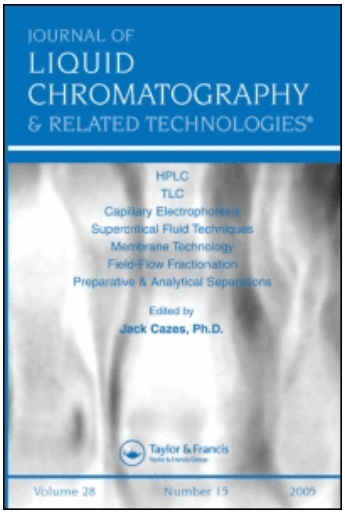

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Abstract: A micromethod was developed and validated for the quantification of lutein and β-carotene in olive oils. The compounds are liquid-liquid extracted with *n*-hexane and *n,n*–dimethylformamide (DMF), by vortex mixing and centrifugation. The *n*-hexane phase retains β-carotene, whereas the DMF phase retains lutein. This last fraction is concentrated and analyzed by RP-HPLC/diode-array, with a gradient of acetonitrile, water, and ethyl acetate, using β-apo-8′-carotenal as I.S. β-carotene is quantified by UV/Vis measurements at 454 nm, by the external standard method. The method shows appropriate inter- and intra-day reproducibility, accuracy, and linearity for both compounds, allowing fast analysis of several samples simultaneously, with a relatively small consumption of organic solvents. The analytical methodology was applied to several samples of commercial Portuguese PDO olive oils.

Keywords: Olive oil, RP-HPLC, Lutein, β-Carotene, Internal standard, Micromethod

INTRODUCTION

Several epidemiologic and clinical studies have shown that carotenoids have an antioxidant and provitamin A activity, together with an ability to improve the function of certain organs and immune system and to prevent some diseases (cardiovascular and cancer).[1] Carotenoids can be found in virgin

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olive oil, together with other natural antioxidants like tocopherols, phenols, and flavonoids, because only physical processes are used during extraction.

The carotenoids analysis in olive oil has been described by several analytical methods. The most frequently cited, reported by Minguez-Mosquera et al.,[2] uses a liquid-liquid extraction method where, despite the good recoveries achieved, the high solvent consumption is a drawback. The solid-phase extraction (SPE) is a quicker and simple alternative compared to classic liquid-liquid extractions, but, due to the sometimes low selectivity for specific compounds and high saturation with lipidic samples, should only be used for sample purification or preconcentration.[2] Most other authors results are still being based on the Minguez-Mosquera et al.[2] method, where *n*-hexane and dimethylformamide (DMF) are used: the *n*-hexane phase retains the β-carotene fraction, whereas the DMF phase retains lutein.[2,3]

The separation methods usually described for carotenoids analysis in olive oil are mainly chromatographic: thin-layer chromatography (TLC),[4–7] reversed phase HPLC[2,3,8,9] and, more recently, normal phase HPLC.[10] TLC, however, is not efficient for carotenoids quantitative analysis due to the high risk of degradation and isomerisation. HPLC reversed phase systems, with C_{18} columns, have been preferred due to their lower hydrophobic interactions with the compounds, compatibility with the majority of the solvents, and with the carotenoids polarities. Lutein is usually detected by UV or diode array detectors, the latter providing useful information for identification and peak purity. The β-carotene is determined in parallel by spectrophotometry.

In this paper, a faster methodology is proposed and validated for the analysis of the main carotenoids in olive oil, namely lutein and β-carotene. The main objectives were to reduce the general high consumption of organic solvents, to increase the number of samples analyzed simultaneously, to achieve a faster separation, essential to avoid their degradation, and an increased precision by introducing an internal standard in the lutein chromatographic analysis.

EXPERIMENTAL

Reagents and Standards

Tetrahydrofuran (THF), 2-propanol, *n,n*-dimethylformamide (DMF), and anhydrous sodium sulphate were all p.a. from Merck (Germany) and butylated hydroxytoluene (BHT) was from Sigma-Aldrich (Spain). Acetonitrile and ethyl acetate (HPLC grade) were also from Sigma-Aldrich. Demineralised water was purified in a Seral system, Seraldest LFM 20 (France).

The standards lutein (xantophyll alfalfa, 90%, HPLC), all-E-β-carotene (97%, HPLC) and the internal standard, β-apo-8′-carotenal (96%, UV),

were provided by Fluka (Spain). Stock solutions were prepared in *n*-hexane and 2-propanol (99:1), with 10% tetrahydrofuran[10] and 0.1% BHT, and stored at −18°C. The concentration of the stock solutions was determined daily by absorbency measurements of diluted solutions, on a Shimadzu UV 160A spectrophotometer (Japan), in accordance with published absorption coefficients in hexane ($A^{1\%}_{1cm}$): 2589 for lutein at 445 nm and 2592 for β-carotene at 454 nm.[11]

Individual working solutions were prepared for calibration purposes from the stock solutions, by dilution in hexane. The internal standard solution was prepared at 1.7 μg/mL. All working standard solutions were submitted to the extraction protocol described above, as for samples. All the procedures occurred under red light.

Samples

Twenty-five commercial Portuguese PDO olive oils were evaluated, all being classified as extra virgin (physically extracted and with less than 0.7% free fatty acids), covering the five Portuguese PDO origins: Trás-os-Montes (n = 14), Beira Interior (n = 2), Ribatejo (n = 2), Norte Alentejano (n = 2), and Moura (n = 5). Samples were kept in their original bottles (glass) and stored protected from the natural light at room temperature.

Extraction Procedure

Olive oil samples (1 g), accurately weighed, were dissolved in 15 mL of DMF and extracted with 5 mL of *n*-hexane, in crew top glass vials (Supelco, USA). The mixture was homogenized, vortexed, and centrifuged (3 min, 2370 g) (Heraeus Sepatech, Germany). The hexane layer was separated and the extraction repeated four more times. The two phases (DMF and *n*-hexane) were treated separately.

A 5 mL amount of sodium sulphate solution (2%, 0°C) was added to the DMF phase and again extracted with 2 × 10 mL of hexane/ethyl ether (1:1). After homogenization by vortex mixing and centrifuged (3 min, 2370 g), the upper phase was dried with anhydrous sodium sulphate and then evaporated under a gentle nitrogen flow at 40°C, until almost dry. A 200 μL amount of internal standard solution (1.7 μg/mL) was added to the residue and evaporated to dryness. The extract was reconstituted with 200 μL of acetonitrile/ethyl acetate (1:1) with 0.1% BHT. All extracts were stored at −18°C, being injected in the HPLC system within two days.

The five *n*-hexane phases were combined, dehumidified with anhydrous sodium sulphate, and the volume reduced to 7.0 mL under a gentle nitrogen flow at 40°C. The β-carotene concentration was immediately measured on a

Shimadzu spectrophotometer, model UV 160A (Japan), with quartz cells of 1 cm length.

All extracting solvents contained 0.01% BHT and the samples fractions were kept at 0°C during the several steps of analysis. Also, all the procedures were performed under a red light.

Chromatography

Lutein contents were determined by HPLC and its quantification was performed by using β-apo-8′-carotenal as internal standard. The HPLC system presented two PU-980 pumps and an MD-910 diode-array detector (DAD), scanning from 195 to 650 nm. Samples were injected in a Reodhyne valve, with 10 μL loop, by means of a Hamilton syringe.

The chromatographic separation was achieved with a reversed phase C_{18} column (250 × 4.6 mm i.d, 5 μm) from Phenomenex (USA). The solvent system used was a gradient of 10% H_2O in acetonitrile (v/v) (A) and ethyl acetate (B). The gradient was as follows: from 0 to 1 min, 0% B; from 1 to 30 min linear gradient to 60% B; from 30 to 40 min, 60% B; from 40 to 45 min linear gradient to 0% B. The flow-rate was 1.0 mL/min, at room temperature.

The DAD measurements were recorded by the data handling system-Borwin™ PDA Controller Software (JMBS, France) 1.50 version, and the chromatograms recorded at 450 nm for both lutein and internal standard. Peak identification and purity was based on the DAD spectroscopic data, retention time and spectral fine.[3]

Spectrophotometry

The β-carotene concentration was calculated directly from the UV measures on the *n*-hexane solutions, at 454 nm, and the quantification obtained by correlation with an external standard calibration curve, based on standards extracted in accordance with the sample's protocol.

RESULTS AND DISCUSSION

Extraction Method

Lutein and β-carotene are usually extracted with organic solvents, with or without previous saponification. Olive oils chemical nature is, however, a major disadvantage because of their high lipid load, which must be eliminated prior to sample injection in reversed phase mode. The more frequently

described extraction method for olive oil was proposed by Minguez-Mosquera et al.,[2] where a liquid-liquid extraction methodology is used. Despite being accurate, the method uses classical liquid-liquid ampoule extractions with high organic solvent volumes, enabling the simultaneous extraction of several samples and, above all, implicating a large solvent disposal with implications for both health and environment.

In order to achieve a fast extraction, together with a reduced solvent consumption, a micromethod was developed based on the above mentioned one.[2] In addition to a 10-fold reduction in the sample and reagents amounts, several adjustments were performed. All the extraction procedure was transferred to microvials, since the extractions were performed by means of vortex mixing, and phase separation was achieved by centrifugation. Also, the concentration and evaporation steps were made under a nitrogen stream, enabling multiple sample concentration.

During the initial tests on the spectrophotometric quantification of β-carotene in the *n*-hexane fraction, high variations between consecutive lectures of the same extract were detected, indicating the presence of phase impurities, imperceptible to the eye. The inconsistency was solved by dehumidifying the *n*-hexane phase with anhydrous sodium sulphate, before the concentration step, eliminating any vestiges of dissolved water. The final volume of the *n*-hexane phase was adjusted to 7.0 mL in order to allow two accurate spectrophotometric readings. Also, a tentative reduction on the number of extraction steps was proposed. It was verified, that with only four n-hexane extractions, a residual β-carotene peak was still detected in the HPLC chromatograms.

The xantophyll lutein was extracted from the DMF phase with *n*-hexane/ethyl ether solution (1:1 v/v). A single extraction, as initially proposed by Minguez-Mosquera,[2] proved to be insufficient and 2×10 mL volumes were used.

The addition of an antioxidant during all steps of the procedure is important to prevent carotenoids oxidation. The more frequently described antioxidant is BHT.[12,13] Since there were no chromatographic interferences, all solvents were, therefore, supplemented with 0.01% BHT.

Internal Standard

An I.S. is essential in the control of the extraction's reproducibility. It was our purpose to add an internal standard for lutein quantification, despite knowing that, for β-carotene, only the external standard method was possible.

β-Apo-8′-carotenal is frequently described as internal standard in carotenoids quantification methods in non-lipidic samples. However, due to the protocol's design and its solubility in *n*-hexane, it was partially extracted together with β-carotene, with a remaining amount of only about 25% of internal standard being chromatographically detected. Therefore, the

internal standard was used to control the final steps of the methodology, being added after total detachment of the two phases, in order to guarantee its full residence in the lutein phase.

Chromatographic Conditions

The generally used chromatographic conditions[2] consist of a relatively complex two phase eluent system, with two ion pair reagents in water (tetrabutylammonium and ammonium acetate), acetone, and methanol with adjusted pH. In order to speed the eluent preparation process, several alternatives were tested. A good peak resolution and clear chromatograms were obtained with the system proposed by Mendes-Pinto,[14] an acetonitrile:water solution (9:1, v/v) with 0.01% BHT (A) and ethyl acetate (B). The gradient used is detailed in the Experimental Section. This eluent system allows a good separation of lutein (RT = 14.2 min.) and internal standard (RT = 21.5 min.) and also the detection of others compounds equally well separated that, attending to their maximum of absorption, seem to be xantophylls and chlorophylls (Figure 1). The confirmation of peak purity within samples was confirmed by their UV spectra, as displayed in Figure 2.

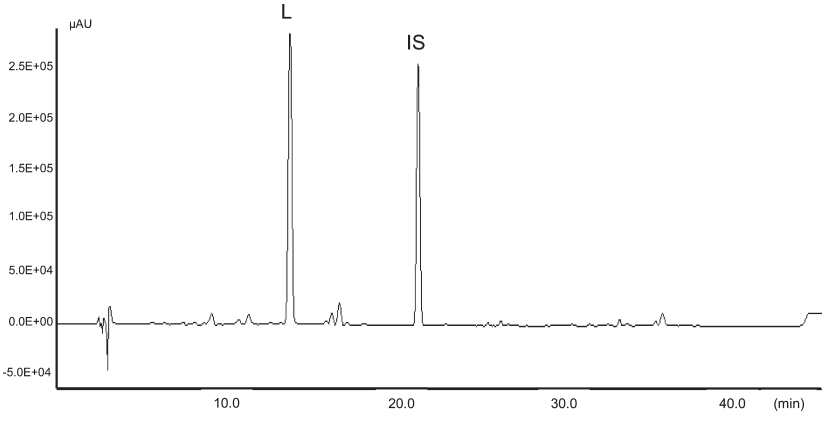

Figure 1. Chromatogram of an PDO extra virgin olive oil from Trás-os-Montes; Lutein – L, apo-8′-carotenal – IS.

Injection Method

Automatic injection was tested, with and without refrigeration, but a high degradation was observed between consecutive injections of the same sample, as displayed in Table 1. This fact must be related with the carotenoids

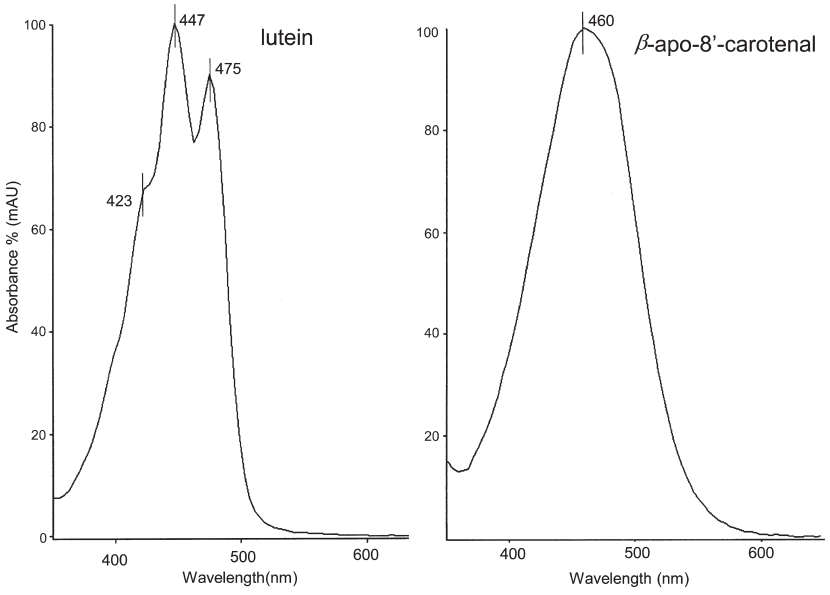

Figure 2. Absorption spectra of lutein and β-apo-8′-carotenal (I.S.) extracted from one olive oil sample.

Table 1. Lutein degradation according to the injection method

		Lutein standard (arbitrary units)		
Injection's method	Preservation	1st Injection	2nd Injection	%
Automatic	Room temperature	1639994	1265727	77
	Refrigerated (±10°C)	1628611	1408686	86
Manual	−18°C	1624314	1590988	98

instability, and additional BHT supplementation was ineffective. In order to avoid it, the samples must be conserved at −18°C between injections.

Method Validation

The extraction micromethod thus developed was evaluated for linearity range, detection limit (LOD), quantification limit (LOQ), precision, and recovery.

The method's linearity, for both lutein and β-carotene, was tested with standard solutions prepared as described in the Experimental section. The standard solutions were submitted to complete extraction as described for

Table 2. Lutein and β-carotene detection and quantification limits

	Linearity range			LOD		LOQ	
	μg/mL (injected)	mg/kg (sample)	Correlation R^2	μg/mL (injected)	mg/kg (sample)	μg/mL (injected)	mg/kg (sample)
Lutein	0.35–34	0.05–6.8	0.9983	0.11	0.01	0.35	0.05
β-carotene	0.02–2.23[a]	0.10–15.6	0.9999	—	—	0.09	0.44

[a]Tested ranged.

samples. The linearity range, in μg/mL of injected standards for each compound, is displayed in Table 2. These values can be converted into sample concentration, at mg/kg, by assuming the extraction of a 1 g sample amount (Table 2). The calibration curves for β-carotene were constructed by plotting Abs measures, at 454 nm, against the standard concentration. For lutein, the lutein/internal standard ratio of the arbitrary area units obtained from the DAD chromatograms at 450 nm were used. High correlation coefficients were obtained for both compounds.

Lutein detection limit was determined as three times the RSD of the chromatographic background noise for a 10 μL injection volume. The quantification limit was equally calculated as ten times the same RSD. For β-carotene, the quantification limit calculation was based on successive dilutions of a sample extract within stable spectrophotometric readings. Both limits are present in Table 2. These values are in accordance with Cichelli et al.,[8] who also reported a LOD of 0.01 mg/kg for lutein in olive oil by using the liquid-liquid extraction described by Minguez-Mosquera et al.[2] No limit of quantification based on this methodology was found in literature.

Intra-day and inter-day reproducibility studies were carried out to evaluate the precision of the proposed methodology. In both studies, three aliquots of the same sample were taken (Table 3). The intra-day reproducibility and inter-day reproducibility values for both compounds were below 5% and 10%, respectively, indicating that this micromethod can be considered

Table 3. Evaluation of the method's precision and accuracy

	Lutein	β-carotene
Reproducibility (RSD)		
Intra-day (n = 3)	3.3	1.3
Inter-day (n = 3 × 3)	7.0	3.3
Mean recovery % (n = 3)	97	101

Table 4. Content of lutein and β-carotene in Portuguese extra virgin PDO olive oils

PDO regions	Lutein (mean ± sd (mg/kg))	β-Carotene (mean ± sd (mg/kg))
Trás-os-Montes	2.32 ± 1.23	5.16 ± 1.03
Beira Interior	2.11 ± 0.15	3.84 ± 0.57
Ribatejo	2.29 ± 0.94	5.39 ± 0.16
Norte Alentejano	2.65 ± 0.20	5.24 ± 0.35
Moura	2.64 ± 0.58	4.84 ± 0.42

precise. The internal standard addition significantly increased the lutein precision (data not shown).

In the absence of a reference olive oil sample, recovery assays were performed by the addition of known amounts of lutein and β-carotene standards in triplicate, corresponding to about 40% of the mean sample amounts (Table 3). The mean recovery results obtained are better than those reported by Minguez-Mosquera et al.[2] (106%) and Cichelli et al.[8] (95%). No references of β-carotene recoveries in olive oil were found in literature.

Method Application

The validated methodology was developed for a rapid and accurate quantification of lutein and β-carotene in olive oils. A total of 25 extra virgin olive oil samples from the five Portuguese "Protected Denomination of Origin" (PDO) were analyzed, with the results reported in Table 4.

The achieved results show that lutein contents are similar in the different regions, but for β-carotene some differences can be found. Our results are within those reported by other authors.[2–10] The method will be applied to a higher number of samples in order to evaluate the significance of the observed variations between geographical regions.

REFERENCES

1. Tapiero, H.; Townsend, D.M.; Tew, K.D. Biomed. Pharmaceut. **2004**, *58*, 100–110.
2. Minguez-Mosquera, M.I.; Gandul-Rojas, D.; Gallardo-Guerrero, M.L. J. Agric. Food Chem. **1992**, *40*, 60–63.
3. Gandul-Rojas, B.; Roca, M.; Minguez-Mosquera, M.I. J. Am. Oil Chem. Soc. **2000**, *77*, 853–858.
4. Ranalli, A.; Ita. J. Food Sci. **1992**, *1*, 53–57.
5. Ranalli, A.; Modesti, G.; Patumi, M.; Fontanazza, G. Food Chem. **2000**, *69*, 37–46.
6. Ranalli, A.; Cabras, P.; Iannucci, E.; Contento, S. Food Chem. **2001**, *73*, 445–451.

7. Ranalli, A.; Malfati, A.; Lucera, L.; Contento, E.; Sotiriou, E. Food Res. Int. **2005**, *38*, 873–878.
8. Cichelli, A.; Pertesana, G.J. Chromatogr. A **2004**, *1046*, 141–146.
9. Giuffrida, D.; Salvo, F.; Salvo, A.; La Pêra, L.; Dugo, G. Food Chem. **2007**, *101*, 833–837.
10. Psomiadou, E.; Tsimidou, M. J. Sci. Food Agric. **2001**, *81*, 640–647.
11. Craft, N.; Soares, J. J. Agric. Food Chem. **1992**, *40*, 431–434.
12. Feltl, L.; Pacákovo, V.; Stulík, K.; Volka, K. Curr. Anal. Chem. **2005**, *1*, 93–102.
13. Oliver, J.; Palou, A. J. Chromatogr. A **2000**, *881*, 543–555.
14. Mendes-Pinto, M.M.; Ferreira, A.C.S.; Oliveira, M.B.P.P.; Guedes de Pinho, P.J. Agric. Food Chem. **2004**, *52*, 3182–3188.

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