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Original Article

# The potential of low temperature extraction method for analysis of polycyclic aromatic hydrocarbons in refined olive and refined pomace olive oils by HPLC/FLD

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## ABSTRACT

**Background and Objectives:** A method was developed and validated for determining 15 polycyclic aromatic hydrocarbons (PAHs) in refined olive and refined pomace olive oils using high performance liquid chromatography coupled with a fluorescence detector.

**Materials and Methods:** The sample preparation involved liquid–liquid extraction with organic solvent and low temperature clean-up. The low temperature allowed most of the lipids in the extract to be removed. Then alumina-N and $NH_2$ solid phase extraction cartridges were used for further clean-up.

**Results:** The results showed that the obtained chromatogram of extract was free of significant interferences. The PAH recoveries ranged from 81% to 114 %. Limit of detection and limit of quantitation for individual PAHs were 0.09-1.97 µgkg-1 and 0.29-5.99 µgkg-1, respectively.

**Conclusions:** The performance of the present method was evaluated for determining PAHs in various types of real refined olive and refined pomace olive oil samples, and suitable results were obtained. The variable levels of PAHs were detected to range from 0.29 to 8.21µgkg-1 in real samples.

**Keywords:** Polycyclic aromatic hydrocarbons (PAHs), Refined olive oil, Refined pomace olive oil, Low temperature extraction, High performance liquid chromatography with fluorescence detection (HPLC/FLD)

## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are persistent chemicals with two or more fused aromatic rings, and are highly stable contaminants that occur in soil, air, and food. They are a very important group of chemical carcinogens, 16 of which have been selected as priority pollutants by the United States Environmental Protection Agency (EPA) on the basis of their occurrence and carcinogenicity. The main sources for the human intake of PAHs are from the ambient air, drinking water, and food. Edible oils and fats are the largest contributory sources because of their lipophilic nature (1, 2). There are many organizations worldwide that set upper limit values for PAHs in fats and oils. For example, European Union (No. 835/2011) has set a maximum level in fat and oils of 2 µgkg$^{-1}$ for benzo[a]pyreneand, and 10 µgkg$^{-1}$ for the sum of 4PAH, benzo[a]pyrene, benzo[a]anthracene, benzo[b]fluoranthene, and chrysene (3).

The presence of polycyclic aromatic hydrocarbons (PAHs) in olive oils is caused by contamination on the skin of olives and contamination of oil during processing in the oil mill (4, 5). PAHs may also be formed directly in crude olive pomace oil as a result of drying heat process before the solvent extraction process obtains the residual oil, and consequently, higher PAH levels are usually detected in the crude oils obtained from pomace oil. This crude oil is not

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used directly for human consumption; rather it is refined to remove unwanted components and undesirable organoleptic properties. Olive oils contain high amounts of squalene (800–8000mgkg$^{-1}$). Squalene is isomerized in bleaching step, and decomposes the steroidal alcohols, yielding a great number of unsaturated hydrocarbons with cyclic moieties, which have polarities similar to those of PAHs (6-9).

Analysis of PAHs in vegetable oils is problematic owing to the complexity of the fat and oil matrix and the lipophilic nature of PAHs (10-13). Different procedures have been applied for sample preparation such as liquid–liquid extraction (14), solid-phase extraction (SPE) (15-16), solid-phase microextraction (17-19), supercritical-fluid extraction (11), and microwave-assisted extraction (20). Most of these techniques are complicated and time-consuming, and require large volumes of organic solvents. In contrast, the low-temperature clean-up method is one of the excellent techniques for eliminating fat interferences from the fat and oil samples (21-24). High performance liquid chromatography with fluorescence (25-27) or ultraviolet (UV) detection (28), gas chromatography–mass spectrometry (GC–MS) (29), and GC–MS–MS (30) are often used for the quantitative determination of PAHs in olive oils. HPLC-FLD methods are sensitive and most widely used assays (31). There are standardized methods based on the isolation of PAH fraction by column chromatography on SPE and analysis by HPLC using fluorescence detector (32-33); however, these methods are not applicable to refined olive pomace oil because of the interferences by different compounds in the HPLC analysis.

The main aim of this study was to develop and optimize a simple analytical method for quantifying PAHs in refined olive and refined pomace olive oils. The method consisted of the isolation of the hydrocarbon fraction, clean-up of PAH fraction using two SPE steps, and subsequent analysis by reverse-phase HPLC using a programmable wavelength fluorescent detector. The sample preparation was based on the extraction of PAHs from oil samples with acetonitrile/acetone solvent mixture and clean-up with alumina-N cartridge, the method described by Payanan et al. (2013). For specializing this method for refined olive and refined pomace olive oils, the second clean-up step with $NH_2$ cartridge was applied (34). Then the method was validated to determine 15 PAHs that were priority pollutants by HPLC with fluorescence detection in refined olive and refined pomace olive oils. The PAHs recovery, limit of detection and limit of quantitation were also calculated. The method was applied to various brands of refined pomace olive and refined olive oil samples.

## Materials and Methods

### Experimental

**Reagent and chemicals:** The standard mixture of 16 EPA PAHs (PAH-mix 4S8743) was obtained from Sigma Aldrich (Bellefonte, PA). Hexane, acetone, acetonitrile, dichloromethane, and toluene solvents were HPLC-graded and purchased from Merck (Darmstadt, Germany). Deionized water was purified on a Milli-Q system (Millipore, Billerica, MA, USA). The stock and working standard solutions of PAHs were prepared in acetonitrile with the concentrations of 200 µgL$^{-1}$ and 50 µgL$^{-1}$, respectively. Eight standard solutions of PAHs in acetonitrile were prepared from the working standard solution for the calibration curve. They were stored at 4ºC in darkness. The oil samples were bought from local supermarkets in Iran and stored at room temperature until analysis.

**Instrument and analytical conditions:** The HPLC equipment, a YL 9100 HPLC system, consisted of a YL 9101 vacuum degasser for the mobile phase solvents, a YL 9110 quaternary pump, a YL 9130 column compartment, and an FP-2020 plus programmable fluorescence detector co-operated with YL Clarity software program (Young Lin, Korea). A ZORBAX Eclipse (150 mm× 4.6 mm i.d., 5 µm particle size; Agilent Technologies, USA) was used together with a $C_{18}$ guard column (10 mm× 2.1 mm i.d.) for chromatographic analyses. Injection volume was 20 µL. Separation was performed at 35◦C using the gradient described in Table 1.

**Table 1.** Gradient elution program for 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 |

For the PAHs determination by fluorescence detector (FLD), the following programmed excitation and emission wavelengths (Ex/Em) were used: 270/324 nm (NPH, ACE, FL) at start, 248/375 nm (PHE, ANT) to 12.8 min, 280/462 nm (FT) to 16.8 min, 270/385 nm (PYR, BaA, CHR) to 18.1 min, 256/446 nm (BbF) to 28 min, 292/410 nm (BkF, BaP, DahA, BghiP) to 31.2 min, and 270/470 nm (IP) to 38 min. Alumina-N cartridges were Waters (Milford, MA, USA). The $NH_2$ cartridges 500 mg, 6 mL, LN 110924 was purchased from (Anpel, China). Additional equipment included an ultrasonic bath (Elma, Germany), an oven (Heraeus, Germany), a tabletop centrifuge (Dynamca, United Kngdom), a vortex mixer (Velp Scientifica, Italy), and a scale (Mettler Toledo, Switzerland).

**Low-temperature extraction and alumina-N SPE clean-up**: One gram of the oil sample was weighed into a 100 mL centrifuge tube, and 200 µL of the working standard solution (50 $\mu gL^{-1}$) and 8 mL of a 4:1 (v/v) solvent mixture of acetonitrile/acetone were added and shaken for10 min. Then the solution was kept in the freezer at -18°C to -25°C for 24 h to allow the fat to precipitate. The extract was removed from the fat precipitate, and the residue was re-extracted with fresh solvent. The total extract was evaporated to dryness; the final residue was dissolved in 1 mL of 1:1 (v/v) of hexane/dichloromethane, purified by passage through alumina-N cartridge, and eluted with 10 mL of 1:1 (v/v) of hexane/dichloromethane. The resulting eluent was evaporated to the approximate volume of 0.50 mL.

**$NH_2$ SPE cartridge clean-up:** The $NH_2$ cartridge was conditioned by passing 30 mL of hexane under vacuum, and the concentrated solution obtained from the alumina-N cartridge was charged onto the cartridge. The column was then eluted with 25 mL of hexane/toluene (70:30), and the eluent evaporated in a rotary evaporator under vacuum just to dryness. The residue was re-dissolved in 250 µL of acetonitrile for HPLC-FLD analysis.

**Statistical analysis:** The experiments were designed by a completely randomized design. All results were the average of three separate experiments. Linear least-squares regression equations were used for calibration curves. Standard deviation of the PAH contents was calculated by the SPSS software (ver. 18.0; SPSS Inc., Chicago, IL, USA).

## Results

**Low-temperature extraction and SPE clean-up:** The extraction was typically achieved by adding an organic solvent for extraction, and subsequently, freezing the solution at -25°C. After freezing for 24 h the fat content was frozen out, and the extracted organic solvent was simply separated off. Then the oil residue in the extract was removed with the alumina-N SPE cartridge; however, huge interference peaks in the subsequent HPLC chromatograms for the detection of PAHs were obtained (Fig. 1a). Therefore, the use of $NH_2$ SPE cartridge was evaluated. The extract obtained was free of significant interferences (Fig. 1b), and the highest interference was removed with the $NH_2$ SPE cartridge.

**Validation of the method**: Limits of detection (LODs), limits of quantification (LOQs), linear ranges, and recoveries of PAHs were calculated under optimal conditions. The external standard calibration was chosen to quantify the analyte values for HPLC/FLD technique using eight multi-component calibration standards (0.01-90 $\mu gl^{-1}$). The complete description of standard linearity supported by regression data is shown in Table 2. Analysis of the PAHs showed a linear relationship with high linear regression coefficients of determination for all of 15the PAHs ($R^2 > 0.9929$). The PAH recoveries were in the range of 81–114%, and the LODs and LOQs were in the range of 0.2-3.2 $\mu gkg^{-1}$ and 0.3-6$\mu gkg^{-1}$, respectively.

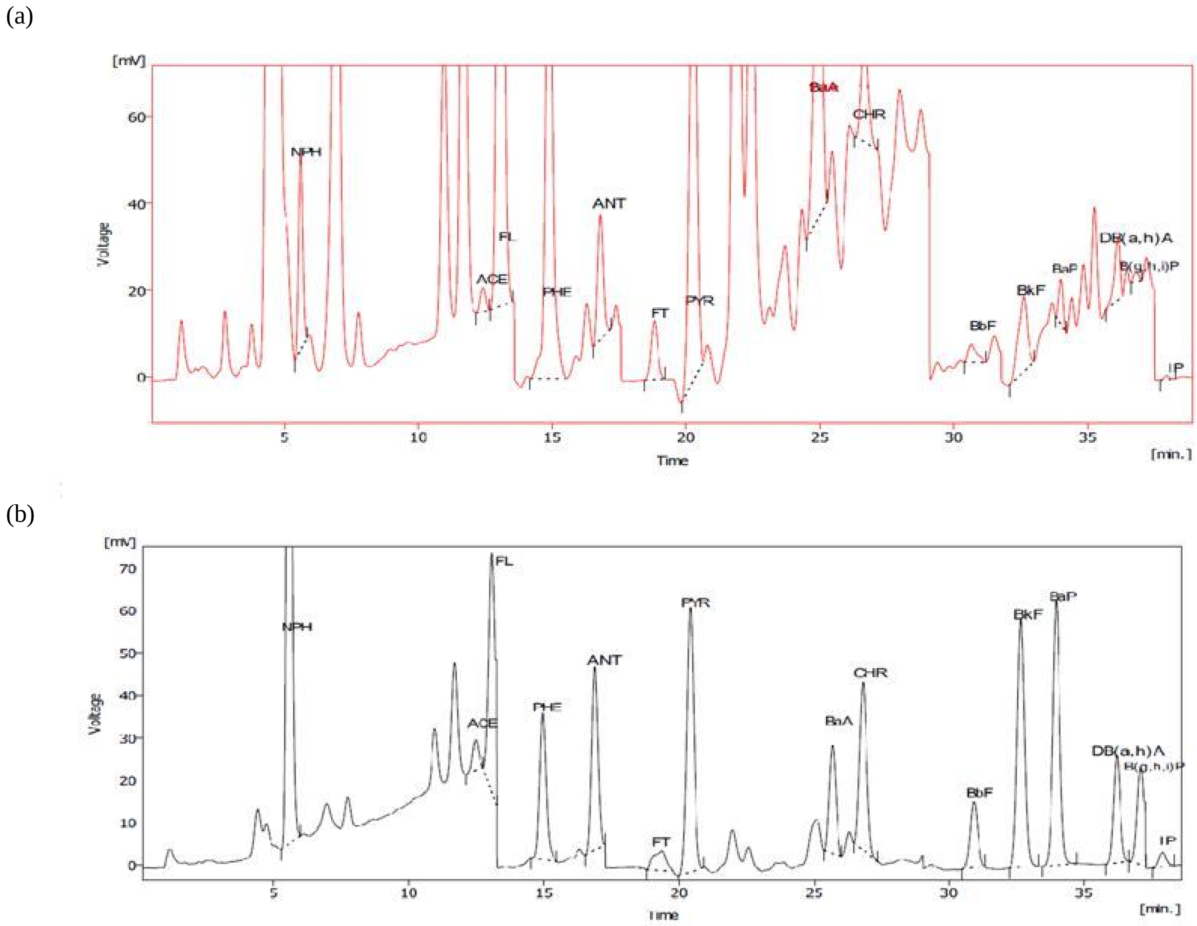

**Fig 1.** Chromatograms of HPLC-FLD analysis of PAHs after low temperature extraction method: (a) no $NH_2$ SPE cleanup and (b) followed by $NH_2$ SPE cleanup.

spiked PAHs solution concentration: 1 $\mu gL^{-1}$ 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.

**Table 2.** Performance criteria of method for determining PAHs; LOD, LOQ in $\mu gkg^{-1}$, instrument linearity in $\mu gL^{-1}$, and recoveries in %

| PAH | Linearity | | Recovery [a] ±RSD (%) | LOD ($\mu gkg^{-1}$) | LOQ ($\mu gkg^{-1}$) |
|---|---|---|---|---|---|
| | Range ($\mu gL^{-1}$) | $R^2$ | | | |
| NPH | 0.01-30 | 0.9945 | 103±10 | 0.68 | 2.08 |
| ACE | 0.01-60 | 0.9997 | 114±6 | 0.29 | 0.88 |
| FL | 0.01-30 | 0.9985 | 113±4 | 0.12 | 0.35 |
| PHE | 0.01-60 | 0.9996 | 108±5 | 0.66 | 1.99 |
| ANT | 0.01-20 | 0.9995 | 105±3 | 0.11 | 0.35 |
| FT | 0.01-90 | 0.9995 | 85±5 | 1.97 | 5.99 |
| PYR | 0.01-30 | 0.9998 | 86±4 | 0.32 | 0.99 |
| BaA | 0.01-60 | 0.9988 | 101±7 | 0.16 | 0.48 |
| CHR | 0.01-30 | 0.9991 | 98±8 | 0.18 | 0.55 |
| BbF | 0.01-30 | 0.9979 | 84±3 | 0.28 | 0.85 |
| BkF | 0.01-12 | 0.9988 | 93±5 | 0.09 | 0.29 |
| BaP | 0.01-12 | 0.9964 | 92±4 | 0.09 | 0.29 |
| DBahA | 0.01-18 | 0.9989 | 81±6 | 0.15 | 0.46 |
| BghiP | 0.01-60 | 0.9989 | 100±7 | 0.59 | 1.80 |
| IP | 0.25-90 | 0.9929 | 86±10 | 0.87 | 2.65 |

[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 of 5 and 10 $\mu gkg^{-1}$ ± relative standard deviation (n = 3)

**Table 3.** Comparing the limit of detection and limit of quantitation for PAHs in vegetable oil for this work and in relation to the ones reported from other methods

| Target analytes | Clean-up | Analysis method | LOQs (mg $kg^{-1}$) | References |
|---|---|---|---|---|
| 15 + 1 EU PAHs | Solid phase micro-extraction (SPME) | GC × GC TOFMS | 0.4-3.7 | 15 |
| 16 EPA PAHs | Solid phase extraction (C18) | HPLC-FLD | 0.3-6 | 10 |
| 16 EPA PAHs | SPE (silica gel) | GC-MS | 0.3-3 | 42 |
| 16 EPA PAHs | Low temperature clean-up and SPE | HPLC-FLD | 0.25–6.25 | 21 |
| BaP | Solid phase clean-up (C18 and Florisil) | GC-MS | 1 | 38 |
| Heavy 8PAHs | Supercritical fluid extraction | HPLC-FLD | 0.2-21 | 11 |

**Determining PAHs in real samples:** The proposed method was applied to the analysis of refined pomace olive and refined olive oil samples. All analyses were done at least in triplicate. The mean content of individual PAHs and the total sum of 15 PAHs content in the analyzed samples are presented in Table 4. NPH, ACE, FL and PHE were the compounds present to a greater extent. ANT, PYR, BaA, and CHR were also found at higher concentrations. None of the oil samples contained any important PAH content and exceeded the maximum limit for BaP and 4PAHs.

**Table 4.** PAHs contents (μg $kg^{-1}$) obtained in the analysis of refined olive and refined pomace olive oils by HPLC/FLD

| | Mean concentrations of PAHs ± standard deviation | |
|---|---|---|
| | Refined pomace olive oil (n=5) | Refined olive oil (n=5) |
| NPH | 8.21±3.23 | 7.25±3.00 |
| ACE | 7.53±4.05 | 6.10±3.05 |
| FL | 3.72±2.03 | 2.52±1.02 |
| PHE | 2.10±2.00 | 2.33±1.20 |
| ANT | 1.17±1.51 | 1.75±0.40 |
| FT | <LOQ (5.99) | <LOQ (5.99) |
| PYR | 1.74±2.50 | <LOQ (0.99) |
| BaA | 1.33±0.36 | 0.50±0.21 |
| CHR | 1.40±0.51 | 1.35±0.33 |
| BbF | <LOQ (0.85) | <LOQ (0.85) |
| BkF | 0.29±0.51 | <LOQ (0.29) |
| BaP | 0.30±0.35 | <LOQ (0.29) |
| DBaA | 0.58±1.10 | 0.70±0.30 |
| BghiP | <LOQ (1.80) | <LOQ (1.80) |
| IP | <LOQ (2.65) | <LOQ (2.65) |

[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.
<LOQ: lower than quantification limit

## Discussion

**PAH extraction and clean-up:** The low-temperature clean-up method is a potentially excellent technique for eliminating fat interferences from the fat and oil samples. This technique is simple, allows a high sample throughput, and utilizes a low amount of organic solvent (21-23). After this step, the highest interferences could be removed with the alumina-N SPE cartridge (4, 7, 35, 36); however, huge interference peaks still remained at the beginning (Fig. 1a). In addition, the heavy PAHs could not be reliably identified in Fig. 1a because of the presence of too many interfering peaks. These peaks were due to the interferences provided by the oil matrix and the existence of unsaturated hydrocarbons with cyclic moieties due to the squalene isomerization and steroidal alcohol decomposition in the refined pomace olive and refined olive oils (37-38). The use of $NH_2$ SPE cartridge allowed the non-aromatic hydrocarbons be eluted with hexane solvent but the heavy PAHs were left on the stationary phase because of the major interaction with the amino groups. Then the heavy PAHs were displaced with toluene aromatic solvent, and finally, the extract was free of significant interferences (Fig. 1b) because the highest interference was removed with the $NH_2$ SPE cartridge (34, 39-40).

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

In order to evaluate repeatability and recovery, blank samples of oils were spiked at two levels for all PAHs: 5 and 10 µgkg$^{-1}$. Repeatability was evaluated by performing three analyses the same day under the same conditions. The recoveries varied between 81 and 114% with 3-10 % of RSD, and were in the range set for BaP (50–120%) in Regulation 835/2011 (Table 2).

The LOD and LOQ defined as the concentration of the analyte producing the signal-to-noise ratio of 3 and 10, were obtained from the standard deviation of the blank samples (n = 20) and the slope of the calibration curve.The LODs and LOQs were in the range of 0.09-1.97 µgkg-1 and 0.29-5.99 µgkg-1, respectively. The LOQs for 12 out of 15 PAHs were all below 2 µgkg-1. For BaP, the LOQ (0.29 µgkg-1) was lower than that of the required maximum level by the EU regulations (2 µgkg-1). In addition, the LOQs of this method were broadly comparable with those reported by other researchers' techniques (Table 3).

**Analysis of oil samples:** The PAHs concentration in the refined pomace olive and refined olive oil samples were in the range of 0.29-8.21 µgkg-$^{1}$ and 0.50-7.25 µgkg-$^{1}$, respectively, and surprisingly, the refined pomace olive oil contained high concentrations of all target PAHs. NPH, ACE, FL and PHE were the compounds present to a greater extent. ANT, PYR, BaA, and CHR were also found at higher concentrations. The rest of PAHs were detected at lower concentrations in all samples. FT, BbF, BghiP and IP in the refined pomace olive oil samples and FT, PYR, BbF, BkF, BaP, BghiP and IP in the refined olive oil samples were below the LOQ. The present and other authors have found similar results (21, 41). Mean content of the sum of 4PAHs (BaP, CHR, BaA, and BbF) in the refined pomace olive and refined olive oil samples was below 10 µgkg$^{-1}$. None of the oil samples contained any important PAH content and exceeded the maximum limit for BaP and 4PAHs, set by the EU regulations for oils and fats.

From the experimental results, it can be concluded that the method described by Paynan et al. (2013) modified from Moreda et al.'s (2004) work could be suitable for determining PAHs in refined olive and refined pomace olive oils. The highest interference in the olive and refined pomace olive oil samples was removed with the $NH_2$ SPE cartridge. In this study, a method was developed and validated for determining 15 mutagenic and carcinogenic PAHs as well as two EPA indicators: PAHs in refined pomace olive and refined olive oils. The HPLC/FLD was selected as a powerful instrumental technique. The linearity, recoveries, LOD, LOQ, and RSD% of the developed procedure demonstrated its suitability for routine monitoring of PAHs in refined olive and refined pomace olive oils.

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