Multidimensional Gas Chromatography Techniques and Sample Preparation: Crucial Aspects in Mineral Oil Hydrocarbons Routine Analysis

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Mineral oil hydrocarbons (MOSH and MOAH) analysis in food presents an analytical challenge requiring advanced chromatographic techniques and carefully designed sample preparation methods. MOSH and MOAH are routinely analyzed by hyphenated liquid-gas chromatography–flame ionization detection (LC-GC-FID), but the need for more detailed information on their composition for accurate risk assessment has recently driven the adoption of even more sophisticated techniques in routine laboratories. Notably, the use of GC×GC has become almost routine, allowing to unravel the complexity of mineral oil hydrocarbons composition while also reducing the uncertainty associated with data integration and interpretation. Nevertheless, the uncertainty related to the sample preparation steps, particularly extraction/saponification and epoxidation, still requires improvement. In this work, recent advances in MOSH and MOAH sample preparation and analytical determination are critically discussed.

MINERAL OIL HYDROCARBONS (MOH) are complex mixtures of petrogenic-origin contaminants consisting of two main classes, namely the mineral oil saturated hydrocarbons (MOSH) and the mineral oil aromatic hydrocarbons (MOAH), which pose different toxicological concerns and analytical challenges (1). Since 2009, a robust hyphenated liquid-gas chromatography–flame ionization detection (LC–GC–FID) method has been introduced to separate MOSH from MOAH (as well as both fractions from the coextracted compounds) and quantify them separately (2). In this method, a series of internal standards (ISs) are added to quantify MOSH and MOAH and to control the different steps of the analytical workflow, including LC separation, online transfer from LC to GC, and GC separation. Verification of the method involves checking the presence of these ISs and their relative ratios. The ISs routinely used today are cholestane (Cho), cyclohexyl-cyclohexane (Cycy), *n*-C11, *n*-C13, tri-tert-butyl benzene (TBB), pentylbenzene (5B), 1- and 2-methyl naphthalenes (MNs), and perylene (Per). Cho indicates the end of the MOSH fraction elution from the LC column, although Cycy has been proven a better marker for the LC separation (3,4). TBB and Per mark the beginning and the end of the MOAH LC elution band. Di(2-ethylhexyl) benzene (DEHB) was pointed as a more suitable marker but elutes in a crowded area of the GC chromatogram, preventing its routine use. Volatility losses of MOSH and MOAH during the online transfer from the LC to the GC or during offline evaporation are detected by *n*-C11 and 5B, respectively. Quantification of the total contamination is based on Cycy and *n*-C13 for MOSH and, 1- and 2-MN for MOAH. The quality control of the method relies on maintaining specific ratios between these standards in each sample.

Despite the well-designed method, the uncertainty associated with MOH analysis remains relatively high. This is mainly due to data interpretation and integration, estimated to account for more than 20% of the total variability (5-8). To address this, the use of GC×GC has been proposed to support interpretation and facilitate integration (9-11). Additionally, the Joint Research Center (JRC) has published a guidance document on data interpretation and integration (focusing at that time on MOH analysis in infant formula) (12).

However, the reliability of MOH analysis heavily depends on the sample preparation steps prior to the LC-GC-FID analysis, which includes extraction/enrichment and purification of MOSH and MOAH from interferences deriving from the contaminated matrix. So far, attempts to control determination uncertainty have focused on harmonizing and standardizing sample treatment methods (4,13-18).

The Role of Comprehensive Two-dimensional Gas Chromatography (GC×GC)

GC×GC was introduced in the field of MOSH and MOAH in 2009 to improve the characterization of what is hidden behind the unresolved complex mixtures generated by the high number of isomers present in the

MOSH and MOAH fractions, and to support the verification of the presence or absence of naturally occurring interferences (19). The initial role of GC×GC was only qualitative, but more recently, dedicated software has been developed (20-22) and validated (23) for reliable quantification in 2D chromatograms. Indeed, the 2D plot obtained by GC×GC allows for (i) better characterizing the unresolved complex mixture of MOH in its sub-classes (Fig 1[a]); (ii) reducing the uncertainties related to the baseline positioning in LC–GC–FID chromatograms (Fig 1[b]), as the bleeding is chromatographically separated from the MOSH and MOAH fractions; (iii) reliably identifying and quantifying the ISs for quantification in particularly cumbersome matrices (Fig 1[c]); (iv) supporting plot interpretation using both 2D or 3D visualization, especially regarding the removal of riding peaks (which can also be partially chromatographically separated) (Fig 1[d]).

A more detailed discussion on the benefits of using GC×GC is reported in a recent review (19). It is important to stress that while a simple GC×GC–FID, equipped with a suitable injection technique that does not cause discrimination towards highly boiling compounds (such as on-column injection), is sufficient for most of the confirmation purposes mentioned, coupling with a mass spectrometer detector (MS) remains important for additional confirmation purposes and for the detection of markers.

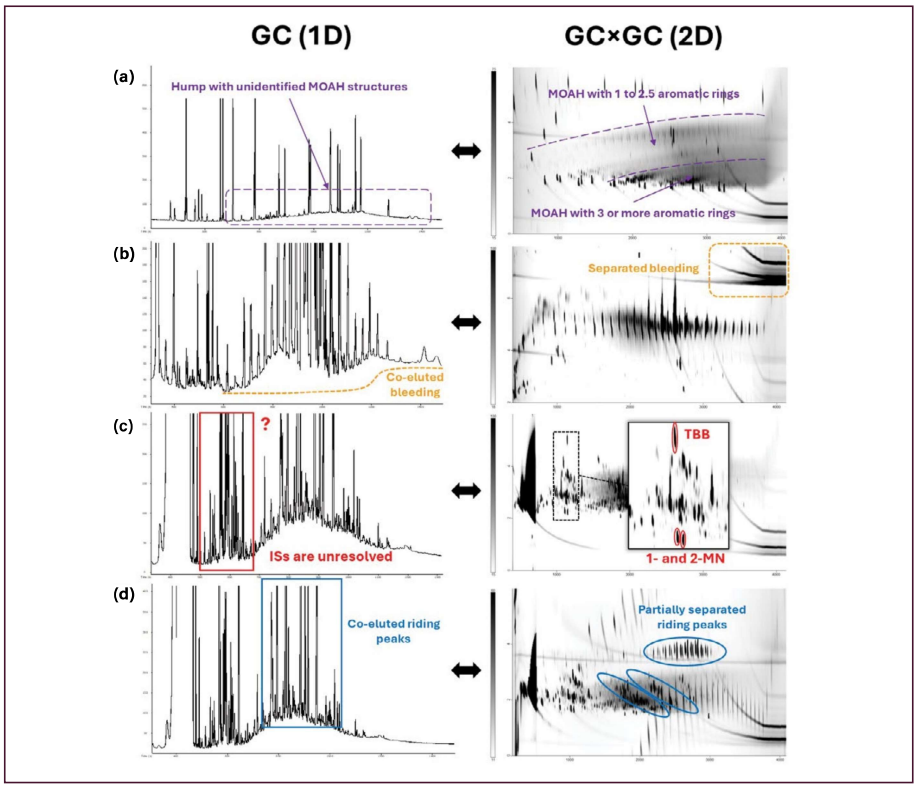

FIGURE 1: Examples of samples analyzed in GC–FID and GC×GC–FID to pinpoint the advantages of the use of GC×GC over GC in MOH analysis. (a) GC (1D) no information on the sub-classes, GC×GC: sub-classes characterization (example of MOAH separated based on the aromatic ring numbers); (b) GC (1D) problems in defining the baseline, GC×GC elution of the bleeding in a chromatographic area of no-interest; (c) GC (1D) impossible determination of the internal standards due to coelution, GC×GC separation and more accurate quantification of the ISs; (d) GC (1D) difficult interpretation of the contribution of the interferences to the total hump, GC×GC easier interpretation of the interference compounds in the 2D space.

The Key Role of Sample Preparation: Saponification and Purification

Despite attempts at harmonization through the establishment of a decision tree for the analytical workflow published by the JRC, interlaboratory uncertainty can still be as high as 60% (14,15). Therefore, a process of standardizing some analytical methods has started, notably for infant formula (17,18) and vegetable oils and fats (16). In both these methods, a saponification step is mandatory to reach the sensitivity required. However, this step has been shown to contribute additional variability due to an uneven distribution of the MOAH ISs (namely TBB and MNs, which should have a ratio of 1.0) in the different solvent phases (4, 24). The JRC Guidance suggests using TBB rather than MNs when the ratio is not respected, as TBB is claimed to be better extracted than the MNs, and reports that a ratio around 1.15 is commonly found (4). In the recently published ISO-20122/2024 method (16), a TBB/2MN ratio up to 1.25 is tolerated. Contrary to the initial purpose of standardization, this situation contributes to the overall uncertainty of the results, intrinsically accepting uncertainty of up to 25% on top of the variability due to the interpretation and integration of the chromatograms.

Very recently Bauwens and colleagues published a paper focused on improving the saponification step with the goal of minimizing the deviation of the TBB/MNs ratio from 1 (25). The results clearly showed that the different partition of the TBB and MNs in the extraction solvent was mainly due to the saponification conditions (solvent and ionic strength), along with a strong matrix effect (Figure 2[a] and [b]).

The conditions tested were selected based on different saponification methods reported in the literature (25). To evaluate the ISs distribution trend, the following solvents or mixtures proportions were tested: pure MeOH, EtOH/H_2O 4/1, 1/1, and 1/4 (v/v). Ratios closer to 1 were obtained with KOH 2M in EtOH/H_2O 1/1 (v/v), while high deviations were obtained with the use of saturated KOH MeOH solution and EtOH/H_2O 4/1 (v/v) solution, which showed a much higher extraction of TBB. The partition of Per was also significantly impacted, a factor that is rarely assessed as lost during epoxidation. Despite using the best solvent mixture from

TABLE I: Comparison of the TBB/2MN ratio obtained when using KOH 2 M in EtOH/H_2O 1/1 (v/v) to the tolerance level admitted in the DGC/ISO-20122 method (16) and JRC Guidance (4). * Above the threshold of the updated JRC guidance (2023).

Matrix	Target TBB/2MN ratio	Max. TBB/2MN ratio – JRC guidance	Max. TBB/1MN ratio – ISO-20122/2024	TBB/2MN ratio obtained with ISO-20122/2024	TBB/2MN ratio with KOH 2 M in EtOH/ H2O 1/1 (v/v)
SFO	1	1.15	1.25	1.13 ± 0.02	1.04 ± 0.02
CCNO	1	1.15	1.25	1.11 ± 0.02	1.04 ± 0.01
RSO	1	1.15	1.25	1.18 ± 0.00*	1.05 ± 0.02
PO	1	1.15	1.25	1.17 ± 0.02*	1.06 ± 0.00
EVOO	1	1.15	1.25	1.10 ± 0.07	1.04 ± 0.02
AVERAGE	**1**	**1.15**	**1.25**	**1.14 ± 0.04**	**1.05 ± 0.01**

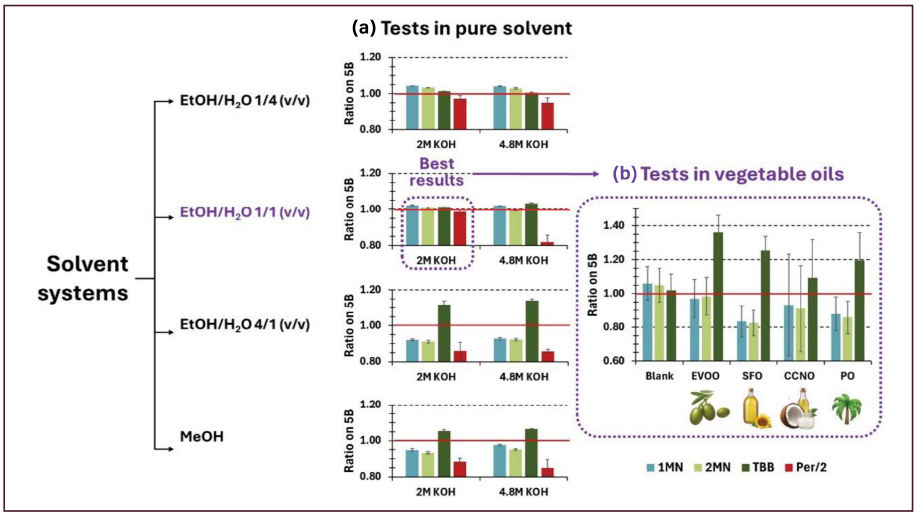

FIGURE 2: Ratios between each MOAH internal standards (1MN, 2MN, TBB or Per/2) and 5B in hexane after (a) Liquid-liquid partition from different solvent mixtures proposed for the KOH solution; (b) Saponification of different oils with 2M KOH in EtOH/H_2O (1/1 v/v). The data are represented as the mean and the standard deviation of four replicates.

the preliminary experiments, i.e., KOH 2 M in EtOH/H_2O 1/1 (v/v), different oil samples significantly impacted the ISs ratios (Figure 2[b]). Different washing procedures were tested to extract the unsaponifiable, without any impact on the IS ratios. Therefore, the use of only H_2O was maintained to limit the use of organic solvents. Finally, the proposed saponification method (2M EtOH/H_2O (1/1 v/v) + 10 mL C6 and washing with 20 mL of H_2O) was compared with the recently approved ISO-20122/2024 method, proving much more robust results in terms of ISs ratios, mainly referring to the monitored ratio TBB/2MN (Table 1) and recoveries in the 80–110 % range for all the tested oils and fats. Noteworthy, the TBB/2MN ratio consistently averaged around 1.05 ± 0.01, significantly lower than the 1.15 or 1.25 tolerated by the JRC Guidance and the DGF/ISO-20122 method, respectively.

Besides saponification, another sample preparation step that contributes to the uncertainty of the quantification is the purification of biogenic interferences, particularly those co-eluting with MOAH. To separate these natural interfering olefins, an epoxidation reaction is generally applied. This reaction introduces an epoxy bond at one or more double bonds in the olefin, increasing its polarity and thereby enhancing their retention in the LC compared to MOAH. Different epoxidation procedures can be applied, varying by the reagent used (*meta*-chloroperbenzoic acid or performic acid), the solvent (*n*-hexane or chloroform), and consequently, the reaction kinetics and purification efficiency (26).

However, epoxidation is a non-selective reaction that affects MOAH and MOAH ISs. Therefore, its robustness is limited, requiring precise control of time and temperature for reproducibility, especially with faster reaction kinetics. Additionally, it leads to variable MOAH losses based on their composition. Moreover, MOAH quantification standards (TBB and 2MN) exhibit different epoxidation rates, meaning that the choice between TBB and 2MN may lead to inconsistent results.

Consequently, there is a current need to develop alternative purification methods that offer enhanced robustness compared to reaction-based approaches. Such an initiative was recently presented by Gorska and colleagues during the 2024 AOCS Annual Meeting & Expo (27), whose results demonstrated that common major interferences such as squalene or carotenoids could be effectively removed using LC purification methods. This approach also achieved high and consistent recoveries for spiked standard compounds and MOAH, which endorses this as a promising direction for further investigation and development. A chromatographic method in place of chemical reaction-based purification would be a desired improvement, enhancing overall robustness. In addition to robustness improvement, this purification method has the advantage of requiring only standard equipment used for common MOH analyses, specifically LC instrumentation.

Conclusions

The use of advanced chromatographic techniques, although fundamental to improving the robustness of the method and enhancing the level of understanding of the contamination observed, cannot disregard the application of robust and properly optimized sample preparation methods for accurate final quan-

tification. This is particularly true in highly complex matrices where multiple sample preparation steps are required to purify the fraction of interest as in the case of many food samples, for instance, fats and oils or spices.

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Declaration of interest statement

The authors have no financial or proprietary interests in any material discussed in this article. The authors have no relevant non-financial interests to disclose.

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