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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 wt.% 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 was 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.

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

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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 wt.% 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 wt.% and 8.3 wt.%. 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 150 °C during 10 min. 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 °C 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, 39–36 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