Purification of mineral oil aromatic hydrocarbons and separation based on the number of aromatic rings using a liquid chromatography silica column. An alternative to epoxidation.

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Abstract

The analysis of mineral oil aromatic hydrocarbons (MOAH) in vegetable oils is currently associated with high uncertainty due to various factors ranging from sample preparation to data interpretation. One significant factor is the coelution of biogenic compounds of terpenic origin with the MOAH fraction during chromatographic analysis. The common purification method is epoxidation, a chemical reaction that changes the polarity of the interferences, allowing their separation from MOAH. However, this reaction is non-selective and can lead to losses of both MOAH and internal standards. This variation is especially noticeable when using epoxidation procedures with higher reaction kinetics, such as those employing performic acid. MOAH losses also vary depending on their composition, which is unpredictable. Furthermore, 2-methylnaphthalene (2MN) and 1,3,5-tri-tert-butylbenzene (TBB), which are the common quantification standards, are lost at different rates. As a consequence, the final MOAH quantity will vary both depending on the standard used, and on its initial composition. This work presents a new purification approach based on liquid chromatography fractionation on silica using the same column and eluents as for the usual MOSH/MOAH fractionation. This approach efficiently removes squalene, carotenes, and derivatives, without inducing inconsistent losses between the internal standards and MOAH as epoxidation does. On average, MOAH recovery using this new method was 94% (± 8%) in coconut, palm, sunflower, and olive oil, using different MOAH sources and concentrations. Additionally, perspectives are presented regarding the separate analysis and quantification of mono-/diaromatic MOAH and other polyaromatic MOAH. This is of particular interest as these two sub-fractions are associated with different toxicological properties.

Keywords: mineral oil aromatic hydrocarbons; comprehensive gas chromatography, high-performance liquid chromatography; purification; vegetable oils; interferences

1. Introduction

Mineral oil hydrocarbons (MOH) are ubiquitous food contaminants deriving from petroleum products which are known since the nineties [1], and for which the interest has risen in 2008/2009 after sunflower oil imported from Ukraine was found to be contaminated at levels >1000 mg/kg [2]. They are a large family of molecules that is commonly subdivided into two major groups: mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH). MOSH consist of alkylated linear alkanes and nude or alkylated cyclic alkanes, while MOAH of branched polyaromatic hydrocarbons [3].

MOSH and MOAH have different toxicological properties. Although these can be affected by the molecular composition of the two fractions, what is currently described is that some MOSH tend to accumulate in human tissues, while MOAH contain potentially mutagenic and genotoxic compounds, with MOAH containing three aromatic rings or more that are the most concerning [3]. The presence of MOAH is considered of concern in food, and its content is therefore currently limited by a statement from the Standing Committee on Plants, Animals, Foods and Feed (SCoPAFF). This limit depends on the fat content of the food matrix, and the higher this content, the higher the tolerance as MOH are lipophilic [4].

The common technique to analyze MOSH and MOAH is on-line high performance liquid chromatography hyphenated with gas chromatography and flame ionization detection (HPLC-GC-FID) [5]. Off-line HPLC/GC-FID can also be employed as an alternative, which offers the possibility of using the HPLC and GC parts separately, not limiting the use of the instruments to the MOSH/MOAH application only. The main disadvantages of off-line systems are however the lower sample throughput, the increased risk of sample contamination, and the reduced sensitivity (which can be more or less adjusted by implementing large volume injection approaches in GC).

There is, unfortunately, still a very high uncertainty related to MOSH/MOAH determination and, therefore, also, a fairly low reliability [6]. For MOAH, the factors that contribute the most to the uncertainty are the enrichment step (saponification) [7], the coelution of interferences, and, consequently the interpretation of the chromatograms [8]. In the case of vegetable oils, these

interferences are typically of terpenic nature, and, when they are present in abundance compared to MOAH, they cover the latter's hump, making determination of the contamination difficult or impossible [9].

Purification methods have been developed to eliminate (at least partially) these interferences. They are based on an epoxidation reaction, which creates epoxy bonds at the level of double bonds present in the interfering compounds (as they are most generally olefins). The available procedures notably differ on the epoxidizing agent and the used solvent, as well as the purification efficiency [10,11]. The most commonly applied procedure, included in the ISO 20122:2024 method for MOSH/MOAH analysis in vegetable oils, involves *meta*-chloroperbenzoic acid (mCPBA) as epoxidation reagent. However, this reagent is not sold pure as potentially explosive beyond 85% purity, and therefore often introduces undesirable impurities in the sample [9]. This issue led to the development of another method, where mCPBA was substituted by performic acid [11]. This method showed two major advantages being the reduced impurities, as well as higher efficiency of purification. Nevertheless, a major issue with epoxidation is that it is not selective, and MOAH also tend to be lost to an estimated total percentage of 20-35%, with compounds having a higher number of aromatic rings being epoxidized (and therefore lost) more easily [9,12].

Another way to deal with interferences consists in using GC×GC, which is recommended as a confirmation method when doubts persist on the nature of the integrated GC hump [13]. However, although it can help locating the interferences on the chromatogram, it has only limited power in separating the interferences from MOH [14]. Alternative methods for purification are therefore currently limited to LC approaches. For example, Zoccali and cowokers [15] used an on-line LC-LC-GC system, incorporating two silica columns and a silver ion column connected in series, to separate solely squalene and its isomers from the MOAH fraction. Later, Biedermann and coworkers [16] succeeded in isolating biogenic interferences from MOAH with more than three aromatic rings in infant formula. They achieved this by combining the standard LC-based MOSH/MOAH fractionation (on silica) with an additional donor-acceptor complex chromatography (DACC) fractionation step, specifically collecting the ≥ 3 aromatic ring fraction (≥ 3AR). Lommatzsch and others [17] subsequently

confirmed the reduction of certain terpenes, such as squalenes and carotenes, from ≥ 3AR in samples like olive oil, a terpene mixture, and infant formula. However, the method was unable to eliminate interferences coeluting with mono-/diaromatic fraction (< 3AR), which required an epoxidation step. The latter work also explored the possibility of quantifying <3 AR and ≥ 3AR separately, with results consistent with those obtained by GC×GC-FID for batching oil, infant formula, and rice samples, although some unexplained discrepancies were observed for 2 out of the 5 rice samples analyzed.

In line with evaluating alternative purification methods, this work investigated the use of the same HPLC conditions (column and eluents) as commonly used for MOSH/MOAH fractionation for the purification of terpenic interferences in the MOAH fraction of vegetable oils, and comparing it to the results obtained by two epoxidation procedures reported in the ISO 20122:2024 method (one of them being reported in the annexes, but not validated). Additionally, it demonstrated the potential for quantifying MDAF and TPAF separately using an HPLC silica column.

2. Materials and Methods

2.1. Solvents, standards, reagents, and samples

Dichloromethane LiChrosolv® was provided by Merck (Darmstadt, Germany). *n*-Hexane and acetone for HPLC were purchased from Biosolve Chemicals (Dieuze, France). To increase its purity, *n*-hexane was distilled before use. Ethanol (99.8%, for HPLC, absolute) was from Thermo Scientific Chemicals (Loughborough, United Kingdom).

Sodium sulfate (≥ 99.0%), sodium carbonate (≥ 99.5%), 3-chloroperbenzoic acid (m-CBPA, ≤ 77%), bis(2-ethylhexyl) maleate (90%), potassium hydroxide (90%), sodium thiosulfate pentahydrate, *ortho*-phosphoric acid (85%, w/w), Emprove® Essential formic acid (98-100%), bis(2-ethylhexyl)sebacate (for synthesis), squalene (≥ 98%), β-caryophyllene (≥ 80%), lycopene (≥ 98%), and trans-β-carotene (95%) were all from Merck-MilliporeSigma (Overijse, Belgium). Hydrogen peroxide (50%, aqueous solution) was provided by Bernd Kraft (Duisburg, Germany). Coronene (Cor) was purchased from LGC Standards (Teddington, United Kingdom. β-carotene European Pharmacopeia

Reference Standard European pharmacopeia reference standard was from the European Directorate for the Quality of Medicines & HealthCare of the Council of Europe (Strasbourg, France).

The MOSH and MOAH internal standards (IS, Restek #31070), the MOSH/MOAH retention time (RT) standard (Restek, #31076), and and the 610 PAH Calibration Mix A (Restek #31264) were kindly provided by Restek (Neukirchen-Vlun, Germany). The IS consisted of 600 µg mL^{-1} 5-α-cholestane, 300 µg mL^{-1} *n*-C11, 150 µg mL^{-1} *n*-C13, 300 µg mL^{-1} cyclohexyl cyclohexane, 300 µg mL−11-methyl naphthalene (1MN), 300 µg mL^{-1} 2-methylnaphthalene (2MN), 300 mg mL^{-1} tri-tert-butyl benzene (TBB), and 600 µg mL^{-1} perylene, in toluene. The RT standard contained *n*-C10, *n*-C13, *n*-C20, *n*-C24, *n*-C25, *n*-C35, *n*-C40, and *n*-C50 at 100 µg mL^{-1} in cyclohexane. The composition of the 610 PAH Calibration Mix A was the following: 1000 µg mL^{-1} acenaphthene (Ap), 1000 µg mL^{-1} acenaphtylene (Ac), 1000 µg mL^{-1} anthracene (A), 500 µg mL^{-1} benz[a]anthracene (BaA), 500 µg mL^{-1} benzo[a]pyrene (BaP), 500 µg mL^{-1} benzo[b]fluoranthene (BbF), 500 µg mL^{-1} benzo[k]fluoranthene (BkF), 500 µg mL^{-1} benzo[g,h,i]perylene (BghiP), 500 µg mL^{-1} chrysene (Ch), 500 µg mL^{-1} dibenz[a,h]anthracene (DBahA), 500 µg mL^{-1} fluoranthene (Fl), 1000 µg mL^{-1} fluorene (F), 500 µg mL^{-1} indeno[1,2,3-cd]pyrene (IP), 1000 µg mL^{-1} naphthalene (Na), 500 µg mL^{-1} phenanthrene (Pa), 500 µg mL^{-1} pyrene (P).

SN100 and SN500 Aromatic Extracts (73 g ± 7 g MOAH/100g and 63 g ± 5 g MOAH/100g, respectively), Gravex (23 g ± 2 g MOAH/100 g), Heavy Vacuum Gas Oil (HVGO), bleached sunflower oil, refined coconut oil, and palm oil were obtained from collaborators. Sternel motor oil (48 g MOAH/100 g) was found in a private home. Olive oil spiked with Shell Gravex 912 (37.55 mg MOAH/kg) was received for an interlaboratory trial organized by the JRC and reused [6].

Standard solutions for the evaluation of the LC purification were all performed in *n*-hexane. For the first optimization steps, comprising the evaluation of elution order using *n*-hexane as eluent, a mix of 1.5-3 mg/L MOSH/MOAH IS, 0.5-1.0 mg/L PAH 610 Calibration Mix A, 0.5 mg/L Cor, and 50 mg/L HVGO was prepared (solution A). For the optimization of the gradient in order to purify biogenic interferences, solution A was spiked with squalene, lycopene, β-carotene, trans β-carotene, β-caryophyllene at 5 mg/L (solution B), and at 25 mg/L (solution C). For the evaluation of MOAH

recovery, hexanic solutions of Gravex, HVGO, and Sternel motor oil were prepared at 5 mg/L of MOAH, and 1.5-3.0 mg/L of MOSH/MOAH IS.

2.2. Sample preparation

All glassware and material were carefully washed with acetone and *n*-hexane prior to use.

2.2.1. Saponification of vegetable oils

The saponification/extraction procedure was based on the work of Bauwens and Purcaro [7], with some adaptations. One and a half gram of vegetable oil was weighed in PTFE vessels for microwave extraction. Then, 15 mL *n*-hexane, and 15 mL of a 2M KOH solution in a 1:1 mixture of ethanol and water (v:v) were added, as well as a magnetic stirring bar. Only for the JRC olive oil, 15 µL of MOSH/MOAH IS were added during this step. The vials were enclosed in 100 mL PTFE microwave vessels and submitted to a saponification/extraction process in the ETHOS™ X microwave equipped with an SR-12 eT TFM rotor (Milestone Srl, Bergamo, Italy). The temperature program was the following: 5 min of preheating until 60°C, then 30 min at 60 °C. Once the program was over, the vessels were left to cool until 40°C. Thirty milliliters of water were then gently added, and the vessels were put inside the refrigerator for 20 min to enhance phase separation. The hexanic phase (~ 15 mL) was then transferred to a clean evaporating flask, and the hydroalcoholic phase was reextracted with 7.5 mL of *n*-hexane. To this extract two drops of bis(2-ethylhexyl)sebacate were added, and the solution was concentrated to 3 mL using a flow of nitrogen. This concentrated extract was then spiked with 10 µL MOSH/MOAH IS (apart from the olive oil), 5 or 10 µL of 610 PAH Calibration Mix A (containing 16 PAHs), and 5 µL of a solution of Cor (1 mg/mL). The spiking with pre-diluted mineral oil (hexanic solution at 4 g/L) was also performed at this step (not for olive oil), reaching a final spiking concentration of MOAH ranging from 7.5 mg/kg to 64 mg/kg (specified for each sample in the text). The sample was then divided into three equal parts and either transferred to 30 mL glass vials closed with screw caps containing a PTFE septum for further epoxidation, or to fresh autosampler vials to perform the LC purification described in section 2.3.

2.2.2. Purification from biogenic interferences

After saponification, liquid/liquid extraction, and concentration of the sample, it was submitted to one of the following epoxidation methods, based on the protocols of the ISO 20122:2024 method. All procedures were performed manually. The LC purification is described in **section 2.3**.

Epoxidation with m-CPBA

One milliliter of ethanolic *m*-CPBA solution (1 g *m*-CPBA in 10 mL ethanol) was added to the obtained extract, the vial was closed, and the solution was stirred for 20 min at 40 °C at e.g. 500 rpm. Five hundred microliters of ethanol were then added as well as 2 mL of a solution of 5 % (w/v) sodium thiosulfate and 5% (w/v) of sodium carbonate in water. The solution was mixed thoroughly for one minute to deactivate the remaining *m*-CPBA, then let to rest until full phase separation. The upper phase (~500 µL) was then transferred to a sample vial containing a spatula tip of sodium sulfate.

Epoxidation with performic acid in n-hexane

Seven hundred fifty microliters of a formic acid solution acidified with 10% phosphoric acid (85%, v/v) and 750 µl of aqueous hydrogen peroxide (50%, w/v) were added to the sample. The vial was closed with the screw cap at 65°C and shaken (e.g., 500 rpm) at 65 °C for 30 minutes. To stop the reaction and induce phase separation, 10 mL of water were added, then the vial was shaken thoroughly for one minute. After phase separation, approximately 500 µL of the upper phase were transferred to a sample vial containing a spatula tip of sodium sulfate.

2.3 Instrumental analysis

2.3.1. HPLC/GC×GC-FID/TOFMS

An off-line HPLC/GC×GC-FID/TOFMS, depicted in **Figure S1**, was used to perform the fractionations/collections and analyses of samples. The HPLC part was composed of the Agilent 1260 Infinity II HPLC, equipped with an isocratic pump G7110B and a Variable Wavelength Detector acquiring at 230 nm (Agilent Technologies, Waldbronn, Germany). The used HPLC column was an Allure silica 250 mm × 2.1 mm i.d. × 5 µm d_p, with a pore size of 60 Å (Restek). Mobile phases were *n*-hexane (C6) and dichloromethane (DCM).

For the fractionation of MOSH and MOAH, the elution gradient (**gradient 1**, being the routine gradient for MOSH/MOAH separation) was 0 min 100% C6; 1.5–6 min 65% C6 35% DCM at 0.3 mL min^{-1}. At 6.10 min the column was backflushed with 100% DCM for 9 min at 0.5 mL min^{-1}. The flow was then switched to forwarding mode to re-equilibrate the column with 100% C6 10 min at 0.5 mL min^{-1} and 5 min at 0.3 mL min^{-1} until the following analysis. The MOSH and MOAH fractions were collected into fresh vials between 2 and 3.5 min, and 4.4 and 5.9 min, respectively, using the HPLC collection tool of the PAL3 Autosampler (PAL System from CTC, Switzerland). The volume of the collected fractions was 450 µL.

To perform the purification of MOAH, as well as the separation by number of aromatic rings, the MOAH fraction was concentrated to 100 µL. It was then reinjected onto the same HPLC column for a second fractionation with a different gradient (**gradient 2**, see later), and the fraction(s) of interest were collected into fresh vials, before undergoing concentration to be injected for a final MOSH/MOAH fractionation. More specifically, **gradient 2** was: 0 min 100% C6; 1.5–14.5 min 98% C6 2% DCM; 14.5-23 min 95% C6 5% DCM at 0.3 mL min^{-1}. At 23.10 min the column was backflushed with 100% DCM for 9 min at 0.5 mL min^{-1}. The flow was then switched to forwarding mode to re-equilibrate the column with 100% C6 10 min at 0.5 mL min^{-1} and 5 min at 0.3 mL min^{-1} until the following analysis. The purified MOAH fraction was eluted between 3.5 and 20.5 min. For a separation in 1-2 and 3+ ring fraction, the cut was performed at 9.5 min, with each of the two resulting fractions collected in separate vials. The collected fractions were reinjected (after concentration) for a second MOSH/MOAH fractionation (**gradient 1**), before being injected into the GC(× GC) system.

The GC and GC × GC system consisted of a Pegasus BT 4D GC × GC ToFMS (LECO, St. Joseph, MI, USA). The latter is composed of an Agilent 8890 gas chromatograph, equipped with a secondary oven and a quad-jet dual-stage thermal modulator, a time-of-flight MS (TOFMS) and an FID. Two different injectors were used for the FID and the TOFMS line, respectively a cold on-column (COC) and a split/splitless (SSL) (from Agilent). Large volume injection was performed by solvent flooding in the FID line (up to 20 µL), while the SSL was operated in splitless mode (injection volume

of 1-2 µL). The delivery of the sample into the injection port was performed using the same autosampler as for the HPLC, but with a GC injection tool.

The COC was connected to a Rxi Guard column (4 m × 0.53 mm), followed by a Rxi-17SilMS (15 m × 0.25 mm × 0.25 µm) for the 1D separation, and a Rxi-1MS HT (0.8 m × 0.15 mm × 0.15 µm) for the 2D separation (kindly provided by Restek). The SSL was connected directly to the 1D separation column (*i.e.*, no guard column), with the 1D and 2D columns being the same as in the FID line, and with a retention gap (FS Deactivated, 0.55 m × 0.25 mm) connected after the 2D column (from Agilent).

For 1D analysis, the modulator was switched off and a 5 °C positive offset was applied to the modulator and the secondary oven. The program started at 59 °C for 5 min, increased to 350 °C at 20 °C min^{-1}, and held 350 °C for 5 min. For 2D analysis, the oven program of the primary oven was 59 °C for 5 min, increased to 350 °C at 5 °C min^{-1}, and held 5 min at 350 °C. The secondary oven program was the same as for the primary oven, with a positive offset of 5 °C. A 15 °C positive offset was applied for the modulator. Modulation was performed every 6 s, applying variable hot and cold pulse durations. The carrier gas was helium, supplied in constant flow mode at 1.7 mL min^{-1} for the FID line, and 1.3 mL min^{-1} for the MS line.

The parameters of the FID were 40 mL min^{-1} for the H_2 flow, 400.0 mL min^{-1} for the air flow, 30.0 mL min^{-1} for the make-up gas flow, 370 °C temperature. The parameters of the MS were 40-700 m/z for the acquired range, 340 °C for the MS interface temperature, 250 °C for the ion source temperature, and the ionization mode was electron ionisation at 70 eV. The data acquisition frequency was 200 Hz in 2D and 20 Hz in 1D for both FID and MS. The start of the MS acquisition was delayed to 180 seconds after the injection, corresponding to the end of the elution of the solvent peak.

Data acquisition and integration were performed using ChromaTOF Version 5 for MOSH/MOAH (LECO, USA). The general procedure was similar to the one described by Bauwens et al. [18,19] and followed the recommendations of the updated JRC guidance [13].

3. Results and Discussion

3.1. Evaluation of the epoxidation methods

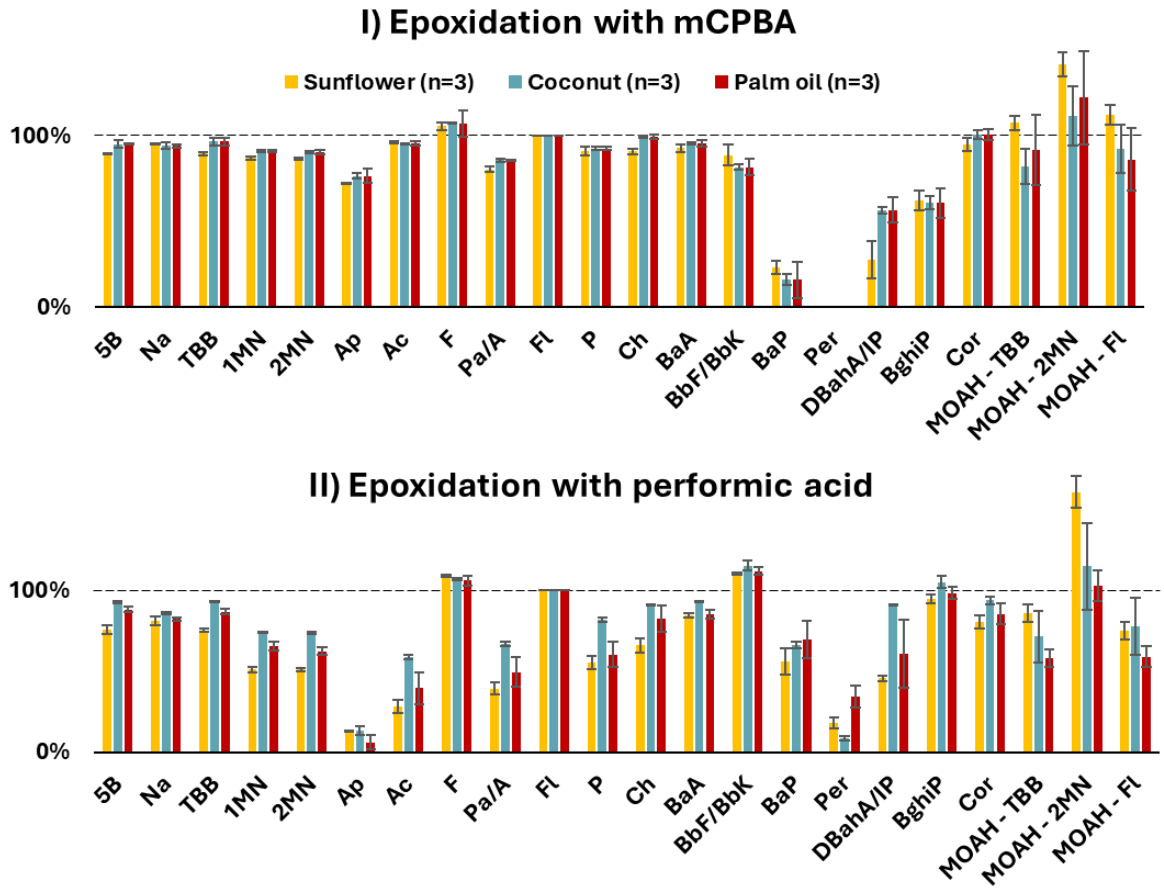
The first step of this work consisted in comparing the two epoxidation methods that are presented in the ISO 20122:2024 method for eliminating olefinic interferences from the MOAH fraction (*i.e.*, epoxidation with ethanolic *m*-CPBA, and with performic acid). The comparison was made regarding the purification efficiency and their impact on the final MOAH quantification in three vegetable oils, namely palm, sunflower, and coconut oil. These matrices were selected as they cover a wide range of interfering compounds, *i.e.*, sesqui-, di-, tri-, and tetraterpenes. Additionally, palm oil is relevant as it contains overloading amounts of carotenoids, which is not the case with other oils (see **Figure S2**).

In terms of results, the visual inspection of the GC chromatograms of the epoxidized coconut, sunflower, and palm oil extracts showed no residual interferences, regardless of the epoxidation procedure used (**Figure S3)**. The efficiency of the purification was therefore considered satisfactory in these cases. However, these results should not be generalized, as persistent interferences may occur in other matrices, depending on the biogenic interferences they contain [9].

In fact, as described by [11], the inability to remove certain biogenic compounds is related to the π-electron density they have on the double bond being attacked. Compounds with highly conjugated systems, such as squalene and β-carotene, undergo epoxidation readily because of the high π-electron density they possess. In contrast, π-electron-deficient olefins like 1-octadecene react much more slowly. This slow reaction can also apply to some terpenes (typically monoterpenes). Examples of matrices containing compounds difficult to epoxidize interferences are spices, as well as some vegetable extracts such as essential oils. Highly refined palm oil, but also other refined edible oils, are also known as being difficult to purify [9,11]. Some persistent interferences are formed during the refining process. In this study, the palm oil was only weakly refined (retaining a strong red-orange color), which could explain the absence of residual interferences after purification.

Concerning the impact on MOAH quantification, **Figure 1** presents the relative recoveries of polycyclic aromatic compounds (PACs) and MOAH following the different epoxidation methods. Fl (was used as the internal standard for PACs, while TBB and 2MN were additionally used and compared

for MOAH. Fl is not a typical MOSH/MOAH standard, but its low epoxidation rate [11] makes it a suitable reference for estimating the loss of other compounds.



III) Average MOAH recovery (± standard deviation)

	mCPBA	Performic Acid
A) TBB	94% ± 16%	72% ± 15%
B) 2MN	125% ± 21%	126% ± 30%
C) FL	97% ± 17%	70% ± 17%

Figure 1 *– Relative recoveries of polycyclic aromatic compounds (PACs) and MOAH after I) Epoxidation with mCPBA, and II) epoxidation with performic acid. PACs are classified from left to right by increasing number of aromatic rings, and their recovery was obtained using fluoranthene (Fl) as the internal standard (IS). For the quantification of the MOAH, spiked at 12.8 mg/kg, three ISs where evaluated (TBB, 2MN, Fl). The average MOAH recovery all matrices merged are given in III). When chromatographic separation was insufficient, the combined recovery of the coeluted compounds was calculated (i.e., Pa/A).*

As can be seen, the recoveries of PACs differ significantly depending on the epoxidation method and the compound's structure, with performic acid generally leading to higher losses, consistent with previous findings [11]. MOAH recovery is also, on average, lower using this method, which was expected because 1) the sample contains MOAH compounds with more than three aromatic rings [16], and 2) the method using performic acid is known to be more efficient at epoxidizing interferences, which also results in greater epoxidation of MOAH. Furthermore, TBB and 2MN are epoxidized at different rates, with 2MN being lost more significantly than TBB [11], leading to diverging quantification results depending on the chosen standard. This difference is particularly pronounced when using performic acid.

Moreover, since epoxidation is a purification method that relies on a chemical reaction, strict control of time and temperature is required; otherwise, it may lead to irreproducible results between laboratories and operators [16]. This is critical both from a quantitative perspective and for safety. Quantitatively, any deviation in time or temperature will affect the reaction progress, potentially altering the internal standard ratios and leading to inconsistent MOAH losses. From a safety perspective, epoxidation produces carbon dioxide. If the reaction time or temperature is not properly controlled, excessive gas formation can cause overpressure in the glassware, potentially resulting in explosions.

Using an automated sample preparation system simplifies control of reaction conditions, theoretically providing more reproducible results. However, not all laboratories have access to such systems, making it important to search and propose alternative robust purification methods that do not rely on chemical reactions. This was the aim of the following parts of this work.

3.2. LC purification method

3.2.1. Preliminary optimization

Preliminary HPLC separation trials using the usual silica column employed for MOSH/MOAH separation were performed with a mixture of MOSH/MOAH standards, PAHs, and HVGO, containing MOAH compounds with up to 6 aromatic rings.

The mixture was first eluted with only *n*-hexane, and each elution window of 90s was analysed by GC×GC-MS. As expected, on the one hand, MOSH elution times remained similar to the ones obtained with the classical gradient used for MOSH/MOAH separation; on the other hand, the MOAH fraction became more spread. It is noteworthy that, within a group of PACs with the same number of aromatic rings, increasing the degree of alkylation (i.e., decreasing polarity) reduces retention times in LC, causing alkylated PACs to elute earlier than their corresponding PAHs, both because of decreased polarity and higher size exclusion effects.

The ultimate goal was to evaluate the elution of the common MOAH interferences; therefore, squalene, lycopene, β-carotene, trans-β-carotene, and β-caryophyllene were added to the mixture as well. The four first olefins were eluted at the end of the MOAH extended fraction, while and β-caryophyllene was only slightly retained and eluted with the mono- and diaromatic MOAH. These results appeared very promising, but the elution with only *n*-hexane was considered not feasible as the high rings MOAH (e.g., BaP, DBahA, BghiP) were too retained (even 40 min).

Therefore, several trials involving DCM at different percentages were tested to optimize the elution. Eventually, a method lasting around 21 min was developed, with a gradual increase of DCM to until 5%, which fully excluded squalene from the polyaromatic MOAH fraction. Noteworthy, as squalene was eluting close to DBahA and IP, the latter were excluded from the collected fraction.

Figure 2 illustrates the purification obtained in palm oil using the developed HPLC gradient. Squalene and carotenoids were removed significantly. The selected gradient was translated in the LC/GC×GC-FID/MS platform and further evaluated.

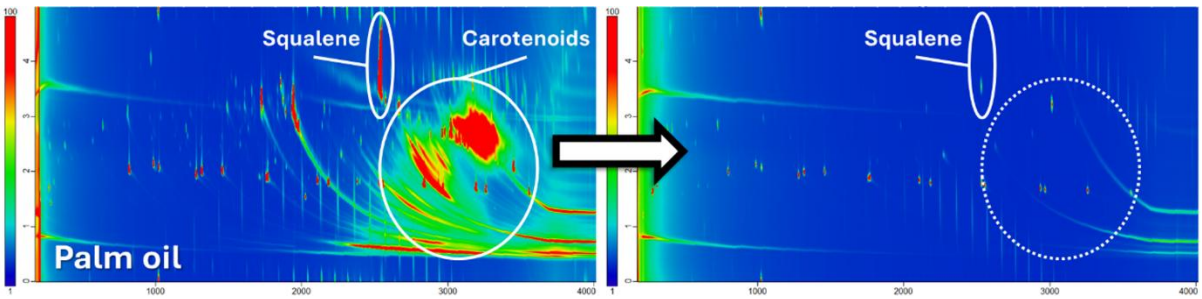


***Figure 2** – Purification of interferences from the MOAH fraction of palm oil using HPLC purification.*

3.2.2. Evaluation of the LC purification method for total MOAH

With the configuration present at the time of the experiments in our lab, the main limitation was that only fractions that could be accommodated in 1.5 mL autosampler vials could be collected. This meant that the purified MOAH fraction was collected in four different vials and care should be taken to quantitatively merge all the fractions. Nevertheless, work on the direction of a single collection is ongoing, and by adapting the system it is feasible to simplify this collection step.

The performances of the optimized LC method were evaluated by increasing sample complexity starting from the recoveries obtained on a mixture of PAHs plus the MOAH internal standards in *n*-hexane (n=3), then pure MOAH (n=3), and finally on different edible oils spiked with different MOAH sources (n=2 for each oil).

Exhaustive recoveries were obtained for all the PAHs and PACs standards in *n*-hexane, except for IP (6 rings) and DBahA (5 rings) as aforementioned. Interestingly, BghiP (6 rings) and Cor (7 rings) showed instead quantitative recoveries (**Figure 3)**. In the routine method > 5 rings are also partially lost [16], but at different proportions, indeed a recovery of 21.9 % was reported for benzo[rst]pentaphene (which presents a structure very similar to DBahA), and 66.8% was reported for Cor, for which we had a quantitative recovery. Furthermore, non-alkylated PACs typically elute before their alkylated counterparts because of size-exclusion effects, although very weakly alkylated compounds can be also eluted later (MNs typically elute later than naphthalene, as will be shown in **paragraphs 3.2.3** and **3.2.4**). Therefore, alkylated pentaromatics will still be recovered, as well as some more heavily alkylated hexa- and heptaromatics. This is further discussed in **paragraph 3.2.4.**

Worthy to stress, as reported also by Biedermann *et al*. [16], that it is very unlikely to have MOAH with >5 rings above the limit of quantification in real-world samples [3].

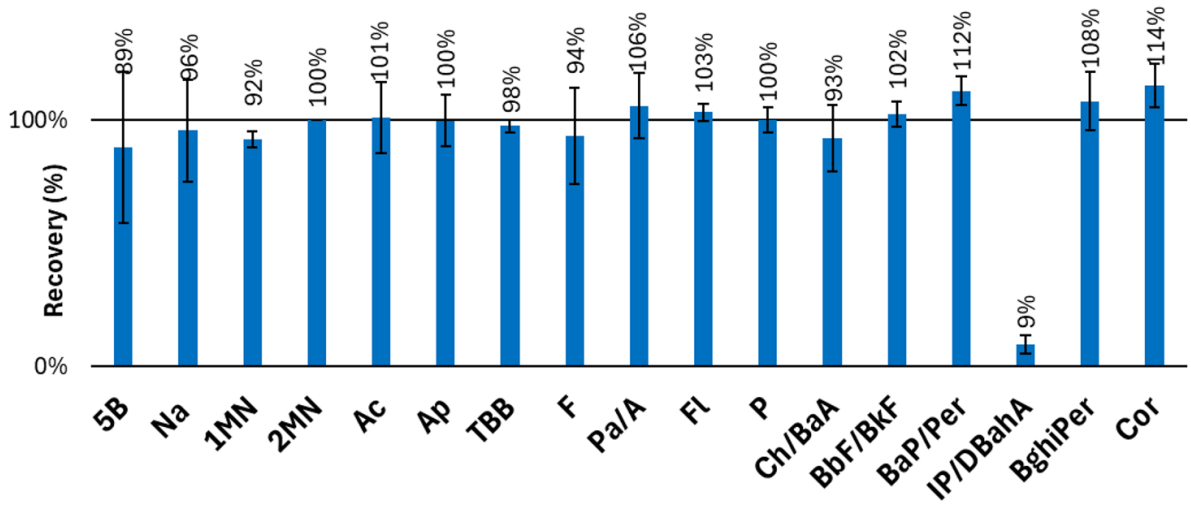


Figure 3 *– Recovery (%) (n=3) after LC purification of different standard polycyclic aromatic compounds (2.5-5 mg/L) using 2MN as an internal standard.*

The evaluation continued considering three pure MOAH sources dissolved in *n*-hexane covering different distributions, namely Gravex, Sternel motor oil, HVGO. The GC×GC profile of these MOAH sources is reported in **Figure S4**. Gravex yielded an average recovery of 117% ± 2%, Sternel motor oil of 89% ± 12%, and HVGO of 116% ± 17%. These results met the requirements of the JRC guidance [13].

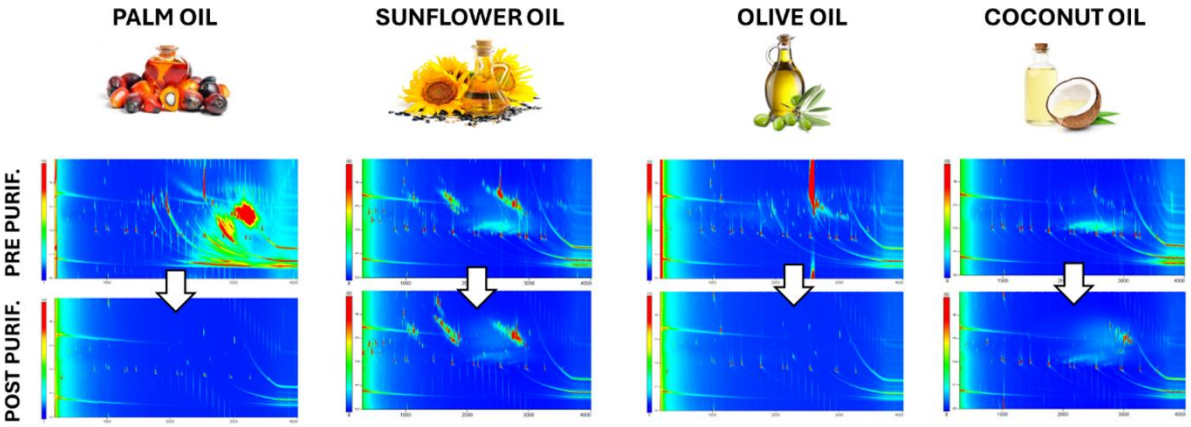


Figure 4 *– GC×GC-FID chromatograms of the MOAH fractions of different vegetable oils (palm, sunflower, olive and coconut oil) before and after the LC purification process, and MOAH recovery (%) following the purification (average ± standard deviation).*

Finally, the HPLC purification method was applied to four different vegetable oils of common use (*i.e.*, palm, sunflower, olive, and coconut oils) after a saponification/extraction procedure (**Figure 4**). In terms of recovery evaluation, the first three oils were spiked in-house: palm oil with a mixture of Gravex, SN100 Aromatic Extract, and SN500 Aromatic Extract (7.5 mg MOAH/kg), coconut oil with Sternel motor oil (64 mg MOAH/kg), and sunflower oil also with Sternel motor oil (48 mg MOAH/kg). The spiked olive oil was sourced from an interlaboratory trial and contained 37.55 mg/kg MOAH (quantified using TBB). The idea behind the use of different MOAH compositions and concentrations was to evaluate whether these two parameters had a significant influence on the recovery, which showed to not be the case here.. Furthermore, MOAH recoveries were once again consistent with the requirements of the updated JRC guidance [13]. Indeed, the MOAH recoveries were as follow: 92 ± 8 % for palm oil, 93 ± 4 % for sunflower oil, 98 ± 6 % for olive oil, and 94 ± 2 % for coconut oil

The method was efficient for removing more polar interferences such as carotenes and squalene (or derivatives) from palm and olive oil, giving at the same time excellent recoveries (i.e., 92% ± 8% and 98% ± 6%, respectively). For palm oil, the application of the epoxidation purification led to a recovery of 92 % ± 21 % and 58 % ± 5% using mCPBA and performic acid, respectively (TBB being used as the internal standard). For the olive oil sample, epoxidation was not performed due to a lack of sample, but it was considered that the effect of variability and uncertainty associated with the epoxidation step was well illustrated in the JRC report [6].

In the case of coconut oil, additional purification was not really needed, but it can be assumed that routine lab with automatic sample prep, will just run all the edible oils including the epoxidation step as needed in the majority of the cases. In this regard, the use of the proposed purification allowed for a quantitative recovery of the added MOAH. Indeed, recovery of 94% ± 2% was obtained compared to 82% ± 10% and 71 ± 16% obtained with epoxidation with mCPBA and performic acid, respectively. In the coconut oil chromatogram, as can be seen, most probably a contamination occurred during manipulation which led also to a higher content of interfering compounds, but this did not impact the final result.

Finally, the LC purification method did not fully succeed in removing smaller terpenes from sunflower oil (**Figure 4**), for which further investigation will be necessary. Nevertheless, a possible workflow (although more complicated) is presented in the next paragraph.

3.2.3. Separation of the 1-2.5 rings and the ≥3 rings MOAH

Studying the elution of the MOAH in the silica columns it was also possible to highlight that a rather good separation between the 1-2.5 rings MOAH and the ≥3 rings was possible, with 2.5 rings corresponding to compounds having two aromatic rings and one that is partially saturated. Indeed, collecting the fraction between 3.5 min and 9.5 min, separately from the remaining elution time, it was possible to separate the MOAH into the two toxicologically-different relevant MOAH sub-classes. In particular, in sunflower oil, the terpenic interferences were split between the two fractions having a much lower amount in the >3 fraction, thus facilitating the integration of the latter without having to resort to epoxidation, which mainly impacts this class of MOAH (**Figure 5**) [12]. On the contrary, if necessary, the <3 rings can undergo epoxidation to try to further reduce the number of remaining interferences, as this MOAH are not highly impacted by epoxidation. The possibility to separate the MOAH sub-fractions in the LC elution gives the possibility to quantify them also using the more conventional monodimensional GC, thus opening to the possibility of monitoring on a routine basis the occurrence of both sub-fractions as required by the latest EFSA opinion from 2023 on MOSH/MOAH [3].

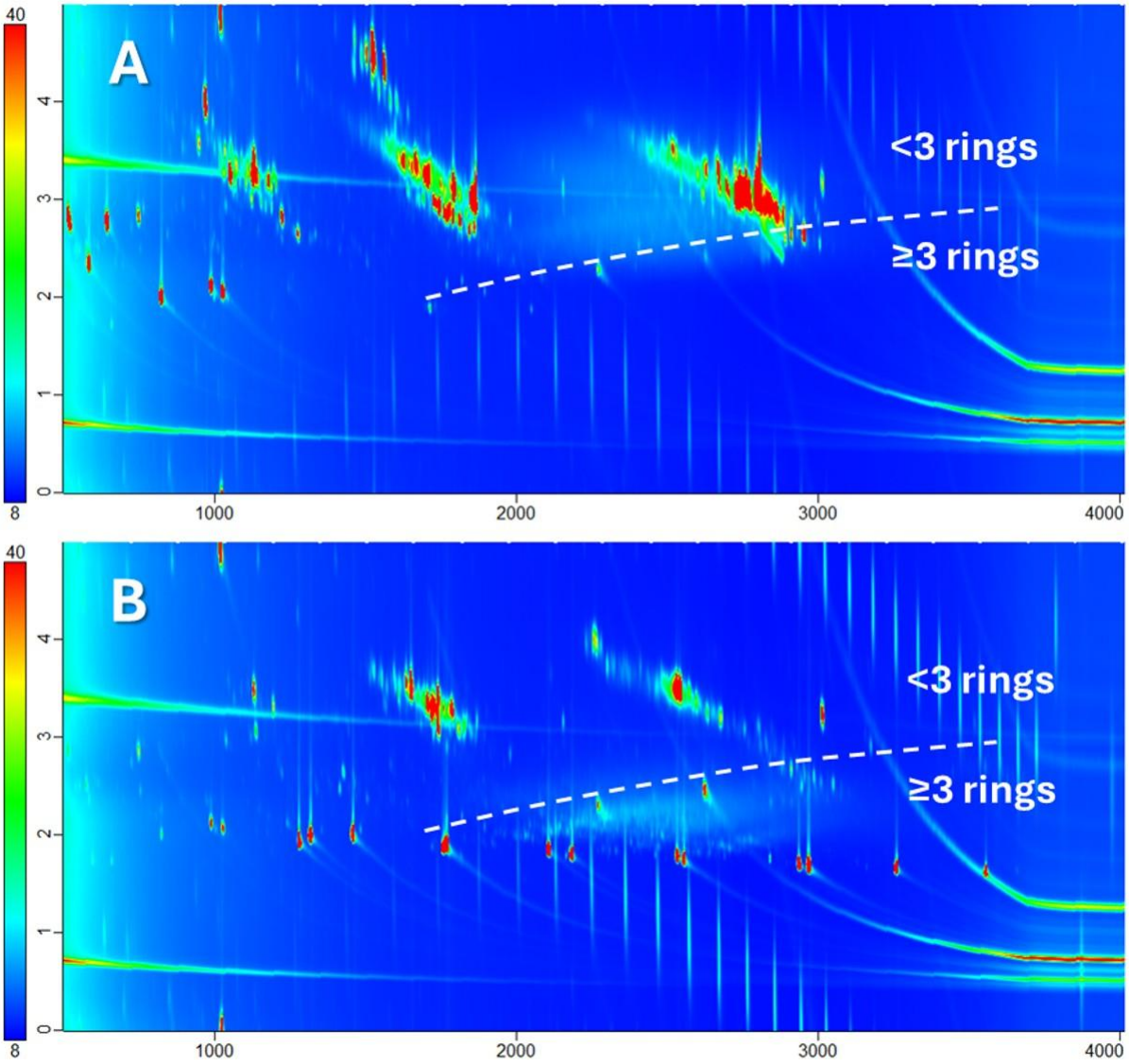


Figure 5 – *GC×GC-FID chromatograms of the < 3(A) and ≥ 3 (B) ring MOAH fractions from sunflower oil obtained using the LC purification gradient.*

3.2.4. Control of the LC elution.

The efficiency of the HPLC purification as well as of the separation of MOAH compounds based on the number of aromatic rings heavily relies on the proper optimization of the elution conditions and collection windows. Such an optimization can be performed with the help of a standard mixture of polyaromatic compounds (such as PAHs), which give a signal in the UV detection in any online platform routinely used in MOSH and MOAH analysis, as shown in **Figure 6**.

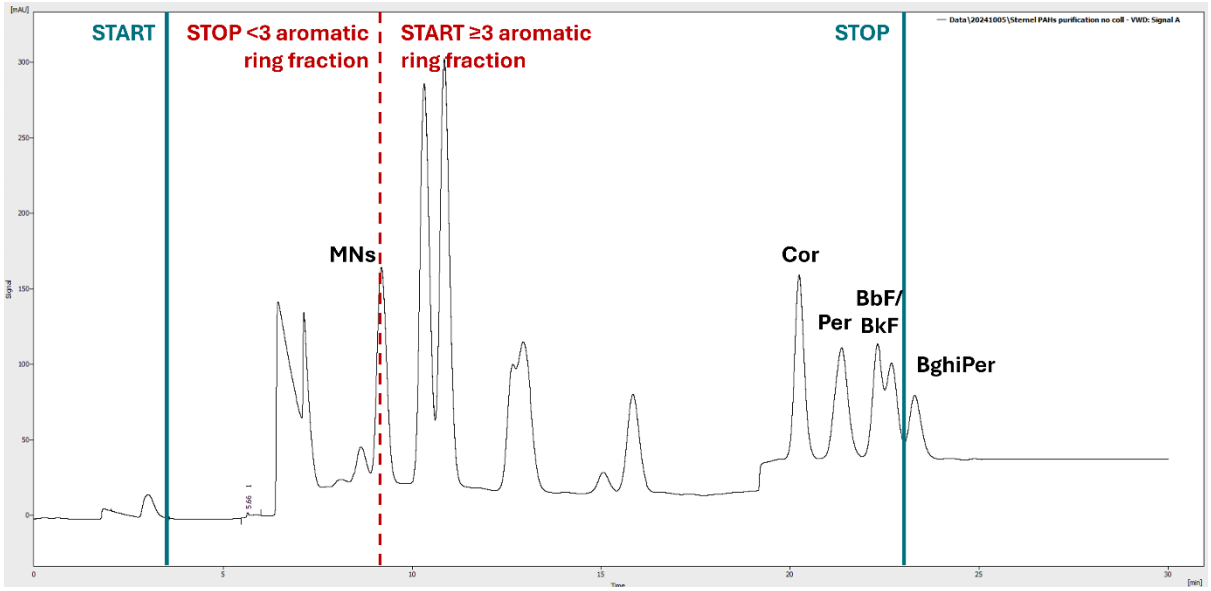


Figure 6 – *HPLC-UV chromatogram (230 nm) of a mixture of MOAH IS and PAHs during the HPLC purification step.*

BbF and BkF peaks were identified as good end markers of the window to be collected, to ensure satisfactory purification. Futhermore, this end window to recover all MOAH compounds that are relevant in food [16]; only 6-7 ring compounds were (partially) lost (**figure 6**).

Regarding the separation of the 1-2 and 3+ rings, the split of the collection window was performed around the MNs' peaks. This approach allowed for the isolation of a MOAH fraction containing compounds with 1 to 2.5 rings, with minimal amounts of 3+ ring compounds (**figure 7B**), since most alkylated PACs elute before their parent PAH due to size-exclusion effects. However, as stated earlier, weakly alkylated compounds can elute later than the parent compound. For instance, naphthalene is fully recovered in the 1-2 ring fraction, while MNs (corresponding to a naphthalene structure with an extra methyl group) tend to tail into the 3+ ring fraction, meaning that they elute later.

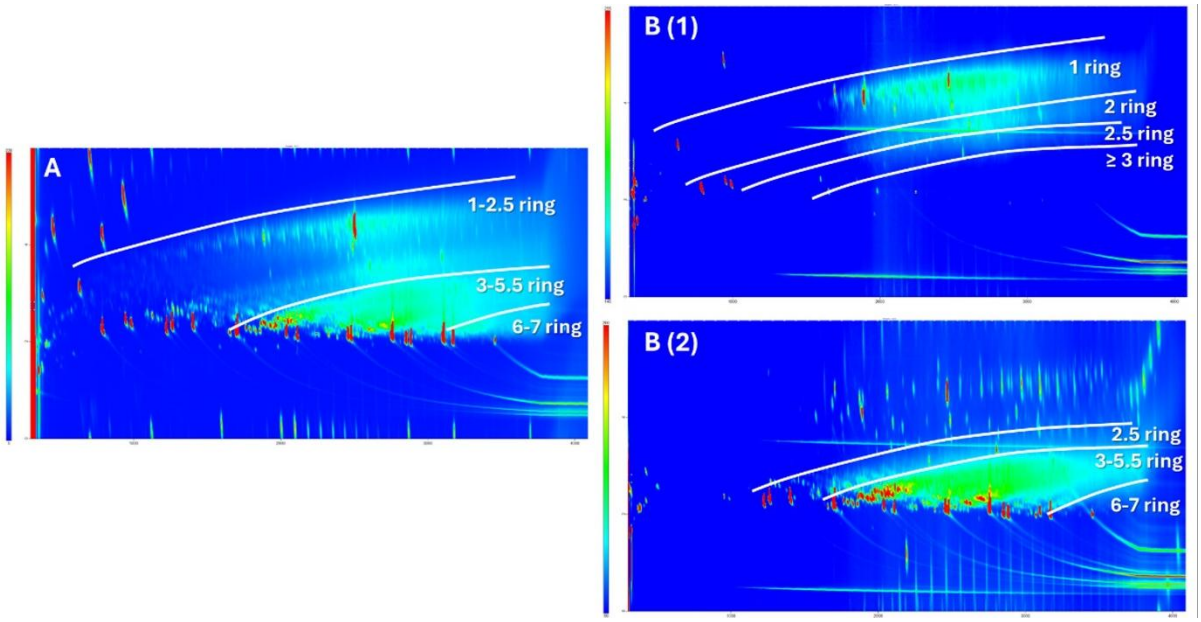


***Figure** 7 – MOAH from heavy vacuum gas oil A) before LC purification, B) after LC purification (1) 1-2 fraction and (2) ≥ 3 ring fraction.*

4. Conclusion, limitations, and perspectives

The removal of biogenic interferences from the MOAH fraction is one of the major challenges in MOAH analysis, as their profile and abundance can vary significantly between matrices. The most widely used approach involves applying an epoxidation step to the sample, which, although efficient in removing π-electron-dense interfering compounds, suffers from a lack of selectivity, leading to MOAH and internal standard losses.

In this work, we evaluated an alternative purification method based on normal-phase HPLC purification, employing the same eluents and LC-silica column conventionally used for MOSH/MOAH analysis. This represents the main advantage of the proposed method, as no modification is needed to the system used routinely for MOSH and MOAH analysis.

This method proved efficient in removing carotenes, squalene, and derivatives, which are notably abundant in palm and olive oils. Additionally, PACs recovery was more consistent and predictable compared to epoxidation methods, where manual steps in time and temperature control often lead to irreproducible results. In terms of MOAH quantification, the analytical performance met the requirements of the JRC guidance for MOSH/MOAH analysis. The proposed method also offers the

advantage of separating MOAH by the number of aromatic rings, allowing for the separate quantification of 1-2 and >3 rings, either in GC or GC×GC.

Nevertheless, further work is needed to fully validate the proposed method, applying it to a larger variety of samples, thus covering a more extensive distribution and quantity of possible occurring interferences. Although a quality control strategy has been proposed, the method needs to be tested in other laboratories, using different systems to assess its robustness, as possible differences in the dwell time of the LC system and dead volumes in the connections can slightly impact the fractions windows.

At present, the high volume of the purified MOAH fraction (about 5 mL) is one of the main limitations of the proposed method. New trials are ongoing to reduce this volume without impacting the purification.

Regarding automation, the primary challenge lies in concentrating the collected fractions for quantitative reinjection into the HPLC system. This issue can be resolved by incorporating commercially available automated evaporation modules, which are already employed by some laboratories for automated MOSH/MOAH sample preparation. Additionally, using conical-bottom vials would eliminate the need for manual intervention.

Finally, additional investigation is needed to improve the purification of the remaining terpenic compounds (notably in sunflower oil). Other column stationary phases will also be investigated for this purpose, although this would complicate the design of the entire method, requiring the connection of two different columns.

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Declaration of interest statement

The authors report there are no competing interests to declare.

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