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Analytical Methods

Rapid determination of polycyclic aromatic hydrocarbons in natural tocopherols by high-performance liquid chromatography with fluorescence detection

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Abstract

A simple and rapid method has been developed and validated for the determination of seventeen polycyclic aromatic hydrocarbons (PAHs) in natural tocopherol products. Samples were dissolved in *n*-hexane, cleaned by an alumina column, and separated and determined by reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection. The recoveries were greater than 77.9%, except for the lowest molecular weight PAHs (Na, 1-Me, 2-Me, AC, F) which were between 15.9% and 75.8%. The limits of quantification were less than 0.38 ng/g for the heavy PAHs and less than 1.50 ng/g for the light PAHs. Good repeatabilities were achieved with RSD less than 10.7% for all the objective compounds. This method has been applied to evaluating PAHs contents in various natural tocopherol products and controlling natural tocopherol product quality.

Keywords: Polycyclic aromatic hydrocarbons; Natural tocopherol; Alumina column; HPLC

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are well-known organic compounds derived from incomplete combustion of organic matters (Lage-Yusty & Cortize Daviña, 2004; Moret & Conte, 2000; Moret & Conte, 2002). Recently, they have been the subject of much health concern and have been included in the American Environmental Protection Agency (EPA) priority pollutant list, due to their mutagenic and carcinogenic properties (U.S. EPA, Vol. 49).

In particular, because of the wide distribution of PAHs in the environment and their lipophilic nature, vegetable oils are prone to PAHs contamination through oilseeds drying procedure and extraction solvents (Dorthe, Ramberti, & Thienpont, 2000; Moret & Conte, 1998; Weißhaar, 2002). Recently, we have known some reports of natural tocopherols contaminated by PAHs, wherein 526, 3646 and 5560 ng/g PAHs were detected for samples from three major manufacturers, respectively (U.S. Patent No. 5,635,189). Production of natural tocopherols used vegetable oil deodorizer distillates as raw materials therefore, PAHs have well been concentrated in natural tocopherols.

Different PAHs maximum limits in vegetable oils have been proposed by several organizations in many countries. Spain has set a legal limit for PAHs in olive pomace oil in July, 2001 with a limit of 2 ng/g for each of the following PAHs: benzo(*a*)anthracene, benzo(*e*)pyrene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthen, benzo(*a*)pyrene, dibenzo(*a,h*)anthracene, benzo(*g,h,i*)perylene, indeno(*1,2,3-cd*)pyrene, and the limit of 5 ng/g for the sum of them, which were also accepted by UK and Ireland (International Olive Oil Council, Resolution no. RES-4/85-IV/01). These limits have been applied to PAHs in natural tocopherols. Natural tocopherols

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preparations, either used by them as a nutritional supplement or added to other foods, food additives, pharmaceuticals, skin care products, could prove to be a significant source of human PAHs intake, which demands a control of PAHs in this type of product (U.S. Patent No. 5,635,189).

Most methods have been developed to determine PAHs residues in oils. The widely used traditional PAHs extraction processes consist of saponification (Gfrerer & Lankmayr, 2003), liquid–liquid partition (Guillén, Sopelana, & Palencia, 2004; Pupin & Figueiredo-Toledo, 1996), caffeine complexation (Kolarovic & Traitler, 1982), followed by clean-up steps, e.g., packed column chromatography or solid phase extraction column (Barranco et al., 2003; Gfrerer & Lankmayr, 2003; Pupin & Figueiredo-Toledo, 1996; Vazque-Troche, García-Falcoón, González-Amigo, Lage-Yusty, & Simal-Lozano, 2000). These methods are time consuming, and require a large volume of organic solvent with formation of emulsion, so that they are not suitable for the routine analysis. Moreover, we faced irregular recoveries, bad repeatabilities and many interfering peaks on the chromatograms when we applied the above methods to analyze PAHs residues in natural tocopherols. The reason resulting to these disadvantages was short of perfect sample preparation procedures, and which could be overcome by some new sample preparation methods which combined extraction steps with clean-up steps. For instance, solid phase extraction (SPE) (Bogusz, El-Hajj, Ehaideb, Hassan, & Al-Tufail, 2004; Moreda, Rodríguez-Acuña, Pérez-Camino, & Cert, 2004; Moret & Conte, 2002; Weißhaar, 2002) and supercritical fluid extraction (SFE) (Lage-Yusty & Cortize Daviña, 2004) have been used to process the lipid matrices samples for the determination of PAHs residues. But some of these have high cost. Regarding analytical determination, reversed-phase HPLC coupled with fluorescence detector together with gas chromatography (GC) coupled with mass spectrometry (MS) are the most powerful techniques for sensitive and selective determination of PAHs.

To the best of our knowledge, there are no methods for the determination of PAHs in natural tocopherols to have been reported. The principal difficulties to analyze PAHs in natural tocopherols are the low levels (ng/g) and diversity of potential interferences present, mainly including tocopherols, squalene, neutral oil, fatty acids, aliphatic esters, and phytosterols, etc. In this paper a relatively simple and low-cost procedure was proposed, which consists of alumina column chromatography followed by reversed-phase HPLC with fluorescence detection, to analyze PAHs in natural tocopherols (54.0% mixed tocopherols containing α-, β-, γ-, and δ-tocopherol; high-purity mixed tocopherols; high-purity α-tocopherol) and to examine specific conditions.

2. Experimental

2.1. Instruments

The Waters 2695 alliance chromatographic system was equipped with a vacuum degasser, a quaternary pump, an autosampler and a Waters 2475 fluorescence detector. The HPLC system was controlled and the data were processed by Waters Empower software. A reversed-phase Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm I.D., 5 μm, Agilent Technologies, USA) with guard column Zorbax Eclipse XDB C18 (12.5 mm × 4.6 mm I.D., 5 μm, Agilent Technologies, USA) were used. The XDB-C18 column was held at 35 ± 1 °C with a column heater.

2.2. Samples

Samples of natural tocopherols (54.0% mixed tocopherols (Jiangsu Taixing Newlight Biological Products Plant, Jiangsu, China), 90.5% mixed tocopherols and 98.0% α-tocopherol (Zhejiang Worldbest Pharmaceuticals Technology Development Co., Ltd.)) were commercially available.

Unfortified natural tocopherol samples were prepared from 98.0% α-tocopherol by treatment with 5% activated carbon in order to obtain a sample with a low content of PAHs. The treated sample was analyzed for residual PAHs and used as unfortified sample of the standard addition technique.

2.3. Reagents and chemicals

The acetonitrile (MeCN) was HPLC grade (Merck, Darmstadt, Germany). *n*-Hexane was HPLC-UV grade (Burdick & Jackson, Honeywell, USA). Water was supplied by a Milli-Q water purification system from Millipore (Molsheim, France). Neutral alumina was analytical reagent (100–200 mesh, Shanghai Wusi Reagent Company, Shanghai, China). Activity grade I alumina was obtained by activation at 525 ± 1 °C for 4 h. Activity grade IV alumina was obtained by deactivation of grade I alumina by addition of 10% water. Determination of activity was performed by Brockmann method. Anhydrous sodium sulfate was analytical reagent (Shanghai Reagent Company, Shanghai, China). Nitrogen (>99.995%) was from Hangzhou Dianhua Company (Hangzhou, Zhejiang, China). Activated carbon was from Norit (Nederland B.V, USA).

2.4. Preparation of standards

The standard PAHs mixture from Supelco (Bellefonte, PA, USA) in benzene–dichloromethane (1:1, v/v) solution contained 2000 μg/ml each of naphthalene (Na), 1-methylnaphthalene (1-Me), 2-methylnaphthalene (2-Me), acenaphthene (Ac), fluorene (F), phenanthrene (Phe), anthracene (Ant), fluoranthene (Fl), pyrene (Pyr), benz(a)anthracene (BaA), chrysene (Chr), benzo(*b*)fluoranthene (BbF), benzo(*k*)fluoranthen (BkF), benzo(*a*)pyrene (BaP), dibenzo(*a,h*)anthracene (DbahA), benzo(*g,h,i*)perylene (BghiP), indeno(*1,2,3-cd*)pyrene (IP). Stock solutions containing 200.0 ng/ml of each PAHs were prepared by dilution of above standard mixture in acetonitrile and stored at 4 °C in

darkness to avoid possible light degradation. The calibration solutions were prepared daily, by appropriate dilution of the acetonitrile stock solutions.

2.5. Procedure

2.5.1. Sample preparation

A glass chromatographic column (300 mm × 15 mm I.D.) was half filled with *n*-hexane and 22 g of alumina (activity grade IV) mixed with a little *n*-hexane was rapidly added. A layer of about 30 mm of anhydrous sodium sulfate was added on top of the column and *n*-hexane was dropped out until it was at the same level as the top of this layer.

Samples (2.0 g) of natural tocopherols were exactly weighed into 10 ml volumetric flask and diluted to scale with *n*-hexane; then 2.0 ml (equivalent to 0.4 g tocopherols) of the diluted sample solution was loaded onto the alumina column, and the column was eluted with *n*-hexane at a flow rate of 1 ml/min approximately. The first 10 ml was discarded, and the next 80 ml was collected in a 100 ml round-bottom flask. The eluate was evaporated to about 2–4 ml by a rotary vacuum evaporator at 40 °C and transferred to a 5 ml vial. The round-bottom flask was rinsed with about 1.0 ml *n*-hexane and transferred to the vial again. The evaporation was continued to dryness at 28 °C under a gentle stream of nitrogen. The residues were reconstituted in 0.5 ml acetonitrile and filtered through 0.45 μm polytetrafluoroethylene (PTFE) filters (Millipore, Bedford, USA).

2.5.2. Liquid chromatography method

An aliquot (10 μl) of the acetonitrile solution was injected into HPLC system and eluted with the mobile phase consisting of acetonitrile (A) water (B) at 1.0 ml/min. The gradient elution program was as follows: 0 → 13 min, 40% A isocratic; 13 → 22 min, linear gradient 40% → 34% A; 22 → 32 min linear gradient 34% A → 16% A; and finally, back to the initial condition and recondition the column. The wavelength program of fluorescence detector was listed in Table 1.

Table 1
Excitation and emission wavelength program for RP-HPLC fluorescence detection

Time (min)	Excitation wavelength (nm)	Emission wavelength (nm)	PAHs detected
0	275	330	Na, 1-Me, 2-Me, Ac, F
11.0	250	366	Phe
12.2	250	402	Ant
14.3	280	460	Fl
16.3	270	390	Pyr
20.0	270	410	Chr, BaA
22.3	300	440	BbF, BkF, BaP, DbahA, BghiP, IP

3. Results and discussion

3.1. Confirmation of the sample preparation process

Firstly, a standard solution of PAHs (20.0 ng/ml, 2.0 ml) was loaded onto the alumina column, and several fractions, 10 ml each were collected using 100 ml *n*-hexane as elution solvent in order to ensure the fractions containing PAHs to be collected without other interferences. Each fraction was evaporated and reconstituted in 0.5 ml of acetonitrile prior to its analysis by RP-HPLC. Fig. 1 shows the elution curves of seventeen PAHs. As shown in Fig. 1, the PAHs components were not eluted by the first fraction of 10 ml, and began to be eluted gradually from the second fraction of 10 ml. The elution of Na, 1-Me, 2-Me, Ac + F reached the largest quantity by the third fraction of 10 ml; the elution of Phe, Ant, Fl, Pyr, BaA, Chr, BbF, BkF, BaP reached the largest quantity by the fourth fraction of 10 ml; the elution of BghiP + IP reached the largest quantity by the fifth fraction of 10 ml; the elution of DbahA which was the PAH most retained by alumina reached the largest quantity by the sixth fraction of 10 ml. All PAHs components were completely eluted by the ninth fraction of solvent.

Secondly, the elution condition of PAHs in different samples of natural tocopherols (54.0% mixed tocopherols, 98.0% α-tocopherol) was also assayed to examine if or not tocopherol matrices affected the elution of PAHs. Without PAHs in the first fraction of 10 ml which mainly contains the more apolar compounds (alkane) for all kinds of tocopherols, the PAHs were eluted by the next 80 ml elution solvent, except that PAHs in 54.0% mixed tocopherols were completely eluted by the next 70 ml elution solvent owing to more impurities in the tocopherols reducing column efficiency. Therefore, we collected from 10 ml to 90 ml fraction of eluate when actual samples were analyzed. Different polarity elution solvent (*n*-hexane with different percentages of dichloromethane) was also tested. When a more polar solvent was used, the PAHs were eluted within a shorter time, however, which led to a poorer separation between tocopherols and PAHs and more residue insoluble in acetonitrile after evaporation. The PAHs and tocopherols matrices had similar low-polarity, so that the more polar solvent which was strong solvent for alumina caused poor separation. Therefore, pure *n*-hexane was chosen as elution solvent.

3.2. Separation of PAHs

The elution gradient was a compromise between peak separation and run time. But among the seventeen PAHs, Ac and F, together with BghiP and IP, were not separated each other, so there are fifteen peaks in the HPLC-FL chromatogram (Fig. 2). For some PAHs the optimal excitation and emission wavelengths could not be used, so that seven wavelength shifts were adopted which were a compromise between the responses of all PAHs and interfering

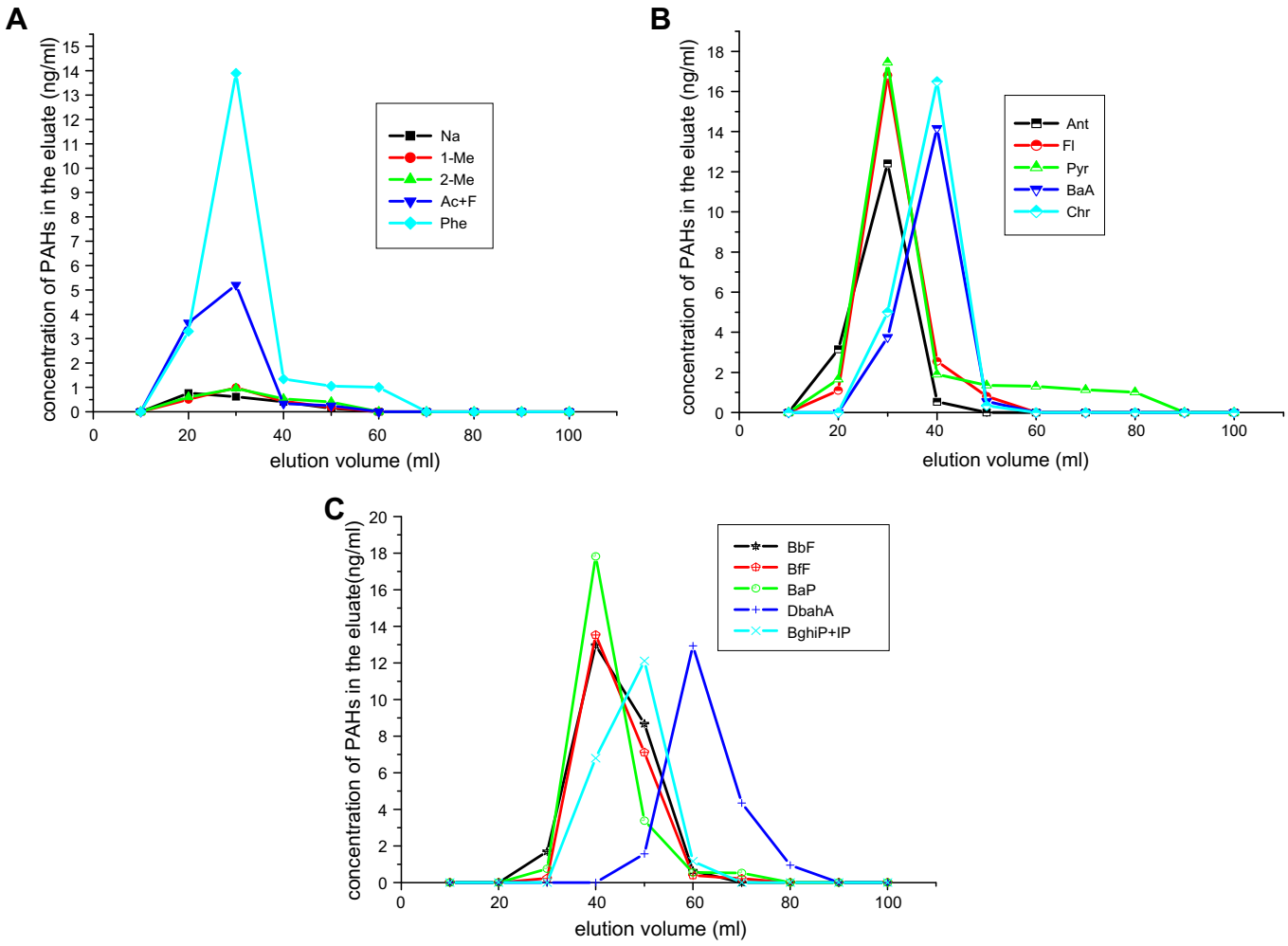

Fig. 1. Elution curves of seventeen PAHs from the alumina column when 2.0 ml (20.0 ng/ml) of a mixed standard solution was loaded.

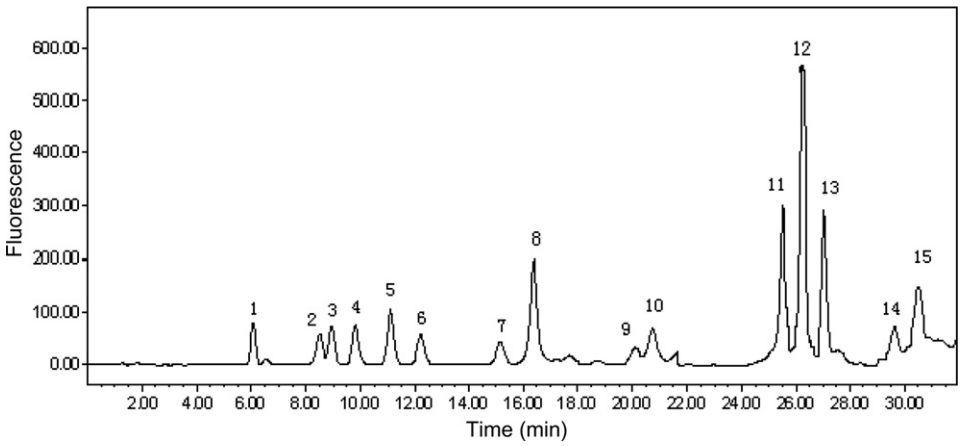

Fig. 2. HPLC-FL chromatogram of 54.0% mixed tocopherols. Peak: 1, Na; 2, 1-Me; 3, 2-Me; 4, Ac+F; 5, Phe; 6, Ant; 7, Fl; 8, Pyr; 9, Chr; 10, BaA; 11, BbF; 12, BkF; 13, BaP; 14, DbahA; 15, BghiP+IP.

peaks in the chromatogram, as well as acceptable separation between peaks. Fig. 2 shows a typical HPLC-FL chromatogram of 54.0% mixed tocopherols for PAHs. It can be seen that the separation was well for the seventeen PAHs, except for Ac and F, and BghiP and IP. The calibration curve was obtained by regression of peak area with

standard solution concentration. All calibration curves were highly linear (with correlation coefficient $r > 0.9998$) in the range of concentration examined ($\leqslant$200 ng/ml).

3.3. Validation of the method

3.3.1. Detection and quantification limits

To reach the detectability required, it was necessary to prepare at least 0.4 g tocopherols, and the PAHs residue had to be dissolved in 0.5 ml acetonitrile. The limit of detection (LOD) and the limit of quantification (LOQ) of the method were calculated from the signal-to-noise (S/N) ratio. The LOD corresponded to the analyte amount for which the S/N ratio of the peak area was equal to 3, and LOQ corresponded to a S/N ratio of 10. LOD and LOQ of the method were 0.01–0.11 ng/g and 0.04 to 0.38 ng/g for the heavy PAHs (BbF, BkF, BaP, DbahA, BghiP + IP), and 0.11–0.45 ng/g and 0.36 to 1.50 ng/g for the light PAHs (Na, 1-Me, 2-Me, Ac + F, Phe, Ant, Fl, Pyr, Chr, BaA), respectively, which were low enough to fulfill with the requirements proposed by various organizations.

3.3.2. Repeatability and recovery

To test the repeatability of the method, five replicate unfortified natural tocopherol samples and the calibration samples by spiking PAHs at two concentration levels (50 ng/g, 500 ng/g) into unfortified natural tocopherol samples were applied to the entire analytical procedure. As shown in Table 2, the method had good repeatability expressed by the RSD% (Relative standard deviation) which was less than 10.7% in all cases.

In order to evaluate the accuracy of the method, the recoveries were determined by the standard addition technique. The calibration samples by spiking PAHs at two concentration levels (50 ng/g, 500 ng/g) into unfortified natural tocopherol samples were analyzed 5 replicates. To calculate the percentage recoveries, the results had been corrected by comparing the concentrations before and after standard addition. The mean absolute recoveries presented in Table 2 were above 77.9%, except for the lowest molecular weight PAHs (Na, 1-Me, 2-Me, AC, F), which were between 15.9% and 75.8% owing to their high volatility and partially losing during the evaporation step. The results of low recoveries for the lowest molecular weight PAHs in other matrices were reported in the literature (Barranco et al., 2003; Moret & Conte, 1998,2002) for the same reason.

3.4. Application of the method to commercial sample analysis

The results for applying the method to different kinds of tocopherol samples (54.0% mixed tocopherol, high-purity mixed tocopherol, high-purity α-tocopherol) are shown in Table 3. If some samples with expected high PAHs levels outside the linear range levels had to be analyzed, it was necessary to dilute those samples. The method had good repeatability for the three kinds of tocopherols. Fig. 2 shows the HPLC chromatograms of 54.0% mixed tocopherols, and the HPLC chromatogram appears clean. As a result, the method was suitable to analyze different tocopherol samples.

Table 3 shows that 54.0% mixed tocopherols contained the highest amounts of PAHs (5445.9 ng/g tocopherol for

Table 2
Repeatability and recoveries of the method from five replicate the spiked samples

PAHs	Unfortified sample		Spiked sample					
			Level I[a]			Level II[b]		
	Mean value (ng/g)	RSD (%)	Mean value (ng/g^{-})	RSD (%)	Average recovery [c]	Mean value (ng/g^{-})	RSD (%)	Average recovery[c]
Na	43.5 ± 3.4	7.8	51.4 ± 4.3	8.4	15.9 ± 5.5	166.8 ± 9.7	5.8	24.7 ± 1.9
1-Me	49.8 ± 2.6	5.1	59.8 ± 5.0	8.3	20.1 ± 4.9	249.2 ± 4.8	1.9	39.9 ± 0.9
2-Me	44.0 ± 2.8	6.3	53.9 ± 5.0	9.3	19.9 ± 3.7	195.1 ± 6.3	3.2	30.2 ± 1.3
Ac + F	4.5 ± 0.2	5.1	42.4 ± 3.5	8.3	75.8 ± 7.1	315.2 ± 9.7	3.1	62.1 ± 1.9
Phe	51.7 ± 4.2	8.0	107.8 ± 11. 0	10.7	112.2 ± 9.9	547.8 ± 20.9	3.8	99.2 ± 4.2
Ant	0.69 ± 0.03	4.8	41.4 ± 4.0	9.8	81.4 ± 8.1	436.1 ± 26.2	5.6	87.1 ± 5.2
Fl	3.8 ± 0.2	5.7	51.8 ± 3.7	7.1	96.1 ± 7.4	568.1 ± 57.9	10.2	112.9 ± 1.6
Pyr	8.7 ± 0.2	2.7	64.1 ± 4.7	7.4	110.7 ± 9.5	526.54 ± 15.5	2.9	103.6 ± 3.1
Chr	n.d.	n.d.	44.1 ± 1.3	2.9	88.1 ± 2.6	453.2 ± 11.3	2.5	90.6 ± 2.2
BaA	n.d.	n.d.	50.4 ± 4.9	9.8	100.8 ± 10.9	488.2 ± 20.4	4.2	97.6 ± 4.1
BbF	0.34 ± 0.03	9.1	51.2 ± 2.5	4.9	101.8 ± 4.9	453.0 ± 32.0	7.1	90.5 ± 6.4
BkF	0.32 ± 0.03	8.4	39.5 ± 3.8	9.6	78.3 ± 7.6	410.7 ± 12.9	3.1	82.0 ± 2.6
BaP	0.30 ± 0.02	5.7	39.3 ± 1.8	4.5	77.9 ± 3.6	394.2 ± 21.8	5.5	78.8 ± 4.4
DbahA	n.d.	n.d.	46.8 ± 1.0	2.2	93.2 ± 1.9	483.4 ± 20.3	4.2	96.7 ± 4.0
BghiP + IP	n.d.	n.d.	40.7 ± 1.3	3.2	81.3 ± 2.6	415.3 ± 21.3	5.1	83.1 ± 4.3

RSD, relative standard deviation; n.d., not detected; SD, standard deviation.
[a] 50 ng PAHs standard were added in per gram tocopherol.
[b] 500 ng PAHs standard were added in per gram tocopherol.
[c] Mean ± SD expressed as % of recovery.

Table 3
Concentrations of PAHs in three different kinds of commercial tocopherol product

PAHs	54.0% mixed tocopherols		90.5% mixed tocopherols		98.0% α-tocopherol	
	Mean value (ng/g)	RSD (%)	Mean value (ng/g)	RSD (%)	Mean value (ng/g)	RSD (%)
Light PAHs						
Na	1037.1 ± 109.5	10.7	26.7 ± 2.1	7.9	45.6 ± 3.8	8.3
1-Me	829.0 ± 61.4	7.4	20.5 ± 0.9	4.2	52.5 ± 2.6	4.9
2-Me	787.4 ± 61.0	7.7	11.1 ± 1.0	9.4	21.1 ± 1.8	8.4
Ac + F	208.7 ± 13.6	6.5	4.6 ± 0.3	6.9	12.8 ± 0.9	6.7
Phe	1148.9 ± 80.2	7.0	37.3 ± 3.6	9.6	125.1 ± 8.0	6.4
Ant	189.6 ± 15.5	8.2	3.0 ± 0.3	8.5	5.4 ± 0.5	9.2
Fl	541.5 ± 45.3	8.4	27.0 ± 0.6	2.0	15.0 ± 1.4	9.0
Pyr	284.3 ± 26.8	9.4	63.9 ± 6.4	10.0	26.7 ± 1.4	5.4
Chr	297.1 ± 30.6	10.3	102.1 ± 5.3	5.2	7.8 ± 0.8	10.0
BaA	122.2 ± 10.6	8.7	70.3 ± 6.9	9.8	5.2 ± 0.3	5.2
Total (Light PAHs)	5445.9		366.4		317.1	
Heavy PAHs						
BbF	832.8 ± 46.8	5.6	428.4 ± 7.4	1.7	17.6 ± 2.1	11.9
BkF	298.1 ± 18.2	6.1	125.6 ± 2.9	2.3	8.3 ± 0.4	5.1
BaP	469.5 ± 36.8	7.8	315.0 ± 9.5	3.0	7.7 ± 0.8	10.6
DbahA	316.4 ± 25.1	7.9	151.3 ± 3.5	2.3	46.8 ± 1.0	2.2
BghiP + IP	357.8 ± 28.4	7.9	272.4 ± 7.6	2.8	6.7 ± 0.6	8.6
Total (Heavy PAHs)	2274.8		1292.8		87.0	
Total	7720.7		1659.2		404.1	

the light PAHs and up to 2274.8 ng/g tocopherol for the heavy PAHs), high-purity mixed tocopherols also contained relatively high level of PAHs (366.4 ng/g tocopherol for the light PAHs, 1292.8 ng/g tocopherol for the heavy PAHs), and high-purity α-tocopherol contained the lowest level of PAHs wherein the level of the heavy PAHs was reduced to 87.0 ng/g and the light PAHs was still high (317.1 ng/g). In general the PAHs contents in natural tocopherols were greatly above the maximum limits that various organizations proposed for oils. The results obtained from the three kinds of tocopherols demonstrate that quality control is required.

4. Conclusions

A simple and rapid method has been developed and validated for the determination of seventeen PAHs in natural tocopherol products, wherein alumina column chromatography was used for the separation of them from different kinds of natural tocopherols and PAHs were separated and determined by reversed-phase high-performance liquid chromatography with fluorescence detection. The method showed good recovery and repeatability. Limits of quantification ranged from 0.04 to 1.5 ng/g for the seventeen PAHs. The whole procedure required approximately 2 h (including sample preparation and analysis by HPLC) and it reduced amounts of solvents and samples compared with the traditional methods. All these facts made the method applicable to routine industrial analysis and quality control of different kinds of natural tocopherols.

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