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Short communication

Simultaneous determination of α-tocopherol and β-carotene in olive oil by reversed-phase high-performance liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatographic method was developed for the determination, in one run, of α-tocopherol and β-carotene in virgin olive oil. The method involved a rapid saponification and a later extraction with a mixture of hexane–ethyl acetate. The chromatographic system consists of an ODS-2 column with a mobile phase of methanol–water–butanol and a diode-array detector. Linearity, precision, recovery and sensitivity were satisfactory. The main advantage of the proposed method is the speed and simultaneous determination of both compounds at the same time.

Keywords: Olive oil; Food analysis; Vitamins; Tocopherols; Carotenes; Antioxidants

1. Introduction

Virgin olive oil, which is obtained from the fruits of *Olea europaea* by mechanical means and undergoes no chemical treatment, is one of the few vegetable oils consumed in its natural state. Consequently it is a good source of antioxidants such as polyphenols, α-tocopherol and β-carotene [1]. The levels of these components in the oil depend on genetic, agronomic and environmental factors [2–5]. α-Tocopherol is the most active form of vitamin E in vivo [6] and β-carotene is the most important provitamin A source [7,8]. Both are involved in the oxidative stability [1,4,6,9] of the oil and have a protective role against cancer and cardiovascular diseases [10,11].

Prior to the analysis of carotenoids and tocopherols in oils, saponification has often been used to remove triacylglycerides and chlorophylls [12,13]. It is claimed that, after saponification, high-performance liquid chromatography (HPLC) methods have a greater specificity than colorimetric or fluorimetric procedures. Although some papers deal with the HPLC analysis of each compound separately [14–16], few report the quantification of both vitamins in one single run, and normal-phase (NP) HPLC methods are usually employed [4,5]. Reversed-phase (RP) HPLC versus NP-HPLC offers certain practical advantages, such as better column stability, reproducibility of retention times and faster equilibration [17,18].

The aim of this study was to develop and validate a rapid RP-HPLC method for the simultaneous

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determination of α-tocopherol and β-carotene in olive oil. On the other hand, this work attempts to minimize the amount of solvents used for the extraction, so the method may be suitable for the routine determination of these components in oil.

2. Experimental

2.1. Equipment

HPLC separation was performed with a Hewlett-Packard liquid chromatographic system (Waldbron, Germany) equipped with a HP-1050 pump system, and a Rheodyne Model 7125 injector (Cotati, CA, USA) with a final loop volume of 500 μl. A HP-1040M photodiode-array detection (DAD) system was also used. The data were stored and processed by an HPLC Chemstation (Dos Series, Hewlett-Packard). The column was a Tracer Extrasil ODS-2 (150×4.0 mm I.D., 5 μm particle size) (Tracer Analítica, Barcelona, Spain) protected by a guard cartridge system (Tracer, C_{18}, 5 μm).

A Labo Rota S-300 rotatory evaporator (Resona techins, Switzerland) with a Labo Term SW200 and a p-Selecta Unitronic-OR bath with a safety thermostat were used for sample preparation.

2.2. Reagents and standards

All standard compounds and the ascorbic acid were purchased from Sigma (St. Louis, MO, USA). A stock standard solution of α-tocopherol (2 mg/ml) was prepared in ethanol and another of β-carotene in hexane (0.5 mg/ml). Working standard solutions were prepared in methanol from the stock standard solutions every week, and were stored at −20°C in amber-coloured bottles for no longer than a month. A calibration graph was prepared just before the analysis. Ultra-pure water generated by the Milli-Q system (Millipore, Bedford, MA, USA) was used. HPLC-grade methanol, *n*-hexane and 1-butanol were obtained from SDS (Peypin, France). All other reagents, of analytical grade, such as absolute ethanol, 85% potassium hydroxide, sodium chloride and ethyl acetate were obtained from Panreac (Barcelona, Spain).

2.3. Sample preparation

Several samples of olive oil and virgin olive oil were analysed. Amber-coloured material, infrared light and the addition of an antioxidant (ascorbic acid) were necessary to prevent the loss of liposoluble vitamins during sample saponification and extraction. A 400-mg sample of oil was accurately weighed in a centrifugation screw-capped tube and then 0.2 g of ascorbic acid, 15 ml of absolute ethanol and 4 ml of 76% potassium hydroxide solution were added under a stream of nitrogen. The tubes were incubated at 70°C for 30 min with slow constant stirring.

A 5-ml volume of sodium chloride (25 g/l) was added after cooling and the suspension was extracted three times with 15-ml portions of *n*-hexane–ethyl acetate (85:15, v/v). The organic phase was evaporated to dryness at 40°C and the residue was dissolved in 0.5 ml of methanol. After passing through a 0.45-μm filter, the samples were injected for chromatographic analysis. They were kept at −20°C before injection but they only remained stable for a week.

2.4. Chromatographic conditions and quantification

The method involved one linear gradient and two isocratic steps. Solvent A was methanol, solvent B was Milli-Q water and solvent C was butanol. We began with A–B–C (92:3:5) for 3 min and went on to A–C (92:8) in 1 min. The composition was then held for 5 min and the system returned to the initial conditions. In 10 min the system was ready for a new injection. The analytical column was maintained at 45°C and the elution was performed at a flow-rate of 2 ml/min. A 50-μl aliquot of sample was injected. Optimum wavelengths were selected for the detection of α-tocopherol (292 nm) and β-carotene (450 nm).

To determine the compounds in the sample, the working standard solutions were always analysed along with the samples, and peak-area ratios were

used for calculations following the external standard method.

3. Results and discussion

3.1. Sample preparation

The official US Food and Drug Administration procedure for the determination of β-carotene in foods is cumbersome and time-consuming [19] because many steps are involved. Instead, the method proposed here is quicker because separating funnels to obtain the insaponifiable matter are not needed. Moreover, we attempted to minimize the amount of sample and solvents used, in order to simplify the process.

In a study to analyse the effects of sample preparation on fat soluble vitamins and carotenoid concentration, significant losses of α-tocopherol and isomerisation of β-carotene occurred. However, these losses can be reduced by using antioxidants during saponification [13]. We chose ascorbic acid because it does not interfere with the chromatographic analysis.

3.2. Dissolution of sample and standards

The choice of extraction and injection solvents of carotenoids and fat soluble vitamins plays a central role in the analysis. Many solvents and mixtures were tested for the ability to extract the insaponifiable matter. Finally a mixture of hexane–ethyl acetate was selected because it was a better extractand for β-carotene and α-tocopherol than hexane alone. We used HPLC-grade hexane because low-purity hexane could have induced isomerization of β-carotene and promoted artefact formation [20].

The injection solvent for the analysis of carotenoids by HPLC was carefully selected so that chromatographic artifacts were not produced. Khachick et al. [22] concluded that the polarity and solubility properties of injection solvent and mobile phase should be compatible in order to avoid peak distortion. Methanol was used because it was the major component in the mobile phase. Although β-carotene is significantly less soluble in methanol than in other solvents, that did not pose a problem since very low concentrations were used.

3.3. Mobile phase

Various mobile phases have been studied in order to optimise the simultaneous elution of both components. Since α-tocopherol and β-carotene are insoluble in water, the primary constituent in the mobile phase should be a weak organic solvent with low viscosity, which limits the choices to methanol and acetonitrile. Methanol has been recommended in several reports [23,24] because separations using acetonitrile or acetonitrile-based solvents have generally resulted in lower recoveries than those using methanol or methanol-based solvents. In order to achieve the desired retention and to increase solubility and selectivity, stronger organic modifiers such as tetrahydrofuran, ethyl acetate and chloroform were tested but butanol was found to be the most suitable since peak distortions did not occur.

3.4. Identification and quantification

The method proposed is specific for the determination of α-tocopherol and β-carotene in olive oils. The typical chromatogram obtained from the samples was relatively simple (Fig. 1), showing high resolution and a positive identification of both compounds in less than 8 min. Compounds were identified on the basis of retention time by comparison with standard solutions and through spectroscopic analysis. Standard calibration has always been a major source of errors when determining β-carotene in foods [20]. Since carotene standards are highly susceptible to oxidation and degradation, commercially available standards are often less pure than expected. Therefore, the stock standard solution of β-carotene was analysed separately by HPLC–DAD.

An internal standard method is unnecessary [20,21]. No improvement in precision was detected when β-apo-γ-carotenal was used as such, so the external standard method was simpler and more useful.

The calibration curves were prepared with standard solutions of each compound at levels similar to those contained in the oils. The method was linear in the range tested, between 20 and 400 μg/ml for

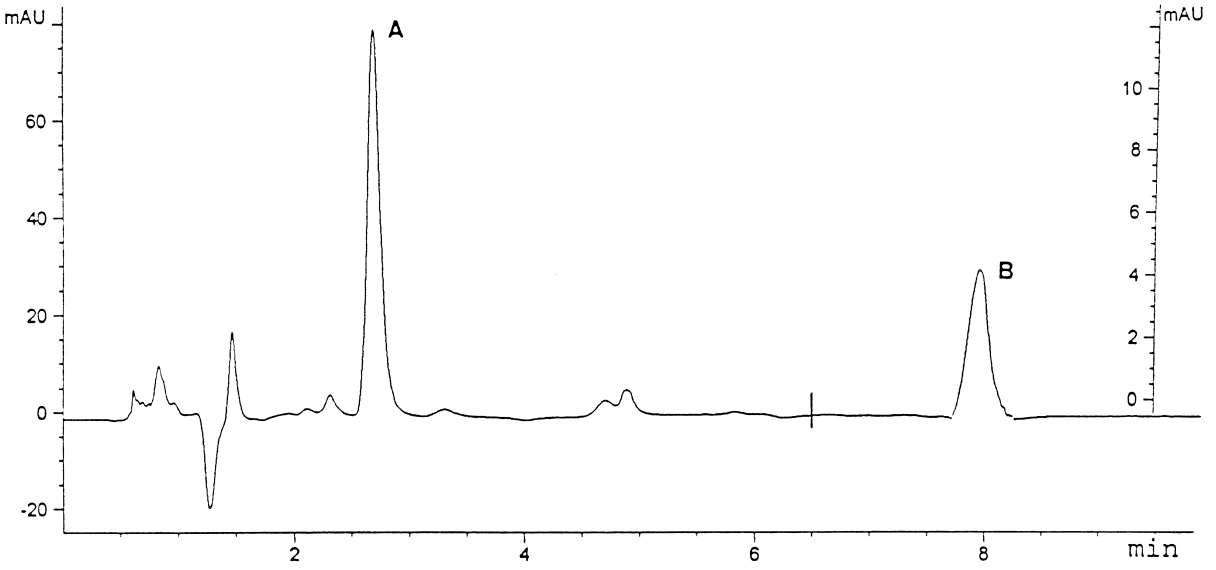

Fig. 1. HPLC chromatogram of α-tocopherol (A) and β-carotene (B) in a virgin olive oil sample.

α-tocopherol and between 0.5 and 5 μg/ml for β-carotene. Linearity was verified by analysing the variance of the regression. The determination coefficient (r^2) value was >0.999 for both compounds.

Eight determinations of α-tocopherol and β-carotene were performed in the sample oil using the same reagents and instruments in order to study the precision of the method. It was measured by calculating the standard deviation (SD) and relative standard deviation (RSD). The sample contained 86.6 mg/kg of α-tocopherol and 2.75 mg/kg of β-carotene. Within the same run, the RSDs for α-tocopherol and β-carotene were 4.44% and 4.27%, respectively. These results met the acceptable precision standards proposed by Horwitz [25] for analyte concentrations of about 0.2 mg/g.

Recovery was tested with the standard addition procedure using three addition levels for each compound (Table 1). Three determinations were performed for each addition level. Average recoveries were 96.5% for α-tocopherol and 107.1% for β-carotene. The detection limit (DL) and the quantification limit (QL) were studied in order to check the sensitivity of the method under the working conditions proposed. The sensitivity results obtained according to USP criteria [26] were similar for both compounds. The DLs for α-tocopherol and β-carotene were 11.5 ng and 15.5 ng, respectively, and

Table 1
Results of the recovery studies in virgin olive oil

Compound	Initial content (mg/kg)	Content after addition (mg/kg)	Mean recovery (%)
α-Tocopherol	100.69	144.83	85.09
		169.32	93.59
		187.19	99.36
Mean			96.50
β-Carotene	1.93	3.68	108.47
		4.98	107.32
		5.93	106.90
Mean			107.10

the QLs were 23.0 ng for α-tocopherol and 31.0 ng for β-carotene.

The method proposed allows a rapid and complete determination of α-tocopherol and β-carotene in olive oil by detection at specific absorption wavelengths for each vitamin. It is faster than conventional saponification methods and the chromatographic separation takes only 8 min. Its sensitivity, precision and accuracy are very satisfactory and allow routine quantification of these compounds in olive oil; furthermore, the method may be used to analyse them in any type of oil sample.

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