Scientific Papers. Series A. Agronomy, Vol. LXVII, No. 2, 2024
ISSN 2285-5785; ISSN CD-ROM 2285-5793; ISSN Online 2285-5807; ISSN-L 2285-5785

COMPARISON OF SOME EXTRACTION TECHNIQUES FOR THE DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) FROM OILSEEDS BY GC-MS/MS

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) constitute a class of organic pollutants with potential risk to human health and can contaminate the oilseeds crops during the growth period and/or during the drying/roasting processes. In order to determine the 4 PAHs regulated (Commission Regulation (EU) No 835/2011) in sunflower seeds by GC-MS/MS, two preparation techniques were evaluated: QuEChERS extraction with modifications and saponification with liquid-liquid extraction. Different factors were studied to isolate PAHs: type of solvent/salt, quantity of reagents/solvents, stirring mode, etc. Acetonitrile extracts were purified by freeze-combined with d-SPE QuEChERS. The comparison of the preparation techniques was evaluated in terms of recovery (50-120%) and co-extract residue values (≤ 2 mg/mL). QuEChERS extraction was selected as the optimal variant, obtaining the lowest co-extract residue values (≤ 0.5 mg/mL) and recoveries between 94.62-102.41%. This methodology was also verified on other samples: sunflower seeds with different fat content, sunflower seed core, pumpkin seeds, flaxseeds, rapeseeds, sesame etc. No PAHs were detected in the analysed samples.

Key words*: gas chromatography, oleaginous seeds, polycyclic aromatic hydrocarbons, QuEChERS extraction, sunflower seeds.*

INTRODUCTION

Vegetable oils are widely used, both for direct human consumption (salads), but also as a medium used for frying food or are incorporated into various food products (cakes, bread, biscuits, etc.) or to obtain products such as: margarine, mayonnaise, etc., thus becoming a major source of dietary PAH exposure. Due to their lipophilic character and their widespread distribution in the natural environment, the level of PAH contamination in oilseeds and their derived processed products has become a serious concern, as up to 15-50% of the absorption and dietary exposure to PAHs from food is attributed to these groups of contaminated foods (Veyrand et al., 2013).

Aware of the toxic effects of PAHs on human health, since 2002 the European Commission (EC) has introduced recommendations and regulations (SCF, 2002; EFSA, 2008) that requires to monitor and to establish maximum permissible limits of these toxic compounds. PAHs represent a major group of chemicals being considered cancer-inducing agents. Several PAHs have been assessed as a potential carcinogenic by the International Agency for Research on Cancer (IARC, 2010). European legislation (Regulation no 835/2011 with subsequent amendments 1327/2014; 1993/2015; 1125/2015; 1255/2020) established maximum limits for 4 PAHs: benzo(a)pyrene (BaP), chrysene (Chr), benzo(a)anthracene (BaA), benzo(b) fluoranthene (BbF) and a separate maximum level for benzo(a)pyrene (BaP), which is the most studied PAH and it is used as a marker of toxicity and occurrence of PAHs in food. Hundreds of PAHs consist of at least two aromatic rings linked together. The chemical structures of the 4 PAHs are shown in Figure 1.

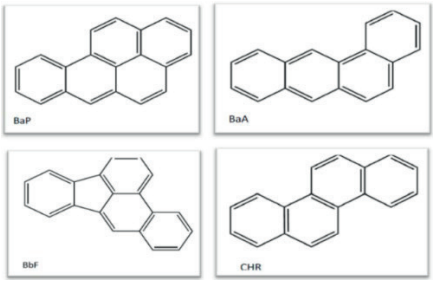

Figure 1. Chemical structure of the 4 PAHs for which maximum limits are set (EFSA, 2008)

PAHs contain several isomers that refer to the same molecular formula, but to distinct configurations. For example, BaP and BbF, like BaA and ChR have identical molecular masses but different structures (Table 1).

Table 1. PAH identification
(IARC, 2010; Sampaio et al., 2021)

HAP abbreviation	Chemical formula	Molecular weight (g/mol)	Boiling point (°C)	Melting point (°C)	Classification IARC*
BaP	$C_{20}H_{12}$	252.3	495	179	1
BaA	$C_{18}H_{12}$	228.3	438	158	2B
CHR	$C_{18}H_{12}$	228.3	448	254	2B
BbF	$C_{20}H_{12}$	252.3	481	168	2B

*IARC: 1 - carcinogenic to humans; 2B - possible carcinogenic to humans

The quantification of PAHs in food is a difficult task, not only because of the complexity and diversity of food matrices, but especially because of their physicochemical properties (Sun et al., 2019). The samples differ widely in both composition and PAH contamination, from trace amounts to thousands of µg/kg in product (Sun and Wu, 2020). Extraction and purification steps play a critical role in the success of an assay to improve selectivity and sensitivity. In these steps, the analytes are transferred from the matrix into an extract, usually in an organic solvent, as free as possible from interfering compounds and compatible with the chromatographic system and the detection method. The selection of the most suitable analytical protocol should be based on the type of food product (type of matrix), the structure and the physicochemical properties of the PAHs (Sun et al., 2019; Wu et al., 2020).

The current trend is to develop new concepts in sample preparation in order to provide a faster and more efficient way to process samples.

In this context, the present study considered to establish the optimal conditions for two preparation techniques of oilseed extracts: method I- QuEChERS (Quick, Easy, Cheap, Efficient, Robust and Safe) extraction and method II- saponification by liquid-liquid extraction (LL), followed by purification by d-SPE QuEChERS. Different factors were studied for each preparation method: type of extraction salt, volume of solvent/purified extract, type of solvent, etc. (method I) and quantity of reagents/ solvents, stirring method, freezing time (method II), respectively.

We aim to find an optimal protocol for extracting and purifying extracts from oilseeds, using low amounts of chemical reagents, eliminating or replacing highly toxic reagents, obtaining minimal amounts of analytical waste, and in agreement with the principles of green chemistry.

MATERIALS AND METHODS

Experimental materials

In this study, three varieties of sunflower seeds with different fat content: S1- 46.39%, S2- 42.06%, S5- 48.44% (Figure 2), and other oilseeds purchased from the Romanian market: mixture of sunflower and pumpkin seeds (MSP), hulled sunflower seeds (HS), white sesame seeds (WS), linseed (L), hulled pumpkin seeds (HP) and rapeseed (R) (Figure 3) were used.

Figure 2. Sunflower seeds, natural and ground, with different fat content (S1, S2, S5)

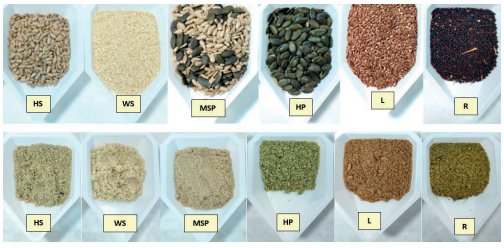

Figure 3. Oilseeds (natural and ground) sold on the Bucharest market

The samples used were processed unspiked or spiked with PAHs solution in different concentrations (2, 5, 10 µg/kg).

Standards, solvents and reagents

The following standards were used: reference standard of native PAHs (mixture of 4 PAHs) in isooctane (EFSA-4 Native PAH, ES-5542, 5 µg/mL); reference standard for labelled PAHs (mixture of 4 ^{13}C-labelled PAHs) in nonane (EFSA-4 ^{13}PAH, ES-5540, 5 µg/mL), both from Cambridge Isotope Laboratories, Inc, USA.

As injection standard (ISI), solution of 9-fluorobenzo[k]fluoranthene (FBkF) in toluene of 100 µg/mL concentration was used which was added to the sample extract prior to injection into the GC-MS/MS.

Solvents of chromatographic purity were used: acetonitrile, n-hexane, methanol, purchased from VWR Chemicals, Belgium. QuEChERS extraction salt kits were used: citrate salts (EN)- 4 g $MgSO_4$ + 1 g NaCl + 0.5 g Na_2H-citrate x 1.5 H_2O- disodium hydrogen citrate sesqui-hydrate rate + 1 g Na_3-citrate x 2 H_2O- trisodium citrate dihydrate; acetate salts (AOAC) - 6 g $MgSO_4$ + 1.5 g CH_3COONa; 4 g $MgSO_4$ + 1 g NaCl (ORIGINAL), purchased from Macherey-Nagel GmbH & Co, Germany/Agilent Technologies, USA/Thermo-Scientific, USA.

In order to purify the extracts, d-SPE-QuEChERS purification kits from Agilent were used: 0.15 g PSA + 0.9 g $MgSO_4$ (5982-5056CH); Enhanced Matrix Removal-Lipid (EMR-Lipid 5982-1010 + EMR-Lipid 5982-0101). Z-Sep$^+$ (Supel QuE Z-Sep$^+$ Bulk, 55418-U, Sigma Aldrich St Louis, USA) was used as well as sorbent for purification. Anhydrous magnesium sulphate ($MgSO_4$) of analytical grade was purchased from Merck, KGaA, Germany.

Disposable ceramic homogenizers (Thermo Fisher Scientific) for 15- and 50-ml tubes were used to homogenize the samples.

Sample preparation

Two extraction methods for the PAH determination from oilseed matrices, were carried out, for both d-SPE QuEChERS purification being used.

Method I - QuEChERS extraction with modifications

For this method 15 experimental variants were performed (Table 2) with the variation of extraction salts, volume of solvent/extract, the type of solvent in combination with the type of sorbent and the weight of sample used.

Table 2. Experimental variants - Method I

Crt. No.	Variable factor			Variant	QuEChERS method with modification (Method I)
1.	QuEChERS extraction salt	EN		V1	5 g sample weighed in a 50 mL centrifuge tube + 10 mL water + 10 mL hexane (H) + QuEChERS salts → vortexing/homogenization → centrifugation → hexane evaporation → collecting the residue with 10 mL acetonitrile (ACN) → vortexing/homogenization → freezing 1-3 h → filtration through quartz wool → 3.5 mL extract purified by EMR-Lipid
		AOAC		V2	
		ORIGINAL		V3	
2.	Volume of ACN/purified extract	10 mL/3.5 mL		V4	5 g sample weighed in a 50 mL centrifuge tube + 10 mL water + 10 mL H + QuEChERS citrate salts → vortexing/homogenization → centrifugation → hexane evaporation → collecting residue with 5-10 mL ACN → vortexing/homogenization → freezing 1-3 h → filtration by quartz wool → 3.5/5 mL extract purified by EMR-Lipid
		10 mL/5 mL		V5	
		5 mL/3.5 mL		V6	
		5 mL/5 mL		V7	
3.	d-SPE QuEChERS Solvent type/sorbent type	H	EMR	V8	5 g sample weighed in a 50 mL centrifuge tube + 10 mL water + 10 mL H/ACN + QuEChERS citrate salts → vortexing/homogenization → centrifugation → hexane evaporation → collecting the residue with 10 mL ACN → vortexing/homogenization → freezing 1-3 h → filtration through quartz wool → 3.5 mL extract purified by EMR-Lipid/500 mg Z-Sep$^+$
			Z-Sep$^+$	V9	
		ACN	EMR	V10	
			Z-Sep$^+$	V11	
4.	Weight of sample	2 g		V12	2/3/4/5 g sample weighed in a 50 mL centrifuge tube + 10 mL water + 10 mL H + QuEChERS citrate salts → vortexing/homogenization → centrifugation → hexane evaporation → collecting the residue with 10 mL ACN → vortexing/homogenization → freezing 24 h → filtration through quartz wool → 3.5 mL extract purified by 500 mg Z-Sep$^+$ + 300 mg $MgSO_4$
		3 g		V13	
		4 g		V14	
		5 g		V15	

Method II - Saponification and liquid-liquid extraction (LL)

The variants carried out (Table 3) in this method were aimed at optimizing the saponification and LL extraction steps with the reduction of the volume of organic reagents/solvents, with the variation of the shaking method of the extracts during the saponification and with the variation of the freezing time of the extracts before d-SPE QuEChERS purification.

To evaluate the performance of each method, the following factors were considered: obtaining colourless and clean extracts, with values of co-extract residues as low as possible (≤ 2 mg/mL), increasing the extraction efficiency by obtaining good recoveries of the 4 PAHs, which should fall within the limits imposed by European regulations no 836/2011 (50-120%).

To investigate the removal of the compounds from the matrix, after the sample extraction and purification procedure, the co-extractive residue of the sample was determined gravimetrically. The co-extraction residue weight was determined by evaporating 1 mL of the final sample extract under a nitrogen atmosphere.

PAHs analysis by GC-MS/MS was performed using a gas chromatograph (Trace GC Ultra) coupled with tandem triple quadrupole mass spectrometer (TSQ Quantum XLS), both from Thermo Fisher Scientific (USA).

Table 3. Experimental variants - Method II

Crt. No.	Variable factor		Variant	Working method (Method II)
1.	Quantities of solvents used in the saponification stage	200 mL CH_3ONa 200 mL hexane 200 mL methanol + water	V1	2 g sample weighed in 250 mL Erlenmeyer flask + 200/100 mL methanolic solution of sodium methoxide (CH_3ONa) → bath stirring (V3/30 min, 60°C) → cooling to room temperature → extraction with hexane (H) (2 x 100/50 mL) → extract washing with a mixture of methanol + water (4:1, v/v) (2 x 100/50 mL) → concentration to a volume of 6 mL → purification by d-SPE QuEChERS (0.15 g PSA + 0.9 g $MgSO_4$)
		100 mL CH_3ONa 100 mL hexane 100 mL methanol + water	V2	
2.	Stirring method of the extract during saponification	Water bath with heating and ultrasonic stirring	V3	2 g sample weighed in 250 mL Erlenmeyer flask + 100 mL CH_3ONa solution → bath stirring (30 min, 60°C) → cooling to room temperature → extraction with H (2 x 50 mL) → extract washing with a mixture of methanol + water (4:1, v/v) (2 x 50 mL) → concentration to a volume of 6 mL → purification by d-SPE QuEChERS (0.15 g PSA + 0.9 g $MgSO_4$)
		Bath with heating and magnetic stirring	V4	
		Water bath with heating and mechanical stirring	V5	
3.	Freezing time	No freezing + EMR-Lipid	V6	2 g sample weighed in 250 mL Erlenmeyer flask + 100 mL CH_3ONa solution → bath stirring (V5/30 min, 60°C) → cooling to room temperature → extraction with H (2 x 50 mL) → extract washing with mixture of methanol + water (4:1, v/v) (2 x 50 mL) → concentration until H evaporation → collecting residue with 2 x 5 mL ACN → vigorous vortexing → purification with and without freezing → filtration through quartz wool → purification by EMR-Lipid (5 mL)
		Freezing 24 h + EMR-Lipid	V7	

Injection of extracts was performed with an autosampler (Thermo Fisher Scientific, USA), and analytical separation was achieved on a TraceGOLD TG-PAH capillary column (30 m × 0.25 mm I.D. x 0.10 μm) from Thermo Fisher Scientific (USA). GC separation was initiated by volatilizing the sample in an injector (Right PTV), heated to 70°C, in splitless mode. The oven temperature program was: initial temperature 80°C, held for 1 min, an increased with 15°C/min on ramp 1 to 210°C, a 25°C/min increase on ramp 2 to 260°C, a 5°C/min increase on ramp 3 to 305°C (held 2 min), then an increase with 25°C/min on ramp 4 to 350°C (held 5 min). The temperatures of the transfer line and for the ion source were 340°C and 300°C, respectively. Total acquisition time was 30.67 min. The MS/MS operated using the electron ionization (EI) technique, in SRM mode, with the precursor ions fragmentation into product ions. Table 4 lists precursor-to-product ion transitions (MS/MS transitions).

Table 4. Parameters for the analysis of PAHs from oilseeds, by GC-MS/MS

PAH/labelled PAH/ISI	Precursor ion (*m/z*)	Product ion (*m/z*) and quantification ions (*m/z*) with bold
BaA	228	202, **226**
$^{13}C_6$ (BaA-IS)	234	208, **232**
CHR	228	202, **226**
$^{13}C_6$(CHR- IS)	234	208, **232**
FBkF	270	249, **268**
BbF	252	226, **250**
$^{13}C_6$ (BbF-IS)	258	232, **256**
BaP	252	226, **250**
$^{13}C_4$ (BaP-IS)	256	228, **254**

As collision gas Argon was used. Xcalibur software was used for data processing. To each sample, labelled PAHs were added from the beginning, therefore it compensates the analyte losses that might occur during preparation.
PAHs were quantified based on a calibration curve (PAH/PAH-IS area ratio as a function of PAH/PAH-IS concentration ratio), with peaks identified based on MS response and GC retention time. Each sample was analysed using the same analytical conditions.

Statistical analysis

Results for the co-extract residues were expressed as mean ± standard deviation (sd) and were statistically analysed by using Minitab statistical software version 20. One-way analysis of variance (ANOVA test), followed by Tukey's test were used to evaluate the statistical significance between results. The chosen level of significance was set at $p < 0.05$.

RESULTS AND DISCUSSIONS

The higher amount of fat in oilseeds and their derivative products has become one of the major challenges in the laboratory analysis of PAHs. Inefficient separation of these substances may adversely affect the identification and/or quantification of PAHs. The separation of PAHs from high-fat food samples (Alomirah et al., 2010; Dost & Ideli, 2012; Mohammadi et al., 2020; Sánchez-Arévalo et al., 2020) before the further steps of the analytical process is a current and difficult problem for which efforts are being made to develop new preparation methods. Even

with the use of advanced mass spectrometry-based techniques, extensive steps are required to extract and purify PAHs from complex fatty matrices (Parrilla Vázquez et al., 2016). To establish the optimal method for PAHs determination, the S1 sunflower seeds were used in experiments.

Method I - QuEChERS extraction with modifications

QuEChERS extraction salt type

In these variants (V1-V3), three extraction salt kits were used: EN, AOAC and original salts and the results are presented in Table 5.

From a visual point of view, the extracts from the proposed variants were clean and colourless and from a gravimetric point of view, the co-extract residues varied between 0.77-0.91 mg/mL, fulfilling the imposed criterion (≤ 2 mg co-extract/mL). The lowest values were obtained in the case of V1 with the use of citrate salts. No PAHs were detected in the sunflower seeds (S1) studied.

The average recoveries obtained for the samples spiked with 5 µg/kg PAHs solution ranged between 98.23-118.61%, fulfilling the criterion imposed by the European regulation.

Table 5. Results obtained in V1, V2, V3 (Method I)

Variant/ co-extract, mg/mL	PAH, µg/kg (mean ± sd)		Recovery, % (mean ± sd)	RSD (%)
	Spiked – 5 µg/kg			
V1 (EN)/ 0.77 ± 0.02[b]	BaA	5.27 ± 0.15	105.44 ± 3.09	2.93
	Chr	5.40 ± 0.11	108.05 ± 2.13	1.97
	BbF	5.16 ± 0.26	103.13 ± 5.17	5.01
	BaP	4.91 ± 0.12	98.23 ± 2.37	2.42
V2 (AOAC)/ 0.91 ± 0.03[a]	BaA	5.93 ± 0.12	118.61 ± 2.40	2.03
	Chr	5.12 ± 0.05	102.44 ± 0.97	0.94
	BbF	5.41 ± 0.13	108.17 ± 2.60	2.40
	BaP	5.88 ± 0.17	117.67 ± 3.42	2.91
V3 (ORIG.) 0.79 ± 0.01[b]	BaA	4.99 ± 0.08	98.77 ± 0.77	1.53
	Chr	5.65 ± 0.35	113.06 ± 6.93	6.13
	BbF	5.13 ± 0.15	102.64 ± 2.99	2.91
	BaP	5.32 ± 0.43	106.31 ± 8.57	8.06

Values followed by different letters are statistically different ($p < 0.05$)

The highest co-extract residue value was determined for V2, when acetate salts were used, but the values for all variants were withing the criterion settle by EC. Since no significant difference ($p > 0.05$) was registered between the values of co-extract residues obtained with V1 and V3, when choosing the optimal variant to continue the experiments the relative standard deviation (RSD) was considered. In the case of citrate salts (V1) it was obtained lower RSD values (<5.01%) than in the case of original method (V3) (1.53-8.06%), therefore this variant was selected as optimal for an efficient extraction and used in further proposed experiments.

Volume of ACN/purified extract

Taking into account the protocol from the previous variants and aiming to obtain clean extracts, with an amount of co-extract residues as low as possible, it is observed (Figure 4) that collecting the extract after hexane evaporation with a larger volume of solvent (10 mL ACN/V4/V5) the lowest co-extract residue values were obtained compared to displacing a smaller volume (5 mL/V6/V7). ACN is known to be a medium-polarity solvent which extract PAHs with weak polarity and are capable to dissolve it (Payanan et al., 2013; Sun and Wu, 2020).

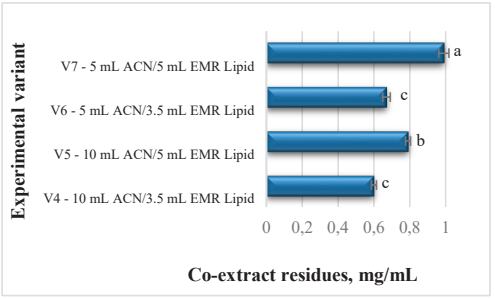

Figure 4. Co-extract residues from V4-V7 (Method I) Columns followed by different letters are significantly different ($p < 0.05$)

By comparing V4 with V5 co-extract residues it was observed that when a smaller volume of extract (3.5 mL) was used for purification a significant lower value of co-extract residues (0.60 mg/mL) was determined (Figure 5). The results obtained in V4 are also confirmed by the areas, S/N, the higher chromatographic peak intensities obtained in this variant, which were higher than the ones obtained in the variants V5, V6, and V7.

Also, when it comes to recoveries (Figure 6), the use of a larger volume of ACN (10 mL/V4) and a lower volume of extract (3.5 mL) led to good results for samples spiked with 5 µg/kg PAH (96.82-112.50%).

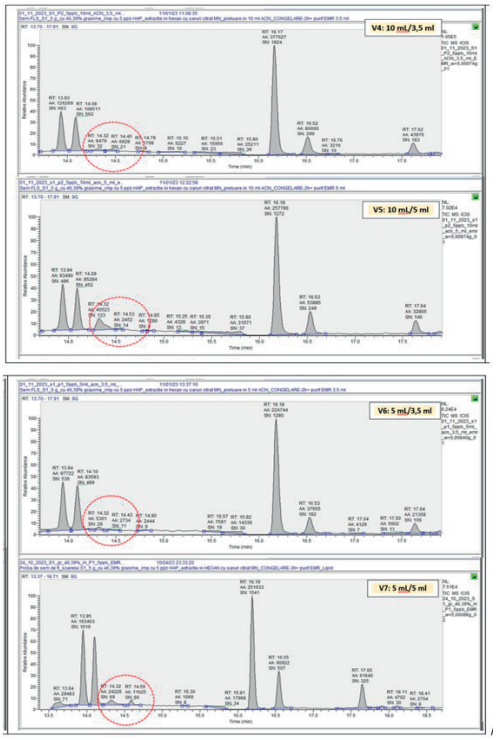

Figure 5. Chromatograms of extracts from V4-V7 (method I)

The recovery obtained for Chr did not fall within the required range (50-120%) in the case of V6. A sufficiently cleaned-up extract was obtained for V4 variant, and based on these results and the obtained recoveries, this variant was selected as optimal and it was used in the following experiments.

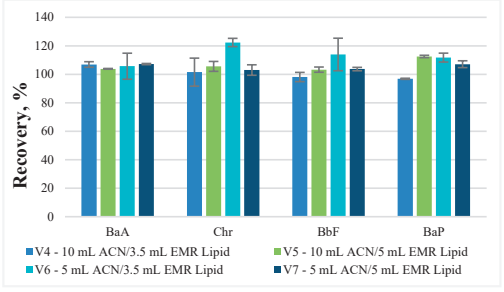

Figure 6. PAH recoveries for V4-V7 (method I)

Payanan et al. (2013) also showed that when a higher volume of ACN (8-10 mL) was used for extraction, the PAHs recoveries were better than when using 4 mL ACN.

d-SPE QuEChERS solvent type/sorbent type

For the QuEChERS extractions the following solvents were used: hexane (H) and acetonitrile (ACN), and for the d-SPE QuEChERS purification two types of sorbents were used: EMR-Lipid (V8, V10) and Z-Sep$^+$ (V9, V11).

EMR-Lipid is a new adsorbent salt which has the purpose to remove lipids from the food matrix, while Z-Sep$^+$ is a silica support coated with zirconium dioxide sorbent used as well for lipids adsorption (Sun and Wu, 2020; Belarbi et al., 2021).

Unspiked samples and spiked samples with 5 and 10 µg/kg PAH solution were analysed. The co-extract residues and recoveries results obtained in the experimental variants are presented in Figures 7 and 8.

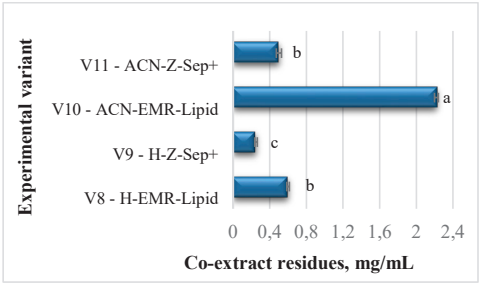

Figure 7. Co-extract residues for V8-V11 (method I) Columns followed by different letters are significantly different ($p < 0.05$)

In all variants except V10, the extracts were clear, colourless. In V10, the final extract showed a yellowish colour, which is also reflected by the higher value of the determined co-extract residue (2.22 mg/mL), exceeding the imposed criterion (≤ 2 mg/mL).

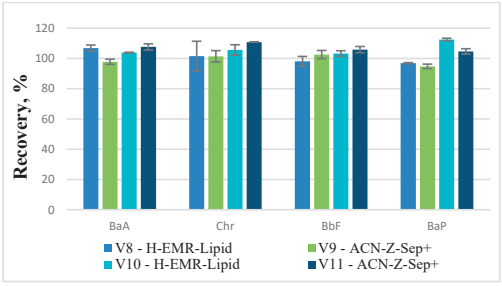

Figure 8. PAH recoveries for V8-V11 (method I)

Comparing the results of the variants with H extraction (V8, V9) with those with ACN

extraction (V10, V11) it was observed that by using H the lowest amounts of co-extract residues were determined for both types of sorbents (0.25 mg/mL/Z-Sep$^+$/V9; 0.60 mg/mL/ EMR-Lipid/V8). The lower values of co-extract residues in the case of H extraction determined cleaner extracts. These results were correlated with the higher values of the chromatographic peak intensities of the 4 PAHs (areas, S/N, etc.). The recoveries determined for the spiked samples ranged between 94.75-106.88%, falling within the required range of 50-120% (Figure 8). Based on these results, the variant with H and Z-Sep$^+$ was consider optimal and was used in the following experiments.

Weight of sample

In order to investigate the influence of sample weight on the co-extract residue and recoveries, different weights of S1 sample (2, 3, 4 and 5 g) were studied, maintaining the previously established optimal parameters. The extracts were subjected to a longer freezing time (24 h) and during the d-SPE QuEChERS purification with Z-Sep$^+$ sorbent, $MgSO_4$ was added in order to remove any water traces from the final extract.

Regardless of the sample weight used, it is found that the values of the co-extract residues determined are similar (0.21-0.22 mg/mL) ($p > 0.05$) (Table 6).

Table 6. Results obtained for V12-V15

Variant/ co-extract, mg/mL	PAH, µg/kg (mean ± sd) Spiked - 5 µg/kg		Recovery, % (mean ± sd)	Ion ratio status
V12/ 0.21 ± 0.02^a	BaA	2.12 ± 0.09	84.89 ± 3.70	Failled/Not used
	Chr	2.11 ± 0.36	84.50 ± 14.43	Passed/Failled
	BbF	2.52 ± 0.02	100.99 ± 0.83	Failled/Coelution Failure
	BaP	2.27 ± 0.01	90.78 ± 0.51	Passed/Passed
V13/ 0.22 ± 0.01^a	BaA	2.63 ± 0.01	105.09 ± 0.39	Passed/Not used
	Chr	2.24 ± 0.07	89.79 ± 2.64	Passed/Passed
	BbF	2.51 ± 0.01	100.36 ± 0.56	Failled/Coelution Failure
	BaP	2.65 ± 0.06	106.16 ± 2.30	Passed/Passed
V14/ 0.21 ± 0.01^a	BaA	2.39 ± 0.00	95.65 ± 0.07	Passed/Passed
	Chr	2.44 ± 0.03	97.54 ± 1.25	Passed/Passed
	BbF	2.50 ± 0.07	100.06 ± 2.76	Coelution/Failure/Passed
	BaP	2.47 ± 0.08	98.71 ± 3.18	Passed/Passed
V15/ 0.21 ± 0.01^a	BaA	2.56 ± 0.00	102.41 ± 0.07	Passed/Passed
	Chr	2.45 ± 0.01	98.05 ± 0.23	Passed/Passed
	BbF	2.50 ± 0.01	99.96 ± 0.35	Passed/Passed
	BaP	2.37 ± 0.01	94.62 ± 0.55	Passed/Passed

Values followed by different letters are statistically different ($p < 0.05$)

The recovery values of the 4 PAHs, in the case of the experimental variants, ranged between 84.50-106.16%, falling within the imposed criterion (50-120%).

By using a lower amount of sample (< 5 g) the criteria specified in the processing method for qualifier ions, specific to BbF, Chr and BaA compounds (ion ratio confirmation, target ratio, qualifier ion coelution, etc.) were not confirmed. The status obtained for these compounds during quantification was "Failed/Coelution Failure/ Not used". In the case of V4 in which 5 g of sample were used, the criteria imposed for the qualification ions specific to the 4 PAHs were met, resulting the "Passed" status. In the case of this variant, the best results were obtained for the determined concentration (2.37-2.56 µg/kg), recovery (94.62-102.41%), as well as the lowest values of standard deviations. Additionally, comparing V15 (24 h freezing) with V9 (1-3 h freezing) from previous experiments, it is observed that the application of a longer freezing time caused a reduction of co-extracts by 16% and therefore V15 was considered as the optimal procedure for Method I. Similarly, Payanan et al. (2013) showed that a 24 h freezing time eliminates the fat from the extract, without influencing the PAH content of sample.

Functionality of the method I/V15 on other types of oilseeds

Based on V15/Method I, the oilseeds shown in Figure 3 were processed.

These seeds were processed unspiked and spiked with 10 µg/kg. No PAHs were quantified in the analysed oilseeds. The average values (n=4) of co-extract residues determined from oilseeds varied between 0.14-1.35 mg/mL, falling within the imposed criterion (Figure 9). A significant higher value was obtained for white sesame seeds.

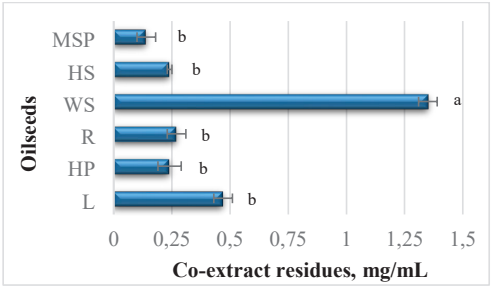

Figure 9. Co-extract residues (mean ± sd) for different oilseeds (V15/method I). Columns followed by different letters are significantly different ($p < 0.05$)

From the point of view of extraction efficiency, the obtained results showed that very good recoveries were determined for all 4 PAHs, in the case of HP seeds (100.32-100.97%) and MSP seeds (97.28-101.23%). For the other

seeds (L, R, WS, HS), the recoveries ranged between 93.25-110.71% in the case of BaA, Chr and BaP compounds, and for BbF, the recovery values exceeded the value of 120%.

Method II – Saponification and liquid-liquid extraction (LL)

Similar to Method I, the variable factors presented in Table 3 were evaluated, from the point of view of co-extractive residues and recoveries by using the S1 sunflower seed sample spiked with 5 and/or 10 µg/kg PAH solutions. The results for co-extractive residue, mean content and recoveries are presented in Table 7.

Table 7. Results obtained for V1-V7 variants/Method II

Variant/ co-extract, mg/mL	PAH, µg/kg (mean ± sd)		Recovery, % (mean ± sd)
V1/ 66.53 ± 5.53[a]	**Spiked - 5 µg/kg**		
	BaA	5.18 ± 0.11	103.66 ± 2.18
	Chr	5.00 ± 0.00	100.00 ± 0.09
	BaA	5.43 ± 0.29	108.57 ± 5.88
	BaP	5.14 ± 0.02	102.83 ± 0.43
	Spiked - 10 µg/kg		
	BaA	10.69 ± 0.17	106.94 ± 1.69
	Chr	10.83 ± 0.04	108.31 ± 0.37
	BaA	10.52 ± 0.58	105.24 ± 5.78
	BaP	10.78 ± 0.18	107.78 ± 1.77
V2/V3 47.18 ± 6.50[b]	**Spiked - 5 µg/kg**		
	BaA	5.26 ± 0.04	105.10 ± 0.85
	Chr	5.09 ± 0.06	101.74 ± 1.12
	BaA	4.94 ± 0.28	98.87 ± 5.54
	BaP	5.16 ± 0.18	103.11 ± 3.53
	Spiked - 10 µg/kg		
	BaA	10.30 ± 0.18	103.02 ± 1.82
	Chr	10.34 ± 0.09	103.43 ± 0.93
	BaA	9.76 ± 0.11	97.56 ± 1.06
	BaP	9.96 ± 0.01	99.59 ± 0.10
V4/ 39.02 ± 0.08[b]	**Spiked - 5 µg/kg**		
	BaA	4.92 ± 0.60	98.34 ± 12
	Chr	4.78 ± 0.07	95.68 ± 1.48
	BaA	5.11 ± 0.13	102.17 ± 2.58
	BaP	5.03 ± 0.17	100.65 ± 3.48
	Spiked - 10 µg/kg		
	BaA	10.42 ± 0.17	104.15 ± 1.69
	Chr	10.15 ± 0.35	101.51 ± 3.47
	BaA	10.69 ± 0.15	106.93 ± 1.49
	BaP	10.13 ± 0.30	101.32 ± 3.05
V5/ 38.24 ± 0.30[b]	**Spiked - 5 µg/kg**		
	BaA	5.20 ± 0.02	104.09 ± 0.46
	Chr	5.40 ± 0.34	107.98 ± 6.73
	BaA	5.49 ± 0.09	109.73 ± 1.90
	BaP	5.09 ± 0.14	101.72 ± 2.82
	Spiked - 10 µg/kg		
	BaA	9.95 ± 0.22	99.47 ± 2.21
	Chr	9.41 ± 0.00	94.15 ± 0.01
	BaA	10.08 ± 0.06	100.77 ± 0.58
	BaP	10.39 ± 0.35	103.89 ± 3.50
V6/ 2.42 ± 0.13[c]	**Spiked - 10 µg/kg**		
	BaA	10.26 ± 0.37	102.61 ± 3.69
	Chr	8.62 ± 0.58	86.19 ± 5.80
	BaA	10.37 ± 0.63	103.73 ± 6.33
	BaP	8.06 ± 0.21	80.60 ± 2.11
V7/ 1.10 ± 0.04[c]	**Spiked - 10 µg/kg**		
	BaA	9.27 ± 0.01	92.66 ± 0.14
	Chr	10.01 ± 0.05	100.07 ± 0.50
	BaA	10.51 ± 0.37	105.09 ± 3.72
	BaP	9.75 ± 0.67	97.52 ± 6.70

Values followed by different letters are statistically different ($p < 0.05$)

Although the final extracts from V1-V7 were clean, colourless, the determined values of co-extract residues varied on average between 1.10-66.53 mg/mL, exceeding, in the case of V1-V6, the criterion imposed for co-extracts.

Comparing V6 with V5 it was observed that when extract is obtained with ACN and EMR-Lipid (V6) in the d-SPE QuECHERS purification, a reduction of co-extracts by about 93% resulted compared to V5 in which the extracts were obtained in hexane and were purified with PSA. This could be an explanation for the higher amounts of co-extract residues in V1-V5. The fats are solubilized in hexane and their existence in the extract determines the inefficiency of the purification (Sánchez-Arévalo et al., 2020).

After running the extracts from these variants to GC-MS/MS, a significant loss of sensitivity was observed after a small number of injections, as well as a build-up of matrix co-extraction compound residues in the GC liner, causing the repeated maintenance of the GC-MS, with the change of the liner and the chromatographic column.

Figure 10 shows images of the liner before (a) and after its change (b), after a number of 40-50 injections.

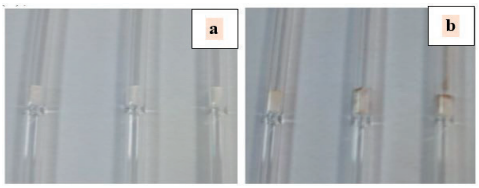

Figure 10. Liners before (a) and after (b) injection of extracts

It was also observed that when EMR-Lipid sorbent combined with extract freezing in ACN (V7) were used in d-SPE QuEChERS purification it led to a much lower co-extract residues (1.10 mg/mL) compared to the non-freezing variant V6 (2.51 mg/mL). The reduction of the co-extract amount in V7 could be explained by the fact that the fat present in ACN extracts subjected to low temperature (freezing), solidifies/ precipitates and can later be separated by centrifugation or filtration. Freezing removes most of the lipids, waxes and sugars, as well as other components with low solubility in ACN, which can adversely affect

the robustness of the GC-MS/MS analysis. Highly lipophilic compounds such as tri-, di-, mono-glycerides and free fatty acids could be removed by freezing, some lipidic compounds being able to precipitate (Payanan et al., 2013; Parrilla Vázquez et al., 2016).
Taking into account all the inconveniences that appeared in variants V1-V6 within Method II, variant V7 was selected as optimal.

Functionality of the method II/V7 on other types of oilseeds

Based on V7 conditions, 3 types of sunflower seeds (S1, S2, S5) with different fat content (Figure 2) and three other types of oilseeds, L, R, HP (Figure 3) were processed. The mean values (n=2) of co-extract residues determined from oilseeds ranged between 1.07-2.04 mg/mL (Figure 11).

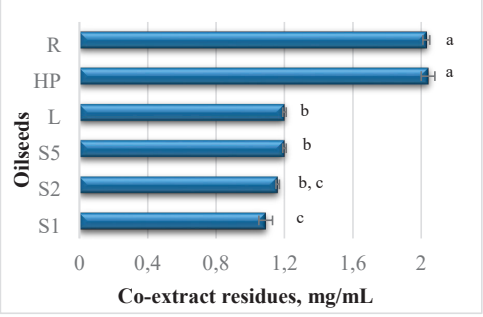

Figure 11. Co-extract residues (mean ± sd) for different oilseeds (V7/Method II). Columns followed by different letters are significantly different ($p < 0.05$)

No PAHs were identified in the analysed oilseeds. Recoveries obtained for samples spiked with 10 µg/kg PAH solution (sunflower seeds) were very good for all 4 PAHs, S1: 92.66-105.09%; S2: 93.92-105.47%; S5: 96.72-111.74%, meeting the criterion for recovery according to Reg. EU 836/2011.

CONCLUSIONS

Comparing the results, from the point of view of co-extractive residues and recoveries obtained, V15/Method I was selected as the optimal protocol for oilseeds processing for the determination of PAHs by GC-MS/MS.
The recoveries (50-120%) and co-extract residue values (≤ 2 mg/mL) determined by this variant fell within the required criteria. The final processing protocol consists of using 5 g of sample, 10 mL of water, 10 mL of hexane as extraction solvent and QuEChERS citrate salts; hexane evaporation followed by collecting the residue with 10 mL ACN and then the purification is performed by freezing the extract for 24 h and afterwards 3.5 mL of extract is purified by d-SPE QuEChERS, using the Z-Sep$^+$ sorbent. Also, this selected variant (QuEChERS extraction and purification) is easier to apply, causes minimal amounts of analytical waste, requires low amounts of chemical reagents, eliminates highly toxic reagents, and is much faster compared to V7/Method II (saponification with LL extraction).
Further research will be carried out in order to obtain better recoveries for BbF and to validate the method.

ACKNOWLEDGEMENTS

This research work was carried out with the support of Ministry of Research, Innovation and Digitalization, Core Programme PN 23 01, contract 39N/16.01.2023, project PN 23 01 03 01.

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