Food Chemistry 211 (2016) 465–473

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Multiresponse optimization of an extraction procedure of carnosol and rosmarinic and carnosic acids from rosemary

Gerlon de A.R. Oliveira [a], Anselmo E. de Oliveira [a,*], Edemílson C. da Conceição [b], Maria I.G. Leles [a]

[a] Instituto de Química, UFG, 74690-900 Goiânia, GO, Brazil
[b] Faculdade de Farmácia, UFG, 74605-220 Goiânia, GO, Brazil

ARTICLE INFO

Article history:
Received 1 July 2015
Received in revised form 1 February 2016
Accepted 6 May 2016
Available online 7 May 2016

Keywords:
Rosemary
Antioxidants
Conventional extraction
RSM
Desirability function

ABSTRACT

A green solvent-based optimization for rosmarinic acid (RA), carnosol (COH), and carnosic acid (CA) extraction, the three main antioxidants from rosemary, was performed. The conventional solid-liquid extraction was optimized using a central composite design (CCD) followed by the desirability approach. In the CCD analysis the quantitative effects of extraction time (4.8–55.2 min), liquid-to-solid ratio (4.6–21.4 mL g^{-1}), and ethanol content (44.8–95.2% v/v) were determined for the extracted amount of antioxidants, their concentrations in the extract, and the extraction yield. Samples were analyzed by HPLC and the antioxidants were identified by comparison with pure standard retention times and UV spectra. The desirability function that simultaneously maximizes the antioxidants extraction and their concentrations in the final product was validated. The extraction using a hydroalcoholic solution 70% v/v, at low liquid-to-solid ratio (5 mL g^{-1}), and after 55-min yielded an antioxidant recovery rate of 89.8%, and a final product 4.75 times richer in the main antioxidants than the raw material.

1. Introduction

Rosemary is a plant known worldwide as a culinary spice and a natural preservative due to its high antioxidant and antimicrobial activities. These activities are related to the presence of phenolic compounds, mainly rosmarinic acid (RA) and diterpenes such as carnosic acid (CA) and carnosol (COH) (Collins & Charles, 1987; Moreno, Scheyer, Romano, & Vojnov, 2006).

Rosemary extract is an effective natural food preservative as on the stabilisation of sunflower oil (Urbančič, Kolar, Dimitrijević, Demšar, & Vidrih, 2014), and pork batter formulation (Hernández-Hernández, Ponce-Alquicira, Jaramillo-Flores, & Legarreta, 2009), for instance. Besides, refrigerated raw meat from animals like gilt-head seabream (Hernández, García García, Jordán, & Hernández, 2014) and sheep (Nieto, Estrada, Jordán, Garrido, & Bañón, 2011) fed with rosemary extract had extended shelf life.

The operational conditions such as extraction time, liquid-to-solid ratio, and the kind of extracting solvent for the extraction method play an important role in the conventional extraction process. Conventional solid-liquid extraction is widely used for antioxidants, plus is safe, cheap, and easy to scale up (Hernández-Hernández et al., 2009; Mulinacci et al., 2011). Extraction techniques like ultrasound and microwave assisted (Rodríguez-Rojo, Visentin, Maestri, & Cocero, 2012), or the ones using supercritical fluid (Carvalho, Moura, Rosa, & Meireles, 2005; Herrero, Plaza, Cifuentes, & Ibáñez, 2010) are mainly employed to improve the extractive process efficiency and final product quality.

The correct choice of the operational conditions leads to a higher recovery rate of the compounds of interest and a more concentrated final product (extract) using less energy, time, raw material, and solvent. All of these factors need to be optimized for antioxidant extraction of different plants or even for the same plant if it has undergone different pretreatments. Differences in matrix structure and plant composition may also require changes to the extraction process (Dorta, Lobo, & González, 2013).

Response surface methodology (RSM), proposed by Box and Wilson in 1951 (Box & Wilson, 1992), has been widely used as a statistical approach to optimize liquid-solid extractions (refer, for instance, to Pap et al., 2013; Xi & Wang, 2013). Central composite design (CCD) is one of the most popular experimental designs because it is efficient, very flexible, and can be run sequentially. Simultaneous optimization of multiple responses, e.g. the recovered amounts of many antioxidants after an extraction process, has been performed using desirability functions (Ghafoor, Choi, Jeon, & Jo, 2009; Hossain et al., 2012).

RA, COH, and CA antioxidant extraction from rosemary and other spices have been reported in the literature. Kim et al. (2010) have proposed an optimized process for RA extraction from

* Corresponding author.
E-mail address: elcana@ufg.br (A.E. de Oliveira).

http://dx.doi.org/10.1016/j.foodchem.2016.05.042
0308-8146/

Melissa officinalis using methanol. Hossain et al. (2012) have optimized the ultrasound assisted extraction of marjoram antioxidants including RA, COH, and CA, using methanol. Several methods have been optimized for antioxidant extraction from rosemary, such as ultrasound assisted extraction (Paniwnyk, Cai, Albu, Mason, & Cole, 2009), accelerated solvent extraction (Hossain, Barry-Ryan, Martin-Diana, & Brunton, 2011), pressurized green solvent extraction (Herrero et al., 2010), and CO_2 supercritical fluid extraction (SFE) (Herrero et al., 2010; Visentín, Cismondi, & Maestri, 2011). Other studies have reported rosemary extraction without response surface modeling and optimization (Babovic et al., 2010; Couto et al., 2012).

This present paper reports a CCD optimization for the conventional solid-liquid extraction of the main antioxidants from rosemary using a desirability approach. A green solvent-based optimization was performed for RA, COH, and CA extraction, the three main antioxidants from rosemary. In the CCD analysis the quantitative effects of extraction time, liquid-to-solid ratio, and ethanol content were determined for the extracted amount and concentration in the extract of RA, COH, and CA, and the extraction yield. Next, the desirability function that simultaneously maximizes the antioxidants extraction and their concentrations in the final product was validated. Lastly, a RA, COH, and CA enriched rosemary extract was obtained with a high recovery.

2. Materials and methods

Two extractive methods were carried out in this work. In the first one, due to the importance of knowing the total antioxidant content available for extraction, high dilution and ultrasound were employed to quantitatively extract RA, COH, and CA from the powder. In the second method, conventional extraction using green solvents (water and ethanol) and low liquid-to-solid ratio were optimized to be suitable for future applications in the food field.

2.1. Materials

Rosmarinic acid (98%) was purchased from Sigma (Germany). Carnosic acid and carnosol, both with over 95% purity, were obtained from Chromadex (USA). Methanol was of HPLC grade (Tedia, Brazil). Acetic acid and absolute ethanol were from Vetec (Brazil). Ultrapure water (Millipore, USA) was also used. Fine rosemary powder (dried ground leaves from Morocco, having particle size distribution as follows: <0.125 mm: 28.2%; 0.125–0.180 mm: 25.7%; 0.180–0.250 mm: 26.0%; and 0.250–0.425 mm: 20.1%; average particle size of 181 μm, and 8.4% of volatile) was purchased from Santosflora (Brazil). RA, COH, and CA were quantified in the powder.

2.2. Quantification of antioxidant compounds in the rosemary powder

Approximately 200.00 mg of rosemary powder were weighed and transferred to a 50.0 mL volumetric flask. The extractor liquid was added to the flask and the extraction was carried out for 10 min using an ultrasound bath (Unique, Brazil). Water, methanol, ethanol, acetone, and their aqueous mixtures (Fig. 1) were employed in trials to quantitatively extract the three main rosemary antioxidants. Following the extraction, samples were filtered through a 0.45 μm Durapore® PVDF membrane (Millipore, Brazil),

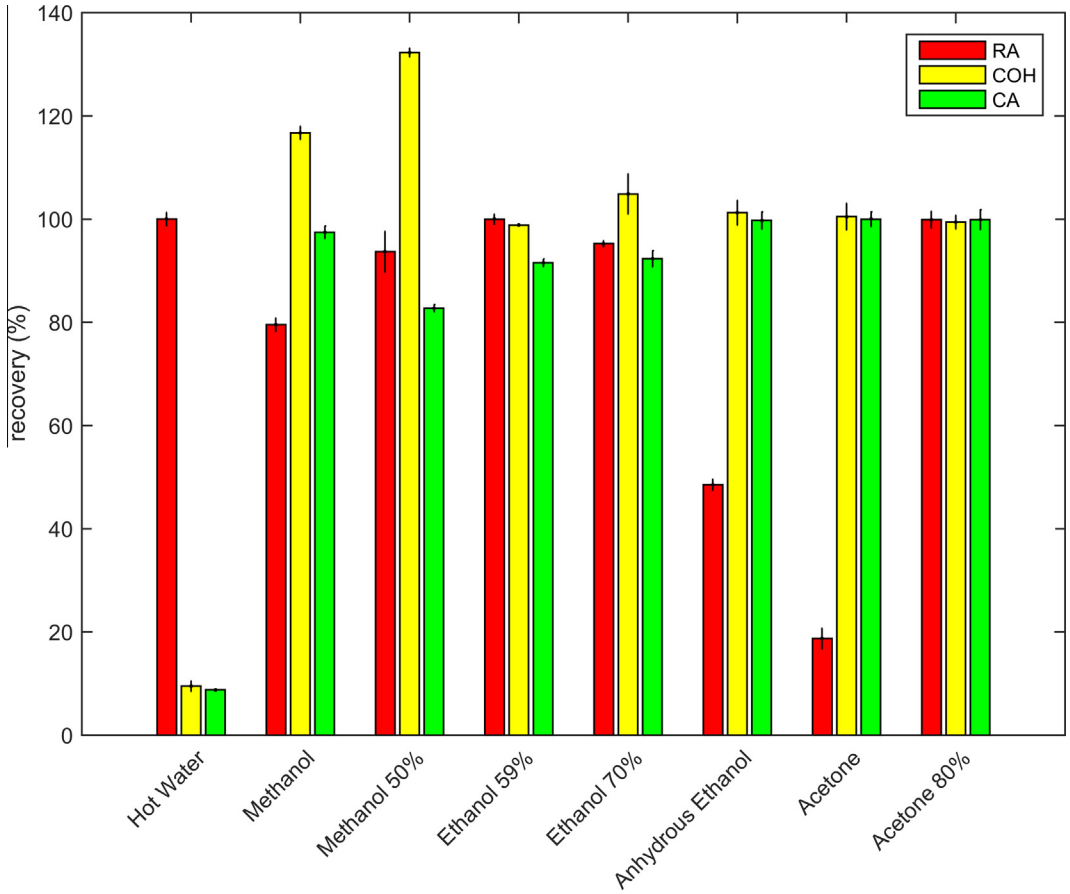

Fig. 1. RA, COH, and CA recovery from rosemary powder using different extraction solvents. Error bars represent mean ± 1 standard deviation.

diluted three times with acetic acid 1% v/v, and immediately injected into the chromatograph.

2.3. Conventional solvent extraction process

Extraction was carried out in a sealed 30 mL cylindrical flask, fastened to a shaker table operating at 70 rpm at room temperature (25 °C). Rosemary powder was extracted with 16.0 mL of a hydroalcoholic solution (ethanol content in Table 1). Extraction time, ethanol content, and liquid-to-solid ratio (amount of rosemary powder in the hydroalcoholic solution) were optimized using a RSM based on a CCD, as shows Table 1. Twenty experiments were randomly performed, including six central point replicates. After the extraction, samples were centrifuged (5000 rpm × 5 min) and filtered using a 0.45 μm PVDF Durapore® membrane (Millipore, Brazil). An aliquot of the filtered extract was separated for dry residue and HPLC analysis. Liquid extracts were diluted ten times with acetone 80% v/v, then diluted three times with acetic acid 1% v/v, and immediately injected into the chromatograph.

2.4. Extraction yield

Dry residue was obtained after 4 mL of the filtered extract were dried at 110 °C during 12 h. The Extraction yield was expressed as the amount of dissolved matter (measured as dry residue) expressed as the percentage of the initial amount of powder.

2.5. HPLC analysis

Samples were analyzed by HPLC using a Waters e2695 chromatograph, equipped with a 2998 PDA detector and an autosampler. The method was adapted from the literature (Cuvelier, Berset, & Richard, 1994) and validated following ICH recommendations (ICH, 2005). The column, a Zorbax XDB C18 (25 cm × 4.6 mm × 5 μm), and the injector were maintained at 30 °C and 4 °C, respectively. Injection volume was 20 μL. The detector operated from 190 to 400 nm. RA was quantified at 330 nm and diterpenes at 280 nm. The mobile phase consisted of a linear gradient using methanol and water acidified with 1% v/v of acetic acid. Time (% of methanol): 0–7 min (40); 7–11 min (40 → 80); 11–23 min (80); 23–24 min (80 → 90); 24–28 min (90 → 40); 28–33 min (40). Data were processed using Empower software (version 2.0 from Waters, USA). RA, COH, and CA contents were determined both in the rosemary powder and in the extracts, on a dry basis. Antioxidants were identified by comparison with pure standard retention times and UV spectra. Quantification was based on peak areas via the external standard method.

2.6. Experimental design

CCD reduces the number of experiments to close to the 2-level full factorial design, and the rotability requirement for m variables is satisfied when $\alpha = 2^{m/4}$. Results, y, can be fitted to the following second order polynomial equation as a function of m coded independent variables

$$y = \beta_0 + \sum_{i=1}^{m} \beta_i X_i + \sum_{i=1:j=1}^{m} \beta_{ij} X_i X_j \quad (1)$$

where β_0 is a constant, β_i are the linear coefficients, β_{ij} are the quadratic coefficients when $i = j$, and the interactive/synergic coefficients when $i \neq j$. Analysis of variance (ANOVA) is carried out for each regression model and non-significant effects are not considered after statistical analysis. New regression coefficients are then recalculated to the final regression model with no evidence of lack-of-fit. Optimal conditions are found using a global desirability function

$$D = (d_1 \cdot d_2 \cdot \ldots \cdot d_k)^{1/k} \quad (2)$$

Table 1
Central composite design for the antioxidant extraction from rosemary powder.

Variables (coded factors)				Levels							
				−1.682		−1		0		1	+1.682
Extraction time[a] (*ExT*)				4.8		15.0		30.0		45.0	55.2
Liquid-to-solid ratio[b] (*LtS*)				4.6		8.0		13.0		18.0	21.4
Ethanol content[c] (*EtC*)				44.8		55.0		70.0		85.0	95.2
Run	*ExT*	*LtS*	*EtC*	Extracted amount (% w/w)			Concentration in the extract (% w/w)				Extraction yield (% w/w)
				RA	COH	CA	RA	COH	CA	Total	
1	−1	−1	−1	0.67	0.33	1.52	3.54	1.75	8.04	13.33	18.95
2	1	−1	−1	0.73	0.44	1.49	3.69	2.22	7.56	13.47	19.65
3	−1	1	−1	0.74	0.37	1.82	3.15	1.58	7.73	12.45	23.58
4	1	1	−1	0.74	0.44	1.74	3.10	1.84	7.31	12.26	23.77
5	−1	−1	1	0.45	0.37	2.06	2.70	2.20	12.31	17.21	16.72
6	1	−1	1	0.55	0.38	1.99	3.32	2.31	12.01	17.64	16.55
7	−1	1	1	0.59	0.42	2.23	3.15	2.23	11.98	17.36	18.57
8	1	1	1	0.58	0.39	2.04	2.87	1.93	10.16	14.96	20.04
9	−1.682	0	0	0.66	0.37	1.97	3.57	2.02	10.63	16.23	18.48
10	1.682	0	0	0.70	0.40	1.92	3.15	1.82	8.66	13.62	22.14
11	0	−1.682	0	0.64	0.38	1.81	3.63	2.13	10.22	15.97	17.76
12	0	1.682	0	0.74	0.39	2.02	3.28	1.74	8.93	13.96	22.56
13	0	0	−1.682	0.73	0.36	1.18	3.70	1.85	6.02	11.57	19.65
14	0	0	1.682	0.28	0.37	2.06	1.98	2.62	14.57	19.16	14.12
15	0	0	0	0.71	0.39	1.97	3.36	1.85	9.31	14.52	21.14
16	0	0	0	0.70	0.38	1.98	3.37	1.83	9.61	14.81	20.65
17	0	0	0	0.69	0.38	1.94	3.30	1.84	9.32	14.46	20.79
18	0	0	0	0.70	0.39	1.98	3.26	1.79	9.23	14.29	21.50
19	0	0	0	0.70	0.39	1.96	3.40	1.88	9.49	14.78	20.65
20	0	0	0	0.72	0.39	1.98	3.48	1.90	9.60	14.98	20.65

[a] minutes.
[b] mL g^{-1}.
[c] % (v/v).

where d_k is an individual desirability function for each of the k responses. D is then a function of the extracted amounts of RA, COH, CA, and of their concentrations in the extract. Six responses were validated at the desirable point for the resulting CCD model.

3. Results and discussion

3.1. Raw material content of antioxidants

Recovery tests using 8 different liquid solvents were run in order of extracting the three main rosemary antioxidants after 10 min ultrasound assisted single-step procedure, Fig. 1. Extraction recovery of 100% was assumed for the most efficient solvent, except for the COH extraction since it can result from CA degradation in polar solvents (Wenkert, Fuchs, & McChesney, 1965). COH recovery in anhydrous ethanol, pure acetone, and acetone 80% v/v were averaged to 100% owed to the fact that these three solvents extracted equivalent COH quantities. Solvents having the highest polarities extracted significantly higher amounts of RA. On the other hand, the ones having the lowest polarities extracted, preferably, CA. Both acetone and anhydrous ethanol quantitatively extracted CA and COH. RA was quantitatively extracted using ethanol 59% v/v and hot water. The three main antioxidants were quantitatively extracted from the rosemary powder in a single-step procedure using acetone 80% v/v. Therefore, this solvent was chosen as the extraction solvent for quantitative analysis. The method was validated following ICH recommendations (ICH, 2005).

The procedure established to quantify the antioxidants used approximately 200.0 mg of rosemary powder, and the relative standard deviations (RSDs) were below 3%. Confidence limits for the mean for the concentration of the main rosemary antioxidants, after five replicates on a wet weight basis and $P = 0.05$, were 0.75 ± 0.01% w/w for RA, 0.37 ± 0.01% w/w for COH, and 2.14 ± 0.04% w/w for CA, which corresponds to a total of 3.26 ± 0.04% w/w. Okamura, Fujimoto, Kuwabara, and Yagi (1994) found 4.21% w/w of CA and 0.39% w/w of COH, and Wang, Provan, and Helliwell (2004) found 1.1% of RA in dried rosemary leaves. Mulinacci et al. (2011) found 1.42% w/w/ of CA, the major antioxidant in *Rosmarinus officinalis*. These different values reported on the literature for the rosemary antioxidant contents are owed to the raw material employed and also to the extraction processes.

3.2. Conventional extraction

A CCD for $m = 3$ variables and $\alpha = 1.682$ was run, according to the variable ranges in Table 1, involving 20 experiments in a full factorial design. Six replicates at the center point were used to determine the experimental error. For each experiment the extracted amount of RA, COH, and CA (recovery), their concentrations in the extract on dry basis, calculated using HPLC peak areas, as well as the extraction yield are also expressed in Table 1. The concentration in the extract is the relative percentage of the three assessed antioxidants in the total amount of extract, calculated using the extraction yield procedure.

Since liquid-to-solid ratios varied according to Table 1 values and the hydroalcoholic volume was fixed at 16.00 mL, rosemary powder masses employed in the factorial design experiments ranged from 747.8 to 1337.5 mg. The three responses varied greatly in the full factorial part of CCD experiments presenting RSDs ranging from 7.16% to 23.79%. On the other hand, values lower than 2.3% were verified using the six replicates at the center point. Squared product-moment correlation coefficients, r^2, for the response functions are shown in Table 2. Explanatory variables for the model functions were significant at $P < 0.1$.

3.3. Extracted amount of the antioxidants

The ethanol content (*EtC*) was the most important variable (β_3 in Table 2) for the extracted amount of RA and CA. RA recovery is favored when the ethanol content is below 70% v/v, Fig 2a. Since the negative sign for the interactive/synergic coefficient indicates antagonistic effect between two antioxidants, the synergic effect between liquid-to-solid ratio and extraction time ($\beta_{12} < 0$ in Table 2) accounts for the fact that low liquid-to-solid ratios and short extraction times may not be enough to extract all RA. The design model for the extracted amount of CA, Table 2, shows that optimal extraction is achieved when ethanol content is above 70% v/v, Fig. 2c. The small and negative extraction time effect result from the fact that CA may be converted to COH and to other chemical compounds in polar solvents (Mulinacci et al., 2011; Wenkert et al., 1965). The COH extracted amount increases when the extraction time increases, and the ethanol content is on the lower level (Fig. 2b).

A high antioxidant recovery is accomplished in the overall using ethanol content 70% v/v. Hydroalcoholic solvent has many advantages when compared to other extraction solvents like methanol, DMSO, dichloromethane, butanone, and ethyl acetate employed for the rosemary antioxidant extraction (Albu, Joyce, Paniwnyk, Lorimer, & Mason, 2004; Mulinacci et al., 2011). Such advantages include low cost, low toxicity to consumers and technicians, and environment benefits.

Conventional extraction of rosemary antioxidants at room temperature ranging from 1 to 10 h have been reported (Del Baño

Table 2
Regression coefficients for the second order polynomial models (with coded variables).

Model parameter	Extracted amount			Concentration in the extract			Extraction yield
	RA	COH	CA	RA	COH	CA	
β_0	0.696	0.386	1.959	3.381	1.881	9.459	20.750
β_1 (*Ext*)	0.015	0.015	−0.034	–	–	−0.463	0.611
β_2 (*LtS*)	0.030	0.009	0.081	−0.113	−0.113	−0.359	1.622
β_3 (*EtC*)	−0.108	–	0.235	−0.318	0.189	2.211	−1.712
β_{33} (EtC^2)	−0.068	–	−0.113	−0.192	0.126	0.256	−1.251
β_{12} (*Ext* × *LtS*)	−0.021	−0.010	–	−0.138	−0.076	–	–
β_{13} (*Ext* × *EtC*)	–	−0.023	–	–	−0.117	–	–
β_{23} (*LtS* × *EtC*)	–	–	−0.042	–	–	–	−0.427
r^2	0.964	0.836	0.973	0.760	0.909	0.965	0.948
Adjusted r^2	0.951	0.792	0.963	0.696	0.877	0.956	0.929
Std. error	0.026	0.011	0.046	0.218	0.087	0.417	0.645
F-value	74.238	19.129	101.277	11.890	27.997	104.212	50.768
F of signification	1.42E−09	9.35E−06	1.75E−10	1.49E−04	8.07E−07	9.37E−11	1.77E−08

Non-significant model parameters ($P > 0.10$) were not included.

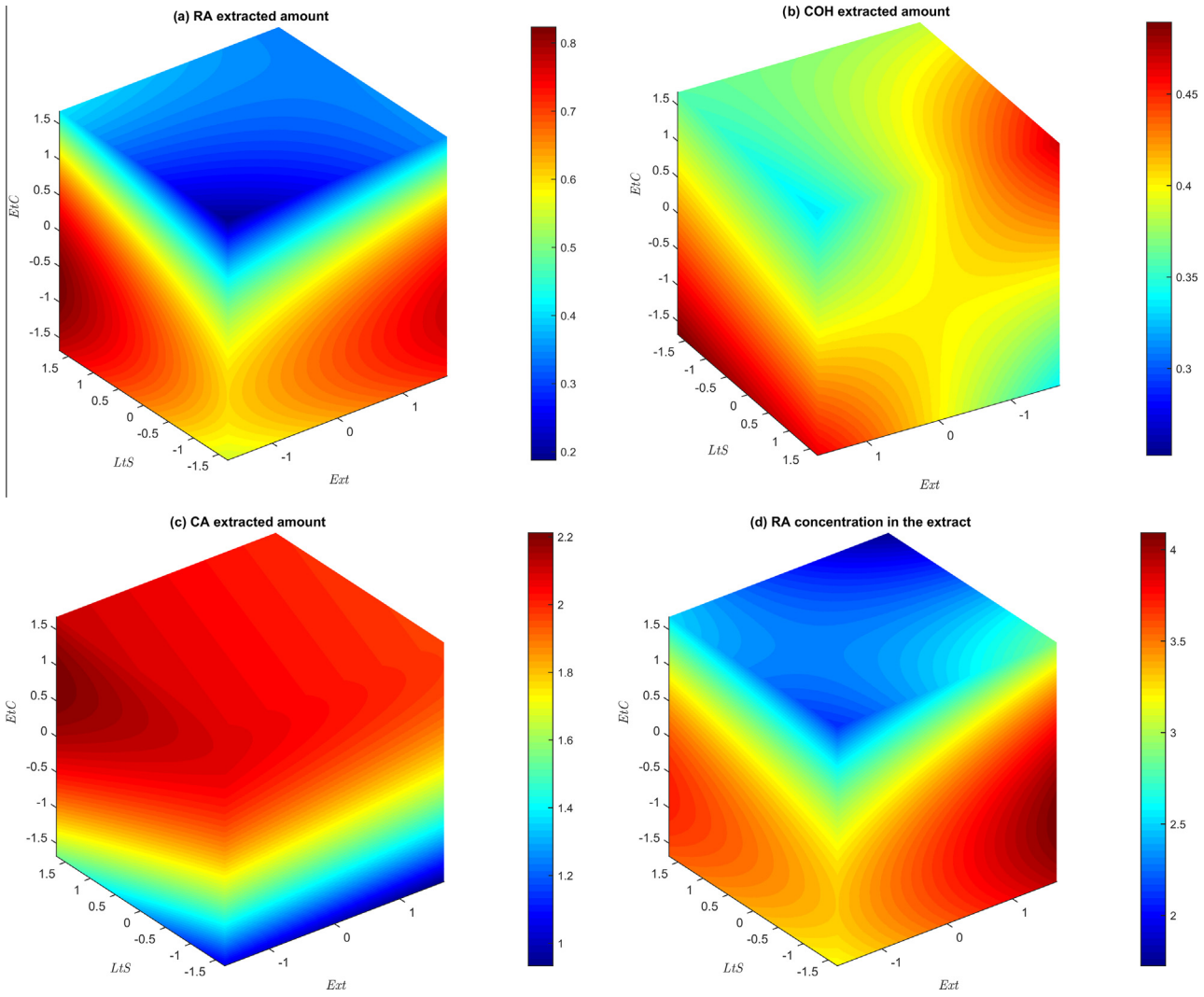

Fig. 2. Response surfaces in 4D plots for the extracted amount and concentration in the extract of RA, COH, and CA, and extraction yield versus the coded variables extraction time (*ExT*), liquid-to-solid ratio (*LtS*), and ethanol content (*EtC*). The scale bars represents range values for each response (% w/w).

et al., 2003; Hernández-Hernández et al., 2009; Rodríguez-Rojo et al., 2012). In our work, an extraction time of less than 1 h was enough to efficiently extract all the three main antioxidants. This may be due to the 181 μm average particle-size of rosemary powder, which was thinner than the ones reported in the literature (Carvalho et al., 2005; Herrero et al., 2010; Rodríguez-Rojo et al., 2012; Visentín et al., 2011). Unless agglomeration occurs, smaller particle sizes tend to increase the extraction of several compounds. This work presents recovery rates above 90% that were obtained after 30 min extraction time, room temperature, and using single-step extractions without ultrasound apparatus, as for the runs 12 and 15 to 20 (Table 1). Small average particle-sizes and hydroalcoholic solution 70% v/v might also have contributed to such performance. However, an experimental condition set up to simultaneously extract the three antioxidants may also extract undesirable constituents from rosemary powder, yielding a low concentrated extract.

3.4. Antioxidant concentration in the extract

The antioxidant concentration in the extract is important when choosing an extractive process. A high concentrated extract result from high antioxidant recovery rate and low extraction yield. In order to evaluate the antioxidant concentration in the extract and the extraction yield, 4D response surfaces were modeled and can be seen in Fig. 2d–g.

The higher the ethanol content, the lower the extraction yield (Table 2 and Fig. 2g). As a consequence, COH and CA concentrations in the extract increase (Fig. 2e and f). On the other hand, less than half of the RA present in the rosemary powder was extracted in such conditions (Fig. 2a and run 14 in Table 1) resulting in a low concentrated RA extract (Fig. 2d). Extracts having high RA contents can be obtained when both high extraction time and low liquid-to-solid ratio are used. As such, an optimum ethanol content combined with high extraction time and low liquid-to-solid ratio must be employed in order of obtaining a high concentration in the extract for COH, CA, and RA, simultaneously.

The higher total concentration in the extract, 19.16% w/w (run 14, Table 1), was obtained when using the higher ethanol content. Similar results for the total concentration in the extract were found when using supercritical fluids or high pressurized and heated liquids (Carvalho et al., 2005; Herrero et al., 2010; Sánchez-Camargo et al., 2014; Visentín et al., 2011). When employing single-step extractions, values below 17% w/w were found (Carvalho et al., 2005; Del Baño et al., 2003; Rodríguez-Rojo et al., 2012). In the present work, total concentration in the extract of 19.16% w/w and extraction yield of 14.12% w/w were obtained (Table 2, run 14).

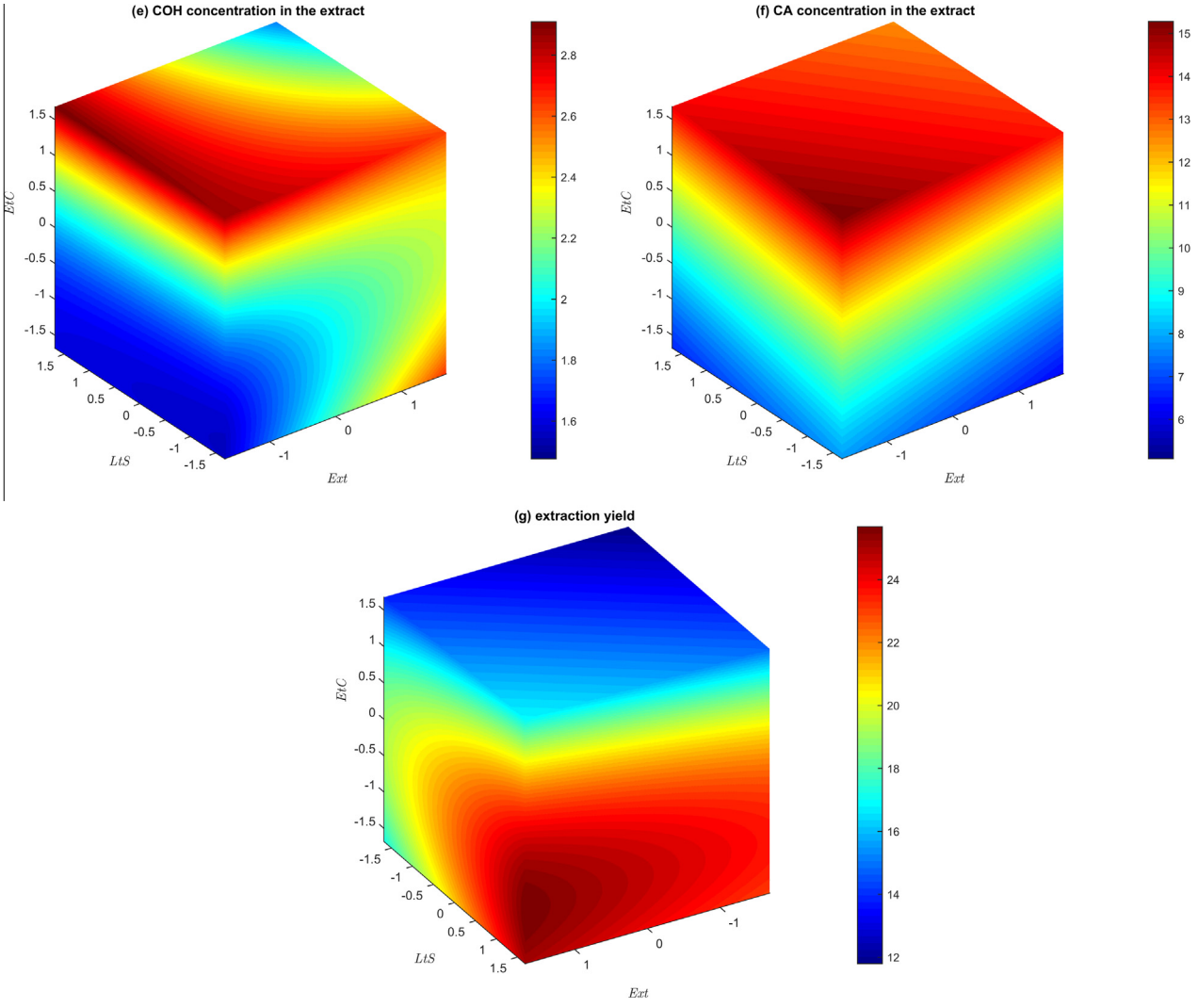

Fig. 2 (*continued*)

On the literature, total antioxidants concentration in the rosemary extracts using supercritical fluid extraction (SFE) ranged from 8.68 to 40.89% w/w (Carvalho et al., 2005; Herrero et al., 2010; Sánchez-Camargo et al., 2014). These works reported extraction yields ranging from 3.3 to 6.5% w/w. SFE presents some advantages such as leading, at suitable conditions, to purer extracts than the ones obtained using one step conventional extraction, and employing low amount, or none, of organic solvents. On the other hand, due to the selectivity of the extraction process, high concentrated extracts do not contain a high amount of all antioxidants. As an example, SFE using ethanol as cosolvent led to an extract having 37.8% w/w of diterpenes (COH and CA), but the RA content was below the limit of quantification (Herrero et al., 2010). Finally, the instruments and equipments necessary to run SFE are more expensive when compared to ones employed in conventional extractions (Pereira & Meireles, 2007).

The antioxidant activity of rosemary extracts may be due to the combining activities of its main antioxidants, RA and CA (Romano, Abadi, Repetto, Vojnov, & Moreno, 2009). Rosemary antioxidant properties in aqueous medium are mainly due to RA, meanwhile CA and COH are the main rosemary antioxidants acting on lipidic systems (Del Baño et al., 2003). As a consequence, food presenting both lipidic and aqueous phases can take advantage on the reduction of oxidation when rosemary extract containing the three main antioxidants are employed (Terpinc, Bezjak, & Abramovič, 2009).

3.5. Desirability and final product

The optimal conditions for an extraction process may vary as a result of different product features or process parameters that need to be chosen following certain commercial interests. An industry may focus on high antioxidants recovery or may prefer a more concentrated final product, for instance. In our work the highest total concentration in the extract was obtained using ethanol 95.2% v/v, having low RA recovery. The high RA extracting conditions (hydroalcoholic solution 70% v/v combined with high *ExT* or high *LtS*) yielded a less concentrated extract since it increased the extraction yield. For model validation, a condition in which antioxidant extraction and antioxidant concentration in the final product were both considered was found via desirability functions.

Six individual desirability conditions were set up based on both the extracted amount (d_1 to d_3) and the concentration in the extract (d_4 to d_6) of RA, COH, and CA. Denominators in Eqs. (1) to (3) represent the raw material content of each antioxidant (Section 3.1), and in Eqs. (4) to (6) they represent the maximum response for each respective response function.

$$d_1 = \frac{\text{RA extracted amount}}{0.75} \quad (3)$$

$$d_2 = \frac{\text{COH extracted amount}}{0.37} \quad (4)$$

$$d_3 = \frac{\text{CA extracted amount}}{2.14} \quad (5)$$

$$d_4 = \frac{\text{RA concentration in the extract}}{4.10} \quad (6)$$

$$d_5 = \frac{\text{COH concentration in the extract}}{2.93} \quad (7)$$

$$d_6 = \frac{\text{CA concentration in the extract}}{15.36} \quad (8)$$

Global desirabilities, D, obtained by the geometric means of these six individual desirabilities, according to Eqs. (2), and (3) to (8), were calculated for each of the twenty CCD experiments listed in Table 1. A model for expressing the relationship between process parameters and the global desirability function resulted in the following equation:

$$D = 0.815 + 0.019\ EtC - 0.023\ ExT \times LtS - 0.014\ ExT \times EtC - 0.033\ EtC^2 \quad (9)$$

Only significant effects ($P < 0.10$) were considered in Eq. (9). A r^2 equals to 0.8228 as well as $P < 0.0001$ were found for model significance. Fig. 3 shows a 4D plot of the estimated global desirability versus the three extraction parameters evaluated. The closer the value of D is to unity, the more desirable it is.

The global desirability function in Eq. (9), and plotted in Fig. 3, indicates that there are two high desirability regions ($D \approx 0.9$). The desirable region on the left of Fig. 3 is characterized by high liquid-to-solid ratio, low extraction time, and ethanol content around 80% v/v. The other desirable region (right side of Fig. 3) is characterized by low liquid-to-solid ratio, high extraction time, and ethanol content around 70% v/v. Since the disruption of cells is not expected in conventional extraction (Ranjan, Patil, & Moholkar, 2010), the two desirable regions in Fig. 3 result, mainly, from the diffusion of the solutes. As a consequence, the solvent effect dependent terms in Table 2 were statistically significant ($P > 0.10$), emphasizing the solvent selectivity expected in diffusion processes (Ranjan et al., 2010).

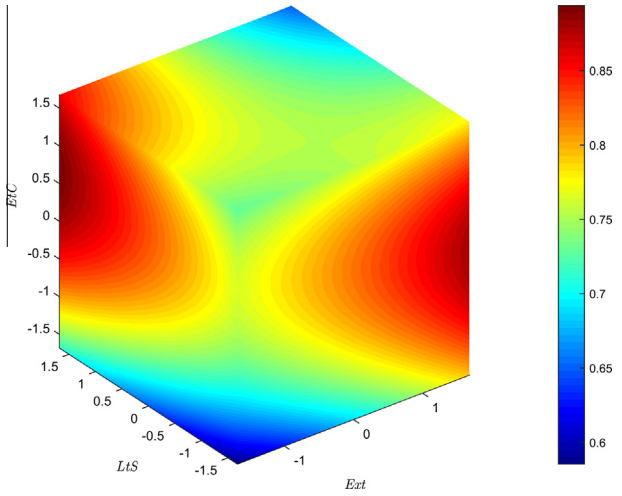

Fig. 3. Desirability (D), versus the coded variables for extraction time (ExT), liquid-to-solid ratio (LtS), and ethanol content (EtC). The scale bar represents values ranging from 0.58 to 0.89.

The desirable region on the left side of Fig. 3 is acquired using low extraction time and high solvent extractor volumes to account for the proper bulk convection. On the other hand, the desirable region on the right side results from high extraction times allowing for an effective diffusion of RA. In this case, convection is hampered due to the high density of solids in the extractor solvent (low liquid-to-solid ratio). However, when high extraction times are employed in conjunction with high liquid-to-solid ratios, it lowers the process desirability (back part of the cube of Fig. 3). This fact is a consequence of the lowering in the final product concentration owed to the diffusion of other constituents (high extraction yield).

The desirable region on the right side of Fig. 3 was chosen for model validation since a low liquid-to-solid ratio yields a less diluted extract, when comparing to an extraction using a high liquid-to-solid ratio. Plus, it brings economic benefits in subsequent concentration and drying steps.

Inside the chosen region (right side of Fig. 3), high extraction times are correlated with high D values. The model functions for the extraction amount and concentration in the extract, Table 2, indicate that both RA and COH increase when increasing the extraction time, but CA reduces. After 55 min of extraction COH is completely extracted. As such, the increase in COH, predicted by the extraction amount function, is a result of the COH generated from the CA degradation. The extracted amount of RA would not be expected to significantly increase once 97.6% of RA has already been extracted after 55 min. In addition, after 55 min a further decrease in the concentration in the extract would be expected as a consequence of an increase in the extraction yield. Based on these facts, there were no scientific evidences of experimental improvements extrapolating extraction time further than 55 min.

Considering the fact that the desirability model for the experimental design supports low liquid-to-solid ratios to achieve maximum desirability, 5 mL g^{-1} ratio was chosen as an optimum liquid-to-solid ratio. The use of even more concentrated mixtures should not significantly increase the value of D. In addition, concentrated mixtures are difficult to be homogenized.

Once optimal conditions for extraction time and liquid-to-solid ratio were determined, the optimum ethanol content was set at 70% v/v. This optimum condition presented a D value of 0.88, which represents a compromise between the recovery of the antioxidants and their concentrations in the extract.

Predicted and experimental values were obtained for the optimized conventional extraction, as can be seen in Table 3. The 95% confidence interval for experimental values measured in triplicates are in agreement with their predicted values (t test), except

Table 3
Predicted and experimental values at optimum conditions: 55 min extraction time, 5 mL g^{-1} liquid-to-solid ratio, and 70% v/v ethanol content.

	Extracted amount (% w/w)			Concentration in the extract (% w/w)				Extraction yield (% w/w d.b.)
	RA	COH	CA	RA	COH	CA	Total	
Predicted	0.73	0.42	1.78	3.92	2.26	9.24	15.42	19.23
Experimental[a]	0.74 ± 0.01	0.42 ± 0.02	1.77 ± 0.03	3.92 ± 0.06	2.22 ± 0.12	9.34 ± 0.17	15.49 ± 0.29	18.90 ± 0.06

[a] 95% confidence interval for the mean.

for the extraction yield (relative error below 2%), reinforcing the experimental design strategy employed.

After 55 min of conventional extraction, 89.8% of the total antioxidants assessed were extracted, yielding a final product with total concentration in the extract of 15.49% w/w. RA, COH, and CA concentration in this extract were, respectively, 3.92%, 2.22%, and 9.34% w/w. The final product is 4.75 times richer in the assayed antioxidants when compared to the raw material, on dry basis. In addition, different experimental setups targeting a high extraction recovery rate or an enriched antioxidant extract can be applied from the CCD runs of Table 2.

4. Conclusions

An experimental design followed by a desirability model proved to be an effective way to simultaneously maximize the extraction of the three main rosemary antioxidants and their concentration in the final product. The concentration in the extract and extraction yield for RA, COH, and CA were fitted with second-order polynomial models to the extraction time, liquid-to-solid ratio, and ethanol content.

The use of green solvents and low liquid-to-solid ratio produced a high antioxidant recovery rate and the total concentration in the extract in the final product was richer in RA, COH, and CA than the raw material. The proposed experimental design for the conventional extraction can produce an enriched extract having antioxidant contents comparable to those obtained with supercritical fluids or high pressurized and heated liquids, reported in the literature.

Acknowledgements

The authors acknowledge the financial support of CAPES, CNPq, and FAPEG. G.A.R.O. thanks CAPES for graduate student fellowship.

References

Albu, S., Joyce, E., Paniwnyk, L., Lorimer, J. P., & Mason, T. J. (2004). Potential for the use of ultrasound in the extraction of antioxidants from Rosmarinus officinalis for the food and pharmaceutical industry. *Ultrasonics Sonochemistry, 11*(3–4), 261–265. http://dx.doi.org/10.1016/j.ultsonch.2004.01.015.

Babovic, N., Djilas, S., Jadranin, M., Vajs, V., Ivanovic, J., Petrovic, S., & Zizovic, I. (2010). Supercritical carbon dioxide extraction of antioxidant fractions from selected Lamiaceae herbs and their antioxidant capacity. *Innovative Food Science & Emerging Technologies, 11*(1), 98–107. http://dx.doi.org/10.1016/j.ifset.2009.08.013.

Box, G. E. P., & Wilson, K. B. (1992). On the experimental attainment of optimum conditions. *Journal of the Royal Statistical Society, 13*, 270–310. http://dx.doi.org/10.1007/978-1-4612-4380-9_23.

Carvalho, R. N., Moura, L. S., Rosa, P. T. V., & Meireles, M. A. A. (2005). Supercritical fluid extraction from rosemary (Rosmarinus officinalis): Kinetic data, extract's global yield, composition, and antioxidant activity. *The Journal of Supercritical Fluids, 35*(3), 197–204. http://dx.doi.org/10.1016/j.supflu.2005.01.009.

Collins, M. A., & Charles, H. P. (1987). Antimicrobial activity of carnosol and ursolic acid: two anti-oxidant constituents of *Rosmarinus officinalis* L.. *Food Microbiology, 4*(4), 311–315. http://dx.doi.org/10.1016/S0740-0020(87)80005-9.

Couto, R. O., Conceição, E. C., Chaul, L. T., Oliveira, E. M. S., Martins, F. S., Bara, M. T. F., & Paula, J. R. (2012). Spray-dried rosemary extracts: Physicochemical and antioxidant properties. *Food Chemistry, 131*(1), 99–105. http://dx.doi.org/10.1016/j.foodchem.2011.08.036.

Cuvelier, M. E., Berset, C., & Richard, H. (1994). Antioxidant constituents in Sage (*Salvia officinalis*). *Journal of Agricultural and Food Chemistry, 42*(3), 665–669. http://dx.doi.org/10.1021/jf00039a012.

Del Baño, M. J., Lorente, J., Castillo, J., Benavente-García, O., del Río, J. A., Ortuño, A., & Gerard, D. (2003). Phenolic diterpenes, flavones, and rosmarinic acid distribution during the development of leaves flowers stems and roots of *Rosmarinus officinalis* antioxidant activity. *Journal of Agricultural and Food Chemistry, 51*(15), 4247–4253. http://dx.doi.org/10.1021/jf0300745.

Dorta, E., Lobo, M. G., & González, M. (2013). Optimization of factors affecting extraction of antioxidants from mango seed. *Food and Bioprocess Technology, 6*(4), 1067–1081. http://dx.doi.org/10.1007/s11947-011-0750-0.

Ghafoor, K., Choi, Y. H., Jeon, J. Y., & Jo, I. H. (2009). Optimization of ultrasound-assisted extraction of phenolic compounds, antioxidants, and anthocyanins from grape (*Vitis vinifera*) seeds. *Journal of Agricultural and Food Chemistry, 57*(11), 4988–4994. http://dx.doi.org/10.1021/jf9001439.

Hernández, A., García García, B., Jordán, M. J., & Hernández, M. D. (2014). Improved conservation of gilthead seabream (*Sparus aurata*) in ice storage. The influence of doses of rosemary extract added to feed. *Aquaculture, 426–427*, 31–40. http://dx.doi.org/10.1016/j.aquaculture.2014.01.018.

Hernández-Hernández, E., Ponce-Alquicira, E., Jaramillo-Flores, M. E., & Legarreta, I. G. (2009). Antioxidant effect rosemary (*Rosmarinus officinalis* L.) and oregano (*Origanum vulgare* L.) extracts on TBARS and colour of model raw pork batters. *Meat Science, 81*(2), 410–417. http://dx.doi.org/10.1016/j.meatsci.2008.09.004.

Herrero, M., Plaza, M., Cifuentes, A., & Ibáñez, E. (2010). Green processes for the extraction of bioactives from Rosemary: Chemical and functional characterization via ultra-performance liquid chromatography-tandem mass spectrometry and in-vitro assays. *Journal of Chromatography A, 1217*(16), 2512–2520. http://dx.doi.org/10.1016/j.chroma.2009.11.032.

Hossain, M. B., Barry-Ryan, C., Martin-Diana, A. B., & Brunton, N. P. (2011). Optimisation of accelerated solvent extraction of antioxidant compounds from rosemary (*Rosmarinus officinalis* L.), marjoram (*Origanum majorana* L.) and oregano (*Origanum vulgare* L.) using response surface methodology. *Food Chemistry, 126*(1), 339–346. http://dx.doi.org/10.1016/j.foodchem.2010.10.076.

Hossain, M. B., Brunton, N. P., Patras, A., Tiwari, B., O'Donnell, C. P., Martin-Diana, A. B., & Barry-Ryan, C. (2012). Optimization of ultrasound assisted extraction of antioxidant compounds from marjoram (*Origanum majorana* L.) using response surface methodology. *Ultrasonics Sonochemistry, 19*(3), 582–590. http://dx.doi.org/10.1016/j.ultsonch.2011.11.001.

ICH (2005). *Validation of analytical procedures: text and methodology Q2 (R1). IFPMA: Geneva, (Step 4)*. Retrieved from http://c11bw1rt.securesites.net/ich/q/q2r2_97_10_28e.pdf.

Kim, S., Yun, E. J., Bak, J. S., Lee, H., Lee, S. J., Kim, C. T., & Kim, K. H. (2010). Response surface optimised extraction and chromatographic purification of rosmarinic acid from *Melissa officinalis* leaves. *Food Chemistry, 121*(2), 521–526. http://dx.doi.org/10.1016/j.foodchem.2009.12.040.

Moreno, S., Scheyer, T., Romano, C. S., & Vojnov, A. A. (2006). Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. *Free Radical Research, 40*(2), 223–231. http://dx.doi.org/10.1080/10715760500473834.

Mulinacci, N., Innocenti, M., Bellumori, M., Giaccherini, C., Martini, V., & Michelozzi, M. (2011). Storage method, drying processes and extraction procedures strongly affect the phenolic fraction of rosemary leaves: An HPLC/DAD/MS study. *Talanta, 85*(1), 167–176. http://dx.doi.org/10.1016/j.talanta.2011.03.050.

Nieto, G., Estrada, M., Jordán, M. J., Garrido, M. D., & Bañón, S. (2011). Effects in ewe diet of rosemary by-product on lipid oxidation and the eating quality of cooked lamb under retail display conditions. *Food Chemistry, 124*(4), 1423–1429. http://dx.doi.org/10.1016/j.foodchem.2010.07.102.

Okamura, N., Fujimoto, Y., Kuwabara, S., & Yagi, A. (1994). High-performance liquid chromatographic determination of carnosic acid and carnosol in *Rosmarinus officinalis* and *Salvia officinalis*. *Journal of Chromatography A, 679*(2), 381–386. http://dx.doi.org/10.1016/0021-9673(94)80582-2.

Paniwnyk, L., Cai, H., Albu, S., Mason, T. J., & Cole, R. (2009). The enhancement and scale up of the extraction of anti-oxidants from *Rosmarinus officinalis* using ultrasound. *Ultrasonics Sonochemistry, 16*(2), 287–292. http://dx.doi.org/10.1016/j.ultsonch.2008.06.007.

Pap, N., Beszédes, S., Pongrácz, E., Myllykoski, L., Gábor, M., Gyimes, E., & Keiski, R. L. (2013). Microwave-assisted extraction of anthocyanins from black currant marc. *Food and Bioprocess Technology, 6*(10), 2666–2674. http://dx.doi.org/10.1007/s11947-012-0964-9.

Pereira, C. G., & Meireles, M. A. A. (2007). Economic analysis of rosemary, fennel and anise essential oils obtained by supercritical fluid extraction. *Flavour and Fragrance Journal, 22*(5), 407–413. http://dx.doi.org/10.1002/ffj.1813.

Ranjan, A., Patil, C., & Moholkar, V. S. (2010). Mechanistic assessment of microalgal lipid extraction. *Industrial & Engineering Chemistry Research, 49*(6), 2979–2985. http://dx.doi.org/10.1021/ie9016557.

Rodríguez-Rojo, S., Visentin, A., Maestri, D., & Cocero, M. J. (2012). Assisted extraction of rosemary antioxidants with green solvents. *Journal of Food Engineering, 109*(1), 98–103. http://dx.doi.org/10.1016/j.jfoodeng.2011.09.029.

Romano, C. S., Abadi, K., Repetto, V., Vojnov, A. A., & Moreno, S. (2009). Synergistic antioxidant and antibacterial activity of rosemary plus butylated derivatives. *Food Chemistry, 115*(2), 456–461. http://dx.doi.org/10.1016/j.foodchem.2008.12.029.

Sánchez-Camargo, A. D. P., Valdés, A., Sullini, G., García-Cañas, V., Cifuentes, A., Ibáñez, E., & Herrero, M. (2014). Two-step sequential supercritical fluid extracts from rosemary with enhanced anti-proliferative activity. *Journal of Functional Foods, 11*, 293–303. http://dx.doi.org/10.1016/j.jff.2014.10.014.

Terpinc, P., Bezjak, M., & Abramovič, H. (2009). A kinetic model for evaluation of the antioxidant activity of several rosemary extracts. *Food Chemistry, 115*(2), 740–744. http://dx.doi.org/10.1016/j.foodchem.2008.12.033.

Urbančič, S., Kolar, M. H., Dimitrijević, D., Demšar, L., & Vidrih, R. (2014). Stabilisation of sunflower oil and reduction of acrylamide formation of potato with rosemary extract during deep-fat frying. *LWT – Food Science and Technology, 57*(2), 671–678. http://dx.doi.org/10.1016/j.lwt.2013.11.002.

Visentín, A., Cismondi, M., & Maestri, D. (2011). Supercritical CO_2 fractionation of rosemary ethanolic oleoresins as a method to improve carnosic acid recovery. *Innovative Food Science & Emerging Technologies, 12*(2), 142–145. http://dx.doi.org/10.1016/j.ifset.2011.01.004.

Wang, H., Provan, G. J., & Helliwell, K. (2004). Determination of rosmarinic acid and caffeic acid in aromatic herbs by HPLC. *Food Chemistry, 87*(2), 307–311. http://dx.doi.org/10.1016/j.foodchem.2003.12.029.

Wenkert, E., Fuchs, A., & McChesney, J. D. (1965). Chemical artifacts from the family labiatae. *The Journal of Organic Chemistry, 30*(9), 2931–2934. http://dx.doi.org/10.1021/jo01020a012.

Xi, J., & Wang, B. (2013). Optimization of ultrahigh-pressure extraction of polyphenolic antioxidants from green tea by response surface methodology. *Food and Bioprocess Technology, 6*(9), 2538–2546. http://dx.doi.org/10.1007/s11947-012-0891-9.