol (Fig. 5). Ethanol 99.5 % (v/v) was selected to selectively extract CA, and the eutectic solvent AmAc:LA (1:2) with 20 % H_2O (m/m) to extract RA. Fig. 5 shows the CA and RA yields of the sequential extraction processes performed considering the two sequential options. Option 1 involved an initial extraction using the eutectic mixture, followed by a subsequent extraction using ethanol. Conversely, in option 2, the first extraction utilized ethanol, followed by an extraction using the eutectic mixture.

The extraction of RA by the eutectic solvent showed a relatively similar yield in both options (Fig. 5A and B). AmAc:LA extracted in the first step 9.6 ± 0.1 $mg_{RA}.g^{-1}_{biomass}$, while its use in option 2 recovered 87.7 % of this value, which is 8.4 ± 0.3 $mg_{RA}.g^{-1}_{biomass}$. This result indicates that RA is stable enough to withstand the ultrasonic waves and the temperature of 78 °C, reached if ethanol is used as the first solvent.

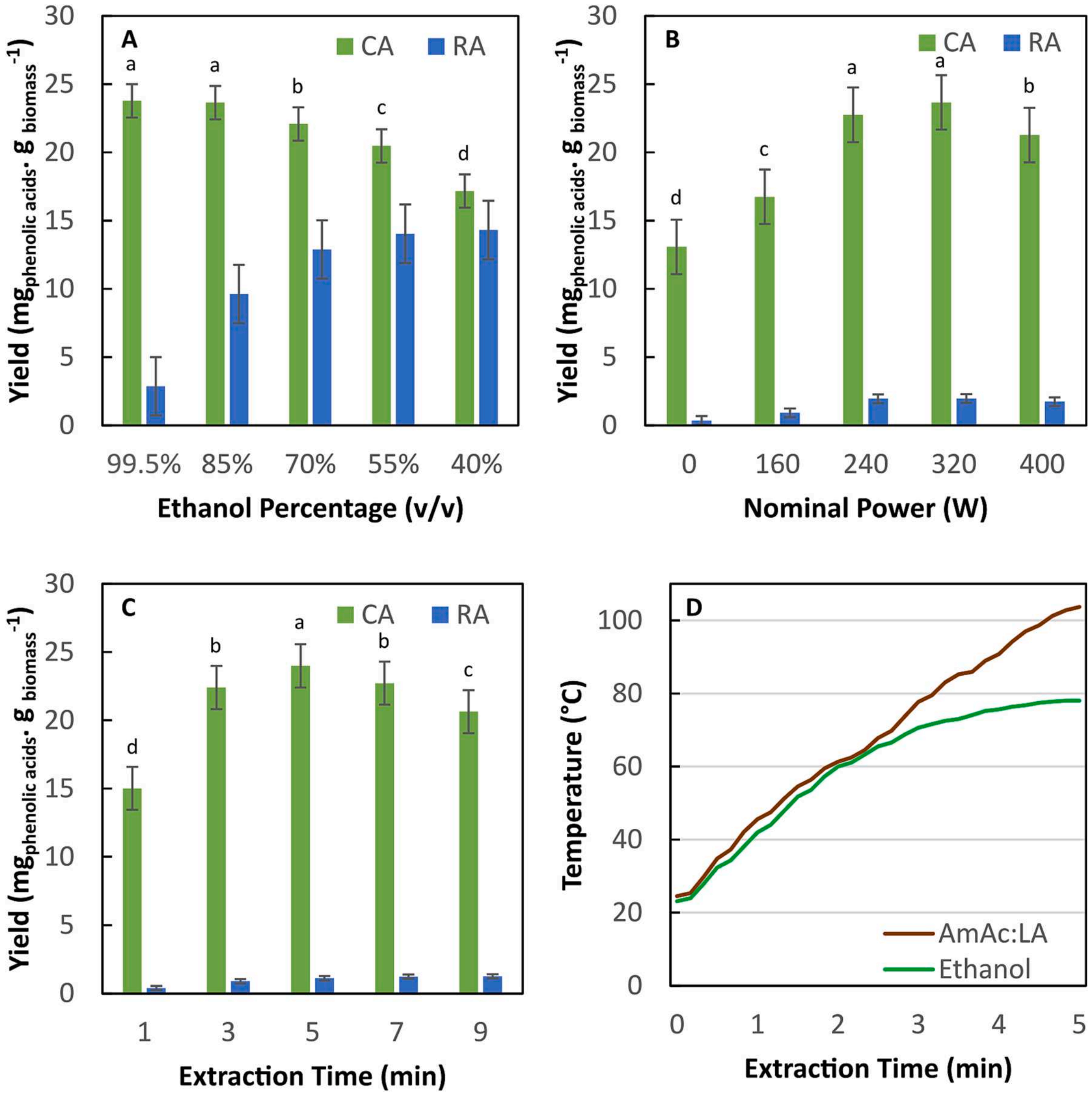

Fig. 4. Carnosic acid (CA) and Rosmarinic acid (RA) yield ($mg_{phenolic\ acids}.g^{-1}_{biomass}$) for rosemary extracts obtained from UAE_{probe} using ethanol. (A) Different ethanol percentages at 240 W, and 5 min; (B) Different UAE nominal powers at 99.5 % (v/v), and 5 min; (C): Different UAE extraction times at 99.5 % (v/v), and 240 W; (D): Extractive medium temperature for ethanol (99.5 % (v/v), 240 W) and AmAc:LA (1:2; 20 % H_2O (m/m), 320 W). Distinct lowercase letters in the same graph represent different significant groups in one-way ANOVA ($p < 0.05$) Tukey's posthoc test.

Ethanolic solvent obtained a smaller RA yield than AmAc:LA regardless of the order of the process.

Otherwise, the sequential UAE process substantially affected the CA yields. The use of ethanol in the first step obtained 24.0 ± 0.3 $mg_{CA}.g^{-1}_{biomass}$ (option 2), while its use in the second step reduced the yield to 6.9 ± 0.1 $mg_{CA}.g^{-1}_{biomass}$ (option 1), representing only 28.7 % of the yield obtained in option 2. This outcome makes sense for CA since it can be converted into carnosol and rosmanol according to the time and temperature employed (Birtić, Dussort, Pierre, Bily, & Roller, 2015). The extraction medium utilizing AmAc:LA reached the maximum temperature of 103.7 °C with a relatively linear increase (Fig. 4D).

In that case, unlike with ethanol, the solvent's boiling point does not limit the temperature increase. Indeed, this is one of the most significant advantages of eutectic solvents. Their capacity to withstand higher temperatures at atmospheric pressure permits their selection as non-volatile alternatives to conventional solvents, improving extraction performance. Moreover, high temperatures decrease the solvent's viscosity and enhance the target compound's mass transfer (Hansen et al., 2021).

The temperature increase while using AmAc:LA for RA extraction (option 1) probably thermally degraded the CA, resulting in its lower recovery in the second stage of the process using ethanol. (Erkan et al., 2009) also observed rapid degradation of carnosic acid in sunflower oil above 80 °C. In this sense, extracting CA in the first step with ethanol and then RA in the second step with AmAc:LA seems to be the most appropriate scenario. Once AmAc:LA (1:2) 20 % H2O (m/m) reaches a temperature of around 105 °C during the UAE, we can conclude that the difference in yield observed between Fig. 5A and B is due to the thermal effect. While ethanol reaches a temperature of 78 °C during UAE, the 5 min duration is short enough to avoid large losses of CA.

Ultrasound was more efficient than conventional methods for extracting phenolic acids from rosemary. Fig. 5C and 5D present the results of the same extraction sequence, according to option 2, using maceration and stirring as extraction techniques. The UAE obtained much higher yields in only 10 min versus 48 h of stirring. Maceration was the method with the lowest yield, 13.8 ± 0.8 $mg_{CA}.g^{-1}_{biomass}$ using ethanol 99.5 % (v/v) in the first 24 h and 4.0 ± 0.5 $mg_{RA}.g^{-1}_{biomass}$ using AmAc:LA (1:2) 20 % H_2O (m/m) in the next 24 h sequential step.

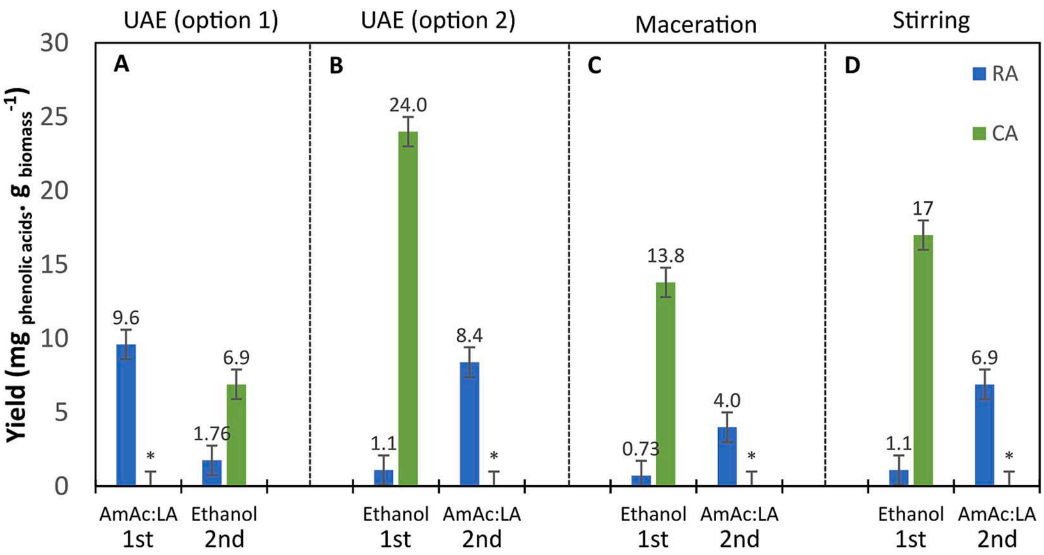

Fig. 5. Phenolic acid yields ($mg_{phenolic\ acids} \cdot g_{biomass}^{-1}$) obtained from rosemary through UAE_{probe} and conventional sequential extraction processes. (A) UAE Option 1: AmAc:LA (1:2) 20 % H_2O (m/m), 320 W, and 5 min, followed by Ethanol 99.5 % (v/v), 240 W, and 5 min; (B) UAE Option 2: Ethanol 99.5 % (v/v), 240 W, and 5 min, followed by AmAc:LA (1:2) 20 % H_2O (m/m), 320 W, and 5 min. (C) Maceration: Ethanol 99.5 % (v/v), 24 h, followed by AmAc:LA (1:2) 20 % H_2O (m/m), 24 h. (D) Stirring: Ethanol 99.5 % (v/v), 24 h, followed by AmAc:LA (1:2) 20 % H_2O (m/m), 24 h. RA: Rosmarinic acid. CA: Carnosic acid. * Non-detected.

Stirring itself extracted 17 ± 1 $mg_{CA} \cdot g_{biomass}^{-1}$ using ethanol 99.5 (v/v) and 6.9 ± 0.2 $mg_{RA} \cdot g_{biomass}^{-1}$ applying AmAc:LA (1:2) 20 % H_2O (m/m). Indeed, we expected conventional methods to obtain a lower yield than UAE since they employ room temperature and no mechanical shear forces. However, these methods confirmed that the selectivity of the solvents is consistent, regardless of the extraction technique employed.

The extract color also changed according to the solvent used and the extracted compounds (**Figure SM1**). The extract in AmAc:LA showed a brownish-orange color, while the ethanolic extract showed a light green color, probably due to the presence of chlorophyll, which, in this case, is considered a contaminant. Hence, it is essential to ensure that we specify the selective nature of the ethanolic fraction not only for carnosic terpenes but also for chlorophyll.

3.5. *Thermal stability of extracted compounds*

Fig. 6A and 6E present the thermal stability of CA in ethanol 99.5 % (v/v) and RA in AmAc:LA (1:2) 20 % H_2O (m/m), at different temperatures (55, 80, and 105 °C). CA degraded rapidly at elevated temperatures (≥80 °C).

CA degradation passes through an intermediate compound where its phenolic hydroxyls are oxidized to a quinone group. The carnosic acid quinone intermediate is further converted to carnosol by isomerization or rosmanol by nucleophilic reaction (Marrero, Andrés, & Luis, 2002; Nieto et al., 2018). **Figure SM2** illustrates the oxidation of CA into its derivatives. Although CA derivatives also exhibit bioactive and antioxidant properties, extracting undegraded CA allows the extract to withstand additional oxidation steps.

Furthermore, extracts submitted to the thermal stability analysis presented an additional peak on the UHPLC chromatogram with a retention time of 5.30 min, a UV spectrum similar to rosmanol at λ_{max} of 288.6 nm, and a mass of $[M-H]^-$ 373 *m/z*, which we identified as 7-ethoxyrosmanol (rosmanol ethyl ether) (Zhang et al., 2012). In this sense, since we performed the extraction in 99.5 % (v/v) ethanol, the temperature increase probably promoted the conversion of rosmanol into 7-ethoxyrosmanol via nucleophilic attack of carbon 7 in its structure, with water as the leaving group (**Figure SM2**) (Marrero et al., 2002).

In fact, as the CA concentration declines, the 7-ethoxyrosmanol concentration starts to elevate rapidly, as observed in the first 4 h at 105 °C and throughout the entire analysis at 80 °C (Fig. 6A and D). At 55 °C, rosmanol and 7-ethoxyrosmanol concentrations remained stable, while carnosol mildly increased. That indicates that milder temperatures may favor the conversion of CA to carnosol rather than rosmanol. Carnosol was also the most susceptible CA derivative to decomposition by increasing temperature. The extract subjected to 105 °C for 2 h and the one exposed to 80 °C for 8 h showed the highest carnosol content. However, after these exposure times, the carnosol content rapidly decreased in extracts subjected to prolonged exposure at both temperatures (Fig. 6B). Rosmanol showed degradation only at 105 °C after 3 h of heating (Fig. 6C). 7-Ethoxyrosmanol increased its concentration over time for all the temperatures studied, making it the most temperature-resistant carnosic derivative among the compounds we evaluated.

RA also proved relatively stable at elevated temperatures (Fig. 6E), withstanding both extraction steps in UAE and the thermal stability assay without significant yield losses. In addition to its innate degradation resistance to temperature increase, since we evaluated RA in AmAc:LA, there is a possibility that this eutectic solvent acts as a protective agent against thermal degradation.

4. Conclusions

In this study, we developed a novel selective extraction procedure employing ethanol and AmAc:LA for the extraction of CA and RA, respectively, from rosemary. UAE's extraction sequence and parameters are paramount in maintaining CA stability, given its susceptibility to degradation above 80 °C. A short UAE duration (10 min total) resulted in higher yields than 48 h maceration and stirring methods.

The ethanolic extract (99.5 % v/v, 240 W, 5 min, 78 °C max.) resulted in a CA yield of 24.0 ± 0.3 $mg_{CA} \cdot g_{biomass}^{-1}$. Furthermore, the sequential extraction utilizing eutectic solvent (AmAc:LA (1:2), 20 % H_2O (m/m), 320 W, 5 min, 103.7 °C max) resulted in a RA yield of 8.4 ± 0.3 $mg_{RA} \cdot g_{biomass}^{-1}$.

Consequently, the combined utilization of UAE and eutectic solvents demonstrated significant outcomes in the selective extraction of the primary phenolic acids from rosemary, without requiring further purification steps. This approach can reduce production time and increase the economic value of plant extracts.

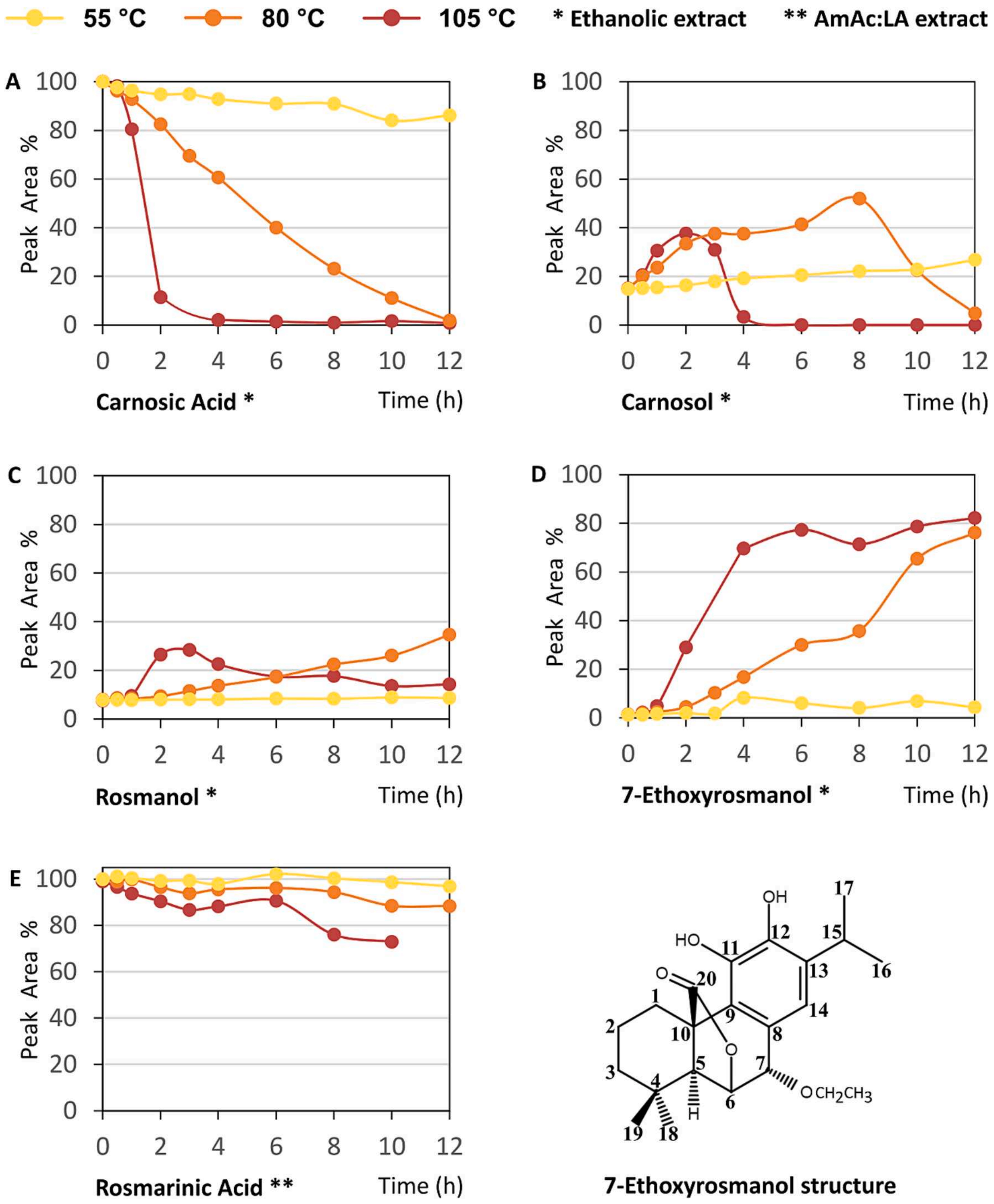

Fig. 6. Thermal stability of rosemary compounds at 55 °C, 80 °C, and 105 °C. (A) Carnosic acid in ethanol 99.5 % (v/v). (B) Carnosol in ethanol 99.5 % (v/v). (C) Rosmanol in ethanol 99.5 % (v/v). (D) 7-Ethoxyrosmanol in ethanol 99.5 % (v/v). (E) Rosmarinic Acid in AmAc:LA (1:2) 20 % H_2O (m/m).

CRediT authorship contribution statement

Rodrigo S. Pizani: Validation, Formal analysis, Investigation, Visualization, Methodology, Writing – original draft, Writing – review & editing. **Juliane Viganó:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Letícia S. Contieri:** Formal analysis, Investigation. **Monique M. Strieder:** Methodology, Supervision, Writing – original draft, Writing – review & editing. **Renan K. Kamikawashi:** Formal analysis, Methodology, Writing – review & editing. **Wagner Vilegas:** Formal analysis, Methodology, Writing – review & editing. **Leonardo M. de Souza Mesquita:** Conceptualization, Methodology, Supervision, Investigation, Writing – review & editing. **Maurício A. Rostagno:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137546.

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