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Solid-phase clean-up in the liquid chromatographic determination of polycyclic aromatic hydrocarbons in edible oils

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Abstract

A solid-phase extraction (SPE) method for sample clean-up, followed by reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection is reported for the determination of polycyclic aromatic hydrocarbons (PAHs) in edible oils. The effects of experimental variables, such as washing and elution solvents, sample solvent and drying time have been studied using C_{18} cartridges. Recoveries and selectivity using other sorbent materials (C_8, C_2, CH, PH and NH_2) were also examined, with C_{18} being the best one. The recoveries ranged between 50 and 103% depending on the molecular mass of the PAH. The limits of quantitation were lower than 1 ng/g for most PAHs and good precision was achieved. The method was validated using certified reference materials.

Keywords: Solid-phase extraction; Oils; Clean-up methods; Edible oils; Polynuclear aromatic hydrocarbons

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a well-known group of chemical contaminants with a wide distribution in the environment and considered to be human carcinogens [1]. It has been estimated that human intake of PAHs from food is considerably higher than that from ambient air or drinking water, edible oils and fats being the most contributing sources because of their lipophilic nature [2].

More than 100 PAHs have been found in nature; however, only 16 have been selected as priority pollutants by the United States Environmental Protection Agency (EPA) on the basis of their occurrence and carcinogenicity [3]. There is no directive of the European Union establishing legal limits for these compounds in oils and fats. For instance, Germany, Austria and Switzerland have adopted a legal limit of 1 ng/g for benzo(*a*)pyrene (BaP) content in smoked foodstuff, and recently Spain has set a limit of 2 ng/g in olive residue oils for some of these compounds [4,5]. This lack of a legal limit in edible oils and fats has led some organisations to set their own recommended limits. In this sense, the German Society for Fat Science has established the following limits: 25 ng/g for total PAHs and 5 ng/g for the heavy fraction [6]. Taking these facts into account, there is a need to design

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analytical methods which reach these limits of detection. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been the most used chromatographic techniques in order to separate and quantify PAHs extracted from foodstuff [7–9]. In this study HPLC with fluorescence detection was used, due to its selectivity and sensitivity. However, acenaphtylene has too low a fluorescence quantum yield, that makes impossible its quantitation by fluorescence [10].

Most of the methods applied to the determination of PAHs in oils and fats involve a saponification step with KOH–methanolic solution and a liquid–liquid extraction (LLE) with hexane, cyclohexane or isooctane; then, the extract is cleaned up on a packed column (silica, alumina, Sephadex or Florisil) [7,11]. In order to avoid the complex and time consuming alkaline digestion, a liquid–liquid partition method with dimethylformamide (DMF)–water (9:1, v/v) or dimethyl sulfoxide (DMSO) has been used to obtain good recoveries [12–15]. Adding water to the extract allows back-extraction to an apolar solvent such as hexane or cyclohexane. However, more purification steps (column chromatography with silica, alumina, Sephadex LH-20 . . .) are still needed to get extracts clean enough for chromatographic analysis [13,16].

Solid-phase extraction (SPE) with silica has been successfully used instead of packed chromatography columns [17]. This technique has a potentially excellent selectivity due to a wide range of available sorbents and a wide variety of extraction conditions that may be used to achieve the desired separation [18]. Moreover, lower amounts of solvents and shorter analysis times are required. In this work, the use of SPE with reversed-phase sorbents is proposed as a substitute of back-extraction and column chromatography clean-up steps for the extraction of PAHs from edible oils.

2. Experimental

2.1. Reagents and chemicals

Acetonitrile, methanol, tetrahydrofuran (THF), acetone, diethylether and hexane from Romil Chemical (Heidelberg, Germany) were of HPLC grade. Water was purified on a Milli-Q system from Millipore (Bedford, MA, USA). *N*,*N*-Dimethylformamide (DMF) from Fluka (Buchs, Switzerland) was of analytical grade and nitrogen (>99.995%) was from Air Liquide (Madrid, Spain).

The standard polynuclear aromatic hydrocarbons mix from Supelco (Bellefonte, PA, USA) consisted of a solution in acetonitrile–methanol (90:10, v/v) of naphthalene (Na) 500 μg/ml, acenaphtylene (Ap) 500 μg/ml, acenaphthene (Ac) 1000 μg/ml, fluorene (F) 100 μg/ml, phenanthrene (Phe) 40 μg/ml, anthracene (Ant) 20 μg/ml, fluoranthene (Fl) 50 μg/ml, pyrene (Pyr) 100 μg/ml, benzo[*a*]anthracene (BaA) 50 μg/ml, chrysene (Chr) 50 μg/ml, benzo[*b*]fluoranthene (BbF) 20 μg/ml, benzo[*k*]-fluoranthene (BkF) 20 μg/ml, benzo[*a*]pyrene (BaP) 50 μg/ml, dibenzo[*a*,*h*]anthracene (DbahA) 200 μg/ml, benzo[*g*,*h*,*i*]perylene (BghiP) 80 μg/ml and indeno[1,2,3-*c*,*d*]pyrene (IP) 50 μg/ml. Stock solutions containing 0.25 μg/ml of BaP were prepared by dilution of this standard mix in hexane or acetonitrile and stored at 4 °C in darkness. These stock solutions were stable for almost 3 months. The different calibration solutions were prepared daily, by appropriate dilution of the acetonitrile stock solution.

2.2. Instrumentation

The solid-phase extraction was performed in a 12-port Visiprep solid-phase extraction Vacuum Manifold (Supelco). The SPE cartridges were Varian Bond-Elut (Barcelona, Spain) octadecyl (C_{18}) silica bonded phase (100, 500 or 1000 mg) and octyl (C_8), ethyl (C_2), cyclohexyl (CH), phenyl (PH) and aminopropyl (NH_2) silica bonded phases (100 mg). The extracts were evaporated to dryness using a Zymark Turbo Vap LV evaporator (Hopkinton, MA, USA), provided with nitrogen blowdown and a water bath at 28 °C. After solvent evaporation, the final extract was filtered through a Waters 0.45 μm GHP filter (Milford, MA, USA) prior to injection in HPLC.

HPLC was used for the determination and quantitation of each PAH. The chromatographic system consisted of a Hewlett-Packard Series 1100 system, equipped with a vacuum degasser, quaternary pump, autosampler and fluorescence detector, connected to a HP Chemstation software. This system allows the

obtention of fluorescence spectra along the chromatogram and comparing spectra from different chromatograms, obtaining a match factor. Excitation and emission wavelengths were programmed as reported in Table 1. A reversed-phase Vydac (Hesperia, CA, USA) C_{18} column (250×4.6 mm I.D., 5 μm) and a Waters (Barcelona, Spain) Nova-Pak C_{18} guard column (20×3.9 mm I.D., 4 μm) were used. A Perkin-Elmer oven LC 101 (Norwalk, CT, USA) was used to maintain the temperature of the column constant at 35 °C. Solvents that constituted the mobile phase were A (acetonitrile) and B (water). The elution conditions applied were: 0–10 min, 50% A isocratic; 10–24 min, linear gradient 50% A–100% A; 24–35 min, 100% A isocratic; and finally, back to the initial conditions and recondition the column. The flow-rate was 1 ml/min and the injection volume was 30 μl.

2.3. Samples

Samples of edible oils (crude and refined coconut oil, crude sunflower oil, palm oil, palm kernel oil, palm stearine, olive oil and residue olive oil) were commercially available.

In order to validate the method, two certified reference materials from BCR/IRMM (Geel, Belgium) were used; a highly refined coconut oil (CRM 459), which has been considered as a ''blank'' oil (certified values in Table 3) and a coconut oil artificially fortified with six selected PAHs in the lower ng/g level (CRM 458; certified values in Table 3).

Aliquots (0.5 g) of edible oils were diluted with 5 ml of hexane and extracted twice with 5 ml of DMF–water (9:1, v/v). The combined extracts were diluted with water until they reached a 1:1 (v/v) proportion before SPE clean-up. SPE cartridges were activated with 5 ml of methanol and 5 ml of the sample solvent, a mixture of DMF–water (1:1, v/v). Then, the sample was loaded and the sides of the vessel were washed with 10 ml of sample solvent that were passed through the cartridge. Finally, the sorbent was washed with 10 ml of an adequate washing solvent, dried and PAHs were eluted with 4 ml of a convenient elution liquid. The SPE extracts were evaporated to dryness, redissolved in 0.2 ml of acetonitrile, filtered and injected into the chromatograph.

Table 1
Excitation and emission wavelength program

Time (min)	Excitation (nm)	Emission (nm)	PAH detected
0.0	275	330	Na, Ac, F
17.3	250	366	Phe
18.8	250	400	Ant
20.0	270	460	Fl
		390	Pyr, BaA, Chr
25.4	255	410	BbF, BkF, BaP
28.5	290	410	DbahA, BghiP
		500	IP

3. Results and discussion

3.1. Optimisation of the extraction procedure

Partitioning of edible oils between hexane and pure DMF was confirmed to be adequate by experiments using standard solutions. The presence of water together with DMF decreased the recoveries of all PAHs, but up to 10% of water could be used without significant losses in PAH recoveries.

Previous studies of PAH extraction from other food matrices, in which C_{18} cartridges were used, indicated that this sorbent was efficient to clean-up aqueous extracts [19]. Thus, dilution with water of DMF extracts was performed and the use of C_{18} sorbent was assayed. Several SPE parameters had to be examined to establish the optimum conditions, in a first stage with standard solutions and after with a sample of 0.5 g of crude coconut oil.

The concentration of the organic solvent in the sample solvent is a critical parameter, because, if it is too low, it may not be enough to solubilize the heavy PAHs, whereas if it is too high, the breakthrough volume for the light PAHs will be too low, and they will not be retained on the SPE cartridge [18]. Therefore, several percentages of DMF were studied. Fig. 1 shows that extraction yield increased with the water content of the sample solvent. The first four PAHs presented low recoveries due to their volatility and they were lost during the evaporation step. These losses could be reduced using other concentration

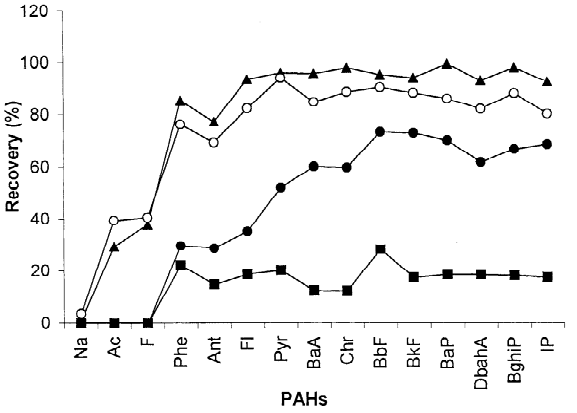

Fig. 1. Effect of DMF content of sample solvent on the SPE recoveries for a PAH standard solution containing 2.5 ng/ml of BaP. ○, DMF–water (1:2, v/v); ▲, DMF–water (1:1, v/v); ●, DMF–water (2:1, v/v); ■, DMF–water (5:1, v/v). PAH abbreviations as indicated in the Experimental.

methods such as a Kuderna Danish apparatus. The maximum recoveries were reached for a DMF–water volume relation of 1:1.

In order to get a cleaner final extract with less interferences, different THF–water and acetone–water mixtures, and an acid (H_3PO_4 1 *M*) and a basic (NaOH 1 *M*) aqueous solution were tested as washing solvents. No improvements in selectivity were observed with any of these washing solvents in relation to the use of pure water when coconut oil samples were treated. Therefore, water was chosen as washing solvent. This washing step could be obviated, because little additional improvement in selectivity was observed.

Once the analytes are retained and the sorbent is washed, a sorbent drying step is needed to remove water before elution; otherwise, if an apolar solvent is used for elution worse recoveries and repeatabilities would be obtained [18]. In addition, the presence of water in the final extract makes difficult the evaporation step, because a low temperature is needed in order to avoid losses by volatilization of the lighter PAHs. After 10 min of drying in the vacuum system no more water was observed.

Next, solvents of different polarity (acetonitrile, methanol, acetone, THF, diethylether and hexane) were checked for the elution step. The best recoveries were obtained when hexane was used. The lowest molecular mass PAHs (Na, Ap, Ac, Fl, Phe and Ant) reached better recoveries with hexane due to its low vapour pressure, which reduces losses by volatilization during the evaporation step. The heavy PAHs, with 4–6 aromatic rings, have low polarities, so they were better eluted with apolar solvents (THF, diethylether and hexane). The order of extraction recoveries for most PAHs were as follows: hexane> diethylether~THF>acetonitrile~methanol>acetone. Acetone was an exception in this logical order, providing recoveries lower than those solvents of less eluotropic strength on C_{18}, like acetonitrile or methanol. No explanation for this unexpected behaviour was found. When a sample of crude coconut oil was tested using these solvents, similar selectivities were observed. Taking into account these results, hexane was selected as the best eluting solvent, the recoveries being around 90% for almost all PAHs.

Several SPE sorbents were tested using the optimum conditions. C_{18} and C_8 sorbents showed the same behaviour, so they can be indistinctly used. With CH sorbent, good recoveries were obtained for the PAHs of 4–6 rings, but the low molecular mass ones were less retained. With more polar sorbents (such as C_2, PH, and NH_2) poor recoveries were obtained, because PAHs were not retained. The selectivity provided by C_{18} and C_8 sorbents was assayed with coconut oil and no significant differences were observed, so the study was continued with the C_{18} cartridge.

Finally, the amount of sample was evaluated. The whole procedure was applied to different amounts of coconut oil (0.1 and 0.5 g; 0.1, 0.5 and 1 g; and 0.5 and 1 g) with cartridges of different mass of sorbent (100, 500 and 1000 mg, respectively). When cartridges of 100 mg were used, the recoveries were lower, probably due to an overloading of active sites of the sorbent. Only with cartridges of 1000 mg, it was possible to analyse 1 g of oil, but with 0.5 g of coconut oil and 500 mg of C_{18} sorbent, good recoveries and the sensibility required were achieved.

Thus, the procedure finally proposed for the SPE of PAHs in edible oils included washing the C_{18} sorbent with water, drying during 10 min and eluting with pure hexane.

3.2. Validation of the method

The proposed method was validated following the Eurachem guideline [20]. The selectivity of the method was assessed evaluating the purity of PAH chromatographic peaks, and comparing the excitation and emission spectra of each PAH peak in the chromatogram of the different edible oils assayed with those obtained with standard solutions. In most cases, the purity of the peak and the grade of match with the spectra of standards were greater than 96%. Low spectral matches were only observed for some PAHs whose concentrations were below their limit of quantitation. A certified reference material of a ''blank'' (highly refined) coconut oil (CRM 459) was also used to assess the selectivity. Fig. 2 shows the chromatograms obtained for this blank oil and that for the same oil fortified at a low concentration level. No interferences were found in these chromatograms for the six certified PAHs of CRM 459; little amounts of pyrene and chrysene, below their certified values, were found in this ''blank'' oil (certified values <0.9 and <0.6 ng/g; found values 0.6 and 0.4 ng/g, respectively). Some peaks due to the presence of other not certified PAHs appeared in the case of the blank oil: phenanthrene (~0.4 ng/g), anthracene (0.3 ng/g), fluoranthene (0.8 ng/g) and benzo(*a*)anthracene (0.4 ng/g).

The same reference material (CRM 459) was used to evaluate limits of quantitation, linear ranges, recoveries and repeatabilities (within-day and between-days) of the proposed method. These results are given in Tables 2 and 3.

The limits of quantitation were less than 1 ng/g for most PAHs and low enough to fulfil the legal requirements. Limits of quantitation were also determined for sunflower oil and olive oil, obtaining similar results to coconut oil. The concentrations of PAHs in all kinds of oil samples analysed were inside the linear range except for the crude coconut and the olive residue oil, which had a large PAH content, so it was necessary to dilute the sample. The precision of the method, expressed by the coefficient of variation (C.V.), was estimated by measuring the within-day and between-day repeatabilities at three different concentration levels. Intra-day repeatabilities and inter-day repeatabilities were in all cases less than 8.0%. Only naphthalene gave rise to a higher value (32%) due to its high volatility. These results were similar to those obtained by other authors with silica cartridges [10,12,21].

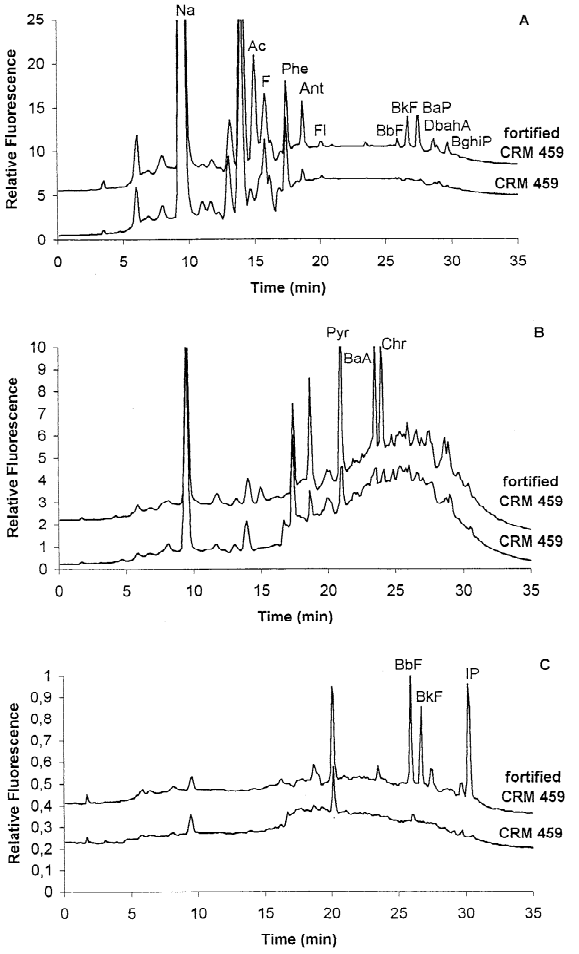

Fig. 2. Chromatograms of a highly refined coconut oil (CRM 459, PAHs certified values in Table 3) and this oil fortified at a level corresponding to 2.5 ng/g of BaP. (A) Fluorescence program as in Table 1, (B) excitation wavelength as in Table 1 and emission wavelength always 390 nm, and (C) excitation wavelength as in Table 1 and emission wavelength always 500 nm. PAH abbreviations as indicated in the Experimental.

A reference material of a coconut oil with certified concentrations for certain PAHs (CRM 458) was used to evaluate the accuracy of the method. For the six certified PAHs, differences lower than 14% were achieved (Table 3). For the rest of the PAHs, the accuracy was assessed using the reference material

Table 2
Limit of quantitation, working linear range and precision of the method

PAHs	Limit of quantitation (ng/g)[a]	Linear range (ng/g)	Intra-day repeatibility[b]	Inter-day repeatibility[c]
Ac	6	6–200	4.8	2.3
F	2.5	2.5–50	5.2	4.9
Phe	1	1–40	6.1	5.5
Ant	0.3	0.3–50	3.9	1.7
Fl	0.8	0.8–100	3.6	3.2
Pyr	0.6	0.6–50	2.9	2.3
BaA	0.3	0.3–125	2.5	2.0
Chr	0.3	0.3–125	3.3	1.8
BbF	0.4	0.4–50	4.4	3.5
BkF	0.3	0.3–50	3.9	3.6
BaP	0.3	0.3–50	3.1	2.4
DbahA	0.6	0.6–200	5.5	4.5
BghiP	0.4	0.4–80	4.5	3.4
IP	0.3	0.3–50	4.8	4.6

[a] Limit of quantitation was calculated as the lowest concentration that can be determined with an acceptable level of uncertainty (10%).
[b] Mean of the within-day repeatibilities ($n=10$) obtained for the three different concentration levels assayed.
[c] Mean of the between-day repeatibility ($n=4$) obtained for the three different concentration levels assayed.

of a ''blank'' coconut oil (CRM 459) which was fortified at two different concentration levels. The mean absolute recoveries were above 80% for all PAHs, except for the lowest molecular mass PAHs (Ac, F, Phe and Ant), that are more volatile and they are partially lost during the evaporation step.

This method was applied to determine PAHs in 47 samples of nine different kinds of edible oils and

Table 3
Accuracy of the method. Reference material CRM 458 is a coconut oil artificially fortified with six selected PAHs and reference material CRM 459 is a highly refined coconut (certified values: <0.9 ng/g of Pyr, <0.6 ng/g of Chr, <0.2 ng/g of BkF, <0.3 ng/g of BaP, <0.2 ng/g of BghiP and <0.2 ng/g of IP)

PAHs	Reference material CRM 458		Reference material CRM 459	
	Certified values (ng/g)[a]	Measured (ng/g) ($n=5$)[a]	Fortified at 1.25 ng/g of BaP (%)[b]	Fortified at 12.5 ng/g of BaP (%)[b]
Ac			51±3	53±3
F			54±8	60±3
Phe			70±2	69±5
Ant			76±5	82±2
Fl			81±5	87±2
Pyr	9.4±1.5	10.0±1.1	102±6	87±2
BaA			88±4	90±1
Chr	4.9±0.4	4.8±0.2	98±6	92±2
BbF			98±10	87±3
BkF	1.9±0.2	1.79±0.03	91±5	91±3
BaP	0.93±0.09	0.83±0.03	94±3	94±2
DbahA			87±6	87±5
BghiP	0.97±0.07	0.89±0.04	94±4	87±3
IP	1.00±0.07	0.92±0.04	94±5	88±5

[a] Mean±confidence interval for a 95% confidence level.
[b] Mean±standard deviation ($n=10$) expressed as % of recovery.

Table 4
Concentrations (ng/g) of PAHs in different oils analyzed: crude (three samples) and refined (two samples) coconut oil, crude (four samples) and refined (four samples) sunflower oil, palm oil (two samples), palm stearine (one sample), palm kernel oil (two samples), olive oil (15 samples) and olive residue oil (14 samples)

PAHs	Range of positive values	Mean
Ac	37–68[a]	50
F	3.1–264	42
Phe	2.1–1145	176
Ant	0.4–230	34
Fl	1.1–464	83
Pyr	2.9–452	94
BaA	0.3–192	27
Chr	1.3–315	46
BbF	0.6–129	20
BkF	0.4–29	7.0
BaP	0.5–136	13
DbahA	0.8–8.3	2.0
BghiP	0.4–81	7.8
IP	0.3–52	5.3

[a] Positive values found only in crude coconut oil.

good results were obtained (Tables 4 and 5). It is remarkable the great reduction in the amount of PAHs found in the samples of olive residue oil collected after the new regulation set in Spain in July 2001.

4. Conclusion

A rapid SPE method was proposed for the isolation and purification of PAHs from edible oils. The method showed excellent recoveries and precisions and limits of quantitation below the ng/g level for most of the PAHs analysed. The good selectivity obtained allows the method to be applied to all types of edible oils. The whole procedure requires 80 min (including liquid–liquid extraction, SPE and analysis by HPLC) and it does not need large volumes of solvents. All these facts made this method suitable for routine analysis.

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Table 5
Concentrations (ng/g) of PAHs in different oils analyzed

PAHs	Crude coconut oil	Refined coconut oil	Crude sunflower oil	Palm oil	Palm stearine	Palm kernel oil	Olive oil	Olive residue oil[a]	Olive residue oil[b]
Ac	68	<1.5[c]	<6[d]	<1.5[c]	<6[d]	<6[d]	<6[d]	<6[d]	<1.5[c]
F	264	5.3	8.6	5.4	4.5	6.6	3.1	35	<2.5[d]
Phe	1145	11	26	37	15	64	10	99	<0.4[c]
Ant	230	1.8	3.1	3.3	1.0	8.9	0.4	22	0.4
Fl	464	5.7	11	3.7	1.1	24	3.3	233	1.2
Pyr	452	8.9	9.3	5.5	<0.3[c]	13	3.9	254	2.9
BaA	31	0.3	4.3	<0.3[d]	<0.1[c]	<0.1[c]	<0.1[c]	97	0.5
Chr	47	1.6	4.3	<0.3[d]	<0.2[c]	<0.2[c]	1.3	217	3.3
BbF	29	<0.4[d]	3.9	<0.4[d]	<0.2[c]	<0.4[d]	0.6	67	1.7
BkF	9.6	<0.3[d]	1.8	<0.3[d]	0.4	<0.3[d]	<0.3[d]	16	<0.3[d]
BaP	26	1.7	3.6	1.1	0.7	<0.3[d]	0.5	67	2.6
DbahA	1.7	0.8	<0.6[d]	1.7	2.6	<0.3[c]	<0.3[c]	3.6	1.6
BghiP	30	0.4	4.3	1.6	0.8	1.0	0.9	30	1.4
IP	17	1.5	3.7	0.8	0.5	0.3	0.3	18	<0.3[d]

[a] Olive residue oil from market before summer 2001.
[b] Olive residue oil from market after summer 2001.
[c] LOD, limit of detection.
[d] LOQ, limit of quantitation.

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