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# Optimization of a simple, effective, and greener methodology for polycyclic aromatic hydrocarbon extraction from human adipose tissue†

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Polycyclic aromatic hydrocarbons (PAHs) are environmentally persistent organic pollutants formed during incomplete combustion and pyrolysis processes. Humans are continuously exposed to PAHs which are linked to severe health effects such as diabetes, cancer, infertility, and poor foetal development, amongst others. PAHs are lipophilic compounds prone to accumulating in adipose tissue. Even though adipose tissue is the ideal matrix to assess over time accumulation of lipophilic pollutants, only a few analytical methods have been developed for this matrix. Aiming to reduce the existent gap, a method for the extraction of PAHs from adipose tissue samples using ultrasound-assisted extraction (UAE) was developed. The behaviour of PAHs (retention, adsorption, and volatilization) over several steps of the analytical procedure was studied. Validation tests were performed on the optimized method. PAHs were quantified using a high performance liquid chromatography (HPLC) system equipped with a photodiode array (PDA) and fluorescence (FLD) detector inline. The method achieved a low matrix effect and presents low method detection (MDL) and quantification (MQL) limits, showing suitability for a selective and sensitive determination of PAHs in adipose tissue. The extraction is performed with 0.4 g of adipose tissue and 6 mL of *n*-hexane and it does not require clean-up afterwards. Additionally, an Eco-Scale score of 74 and an Analytical GREEnness score of 0.66 were obtained. The method achieved is effective, simpler, greener, and easy to perform, being an alternative to conventional extraction methods. Furthermore, this method can be used as a multi-analyte methodology since it has been previously validated by the authors for the analysis of other lipophilic compounds. Naphthalene (Naph), acenaphthene (Ace), fluorene (Flu), phenanthrene (Phe), anthracene (Ant), fluoranthene (Fln), pyrene (Pyr) and benzo[*k*]fluoranthene (B[*k*]Ft) were found in all the tested adipose tissue samples.

## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are environmentally persistent organic pollutants[2] formed on a large scale from anthropogenic activities during incomplete combustion and pyrolysis processes.[3,4] A group of sixteen PAHs [naphthalene (Naph), acenaphthene (Ace), acenaphthylene (Acy), phenanthrene (Phe), fluorene (Flu), anthracene (Ant), fluoranthene (Fln), pyrene (Pyr), benz[*a*]anthracene (B[*a*]A), chrysene (Chry), benzo[*b*]fluoranthene (B[*b*]Ft), benzo[*k*]fluoranthene (B[*k*]Ft), benzo[*a*]pyrene (B[*a*]P), indeno[1,2,3-*cd*]pyrene (InP), dibenz[*a*,*h*]anthracene (DB[*a*,*h*]A) and benzo[*g*,*h*,*i*]perylene (B[*g*,*h*,*i*]P)] have been classified as carcinogens or possible carcinogens and priority pollutants by the International Agency for Research on Cancer and the European Union,[5] respectively. Humans are continuously exposed to PAHs, either by ingestion, inhalation, or dermal contact. As lipophilic compounds, PAHs tend to accumulate in adipose tissue. Severe health effects are associated with PAH exposure, for instance, diabetes, oxidative stress, inflammation, cancer, infertility, poor foetal development and cardiovascular diseases.[3,4] Despite adipose tissue being accepted as the ideal matrix to assess over time accumulation of lipophilic pollutants, not many analytical methods have been developed using this matrix, especially for PAHs. The main reasons may include the invasive sample collection procedure (necessity of medical intervention) and the challenges that a high fat and complex matrix can bring.[6] Most biomonitoring studies are performed with serum or urine samples.[6] Serum

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samples are an excellent representative of recent exposure. On the other hand, urine samples allow the analysis of PAH metabolites and are easily obtained.[7] However, neither is a good or accurate representative of long-term exposure. Hence, the measurement of PAHs in adipose tissue is important to fully evaluate their accumulation in the human body.

The development of an analytical method with adequate sensitivity and selectivity, which follows the principles of a green analytical methodology is not always easy, especially when this method must also be capable of retrieving small amounts of low polarity and highly lipophilic compounds using a small amount of sample. Moreover, the development of multi-compound methods that fulfil the criteria previously mentioned are not just encouraged but desirable. Extraction techniques such as Soxhlet, liquid–liquid extraction (LLE) and solid–liquid extraction (SLE) are conventional methods, which are time-consuming and require large quantities of solvents.[6] Furthermore, these are often paired with gel permeation chromatography (GPC) or solid-phase extraction (SPE) as clean-up procedures, adding to the duration and the cost of the analytical method.[6] On the other hand, ultrasound-assisted extraction (UAE) is less time-consuming, can be performed at low temperature with small amounts of sample and solvents and has low energy requirements. Furthermore, the UAE probe-type has been reported to be more efficient than ultrasound baths, as the probe is in direct contact with the solvent, which increases the contact area and the mass transference while maintaining the quality of the extract.[8]

Considering the above-mentioned, the present study intends to develop a quick and simple method with a low solvent requirement for the quantification of PAHs in adipose tissue using UAE.[1] Furthermore, the developed method should be suitable for the simultaneous extraction of other lipophilic pollutants, as the authors aim to extend their previously validated UAE methodology[1] to PAHs and also other compounds.

# 2. Experimental

## 2.1. Sampling

Samples of subcutaneous adipose tissue were collected between 2009 and 2010 at Hospital de São João (Porto, Portugal) General Surgery Department from female patients undergoing bariatric surgery. This research followed the Declaration of Helsinki, being previously approved by the hospital's ethics committee (CE 146-09) and all participants provided written informed consent. Until analysis, the samples were stored at −80 °C.

## 2.2. Chemicals and reagents

*n*-Hexane and ethyl acetate assay >99.8% of chromatographic grade were obtained from Merck (Darmstadt, Germany), acetonitrile (ACN) assay >99.8% of chromatographic grade was acquired from VWR Chemicals (Fontenay-sous-Bois, France), dichloromethane (DCM) and toluene >99% were purchased from Carlos ERBA (Val-de-Reuil, France), acetone (99.7%) was obtained from LabChem (Zelienople, Pennsylvania, USA), and isopropanol (99.9%) and methanol (>99.9%) were purchased from HoneyWell (Charlotte, North Carolina, USA). Deionized water was produced using an Elix apparatus, 15.0 MΩ cm resistivity and purified with a Simplicity 185 system, 18.2 MΩ cm resistivity (Millipore, Molsheim, France).

The target PAHs which include benzo[*j*]fluoranthene (B[*j*]Ft) at 2000 μg $mL^{-1}$, dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P) at 2000 μg $mL^{-1}$ and the certified EPA 610 mixture standards with naphthalene (Naph) at 1000 μg $mL^{-1}$, acenaphthylene (Acy) at 2000 μg $mL^{-1}$, acenaphthene (Ace) at 1000 μg $mL^{-1}$, fluorene (Flu) at 199.9 μg $mL^{-1}$, phenanthrene (Phe) at 99.8 μg $mL^{-1}$, anthracene (Ant) at 100.0 μg $mL^{-1}$, fluoranthene (Fln) at 200.1 μg $mL^{-1}$, pyrene (Pyr) at 99.9 μg $mL^{-1}$, benz[*a*]anthracene (B[*a*]A) at 100.1 μg $mL^{-1}$, chrysene (Chry) at 100.0 μg $mL^{-1}$, benzo[*b*]fluoranthene (B[*b*]Ft) at 200.2 μg $mL^{-1}$, benzo[*k*]fluoranthene (B[*k*]Ft) at 99.9 μg $mL^{-1}$, benzo[*a*]pyrene (B[*a*]P) at 100.0 μg $mL^{-1}$, dibenz[*a*,*h*]anthracene (DB[*a*,*h*]A) at 200.0 μg $mL^{-1}$, benzo[*g*,*h*,*i*]perylene (B[*g*,*h*,*i*]P) at 200.0 μg $mL^{-1}$ and indeno[1,2,3-*cd*]pyrene (InP) at 100.1 μg $mL^{-1}$ were purchased from Supelco (Bellefonte, PA, USA). Intermediate solutions were prepared in ACN by diluting stock standard solutions and stored in amber vials at −20 °C prior to usage. These solutions were used to prepare the standards for plotting the calibration curves (S) and for optimization tests. The compositions for each prepared standard S are shown in Table SM1-ESI.†

Dispersive SPE 2 mL fatty samples AOAC containing 150 mg of $MgSO_4$, 50 mg of C18 and 50 mg of primary secondary amine (PSA) and C18 endcapped bulk (C18EC) sorbent were obtained from Agilent Technologies (California, USA) and Supel QuEZ-Sep + bulk (Z-Sep) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

## 2.3. Analytical method optimization

Small details are often disregarded in the descriptions of analytical methods; however missing those small steps can considerably affect the performance of the methodology. As such, the recovery of PAHs was assessed in several steps of the extraction procedure (Fig. 1), namely sample preparation, UAE, clean-up, sample concentration, solvent exchange and filtration. The detailed composition of the PAH standard mixtures used in this paper can be found in Table SM1-ESI.†

**2.3.1. Retention of PAHs in the syringe filter.** To avoid particles damaging the LC column and system, samples are often filtered before HPLC injection[9] (Fig. 1 step VI). To verify if PAHs are retained in the filter, 200 μL of PAH standard S8 was filtered through a 0.22 μm PTFE syringe filter, transferred to an insert, placed inside an amber vial, and injected in a HPLC system. The obtained peak areas were compared with those of the same standard without being filtered.

**2.3.2. PAH redissolution.** Another important issue is the strong adsorption to the vial glass surface of the heavier PAHs.[10] Therefore, ACN, ultra-pure water, and ACN/ultra-pure water mixtures (75 : 25%; 50 : 50%, and 25 : 75%) were tested for the redissolution of PAHs after $N_2$ evaporation. Additionally, it was also tested if acidification of the organic solvent (ACN with 0.1% formic acid) would improve PAH recoveries, since sorption coefficients are influenced by pH.[11] Thus, 50 μL of PAH standard

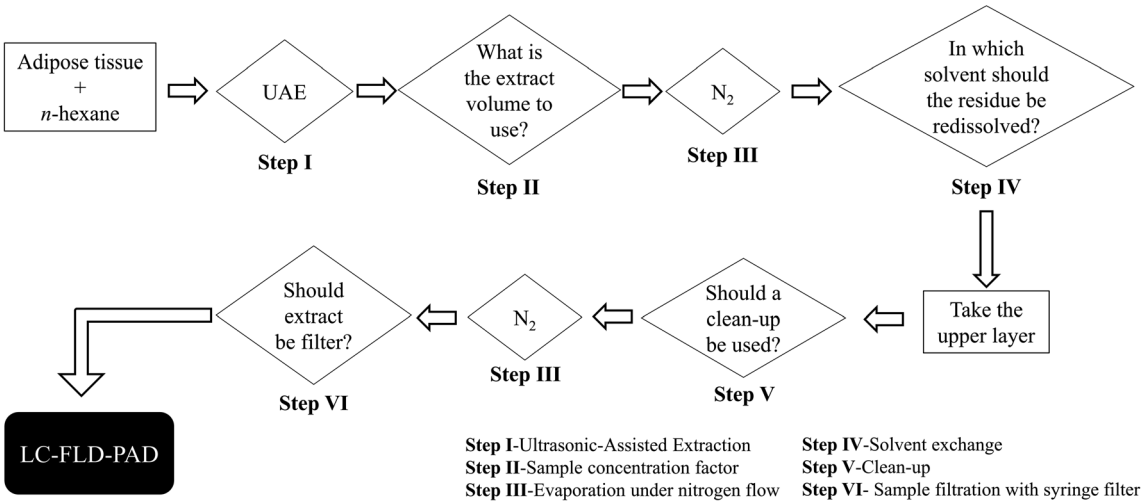


Fig. 1 Flowchart of the tested steps for PAH extraction from human adipose tissue.

S8 were dried under $N_2$ in a vial and redissolved in 50 μL of the correspondent tested solvent. After transference to an insert and placement inside an amber vial, each one underwent HPLC analysis. The obtained peak areas were compared with those of the same standard without being evaporated. As PAHs are photosensitive,[10] these tests were performed in clear and amber vials.

**2.3.3. Loss of PAHs in the evaporation.** Nitrogen is often used to concentrate an extract or change an extract solvent (Fig. 1 step III). However, analyte loss can occur during this process, particularly with volatile PAHs (Naph, methylnaphthalenes, Acy, Ace).[10] To avoid the loss of volatile compounds a keeper (high-boiling-point solvent) can be added prior to solvent evaporation.[10] Methanol and isopropanol were tested as potential keepers for PAHs, as these were reported before in other studies.[12] Moreover, one factor often neglected is the height of the glass vial used during evaporation in a stream of $N_2$ and consequently the distance from the needle to the solvent and the superficial area. Three vial sizes were tested: (a) 1.5 mL capacity (height = 3 cm); (b) 4 mL capacity (height = 4.5 cm); and (c) 8 mL capacity (height = 6 cm) (Fig. SM1-ESI†). In each vial, to 50 μL of PAH standard S8 10 μL of methanol or isopropanol was added . The vials were dried under $N_2$, redissolved in 50 μL of ACN, transferred to a 250 μL insert, placed inside a 1.5 mL amber vial, and injected into a HPLC system. The procedure was repeated for each vial without the addition of keepers. The obtained peak areas were compared with those of the same standard without being evaporated.

**2.3.4. Ultrasonic-assisted extraction.** Because adipose tissue is a fatty matrix, the extraction of small amounts of contaminants with low polarity and high lipophilicity while guaranteeing the sensitivity and selectivity of the method is not easy.[6] An UAE methodology for the quantification of lipophilic compounds, namely synthetic musks, organochlorine, and organophosphorus pesticides in adipose tissue was already validated by the authors.[1] Nonetheless, aiming to achieve an experimental procedure capable of analysing a wide range of lipophilic contaminants in a single extraction, the methodology developed previously by Sousa *et al.*[1] was followed with some modifications. Briefly, 0.4 g of adipose tissue was homogenized for 60 seconds with 6 mL of *n*-hexane in an ultrasonic processor (Sonics & Materials VCX750) at 30% amplitude. After this, the extracts were centrifuged at 2575*g* for 5 minutes and an aliquot (3.5 mL) of the extract was dried under $N_2$. An exchange of solvent was performed, as the eluents of the HPLC mobile phase are ACN and ultra-pure water. Exchanging to a more polar solvent can also provide an extract lower in fat content. Therefore, the residue was redissolved with 1 mL of ACN, centrifuged, the upper layer was separated from the fat fraction and transferred to a vial, dried under $N_2$ and finally re-dissolved in 140 μL of ACN. The extracts were filtered through a 0.22 μm PTFE syringe filter, transferred to an insert, placed inside a vial, and subjected to HPLC analysis.

After sample spiking, analyte adsorption onto the sample should be guaranteed. As such, the time between the spiking of the sample and UAE extraction impact accuracy was studied (Fig. 1 step I and Section 2.3.4.2). Furthermore, PAHs are thermosensitive;[10] hence analyte loss may occur during UAE as the probe heats up during the extraction.[8] Therefore, the impact of the probe temperature on accuracy was also tested (Section 2.3.4.1). The results were compared to those of the corresponding standard prepared in solvent or in the human adipose tissue extract (matrix without fortification – blank).

*2.3.4.1. Effect of UAE on PAH extraction.* The PAH standard was placed inside an amber vial for an analysed concentration of S6 (Table SM1-ESI†), dried under $N_2$ and subjected to UAE as previously described (Section 2.3.4).

*2.3.4.2. Spiking conditions.* To four amber flasks, 0.4 g of the adipose tissue sample was added. Then, the samples were spiked with the PAH standard (for the S3, S4, S6 or S8 analysed concentration), dried under $N_2$, and followed the methodology previously described (Section 2.3.4). This procedure was

 

repeated except that after being spiked the samples were kept overnight at −20 °C, after which the methodology previously described (Section 2.3.4) was followed.

**2.3.5. Clean-up.** Clean-up steps are usually included to remove possible interferents, as such the usage after UAE of a dispersive SPE for fatty samples with additional C18EC and Z-Sep was assessed (Fig. 1 step V). A sample of adipose tissue was prepared as described in Section 2.3.4. After UAE, an aliquot (4 mL) of extract was spiked with the PAH standard (for the S8 analysed concentration) and divided into 2 clean-up tubes each containing 50 mg PSA, 150 mg $MgSO_4$, 100 mg C18EC and 50 mg Z-Sep (2 mL of extract each) and vortexed for 2 minutes. After centrifugation at 3260*g* for 10 minutes, extract was taken from both clean-up tubes to a total of 3.5 mL, and the methodology previously described (Section 2.3.4) was followed for the remaining procedure. The results were compared to those of the corresponding standard prepared in human adipose tissue extract (matrix without fortification – blank).

**2.3.6. Sample concentration factor.** One disadvantage of working with invasive human biological samples such as adipose tissue is the amount of sample available. The collection of adipose tissue occurs under medical intervention,[13] which consequently means a low and unpredictable amount of tissue. Hence, a high sample concentration factor is desirable as it allows the usage of a low amount of sample tissue and enriches the analyte signal present at low concentrations. However, the matrix effect can significantly impact the performance of an analytical method, either by causing a loss (suppression signal) or an increase (enhancement signal) in the response.[14] Moreover, as the concentration factor increases, the matrix effect tends to get higher and the concentration of interferents also increases. The presence of interferents' peaks can compromise the quantification of analytes during the HPLC analysis. Additionally, the analysis of biological samples with high fat content becomes problematic. The extraction and isolation of analytes with low polarity and high lipophilicity with low interferents and a good sensitivity and selectivity present a challenge.[6]

Six adipose tissue extracts (matrix without fortification – blanks) were obtained following the same procedure described in Section 2.3.4 until the extract filtration step (Fig. 1 step IV). The residues were then redissolved with an appropriate amount of ACN to obtain the desirable concentration factor, being 1, 5, 10, 15, 20 or 25 times. Before HPLC analysis, the extracts were filtered as described previously. The chromatograms for all the tested concentration factors were overlayed with a chromatogram of the S8 standard PAH mixture prepared in solvent (Table SM1-ESI†).

To study the influence of fat content on the extraction procedure, three adipose tissue samples were fortified with the same amount of PAH standard and UAE was performed as described in Section 2.3.4. After this, different volumes of adipose tissue extract were taken from each extraction vial and transferred to new vials (3.5 mL-higher concentration factor, 2.0 mL-medium concentration factor, or 0.2 mL-lower concentration factor, Fig. 1-step II) and the methodology previously described was followed for the remaining procedure (Section 2.3.4). The analysed concentrations were S3, S6, and S8 for the lower, medium, and higher concentration factors, respectively (Table SM1-ESI†). The results were compared with those of the respective standard prepared in the human adipose tissue extract (matrix without fortification – blank) to determine PAH recoveries. The fat content for each assay was measured gravimetrically.[15]

**2.3.7. Solvent exchange.** For HPLC analysis the injected extract should be diluted in ACN; however during the solvent exchange (Fig. 1 step IV), from *n*-hexane (highly non-polar solvent) to ACN (highly polar solvent), it is possible that some PAHs would be kept in the bottom layer (fat residue), particularly the heavier ones. Hence, it might be necessary to use a different solvent with intermediate polarity (between that of *n*-hexane and ACN) to facilitate the transfer of PAHs from *n*-hexane to ACN. Different solvents were considered, specifically ACN, toluene, DCM, acetone, ethyl acetate[15] and mixtures of these with ACN (50 : 50%, v/v). A total of 9 solvents or solvent mixtures were tested. Sample preparation of adipose tissue and UAE extraction were performed as described previously (Section 2.3.4). After UAE, 200 μL of extract were taken for 9 separate vials and the PAH standard mixture was added for a S8 analysed concentration (Table SM1-ESI†). The fortified extracts were evaporated under a $N_2$ flow and the residues were redissolved in 200 μL of each solvent or solvent mixture (*i.e.* ACN, toluene, DCM, acetone, ethyl acetate and ACN : toluene 50 : 50% v/v, ACN : DCM 50 : 50% v/v, ACN : acetone 50 : 50% v/v or ACN : ethyl acetate 50 : 50% v/v). After vortexing and centrifugation, the resulting upper layer was separated from the fat fraction, placed in a vial and dried under $N_2$. The residue was re-dissolved in 140 μL of ACN and prepared for HPLC analysis, as described in Section 2.3.4. The results were compared with those of the respective standard prepared in human adipose tissue extract (matrix without fortification – blank). The signal suppression/signal enhancement was calculated according to Paíga *et al.*[16] The fat content for each assay was measured gravimetrically.[15]

### 2.4. Liquid chromatography analysis

The chromatographic analysis of PAHs was performed according to Ramalhosa *et al.*[17] A Shimadzu LC system (Shimadzu Corporation, Kyoto, Japan) was equipped with a degasser (DGU-20 A5R), a delivery pump (LC 20AD), an autosampler (SIL 20AHT), a system controller (CBM-20A), a photodiode array (PDA) (SPD-M20A) and fluorescence (FLD) (RF-10AXL) detectors inline. The separation and analysis of PAHs was carried out with a C18 column, CC150/4Nucleosil100-5C18PAH; 5 μm particle size and with 150 mm length and 4.0 mm of internal diameter from Macherey-Nagel (Duren, Germany). The LC system operation and data generation were performed with LabSolution software version 5.82 (Shimadzu, Kyoto, Japan). The LC oven was set to 25 °C and the injection volume was 20 μL. Ultra-pure water and ACN were eluents A and B, respectively. These eluents were filtered through a 0.22 μm nylon membrane filter 47 mm (Fioroni Filters, Ingré, France) using a Dinko D-95 vacuum pump (Barcelona, Spain) and degassed for 15 min in an ultrasonic bath (Selecta P, Barcelona, Spain). Before

chromatographic analysis, standards and sample extracts were filtered through a 0.22 μm PTFE syringe filter, 13 mm (Specanalitica, Carcavelos, Portugal).

The chromatographic program was as follows: beginning with 50% of eluent B for 5 min, a linear increase to 100% of eluent B in 15 min and holding for 16 min and lastly, the mobile phase returning to the initial composition in 2 min. The total run time was 45 min with a flow rate of 0.8 mL $min^{-1}$. Each PAH was detected at its optimum excitation/emission wavelength pair: 260/315 nm for Naph, Ace, and Flu; 260/366 nm for Phe; 260/430 nm for Ant, Fln, Pyr, B[*a*]A, Chry, B[*b*]Ft, B[*j*]Ft, B[*k*]Ft, B[*a*]P, DB[*a*,*h*]A, B[*g*,*h*,*i*]P, and DB[*a*,*l*]P; and 290/505 nm for InP.[17] On the other hand, Acy was detected at 229 nm in PDA since it has limited fluorescence. A chromatogram for PAH standard mixture S8 (Table SM1-ESI†) is shown in Fig. SM2-ESI.†

### 2.5. Method validation and greenness assessment

Method validation was performed by the measurement of several parameters such as linearity, accuracy, repeatability (intraday precision), intermediate precision (interday precision), matrix effect and expanded combined uncertainty (*Ur*,*tot*), according to Sousa *et al.*[1] Solvent and matrix-matched calibration curves were plotted using 10 PAH standards (S1 to S10 Table SM1-ESI†). Linearity was set to coefficient of determination >0.99. The method detection limit (MDL) and method quantification limit (MQL) were obtained, respectively, from 3 and 10 times the ratio of the standard deviation of the lower standard and the slope of the calibration curve. Accuracy was assessed, in triplicate, at four fortification levels (S3, S4, S6 and S8, Table SM1-ESI†). Repeatability and intermediate precision were measured, in triplicate, at four concentrations (S3, S4, S8 and S10, Table SM1-ESI†). The matrix effect was determined by comparing the same PAH standard prepared in solvent and in the human adipose tissue extract (matrix without fortification – blank). The signal suppression/signal enhancement was determined with the matrix effect according to Paíga *et al.*[16] The expanded combined uncertainty (*Ur*,*tot*) was calculated for S4 and S8 PAH standard mixtures (Table SM1-ESI†) considering a confidence level of 95% and coverage factor *k* of 2, according to Nagyová *et al.*[14]

Nowadays the demand for cleaner, simple, sustainable, and greener analytical methods is increasing. As such, the environmental impacts of these procedures were estimated according to the Eco-Scale approach as described by Gałuszka *et al.*[18] Points are assigned to the analytical procedure based on the amount and toxicity of reagents used, energy requirements, waste generated and others. The Eco-scale value is obtained by subtracting these points (or penalty points) from 100, the ideal green analysis value. A method can be classified in three ways: either as "excellent green analysis" with a score higher than 75, as "acceptable green analysis" with a score between 50 and 75 or as "inadequate green analysis" with a score below 50. Penalty points were attributed following the criteria described in Gałuszka *et al.*[18] Additionally, the greenness of the selected method was also measured with the Analytical GREEnness (AGREE) approach by Pena-Pereira *et al.*[19] (available at **https://mostwiedzy.pl/en/wojciech-wojnowski,174235-1/AGREE**), based on the 12 principles for green analytical chemistry:[19] (1) direct analytical techniques should be applied to avoid sample treatment; (2) minimal sample size and minimal number of samples are goals; (3) *in situ* measurements should be performed; (4) integration of analytical processes and operations saves energy and reduces the use of reagents; (5) automated and miniaturized methods should be selected; (6) derivatization should be avoided; (7) generation of a large volume of analytical waste should be avoided and proper management of analytical waste should be provided; (8) multi-analyte or multi-parameter methods are preferred *versus* methods using one analyte at a time; (9) the use of energy should be minimized; (10) reagents obtained from renewable sources should be preferred; (11) toxic reagents should be eliminated or replaced and (12) the safety of the operator should be increased. In this tool scores from 0 to 1 are assigned to each principle and the average score determines the greenness of the method; the higher the score the greener the method. Furthermore, a colour scale of red-yellow-green indicates how the method reflects each principle.

### 2.6. Application to human adipose tissue

To confirm the effectiveness of the method, PAHs were quantified in adipose tissue from six Portuguese women. The PAHs were expressed as ng $g^{-1}$ of adipose tissue and as ng $g^{-1}$ lipid in wet weight (ww). The values were adjusted according to PAH accuracy.

### 2.7. Statistical analysis

Student's *t*-test was performed using GraphPad Prism 6.01 software (La Jolla, CA, USA) to determine differences between conditions, which were considered significant when $p < 0.05$.

## 3. Results and discussion

### 3.1. Analytical method optimization

**3.1.1. Loss of PAHs in the syringe filter and by adsorption and volatilization.** The polymers used in the manufacture of microfilters could interact with organic chemicals and consequently retain the analyte within the filter.[20] Some report the retention of organic compounds (*e.g.* organic micropollutants) in PTFE filters.[20] In this study, no differences were observed between the filtered and non-filtered standards, meaning that the PAHs studied are not retained in the PTFE filter (Fig. SM3-ESI†). In the Dong *et al.*[20] study organic micropollutant recoveries were diminished after extract filtration. However, there water was used as solvent and, subsequently, tests showed that adding methanol (organic solvent) to the solvent reduced the analyte retention in the PTFE filter. In the present study ACN was the solvent used, which may be one reason for PAHs not being retained in the filter.

Solubility of PAHs in water decreases as their molecular weight increases.[21] The highest loss of PAHs was shown when 100% of ultra-pure water was used as the redissolving solvent (between 89.7 and 100%, Table SM2-ESI†). The loss decreased

 

as the percentage of ACN increased. Furthermore, with 100% of ACN, heavier PAHs (Fln, Pyr, B[*a*]A, Chry, B[*b*]Ft, B[*k*]Ft, B[*j*]Ft, B[*a*]P, DB[*a*,*l*]P, DB[*a*,*h*]A, B[*g*,*h*,*i*]P, and InP) showed a loss lower than 10%, while Phe and Ant, around 55% and the most volatiles PAHs presented the highest loss percentage (between 92.1% for Fln and 100% for Naph). However, the volatile PAHs (Naph, Acy, Ace, and Flu) showed poor recovery regardless of the solvent used. The acidification of ACN did not improve the results (Fig. SM4 and Table SM2-ESI†), nor did the addition of methanol as a keeper (data not shown). Some PAHs showed a slight improvement in their recovery with isopropanol as a keeper when evaporation occurred in a 1.5 mL or an 8 mL capacity vial (Fig. SM5–SM7-ESI†). However, the recoveries for the most volatile PAHs (Naph, Acy, Ace, and Flu) were still low, which might be due to the proximity of the needle to the standard in the 1.5 mL capacity vial (ranging from 0.4 to 99% of loss) or the high superficial area in the 8 mL capacity vial (ranging from −4.6 to 100% of loss), whereas, in the 4 mL capacity vial, all PAHs could be recovered with high recovery rates (ranging from −3.8 to 18% of loss). Other studies[10,12] showed better PAH recoveries after the addition of keepers before evaporation. However, these studies did not consider the vial capacity in which evaporation was performed. Our results showed that when using a 4 mL capacity vial, PAH recoveries were not altered by the addition of isopropanol. Hence, the addition of a keeper is unnecessary while using this vial size (Fig. SM5–SM7-ESI†).

**3.1.2. Spiking conditions and effect of ultrasound-assisted extraction.** The test of the probe during UAE without adipose tissue showed recoveries higher than 81% (Fig. SM8-ESI†) for all PAHs with the exception of Naph, Acy, Ace, and Flu. In the absence of adipose tissue, volatile PAHs may be more affected by the heating of the probe and volatilized.[8] Recoveries between 22 and 78% were achieved when adipose tissue was spiked and stored overnight at −20 °C before UAE, with the lowest values being obtained for heavier PAHs. Similar recoveries were obtained when spiking took place briefly before UAE (no time at −20 °C). Here accuracy values were between 22 and 91% (except for DB(*a*,*l*)P). Hence, it can be assumed that PAHs are rapidly adsorbed by the adipose tissue and is unnecessary to store the sample overnight for the spiked analytes to be incorporated into the sample. Additionally, recoveries lower than 50% were obtained for heavy PAHs in the assays performed with adipose tissue (Fig. SM8-ESI†). Because adipose tissue is a complex matrix, it may be possible that these PAHs are retained within the fat extract, since their high lipophilicity and good recoveries were achieved in the assay performed without adipose tissue. To the authors' knowledge, UAE probe-type extraction has not yet been applied to the extraction of PAHs as the ultrasound bath type has.[22–25] However, the UAE probe-type has been used for the extraction of bioactive compounds,[8,26] allowing the successful recovery of analytes without compromising the quality of the extract.

**3.1.3. Clean-up and influence of the sample concentration factor.** Naph, Acy, Fln and Pyr were the most affected by the clean-up, with recoveries below 17% (Table SM3-ESI†). The remaining PAHs showed recoveries between 53 and 103%, showing that the clean-up by itself retrieves a substantial amount of PAHs. This has been reported when the C18 sorbent is used.[27] Additionally, the use of clean-up neither reduced the number of interferents nor improved the chromatographic sensitivity. Since the method without a clean-up step allows a suitable chromatographic analysis, a clean-up step is not necessary, which also reduces sample preparation time.

As expected, a slight increase in the average recovery of PAHs from adipose tissue was observed as the amount of fat present in the extract diminishes (between 51.9 and 65.6%, Table SM4-ESI†). Nonetheless, despite the presence of non-analyte peaks with areas proportional to the concentration factor, these did not overlap the analytes peaks (Fig. SM9-ESI†). So, the higher concentration factor (25 times, with 3.5 mL of extract) can be selected, as it increases HPLC sensitivity without compromising selectivity.

**3.1.4. Influence of the solvent exchange.** The affinity of heavier PAHs to ACN may not be enough to remove them from the fat layer, since as mentioned PAHs are highly lipophilic compounds and their solubility in water declines as the molecular weight increases.[21] ACN, ethyl acetate, acetone, toluene, and DCM were evaluated regarding accuracy, fat content of the final extract, and signal suppression/enhancement (Fig. 2, SM10 and Table SM5-ESI†).

Accuracy was higher when ethyl acetate (71–104%), DCM (64–100%), toluene (75–101%), or the mixtures ACN/DCM (50 : 50%, v/v) (68–99%) and ACN/toluene (50 : 50%, v/v) (80–116%) were used (Fig. 2, average recoveries). Regarding fat content (Fig. 2), the usage of ACN (0.5 mg of fat, recovery from 45–81%) and the mixtures ACN/ethyl acetate (50 : 50%, v/v) (9.1 mg of fat, recovery from 56–83%) and ACN/acetone (50 : 50%, v/v) (2.8 mg of fat, recovery from 33–81%) resulted in extracts with a lower amount of fat (initial fat content of 26.6 mg). Furthermore, in these three assays extracts with two layers were observed: a fat layer in the bottom and a clear extract in the upper layer, which allowed the retrieval of an extract with fewer interferent peaks and fat content (upper layer). Consequently, the lowest signal suppression/enhancement effects (Table SM5) were also observed with ACN (between −6.3 and 2.2%, except for Ace and Flu) and the mixtures ACN/ethyl acetate (50 : 50%, v/v) (between −62.9 and 80.2%) and ACN/acetone (50 : 50, v/v) (between −19.5 and −4.9, except for Ace and Flu). By looking at the HPLC chromatograms, it can be found that the interferent peaks are similar with the usage of ACN, ethyl acetate, and DCM (Fig. SM10 a, b and e-ESI†) and the respective mixtures (data not shown). When acetone or toluene is used, a big interferent peak appears in HPLC chromatograms (Fig. SM10 c and d-ESI†), while for the acetone and respective mixture, the interferent peak does not interfere with the analysis of PAHs. With toluene and the respective mixture, the interferent peak influences the analysis of Naph.

Considering the results, acetone, toluene, ACN/acetone (50 : 50%, v/v), and ACN/toluene (50 : 50%, v/v) were excluded due to the existence of interfering peaks. On account of the high toxicity of DCM, all solvents that had it in their composition were also excluded. Regarding the remaining solvents, the lowest signal suppression/enhancement effects (Table SM5-ESI†) and lowest amount of fat (0.5 mg, Fig. 2) were found with

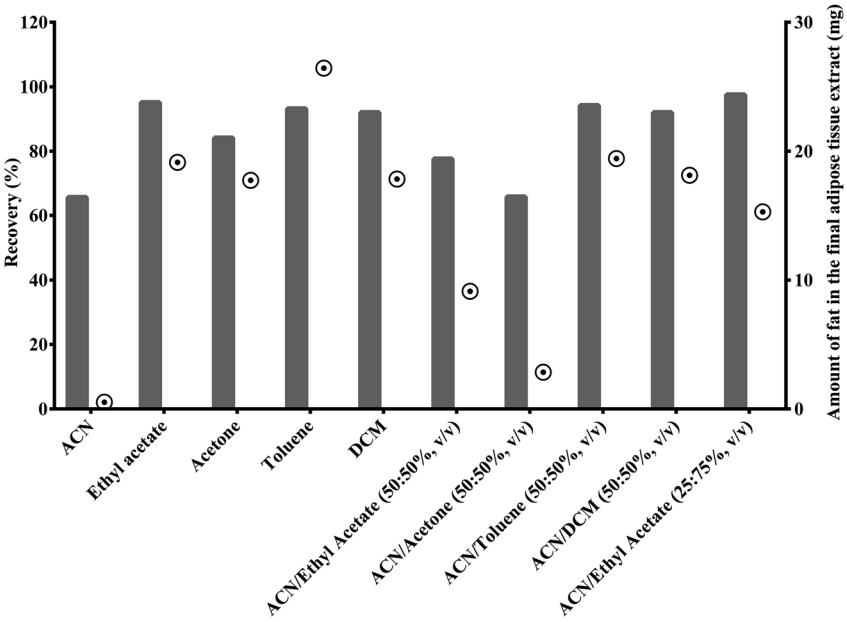


Fig. 2 Influence of different exchange solvent on PAH average recovery (%, grey bars) and amount of fat in the final adipose tissue extract (mg, black circles).

ACN. On the other hand, with ethyl acetate higher recoveries were achieved (71–104%, Fig. 2). Accordingly, the mixture ACN/ethyl acetate (50 : 50%, v/v) provided lower recoveries (56–83%) and lower amount of fat (9.1 mg) than ethyl acetate. Therefore, another proportion of the mixture ACN/ethyl acetate (25 : 75%, v/v) was tested. Accuracy increased significantly (87–102%); however the signal suppression effect and fat content (15.2 mg) increased as well. Nonetheless, two layers were still observed and no changes were observed in the HPLC chromatograms compared to the mixture ACN/ethyl acetate (50 : 50%, v/v). Hence, two solvents were selected for method validation in adipose tissue: Method 1 with ACN which allows a lower matrix effect and lower fat content and Method 2 ACN/ethyl acetate (25 : 75/, v/v) as it provides higher recoveries.

### 3.2. Method validation and greenness assessment

The two selected methods were validated for each individual analyte and validation parameters are presented in Table 1. The HPLC method repeatability and intermediate precision were confirmed, standing at 0.08 to 4% and 0.9 to 10%, respectively. The signal suppression/enhancement response obtained was between −8.1 and 7.9% for Method 1 and between −96.5 and −48.1% for Method 2. In Method 1, Naph, Acy, Flu, Phe, Ant, and Pyr presented a signal suppression signal, while all other PAHs showed a signal enhancement signal; whereas, in Method 2 all PAHs had a signal suppression signal. According to European Commission guidelines, method signal suppression or enhancement should be ideally below 20%.[28] Considering this, only Method 1 abides by this criterion. Good linearity was achieved in both methods (coefficients of determination ≥ 0.99). The MDL and MQL ranged, respectively, from 0.04 to 1 ng $g^{-1}$ and from 0.1 to 4 ng $g^{-1}$, except for Acy with a MDL of 12 ng $g^{-1}$ and MQL of 39 ng $g^{-1}$ for Method 1. As for Method 2, the MDL and MQL were, respectively, between 0.9 and 10 ng $g^{-1}$ and from 3 to 28 ng $g^{-1}$, except for Naph and Acy with a MDL of 275 and 99 ng $g^{-1}$, respectively and MQL of 918 and 330 ng $g^{-1}$, respectively. Accuracy was between 18 and 113% for Method 1 and n.d. and 116% for Method 2. The European Commission recommends that average recoveries be between 70 and 120% with a relative standard deviation of less than 20%. However, recoveries between 30 and 140% are still acceptable in some particular cases, if the criterion for consistency is respected (relative standard deviation ≤ 20%).[28] In Method 1, two PAHs (DB[*a*,*l*]P and B[*g*,*h*,*i*]P) showed average recoveries below 30%, while in Method 2, for six PAHs (Acy, B[*a*]A, Chry, DB[*a*,*l*]P, B[*g*,*h*,*i*]P and InP) it was not possible to obtain recoveries at the lowest fortification levels tested (S3 and S4). Finally, the obtained values of *Ur*,*tot* were between 3 and 23% for Method 1 and ranged from 1 to 1153% for Method 2. Since the criterion for acceptable *Ur*,*tot* by the European Network of Forensic Science Institutes is *Ur*,*tot* < 20% (for diagnostics purposes)[29] and by the European Commission is *Ur*,*tot* < 50%,[28] only Method 1 has an adequate *Ur*,*tot* for the intended purpose.

The present environmental crisis calls for scientists to take particular care in the development and application of analytical methods. Techniques low on reagent amounts, hazards, energy, and waste are encouraged and desirable.[30] The Eco-scale approach presented by Gałuszka *et al.*[18] allows a simple way to classify an analytical method. A score of 74 and 70 points was obtained respectively for Method 1 and 2, which means both methods are considered an "acceptable green analysis" (ESI-Table SM6†).

Despite better recoveries, Method 2 presents a much higher MDL and MQL than Method 1, as such not allowing the detection of small amounts of PAHs, which is related to the high

 

Table 1 Method validation parameters for PAHs in human adipose tissue spiked at different levels for the selected methods[a]

| | Signal suppression/ signal enhancement (%) | | Coefficient of determination | | MDL (ng g$^{-1}$) | | MQL (ng g$^{-1}$) | | *Ur*,*tot* (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | S4 | S8 | S4 | S8 |
| PAHs | Method 1 | Method 2 | Method 1 | Method 2 | Method 1 | Method 2 | Method 1 | Method 2 | Method 1 | | Method 2 | |
| Naph | −8.1 | −48.1 | 0.99993 | 0.99957 | 1 | 275 | 4 | 918 | 11 | 3 | 243 | 3 |
| Acy | −2.2 | −87.6 | 0.99939 | 0.99603 | 12 | 99 | 39 | 330 | 11 | 6 | 36 | 1 |
| Ace | 1.4 | −84.8 | 0.99998 | 0.99458 | 0.6 | 9 | 2 | 28 | 9 | 5 | 41 | 4 |
| Flu | −1.9 | −80.0 | 0.99995 | 0.99889 | 0.2 | 2 | 0.6 | 8 | 23 | 6 | 29 | 5 |
| Phe | −0.7 | −62.2 | 0.99985 | 0.99753 | 0.2 | 7 | 0.6 | 23 | 11 | 8 | 588 | 59 |
| Ant | −0.1 | −83.6 | 0.99999 | 0.99875 | 0.04 | 0.9 | 0.1 | 3 | 8 | 5 | 17 | 5 |
| Fln | 1.8 | −88.0 | 0.99996 | 0.99823 | 0.2 | 3 | 0.6 | 10 | 19 | 4 | 57 | 8 |
| Pyr | −0.9 | −90.1 | 0.99970 | 0.99800 | 0.2 | 4 | 0.8 | 12 | 3 | 6 | 350 | 149 |
| B[*a*]A | 0.9 | −89.4 | 0.99996 | 0.99732 | 0.08 | 1 | 0.3 | 4 | 7 | 5 | 54 | 9 |
| Chry | 7.9 | −89.3 | 0.99969 | 0.99940 | 0.2 | 5 | 0.7 | 16 | 9 | 7 | 1153 | 39 |
| B[*b*]Ft + B[*j*]Ft | 3.0 | −92.8 | 0.99995 | 0.99962 | 0.4 | 2 | 1 | 5 | 10 | 7 | 31 | 3 |
| B[*k*]Ft | 2.0 | −91.7 | 0.99998 | 0.99941 | 0.06 | 2 | 0.2 | 5 | 9 | 4 | 34 | 10 |
| B[*a*]P | 7.6 | −93.5 | 0.99998 | 0.99941 | 0.06 | 1 | 0.2 | 4 | 12 | 3 | 16 | 7 |
| DB[*a*,*l*]P | 6.6 | −96.5 | 0.99992 | 0.99929 | 0.2 | 3 | 0.8 | 9 | 9 | 7 | 19 | 2 |
| DB[*a*,*h*]A | 6.3 | −90.8 | 0.99977 | 0.99955 | 0.4 | 10 | 1 | 32 | 9 | 4 | 425 | 14 |
| B[*g*,*h*,*i*]P | 3.0 | −96.2 | 0.99987 | 0.99963 | 0.3 | 3 | 1 | 11 | 8 | 5 | 136 | 17 |
| InP | 1.7 | −94.2 | 0.99981 | 0.99892 | 0.2 | 1 | 0.7 | 4 | 10 | 4 | 118 | 36 |

| | Accuracy (%) | | | | | | | | Repeatability (%) | | | | Intermediate precision (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Method 1 | | | | Method 2 | | | | | | | | | | | |
| PAHs | S3 | S4 | S6 | S8 | S3 | S4 | S6 | S8 | S3 | S4 | S8 | S10 | S3 | S4 | S8 | S10 |
| Naph | 113 | 89 | 80 | 56 | 94 | 88 | 89 | 81 | 2 | 2 | 0.4 | 0.4 | 3 | 6 | 1 | 1 |
| Acy | 46 | 58 | 68 | 75 | n.d. | n.d. | 73 | 93 | 0.6 | 0.8 | 0.3 | 0.2 | 2 | 2 | 2 | 0.9 |
| Ace | 66 | 62 | 60 | 54 | 116 | 92 | 64 | 113 | 2 | 2 | 0.8 | 0.8 | 4 | 4 | 2 | 2 |
| Flu | 79 | 75 | 69 | 62 | 93 | 84 | 64 | 110 | 3 | 1 | 0.3 | 0.2 | 4 | 3 | 2 | 1 |
| Phe | 78 | 83 | 80 | 71 | 104 | 95 | 80 | 96 | 1 | 0.8 | 0.9 | 0.7 | 10 | 4 | 2 | 1 |
| Ant | 64 | 60 | 57 | 55 | 61 | 71 | 59 | 67 | 3 | 2 | 0.7 | 0.8 | 3 | 4 | 2 | 1 |
| Fln | 74 | 60 | 57 | 54 | 59 | 73 | 74 | 75 | 3 | 2 | 0.8 | 0.7 | 4 | 3 | 2 | 2 |
| Pyr | 67 | 63 | 55 | 50 | 74 | 69 | 69 | 71 | 4 | 2 | 1 | 0.6 | 6 | 4 | 2 | 1 |
| B[*a*]A | 55 | 47 | 49 | 48 | n.d. | 88 | 101 | 100 | 4 | 2 | 0.7 | 0.4 | 4 | 3 | 2 | 2 |
| Chry | 55 | 49 | 50 | 47 | n.d. | n.d. | 77 | 84 | 3 | 3 | 0.8 | 0.6 | 9 | 5 | 3 | 1 |
| B[*b*]Ft + B[*j*]Ft | 39 | 39 | 40 | 39 | 58 | 85 | 99 | 97 | 2 | 2 | 0.3 | 0.2 | 3 | 3 | 2 | 1 |
| B[*k*]Ft | 43 | 42 | 41 | 39 | 98 | 100 | 86 | 102 | 2 | 2 | 0.3 | 0.3 | 3 | 3 | 2 | 1 |
| B[*a*]P | 37 | 35 | 34 | 33 | 83 | 102 | 86 | 96 | 4 | 1 | 0.6 | 0.2 | 10 | 8 | 7 | 5 |
| DB[*a*,*l*]P | 24 | 21 | 23 | 22 | n.d. | n.d. | 107 | 106 | 1 | 2 | 0.9 | 0.08 | 8 | 6 | 5 | 4 |
| DB[*a*,*h*]A | 46 | 41 | 38 | 36 | 95 | 96 | 95 | 125 | 4 | 3 | 1 | 0.8 | 7 | 3 | 3 | 3 |
| B[*g*,*h*,*i*]P | 18 | 22 | 24 | 24 | n.d. | n.d. | 96 | 103 | 3 | 4 | 0.8 | 0.3 | 6 | 4 | 2 | 2 |
| InP | 34 | 29 | 31 | 27 | n.d. | n.d. | 92 | 109 | 3 | 4 | 1 | 0.5 | 4 | 6 | 2 | 1 |

[a] Method 1-ACN and Method 2-ACN/ethyl acetate (25 : 75) as exchange solvents. Acenaphthene (Ace); acenaphthylene (Acy); anthracene (Ant); benz[*a*]anthracene (B[*a*]A); benzo[*a*]pyren e(B[*a*]P); benzo[*b*]fluoranthene (B[*b*]Ft); benzo[*g*,*h*,*i*]perylene (B[*g*,*h*,*i*]P); benzo[*j*]fluoranthene (B[*j*]Ft); benzo[*k*]fluoranthene (B[*k*]Ft); chrysene (Chry); dibenz[*a*,*h*]anthracene (DB[*a*,*h*]A); dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P); expanded combined uncertainty (*Ur*,*tot*); fluoranthene (Fln); fluorene (Flu); indeno[1,2,3-*cd*]pyrene (InP); method detection limit (MDL); method quantification limit (MQL); naphthalene (Naph); n.d.-not detected; phenanthrene (Phe); polycyclic aromatic hydrocarbon (PAH); pyrene (Pyr); S – standard. Note: the composition for each standard S is shown in Table SM1 – ESI.

matrix effect observed with the use of ethyl acetate in Method 2. Even though Method 1 presents lower recoveries, it assures the detection and quantification of lower amounts of PAHs. Furthermore, this method fulfils the established criteria for acceptable signal suppression/enhancement and *Ur*,*tot* and it can still be accepted regarding accuracy. Hence, considering the above-mentioned Method 1 (Fig. 3) should be the method chosen for PAH analysis in adipose tissue, as it allows the detection of low amounts of PAHs and a cleaner extract despite its lower recoveries.

The authors are aware that the usage of *n*-hexane seems contradictory in a green methodology approach. However, as mentioned before, the authors previously presented an UAE methodology for the quantification of other lipophilic

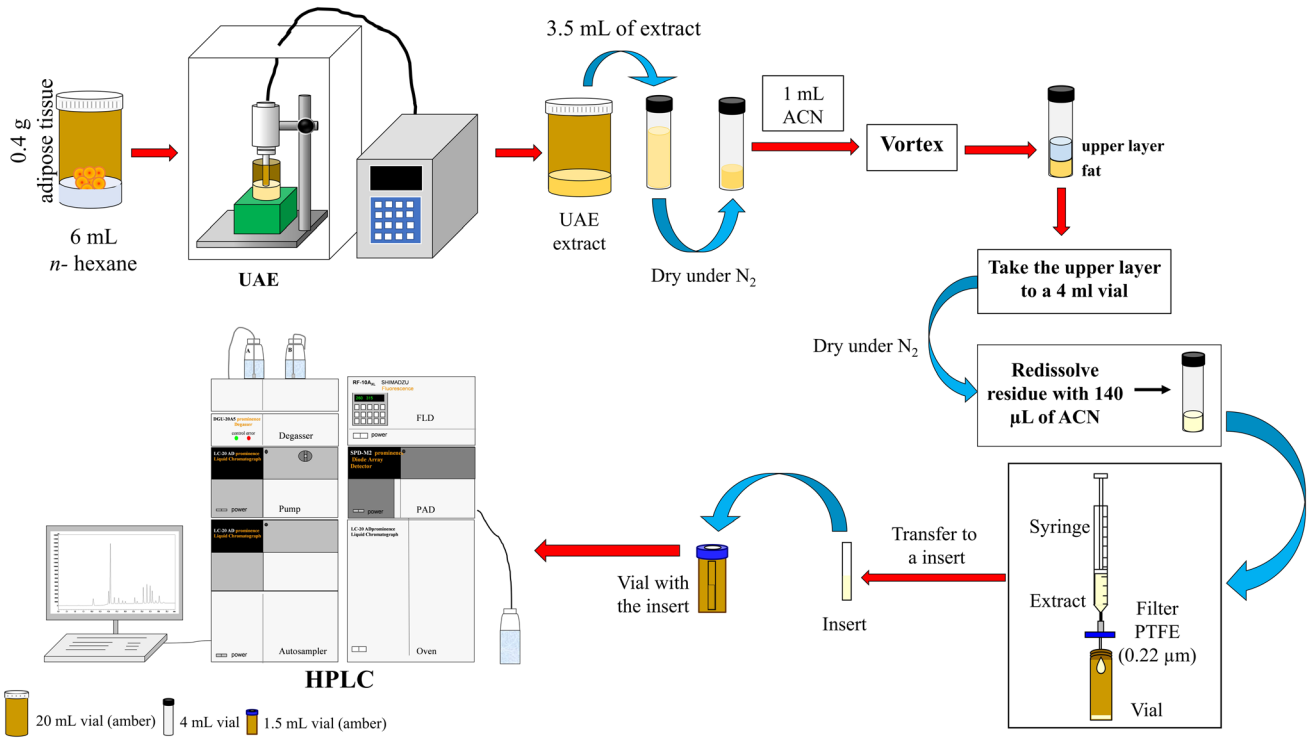


Fig. 3 Flow diagram of the developed method for PAH extraction from human adipose tissue (Method 1).

compounds, namely synthetic musks, organochlorine, and organophosphorus pesticides in adipose tissue,[1] which are best retrieved using *n*-hexane. Now the authors have developed a method for PAH analysis that can be combined with the extraction previously developed. So despite using *n*-hexane, the amount is relatively low (6 mL) and the extraction is a multi-analyte methodology (4 groups of pollutants). Nevertheless, to assure the greenness of the selected method the AGREE tool was also used (Fig. 4). The lowest scores of the method were at the principles 3 and 10 (in red), since the HPLC instrument is not field-portable and the reagents used are not renewable or easily degradable. Principles 1 (in orange) and 9 (in yellow) were scored low, as sample preparation before analysis, even if reduced, is still required and the detection method (HPLC) requires a high energy consumption. The remaining principles were scored higher than 0.75 and are coloured green (Fig. 4). The method needs a low amount of sample (principle 2), sample preparation is not time-consuming nor with many steps and is semi-automatic (principles 4 and 5), there is no derivatization step (principle 6), the amount of waste generated is low (principle 7), several analytes are assessed at the same time (principle 8) and even though toxic solvents are used the amount is low (principles 11 and 12).

Considering the scores obtained in Eco-scale (74) and AGREE (0.66), Method 1 can be considered green.

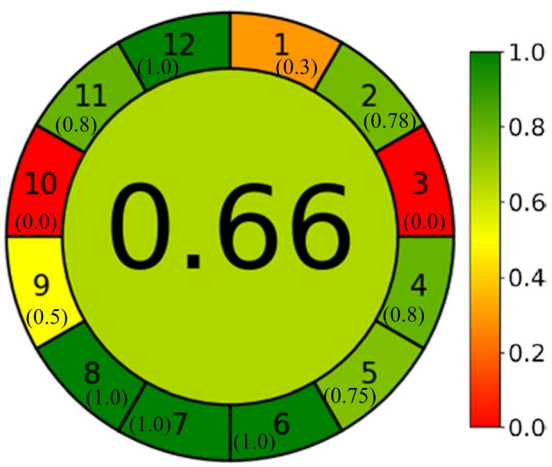


Fig. 4 Results of AGREE analysis for Method 1 (left) and the colour scale for reference (right). The score of each principle is given in parentheses.

### 3.3. Application to human adipose tissue

Method 1 was validated in samples of human adipose tissue of six volunteers. Samples were analysed in duplicate and extracted as described in Section 2.3.4 and Fig. 3.

Eight PAHs were found in all the samples tested, namely Naph, Ace, Flu, Phe, Ant, Fln, Pyr and B[*k*]Ft (Table 2). Moreover, B[*b*]Ft + B[*j*]Ft were detected in two samples. Concentrations of individual PAHs ranged from <0.2 to 31.8 ng $g^{-1}$ of adipose tissue (<0.2 to 43 ng $g^{-1}$ of lipid) and the sum of PAHs from 31.7 to 47.3 ng $g^{-1}$ of adipose tissue (33.7 to 63.9 ng $g^{-1}$ of lipid).

### 3.4. Comparison with other analytical methods for detection in adipose tissue

Eight studies were found for the determination of PAHs in adipose tissue (Table 3). Soxhlet extraction is the most

 

Table 2 Levels of PAHs in human adipose tissue samples (ng $g^{-1}$ of adipose tissue ww and ng $g^{-1}$ lipid ww)[a]

| | Concentration (ng $g^{-1}$ of adipose tissue ww/ng $g^{-1}$ of lipid ww) | | | | | |
|---|---|---|---|---|---|---|
| PAHs | Samples 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
| Naph | 25.8/32.3 | 19.1/20.3 | 31.8/43.0 | 20.6/21.5 | 26.3/35.5 | 18.7/21.5 |
| Acy | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Ace | 3.7/4.6 | 4.4/4.7 | 3.7/5.0 | 4.8/5.0 | 5.5/7.4 | 5.7/6.6 |
| Flu | 2.4/3.0 | 1.9/2.0 | 2.8/3.8 | 2.1/2.2 | 2.6/3.5 | 1.9/2.2 |
| Phe | 4.9/6.1 | 4.0/4.3 | 6.0/8.1 | 4.3/4.5 | 5.2/7.0 | 3.9/4.5 |
| Ant | 0.3/0.4 | 0.3/0.3 | 0.4/0.5 | 0.4/0.4 | 0.3/0.4 | 0.2/0.2 |
| Fln | 1.3/1.6 | 1.6/1.7 | 1.8/2.4 | 1.7/1.8 | 0.8/1.1 | 0.5/0.6 |
| Pyr | <MQL | <MQL | <MQL | <MQL | <MQL | <MDL |
| B[*a*]A | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Chry | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| B[*b*]Ft + B[*j*]Ft | n.d. | n.d. | n.d. | n.d. | <MQL | <MQL |
| B[*k*]Ft | 0.5/0.6 | <MQL | <MQL | <MQL | <MQL | <MQL |
| B[*a*]P | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| DB[*a*,*l*]P | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| DB[*a*,*h*]A | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| B[*g*,*h*,*i*]P | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| InP | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| ∑PAHs | 39.3/49.1 | 31.7/33.7 | 47.3/63.9 | 34.0/35.4 | 41.6/56.2 | 31.8/36.6 |

[a] Acenaphthene (Ace); acenaphthylene (Acy); anthracene (Ant); benz[*a*]anthracene (B[*a*]A); benzo[*a*]pyrene (B[*a*]P); benzo[*b*]fluoranthene (B[*b*]Ft); benzo[*g*,*h*,*i*]perylene (B[*g*,*h*,*i*]P); benzo[*j*]fluoranthene (B[*j*]Ft); benzo[*k*]fluoranthene (B[*k*]Ft); chrysene (Chry); dibenz[*a*,*h*]anthracene (DB[*a*,*h*]A); dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P); expanded combined uncertainty (*Ur*,*tot*); fluoranthene (Fln); fluorene (Flu); indeno[1,2,3-*cd*]pyrene (InP); naphthalene (Naph); n.d.-not detected; phenanthrene (Phe); polycyclic aromatic hydrocarbon (PAH); pyrene (Pyr); ww-wet weight.

frequently used. Salting-out LLE, SLE, homogenisation, and saponification were also reported. Regarding clean-up methods, GPC or SPE are the methods that are commonly used. Extraction techniques such as Soxhlet, LLE, and SLE often involve large quantities of solvents and are additionally time-consuming protocols. GPC requires specific instrumentation and is rather time-consuming, whereas SPE cartridges which are for single-use may lead to a high cost per sample. UAE extraction does not need high amounts of solvent nor is it time-consuming. Additionally, in this work the UAE probe-type was used, which allows for a more effective transference of the analytes to the solvent. Moreover, Soxhlet and GPC are techniques with high energy consumption whereas UAE is not.

Despite the method present in this study being the one showing the lowest recoveries (from the studies that report accuracy), it is also one of those with the lowest MDL and MQL. By looking at the Eco-Scale scores, it can be inferred that the usage of large volumes of hazard solvent highly impacts the score obtained (Table SM6-ESI†).

Table 3 Comparison of the presented method with other analytical methods for PAH detection in adipose tissue

| Sample quantity (g) | PAH analysed (n) | PAH detected (n) | Extraction | Final volume extract (μL) | Detection | Recovery (%) | MDL/MQL (ng $g^{-1}$) | Eco-Scale[a] | Reference |
|---|---|---|---|---|---|---|---|---|---|
| 0.4 | 18 | 8 | UAE | 140 | LC-FLD-PDA | 22–85 | 0.04–1/0.1–4[a] | 74 | Present study (method 1) |
| 0.3 | 13 | 11 | Salting-out LLE + DLLME | 35 | GC-MS | 89–110 | 0.02–0.1/ 0.07–0.4 | 73 | 31 |
| 1–2 | 16 | 8 | Soxhlet + GPC + SPE | 1000 | GC-MS | ns | ns/0.8–2 | 51 | 32 |
| 1–2 | 16 | 16 | Soxhlet + GPC + SPE | 1000 | GC-MS | 68–118 | ns/0.8–2 | 51 | 33 |
| 5 | 15 | ns | Homogeniser + GPC | 1000 | GC–MS/MS | 120–130 | 0.1–7/0.2–13 | 56 | 34 |
| 10 | 9 | 9 | Soxhlet + ns | 1000 | GC-MS | 83–88 | ns | 43 | 35 and 36 |
| 0.1–0.5 | 16 | 4 | SLE + SPE | 500 | GC-TOF MS | ns | ns | 67 | 37 |
| 20 | 9 | 4 | Homogeniser + GPC | 200–600 | GC-MS | ns | 10–14/ns | 6 | 38 and 39 |
| 40–90 | 9 | 6 | Saponification + SPE | 2000 | LC-FLD | ns | ns | 28 | 40 |

[a] Value calculated according to Gałuszka *et al.*;[18,30] (a) except for Acy 12/39 ng $g^{-1}$; dispersive liquid–liquid microextraction (DLLME); fluorescence detector (FLD); gel permeation chromatography (GPC); liquid chromatography (LC); liquid–liquid extraction (LLE); mass spectrometry (MS); method detection limit (MDL); method quantification limit (MQL); not specified (ns); photodiode array detector (PDA); polycyclic aromatic hydrocarbon (PAH); solid–liquid extraction (SLE); solid-phase extraction (SPE); tandem mass spectrometry (MS/MS); time-of-flight (TOF); ultrasound-assisted extraction (UAE).

The studies performed by Lordo *et al.*,[38] Obana *et al.*[40] and Quin *et al.*[35] can be classified as inadequate green analysis, and although studies by Moon *et al.*,[33] Kim *et al.*,[32] and Wang *et al.*[34] achieved the acceptable green analysis status their scores are very near the border of inadequate green analysis. On the other hand, the study performed by Pastor-Belda *et al.*[31] as the one presented in this paper (Method 1) can be classified as acceptable green analysis but it is important to notice that their scores are very close to the excellent green analysis status limit score. Moreover, methodologies from Quin *et al.*,[35] Lordo *et al.*,[38] and Obana *et al.*[40] require a large amount of adipose tissue, *i.e.* 10, 20 and 40–90 g, respectively. With patient care and comfort being top priorities, adipose tissue collection should only occur when there is assurance of no compromise in the anatomopathological evaluation or significant aesthetic impact. A large sample quantity requirement, as the ones mentioned above, may frequently not abide by the conditions outlined for sample collection for most patients, consequently, reducing the number of patients and volunteers included in the studies and the significance of the biomonitoring conclusions.

The method presented in this paper is compliant with the principles of green analytical chemistry numbers: 2, 4, 5, 6, 7, 8, 11 and 12. It requires a small amount of adipose tissue and solvents, not being a time-consuming procedure or with high energy requirements like conventional methods. Additionally, this method can be applicable to other lipophilic analytes.

## 4. Conclusions

Analytical methods for PAH determination in adipose tissue are barely existent, and it is well-known that these pollutants accumulate in adipose tissue and are associated with severe health issues. To help fill the existent gap, an UAE methodology was adapted, tested, and validated for the extraction and determination of PAHs in human adipose tissue. Different conditions and solvents were tested to improve the method performance. Because adipose tissue is a problematic matrix, better recoveries do not translate into a more selective and sensitive method. A high extract fat content and the consequent amount of interferents severely affect the chromatographic analysis, which leads to a high MDL and MQL (lowering sensitivity) and high matrix effect (compromising selectivity) despite adequate accuracy (Method 2). Hence, from the conditions tested the one that allowed a lower MDL, MQL, and signal suppression/enhancement effect was selected (Method 1, Fig. 3). Despite being the one with lower accuracy, this method allows PAHs to be determined in lower amounts and a chromatographic analysis with fewer interferents. The method was validated in human adipose tissue samples and eight PAHs (Naph, Ace, Flu, Phe, Ant, Fln, Pyr, and B[*k*]Ft) were found in all the samples tested.

A faster, simple, reliable, and efficient methodology was achieved (Fig. 3) and classified as a greener alternative to the conventional time and high solvent and energy consuming analytical methods. The present method has been previously validated by the authors for the analysis of other lipophilic compounds. Now it has also been proven suitable for a selective and sensitive determination of PAHs in adipose tissue samples, adding to the need for multi-analyte methodologies. In future work the authors aim to widen this methodology to even more lipophilic compounds.

## Abbreviations

| | |
|---|---|
| Ace | Acenaphthene |
| Acy | Acenaphthylene |
| ACN | Acetonitrile |
| AGREE | Analytical GREEnness |
| Ant | Anthracene |
| B[*a*]A | Benz[*a*]anthracene |
| B[*a*]P | Benzo[*a*]pyrene |
| B[*b*]Ft | Benzo[*b*]fluoranthene |
| B[*g*,*h*,*i*]P | Benzo[*g*,*h*,*i*]perylene |
| B[*j*]Ft | Benzo[*j*]fluoranthene |
| B[*k*]ft | Benzo[*k*]fluoranthene |
| C18EC | C18 endcapped bulk |
| Chry | Chrysene |
| DB[*a*,*h*]A | Dibenz[*a*,*h*]anthracene |
| DB[*a*,*l*]P | Dibenzo[*a*,*l*]pyrene |
| DCM | Dichloromethane |
| EPA | Environmental Protection Agency |
| *Ur*,*Tot* | Expanded combined uncertainty |
| Fln | Fluoranthene |
| Flu | Fluorene |
| FLD | Fluorescence detector |
| GPC | Gel permeation chromatography |
| Inp | Indeno[1,2,3-*cd*]Pyrene |
| LC | Liquid chromatography |
| LLE | Liquid–liquid extraction |
| MDL | Method detection limit |
| MQL | Method quantification limit |
| Naph | Naphthalene |
| Phe | Phenanthrene |
| PAH | Polycyclic aromatic hydrocarbon |
| PTFE | Polytetrafluoroethylene |
| PAD | Photodiode array detector |
| PSA | Primary secondary amine |
| Pyr | Pyrene |
| SLE | Solid–liquid extraction |
| SPE | Solid-phase extraction |
| Z-Sep | SupelQueZ-Sep + bulk |
| UAE | Ultrasound-assisted extraction |
| ww | Wet weight |

## Author contributions

Sara Sousa: conceptualization, methodology, validation, investigation, visualization, writing-original draft. Paula Paíga: conceptualization, formal analysis, writing-review & editing. Diogo Pestana: writing-review & editing. Gil Faria: resources. Conceição Calhau: writing-review & editing. Cristina Delerue-Matos: resources, writing-review & editing. Maria João Ramalhosa: conceptualization, validation, supervision, writing-

 

review & editing. Valentina Fernandes Domingues: conceptualization, validation, supervision, writing-review & editing.

## Conflicts of interest

There are no conflicts to declare.

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