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Rapid determination of benzo(*a*)pyrene in olive oil samples with solid-phase extraction and low-pressure, wide-bore gas chromatography–mass spectrometry and fast liquid chromatography with fluorescence detection

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Abstract

Benzo(*a*)pyrene (B(*a*)P) was extracted from olive oil using solid-phase extraction on columns filled with Florisil and Nucleoprep C18. The extracts were analyzed with GC–MS, using standard capillary column and low-pressure wide-bore column (LP-GC–MS), as well as with HPLC on standard column and short donor–acceptor complex chromatography (DACC) column. Quantitation was done with isotope dilution method (GC–MS and LP-GC–MS) or with internal standard benzo(*k*)fluoranthene (HPLC). Limits of detection were 1 ng/g for GC–MS on standard column, 1.6 ng/g on LP-column, 0.5 ng/g for HPLC on standard column, and 0.3 ng/g on DACC column, respectively. The applied extraction method allowed handling over 50 samples per day and assured recovery over 80%. Matrix solid-phase dispersion, tried as an alternative isolation method, appeared less advantageous. Fast chromatographic methods (LP-GC–MS and HPLC on DACC) made it possible to reduce analysis time to 8 and 5 min, respectively. The method was applied to routine analysis of B(*a*)P in olive oil samples.

Keywords: Olive oil; Solid-phase extraction; Benzo(*a*)pyrene

1. Introduction

Benzo(*a*)pyrene (B(*a*)P), as well as other polycyclic aromatic hydrocarbons (PAH), is generated to environment mainly in combustion process of various biogenic or anthropogenic materials. B(*a*)P is regarded as suspected carcinogen and subjected to monitoring in the environment and food products.

B(*a*)P occurs also in olive–pomace oil. This is associated with several factors during the production process, like: use of contaminated solvent for extraction of the oil from the olive mash, exposure of the mash to gasoline exhaust, or extensive use of heat during solvent evaporation. The same factors cause contamination of grapeseed oil with B(*a*)P [1] as well as high levels of mineral paraffins in olive–pomace oil [2]. International Olive Oil Council recommended in 2001 a value of 2 μg/kg as a maximum tolerable concentration of benzo(*a*)pyrene as well as other PAHs in olive–pomace oil [3]. The same concentration was accepted also by the Food Safety Authority in the UK [4] and Ireland [5]. According to Food Standard Agency, in 2001 in some olive–pomace oil samples, originating from Greece, Spain and Italy, the levels of B(*a*)P ranging from 9 to 43 ng/g were found [4].

Most methods used for isolation of B(*a*)P in oil or fat-containing specimens consist of time consuming procedure, comprising saponification of fats, solvent extraction, and column extraction/clean-up. These methods were reviewed by Moret and Conte [6]. These authors stressed the need of fast analytical methods, based on hyphenation of GC with HPLC or HPLC on coupled columns. van Stijn et al. [7] developed fast HPLC method for determination of PAH in edible oils, using on-line clean-up of sample on a short donor–acceptor complex chromatography column (DACC) with consecutive column switching to the ODS analytical column and fluorescence detection. Moret and Conte [8] isolated PAHs from edible oil using SPE on silica

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cartridges, followed by HPLC with fluorometric detection. International Organization for Standardization published a HPLC method for determination of B(*a*)P in animal and vegetable fats and oils, using reversed phase HPLC and fluorometric detection [9]. In the last years, some authors applied matrix solid-phase dispersion (MSPD) as an isolation method for B(*a*)P and other PAHs from solid or semi-solid samples [10–12]. Both chromatographic methods—GC–MS and HPLC—are regarded as complementary in the analysis of PAHs [13].

The present study followed two purposes. The first was to develop a fast and reliable isolation method, applicable for HPLC and GC analysis. This was undertaken using two isolation techniques: SPE and MSPD. The second purpose was to develop fast GC–MS and HPLC procedures for separation and quantitation of B(*a*)P extracted from olive oil. In the case of GC–MS, the low-pressure gas chromatography (LP-GC) has been applied. This technique was recently successfully introduced in GC–MS by de Zeeuw et al. [14], and applied for fast separation of pesticide residues [15,16] or aromatic hydrocarbons [17]. In the case of HPLC, an attempt was undertaken to accelerate the separation. For this purpose, a fast DACC column was applied for separation, besides a standard ODS column. Generally, the final method should assure a distinct gain in analysis time, without compromising the selectivity and sensitivity.

2. Experimental

2.1. Reagents and materials

C18 Nucleoprep 300-30 (art. 712917.100, Macherey Nagel, Düren, Germany). Florisil (Macherey Nagel, Düren, Germany, 100-60 mesh), rinsed before use consecutively with acetonitrile and hexane/dichlormethane (4:1). Empty plastic syringe tube columns 6 ml with filter at the bottom.

Extra virgin, first-cold pressed olive oil from different manufacturers was used as blank material for preparation of calibration samples. The samples of oil, extracted without any additions with SPE method, were checked with HPLC method applied in the study for the absence of any peaks with retention time corresponding to benzo(*a*)pyrene and benzo(*k*)fluoranthene. Such samples were run along with each series of spiked standards.

Benzo(*a*)pyrene (Cerilliant, Austin, TX, USA) 100 μg/ml acetonitrile, stored under Ar in freezer. Working solutions of B(*a*)P were prepared in acetonitrile (for HPLC) or in hexane (for GC–MS).

Internal standard for GC–MS: deuterated B(*a*)P-D12 (Cerilliant), 200 μg/ml isooctane, 1.2 ml ampoule. Working solution 1 μg/ml was prepared in hexane.

Internal standard for HPLC: benzo(*k*)fluoranthene (Cerilliant), 100 μg/ml acetonitrile. Working solution 1 μg/ml was prepared in acetonitrile.

Mixture of 16 PAH (EPA Method 8310 Stock Standard, Cerilliant), 100 μg/ml acetonitrile. Working solution diluted 1 μg/ml was prepared in acetonitrile.

2.2. Sample pretreatment

2.2.1. Preparation of oil samples for extraction

Twenty-five microliters of working IS solution were added to 5 g of oil and vortexed for 2 min. In the case of spiked standards, 20 μl of appropriate solution of B(*a*)P were additionally added to oil and vortexed for 2 min. After around 30 min, 0.5 g portions of oil were then taken for individual extraction.

2.2.2. Solid-phase column extraction

Five hundred milligrams of C18 Nucleoprep material was put on the bottom of plastic syringe tube, and 1 g of Florisil (MN, 100-60 mesh) was added on the top. Half milliliter of oil, spiked with internal standard (benzo(*k*)fluoranthene, 5 ng/g oil for HPLC, B(*a*)P-D12, 5 ng/g oil for GC–MS) was applied on the column. The column was eluted with 10 ml acetonitrile under low vacuum (1 ml/min). Acetonitrile was collected, evaporated under nitrogen, and reconstituted with 200 μl acetonitrile for HPLC or with 100 μl with hexane for GC–MS analysis. Ten or 20 μl acetonitrile were injected into HPLC, and 1–5 μl were injected into GC–MS.

2.2.3. Matrix solid-phase dispersion

Half gram of oil, spiked with internal standard, was applied on 2 g C18 phase in glass mortar and ground gently with pestle until homogenous and dry. This step took around 15–20 min. The mixture was poured in plastic syringe tube filled on the bottom with 2 g of Florisil, settled gently, and eluted with 12 ml of acetonitrile under low vacuum. The eluate was collected, evaporated under nitrogen

Table 1
Comparison of absolute recoveries of B(*a*)P and B(*a*)-P-D12 extracted with SPE and MSPD and retention times for B(*a*)P determined standard and low-pressure (LP-GC) GC–MS procedure

Extraction	GC	Retention time for IS/B(*a*)P (min)	Percent recovery IS[a]	Percent recovery B(*a*)P[a]
SPE	Standard	35.26/35.33	85 ± 7.2	77 ± 4.4
SPE	LP-GC	5.26/5.30	86 ± 6.0	79 ± 6.4
MSPD	Standard	35.23/35.31	56 ± 11.1	66 ± 8.2
MSPD	LP-GC	4.98/5.12	63 ± 10.0	55 ± 11.0

[a] Mean values ± S.D. from four concentrations. The recoveries were calculated against peak areas for non-extracted compounds, analyzed in the same analytical run.

and reconstituted to 100 μl with hexane. One to five microliters was injected into GC–MS.

2.3. Gas chromatography–mass spectrometry (standard and low-pressure GC)

GC–MS was performed on instrument using two columns. A DB5-5MS column, 30 m × 0.25 mm i.d., film thickness 0.25 μm (J&W Scientific, Folsom, CA, USA) was applied for standard GC–MS. A Rapid MS FS CP-Sil 8 low bleed column, 10 m × 0.53 mm, film thickness 0.50 μm (Varian Chrompack International, Middelburg, The Netherlands) fitted with restrictor 0.6 m × 0.25 mm, installed on the injection end, was used for LP-GC–MS.

For the standard column, following temperature program was used: 1 min at 50 °C, rise 7.5 °C/min to 310 °C, hold for 6 min. Total run time was 42 min. For the rapid MS column, the oven temperature was set isothermally at 250 °C, and the total run time was 8 min.

GC–MS was performed in EI mode. Following ions were monitored: *m/z* 250, 252 and 253 (for B(*a*)P), *m/z* 264 and 265 (for IS). Quantitation was done from the intensity ratios of the ions *m/z* 252 and 264.

2.4. HPLC with fluorescence detection on ODS PAH column and short PI column

HPLC was done on Shimadzu LC 10A instrument, equipped with RF-10Axl fluorescence detector, set at λ_{ex} = 370 nm, λ_{em} = 470 nm. Two columns were applied: CP EcoSpher 4 PAH, 150 mm × 3 mm, and CP ChromSpher π, 20 mm × 3 mm, both from Varian Chrompack International. The former column was specially prepared for the analysis of PAH; the latter column recommended by the manufacturer not for the chromatographic separation, but as a clean-up column. The same column has been used previously [8] for pretreatment of oil samples before HPLC analysis of PAH. Both columns were equipped with the same ChromSep guard cartridges for 3 mm i.d. reversed phase columns (Varian Chrompack International). As a mobile phase acetonitrile–H_2O (85:15) was used at a flow rate of 1.0 ml/min for both columns. The run time for the HPLC on EcoSpher 4 PAH column was 12 min, and for ChromSpher π column was 5 min.

All validation parameters (linearity, limit of detection (LOD), limit of quantitation (LOQ), and confidence range (CR)) were calculated using a BEN 2.0 software [18] for the calculations the analytical limits according to the DIN 32645 [19,20]. The significance level was set at 99%.

Following formulas were used in this software:

$$\mathrm{LOD} = s_{x0} t f_{\alpha} \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{X^2}{Q_x}}$$

$$\mathrm{LOQ} = \mathrm{LOD} + s_{x0} + t f_{\beta} \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{X^2}{Q_x}}$$

Table 2
Validation data for B(*a*)P determined using standard and low-pressure GC–MS and HPLC on standard and short π columns

Column	Retention time for B(*a*)P (min)[a]	Retention time for IS (min)[a]	LOD B(*a*)P (ng/g)	LOQ B(*a*)P (ng/g)	Linearity	Accuracy[a] at 2.5 ng/g (%)	Accuracy[a] at 7 ng/g (%)
Rapid MS FS CP-Sil 8, 10 m × 0.53 mm	5.03 ± 0.14	5.00 ± 0.11	1.6	5.5	$y = 0.01x + 0.01$; $r^2 = 0.99991$		
BD5-5MS, 30 m × 0.21	35.30 ± 0.21	35.23 ± 0.19	1.0	3.4	$y = 0.06x + 0.03$; $r^2 = 0.99959$		
EcoSpher 4 PAH, 150 mm × 3 mm	9.2 ± 0.20	7.7 ± 0.14	0.5	1.7	$y = 0.07x + 0.01$; $r^2 = 0.999837$	92 ± 2.2	88 ± 3.3
ChromSpher π, 20 mm × 3 mm	3.5 ± 0.15	1.9 ± 0.07	0.3	1.1	$y = 0.064x + 0.002$; $r^2 = 0.99935$	103 ± 3	108 ± 4

[a] Mean ± S.D. from all experiments.

$$\mathrm{CR} = \pm s_{x0} t f_\alpha \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{x - X^2}{Q_x}}$$

where s_{x0} is the standard deviation of determination, t the quantile of F-distribution, α the probability of the alpha-errors (false positive), β the probability of the beta-errors (false negative), n the number of calibration points, m the number of measurements, Q the sum of squared deviations, x the individual value, and X the mean of all values.

3. Results and discussion

3.1. SPE versus MSPD

In the pilot experiments, the efficiency of both extraction methods used—SPE and MSPD—were compared. The samples of oil, spiked with B(*a*)P to the concentrations of 10, 20, 40, and 60 ng/g and with internal standard B(*a*)P-D12, to the concentration of 5 ng/g, were extracted with both methods. The extracts were analyzed with LP-GC–MS and standard GC–MS. The determinations were performed in triplicate for each concentration. Table 1 shows the results of this experiment.

Daily practical experience showed that SPE was simpler, faster, and less proned to interindividual variability than MSPD. Each sample, extracted with MSPD, should be treated individually, without the possibility of serial work. Additionally, the recovery of SPE was consistently higher and more precise. For these reasons, SPE was selected as an isolation method for further experiments. Nevertheless, it was proven that MSPD can be certainly used for solid or semi-solid fat samples and is much simpler than the standard methods, requiring saponification, extraction, and clean-up steps.

3.2. Standard GC–MS versus LP-GC–MS

Comparative experiments using both GC–MS methods were performed in three series for each method, on different days. Oil samples, spiked with B(*a*)P to the concentration of 0, 5, 10, 20, 30 and 50 ng/g and with IS were used. Table 2 shows the comparison of retention times for B(*a*)P as well as validation data for both GC methods applied. Since B(*a*)P on low-pressure column eluted in about 5–6 min, the total run time in this method was only 8 min, whereas in standard GC it was extended to 42 min. The speed of analysis was achieved at the cost of selectivity; the peak of B(*a*)P was quite broad, and the N_{eff} value in applied conditions was around 3300 (Fig. 1). Additionally, the sensitivity of standard GC–MS was slightly higher. For the target analysis, however, the column selectivity was sufficient, and no interference from matrix compounds has been observed in authentic oil samples of various countries of origin. Therefore, LP-GC–MS was selected as a gas chromatographic method of choice for the analysis of B(*a*)P.

3.3. HPLC on standard PAH columns versus short π columns

Experiments with standard HPLC column (CP EcoSpher 4 PAH, 150 mm × 3 mm) and fast column (ChromSpher π, 20 mm × 3 mm) were performed in four series, on different days. Each series consisted of oil samples, spiked with B(*a*)P to the concentrations of 0, 0.5, 1.0, 2.0, 5.0 and 10.0 ng/g and with IS (5 ng/g).

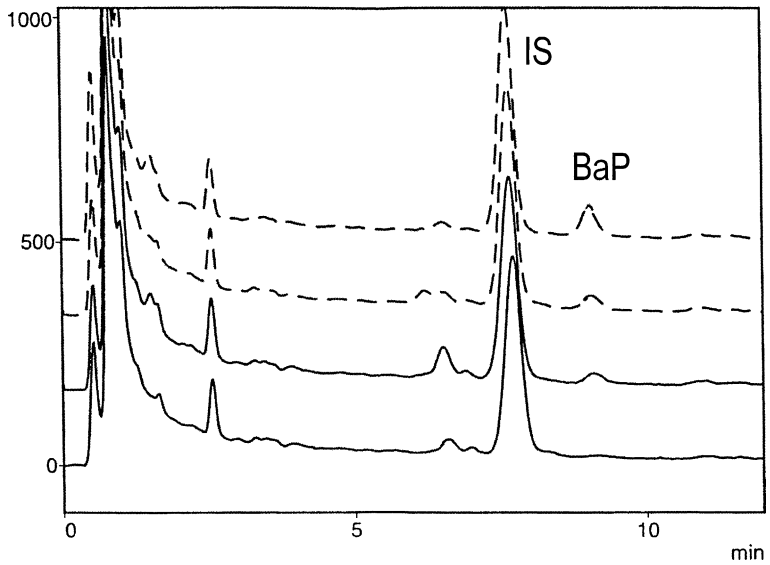

Fig. 1. HPLC chromatogram of blank oil sample (lowest trace), as well as samples spiked with B(*a*)P to the concentrations of 0.5, 1.0, and 2.0 ng/g and with IS (benzo(*k*)fluoranthene) to the concentration of 5 ng/g. Retention time: IS, 7.7 min; B(*a*)P, 9.2 min. Column: CP EcoSpher 4 PAH, 150 mm × 3 mm.

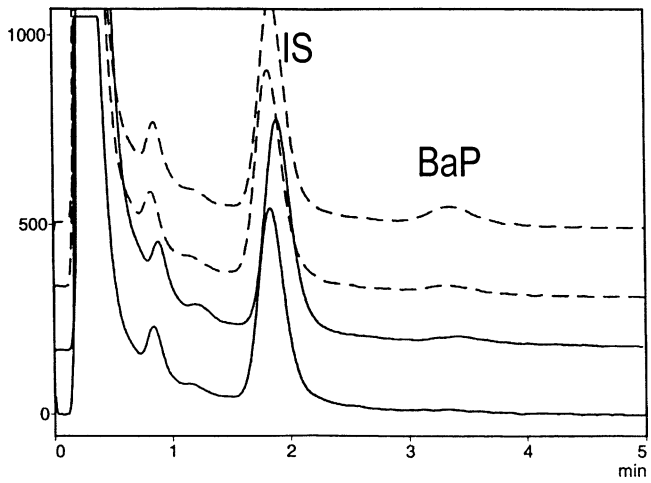

Fig. 2. HPLC chromatogram of blank oil sample (lowest trace), as well as samples spiked with B(*a*)P to the concentrations of 0.5, 1.0, and 2.0 ng/g and with IS (benzo(*k*)fluoranthene) to the concentration of 5 ng/g. Retention time: IS, 1.85 min; B(*a*)P, 3.35 min. Column: CP ChromSpher π, 20 mm × 3 mm.

Oils samples were extracted according to the developed SPE procedure in duplicate. Hence, 48 individual samples were determined using both columns. For accuracy testing, three oil samples were spiked in duplicate to the target concentrations of 0, 2.5 and 7.0 ng/g. The samples were given to the analysis as unknown and analyzed twice on two different days. Mean recoveries of B(*a*)P and IS, calculated from all examined samples in this series of experiments, amounted to 84 ± 4.0, and 89 ± 3.1, respectively. Table 2 presents the validation data for both columns.

Figs. 2 and 3 show typical chromatograms of extracts observed using standard and fast HPLC column. For each column, the chromatograms of blank oil extract, containing only IS, as well as chromatograms of extracts of oil spiked to the concentration of 0.5 ng/ng (around detection limit level), 1 and 2 ng/g (reporting level at maximum tolerable concentration) were presented. Both columns performed well, allowing baseline separation of B(*a*)P and IS. It was proven in the course of routine analysis of numerous samples, that no interfering peaks originating from oil matrix were observed in the relevant elution range. In order to prove the selectivity of the method, a mixture of PAHs, containing B(*a*)P, benzo(*k*)fluoranthene (IS), as well as 14 other compounds, was injected on both columns. The chromatograms are presented in Fig. 4. It was demonstrated, that the peaks of other PAHs did not interfere with B(*a*)P and IS on EcoSpher 4 PAH (15 mm × 3 mm) column. In the case of ChromSpher π (20 mm × 3 mm) column, however, the peak of IS (benzo(*k*)fluoranthene) was not fully resolved from the peak of benzo(*b*)fluoranthene. Nevertheless, the peak of B(*a*)P was fully separated from other PAHs. It should be noted, that in all examined olive oil samples both peaks—B(*a*)P and IS—were fully separated from all other peaks, and the parallel use of both HPLC columns for the analysis on B(*a*)P showed that the short ChromSpher π column gave virtually the same results as the analytical column. Therefore, this column, which has been developed for the clean-up of oil sample, may be applied for the fast target analysis. The use of benzo(*k*)fluoranthene as IS was substantiated by its close structural relation and very good chromatographic properties in relation to the analyte,

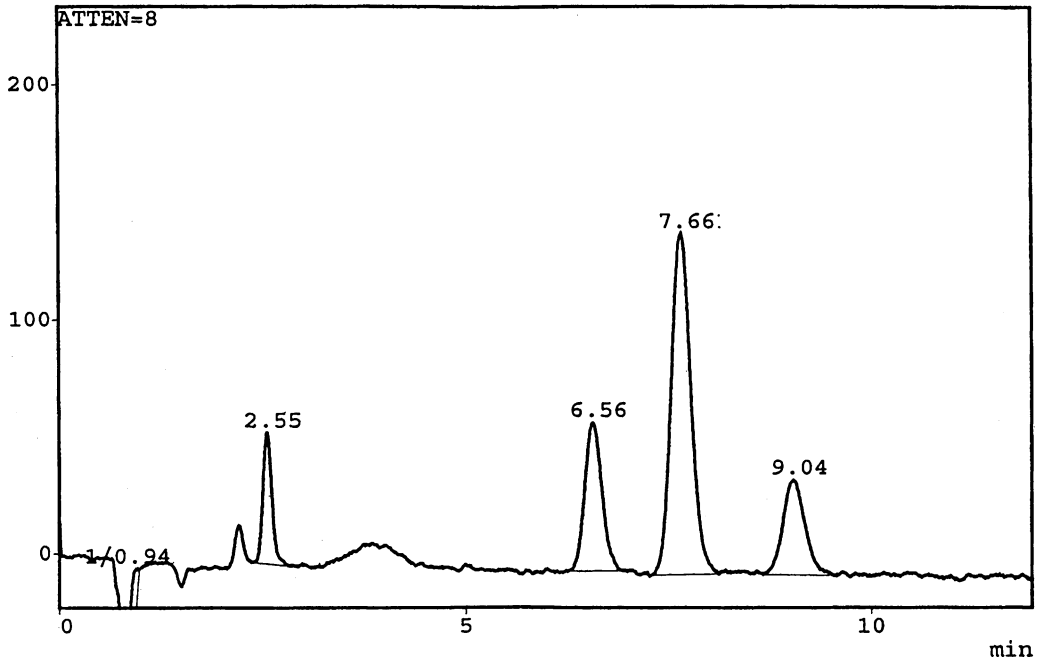

Fig. 3. HPLC chromatogram of a mixture of PAH on CP EcoSpher 4 PAH, 150 mm × 3 mm column. Retention time: benzo(*b*)fluoranthene, 6.56 min; benzo(*k*)fluoranthene, 7.66 min; benzo(*a*)pyrene, 9.04 min. HPLC conditions as for determination of B(*a*)P.

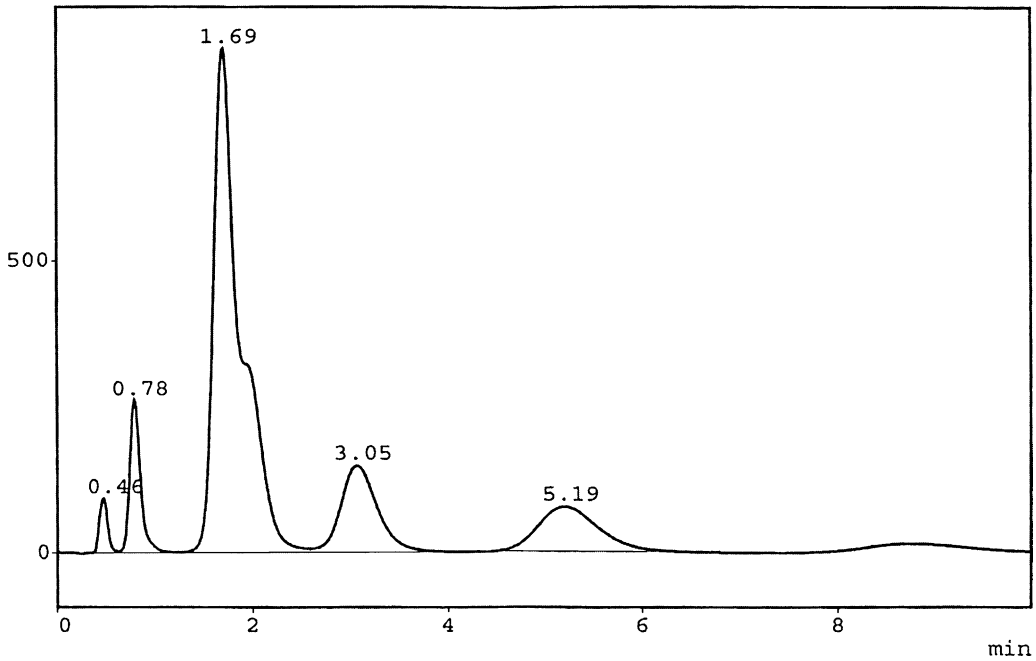

Fig. 4. HPLC chromatogram of a mixture of PAH on CP ChromSpher π, 20 mm × 3 mm column. Retention time: benzo(*b*)fluoranthene and benzo(*k*)fluoranthene, 1.69 min; benzo(*a*)pyrene, 3.05 min; dibenzo(*a*,*h*)anthracene, 5.19 min. HPLC conditions as for determination of B(*a*)P.

like high delectability and baseline separation. Nevertheless, when high amount of B(*a*)P is present in the sample, the presence of benzo(*k*)fluoranthene may be also expected. This may lead to the calculation of erroneously low concentration of B(*a*)P. Therefore, in such cases the analysis should be repeated without addition of IS. Confirmatory GC–MS analysis is also recommended.

4. Conclusions

Column extraction method applied, based on combined Florisil and ODS solid-phase extraction, assured high recoveries of benzo(*a*)pyrene, its deuterated analogue, and benzo(*k*)fluoranthene, used as internal standard for HPLC. The procedure is simple and allows handling 50–100 samples in one workday. Matrix solid-phase dispersion was more laborious and may be applied for extraction of solid or semi-solid samples.

HPLC on short ChromSpher π columns allowed detecting benzo(*a*)pyrene at the same detection level as standard HPLC column but in much reduced analysis time. This column is not suitable for separation of complex PAHs mixtures, but is applicable for the analysis of oil samples on the presence of benzo(*a*)pyrene. Low-pressure GC–MS reduced the analysis time of benzo(*a*)pyrene fivefold, in comparison with the standard GC–MS. The method is recommended as a confirmation of positive results obtained with HPLC.

Generally, the method developed allow very fast and sensitive determination of benzo(*a*)pyrene in olive oil samples. The method may be also applied for other PAHs as well as for other edible oils.

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