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Dissertation Title

"Development and validation of rapid sample preparation protocols for high-sensitivity multidimensional determination and source identification of hydrocarbon contaminants in complex food matrices"

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"If we knew what we were doing,
it would not be called research"

-Albert Einstein-

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List of Abbreviations

1MN	1-methyl naphtalene
2MN	2-methyl naphtalene
5B	Pentyl benzene
ADI	Acceptable daily intake
ANSES	French Agency for Food, Environmental and Occupational Health & Safety
BaP	Benzo [a]pyrene
BfR	German Federal Institute for Risk Assessment
BMEL	German Ministry for Nutrition, Agriculture and Consumer Protection
bw	Body weight
Cho	Cholestane
CyCy	Cyclohexyl cyclohexane
DI	Direct immersion
DIPN	Diisopropylnaphthalene
DLLME	Dispersive liquid-liquid microextraction
EFSA	European Food Safety Authority
ELSD	Evaporative light scattering detector
EVOO	Exta virgin olive oil
FAO/WHO	Food Agriculture Organization/World Health Organization
FCMs	Food Contact Materials
FID	Flame ionization detector
FLD	Fluorescence
GC	Gas chromatography
GMP	Good Manufacturing Practices
HOSO	High oleic sunflower oil
HPLC	High performance liquid chromatography
HS	Head space
IS	Internal standard
JECFA	Joint Expert Committe on Food Additives
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOAEL	Lowest observed Adverse Effect Level
LOQ	Limit of quantification
MAE	Microwave assisted extraction
MAS	Microwave assisted saponification
MASE	Membrane-assisted solvent extraction
m-CBPA	*m*-chloroperbenzoic acid
MOAH	Mineral oil aromatic hydrocarbon
MOE	Margin of Exposure
MOH	Mineral oil hydrocarbon
MOSH	Mineral oil saturated hydrocarbon
MS	Mass spectrometry
MSPD	Matrix-solid phase dispersion
MSPE	Magnetic solid phase extraction

NIAS	Non-intentionally added substances
NOAEL	No Observed Adverse Effect Level
OMG	Overall Migration Limit
PAH	Polycyclic aromatic hydrocarbons
PAO	Poly(alpha)olefins
PE	Polyethylene
Per	Perylene
PET	Polyethylene terephthalate
PLE	Pressurized liquid extraction
POMH	Polyolefin monounsaturated hydrocarbons
POSH	Polyolefin oligometic saturated hydrocarbons
PP	Polypropylene
RP	Reference Point
SCF	Scientific committee on Food
SFE	Supercritical fluid extraction
SIM	Single ion monitoring
SML	Specific Migration Limit
SPE	Solid phase extraction
SPME	Solid phase microextraction
SVE	Solvent vapor exit
TBB	1,3,5-tritert-butylbenzene
UCM	Unresolved complex mixture
UV	Ultraviolet

Summary

The hydrocarbon contamination is ubiquitous and can occur at any stage of food production. In the present work the focus is given on mineral oil hydrocarbons (MOHs) and polyolefin oligomeric hydrocarbons (POHs). MOHs are complex mixtures of compounds, mainly deriving from crude petroleum through distillation process and various refining steps. They consist of mineral oil saturated hydrocarbons (MOSH) that includes *n*-alkanes, isoalkanes and napthenes, and mineral oil aromatic hydrocarbons (MOAH), containing one or more benzene rings, mostly alkylated. The MOSH fraction may include polyolefin oligomeric saturated hydrocarbons (POSH), oligomers of polyolefins, that can migrate from plastic materials, as well as poly-alpha olefins (PAO) from hot melt adhesives.

The MOSH toxicity is controversial and still under investigation because of the lack of data on tissue accumulation and on exposure, especially on the background levels. MOAH are recognized to contain possible carcinogenic and mutagenic compounds, so their occurrence should be eliminated. This particular situation explains also the missing of legal limits for the MOH.

Depending on the complexity of the matrix, the sample preparation can be more or less challenging and requires a multiple steps to extract the analytes and eliminate the interferences, in order to obtain an adequate sensitivity and reliable quantification.

The aim of this Ph.D. work was the validation of a rapid and solvent-sparing methods, based on microwave assisted saponification followed by on-line HPLC-GC-FID, for MOSH/POH and MOAH determination in different complex food matrices. For all matrices (fish products, pesto sauce, cocoa powder and infant formula), methods with good performance characteristics in terms of LOQ, recovery and repeatability well within the JRC requirements, were obtained. Subsequently the methods were applied to a range of different samples.

The first part of the present work regarded the contamination present in fish products. It was found that the fish of low-fat content had no detectable MOH contamination, while fatty fish had variable MOH contamination levels. From the comparison of the chromatographic traces between the packaging and the corresponding packaged products, it was concluded that, except for a couple of vacuum-packed samples, there was no appreciable POH migration from the packaging to the food.

The second part of the work regarded the analysis of Pesto sauce and related ingredients. It was observed that the total MOH content found in pesto samples was clearly determined by high oleic sunflower oil (HOSO) contamination, followed by cashews (due to transport/storage in jute bags) and extra virgin olive oil (EVOO).

The third part of this Ph.D. work was focused on the monitoring of the presence of these contaminants in a wide range of cocoa powder, both in the product and the packaging, from two different markets (Italy and North Macedonia). The results obtained showed that the concentration of MOH and POSH can vary widely concluding that possible sources of contamination are numerous. In particular, the samples in PP bags purchased from the North Macedonian market showed a much higher POH migration in comparison with those purchased from the Italian market.

As well, the fourth part regarded the cocoa powder, aimed to evaluate if the type of packaging used (primary and secondary packaging in virgin paperboard and tertiary packaging in PP film) can act as a barrier against the migration of MOH from the external environment. Migration possible occurring during the secondary shelf life of the product was also investigated for the first time. From the results obtained it was concluded that the tertiary packaging in PP plays a very important role in limiting the contamination coming from the external environment, also once the product was opened.

Finally, the last part of the present work was conducted on infant formula (milk powder) where no MOAH contamination was found in none of the samples analyzed, while the presence of MOSH was given by the presence of vegetable oils used in the formulation of the product. POSH contamination was detected in all the products in contact with a plastic layer, while the samples packaged in metal cans resulted free from POSH.

Chapter 1. INTRODUCTION

1.1. Hydrocarbon contaminants

Hydrocarbon contamination can occur at any stage of food production, such as environmental contamination, farming practices, food transformation processes (domestic and industrial), and migration from food packaging. There are different classes of hydrocarbons that can contaminate the food matrices. The first class of contaminants is represented by mineral oil hydrocarbons (MOH), including saturated and aromatic hydrocarbons, originating from petrogenic processes. The second class of hydrocarbons in represented by hydrocarbons of synthetic origin (usually quantified together with the MOH) comprising polyolefin oligomeric hydrocarbons (POH) formed during the polymerization process of polyolefins or during polymer degradation, as well as poly alpha olefins (PAO) entering the composition of certain adhesives. Another important class of hydrocarbon contaminants is represented by non-alkylated polycyclic aromatic hydrocarbons (PAH), generally present at low amount when the contamination is of petrogenic origin (mineral oil). Large amount of PAHs can instead be generated by incomplete pyrogenic processes.
These classes of contaminants, which can contaminate food from different sources, are thoroughly discussed in the next paragraphs.

1.1.1. Mineral oil hydrocarbons (MOH)

1.1.1.1. Origin, chemical characteristics and source of contamination

Mineral oil hydrocarbons (MOH) are known to be complex mixture of isomers, mostly deriving from crude mineral oil which undergo to the process of petroleum refinement, and in a small part from the synthesis of coal, natural gas or biomass. MOH can be divided into three families:

- Paraffins including both linear and branched alkanes;
- Naphtenes including non alkylated and alkylated cycloalkanes, mainly cyclopentanes and cyclohexanes, with systems based on one or more rings;
- Aromatics containing almost one or more benzene rings, featured mainly by alky-substituted. Only a minor part is characterized by non alkylated polyaromatic hydrocarbons, known as PAHs mostly deriving from pyrogenesis process [1].

Paraffins and naphtenes are classified as MOSH (Mineral Oil Saturated Hydrocarbons) (figure 1), while aromatics are classified as MOAH (Mineral Oil Aromatic Hydrocarbons) (figure 2).

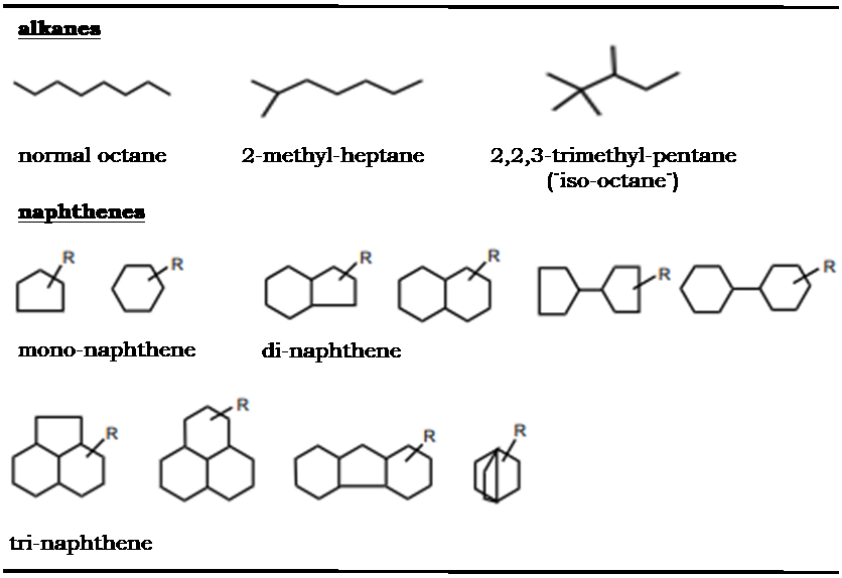

Figure 1. Examples of aliphatic and cyclic hydrocarbons, characterizing the MOSH fraction.

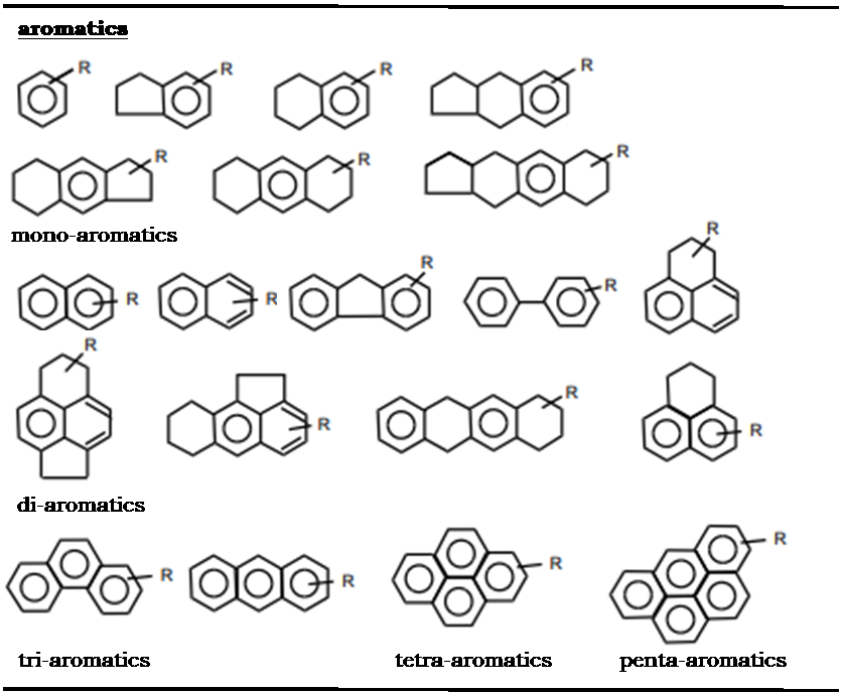

Figure 2. Examples of aromatic hydrocarbons, characterizing MOAH fraction.

MOH may also include minor amounts of nitrogen- and sulphur-containing compounds [1].

The distillation products of crude oil are usually grouped into three categories:

- light distillate including liquefied propane gas, gasoline and naphtha;
- middle distillate consisting kerosene, diesel and solvents;

- heavy distillate and residues including heavy fuel, lubricating oils, solid paraffins.

These products are characterized by different molecular mass distributions which influence their physical properties, such as boiling range, viscosity, density, etc. The products of the refining process are opportunely blended to obtain mixtures with the required characteristics for desired end uses [1], [2].
Hydrocarbons naturally occurring in food (primarily *n*-alkanes of odd-numbered carbons from *n*-C_{21} to *n*-C_{35} and hydrocarbons of terpenic origin) and oligomeric hydrocarbons released from polyolefins (largely consisting of branched alkanes) are not included in MOH products [3].
MOH may be present in food at different concentration according to the food nature and the source of contamination. Specific oils are normally used in the food manufacturing field for certain applications and considering the totality of the food supply chain, the transfer of mineral oils can occur easily [4]. The oils considered as food grade are highly refined, since they are obtained after processes that assure the elimination, or at least the limitation, of the MOAH fraction [5]. Processed foods can be contaminated with food grade mineral oils largely used in the food industry as lubricants, release agents, dust suppressants for grain or animal feed, protective coatings for raw fruits and vegetable and in some cases as additives (microcrystalline waxes used, for example, as glazing agents in confectionary and frozen meat) [6]. Due to their carcinogenic effect, MOAH are almost completely absent in food-grade mineral oils, but present in technical-grade lubricants [7]. Mineral oil contamination could occur also during harvesting, transportation and storage processes and the application of non-food grade lubricants for machinery in contact with food must be considered as a route of contamination [8].
Environmental contamination is due to the contribution of air, the aquatic ecosystem and soil. Exhaust gases from vehicles and domestic heating seem to be the main causes of higher levels of contamination in urban zones [9] with respect to that found in rural areas.
Another important source of contamination are food contact materials. Grob et al. [10] found an important amount of mineral oil contamination (10-100 mg/kg) in different samples such as, nuts, coffee, cocoa beans and chocolate, rice transported in jute bags. It occurs because raw jute and sisal fibers are treated with an aqueous emulsion of a mineral oil fraction, known as batching oil, in order to soften them and to facilitate their spinning. Furthermore, the mineral oil

used as batching oil contains 23% of aromatics, a reason why it could not have been called of 'food grade quality' [10]. In 1998, a special criteria for the manufacture of jute bags used in the packaging of selected food materials (cocoa beans, coffee beans and shelled nuts) was adopted by the International Jute Organization (IJO) which established that the oil should only contain non-toxic ingredients and specify the limits for the presence of unsaponifiable material in the bags (less than 1250 mg/kg jute fiber) [11]. This limit was provided, taking into account the non-saponifiable matter of vegetable oils used for batching. In 2004, EFSA concluded that the use of edible rice bran oil, palm oil or any other vegetable oil would not result in food contamination with mineral hydrocarbons [12]. Serra Bonvehì & Coll [13] found the presence of MOSH in cocoa butter that was directly attributed to jute batching oil when cocoa beans were transported and stored in jute bags with contamination characterized by the presence of hydrocarbons in the range from *n*-C_{14} to *n*-C_{22} [13].

One of the main issues regarding the contamination of food matrices with MOH is the migration of mineral oils from recycled paperboard into dry foods [14]. Paper and cardboard are widely used as packaging materials for dry solid foods because of their abundant sources, renewability and natural degradation. However, some organic substances, which are introduced during the paper packaging materials manufacturing and the printing processes, can affect human health. When these materials come into contact with food, harmful substances can directly or indirectly migrate [15]. Mineral oil migration from paperboard surface to dry food has been an issue that many studies dealt with during the last decades [14], [16]–[18]. The study conducted by Droz and collaborators [16] described the contamination with MOSH in powdered baby milk and other dry food packed in cardboard boxes and stored for several weeks. Since recycled paperboard is not used for liquid food contact, migration is limited to the components of sufficient volatility enabling transfer through the gas phase, which is up to about *n*-C_{24} [19].

Cardboard used in food packaging is frequently produced using recycled paper which often includes printed newspaper, magazines and other graphic paper. Most commonly used newspaper inks contain mineral oils which are not removed during the recycling process and therefore enter cardboard food packaging. Therefore, the offset printing ink are of primary concern and particularly those used for newspaper [17]. In order to reduce the levels of MOH in the paperboard food packaging, many paper and ink industries mobilized to resolve the problem.

Nevertheless, printing inks are not the only source of MOHs in paper and cardboard food packaging. The hot-melt glues and adhesives used to stick the paper and cardboard boxes used as food packages consist partly of hydrocarbons that can migrate into food [20], [21]. In 2017, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) published an opinion on 'Migration of mineral oil compounds into food from recycled paper and cardboard packaging'. ANSES recommends the use of printing inks, glues, additives and processing aids MOAH-free in the paper and cardboard packaging industries to prevent the high content of MOH in recycled paperboard and strongly recommends to limit the consumer exposure to MOH - and more particularly to MOAH - by different ways, such as limiting the sources of MOH in paper and board, optimizing the recycling process and lastly by using barriers to limit the migration of MOH from packaging into the food [22].

1.1.1.2. Toxicological assessment

The toxicity of the mineral oil in human depends on the distribution of the molecular weights in the paraffinic fraction, as well as on the refining treatment they undergo. The MOSH toxicity is controversial and still under investigation because of the lack of data on tissue accumulation and on exposure, especially on the background levels. MOAH are recognized to contain possible carcinogenic and mutagenic compounds, so their occurrence should be eliminated. This particular situation explains also the missing of legal limits for the MOH.

Food contamination with MOH became a subject of interest in the early nineties, when the 'white mineral oils' were still considered 'food grade'. 'White oils' are generally free from MOAH, consisting only of MOSH. In 2002, the Joint FAO/WHO (Food Agriculture Organization/World Health Organization) Expert Committee on Food Additives (JECFA) re-evaluated white mineral oils and waxes from their previous assessment in 1995 [23] establishing acceptable daily intake (ADIs) according to chronic experiments performed in Fischer 344 rats. It was demonstrated that an intake concentration between 0.01-20 mg/kg (depending on the type and viscosity of the oil) causes side effects on rats, which developed liver granuloma and damage to mesenteric lymph nodes [24], [25]. Later it was demonstrated that granuloma formation in Fischer 344 rats has been correlated to the presence of high proportion of *n*-alkanes in the MOH mixture. Humans are normally exposed to high concentrations of natural *n*-alkanes, but no bioaccumulation has been detected. This indicates that humans metabolize

efficiently *n*-alkanes and that granuloma formation in humans, because of exposure to mineral oils, is uncertain. MOSH accumulation in human tissues is a concerning issue, in fact several studies demonstrated the presence of MOSH in fat human milk, subcutaneous adipose tissue in women [26], [27]. Barp et al. [28] investigated the concentration and the molecular distribution of MOSH in several human tissues, demonstrating that MOH is by far the contaminant present at the highest amount in the human body. The amount of MOSH accumulated in human tissue seems high compared to exposure observed in shorter term animal experiments [28].

Recently, new studies on the accumulation and toxicity of MOSH in animals (female Fischer 344 rats) were published [29]–[31]. The authors of these studies were strongly dubious about the possibility of transporting the results from rats to humans because the data were different in concentrations and distribution of MOSH compared to previous results in the same tissues, focusing on human tissues. They also suggested that hepatic microgranuloma could be not the most relevant critical effect on which to base future ADI values.

In 2012, EFSA affirmed that MOAH with three or more, non- or simple alkylated, aromatic rings may be mutagenic and carcinogenic and that highly alkylated MOAH can act as tumor promoters, although they are not carcinogenic themselves. Mono- and diaromatic MOAH seem to have little toxicological relevance, because no genotoxic constituent is known, whereas some simple MOAH, such as naphthalene, are carcinogenic due to a non-genotoxic mode of action. The predominant fraction of MOAH detected in food is constituted by a single ring and a small part has two rings, whereas the MOAH with three aromatic rings usually represent a small part. For instance, MOAH derived from recycled board and printing inks almost exclusively have one or two rings- for this reason it is important to distinguish between MOAH that have one-two rings and MOAH with higher number of aromatic rings [32].

1.1.1.3. ADI values and proposed limits

In 1995 the Scientific Committee on Food (SCF) established temporary ADI of 0-4 mg/kg bw (body weight) for paraffinic oils and an ADI of 0-20 mg/kg bw for waxes, largely consisting of *n*-alkanes [33].

In 2002, the JECFA re-evaluated white mineral oils and waxes from their previous assessment in 1995 and established new ADIs. In details, JECFA specified the following ADI values:

- 0-20 mg/kg bw for mixtures of highly refined paraffinic and naphthenic liquid hydrocarbons with boiling points above 350 °C (high viscosity);
- 0-10 mg/kg bw for medium and low viscosity mineral oil of class I (molecular weights about 480-500 Da corresponding to *n*-C_{34-36}; mixture of highly refined paraffinic and naphthenic liquid hydrocarbons with boiling point above 200 °C);
- 0-0.01 mg/kg bw for medium and low viscosity mineral oils of Class II and III (molecular weights about 300-480 Da corresponding to *n*-C_{17-25}; mixture of highly refined paraffinic and naphthenic liquid hydrocarbons with boiling points above 200 °C). Based on the fact that mineral oils migrating from recycled paperboard packaging mostly falls in the latter range for which ADI value of 0.01 mg/kg was set, a specific migration limit of 0.6 mg/kg MOSH in food has been derived [3].

This limit was derived from the convention that a reference person of 60 kg eats every day 1 kg of food in contact with the packaging material containing the target compounds.

Later, in 2008, as a result of the Ukrainian sunflower oil contamination case (above 1000 mg/kg found), the European Commission applied legal limit of 50 mg/kg for mineral paraffines in Ukrainian sunflower oil [34]. This limit was withdrawn in 2014 since non-compliant samples has been reported in 2010-2014 period [6].

An EFSA opinion in 2009 established a new ADI of 12 mg/kg bw per day for high viscosity white mineral oils, based on no-observed-adverse-effect level (NOAEL) of 1200 mg/kg bw per day. This value substitute 0-4 mg/kg bw per day set by SCF in 1995. In subsequent years, the study on hydrocarbons of class II and III has been deepened in order to clarify the relationship between toxicity and carbon chain length [35]. After several studies, in 2012 the German Federal Institute for Risk Assessment (BfR), demonstrated that saturated hydrocarbons with carbon chain from *n*-C_{10} to *n*-C_{16} are not accumulated in tissues and it was concluded that transfer of these substances into food should not exceed 12 mg/kg [36]. Furthermore, a limit of 4 mg/kg of food for MOSH from *n*-C_{16} to *n*-C_{20} was established [32].

In 2012 EFSA published an Opinion about mineral oils in food, considering ADI values previously stated by SCF and by JECFA as valid and introduced a

Reference Point (RP) for the calculation on MOE (Margin of Exposure) [1]. Regarding MOSH, two different levels of RP were highlighted:

- Base exposure level: the RP was set equal to the NOAEL of 19 mg/kg per day;
- High exposure level: the RP was set equal to LOAEL (Lowest Observed Adverse Effect Level) of 45 mg/kg per day.

Concerning the MOAH fraction, EFSA declared that the RP value to calculate MOE cannot be established.

After the publication of the EFSA opinion, JECFA withdrew the temporary ADI for Class II and III of MOAH free mineral oils [3], for which a limit of 0.6 mg/kg for MOSH and 0.15 mg/kg for MOAH in food had been derived [17].

Meanwhile, in 2011 the German Ministry for Nutrition, Agriculture and Consumer Protection (BMEL) emitted a draft (I draft) ordinance on mineral oils, setting migration limits for both MOSH (0.6 mg/kg) and MOAH (0.15 mg/kg), but was never applied. Later, in 2013, the BMEL published a second draft (II draft) proposal setting a MOAH maximum migration limit of 0.15 mg/kg. In 2014, a new draft ordinance (III draft) provided maximum limits for mineral oil residues in food packaging made of recycled fibers and food in contact with packaging. It established maximum limits of 24 mg/kg and 6 mg/ kg for MOSH and MOAH respectively, in the range of *n*-C_{16-25} (contact with dry food) or *n*-C_{16-35} (contact with wet food). When the maximum limits in the packaging materials exceeded, a packaging may still be placed on the market if migration values for food do not exceed 2 mg/kg for MOSH (*n*-C_{20-25}) and 0.5 mg/kg for MOAH (*n*-C_{16-35}). In 2017, the BMEL announced the fourth draft of a 22^{sd} ordinance on the modification of the German Consumer Goods Ordinance, in particular, the limit for MOSH (2 mg/kg) has been removed (remained the 0.5 mg/kg limit for the MOAH, as a consequence of migration from food packaging made of recycled paperboard) and it recommended the introduction of functional barriers to reduce the migration of chemicals from recycled paper and board used in contact with food [37].

In 2018, the Scientific Committee (SciCom) of the Belgian Food Safety Agency (FAVV-AFSCA) published an advice with action threshold for MOH. Threshold varying from 5 to 150 mg/kg were established for MOSH in relation to the food type, and, for MOAH it was not possible to define a threshold due to the limited toxicological information. For animal and vegetable fats and oils, an action threshold of 100 mg/kg was established for MOSH [38].

In 2019, based on background levels found in a large number of samples, the representatives of the food control authorities of the federal states and the Food Federation of Germany suggested the use of "benchmark levels" for MOH contents (table 1). After the first publication in April 2019, a further product category was added in June 2020 [39].

Table 1. Common MOH-Benchmark levels (Adapted from [39]).

LAV and Lebensmittelverband: Common MOH-Benchmark levels (June 2020)			
Product group Food category (consumer product)	MOSH and analogs (mg/kg) *n*-C_{10-50}	MOAH (mg/kg) *n*-C_{10-50}	Notes on use*
Vegetable oils.(such as rapeseed oil, linseed oil, olive oil) (excluding oils/fats of tropical plants and soya oil)	13	n.q.**	These benckmark has been extracted from tropical plants (e.g. coconut oil) due to an insufficient base levels are not intended for application to oils/fats that have of statistical data (in Dec. 2018)
Bread and biscuits, fine pastries, cereal products and cereal-based products, cereals, rice, pasta	6	n.q.	Not to raw commodities of raw doughs
Confectionery (sugar confectionery except chewing gum), chocolate and cocoa-based confectionery	9	n.q.	
Nuts, tree nuts, oilseeds, cocoanut, peanuts and dried fruit, including mixtures	4	n.q.	

*Notes on the food groups recorded/on non-recorded products and limitations/if appropriate on reasons, the basis of the data or other special features
**n.q. not quantibiable

In 2020, the BMEL announced an updated version of the 22sd ordinance amending the Consumer Goods Ordinance concerning the use of recycled paper in manufacturing food contact materials (FCMs). The ordinance requires the use of functional barriers when recycle paper is used in manufacturing FCMs. An exclusion to this requirement would be settled if the manufacturer can demonstrate that MOAH migration does not take place above:

- 0.5 mg/kg of MOAH for food;
- 0.15 mg/kg of MOAH for food simulants [40].

In 2021, the representatives of the food control authorities of the federal states and the Food Federation of Germany extended the use of "benchmark levels" for MOH contents, introducing new product categories (table 2)[41].

Table 2. Common MOH-Benchmark levels (Adapted from [41]).

LAV and Lebensmittelverband: Common MOH-Benchmark levels (UPDATE August 2021)			
Product group Food category (consumer product)	MOSH and analogs (mg/kg) *n*-C_{10-50}	MOAH (mg/kg) *n*-C_{10-50}	Notes on the application* MOH orientation values are always to be appied in conjunction with the definition described
Vegetable oils and fats (such as rapeseed oil, sunflower oil, soya oil, linseed oil, olive oil and margarines) (excluding oils/fats of tropical plants)	13	n.q.**	Not for use with oils/fats obtained from tropical plants (e.g. coconut oil)
Bread and biscuits, fine pastries, cereal products and cereal-based products, cereals, rice, pasta	6	n.q.	Not to raw commodities of raw doughs
Confectionery (sugar confectionery except chewing gum), chocolate and cocoa-based confectionery	9	n.q.	
Nuts, oilseeds, coconut, peanuts and dried fruit, including mixtures thereof	4	n.q.	
Desserts (ready to eat) and ice cream	4	n.q.	
Ice cream with fat-based coatings, glazes and couvertures (whether or not in pieces on/in ice cream, on/in wafers)	10	n.q.	
Meat, meat preparations and meat products (including sausages)	9	n.q.	Not for firm raw sausages with cheese, with cheese or pepper casings; not for meat preparations in oil-based marinades
Fish and fish products (including canned fish in aqueous infusion/own juice)	4	n.q.	Not for canned fish and fish products in oil or oil-based sauces and dips; not for crustaceans and molluscs and product thereof
Milk and mil products (such as cream, butter, yoghurt, cheese) including preparations thereof	22 mg/kg milk fat	n.q.	Assessment is made in all milk products and preparations in relation to the milk fat content; not for mixed spreadable fat products

*Notes on the food groups covered/on products not covered and delimitations/on justifications or other special features
**n.q. not quantibiable

1.1.1.4. Sample preparation

Depending on the complexity of the matrix, the sample preparation can be more or less challenging and requires various techniques to extract the analytes of interest and to eliminate any interferences, in order to obtain an adequate sensitivity and reliable quantification. The sample preparation is a very important and essential step of the entire analytical process and it is divided into several steps:

- Sampling: represents the first stage of the analytical process and is crucial for the correct interpretation of the results. Furthermore, an error at this stage cannot be corrected in any way and affects the entire analytical process;
- Extraction: is a procedure that allows to isolate the analyte of interest from the mass of the matrix;
- Purification: is a procedure able to remove any co-extracted interferers;
- Concentration/dilution [42].

Extraction and purification are the crucial steps allowing the correct quantitative analysis.

In respect of the matrices there are different extraction techniques. Usually, liquid-liquid extraction (LLE) with hexane is the most used method for liquid samples, such as wine, water, beverages, etc. This extraction can be followed or replaced by solid phase extraction (SPE). The presence of water in some wet samples represents a perfect barrier for example against the mineral oil hydrocarbons extraction from particles using non-polar solvent, so for this reason a dehydration is required [43]. To extract the superficial contaminants from dry foods a solvent extraction with *n*-hexane was applied [14]. Later Moret and collaborators [44] proposed an alternative pressurized liquid extraction (PLE) in order to reach a complete extraction of MOH from dry foods. This technique also was applied to extract the mineral oil contamination from paper and cardboard intended for food contact by the same author [45]. For samples such as edible oils, fatty foods or foods with complex matrix sample dilution or saponification, before enrichment and purification, is usually applied. The first approach used to remove the fat was a traditional saponification followed by unsaponifiable extraction for determination of mineral oil hydrocarbons or *n*-alkanes naturally present in different matrices [46]. As an alternative to a time and solvent consuming traditional method of saponification, a rapid microwave assisted saponification (MAS) was developed by Moret and collaborators [47] for the determination of mineral oils in cereal-based foods and, more recently, on fish products [48]. Also, activated silica gel allows for fat retention [49].

MOH are a complex mixture of a huge amount of isomeric substances, consequently during analysis they cannot be separate in individual compounds. A typical chromatographic profile is characterized by 'humps', consisting of a large number of unresolved peaks, and or *n*-alkanes. MOSH total area has to be cleared of endogenous *n*-alkanes from *n*-C_{21} to *n*-C_{35} giving sharp peaks on the top of MOSH fraction with a typical profile where odd terms prevail on even ones. In addition, MOAH form one or more humps of unresolved peaks, showing the same range of volatility of saturated fraction, which means they originate from the same mineral oil fraction obtained from a distillation process [1], [49], [50]. Plant samples and certain foods may contain a high amount of *n*-alkanes naturally present, which can lead to incorrect quantification of the MOSH fraction. This problem can be overcome by introducing a purification step on activated aluminum oxide, which retains long chain *n*-alkanes [43], [51]–[53]. On the other hand, the presence of high amounts of naturally occurring olefins, such as squalene and its isomerization product, sterens and carotenoids, can

lead to incorrect quantification of the MOAH fraction. The first method to solve this problem, proposed by Wagner et al. [54] was the bromination performed with a bromine solution in chloroform. Lately, Biedermann et al. [50] introduced the epoxidation which involves derivatization to increase the olefins polarity and hence their retention, beyond that of MOAH. Recently, Nestola & Schmidt [55] reported some modification in the conditions of epoxidation and developed an automated method to simplify the manual operations and to have higher control of the reaction. Epoxidation can be performed on the row extracts or on edible oil samples before analytical determination. In figure 3 are reported the three protocols used to eliminate the olefins from the MOAH fraction.

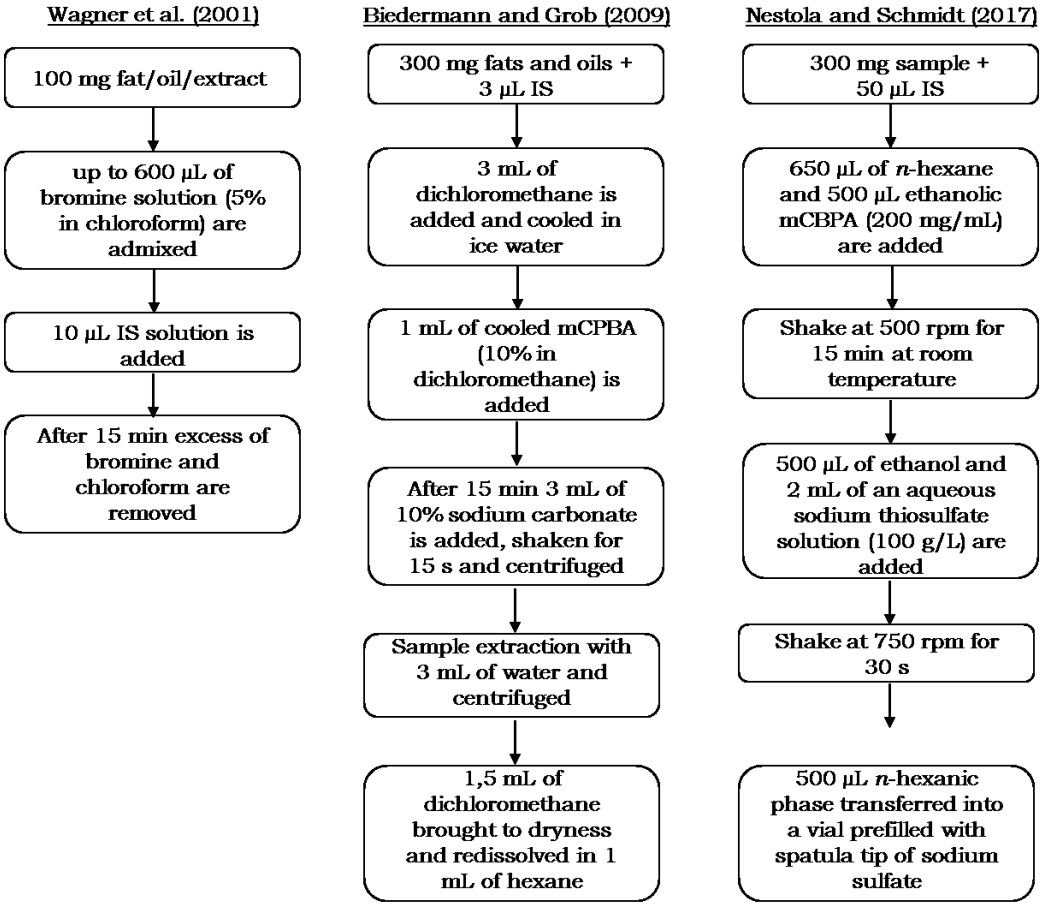

Figure 3. Different protocols used to eliminate the olefins from the MOAH fraction.

In order to reduce the risk of an external contamination during sample preparation it is important to take into consideration some precautions, such as avoiding sample contact with plastic materials. All glassware must be accurately cleaned, and the solvent be distilled to eliminate the eventual presence of interferences [49].

1.1.1.5. Analytical determination

The most suitable analytical method for MOH determination in food is capillary gas-chromatography (GC), coupled with a flame ionization detector (FID). Sometimes GC coupled with a mass spectrometer (MS) has been proposed in mineral oil analysis, but it is not a suitable technique for petroleum hydrocarbon quantification. A MS may give very different responses for two different hydrocarbon compounds of the same mass, while the FID response is proportional to the mass of the hydrocarbon present (*n*-alkanes, olefin, aromatic) [56]. Nevertheless, GC-MS methods (Single Ion Monitoring – SIM mode) can give specific information on selected polycyclic aromatic hydrocarbons (PAH) and hopanes considered as petroleum biomarker [57], [58].
MOH separation into MOSH and MOAH fraction can be performed both off-line (on an HPLC column; solid phase extraction (SPE) cartridge; larger glass column filled with a suitable sorbent) [53], [59]–[63] or on-line (on an HPLC column) [50], [64], [65].

Off-line methods

Off-line methods make use of SPE glass cartridges (or larger glass columns) filled with non-activated silica gel [66] or silver (Ag) silica gel [63], or mixed activated and silver coated silica gel [53], [62]. Moret et al. [62] employed the silver silica gel for MOSH and MOAH determination, in cardboard and dry foods. This method involves the use of an SPE cartridge manually packed with 1 g of silver silica (10%) The eluted MOSH and MOAH fractions are concentrated before large volume injection into GC system [62]. Later, a manual method suitable for analyzing also vegetable oils, was described by Fieslier et al. [63]. To exploit the higher retention power of activated silica towards wax esters, a mixed bed (3 g) of activated silica/silver silica 0.3% was used.
Off-line SPE-NP-HPLC technique coupled with an evaporative light scattering detector (ELSD) was developed to determine the aromatic hydrocarbons in commercial lubricants [67].

On-line methods

The on-line method is the most suitable technique for mineral oil determination, is widely used for routine analysis and, as reported by Biedermann & Grob [43] offers important advantages compared to off-line method. It separate MOH fractions in robust manner, enables direct injection of a large aliquots of row

extracts, avoiding contamination of the sample during sample preparation and is fully automated.

The first method (LC-LC-GC system) for MOAH determination was developed in 1996. Since it is complicated, requires a silica column, an online solvent evaporator and amino column it was never applied for routine analysis [68].

In 2009 a routine on-line high-performance liquid chromatography (HPLC)-GC-FID method was proposed, which nowadays is the most applied one for routine analysis of MOH. The liquid chromatography HPLC column provides for MOSH and MOAH separation, using a gradient (100% hexane for 0.1 min and reaching a 30% dichloromethane after 2 min, with a flow of 300 µL/min). After the elution of separated MOSH and MOAH fractions a backflush with dichloromethane to eliminate the retained fat, is performed. Each fraction is sent to the GC through a retention gap by exploiting partially concurrent eluent evaporation using a Y-interface (figure 5) [43]. The Y-interface is a variant of the classical on-column interface with the advantages of avoiding memory effects due to the slow transfer of the HPLC eluent [43]. Partially concurrent eluent evaporation is the most suitable transfer technique: the HPLC eluent is introduced into the retention gap through the interface (figure 4); at a speed that slightly exceeds the eluent evaporation rate in order to allow the evaporation of the solvent. The size of the retention gap has to allow retention of the flooding eluent to exploit solvent trapping and retention of the more volatile components [49], [69].

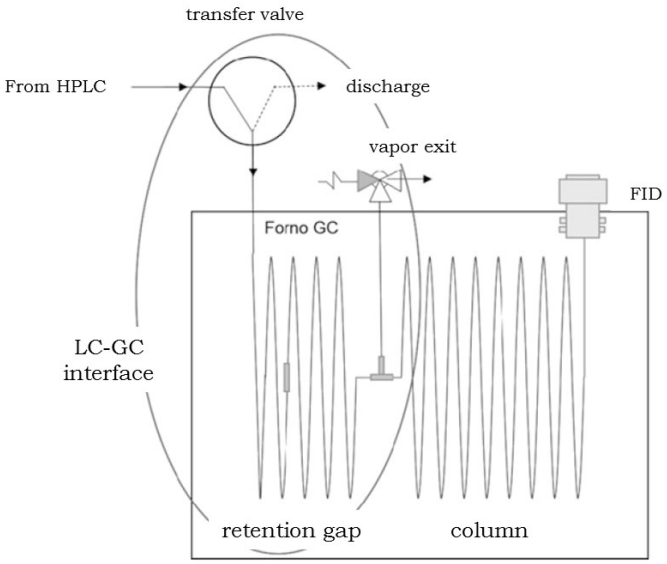

Figure 4. LC-GC interface based on the retention gap technique [42].

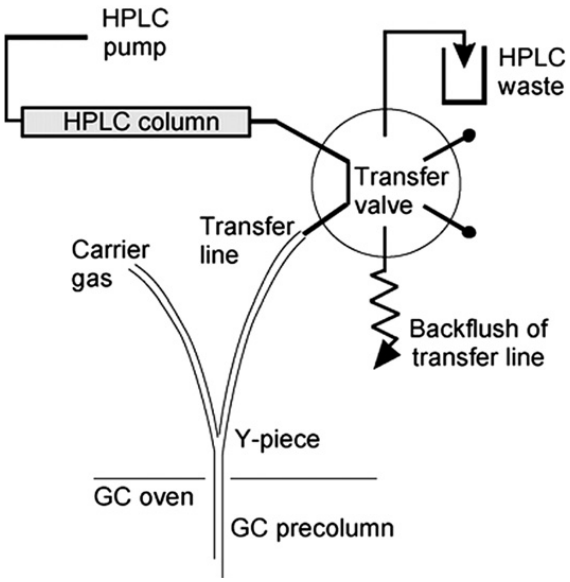

Figure 5. Y-interface (Adapted from [43]).

Over the years, several interfaces for on-line HPLC-GC were used, mostly for different designs for fully and partially concurrent eluent evaporation.

The first interface with concurrent eluent evaporation was the loop-type interface (figure 6). This interface is based on a switching valve equipped with a loop of the size as a fraction to transfer. By switching the valve, the sample is driven by the carrier gas into a retention gap at a temperature above the eluent boiling point. The solvent evaporates concurrently during its introduction and the transfer occurs at a speed corresponding to the evaporation rate [49]. This interface is not suitable for mineral oil analysis due to the high losses of more volatile hydrocarbons.

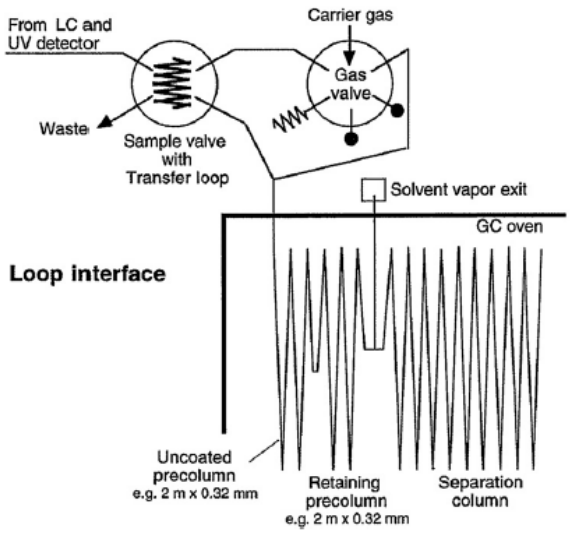

Figure 6. Loop type interface for LC-GC transfer involving concurrent eluent evaporation (Adapted from [70]).

To overcome this problem, Grob & Bronz [71] proposed the wire interface (figure 7) where evaporation occurrs concurrently in a heated capillary into which a piece of wire is introduced to avoid solvent shooting during evaporation. This interface was used for the determination of mineral paraffins in the oil phase of canned sea foods and in vegetable oils [72]–[74].

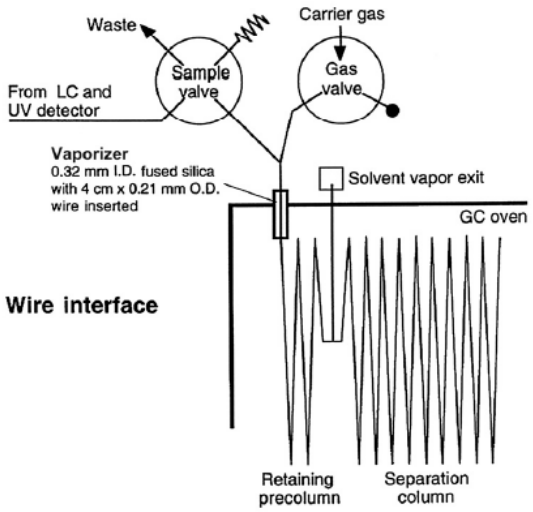

Figure 7. Wire interface for concurrent evaporation (Adapted from [70]).

Partially concurrent eluent evaporation is the most suitable transfer technique when samples contain hydrocarbons more volatile than *n*-C_{13}. For this propose on-column interface (figure 8) was introduced; the eluent is introduced into the retention gap at a speed that slightly exceeds the eluent evaporation rate. High-boiling solutes, which at the end of the transfer are spread along the flooded retention gap, are reconcentrated at the inlet of the separation column by the retention gap effect [49].

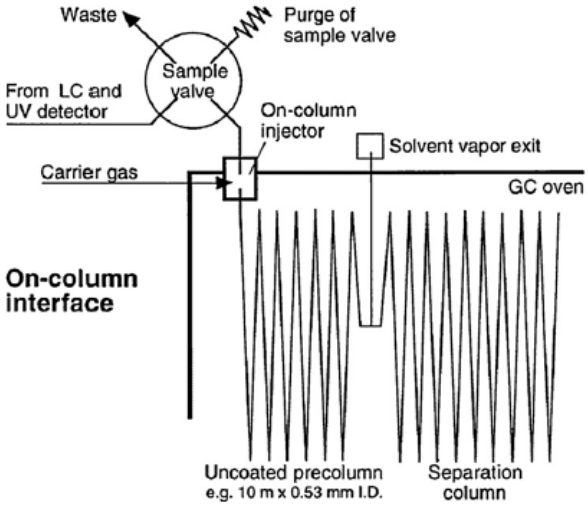

Figure 8. On-column interface, mostly used for transfer of wetting liquids by retention gap technique (partially concurrent evaporation) (Adapted from [70]).

Later the on-column interface was replaced by Y-interface developed by Biedermann & Grob [50], described above.

Quantitative determination of MOH can be performed by external calibration with a mineral oil standard (when sample components interfere with the internal standards) or by comparing the area of the internal standard and the MOH. For the last one, Biedermann & Grob [43] proposed a mixture of different components to verify the MOSH and MOAH separation and to perform the quantitative analysis. The standard solution for MOSH fraction involves *n*-C_{11}, which allows to control the loss of volatiles; *n*-C_{13} being eluted closely to the cyclohexyl cyclohexane (CyCy), the latter is taken for the quantification; cholestane (Cho) that serves to control the end of the MOSH window. On the other hand, the standard solution for MOAH fraction involves *n*-pentyl benzene (5B) used to control the loss of volatiles; 1- and 2-methylnaphtene (MN) that are selected to identify possible coelution with sample components; tri-tert-butyl benzene (TBB) and perylene (Per) to identify the beginning and the end of the MOAH fraction, respectively. However, more recently, Biedermann et al. [75] proposed a new internal standard, di(2-ethylhexyl) benzene (DEHB) to identify the beginning of the MOAH fraction in order to start the transfer of the MOAH immediately after the MOSH. This was because they found that some long chain alkyl benzenes were eluted earlier than TBB. Moreover, the authors also stated that CyCy could be used to determine to end of the MOSH fraction because it eluted slightly after Cho [75].

Recently, Zoccali et al. [76] proposed a multidimensional method for the determination of MOH in edible oils. Online HPLC-HPLC-GC chromatography was coupled with a dual detector, FID for quantification, and a triple quadrupole MS for identification of specific markers to confirm the petrogenic origin of the contamination. A silica column provided the MOSH/MOAH fraction, while the second column (silver-ion) retained the interfering olefins, which usually need a specific sample preparation [76].

The best technique for a detailed characterization of complex mixtures of hydrocarbons is comprehensive two-dimensional GC (GC×GC) technique [77]–[79], offering an outstanding separation power and high sensitivity thanks to re-concentration at the end of the first separation column. The disadvantage of this technique is the non-possibility of a complete separation of MOSH and MOAH, since naphthenic hydrocarbons are coeluted with highly alkylated aromatic components. In particular, four- and five-ring saturated hydrocarbons (such as

steranes, hopanes, and bicyclic sesquiterpanes) coelute with the highly alkylated two- and three-aromatics [80], [81]. Biedermann & Grob described a GC×GC method for characterizing MOAH by ring number and degree of alkylation after LC pre-separation [82].

1.2. Migration and barrier to mineral oil hydrocarbons

1.2.1. Migration

The term '*migration*' is used to describe the mass transfer of chemical compounds from food packaging into the food. Migration of substances is a diffusion process governed by kinetic and thermodynamic dimensions, described by mathematics from Fick's first and second law [83], [84].

$$Fick's\ 1st\ law: F = -D_p(\delta C_p/\delta x)$$

$$Fick's\ 2nd\ law: (\delta C_p/dt) = D_p(\delta^2 C_p/\delta x^2)$$

Where F is the rate of transport per unit area of the polymer, D_p is the diffusion coefficient of migrant in the polymer (cm^2/s), C_p is the migrant concentration in the polymer (mg/g), x is the polymer-food interface (cm) and t is the elapsed time (s). The diffusion coefficient is the only parameter that must be measured with kinetic experiment, in which the temperature is also involved [83]. In this process, a potential migration constituent gradually transfers from the packaging to the food. A point of equilibrium could be reached if the concentration of the constituent stays constant in food and packaging.

Migration can occur in three different ways (migration via gas phase; migration by contact of diffusive substances; migration by contact of non-diffusive substances) [85] and is affected by different parameters that can be referred to:

- the packaging material (type, superficial area, thickness, etc.) and the concentration of potential migrants in the packaging, which determines the extent of any migration;
- the type of food in contact with packaging material (solid foods make only limited contact, while liquids make more extensive contact). The presence of barrier layer may retard or prevent migration [86];
- the conditions of contact between food and packaging. The most important affecting migration are the time and temperature of contact. Temperature has a direct influence on rate and extent of migration,

and migration is faster when the temperature is higher, thus achieving a rapid establishment of equilibrium [87].

1.2.1.1. Food contact material (FCM) legislation

Migration can be defined as the mass transfer phenomenon, which occurs from FCMs to food and vice versa. Food, packaging, and environment, can interact with each other. The most dangerous interactions are those between food and environment, both for food hygiene and to the maintenance of food organoleptic characteristics. However, the food and packaging interaction has been of particular concern to the consumers, lawmakers and industries, due to the use of synthetic materials and new chemical substances used in packaging manufacturing. [85].

Food packaging represents the most prevalent FCM and it is regulated under the EU framework regulation EC 1935/2004. The framework covers all food contact materials and sets general safety requirements for the manufacturing, processing and distribution of all FCMs. Art. 3 of the present regulation introduced the migration subject stating that FCM under normal and foreseeable conditions should not transfer their constituents to food in quantities which could endanger human health, bring an unacceptable change in the composition of the food or bring deterioration in the organoleptic characteristics. FCMs have to be produced in compliance with the Good Manufacturing Practices (GMP) reported in the regulation EC 2023/2006 [88] that introduces them as compulsory for packaging production to ensure quality and safety. The materials that are included in the list of the framework regulation must be produced with raw materials and recognized ingredients that are considered safe by the law and are included in positive lists.

Since the Framework Regulation 1935/2004 does not provide specific requests for paper and board materials, some country of European Union enacted national requirements. In 2012, the Confederation of European Paper Industries (CEPI) developed the 'Industry guideline for the compliance of paper and board materials and articles for food contact', an operation guide for industry to comply with Reg. 1935/2004. This document lists raw materials and substances allowed in paper and board [89]. Recently, in 2019, CEPI published an update of the guideline after the revision by organization and authorities at both European and national level, becoming a reference for the paper and board food packaging value chain [90]. In 2021, The Federal Institute for Risk assessment (BfR)

published a new Recommendation XXXVI about 'Paper and broad intended for food contact' that the countries with no national laws recognized as a reference [91].

On the other hand, the plastics, different from the paper and board, are subjected to framework regulation 1935/2004. In addition to the general legislation, the European Union leads specific requirement for plastic materials used to produce food packaging. Commission Regulation (EU) n. 10/2011 [92] came into force on May 2011 replacing the plastic Directive 2002/72/EC. It regulates all plastic products intended for food contact, such as mono and multilayer plastic articles, as well as coatings of plastic and gaskets of glass jar closures. As reported in the Annex I of the Regulation 10/2011, the plastic materials have to be produced only with substances included in a positive list. To ensure the protection of the health of the consumer and to avoid any contamination of foodstuff, the Regulation establishes two types of migration limits:

- overall migration limit (OMG) of 60 mg/kg of food; is a limit to control the potential transfer of substances from packaging into food and it regards all chemicals in the material. It is a measure for the inertness of the material;
- specific migration limit (SML); applied to individual authorized substances and fixed on the basis of the toxicological evaluation of the substance. European authorities assume that an ideal cubic container (with a side of 1 dm^2) can lead to the maximum amount of 10 mg, from each side, to food contained in it (1 dm^3 or 1 L with a density of 1 kg/m^3 corresponding to the mass of 1 kg). Therefore, the limit is expressed in mg/kg of food in contact with food packaging. When the limit refers to the non-filled object or to the object with a capacity greater than 10 L or less than 500 mL, the limit is converted in mg/dm^2 (mg/dm^2=mg/kg x 6) [85].

1.2.1.2. Migration tests

Food packaging legislation have the object to protect the consumer by controlling the contamination of the food by chemical substances transferred from the packaging materials, so standard migration tests are recognized as an important tool to evaluate compliance of food packaging with the migration limits. Regulation No 10/2011 (EC) gives the basis to carry out conventional migration

tests. It provides the necessary data of time and temperature needed to conduct the experiments using both food and food simulants. The six food simulants are listed in table 3. A (for water containing food), B (for water containing food that have a pH value below 4.5) and C (used for alcoholic foods with an alcohol content of up to 20%, and those food that contain a relevant amount of organic ingredients that render the food more lipophilic) are assigned for foods that have a hydrophilic character, while D1 (used for alcoholic foods with an alcohol content of above 20% and for oil and water emulsions) and D2 (used for foods that contain free fats at the surface) are assigned for food that have a lipophilic character. Simulant E is assigned for testing specific migration into dry foods [93].

Table 3. Food simulant suggested for migration tests by Reg. 10/2011 (Adapted from [92]).

Food Simulant	Composition	Simulated Food
A	Ethanol 10% (v/v)	Hydrophilic foods
B	Acetic acid 3% (w/v)	Hydrophilic foods (pH <4.5)
C	Ethanol 20% (v/v)	Hydrophilic foods (alcohol content <20%) and lipophilic foods (relevant amount of organic ingredients)
D1	Ethanol 50% (v/v)	Alcoholic foods (alcohol content > 20%) and oil–in–water emulsion
D2	Vegetable oil (unsaponifiable matter <1%)	Foods with free fats at the surface
E	poly(2,6–diphenyl–p–phenylene oxide), particle size 60–80 mesh, pore size 200 nm (Tenax®)	Dry foods

The UNI-EN 1186:2003 standard introduced first the guidance to select appropriate methods to determine migration in food simulants. The methods proposed are:

- *Diving method*: the surface of the test material has to be fixed to a metal support and then immersed in a filled vessel of the simulant chosen for the test;
- *Fill method*: used for fillable packages, to use in similar conditions to real use;
- *Single side method:* used for materials where only one side comes in contact with food, the material is placed into a completely hermetic steel cell or in a closable Petri dishes and covered by the food simulant;
- *Other methods* for special cases (capsules, gaskets, caps etc.).

Food simulants must reflect the actual migration within narrow margins, providing valid data in a fast and simple manner [94]. Liquid simulants are not compatible with paper and board testing as they penetrate the material and their use would lead to an extraction rather than a one-side migration test. The European Standard EN14338 and CEPI suggest the use of Tenax® for testing this type of material [95]. Tenax® (poly 2,6-diphenyl-p-phenylene oxide), included in the EU legislation (simulant E) is the most commonly referred simulant for testing migration at high temperatures simulating fatty foods, and found wide use in migration testing of contaminants from paper and board [96]–[102].

The Regulation No 10/2011 provides the necessary data of time and temperature needed to conduct the experiments using both food and food simulants. The conditions must be representative for the worst foreseeable conditions of the intended use of the material or article and if the worst foreseeable conditions are represented by temperature exceeding 100 °C, the migration test should be done using the food simulant E. When these conditions are not covered by the regulation, the testing time and temperature parameters should be conducted using the Arrhenius equation:

$$t_2 = t_1 * Exp\,[(-E_a/R) * (1/T_1 - 1/T_2)]$$

Where t_1 is time at temperature of use, t_2 is testing time, E_a is activation energy, R is a gas constant (8.31 J/K/Mol), T_1 is contact temperature at use expressed in Kelvin (e.g. 298K= 25 °C) and T_2 is testing temperature in Kelvin.

The importance of this formula lies in the possibility of changing the test temperature to reduce the test time.

1.2.2. Barriers against mineral oil hydrocarbons

The Regulation No 10/2011 consolidates the definition of functional barrier, i.e. one or more layers of any type of material which ensures that the final packaging material does not transfer their constituents to food in quantities which could endanger human health, bring an unacceptable change in composition or bring a deterioration of the packed food.

The barrier properties of a packaging depend on the nature of the material of which are made. Glass and metals with adequate thickness provide a total barrier, i.e. preventing any migrating substance from reaching the food. Other materials, mainly plastics, offer selective barriers towards some migrants based on multiple factors.

Barrier materials have to be tested to evaluate their efficiency. According to Biedermann & Grob [103], the barrier efficiency is defined as the reduction of migration at the end of the shelf life of a product protected by a barrier layer compared with the migration in a product packed without the barrier layer. There are four type of methods that can be used to perform migration tests of barrier materials: migration cell method [104]–[106], sandwich [86], dynamic [107] and gravimetric [108] method. The basic method makes use of a migration cell (usually a glass cell) in which is positioned a specimen of the cardboard (which can be spiked with mineral oil), the barrier material (if separated from the cardboard) and the simulant (mostly Tenax®). Conditions used are usually those reported by method DIN EN 14338:2004, while test conditions are those described in the Regulation No 10/2011.

The experiment for testing the barrier material can be performed by:

- Migration experiments are used for testing the real packaging material and to evaluate the quantity of constituents migrating to foodstuffs or to food simulants. An example of this method application is reported in the study conducted by Guazzotti et al. [106] where the aim was to evaluate the effectiveness of a starch-coating for paperboard packaging preventing the migration of *n*-alkanes and MOSH. Different conditions of time and temperature were used for this migration test and Tenax® was used as food simulant;
- Permeation experiments are substantially conducted in the same way as the migration experiments, but the migrants (real or surrogates) are spiked at higher concentrations than normal in the donor phase, to represent the worst case of contamination. The main disadvantage is that the permeation rate depends on the film thickness of the barrier material and on the concentration of the migrant. Another disadvantage is that swelling effects can compete with migration, if migrants have low vapour pressure [109]. Biedermann & Grob [103] used this approach to find a method for cheeking the barrier efficiency of different materials. The usage of surrogate compounds instead of mineral oil mixtures allowed the authors to obtain much better detection limits of those obtained in previous works, as the one conducted by Fiselier & Grob [86], in which they used mineral oil mixtures;

- Lag time experiment is initially organized as a permeation experiment, but it uses a constant concentration on the donor side of the permeation cell. The receptor collects the permeated substances that are extracted and analysed to obtain the permeation rate. Usually, the donor is represented by a gas stream spiked with substances as mineral oil mixture or surrogate substances, so the starting point of the migration is clearly defined. The lag time is an important parameter for barrier materials, because it has to be longer than the shelf life of the packed product. In this case, the functional barrier can be considered satisfactory. Moreover, it is a pure kinetic parameter for a permeant/barrier combination and it is independent from the food, i.e. partition coefficients do not affect the lag time, in fact it can be calculated knowing only film thickness (l) and the diffusion coefficient (D_p) [109]. Ewender & Welle [110], set up an automated permeation testing method in order to determine the lag time of high-barrier films. They used a 12 μm polyethylene terephthalate (PET) film, as the literature reports good barrier behaviour of this material, placed between the two sides of a permeation cell made of aluminium. The authors concluded that the investigated PET film is an effective barrier towards MOH, because provides a lag time longer than the shelf life of the packed foods, calculated experimentally.

A lot of scientific works have turned their attention towards solutions to reduce migration of mineral oils to foods. In table 4 and 5 are summarized the plastic and bio-based barrier materials.

Table 4. Plastic barrier materials against mineral oil migration.

Barrier Materials	Testing Method	Conclusions	Ref.
LDPE/LLDPE (50 μm) LLDPE/HDPE (60 μm) OPP (30-50 μm) PA (20 μm) PET (12 μm)	Sandwich method, the donor pack (sheet of paper loaded with a solvent for printing inks) on one side of the tested barrier and the receptor (polyethylene film) on the other.	PE films showed low barrier properties. PP and PET films showed in contrast far beyond needed barrier properties.	[86]
HDPE (20.3/23.8 μm) OPP (19.7 - 42.5 μm) PET (5.5/7.6 μm) OPET (12.4 μm) OPA (14.9 μm)	Automated method, a paper disk soaked with the contaminant solution is placed into a permeation cell in contact with the tested film. A nitrogen flow moves continuously the permeated substances to pre-trap and then to a GC-FID.	PET and PA films are effective barriers towards mineral oil migration. Another important conclusion of this study is the developing of a method to evaluate the permeation rate of mineral oil components.	[111]
Various material of modest barrier efficiency (OPP, PVOH, PA)	Sandwich method, it was used the method descripted by Fiselier & Grob [86], changing the receptor with a silicone paper and the mineral oil solution with surrogate substances.	The aim of the study was to find a method for specifying and checking the efficiency of a barrier.	[103]
Various type of internal bag (mono or multilayer) Constituted by: Paper, aluminium, PE, PP, PET, PET-G, PA, EVOH, EVA, cellophane, PMMA and PVDC.	Sandwich method, it was used the method descripted by Biedermann & Grob [103].	The main conclusion of this study was the establishing of classes of barrier efficiencies, from 1 (barrier with no relevant efficiency) to 5 (complete barrier, aluminium foil).	[112]
PET (12 μm)	Automated method descripted by Ewender et al. [111]. The lower part of the permeation cell was	Developing of an automated method for the determination of lag times for high-barrier films.	[110]

	spiked with *n*-alkanes.		
PVOH coating (5 μm) Multilayer of polyester (7 μm) Flexo-printed polyacrylate layer (5-8 μm) Active carbon as adsorbent and PVOH coating (2 μm)	Sandwich method, it was used the method descripted by Biedermann & Grob [103].	Barrier tests was not adequate to evaluate the efficiency of the active carbon as incorporated adsorbent but appeared to be a promising way to reduce migration. Two of the three tested barrier kept the MOH migration low.	[20]
BOPP base film coated with: -no coating -Acrylic/Acrylic (25 μm) -PVdC (LTS) (26 μm) -Acrylic/PVdC (26 μm) -Acrylic (LTS) (25 μm) -Functional coating with MO barrier (LTS) (25 μm)	Basic method, use of a glass cell with Tenax as food simulant in contact with the barrier film. A head space was present between the cardboard and the film.	The not-coated film and the film coated with Acrylic/acrylic showed the worst performance. The last sample (mineral oil barrier coating (LTS)) showed the best result.	[104]
BOPP film (20 μm) Metallized BOPP film (20 μm) BOPP/EVOH multilayer film (20 μm) BOPP coated with Acrylic on both sides (31 μm) BOPP film PVDC/acrylic coated (25 μm) BOPET (12 μm)	Automated method descripted by Ewender et al. [111]. The lower part of the permeation cell was spiked with representative substances for MOSH and MOAH.	Not-coated BOPP provided no barrier against mineral oil migration. BOPET showed very good barrier properties, as a polar material. All other tested films provided insufficient barrier properties.	[107]
PET-coated boards and uncoated boards.	Cup test method, testing cups were filled with	A method for testing the mineral oil barrier properties of	[113]

	Tenax or powdered sugar and closed with the tested barrier material, placed into the desiccator together with the surrogate compounds (simulating mineral oils).	packaging materials was developed. The obtained migration results of PET-coated packaging were in accordance with literature.	
Biaxial oriented polyamide 6 (Ultramid) (12 μm)	Automated method descripted by Ewender et al. [111]. The lower part of the permeation cell was spiked with *n*-alkanes and 1-alcohols.	The tested barrier film was a sufficient barrier towards organic compound migration.	[114]
Functionalized recycled paper with PMMA and cyclic olefin copolymer (386.1 ± 12.9/439.8 ± 12.4 μm)	Basic method, it was used a glass migration cell filled with the paperboard sample in contact with Tenax as food simulant.	The proposed treatment reduced the MOHs migration below the limits proposed by BMEL. Moreover, it improves water and grease resistance.	[105]

Polyethylene (PE); Low Density Polyethylene (LDPE); Linear Low Density Polyethylene (LLDPE); High Density Polyethylene (HDPE); Oriented Polypropylene (OPP); Polyamide (PA); Polyethylene Terephtalate (PET); Oriented Polyethylene Terephtalate (OPET); Oriented Polyamide (OPA); Polypropylene (PP); Polyethylene Terephtalate Glycol (PET-G); Ethylene Vinyl Alcohol (EVOH); Ethylene Vinyl Acetate (EVA); Poly(Methyl Methacrylate) (PMMA); Poyvinyldene chloride (PVDC); Polyvinyl Alcohol (PVOH); Biaxially Oriented Polypropylene (BOPP); Biaxially Oriented Polyethylene Terephtalate (BOPET).

Table 5. Bio-based and edible barrier materials against mineral oil migration.

Barrier Bio-based Materials	Testing Method	Conclusions	Ref.
Paperboard coated with: **-Maize cationic waxy starch** **-Maize cationic starch** **-Cationic starch mixture with high amylose content** **Other three coating were prepared with the addition of sorbitol as plasticizer (3.8–14.2 μm)**	Basic method, it was used a glass migration cell into a thermostatic oven. A round piece of spiked (*n*-alkanes C_{10}-C_{40}) paper was covered by Tenax, as food simulant, on its food contact side.	Coated paper demonstrated the effectiveness against migration of *n*-alkanes (C_{18}-C_{26}). No significant differences between different starch composition were observed.	[106]
Cellulose-based film (Cellophane and Natureflex) (20-30 μm) **BOPP films coated with:** **-PVdC on both sides** **-Acrylic coating on one side** **-Acrylic coating on both sides**	Sandwich method, the donor material (virgin cardboard spiked with paraffinic mineral oil) was placed in direct contact of one side of the barrier material and, on the other side was placed the receptor material (filtration paper). The pack was wrapped into metal foil.	Cellulose -based films showed excellent barrier properties against mineral oil migration, due to the structural composition of this films. BOPP films did not have sufficient barrier properties. The acrylic coated BOPP film, with a modification on the density of the material, improved its barrier property.	[115]
Paperboard coated with different composition of the following ingredients: **-low-viscosity potato starch** **- latex** **-barrier-grade kaolin pigment** **-carboxymethyl cellulose (CMC)**	Gravimetric method, measurement dishes were filled with heptane as mineral oil simulant and closed with the coated paperboard with the coated side towards the inside. The assembled dishes were weighted at 0, 1,3,5 and 24 hours to determine the decrease in weight.	The starch was recognized as the main responsible for the plasticity and hydrophilicity of the coating, giving to the packaging great barrier properties.	[108]
Uncoated Bio-HDPE film and Bio-HDPE	Cup test method, proposed by Liane et al. [113].	Uncoated Bio-HDPE showed insufficient	

coated with: **-TEMPO-CNFs** **-HPX** **-HPC**	The used surrogates were *n*-decane, isobutylbenzene, 1- cyclohexylbutane, 1-cyclohexylheptane, and 1-cyclohexyldecane.	results against mineral oil migration. However, Bio-HDPE coated with the tested materials, improved considerably the film performance, in particular TEMPO-CNF.	[116]
Fresh fiber paper and recycled fiber paper coated with chitosan coating solution (98/199 μm) and sodium-alginate coating solution (98/198 μm)	Both migration and permeation experiments were performed. For the first, a simple set-up was used, with a migration cell filed with Tenax and the tested material with the coated side facing the simulant. For the second, it was used a single-sided experiment and a two-sided experiment.	With the application of this bio-based materials, the permeability, migration and transmission properties of the paper-based packaging were substantially improved.	[117]

(2,2,6,6-tetramethylpiperidin-1-yl)oxy Oxidized Cellulose Nanofibrils (TEMPO-CNFs); Hydroxypropylated Xylan (HPX); Hydroxypropylated Cellulose (HPC)

1.2.2.1. Barrier materials against MOH currently available on the market

Many barrier materials have been proposed on the market as a solution against mineral oil migration. The company "Smurfit Kappa" proposed a solution to prevent mineral oil migration from cardboard packaging to food based on the work of Lommatzsch et al. [20]. The packaging technology called "Catcher Board MB12" can be applied to solid boards and corrugated and it does not require any changes to the existing production process of the packaging.

Treofan, for the mineral oil migration problem, introduced a functional barrier material, called Treofan Mineral Oil Barrier Film, that provides a lag time up to 128 weeks at room temperature, much longer than the one provided by other mineral oil barrier films. It also provides a barrier against water vapor and aroma transmission. It is mainly recommended for dry foodstuffs like pasta, cereals and bakery.

Another barrier material was proposed by Mondi and it is called "Miprotex", a paper-based solution that prevents the migration of mineral oils. It is a cost-efficient alternative to traditional high-barrier materials and also provides barrier capabilities against aroma, grease, oxygen and water vapor. Its applications include a wide variety of foods, from baking mixture to snacks and from pasta to pizza box.

Innovia company developed different packaging solutions and, among them, the Propafilm™ Strata, a high-barrier mono-material-web recyclable film that provides protection against oxygen and water vapor, even at high relative humidity, and against mineral oil migration.

A bio-based material, biaxially oriented PLA based, called NATIVIA® was introduced in the market by Taghleef Industries. It is made of polylactic acid from NatureWorks and, thanks to the bio-based content and the lower consumption of energy, it has a lower carbon footprint compared to other packaging plastic materials. Its properties include high mechanical strength, high moisture transmission rate and effective barrier against MOH migration.

1.3. Oligomers

The Union Guidelines of Regulation (EU) N. 10/2011 defines the oligomers as *'substances consisting of a finite number of repeating units which has a molecular weight of less than 1000 Da'*. As potential migrants are considered the molecules with weight <1000Da [93]. The main sources of production of these substances

are the incomplete polymerization and hydrolytic or thermal degradation of the polymer chain during the polymerization process. Degradation of the polymer by irradiation energy is another way of oligomer formation. In addition, during the main process of food treatment, such as sterilization, microwave, ozone treatments or UV-light, this type of degradation may occur [118].
Even though often defined as non-intentionally added substances (NIAS) oligomers are intrinsic part of the molecular weight distribution of the polymer [119]. Oligomers are only regulated by Framework Regulation 1935/2004, in particular by Art. 3 requiring that the migrates must not endanger human health. Missing specific regulation means that the producers must perform the compliance work themselves.

1.3.1. Polyolefin oligomeric hydrocarbons (POH)

1.3.1.1. Origin, chemical characteristics and legislation

Polyolefins are a family of polyethylene (PE) and polypropylene (PP) thermoplastics. They are produced mainly from oil and natural gas by a process of polymerization of ethylene and propylene, respectively. Polyolefins contain oligomers in a form of linear and branched alkanes and alkenes (no aromatics) (figure 9). Their versatility has made them one of the most popular plastic in use. In Art. 3 of Regulation (EC) 1935/2004, general rules concerning the presence of undesirable compounds in food are specified [120]. Regulation (EU) 10/2011 establishes more precise parameters. European commission (2011) [92] introduced an OML of 10 mg/dm^2 to cover the complete migration of all substances and this limit can be monitored through migration tests, which use food simulants at different time and temperature conditions.
Polyolefins may release oligomers into foods and they largely consist of saturated hydrocarbons called POSH. The presence of POSH into the food and food packaging samples can be easily confused with MOSH, because both consist of highly isomerized branched and possibly cyclic hydrocarbons, both forming humps of unresolved components in gas chromatography.

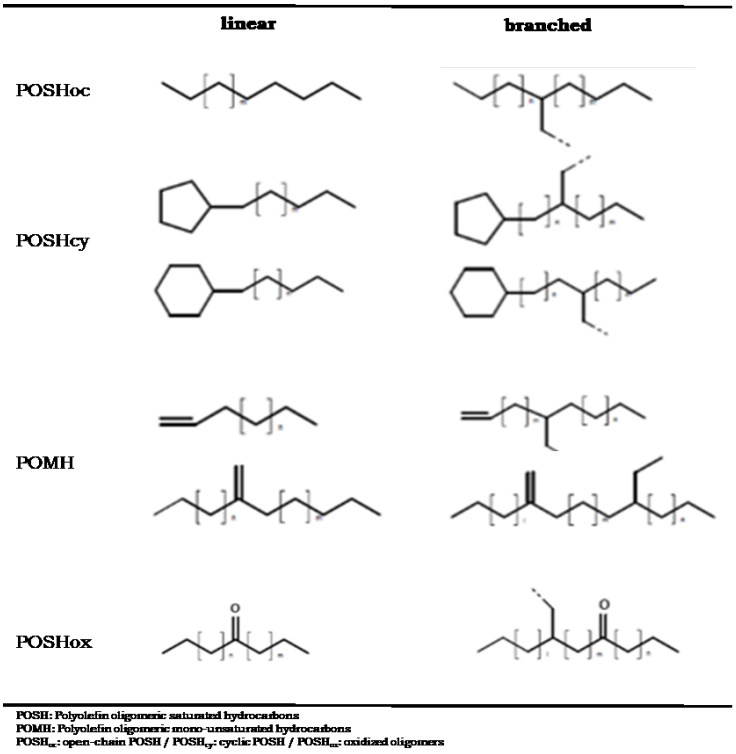

Figure 9. Examples of polyolefins (Adapted from [121]).

Polyolefin oligomers are mentioned in the EFSA scientific opinion as a source of hydrocarbon contamination in food. Some important statements about POSH are reported below:

- 'Sources, occurrence and exposure assessment: polyolefins contain low molecular weight oligomers, including POSH and olefins. In most polyolefins POSH largely consist of branched components forming patterns of unresolved peaks. Depending on the type of food (dry, aqueous or fatty), the polymer type and thickness of the polyolefin layer, as well as the nature of contact with the packed food (time, temperature) these oligomers can migrate into the food at levels from the low ppm range (1-10 mg/kg) up to overall migration limit of 60 mg/kg';
- 'Advice on future monitoring: Distinction of hydrocarbons, such as Poly Alpha Olefins (PAO) and POSH, from MOSH, if possible';
- 'Conclusions: Contamination with polyolefin oligomeric saturate hydrocarbons (POSH), e.g. from plastic bags, heat sealable layers or adhesives, may interfere with POSH analysis' [1].

Since specific regulation for POSH toxicity is missing, Biedermann-Brem et al. [122] affirm that it would be prudent to evaluate POH in the same way as MOSH (see paragraph 1.1.1.2.). From this point of view, since the sum of the two is

relevant, the problem that the POH and MOSH cannot be qualitatively separated is of minor importance. Assuming the same ADI for the POH as JECFA [3] specified for the MOSH and applying the appropriate conventions for specific migration limits, up to 0.6 mg/kg, POH could be considered as safe. However, the concentrations found in foods packed into plastic bags with internal polyolefin layer often exceeded this value, frequently by some 10 times [122]. Nevertheless, the temporary ADI value of 0.01 mg/kg bw per day, proposed in 2002 by JECFA has been withdrawn in 2013 [123] for lack of toxicological data confirming the proposed ADI.

1.3.1.2. Analytical determination

Polyolefin's oligomer characterization is still little discussed, due to the lack of publications on this topic and the complexity of this class of substances. The method developed for analysis of mineral oil hydrocarbons by Biedermann & Grob [43], [69] was used for POH analysis by Biedermann-Brem and collaborators [122]. Samples were analyzed using on-line coupled HPLC-GC-FID. Nevertheless, using on-line HPLC-GC-FID it is not possible to distinguish between MOSH and POH. Recently, Lommatzsch et al. [124] developed on-line HPLC-HPLC(Ag^+)-GC method, able to separate POH into POSH and polyolefin monounsaturated hydrocarbons (POMH). To obtain additional information about the composition of POH from different polyolefin samples Lommatzsch et al. [124] used characterization by comprehensive two-dimensional GC (GC×GC-FID and GC×GC-MS). Biedermann & Grob [82] reported that comprehensive two-dimensional GC×GC-FID is able to produce plots distinguishing MOSH from POSH and characterizing the degree of raffination of a mineral oil using a phenyl methyl polysiloxane in the first dimension and dimethyl polysiloxane in the second dimension.

1.3.2. Poly(alpha)olefins (PAO)

PAO are isoparaffins with short main chains and long side chains. The main constituents of synthetic motor lubricant oil or lubricants in the food sector are low molecular weight PAOs and higher molecular weight PAOs (resins) are used in glues (e.g. hotmelts) [125]. They are of synthetic origin, mainly co-eluting with MOSHs (sometimes even with MOAHs) and can migrate from "hotmelt" type adhesives used to seal packaging [126]. Due to the lack of publications on this topic PAO characterization is still discussed. PAOs are evaluated together with

POSH and MOSH, because at the moment there are no methods in the literature able to analyze only PAOs, but they can be distinguished from MOH by using comprehensive GC. Barp and collaborators [126] found contamination with PAO in semolina pasta packed in boxes closed with hot-melt adhesives. For the analytical determination the authors used on-line LC-GC-FID, but an accurate quantification of the contribution of the hot-melt adhesives was not possible due to the high interference in the chromatogram of the MOH from recycled paperboard.

1.4. Polycyclic aromatic hydrocarbons (PAH)

1.4.1. Origin, chemical characteristics and legislation

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds containing 2-6 fused aromatic rings with linear, cluster or angular arrangement, and can present substituting group, such as alkyl, aryl, nitro, amino or heterocyclic ring. Figure 10 shows the chemical structure and abbreviations of the main PAHs considered by European legislation.

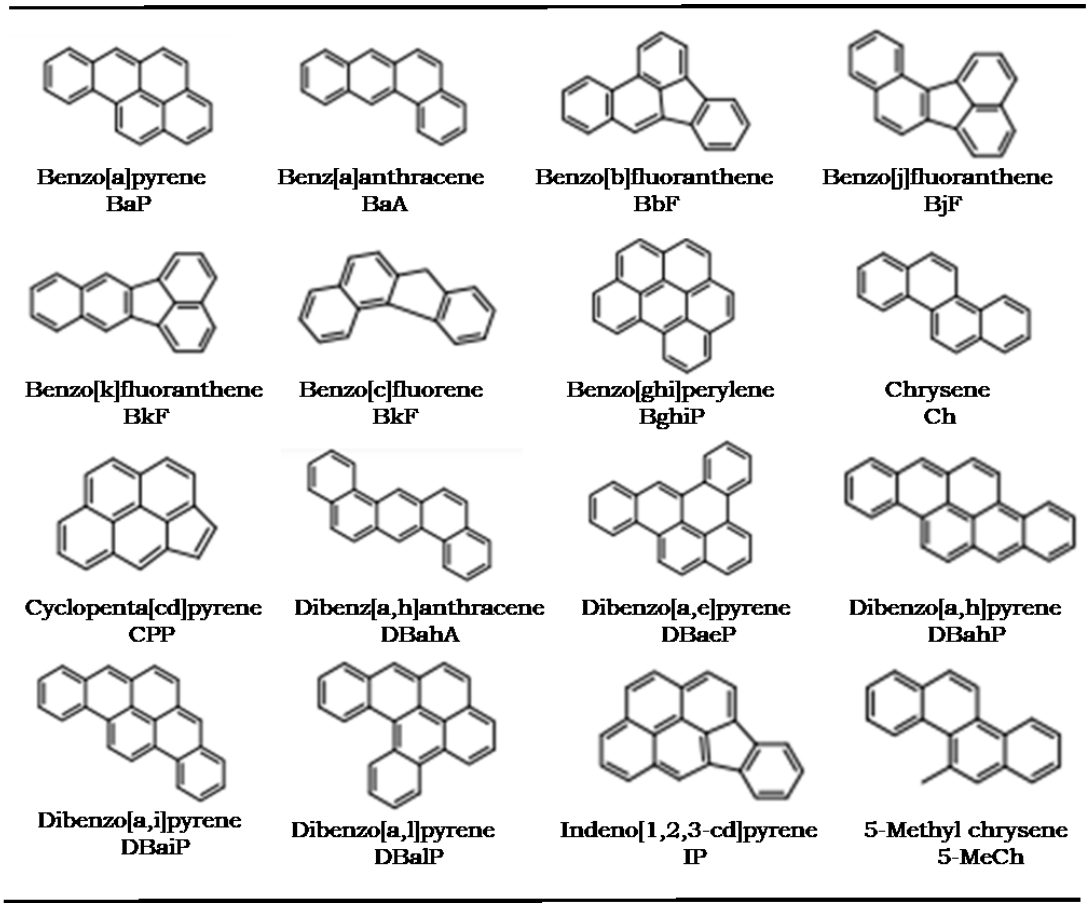

Figure 10. Chemical structure and abbreviations of 16 priority EU PAHs.

PAHs are formed at high temperature (500-700 °C) during incomplete combustion or pyrolysis of organic matter and the major emission sources in the atmosphere are mainly related to human activities, like domestic wood burning, emission from motors and municipal waste combustion [127], [128], but there are also natural sources as forest fires, volcanic eruptions and hydrothermal processes [49], [129]. PAHs can be also formed at low temperature (100-150 °C) but it requires a period on the geological time scale. This process originates a large number of alkylated PAHs, as in the case of natural fossil fuel formation. Thus, the alkylated/non-alkylated PAHs ratio could be an indicator of the contamination source [6], [49].

Food can be contaminated by environmental PAHs that are present in the air (by deposition), soil (by transfer) or water (by deposition and transfer), or during processing and cooking. However, the major sources of PAHs are technological processing involving heating processes (drying and smoking) or cooking food at high temperatures (grilling, roasting, toasting) [130]. The level of contamination is related to the time and temperature of processing, the distance from the heating source, the kind of process, the type of fuel used and the amount of fat present in the food [6], [49]. Fatty foods are one of the main sources of PAHs in our diet, due to the lipophilic nature of these contaminants. According to the SFC in 2002 [130] and the European Food Safety Authority (EFSA) in 2007 [131], the main PAH dietary intake for humans is attributable to vegetable oils and fats. PAHs are compounds that involves potential health risks because of mutagenic, carcinogenic and endocrine disrupting effects of some of them [127]. The structure, the size and the chemical nature of the particles where PAHs are adsorbed determine lung adsorption, while the adsorption in the intestine mucosa is mainly affected by the diet composition (enhanced by lipophilic food and inhibited by high-fiber foods) [6]. PAHs presenting 4-6 rings are considered the most harmful, in particular benzo[a]pyrene (BaP) which can cause infertility and development disorders. Nevertheless, not all the PAH congeners have the same activity. SFC reported that only 15 of 33 considered PAHs showed clear evidence of both genotoxicity and carcinogenicity; some were genotoxic but not carcinogenic, other were non-carcinogenic, but may act as synergists [130]. EC Regulation 1881/2006 [132] harmonized the PAHs legislation among EU Member States and fixed a limit for the presence of BaP only, used as a marker for the presence of genotoxic and carcinogenic PAHs. In 2005 European Authorities recommended that all the Member States should investigate the presence of the

15 PAHs underlined by SCF plus the one (BcF) considered by JECFA (called the 16 EU PAHs) [133] to review the limits already set. Later, in 2011 was published a new Regulation (Reg. 835/2011) that fixed new limits for BaP and PAH4 for several food classes [134].

1.4.2. Sample preparation and analytical determination

There is still a lack of standardized methods for the analysis of PAHs. However, a numerous in-house validated method are reported in the literature. Since PAHs are usually present in food at very low concentration, sample preparation methods consist of several extraction and purification steps to reach low detection limits. Over the years, many methods for the PAHs analysis in edible oils and fatty sample extracts have been proposed. SPE is probably the most widely employed technique performing a unique extraction and clean-up step using different sorbent phases (C18/Florisil®, polystyrene/divinylbenzene (PS/DVB), silica). The same sorbent phases are employed for matrix-solid phase dispersion (MSPD). Solid phase microextraction (SPME) both in the head-space (HS) and direct immersion (DI) mode was also explored for PAH analysis in liquid and solid samples. Magnetic-solid-phase extraction (MSPE), which uses a magnetic material as an adsorbent, has recently been developed for PAHs extraction. PLE, supercritical fluid extraction (SFE), microwave assisted extraction (MAE), membrane-assisted solvent extraction (MASE), and dispersive liquid-liquid microextraction (DLLME) are sample preparation techniques generally used for PAHs extraction from solid matrices [6], [135].

PAHs are often separated and quantified using either GC-FID or coupled to MS or HPLC-ultraviolet (UV) or fluorescence (FLD) detection or coupled to MS [131]. FLD allows to obtain high sensitivity, selectivity and high resolution and it allows to minimize the sample preparation by injecting partially purified samples. GC-MS has some advantages over LC-FLD, particularly on terms of resolution. Thus, GC possesses a high resolving power and MS provides high mass selectivity and structural information. Moreover, GC-MS enables the determination of non-fluorescent and poorly fluorescent PAHs [136]. Coupled techniques, such as LC-GC, LC-LC-GC, or LC-LC, were proposed to reduce sample manipulation and risk of cross-contamination. Moreover, a multidimensional comprehensive technique such as GC×GC have also been applied for PAHs determination in food [137].

AIM OF THE WORK

The main purpose of this PhD thesis was the optimization and validation of rapid, high-sensitivity and solvent-sparing sample preparation protocols (based on microwave assisted saponification and epoxidation, followed by on-line HPLC-GC-FID method), for the determination of mineral oil and related hydrocarbon contaminants in complex food matrices (fish products, pesto sauce, cocoa powder, infant formula).

The other purposes of the present work were to collect data on the contamination levels in the investigated matrices, to identify the main sources of contamination, and to assess the impact of migration from food packaging.

Chapter 2. ON-LINE HPLC-GC-FID DETERMINATION OF HYDROCARBON CONTAMINANTS IN FRESH AND PACKAGED FISH AND FISH PRODUCTS

- ✓ Srbinovska, A., Conchione, C., Lucci, P., Moret, S. **(2021).** On-line HP(LC)-GC-FID determination of hydrocarbon contaminants in fresh and packaged fish and fish products. *Journal of AOAC INTERNATIONAL,* 104(2), 267-273.

2.1. Introduction

Mineral oil hydrocarbons (MOH) are complex mixtures of hydrocarbons of petrogenic origin, which may contaminate foods throughout different routes. Due to their different toxicological relevance, it is important to distinguish mineral oil saturated hydrocarbons (MOSH), comprising linear, branched and cyclic saturated hydrocarbons, from mineral oil aromatic hydrocarbons (MOAH) of different ring number and alkylation degree. MOSH are of concern due to their ability to accumulate in different organs and tissue in humans, while MOAH may comprise carcinogenic and genotoxic substances ($\geq$3 aromatic rings and low alkylation degree) [32].

The reference method for MOSH and MOAH analysis is on-line liquid chromatography-gas chromatography, followed by flame ionization detection (LC-GC-FID), as firstly described by Biedermann et al. [50]. With the same analysis it is also possible to highlight the presence of polyolefin oligomeric hydrocarbons (POH) [122] which may migrate from polyolefin packaging (PE and PP) to food. Since POH co-elute with the MOSH fraction, on-line LC-GC-FID only allows for partial POH/MOSH discrimination, based on the chromatographic profile.

Although there has long been evidence of accumulation of aliphatic hydrocarbons in the tissue of different fish species, data on the presence of MOH in fish and seafood is relatively scarce. Mironov et al. [138] found that biogenic and branched hydrocarbons, as well as unresolved complex mixture (UCM) indicative of petrogenic contamination, accumulated in several fish species caught in the Straits of Malacca and the Mediterranean Sea. In freshwater fish from populated regions the sources could include dust washed into the water or debris/extracts from road tar [1].

The fat extracts of 40 fishes, randomly collected from the Swiss market in 1996, contained 20-800 mg/kg of MOH (on average 350 mg/kg), 3-150 mg/kg referring to the whole fish, with no difference between salt and freshwater fish. The molecular mass distributions were centered between *n*-C_{17} and *n*-C_{28}. None of the sample was from an area with a known oil spill [1], [72].

Moret et al. [5] reported in the fat of a lake fish 220 mg/kg of MOSH, 25 mg/kg of naphthalenes (11%) and little 3-ring aromatics.

Comprehensive two-dimensional GC-time-of-flight-mass-spectrometry was used by Booth et al. [139] to resolve and identify individual petroleum-derived hydrocarbons in UCMs, accumulated by mussels (*Mytilus edulis*). Mussels

collected around the UK coast exhibiting impaired health contained larger amounts of aromatic hydrocarbon UCMs compared to healthy mussels. The UCMs (up to 125 mg/kg dry tissue) contained branched alkylbenzenes, tetralins, indanes and indenes.

Colombo et al. [140] studied the bioaccumulation dynamics of *n*-$C_{12–25}$ alkanes, isoprenoids and unresolved aliphatic hydrocarbons in a detritivorous fish (*Prochilodus lineatus*) collected from 1999 to 2005 in the sewage impacted Buenos Aires coastal area. Fish muscles contained huge amounts of *n*-$C_{12–25}$ and UCM (399 ± 288 fresh weight, corresponding to 1567 ± 802 mg/kg lipid weight) reflecting the chronic bioaccumulation of fossil fuels from sewage particulates.

More recently, Reid & Budge [141] found that fish used in the production of fish oil supplements accumulated petrogenic hydrocarbons in tts tissues, which were not removed by minimal oil refining. Weathered petroleum hydrocarbons in the form of a UCM were found at 523 mg/kg in a virgin Alaskan salmon oil supplement. Highly refined supplements were free of contamination.

In wild fish the presence of MOH can be the results of bioconcentration from contaminated water (e.g. after an oil spill) or biomagnification through the food chain. Contamination can also come through the feed [54], [73], thereby extending contamination to farmed fish. Mineral oils are indeed used as binders for additives, as lubricants in the preparation of pellets and to reduce their dustiness, increase their resistance to humidity and control their content in water. Excluding fraud, causes of accidental contamination can also occur during feed production, processing (e.g. leaks of lubricating oils) or transport [1]. Moret et al. (unpublished) reported for 19 feeds sampled in 4 different rainbow trout breeding in Friuli Venezia Giulia region, MOSH level comprised between 3.7 and 221.5 mg/kg (on average 27.2 mg/kg). Levels in the trout fillet ranged from 0.3 to 6.9 mg/kg fresh weight (on average 1.9 mg/kg). Van Heyst et al. [38] found MOSH/MOAH levels lower than 0.5 mg/kg in four cods and three soles.

In addition to the contamination already present in the fish, migration from packaging can cause further contamination. A survey carried out on canned fish by Grob et al. [72], showed MOSH levels mostly around 100 mg/kg in the oil phase (maximum 820 mg/kg) and 10-15 mg/kg in the sea foods (maximum 370 mg/kg). Mineral oils used to lubricate sheets of metal as they are cut and shaped into can bodies and ends were identified as one of the potential sources of contamination. Also, polyolefin which is used as primary packaging, may determine POH migration under favorable condition.

Sample preparation before analytical determination of mineral oils represents a fundamental step of the entire procedure. Depending on the food type, different sample preparation protocols based on solvent extraction or traditional saponification, followed by further sample purification (when necessary), have been applied over the last years [6], [142]. In 2019, the JRC [143] recommended to follow a decision tree to select proper auxiliary methods for removing interference and reaching adequate sensitivity. It also gave indications on data reporting (defined MOSH and MOAH C-fractions) and reported different performance requirements for different food categories (in terms of method recovery, intermediate precision and limit of detection). Regarding extraction from fish tissue, saponification represents an advantageous solution which enables for simultaneous extraction and purification from triglycerides. Nevertheless, saponification, when carried out with traditional methods, is time consuming and requires a large amount of solvent.

Based on these factors, the first aim of this work was to validate a rapid, solvent-sparing method, based on microwave assisted saponification/epoxidation followed by on-line LC-GC-FID, for MOSH/POH and MOAH determination in fish samples. The validated method was applied to a range of different fish samples, in order to collect more data on the contamination levels of this food category, in agreement with requirements of Commission Recommendation (EU) 2017/84 of 16 January 2017 on the monitoring of MOH in food and in materials and articles intended to come into contact with food. Attention was focused on salmon samples of different fat content (wild and farmed), packaged under different conditions.

2.2. Materials and methods

2.2.1. Chemicals and materials

n-Hexane and dichloromethane (distilled before use), were purchased from Sigma–Aldrich (Milan, Italy). Ethanol was of HPLC grade. Water was purified with a Milli-Q System (Millipore, Bedford, MA). Glassware and other materials were carefully rinsed with acetone and hexane just before use.

2.2.2. Standards

The C_{10-40} *n*-alkane standard mixture used to check GC performance, was from Fluka Sigma–Aldrich. The Gravex solution used for sample spiking was kindly furnished by a producer.

The working internal standard solution (IS), was purchased by Restek (Milan, Italy) and consisted of: 5-α-cholestane (Cho) and perylene (Per) at 0.6 mg/mL, 1,3,5-tritert-butylbenzene (TBB), *n*-C_{11}, cyclohexyl cyclohexane (CyCy), pentyl benzene (5B), 1-methyl naphthalene (1MN), 2-methyl naphthalene (2MN) at 0.30 mg/mL, and *n*-C_{13} at 0.15 mg/mL in toluene.
Before samples analysis, standard solutions were injected to check method performance.

2.2.3. Sampling

Samples of both fresh and packaged fish were taken from the Italian market. With the term "fresh" we mean that the fish had not been previously frozen. Fresh fish was sold unpackaged, but this does not mean that it had not previously been in contact with packaging materials capable of releasing hydrocarbon contaminants. Table 6 reports information on the food and the packaging.

Table 6. Sample description.

Code	Product type	Origin	Wild (W)/ Farmed (F)	Fat (%) (label)	Packaging mode	Primary packaging
SA1	Fresh salmon fillet	Norvey	F	NA	None	None
SA2	Fresh salmon fillet	Norvey	F	NA	None	None
SA3	Salmon fillet	Norvey	F	NA	Protective atmosphere	Pl (PET)
SA4	Smoked sliced salmon	Norvey	F	11	Vacuum packed	RP/Al-Pl
SA5	Smoked salmon fillet	Scotland	F	7.2	Vacuum packed	RP/Al-Pl
SA6	Smoked salmon fillet	Northeast Pacific	W	1.5	Vacuum packed	Pl (PE)
SA7	Smoked salmon fillet	Northeast Pacific	W	1.5	Vacuum packed	Pl
SA8	Smoked salmon fillet	Scotland	F	11	Vacuum packed	RP/Al-Pl
SA9	Smoked sliced salmon	Norvey	F	14	Vacuum packed	RP/Al-Pl
SA10	Smoked sliced salmon	Norvey	F	14.5	Vacuum packed	RP/Al-Pl
SA11	Salmon tartare	Faroe Islands	F	NA	Protective atmosphere	Pl (PET)
FL1	Fresh flounder fillet	Northeast Pacific	W	NA	None	None

FL2	Frozen flounder fillet	Northeast Pacific	W	1.1	Plastic bag	LDPE
SO1	Fresh sole fillet	Northeast Pacific	W	NA	None	None
CO1	Frozen cod fillet	Northeast Pacific	NA	0.1	Vacuum packed	Pl
SB1	Frozen seabass fillet	Turkey	F	2.7	Plastic bag	PA/EVOH/PE
SW	Smoked sliced swordfish	Central-western Pacific	W	9.4	Vacuum packed	Pl/Al
TU1	Frozen tuna fillet	Southeast Pacific	W	1.1	Vacuum packed	LDPE
TU2	Tuna tartare	Central-western Pacific	W	10	Protective atmosphere	Pl (PET)
SBF	Sea bream fillet ready to cook	NA	NA	7.2	Plastic film for oven cooking	PET
RCS	Ready-to-eat rice with cuttlefish and shrimp (10%)	NA	NA	3	Plastic container for microwave heating	PP

NA = Not available; Pl, plastic (not specified); RP, recycled paperboard; Al, aluminum

2.2.4. Sample preparation

For packaging extraction the method proposed by Biedermann-Brem et al. [122] was used. About 300 mg of the sample cut into little pieces were added with 20 µL of the IS mixture and extracted with 10 mL of *n*-hexane overnight (under magnetic stirring). An aliquot of the extract corresponding to about 20 µL was directly injected into the LC-GC-FID system.

For food samples, 5 grams of the fish sample, accurately homogenized with a laboratory mill (IKA A10 analytical mill), were directly weighed into a Teflon-lined vessel (Green Chem plus, CEM Corporation), added with 5 µL of the IS mixture, 10 mL of saturated methanolic potassium hydroxide, and 10 mL of *n*-hexane.

After closure, the extraction cells were positioned into a microwave extractor (Mars, CEM Corporation, Matthews, NC) able to process up to 14 samples simultaneously. MAS condition (120 °C for 20 min) were the same described for PAH extraction from fish tissue and propolis [144], [145] and later validated for MOH determination on cereal-based products [47]. After MAS and cooling at ambient temperature, the sample was washed (in the extraction cell) with 40 mL of water followed by 2 mL of methanol, letting the solvent to flow along the walls of the extraction cell. After a rest at -20 °C for at least 30 min, 5 mL of the sample extract in *n*-hexane was withdrawn and concentrated to 1 mL. Of this, 300 µL were transferred to an autosampler vial for direct MOSH analysis. If MOAH presence was suspected, the remaining extract (700 µL) underwent epoxidation according to a slightly modified Nestola & Schmidt [55] protocol. In

brief, 700 µL of the sample extract was added with 500 µL of an ethanolic *m*CBPA 20% solution and placed into an agitator at a speed of 500 rpm for 15 min at room temperature. Afterward, 2 mL of an aqueous sodium thiosulfate 10% solution and 500 µL of ethanol were added. The vial vas shaken for 2 minutes. Five hundred microliters of the hexane phase were transferred into an autosampler vial prefilled with a spatula tip of sodium sulfate, and then injected into LC-GC-FID system.

2.2.5. LC-GC-FID analysis

On-line LC-GC-FID analysis was performed on a LC-GC 9000 from Brechbühler (Zurich, Switzerland). The LC column was 25 cm × 2.1 mm i.d. packed with Lichrospher Si 60, 5 µm (DGB, Schlossboeckelheim, Germany). The GC was a double channels Trace 1310 series from Thermo Scientific (Milan, Italy). Both channels consisted of carrier gas line, Y-interface, retention gap and GC column. The transfer of the LC fraction into the GC was carried out through a Y-interface [50], using the retention gap technique. A 10 m × 0.53 mm i.d. uncoated, deactivated pre-column was followed by a steel T-piece union connected to the solvent vapor exit and 15 m × 0.25 mm i.d. separation column, and coated with a 0.15 µm film of PS-255 (1% vinyl, 99% methyl polysiloxane; Mega, Italy). The FID (sampling frequency of 50 Hz) and solvent vapor exit were heated at 360 and 140 °C, respectively.

The sample was eluted at 300 µL/min using a gradient starting with *n*-hexane (0.1 min hold) and reaching 30% of dichloromethane after 0.5 min. The MOSH and the MOAH fractions were eluted from 2.0 to 3.5 min, and from 3.8 to 5.3 min, respectively. Six minutes after the injection, the column was backflushed with dichloromethane at 500 µL/min for 9 min and then reconditioned at 700 µL/min with *n*-hexane for 6.5 min and at 300 µL/min for 1.5 min. The GC run was speeded up by increasing the oven temperature program at 20 °C/min from 55 to 350 °C (40 °C/min for some chromatographic runs) and using hydrogen as a carrier at a constant pressure of 60 kPa. During the transfer the pressure was increased at 90 kPa. The MOSH area was determined by the integration of the whole hump of largely unresolved peaks, subtracted of the endogenous *n*-alkanes. All sharp peaks standing on the top of the MOAH "hump" were subtracted from the total area. The position of the baseline was assessed by procedural blank runs obtained on the same day. Quantification was based on ISs.

2.2.6. Recovery and repeatability tests

A Gravex mineral oil standard (containing 73% of MOSH and 27% of MOAH in the *n*-$C_{16–25}$ range) was used for recovery tests. The recoveries were calculated by comparing the amount found in the spiked sample (the unspiked sample had no detectable contamination in the range *n*-$C_{16–25}$), with the amount of standard used to spike the sample. The sample was finely ground, weighed directly into the extraction vessel and added with the mineral oil standard dissolved in about 5 mL of pentane, and gently stirred for 30 min to uniformly distribute the added mineral oil (the added solvent evaporated during stirring) and was left to age for 72 h before MAS. Recovery tests were performed (during 4 different days) at two different fortification levels (1.0 and 5.0 mg/kg). Inter-day repeatability was calculated both on the added Gravex and on the pre-existing contamination.

2.3. Results and discussions

2.3.1. Method performance

Linearity of the on-line HPLC-GC-FID method was previously verified by Barp et al. [65] for a mixture of paraffin oil and offset printing ink in *n*-hexane (in the range 1–50 μg/mL each).

To verify GC performance, the response ratio of *n*-C_{50} to *n*-C_{20} was checked daily (every few chromatographic runs). It was around 0.9-1.0, well within the range (0.8 and 1.2) indicated by the JRC guidelines [143].

Since no certified reference material is commercially available, method accuracy was assessed by the standard addition procedure described in Materials and Methods (paragraph 2.2.).

Figure 11 shows the chromatographic trace of the tuna sample (TU2) used for both recovery and repeatability tests. This sample (indicated as “unspiked” in figure 11) had no detectable contamination in the range *n*-C_{13-25}, corresponding to the elution range of the Gravex used for recovery tests, but had a pre-existing contamination in the range *n*-C_{25-50}. The choice went to this sample, rather than to one completely free of contamination, because in this way it was possible to use the same tests for calculating both recoveries and precision of the method.

To assess recovery, 6 aliquots of this tuna sample were spiked with 1.0 mg/kg of Gravex, corresponding to 0.7 mg/kg of MOSH and 0.3 mg/kg of MOAH, while other 4 aliquots were spiked with 5.0 mg/kg of Gravex corresponding to 3.7 mg/kg of MOSH and 1.4 mg/kg of MOAH.

Practically quantitative recoveries were obtained for both the spiking levels: on average 103% for the MOSH and 99% for the MOAH at the lowest spiking level (6 replicates), and 106% for the MOSH and 105% for the MOAH at the highest spiking level (4 replicates). Relative standard deviation (RSD) was always lower than 8.

Good intermediate precision (RSD = 13) was also found when analyzing (on 4 different days) pre-existing MOSH contamination in the range *n*-C_{25-50} in the 10 aliquots of the same TU2 sample used for recovery tests.

Figure 11 reports for both MOSH and MOAH an overlay of the LC-GC-FID trace of the unspiked sample (TU2), and of the samples fortified with 1.0 and 5.0 mg/kg of Gravex.

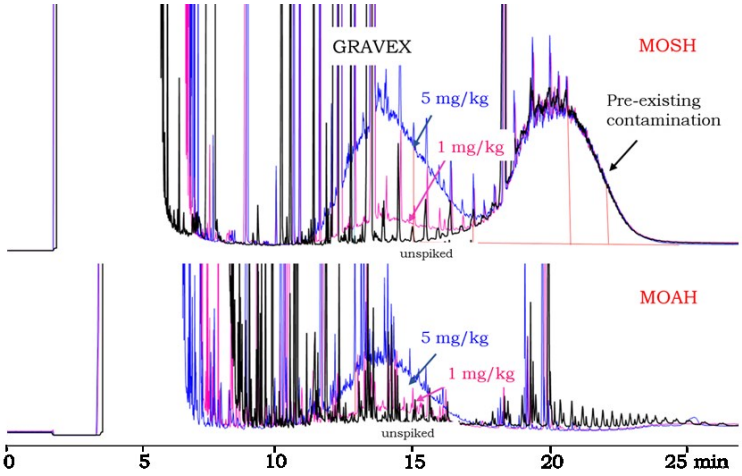

Figure 11. Overlay of MOSH and MOAH LC-GC-FID chromatograms of tuna sample (TU2): unspiked (black line), spiked with 1.0 (pink line) and 5.0 mg/kg (blue line) of Gravex (27% of MOAH).

Although the limit of quantification (LOQ) for mineral oil analysis is closely related to the molecular weight distribution of the contamination (width of the "hump"), an approximate estimation was made by extrapolating the value from an injection of the sample near the detection limit, and based on a signal-to-noise ratio of 10. When processing the sample as described, a LOQ around 0.1 mg/kg for each *C*-fraction was obtained.

2.3.2. Application to fish products

Table 7 shows MOH contents (expressed in mg/kg) for the 20 samples analyzed (18 fish samples and 2 fish-based ready-to eat meals). In accordance with the JRC guidance on sampling, analysis and data reporting for the monitoring of MOH in food and food contact materials [143], results were divided into 6

fractions for the MOSH and 4 for the MOAH. Total MOSH and MOAH (*n*-C_{10-50}), obtained by summing up each single C-fraction, are also reported.

Table 7. MOSH/POH and MOAH content (mg/kg) of the analyzed fish products.

Sample code	MOSH (mg/kg)							MOAH (mg/kg)				
	***n*-C_{10-16}**	***n*-C_{16-20}**	***n*-C_{20-25}**	***n*-C_{25-35}**	***n*-C_{35-40}**	***n*-C_{40-50}**	***n*-C_{10-50}**	***n*-C_{10-16}**	***n*-C_{16-25}**	***n*-C_{25-35}**	***n*-C_{35-40}**	***n*-C_{10-50}**
SA1	0.5	1.3	0.9	0.5	0.2	<LOQ	**3.4**	0.1	1.2	0.1	<LOQ	**1.4**
SA2	0.3	1.1	0.7	0.2	<LOQ	<LOQ	**2.3**	0.1	0.5	0.1	<LOQ	**0.7**
SA3	0.3	0.6	0.4	0.2	<LOQ	<LOQ	**1.6**	0.1	0.6	0.1	<LOQ	**0.8**
SA4	0.3	0.7	0.8	1.4	0.5	0.6	**4.3**	0.1	0.6	0.2	0.1	**0.9**
SA5	<LOQ	0.2	0.2	0.1	<LOQ	<LOQ	**0.5**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
SA6	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
SA7	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
SA8	<LOQ	0.3	0.4	0.8	0.2	<LOQ	**1.7**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
SA9	<LOQ	0.2	0.4	0.3	<LOQ	<LOQ	**1.1**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
SA10	<LOQ	0.2	0.3	0.8	<LOQ	<LOQ	**1.3**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
SA11	<LOQ	0.2	0.3	<LOQ	<LOQ	<LOQ	**0.6**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
FL1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
FL2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
SO1	<LOQ	<LOQ	<LOQ	0.1	<LOQ	<LOQ	**0.2**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
CO1	<LOQ	<LOQ	<LOQ	0.1	<LOQ	<LOQ	**0.2**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
SB1	<LOQ	0.3	0.4	<LOQ	<LOQ	<LOQ	**0.8**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
SW	<LOQ	0.3	0.9	1.4	0.3	0.1	**3.1**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
TU1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
TU2	<LOQ	<LOQ	0.2	2.4	1.1	0.2	**4.0**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
SBF	1.1	0.3	0.3	0.1	<LOQ	>LOQ	**1.9**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**
RCS	1.3	0.8	0.5	4.2	1.7	0.9	**9.4**	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**

Fish may bioaccumulate huge amount of *n*-alkanes from the aquatic environment, which cannot be considered of petrogenic origin, and were therefore subtracted from total contamination. As visible from MOSH traces of figure 12, and in agreement with Moret at al. [5], predominant *n*-alkanes are generally: *n*-C_{15}; *n*-C_{17} and *n*-C_{19}.

Farmed salmon (nine samples) had total MOSH (*n*-C_{10-50}) between 0.5 and 4.3 mg/kg (on average 1.9 mg/kg) and total MOAH comprised between the LOQ and 1.4 mg/kg (four of the nine samples contained MOAH after epoxidation). Wild salmons (two samples) had no detectable MOSH and MOAH. The two

uncontaminated samples (SA6 and SA7) were both fished in the North-East Pacific, vacuum packed and frozen.

Figure 12 reports a selection of LC-GC-FID traces showing different contamination profiles, probably related to different sources. SA2 is a fresh farmed salmon fillet (not packed) with a typical "hump" or UCM (2.3 mg/kg) in the range *n*-C_{13-28} (centered on *n*-C_{18}), visible at lower concentration in all farmed salmons and absent in wild fish samples. Even though no clear correlation between the MOH contamination and fat content was found, samples with less than 1.5% of fat resulted practically clean. Further investigations are needed to clarify if this contamination can be the result of bioaccumulation from the feed. Notably, the contamination is accompanied by MOAH in the same molecular weight range. Presence of MOAH in fish tissue and mussels has been previously reported [5], [139].

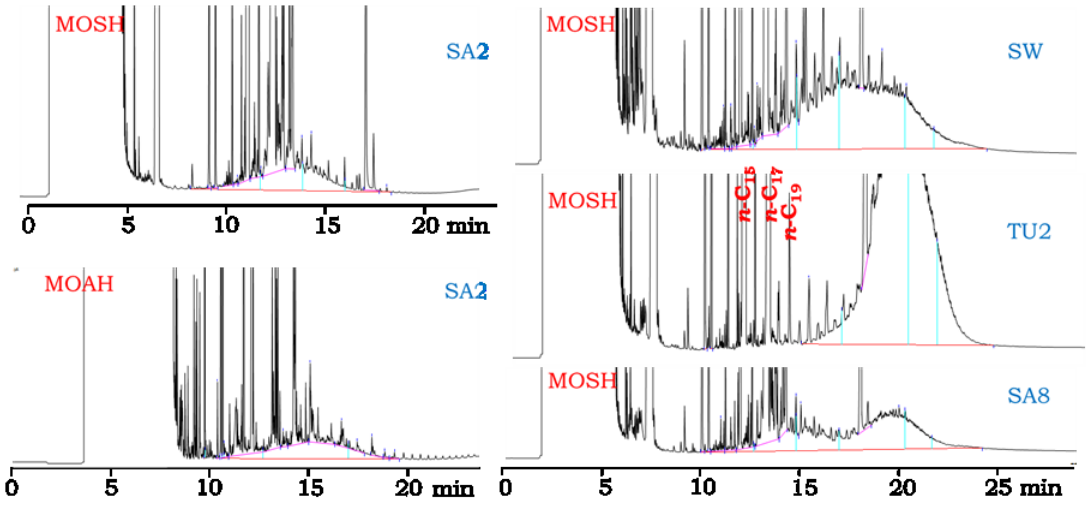

Figure 12. LC-GC-FID chromatograms of selected contaminated fish samples.

SW is a sliced, vacuum-packed, swordfish (3.1 mg/kg of total MOSH/POH), having a contamination profile only partially resembling that of the packaging (POH) (not reported). The tuna tartare (TU2) packed under protective atmosphere (PET), shows a contamination (4.0 mg/kg) from around *n*-C_{25} to beyond *n*-C_{40} (centered on *n*-C_{32}), not accompanied by MOAH. The origin of the contamination could be a food grade lubricant contaminating the product during cutting operation. A similar contamination (1.0 mg/kg in the range *n*-C_{25-40}) was also present in SA8 (vacuum-packed salmon sample, farmed), where it was also evident a little contamination (0.7 mg/kg) in the range *n*-C_{13-25} (as for other farmed salmons).

2.3.4. POH in fish packaging

Table 8 reports POH data of polyolefin packaging in direct contact with the food. Even though, as previously reported, extraction of POH is practically quantitative for the terms eluting up to *n*-C_{35} [146], results beyond *n*-C_{35} (up to *n*-C_{50}) were also reported.

One of the samples (SW_P) showed a MOSH hump in the range *n*-C_{10-20}, while sample SA8_P showed an atypical contamination in the range *n*-C_{35-50}. The other plastic films (mainly made of PE, LDPE) showed typical POH patterns with total POH *n*-C_{10-35} comprised between 205 (SA6_P) and 420 mg/kg (FL2_P), while the PP container (RCF_P) resulted contaminated with 640 mg/kg of POH *n*-C_{10-35}.

Table 8. POH content (mg/kg) of polyolefin packaging in contact with the fish.

	POH (mg/kg)							
Sample code	***n*-C_{10-16}**	***n*-C_{16-20}**	***n*-C_{20-25}**	***n*-C_{25-35}**	***n*-C_{35-40}**	***n*-C_{40-50}**	***n*-C_{10-35}**	***n*-C_{10-50}**
SA4_P	53	57	77	134	174	377	321	872
SA5_P	27	58	61	216	140	212	363	714
SA6_P	30	34	35	105	55	116	205	376
SA7_P	34	28	47	121	79	161	230	470
SA8_P	37	101	91	n.q.	n.q.	n.q.	n.q.	n.q.
SA9_P	27	69	69	227	147	245	393	785
SA10_P	13	56	58	198	88	152	324	564
FL2_P	84	61	122	152	192	365	420	976
CO1_P	30	26	47	105	67	137	209	412
SB1_P	60	64	88	188	102	165	400	667
SW_P	n.q.	n.q.	88	379	191	207	n.q.	n.q.
TU1_P	42	46	61	138	80	153	287	519
RCS_P	75	115	132	318	144	263	640	1046

n.q.: not quantifiable due to the presence of interference

2.3.5. POH migration from packaging to fish

From the comparison between the LC-GC-FID traces of the packaging and those of the corresponding packed products, it was concluded that, except for a couple of vacuum-packed samples (SA4 and SW), there was no appreciable POH migration from the packaging to the food.

SA4 is a sliced smoked salmon, raised in Norway, with a high lipid content (11%) and vacuum packed in mixed plastic packaging with a composition not explicitly stated by the manufacturer. Figure 13 shows the LC-GC-FID traces of the product and the relative plastic packaging. From the comparison of the traces, it can be concluded that the contamination found in the sample is probably the result of a pre-existing contamination in the fish product (first part of the trace), present in all farmed salmons, and of a secondary contamination originating from POH migrated from the packaging to the food (well evident in the second part of the chromatographic trace).

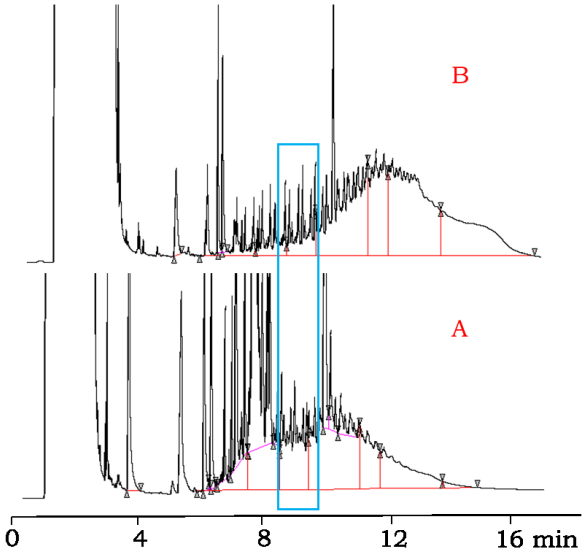

Figure 13. LC-GC-FID chromatograms of MOSH/POH fraction of (A) vacuum-packed sliced salmon (SA4) and (B) of the corresponding packaging. The blue rectangle highlights POH clearly visible in both the food and the packaging.

Even though the low storage temperature (4 °C) and the relatively low storage time should prevent high migration rate, the relatively high fat content, the high packaging-to-food weight ratio, and the intimate packaging-to-food contact (vacuum-packed), are factors that certainly could favor POH migration.

Nevertheless, of the 5 vacuum-packed sliced fish samples, only two evidenced POH migrations. All other fish products, except for one ready-to-eat meal (RCS), showed no POH migration. Despite the high levels found in the LDPE packaging FL2_P, no detectable migration was found in the fish. This result can be explained by the fact that the fish fillet was stored at -18 °C, packed in plastic bags with limited packaging-to-food contact, and had a reduced lipid content (1.1%).

Regarding the two ready-to-eat fish-based meals: the first was sea bream fillet (SBF) packed in a PET film intended to be used for oven cooking (190 °C for 22

min), the second was a seasoned rice with cuttlefish and shrimps (RCS), packed in a PP container intended to be microwave-heated (according to time and temperature conditions provided on the label) before consumption. SBF resulted contaminated with 1.9 mg/kg of MOSH in the range *n*-C_{10-35}. As expected, no POH migration was evidenced in this case.

Regarding sample RCS, figure 14 shows the LC-GC-FID traces of the PP container and of the food after microwave heating. POH migration is well evident in the range *n*-C_{10-25} (2.6 mg/kg). The hump in the second part (extending from *n*-C_{24} to *n*-C_{40}, centered on *n*-C_{34}) of the trace could origin from the rice.

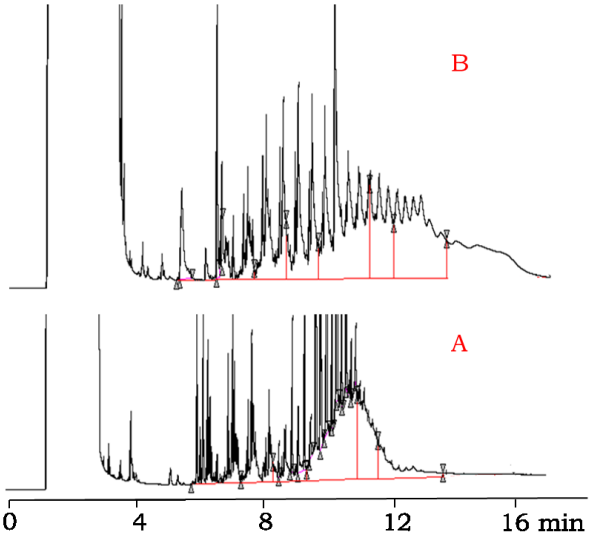

Figure 14. LC-GC-FID chromatograms of the MOSH/POH fraction of (A) ready-to-eat fish-based meal (RCS) and (B) of corresponding packaging.

2.4. Conclusions

A rapid and solvent sparing sample preparation protocol integrated to on-line HPLC-GC-FID determination of MOH/POH has been validated on fish samples. Recovery and repeatability characteristics, as well as LOQ values, resulted in line with the performance requirements reported in the JRC guidance [143].

The highest contamination (9.4 mg/kg of total MOSH/POH) was found in a ready-to-eat rice with cuttlefish and shrimps.

In general fish of low-fat content had no detectable contamination, while fatty fish had variable contamination levels, with maximum MOSH of 4.3 mg/kg. Compared to wild fishes, farmed fishes have a higher fat content, due to artificial feeding and less physical activity, and this allows for a greater accumulation of lipophilic contaminants such as MOH. Furthermore, feeds may represent an important source of contamination.

MOAH presence was evidenced in 4 of the 20 samples with a maximum of 1.4 mg/kg. Further investigations involving comprehensive GC are needed to elucidate MOAH composition.

Even though POH migration in packaged fish is generally negligible, mainly due the low storage temperature, under certain favorable condition, it may occasionally occur in sliced vacuum-packed products.

Chapter 3. MINERAL OIL CONTAMINATION IN PESTO SAUCES OF THE ITALIAN MARKET AND RELATED INGREDIENTS

- ✓ Srbinovska, A., Gasparotto, L., Conchione, C., Menegoz Ursol, L., Moret, S. Mineral oil contamination in Pesto sauces of the Italian market and related ingredients. *Paper drafting*.

3.1. Introduction

Basil Pesto is a traditional Ligurian (North-West region of Italy) sauce, with peculiar organoleptic features, resulting from a balanced contribution of a number of ingredients. According with the Consortium of Genoese Pesto, it can be defined "Pesto alla genovese" only if composed of the following seven ingredients: at least 25% of Genoese PDO (protected denomination of origin) basil, Italian extra virgin olive oil (even better if of Ligurian Riviera), very seasoned Parmigiano Reggiano, Pecorino PDO (Fiore Sardo), garlic, coarse salt and pine nuts from the Mediterranean area. In Italy Basil Pesto is the second most popular pasta sauce, after tomato sauce.

Most of commercial products available on the market are replaced in the original recipe.

One of the fundamental ingredients for Pesto are pine nuts, whose cost is very high. In fact, due to the worldwide diffusion of an infesting pine bug (*Leptoglossus Occidentalis*), the pinecones have begun to become infertile, so that from 2011 to 2015 the production of pine nuts decreased from 34 to 10 thousand tons. Added to this is the growing unsustainability of low-cost pine nut harvesting in countries such as China, Korea, Russia and Pakistan (cause of deforestation and threats to the ecosystem). By analyzing the labels of the Pesto offered for sale by the major brands of ready-made sauces, it emerged that pine nut were present in 11 of the 12 samples analyzed, but always in moderate quantities (from 0.5 to 2%), and that cashew is the main substitute. All the commercial brands of Pesto sauce examined contained sunflower oil. Extra virgin olive oil was present at concentration comprised between 1.0 and 19%.

Mineral oils are ubiquitous contaminants largely present in vegetable oils and in a wide range of food products as consequence of environmental or processing contamination, and/or migration from food packaging [6]. Even though to date no legal limits for the presence of this contaminants in food have been settled, very restrictive limits (e.g. 2 mg/kg for MOSH and 0.5 mg/kg for MOAH) have been often requested by the large retail chain. Based on background contamination levels, recently, the German food control Authorities and the food industry published benchmark levels for mineral oil hydrocarbons and their structural analogues in different food type. For vegetable oils, which account for about 50% of the Pesto weight, the MOSH level was set 13 mg/kg, while the MOAH should be lower than 0.5 mg/kg [41].

Due to the high complexity of Pesto sauce and the presence of interferences (triglycerides, olefins and endogenous *n*-alkanes), which complicate a correct quantification of the contamination, mineral oil determination in Pesto sauce is particularly challenging, especially when low detection limits (0.5 mg/kg) are requested.

The main objective of this work was to investigate on the presence of mineral oil in Pesto sauces from the Italian market and on possible sources of contamination. To this purpose, an on-line HPLC-GC-FID method, preceded by MAS to reach high sensitivity (0.5 mg/kg), and epoxidation to eliminate interference by olefins, and, when necessary by *n*-alkanes removal (through activated alumina), was validated and applied to analyze 12 Pesto samples purchased from the Italian market. Four additional samples, appositely produced in a pilot plant, and related ingredients, were also analyzed in order to investigate their impact to the final contamination.

3.2. Material and methods

3.2.1. Reagents and standards

Dichloromethane, *n*-hexane (both distilled before use), potassium hydroxide, *m*-chloroperbenzoic acid (CBPA), sodium thiosulfate, sodium sulphate, methanol and toluene, were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Pure water was obtained with a Milli-Q system from Millipore (Bedford, Massachusetts, USA). Ethanol was acquired from VWR (Radnor, Pennsylvania, USA).

To verify the GC performances, a *n*-C_{10-40} standard mixture (containing even-numbered alkanes plus *n*-C_{50}, was purchased by Restek (Bellefonte, Pennsylvania, USA). To check the LC-GC performance and MOSH and MOAH separation, as described by Biedermann & Grob [69], the internal standard solution (IS) in toluene from Restek was used. It includes *n*-C_{13} at 0.15 mg/mL, 1,3,5-tritert-butylbenzene (TBB), *n*-C_{11}, cyclohexyl cyclohexane (CyCy), pentyl benzene (5B), 1-methyl naphthalene (1MN), 2-methyl naphthalene (2MN) at 0.30 mg/mL and 5-α-cholestane (Cho) and perylene (Per) at 0.6 mg/mL. The same standard solution was used also for sample quantification.

The Gravex (a mineral oil consisting 27% of MOAH, with a chromatographic profile, extending from *n*-C_{13} to *n*-C_{25} and centred on *n*-C_{18}) was supplied by a manufacturer and was used to prepare the standard solution to fortify the samples used to perform recovery tests.

3.2.2. Samples

Four basil Pesto samples were appositely produced for this study in a pilot plant using typical ingredients, previously screened for having significant MOH content, and recipe that could be used for real production. For each one of the four batches of ingredients, the main ingredients, e.g. high oleic sunflower oil (HOSO), extra virgin olive oil (EVOO), basil, cashews, grana and pecorino cheese, as well as the final product, were analyzed.

Twelve commercial basil Pesto samples of different brands were purchased from the Italian market and analyzed. All the samples, except 3 which were packaged in plastic tray, were in glass jars.

3.2.3. Sample preparation

Microwave-assisted saponification was performed in a 14-position MARS 5 extractor (CEM, Matthews, NC, USA). After homogenizing the sample with a Politron (IKA® T18 basic ULTRA-TURRAX®, Germany) in order to avoid oil separation and hence the possibility of taking a sample that was not representative, 1 g of the Pesto sample was directly weighed in the extraction cell and added with the IS (10 µL), 10 mL of saturated KOH in CH_3OH (for saponification) and 10 mL of *n*-hexane (for simultaneous extraction of the unsaponifiable matter). The saponification was carried out at 120 °C for 20 min (under magnetic stirring). After cooling, an in-tube wash with water and methanol was performed. To this purpose 40 mL of water and 2-3 mL of methanol were introduced in the extraction cell without mixing (letting them to flow along the cell wall) and, after a 30 min rest at -20 °C, the upper hexane phase was withdrawn and concentrated to 700 µL.

The MAS conditions were the same for all ingredients analyzed, but the protocols differed in terms of initial quantity of the sample and KOH concentration. For EVOO and HOSO, the introduction of a washing step before epoxidation was necessary in order to avoid formation of gel during the concentration step after MAS [147]. To this purpose, the upper hexane extract was taken to 4 mL and then washed in a test tube as described in figure 15. After vortexing (2 min) and phase separation, the hexane extract was further concentrated to 700 µL before epoxidation.

For epoxidation the concentrated sample extract was added with 0.5 mL of *m*-CBPA 20% in ethanol solution and mixed for 15 min at room temperature. After the addition of 2 mL of sodium thiosulfate (10%) and 0.5 mL of ethanol, the vial

was shaken for 2 min and around 500 µL of the upper phase were transferred into an autosampler vial and added with a spatula tip of sodium sulfate. Once obtained the final extract for Pesto samples, as well as for other sample matrices, an aliquot of 100 µL, corresponding to around 140 mg of the initial sample, was injected for MOAH determination in order to reach the sensitivity required.

Even though the instrument used was a double channel able to perform simultaneous MOSH and MOAH analysis from the same HPLC run, in most cases MOSH determination required a separate run and a lower volume of the sample extract (generally 30 µL) was injected in order to avoid sample overloading by endogenous *n*-alkane. When necessary, e.g. Pesto and basil samples very rich in endogenous *n*-alkanes, a further purification was applied to the MOSH fraction. After being separated and manually collected (at the outlet of the transfer valve) during the same HPLC run used for the MOAH determination, the MOSH fraction (450 µL) was directly loaded onto a glass cartridge filled with 1 g of aluminium oxide (Alox), activated overnight at 500 °C. The cartridge was then eluted with further 5 mL of *n*-hexane, collected together with the loading volume into a conical shaped vial, and gently evaporated to a final volume of 200 µL. The sample extract was then transferred into an autosampler vial, and 100 µL were injected into the on-line HPLC-GC-FID apparatus for MOSH determination.

Figure 15 schematizes the protocols used for the different ingredients which required some minor modification, depending on the matrix type.

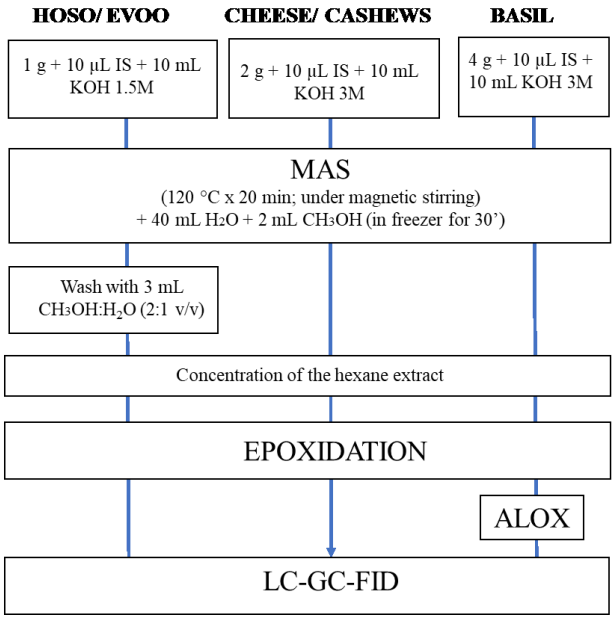

Figure 15. Protocols used for Pesto ingredients.

3.2.4. LC-GC-FID analysis

Analytical determination was carried out with an LC-GC 9000 from Brechbühler (Zurich, Switzerland) consisting of an HPLC Phoenix 9000 by Brechbühler and a GC Trace 1310 series by Thermo Fisher Scientific (Waltham, Massachusetts, USA). The sample was eluted at 300 mL/min using a gradient starting with hexane (0.1 min hold) and reaching 30% of dichloromethane after 0.5 min. The MOSH and the MOAH fractions were eluted from 2.0 to 3.5 min, and from 3.8 to 5.3 min, respectively. Six minutes after the injection, the column was backflushed with dichloromethane at 500 mL/min for 9 min and then reconditioned at 700 mL/min with hexane for 6.5 min and at 300 mL/min for 1.5 min. The HPLC column was 25 cm × 2.1 mm i.d. packed with Lichrospher Si 60, 5 µm of particle size (DGB, Schlossboeckelheim, Germany). HPLC-GC transfer occurred by exploiting partially concurrent eluent evaporation through an Y-interface using a retention gap technique [50]. The GC, double channel, consists of two PS-255 (1% vinyl, 99% methyl) GC columns, 15 m × 0.25 mm i.d. of 0.15 µm film thickness (Mega, Milan, Italy), connected via a T-piece union to a 10 m × 0.53 mm i.d. deactivated pre-columns (retention gap) and to a solvent vapor exits (SVE). The FID (sampling frequency of 50 Hz) and the SVE were heated at 360 and 140 °C, respectively. The GC run was accelerated by increasing the oven temperature program from 55 to 350 °C (20 °C/min) and using hydrogen as carrier at a constant pressure of 60 kPa. During the transfer the pressure was increased to 90 kPa. Data were acquired and processed by Chromeleon 7 software. The MOSH area was determined by the integration of the whole hump of largely unresolved peaks, subtracting the endogenous *n*-alkanes (when present). All sharp peaks standing on the top of the MOAH "hump" were subtracted from the total area. The position of the baseline was assessed by procedural blank runs obtained on the same day. Quantification was based on the internal standards: *n*-C_{13} for the MOSH and 5B for the MOAH.

3.3. Results and discussions

3.3.1. Method optimization and performance

Due to the complexity of the matrix, the presence of high percentage of vegetable oil, and the lack of a harmonized sample preparation protocol, mineral oil analysis in Pesto sauce can be particularly difficult. Saponification represents a

valid enrichment/purification method, which can be conveniently performed using microwave energy [47], [48].

With some little modifications, mainly concerning the amount of sample processed and the KOH solution used, the method applied on Pesto samples and related ingredients was the same previously applied by Srbinovska et al. [48] for MOSH and MOAH determination in fish products.

Due to the interference by olefins, epoxidation was mandatory. Figure 16 shows from the top to the bottom the chromatograms of the MOAH fractions of a basil sample (A), a cashew (B), a sunflower oil (C), a Grana cheese (D), all before epoxidation. The two bottom chromatograms refer to a Pesto sample before (E) and after (F) epoxidation, and well evidence as all the main ingredients contain interfering olefins (eluting in different part of the chromatographic trace), that can be eliminated after epoxidation.

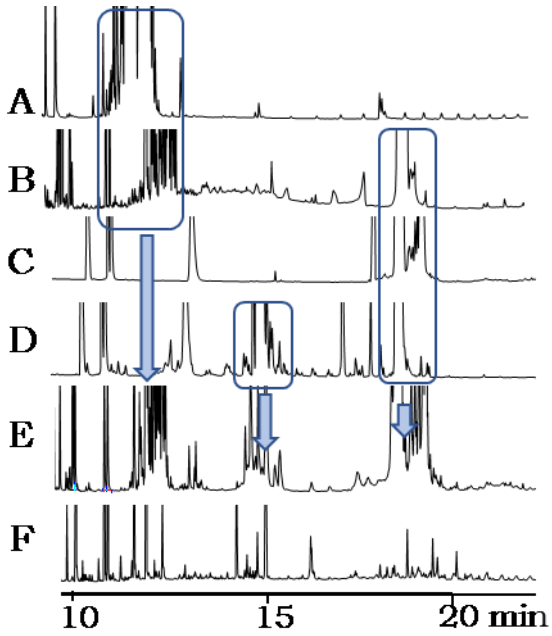

Figure 16. HPLC-GC-FID chromatograms of the MOAH fraction of a basil (A), a cashew (B), sunflower oil (C), a Grana Cheese (D) and Pesto sauce (E and F), before (A-E) and after (F) epoxidation.

The passage on an alumina bed revealed to be necessary for accurate MOSH quantification at levels lower than 2-5 mg/kg (depending on the endogenous *n*-alkane content) and was applied to all basil and Pesto samples.

Figure 17 shows an example of a Pesto sample with a contamination around 3 mg/kg and a content of endogenous *n*-alkanes around 113 mg/kg.

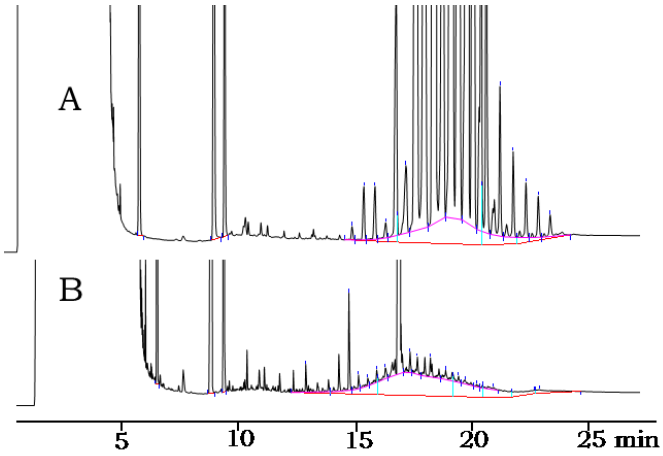

Figure 17. HPLC-GC-FID chromatograms of the MOSH fraction of a Pesto sample that, after MAS and epoxidation underwent: (A) direct HPLC-GC-FID (injection 20 µL); (B) HPLC fractionation followed by SPE on 1 g of Alox and HPLC-GC-FID analysis.

The top trace (A) in figure 17, which refers to a sample extract (after MAS and epoxidation), injected directly at amount corresponding to about 30 mg of the food sample, clearly shows *n*-alkanes which are not separated valley to valley on the top of the hump. When this happens, the peaks related to these biogenic compounds tend to overlap, making difficult to draw the upper contour line of the MOSH hump. In this specific situation, integration is still possible, but doubts remain about data reliability, i.e. possible overestimation of the contamination. A possible solution is to further reduce the amount of sample injected, but this would lead to further sensitivity loss. For this reason, the best alternative is to introduce a passage on Alox to eliminate interference by endogenous *n*-alkanes over *n*-C_{23}. As reported in previous works [51], [53], sample elution through a cartridge of activated Alox allows to retain linear alkanes with more than 20 carbon atoms, while isoalkanes and naphtenes elute unretained. Hence, considering the distribution of plants *n*-alkanes within the range *n*-$C_{21\text{-}33}$ [148], its application on vegetable samples is feasible and for this reason it was applied to the Pesto samples, following the protocol described in paragraph 3.2.3., and obtaining the result shown in figure 17B. Comparative trials, performed in duplicate on 4 different Pesto samples, confirmed a systematic overestimation when avoiding Alox purification.

Usually, the Alox protocol is directly applied to samples or sample extracts containing fat [149], so relatively high amount of this sorbent, used alone or in combination with activated silica gel (and hence high volumes of the elution solvent), is required. Different from previous works, in this application we applied the Alox protocol directly to the MOSH fraction manually collected during the same HPLC run used for MOAH quantification, which, being free from fat,

required very little sorbent and solvent amount. Using the described protocol, we were able to inject an amount of sample, corresponding to about 70 mg, reaching a LOQ of 1 mg/kg on the entire hump, which was considered enough for the MOSH.

Recovery and repeatability tests on Pesto samples were performed on a sample free from contamination in the range *n*-C_{13-25} (where Gravex hydrocarbons elute), but with 1.6 mg/kg of pre-existing MOSH and non-detectable MOAH in the range *n*-C_{25-50}. The spiking was performed in quadruplicate with 2 and 10 mg/kg of Gravex solution, corresponding to 1.5 e 7.3 mg/kg of MOSH and to 0.5 and 2.7 mg/kg of MOAH. Recoveries were within the 70 and 120% interval indicated by the JRC guidance [143]. Intermediate repeatability, assessed on 6 replicates of the same Pesto sample with a pre-existing *n*-$C_{25–50}$ MOSH contamination of 1.9 mg/kg (and no MOAH), as well as on the samples spiked with Gravex (to evaluate MOAH repeatability), gave RSD values always well below the maximum level (20%) required by the JRC [143]. Based on repeatability data calculated for the single C-fractions, a LOQ around 0.2 mg/kg was obtained, while total LOQ was assessed at 0.5 mg/kg. As reported by Menegoz Ursol et al. [147] for EVOO, the liquid-liquid partition step, that follows the saponification, determined a different partition of the IS of the MOAH fraction in the hexane phase. This behavior explains the different recoveries observed when using different ISs for the MOAH quantification. Nevertheless, the different MOAH values obtained when quantifying with different IS, realigned when correcting them for the recovery. Since, practically quantitative recoveries, independently on the matrix, were obtained when using 5B (97-101%), it was selected as IS for the MOAH quantification (thus avoiding correction for the recovery).

Recoveries at the same spiking levels (2 and 10 mg/kg of Gravex solution) were in fact evaluated for the main individual ingredients used for Pesto production, except for cashews (due to the impossibility to find samples without pre-existing contamination in the range of interest for the fortification), and basil (which had very low contamination). Recoveries obtained using internal standards *n*-C_{13} for the MOSH, and 5B for the MOAH, are resumed in table 9.

Table 9. Mean recovery and RSD for Pesto sample and related ingredients.

	MOSH		MOAH	
Matrix	Recovery % (mean)	RSD (%)	Recovery % (mean)	RSD (%)
HOSO	94.8	0.7	96.9	4.6
EVOO	97.2	4.5	97.9	5.0
CHEESE	106.4	3.4	101.0	10.0
BASIL	-	-	-	-
CASHEWS	-	-	-	-
PESTO	104.7	8.0	100.5	4.7
PESTO*	109.0	4.7	-	-

*passage on Alox

3.3.2. MOH in Pesto ingredients

Data obtained from ingredients clearly indicated that the most contaminated ones were cashew followed by vegetable oils. Other ingredients (cheese and basil) had contamination level near or below the quantification limit.

Data on cashew contamination are lacking, but it is well known that, they are usually stored and transported in jute bags. Traditionally, jute and sisal fiber for manufacturing sacks, are batched with about 7% of mineral oil to improve their spinning properties [10], and the volatile part of this oil can be transferred to the food. To limit possible contamination, in 1998 the International Jute Organisation (IJO) established a maximum limit of 1250 mg/kg for non-saponifiable matter extracted from jute bag. This limit was provided, taking into account the non-saponifiable matter of vegetable oils used for batching. After this action, the contamination of foods arriving in Europe dropped, but did not disappear.

Cashew samples had average MOSH and MOAH contamination of 19.6 and 4.5 mg/kg, respectively. Figure 18 shows an overlay of the MOSH/MOAH chromatograms of 2 cashews samples. As visible from the HPLC-GC-FID traces, MOSH contamination ranged from *n*-C_{13} to *n*-C_{40} and includes a first hump from *n*-C_{13} to *n*-C_{25} (centered on *n*-C_{18}), accompanied by MOAH (17-20%) of the same molecular range, as typical of samples contaminated with batching oil, and a second hump centered around *n*-C_{28}. This second hump (not accompanied by MOAH), probably has a different origin, which remained unknown.

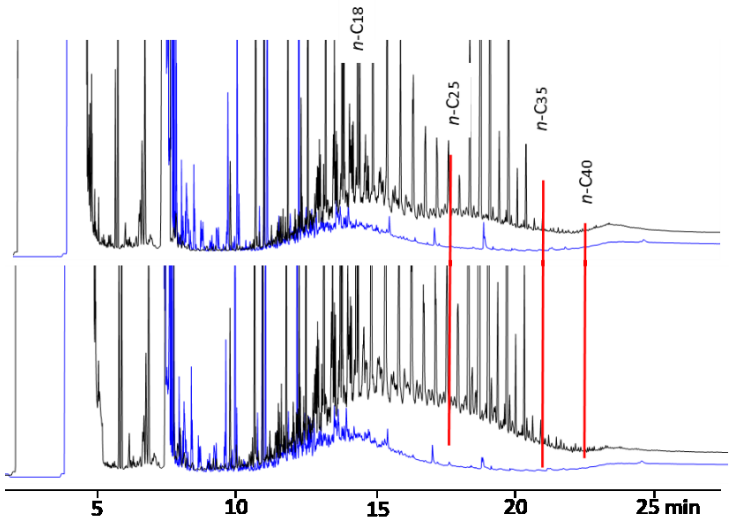

Figure 18. Overlay of MOSH and MOAH chromatograms of 2 cashew samples.

Vegetable oils represent one the most important contributors to mineral oil intake through the diet, and a background contamination is generally found in all vegetable oils. Usually, in commercial refined sunflower oils, concentrations of MOSH ranges between 2.7 and 32 mg/kg, on average 11.2 mg/kg [8]. Gharbi et al. [150], reported average MOSH content of 19.1 mg/kg in Tunisian extra virgin olive oils from the market, while MOAH were not detected (LOQ around 2 mg/kg). The authors used on-line HPLC-GC-FID without sample pre-enrichment. A more recent investigation on EVOO from the Italian market, performed with the high sensitivity method used in this work [147], reported similar MOSH levels (on average 22.9 mg/kg), but well detectable MOAH (on average 3.3 mg/kg). These higher MOAH values are probably the results of the lower quantification limit reached thanks to the sample enrichment obtained with MAS.

EVOO and HOSO samples had on average 16.7 and 17.3 mg/kg of total MOSH, and MOAH around 3.0 mg/kg. They had a similar contamination profile from *n*-C_{25} to *n*-C_{50}, but centered on *n*-C_{33} for the HOSO and on *n*-C_{28} for the EVOO. Since EVOO was added at very low amounts (1%), its contribution to the total contamination of Pesto sauce was in this case negligible, while HOSO contribution (which represented 45% of the total weight), was relevant.

Basil is, together with sunflower oil, the main ingredient of Pesto sauce (30%). Of the 4 basil samples analyzed only one had detectable MOSH contamination (at level lower than 0.7 mg/kg), while none of them presented MOAH. Similarly, cheese sample had MOSH level around 0.5 mg/kg and were free from MOAH.

Figure 19 reports MOSH and MOAH data (divided into C-fraction) obtained for the 4 batches of ingredients and for the corresponding Pesto samples produced

in the pilot plant. It also reports the contribution to the MOH contamination given by each ingredient. MOSH and MOAH values of each ingredient have been normalized taking into account the percentage in the final product.

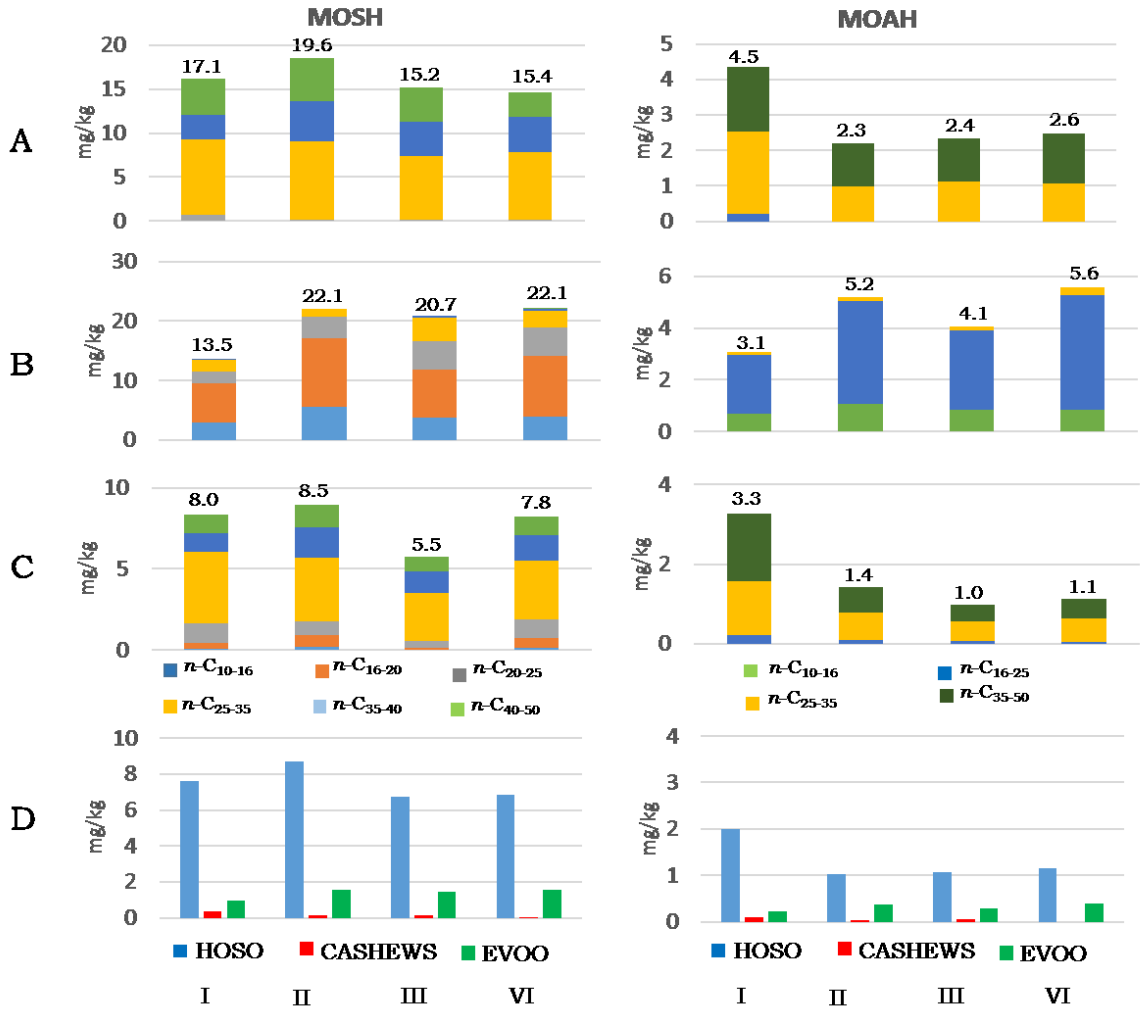

Figure 19. MOSH and MOAH content in: (A) HOSO, (B) cashew, (C) corresponding Pesto samples, (D) ingredients contribution (mg/kg) to total MOAH and MOAH contamination (based on ingredient percentage) for all 4 batches (I, II, III, IV).

Total MOH found in Basil Pesto samples is clearly determined by HOSO contamination, which provided the greatest contamination (80-85%), followed by cashews. Due to the low amount at which they were present in the final product (extra virgin olive oil), or the very low contamination level, other ingredients had little impact on the final contamination. By summing up the MOSH brought by the ingredients, and comparing them to those found in the final product, a good correspondence (maximum deviation of 30% with respect to the expected value) was found, which confirmed that the analytical protocols used for the different matrices give reliable results and that the contribution due to food processing is negligible.

3.3.3. MOH content in Pesto sauce samples from the market

Table 10 summarizes the MOH concentration (expressed in mg/kg) found in 12 Pesto samples purchased from the Italian market.

Table 10. MOSH and MOAH content (mg/kg) of the analyzed Pesto samples.

Sample	MOSH (mg/kg)							MOAH (mg/kg)				
	n-C_{10-16}	*n*-C_{16-20}	*n*-C_{20-25}	*n*-C_{25-35}	*n*-C_{35-40}	*n*-C_{40-50}	*n*-C_{10-50}	*n*-C_{10-16}	*n*-C_{16-25}	*n*-C_{25-35}	*n*-C_{35-50}	*n*-C_{10-50}
P1	<0.2	<0.2	0.5	1.0	0.2	<0.2	**1.7**	<0.2	<0.2	<0.2	<0.2	**<0.5**
P2	<0.2	<0.2	0.5	1.3	0.9	0.2	**3.1**	<0.2	<0.2	<0.2	<0.2	**<0.5**
P3	0.9	3.0	2.7	2.1	0.7	0.6	**9.9**	0.3	1.0	<0.2	<0.2	**1.3**
P4	0.8	1.5	1.7	2.2	0.4	<0.2	**6.6**	<0.2	<0.2	<0.2	<0.2	**<0.5**
P5	0.9	2.4	2.7	2.7	0.4	<0.2	**9.1**	0.2	0.5	<0.2	<0.2	**0.7**
P6	0.4	1.9	2.4	2.0	0.2	<0.2	**6.9**	0.2	0.6	<0.2	<0.2	**0.8**
P7	<0.2	0.2	1.0	1.4	<0.2	<0.2	**2.7**	<0.2	<0.2	<0.2	<0.2	**<0.5**
P8	<0.2	<0.2	0.7	2.7	0.6	<0.2	**4.2**	<0.2	<0.2	<0.2	<0.2	**<0.5**
P9	<0.2	<0.2	0.3	2.0	0.5	0.4	**3.3**	<0.2	<0.2	0.4	0.3	**0.8**
P10	<0.2	0.2	1.0	5.3	2.1	1.5	**10.0**	<0.2	<0.2	0.6	0.5	**1.1**
P11	<0.2	0.2	0.7	3.4	0.8	0.6	**5.8**	<0.2	<0.2	0.6	0.4	**1.1**
P12	<0.2	<0.2	0.7	2.4	0.5	0.3	**4.1**	<0.2	0.2	0.7	0.2	**1.1**

According to the JRC guidance [143], the quantification was carried out for each C-fraction. Table 10 reports also the total *n*-C_{10-50} concentrations. Due to the presence of a huge amount of *n*-alkanes naturally present in the samples, the passage on Alox was mandatory to correctly quantify the MOSH.

MOSH contamination ranged from 1.7 to 10.0 mg/kg (on average 5.6 mg/kg) while MOAH contamination from <LOQ to 1.3 mg/kg (on average 0.6 mg/kg). All the samples showed a main contamination in the range *n*-C_{25-50}, as typical for HOSO, while 3 of the 12 samples clearly showed a hump in the range *n*-C_{13-25}, as typical for cashew transported in jute bags.

Figure 20A, shows the MOSH trace of one of these samples, where it is well visible a first hump in the range *n*-C_{25-50}, with the same profile, the same MOSH/MOAH ratio, of a cashew sample. Furthermore, both samples were characterized by the presence of pristane and phytane, which are considered markers of the presence of batching oil. All these similarities allowed to conclude that part of the contamination found in this sample comes from cashew transported/stored in jute bags treated with batching oil. The second hump in the range *n*-C_{25-50}, is instead typical of vegetable oils.

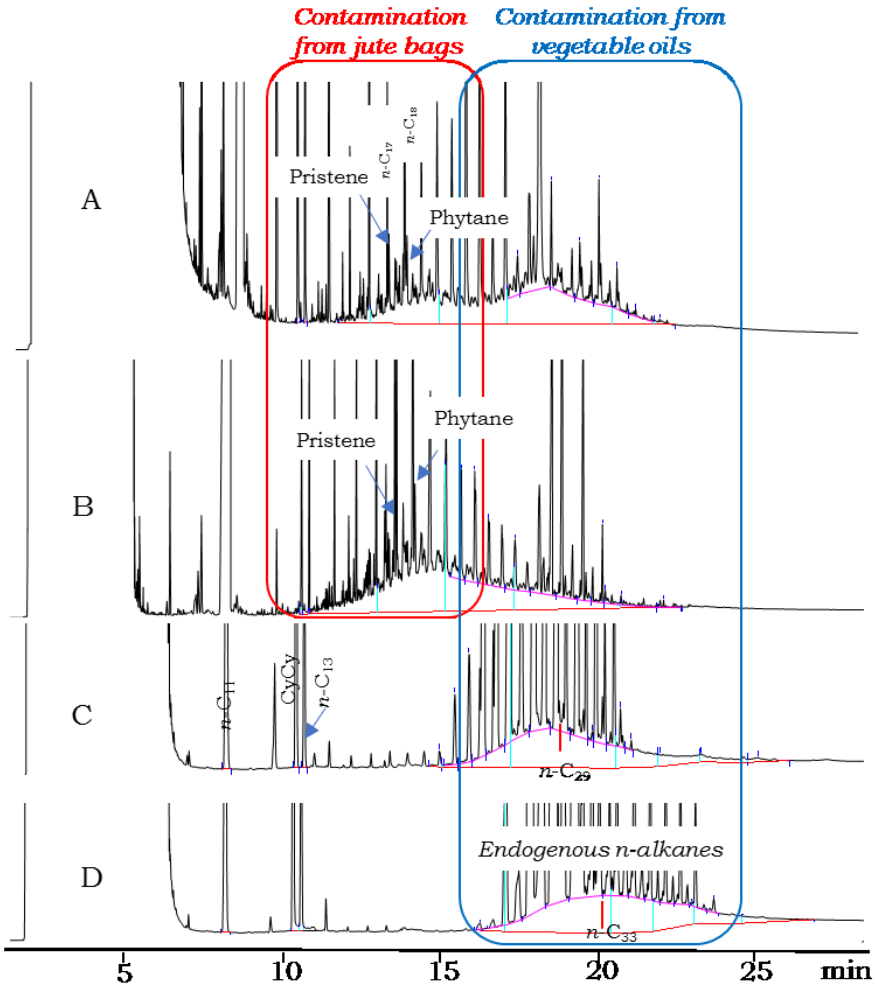

Figure 20. MOSH chromatograms of (A) Pesto sample from the market, (B) cashew sample, (C) EVOO, (D) HOSO:

3.4. Conclusions

Basil Pesto sauce represents a complex food matrix and matrix tailored sample preparation protocols are needed for reliable MOH quantification. A rapid and high sensitivity method for MOH analysis was optimized and validated for Pesto samples. The method consists of MAS followed by epoxidation and passage on alumina to assure enrichment/purification of the extract before on-line HPLC-GC-FID analysis. The performance of the method was found to be in line with the requirements of the JRC guidance. To evaluate the MOH contribution given by each ingredient to the final product, methods for the analysis of the main ingredients were also optimized. Total MOH content found in Pesto samples is clearly determined by HOSO contamination, which provides the greatest contamination, followed by cashews and EVOO. Pecorino and Grana cheese do not contribute to the contamination of the finished product. The contribution due to food processing is negligible. Pesto samples from the Italian market had

average MOSH contamination below 10 mg/kg, but 50% of the samples contained detectable MOAH (0.7-1.3 mg/kg). Even though the contamination found are not particularly worrying, mitigation actions to reduce contamination (especially for the MOAH) are possible, and should be directed to a careful control of the 2 most critical ingredients (vegetable oils and cashews), making producing countries aware of the problem related to jute bags.

Chapter 4. HIGH SENSITIVITY DETERMINATION OF MINERAL OILS AND OLEFIN OLIGOMERS IN COCOA POWDER: METHOD VALIDATION AND MARKET SURVEY

- ✓ Srbinovska, A., Conchione, C., Menegoz Ursol, L., Celaj, F., Moret, S. High sensitivity determination of mineral oils and olefin oligomers in cocoa powder: method validation and market survey. *Paper drafting*.

4.1. Introduction

Mineral oils (MOH, mineral oil hydrocarbons) are complex mixtures of hydrocarbons of petrogenic origin, which can be divided into 2 classes of different toxicological relevance: saturated hydrocarbons (called MOSH, mineral oil saturated hydrocarbons) comprising *n*-alkanes, isoalkanes and cycloalkanes, and aromatic hydrocarbons (called MOAH, mineral oil aromatic hydrocarbons), comprising compounds with one or more benzene rings (mainly 1-3) with various alkyl substituents. While the former are of concern due to their proven ability to bio-accumulate in human organs and tissues, the latter are even more worrying as include suspected carcinogens and genotoxic, in particular compounds with 3 and more benzene rings and low alkylation degree [32].

Mineral oils can enter into the food chain through different routes: the polluted environment, harvesting practices, intentional use (lubricating, release agents), accidental contamination, and last but not least, via food contact materials.

Starting from the beginning of nineties, cocoa beans and cocoa products have been often found contaminated with residues of mineral oils. Amounts up to 100 mg/kg were found in cocoa beans (and other seeds and nuts) stored and/or transported in jute bags treated with mineral batching oil [10]. To solve this problem, in 1998, the International Jute Organisation (IJO) adopted special criteria for the manufacture of jute bags recommending the use of batching oil containing non-toxic ingredients, and introducing a maximum limit of 1250 mg/kg for the unsaponifiable matter to rule out the use batching oil of mineral origin [11]. Since then, jute bags for the European market are mainly batched with plant oils and certified mineral-oil free bags exist, even if they are not used in all origins [1], [13].

Also, migration from the packaging can be cause of mineral oil contamination. Migration can come from the packaging made of recycled paper or from printing inks, as well as from the external environment. If polyolefin packaging, or hot melts adhesives are used, contamination with polyolefin oligomeric hydrocarbons (POH) or polyalphaolefins (PAO) may be present. In the absence of a functional barrier, due to its high fat content and powdered form, cocoa powder is particularly prone to be contaminated via migration from the packaging or the external environment. Mineral oils, as known are also processing contaminants, and are widely used as lubricant, so that other sources of contamination cannot be ruled out.

Currently there are no legal limits for MOSH and MOAH, even if the large-scale distribution frequently asks for very restrictive MOSH levels, not consistent with the background levels commonly found in food. Concerning the MOAH a maximum limit of 0.5 mg/kg is often required. In Germany, representatives of the federal state control authorities (LAV) together with the leading association of the German food sector (Lebensmittelverband Deutschland e. V. Food Federation Germany, formerly BLL), recommend the use of "benchmark levels" developed as part of a project joint data collection started in June 2016 (revised in August 2021) [41]. These levels are not based on toxicological or exposure assessments, nor should be intended as legal limits, but reproduce the current state of the art on the background levels present in the different food groups. A value of 9 mg/kg for MOSH and lower than the limit of quantification (LOQ<1.0 mg/kg) for the MOAH, has been defined for chocolate and cocoa-based products.

The analytical determination of mineral oils and related synthetic hydrocarbons (POH and PAO) is carried out by gas-chromatography (GC) connected to a flame ionization detector (FID). The reference method is the on-line high performance liquid chromatography (HPLC)-GC-FID method developed by Biedermann et al. [50], which, depending on the complexity of the matrix, potential interferences and the sensitivity required, must be preceded by suitable sample preparation. Microwave assisted saponification (MAS), has been proven a powerful sample preparation technique to be applied before on-line HPLC-GC-FID determination [47], [48], [147]. It allows to process up to 14 samples simultaneously, with minimal solvent consumption and need for sample post treatment.

Based on this background, the aim of this work was to validate a method based on MAS, followed by epoxidation and on-line HPLC-GC-FID, for a rapid, solvent-sparing and high sensitivity determination of mineral oils and related hydrocarbons in cocoa powder. Then the validated method was used to monitor the presence on these contaminants in a wide range of cocoa powder (food and corresponding packaging) marketed in Italy, and in an extra EU country (North Macedonia).

4.2. Materials and methods

4.2.1. Reagents and chemicals

n-Hexane and dichloromethane (both distilled before use), *n*-pentane, *m*-chloroperbenzoic acid, potassium hydroxide, sodium thiosulfate, sodium sulfate, methanol and toluene, were obtained from Sigma-Aldrich (St. Louis, Missouri,

USA). Ethanol was purchased from Supelco (Bellefonte, Pennsylvania, USA) and pure water was obtained with a Milli-Q system from Millipore (Bedford, Massachusetts, USA).

4.2.2. Standards

To verify the GC performances, a C_{10}-C_{40} even-numbered *n*-alkane standard mixture from Restek (Bellefonte, Pennsylvania, USA), was used. The internal standard solution (IS) in toluene used for sample quantification and HPLC-GC performance verification was also from Restek and contained: *n*-C_{13} at 0.15 mg/mL, 1,3,5-tritert-butylbenzene (TBB), *n*-C11, cyclohexyl cyclohexane (CyCy), pentyl benzene (5B), 1-methyl naphthalene (1MN), 2-methyl naphthalene (2MN) at 0.30 mg/mL and 5-α-cholestane (Cho) and perylene (Per) at 0.6 mg/mL.
The Gravex (a mineral oil consisting of 27% of MOAH, with a chromatographic profile centred on *n*-C_{18}, extending from *n*-C_{13} to *n*-C_{25}) used for recovery tests at different spiking levels was supplied by a manufacturer.

4.2.3. Instrumentation

The on-line HPLC-GC-FID system was an LC-GC 9000 from Brechbühler (Zurich, Switzerland). It consisted of an HPLC Phoenix 9000 pump from Brechbühler (Zurich, Switzerland) and a GC Trace 1310 series from Thermo Fisher Scientific (Waltham, Massachusetts, USA).
The HPLC column was a 25 cm × 2.1 mm i.d. packed with Lichrospher Si-60, 5 µm particle size (DGB, Schlossboeckelheim, Germany). MOSH and MOAH elution was accomplished using a gradient starting from 100% *n*-hexane (0.1 min hold) and reaching a *n*-hexane/dichloromethane ratio of 70/30 after 0.5 min, with a flow rate of 300 µL/min. Six minutes after the injection, the column was backflushed with dichloromethane (500 µL/min for 9 min) and then reconditioned for 6.5 min with *n*-hexane at 700 µL/min and at 300 µL/min for 1.5 min.
The GC was a double channel able to perform MOSH and MOAH analyses simultaneously, from the same HPLC run. Each GC channel consisted of a carrier gas line, a deactivated retention gap (10 m × 0.53 mm i.d.) followed by a steel T-piece connected to a 15 m × 0.25 mm i.d. PS-255 (1% vinyl, 99% methyl polysiloxane) separation column (0.15 µm of film thickness) and to a solvent vapour exit (SVE). Both the retention gap and the separation column were purchased from Mega (Milan, Italy). The SVE (which remained open during the

transfer) and the FID were heated at 140 and 360 °C, respectively. The HPLC-GC transfer occurred via an Y-interface connecting the carrier gas line and the HPLC line to the retention gap, in order to exploit partially concurrent eluent evaporation to improve volatile retention through the solvent trapping effect, as described by Biedermann et al. [50].

The GC oven temperature gradient started from 55 °C and was raised to 350 °C at a rate of 20 °C/min. Hydrogen was used as carrier gas at a constant pressure of 60 kPa (during HPLC transfer the pressure was raised to 90 kPa).

The Microwave Extraction System used for simultaneous sample saponification and unsaponifiable extraction was a MARS 5 from CEM Corporation (Matthews, North Carolina, USA), equipped with GreenChem Plus Teflon vessels able to host 14 samples simultaneously.

4.2.4. Samples

To monitor MOH contamination in cocoa powders, 15 samples from the Italian market and 6 samples from the North Macedonian market were collected and analyzed. Products, all of different brands, were classified based on the type of packaging. Packaging description is reported in table 11.

Table 11. Packaging types.

Packaging type	Primary packaging	Secondary packaging	Tertiary packaging	Packaging code
A	plastic bag	virgin cardboard		Pl_VC
B	plastic bag	recycled cardboard		Pl_RC
C	virgin paper bag internally laminated with PE	virgin cardboard		VP/PE_VC
D	virgin paper bag internally laminated with PE	recycled cardboard		VP/PE_RC
E	virgin paperbag	virgin cardboard	PP film	VP_VC_PP
F	virgin cardboard can internally coated with Al			VC/Al
G	plastic bag			Pl

Pl, plastic; VC, virgin cardboard; RC, recycled carrdboard; VP, virgin paper; PE, polyethylene; PP, polypropylene; Al, aluminium

4.2.5. Sample preparation

For paper packaging, the extraction method proposed by Lorenzini et al. [19] was used. About 1 g of each samples, cut into little pieces, was added with 20 µL of IS mixture, and extracted with 10 mL of *n*-hexane/ethanol (1:1 v/v). The samples were extracted at ambient temperature for 2 h and under magnetic stirring for the first and the last 5 min. The extract was then added with 10 mL of water to separate the ethanol from the hexane phase. For plastic packaging, instead, 300 mg of each sample, cut into little pieces, was added with 20 µL of IS mixture and

extracted overnight with 10 mL of hexane (under magnetic stirring), as reported by Biedermann-Brem et al. [122]. For both, paper and plastic packaging, an aliquot of the extract was directly injected into the on-line LC-GC-FID system.

For cocoa powder samples, 4 g of sample were directly weighted into a Teflon vessel (Green Chem plus, CEM Corporation) and added with 20 µL of IS, 10 mL of *n*-hexane and 10 mL of a 1.5 M methanolic KOH solution. After a pre-heating step (2 min), MAS was carried out at 120 °C for 20 min under magnetic stirring. After cooling at ambient temperature, 40 mL of water and 3 mL of methanol were added to the extraction vessel (without mixing), which was then placed at -20 °C for 30 min. Six milliliters of the sample extract were later concentrated at 4 mL and washed with 3 mL of a CH_3OH/H_2O mixture 2:1 (v/v). The organic phase was collected and concentrated to 700 µL and subjected to epoxidation according to a slightly modified Nestola & Schmidt [55] protocol: 500 µL of an ethanolic *m*-CPBA solution (20% m/v) were added to the extract previously concentrated (700 µL) and placed into an agitator at room temperature (500 rpm for 15 min). Then, 2 mL of aqueous sodium thiosulfate solution (10% m/v) to stop the reaction and 500 µL of ethanol were added. The vial was shaken for 3 min. Finally, 500 µL were transferred into an autosampler vial containing a spatula tip of anhydrous sodium sulphate and injected (100 µL).

4.2.6. Method validation

Recovery tests were performed on a sample spiked with Gravex (containing 73% of MOSH and 27% of MOAH in the range *n*-C_{16-25}) at two different levels (3 and 10 mg/kg of total MOH). Four replicate samples for each spiking level were directly weighted into the extraction vessel an added with the known amount of Gravex solution dissolved in 5 mL of pentane. Subsequently, the vessels were gently stirred to uniformly distribute the mineral oil standard on the whole sample matrix and were left to age for 48 h (in order to allow analyte-matrix interactions and solvent evaporation) before performing the MAS. Percentage recoveries were calculated by comparing the amount found in the spiked samples with those used to spike the samples. The native sample used for recovery test was free of contamination in the range *n*-C_{16-25}.

Inter-day repeatability was assessed on two cocoa powders with low and high total MOH *n*-C_{10-50}.

The LOQ for single C-fraction and for total MOSH and MOAH were assessed in accordance with the SANTE guidance [151] which states that LOQ is the lowest

spiked level meeting the method performance criteria for recovery and precision. The performance criteria were those reported in the JRC guidelines [143], which requires recoveries comprised between 70 and 120% and intermediate repeatability with residual standard deviation (RSD) lower than 20%.

4.3. Results and discussions

4.3.1. Method validation

The MAS procedure was optimized by testing different amounts of the sample (taking into account the sensitivity required), different saponification conditions (KOH concentration and solvent volumes) and evaluating the opportunity to introduce an additional wash before the epoxidation. The final procedure, described in paragraph 4.2.5., includes a further washing step with a CH_3OH/H_2O mixture 2:1 (v/v), necessary to avoid problems of gel formation during the concentration step which preceded the epoxidation.

To assess method recovery, 4 different aliquots of the same cocoa powder were spiked with 3 mg/kg of Gravex, corresponding to 2.2 mg/kg for the MOSH and 0.8 mg/kg for the MOAH, while another 4 aliquots were spiked with 10 mg/kg of Gravex, corresponding to 7.3 mg/kg for MOAH and 2.7 mg/kg for MOAH. The samples so prepared in 2 different sessions, underwent the entire analytical procedure.

Figure 21 shows the MOSH and MOAH traces of the Gravex standard (at the highest spiking level) added to the sample for recovery test (on the left), and an overlay of the chromatographic traces of the cocoa powder before and after the spiking at two different levels (on the right).

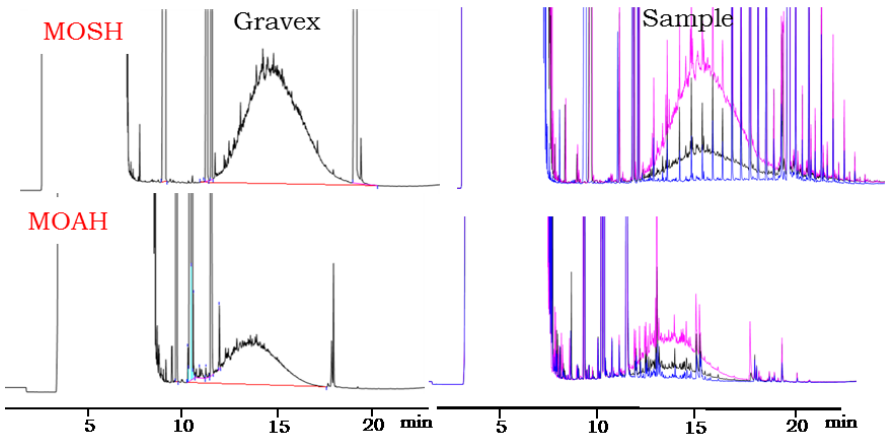

Figure 21. On the right: overlay of MOSH and MOAH chromatograms of cocoa powder sample (E1): unspiked (blue line), spiked with 3.0 (black line) and 10.0 mg/kg (violet line) of Gravex. On the left MOSH and MOAH chromatograms of the Gravex standard at the same concentration of that added to the sample for the highest spiking level.

Table 12 shows the recoveries calculated (at two different spiking levels), using CyCy as IS for the MOSH, and 5B, 1-MN, 2-MN and TBB for the MOAH. All recoveries were within the 70-120% range required by the JRC guidance [143], with RSD within the 20% (except for TBB at the lowest spiking level). While average MOSH recovery was practically quantitative (98%), average MOAH recovery ranged from 73% for TBB to 106% for 1-MN. As reported by Menegoz Ursol et al. [147], saponification leads to significantly different partitioning of the 4 MOAH IS between the aqueous/alcoholic phase and the hexane phase, and hence to different MOAH recoveries (when using different IS for MOAH quantification). Even though by correcting the raw data for the recovery a good data alignment was obtained for all the cocoa powder samples, regardless of the IS used for the MOAH fraction, it was chosen to use 2-MN (average recovery 105%) as IS for the MOAH fraction.

Table 12. Recovery and RSD at different fortification levels.

Sample	n° replicates	MOSH added (mg/kg)	Recovery % (mean)		RSD (%)		MOAH added (mg/kg)	Recovery % (mean)				RSD (%)			
			CyCy	*n*-C_{13}	CyCy	*n*-C_{13}		5B	1-MN	2-MN	TBB	5B	1-MN	2-MN	TBB
IE1_1	4	2.2	95.4	88.1	18.5	16.7	0.8	85.2	104	103.4	71.1	15.1	12.3	12.2	23.1
IE1_2	4	7.3	100.7	93.1	3.9	4.2	2.7	93.1	108.8	106.5	76.3	4.3	3.5	3.9	3.5
Mean	8		98.1	90.6				89.2	106.4	105.0	73.7	9.7	7.9	8.1	13.3

Intermediate repeatability was assessed on two different samples with a total MOH contamination of 2.0 mg/kg (of which 0.3 mg/kg of MOAH) and 53.6 mg/kg (of which 4.5 of MOAH).

In agreement with the JRC guidance for data reporting, table 13 shows, for each C-fraction, the mean concentration (mg/kg) obtained from the 8 replicate samples, and the related RSD (%).

Table 13. Repeatability.

Sample	n° replicates	C-fraction	Mean concentration (mg/kg)	RSD%	C-fraction	Mean concentration (mg/kg)				RSD%			
			CyCy	CyCy		5B	1-MN	2-MN	TBB	5B	1-MN	2-MN	TBB
IE1	8	*n*-C_{10-16}	0.31	13.3	*n*-C_{10-16}	0.04	0.05	0.04	0.03	14.3	17.3	14.0	13.5
		n-C_{16-20}	0.49	11.9	*n*-C_{16-25}	0.21	0.24	0.23	0.17	15.1	10.1	12.9	14.1
		n-C_{20-25}	0.31	5.2	*n*-C_{25-35}	0.01	0.01	0.01	0.01	43.0	30.3	40.5	41.2
		n-C_{25-35}	0.45	6.2	*n*-C_{35-50}								
		n-C_{35-40}	0.07	11.0									
		n-C_{40-50}	0.05	63.3									
		n-C_{10-50}	1.67	7.1	*n*-C_{10-50}	0.25	0.33	0.29	0.20	15.0	10.9	12.9	13.9
IE3	8	*n*-C_{10-16}	25.33	5.2	*n*-C_{10-16}	1.43	1.73	1.62	1.08	14.4	12.2	11.4	11.3
		n-C_{16-20}	22.95	7.9	*n*-C_{16-25}	2.46	2.98	2.79	1.87	12.8	10.5	9.7	9.8
		n-C_{20-25}	0.43	14.1	*n*-C_{25-35}	0.07	0.08	0.07	0.05	26.0	24.5	23.9	24.3
		n-C_{25-35}	0.31	19.3	*n*-C_{35-50}								
		n-C_{35-40}	0.07	128.3									
		n-C_{40-50}	0.06	108.1									
		n-C_{10-50}	49.12	6.5	*n*-C_{10-50}	3.95	4.78	4.49	3.00	13.5	11.2	10.4	10.4

Good intermediate precision was found for both the samples. More precisely, total *n*-C_{10-50} RSD (%) were below 13%. Moreover, considering the individual C-fractions, a LOQ of 0.2 mg/kg was established based on the RSD lower than 20% associated to this level, while the total LOQ was set at 0.5 mg/kg.

4.3.2. MOH/POH content of cocoa powder samples and related packaging

Table 14 reports MOSH/POH and MOAH concentrations (expressed in mg/kg) of 21 cocoa powder samples of different brands and related primary and secondary packaging, taken from the Italian (15 samples) and the North Macedonian (6 samples) market, sorted according to the packaging type. All the samples were analyzed in double and results are the average of the two replicates.

Seven different packaging solutions (described in table 11) were identified: 6 for the Italian market (A-F) and 2 from the North Macedonian market (B, G).

Following the indication given in the JRC guidance on sampling, analysis and data reporting [143], food results were divided into 6 *C*-fractions for the MOSH and 4 *C*-fractions for the MOAH. Total *n*-C_{10-50} concentration (calculated by integrating the signal over the whole carbon atom range) was also reported. Due to the co-elution with the MOSH, POH presence was not always easily identifiable

in the chromatographic trace, and a separate POH/MOSH quantification in the food was not possible. Since in food MOSH contamination generally prevails on POH contamination, data were expressed as MOSH/POH.

For the primary packaging in direct contact with the food, *n*-C_{10-35} concentration was reported (under direct contact condition hydrocarbons up to *n*-C_{35} may easily migrate), while for the secondary packaging both *n*-C_{10-25} and *n*-C_{10-35} concentrations were reported (the former reflects hydrocarbons which may migrate via gas phase at room temperature). Since MOSH and MOAH contamination (migrated from secondary packaging of recycled cardboard) was also present in many primary plastic packaging, data reported for these samples in table 14 were expressed as POH/MOSH (where POH generally prevail on MOSH) and MOAH.

Table 14. MOH and POH content (mg/kg) of the analyzed cocoa powder samples and corresponding packaging.

Sample code*	Food product (cocoa powder)								Primary packaging	Secondary packaging		Food product (cocoa powder)						Primary packaging	Secondary packaging	
	MOSH/POH (mg/kg)								POH/MOSH (mg/kg)	MOSH (mg/kg)		MOAH (mg/kg)								
	n-C_{10-16}	*n*-C_{16-20}	*n*-C_{20-25}	*n*-C_{25-35}	*n*-C_{35-40}	*n*-C_{40-50}	*n*-C_{10-25}	*n*-C_{10-50}	*n*-C_{10-35}	*n*-C_{10-25}	*n*-C_{10-35}	*n*-C_{10-16}	*n*-C_{16-25}	*n*-C_{25-35}	*n*-C_{35-50}	*n*-C_{10-25}	*n*-C_{10-50}	*n*-C_{10-35}	*n*-C_{10-25}	*n*-C_{10-35}
IA1	0.7	1.4	1.3	2.4	0.8	0.8	**3.5**	**7.6**	395	40	56	<0.2	0.2	<0.2	<0.2	**0.3**	**<0.5**	<20	8	9
IA2	0.7	0.8	0.8	1.1	0.4	0.7	**2.3**	**4.4**	223	9	22	<0.2	0.3	<0.2	<0.2	**0.4**	**<0.5**	<20	<3	<5
IA3	0.8	1.1	0.7	1.2	0.5	0.7	**2.6**	**5.0**	256	15	33	<0.2	0.2	<0.2	<0.2	**0.4**	**<0.5**	<20	<3	<5
IA4	0.6	0.9	0.5	1.8	1.0	1.7	**2.0**	**6.5**	433	55	132	<0.2	0.2	<0.2	<0.2	**0.3**	**<0.5**	<20	<3	<5
IB1	1.1	1.2	0.6	0.9	0.3	0.3	**2.9**	**4.5**	713	157	322	<0.2	0.3	<0.2	<0.2	**0.4**	**<0.5**	<20	36	65
IB2	1.4	2.6	0.8	2.1	0.6	0.5	**4.8**	**8.0**	505	299	638	0.2	0.8	<0.2	<0.2	**1.0**	**1.0**	<20	36	62
MB1	7.8	16.8	1.9	1.4	0.3	0.2	**26.5**	**28.4**	3544	328	651	1.1	1.7	<0.2	<0.2	**0.4**	**2.7**	180	60	95
MB2	2.1	3.2	1.8	5.5	0.8	0.5	**7.0**	**13.9**	1596	104	329	0.4	0.4	<0.2	<0.2	**0.8**	**0.8**	25	14	29
MB3	5.8	3.2	1.0	0.7	<0.2	<0.2	**10.0**	**10.7**	2845	119	408	0.2	0.3	<0.2	<0.2	**0.4**	**0.8**	87	20	46
MB4	2.7	9.2	10.1	31.9	10.1	5.2	**22.0**	**69.2**	1455	158	509	0.6	3.2	7.2	4.8	**15.7**	**15.7**	52	25	57
MB5	3.1	3.0	2.3	5.0	0.9	0.5	**8.4**	**14.8**	3338	248	511	0.3	0.5	0.5	<0.2	**1.3**	**1.3**	132	25	43
MB6**	1.0	1.3	2.1	12.1	1.7	0.9	**4.3**	**19.1**	1398	-	-	0.2	0.3	0.4	0.4	**1.2**	**0.8**	<20	-	-
IC1	2.0	4.8	2.8	6.6	1.9	1.7	**9.6**	**19.8**	422	15	63	0.2	1.0	<0.2	<0.2	**1.2**	**1.2**	<20	3	<5
IC2	2.3	3.9	3.5	16.7	8.1	6.0	**9.7**	**40.6**	567	14	28	0.2	1.0	0.3	<0.2	**1.5**	**1.5**	<20	<3	<5
ID1	9.8	25.2	5.2	7.7	2.2	2.0	**40.3**	**52.2**	438	133	414	0.7	3.8	0.5	<0.2	**5.0**	**5.1**	28	21	50
ID2	5.7	13.9	5.2	5.2	2.1	2.2	**24.8**	**34.3**	184	100	337	0.4	1.8	<0.2	<0.2	**2.2**	**22.0**	<20	33	71
ID3	7.6	8.3	3.6	8.0	4.2	4.3	**19.5**	**36.1**	145	81	296	0.4	1.5	<0.2	<0.2	**1.9**	**1.9**	<20	18	44
IE1	0.3	0.4	0.2	0.3	<0.2	<0.2	**0.9**	**1.2**	<3	<3	<5	<0.2	0.2	<0.2	<0.2	**0.2**	**<0.5**	<3	<3	<5
IE2	0.3	0.6	0.3	0.4	<0.2	<0.2	**1.2**	**1.7**	<3	<3	<5	<0.2	0.2	<0.2	<0.2	**0.4**	**<0.5**	<3	<3	<5
IE3	22.4	25.6	0.6	0.5	<0.2	<0.2	**48.7**	**49.2**	<3	<3	<5	1.6	3.5	<0.2	<0.2	**5.1**	**5.2**	<3	<3	<5
IF1	0.9	2.0	2.2	4.0	1.9	1.3	**5.1**	**12.2**	-	-	-	<0.2	0.4	<0.2	<0.2	**0.6**	**0.6**	-	-	-

*Sample code indicates origin (I= Italy; M= North Macedonia); packaging type (A-F, see table 11) and sample number
**Sample without the secondary packaging

Total *n*-C_{10-50} MOSH/POH contamination in the food products from the Italian market ranged from 1.2 to 52.2 mg/kg (on average 18.9 mg/kg), while MOAH contamination ranged from <0.5 to 5.0 mg/kg (on average 1.5 mg/kg). The products from the North Macedonian market had higher contamination comprised between 8.0 and 69.2 mg/kg (on average 26.0 mg/kg) for the MOSH, and between <LOQ (0.5 mg/kg) and 15.7 mg/kg (on average 3.9 mg/kg) for the MOAH. When compared to the reference benchmark levels recently agreed by representatives of the federal states and producer association, within the framework of a joint project based on current data [41], 7 samples of the 15 from the Italian market exceeded the 9 mg/kg level established for the sum of MOSH and MOSH analogous (comprising POH), and the same samples plus sample IB2 exceeded the MOAH quantification limit (0.5 mg/kg). All of the 6 samples from the North Macedonian market exceeded the MOSH benchmark level and had detectable MOAH.

In order to evaluate the influence of the packaging choice on food contamination, both the food and the corresponding packaging were analyzed, and all the chromatographic traces were carefully examined. Besides a pre-existing contamination due to different sources (lubricants, transport in jute bags, etc.), well visible in some of the samples, a systematic contamination with POH migrated from polyolefin primary packaging and MOH migrated from secondary packaging made of recycled cardboard was highlighted in many sample, depending on the packaging type.

As reported in table 11, primary packaging in direct contact with the food consisted of bags made of plastic or virgin paperboard (internally laminated with PE or not), while both virgin and recycled paperboard were used as secondary packaging.

Except for sample IB1, all plastic primary packaging used in combination with secondary packaging made of recycled cardboard contained some MOSH (up to around *n*-C_{25}) migrated from the secondary packaging, so data reported in table 14 can be affected by this migration. Total POH/MOSH *n*-C_{10-35} ranged from 223 mg/kg) to 3544 mg/kg (on average 1392 mg/kg), while MOAH were always lower than the LOQ (20 mg/kg), except for samples MB1-MB5 with a secondary packaging of recycled cardboard, which had MOAH *n*-C_{10-25} comprised between 25 to 180 mg/kg. Except for sample ID3, also virgin paper bags internally laminated with plastic showed clear MOSH contamination (up to *n*-C_{25}) from the secondary packaging made of recycled cardboard.

Secondary packaging made of virgin cardboard had MOSH *n*-C_{10-35} content ranging from below the LOQ (3 mg/kg) to 132 mg/kg (on average 39 mg/kg), and MOAH always lower than the LOQ (5 mg/kg), while secondary packaging made of recycled cardboard showed MOSH content from 322 to 638 mg/kg (on average 401 mg/kg) and MOAH content from 44 to 71 mg/kg (on average 58 mg/kg).
Data reported in table 14 clearly indicate as food samples with a secondary packaging made of recycled cardboard (packaging type B and D) are more prone to be contaminated with MOSH and MOAH *n*-C_{10-25} (which is the carbon atom interval more subjected to MOH migration from recycled cardboard). MOAH contamination was also confirmed by the presence of diisopropylnaphthalene (DIPN). Of course, not all the contamination found in this molecular range comes from packaging migration, but it can also derive from pre-existing contamination due for example to migration from jute bags used for cocoa bean transport and storage. Is this probably the case of samples IE3, IC1 and IC2 which had a secondary packaging made of virgin cardboard. IE3 had also a tertiary packaging made of a polypropylene (PP) film, which, as demonstrated in another study (Srbinoska et al., in preparation), prevent/limit migration from transport boxes usually made of recycled cardboard (and from the external environment). In all the three cases migration from transport boxes or from the external environment was excluded due to the absence of DIPN markers in the MOAH fraction.
It follows a more detailed description of the impact due to the different sources of contamination.

4.3.3. Migration from the primary packaging

Regarding the primary packaging, we have to distinguish them based on the material: plastic (of different types) or paper bags internally laminated or not with plastic. Even though the plastic type was not declared by the producers, as suggested by Biedermann & Grob [69], some information can be obtained by the chromatographic profiles.
Figure 22 shows a selection of HPLC-GC-FID traces of different primary packaging and of the corresponding food product. The lower traces refer to the primary packaging, while the upper ones to the cocoa powder.

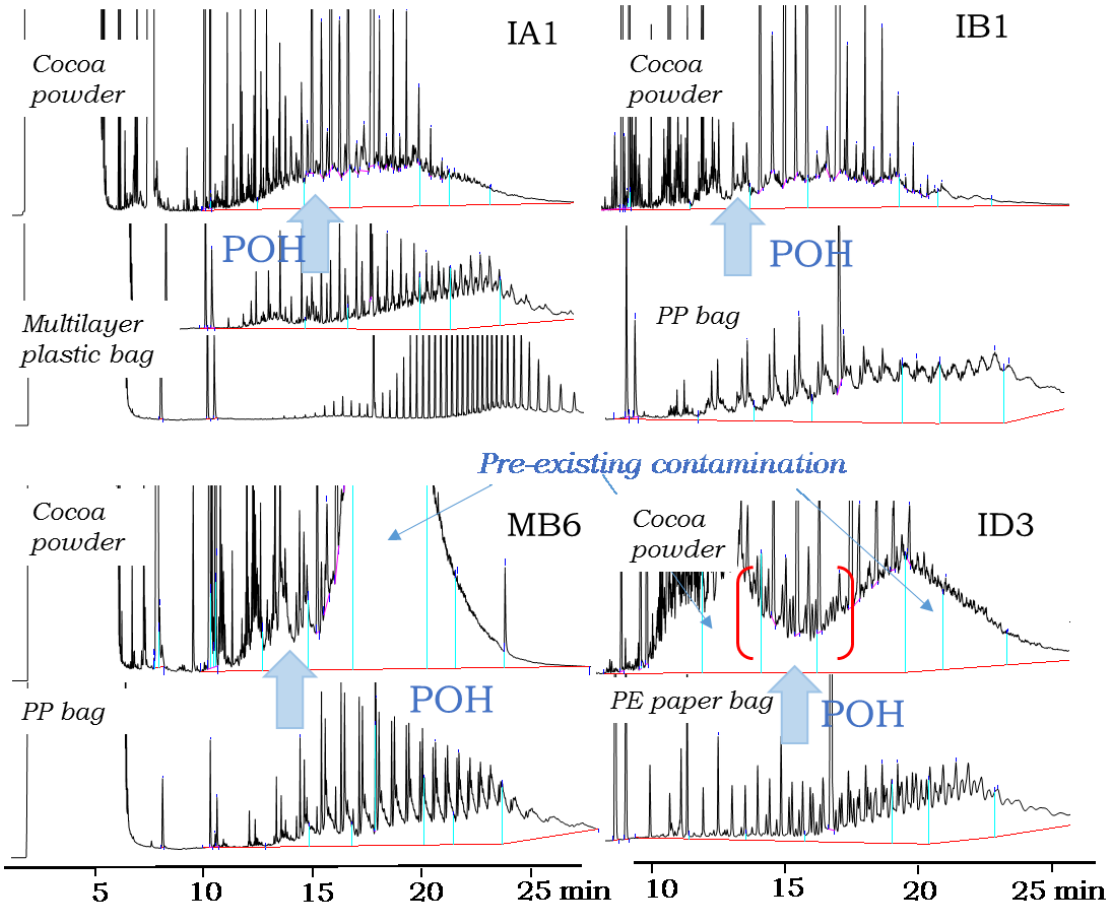

Figure 22. Examples of POH migration from the primary packaging.

All packaging types, except packaging type E, which had a primary packaging made of virgin paper not laminated with plastic, showed possible POH migration from the plastic bag or the paper bag internally laminated with plastic.

Of the 6 plastic bags of the Italian market (packaging type IA and IB, most of which were probably multilayer material), only sample IB1, whose trace is visible in figure 22, had the typical pattern of polypropylene (PP), while all the 6 samples of the North Macedonian market (packaging type MB) were clearly made of PP. The rest of the plastic bags from the Italian market had a profile compatible with that of polyethylene (PE), as in the case of the middle IA1 chromatogram of figure 22. In the case of sample IA1 it was possible to separate two layers that were analyzed separately. Both cocoa powder IA1 and IB1 showed a POH distribution similar to that found in the corresponding primary packaging made of PE and PP, respectively. It is interesting to observe as relevant migration of PP oligomers in sample IB1 (and in many other samples not shown) occurred up to *n*-C_{35} (in agreement with what foreseen for migration under wet contact conditions). POH migration (from the PP plastic bag) was also evident in cocoa powder MB6 which had no secondary packaging and showed a pure POH contamination in the range

n-C_{10-20} (around 2 mg/kg) and a mixed contamination in the range *n*-C_{20-50} (16.9 mg/kg), where a pre-existing MOSH contamination, probably due to a lubricant, prevailed. MOSH contamination was also accompanied by MOAH (0.8 mg/kg) in the same molecular range. A similar POH migration was observed in all the samples of the Macedonian market. The primary packaging of ID3 was a virgin paper bag internally laminated with plastic and had the typical POH pattern visible in figure 22. According with the information furnished by one producer, the plastic layer usually consists of PE/polyethylene terephthalate (PET). A similar pattern is visible in the central part of the chromatogram of cocoa powder ID3, which resulted already contaminated with MOSH from two different pre-existing sources.

It is interesting to observe that plastic bag from the North Macedonian market had by far higher POH content than those from the Italian market, and consequently also migration into the food was more evident. Figure 23 compares POSH/MOSH content of the different plastic packaging and shows as by subtracting the contribution due to the MOSH migrated from the secondary packaging to the total POH/MOSH area up to *n*-C_{10-35}, it was possible to perform a rough separate estimation of POH and MOH in the plastic primary packaging. As expected, plastic bags in contact with virgin cardboard (packaging type IA), or without secondary packaging (MB6) contained only POH, while all those with a secondary packaging of recycled cardboard (MB1-MB5, IB2), except IB1, showed some MOSH and MOAH migrated from the cardboard box. MOAH chromatograms are not reported, but their presence in the plastic primary packaging, together with that of DIPN, confirmed that the source was migration from recycled cardboard.

Based on these results, it was calculated that the plastic bags from the North Macedonian market had on average POH concentration 4 times higher than those of the Italian market. From figure 23 it is well evident that the latter are also less prone to be contaminated with MOSH.

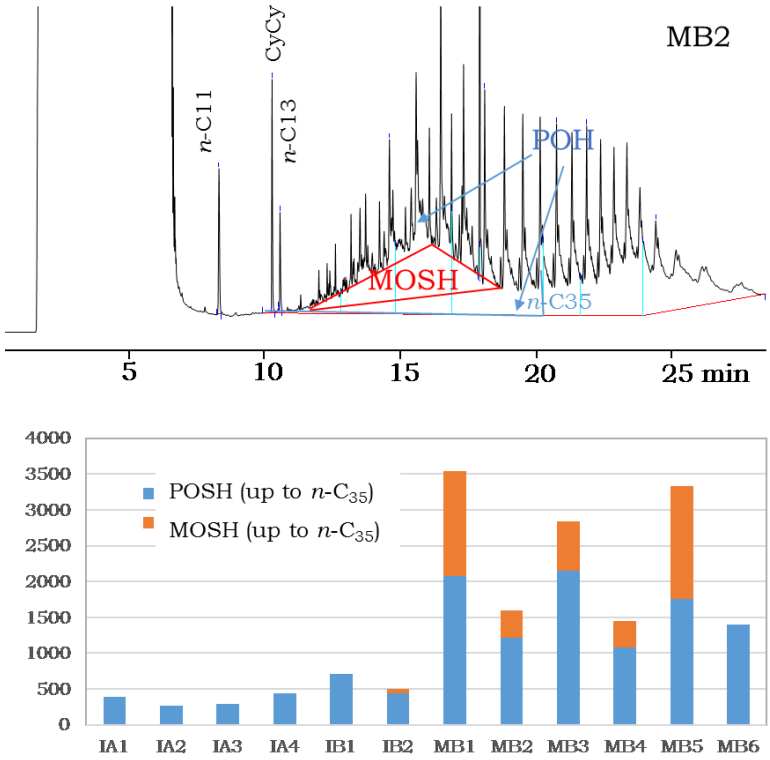

Figure 23. POH/MOSH distribution in plastic primary packaging from Italian and North Macedonian market.

4.3.4. MOH migration from secondary packaging made of recycled cardboard

Different works reported migration of hydrocarbons up to *n*-C_{24-25} from recycled cardboards into the food [126], [152]–[154] and underlined the necessity to use recycled cardboard only in association with a functional barrier.

Figures 24 reports LC-GC-FID chromatograms of selected cocoa powder samples (upper traces) with different primary packaging type (middle traces), and a secondary packaging made of recycled cardboard (lower traces).

The MOSH traces of the secondary packaging are typical and show a first hump in the range *n*-C_{13-25} (centered on *n*-C_{18}), due to residue of printing ink, and a second more enlarged hump including paraffins used for cardboard treatment. In sample MB3, most of the first hump is no more visible because most of the more volatile hydrocarbons have already migrated. This cocoa powder was indeed sampled and analyzed very close to the expiration date. The chromatographic profiles of all the primary packaging and of cocoa powder samples showed the same hump, centered on *n*-C_{18}, found in recycled cardboard, demonstrating as MOH migration occurred from the secondary to the primary packaging and then

to the food. MOSH contamination was also accompanied by MOAH of the same volatility range and by DIPN, as typical for residue of printing inks.

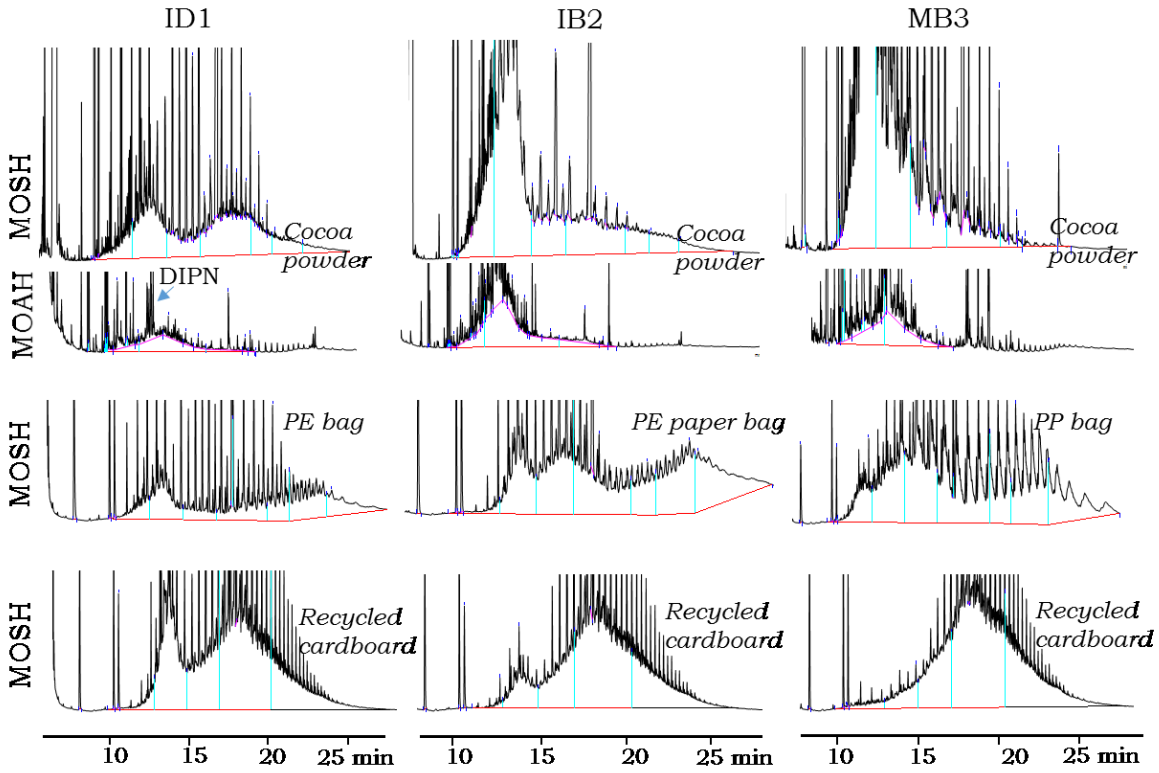

Figure 24. Examples of MOH migration from the secondary packaging.

ID1 primary packaging consisted of a paper bag internally laminated with PE, while cocoa powder IB2 had a primary plastic bag (PE based) that, different from that of sample IB1 (see figure 22), allowed for MOH migration into the primary packaging and then to the food. MB3 primary packaging was a PP bag from the North Macedonian market. An important POH contamination, recognizable from the typical clustered peaks (extending up to *n*-C_{35}), was also evident in sample MB3. All these primary packaging types, used in combination recycled cardboard, expose the food product to MOSH and MOAH contamination. PP bag from the North Macedonian market gave the worst results, while plastic bag from the Italian marked gave different results demonstrating that in some cases a functional barrier was probably used.

4.3.5. Contamination already present in the cocoa powder

Beyond migration from the food packaging, chromatographic profiles revealed the presence of humps of different molecular range, clearly indicating a pre-existing contamination from different sources. Figure 25 shows a selection of traces with

different contamination profiles and a tentative identification of the contamination source.

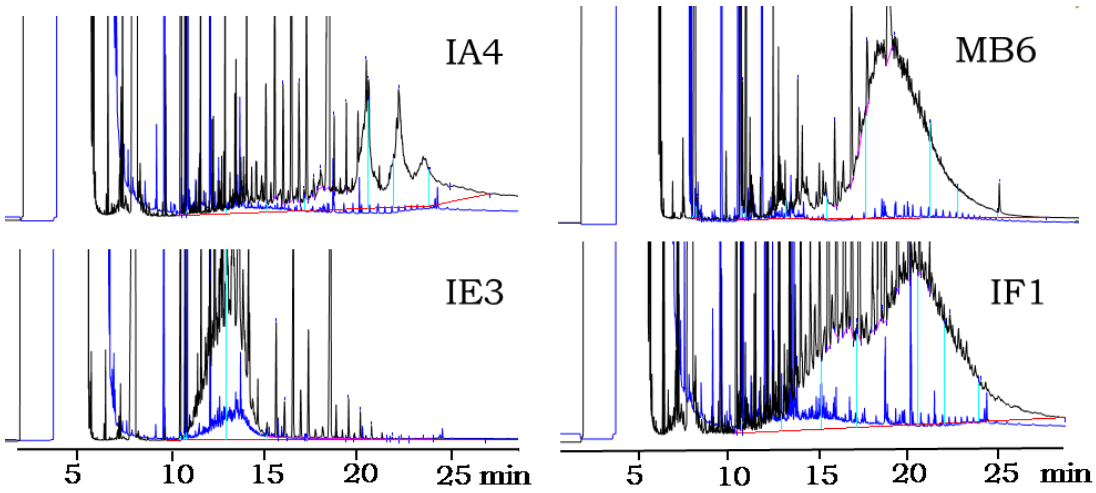

Figure 25. LC-GC-FID chromatograms of a selection of samples with pre-existing contamination (in black MOSH traces, in blue MOAH traces).

Cocoa powder IA4 evidenced a contamination with poly(alpha)olefins (PAO) of high molecular weight (in the range between *n*-C_{30} and *n*-C_{50}), which probably comes from a synthetic lubricant.

Sample MB6 shows instead a contamination ranging from *n*-C_{22} to *n*-C_{45} (centered on *n*-C_{29}) which could derive from a lubricants used during food processing.

Sample IE3 had a contamination ranging from *n*-C_{13} to *n*-C_{23} and centered on *n*-C_{17}, which could derive from transport/storage of cocoa beans in contaminated jute bags [10]. Finally, samples IF1 showed 2 partially fused humps standing from *n*-C_{13} to beyond *n*-C_{50} (one centered on *n*-C_{23} and the other on *n*-C_{35}), who's origin could probably due to processing lubricants.

4.4. Conclusions

Cocoa powder matrix is characterized by the presence of interfering compounds such as triglycerides and olefins, thus it requires an adequate sample preparation for reliable MOSH/POSH and MOAH quantification at low quantification limits. Based on previous work on bakery and fish products, an analytical protocol, based on microwave assisted saponification was optimized and validated. The method demonstrated good performance characteristics in terms of recoveries, inter-day repeatability and LOQ in line with requirements of the JRC guidance. The results obtained from the monitoring of contamination in products of the Italian and North Macedonian market showed that MOSH/POSH

and MOAH concentrations may vary widely. Chromatographic profiles revealed the presence of humps with different molecular range, which indicate different contamination sources. Based on the chromatographic profiles obtained, it was concluded that possible sources of contamination are several. Migration from secondary packaging made of recycled paperboard still represents one of the most important contamination source for those products packaged in primary packaging without barrier properties. Particularly product in PP bags from the North Macedonian market showed high POH migration. A more conscious choice of the type of packaging could limit contamination. Pre-existing contamination is in most cases the most important source of contamination (from a quantitative point of view). Further studies are needed to assess the impact of pre-existing contamination and to identify the most important sources of contamination.

Chapter 5. MINERAL OIL MIGRATION IN COCOA POWDER UNDER WAREHOUSE AND DOMESTIC STORAGE CONDITIONS: A CASE STUDY

- ✓ Srbinovska, A., Conchione, C., Moret, S. Mineral oil migration in cocoa powder under warehouse and domestic storage condition: a case study. *Paper drafting.*

5.1. Introduction

Cocoa (*Theobroma cacao L.*) is one of the main ingredients used in pastry, confectionery and bakery products. Cocoa products (cocoa powder and butter) are obtained from the cocoa beans, which undergo fermentation, drying, deshelling and roasting processes [155]. The cocoa butter (fatty fraction) and the cocoa cake (partially defatted fraction) are obtained by grounding and liquefying, using heat, the roasted and unshelled cocoa beans. The natural cocoa powder is obtained by pulverization process of the dried cocoa cake [156]. These products are used as ingredients in chocolate production [157].

According to the information gathered on the web and by some producers, cocoa powder has a primary shelf life of at least two to three years, which can extend beyond its expiration date. Even if not declared on the label, it is commonly suggested that, if maintained in a dry and cool place, after opening, the product should be consumed within a year (secondary shelf life). While the term primary shelf life refers to the intact products in its original packaging, the term secondary shelf life refers to the opened product.

Depending on the food type and composition, and type of packaging, household management of opened products by consumers can lead to more or less rapid quality and safety decay. Among possible causes of safety decay, migration of mineral oils during household storage after pack opening, has not been studied so far. Unless the product is protected by a barrier material, due to its powdered form and relatively high fat content (~25%), cocoa powder is particularly prone to be contaminated with mineral oil hydrocarbons (MOH) via gas-phase migration from recycled paperboard.

MOH are complex mixture of isomers, originating from petrogenic processes, which may be separated into two fractions of different toxicological relevance [1], [32]: saturated (MOSH) and aromatic (MOAH) hydrocarbons. The former include paraffins (linear and branched alkanes) and predominantly alkylated naphthenes (cyclic alkanes) which may bioaccumulate in human tissue and organs, while the latter includes mono- or polyaromatic compounds with an alkylation degree greater than 98% [143], included suspected carcinogenic and genotoxic substances.

In addition to possible migration occurring during storage of the intact packaging (at the warehouse of the producer or of a store, or on the shelf of the store or on the pantry of the consumer), an increased migration may occur during storage at

home, especially when the opened product is left in contact with products packaged in recycled cardboard in the same pantry. Indeed, contamination with hydrocarbons up to *n*-C_{24} deriving from recycled cardboard, which are volatile enough to migrate through gas phase eventually ending up into the food, was reported by Lorenzini et al. [19].

Being often in contact with plastic packaging, cocoa powder can also be contaminated with polyolefin oligomeric hydrocarbons (POH) of synthetic origin.

Based on the result of a previous survey on MOH/POH contamination of cocoa powder and corresponding packaging from the Italian and the North Macedonian market (Srbinovska et al., in progress), it emerged that a correct choice of the packaging has an important impact in preventing possible migration from secondary/tertiary packaging or the external environment. Almost all samples resulted clearly contaminated with POH migrated from the primary packaging, mostly consisting of a bag made of plastic or virgin paper internally laminated with plastic, in direct contact with the food. MOH contamination was also well evident in many samples with a secondary packaging of recycled cardboard. Seven different packaging types were identified, and among these also the one chosen as a case study in this work.

The present study was carried out in collaboration with a producer of cocoa powder, and the packaging type investigated consisted of a virgin paper bag, contained in a virgin paperboard box, wrapped in a polypropylene (PP). Such packaging combination has the advantage of not exposing the product to POH migration. Furthermore, the outer PP film contributes to reduce possible migration from the transport box (recycled cardboard) or the external environment.

The aim of the present work was therefore to verify if the packaging under evaluation could act as an effective barrier against MOH migration from the transport box, where the packaged products may be stored for several months, and to evaluate for the first time migration that may occur in the home pantry, after opening the package (during the secondary shelf life of the product).

5.2. Materials and methods

5.2.1. Reagents and standards

Dichloromethane, *n*-hexane (both distilled before use), *n*-pentane, *m*-chloroperbenzoic acid, potassium hydroxide, sodium thiosulfate, sodium sulfate, methanol and toluene, were obtained from Sigma-Aldrich (St. Louis, Missouri,

USA). Ethanol was purchased from VWR (Radnor, Pennsylvania, USA). Pure water was obtained with a Milli-Q system from Millipore (Bedford, Massachusetts, USA).

To verify the LC-GC performance and to check the MOSH and MOAH separation, as described by Biedermann & Grob [69], the internal standard solution (IS) that include *n*-C_{13} at 0.15 mg/mL, 1,3,5-tritert-butylbenzene (TBB), *n*-C11, cyclohexyl cyclohexane (CyCy), pentyl benzene (5B), 1-methyl naphthalene (1MN), 2-methyl naphthalene (2MN) at 0.30 mg/mL and 5-α-cholestane (Cho) and perylene (Per) at 0.6 mg/mL was used (purchased by Restek, Bellefonte, Pennsylvania, USA). The same standard solution was used also for sample quantification. To verify the GC performances, a *n*-C_{10}-C_{40} and *n*-C_{50} standard mixture (from Restek), containing *n*-alkanes with even-numbered carbon atom, was used. All solutions were stored at -18 °C in toluene.

The Gravex (a mineral oil consisting 27% of MOAH, with a chromatographic profile, extending from *n*-C_{13} to *n*-C_{25} and centred on *n*-C_{18}) was supplied by a manufacturer and was used to prepare the standard solution to fortify a virgin cardboard for the accelerated migration tests.

5.2.2. Instrumentation

For sample saponification a Microwave Extraction System MARS5 (CEM Corporation, Matthews, North Carolina, USA) equipped with GreenChem Plus Teflon vessels (able to host 14 samples simultaneously), was used.

Sample analyses were carried out on a double channel LC-GC 9000 apparatus, from Brechbühler (Zurich, Switzerland), connected to two flame ionization detectors (one for each channel). The LC column was a 25 cm × 2.1 mm i.d., packed with Lichrospher Si-60, 5 µm particle size (DGB, Schlossboeckelheim, Germany). HPLC-GC transfer was carried out through a Y-interface using the retention gap technique [50].

Both the GC channels consisted of an uncoated/deactivated pre column of 10 m × 0.53 mm i.d., followed by a steel T-piece connected to a 15 m × 0.25 mm i.d. separation column (PS-255, 0.15 µm of film thickness from Mega, Italy), and to the solvent vapour exit (SVE). HPLC and GC conditions as well other details are reported in a previous work (Srbinovska et al., in progress). Data was acquired and processed by Chromeleon 7 software.

5.2.3. Samples

All samples were directly supplied by an Italian producer. The packaging type taken into consideration for the case study was that commonly used by the producer for cocoa powder at the time of the investigation, and consisted of a virgin paper bag as primary packaging, a virgin cardboard box as secondary packaging, and a polypropylene (PP) film as tertiary packaging.
Cocoa powder samples from three different lot (A, B, C) were used to monitor possible migration from the transport box (quaternary packaging made of recycled corrugated cardboard) during 6 months of storage. Longer time were not considered since, as indicated by the producer, it is very difficult to exceed a 6-months storage into the quaternary packaging.
Samples used for migration test were all of the same lot (D), with a low pre-existing contamination.

5.2.4. Sample preparation

For cocoa powder samples the protocol used for sample preparation was the same as that used in the previous work conducted by Srbinovska et al., (in process, Chapter 4). Briefly, samples were weighed inside the MARS Teflon vessels, added with IS, 10 mL of *n*-hexane and 10 mL of methanolic KOH solution. Microwave assisted saponification (MAS) was performed at 120 °C for 20 min, and subsequently two rapid washings, one in the extraction vessel and the second one in a 10 mL vial, with a CH_3OH/H_2O mixture 2:1 (v/v), were performed. Then the recovered organic phase was reconcentrated to 700 µL and subjected to epoxidation following the Nestola & Schmidt [55] protocol. Epoxidation was performed for 15 min with a 20% (m/v) ethanolic *m*-CPBA solution and then stopped with a 10% (m/v) aqueous sodium thiosulphate solution. The sample was then transferred into an autosampler vial and injected.

5.2.5. Migration from the transport box

In order to verify whether during the storage of the product there could be some migration of mineral oils from the quaternary packaging (recycled fibers) to the cocoa powder, the samples were taken at three different moments during storage: at time 0 (immediately after packaging), and after 3 and 6 months of storage.
Figure 26 shows a schematic representation of how the sampling was performed and the type of packaging.

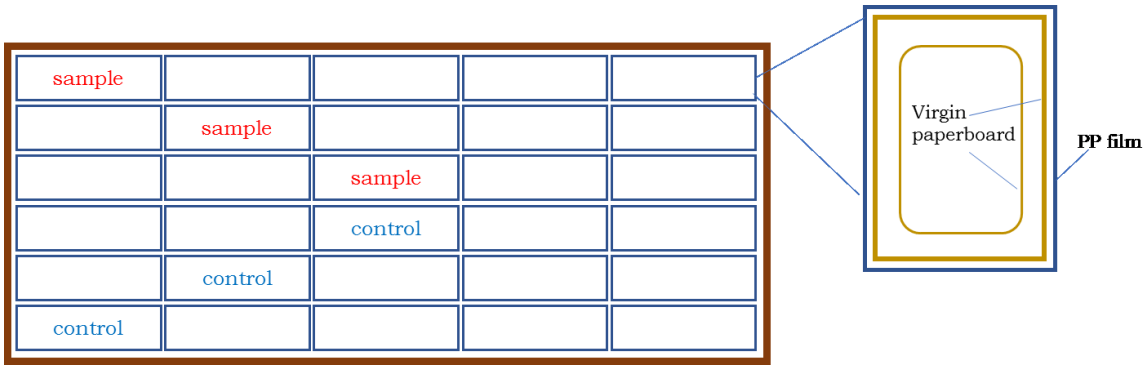

Figure 26. Schematic representation of the sampling and packaging type.

As indicated in figure 26, each transport box contained 30 packages (containing 75 g of cocoa powder each). For each of the 3 lots sampled, 3 transport boxes were stored in warehouse of the producer and used for the three sampling at different times. For each sampling time, the packages of cocoa powder were taken from 3 different positions in the transport box (as indicated in figure 26), the contents of which were mixed in order to obtain a representative sample. The control samples, taken in a symmetrical position at the time of collection, were wrapped in aluminum foil and stored separately to verify possible differences between the sample in direct contact with the quaternary packaging and the more distant one, if significant differences were found among samples stored for different periods.

5.2.6. Accelerated migration tests in a glass cell under worst case condition

To evaluate the migration behavior and the efficacy of the tertiary packaging (PP film) to prevent migration from the external environment, accelerated migration tests were carried out by inserting the cocoa boxes into a sealed glass cell. A strip of virgin paper (29 cm × 3 cm), with negligible contamination levels, was spiked with a known quantity of Gravex solution (100 mg/mL) and inserted into the glass cell.

The migration tests were carried out on an intact (unopened) cocoa powder package before storage (control sample) and on the following samples after storage for 20 days at 40 °C: i) intact cocoa powder package, ii) opened package with the tertiary packaging (PP film) and iii) opened package without the tertiary packaging. Figure 27 shows the glass cell containing the cocoa powder boxes.

Figure 27. Glass cell used for migration tests.

5.2.7. Migration occurring in the pantry

To evaluate the migration behavior under more realistic conditions, even if not standardized, migration tests were carried out by storing the cocoa packages (same product and lot used for the accelerated migration tests), in a specially prepared pantry filled with a non-standardized quantity of other products packaged in recycled paper.

The samples were picked up monthly (T0, T1, T2, T3, T4), respectively at time 0 (control), and after 1, 2, 3 and 4 months of storage at room temperature. The tests were carried out on: 4 intact cocoa powder packages (control samples D_P1) wrapped in aluminum in order to prevent any migration from the external environment, 4 intact packages (D_P2), 4 unopened packages without the PP film (D_P3), 4 opened packages with the PP film (D_P4) and 4 opened packages without the PP film (D_P5). Figure 28 shows the position of the cocoa powder boxes into the pantry.

Figure 28. Position of the cocoa powder boxes into the pantry.

5.3. Results and discussions

5.3.1. Contamination monitoring during storage in quaternary packaging

It is well known that in the absence of a functional barrier migration can easily occur from the transport box through the paperboard packaging to the food [69], [158].

Unlike other packaging combinations tested so far, the one under consideration has an outer PP film (tertiary packaging), which could prevent or slow down migration from the outside.

To evaluate the efficacy of the tertiary packaging (PP) in preventing mineral oil contamination from the recycled cardboard, cocoa powder samples from three different lots (A, B, C) were analyzed during six months of storage at the producer warehouse, picking them up every three months.

The corrugated transport boxes had a weight of 220 g each and contained 2.25 kg of cocoa powder (30 × 75 g). They had an initial MOSH *n*-C_{10-25} contamination of 122 mg/kg (408 mg/kg in the range *n*-C_{10-35}), and a MOAH *n*-C_{10-25} contamination of 9 mg/kg (37 mg/kg in the range *n*-C_{10-35}). Based on this information and considering that only hydrocarbons up to *n*-C_{25} have sufficient volatility to migrate from to box to the food, a maximum potential migration of 12 and 0.5 mg/kg was calculated for MOSH and MOAH (*n*-C_{10-25}), respectively. This calculation was made by considering a uniform distribution of the contamination but, of course, higher contamination was expected into the packages in direct contact with the transport box, particularly in those placed at the corners of the box, having a greater surface area in contact with the recycled cardboard.

As can be seen in figure 29, that reports the total MOH content (mg/kg), the samples showed different initial contamination levels, due to different pre-existing contamination levels. As the storage period proceeded, there was no appreciable increase in the level of contamination, indicating that under the condition tested there is no appreciable migration of MOH from the quaternary packaging made of recycled cardboard to the product. The samples of lot C showed the highest contamination; 8.1 and 2.3 mg/kg for MOSH and MOAH respectively (at time zero), while the other two samples had MOSH around 1.0 mg/kg and no MOAH.

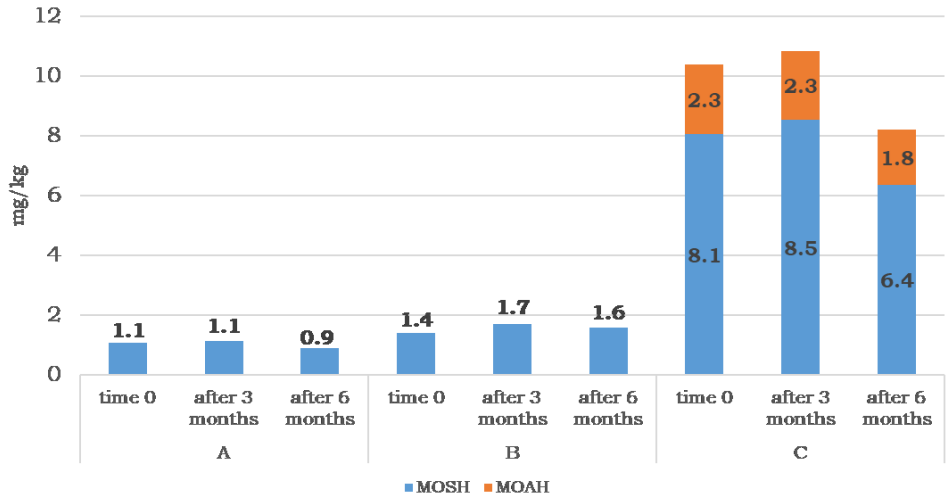

Figure 29. Total MOH levels (mg/kg) in cocoa powder samples stored in the quaternary packaging for six months.

5.3.2. Accelerated migration tests in a glass cell

In order to evaluate the migration behavior and the efficacy of the PP film in preventing contamination from the external environment, accelerated migration tests under worst case condition (very high contamination level), were carried out by inserting the cocoa powder box in a sealed glass cell together with a donor strip (virgin paper spiked with a Gravex solution), simulating three different storage conditions as reported in material and methods. Migration tests were carried out at 40 °C for 20 days, in order to simulate (according to the Arrhenius equation) a storage corresponding to about 3 months at room temperature.

Figure 30 shows the MOSH and MOAH traces of the intact sample before storage (D_A) and of samples D_B, D_C and D_D.

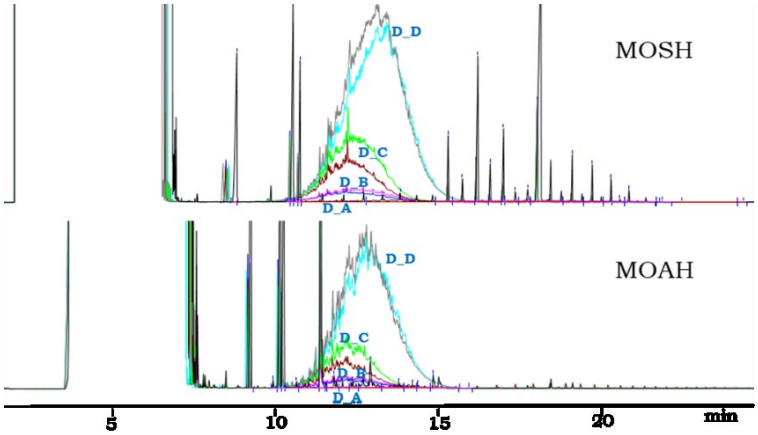

Figure 30. LC-GC-FID traces of the MOSH/MOAH fraction of the samples subjected to accelerated migration test in a glass cell (40 °C x 20 days). (D_A) cocoa unopened package before storage; (D_B) unopened cocoa package; (D_C) opened cocoa package with PP; (D_D) opened cocoa package without PP.

Assuming that all the mineral oil deposited on the donor paperboard migrates into the cocoa powder, it was calculated that the level of fortification used could determine a maximum potential migration of 1333 mg/kg for the MOSH and 360 mg/kg for the MOAH. Such contamination levels are very high compared to those encountered in a real situation, but may well stress the barrier properties of the packaging.

A contamination of 26.5 mg/kg for MOSH and 8.7 mg/kg for MOAH were found in the unopened package. In the opened package with the PP film the levels found were 109.8 and 29.9 mg/kg for MOSH and MOAH, respectively, while they were 365.8 and 126.7 mg/kg for the opened package without the PP film. For the latter, about 35% of the potential migrated into the cocoa powder, differently from the 10% observed for the opened package with the PP film, indicating the feasibility of the PP as a good barrier against mineral oil migration, suitable for preventing cross-contamination from the external environment during product storage. This is confirmed also in the unopened package with PP where the migration reached less than 3%, i.e. levels 10 times lower than for D_D (opened cocoa package without PP).

Figure 31 shows the percentage of the potential migration calculated for cocoa powder samples and for the donor strip. To obtain this last data, the donor paper strip, fortified with the Gravex solution, was analyzed after the accelerated migration tests, in order to evaluate the quantity of Gravex remained in the strip, and consequently to obtain the amount migrated (negative migration). As can be observed from the graph, only a part of the mineral oils migrated from the cardboard, arrived in the cocoa powder (positive migration), the remaining part probably remained partly in the headspace and partly distributed in the packaging. As the protection determined by the packaging decreased (passing from D_B to D_D) it was observed that the difference between the percentage migrated into the cocoa powder and that migrated from the donor strip decreased, i.e. a greater part of what migrates from the donor strip reached the cocoa powder (increases positive migration).

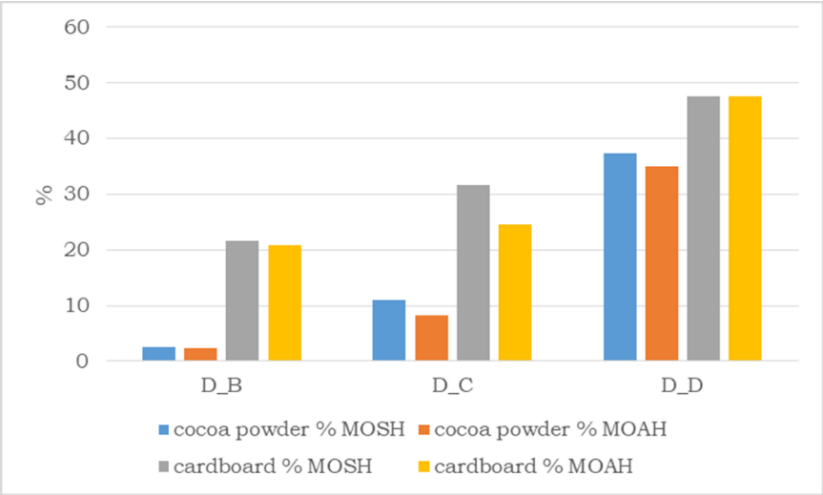

Figure 31. Percentage of potential migration (%) referred to positive migration in cocoa and negative migration from the donor paper strip.

5.3.3. Migration in the pantry under real condition

As reported in Materials and Methods, migration under domestic condition (ambient temperature) was also followed for 4 months in a pantry filled with other food products, some of which packed in recycled cardboard.

Figure 32 shows the total MOSH and MOAH concentration (mg/kg) obtained, minus the average value found in the control samples (this explains some negative values).

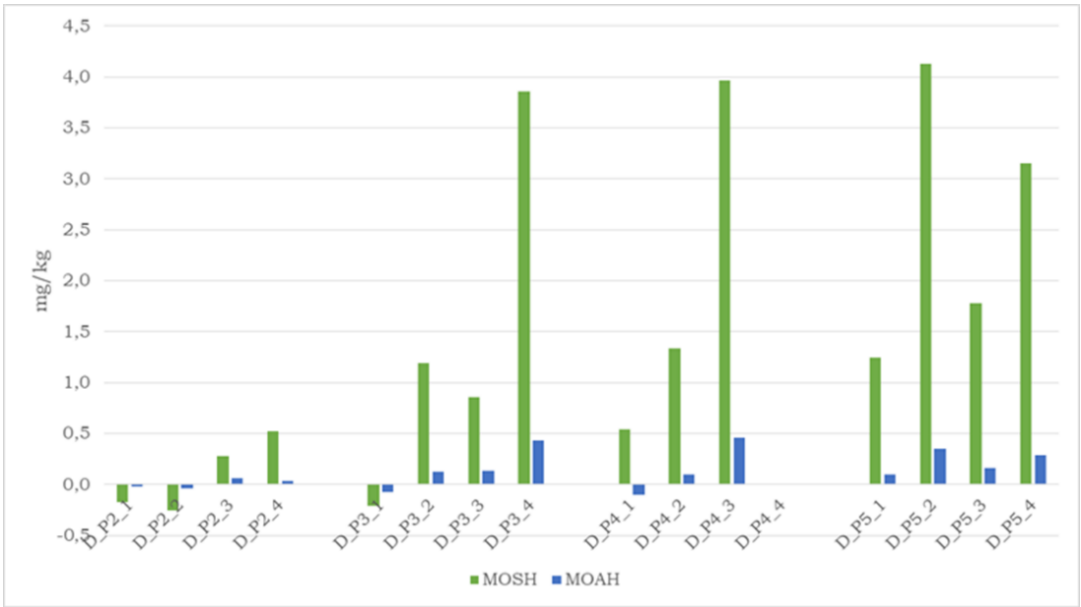

Figure 32. Total MOSH and MOAH levels (mg/kg) of cocoa powder after storage (1-4 months) in the pantry.

As can be seen from the results shown in the graph, all the cocoa samples in an intact package (D_P2) did not show relevant increase due to migration from the external environment, while the samples in an opened package and without PP

film (D_P5) showed on average, the highest levels of migration. Also, in this case, the protective effect of the tertiary packaging (PP film) against migration from the external environment was confirmed. Except for the samples in an intact package, a high variability is observed within the individual groups of samples. Contamination does not grow linearly with increasing the storage time, although compared to T1 (1 month of storage), samples with longer storage times generally show higher levels of migration. This variability could be due to the non-homogeneous position of the individual packages within the pantry and therefore to a non-uniform exposure to the source of contamination, randomly located within the pantry.

Figure 33 shows the LC-GC-FID chromatograms obtained for samples D_P2-D_P5 after 2 months of storage. It can be observed that as the protection given by the packaging decreases (from D_P2 to D_P5), the amount of contamination present in the cocoa powder increases during the storage time.

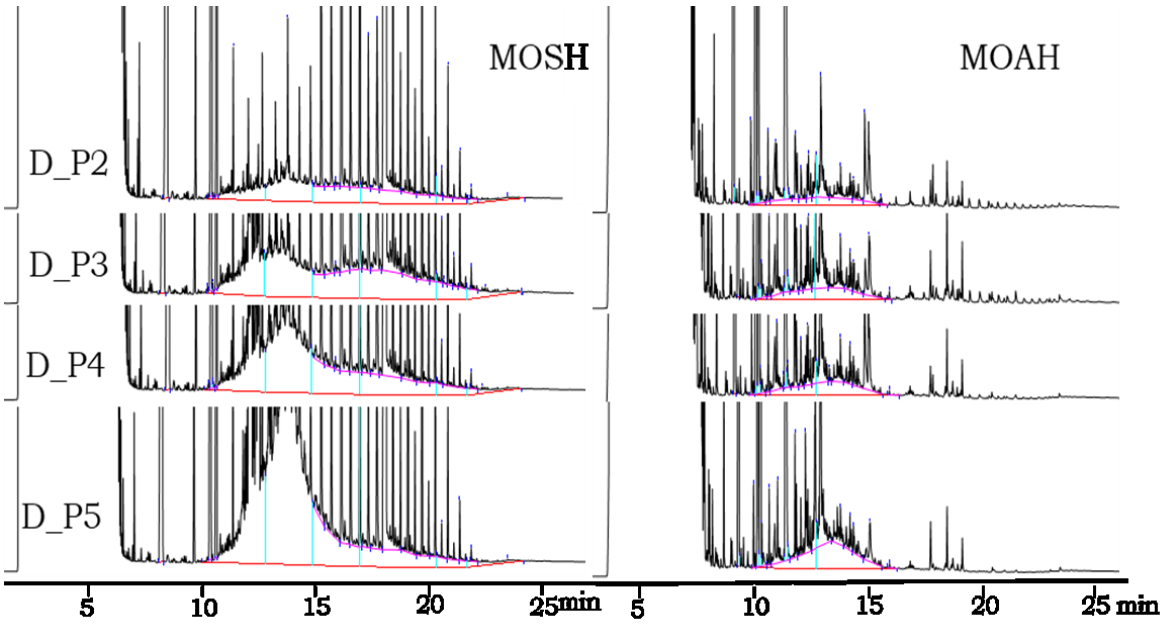

Figure 33. LC-GC-FID traces of the MOAH and MOAH fraction of cocoa powder stored for 2 months in the pantry in : intact package (D_P2), closed package without tertiary PP packaging (D_P3), open package with PP (D_P4), open package without PP (D_P5):

5.4. Conclusions

The tested packaging system, consisting of a primary packaging in virgin paper, a secondary packaging in virgin paper and a tertiary packaging in PP film, resulted to be effective in limiting the contamination coming from the external environment, and, in this regard, the presence of tertiary packaging (PP film) plays a very important role. The storage tests carried out in the pantry and the

accelerated migration tests have shown how inappropriate storage of the product in the pantry, once the package is opened, can expose the product to contamination. A more precise warning on how to store the product, communicated through the label, could direct the consumer towards more suitable behaviors for the protection of their health.

Chapter 6. MINERAL OIL CONTAMINATION IN INFANT FORMULA FROM THE ITALIAN MARKET

6.1. Introduction

Mineral oil hydrocarbons (MOH) are complex mixtures of hydrocarbons, divided into two major classes, namely mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH). While MOSH are investigated due to their ability to accumulate in human organ and tissues, MOAH are of major concern because may include the presence of genotoxic and carcinogenic 3-7 ring polycyclic aromatic compounds [1], and can act as endocrine disruptors [159].

MOH are ubiquitously present in a wide variety of petroleum derivative products in many fields (fuel, solvent, white oil, lubrication oils, etc.). Food is often contaminated with MOH during harvesting, processing, transportation and storage, and sources of contamination include packaging materials, food additives, processing aid and lubricants.

Currently there are no maximum limits for MOSH and MOAH in food within European legislation. In the absence of European regulation, representatives of the food control authorities of the federal states (LAV) together with the food industry (Lebensmittelverband) recommend the use of "benchmark levels" for the content of mineral oil hydrocarbons (MOH) and their structural analogues [41]. For baby food the general opinion is that neither MOSH nor MOAH should be present in measurable amounts in any samples (<LOQ).

Because of the low body weight and increased susceptibility of infants to xenobiotics, the presence of contaminants in baby foods is of great concern, so that much more restrictive legal limits are usually set than in conventional foods. Milk powder products play an important role in providing essential nutrients to infants who, for different reasons, cannot be breastfed.

The first data on mineral oils and POSH content in infant formula have been reported by Droz & Grob [16] and Biedermann et al. [43].

Zhang et al. [160] investigated MOSH/POSH and MOAH in 50 milk powder products packed in different types of packaging, confirming the presence of MOSH/POSH in 33 samples and MOAH in none of the analyzed products.

One factor that contributes to MOSH/POSH contamination is the fat content, and the authors found that a higher fat content was associated to a higher contamination levels in the samples. In agreement with what reported by Wan et al [161], the authors also observed that the infant formula packed in metal cans had the lowest MOSH content and was almost free from POSH.

In 2019 Foodwatch reported the detection of MOAH in batches of infant and follow-on formula in France, Germany and the Netherlands, attracting enormous public attention [162]. Following this publication, the European Commission asked Member States to analyze the concerned batches and to investigate possible contamination sources and gave mandated EFSA to perform a rapid assessment on the health risks related to the presence of MOAH in infant and follow-on formula [163]. Quantified MOAH levels were in the range 0.2-3 mg/kg. Due to the complex analytical methods, there was uncertainty on the reported levels. At the same time the Directorate General for Health and Food Safety (DG SANTE) of the European Commission requested to the Joint Research Centre (JRC) to organize an exploratory interlaboratory comparison (which involved 27 participants from 8 European countries) to develop a harmonized method for the determination of MOAH in infant formula. In 2020 the JRC published a technical report with the results of the interlaboratory comparison [164], according to which a revised protocol will be validated in a next ring trial.
In 2020, Sui and collaborators [165] published a work on survey of MOH in infant formula from the Chinese market and found 17 of 51 samples contaminated with MOSH and trace levels of MOAH (≤0.7 mg/kg), while Liu et al. [166] find MOSH/POSH ranging from 0.61 to 5.46 mg/kg in 10 commercial milk powders.
The aim of the present work was to validate a rapid, solvent-sparing and high sensitivity method for MOH determination in infant formula. Then the validated method was used to analyze 13 milk powder samples purchased from the Italian market.

6.2. Materials and methods

6.2.1. Reagents and chemicals

n-Hexane and dichloromethane (both distilled before use), *n*-pentane, *m*-chloroperbenzoic acid, potassium hydroxide, sodium thiosulfate, sodium sulfate, methanol and toluene, were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Ethanol was purchased from Supelco (Bellefonte, Pennsylvania, USA) and pure water was obtained with a Milli-Q system from Millipore (Bedford, Massachusetts, USA).

6.2.2. Standards

The C_{10}-C_{40} *n*-alkane standard mixture (even-numbered) used to verify the GC performances, was from Restek (Bellefonte, Pennsylvania, USA) as the internal standard solution (IS) used for sample quantification and HPLC-GC performance verification. The latter contained: *n*-C_{13} at 0.15 mg/mL, 1,3,5-tritert-butylbenzene (TBB), *n*-C11, cyclohexyl cyclohexane (CyCy), pentyl benzene (5B), 1-methyl naphthalene (1MN), 2-methyl naphthalene (2MN) at 0.30 mg/mL and 5-α-cholestane (Cho) and perylene (Per) at 0.6 mg/mL. The IS solution was in toluene and was stored at -18 °C.

The Gravex (a mineral oil consisting 27% of MOAH, with a chromatographic profile centred on *n*-C_{18}, extending from *n*-C_{13} to *n*-C_{25}) used for recovery tests at different spiking levels was supplied by a manufacturer.

6.2.3. Instrumentation

The on-line HPLC-GC-FID system was an LC-GC 9000 from Brechbühler (Zurich, Switzerland). It consisted of an HPLC Phoenix 9000 pump from Brechbühler (Zurich, Switzerland) and a GC Trace 1310 series from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

The HPLC column was a 25 cm × 2.1 mm i.d. packed with Lichrospher Si-60, 5 µm particle size (DGB, Schlossboeckelheim, Germany). MOSH and MOAH elution was accomplished using a gradient starting with 100% *n*-hexane (0.1 min hold) and reaching a *n*-hexane/dichloromethane ratio of 70/30 after 0.5 min, with a flow rate of 300 µL/min. Six minutes after the injection, the column was backflushed with dichloromethane (500 µL/min for 9 min) and then reconditioned for 6.5 min with *n*-hexane at 700 µL/min and at 300 µL/min for 1.5 min. The HPLC-GC transfer occurred via an Y-interface connecting the carrier gas line and the HPLC line to the retention gap, in order to exploit partially concurrent eluent evaporation to improve volatile retention through the solvent trapping effect, as described by Biedermann et al. [50]. The GC had a double channels configuration able to perform MOSH and MOAH analyses simultaneously, from the same HPLC run. Each GC channel consisted of a carrier gas line, a deactivated retention gap (10 m × 0.53 mm i.d.) followed by a steel T-piece connected to a 15 m × 0.25 mm i.d. PS-255 (1% vinyl, 99% methyl polysiloxane) separation column (0.15 µm of film thickness) and to a solvent vapour exit (SVE). The SVE (which remained open during the transfer) and the FID were heated at 140 and 360 °C, respectively. Both the retention gap and the

separation column were purchased from Mega (Milan, Italy). The GC oven temperature gradient started from 55 °C and was raised to 350 °C at a rate of 20 °C/min. Hydrogen was used as carrier gas at a constant pressure of 60 kPa (during HPLC transfer the pressure was raised to 90 kPa).

The Microwave Extraction System used for simultaneous sample saponification and unsaponifiable extraction was a MARS from CEM Corporation (Matthews, North Carolina, USA), equipped with GreenChem Plus Teflon vessels able to host 14 samples simultaneously. To ensure a uniform distribution of energy, the system is equipped with a turntable. One of the 14 positions is reserved for the pilot vessel, in which, in addition to the sample, the optical fiber probe (Probe RTP-300 Plus; range from 40 to 250 °C) for temperature control and a sensor for pressure control (ESP-1500 Plus), was also inserted.

6.2.4. Samples

To monitor MOH contamination in milk powder, 13 samples from the Italian market were purchased and analyzed. The samples, all of different brands, were classified based on the brand and the type of packaging. Table 15 reports the sample description.

Table 15. Sample description.

Brand	n° samples	Sample code	Primary packaging	Secondary packaging
A, D, E, L	6	LP1; LP2; LP3; LP4; LP5; LP6	aluminum bag laminated with plastic	recycled cardboard
C, H	3	LP7; LP8; LP9	can in virgin cardboard internally coated with plasticized aluminum film, top closure in plasticized aluminum and plastic cap	
F	1	LL10	can in recycledcardboard internally coated with plasticized aluminum film, top closure in plasticized aluminum and plastic cap	
B, G	3	LP11; LP12; LP13	metal can with aluminum closure and plastic cup	

6.2.5. Sample preparation

The protocol used was the same previously used for cereal based and fish products [47], [48]. Briefly, 4 g of powder milk were directly weighted into a Teflon vessel (Green Chem plus, CEM Corporation) and added with IS (20 µL), 10 mL of *n*-hexane and 10 mL of a saturated methanolic KOH solution. MAS was carried out at 120 °C for 20 min under magnetic stirring. After cooling at ambient temperature, 40 mL of water and 3 mL of methanol were added to the extraction vessel (without mixing), which was then placed at -20 °C for 30 min.

Five milliliters of the sample extract were then concentrated at 1 mL and an aliquot (100 µL) were directly injected into the HPLC-GC-FID system.
In order to eliminate interfering olefins from the MOAH fraction, an epoxidation procedure according to the protocol Nestola & Schmidt [55] was applied on the saponified sample. Another aliquot (500 µL) of the saponified extract was added with 500 µL of an ethanolic *m*-CPBA solution (20% m/v) and 450 µL of *n*-hexane and placed into an agitator at room temperature (500 rpm for 15 min). Subsequently, 2 mL of aqueous sodium thiosulfate solution (10% m/v) to stop the reaction and 500 µL of ethanol were added. The vial was shaken for 2 min. Finally, 500 µL were transferred into an autosampler vial containing a spatula tip of anhydrous sodium sulphate and then injected (100 µL) into the HPLC-GC-FID system.

6.2.6. Method validation

Inter-day repeatability was assessed on one milk powder sample (LP13) and was achieved by eight replicates carried out in four different sessions by two different operators.
Recovery tests were performed on the same sample spiked at two different levels (3 and 10 mg/kg of total MOH) with Gravex (containing 73% of MOSH and 27% of MOAH in the range *n*-C_{16-25}). Four replicates for each spiking level were conducted by weighting directly into the extraction vessel the samples and added with known amount of Gravex solution dissolved in 5 mL of pentane. Subsequently, in order to allow analyte-matrix interactions and solvent evaporation the vessels were gently stirred to uniformly distribute the mineral oil standard on the whole sample and were left to age for 48 h before performing the saponification. The sample used for recovery test was free of contamination in the range *n*-C_{16-25}.
To check method performance, compliance with the requirements of the JRC guidance (RSD <20%; recovery between 70% and 120%) was verified [143]. LOQ values were assessed following the SANTE guidance [151].

6.3. Results and discussions

6.3.1. Method optimization and validation

To optimize the saponification step various MAS tests were carried out by varying the quantity of the initial sample and the concentration of KOH solution. Initially 4 g of samples were added with KOH 1.5 M. The hydrolysis of the fats resulted to

be incomplete as, after having completely evaporated the *n*-hexane from the extract, a residual amount of fat was still present. Subsequently, tests were carried out with 3 different quantities of initial sample (1, 2 and 4 g) using a saturated KOH methanol solution. In all three conditions, 10 mL of saturated KOH was sufficient to break the analyte-matrix interactions and completely saponify the fat, so as to allow an exhaustive extraction of the analytes. At this point, to achieve greater sensitivity, it was chosen to process 4 g of sample. All milk powder samples have been epoxidized using the protocol described in the paragraph 6.2.5. Epoxidation was mandatory to remove olefins which otherwise coelute with the MOAH. Figure 34 shows, for a number of samples, the comparison of the MOSH concentration obtained by injecting the saponified samples before and after epoxidation.

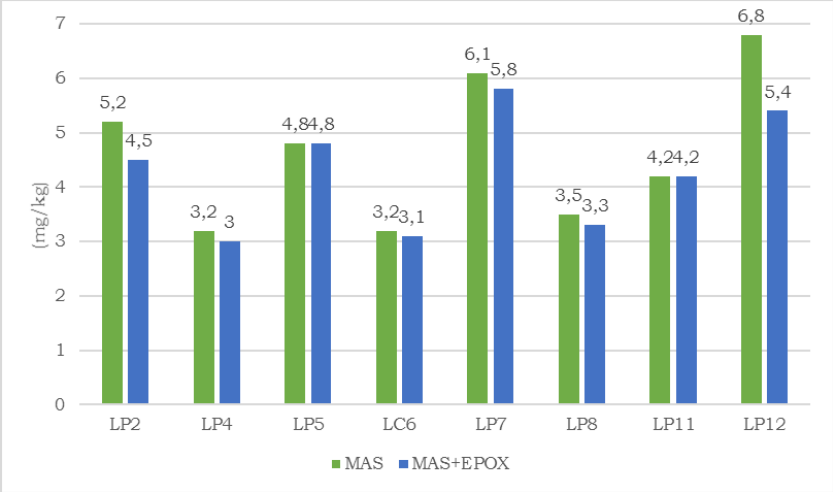

Figure 34. Comparison of the total *n*-C_{10-50} MOSH concentration (mg/kg) of the saponified samples (MAS) and of the saponified and epoxidized sample (SAP+EPOX).

As can be noticed from figure 34, the concentrations of the MOSH fraction in the saponified and saponified and epoxidized sample are generally in line with each other. Some saponified and epoxidized samples showed a slightly lower concentration value than the corresponding saponified ones, which can be explained by the fact that the epoxidation process also eliminates olefins which partially coelute in the MOSH fraction [143].

Repeatability and recovery tests were performed on sample LP13 with a pre-existing MOSH contamination in the range *n*-C_{25-50} (4.5 mg/kg) and free of contamination in the range *n*-C_{16-25}, where Gravex solution elutes. Intermediate repeatability, assessed on 8 replicates performed in 4 different days by 2 operators, gave RSD values of 5.4%, well within the range of the 20% variability required by the JRC guidelines [143]. For recovery tests two spiking levels were

performed in quadruplicate with 2 and 10 mg/kg of Gravex solution (1.5 e 7.3 mg/kg of MOSH and to 0.5 and 2.7 mg/kg of MOAH). Also, the recoveries were well within the range (70-120%) indicated by the JRC guidance [143], obtaining 98% for MOSH, using the internal standard *n*-C_{13} and 97% for MOAH, using the internal standard 1-MN. In figure 35 are reported from the top to the bottom the chromatograph traces of MOSH and MOAH of an unspiked sample after MAS and samples after MAS and EPOX spiked with 2 mg/kg and with 10 mg/kg of total MOH.

The JRC guidelines indicate for this type of matrix a maximum LOQ of 1 mg/kg and a target LOQ of 0.2 mg/kg. By injecting an amount of the final sample extract corresponding to 100 mg of the original sample, it was easy to satisfy the JRC requirements reaching a LOQ of 0.2 mg/kg for single C-fraction. The limit of quantification (LOQ) of the total fraction *n*-$C_{10\text{-}50}$ depends on the distribution of the contamination, but was assessed around 0.5 mg/kg. Due to the presence of residual olefin (in the range *n*-$C_{26\text{-}50}$), particularly resistant to epoxidation, in some of the samples, it was necessary to slightly rise the LOQ value for the MOAH. It is important to observe that in two of the samples analyzed an interference by residual olefins was observed after epoxidation. This is the reason why a higher LOQ was reported for these two samples.

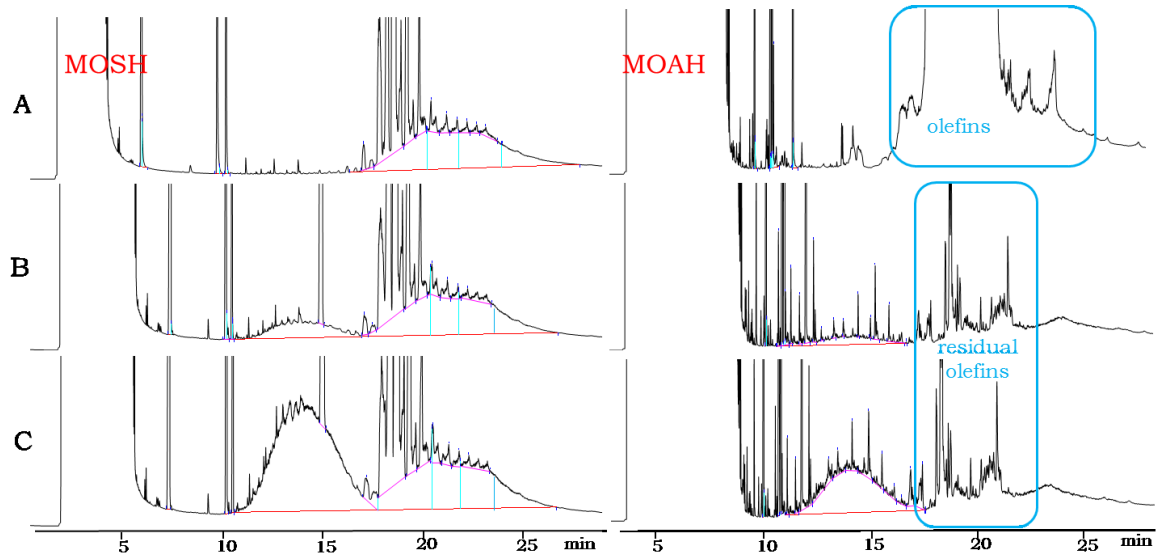

Figure 35. Chromatogram traces of MOSH and MOAH of: A) unspiked sample after MAS; B) sample spiked with 2 mg/kg of total MOH after MAS and EPOX; C) sample spiked with 10 mg/kg of total MOH after MAS and EPOX.

6.3.2. MOH content in milk powder samples

Thirteen samples of 9 different brands were analyzed. Six products were packaged in primary packaging consisting of an aluminum bag, internally

laminated with a plastic film, and a secondary packaging consisting of recycled cardboard (I). Three samples consisted of a virgin cardboard can, internally coated with a plasticized aluminum film, top closure in plasticized aluminum and a plastic cap (II). Only one cardboard can was of recycled fiber (III). Three samples consisted of a metal can with aluminum closure and a plastic cup (VI). Powdered milk is consumed after reconstitution, therefore it has to be specified that analyses were conducted on the product as it is. Table 16 summarizes the MOH concentration (expressed in mg/kg) found in 13 samples purchased from the market.

Table 16. MOSH and MOAH content (mg/kg) of the analyzed milk powder samples.

Sample code	Brand	Packaging type	Fat content %	MOSH/POSH (mg/kg)							MOAH (mg/kg)				
				n-C_{10-16}	*n*-C_{16-20}	*n*-C_{20-25}	*n*-C_{25-35}	*n*-C_{35-40}	*n*-C_{40-50}	*n*-C_{10-50}	*n*-C_{10-16}	*n*-C_{16-25}	*n*-C_{25-35}	*n*-C_{35-50}	*n*-C_{10-50}
LP1	A	I	24.1	0.4	0.7	0.6	3.0	1.0	1.0	**6.7**	<0.2	<0.2	<0.2	<0.2	<0.5
LP2	A	I	24.1	0.3	0.4	0.5	2.0	0.6	0.7	**4.5**	<0.2	<0.2	<0.2	<0.2	<0.5
LP3	D	I	22.9	<0.2	0.4	0.2	1.6	0.5	0.5	**3.3**	<0.2	<0.2	<0.2	<0.2	<0.5
LP4	E	I	26.4	0.2	0.3	0.6	1.3	0.3	0.2	**3.0**	<0.2	<0.2	<0.2	<0.2	<0.5
LP5	L	I	20.5	<0.2	0.4	0.3	2.2	0.9	0.9	**4.8**	<0.2	<0.2	<0.2	<0.2	<0.5
LP6	L	I	17.7	<0.2	0.3	0.2	1.4	0.5	0.6	**3.1**	<0.2	<0.2	<0.2	<0.2	<0.5
LP7	C	II	23.8	0.2	0.4	0.6	3.0	0.9	0.8	**5.8**	<0.2	<0.2	<0.2	<0.2	<0.5
LP8	C	II	23.8	<0.2	0.3	0.3	1.5	0.5	0.5	**3.3**	<0.2	<0.2	<0.2	<0.2	<0.5
LP9	H	II	22.2	0.2	0.5	0.7	4.4	1.6	1.3	**8.5**	<0.2	<0.2	<0.2	<0.2	<0.5
LL10	F	III	26.7	0.5	2.1	2.7	2.0	0.7	0.6	**8.5**	<0.2	<0.2	<0.2	<0.2	<0.5
LP11	B	IV	23.6	<0.2	<0.2	0.3	2.6	0.6	0.8	**4.2**	<0.2	<0.2	<0.2	<0.2	<0.5
LP12	B	IV	23.6	<0.2	<0.2	0.4	3.0	0.9	0.9	**5.4**	<0.2	<0.2	<0.4	<0.3	<1.0
LP13	G	IV	23.6	<0.2	<0.2	<0.2	1.7	1.2	1.6	**4.5**	<0.2	<0.2	<0.4	<0.3	<1.0

The quantification was carried out using the internal standards *n*-C_{13} and 1-MN for MOSH/POSH and MOAH respectively and was carried out for each C-fraction. MOSH/POSH contamination ranged from 3.0 to 8.5 mg/kg (on average 5.1 mg/kg), while MOAH contamination was lower than LOQ. The samples packaged in the primary packaging consisting of the aluminum bag internally laminated with plastic material and secondary packaging in recycled cardboard, have levels of total MOSH contamination (*n*-C_{10-50}) ranging from 3.0 to 6.7 mg/kg (on average 4.6 mg/kg). The samples contained in the can in virgin cardboard internally coated with a plasticized aluminum film show levels of total MOSH contamination from 3.3 to 8.5 mg/kg (on average 5.9 mg/kg). The figure 36 shows the MOSH chromatograms relative to the samples divided per packaging type. As can be seen from the figure LP2 (type I), LP5 (type I) and LP7 (type II), POSH contamination is well evident in the range *n*-C_{12-20}. These oligomers migrated from the plastic film in contact with the food and coeluted together with

the MOSH. The samples contained in the metal can did not show POSH contamination as there is no plastic material in contact with the food matrix, as can be seen from the chromatogram in figure 36 related to the sample LP12 (type IV). The sample LL10 (type III) was the only sample contained in the can of recycled cardboard internally coated with plasticized aluminum film. The narrow hump visible in the profile of the MOSH chromatogram, however, does not come from recycled cardboard (this is also confirmed by the fact that it is not accompanied by MOAH of the same molecular range), it probably belongs to some component used in the formulation of the product. The hump present in all samples in the range from *n*-C_{20} to over *n*-C_{50} derives probably from the vegetable oil (for example palm oil) used for product formulation.

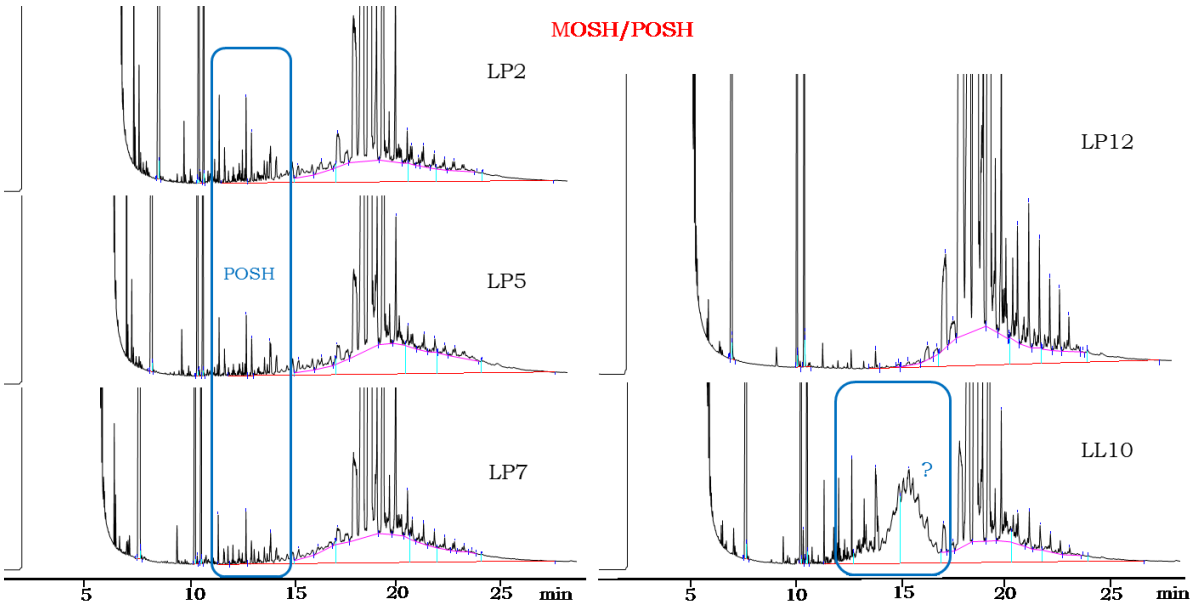

Figure 36. Chromatogram traces relative to MOSH/POSH contamination in samples with different packaging type: LP2 (type I), LP5 (type I), LP7 (type II), LP12 (type IV) and LL10 (type III).

The purchased milk powder samples had an average fat content around 23.3%. The presence of fat within the food matrix can influence the migration, in fact a higher fat content corresponds to a higher migration. The figure 37 shows the linear correlation graph between the percentage of fat and the MOSH concentration relative to the interval *n*-$C_{10\text{-}50}$ (A) and the percentage of fat and the relative MOSH concentration in the interval *n*-$C_{10\text{-}25}$ (B).

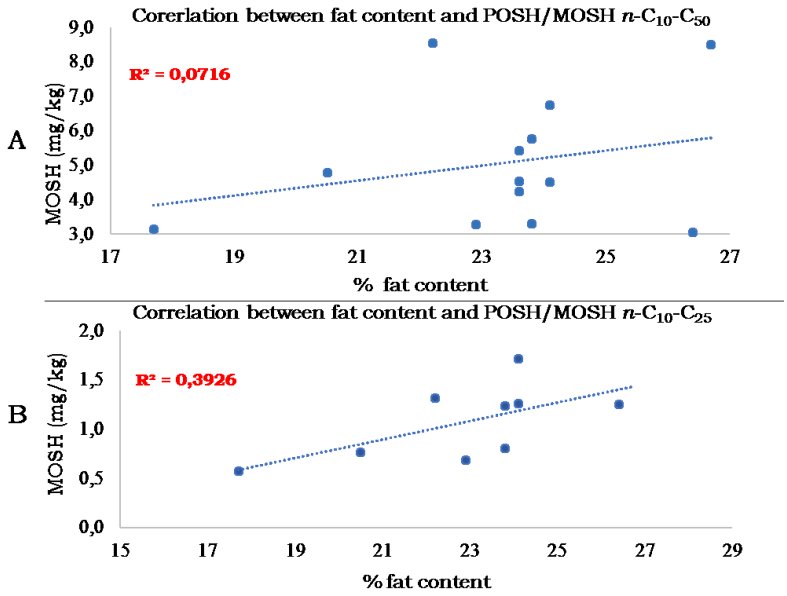

Figure 37. Comparison between the percentage of fat content and the MOSH concentration (mg/kg).

In order to better observe the possible correlations of the contamination with the presence of a plastic film in direct contact with the sample, and with the presence or absence of palm oil, in table 17 are reported the values of *n*-C_{10-25} and *n*-C_{25-50} of the milk powder samples. These considerations arise from the observation that the POSH coming from the packaging are generally present in the range *n*-C_{10-25}, while the MOSH contamination given by the vegetable oil are generally present in the range *n*-C_{25-50}. As regards the presence of POSH, the presence was evident in all the samples with plastic film in contact with the product, while the 3 samples in metal cans did not show the typical profile of PE oligomers. Even if the number of samples without the plastic film in contact is very low (only 3 samples), from the comparison of the data reported in table 17, it can be seen that the presence of the plastic material is responsible for migration of levels around 1.1 mg/kg (average), against the value of 0.3 mg/kg found in the samples in metal cans. Seven samples of total thirteen contained palm oil, while the other six did not contain palm oil. The MOSH levels of the samples that do not contain palm oil were found to be only slightly lower than those that contain palm oil, indicating that the other vegetable oils (sunflower oil, rapeseed oil, extra virgin olive oil, etc.) can lead to contamination and the presence or absence of palm oil did not significantly affect the contamination level.

Table 17. Comparison of the contamination between samples in contact with the plastic material and/or with the presence of palm oil.

sample	POSH/MOSH *n*-C_{10-25} (mg/kg) plastic film contact		MOSH *n*-C_{25-50} (mg/kg) palm oil presence	
	yes	no	yes	no
LP1	1.7		5.0	
LP2	1.3		3.3	
LP3	0.7			2.6
LP4	1.3			1.8
LP5	0.8		4.0	
LP6	0.6		2.6	
LP7	1.2		4.5	
LP8	0.8		2.5	
LP9	1.3		7.2	
LL10	n.q.*			3.3
LP11		0.3		4.0
LP12		0.5		4.9
LP13		n.q.*		4.5
mean	1.1	0.3	4.2	3.5
min	n.q.	n.q.*	2.6	1.8
max	1.7	0.5	7.2	4.5

*non quantifiable

6.4. Conclusions

A rapid, solvent-sparing and high sensitivity on-line HPLC-GC-FID method for MOH determination in infant formula was optimized and validated obtaining the performance of the method well in line with the requirements of the JRC guidance in terms of recovery, repeatability and LOQ. Total MOSH/POSH content found in milk powder samples was in the range between 3.0 and 8.5 mg/kg (on average 5.1 mg/kg), while MOAH contamination was always lower than the LOQ. Epoxidation was not always able to completely eliminate interfering olefins, and further investigation are needed to improve this clean-up step. The milk powder packaged in metal cans resulted to be free of POSH contamination in the range between *n*-C_{10-25}, while the other three types of packaging contained POSH derived from the plastic material in contact with the product. The presence of vegetable oils (especially palm oil) often used in the formulation of the product is instead responsible for the contamination with MOSH in the range *n*-C_{25-50}.

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LIST OF PUBLICATIONS

Publications on international peer reviewed journals

- ✓ Menegoz Ursol, L., Conchione, C., Srbinovska, A., Moret S. **(2022)**. Optimization and validation of microwave assisted saponification (MAS) followed by epoxidation for high-sensitivity determination of mineral oil aromatic hydrocarbons (MOAH) in extra virgin olive oil. *Food chemistry*, 370, 15 February 2022.
- ✓ Valli, E., Milani, A., Srbinovska, A., Moret, E., Moret, S., Bendini, A., Moreda, W., Toschi, T. G., Lucci, P. **(2021)**. In-House validation of a SPE-GC-FID method for the detection of free and esterified hydrohylated minor compound in virgin olive oils. *Foods*, 10(6), 1260.
- ✓ Srbinovska, A., Conchione, C., Lucci, P., Moret, S. **(2021)**. On-line HP(LC)-GC-FID determination of hydrocarbon contaminants in fresh and packaged fish and fish products. *The Journal of AOAC INTERNATIONAL*, 104(2), 267-273.
- ✓ Srbinovska, A., Conchione, C., Lucci, P., Moret, S. **(2020)**. Occurrence on *n*-alkanes in vegetable oils and their analytical determination. *Foods,* 9, 1546.
- ✓ Moret, S., Conchione, C., Srbinovska, A., Lucci, P. **(2019)**. Microwave-based technique for fast and reliable extraction of organic contaminants from food, with a special focus on hydrocarbon contaminants. *Foods*, 8(10), 503.

In progress manuscripts

- o Srbinovska, A., Gasparotto, L., Conchione, C., Menegoz Ursol, L., Moret, S. Mineral oil contamination in Pesto sauces of the Italian market and related ingredients. *Paper drafting.*
- o Srbinovska, A., Conchione, C., Menegoz Ursol, L., Celaj, F., Moret, S. High sensitivity determination of mineral oils and olefin oligomers in cocoa powder: method validation and market survey. *Paper drafting.*
- o Srbinovska, A., Conchione, C., Moret, S. Mineral oil migrationin cocoa powder under warehouse and domestic storage condition: a case study. *Paper drafting.*

Book Chapters

- ✓ Lucci, P., Srbinovska, A., Fiorini, D., Milani, A., Pacetti, D., Moret, S. **(2021)**. Chapter 1: Application of gas chromatography for quality and authenticity assessment of olive oil. In book: '*Chromatographic and Related Separation Techniques Volume B: Relevant Applications'.* Editor: Guillem Campmajó and Oscar Núñez, World Scientific Publishing.

Contributions to national and international conferences

- ✓ Lucci, P., Valli, E., Milani, A., Srbinovska, A., Moret, E., Moret, S., Bendini, A., Moreda, W., Toschi, T. G. **(2021)**. SPE-GC-FID method for detection of free and esterified hydrohylated minor compounds in virgin olive oil. *18th Euro Fed Lipid Congress and Expo,* On-line meeting. Oral communication.
- ✓ Menegoz Ursol, L., Conchione, C., Srbinovska, A., Moret, S. **(2021)**. Rapid and high-sensitivity determination of mineral oil aromatic hydrocarbons (MOAH) in extra virgin olive oil (EVOO). Method validation and first results. *18th Euro Fed Lipid Congress and Expo,* On-line meeting. Oral communication.
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- ✓ Moret, S., Conchione, C., Rigo, V., Srbinovska, A. **(2019)**. Hydrocarbon contaminants (MOH and POH) in fish products. *Seminar: Process-Related Compounds and Natural Toxins, Gembloux, Belgium*. Poster.
- ✓ Srbinovska, A., Moret, E., Conchione, C., Milani, A., Lucci, P., Conte, L., Moret, S. **(2019)**. Microwave assisted saponification (MAS) for sterol determination in vegetable oils: First results. *17th Euro Fed Lipid Congress and Expo, Seville, Spain.* Poster.
- ✓ Nartea, A., Conchione, C., Srbinovska, A., Moret, S. **(2019)**. Evaluation of possible sources of mineral oil contamination in olives and extra virgin olive oil. *17th Euro Fed Lipid Congress and Expo, Seville, Spain.* Poster.
- ✓ Srbinovska, A. **(2019)**. Development of integrated, multidimensional approaches for food safety assessment, with special focus on

contaminants from food packaging. *XXIV Workshop on the Development in the Italian Ph.D. Research on Food Science, Technology and Biotechnology, Florence, Italy.* Poster.

- ✓ Srbinovska, A., Conchione, C., Lucci, P., Conte, L., Moret, S. **(2018)**. Validation of an off-line SPE-GC-FID method for the determination of n-alkanes in vegetable oils and comparison with the on-line LC-GC-FID method. *Congresso scientifico sullo studio dei grassi e degli oli, Bari, Italy*. Poster.

Awards

- ✓ February 2019 'First classified' for the master's thesis. Award Ranieri Filo della Torre, *Rome, Italy*.
- ✓ June 2018. 'Young researcher'. Assignee of one of the free congress registrations SISSG.