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Liquid chromatographic method for the analysis of tocopherols in malt sprouts with supercritical fluid extraction

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Abstract

A simple, specific and sensitive high-performance liquid chromatographic method has been developed for the determination of tocopherols in malt sprouts. A supercritical fluid extraction (SFE) procedure was used to isolate tocopherols from the vegetal matrix before quantitative analysis. The analytes were separated on a Zorbax reversed-phase column using methanol–water as mobile phase and quantified by measuring its fluorescence at λ_{em}=328 nm after excitation of the analytes at λ_{exc}=303 nm. The limits of detection for α-, γ- and δ-tocopherols were 0.04, 0.05, and 0.05 μg/ml, respectively. The calibration graphs of the method were linear from 0.1 to 1.5, 0.2 to 2.5, and 0.2 to 2.0 μg/ml, for α-, γ- and δ-tocopherols, respectively. This SFE and HPLC procedure is simple, precise and accurate for the determination of tocopherols in malt sprouts.

Keywords: Supercritical fluid extraction; Extraction methods; Food analysis; Tocopherols; Vitamins

1. Introduction

Vitamin E is an important natural antioxidant in foods, especially those rich in polyunsaturated fatty acids [1]. Due to its role as a scavenger of free radicals, vitamin E is also believed to protect our bodies against degenerative malfunctions, mainly cancer and cardiovascular diseases [2]. Natural vitamin E is composed of eight chemical compounds: α-, β-, γ- and δ-tocopherols and four corresponding tocotrienols. Different methods are described in the literature for analysis of vitamin E by gas chromatography (GC) [3–5] as well as by high-performance liquid chromatography (HPLC) [6–9]. This report describes the development of a method for the extraction of tocopherols in malt sprouts using supercritical fluid extraction (SFE), followed by reversed-phase HPLC separation and fluorimetric detection.

2. Experimental

2.1. Chemicals and reagents

α-, γ- and δ-tocopherols were purchased from Fluka (Buchs, Switzerland). The concentration of each tocopherol standard solution can be determined in according to the European standard method. Methanol (HPLC grade) was obtained from Carlo Erba (Milan, Italy). Butylated hydroxytoluene

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(BHT) was purchased from Sigma–Aldrich (Milan, Italy). All SFE experiments were completed with supercritical fluid-grade carbon dioxide purchased from Rivoira (Chieti, Italy). Modifiers for SFE experiments were HPLC-grade solvents. Malt sprouts were kindly supplied by Agroalimentare Sud (Melfi, Italy). Water (HPLC grade) was obtained by an ELIX 3 Water System and passage through a Milli-Q Academic water-purification system (Millipore, Bedford, MA, USA).

2.2. High-performance liquid chromatography

HPLC analysis was carried out using a chromatographic system composed of a Model 515 pump (Waters, Milford, MA, USA) and a Model LS 30 Luminescence Spectrometer (Perkin-Elmer). A Model 7725i sample injector (Rheodyne, Cotati, CA, USA) equipped with a 20-μl loop was used. Chromatographic data management was automated using a Millennium^{32} chromatography manager data acquisition system (Waters). Chromatographic separations were carried out using 250×4.6 mm I.D. Zorbax reversed-phase analytical column, 5 μm particle size (Rockland Technologies, Nuenen, The Netherlands) equipped with a 20×4.6 mm Supelguard LC-18 precolumn (Supelco, Bellefonte, PA, USA). Detection was carried out by measuring the fluorescence at λ_{em}=328 nm after excitation of the analytes at λ_{exc}= 303 nm. The mobile phase consisted of a mixture of methanol and water (98:2, v/v). Methanol prior to use was filtered through an FA 0.5 μm filter (Millipore). The mobile phase was prepared daily, was degassed using an in-line degasser (Waters) and delivered at a flow-rate of 2.0 ml/min at room temperature.

2.3. Standard solutions and calibration curves

Stock solutions of α-, γ- and δ-tocopherols were prepared by dissolving 10 mg of each analyte in 10 ml of methanol containing 0.05% of BHT. BHT is added to prevent tocopherol oxidation. These solutions were stored in aluminum foil-covered containers and kept at −20°C. Standard solutions, each containing the three analytes, were prepared in the concentration range of 0.1 to 1.5 μg/ml for α-tocopherol, 0.2 to 2.0 μg/ml for γ-tocopherol, and 0.2 to 2.0 μg/ml for δ-tocopherol. For each solution the concentration of the other two analytes was kept constant at 1.0 μg/ml. The calibration curves were obtained by plotting the peak height of each analyte versus its concentration.

2.4. Sample preparation

After reception, malt sprouts were extracted as received using a HP-7680 supercritical fluid extractor (Agilent Technologies, Milan, Italy). A typical extraction run involved the extraction of about 1 g of malt sprouts. The optimal extraction conditions were determined to be 250 bar, 80°C, carbon dioxide flow-rate: 1 ml/min. Trap temperature was set at 25°C. Adsorbing solid-phase material (ODS) was used to recover extracted compounds from the SFE procedure. The quantitative trapping of tocopherols was checked during all the work by sparging the carbon dioxide leaving the trap in a methanol–0.05% BHT solution and determining its tocopherol content, no tocopherols were found in this solution. Methanol at a flow-rate of 1.5 ml/min was used as rinsing solvent for tocopherols and was collected in amber vials. Aliquots of methanolic solutions were used for HPLC analysis.

3. Results and discussion

Structures of the investigated three tocopherols are depicted in Fig. 1. SFE is widely perceived as a technique for the extraction of low to moderately polar compounds. Recent studies have shown that the use of supercritical fluids as an extraction media provides a powerful alternative to traditional extraction methods [10,11]. The potential benefits of SFE include: faster analysis; reduced sample handling; protection from degradation by light, heat, or oxygen; high loadability of samples and possibility of trace analysis; elimination of hazardous and/or expensive solvents. The gentle extraction conditions used in SFE compared to more traditional extractions such as Soxhlet extraction also provides greater assurance against chemical reaction not taking place during the extraction.

Carbon dioxide is the most popular choice of

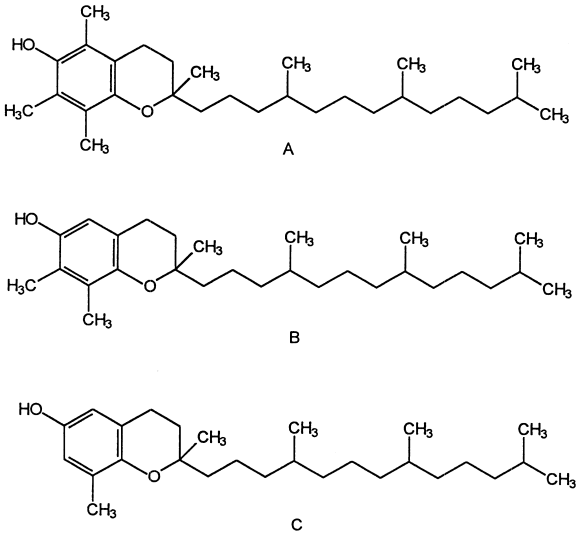

Fig. 1. Chemical structures of α-tocopherol (A), γ-tocopherol (B), and δ-tocopherol (C).

supercritical fluid for extraction of target analytes from solid or liquid matrices [12–16]. However, it should be noted that even a high solubility of analytes in the supercritical fluid does not always ensure their quantitative extraction, unless proper extraction conditions and extraction time are used [17]. The location of the analyte in or on the matrix is critical: when the analyte is inside the matrix it is not easily accessible to carbon dioxide and the extraction process is made in three steps: disruption of the interaction of the analyte with the active sites; diffusion of the analyte through the solid matrix and solubilization in the supercritical fluid [18,19]. The rate of the first and second step can vary according to the nature of the interactions between the analyte and the solid matrix, and according to the characteristics of the solid matrix, particularly for what concerns the solubility of the supercritical fluids in the solid matrix. These features can greatly lengthen the extraction time and can require that the extraction pressure is significantly increased. The conditions reported in the literature for the extraction of vegetable matrix with supercritical carbon dioxide are in the range from 80 to 300 bar and from 35 to 90°C [12,13]. In this work extraction of malt sprouts was performed at pressure from 100 to 300 bar and 40 to 80°C. The use of a carbon dioxide modifier (methanol) gave no improvement in the extraction efficiency. For an extraction time of 180 min the total tocopherol yield was 21 μg/g of malt sprouts at 250 bar and 80°C; 14 μg/g of malt sprout at 250 bar and 40°C and 8 μg/g at 117 bar 40°C, in all the tests the carbon dioxide flow-rate was 1 ml/min. Lower carbon dioxide flow-rates were tested, but, irrespective of the pressure and temperature conditions, lower tocopherol yields were obtained.

To evaluate the effectiveness of the process, repeated extractions were performed in dynamic mode on the same sample, up to a total extraction time of 360 min. After each extraction step the rinsing of the trap was repeated five times, and the tocopherol content of each amber vial was determined, showing that no tocopherol was detected in the samples coming from the last two rinsing steps. Fig. 2 shows the results of six repeated extraction steps of malt sprouts with carbon dioxide. It is evident that almost complete extraction is attained after 270 min.

The calibration curves for HPLC analysis were obtained by plotting the peak height of each analyte versus its concentration. The calibration graph for α-tocopherol was linear from 0.1 to 1.5 μg/ml, for δ-tocopherol from 0.2 to 3.5 μg/ml and for γ-tocopherol from 0.2 to 2.0 μg/ml. The equations, obtained through regressional analysis of data for the above standard solutions (each datum is the average of a minimum number of seven determinations) were: for α-tocopherol $y = 1.56 \cdot 10^6 x - 9.50 \cdot 10^4$

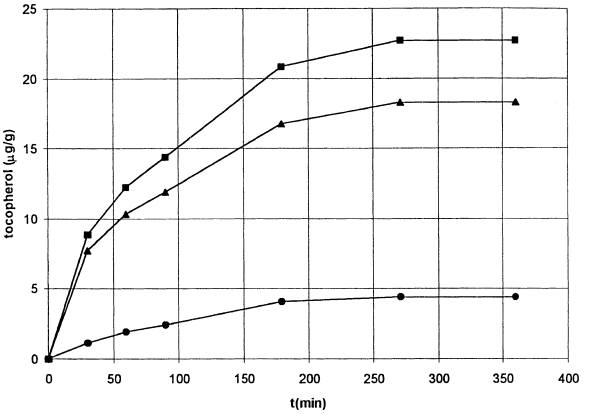

Fig. 2. SFE profiles of tocopherols from malt sprouts. ■, Total tocopherols; ▲, α-tocopherol; ●, γ-tocopherol.

($r = 0.994$); for γ-tocopherol $y = 5.45 \cdot 10^6 x + 2.90 \cdot 10^5$ ($r = 0.999$), and for δ-tocopherols $y = 9.36 \cdot 10^6 x + 1.70 \cdot 10^4$ ($r = 0.999$); were y is the peak height in the arbitrary units of the Millennium[32] chromatography manager data acquisition system and x is the analyte concentration (μg/ml). From these equations the concentration of the analytes can be determined. The precision and accuracy of the method was determined by preparing pools of standard solutions containing α-, γ- and δ-tocopherols at seven different concentrations. The values for α-, γ- and δ-tocopherols for each standard concentration were determined by seven repeated analyses. The RSD values for the slope were 2.3% for α-tocopherol, 1.8% for γ-tocopherol and 2.5% for δ-tocopherol. The standard errors were comprised between 1.3 and 1.7% for α-tocopherol; between 2.1 and 2.4% for γ-tocopherol and between 1.7 and 2.0% for δ-tocopherol. Retention times for α-, γ- and δ-tocopherols were 10.08, 8.38, and 6.93 min, respectively. No endogenous matrix components were observed near the retention times corresponding to α-, γ- and δ-tocopherols.

Representative chromatograms of SFE extracts obtained under the conditions described above for α-, γ- and δ-tocopherol standard and samples of malt sprouts extracted are shown in Fig. 3. The limits of detection for α-, γ- and δ-tocopherols were 0.04, 0.05, and 0.05 μg/ml, respectively.

The sample preparation described in this paper introduces a highly selective procedure of supercritical fluid extraction with carbon dioxide of tocopherols. The performance of the method was checked extracting the same malt sprouts lot with different liquid solvents (i.e. acetone, ethanol, methanol, chloroform) and it was found that the extraction with supercritical carbon dioxide gives results comparable with those obtained using acetone and better than those obtained using the other organic solvents. In addition, in the supercritical carbon dioxide extracts, lower amounts of interfering compounds are found in comparison with the organic solvent extracts and it is possible to avoid the saponification step.

After supercritical carbon dioxide extraction exhaust malt sprouts were again extracted with acetone, in order to evaluate the residual tocopherol content. Results obtained by HPLC analysis of these samples after dryness and dissolution in mobile phase showed the absence of tocopherols. The optimization of the analytical procedure has been carried out by varying the following: reversed-phase column used, mobile phase composition, flow-rate, monitoring wavelength. The degree of reproducibility of the results obtained through small deliberate variations in method parameters and by changing instruments and operators has been very satisfactory. The procedure described is very simple and rapid and should be of value for the quantitation of these analytes in malt sprouts.

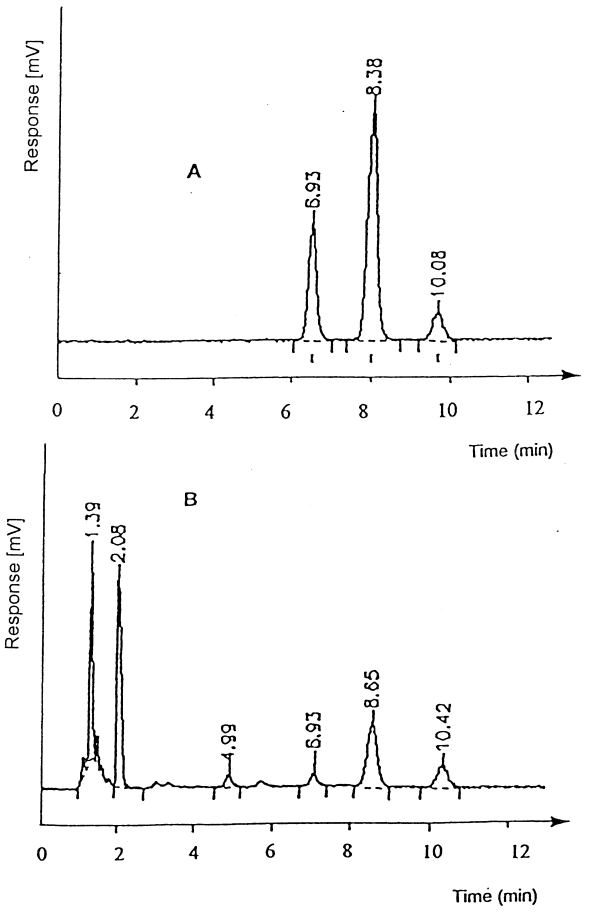

Fig. 3. HPLC profiles of a standard solution (A) and a sample extract (B).

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