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Ultrasonication extraction and gel permeation chromatography clean-up for the determination of polycyclic aromatic hydrocarbons in edible oil by an isotope dilution gas chromatography–mass spectrometry

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ABSTRACT

An analytical method for the determination of US EPA priority pollutant 16 polycyclic aromatic hydrocarbons (PAHs) in edible oil was developed by an isotope dilution gas chromatography–mass spectrometry (GC–MS). Extraction was performed with ultrasonication mode using acetonitrile as solvent, and subsequent clean-up was applied using narrow gel permeation chromatographic column. Three deuterated PAHs surrogate standards were used as internal standards for quantification and analytical quality control. The limits of quantification (LOQs) were globally below 0.5 ng/g, the recoveries were in the range of 81–96%, and the relative standard deviations (RSDs) were lower than 20%. Further trueness assessment of the method was also verified through participation in international cocoa butter proficiency test (T0638) organised by the FAPAS with excellent results in 2008. The results obtained with the described method were satisfying ($z \leq 2$). The method has been applied to determine PAH in real edible oil samples.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), organic compounds containing two or more fused carboxylic aromatic rings, are highly stable contaminants that occur in soil, air, and food. They are a very important group of chemical carcinogens, and 16 of these have been selected as priority pollutants by the United States Environmental Protection Agency (EPA) on the basis of their occurrence and carcinogenicity [1].

Human intake of PAH from food is believed to be higher than that from ambient air or drinking water. Edible oils and fats are the largest contributory sources because of their lipophilic nature. Edible oils and fats are contaminated by technological processes such as smoke-drying of oil seeds, or indirectly by environmental sources such as exhaust gases from traffic or other combustion-derived atmospheric particles deposited on the crops during growing. The presence of PAH in edible oil, and fats has been reported by several investigators [2].

The European Scientific Committee on Food (SCF) recommended that one uses benzo[a]pyrene as a marker for the carcinogenic PAHs in food and to evaluate the risk assessment of the carcinogenicity of PAHs in food on the basis of the level of benzo[a]pyrene [3]. In December 2006, the European Commission maximum residue levels (MRL) for benzo[a]pyrene were adapted for oils and fats intended for human consumption or for use as an ingredient in foods (2 μg/kg wet weight) [4]. MRL for benzo[a]pyrene are set at 2 μg/kg in edible oil in South Korea. MRL for benzo[a]pyrene are set at 10 μg/kg in edible oil according to the national standard in China [5].

The very low concentration levels set by regulatory bodies and the complex matrix nature of vegetable oils potentially containing PAHs has raised the need to develop simple, sensitive, selective, accurate analytical methods for their routine analysis.

Because of its selectivity and sensitivity high-performance liquid chromatography and fluorimetric detection (HPLC-FLD) has been used extensively to determine PAHs from oil [6–10]. It is, however, impossible to quantify acenaphthylene by HPLC with fluorescence detection [11].

Recently, gas chromatography coupled to mass spectrometry (GC–MS) is the technique of choice for determining PAHs in oil samples [12–14], because deuterated PAHs surrogate standards can be utilized for tracing and compensating analyte losses during the particular stages of analytical procedure, which make the results more accurate.

Traditionally, the sample preparation of PAHs has relied on procedures generally consisting of an extraction step (e.g. liquid–liquid

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partition or saponification) followed by one or more purification procedures (e.g. column chromatography or solid phase extraction (SPE), and gel permeation chromatography (GPC)).

Ultrasound-assisted extraction is an expeditious, inexpensive and efficient alternative to conventional extraction techniques. Use of ultrasonication for extraction PAH from soils has been reported by several authors [15,16].

As it is already known, lipidic compounds can significantly reduce the performance of GC–MS due to accumulation in the injection port, column and ionization source. Because of the complexity of the biological matrices mentioned above, the presence of interfering compounds in the extract requires an intensive clean-up before extracted samples can be submitted to the separation and determination step. GPC clean-up is a clear alternative for PAH determination in large molecule oils matrix. Marinez-Lopez et al. [10] and Ballesteros et al. [18] established a method for the determination of PAH in olive oil by means of two GPC steel columns clean-up using CH_2Cl_2 as mobile phase. Fromberg et al. [13] proposed the use of ethyl acetate–cyclohexane (1:1, v/v) for dissolving vegetable oil, followed by purification on GPC with ethyl acetate–cyclohexane (1:1, v/v) consumption at least 115 mL when using ethyl acetate–cyclohexane (1:1, v/v) as mobile phase.

By combining the advantages of many methods, we developed a robust and reliable analytical method, separated acetonitrile extraction with ultrasound-assisted extraction and followed by a clean-up procedure using a narrow and short GPC clean-up. The use of GPC solvent was reduced until around 60 mL. Three deuterium-labeled PAHs (surrogate standards) were used for analytical quality control and quantification. Employing of retention time locking (RTL) software ensured retention time repeatability after column maintenance (such as cutting the head of column). We used this method to analyze PAHs in different oils, including corn oil, peanut oil, olive oil, cocoa butter and pepper oil.

2. Experiments

2.1. Chemical and materials

The isotope internal standard, phenanthrene-d10, anthracene-d10, and benzo(a)pyrene-D12, were supplied from Dr. Ehrenstorfer GmbH (Augsburg, Germany); pesticide-quality solvents (cyclohexane, ethyl acetate, and acetonitrile, dichloromethane) were supplied by honeywell (B&J, Muskegon, MI 49442, USA), PAH standard mix solutions at 2000 g/L in cyclohexane were from Supelco (Bellefonte, PA, USA), and were stored in a freezer. Working standard solutions were prepared by appropriate dilution with ethyl acetate–cyclohexane (1:1, v/v) and stored under refrigeration at 4 °C.

According to EU commission regulation 333/2007, containers shall be rinsed with high purity acetone or hexane before use to minimize the risk of contamination. Wherever possible, apparatus and equipment coming into contact with the sample shall be made of inert materials such as aluminum, glass or polished stainless steel. Plastics such as polypropylene or PTFE shall be avoided because the analyte can adsorb onto these materials [17].

2.2. Instrumentation

Gas chromatography–mass spectrometry: GC–MS analyses were carried out on an Agilent7890 gas chromatograph and 5975 C mass spectrometer equipped with a split/splitless injector and a model 7683 B autosampler (Agilent Technologies, Little Falls, DE, USA), fitted with an HP-5MS fused-silica capillary GC column (30 m × 0.25 mm I.D., 0.25 μm film thickness).

Gel permeation chromatograph:ASPEC Xli-GPC (GilsonA.S. 19, avenue des Entrepreneurs, BP 145, F-95400 VILLIERS LE BEL, France) consisted of a autosampler, a solvent delivery module, a UV–vis152 detector with multiple wavelength operation (set at 254 nm), a fraction collector, and self-made GPC glass clean-up columns (15 mm I.D. × 250 mm) packed with pre-swollen and washed Bio-Beads S-X3 resin corresponding to 11.5 g of dry material.

Model KQ-500DB ultrasonic cleaning bath (kunshan ultrasonic instrument company, PR China) was used for the ultrasonication extraction of PAH in oil into the solvent. A rotary evaporator R-114 (Buchi, Flawil, Switzerland) was available for extracts' concentration.

2.3. Sample extraction and clean-up

Samples were stored at room temperature until analysis. Four grams of oil samples was weighed and spiked with 10 ng of surrogate standards and transferred into 50 mL glass centrifuge tubes. For recovery studies, the samples were fortified with the PAH and labeled internal standard working standard solutions, well mixed and equilibrated for 4 h to ensure that PAHs were homogenously distributed throughout the sample. 10 mL of acetonitrile was added and the cap was screwed on. The sample was shaken vigorously using the mixer for 5 min, and the tube was put in an ultrasonic water bath for 10 min. Top layer was carefully transferred into a glass flask with Pasteur pipette; the extraction was repeated twice with further 20 mL acetonitrile. All extracts were collected and concentrated to nearly dryness using rotary evaporator and further evaporated to just dryness under a nitrogen stream, and the residue was re-dissolved with 4 mL of ethyl acetate–cyclohexane (1:1, v/v). The re-dissolved sample was transferred into a 5 mL vial and then 1 mL was injected in the GPC system. Ethyl acetate–cyclohexane (1:1, v/v) was used as the mobile phase of the GPC system at a column flow of 3 mL/min. The representative fraction containing the target PAH was collected from 10 to 18 min (approximately 24 mL). The GPC fraction was evaporated to a small volume and then taken to nearly dryness under a nitrogen stream, re-dissolved in 0.5 mL ethyl acetate–cyclohexane (1:1, v/v), and finally transferred into an autosampler vial for GC-MS analysis.

2.4. GC-MS analysis

Operating conditions for the GC were: helium (99.999% purity) carrier gas, constant pressure at 20 psi (1 psi = 6894.76 Pa); the oven temperature was programmed at 70 °C for 2 min, ramped to 150 °C at 25 °C/min, then to 200 °C at 3 °C/min, and then to 280 °C at 8 °C/min and held for 10 min. The total GC analysis time was of 41.867 min. The injection port temperature was 300 °C. The injection volume was 2 μL. The MS was operated in electron ionization (EI) mode at 70 eV with a transfer line temperature of 280 °C, ion source 230 °C, quadrupole temperature 150 °C. Solvent delay was set to 4 min. Selected ion monitoring mode (SIM) was used for the qualitative and quantitative determination of PAH. Agilent Chemstation with Retention Time Locking (RTL) software was used for instrumental control and chromatographic data processing.

3. Results and discussion

3.1. Extraction

Several methods have been described regarding the extraction of PAHs in oils. Among them, supercritical fluid extraction with CO_2 as extractor [6], DMF-water (9:1) [9], acetonitrile [10,18], and cyclohexane–ethyl acetate 1:1 (v/v) [13] have been used for extracting PAH in oils.

Five organic solvents (cyclohexane, ethyl acetate, hexane, dichloromethane, acetonitrile) were investigated as extractant for the extracting PAHs from oil. Cyclohexane–ethyl acetate was found to be efficient for extraction of the PAHs. But for oil sample, ethyl acetate can extract overabundant lipids. Acetonitrile can extract fewer lipids compared with other solvents, and acetonitrile layer can be separated from oil layer. The efficiencies of extraction for PAH were very satisfying. Therefore, acetonitrile was selected as the extraction solvent. Ultrasonication extraction mode is a good choice for liquid sample. The influence of extremely high effective temperatures, which results in increased solubility and diffusivity, and pressures, which favour penetration and transport, at the interphase between organic solution subject to ultrasonic energy and oil matrix, result in a high extractive power in a short time. Therefore, ultrasound-assisted extraction methodologies were used for the extraction of PAH and 10 min extraction time was selected for subsequent experiments [7].

In order to limit the loss, the evaporation to just dryness under a gentle stream of nitrogen at moderated temperature (40 °C) was preferred; precautions were taken to stop the nitrogen flow as soon as the solvent was evaporated.

3.2. Clean-up

Several methods have been described regarding the purification of PAHs in oil. Among many purification protocols, silica gel [8], polystyrene–divinylbenzene SPE [15], and gel permeation chromatography [10,13,18] are the most popular ones.

In order to reduce the oil co-extractives, GPC was chosen. GPC has been used to remove co-extractive large molecular interferences such as fatty and wax, on the basis of great difference in molecular size between them and the target compounds. The oil was a very complex matrix. The compounds of high size (pigment and lipidic material) in oil can be eluted earlier than compounds of lower size (PAH). The GPC clean-up can decrease the presence of interferents in the final extract and also avoid the deterioration of the chromatographic column and contamination of the ion source of MS.

Although the use of high capacity GPC column (with I.D. 25 mm) can increase sample capacity, we preferred employing of a relatively "narrow-bore" column (with I.D. 15 mm) for purification of extracts owing to following reasons: (1) decreased elution volumes and (2) lower cost of GPC column. Considering the hazard posed by the chlorinated solvent, ethyl acetate–cyclohexane (50:50, v/v) was examined as an alternative mobile phase in the next experiments. To investigate separation efficiency of GPC, 1 mL of mixed standard dilution and the extracts of sample were injected into the GPC system. The elution was carried out with ethyl acetate–cyclohexane (50:50, v/v) at a flow rate of 3 mL/min. The GPC chromatograms for different oils were shown in Fig. 1. Then the discarded fraction and collected fraction were obtained on the basis of the chromatogram. The interferents were almost eluted in 11 min, the standards began to be outflowed from the GPC column at 10 min and the target analytes were completely eluted in 10–18 min.

3.3. Selection of GC–MS parameters

3.3.1. Selection of MS parameters

With the use of a MS-detector, stable isotope-labelled materials of analogous of the native analyte are convenient internal standards. They can be used for tracing and compensating analyte losses during the particular stages of analytical procedure [20]. The labeled standards allow quantification of trace quantities of PAH analytes with a high precision. For this purpose a technique known as isotope dilution mass spectrometry was used. Full scan mass spectra of benzo(a)pyrene-d12 is shown in Fig. 2. Improved accuracy of measurement can be obtained by the use of dilution technique.

3.3.2. Identification and confirmation criteria

For the identification of the target compounds, a relative retention time window (RRTW) was used, i.e. the ratio of the chro-

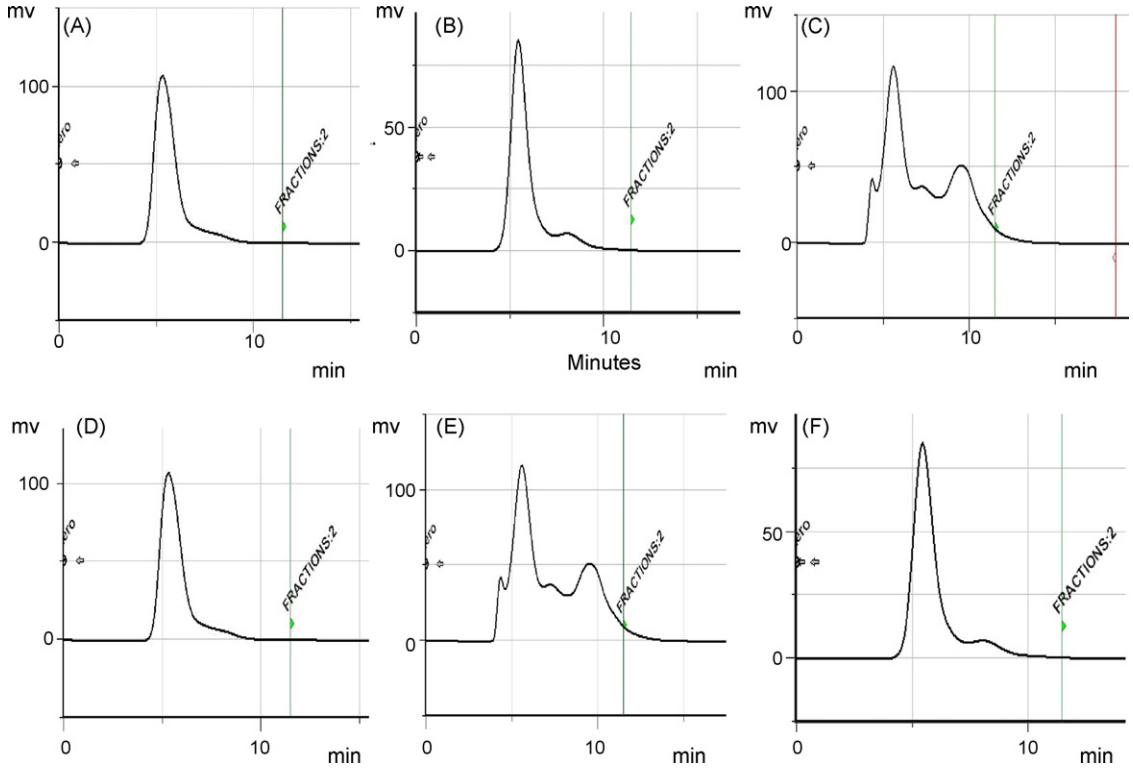

Fig. 1. GPC chromatograms of the oil samples at 254 nm: (A) corn oil, (B) peanut oil, (C) olive oil, (D) pepper oil (E) grape seed oil, and (F) butter oil.

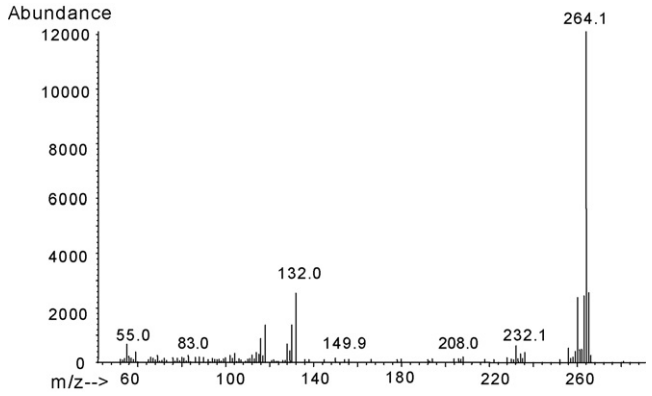

Fig. 2. Full scan mass spectrum of benzo(a)pyrene-d12.

Table 1
Maximum permitted tolerance values for relative ion intensities using a range of mass spectrometric techniques.

Relative intensity (% of base peak)	EI-GC-MS (relative)
>50%	±10%
>20–50%	±15%
>10–20%	±20%
≤10%	±50%

matographic retention time window of the analyte to that of the I.S. The RRTW of the analyte was matched based on the calibration standard at a tolerance of ±0.5%. The final confirmation of a target compound, firstly identified by RRTW, was done by the use of identification points (IPs) established in the Commission Decision 2002/657/CE [19]. The number of IPs achieved in GC–MS is four, corresponding to four ions, should be monitored and fit the identification criteria, listed in Table 1.

The specific MS parameters are described in Table 2 for all the target PAH total ion chromatogram of 16 PAH is shown in Fig. 3.

3.3.3. Optimization of the GC condition

The RTL pesticide library software was used to eliminate the tedious SIM method retention time and ion groups updating process after column maintenance (such as cutting the head of the column. The retention time (t_R) of pesticides can be relocked to the initial times when as much as 5 m of the column is cut. The recent review [21] illustrates the RTL basis and practical aspects together with brief description of some application. We [22,23] have applied the technique to pesticide residues analysis. To the best of our knowledge, it is the first time that this technique is reported to be applied for PAH analysis.

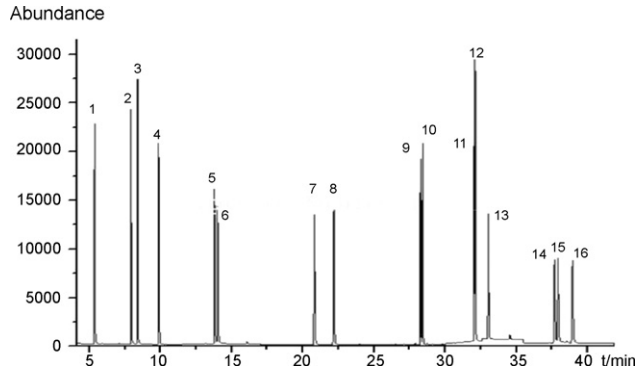

Fig. 3. Total ion chromatogram of 16 PAH, peak number: (1) naphthalene, (2) acenaphthylene, (3) acenaphthene, (4) fluorene, (5) phenanthrene and phenanthrene-d10, (6) anthracene and anthracene-d10, (7) fluoranthene, (8) pyrene, (9) benzo(a)anthracene, (10) chrysene, (11) benzo(b)fluoranthene, (12) benzo(k)fluoranthene, (13) benzo(a)pyrene and benzo(a)pyrene-d12, (14) indeno(1,2,3-cd)pyrene, (15) dibenzo(a,h)anthracene, and (16) benzo(g,hi)perylene.

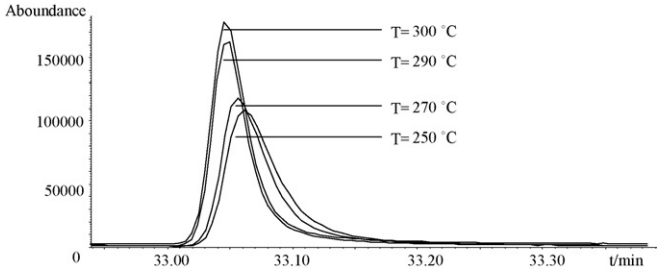

Fig. 4. GC–MS chromatograms of a benzo[a]pyrene with different inlet temperatures: (a) T = 250 °C, (b) T = 270 °C, (c) T = 290 °C and (d) T = 300 °C.

We found that high temperature of the insert caused a slight decrease in the lightest PAHs, but it increased the heavy PAH (dibenzopyrenes) signal significantly. For example, the relative response ratio for benzo(a)pyrene was as follows: T = 250 °C, 59.1%; (b) T = 270 °C, 77.3%; (c) T = 290 °C, 79.8% and (d) T = 300 °C 100%, respectively. The chromatogram is shown in Fig. 4.

3.4. Method performances

The method was validated for linearity, limits of quantification (LOQs), accuracy and precision. Quantification was by peak area relative to that of the IS. Results of the validation are summarized in Table 3. LOQs were estimated on the signal observed at the lowest

Table 2
Retention times, target ions, and qualifier/target relative abundances for PAH.

PAH	Retention time (min)	Target ions (ions ratio)
Naphthalene	5.41	128(100), 127(44.8), 129(33.5), 102(22.5)
Acenaphthylene	8.02	152(100), 151(22.8), 153(16.2), 150(19.3)
Acenaphthene	8.46	153(100), 154(93.2), 152(53.1), 151(24.6)
Fluorene	9.96	166(100), 165(94), 167(17.6), 139(10.5)
Phenanthrene	13.89	178(100), 179(18.8), 176(23.7), 152(13.5)
Anthracene	14.12	178(100), 179(19.2), 176(24.5), 152(12.2)
Fluoranthene	20.97	202(100), 203(19.3), 200(22.1), 101(19.3)
Pyrene	22.34	202(100), 203(20.3), 200(22.1), 101(21.8)
Benzo(a)anthracene	28.45	228(100), 226(28.4), 229(22.6), 227(10.7)
Chrysene	29.60	228(100), 226(31.3), 229(22.6), 113(23.9)
Benzo(b)flouranthene	32.06	252(100), 253(68.6), 250(28.1), 126(55.6)
Benzo(k)fluoranthene	32.14	252(100), 253(24.4), 250(23.5), 126(23.9)
Benzo(a)pyrene	33.1	252(100), 253(27.4), 250(24.1), 126(21.1)
Indeno(1,2,3-c,d)pyrene	38.17	276(100), 277(25.1), 274(22.2), 138(40.2)
Dibenz(a,h)anthracene	38.37	278(100), 276(29.3), 138(32.8), 139(32.5)
Benzo(g,h,i)perylene	39.47	276(100), 277(25.1), 274(23.1), 138(43)

Table 3
Recovery, repeatability relative standard deviation (RSDr) and LOQ for fortified corn oil ($n = 6$).

PAH	Average recovery (%)				RSD	LOQ
	1 μg kg^{-1}	2 μg kg^{-1}	5 μg kg^{-1}	10 μg kg^{-1}	(%)	μg kg^{-1}
Naphthalene	85.0	94.0	82.8	86.8	6.4	0.3
Acenaphthylene	91.8	86.8	89.0	84.8	5.7	0.3
Acenaphthene	84.5	86.3	87.1	85.1	4.6	0.3
Fluorene	86.9	91.3	85.7	82.8	7.7	0.3
Phenanthrene	94.2	85.7	84.3	85.2	5.9	0.3
Anthracene	93.2	88.7	82.7	87.1	5.9	0.3
Fluoranthene	92.0	87.9	96.0	83.0	7.5	0.3
Pyrene	87.2	91.5	94.2	84.3	6.5	0.3
Benzo(a)anthracene	85.3	91.9	81.5	82.1	8.6	0.3
Chrysene	89.3	88.7	90.7	92.5	6.2	0.3
Benzo[b]fluoranthene	93.5	87.8	85.9	88.1	4.9	0.3
Benzo[k]fluoranthene	93.1	87.8	83.8	83.1	7.2	0.3
Benzo[a]pyrene	85.3	77.6	82.5	83.9	10.8	0.3
Indeno[1,2,3-cd]pyrene	92.9	88.8	89.0	94.0	6.0	0.6
Dibenz(a,h)anthracene	86.8	89.2	86.7	93.6	8.1	0.6
Benzo(g,h,i)perylene	96.0	90.4	85.6	85.5	4.0	0.6

point of the calibration curve: calculations were performed on the basis of an extrapolation at S/N = 10 for LOQs. LOQs were globally below 0.5 ng/g. The method was robust for the different oils studied, yielding recoveries in the range of 81–96%, with precision values expressed as relative standard deviation (RSD) lower than 20%, and these results are fully compatible with the EU requirement [19].

3.5. Proficiency test

In order to ensure quality during method validation, further trueness assessment was done to verify the analytical method through participation in inter-laboratory proficiency test 0638 organised by the UK Food Analysis Performance Assessment Scheme (FAPAS) in 2008 [24], and results are presented in Table 4 (our laboratory number was 17). All analytes led to excellent results (z-score values $z \leq 2$). Thus, based on these results, this analytical method showed high precision for PAH analysis.

3.6. Application of the method to real samples

The method proposed was applied to the analysis of real oil samples. Due to the fact that our laboratory was certified by ISO 17025 for food safety analysis, internal quality control criteria were implemented in order to check if the method is under control. A variety of daily operations and internal quality controls were applied in order to assess and ensure the correct working of the system: (i) use of a blank matrix extract to eliminate false positives owing to a possible contamination with PAH from the instrument or the chemicals used during the extraction and clean-up procedure; (ii) use of samples of a spiked blank matrix sample to check the extraction efficiency; and finally (iii) a calibration curve ($R^2 > 0.99$) daily to evaluate sensitivity as well as linearity in the working range of concentrations, and in this way, the influence of matrix effect and instrumental fluctuations on the quantification was avoided. A reagent blank was also injected after each six-sample injection to check for carry-over and to perform a simply cleaning of the chromatographic system. No carry-over phenomena were noticed. These solvent injections showed to be a good option of testing the GC-MS when a high number of relatively dirty samples were being injected. Fig. 5 shows the chromatogram obtained for incurred pepper oil containing 3 μg/kg benzo(a)pyrene.

Table 4
Assigned values, measured concentrations and z-scores obtained for the proficiency test dedicated to analysis of PAH in T0638 cocoa butter oil sample.

Analyte	Median concentration[a]	Measured concentration[b]	z-Score
Benzo[a]pyrene	1.97	2.64	1.6
Indeno[1,2,3-c,d]pyrene	1.19	1.37	0.7
benzo[a]anthracene	3.32	4.77	2.0
Benzo[g,h,i]perylene	1.48	1.50	0.1

[a] Determination based on results from all laboratories.
[b] Determined using the described analytical method.

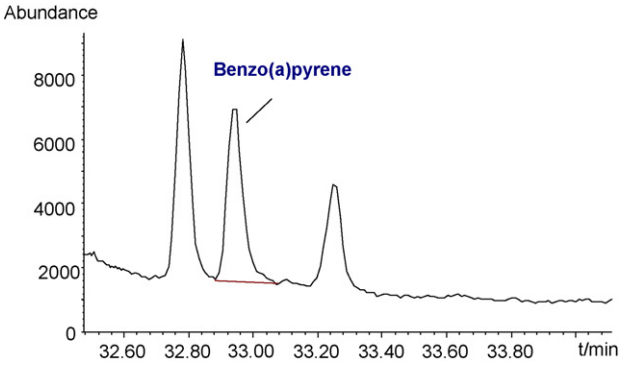

Fig. 5. Chromatogram obtained for incurred pepper oil containing 3 μg/kg Benzo(a)pyrene.

4. Conclusions

We have developed and validated a robust and reliable method to determine PAHs in edible oil by GC–MS. The use of ultrasound-assisted extraction followed by GPC clean-up can efficiently extract the 16 PAH from oil samples providing good recoveries and overall quality parameters. Compared to traditional GPC column, the method significantly reduces the amounts of solvents and GPC resin by use of self-made short and narrow GPC column. For all the PAH, the sensitivity of the method was good enough to ensure a reliable routine determination at levels lower than MRL.

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