Effect of the simultaneous extraction of rosemary and spinach leaves on the antioxidant activity of the extracts

Erika Vázquez, Gonzalo Vicente, Mónica R. García-Risco, Guillermo Reglero and Tiziana Fornari*

Instituto de Investigación en Ciencias de la Alimentación CIAL (CSIC-UAM)
Universidad Autónoma de Madrid, 28049 Madrid, Spain.
Corresponding autor: tiziana.fornari@uam.es Phone: +34 910017927 Fax: +34910017905

ABSTRACT

Several spices and some herbs have received increased attention as sources of effective natural antioxidants. Particularly, rosemary (*Rosmarinus officinalis* L.) is a Lamiaceae plant with high content of phenolic antioxidant substances, and spinach (*Spinacia oleracea* L.) is a natural source of carotenoids (lutein and β-carotene) which were also recognized to possess antioxidant activity.
In this work the simultaneous extraction of leaves of a rosemary/spinach mixture (50 weight % of each plant) was investigated and compared with the extraction of the separate species. Three different extraction techniques were applied: (i) solid-liquid extraction at ambient pressure and using ethanol or hexane at 50°C, (ii) accelerated solvent extraction (ASE) with the same liquid solvents but at higher temperatures (100 and 150°C) and pressure (100 bar); and (iii) supercritical fluid extraction (SFE) with pure CO_2 at 40°C and different pressures (200 and 300 bar).
All extracts obtained were studied in terms of the presence of antioxidant substances. Carotenoids and phenolic compounds were identified and quantified by HPLC analysis. Despite the extraction procedure and conditions applied, the results obtained show a linear behavior in terms of extraction yield and composition of key antioxidant compounds. The antioxidant activity of all samples was determined by the DPPH test and the effect of the simultaneous extraction (the mixed spinach/rosemary plant matrix) on the antioxidant activity of the extracts was analyzed and discussed.

INTRODUCTION

Natural sources of bioactive substances, as well as new industrial approaches to extract and isolate these substances from raw materials, are gaining much attention in the food and pharmaceutical research field. Particularly, the production of plant extracts has received increasing interest in recent decades [1, 2]. Indeed, one key target is the recovery of antioxidant compounds from natural matter [3, 4].
On one side, antioxidants in food play a very important role since oxidation is one of the major causes of food chemical spoilage. Use of synthetic antioxidants has been related to health risks resulting in strict regulation of their use in foods. Further, the increased consumer consciousness of food safety, create a need for identifying alternative, natural and probably safer sources of food antioxidants [5, 6].
On the other side, antioxidants such as vitamins C and E, carotenoids (β-carotene, lycopene, lutein), phenolic compounds and many other substances, are recognized to play a role in helping to prevent diseases such as cancer, cardiovascular disease, Alzheimer's disease and macular degeneration. Antioxidants are thought to help because they can neutralize free radicals [7, 8], which are toxic byproducts of natural cell metabolism. Although the human body naturally produces antioxidants but the process effectiveness declines with age. In this respect, research is increasingly showing that those who eat antioxidant-rich foods reap health benefits.
Among the different natural sources of antioxidants, spices and herbs are gaining much attention [3, 4]. For example, rosemary (*Rosmarinus officinalis* L.) is identified as one of the plants with large content of phenolic antioxidants. Main substances associated with the antioxidant activity of rosemary extracts are the phenolic diterpenes such as carnosol, rosmanol, carnosic acid, methyl carnosate, and phenolic acids such as the rosmarinic and caffeic acids [9-12]. Additionally, Spinach (*Spinacia oleracea* L.) is a natural source of carotenoids, principally lutein and β-carotene [13], which also have antioxidant activity [14, 15]. Carotenoids are natural pigments which are synthesized by plants and are responsible for the bright colors of various fruits and vegetables. β-carotene is the best studied, since it is the most common carotenoid in fruits and vegetables. β-carotene and others carotenoids have shown antioxidant properties *in vitro* and in animal models [14, 16]. Further, mixtures of carotenoids or associations with others antioxidants (e.g. vitamin E) can increase their activity against free radicals [15, 17, 18], and in many studies phenolic compounds demonstrated higher antioxidant activity than antioxidant vitamins and carotenoids [19, 20].

In this work the extraction of leaves of rosemary, spinach and mixtures rosemary/spinach (50 weight % of each plant) was investigated and compared. Rosemary was selected as natural source of phenolic-type antioxidants and spinach as natural source of carotenoid-type antioxidants. Simultaneous extractions of rosemary and spinach leaves were investigated to study the effect of the simultaneous extraction on the recovery of both classes of antioxidants. Extraction of mixed plants is of high interest from an economical point of view since bioactive phytochemicals may act synergistically and thus, a product with particular added value can be obtained from the simultaneous extraction.
Three different extraction procedures, namely solid-liquid extraction, accelerated solvent extraction and supercritical CO_2 extraction were applied. The antioxidant activity of the samples was evaluated by quantification of main phenolic compounds (carnosic acid and rosmarinic acid) and carotenoids (β-carotene and lutein) identified, and by comparing the EC_{50} value of the DPPH test.

MATERIAL AND METHODS

Chemicals and reagents
2, 2- Diphenil-1-pycril hydrazyl hydrate (DPPH, 95% purity) were purchased from Sigma-Aldrich (Madrid, Spain). Carnosic acid (≥96%) was purchased from Alexis Biochemical (Madrid, Spain). Rosmarinic acid (97%) and β-carotene (95%) 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 was HPLC grade from Lab Scan (Dublin, Ireland). CO_2 (N38) was supplied from Carburos Metálicos (Spain). Washed sea sand (particle size 0.25-0.30 mm) was purchased from Panreac (Barcelona, Spain).

Preparation of samples
Plant material consisted of dried leaves (8.3 % w/w moisture) obtained from an herbalist's producer (Murcia, Spain). The leaves were ground in a cooled mill (Lleal Group, Granollers, Spain) and samples were sieving to 200-600 μm size.

Extraction methods
The solid-liquid extractions were carried out using 1 g of sample with 100 mL of ethanol or hexane at 50ºC in a Stuart Orbital S150 shaker apparatus (Bibby Scientific Limited. Stone, UK) during 24 h.
ASE extractions with the two different liquid solvents (ethanol and hexane) were carried out in an Accelerated Solvent Extraction system ASE 350 from Dionex Corporation (Sunnyvale, CA, USA) equipped with a solvent controller unit. Extractions were performed at two different extraction temperatures (100 and 150ºC) during 10 minutes. The cells employed (10 ml capacity) were placed into an oven; each cell was filled with around 1.5 g of solid sample and 1.5 g of sea sand as a sandwich.
Supercritical extractions were carried out in a pilot-plant scale supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylinder extraction. For each experiment, the cell was filled with 0.4-0.5 kg of plant raw material. The extractions were performed at two different pressure of 20 and 30 MPa. Extraction temperature was 313 K in all experimental assays and CO_2 flow was 3.6 kg/h in all experiments (CO_2/plant = 35-45 kg/kg). No fractionation of the extracted material was accomplished. Extract was recovered from separator cell using ethanol.
After extractions, solvent was eliminated by evaporation under vacuum and the extract was dried in a stream of N_2 to constant weight. All experiments were carried out by duplicate. Mean difference between extraction yields obtained in the duplicates were lower than 2.4% in the case of solid-liquid extractions, 6.2% for the ASE assays and 5.1% for the SFE trials. The dried samples obtained were stored at 4ºC until analysis.

HPLC analysis
In order to quantify the carnosic acid content in the rosemary extracts, samples were analyzed employing a HPLC (Varian Pro-star) equipped with a Microsorb-100 C18 column (Varian) of 25 cm × 4.6 mm and 5 μm particle size. The mobile phase consisted of acetonitrile (solvent A) and 0.1% of phosphoric acid in water (solvent B) applying the following gradient: 0–8 min, 23% A, 8-25 min, 75% A, 25-40 min 75% A and the 40-45 min 23% A. Initial conditions were gained in 5 min. 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, 280 and 350 nm. For identification and quantification of β-carotene and lutein, the samples were analyzed employing a HPLC (Agilent 1260 Infinity) equipped with a KROMASIL 100 C18 column (Scharlab) of 25 mm × 4.6 mm and 3.5 μm particle size. The mobile phase is constituted by solvent A, methanol: water:triethylamine (90:10:0.1, v/v/v) and solvent B, methyl-tert-butyl ether: methanol:water:triethylamine

(90:6:4:0.1, v/v/v/v). The gradient applied was 93% A to 0% A during 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 in an oven. 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.

Determination of antioxidant activity

The method consists in the neutralization of free radicals of DPPH by an antioxidant sample. An aliquot (50 µl) of chloroform-ethanol (50:50) solution containing 5-30 µg/ml of rosemary extract, was added to 1.950 µl of DPPH in ethanol (23.5 µg/ml) prepared daily. Reaction was completed after 3 h at room temperature and absorbance was measured at 517 nm in a Nanovette Du 730 UV spectrophotometer (Beckman Coulter, USA). The DPPH concentration in the reaction medium was calculated from a calibration curve determined by linear regression ($y = 0.0265 \cdot x$; $R^2 = 0.9998$). Ethanol was used to adjust zero and DPPH-ethanol solution as a reference sample. The amount of extract necessary to decrease the initial DPPH concentration by 50% or EC50 (µg/ml) was determined and employed to value the antioxidant power of the sample; the lower the EC50, the higher the antioxidant power.

RESULTS AND DISCUSSION

As explained before, three different extraction procedures were investigated (see Table 1): solid-liquid extraction (SLE) at ambient pressure and 50°C employing a Stuart Orbital shaker apparatus, and using polar (ethanol) and non-polar (hexane) solvents; accelerated solvent extraction (ASE) at temperatures higher than the normal boiling point of the solvents employed (ethanol and hexane); and supercritical fluid extraction (SFE) with pure CO_2 at 40°C and two different extraction pressures (200 and 300 bar). In all cases, the raw materials extracted correspond to (i) spinach leaves; (ii) rosemary leaves and (iii) a mixture comprising 50:50 weight spinach and rosemary leaves.

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 (bar)	extraction time	solvent / raw material ratio (kg/kg)
1	SLE	ethanol	50	1.01	24 h	79
2	SLE	hexane	50	1.01	24 h	65
3	ASE	ethanol	100	113	10 min	18
4	ASE	hexane	100	113	10 min	18
5	ASE	ethanol	150	113	10 min	18
6	ASE	hexane	150	113	10 min	18
7	SFE	CO_2	40	200	5 h	33-54
8	SFE	CO_2	40	300	5 h	33-54

Table 2. Global yields. S: spinach leaves; SR: spinach:rosemary (50:50) leaves; R: rosemary leaves.

extraction number	plant matrix		
	S	SR	R
1	10.14	14.24	20.66
2	2.84	4.93	7.39
3	11.24	16.22	19.35
4	4.26	6.65	9.87
5	23.50	26.28	27.63
6	7.16	10.91	15.63
7	1.75	2.66	3.14
8	1.82	2.76	4.45

The global extraction yields obtained in the different extractions accomplished are given in Table 2. As expected, solvent liquid extraction (SLE and ASE) produced higher yields than CO_2-SFE. ASE extracts with similar yields than those obtained with SLE were produced in considerably shorter extraction times and with lower amounts of solvent. Further, despite the temperature employed, extraction yields were higher using ethanol than using hexane.

Figure 1 show the extraction yields obtained in experiments 1 to 8 as a function of the percentage of rosemary leaves present in the plant raw material matrix: 0% corresponds to solely spinach leaves (S); 50% to the mixed matrix spinach:rosemary (50:50) (SR) and 100% corresponds to solely rosemary leaves (R).
As can be observed in Figure 1, despite the extraction method applied or the conditions employed, a linear correlation between the raw material composition and the global yield was obtained. Lineal regression coefficients (R^2) were higher than 0.96 in all cases and thus, it can be stated that the influence of extracting mixed species on global yield is not important. That is, the extraction yield obtained experimentally when processing the mixed leaves (Y_{SR}) correspond to the expected mean values of the yield obtained in the extraction of the separate plants, i.e. $Y_{SR} \approx (Y_S + Y_R) / 2$.

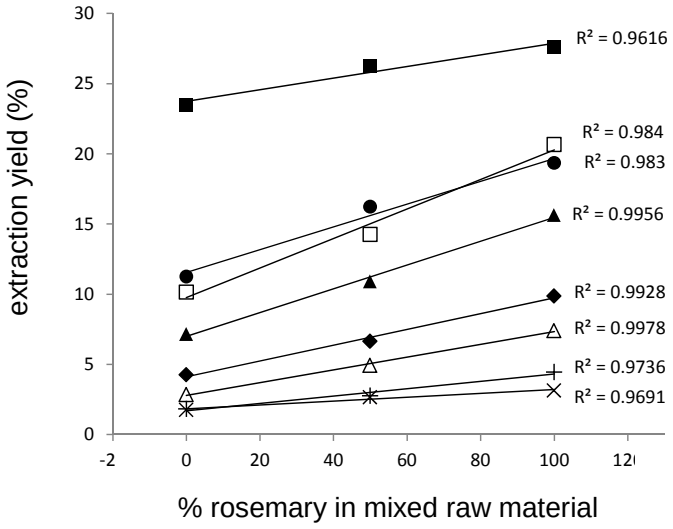

Figure 1. Global extraction yield as a function of the percentage of rosemary leaves present in plant raw material: (□) ext. 1; (△) ext. 2; (●) ext. 3; (◆) ext. 4; (■) ext. 5; (▲) ext. 6; (×) ext. 7; (+) ext. 8.

The concentration (% w/w) of phenolic antioxidants (carnosic acid and rosmarinic acid) and carotenoids (β-carotene and lutein) are reported in Table 3, for all extractions accomplished and for the three different plant matrix employed: S, SR and R.
As expected, despite the extraction procedure applied, the phenolic compounds were not detected to be present in spinach extracts. Additionally, according to the solutes and solvents polarity, CO_2 and hexane were more effective than ethanol to extract β-carotene and carnosic acid, while ethanol was more selective than CO_2 or hexane to extract rosmarinic acid. In fact, rosmarinic acid could not be detected in the extracts obtained with hexane or supercritical CO_2 and similar concentrations of this phenolic acid were obtained in all alcoholic samples.
The higher concentrations of carnosic acid were obtained using hexane as solvent (R and SR extracts); rosmarinic acid was identified only SLE and ASE alcoholic extracts. About the carotenoids quantification, 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 8 in Table 1).
Table 4 gives the linear regression coefficients (R^2) obtained in the correlation of the concentration of the antioxidant compounds (Table 3) with the plant matrix composition. The high R^2 values obtained reveal an insignificant effect of employing the mixed raw material (SR) on the removal of the key antioxidant compounds. That is, the R^2 values close to one suggest that the extraction of a given antioxidant is not enhanced or reduced when the SR mixture is employed as raw material. This effect was observed despite the extraction procedure applied (SLE, ASE or SFE) and the conditions of temperature and pressure employed.

Table 3. Composition (% w/w) of antioxidant compounds identified. S: spinach leaves; SR: spinach:rosemary (50:50) leaves; R: rosemary leaves.

extraction number	plant matrix	carnosic acid	rosmarinic acid	β-carotene	lutein
1	S	-	-	-	-
	SR	5.85	1.24	-	-
	R	10.16	2.44	-	-
2	S	-	-	0.13	-
	SR	9.74	-	0.05	-
	R	16.52	-	-	-
3	S	-	-	3.10	1.92
	SR	4.70	0.84	0.86	0.52
	R	9.36	2.15	-	-
4	S	-	-	7.52	1.17
	SR	10.11	-	1.47	0.78
	R	16.15	-	-	-
5	S	-	-	2.34	0.91
	SR	4.34	1.33	0.98	0.52
	R	8.36	2.95	-	-
6	S	-	-	4.57	0.68
	SR	5.81	-	0.97	0.49
	R	10.42	-	-	-
7	S	-	-	11.38	0.33
	SR	3.02	-	4.1	0.13
	R	5.10	-	-	-
8	S	-	-	13.28	3.63
	SR	5.43	-	5.08	0.29
	R	9.48	-	-	-

The antioxidant capacity of the extracts produced was determined and compared by means of the EC_{50} value obtained using the DPPH test. Figure 2 show the EC_{50} values (μg/ml) of all extracts produced when using 100% rosemary plant matrix when using the different extraction procedures (SLE, ASE and SFE). As can be observed in the figure, a clear relation between the EC_{50} value obtained and the % w/w of phenolic compounds (carnosic acid + rosmarinic acid) present in the samples can be established.

The effect of simultaneous extraction of spinach:rosemary (50:50) leaves on the EC_{50} values of the samples produced is given in Figure 3, in which the data presented in Figure 2 is also depicted as sake of comparison. According to the analysis (based on the DPPH test), the extracts produced with the SR plant matrix show the same trend observed in Figure 2. That is, the antioxidant effect is mainly determined by the concentration of phenolic compounds present in the samples, despite the fact that in some SR samples (namely, the SFE extracts) carotenoids are present in the extract in the same concentration that phenolic compounds (see Table 3).

Table 4. Linear regression coefficients (R^2) obtained in the correlation of the concentration (% w/w) of mean antioxidants identified in the extracts as a function of the percentage of rosemary leaves present in plant material.

extraction number	carnosic acid	rosmarinic acid	β-carotene	lutein
1	0.992	0.999	-	-
2	0.989	-	-	-
3	0.999	0.981	0.924	0.935
4	0.975	-	0.874	0.964
5	0.999	0.996	0.984	0.993
6	0.995	-	0.881	0.939
7	0.989	-	0.974	0.985
8	0.993	-	0.976	0.810

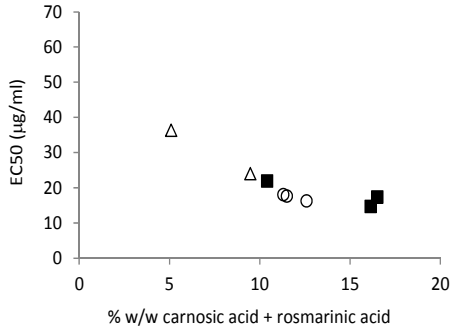

Figure 2. EC_{50} values of R extracts as a function of the % w/w of carnosic acid + rosmarinic acid using SLE, ASE and SFE: (■) hexane; (○) ethanol; (△) $SCCO_2$.

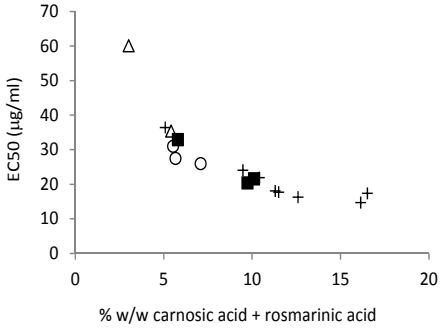

Figure 3. EC_{50} values of SR extracts (SLE, ASE and SFE) as a function of the % w/w of carnosic acid + rosmarinic acid: (■) hexane; (○) ethanol; (△) $SCCO_2$. Symbol (+) represent the data of R extracts (Figure 2).

CONCLUSIONS

The extractions of a mixture (50:50) of rosemary and spinach leaves were compared with the extractions of the separate plant matrixes. Different extraction methods (solid-liquid, pressurized solvent extraction and supercritical extraction) and conditions (solvent, temperature, pressure) were applied. In all cases, extraction yield and concentration of main antioxidants (phenolic compounds and carotenoids) was very close to the mean value expected considering the results of the separate extractions. That is, no influence on the recovery of antioxidant from plant matrix was observed when the mixed rosemary + spinach leaves were extracted.
According to the DPPH test, the antioxidant activity of the extracts is mainly determined by the amount of phenolic compounds (carnosic acid + rosmarinic acid) present in the samples. This result can be supported on the basis of the higher antioxidant activities reported for phenolic compounds in comparison with carotenoids.

ACKNOWLEDGES

This work has been supported by project INNSAMED IPT-300000-2010-34 (subprogram INNPACTO) from Ministerio de Ciencia e Innovación (Spain) and Comunidad Autónoma de Madrid (project ALIBIRD-S2009/AGR-1469).

REFERENCES

[1] POURMORTAZAVI, S.M., HAJIMIRSADEGHI, S.S., J. of Chromatogr. A, 1163, **2007**, p. 2.
[2] HERRERO, M., CIFUENTES, A., IBAÑEZ, E., Food Chem., 98, **2006**, p. 136.
[3] M. SUHAJ, J. Food Compos. Anal., 19, **2006**, p. 531.
[4] AHN, J., GRÜN, I.U., FERNANDO, L.N., J. Food Sci., 67, **2002**, p. 1364.
[5] MCCARTHY, T. L., KERRY, J. P., KERRY, J. F., LYNCH, P. B., BUCKLEY, D. J., Meat Sci., 57 **2001**, p. 177.
[6] WILLIAM, G. M., IATROPOULOS, M. J., WHYSHER, J., Food Chem. Toxicol., 37, **1999**, p. 1027.
[7] LILA, M. A., ANN, N. Y., Acad. Sci., 1114, **2007**, p. 372.
[8] VAINIO, H., WEIDERPASS, E., Nutr. Cancer. 54, **2006**, p. 111.
[9] IBÁNEZ, L., KUBÁTOVÁ, A., SEÑORÁNS, F. J., CAVERO, S., REGLERO, G., HAWTHORNE, S. B., Food Chem., 51, **2003**, p. 375.
[10] CAVERO, S., JAIME, L., MARTÍN-ALVAREZ, P. J., SEÑORÁNS, F.J., REGLERO, G., IBÁÑEZ, E., Eur. Food Res. Technol., 221, **2005**, p. 478.

[11] VICENTE, G., GARCÍA-RISCO, M.R., FORNARI, T., REGLERO, G., Chem. Eng. Technol., Accepted November 2011.
[12] CHANG, C-H., CHYAU, C-C., HSIEH, C-L., WU, Y-Y., KER, Y-B., TSEN, H-Y., PENG, R.Y., Nat. Prod. Res., 22, **2008**, p. 76.
[13] BUNEA, A., ANDJELKOVIC, M., SOCACIU, C., BOBIS, O., NEACSU, M., VERHE´, R., VAN CAMP, J., Food Chem., 108, **2008**, p. 649.
[14] KRINSKY, N. I., Nutrition, 17, **2001**, p. 815.
[15] STAHL, W., SIES, H., Mol. Aspects Med., 24, **2003**, p. 345.
[16] RODRÍGUEZ-AMAYA, D. B., J. Food Compos. Anal., 23, **2010**, p. 726.
[17] KRINSKY, N. I., YEUM, K.-J., Biochem. Bioph. Res. Co., 305, **2003**, p. 754.
[18] EDGE, R., MC GARVEY, D. J., TRUSCOTT, T. G., J. Photoch. Photobio. B., 41, **1997**, p. 189.
[19] RE, R., PELLEGRINI, N., PROREGGENTE, A., PANNALA, A., YANG, M., RICE-EVANS, C., Free Radical Bio. Med., 26, **1999**, p. 1231.
[20] VELIOGLU, Y. S., MAZZA, G., GAO, L., OOMAH, B. D., J. Agric. Food Chem., 46, **1998**, p.4113.