



Optimization of a miniaturized microwave-assisted saponification and extraction method to evaluate mosh and MOAH in biological samples

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ABSTRACT

Mineral oil hydrocarbons (MOH) are a group of lipophilic contaminants composed of thousands of different compounds. The human toxicokinetics (absorption, metabolism, distribution and elimination) of these contaminants depends on their molecular structure but information are still lacking. The primary gap to be filled is the optimization and validation of a method to treat a very small amount of samples, as the ones produced during *in-vitro* studies.

The aim of this study is to develop and validate a miniaturized microwave-assisted saponification and extraction (MASE) method for the evaluation of MOH subclasses in low amounts of biological samples (<400 mg). The method was validated using an LC/GC×GC-FID/MS system in mono- and two-dimensional modes. The results demonstrated excellent recovery ($\geq 94\%$), repeatability, and compliance with JRC (Joint Research Center) guidance criteria for internal standard ratios. Furthermore, the method achieved a limit of quantification as low as 0.4 μg of MOH in the sample, outperforming conventional extraction methods.

1. Introduction

Mineral oil hydrocarbons (MOH) are environmental and processing contaminants of petrogenic origin. They are complex mixtures of lipophilic compounds containing between 10 and 50 carbon atoms [1]. MOH are classified into two main categories, named mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH). The toxicokinetic properties of these compounds—including intestinal absorption, distribution, metabolism, and excretion—can vary significantly across the thousands of substances within each class, underscoring the need to differentiate between subclasses of MOSH and MOAH [2–4]. For a proper evaluation of these differences in MOH toxicokinetics, the absence of an animal model that accurately represents human toxicokinetics [2,4–8], combined with ethical concerns related to animal studies, highlights the need for *in vitro* tests using human cells. However, only *in vitro* tests with single compounds that can

be present in the MOAH fraction have been performed. For instance, the metabolism of some alkylated benzo[a]pyrenes and naphthalenes has been evaluated using a UHPLC-PDA to follow the metabolites formation [9,10]. The evaluation of MOH toxicokinetics using entire mixtures with *in vitro* tests is hindered by the sensitivity of the analytical methods currently available in the literature to determine MOH in biological samples.

In most of the studies conducted to assess MOH levels in human and animal tissues, an extraction of MOH was performed using different proportions of hexane:ethanol mixtures [5,11–14]. However, a major limitation of these methods is the co-extraction of triglycerides (TAGs) from the original sample, which restricts the volume that can be injected into the analytical instrument, ultimately increasing limits of quantification (LOQ) [15,16]. Therefore, an enrichment step performing saponification is highly advisable as lipid content increases. The TAG content varies among tissues and across species. But generally, the lipid

Abbreviations: Cho, cholestane; Cycy, cyclohexyl-cyclohexane; EtOH, ethanol; IS, internal standard; JRC, joint research center; LOQ, limit of quantification; LC/GC×GC-FID/MS, liquid chromatography/gas chromatography-flame ionization detector and liquid chromatography/comprehensive multidimensional gas chromatography-flame ionization detector/mass spectrometry; LC-GC-FID, liquid-gas chromatography-flame ionization detection; mCPBA, m-chloroperbenzoic acid; MeOH, methanol; MN, methyl naphthalene; MASE, microwave-assisted saponification and extraction; MOH, Mineral oil hydrocarbons; MOAH, mineral oil aromatic hydrocarbons; MOSH, Mineral oil saturated hydrocarbons; 5B, pentylbenzene; Per, perylene; RSD, relative standard deviation; TAG, triacylglycerol; TBB, tri-tert butyl benzene.

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content of the spleen and liver ranges between 1 and 5.5 %, in the brain it can reach up to 12 %, and in adipose tissue it may be as high as 92 % [16–19]. Thus, the most challenging scenario to ensure optimized saponification conditions is apply the method to adipose tissue.

To achieve adequate sensitivity, previously applied analytical methods typically require large amounts of biological sample (1–5 g) [5, 11–14,20], which may not always be feasible. For example, when studying the retention and metabolization of compounds using *in vitro* tests, only 10–200 mg of sample is often obtained. In such cases, strategies to decrease the LOQ of the method should be implemented. In this regard, saponification of the co-extracted TAGs allows for a decrease in the LOQ by a factor of ~5 [21–24]. However, it has also been noted that saponification can significantly impact the distribution of internal standards (ISs), which are critical for monitoring performance across the analytical steps and for accurate quantification of MOSH and MOAH [23,25], thus requiring a proper verification of the saponification conditions.

The aim of this study was to develop and validate a miniaturized microwave-assisted saponification and extraction (MASE) method for analysing MOSH and MOAH in small quantities of adipose tissue. The method was designed to be compatible with the small sample weights typically obtained *in vitro* studies, while providing sufficient sensitivity for accurate MOSH and MOAH quantification.

2. Material and methods

2.1. Reagents and standard solutions

Acetone and *n*-hexane HPLC grades were purchased from Biosolve Chemicals (Dieuze, France). Hexane was distilled before use. Sodium sulfate, bis(2-ethylhexyl) sebacate and dichloromethane LiChrosolv® were provided by Merck-MilliporeSigma (Darmstadt, Germany). Ethanol 99.8 % (EtOH) for HPLC was from Thermo Scientific (Waltham, MI, USA) and milli-Q water was obtained with a Millipore system (Bedford, MA, USA). All solvents were tested for purity before use.

The ISs solution (#31070) contained *n*-C13 at 0.15 mg/mL; TBB, *n*-C11, CyCy (cyclohexyl-cyclohexane), 5B (pentylbenzene), 1-MN, and 2-MN (methyl naphthalenes) at 0.30 mg/mL; and Cho (cholestane) and Per (perylene) at 0.60 mg/mL, all dissolved in toluene. To verify the performance of the GC system, the MOSH/MOAH retention time standard mixture (#31076) consisting of the following alkanes: C10, C11, C13, C16, C20, C24, C25, C35, C40, and C50 at 100 mg/L each in cyclohexane was employed. Both standard solutions were generously provided by Restek (Neukirchen-Vluyn, Germany) and stored at –18 °C. A motor oil (Sternel) was used for the recovery test and sensitivity evaluation. This motor oil is characterized by a MOH profile with comparable levels of MOSH and MOAH (44 ± 1 % and 56 ± 1 %, respectively) and with a hydrocarbon distribution ranging from *n*-C₂₀ to *n*-C₃₆ and a peak centered around *n*-C₂₆.

2.2. Sample

To develop the miniaturized MASE method for biological samples, adipose tissue was selected as the biological matrix due to its high TAG content. A homogeneous batch of white adipose tissue was prepared to ensure consistency across all analytical replicates used in the various tests. This step involved grinding chicken skin and removing the connective tissue, which was retained in a colander after melting the sample in an oven at 65 °C for 30 min. The melted adipose tissue was then transferred to glass vials with Teflon-lined caps, previously washed with acetone and hexane, and stored at –18 °C until analysis.

2.3. Miniaturized microwave-assisted saponification and extraction method

The conditions tested were adapted from the method recently

published in [23,25]. The adipose tissue was weighed inside a microwave glass tube (15 mL of capacity), 2.5 mL of *n*-hexane, 5 µL of the ISs solution, and 2.5 mL of a 2 M KOH solution in EtOH:H₂O (1:1; v:v) were added. The tubes were placed into the microwave rotor of 15 positions and the saponification was performed using a SynthWAVE (Milestone Srl, Bergamo, Italy) at 120 °C and 35–40 bar during 20 min, with 5 min of pre-heating. When the tubes reached the ambient temperature, 5 mL of water and some drops of EtOH were added along the walls and were kept in the fridge for 30 min. Once the tubes were at room temperature, the organic phase was recovered, and a re-extraction step was performed by adding an additional 1 mL of *n*-hexane to the microwave tube. After 1 min of vortexing, some drops of EtOH were added to obtain a sharp phase separation. The upper layer was transferred to the tube with the previous extract and concentrated to 1 mL. When the hexane phase was not clear, a washing step was performed with 1 mL of H₂O, mixing both phases with a vortex for 1 min. After centrifuging the tubes for 5 min at 1400 rpm, a clear hexane phase was recovered. The final organic phase collected was concentrated to 500 µL after adding bis(2-ethylhexyl) sebacate as a keeper. Finally, a spatula tip of sodium sulfate was added to remove possible residual water before the LC separation.

Different tests were performed to evaluate the maximum amount of sample that can be subjected to the described miniaturized MASE, resulting in about 400 mg of adipose tissue. ISs distribution, recovery and the repeatability were evaluated using two different mass quantities (*i.e.*, 230 mg and 406 mg) after spiking the samples with Sternel motor oil, at 2.1 µg of MOSH and 2.4 µg of MOAH. The aforementioned evaluations were performed with and without the re-extraction step. All the tests were performed in triplicate.

The linearity and sensitivity of the analytical instrument in 1D and 2D mode (GC×GC) were evaluated. A six-point calibration curve was prepared using Sternel motor oil to cover the range from approximately 10 ng to 2000 ng of MOH injected into the GC system. Each calibration point was prepared in triplicate. The regression curves were obtained by applying the least squares method, and the linearity within the range considered was assessed using visual residue analysis.

The limit of quantification (LOQ) was estimated using the standard deviation obtained from 10 replicates of blank adipose tissue (pulling together the values obtained using 230 mg and 400 mg as no significant differences were highlighted) and injected into the system in 1D mode and 2D mode.

2.4. Mineral oil hydrocarbons determination

The miniaturized MASE method was validated using an LC/GC (×GC)-FID/MS platform [26,27], both in one-dimensional mode (LC/GC-FID), which is considered the routine technique for MOH analysis, and in two-dimensional mode, which provides detailed insights into the MOSH and MOAH subclasses. The LC/GC(×GC)-FID/MS system used in this study for MOH determination was similar to the one described in [23]:

LC separation: MOSH and MOAH fractions were obtained after performing an LC separation using an Allure silica column of 25 cm × 2.1 mm i.d. packed with Si-60, 5 µm particle size (Restek). The LC (Agilent 1260 Infinity) was equipped with a binary pump and a Variable Wavelength Detector acquiring at 230 nm (Agilent Technologies, Waldbronn, Germany). The MOH were eluted as follows: MOSH between 2.0 and 3.5 min and the MOAH between 4.4 and 5.9 min. The fractions were collected in different vials (450 µL each fraction). In this study, 100 µL out of 500 µL of the concentrated hexane phase obtained after applying the miniaturized MASE was injected into the LC to collect MOSH and MOAH in different vials. To decrease the LOQ, this step was performed two times for each analytical replicate, leading to 900 µL (450 µL × 2 collections) of each fraction, which were concentrated under N₂ stream until a volume of ~300 µL.

The GC and GC×GC-MS/FID system: consisted of a Pegasus BT 4D GC×GC time-of-flight (ToF) MS (LECO, St. Joseph, MI, USA). The

configuration and the method were the same as in [20], except for a few changes herein reported in the hardware of the FID line, where a solvent vapour exit (SVE) was added. For the FID line, an on-column injector was used, and an Rxi-retention gap (10 m × 0.53 mm i.d) was connected to the GC×GC set of columns through a steel T-piece, which was linked in turn with a solvent vapor exit to remove the solvent evaporating during partially concurrent eluent evaporation, which was open for 0.55 min. The GC×GC set of columns consisted of a Rxi-17Sil MS (15 m × 0.25 mm i.d. × 0.25 mm) column connected to a Rxi-1 MS HT (0.6 m × 0.15 mm i.d. × 0.15 μm) column, provided by Restek. The carrier gas, helium, was supplied in constant flow mode at 2.0 mL/min to the FID. For the 1D analysis, the GC oven temperature program was: 59 °C (hold 8 min) to 350 °C (hold 5 min) at 15 °C/min. For the 2D analysis, the GC oven temperature program of the primary oven was 59 °C (hold 5 min) to 350 °C (hold 5 min) at 3 °C/min. Regardless of the analysis mode (1D or 2D), a 15 °C positive offset was applied for the modulator and 5 °C for the secondary oven. The modulator was switched off to operate in 1D mode. Modulation was performed every 6 s, applying hot and cold pulses of 1.80 s and 1.20 s of duration, respectively. The FID was set at 370 °C and operated using 40 mL/min of H₂, 400 mL/min of air flow, and 30 mL/min of the make-up gas (N₂).

2.5. Integration and calculations

The software ChromaTOF BT Version 5 for MOSH/MOAH was used to acquire and elaborate the data, following the recommendations of the Joint Research Center (JRC) guidance [1,28]. The areas of MOH were obtained in GC and GC×GC after trimming the interferences from the whole hump of the largely unresolved peaks with automatic smooths applied with the software. The baseline was corrected by subtracting the signal of system blanks obtained on the same day and by using a constant in cases of baseline shift between the blanks and the samples.

The ratios between the peak area obtained for the different ISs were used for quality control as indicated in the JRC guidance [1,28]. The quantification of MOH was performed with the IS method. Cycy was used for MOSH, whereas 2-MN and TBB were considered for MOAH quantification.

3. Results and discussion

As mentioned in the introduction, the presence of TAGs affects the LOQ of the method, as a maximum of 20 mg of TAGs can be safely retained on the LC column without affecting the separation of MOSH/MOAH [15]. Considering that 100 μL are usually injected into the LC, the maximum permissible concentration of TAGs is 0.2 mg/μL. Since the final hexane phase is usually concentrated to 500 μL to achieve the required LOQ, the total TAG content must not exceed 100 mg (0.2 mg/μL × 500 μL) in the final extract to avoid compromising the method's performance. In the case of adipose tissue, which contains approximately 80 % lipids in animals [29] and 84 % in humans [30], the maximum sample weight that can be injected without saponification would be 0.25 mg in 100 μL (corresponding to 125 mg of tissue in 500 μL). Considering a limit of ~50 ng MOH into the column as instrumental LOQ [15], this scenario leads to an LOQ of ~2.5 mg/kg in the adipose tissues. In this regard, LOQs reported in the literature for biological samples are confusing. For instance, different LOQs have been reported for the same extraction method without saponification [5,11], due to the use of different estimation methods. On one side, an LOQ of 1 mg/kg was reported in rat tissues [5] by estimating the LOQ from the RSD of three analyses at 1.3 mg/kg of MOSH in the liver. Conversely, applying the same method to human tissues [11], an LOQ of 2.0 mg/kg was estimated considering the standard deviation at the lowest point (2.7 mg/kg) of a calibration curve. In 2022, the accumulation of MOSH was determined in various rat tissues, with saponification applied to adipose tissue [20]. However, the LOQ was not reported, and it is challenging to derive it due to the lack of clear information on the sample amount used [20].

Another important note when considering the study of MOH retention in tissues is that animal studies have been performed fortifying the diets with different sources of MOSH [5,14,20], without including in the experimental design the possible retention of MOAH. In the case of human data, no MOAH have been detected (LOQ = 2.0 mg/kg) in human tissues, which has been justified by their levels of exposure through the diet and their high tendency to be metabolized [11]. However, some *in vitro* studies with human and rat microsomes have reported that the overall rate of oxidative metabolism decreased with the length of the alkyl substituent, observing a lack of metabolism for long alkylation (*i.e.*, *n*-dodecyl-naphthalene) [4,9,10,31]. It has also been observed that some polycyclic aromatic compounds, which share a similar structure with compounds that can be present in MOAH mixtures, can be retained in hepatocytes [32] and adipocytes [33]. Thus, the lack of detectable MOAH in human tissues [11] could also be due to the sensitivity of the methods previously applied (2.0 mg/kg).

The saponification method proposed in this article was adapted from the protocol described for vegetable oils (1 g) [25] and further adapted to meat (5 g) [23]. Besides the increased LOQ brought by the saponification step, the main goal was to reduce the total solvent volume involved in the procedure, decreasing its contribution to the LOQ level. Indeed, the blank contribution should not exceed 1/3 of the estimated LOQ. As for our experience, the major contribution to the blank level is related to solvent contamination rather than occurring during manipulation. Considering the data of the MASE applied to meat [23], distilled *n*-hexane contributed to ~90 % of the procedural blank contamination (~0.10 mg/L of MOSH), and, therefore, reducing the amount of solvents during the sample preparation step should lead to an improvement in the LOQ.

For the miniaturized method for adipose tissue, the reaction conditions (120 °C for 20 min) used for meat were maintained to ensure complete saponification. Given that the lipid content of adipose tissue (≥80 % [29]) is closer to oils and fats (95–98 % [34]) than to meat (<23 % [23]), the reagent volumes were scaled down by a factor of four to adapt the method to ~220 mg of tissue. For a further reduction of reagents and simplification of the miniaturized MASE, the omission of the re-extraction and washing steps was considered during the optimization of the method.

3.1. Optimization of the miniaturized microwave-assisted saponification and extraction method

First, the maximum sample mass applicable without further adaptation of the miniaturized MASE method was evaluated, testing approximately 230 mg, 400 mg, 500 mg, 700 mg, and 1 g of adipose tissue. Starting from 500 mg, no phase separation occurred anymore; thus, these conditions were discharged. The results showed complete saponification up to 406 ± 4 mg and that washing of the hexane phase with water was unnecessary for 230 ± 5 mg samples, but became essential for 406 ± 4 mg samples to ensure a clean phase before LC injection. The optimization and performance evaluation of the method were done considering both sample weights (230 mg and 406 mg).

3.1.1. Evaluation of the ISs distribution

The saponification conditions and the unsaponifiable extraction may affect the partitioning of the ISs between the two phases formed after the reaction, thus mainly affecting the quantification according to the ISs used to quantify (*e.g.*, TBB or 2MN for MOAH), but it may also impact the recovery based on the MOH sub-class distribution. Therefore, particular attention was given to the ISs ratio, which was evaluated by applying the miniaturized MASE method, with and without the re-extraction step, to 230 mg and 406 mg of adipose tissue. To compare the extraction efficiency of the various ISs, peak areas were normalized against Cycy for MOSH and 5B for MOAH. The expected value of these ratios is 1.00, except for Cho and Per, which are present at a concentration two times higher than the others, therefore, their ratios were

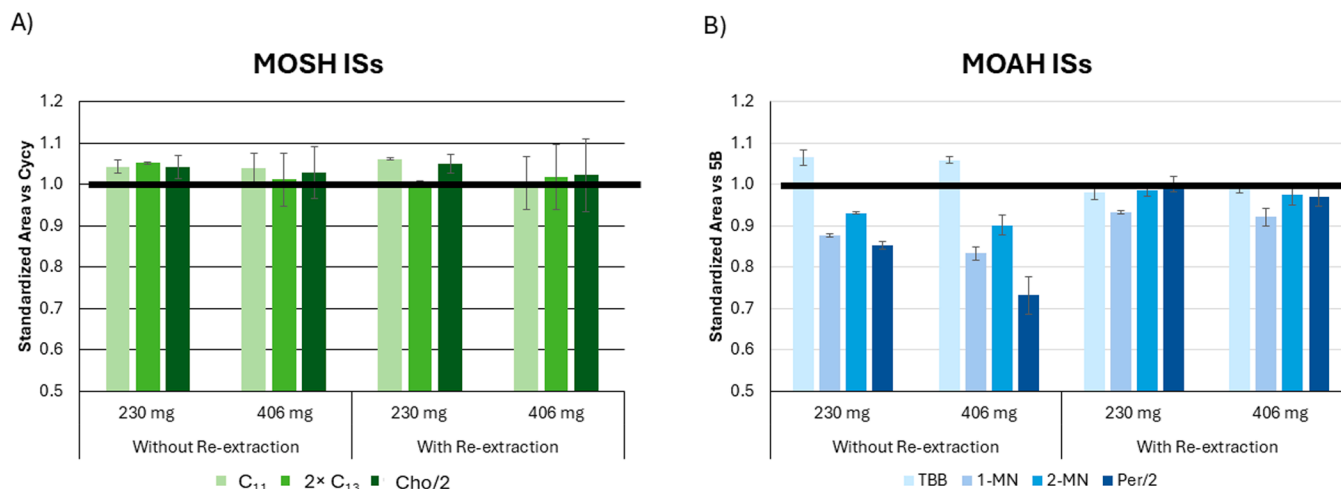


Fig. 1. Comparison of the internal standard distribution obtained after applying miniaturized MASE, with and without the re-extraction step of the hydrophilic phase following the saponification of 230 ± 5 mg and 406 ± 4 mg of adipose tissue. The areas of the different internal standards (ISs) used for the quantification and quality control of MOSH (A) were normalized to the area of CyCy, whereas those used for MOAH (B) were normalized to the area of 5B. Results are presented as the mean and standard deviation of three replicates. The black line represents the expected value (1.00).

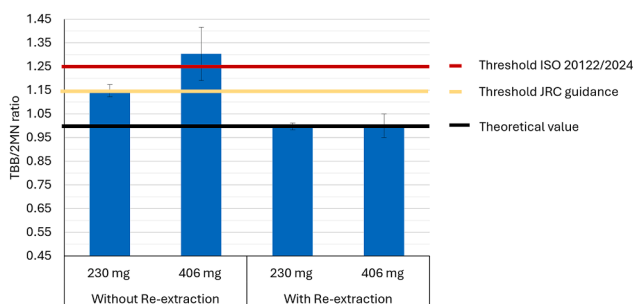


Fig. 2. Comparison of the TBB/2-MN ratio after applying miniaturized MASE, with and without the re-extraction step of the hydrophilic phase following the saponification of 230 ± 5 mg and 406 ± 4 mg of adipose tissue. Results are presented as the mean and the standard deviation of three replicates, together with the theoretical value, and the threshold set by the JRC guidance [1] and ISO-20,122/2024 [21].

divided by 2 to have an expected value of 1.00 for all the ratios. The ratios obtained are presented in Fig. 1.

The results showed that for MOSH ISs, the standardized areas remained consistently close to the theoretical value across all tested conditions. Only minor deviations were observed, and neither the omission of the re-extraction step nor the increase in sample weight (from 230 mg to 406 mg) produced substantial differences. In contrast, MOAH ISs showed greater variability, particularly in the absence of the re-extraction step. Without re-extraction, the ISs areas were generally lower than the theoretical value, impacting especially the recovery of 2-MN and Per, as previously reported in vegetable oils [25,35] and in meat [23]. The under-recovery of 2-MN and Per was even more pronounced when 406 mg of adipose tissue was tested.

Fig. 2 reports the values of the TBB/2-MN ratio, as requested for quality control in the JRC guidance [1] and the ISO method [21], comparing the data obtained with the acceptance levels in the two documents (*i.e.*, 1.15 and 1.25, respectively). The re-extraction step was mandatory to ensure a ratio close to 1.00, both using 230 mg or 406 mg of adipose tissue. Nevertheless, the absence of the re-extraction step had a substantially more pronounced impact on the results when the amount of sample was higher.

3.1.2. Evaluation of the recovery and the repeatability

MOSH and MOAH recoveries after fortifying 230 mg and 406 mg of sample with $4.5 \mu\text{g}$ of MOH ($2.1 \mu\text{g}$ of MOSH plus $2.4 \mu\text{g}$ of MOAH) were $94 \pm 3\%$ for MOSH and $98 \pm 2\%$ for MOAH, regardless of the amount of sample. These results were similar to the ones obtained when applying the original MASE method to meat ($90 \pm 3\%$ and $98 \pm 4\%$) [23]. Obviously, for MOAH quantification, the impact on the TBB/2-MN ratio when avoiding the re-extraction step leads to different recoveries when using one or the other for the calculations. In fact, the use of TBB led to recoveries similar or under the threshold set by the JRC guidance (80 %), whereas the 2-MN represented better the MOAH behaviour. Thus, only the results obtained with 2-MN are included in Fig. 3. MOAH recoveries generally increased slightly when performing the re-extraction step (Fig. 3), following the behavior of the ISs, confirming the necessity of the re-extraction step for reliable quantification of MOAH.

These recovery results confirmed that the conditions applied in the proposed method did not alter the original MOH composition. Two of the most critical factors determining the effectiveness of a saponification method are the reaction temperature and the solvents used. Although MOSH and MOAH are chemically stable at elevated temperatures (Gorska et al. reported 96 % recovery of MOAH after 1 h of distillation at 163°C under 3 mbar [36]), there is still a risk of volatilization of MOHs with shorter carbon chain lengths. In the proposed method, this risk is minimized by performing the saponification in a hermetically closed system under 35–40 bar, which remained under pressure until cooled to room temperature. Regarding solvent selection, the use of a KOH solution in EtOH:H₂O ensured adequate ISs distribution and recoveries, consistent with results previously reported in other studies [23,25].

MOSH and MOAH results obtained for the recovery test were also used to evaluate the repeatability of the miniaturized MASE, expressed as the relative standard deviation (RSD) of the three replicates analyzed for each experimental condition. A comparison of the RSD results with the limits established by the JRC guidance for samples with less and $>4\%$ of fat [1] is schematized in Fig. 4. In all cases, the values of RSD obtained with the tested experimental conditions were lower (0.7 % – 2.5 %) than the thresholds established by the JRC guidance. The repeatability of the method was not impacted by the sample weight, showing an adequate performance for the range of sample's weights studied. No clear improvement in repeatability was observed when applying the re-extraction step after the saponification reaction.

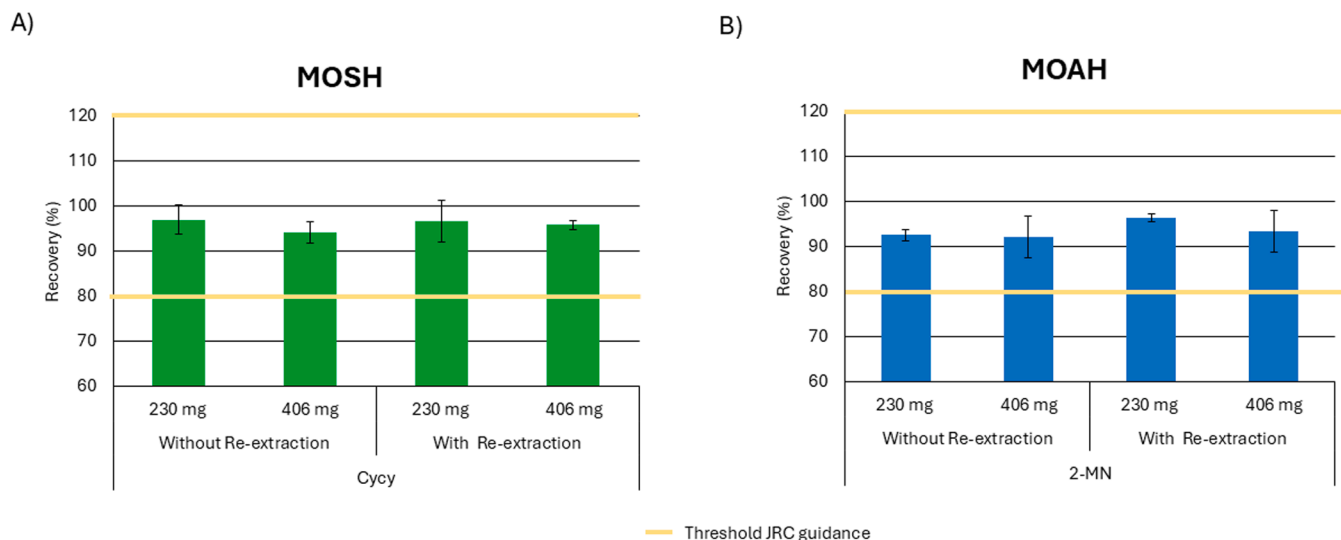


Fig. 3. Recoveries obtained for MOSH (A) and MOAH (B) after applying miniaturized MASE, with and without the re-extraction step of the hydrophilic phase following the saponification of 230 ± 5 mg and 406 ± 4 mg of adipose tissue spiked with Sternal motor oil ($2.1 \mu\text{g}$ of MOSH plus $2.4 \mu\text{g}$ of MOAH). Results were obtained by LC/(GC×GC)-FID in mono-dimensional (1D) and two-dimensional (2D) mode and presented as the mean and the standard deviation of three replicates obtained considering the 1D and 2D results, together with the recovery requirements specified by JCR guidance [1]. For MOSH the quantification was performed with Cycy and for MOAH with 2-MN.

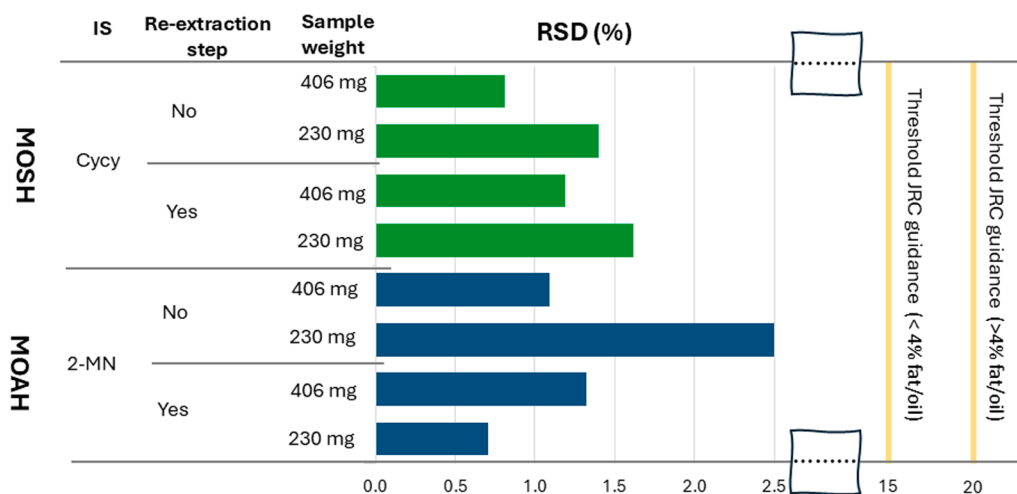


Fig. 4. Relative standard deviation (RSD) obtained for MOSH and MOAH after applying miniaturized MASE, with and without the re-extraction step of the hydrophilic phase following the saponification of 230 ± 5 mg and 406 ± 4 mg of adipose tissue spiked with Sternal motor oil ($2.1 \mu\text{g}$ of MOSH plus $2.4 \mu\text{g}$ of MOAH). Results were obtained by LC/(GC×GC)-FID in mono-dimensional (1D) and two-dimensional (2D) mode and presented as the mean of all the replicates. Vertical yellow lines represent the requirements specified by JCR guidance [1]. For MOSH the quantification was performed with Cycy and for MOAH the calculations were made with 2-MN.

3.3. Evaluation of the limit of quantification

The LOQ of the analytical method depends on various factors, such as the instrument configuration, chromatographic method, sample preparation, and the breadth of the hump. The use of the LC/(GC×GC)-FID system was shown in a previous work to increase the sensitivity by 10 times compared to LC/GC-FID [23]. Compared to the previous configuration, a solvent vapor exit was installed. This allowed the volume of injection into the GC system to be increased from $20 \mu\text{L}$ to $90 \mu\text{L}$ (potentially increasing it to $450 \mu\text{L}$), further improving the system's LOQ. Nevertheless, as discussed in [23], the final LOQ is primarily influenced by the contribution of the procedural blank. Indeed, as expected, the reduction in the volume of hexane used decreased the contamination found in the procedural blank (from $2.1 \mu\text{g}$ to $0.6 \mu\text{g}$ of MOSH absolute amount). The reduction in the blank contamination was

slightly lower (3.5 times) than expected (4 times) due to a higher contamination in the distilled hexane used in this study (0.19 mg/L) compared to that used in [23] (0.12 mg/L).

Additionally, the conversion into concentration in the sample is impacted by the lipid content of the sample, which is the major limiting factor in the quantity that can be injected into the system. Therefore, for comparison purposes with the other methods, initially the LOQ is discussed as an absolute quantity of MOH in the sample processed. The LOQ was extrapolated by calculating the standard deviation obtained from the analysis of ten replicates of blank adipose tissue. The value was multiplied by 10 and the corresponding concentration was extrapolated from the calibration curve, as suggested by the JRC Guidance [1]. The results showed an LOQ in adipose tissue of $0.4 \mu\text{g}$ per sample, with similar values obtained using both 1D and 2D modes. This represented an approximately 3.3-fold improvement compared to the $1.3 \mu\text{g}$ LOQ

Table 1

Comparison between conditions and performance of the miniaturized MASE method and previous extraction and saponification methods applied for MOH evaluation in biological samples.

Method	Sample	Amount of Sample (g)	Volume of Reagents for sample prep (mL)			Estimated time (h)	LOQ (μg of MOH in the sample)
			C ₆	EtOH	H ₂ O		
Miniaturized MASE	Chicken adipose tissue	0.23–0.41	3.5	2	7.25	3	0.4
Extraction [5]	Rat carcass	~1	5	5	10	20	1
Extraction [11]	Human adipose tissue	~1	5	4	10	20	2
Saponification [20]	Rat fatty tissue	Unknown	17.5	15	5	3	Unknown

reported in [23] and it was consistent with the difference observed in the procedural blank. When converting this value to the concentration of MOH in the sample, the improvement was less evident since it also relates to the quantity of sample processed. In the method optimized for meat, an LOQ of 0.3 mg/kg was achieved, as the initial sample mass was 5 g. For the adipose tissue studied here, as the quantity of samples was only ~0.4 g, an LOQ of 1 mg/kg was obtained. Nevertheless, this value was still twice as low as those previously reported in the literature for methods used to evaluate MOSH retention in tissues [11,5], which required a minimum of 1 g of sample. Therefore, the proposed miniaturized MASE method is a more sensitive alternative to conventional extraction methods, requiring a lower amount of sample, reagents, and time.

3.4. Advantages and future perspectives

When comparing the miniaturized MASE method with other approaches previously applied to biological samples for MOH evaluation (Table 1), the proposed method stands out for requiring smaller sample amounts, reduced reagent volumes, and enabling improved quantification of MOHs. Considering these advantages, although this miniaturized MASE method was developed and validated for adipose tissue (up to 406 mg, containing ≥ 80 % of lipids [16,30]), other applications can be anticipated. For instance, it may also be applied to other biological matrices with lower fat contents, such as liver, lung, and brain tissues. For such tissues, an extraction-based method could theoretically be applied to larger sample masses without exceeding the TAG loading limit for the LC column. For example, human liver typically contains 1–5.5 % lipids [18] and mouse liver 2–5 % [29]. Considering the maximum lipid content (5.5 %), up to 1.8 g of liver tissue could be extracted without surpassing the 20 mg TAG limit. However, Barp et al. reported that the retention capacity of the silica LC column for liver and brain lipids was lower than that for other matrices, limiting the injected amount to 20 mg of tissue [11]. This was probably due to the high amount of glycerophospholipids in this kind of matrix [37,38], which could have been eliminated by applying saponification. Thus, this means that a LOQ of 0.3 mg/kg could be reached if the proposed method could be applied to 1.3 g of liver and brain. It remains clear that, when the method will be extended, the performance needs to be verified for the specific matrix, but still the potential is evident.

4. Conclusions

The miniaturized MASE method for the determination of MOSH and MOAH in biological samples demonstrated high analytical performance, fulfilling the JRC guidance criteria for ISs distribution, recovery, and repeatability, even when applied to sample masses as low as 230 mg to 406 mg.

The inclusion of a re-extraction step was shown to be essential for reliable MOAH quantification, particularly at higher sample weights, as it led to a similar extraction of the different ISs (especially TBB and 2-MN). The LOQ achieved with this miniaturized approach was significantly lower than previously reported methods, supporting its suitability for sensitive analysis in scenarios where sample availability is limited,

such as *in vitro* models or biopsy samples where the methods will be applied in the ongoing studies in our research team, for which this method has been developed. Overall, this miniaturized MASE method offers a robust, sensitive, and efficient alternative to conventional extraction techniques, with reduced reagent consumption and processing time, aligning with current trends in green analytical chemistry and advancing the capability for MOH evaluation in biological research and risk assessment.

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CRediT authorship contribution statement

Paula Albendea: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Aleksandra Gorska:** Writing – review & editing, Methodology. **Giorgia Purcaro:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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