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Analytical Methods

# Tocopherols in espresso coffee: Analytical method development and validation

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ABSTRACT

The present paper reports the development and validation of an analytical micro-method for tocopherols quantification in espresso coffee by normal-phase HPLC with fluorescence detection. The proposed method consists in a liquid–liquid extraction with *n*-hexane:ethyl acetate, followed by a clean-up with dimethylformamide to eliminate co-eluting interferences. The method showed good intra- and inter-day precisions (coefficient of variation < 6.5%), good accuracies (98 ± 6%), and high correlation coefficients ($r > 0.999$) for standards subjected to the entire procedure. Only α- and β-tocopherols were identified in the brews. The detection and quantification limits were 0.5 and 1.4 ng/ml, for α-tocopherol, and 0.4 and 1.1 ng/ml, for β-tocopherol, respectively. A mean total tocopherol content (α + β) of 3.5 ± 0.9 μg in commercial espresso coffee blends (30 ml) was detected. The proposed method requires low solvent consumption and proved to be sensitive, precise and accurate.



## 1. Introduction

Coffee is one of the most popular beverages all over the world, due to its organoleptic characteristics and stimulating effects. The antioxidant activity of this brew is also known, being generally associated with the presence of phenolic compounds and melanoidins (Cämmerer & Kroh, 2006; Parras, Martínez-Tomé, Jiménez, & Múrcia, 2007; Svilaas et al., 2004). However, the contribution of other minor components of the brew, such as vitamin E, should not be neglected.

Vitamin E encompasses a group of eight vitamers characterised by a chromanol ring structure with a distinct substitution pattern of methyl groups at positions 5, 7 and 8 of the head group (α-, β-, γ- and δ-) and a 16-carbon phytyl side chain (saturated in tocopherols and with three double bonds in tocotrienols) (Wang & Quinn, 1999). Vitamin E is known as the most effective natural lipid-soluble antioxidant, protecting cell membranes from peroxyl radicals and mutagenic nitrogen oxide species (Gliszczyńska-Świglo & Sikorska, 2004). The prevention of free radical-mediated tissue damage may play an important key role in the pathogenesis delaying of several degenerative diseases, such as cardiovascular disease, cancer, inflammatory diseases, neurological disorders, cataract and in the maintenance of the immune system (Bramley et al., 2000).

Vitamin E is only biosynthesized by plants and, due to its lipophilicity, this group of compounds is naturally found at high levels in edible plant oils (Hammond, 2003), with a variable isomer profile according to the oil identity (Gama, Casal, & Oliveira, 2000).

The presence of tocopherols in coffee oil was reported for the first time by Folstar, Van der Plas, Pilnik, and De Heus (1977). Some studies concerning these compounds in coffee beans have been published, mainly performed by HPLC with fluorescence detection, taking advantage of the compounds native fluorescence (Alves, Casal, & Oliveira, in press; Ayoama et al., 1988; González, Pablos, Martín, Léon-Camacho, & Valdenebro, 2001; Jham, Winkler, Berhow, & Vaughn, 2007; Kölling-Speer, Kurzrock, & Speer, 2006; Ogawa, Kamiya, & Iida, 1989). Recently, the usefulness of tocopherols content and profile as a marker of coffee adulteration with corn was also described by Jham et al. (2007).

Despite being naturally aqueous, coffee brews lipid amount may vary according to the preparation method. While filtered coffee brews contain less than 7 mg of lipids, those prepared by boiling without filtering and espresso coffee may reach up to 160 mg of lipids per cup (Ratnayake, Hollywood, O'Grady & Stavric, 1993). Lipidic compounds can be present either in oil droplets or in coffee particles suspended in the beverage (Urgert et al., 1995).

An elevated consumption of certain coffee brews with a high lipid content (Turkish and boiled coffee) has been negatively connoted, due to its contribution for a higher intake of two hypercholesterolaemic compounds (cafestol and kahweol), present in the coffee beans oil fraction (Gross, Jaccaud, & Huggett, 1997).

Information about tocopherols in coffee brews is, however, extremely scarce. Only one reference (Ogawa et al., 1989) reports a vitamin E mean content in coffee infusion of 7 μg/100 mL.

Espresso coffee is the most appreciated coffee brew in Portugal. It differs from all the other brews, due to its special method of preparation that consists in percolate, in a very short time (30 ± 5 s) and through a ground coffee cake (6.5 ± 1.5 g), a limited

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amount (20–50 ml) of hot water under high pressure (9 ± 2 atm, 90 ± 5 °C), originating a concentrated, intensely flavoured and scented brew, covered by a dense foam layer (Illy & Viani, 2005).

No data about tocopherols amount in espresso coffee was found in literature. Due to the complex chemical composition of espresso, the usual extraction methods used for other matrices were not applicable. Therefore, the aim of this work was to develop and validate an extractive micro-method for tocopherols quantification in this brew by normal-phase HPLC with fluorescence detection.

## 2. Experimental

### 2.1. Chemicals and solutions

Tocopherols (α, β, γ and δ) were purchased from Calbiochem (La Jolla, CA). Standard solutions were prepared in ethanol and kept at −20 °C. Their concentrations were evaluated, by UV measurements, according to their molar absortivities in ethanol (3265, 3725, 3809 and 3515, for α-, β-, γ-, and δ-tocopherols, respectively) (Eitenmiller & Landen, 1999). Dilutions were performed as required for calibration or other purposes.

The internal standard tocol (2-methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol) was obtained from Matreya Inc. (PA, USA). A 250 μg/ml solution was prepared in ethanol and kept at −20 °C. Butylated hydroxytoluene (BHT) was used as antioxidant and was obtained from Aldrich (Madrid, Spain). A 1% working solution of BHT was prepared in ethanol.

HPLC grade *n*-hexane was obtained from Merck (Darmstad, Germany) and 1,4-dioxane from Fluka (Madrid, Spain). Ethyl acetate was from Sigma Aldrich (Steinheim, Germany) and *N*,*N*-dimethylformamide (DMF) from Fluka (Seelze, Germany). All other reagents were of analytical grade.

For extractive and clean-up tests, C18 Sep-Pak® Vac (12 cc/2 g) cartridges were obtained from Waters Co. (MA, USA), Isolute Multimode SPE columns (3 g, custom) from International Sorbent Technology (Hengoed, UK), Florisil 60–100 mesh from Sigma (Steinheim, Germany), ethylenediamine-*N*-propylsilane bonded silica (PSA) from Supelco (Bellefonte, PA, USA), aminopropyl silica gel ($NH_2$) from Fluka (Buchs, Switzerland), Silica gel 60 G, aluminiumoxid 90 ($Al_2O_3$) and Extrelut$^{NT}$ from Merck (Darmstad, Germany). A Visiprep SPE Vacuum Manifold 57030-U from Supelco was used to manipulate cartridges, when necessary.

### 2.2. Samples

A total of ten commercial coffee blends (roasted beans) were randomly purchased in local supermarkets or supplied by a local industrial importer/roaster of coffee. One roasted sample of 100% arabica (Costa Rica) and one of 100% robusta (India) were also supplied by the same industry. Roast degrees were evaluated by photometric analysis with infrared radiation (Colorimeter Colorette 3 from Probat-Werke), being very similar for all the samples (125–139).

All coffee samples were mechanically powdered to pass through a 0.75 mm sieve (Krups 408-75 Coffee Grinder). Standard espresso coffees (30 ml) were prepared in a HL3854 *Espresso Professional* (PHILIPS, The Netherlands) using 6.5 g of ground coffee.

### 2.3. Extraction method

For the extraction experiments and method validation, a homogeneous and representative brew was prepared by homogenisation of fifteen espressos from one commercial sample. Beverage aliquots (5 ml) were taken, spiked with the internal standard (50 μl) and antioxidant (50 μl), homogenised and kept at −20 °C in amber glass vials (SUPELCO, Bellefonte, PA, USA) (for some tests, only 1 ml of these prepared aliquots was used).

The different tested methods are summarised in Table 1. Each extraction procedure was performed in duplicate, simultaneously with a sample aliquot and a standard mixture of tocopherols (mean concentration of 26 ng/ml). The final extracts were taken to dryness under a nitrogen stream and reconstituted in *n*-hexane for the HPLC injections. All operations were performed in the absence of light and using amber glassware.

The final extraction method, sequentially developed from those represented in Table 1 is detailed below.

#### 2.3.1. Liquid–liquid extraction

Espresso aliquots (5 ml) were taken in duplicate, spiked with tocol (50 μl), BHT (50 μl) and methanol (5 ml). After homogenisa-

**Table 1**
Preliminary extraction assays.

| Method | Organic solvents | References |
|---|---|---|
| *Liquid–liquid extraction* | | |
| A | *n*-Hexane (2 × 6 ml) | Ratnayake, Hollywood, O'Grady, and Stavric (1993) |
| B | Absolute ethanol (5 ml) + *n*-hexane (2 × 6 ml) | Lane, Webb, and Acuff (1997) |
| C | Methanol (10 ml) + *n*-hexane (2 × 6 ml) | Pollok and Melchert (2004) |
| D | Dichloromethane (2 × 6 ml) | |
| E | *n*-Hexane:diethyl ether (5:1) (2 × 6 ml) | Melchert and Pabel (2000) |
| F | *n*-Hexane:ethyl acetate (90:10) (2 × 6 ml) | Eitenmiller and Landen (1999) |
| G | Methanol (10 ml) + *n*-hexane/ethyl acetate (90/10) (2 × 6 ml) | |
| H | Modified Folch (Bligh and Dyer) | Bligh and Dyer (1959) |
| *Solid-phase extraction* | | |
| I | (A) Methanol (2 ml, 5 min) + extrelut column (0.5x4 cm); elution: *n*-hexane (6 ml)<br>(B) Silica gel column (0.5x4 cm); elution: *n*-hexane/diethyl ether (5/1) | Melchert and Pabel (2000) |
| J | Extrelut (7 g) (mixed in a mortar, reserved for 5 min); mixture loaded into a glass column (1.0 × 7.0 cm); elution: *n*-hexane (20 ml) | |
| K1–K5 | Methanol (2 ml); C18 Sep-Pak (conditioned with methanol and water); rinsing: water (10 ml); dryness under vacuum; elution (5 ml): *n*-hexane (1), ethyl acetate (2), *n*-hexane/ethyl acetate (90/10) (3), acetonitrile (4) or absolute ethanol (5) | |
| L | Methanol (2 ml); isolute multimode (conditioned with methanol and water); rinsing: water (10 ml); dryness under vacuum<br>Elution (5 ml): *n*-hexane/ethyl acetate (90/10) | |
| *Other* | | |
| M | Saponification | Indyk (1988) |

tion, the extraction was performed twice with 6 + 4 ml of *n*-hexane: ethyl acetate (90:10), vortexing the mixture (2 min) and centrifuging (3 min, 5000 rpm). Organic phases were combined and taken to dryness under a nitrogen stream. The residue was re-suspended in 2.3 ml of *n*-hexane.

*2.3.2. Liquid–liquid clean-up*

The interfering compounds were extracted with 750 μl of DMF by vortexing (2 min). After centrifugation (3 min, 5000 rpm), 1.9 ml of the *n*-hexane layer were transferred to a 2 ml graduated eppendorf and gently reduced to about 300 μl under a nitrogen stream.

At this point, two separated phases are visible again, due to a small quantity of DMF that was partially dissolved in the *n*-hexane phase. After a new centrifugation (10 min, 13,000 rpm), 150 μl of the *n*-hexane layer were transferred to an insert within a 2 ml amber vial and 20 μl portions analysed by HPLC.

*2.4. HPLC equipment*

The chromatographic analysis was carried out in a HPLC integrated system equipped with an AS-950 automated injector, a PU-980 pump, an MD-910 multiwavelength diode-array detector (DAD) connected in series to an FP-920 fluorescence detector (Jasco, Japan), programmed for excitation at 290 and emission at 330 nm (gain 100).

The chromatographic separation of the compounds was achieved with an Inertsil 5 SI column (5 μm, 250 × 3 mm) from Varian (Middelburg, The Netherlands), operating at constant room temperature (21 °C). A mixture of *n*-hexane and 1,4-dioxane (97:3) was used as eluent, performed at a solvent flow rate of 0.7 ml/min. Chromatographic data were analysed using a Borwin-PDA Controller Software (JMBS, France).

The compounds under study were identified by chromatographic comparison with authentic standards, by co-elution and by their UV spectral characteristics (DAD). Peaks purity evaluation on the DAD measurements was based on spectral comparison at three different peak heights. Quantification was performed on the basis of the internal standard method using fluorescence signals.

*2.5. Statistical analysis*

Data were reported as mean ± standard deviation. Data treatment was carried out with Microsoft Excel statistical software (Microsoft Office Excel 2003, Microsoft Corp., Redmond, WA). Student's *t* tests were used for evaluation of differences between fresh and frozen samples.

## 3. Results and discussion

*3.1. Optimisation of the chromatographic separation*

Based on previous studies about vitamin E in coffee beans and oil (Alves et al., in press; Ayoama et al., 1988; Folstar et al., 1977; González et al., 2001; Jham et al., 2007; Kölling-Speer et al., 2006; Ogawa et al., 1989), two main tocopherol isomers were expected to be found in the brews: α and β. Due to the inconsistencies about the γ-tocopherol coffee presence in the beans, one decided to use a normal phase chromatographic column in order to efficiently separate β- and γ-tocopherol (Gama et al., 2000).

A normal phase Supelcosil™ LC-SI (3 μm, 75 × 3 mm) column (Supelco, Bellefonte, PA), with a mobile phase of *n*-hexane/1,4-dioxane (98:2), at a flow rate of 0.7 ml/min, was initially tested for the tocopherols chromatographic separation. This column was already successfully applied to the determination of vitamin E in coffee beans (Alves et al., in press), allowing a good separation of the eight vitamin E compounds of a standard mixture, in less than 8 min.

However, when used to separate the tocopherols of an espresso coffee extract the results were not satisfactory. The short chromatographic run, the small tocopherol contents of the sample and the presence of high amounts of interfering compounds (due to the chemical complexity of the brew), resulted in a very weak peak resolution, being necessary to adjust the chromatographic conditions.

A longer normal phase column was tested with the same purpose (Inertsil 5 SI, 5 μm, 250 × 3 mm, Varian, Middelburg, The Netherlands), with a mobile phase of *n*-hexane/1,4-dioxane in variable proportions of 1,4-dioxane (2–.5%), always in isocratic mode. The chromatographic resolution of the coffee extract was enhanced, especially with the mixture of 3% dioxane in *n*-hexane, allowing an improved separation of some co-eluting interferences in less than 20 min.

The retention times for α-, β-, γ-, δ-tocopherol (standards) and tocol (internal standard) were, respectively, 6.2, 9.7, 10.8, 14.2 and 19.0 min, with mean repeatabilities of 0.7 ± 0.2% and mean reproducibilities of 3.4 ± 0.2%.

*3.2. Choice of the extraction method*

Diversified extraction procedures for vitamin E quantification in several matrices have been described in literature. Except for oils, which can be directly injected onto a normal-phase HPLC column after dilution, in most cases vitamin E must be previously released from the sample matrix and/or concentrated (Eitenmiller & Landen, 1999).

Espresso coffee is an aqueous brew with a complex chemical composition (Illy & Viani, 2005). Tocopherols are lipophilic compounds, expected to exist in small amounts in this beverage. Different extraction procedures were tested in order to choose the most appropriate one to espresso coffee analysis (Table 1).

Being espresso coffee an aqueous matrix and tocopherols lipophilic compounds, direct liquid–liquid extractions with several organic solvents were assayed. Solid-phase extractions, as well as other classic methods (Bligh & Dyer and saponification), were also tested and all compared. In this preliminary phase, the chromatograms were compared in terms of peak relative areas, resolution, peak purity tests (DAD), and standard recoveries.

The organic solvent mainly used in tocopherols extraction is *n*-hexane. In the first assayed method (**A**), this solvent was directly added to the sample, as already used by Ratnayake, Hollywood, O'Grady, and Stavric (1993) to extract total lipids from coffee brews. However, in our case, it was not possible to efficiently mix both phases, since an intermediate emulsified layer appeared during vortexing, being impossible to eliminate it with centrifugation. The high polarity differences and volume ratios of the two liquid phases might responsible. With an incomplete mixture/separation, the compounds extraction would certainly be affected. Therefore, this procedure was slightly modified, according to other references.

In methods **B** and **C**, ethanol and methanol, respectively, were added before the extraction step with *n*-hexane. These water mixing solvents improved protein precipitation and slightly increased the solvent polarity, allowing a better mixture of the phases and a complete separation after centrifugation (Nelis, D'Haese & Vermis, 2000). The use of methanol instead of ethanol provided cleaner chromatograms. However, the chromatographic peaks corresponding to tocopherols were still very impure (DAD).

Other extraction solvents were then tested, namely dichloromethane (method **D**) and *n*-hexane: diethyl ether (5:1) (method **E**). The addition of ethyl acetate to *n*-hexane in levels up to 10% (v/v) is recommended to improve the recoveries of β-tocopherol and tocol (Eitenmiller & Landen, 1999). Therefore, *n*-hexane:ethyl acetate (90:10) was also tested, without (method **F**) and with a previous addition of methanol (10 ml) to the sample (method **G**). The Bligh and Dyer method (**H**) was also tested.

In general, all of these liquid–liquid extractions (**A**–**H**) seemed to be adequate when working with standards (mean recoveries > 95%). However, none provided good and clean chromatograms in what concerns to the coffee brews. Peak resolution was very weak and purity low. Nevertheless, method **G** provided higher peak definition in the retention times expected for tocopherols.

Simultaneously, some solid-phase extraction (SPE) methods were tested. Method **I** uses Extrelut (A) and silica gel (B) columns. A variation of the first part of this method was also performed (method **J**) by direct matrix solid phase dispersion with Extrelut and elution with *n*-hexane.

No chromatographic improvements, comparatively to the liquid–liquid extractions described above, were obtained with these two methods (**I** and **J**). Besides, a slightly decrease in the tocol signal was observed, probably justified by polarity differences between the compounds and, consequently, different behaviours in the presence of Extrelut. Therefore, these methods were discarded.

Other SPE attempts were done, in order to selectively extract the compounds. In method **K**, a C18 Sep-Pak Vac (12 cc/2 g) cartridge was tested with five different eluting solvents (Table 1). This procedure was also tested using Isolute Multimode SPE columns and *n*-hexane:ethyl acetate (90:10) as eluent (method **L**).

In general, when working with standard mixtures, high recoveries for the three compounds were found (>90%) with both methods **K** and **L**. However, when applying the procedures to the coffee samples, it was not possible to isolate tocopherols from co-eluting compounds, being the chromatograms very similar to those obtained with liquid–liquid extractions, presenting weak resolution and low peak purity.

In addition, a simplified saponification (method **M**) (Indyk, 1988) was tested. Also here, the chromatograms showed bad resolved peaks and, moreover, saponification did not allow the use of tocol as internal standard due to its degradation in alkaline conditions and conversion to co-eluting interferences. For those reasons, this method was eliminated.

None of the tested methods (Table 1) was able to isolate the tocopherols from other interfering compounds present in espresso coffee. However, method **G** provided higher chromatographic peak definition, although still very impure. Therefore, it was selected for the following studies.

### 3.3. Choice of the clean-up procedure

Clean-up experiments were carried out with the extract obtained by method **G**, reconstituted in *n*-hexane.

The first attempt consisted in using a silica gel column according with method **I**. Different SPE sorbents were also individually tested, with 150 mg of each $Al_2O_3$, Florisil, $NH_2$, PSA, and silica. The mixtures were vortexed, centrifugated, the liquid phase collected, evaporated, re-suspended (500 μl of *n*-hexane) and injected. None of these methods allowed an efficient separation between tocopherols and other compounds.

Two liquid–liquid clean-ups were also tested, by vortexing extracts with acetonitrile or DMF (3 ml), for 2 min. After centrifugation (3 min, 5000 rpm), the *n*-hexane layers were collected, evaporated, re-suspended (500 μl of *n*-hexane) and injected.

In general, both liquid–liquid clean-up procedures allowed a chromatographic improvement, being DMF the most efficient.

### 3.4. Optimisation of the chosen method

After selecting the extraction procedure (method **G** followed by a liquid–liquid clean-up with DMF) it was necessary to optimise it in order to improve its performance. In the clean-up step with DMF, a small incorporation of this solvent was noticed in the *n*-hexane, even after centrifugation, increasing the evaporation time under the nitrogen stream. For that reason, it was necessary to optimise each step and volume individually. The optimisation steps are described in Table 2, as well as the results achieved in terms of relative areas for α- and β-tocopherol. The isomers γ and δ were not detected. The best option for each step was chosen on the basis of the higher relative peak area.

According to the results described in Table 2, some improvements were done to the extraction procedure. It was possible to reduce the amount of organic solvents to only 5 ml of methanol and 6 + 4 ml of *n*-hexane:ethyl acetate (90:10). The ratio between *n*-hexane and DMF was changed to 3:1, since it gave the better results. After that, *n*-hexane and DMF amounts were readjusted, maintaining the same ratio (2.3 ml and 750 μl, respectively), in order to slightly decrease the solvent consumption.

A complete separation of DMF and *n*-hexane after liquid–liquid clean-up was crucial, otherwise chromatographic peak resolution was impaired. To minimise the DMF retention within the *n*-hexane phase, after the liquid–liquid clean-up and subsequent centrifugation, only 1.9 ml of the *n*-hexane layer was separated to a 2 ml eppendorf. The removed extract (1.9 ml) was gently taken to dryness ($N_2$) till 300 μl. At this point, two separated phases were visible again, due to a small quantity of DMF that was partially dissolved in the *n*-hexane phase. After a new centrifugation (best results for 10 min at 13,000 rpm, Table 2), 150 μl of the *n*-hexane layer were transferred to an insert within a 2 ml amber vial and 20 μl portions analysed by HPLC.

Figs. 1 and 2 show the chromatograms before and after the purification with DMF, respectively. Comparing both figures it is possible to recognise the chromatographic improvement derived from the clean-up step with DMF, with elimination of co-eluting interferences and high peak purities for both tocopherols (α and β) and tocol (diode-array).

The final proposed method is entirely described in Section 2. Although slightly laborious, it allows an accurate quantification of espresso coffee tocopherols, without the interference of other co-eluting compounds initially present in the sample, and with a

**Table 2**
Relative areas achieved under several extraction conditions.

| Extracting conditions | Relative areas | |
|---|---|---|
| | α-Tocopherol | β-Tocopherol |
| *Methanol amount (ml)* | | |
| 10 | 100 | 100 |
| 5 | 100 | 100 |
| 2.5 | 87 | 94 |
| *n-Hexane:ethyl acetate (90:10) amount (ml)* | | |
| 2 × 6 | 100 | 100 |
| 3 × 6 | 100 | 100 |
| 6 + 4 | 100 | 100 |
| *DMF amount for a 3 ml extract (ml)* | | |
| 6 | 23 | 36 |
| 3 | 56 | 69 |
| 1 | 100 | 100 |
| 0.5 | 40 | 55 |
| *Centrifugation time immediately before injection (min;rpm)* | | |
| 5;5000 | 54 | 67 |
| 5;13,000 | 86 | 93 |
| 10;13,000 | 100 | 100 |
| 15;13,000 | 100 | 100 |

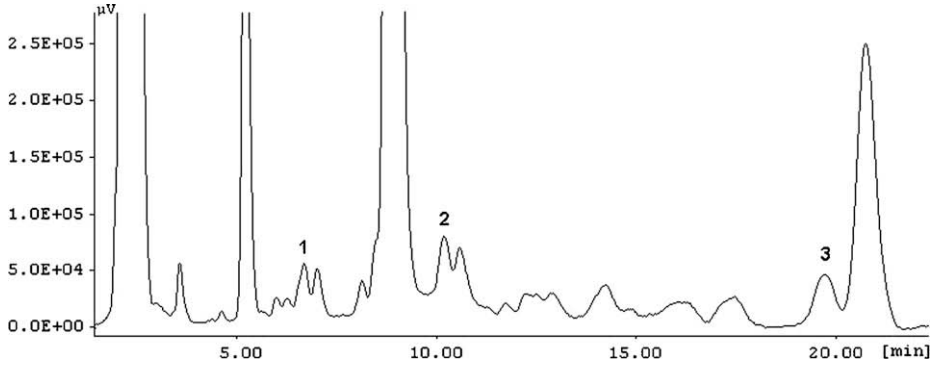


**Fig. 1.** Fluorescence chromatogram before the clean-up with DMF. (1) α-Tocopherol; (2) β-tocopherol and (3) tocol.

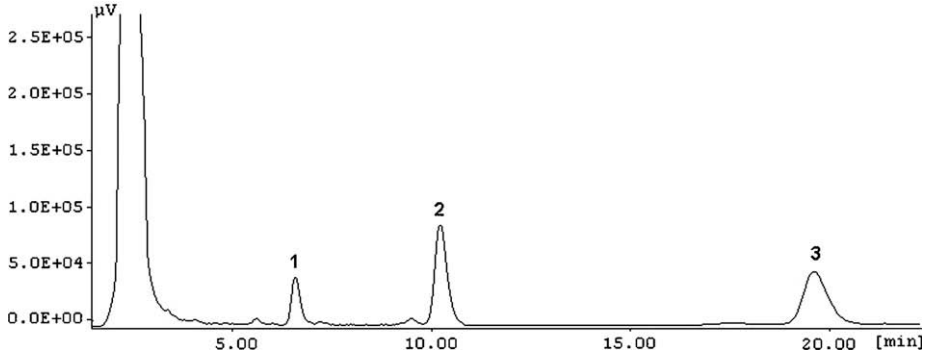


**Fig. 2.** Fluorescence chromatogram after the clean-up with DMF. (1) α-Tocopherol; (2) β-tocopherol; (3) tocol.

minimal solvent consumption. This method avoids any tocopherol mislabelling, easy to occur due to the chemical complexity of coffee brews, as already referred. However, some labour reduction is possible by preparing a high number of samples (See Section 2.3, *Sample preparation*), freezing (−20 °C), and analysing them all together in another day. No significant differences ($p > 0.05$) were found between aliquots of the same espresso analysed freshly or frozen for 2 weeks.

### 3.5. Method validation

#### 3.5.1. Linearity

The linearity range was tested with six different concentrations of α- and β-tocopherol standard solutions, according to the expected levels in the sample (2.6–530 ng/ml for α-tocopherol and 2.6–535 ng/ml for β-tocopherol), subjected to the entire extraction method. Triplicate determinations of each calibration standard were done. The relative average deviations of triplicates were always lower than 2.3%. Calibrations curves were constructed based on the average ratio between the peak areas (arbitrary units) of each compound and the internal standard versus the standard concentration (ng/ml), with high correlation coefficients (>0.999).

#### 3.5.2. Precision

The intra-day precision was evaluated by assaying one sample six times during the same day. The coefficients of variation were 4.5% and 5.8% for α- and β-tocopherol, respectively. The inter-day precision was determined by analysing the same sample in six different days. The coefficients of variation were 4.6% for α-tocopherol and 6.5% for β-tocopherol.

#### 3.5.3. Limit of detection and limit of quantification

The detection and quantification limits were calculated as 3.3 and 10 times the standard deviation of the background noise divided by the slope of the calibration curve, respectively. The detection limits were 0.5 and 0.4 ng/ml, for α- and β-tocopherols, respectively. The quantification limits were 1.4 ng/ml (for α-tocopherol) and 1.1 ng/ml (for β-tocopherol).

#### 3.5.4. Recoveries

In the absence of a certified or standard reference material for coffee brew, simultaneously with low vitamin amounts and high matrix interference, the method accuracy was evaluated by the standard addition procedure (% of recovery). Three different concentration levels of α- and β-tocopherols were added to the sample

**Table 3**
Recovery of α- and β-tocopherols from a spiked espresso coffee.[a]

| Compound | Present (ng/ml) | Added (ng/ml) | Found (ng/ml) | Recovery (%) | CV (%) |
|---|---|---|---|---|---|
| α-Tocopherol | 25.1 ± 1.2 | 15.7 | 40.6 ± 0.9 | 98 | 2.2 |
| | | 31.4 | 51.6 ± 1.1 | 90 | 2.2 |
| | | 47.2 | 65.9 ± 0.3 | 91 | 0.5 |
| β-Tocopherol | 41.9 ± 1.6 | 16.1 | 60.5 ± 1.3 | 104 | 2.2 |
| | | 32.2 | 75.2 ± 1.7 | 102 | 2.3 |
| | | 48.2 | 90.3 ± 0.8 | 100 | 0.9 |

[a] Mean value ± standard deviation; CV: coefficient of variation.

before the extraction method, in triplicate. The average recovery values were 93 ± 4% and 102 ± 2%, for α- and β-tocopherols, respectively. The results are listed in Table 3.

## 4. Vitamin E determination in commercial espresso coffees

In order to verify the applicability of the proposed method, espresso coffees were prepared from ten commercial coffee blends of different brands (unknown composition), plus one 100% arabica coffee and one 100% robusta sample. The extraction method was performed in duplicate and the results can be observed in Table 4.

As expected, the main tocopherols described for coffee beans (α and β isomers) (Alves et al., in press; Ayoama et al., 1988; Folstar et al., 1977; Jham et al., 2007; Kölling-Speer et al., 2006; Ogawa et al., 1989) were identified in the brews. In these samples, and by this analytical methodology, γ- and δ-tocopherols were not detected.

A high variability was found between the tocopherol contents in the ten commercial samples analysed. Expressing the results in ng/ml of espresso coffee, the compounds ranged between 26.6 ± 0.7 and 77.7 ± 2.6 ng/ml, for α-tocopherol, with a mean concentration of 45.5 ± 15.7 ng/ml, and between 39.5 ± 0.1 and 103.7 ± 1.1 ng/ml, for β-tocopherol, with an average concentration of 69.6 ± 22.4 ng/ml.

Regarding the tocopherol contents (μg) per cup (30 ml) (samples 1–10), the amounts varied from 0.80 ± 0.03 to 2.33 ± 0.08 μg, and from 1.18 ± 0.01 to 3.11 ± 0.03 μg, for α- and β-tocopherols, respectively. The average contents were 1.37 ± 0.47 μg, for α-tocopherol, and 2.09 ± 0.67 μg, for β-tocopherol.

In a general way, the commercial espresso cups contained higher amounts of β-tocopherol (approximately the double of α-tocopherol content). However, there were two exceptions (samples 4 and 7), in which the β-tocopherol content was lower or similar to that of α-tocopherol.

The coffee species proportion (arabica and/or robusta) in each commercial blend was not known. In a general way, the arabica roasted beans are known to contain higher tocopherol amounts than robusta ones, especially for β-tocopherol, also associated with a higher lipid content (Ratnayake, Hollywood, O'Grady, & Stavric, 1993). Therefore, the differences between the samples 1 to 10 in Table 4 should be mainly due to the distinct compositions (in terms of coffee species) of the blends used to prepare the brews. One might speculate that samples 4 and 7 should correspond to blends with a high percentage of robusta, in accordance with the results for samples 11 (100% arabica) and 12 (100% robusta) and the results described for beans (Alves et al., in press; Kölling-Speer et al., 2006; Ogawa et al., 1989).

No conclusions can be taken about the roasting influence on the brews tocopherol contents, since the analysed samples showed very similar roast degrees (see Section 2.2 Samples). Nevertheless, reduced tocopherol amounts might be expected for darker roast.

These preliminary results show that it will be interesting to study the contribution of different coffee species (namely, arabica and robusta), as well as the interference of other factors (roasting, brew volume) to the tocopherol contents of espresso coffee.

**Table 4**
Tocopherols contents in commercial espresso coffees.[a]

| Sample | α-Tocopherol (ng/ml) | α-Tocopherol (μg/30 ml) | β-Tocopherol (ng/ml) | β-Tocopherol (μg/30 ml) | Total tocopherols (μg/30 ml) |
|---|---|---|---|---|---|
| 1 | 49.1 ± 2.6 | 1.47 ± 0.08 | 98.4 ± 3.0 | 2.95 ± 0.09 | 4.43 ± 0.17 |
| 2 | 28.7 ± 0.3 | 0.86 ± 0.01 | 71.9 ± 1.4 | 2.16 ± 0.04 | 3.02 ± 0.03 |
| 3 | 31.5 ± 1.1 | 0.95 ± 0.03 | 73.2 ± 1.7 | 2.19 ± 0.05 | 3.14 ± 0.08 |
| 4 | 77.7 ± 2.6 | 2.33 ± 0.08 | 49.1 ± 1.7 | 1.47 ± 0.05 | 3.80 ± 0.13 |
| 5 | 59.1 ± 1.2 | 1.77 ± 0.04 | 90.0 ± 1.5 | 2.70 ± 0.04 | 4.47 ± 0.08 |
| 6 | 40.6 ± 0.6 | 1.22 ± 0.02 | 45.1 ± 0.9 | 1.35 ± 0.03 | 2.57 ± 0.05 |
| 7 | 39.8 ± 0.2 | 1.19 ± 0.01 | 39.5 ± 0.1 | 1.18 ± 0.01 | 2.38 ± 0.00 |
| 8 | 54.8 ± 0.2 | 1.64 ± 0.01 | 103.7 ± 1.1 | 3.11 ± 0.03 | 4.76 ± 0.04 |
| 9 | 47.3 ± 0.6 | 1.42 ± 0.02 | 67.2 ± 0.0 | 2.01 ± 0.01 | 3.43 ± 0.02 |
| 10 | 26.6 ± 0.7 | 0.80 ± 0.03 | 58.1 ± 1.5 | 1.74 ± 0.04 | 2.54 ± 0.02 |
| 11[b] | 81.3 ± 0.6 | 2.44 ± 0.02 | 134.2 ± 0.6 | 4.03 ± 0.02 | 6.46 ± 0.03 |
| 12[c] | 44.1 ± 0.5 | 1.32 ± 0.02 | 30.38 ± 1.2 | 0.91 ± 0.04 | 2.23 ± 0.04 |

[a] Mean value ± standard deviation.
[b] 100% arabica coffee.
[c] 100% robusta coffee.

## 5. Conclusion

The developed micro-method proved to be efficient for a precise and accurate quantification of tocopherols in espresso coffee. Although slightly laborious, the solvent consumption is minimal, being possible to obtain clean chromatograms of tocopherols, without interference of co-eluting compounds initially present in the samples. Besides, a high number of samples can be prepared, frozen (at least for two weaks), and analysed all together in another day, without any loss of compounds.

This extraction method might also be an option for other complex matrices, to which the usual methods do not allow a good quantification.

The contribution of espresso coffee to the recommended daily vitamin E intake (15 mg of α-tocopherol) (Institute of Medicine, 2000) is very small. However, the tocopherols in this beverage, due to their lipophilic nature, might complement the overall antioxidant effect provided by the brew, being consequently involved in the protective effect of moderate coffee consumption against several diseases. An efficient quantification of tocopherols in coffee beverages will certainly contribute to elucidate about composition and antioxidant properties of this brew.

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