Journal of Food Biosciences and Technology,
Islamic Azad University, Science and Research Branch, Vol. 6, No. 2, 77-85, 2016

Determination of Polycyclic Aromatic Hydrocarbons in Olive and Refined Pomace Olive Oils using HPLC/FLD

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Received: 13 February 2016 Accepted: 22 February 2016

ABSTRACT: In this study, an analytical method is developed to determine 15 polycyclic aromatic hydrocarbons (PAHs) in olive and refined pomace olive oils using HPLC coupled with a fluorescence detector. The standardised method of ultrasound-assisted solvent extraction consisted of liquid–liquid extraction with organic solvent and purification on C_{18} and Florisil bonded-phase cartridges was modified for the refined olive and refined pomace olive oils. The modification included the clean-up by solid phase extraction on an amino cartridge eluting with toluene. The limits of detection and limits of quantitation were 0.19-0.97 µg kg^{-1} and 0.57-2.93 µg kg^{-1}, respectively. The PAHs recoveries ranged from 75% to 110% (RSD = 3–8%).The performance of the present method was evaluated for determination of PAHs in various types of olive oils samples, and suitable results were obtained. The variable levels of PAHs were detected ranging from 0.61 to 6.30µgkg^{-1} in real samples.

Keywords: *High Performance Liquid Chromatography with Fluorescence Detection (HPLC/FLD), Modified Ultrasound-assisted Solvent Extraction (MUSAE), Olive Oil, Polycyclic Aromatic Hydrocarbons (PAHs), Refined Pomace Olive Oil.*

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds containing two or more fused aromatic rings made up of carbon and hydrogen atoms. PAHs are formed and released during incomplete combustion or pyrolysis (burning) of organic matter such as soil, wood, coal, waste or food, during industrial processes and other human activities (Wenzl *et al*., 2006). A number of PAHs are known to be carcinogens and/or mutagens, including 16 proposed bythe US Environmental Protection Agency (EPA) and 16 by theEuropean Union. However, referring to theEuropean Food Safety Authority (EFSA) opinion from 2008 and Commission Regulation (EU) No. 835/2011 of 19th August 2011 benzo[α]pyrene is not a suitable marker for the occurrence of the other PAHs in food and hence a system of four specific PAHs (benzo[α]pyrene (BαP), benzo[α]anthracene, benzo[b]fluoranthene and chrysene) would be the mostsuitable indicators of PAHs in food (EC, 2011a, b; EFSA, 2008).

Food is the primary source of human exposure to PAHs for non-smokers and non-occupationally exposed adults, and edible oils and fats are one of the most contributing sources (Cirillo *et al*., 2006; Ibanez *et al*., 2005; Martí-Cid *et al*., 2008). They 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

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(Gertz & Kogelheide, 1994; Moret & Conte, 2000). However, in olive oils the main routes of PAHs contamination have been suggested as the followings: the contact with polluted environment and the drying of the olive pomace in the process of residue oil extraction with the solvent, using combustion fumes of organic matter (Ciecierska & Obiedzinski, 2013; Guillen *et al*., 2004).

The determination of polycyclic aromatic hydrocarbons (PAHs) in olive and refined pomace olive oils is complicated due to the oil matrix and hydrocarbon interferences namely squalene, that affect both extraction efficiency and analytical quality. International standards describe a method for the determination of 15 PAHs in animal and vegetable fats and oils (ISO, 2006). However, this method is not suitable for the analysis of olive pomace oil. Recent studies have shown that this method needs to be improved (Wu & Yu, 2012; Serpe *et al*., 2010; Simon *et al*., 2008; Simon *et al*., 2010).

The main objective of this study was to propose some modifications in standard method of ISO 13753 for determining the15 listed mutagenic and carcinogenic PAHs on Commission Regulation No 835/2011 (Commission Regulation 2011a), as well as two EPA indicator PAHs in olive and refined pomace olive oils. The method of ISO 13753 is based on the extraction of PAHs from oil samples with mixture of acetonitrile/acetone solvents, isolation of the hydrocarbon fraction, and clean-up of PAHs fraction using two C_{18} and florisil SPE steps. The modification was consisted in clean-up by SPE on an amino phase cartridge eluting with toluene for eliminating of interferences peaks (Moreda *et al*., 2004). The liquid chromatography coupled with fluorescence detection (FLD) was selected as detection technique. The modified method has been applied to various types of olive oils (extra virgin, virgin and refined pomace olive oil). The recoveries, limit of detection (LOD) and limit of quantitation (LOQ) of method were also calculated.

Materials and Methods

- *Materials*

The standard mixture of the 16 EPA PAHs (PAH-mix 4S8743) consisted of acenaphthene, acenaphthylene, anthracene, fluoranthene, fluorine, naphthalene, phenanthrene, pyrene, benz [a] anthracene, benzo [b] fluoranthene, benzo [k] fluoranthene, benzo [ghi] perylenebenzo [a] pyrene, chrysene, dibenz [a,h] anthracene and indeno [1,2,3-cd] pyrene 10 ngμL^{-1} in acetonitrile were obtained from Sigma Aldrich (Bellefonte, PA). Acetone, acetonitrile, dichloromethane, hexane, methanol and toluene solvents (HPLC grade) were purchased from Merck (Darmstadt, Germany). Deionized water purified on a Milli-Q system (Millipore, Billerica, MA, USA). The C_{18}-bounded phase cartridges were Sep-Pak C_{18}, 6 mL, 500mg (Waters, Ireland), Florisil- bounded phase cartridges were Chromabond, 3 mL, 500 mg (Macherey-Nagel, Germany). The NH_2 cartridges, 500 mg, 6 mL, were purchased from (Anpel, China).The stock and working standard solutions of PAHs were prepared in acetonitrile with the concentrations of 200μgL^{-1} and 50μgL^{-1}, respectively. Eight different calibration solutions in acetonitrile were prepared from working standard solution for the calibration curve and stored at 4 ºC in darkness. The olive and refined pomace olive oils were bought from the local supermarkets in Iran and stored at room temperature until required for analysis. All of the containers were carefully washed and rinsed with high purity hexane before use to minimize the risk of contamination.

- *Instruments*

HPLC-FLD determination was carried out using an YL 9100 HPLC system

consisted of a YL 9101 vacuum degasser, YL 9110 quaternary pump, YL 9130 column compartment and FP-2020 plus fluorescence detector co-operated with YL Clarity software program (Young Lin, Korea). A ZORBAX Eclipse column; 150 mm× 4.6 mmi.d.,5 µm particle size; (Agilent Technologies, USA) with a C_{18} guard column; 10 mm× 2.1 mm i.d.; was used for chromatographic analyses. Sample preparation was performed using a vortex mixer (VelpScientifica, Italy), an ultrasonic bath (Elma, Germany) and a tabletop centrifuge (Dynamca, United Kingdom).

- *Methods*

- *Ultrasound-assisted solvent extraction*

The procedure proposed by ISO 15753 (2006), with some modifications was used. About 2.5 g of the oil sample was extracted three times with 10 mLmixtures of acetone/acetonitrile (40:60, v/v) by shaking with a vortex mixer for 30 sec, ultrasounicating for 5 min in an ultrasonic bath, and centrifuging (4000 rpm/ 5.0 min).The top layer was carefully removed and evaporated to dryness under the flow of nitrogen.

- *C_{18} SPE clean-up*

The obtained residues were dissolved in 2 mL of extraction solvent, mixed, and centrifuged. The top layerwastransferred on C_{18} cartridgethat was previously conditioned with 24 mL of acetonitrile and methanol. The cartridge was eluted with 5 mL extraction solvent. The SPE extract was evaporated to dryness and dissolved in 1 mL of hexane.

- *Florisil SPE clean-up*

Florisil cartridge was conditioned with 15 mL of dichloromethane and 12 mL of hexane and the extract wastransferred onthe cartridge. PAHs were eluted with 9 mL mixtures of hexane/ dichloromethane solvents (3:1, v/v).These eluted extract was concentrated under the flow of nitrogen to approximately 0.50 mLvolume.

- *NH_2 SPE clean-up*

The NH_2cartridgewasconditioned with 30 mL of hexane under vacuum and the extract obtained fromflorisil cartridge was applied. The cartridge was eluted under vacuum with 25 mL mixtures of hexane/toluene (70:30). The final eluent solution was evaporated by a rotary evaporator under vacuum to dryness and the residuewas re-dissolved in 250 µL of acetonitrile for HPLC-FLD application.

- *HPLC-FLD analysis*

All the chromatographic analyses (the samples and standard solutions) were carried out using YL 9100 HPLC system by a fluorescence detector (HPLC-FLD). A gradient method with flow rate of 1.2 $mLmin^{-1}$ and mobile phases of acetonitrile (A) and acetonitrile/water 50/50 (B) was applied. Separation was performed at 35°C using the gradient described in Table 1. The following programmed excitation and emission wavelengths (Ex/Em) were used for determination of PAHs by the fluorescence detector: 270-324 nm (NPH, ACE, FL) at start, 248-375 nm (PHE, ANT) for12.8 min, 280/462 nm (FT) for 16.8 min, 270/385 nm (PYR, BaA, CHR) for 18.1 min, 256/446 nm (BbF) for 28 min, 292/410 nm (BkF, BaP, DahA, BghiP) for 31.2 min, and 270/470 nm (IP) for 38 min.

Table 1. Gradient elution program for the HPLC separation

Time (min)	Solvent mixture A (%)	Solvent mixture B (%)
0	0	100
5	0	100
27	60	40
36	100	0
41	100	0
43	0	100
45	0	100

- *Statistical analysis*

The experiments were designed by a

completely randomized design. All the results were the average of three separate experiments. Linear least-squares regression equations were used for the calibration curves. Independent student t-test was used to compare means in the refined pomace olive and refined olive oils. For the data analysis, SPSS software (version 22.0; SPSS Inc., Chicago, IL, USA) was used and p-value <0.05 was considered statistically significant.

Results and Discussion

- *Sample preparation and clean-up*

The analytical procedure was based on the method of ISO 15753 (2006) related to PAHs analysis in oils, nevertheless some modifications from Moreda *et al*. (2004) were introduced. Refined olive pomace oil spiked with known concentrations of PAHs was used for the development of the method. The standard method consists in the isolation of the hydrocarbon fraction and clean-up of PAHs fraction using column chromatography on C_{18} and Florisil and analysis by HPLC with fluorescence detector, but the method is not applicable to refined pomace olive oils because of interferences by different compounds during HPLC analysis. In addition, the heavy PAHs could not be firmly identified (in Figure 1a) because of the presence of too many interfering peaks. PAHs fractions contained considerable amounts of interfering compounds such as squalene and unsaturated hydrocarbons with cyclic moieties due to the squalene isomerization and steroidal alcohol decomposition in the refined olive pomace and refined olive oils (Moret & Conte, 2002; Bogusz *et al*., 2004).

The use of NH_2 SPE cartridge allowed the non-aromatic hydrocarbons to be eluted with hexane solvent but the heavy PAHs were left on the stationary phase because of the major interaction with the amino groups. The heavy PAHs were displaced with toluene, and finally, the extract was free of significant interferences (Figure 1b) due to the removal of impurities by the NH_2 SPE cartridge (Moreda *et al*., 2004; Rodríguez-Acuña *et al*., 2007; Rodríguez-Acuña *et al*., 2008).

- *Validation of the assay*

The analysis of the PAHs showed a linear relationship with high linear regression coefficients of determination for all the 15PAHs (R^2> 0.9929). The complete description of standard linearity supported by regression data is shownin Table 2. The results indicated that the developed extraction method provided reasonably good accuracy for the analysis of PAHs in refined olive and olive pomace oil samples in the tested range of concentrations (0.01-90 μgkg^{-1}).

In order to evaluate the repeatability and the recovery, blank samples of the oils were spiked with two levels of all PAHs (5 and 10$\mu gkg^{-1)}$. Reproducibility was evaluated by performing three analyses on the same day under the same conditions. The recoveries varied between 75to 111% with 3 to 8% of relative standard deviation (RSD) and were in the limit set for BaP (50–120%) according to Regulation of 835/2011 (Table 2).

The LOD and LOQ were defined as the concentration of the analyte producing the signal-to-noise ratio of 3 and 10 and were obtained from the standard deviation of the blank samples (n = 20) and the slope of the calibration curve. LOD and LOQ were in the range of 0.19 to 0.97 $\mu gkg^{-1}$and 0.57 to 2.93 μgkg^{-1}, respectively. LOQ for 12 out of 15 PAHs were all below 2 μgkg^{-1}. For BaP, LOQ (0.29 μgkg^{-1}) was less than that of the required maximum level by EU Regulations (2 μgkg^{-1}). In addition, LOQ of this method was broadly comparable with those reported by the techniques of other researchers (Table 3).

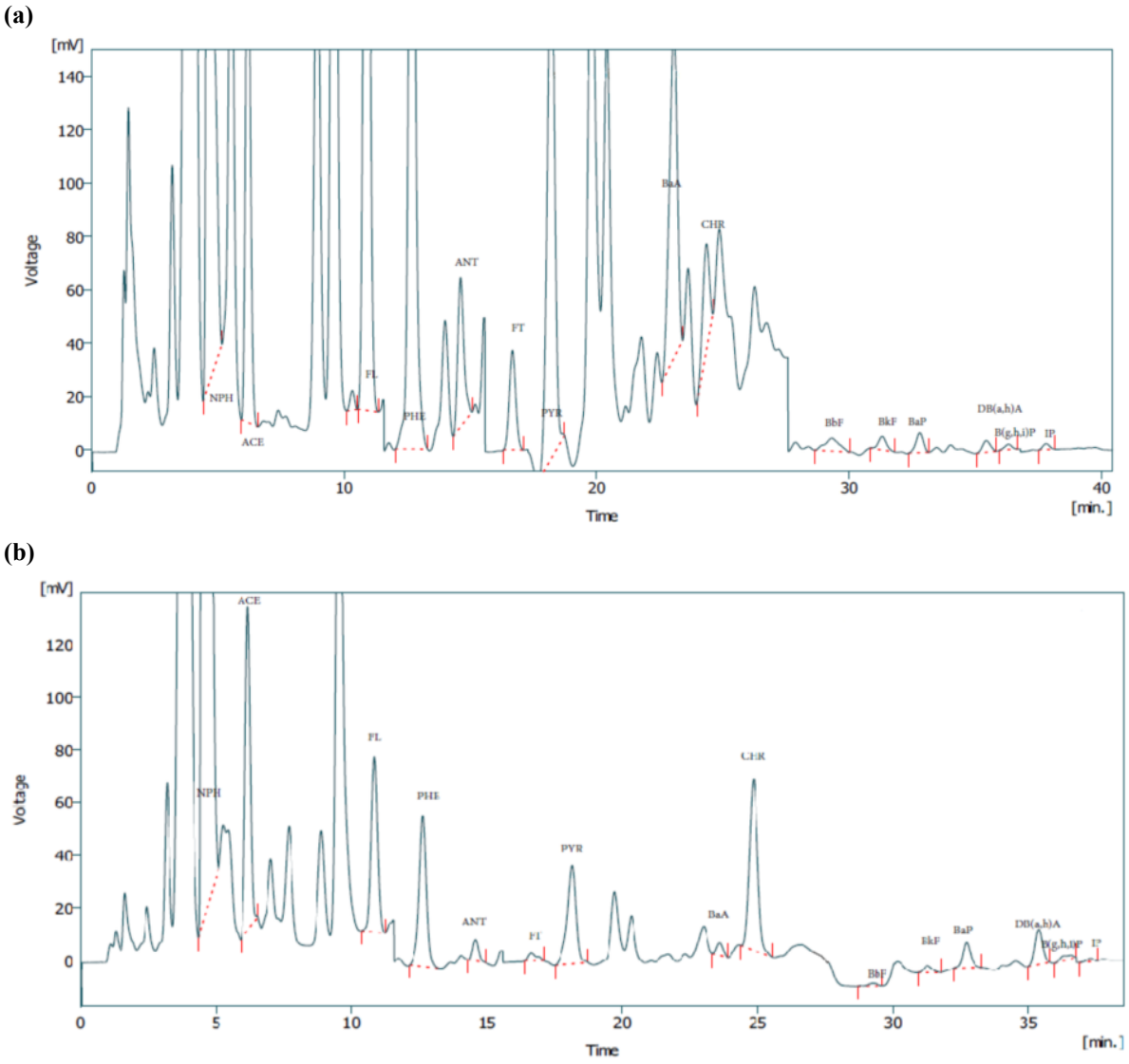

Fig. 1. Chromatograms of HPLC-FLD analysis of PAHs after: UASE extraction method (a), UASE extraction method followed by NH_2 SPE clean up (b).
Spiked PAHs solution concentration: 1 μgL^{-1} of NPH: Naphthalene; ACE: Acenaphthene; FL: Fluorine; PHE: Phenanthrene; ANT: Anthracene; FT: Fluoranthene; PYR: Pyrene; BaA: Benz [a] anthracene; CHR: Chrysene; BbF: Benzo [b] fluoranthene; BkF: Benzo [k] fluoranthene; BaP: Benzo[a]pyrene; DBaA: Dibenz [a, h] anthracene; BghiP: Benzo [ghi] perylene and IP: Indeno [1,2,3-cd]pyrene.

- *Analysis of the oil samples*

The concentrations of the PAHs in the real oil samples are shown in Table 3. The refined pomace olive oils had the total PAHs concentration of 24.41μgkg^{-1} that included light heavy PAHs. Among the heavy PAHs, the concentrations of BaA and CHR were higher. In the extra virgin and virgin olive oils, the total PAHs concentration was 19.05$gkg^{-1}$and concentration of CHR was greater.

The light PAHs were predominant (>87% of the total content) in all the oil samples. BkF, DBaA, Bg, h, iP and IP in the refined olive pomace oil samples and FL, BaA, BbF, BkF, Bg, h, iP and IP in olive oil samples were below the LOQ. The present and other authors found similar results (Wu and Yu,

2012; Payanan et al., 2013). The PAHs concentrations in refined olive pomace and olive oil were not significantly different ($p>0.05$ running a t-test, except PYR in olive oil samples).The BaP concentrations in the samples were lower than the limits imposed by the EU and other European countries.

Table 2. Performance criteria of modified UASE method for the determination of PAHs; LOD, LOQ in μgkg^{-1}, instrument linearity in μgL^{-1} and recoveries in %

PAH[a]	Linearity		Recovery [b] ±RSD (%)	LOD (μgkg^{-1})	LOQ (μgkg^{-1})
	Range (μgL^{-1})	R^2			
NPH	0.01-30	0.9945	102.0±7	0.75	2.28
ACE	0.01-60	0.9997	110.0±5	0.97	2.93
FL	0.01-30	0.9985	111.0±4	0.79	2.39
PHE	0.01-60	0.9996	98.0±3	0.16	0.50
ANT	0.01-20	0.9995	100.0±5	0.25	0.76
FT	0.01-90	0.9995	89.0±7	0.31	0.95
PYR	0.01-30	0.9998	83.0±8	0.19	0.60
BaA	0.01-60	0.9988	100.0±6	0.20	0.58
CHR	0.01-30	0.9991	91.0±4	0.19	0.57
BbF	0.01-30	0.9979	90.0±3	0.20	0.60
BkF	0.01-12	0.9988	95.0±5	0.21	0.62
BaP	0.01-12	0.9964	85.0±6	0.21	0.62
DBahA	0.01-18	0.9989	80.0±3	0.20	0.60
BghiP	0.01-60	0.9989	97.0±4	0.30	0.91
IP	0.25-90	0.9929	75.0±6	0.52	1.59

[a] NPH: Naphthalene; ACE: Acenaphthene; FL: Fluorine; PHE: Phenanthrene; ANT: Anthracene; FT: Fluoranthene; PYR: Pyrene; BaA: Benz[a]anthracene; CHR: Chrysene; BbF: Benzo[b]fluoranthene; BkF: Benzo[k]fluoranthene; BaP: Benzo[a]pyrene; DBaA: Dibenz[a,h]anthracene; BghiP: Benzo[ghi]perylene and IP: Indeno [1,2,3-cd] pyrene.
[b] Mean value for two levels 5 and 10 μgkg^{-1} ± relative standard deviation (n = 3)

Table 3. Comparison of the LOD and LOQ for PAHs in oils derived from this work as compared to that reported from other publications

Target analytes	Clean-up	Analysis method	LOQs (mg kg^{-1})	Reference
16 EPA PAHs	Low temperature clean-up and SPE	HPLC-FLD	0.25–6.25	Payanan et al. 2013
15 + 1 EU PAHs	Solid phase micro-extraction (SPME)	GC × GC TOFMS	0.4-3.7	Purcaro et al. 2007
16 EPA PAHs	SPE (silica gel)	GC-MS	0.3-3	Fromberg et al. 2007
Heavy 8PAHs	Supercritical fluid extraction	HPLC-FLD	0.2-21	LageYusty, CortizoDaviña2005
BaP	Solid phase clean-up (C18 and Florisil)	GC-MS; HPLC (DACC)-FLD	1	Bogusz et al. 2004
16 EPA PAHs	Solid phase extraction (C18)	HPLC-FLD	0.3-6	Barranco et al. 2003

[a] The range includes the lowest and the highest value reported in the related references.

Table 4. PAHs contents (µg kg^{-1}) obtained in the analysis of olive and refined pomace olive oils by modified UASE method

PAH[a]	Mean concentrations of PAHs ± standard deviation	
	Refined pomace olive oil (n=5)	Olive oil (n=5)
NPH	6.30 ±2.80[b]	5.59±4.11[b]
ACE	5.24 ±3.63[b]	5.54±3.90[b]
FL	3.36 ±2.00[b]	<LOQ
PHE	1.84 ±0.92[b]	2.28±1.51[b]
ANT	0.84 ±1.80[b]	1.68±0.82[b]
FT	1.69 ±0.90[b]	1.45±0.50[b]
PYR	1.97 ±1.10[b]	0.61±0.50[c]
BaA	1.20 ±0.13	<LOQ
CHR	1.21 ±0.22[b]	1.28±0.20[b]
BbF	0.76 ±1.10	<LOQ
BkF	<LOQ	<LOQ
BaP	0.64±0.24[b]	0.70±0.30[b]
DBaA	<LOQ	0.62±1.21
BghiP	<LOQ	<LOQ
IP	<LOQ	<LOQ

[a] NPH: Naphthalene; ACE: Acenaphthene; FL: Fluorine; PHE: Phenanthrene; ANT: Anthracene; FT: Fluoranthene; PYR: Pyrene; BaA: Benz[a]anthracene; CHR: Chrysene; BbF: Benzo [b] fluoranthene; BkF: Benzo [k] fluoranthene; BaP: Benzo [a] pyrene; DBaA: Dibenz[a,h]anthracene; BghiP: Benzo [ghi] perylene and IP: Indeno [1,2,3-cd] pyrene.
Mean values of PAHs marked with different letters indicate statistically significant difference between means at α= 0.05 level
<LOQ: lower than quantification limit

Conclusion

This study presented an additional purification step for PAHs analysis in the olive and refined pomace olive oils. The analytical procedure was based on ISO 15753 method (2006); while, some modifications from Moreda *et al*. (2004) were proposed. NH_2 SPE cartridge was used to remove interferences. As compared with the reference method, the highest interferences were removed by NH_2 SPE cartridge and better cleaner chromatograms were obtained and HPLC/FLD was selected as an applicable and powerful instrumental technique for PAHs analysis. The linearity, recoveries, LOD, LOQ, and RSD% of the developed procedure demonstrated its suitability for routine monitoring of PAHs in olive and refined pomace olive oils.

Acknowledgements

This research was carried out with the financial support of Standard Research Institute, Faculty of Food Industry and Agriculture (project no. 91-05).

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