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# EVOLUTION OF HYPHENATED TECHNIQUES FOR MINERAL OIL ANALYSIS IN FOOD

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Non standard abbreviations

CEE: Concurrent eluent evaporation

Cho: Cholestane

Cycy: Cyclohexylcyclohexane

DEHB: Di(2-ethylhexyl)benzene

EU: European Union

EFSA: European Food Safety Authority

FCM: Food contact material

ISs: Internal standards

JRC: Joint Research Center

MAE: Microwave-assised extraction

MOAH: Mineral oil aromatic hydrocarbons

MOH: Mineral oil hydrocarbons

MOSH: Mineral oil saturated hydrocarbons

NOAEL: No observed adverse effect level

PCEE: Partially concurrent eluent evaporation

Per: Perylene

PLE: Pressurized liquid extraction

POSH: Polyolefin oligomeric saturated hydrocarbons

PTV: Programmed temperature vaporizer

SVE: Solvent vapor exit

TAGs: Triacylglycerols

TBB: tri-tert butylbenzene

UCM: Unresolved complex mixture

VUV: Vacuum ultraviolet detector

# Keywords: Aromatic hydrocarbons; Food; Hyphenated chromatographic techniques; Mineral oil; Saturated hydrocarbons.

# ABSTRACT

The occurrence of mineral oil in food is known since the early 90s, and it was discovered by chance in one of the first applications using the hyphenated LC-GC system. Since then, the relationship between hyphenated techniques and mineral oil analysis has been tightly interrelated and successful.

This review aims to show and explain how this mutual interaction has driven the development of the hyphenated techniques on one side and has supported the increase of knowledge on the other, supporting the complex task of mineral oil determination in food.

The paper presents the background of the mineral oil problem in food (a brief history of its finding, toxicology, and occurrence), moving then towards the analytical determination. The development of different hyphenated techniques in relation to mineral oil determination is discussed, focusing mainly on two-dimensional techniques, such as LC-GC. The necessity of additional dimensions, such as LC-LC-GC and comprehensive approaches, such as GC×GC and LC×GC, is also discussed. Finally, the role of the hyphenation with MS is presented.

# 1. Introduction

The determination of mineral oil hydrocarbons (MOH) in food and the development of hyphenated techniques, in particular liquid chromatography with gas chromatography (LC-GC), have always been tightly connected. The two topics have mutually interacted over the years, supporting and driving their mutual development. The first LC-GC hyphenation able to handle the transfer of a large volume of solvent was presented by Grob *et al.* in 1984 (*i.e.*, the on-column interface [1]) and was applied in the determination of degradation products formed by irradiation of food. By chance, a sample of hazelnuts was found unexpectedly contaminated by MOH, present as an unresolved complex mixture (UCM) [2]. Its petrogenic origin was confirmed by the presence of phytane and pristane markers. The contamination was later proved to originate from the jute bags (typically softened with batching oil) where the hazelnuts were stored and transported.

The first systematic study on the contamination of food by MOH appeared in 1991 [3]. Contextually, the LC-GC technique experienced success in the 1990s [4], but it did not encounter the expected success for another decade, until the case of MOH exploded at the public opinion. In 2009 the discoveries of a highly contaminated Ukrainian sunflower oil sample [5] and of significant migration of MOH from recycled paperboard used in food contact material (FCM) [6] led to a renewed popularity of LC-GC. The extremely high amount of MOH found in the Ukrainian sunflower oil (>1000 mg/kg) needed the quantification of the aromatic fraction (MOAH) within the MOH UCM hump (before it was estimated based on the possible origin of the MOH contamination). Therefore, a method to separate the two main fractions of MOH, namely mineral oil saturated hydrocarbons (MOSH) and MOAH, was developed [7]. Such an LC-GC method still represents the reference for MOSH and MOAH determination, as pinpointed by the European Food Safety Authority (EFSA) opinion in 2012 [8], and has led to the rebirth of the long-neglected LC-GC technique. Dealing with the extremely high contamination of the Ukrainian sunflower oil, it was also observed that the on-column interface was affected by a memory effect equivalent to 0.5-3% of the previous transfer. Therefore, a novel interface was developed (*i.e.,* Y-interface), reducing the carryover to 0.02% [9]. Moreover, in the same year, *i.e.,* 2009, the necessity to understand the source of the Ukrainian sunflower oil contamination brought into play another key hyphenated technique, namely comprehensive two-dimensional GC (GC×GC), which allowed to obtain a detailed characterization of the MOSH and MOAH fractions [10]. Since then, both LC-GC and the MOH contamination topics have started to grow in tight connection, followed, more recently, by GC×GC as a confirmatory method (Figure 1). GC×GC has also played a crucial role in adjusting the LC-GC reference method. In fact, in 2017, following detailed GC×GC studies, the mixture of standards selected in 2009 [7] as quality control and for quantification was updated [11]. In 2020, Koch *et al.* used GC×GC to support the development of a method to separate the mono-, di- from the tri- and the poly-aromatics and quantify them separately [12]. In May 2020, a co-authored paper by two recognized institutions, namely the Joint Research Centre (JRC) of the European Commission and the Official Food Cantonal Laboratory of Zurich, clearly stated that GC×GC is the best technique for verification purposes [13], confirming the previous suggestion reported by the EFSA in its opinion on MOH in food published in 2012 [8].

This paper aims to review the fundamental steps through the evolution and implementation of hyphenated techniques as an essential support for understanding the complexity of the MOH contamination in food. First, an introduction to the MOH problem in terms of toxicity and legislation will be provided, as well as a brief summary of the extraction and purification techniques commonly applied. The main topic will then be the application and evolution of hyphenated techniques for both MOSH and MOAH determination in food.

Figure 1 reports the number of papers published since 1989 on the presence of mineral oil in food. The count did not include reviews and papers dealing with toxicological assessment, while studies on the migration from packaging material, even if in simulant, where reported as tightly related to the occurrence in food. Within the papers on MOH determination, the ones using LC-GC are also counted separately, as well as the ones that include GC×GC in their experiments. It appears evident as almost all the papers dealing with MOH determination have relied on the LC-GC hyphenated technique, with an increasing number over the last decade supporting their finding with also GC×GC.

**2. Evolution of the European framework on mineral oil and state-of-the-art on toxicity and exposure.**

The commonly used definition of MOH as a complex mixture counting a large number of structural isomers and divided into two main fractions, namely MOSH (composed by linear, branched paraffins, and alkyl-substituted cycloalkanes) and MOAH (which include mainly alkyl-substituted (poly)aromatic hydrocarbons with a different number of fused rings) was introduced by Biedermann *et al.* in 2009 [7]. This definition was based on the outcomes of the LC-GC separation into the two main fractions. Along with MOSH and MOAH, the analytical separation may detect the presence of the so-called MOH analog mixtures (such as polyolefin oligomers migrated from FCM) and “white mineral oil” (highly refined mineral oil where the presence of aromatic compounds is minimized). The use of both is permitted for certain applications under specific regulated requirements (*e.g*., migration limits or well defined molecular mass distribution). Their presence contributes to the total MOSH determination, summing up to MOSH from other not permitted sources, thus complicating the compliance assessment with legal limits.

As said, the problem of MOH stepped in the light of the public concern in 2008/2009 [5,6], shortly after, in 2012, the EFSA published an Opinion on MOH in food [8]. It highlighted the urgency of the matter, but no definitive conclusions were drawn due to the limited information available. In 2017, the EFSA and the European Union (EU, Commission Recommendation 2017/84 [14]) required the collection of more data to evaluate the risk associated with their intake. However, the Guidance reporting the information on how to report the data were published only in February 2019 by the JRC [15]. In this Guidance, the carbon range considered for the MOSH and MOAH humps was established in the C10-C50 range, requiring to guarantee a response ratio of C50 on C20 between 0.8-1.2 (when a solution at the same concentration for each hydrocarbon is injected). Moreover, the quantification of the UCM hump should be reported in sub-fractions (also called C-fractions), assuring to reach the given targeted limit of quantification (LOQ), recovery, and intermediate precision for each the sub-fraction, regardless of the analytical procedure applied (*i.e.,* on-line or off-line) [15]. An exhaustive overview of the evolution of the proposed limits is reported in [16].

In 2019, after that foodwatch reported the presence of MOAH in infant and follow-on formulas in the German, French and Dutch market [17], the EFSA released a new opinion regarding the risk related to the presence of MOAH in infant formula and foods [18]*.* It wasconcluded that the characterization of the hazards was not possible in the absence of relevant dose-response data and information regarding the presence of the more health-concerning 3-7 ring MOAH.

No regulation exists yet, mainly due to the lack of a complete risk assessment, including knowledge on the MOH toxicity and occurrence data. However, some threshold or benchmark levels in various food products have been set by the Belgian Scientific Committee of the Federal Agency for the Safety of the Food Chain [19], the German Consumer Protection Consortium of the Federal States and the German Federation for Food Law and Food Science (Bund für Lebensmittelrecht und Lebensmittelkunde e.V.) [20].

Moreover, it is important to highlight that white mineral oil has several permitted and regulated uses in the food industry. For instance, Regulation (EU) 1333/2008 allows the use of microcrystalline wax (E 905) and hydrogenated poly-1-decene (E 907) as food additives [21]. White oils are also commonly used in the food industry as mold releasing or anti-caking agents. Some MOH are used as additives in FCM (Regulation (EU) 10/2011) [22], as pesticides (Regulation (EU) 889/2008), and as active substances for plant protection products (Regulation (EU) No 540/2011) [23,24].

# 2.1 Exposure and toxicity

Food represents the highest source of MOH intake for humans, even though the use of MOH is widespread in various other products such as medicines and cosmetics [8]. The primary sources of MOH in food are FCM, processing aids, and technical grade lubricants, as well as environmental or crop contamination. Over time, few studies were conducted to evaluate the exposure and toxicity of MOH, both on animal tissues [25–31] and human tissues [32,33], highlighting bio-accumulation of MOSH in liver, spleen, lymph nodes and adipose tissue. Long discussed was the observed formation of hepatic micro granulomas in Fischer 344 rats, later considered irrelevant to the human species due to differences in terms of MOSH absorption, catabolism, and sensitivity between the two species [34]. Granuloma formation in rats is most probably induced by long-chain *n*-alkanes [28] (usually called waxes), contrarily to what is observed in the human liver, which retains mainly medium and high viscosity cycloalkanes [29,32]. A NOAEL (no observed adverse effect level) of 19 mg/kgbw per day for MOSH [25], is still used as a reference point for the risk assessment of the background exposure scenario. For white mineral oils used as a release or anti-caking agents, a higher NOAEL of 45 mg/kgbw per day was selected (high exposure scenario) [35].

MOAH do not bio-cumulate and are rapidly catabolized via oxidation pathways. Due to reactive intermediates (*e.g.,* epoxides) formed along the oxidation route, they are classified as potentially mutagenic, referring in particular to 3-7 ring compounds non- or poorly alkylated [8]. A high degree of alkylation is thought to reduce their metabolic activation, leading to the formation of non-mutagenic intermediates excreted via bile [36]. However, at present, a conclusive hazard characterization cannot be performed since either dose-response data or a toxicological reference point is not available. Therefore, additional studies to identify the hazards, both carcinogenic and non-carcinogenic, of MOAH are urgently needed.

At the current state, no reference ADI values exist for MOAH. Due to the lack of health-based guidelines, a MOE (margin of exposure) approach has been discussed [35], pinpointing the need for an exhaustive exposure assessment. For this purpose, the European Commission published the Recommendation (EU) No 2017/84 [14] on the monitoring of mineral oil hydrocarbons in food and FCM, followed by the JRC Guidance for data reporting and method performance [15].

# 3. Analytical determination of mineral oil hydrocarbons

Both on-line and off-line methods have been developed over the years for extraction, purification, and final analytical determination.

Although a complete overview of the analytical procedure for MOH determination is out of the scope of the present review, a brief overview of the different sample preparation steps, which includes extraction, purification, and auxiliary techniques, will be given for the sake of completeness on the topic. Besides the extraction step, off-line separation techniques, as well as off-line auxiliary purification methods, will be described to facilitate the understanding and comparison with the hyphenated approaches described in more detail in section 4. The readers are directed towards specific papers and reviews for more information [16,37–39].

# 3.1. Sample Preparation

# Sample preparation represents the first fundamental steps of the entire analytical protocol and often the most critical to assure the overall accuracy of the determination. Although conceptually, sample preparation is carried out to reduce the presence of disturbing matrix components and thus increase sensitivity and accuracy, in the specific case of MOH analysis, a too intense or inappropriate sample preparation can lead to the opposite outcomes. In fact, contamination can occur during sample pre-treatment. No efficient removal of interferents can cause misinterpretation of the chromatograms leading often to overestimation of the contamination. In fact, whether interferents are not efficiently removed, they need to be excluded from the integration of the hump, but the interpretation is not unequivocally harmonized. On the other side, if not carried out properly, sample pre-treatment may lead to the removal of part of the target compounds, leading to underestimation. The volume of solvent and sample manipulation should be reduced as much as possible, blank analyses should be run periodically, and the purity of the solvents needs to be systematically checked to maximize the accuracy of the analyses. Moreover, the cleaning of the reused glassware must be verified and plastic tools must be avoided [15].

The flowchart of the sample preparation before the LC-GC-FID analysis is very different according to the specific food sample analyzed. It can be as simple as a dilution of the liquid sample into an appropriate solvent (*e.g*., olive oil in hexane) or comprise several steps, *e.g.*, solvent extraction, enrichment step, interferents removal for both the MOSH and the MOAH fractions, involving laborious procedure (*e.g*., for palm oil or high fat-content solid food). In this regard, the JRC Guidance reports a useful decision tree to support the analysts in deciding how to treat the samples and support the harmonization of the entire analytical procedure [15].

# *3.1.1. Mineral oil hydrocarbons extraction*

In principle, any method available for fat extraction can be applied, but an in-depth knowledge of the specific matrix and the possible source of contamination may simplify the following purification steps [16]. The sample preparation and, in particular, the extraction step, is highly dependent on the kind of sample to analyze. From an extraction viewpoint, different procedures are necessary whether the samples are liquid or solid, with low- or high-fat content, dry or wet. However, a detailed description of the extraction techniques is out of the scope of this review and the readers are directed toward other references [16,39,40]. Here a brief and general overview of the extraction techniques is described and detailed of the extraction technique used in the applications involving hyphenated techniques is reported in Table S1.

The ideal extraction step should be able to quantitative extract the targeted analytes minimizing co-extraction of interferents. This goal is not easy to achieved for such lipophilic molecules. Indeed, the MOSH and MOAH are co-extracted with the fat fraction. Traditional liquid extraction has been widely applied for MOH extraction from a variety of dry and wet foods. The use of a single extraction solvent, usually hexane, has been proved effective for extraction from dry and low fat-content food; while for an exhaustive extraction from both dry and wet foods, a combination of ethanol and hexane has been suggested [16,39]. The use of automated extraction techniques such as pressurized liquid extraction (PLE) or microwave-assisted extraction (MAE) have been also proposed as a valid alternative to increase the throughput [16,39,40]. A combination of a first hexane extraction followed by ethanol/hexane extraction using PLE has been proposed to discriminate between the MOH contamination deriving from the migration from food packaging and pre-existing contamination in the raw materials of pasta samples [41,42].

Alternatively, acid hydrolysis and saponification have been proposed to maximize the recovery and performing a pre-enrichment step [16,39,43]. The use of MAE to perform saponification and extraction simultaneously was proved highly efficient to maximize the throughput [43].

***3.1.2. Off-line mineral oil saturated hydrocarbons and mineral oil aromatic hydrocarbons purification and separation***

As MOH are coextracted with the fat fraction, a purification step to remove the bulk of the triglycerides (TAGs) and other possible polar compounds needs to be performed. For similarity with the on-line method, which uses a silica LC column, the use of solid-phase extraction (SPE) packed with silica-based sorbent was introduced. Moret *et al.* proposed using 1 g of silica loaded with 10% of silver nitrate packed into a glass SPE cartridge [44,45]. The silver silica had a fat loading capacity of 125 mg per gram of sorbent. The MOSH fraction was eluted first with hexane followed by the MOAH fraction eluted with a mixture of hexane/dichloromethane (1:1 v:v). The method was successful with cardboard and dry food but failed to retain wax esters when dealing with vegetable oils or high-fat content food.

Fiselier *et al.* proposed the use of a glass column packed with 3 g of a mixed sorbent consisting of activated silica and silver silica (0.3%) [46]. The activated silica showed a higher retention power towards waxes without impairing the MOSH and MOAH separation guaranteed by the silver silica. A small amount of toluene added to the hexane/dichloromethane mixture allowed to reduce the retention of MOAH in the silver silica, without affecting the retention capability of the activated silica towards the waxes. Such a method was applied by the German Bundesinstitut für Risikobewertung (BfR) as the off-line reference method [47].

Along with the separation of MOSH and MOAH and them from the bulk of the fat co-extracted, several auxiliary techniques have been proposed over the years for increasing the sensitivity (extract enrichment) and for additional purification from specific interferents of the MOSH or MOAH fractions. These methods can be used in combination with both the off-line or on-line MOSH and MOAH determination procedures. Saponification was proved highly efficient to perform enrichment during the extraction step, thus reducing manipulation and solvent consumption [43]. Simultaneous enrichment and purification procedures were proposed by Zurfluh *et al*. using a glass column packed with a bottom layer containing activated aluminum oxide, silica gel, and silver nitrate (0.3%) and an upper layer consisting of sole silica gel [48]. The LOQ was lowered of ~10-fold by loading 1 g of oil into the column compared to the direct LC-GC-FID procedure. The upper layer retained the TAGs while eluting the MOSH and the MOAH, while the silver silica in the bottom layer allowed the separation between the MOAH and the MOSH. The presence of alumina in the bottom layer caused the long-chain *n*-alkanes to be removed from the MOSH fraction and elute with the MOAH, but they were separated later in the conventional LC-GC step. Wrona *et al*. proposed the use of a sulphuric acid-impregnated silica gel to remove the fatty acid while eluting the MOH [49]. They calculated the recovery spiking an edible oil with paraffin oil, therefore how this method may affect the MOAH fraction is not documented yet.

Plant origin foods may contain a high amount of *n*-alkanes (in the C21-C33 range with the prevalence of the odd-number), which need to be removed when overloading the GC column. Fiselier *et al*. proposed an off-line method based on the retention of the long-chain *n*-alkanes in an LC column (10 cm × 2 mm i.d.) manually packed with aluminum oxide [50]. The same approach was then extended to an on-line LC-LC-GC-FID method [51], later described in more detail.

However, the main problem in terms of interferents is the coelution of naturally present olefins (squalene, carotenoids, and sterenes) or olefins formed during the refining process of vegetable oils and high-fatty food. A small portion of this interferents coelute with MOSH (*i.e*., low unsaturated compounds), but most of them coelute as individual peaks or as a narrow hump in the MOAH fraction. Epoxidation with *meta*-chloroperoxybenzoic acid (mCPBA) was proposed to enhance the polarity of the olefins, thus increasing their retention in the silica column beyond the MOAH fraction [7,52,53]. A loss of 20-35% of MOAH may occur during this purification step, as well as the removal of the interferences may remain incomplete. In the latter case, they need to be recognized and subtracted from the integration of the MOAH UCM [53]. On-line strategies to remove olefins have been proposed as well, and they will be discussed in session 4.

**4. Hyphenated techniques in mineral oil analysis**

**4.1. LC-GC and LC×GC**

***4.1.1. LC-GC***

As aforementioned, LC-GC has a tight connection with MOH analysis, and its evolution has followed the necessities of the specific MOH determination. The development of the LC-GC interfaces has reflected this connection. For a more detailed and theoretical explanation of the different interfaces available, the readers are directed towards the many reviews [4,37,38,54,55] and book chapters [56,57]. Here the main theoretical aspects will be discussed in the context of their evolution and concerning the MOH determination. An overview of the research papers on MOH analysis in food using hyphenated techniques is reported in Supplementary Table S1 and S2.

***LC-GC Interface***

The presence of MOH in food was discovered by chance in 1989 analyzing markers deriving from irradiation of fatty food [2]. The work was mainly focused on the optimization of an LC-GC interface compatible with an LC flow rate of 500 μL/min exploiting the partially concurrent eluent evaporation (PCEE) in the on-column interface. Differently from the fully concurrent eluent evaporation (CEE) mechanism, which is easier to optimize but it is affected by loss and/or broadening of the most volatile compounds (up to ~80-120°C over the transfer temperature), PCEE allows to retain compounds and obtain well-shaped peaks almost starting from the compounds eluted at the transfer temperature [2]. Both PCEE and CEE, as well as solvent flooding evaporation transfer techniques, can be exploited in the on-column retention gap interface, which is based on the use of an uncoated and properly deactivated silica capillary (named retention gap when used with the purpose of retaining volatiles). The main difference between solvent flooding, CEE, and PCEE is the transfer temperature that can be lower, higher, or at the boiling point of the LC eluent. The PCEE transfer mechanism requires a fine tune between the eluent evaporation rate and the LC transfer flow rate to exploit the *solvent trapping effect*. The transfer of the LC eluent must occur at the solvent dew point, and the eluent transfer rate must slightly exceed the evaporation rate. The *solvent trapping effect* occurs when the retention gap is flooded with a thin layer of solvent (~10-40 μm) coming from the LC transfer. The evaporation of the solvents starts from the rear of the flooded zone driven by the carrier gas, which is immediately saturated with the solvent vapors. The volatiles compounds evaporate as well, but they recondense in the solvent present in front. This process allows the refocusing of the volatiles in a narrow band at the head of the analytical column.

The on-column interface used to discharge the solvent vapors through the entire GC column, leading to a very low evaporation rate, *i.e.*, 10-30 μL/min. Such flow rates were compatible only with packed capillary LC column. Based on previous work [58], the use of a solvent vapor exit (SVE), located between the retention gap (~10 m × 0.53 mm) and the analytical column, was introduced to increase the evaporation rate up to 500-800 μL/min. Such a modification allowed to couple a 2 mm i.d. LC column. On the other side, the presence of the SVE might cause the loss of volatile compounds if the SVE was not closed shortly before the completion of the solvent evaporation. A residual flow, through a capillary restrictor, was necessary after the closure of the SVE to avoid the back diffusion of vapors into the capillary column causing distortion of baseline and the presence of ghost peaks. Alternatively, the SVE was maintained open until the complete evaporation of the solvent and the use of a retentive pre-column (3 m × 0.32 mm, with the same stationary phase of the analytical column) located before the SVE was proposed. The latter solution was later abandoned, proving that a restriction at the SVE was working equivalently or even better [59]. The proper moment to close the SVE was defined using the flame method, *i.e.,* lightening the exudate at the exit of the SVE. When the flame went out, the evaporation of the solvent was off. The closure of the SVE was set a few seconds before. Figure 2 reports a scheme of the on-column interface, equipped with an SVE between the retention gap and the analytical column.

Later works moved towards the use of the loop-type interface with CEE, following the preliminary findings that the contamination was generally starting from C14 [60]. The transfer exploiting the CEE mechanism is easier to optimize; thus, it was preferred. The loop-type interface, introduced in 1986 [61], consisted of a multiport transfer valve located between the LC column and the GC injector. The eluent from the LC eluent was collected in a loop of the exact volume of the fraction to be transferred to the GC. Once filled, the valve switched and the carrier gas drove the collected fraction from the loop into the GC retention gap (typically 2-3 m × 0.32 mm), maintained at a temperature equal or above the eluent boiling point. The solvent evaporated concurrently during its introduction, generating a back-pressure which avoided the flooding of the retention gap. The solvent vapors were discharged through overflow via the SVE. The contrast between the pressure generated by the carrier gas and the back-pressure due to the solvent evaporation automatically adjusted the evaporation rate. Moreover, at the end of the transfer, a pressure drop allowed to trigger the signal for the SVE closure. The loop-type interface remained in the scene of MOH analysis for over a decade since easier to optimize: the principle of the transfer itself automatically tuned all the parameters, except the transfer temperature that needed to be optimized.

The necessity to increase the sensitivity, and thus to use a large internal diameter LC column led to the development of the in-line vaporizer or wire-interface in 1995 [62]. This interface partially replaced the loop-type interface during the period between 1995 and 2004 [62–66]. Compared to the loop-type interface, the wire-interface allowed more flexibility in the volume of solvent transferred from the LC, allowing to use LC eluent flow up to 500 μL/min. Moreover, it retained high boiling material in the vaporizing chamber and improved the phase soaking effect to better retain volatiles compared to the loop-type (of about four carbon atoms) [67,68]. The interface consisted of a Y-union connected to a short 0.32 mm i.d. capillary heated at 250-350°C, representing the vaporizer chamber. A piece of wire (about 4 cm in length) was introduced in the capillary to prevent excessively-violent solvent evaporation. The vapors were discharged by overflow through a SVE positioned between a short retaining pre-column (about 1.5 m) and the analytical column. On the other lines, the Y-union was connected to two valves, one connected to the LC exit allowing the transfer of the fraction of interest into the GC, and the other connected to the carrier gas source. During the transfer, the carrier gas was diverted to a restrictor. At the end of the eluent transfer, the carrier gas was switched back into the GC. It entered the vaporizer chamber while a residual flow (regulated by a restrictor on the transfer valve) flushed the LC transfer line minimizing any memory effect. The most critical parameters to be optimized in the wire-interface were the closure of the SVE and the GC oven temperature during transfer, which should be close to the solvent dew point, in order to exploit at its maximum the *phase soaking effect* in the precolumn. This transfer temperature needed a fine experimental adjustment since a 1-2 °C of fluctuation could be critical for the final performance of the system.

Starting from 2001, the use of the on-column interface with PCEE started to reappear, allowing the detection of compounds more volatile than C13 [66]. The few papers published between 2001 and 2004 presented either on-column or wire interface [69,70]. No paper at all was published between 2004 and 2008, while from 2008 the on-column interface (mainly the Y-interface) has become the only interface used for MOH determination, except for a few applications using a PTV based interface [71–73]. In fact, the discovery of MOH migration from recycled packaging, which generated a hump centered in a rather low volatility range, required to assure the analysis of the broadest possible range of volatility, thus leading to the selection of the on-column interface as the best solution [74]. The on-column interface was further improved in 2009 by introducing the Y-interface [9]. Instead of transferring the LC fraction directly into the retention gap located in the GC on-column injector, a Y-union was used to steer the LC eluent into the retention gap. At the same time, the carrier gas arrived through a short capillary connected between the injector and the other branch of the Y-union. Such a configuration allowed to avoid the dead-volume present between the external wall of the transfer capillary (which entered, via the on-column injector, some cm into the retention gap) and the retention gap, responsible for a memory effect equivalent to about 0.5-3 % of the previous transfer. When the transfer from the LC was over, the transfer line was backflushed with the carrier gas. The use of the Y-interface reduced the memory effect below 0.02%.

***LC separation and internal standards***

In the analysis of MOH, the LC separation may have multiple purposes: I) sample preparation, separating the MOH from the bulk of the matrix; II) purification, to remove possible interference from the targeted fraction; III) analytical separation of the compounds of interest, namely MOSH and MOAH, and possibly in sub-classes.

The first purification of MOSH and MOAH from TAGs and more polar sample components can be carried out off-line, as briefly described in section 3, or on-line. The use of a silica LC column represents the optimal solution for the on-line purification of MOH, since normal phase (NP) LC allows a higher loading capacity of fats compared to reversed phase (RP) LC due to mobile phase solubility. Moreover, the solvents used in NPLC are compatible with GC, while the solvents used in RP are more problematic to transfer into the GC [4,37,38,54,55]. In NPLC MOH are eluted first, while the fats are retained into the column. The loading capacity of the silica column depends mainly on the type of solvent used for elution. In 1991, a systematic study on the loading capacity of the different silica gels, used as LC stationary phase, was presented, showing how the capability of a silica column in retaining TAGs was tightly related to the type of solvent used as the mobile phase. Loading capacity of 25 mg of edible oil was reported using a 100 × 2 mm i.d. silica column along with *n*-hexane as the only mobile phase. The addition of a modifier decreased the retention capability (*e.g.,* adding 20% of CH2Cl2 the retaining capacity dropped to 15 mg and to 5 mg with the addition of 1% of methyl-*tert*-butyl ether) [75]. The necessity to increase the sensitivity in MOH determination led to the use of larger capacity columns (such as a 100 × 5 mm i.d. which has ~6-times more packing material than a 100 × 2 mm i.d. column), leading to an estimated 10-folder higher capacity to retain TAGs compared to the 100 × 2 mm i.d. column [62]. However, such a column required a flow rate of 500 μL/min, which was not compatible with the on-column ,thus leading to the development of a new LC-GC coupling solution, namely the wire-interface (described in detail in the previous section) [62].

The 100 mm LC silica column was used over a long period of time for MOH analysis, although MOSH and possibly monounsaturated olefins were eluted together; while MOAH eluted later and were not transferred to the GC [3].

Starting from 2001, a 250 mm × 2 mm i.d., 5 μm *dp* LC silica column was preferred [66]. This column was often coupled with further LC purification using the same separation mechanism (*e.g.*, silica with a different retentive capacity to remove olefins) or a different one for interferences removal (*e.g*., aluminum oxide to remove long-chain n-alkanes) or further fractionation of the MOAH (*e.g*., amino column) (see paragraph §4.2 for a more detailed discussion). But the extra chromatographic plates provided by the 250 mm compared to the 100 mm column were fully exploited starting from 2009 [7]. A column packed with more retaining silica (Lichrosphere compared to previous Spherisorb) was used, which allowed to retain 20 mg of TAGs and still leave enough chromatographic separation space to separate MOSH from MOAH effectively. After elution of the fractions of interest, the column was backflushed to remove TAGs. Initially, methyl-*tert*-butyl ether was successfully used, but the following reconditioning with hexane was too time consuming and used a large amount of solvent due to the low elution strength of hexane. Therefore, dichloromethane was preferred. It was also used in the elution gradient; thus the overall system design was simplified by adding only a backflush valve rather than an additional pump.

This method was very wisely optimized using a series of internal standards (ISs) to mark the start and the end of the MOSH and MOAH fraction eluted from the LC column, to verify the transfer performance at each analysis, and to quantify the MOSH and MOAH fraction (Figure 3). MOSH elute with the front of the solvent, and the long-chain alkanes eluted earlier than the short one due to the size exclusion effect in the silica column. Cholestane (Cho) was used as a marker of the end of the MOSH fraction. Tri-*tert* butyl benzene (TBB) was used as a marker of the beginning of the MOAH fraction (eluting shortly after the MOSH), while perylene (Per) was used as a marker of the end. The efficient MOSH and MOAH LC separation was verified by the absence of Cho in the MOAH and TBB in the MOSH fraction.

Similarly, the complete presence of Per in the MOAH fraction, verified with the UV signal acquired at the exit of the LC column, assured the complete transfer of the MOAH into the GC. Additionally, C12, C14, and C16 were used to verify that no losses of volatiles were occurring during the transfer from the LC into the GC (C12) and to quantify the MOSH hump (either C14 or C16 could be used once verifying that no coelution was present with neither of them). Biphenyl was added to quantify the MOAH fraction, while hexylbenzene monitored evaporation losses during the transfer of the fraction into the GC. Nonylbenzene was used as control of possible coelution when the ratio with the others was not confirmed.

Further studies, in particular on packaging migration (characterized by a very volatile carbon range starting from C10) and on the polyolefin oligomeric saturated hydrocarbon (POSH) led to the modification of the ISs used. The ISs for the MOSH fraction were replaced by cyclohexyl cyclohexane (Cycy) for quantification and with C13 and C11 for verification purposes as they were observed to be less common than the even *n*-alkanes previously selected [76]. Moreover, Cycy and C13 were closely eluted together; thus, they could easily be recognized in the chromatogram. With the same logic, the ISs for the MOAH fraction were replaced by 1- and 2-methylnaphthalene, which created an easily distinguishable peak pair, and with *n*-pentyl benzene as a watchdog for volatile losses. Figure 3 reports the set of standards, firstly selected, and their use [7].

Cho and TBB as markers of the LC elution were revised in 2017 based on the changes in elution performance observed in a heavily used LC column compared to a new one and due to the more accurate information obtained by the use of GC×GC (described in detail in section 4.3) [11]. Di(2-ethylhexyl)benzene (DEHB) was suggested in place of TBB, because it elutes earlier due to the size exclusion effect. DEHB is also visible in the UV trace. However, it elutes in a crowded area of the GC chromatogram; therefore, the authors suggested starting the MOAH fraction exactly after the MOSH one to assure the complete inclusion of the MOAH even after the loss of retention capacity of the LC column. On the MOSH side, it was observed that Cycy is slightly more retained than Cho; thus, the former should be preferred as eluted in a less crowded area of the GC chromatogram [7].

***GC separation***

The use of GC allows to have an elution order that simulates the distribution of the MOH hump based on its production procedure (*i.e.,* mainly distillation). The analytical column selected, since the beginning, has been a dimethyl polysiloxane stationary phase column (or a 5% phenyl column). The chemistry of the stationary phase has to be compatible with the non-polar eluent transferred from the NPLC to assure an effective *phase soaking effect* [56,67,68].

A minimum of stationary phase thickness needs to be selected to provide a sufficient reconcentration factor of the solute band spread in the retention gap during the transfer from the LC. To avoid loss of efficiency in the separation column, the initial band should be up to ~15-30 cm (depending on column length). For instance, a 20 m separation column tolerates an initial solute band of ~20 cm. During the transfer from the LC, the solute band is spread through the entire length of the retention gap. Assuming a 20 m retention gap of the same internal diameter of the analytical column, the initial band should be concentrated of a factor of 100. Diphenyltetramethyldisilazane deactivated retention gaps show a residual retention power comparable to a film thickness of ~2 nm. This means that the stationary phase in the analytical column should be at least 200 nm (or 0.2 μm) to have a 100-fold reconcentration of the solute band. If the internal diameter of the retention gap is larger than the analytical column (generally 0.53 mm vs 0.25 mm), the minimum required film thickness increase linearly with the ratio of the two internal diameters [56]. Typically, with the on-column interface (or Y-interface) and the SVE, a retention gap of a maximum 10 m × 0.53 mm has been used coupled with an analytical column of 10-15 m × 0.25 mm with a film thickness in the 0.13-0.25 μm range (see Supplementary Table S1).

Short analytical columns have been preferred to speed up the elution and have the MOH hump in a narrow band to increase the sensitivity. Although at the beginning, more conventional-length columns were used (*i.e.,* 20-25 m), in later years, the trend has been to reduce the column length to 10-15 m (see Supplementary Table S1). For the same reason, thinner stationary phases have always been preferred to reduce the analysis time and the impact of the bleeding on the hump integration. High-temperature stable columns are highly required in order to elute up to the C50 as required by the JRC Guidance [15], avoiding the impact of the column bleeding.. A noteworthy evolution on the GC side is related to the use of GC×GC hyphenated or not to the LC separation. Such a configuration will be discussed in more detail in section 4.3.

***4.1.2. LC×GC***

Comprehensive LC-GC (LC×GC) has also appeared in the field of MOH determination. Although never applied for the direct determination of MOH extracted from food, it has been presented for the characterization of “food-related” MOH (*i.e.,* petroleum products and white mineral oils supplied for pharmaceutical and food applications) [77–80]. Therefore, the technique is herein briefly described to provide a proof-of-concept of the potentiality of the technique, which most likely will be applied for the determination of MOSH and MOAH extracted from food in a near future.

LC×GC was first introduced by Quigley *et al.* in 2000 [77]. A so-called “drop interface” was applied to analyze volatile organic compounds in water. As the name recalls, the method consisted of transferring a limited amount of sample and it was suitable only for very volatile components. In 2004, de Koning *et al.* proposed the used of an amino column (250 mm × 4.6 mm i.d.) for a group-based fractionation of MOH [78]. The LC column was eluted at 800 μL/min and fractions were transferred every 6 s (total volume of 80 μL) to a 5% phenyl-methylsiloxane column through a PTV interface working in the hot split mode. Three well distinct groups were obtained, namely I) paraffin and mono-, di-, and tri-cycloparaffins; II) alkylbenzene, indanes and tetralines, and indenes; III) naphthalenes, acenapthanes and acenaphtylenes.

Xu *et al.* [79] proposed a rather complicated multi-loop interface (composed of 6 loops of different volumes ranging from 10 to 60 μL) to perform LC×GC of alkylbenzene products. The sub-classes were separated in a μ-LC column consisting of a 250 mm × 0.53 mm i.d., packed with 5 μm *dp* diol-bonded silica sorbent eluted with hexane at a flow rate of 70 μL/min. The content of each loop was subsequently transferred into the GC exploiting the on-column interface.

More recently, García-Cicourel *et al.*[80] proposed an off-line silver phase LC×GC analysis coupled with FID or vacuum ultraviolet (VUV) detector. Although the system was not hyphenated, the data were elaborated as it was, obtaining 2D plots comparable to the one presented by de Koning *et al.* [78]. The white MOH analyzed were fractionated in a silver-silica column (200 mm × 4.6 mm i.d., 5 μm *dp*) at a flow rate of 500 μL/min. Fractions were collected for 20s (corresponding to 166.7 μL) and 1 μL was injected into the GC system. Figure 4 shows a 2D plot where the sample is well-separated into the MOSH fraction (early eluted between 4.7 and 6.5 min) followed by the monoaromatics (6.6-14.6 min) and the di- and poly-aromatics (15-16.6 m). The curved band obtained for the second group of compounds is due to the differential elution of the sub-classes, with the highly-alkylated MOAH eluted earlier than the low-alkylated ones. The same effect, although less pronounced is present in the third group but the LC elution gradient reduced the differences in the elution order.

The coupling with the VUV detector provided an extra level of information, being such a detector very selective towards aromatic compounds and capable of discriminating based on the degree of alkylation [81].

**4.2. LC-LC-GC**

In 1996, Moret *et al*. presented the first approach for MOH analysis in food by LC-LC-GC, with the specific aim of separating MOSH from MOAH [63]. The primary LC silica column allowed the separation of MOH from TAGs using pentane/dichloromethane (9/1 v/v) at 600 µL/min. A large capacity column (250 mm × 4.6 mm I.D., 5 µm *dp*), able to retain up to 150-200 mg of fat, was used to maximize the sensitivity. After the elution of MOH, the primary column was backflushed with dichloromethane. The 6 mL eluting fraction was concentrated on-line through a solvent evaporator (consisting of a 50 mm × 1 mm silica packed bed thermostatted at 40 °C) and driven to a secondary LC aminosilane column (100 mm × 4.6 mm I.D., 5 µm *dp*). The following amino column allowed the separation of MOSH and MOAH, and the latter according to the ring number. Either the total amount of MOAH was determined by backflushing the second column after the elution of the MOSH, or a more detailed fractionation of the MOAH was obtained by multiple-heartcutting transfer of the amino column eluate. The elution order and resolution capability of the LC amino column are shown in Figure 5. A detailed characterization of the MOH contamination was obtained for the first time, although many coelutions still remained. For instance, highly alkylated benzenes partially coeluted with paraffin. Benzothiophene and biphenyl were largely coeluted with naphthalenes. Moreover, a high degree of alkylation reduced the ring-based separation of the MOAH.

This configuration was applied for the analysis of rice and chocolate contaminated by jute batching oil, and in fish and edible oils contaminated by lubricating oil [64]. However, the method was not easy to implement; therefore, it was abandoned and the MOAH were “forgotten” for many years. Only in 2009, the attention came back to MOAH when the straightforward method using a single silica column allowed the separation of the entire MOAH class from MOSH [7]. Since then, the characterization of the MOAH has been mainly faced by GC×GC (See section 4.3). Meanwhile, the advancement on the toxicological side stressed the attention on the 3-7 rings MOAH. To enrich this fraction, Koch *et al.* [12] proposed the use of donor-acceptor complex chromatography to separate mono- and di- aromatics from the tri- and poly-aromatics after a pre-separation of MOSH and MOAH in a silver silica packed column. The fraction of the polyaromatics was collected five times before quantification by LC-GC-FID and characterization by GC×GC-ToFMS.

The use of two different columns in series was also proposed to remove plant originated *n-*alkanes interfering with the MOSH fraction. Based on previous off-line work [50], Fiselier *et al*. used a silica column (250 mm × 2 mm I.D., 5 μm *dp*) to retain TAGs, coupled to a secondary aluminum oxide column (100 mm × 2 mm I.D., 63-200 μm) which was prepared in-house by heating 300 mg of aluminum oxide in a GC oven (400°C for 60 h) [51,82,83]. The aluminum oxide column was flushed with *iso-*octane, thus regenerating the retention capability towards long-chain *n*-alkanes and so allowing its use for many cycles reducing the potential contamination compared to analog off-line methods.

Based on previous works done on the separation of fats and interferents to detect irradiation by-product[84], Grob *et al.* proposed an LC-LC-GC method to remove olefin from the MOH fraction in vegetable oils [85,86]. Two identical columns (250 mm × 2 mm I.D., 5 µm *dp*) packed with differently retaining silica were coupled. The first one (packed with less retentive silica) retained the TAGs, while the second one (packed with a strongly retained silica) allowed the separation of mono-, di- and tri- unsaturated olefins from the MOH. The same approach was used to study MOH contamination in human milk [87,88]. Up to 4 columns were connected in series to analyze egg yolk and automatically remove all the interferences. In the same work, the use of bromination (later replaced by epoxidation) was proposed [66].

The use of different packed sorbent columns was proposed to remove olefins by Lommatszch *et al*. [89] and Zoccali *et al.* [73], from the MOSH and the MOAH fraction, respectively. Both papers reported the use of a silver silica column after a pre-separation of the fats in a silica column, but in the former, the selectivity of the silver silica column was exploited to remove monounsaturated polyolefin oligomers (deriving from packaging materials) from the MOSH fraction [89]; while, in the latter, it was used to remove olefins (mainly squalene isomers) from the MOAH fraction [73]. In both cases, only the fraction of interest was diverted to the second column, while the other fraction was directly transferred into the GC. Moreover, Zoccali *et al.* [73] confirmed the petrogenic origin of the MOH contamination by confirming the presence of hopanes by a simultaneous FID and MS detection.

**4.3. GC×GC and LC-GC×GC**

GC×GC is a technique as old as the MOH problem in food. It was introduced in 1991 by Liu and Phillips [90], but, differently from LC-GC and the MOH issue, it has had exponential grown since its introduction. GC×GC provides a sensitivity gain and a burst in separation power, along with a well-ordered two-dimensional (2D) plot, which allows a group-type separation. For technical details on the technique, the readers are directed towards the many reviews on the topic, *e.g.,* [91–93]. GC×GC has had a big success in the petroleum field for the high complexity of the samples to be separated [93], but it has also had an important impact on food analysis [91]. Despite the similarity of the mixture to be characterized, GC×GC was introduced in the field of MOH analysis only in 2009, when Biedermann *et al.* published two works dealing for the first time with the GC×GC characterization of MOH, meant as a food contaminant [7,10]. But since then, its role in supporting the development of the theme has been rather evident. It has promoted a better comprehension of the topic, allowing a better characterization of the MOSH and MOAH fraction and the understanding of some issues associated with the sample preparation [11,12,30,33,53,89,94]. In the paper introducing the reference LC-GC method for the MOSH and MOAH separation and determination [7], the authors mentioned the use of GC×GC for confirmation purposes; while they fully exploited the potentiality of the technique in a second paper investigating in detail the MOAH fraction of a highly contaminated Ukrainian sunflower oil [10]. A pre-separation of the MOSH and MOAH fraction was necessary, being their ratio usually 4:1; thus, the MOAH would have been barely visible or the MOSH heavily overloaded (which can lead to a distortion of the 2D structure obtained in the 2D plot). Moreover, the pre-separation of the MOSH and MOAH fraction was also necessary to avoid coelution of the four- and five-ring saturated hydrocarbons (*i.e.*, steranes, hopanes, and bicyclic sesquiterpenes) with the highly alkylated two- and three-ring aromatics (Figure 6) [6,10,38]. Within the MOAH fraction, it was highlighted as the sub-class resolution is reduced when partially hydrogenated MOH are analyzed. In fact, the combination of aromatic and saturated rings in the same compound contributes to decrease retention in the second dimension reducing the capability to discriminate among the different numbers of rings. Despite these difficulties, more detailed information on the distribution of the sub-classes can be obtained by GC×GC compared to the LC-GC trace, leading, in 2012, the EFSA to point toward GC×GC as the most effective method to support confirmation and characterization of MOH [8]. Moreover, GC×GC has significantly contributed to the field of MOH, by leading new insights supporting the knowledge on toxicology (*e.g.*, information on the accumulation of the MOSH sub-class and more detailed characterization of specific sources of contamination) and by steering the analytical improvement (*e.g.*, the update LC-GC method).

At the beginning, the so-called normal set was explored, using a non-polar column in the first dimension and a semi-polar one in the second dimension, generally a 20 m × 0.25 mm i.d. × 0.12 μm *df* of PS-255 (1% vinyl-99% dimethyl polysiloxane) followed by a 1.5 m × 0.15 mm i.d. × 0.075 μm *df* 50% diphenyl-polysiloxane as the second dimension column. Starting from 2015, the so-called reverse set, in the specific case, mid-polar (50% diphenyl-polysiloxane) × non-polar (1% vinyl- 99% dimethyl polysiloxane), was preferred [33], based on previous evidence published on petroleum-related products [95,96]. Despite pre-separation of the MOSH and MOAH fraction remained necessary, a remarkable separation was obtained within the MOSH class, thus resulting in the most appropriate set of columns for the studies on the MOSH accumulation in the human body [33], and for more detailed studies on food packaging migration [89,94,97,98]. The reverse set allowed a very good separation between polypropylene POSH and MOSH, being the former more retained in the second column, while the monounsaturated polyolefin oligomers eluted between the two. The cyclic hydrocarbons occupied the 2D space just below the *n-* and *iso*-alkanes and were separated based on the number of cyclic (Figure 7) [97]. Although less resolved compared to the normal set, the MOAH sub-class remained clearly distinguishable also in the reverse set, and well separated based on the number of rings (Figure 7).

GC×GC has been coupled both to FID and MS for confirmatory purposes, while data on quantification performed in GC×GC has been rarely reported [33,98,99]. In 2013, Purcaro *et al.* [99] compared the quantification performed in LC-GC-FID with the one performed using a GC×GC system coupled simultaneously to an FID and an MS to characterize the contamination and quantify it at the same time. Back then, the range of volatility considered was up to C25, corresponding to packaging migration. Such a narrow volatility range was not affected by discrimination problems during split/splitless or PTV injection and eluted largely before the isotherm of the temperature oven program, thus also assuring the 2D structure of the chromatogram. Even if a few samples were analyzed, the quantitative results showed a rather good match between the 1D and 2D integration. A slight underestimation can be observed in the quantification performed on the 2D data. Although not discussed in that paper, this observation can be explained by a problem in the precise definition of the starting and end point of integration of the peaks located on top of the UCM hump (which need to be removed from the final quantification) when analyzed in the 2D space. Indeed, they are integrated all the way down to the baseline, thus including a portion of the UCM hump as well. Therefore, an improvement in the integration algorithm should be desirable to be able to perform reliable 2D quantification without the possibility of deviation due to the specific amount of interferences coeluted with the fraction of interest.

It is also important to highlight that all the papers discussed so far dealing with GC×GC characterization considered the elution up to C40, thus not in compliance anymore with the JRC Guidance publish in early 2019, which required a determination of the MOSH and MOAH UCM hump up to C50. So far, only one paper, recently published by Purcaro *et al*. [100] optimized a GC×GC set up using two-parallel second dimension columns connected to FID and TOF MS for simultaneous quantification and confirmation purposes. The goal of the work was to obtained two 2D plots perfectly superimposable in order to translate the classification defined using the MS information into the FID plot for a more reliable quantification (Figure 8). However, quantification performance was not presented yet.

A completely hyphenated LC-GC×GC platform equipped with an on-column interface was presented by Purcaro *et al*. at the Recent Advance in Food Analysis Conference in late 2019 and the first results are expected to be presented soon[101].

**4.4. MS hyphenation**

Differently from other contaminants, where the MS plays a systematic role, its use in the MOSH and MOAH determination is under continuous debate. On one side, the EU Commission Decision 657/2002 requires the use of an MS for confirmation of contaminants in food [102], but, on the other side, MS cannot be used for MOSH and MOAH quantification and the interpretation of the sole mass spectra may be ambiguous as the fragments generated in electron impact MS are not very selective. Although MS is far more sensitive than FID, calibration of complex mixtures of heterogeneous composition cannot be performed without proper standards. Instead, FID has a relative response factor of virtually 1 for both MOSH and MOAH, rigorously it has a marginally higher response factor for MOAH, but the approximation is more than acceptable [37]. To obtain a detectable hump in the FID, ~50 ng of MOSH or MOAH should be injected (depending on the width of the hump and presence of interferences). This means that to reach the target LOQ required by the JRC for food, *i.e.*, 0.5 mg/kg, a quantity corresponding to ~100 mg of food must be injected. The sensitivity of the system in MOH analysis was estimated as roughly 100-times lower than the sensitivity of the same system for a single gaussian peak. From here, the need to perform large volume injection (or transfer of the entire LC fraction) to achieve the sensitivity required. Moreover, the complete lack of selectivity of the FID required a more intense sample preparation with the threaten of cross-contamination.

Nevertheless, doubts about the nature of the UCM hump remains and a confirmation method has been widely required [103–105]. In particular, Spack *et al.* proposed a GC-MS method to confirm the nature of the UCM hump and to filter out the presence of interferences and thus correct the false positive [103]. However, as also published shortly after by Biedermann *et al.* as a commentary to that work [105], very aspecific fragments were chosen, namely *m/z* 43, 57, 71, and 85 for MOSH and *m/z* 91, 105, 119, and 133 for MOAH. These fragment ions are present in high amounts in all the different possible interferences present from food, such as squalene isomers, carotenoids, sterenes. It appears thus evident that the best solution is to act on the separation step, either chromatographic or during the sample preparation. However, the lack of a confirmatory method remains. GC×GC-MS has been pinpointed as the best possible solution [13,105], but still, resistance remains towards the acceptance of such an instrument.

The MS is instead of great support to detect the presence of markers of specific sources of contamination [97]. For instance, diisopropyl naphthalenes are used as markers of recycled paperboard migration, dibenzothiophene as an indicator of contamination originated from little refined oil. However, the presence of these markers have never been correlated to the extent of the contamination, and most probably cannot.

**5. Concluding remarks**

The fundamental role of hyphenated techniques in supporting the understanding and characterization of the MOH issue has been evident since the beginning, as well as the mutual and positive interaction in their development and advancement. MOH contamination in food represents one of the few applications that fully benefit from more than two chromatographic dimensions. Although from a routine viewpoint, the handling of more than two-dimension (LC-GC and GC×GC) is still rather complicated, it is evident as the regular implementation of additional hyphenations (*e.g*., LC-LC-GC, LC-GC×GC, or even LC-LC-GC×GC) would be highly desirable to fully characterize the MOSH and MOAH fractions, as required by the EU and the EFSA. To achieve such a goal, a tight and mutual collaboration between scientists and instrumental companies is the only way to optimize the system and, at the same time, simplify it improving the remaining weak points, such as automation and software. Considering the long history of successful interactions, it is the authors’ opinion that this perspective is not utopic and it can lead to outstanding results.

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**Conflict of interest**

The authors have declared no conflict of interest.

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**Figure Captions**

**Figure 1**. The number of papers published per year obtained from Scopus (up to May 2020). Reviews and papers on the toxicological aspects were not considered. The number of papers using LC-GC and GCxGC was extrapolated from the total to show their trend separately.

**Figure 2.** Scheme of the on-column interface exploiting the partially concurrent eluent evaporation (PCEE) with the use of a solvent vapor exit (SVE). Reprinted with permission from ref [59].

**Figure 3.** Use of the internal standards in LC-GC-FID analysis. A) elution order in the silica LC column with the internal standards used to mark the end of the MOSH fraction and the beginning and end of the MOAH fraction. Reprinted with permission from ref [37]. B) LC and GC-FID chromatogram of the MOSH and MOAH fractions. Reprinted with permission from ref [7].

**Figure 4**. Comprehensive AgLC × GC-FID chromatogram of mineral oil. Separation into three groups in the first LC dimension is observed: MOSH (4.7–6.5 min), mono-aromatics (6.6–14.6 min) and poly-aromatics (15–16.6 min). Reproduced with permission from ref [80].

**Figure 5**. LC-LC-UV and LC-LC-GC-FID trace obtained from the analysis of a non-refined linseed oil. The different fraction transferred to the GC-FID system are highlightd in the LC-LC-UV trace. Reprinted with permission from ref [63]

**Figure 6** GC×GC‐FID plots of the MOSH and the MOAH in a mixture of oils. Abbreviations: B, Benzene; N, naphthalene; Fluo, fluorene; DBT, dibenzothiophene; BDBT, benzo dibenzothiophene; Phe, phenanthrene; An, anthracene; Flu, fluoranthene; Py, pyrene; Chry, chrysene; BPy, benzopyrene; TAS, triaromatic steranes; Sesqui, bicyclic sesquiterpanes; Cho, cholestane. Reprinted with permission from ref. [10].

**Figure 7**. On the left, LC–GC-FID chromatograms; on the right, GC × GC–MS plots of the sections pointed out in the LC–GC chromatograms. Upper chromatograms, MOSH fraction; lower chromatograms, MOAH from a rice sample. Reprinted with permission from ref [97].

**Figure 8**. Translation of the classification based on the MS trace onto the FID trace.Reprinted from [100]

***Table S1***. References dealing with MOH determination using LC hyphenated techniques (i.e. LC-GC and LC-LC-GC).

***Table S2***. References dealing with MOH determination using GC×GC.