Simultaneous extraction of rosemary and spinach leaves and its effect on the antioxidant activity of products

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Abstract

Mixed vegetal extracts are interesting target of new products as nutraceuticals, superior ingredients for the design of functional food, singular ingredients for cosmetics, etc. In this work the extraction of a mixture of spinach and rosemary leaves (50 % weight of each plant) was investigated in terms of its antioxidant activity, and compared with the extraction of the separate species. Phenolic diterpenes of rosemary and carotenoids of spinach were target compounds due their recognized biological activities. Two different extraction techniques were applied, namely pressurized liquid extraction using hexane at two different temperatures (100 and 150°C) and supercritical fluid extraction with pure carbon dioxide at 40°C and two different pressures (20 and 30 MPa). For each extraction technique and conditions three different raw materials were employed: spinach leaves, rosemary leaves and the mixture 50:50 of spinach and rosemary leaves.

The antioxidant activity of the samples produced were evaluated with the ABTS assay and showed to be enhanced when the species are simultaneously extracted, with antioxidant values around 20% higher than the values corresponding to mixing the extracts obtained by separate. A possible synergic effect between carotenoids and phenolic diterpenes was studied, although no specific synergic activity could be observed. However, the enhanced antioxidant activity could be attributed to a definite increase of the concentration of carnosic acid, which was observed in the samples produced by the simultaneous extraction.

Keywords: Supercritical fluid extraction; Pressurized liquid extraction; Antioxidants; Phenolic diterpenes; Carotenoids; Spinach; Rosemary.

1. Introduction

Antioxidants play a very important role in the food, cosmetic and pharmacy industries [1]. Both phenolic compounds and carotenoids have been identified as important antioxidant compounds present in natural matter. Furthermore, it has been reported that some antioxidants may act synergistically, thus being much more effective response against oxidation. The most studied synergism between antioxidants is between β-carotene and vitamins C and E [2-5].

Numerous plants and herbs have been recognized as a source of natural antioxidants. Among them, rosemary (*Rosmarinus officinalis* L.) is one of the *Lamiaceae* plants with large antioxidant activity. The substances related with its antioxidant activity are phenolic diterpenes such as carnosol, rosmanol, carnosic acid, methyl carnosate, and phenolic acids such as rosmarinic and caffeic acids. Particularly, carnosic acid and carnosol are the most abundant antioxidants present in rosemary extracts [6-10].

On the other side, spinach (*Spinacia oleracea*) is an edible flowering plant (*Amaranthaceae* family) native to central and southwestern of Asia, now cultivated all over the world, which is renowned for its high content of carotenoids. Numerous studies about its anti-carcinogenic, antimicrobial and antioxidant activity of spinach have been reported in recent years [11-13]. Besides carotenoids (mainly lutein and β-carotene) [14], other bioactive substances identified in spinach are phenolic compounds, such as flavonoids and phenolic acids (p-cumaric, gallic and ferulic acids) [12, 15] and fatty acid derivative compounds, such as glycoglycerol lipids [16] and lipoic acid [17].

The extraction of antioxidants from plant matrix could be accomplished by different techniques. Solid-liquid extraction is a traditional and much utilized technology in which varying the solvent the recovery of target molecules could be attained. For example, carotenes are readily extracted using non-polar solvents (hexane, pentane, and petroleum ether) or moderate polar solvents (dichloromethane); phenolic compounds are usually extracted using water [12] and glycoglycerol lipids using ethanol or methanol [16]. As it is well-known, one of the main drawbacks of solid-liquid extraction is the large consumption of organic solvents. In this respect, pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) are intensively investigated as more efficient extraction technologies.

Several works were reported about the extraction of carotenoids of spinach using conventional solid-liquid extraction with different solvents. For example, Bunea et al. [14] determined the content of carotenoids in fresh, stored and processed spinach by using a

solvent mixture comprised by methanol, ethyl acetate and petroleum ether, Pellegrini et al. [18] extracted carotenoids of fresh spinach with acetone, and Simonovska et al. [19] quantified lutein in spinach extracts obtained using water and triethylammonium acetate. However, there is no bibliographic information, according to our knowledge, about the extraction of carotenoids of spinach by SFE or PLE. The latter has been used to extract flavonoids from spinach but no carotenoids were investigated [15] although this technique is readily used to extract these compounds from other vegetal matrix, such as algae or carrot by-products [20-23]. Moreover, there are very few studies focusing on to determine the antioxidant activity of extracts rich in spinach carotenoids.

With respect to the extraction of the phenolic diterpenes of rosemary many publications could be cited. The reader is referred to the works of García-Risco et al. [24], Fornari et al. [25], Herrero et al. [26, 27] or Hossain et al. [28] in which the most important contributions regarding the SFE or PLE of rosemary are discussed.

Mixed vegetal extracts are of high interest as target of new products due to the synergic effects among certain phytochemicals that could produce a much more active response. In this respect, the simultaneous extraction of a mixture of the different vegetal species is of high interest from a processing point of view, since manufacture costs may be considerable reduced. Thus, the product obtained from the extraction of the mixture of species should be of similar (or better) quality than the product obtained by mixing the separate extracts.

In this work, the PLE and SFE of a mixture of spinach and rosemary leaves (50 % weight of each plant) was investigated and compared with the extraction of the separate species, with the target of assess the effect on the antioxidant quality of the products obtained. To our knowledge, this is the first time that the simultaneous extraction of spinach and rosemary leaves is studied. Carotenoids and phenolic diterpenes, due to their lipid affinity, can be readily extracted using non-polar paraffinic solvents, such as pentane, hexane or heptane fractions, so as CO_2, which at 12 MPa and 320 K has a density, and thus solvent power, similar to that of liquid pentane (626 kg/m^3) [29]. Thus, hexane was employed in PLE assays and pure supercritical CO_2 in the SFE experiments.

The extraction yield and recovery of selected antioxidant substances, namely β-carotene and lutein in spinach, and carnosic acid and carnosol in rosemary, were studied in terms of the composition of the plant matter employed as raw material. Additionally, the antioxidant activity of the different extracts was evaluated in order to determine potential synergic effects among these main antioxidants present in these vegetal species.

2. Materials and methods

2.1 Chemicals and reagents

Carnosic acid (≥96 %) and Carnosol were purchased from Alexis Biochemical (Madrid, Spain). β-carotene (95 %), ABTS [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] and potassium persulfate were purchased from Sigma-Aldrich (Madrid, Spain). Lutein (≥95 %) was purchased from Extrasynthese (Genay Cedex, France). Ethanol and phosphoric acid (85 %) were HPLC grade from Panreac. Acetonitrile, methanol and methyl-tert-butyl ether were HPLC grade from Lab Scan (Gliwice, Poland). Triethylamine was purchased from Sigma-Aldrich (Madrid, Spain). CO_2 (N38) was supplied from Air Liquid. Washed sea sand (particle size 0.25-0.30 mm) was purchased from Panreac (Barcelona, Spain).

2.2 Preparation of samples

Plant material consisted of dried leaves obtained from an herbalist's producer (Murcia, Spain). Water content in the spinach and rosemary samples was, respectively, 4.9 % weight and 8.3 % weight. The samples were ground in a cooled mill and were sieving to the appropriate size (between 200 and 600 µm). Thus, similar particle size was obtained for each batch of plant matrix. The 50:50 mixture of spinach and rosemary was obtained by homogenization of same amounts of ground rosemary and spinach.

2.3 Extraction methods

Pressurized liquid extraction (PLE): extractions with liquid hexane were carried out in an ASE 350 system from Dionex Corporation (Sunnyvale, CA, USA) equipped with a solvent controller unit. Hexane was selected as PLE solvent due to the good solubility that carotenoids and carnosic acid exhibit in this solvent.

Each extraction cell (10 ml capacity) was filled with 1 g of solid sample and 1 g of sea sand as a sandwich, and then placed into an oven. Then, the cell was filled with hexane up to a pressure of 1500 psi (which ensures the liquid state of the solvent at both temperatures studied) and was heated-up to the desired temperature. Static extractions were performed at 100 and 150ºC during 10 minutes. After extraction the cell was washed with the solvent and subsequently the solvent was purged from cell using N_2 gas until complete depressurization

was accomplished. The extracts were recovered in glass vials and the solvent was eliminated by evaporation under vacuum and then dried in a stream of N_2. All experiments were carried out by duplicate. The dried samples obtained were stored at 4 ºC in the dark until analysis.

Supercritical fluid extraction (SFE): trials were carried out in a pilot-plant scale supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylinder extraction cell with automatic control of temperature and pressure. A detail explanation of the experimental device can be found elsewhere [30].

For each experiment, the cell was filled, respectively, with 0.42 kg of spinach leaves, 0.50 kg of rosemary leaves and 0.46 kg of the mixture 50:50 spinach + rosemary, which correspond to the mean values of the amounts employed for spinach and rosemary. The extractions were performed at 40°C and two different pressures (20 and 30 MPa) were employed. No cosolvent was employed since both carotenoids and phenolic diterpenes can be satisfactory extracted using pure CO_2. The extraction time was 5 h, no fractionation of the extract was performed and the supercritical solvent (CO_2) flow rate was set to 60 g/min in all experiments. Extraction conditions were selected on the basis of previous works [24, 30] related with SFE of rosemary. Considering the different amount of raw material loaded to the extraction cell, the CO_2/plant ratio were respectively 43, 39 to 36 kg/kg for spinach, rosemary and the spinach + rosemary mixture.

Ethanol was used to wash out the collector vessel and ensure a complete recovery of the material precipitated in the cell. Ethanol was eliminated by evaporation and the homogeneous solid samples obtained were kept at 4°C in the dark until analysis. All experiments were carried out by duplicate.

2.4 HPLC analysis

Quantification of carnosic acid and carnosol: samples were analyzed employing a HPLC (Varian Pro-star) equipped with a MICROSORB-MV-100 C18 column (Varian) of 250 mm × 4.6 mm and 5 µm particle size. The analyses were carried out at ambient temperature (20°C). The mobile phase consisted of acetonitrile (solvent A) and 0.1 % (v/v) of phosphoric acid in water (solvent B) applying the following gradient: 0–8 min, 23 % A and 8-25 min, 77 % A. This last composition was kept for 15 minutes and initial conditions were gained in 5 min. Total time analysis was 45 minutes. The flow rate was constant at 0.7 mL/min. Injection volume was 20 µL and the detection was accomplished by using a diode array detection system Varian storing the signal at a wavelength of 230 and 280 nm.

Quantification of β-carotene and lutein: samples were analyzed employing a HPLC model Agilent 1260 Infinity (Agilent, Santa Clara, USA) equipped with a KROMASIL 100 C18 column (Scharlab, Barcelona, Spain) of 25 mm × 4.6 mm and 3.5 µm particle size. The mobile phase is constituted by solvent A, MeOH:H_2O (90:10) and solvent B, MTBE:MeOH:H_2O (90:6:4). 0.1 % (v/v) of triethylamine was added to both solvents. The gradient started with 93 % A to 0 % A from 0 to 34 min and recovers the initial conditions of the method in 4 min. Total time analysis was 38 minutes. During analysis the column was maintained at 25ºC. The flow rate was constant at 1 mL/min and the injection volume was 20 µL. For detection were assigned the wavelength of 450, 470, 550, 660 nm.

2.5 Determination of antioxidant activity

$ABTS^{\bullet+}$ assay. The $ABTS^{\bullet+}$ assay described by Re et al. [31] was used to measure the antioxidant activity of the extracts. Briefly, $ABTS^{\bullet+}$ radical cation was generated by reacting 7 mmol/l ABTS with 2.45 mmol/l potassium persulfate after incubation at room temperature for 16 h in the dark. The $ABTS^{\bullet+}$ radical solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. 10 µl of each extract (previously dissolved) at four different concentrations was added to 0.990 ml of diluted $ABTS^{\bullet+}$ radical solution. The reaction was allowed to stand until the absorbance reached a plateau, and the absorbance was recorded at 734 nm. Trolox was used as reference standard, and results were expressed as TEAC (Trolox Equivalent Antioxidant Capacity) values (mmol Trolox/g extract). All analyses were done in triplicate.

Synergy assays. Synergy assays were done between carotenoids (β-carotene and lutein) and phenolic compounds (carnosic acid and carnosol) at three different levels. Results were compared with the estimated values calculated to each mixture. Moreover, this synergy assay was also carried out with a mixture of spinach and rosemary extracts.

3. Results and discussion

3.1 Extraction yield

The extraction techniques and conditions investigated are summarized in Table 1. For experiments 1 to 4 of Table 1, the raw materials extracted were (i) spinach leaves, (ii) rosemary leaves and (iii) the mixture comprising 50:50 weight spinach and rosemary leaves.

The extraction yields obtained in the different extractions are given in Table 2 and represented in Figure 1: S denotes spinach leaves (0% rosemary), SR the mixture 50% spinach and 50% rosemary, and R represents 100% rosemary leaves.

While the objective of the present work is the comparison of the simultaneous extraction of spinach + rosemary leaves with the extraction of the isolated species, some conclusions could be derived by comparison of PLE with SFE. As can be observed in Table 2, extraction yields were higher in PLE than in SFE for all vegetal matter extracted. Furthermore, the temperature increase in PLE from 100°C to 150°C produces a significant increase in yield because higher temperature promotes higher analyte solubility, decreases the viscosity and surface tension of solvents, thus improving extraction rate. On the other hand, increasing the extraction pressure from 20 to 30 MPa in SFE produce a minor increase of extraction yield.

The comparison of the yields obtained (both PLE and SFE assays) in the simultaneous extraction of spinach + rosemary leaves with the yield obtained when extracting the isolated species is depicted in Figure 1. A mixture 50:50 weight of each plant was selected since it is probable that the higher deviations from the mean (linear) behavior should be produced for this mixture.

As can be observed in Figure 1, for both extraction methods applied and for all conditions employed, a linear correlation between the composition of the raw material and the extraction yield was obtained. Lineal regression coefficients (R^2) were higher than 0.97 in all cases and thus, it can be stated that the influence of extracting mixed species on extraction yield is not noteworthy. That is, the extraction yields obtained experimentally when processing the mixed leaves ($Y_{SR}^{\exp}$) are very close to the yields calculated as the mean values of the yields obtained in the extraction of the separate plants ($Y_{SR}^{cal} = (Y_S^{\exp} + Y_R^{\exp})/2$).

Figure 2 shows the $Y_{SR}^{\exp}$ and Y_{SR}^{cal} values together with the corresponding (experimental and calculated) standard deviations; for all type of extractions and solvents differences between both values are not significant. Then, despite the extraction procedure or conditions applied, the results obtained suggest that yield is not significantly enhanced or reduced when the mixture spinach/rosemary is extracted in comparison with processing the separate species.

3.2 Antioxidant activity and chemical analysis of samples

The antioxidant activity of the samples obtained by extraction of the mixture of species (SR) was compared with the antioxidant activity of the pure extracts (S and R) and their mixture (S+R).

As can be observed in Table 3 for extractions 1 and 4 of Table 1, rosemary extracts (R) are much more active than spinach extracts (S). Chohan et al. [32] and Tawaha et al. [33] studies showed lower TEAC values for conventional aqueous and methanol rosemary extracts that could be explain for the higher extraction of potent antioxidant components such as carnosic acid or carnosol with SFE or PLE with non-polar solvents. Regarding to spinach activity not many studies have been focused on the study of the antioxidant capacity of lipophilic extracts. In this respect, Isabelle et al. [34] reported similar results using ORAC assay in hexane extracts, whereas Pellegrini et al. [18] observed higher TEAC values in acetone extracts, together with higher carotenoid content, in acetone extracts. Additionally, the sample denoted as S+R in Table 3, which was obtained by mixing equal amounts of S and R, presents TEAC values very close to the corresponding calculated mean value (differences lower than 2.3%). Nevertheless, the product indicated as SR in Table 3, which was obtained by the simultaneous extraction of spinach and rosemary leaves (50:50), resulted in noteworthy higher antioxidant activity. That is, the TEAC values of SR samples are around 20% higher than the TEAC values of the S+R samples. This effect could be attributed to small modifications in the composition of the extracts, due to the presence of both raw materials in the extraction cell, or even to synergistic effect between the antioxidant substances present in spinach and rosemary. In this regard, Hait-Dashan et al. [5] reported a synergistic activity between a spinach extract rich in aromatic polyphenols [32] and some phenolic compounds such as ferulic acid, caffeic acid and epigallocatechin-3-gallate. In order to elucidate whether small increase of certain components or synergistic effect between them explain the higher TEAC values of SR in comparison to S+R mixture, chemical characterization of the extracts was done, the main antioxidant substances in the extracts were identify and the potential synergistic effect between them was investigated using standards.

The concentrations (mg compound / g extract) of phenolic diterpenes (carnosic acid and carnosol) and carotenoids (β-carotene and lutein) were determined for all extracts obtained and are reported in Table 4. Figure 3 shows an example of chromatograms obtained for SFE extracts obtained by processing only spinach leaves, only rosemary leaves and the mixture 50:50 spinach + rosemary leaves. As expected, carnosic acid and carnosol were not detected to be present in spinach extracts and only very low concentrations of carotenoids were

determined in rosemary extracts. Furthermore, taking into account the low polarity of solvents employed, no phenolic acids, such as rosmarinic acid in rosemary or ferulic acid in spinach, were detected in the samples.

The higher concentrations of carnosic acid were obtained using hexane as solvent (R and SR extracts) and carnosol was found in very low concentrations in all samples studied. About the quantification of carotenoids, significant higher concentrations of β-carotene were obtained in the SFE extracts and, according to the higher polarity of lutein in comparison with β-carotene, higher CO_2 density were required to obtain significant concentration of lutein in the SFE extracts (Extraction 4). Nevertheless, in fact higher amounts of β-carotene were extracted using PLE than SFE from spinach leaves in both experiments, although lower contents were detected in PLE extracts due to their higher extraction yields (32.0, 32.7, 19.9 and 24.2 mg/ 100 of dry spinach leaves, corresponding to 1 to 4 experiments). In this way, β-carotene contents of PLE extracts are in agreement with literature data about β-carotene content of spinach leaves [14, 18], whereas lower amounts of lutein were obtained in all the extracts presented in this study. This result could be due to by the fact that more polar solvents, i.e. acetone, is commonly used for total carotenoid determination, and therefore hexane or supercritical CO_2 only produced a partial extraction of lutein.

Table 4 also reports the expected concentration of antioxidant compounds in SR extracts, calculated as the mean value of the concentrations obtained in the extraction of the separate plants (S and R samples). In the case of β-carotene, it can be clearly stated that its extraction is reduced when the mixed raw material is processed, with experimental concentrations around 1.5 times lower in the SFE extracts, and ca. 2.5 times lower in the hexane PLE extracts. Figure 4 shows a comparison of the experimental β-carotene concentrations obtained in SR extracts and the corresponding mean values. Also represented in the figure are the standard deviations obtained, which indicate that differences are significant and thus, it could be accepted the observed decrease of β-carotene extraction in SR samples. In general, this behavior was also observed for lutein, particularly in the case of the SFE extractions.

On the contrary, according to the results given in Table 4 for carnosic acid, it could be argued that the extraction of carnosic acid is enhanced when the mixed material is processed (see Figure 5).

Among target compounds studied, β-carotene and lutein were the main carotenoids identified in spinach extracts, and carnosic acid and carnosol were the main phenolic diterpenes

quantified in rosemary extracts. Thus, synergistic assays between β-carotene or lutein and carnosic acid or carnosol were carry out. The TEAC values obtained for the mixtures of carnosic acid + β-carotene or lutein are given in Table 5 and those corresponding to carnosol + β-carotene or lutein mixtures are reported in Table 6. According to the TEAC values obtained the order of antioxidant capacity of the standards is as follows: carnosic acid > β-carotene > carnosol ≈ lutein. As can be observed in these tables, for all phenolic compound + carotenoid mixtures studied no synergic enhancement of the antioxidant activity was observed when comparing the experimental TEAC value of the mixture with the corresponding calculated mean (linear) TEAC value. On the contrary, it was obtained a general decrease of the TEAC value of the phenolic compound + carotenoid mixture with respect to the corresponding mean theoretical value in certain cases.

As an example, Figure 6 shows the comparison between (a) the variation of TEAC values in carnosic acid + β-carotene mixtures and (b) the TEAC values obtained in the samples produced by extracting spinach, rosemary and a mixture 50:50 of spinach and rosemary leaves (SR) (Extractions 1 and 4 in Table 1). As can be observed in Figure 6, the mixtures of carnosic acid + β-carotene show similar TEAC values than the expected mean values, moreover, TEAC values of S+R showed a similar behavior, whereas the antioxidant activity of SR was significant enhanced in comparison with the expected mean value. Therefore, taking into account the analysis of the composition of the extracts given in Table 6, it could be stated that the observed increase of the antioxidant activity of the SR extracts could be a consequence of an enhancement of the extraction of carnosic acid, produced when both raw materials (spinach and rosemary) are simultaneously extracted, and synergistic effects between carotenoids from spinach and phenolic diterpenes from rosemary could be discarded. Furthermore, Figure 7 shows that the TEAC values of all extracts obtained (S, R, SR and S+R) could be satisfactory correlated with the concentration of carnosic acid present in the sample.

Conclusions

The product obtained from the simultaneous extraction of spinach and rosemary leaves was investigated to ascertain an enhancement of antioxidant activity, due to presumed potential synergic effects between carotenoids from spinach and phenolic diterpenes from rosemary.

PLE using hexane and SFE with pure CO_2 were utilized as extraction technologies; these solvents were selected due to their good affinity to extract carotenoids and carnosic acid.

The product obtained from the extraction of a mixture 50:50 spinach and rosemary leaves (SR) was compared with the extraction of solely spinach (S) and rosemary (R), and with the sample obtained by mixing equal amounts of S and R (S+R sample).

The antioxidant activities of the SR extracts were 20% higher than the antioxidant activities of the S+R samples, which is a very attractive result in order to target new spinach-rosemary mixed products. This effect could be explained by an increase in the concentration of carnosic acid observed in the SR extracts, which was around 10-20% greater than the expected mean values, as not synergic effects between carotenoids (β-carotene and lutein) of spinach and phenolic diterpenes (carnosic acid and carnosol) of rosemary were found.

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Table 1. Methods and conditions employed in the extraction of spinach, rosemary and mixed spinach/rosemary (50:50) leaves.

Extraction number	Extraction method	Solvent	T (°C)	P (MPa)	Extraction time	solvent / raw material ratio (kg/kg)
1	ASE	hexane	100	10	10 min	18
2	ASE	hexane	150	10	10 min	18
3	SFE	CO_2	40	20	5 h	36-43
4	SFE	CO_2	40	30	5 h	36-43

Table 2. Yields obtained (%) in extractions 1 to 4 of Table 1. S: spinach leaves; SR: spinach/rosemary (50:50) leaves; R: rosemary leaves.

Extraction number	Plant matrix		
	S	SR	R
1	4.26 ± 0.33	6.65 ± 0.62	9.87 ± 0.46
2	7.16 ± 0.27	10.91 ± 0.31	15.63 ± 0.62
3	1.75 ± 0.16	2.66 ± 0.11	3.14 ± 0.36
4	1.82 ± 0.16	2.76 ± 0.35	4.45 ± 0.86

Table 3. TEAC values of the samples obtained by extraction of spinach leaves (S), rosemary leaves (R), the mixture of species (SR) and by mixing the pure extracts (S+R).

	Experimental value* (mmol Trolox/g)	Calculated mean value (mmol Trolox/g)
PLE, hexane, 100°C (Ext. 1 in Table 1)		
S	0.229 ± 0.005	
R	0.721 ± 0.018	
SR	0.565 ± 0.004	
S+R	0.475 ± 0.008	0.475
SFE, 30 MPa, 40°C (Ext. 4 in Table 1)		
S	0.109 ± 0.001	
R	0.578 ± 0.009	
SR	0.420 ± 0.006	
S+R	0.352 ± 0.002	0.344

*Mean ± Standard Deviation.

Table 4. Composition (mg of compound / g of extract) of antioxidant compounds identified in the extracts obtained from experiments 1 to 4 of Table 1. S: spinach leaves; SR: spinach/rosemary (50:50) leaves; R: rosemary leaves. EXP: experimental value; MV: calculated mean value.

Extraction number	Plant matrix	Phenolic compounds[a]				Carotenoids[b]			
		carnosic acid		carnosol		β-carotene		lutein	
		EXP	MV	EXP	MV	EXP	MV	EXP	MV
1	S	n.i.	80.75	n.i.	6.82	7.52	3.88	1.49	0.75
	SR	101.13		3.93		1.47		1.01	
	R	161.49		13.64		0.23		n.i.	
2	S	n.i.	52.10	n.i.	4.81	4.57	2.37	0.88	0.45
	SR	58.13		1.81		0.97		0.64	
	R	104.20		9.62		0.17		0.01	
3	S	n.i.	26.44	n.i.		11.38	6.01	0.58	0.29
	SR	30.19		n.i.		4.10		n.i.	
	R	52.88		n.i.		0.64		n.i.	
4	S	n.i.	47.42	n.i.	3.41	13.28	7.10	4.70	2.35
	SR	54.34		0.00		5.08		0.53	
	R	94.84		6.81		0.92		n.i.	

n.i.: non-identified

[a] Mean standard deviations for carnosic acid and carnosol quantification were, respectively, 6.04 and 0.97.

[b] Mean standard deviations for β-carotene and lutein quantification were, respectively, 0.22 and 0.03.

Table 5. TEAC values of carnosic acid, β-carotene, lutein and their mixtures.

(a) Carnosic acid + β-carotene

Carnosic acid (%)	β-carotene (%)	TEAC value (mmol Trolox/g)	
		Experimental value*	Calculated mean value
100	0	5.548 ± 0.076	-
63	37	5.057 ± 0.192	5.079
36	64	4.710 ± 0.101	4.737
15	85	4.513 ± 0.061	4.479
0	100	4.296 ± 0.104	-

(b) Carnosic acid + lutein

Carnosic acid (%)	Lutein (%)	TEAC value (mmol Trolox/g)	
		Experimental value*	Calculated mean value
100	0	5.722 ± 0.154	-
57	43	4.821 ± 0.463	4.911
30	70	4.144 ± 0.142	4.423
13	87	4.049 ± 0.060	4.093
0	100	3.859 ± 0.084	-

*Mean ± Standard Deviation.

Table 6. TEAC values of carnosol, β-carotene, lutein and their mixtures.

(a) Carnosol + β-carotene

Carnosol (%)	β-carotene (%)	TEAC value (mmol Trolox/g)	
		Experimental value*	Calculated mean value
100	0	3.724 ± 0.058	-
75	25	3.542 ± 0.093	3.862
50	50	3.837 ± 0.116	4.000
25	75	3.860 ± 0.137	4.138
0	100	4.276 ± 0.044	-

(b) Carnosol + lutein

Carnosol (%)	Lutein (%)	TEAC value (mmol Trolox/g)	
		Experimental value*	Calculated mean value
100	0	3.884 ± 0.071	-
69	31	3.770 ± 0.182	3.807
43	57	3.589 ± 0.058	3.741
20	80	3.676 ± 0.299	3.683
0	100	3.633 ± 0.040	-

*Mean ± Standard Deviation.

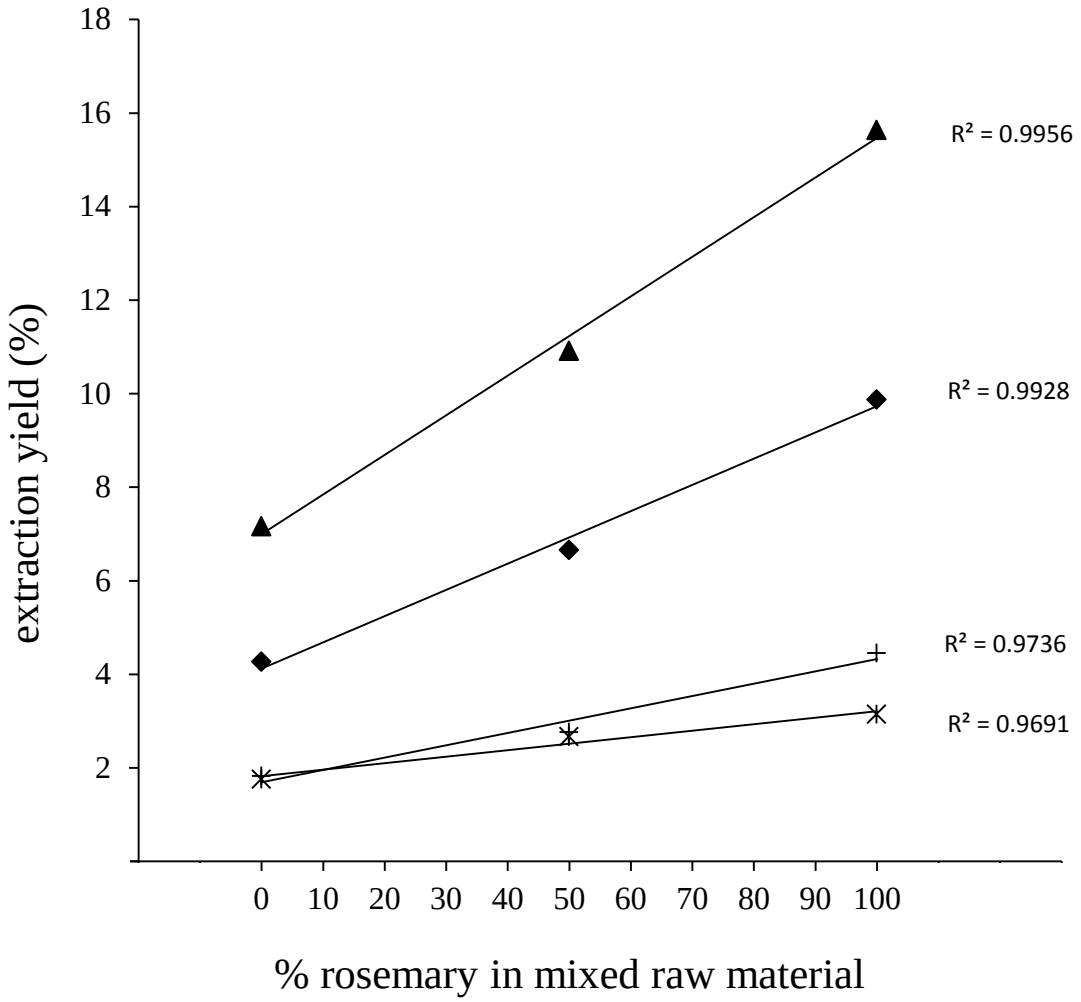

Figure 1. Extraction yield as a function of the percentage of rosemary leaves present in plant raw material: (◆) Hexane ASE at 100°C (Ext. 1); (▲) Hexane ASE at 150°C (Ext. 2); (∗) CO_2 SFE at 20 MPa (Ext. 3); (+) CO_2 SFE at 30 MPa (Ext. 4).

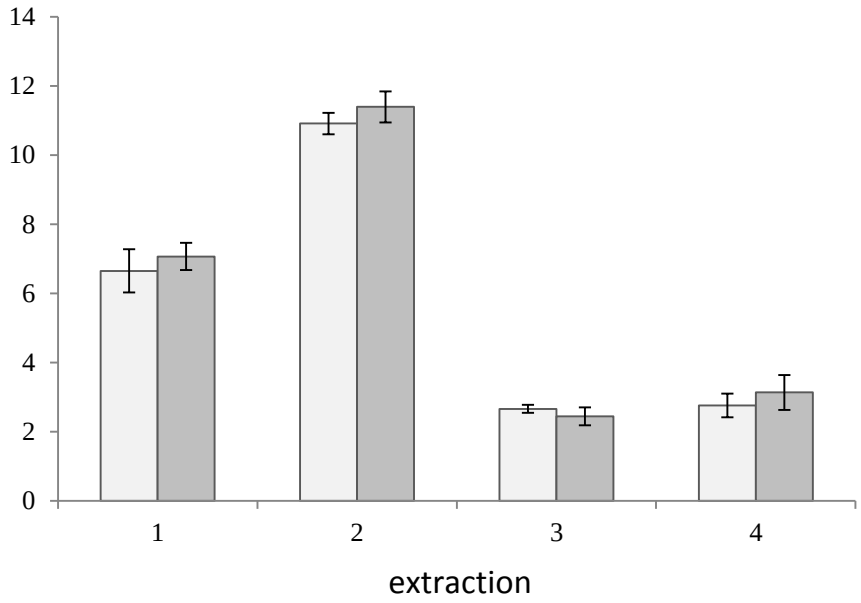

Figure 2. Extraction of spinach/rosemary leaves mixture (50 weight % of each plant): comparison between (▭) experimental yields $Y_{SR}^{\exp}$ and (▭) yields calculated as the mean values of the yields obtained in the extraction of the separate plants ($Y_{SR}^{cal} = (Y_S^{\exp} + Y_R^{\exp})/2$). 1 and 2: PLE; 3 and 4: SFE.

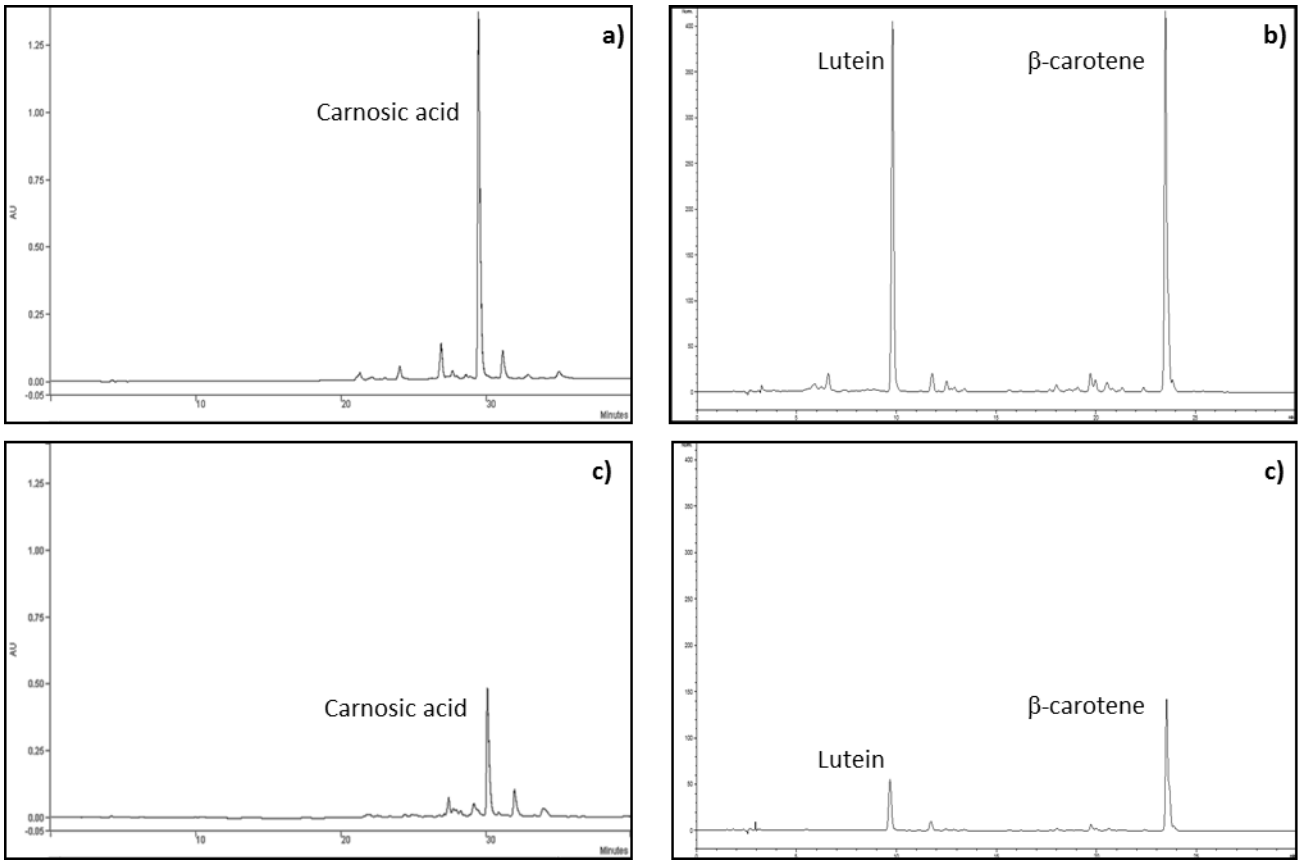

Figure 3. Chromatograms of SFE extracts obtained by processing (a) only rosemary leaves, (b) only spinach leaves and (c) the mixture 50:50 spinach + rosemary.

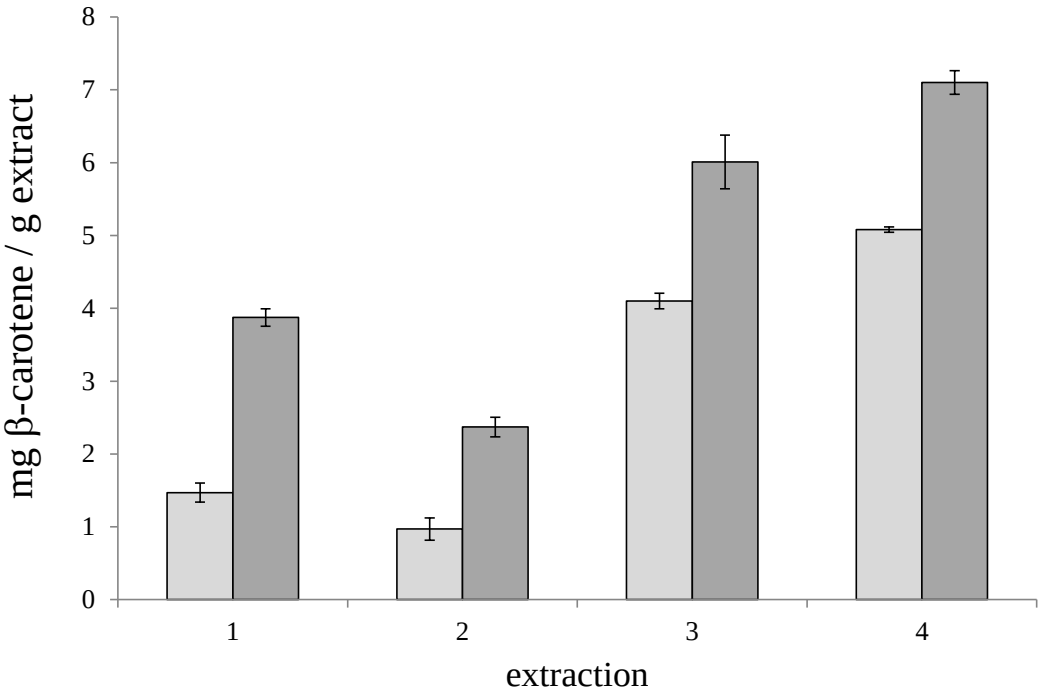

Figure 4. Spinach/rosemary leaves mixture (50 weight % of each plant): comparison between (▭) experimental β-carotene concentrations and (▭) values calculated as the mean values of the concentrations obtained in the extraction of the separate plants. 1 and 2: PLE; 3 and 4: SFE.

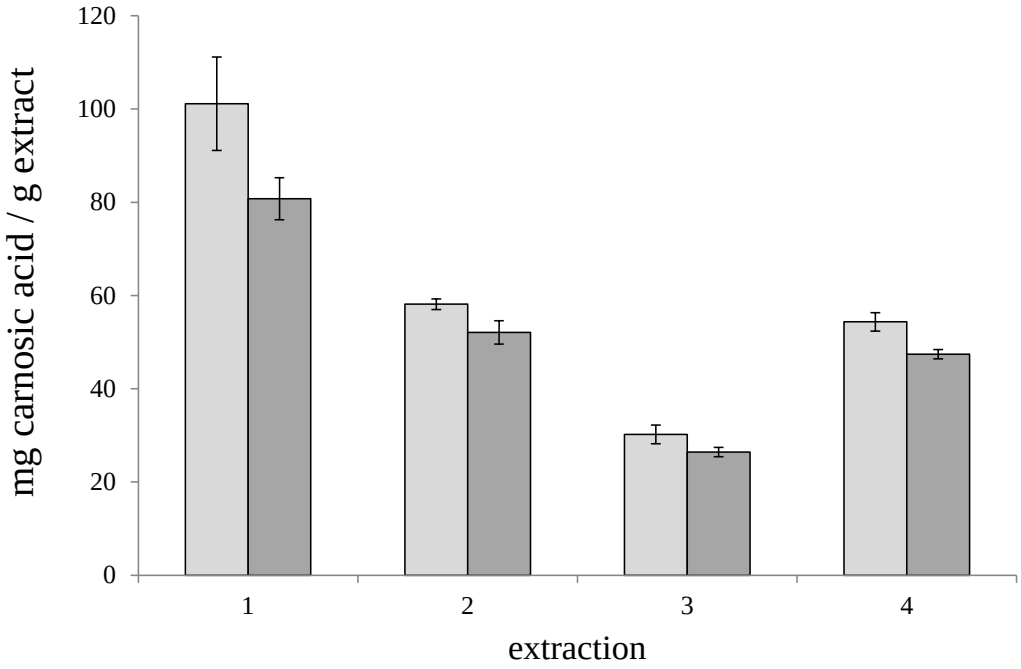

Figure 5. Spinach/rosemary leaves mixture (50 weight % of each plant): comparison between ( ) experimental carnosic acid concentrations and ( ) values calculated as the mean values of the concentrations obtained in the extraction of the separate plants. 1 and 2: PLE; 3 and 4: SFE.

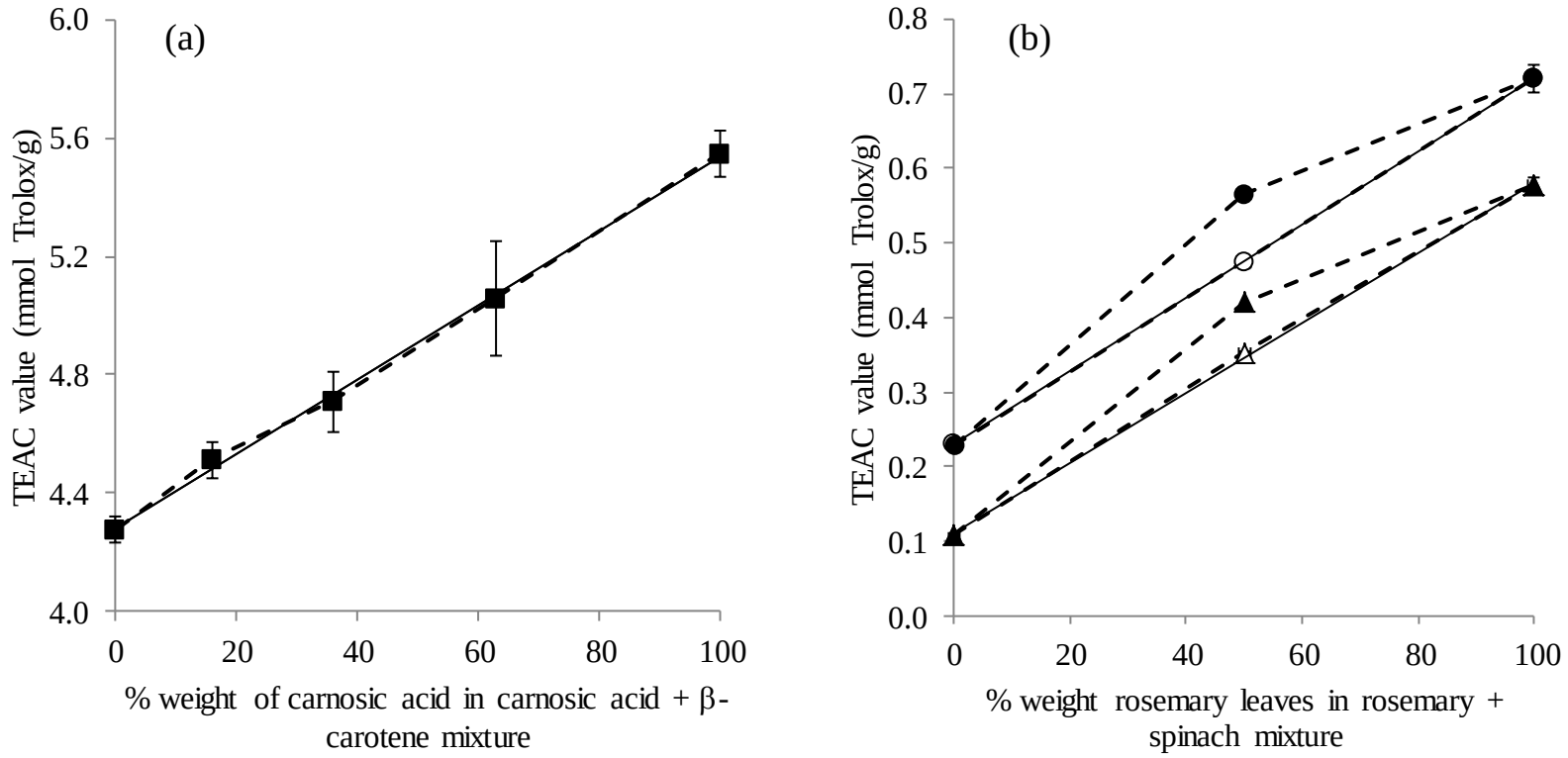

Figure 6. Comparison between (a) the variation of TEAC values in carnosic acid + β-carotene mixtures and (b) the TEAC values obtained in the samples produced by extracting spinach leaves (S), rosemary leaves (R) and a mixture 50:50 of spinach and rosemary leaves (SR). (●) PLE with hexane at 100°C (Ext. 1 in Table 1); (▲) SFE at 30 MPa and 40°C (Ext. 4 in Table 1). Empty symbols represent the mixture of spinach and rosemary extracts (S + R).

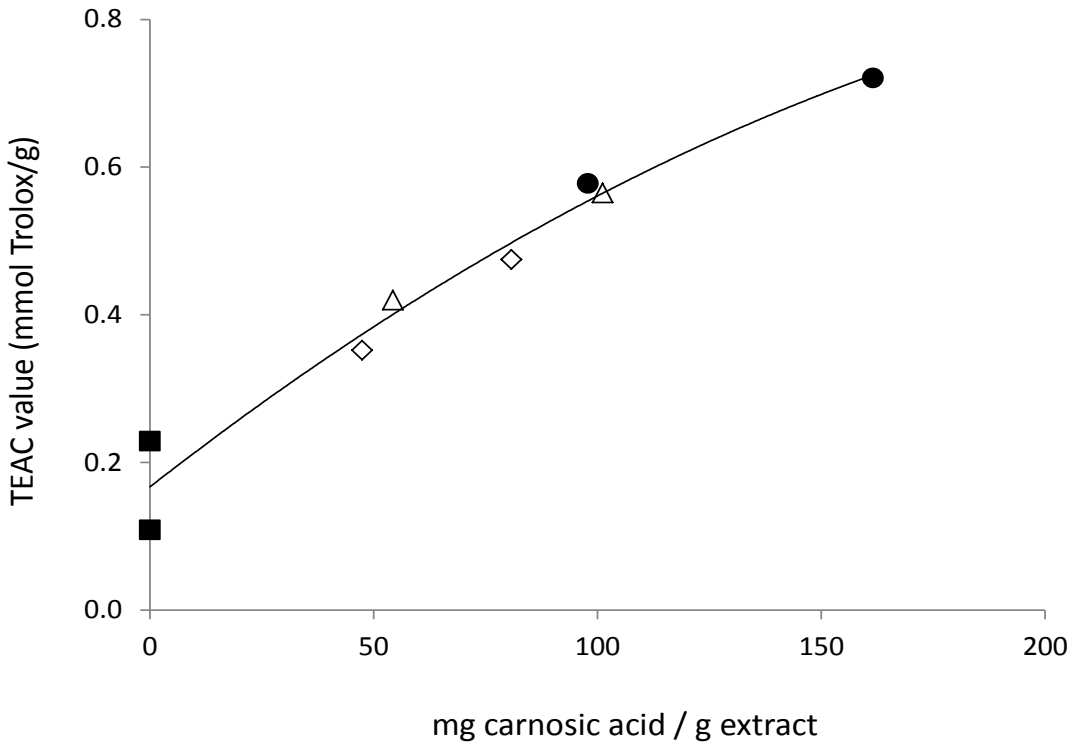

Figure 7. Variation of the TEAC value of the (■) S, (●) R, (△) SR and (◇) S+R extracts obtained from experiments 1 (PLE) and 4 (SFE) of Table 1. Solid line: general trend.