Mineral oil hydrocarbons: development/optimization of analytical methods, investigation of migration from food packaging into semolina and egg pasta, and occurrence in human tissues.

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Laura Barp

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Coordinator: Mara Lucia Stecchini, Full Professor
Department of Food Science
University of Udine, Italy

Supervisor: Sabrina Moret, Associated Professor
Department of Food Science
University of Udine, Italy

Reviewers: Rita Lorenzini, Ph.D.
For Food s.r.l.
Bologna, Italy

Thomas Gude, Ph.D.
Department of Swiss Quality Testing Services
Dietikon, Switzerland

“An expert is a person who has made all the mistakes that can be made in a very narrow field.”
Niels Bohr

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SUMMARY

Mineral oil is a complex mixture of compounds, primarily manufactured from crude petroleum through distillation processes and various refining steps, mainly consisting of MOSH (mineral oil saturated hydrocarbons), including *n*-alkanes, isoalkanes and cycloalkanes (naphtenes), and MOAH (mineral oil aromatic hydrocarbons), mostly alkylated. The MOSH fraction may include polyolefin oligomeric saturated hydrocarbons (POSH), oligomers of polyolefins, which can migrate from plastic bags, heat-sealable layers and other laminates as well as adhesives and plasticizers.

Mineral oils hydrocarbons present in printing inks and recycled paper are sufficiently volatile (< *n*-C_{24}) to migrate from the packaging to the food through the gas phase. Exposure to mineral oil hydrocarbons via packaging and some foods may pose a human health hazard.

For mineral oil determination, sample extracts can be directly injected into the on-line liquid-gas chromatography (LC-GC), where a silica column retains fat and polar interferences, and fractionates MOSH and MOAH. As an alternative to the on-line method, MOSH and MOAH can be separated by off-line solid phase extraction (SPE) and then analyzed by GC with flame ionization detector (FID).

Mineral oil extraction can be more or less demanding, depending on the food composition and source of contamination. Overnight extraction with hexane generally allows for the total extraction of mineral oil migrated from the packaging into dry foods such as pasta, but does not allow for the extraction of pre-existing contamination, firmly embedded into the solid structures. A more laborious and solvent consuming procedure has been proposed for the extraction of total contamination from wet-foods and pasta samples.

This PhD work is divided into three main parts. The first one is dedicated to the investigation of the LC-GC system and extraction methods for mineral oil analysis. In particular, an application of a minor technical improvement on the LC-GC method proposed by Biedermann and co-workers is described. It allowed to largely improve the data throughput, to save time and solvent consumption and to increase sensitivity. In particular, a total time and solvent reduction of 34 % and 23 %, respectively, was obtained by speeding up the gas chromatographic run and reducing the liquid chromatographic reconditioning step. The band broadening occurring in the liquid chromatography column during stop flow in the multi-transfer mode was assessed by comparing the variances of the perylene peak width recorded in the stop-flow and normal modes. A band broadening directly proportional to the stop time of LC pumps was observed, however it did not affect the analysis reliability.

In addition, a direct analytical comparison between two popular multidimensional LC-GC systems, namely the Y-interface (retention gap approach) and the syringe-based (programmed temperature vaporizer -PTV- approach) interface is reported. The two LC-GC methods developed were subjected to validation, in terms of linearity over the calibration range, analyte discrimination, precision, accuracy, limits of detection and quantification. Both LC-GC interfaces provided a satisfactory and comparable performance for the determination of MOSH contamination in food products.

The present work also deals with the development/optimization of a comprehensive two-dimensional gas chromatographic method, with dual detection [FID and mass spectrometric (MS)], for the simultaneous identification and quantification of mineral-oil contaminants in a variety of food products. The quantitative results were compared with those obtained by performing a large volume injection, in a GC-FID system, after the same SPE process and by an on-line liquid-gas chromatography method, with very similar results observed. The presence of a series of unknown compounds, that appeared when using the off-line methods, was investigated using the mass spectrometric data, and were tentatively-identified as esterified fatty acids, most probably derived from vegetable oil based inks.

Concerning the extraction approaches, a pressurized liquid extraction (PLE) method has been optimized for rapid mineral oil determination in cardboard and paper samples. The proposed method involves extraction with hexane (2 cycles) at 60 °C for 5 min, and allows for the processing of up to 6 samples in parallel with minimal sample manipulation and solvent consumption. It gave good repeatability (coefficient of variation lower than 5 %) and practically quantitative extraction yield (less than 2 % of the total contamination found in a third separate cycle). The method was applied to different cardboards and paper materials intended for food contact.

Furthermore, two different PLE methods, one for rapid determination of superficial contamination mostly coming from the packaging, the other for efficient extraction of total contamination from different sources, have been developed and optimized. The two methods presented good performance characteristics in terms of repeatability (relative standard deviation lower than 5 %) and recoveries (higher then 96 %). To show their potentiality, the two methods have been applied in combination on semolina pasta and rice packed in direct contact with recycled cardboard.

The second part of this PhD thesis concerns the mineral oil migration into dry semolina and egg pasta packed in recycled and virgin paperboard and in plastic film at room temperature. In particular, migration kinetics has been monitored for up to 2 years, focusing on the influence of time, storage conditions, food packaging material and food characteristics. Mineral oil migration from packaging to food is a rapid phenomenon and reaches considerable levels already after 1 month from packaging time, in particular if recycled paperboard was used. A significant contribution to food contamination coming from packaging can be due to adhesives applied to close the boxes. Not only the packaging, but also the external environment contributed to the total food contamination. Food characteristics seem to influence mineral oil migration from packaging: the egg pasta reached higher levels of contamination than the semolina pasta, due to its higher fat content.

Migration tests were performed also under accelerated conditions (40 and 60 °C for paperboard and plastic film, respectively, up to 30 days) in order to find a correlation with the migration during the shelf-life of the product. The migration kinetic is accelerated with respect to that at room temperature, in particular for the heavier hydrocarbons, but the trend is very similar: the migration of volatile hydrocarbons up to C_{20} was very fast and complete in a short time.

Finally, the third part of this work is dedicated to the investigation of mineral oil content in human tissues (fat tissue, mesenteric lymph nodes, spleen, liver, lungs, kidneys, heart and brain) focusing on its concentrations and molecular mass distribution. After the development of an appropriate extraction method, mineral oil contents found in human tissues have been correlated with some information on each individual's physical features and clinical history in order to identify the possible sources of contamination. For a quarter of the subjects (n = 37), a total amount of MOSH above 5g/body was calculated. The composition found in tissues seems determined not so much by the mineral oils the individuals are exposed to, but more so by the selectivity of the uptake, evaporation and metabolic elimination.

RIASSUNTO

Per "olio minerale" si intende una complessa miscela di composti ottenuti principalmente dalla distillazione e raffinazione del petrolio, che comprendono idrocarburi saturi (MOSH, *mineral oil saturated hydrocarbons*) inclusi *n*-alcani, isoalcani e ciclo alcani (nafteni), e idrocarburi aromatici (MOAH, *mineral oil aromatic hydrocarbons*) che sono per la maggior parte alchilati. La frazione MOSH può includere anche oligomeri di poliolefine (POSH, *polyolefin oligomeric saturated hydrocarbons*), che possono migrare da materiali plastici, resine termosaldabili, adesivi e plasticizzanti.
Gli oli minerali presenti negli inchiostri della stampa e nel cartone riciclato sono sufficientemente volatili (< *n*-C_{24}) da migrare dall'imballaggio all'alimento. L'esposizione agli idrocarburi di origine minerale dovuta agli imballaggi e al cibo rappresenta un pericolo per la salute umana.
Per l'analisi dell'olio minerale, l'estratto ottenuto dal campione in questione può essere direttamente iniettato in un sistema accoppiato LC-GC (*liquid-gas chromatography*), costituito da una colonna di silice che trattiene il grasso e gli interferenti polari e permette la separazione dei MOSH dai MOAH. In alternativa al metodo *on-line*, MOSH e MOAH possono essere separati *off-line* con un'estrazione in fase solida (SPE) e analizzati poi con GC e rivelatore a ionizzazione di fiamma (FID, *flame ionization detector*).
L'estrazione dell'olio minerale può essere un processo più o meno complicato a seconda della composizione della matrice e della fonte di contaminazione. Generalmente, un'estrazione con esano per una notte permette il recupero totale dell'olio minerale migrato dall'imballaggio in alimenti secchi come la pasta, ma non permette un'estrazione quantitativa di una contaminazione pre-esistente, intrappolata nella matrice. Una procedura più laboriosa è stata proposta per l'estrazione della contaminazione totale di olio minerale da campioni "umidi".
Questa tesi di dottorato è divisa in tre parti. La prima parte è dedicata ad aspetti analitici e all'estrazione dell'olio minerale. In particolare, viene descritto un miglioramento tecnico, apportato al metodo già proposto da Biedermann e collaboratori, che permette di aumentare la sensibilità, di ridurre il tempo di analisi del 34 % e il consumo di solventi del 23 %, velocizzando la corsa cromatografica e riducendo la fase di ricondizionamento della colonna LC. E' stato valutato anche l'allargamento della banda nella colonna LC durante l'arresto del flusso (*stop-flow*) eseguito per trasferire più frazioni al sistema GC con un'unica iniezione. Tale valutazione è stata fatta confrontando la varianza della larghezza del picco del perilene registrato utilizzando lo *stop-flow* e la modalità normale. L'allargamento di banda è risultato direttamente proporzionale al tempo in cui la pompa LC viene lasciata in stop, e tuttavia tale allargamento non sembra influenzare l'affidabilità dell'analisi.
Inoltre, è stato fatto un confronto tra due sistemi LC-GC con diversi tipi di interfaccia: uno strumento con la Y-*interface* basata sulla tecnica del *retention gap* e l'altro con un vaporizzatore a temperatura programmata (PTV, *programmed temperature vaporizer*). I due diversi metodi sviluppati sono stati sottoposti a validazione in termini di linearità, discriminazione, precisione, accuratezza, limiti di quantificazione e di rilevabilità. Entrambe le interfacce hanno dato risultati soddisfacenti e confrontabili tra loro durante la determinazione di MOSH in prodotti alimentari.
In questo lavoro è riportato anche lo sviluppo/ottimizzazione di una tecnica *comprehensive* GCxGC con doppio rivelatore (FID e spettrometria di massa) per una simultanea identificazione e quantificazione di olio minerale in prodotti alimentari. I risultati quantitativi sono stati confrontati con quelli ottenuti con analisi in GC-FID, previa separazione di MOSH e MOAH con SPE, e con il metodo on-line LC-GC, ottenendo valori del tutto simili. Con l'utilizzo del metodo *off-line* SPE-GC-FID si è riscontrata la presenza di composti sconosciuti nel tracciato gas cromatografico, che sono stati poi identificati come esteri degli acidi grassi, molto probabilmente derivanti dall'utilizzo di inchiostri a base di oli vegetali.

Per quanto riguarda le tecniche di estrazione, è stato ottimizzato un metodo PLE (*pressurized liquid extraction*) per la determinazione dell'olio minerale in campioni di carta e cartone. Il metodo proposto prevede un'estrazione con esano (2 cicli) a 60 °C per 5 minuti e permette di processare sei campioni contemporaneamente con una minima manipolazione del campione e un basso consumo di solvente. Tale metodo presenta una buona ripetibilità (coefficiente di variazione inferiore al 5 %) e un'estrazione praticamente quantitativa (meno del 2 % della contaminazione totale è stata ritrovata in un terzo ciclo separato). Il metodo è stato, quindi, applicato a differenti tipi di cartone e carta utilizzati per il contatto con alimenti.
In aggiunta a ciò, sono stati sviluppati anche due differenti metodi PLE per alimenti secchi con basso contenuto di grasso: uno per la determinazione di olio minerale superficiale, per la maggior parte proveniente da migrazione da imballaggio, e l'altro per l'estrazione della contaminazione totale proveniente da differenti fonti. I due metodi presentano una buona ripetibilità (deviazione standard inferiore al 5 %) e ottimi recuperi (oltre il 96 %). Per sottolineare la potenzialità di questi metodi, essi sono stati applicati in combinazione su campioni di riso e di pasta di semola tenuti a contatto diretto con cartone riciclato.
La seconda parte di questa tesi riguarda la migrazione di olio minerale in pasta secca (di semola e all'uovo) confezionata in imballaggi di cartone riciclato, in fibra vergine e in pacchi di film plastico, tenuti a temperatura ambiente. In particolare, le cinetiche di migrazione sono state monitorate nell'arco di due anni, focalizzando l'attenzione sull'influenza del tempo, delle condizioni di stoccaggio, sul materiale dell'imballaggio e sulle caratteristiche dell'alimento. La migrazione dell'olio minerale dall'imballaggi all'alimento è un processo rapido che può raggiungere considerevoli livelli di contaminazione già dopo un mese dal momento del confezionamento, soprattutto se viene utilizzato il cartone riciclato. La presenza di colla utilizzata per la chiusura delle confezioni sembra contribuire in modo significativo alla contaminazione totale dell'alimento confezionato. Non solo l'imballaggio, ma anche l'ambiente di stoccaggio circostante al campione incide sulla contaminazione totale dell'alimento. La quantità di grasso dell'alimento, inoltre, influenza la migrazione dell'olio minerale dall'imballaggio.
Sono stati eseguiti anche test di migrazione accelerata a temperature di 40 °C per imballaggi di cartone e 60 °C per i pacchi in film plastico, della durata di 30 giorni, per cercare una correlazione con la migrazione durante la *shelf-life* del prodotto. Le cinetiche di migrazione sono ovviamente accelerate rispetto a quelle ottenute a temperatura ambiente, soprattutto per gli idrocarburi a più alto peso molecolare. In generale, però, l'andamento rimane simile, con una migrazione degli idrocarburi fino al *n*-C_{20} molto rapida e completa in poco tempo.
Infine, la terza parte di questa tesi è dedicata all'indagine del contenuto di olio minerale in tessuti umani (tessuto adiposo, linfonodi mesenterici, milza, fegato, polmoni, reni, cervello e cuore) focalizzando l'attenzione sulla sua concentrazione e distribuzione dei pesi molecolari. In seguito allo sviluppo di un appropriato metodo di estrazione, il contenuto di olio minerale ritrovato nei vari tessuti umani è stato correlato con informazioni personali e cliniche dell'individuo a cui appartenevano, allo scopo di identificare una possibile fonte di contaminazione. Per un quarto dei soggetti analizzati (n = 37), sono stati trovati livelli di MOSH maggiori a 5 g per persona. La composizione della contaminazione ritrovata nei tessuti umani sembra determinata non tanto dall'olio minerale a cui gli individui sono stati esposti, quanto dalla selettività dovuta a processi metabolici.

INTRODUCTION

1. Mineral oil: general aspects

1.1. Chemical characteristics

Petroleum products are complex mixtures which contain a wide variety of elements combined in various forms. The principal constituents are hydrocarbons and compounds which contain heteroatoms. In the refining process petroleum products are strongly enriched with hydrocarbons, leaving most crude-based inorganic materials and other types of organic compounds containing sulfur, nitrogen and oxygen in the residual. Petroleum hydrocarbons can be divided into three families:

- saturated hydrocarbons involve aliphatic hydrocarbons (open-chain compounds) which can be linear or branched (alkanes and isoalkanes called also paraffins and isoparaffins) and cyclic compounds (cycloalkanes or naphtenes). The latter contain one or more rings with possible lateral alkylation (Figure 1). The term MOSH indicates mineral oil saturated hydrocarbons.
- Unsaturated hydrocarbons are mainly alkenes (olefins), which are hydrocarbons with less hydrogen, carbon for carbon, than the corresponding alkane, due to the occurrence of one or more double bonds between carbon atoms in the structure. Alkynes (triple bonds between carbon atoms) are not commonly found in petroleum hydrocarbons.

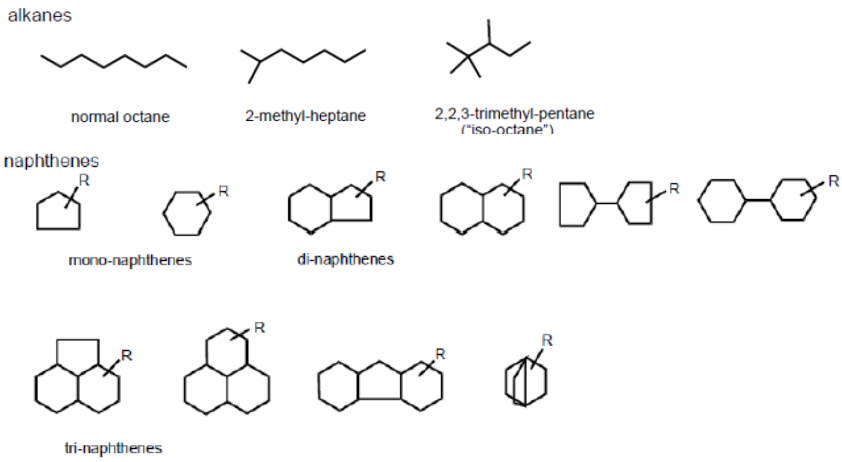

Figure 1. Examples of aliphatic hydrocarbons which can be found in mineral oil (EFSA, 2012).

- Aromatic hydrocarbons contain one or more benzene rings (six-membered carbon ring with the chemical formula C_6H_6 and with three carbon-to-carbon single bonds that alternate with three carbon-to-carbon double bonds) as structural components. The alkylated benzene rings are predominant with respect to the non-alkylated ones (Figure 2). The term MOAH indicates mineral oil aromatic hydrocarbons.

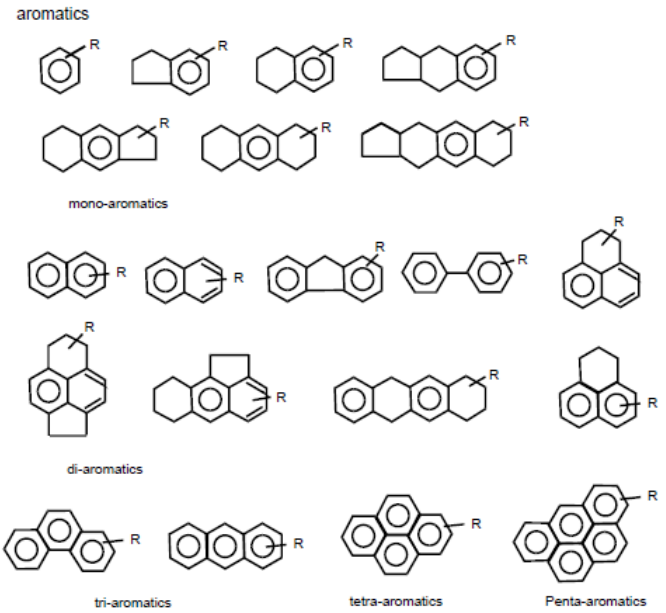

Figure 2. Examples of aromatic hydrocarbons which can be found in mineral oil (EFSA, 2012).

A key feature of petroleum hydrocarbons is that they typically have a large number of isomers (compounds that have the same elemental formula but have a different structural configuration) which account for the high degree of complexity of petroleum mixture. In general, as the carbon number increases, the number of possible isomers increases rapidly.

Organic compounds containing sulfur, nitrogen and oxygen may be encountered at significant concentrations in crude oil. Metals are also encountered in petroleum fuel mixtures in the form of salts or carboxylic acid.

Petroleum fuel mixtures are produced from crude oil through a variety of refining and blending processes (Figure 3). After the treatment which removes dissolved gas, dirt and water, crude oil is distilled and a variety of petroleum product fractions is formed. The fractions can be directly used or their hydrocarbons composition can be altered through cracking and/or reforming. Cracking is a process that converts long-chain alkanes into smaller alkanes, alkenes and some hydrogen. It is this process which accounts for the occurrence of alkenes in petroleum fuel mixtures. Reforming is a process that converts aliphatics into aromatics. Composition of distillation fractions can also be altered by removal or conversion of undesirable components, or by addition of desirable compounds (Potter et al., 1998; EFSA, 2012).

The distillation products of crude oil are usually grouped into three categories (EFSA, 2012):

- light distillates: liquefied propane gas or LPG, gasoline, naphta;
- middle distillates: kerosene, diesel, solvents;
- heavy distillates: heavy fuel oil, lubricating oils, solid paraffin waxes, asphaltic materials.

These products are characterized by different molecular mass distributions which influence their physical properties, such as boiling range, density, viscosity, etc. The products of the refining process are opportunely blended to obtain mixtures with the required characteristics for desired end uses (Walters et al., 1994; Potter et al., 1998; EFSA, 2012).

The wide use of these products in different sectors contributes to a widespread environmental contamination. When petroleum products are released into the environment, they can dissolve in water, volatilize and biodegrade. Each process affects hydrocarbons families differently

(Potter et al., 1998). Exhaust gases from vehicles and domestic heating seem to be the main causes of higher levels of contamination in urban zones (Neukom et al., 2002) with respect to that found in rural areas.

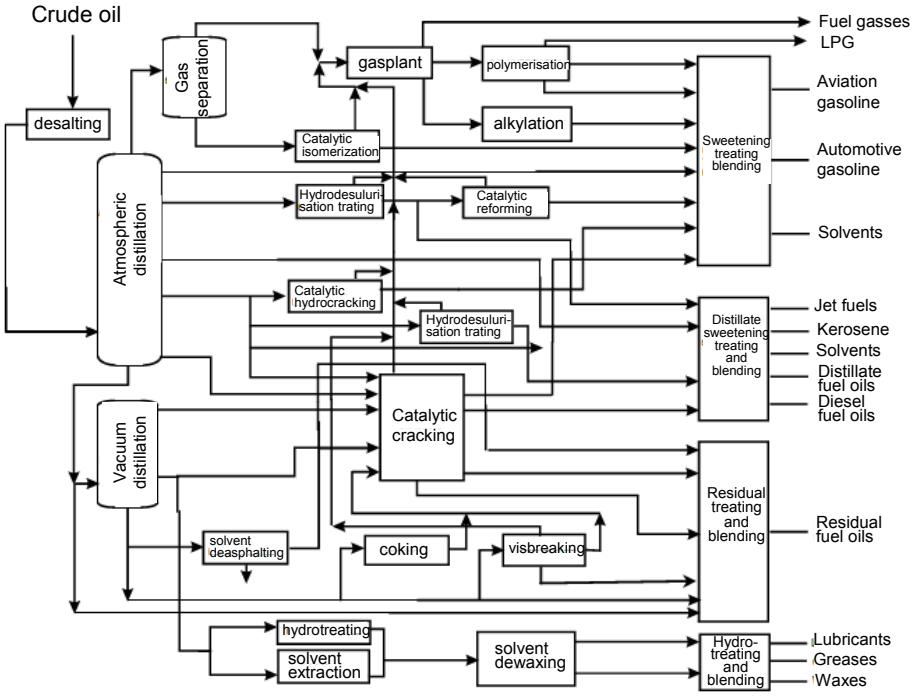

Figure 3. Schematic flow diagram of a typical integrated oil refinery (EFSA, 2012).

Food contamination mostly involves "food grade" mineral oils and solvents. The first are highly refined petroleum products suitable for contact with food because of the removal of the aromatic fraction. They have different molecular mass distributions, ranging from C_8 to C_{40}, and are used, for example, in food processing, as protective coating for raw fruits and vegetable, release agents in bakery products, components of food packaging, etc. The solvents are petroleum products ranging from C_8 up to C_{20}, containing from 0 to 99 % aromatics and are used as additives in inks and paints (EFSA, 2012). In the recent years, the attention has been focused on the use of mineral oil based solvents in the printing of packaging, in particular due to the possible migration into food and the concern of toxicity of the MOAH fraction (Biedermann and Grob, 2010).

1.2. Toxicity and exposure

Mineral oil toxicity has been a much studied subject from several years, but still remains a greatly debated topic.

Mineral oils obtained from non refined petroleum products (motor oils, hydraulic oils, etc.) can contain high amounts of aromatic hydrocarbons (mainly alkylated), heavy metals and several additives, added to improve physic-chemical properties, the stability of the product and to obtain the desired characteristics (Henry, 1998). These mineral oils are classified as carcinogenic for humans, especially for their aromatic content (IARC, 1987). Lubricating oils can contain up to 20 % of additives, some of which are included in a list of dangerous substances drawn up by the European Commission (Henry, 1998).

Highly refined mineral oils seem to be non-carcinogenic for humans (even if some hydrocarbons seem to act as co-carcinogenic) (EFSA, 2012). However, the administering of 0.01-20 mg/kg body weight (b.w.) (depending on the type of mineral oil and its viscosity) cause

undesirable effects, in particular in *Fisher 344* rats, such as formation of granulomas in liver and inflammations of mesenteric lymph nodes (Nash et al., 1996; Shoda et al., 1997). It has been demonstrated that only a small percentage of ingested mineral oils is actually absorbed in the gastrointestinal tract. The absorption depends on the molecular structure and is inversely proportional to the length of the carbon chain (Low et al., 1996). After administration of a single dose to rats, the retention of the aliphatic hydrocarbons was inversely proportional to the number of carbon atoms and ranged from 60 % for C_{14} to 5 % for C_{28} compounds, independent of the hydrocarbon type (alkanes, alkenes or alkynes) and the dose rate (Albro and Fishbein, 1970). Cyclic and branched hydrocarbons are absorbed easier than linear hydrocarbons and the accumulation is more consistent for heavy hydrocarbons with a carbon chain from C_{20} to C_{35} (Scotter et al., 2003). The absorption rates were determined in rats and pigs: 52 % for MOSH with a high proportion of cycloalkanes when the average carbon number was 20 and 20 % if the average carbon number was 28, with lower absorption if the dose was reduced. There is no conclusive data on the relative absorption rates of alkanes of differing structure (Tulliez, 1986). The metabolism of some branched and cyclic alkanes has been investigated (e.g. Le Bon et al., 1988; Halladay et al., 2002), but little is known about the rate of degradation for structurally differing hydrocarbons, in particular about structures which are virtually not degradable. *n*-Alkanes are metabolized to fatty alcohols and then fatty acids in the small intestine and the liver through the cytochrome P450 system (Ichihara et al., 1981; Perdu-Durand and Tulliez, 1985).

Mean hepatic concentrations in male and female *Fischer 344* rats (considered most sensitive) of about 2300 mg/kg and 11000 mg/kg, respectively, after feeding them a diet containing 20000 mg/kg mineral oil for 90 days, were determined by Baldwin et al. (1992). The concentrations in mesenteric lymph nodes were approximately half of those found in the liver. Firriolo et al. (1995) measured MOSH concentrations of 5600 and 1700 mg/kg in the liver of *Fischer 344* and *Sprague Dawley* rats, respectively, after a diet containing 2000 mg/kg MOSH for 90 days. After the administration of a ten times higher dose, 8200 and 4100 mg/kg MOSH were measured in the liver. The MOSH ranged from C_{18} to C_{30}, with a mean molecular mass of 350 Da. Smith et al. (1996) found 600-4300 mg/kg MOSH in the liver and up to 3300 mg/kg in the mesenteric lymph nodes of female *Fischer 344* rats fed a diet for 90 days containing 20000 mg/kg MOH. Their spleens and kidneys contained less than 100 mg/kg MOSH. Scotter et al. (2003) analyzed the liver, intestines, heart, kidneys, cervical lymph nodes and mesenteric lymph nodes after the administration of a diet containing 20000 mg/kg of various mineral waxes and white oils for 90 days to female *Fischer 344* rats. MOSH, mainly ranging from C_{20} to C_{35}, were detected in the small intestine, heart and kidneys at concentrations between 100 and 7500 mg/kg.

Dose-related accumulation of MOSH in the liver and mesenteric lymph nodes was observed after repeated exposure of *Fischer 344* rats to mineral oils and some waxes. For oils specified as Class II and III by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2002), the formation of granulomas or microgranulomas, i.e. droplets containing MOSH surrounded by proteins, was observed at elevated doses (Smith et al., 1996; Fleming et al., 1998; Carlton et al., 2001). In some cases, these granulomas caused inflammation of the mesenteric lymph nodes, but such inflammations were not observed in other rat strains and other species (Firriolo et al., 1995; Griffis et al., 2010) and granulomas formation was non-specific, adaptative and not progressing to more severe pathological effects (Carlton et al., 2001; EFSA, 2009).

Studies on human toxicity are still limited, but some of them have demonstrated the presence of reactions similar to that observed in rat tissues (Fleming et al., 1998; Carlton et al., 2001). Further investigations are necessary to compare reactions observed in rats and in humans and to identify an appropriate “animal model” to better evaluate the exposure (Miller et al., 1996).

In 2012, the European Food Safety Authority (EFSA) published an opinion on MOH in food, focusing on exposure and toxicology (EFSA, 2012). The crucial toxicological end point is that MOSH may be accumulated in human tissue and form microgranulomas, and that MOAH may be mutagenic and carcinogenic, and therefore of potential concern. No values for tolerable daily intakes (TDIs) were specified, neither for MOAH, since no safe dose can be defined for

genotoxic compounds, nor for MOSH due to insufficient data, particularly with regard to accumulation. Estimated MOSH exposure ranged from 0.03 to 0.3 mg/kg b.w. per day, with higher exposure of children. This means that an adult of 60 kg b.w. may be exposed to 18 mg MOSH per day, adding up to 6.6 g per year.
Transfer of animal data to humans faces uncertainties. Firstly, animal tests last for far less time than human lives and, therefore, accumulation is not adequately reflected. In none of the animal tests a steady state concentration in tissue was reached. Secondly, it is unknown whether granulomas formation is merely determined by the MOSH concentration in a tissue (a kind of oversaturation) or also other factors.
Granulomas in human tissues have been described in literature for a long time (e.g. by Boitnott and Margolis, 1970; Nochomovitz et al., 1975; Blewitt et al., 1977; Dincsoy et al., 1982; Wanless and Geddie, 1985). Cruickshank (1984) found that the MOH content in spleens without detectable granulomas was lower (100-300 mg/kg tissue) compared to those with a high incidence (3400-4200 mg/kg tissue; 3 samples each). In a subsequent publication, Cruickshank and Thomas (1984) found a higher incidence in the spleen (approximately 80 %) compared to the liver (approximately 40 %). A correlation of the incidence and severity of granulomas with age for the liver but not for the spleen was found by Wanless and Geddie (1985). No correlation was found with diseases.
Noti et al. (2003) determined mineral oil hydrocarbons in human milk. The mean concentration was 95 mg/kg (n=33), with a maximum at 1300 mg/kg in the fat fraction. Continuing breast feeding decreased the concentrations several fold, but application of breast salves increased it. As a consequence, the exposure of babies not only through milk but also through breast feeding by directly licking off salves (often consisting of petroleum jelly) from the breast is of concern (Noti et al., 2003).
In abdominal fat from 144 women living in Austria collected during a Caesarean section, MOH concentrations ranged from 15 to 360 mg/kg of fat (average of 60.7 mg/kg). Milk fat from days 4 and 20 after birth contained a mean value of 45 and 22 mg/kg of MOSH, respectively, with a maximum of 355 mg/kg (Concin et al., 2008). MOSH distribution was largely identical in all fat tissues and milk samples, ranging from *n*-C_{17} to *n*-C_{32} and centered at *n*-C_{23}/C_{24}. Since the mineral oil products humans are exposed to range from much smaller to much higher molecular mass, it seems that a selective uptake, elimination by evaporation and metabolic degradation influence the MOH composition found in tissues.
In order to identify the most relevant sources, MOSH concentration in fat tissues were correlated with women's personal data, nutritional habits and cosmetics use. The age was identified as the predominant predictor for accumulated MOSH, supporting the assumption that part of the MOSH are persistent for a long time, possibly accentuated by a wider use of mineral oils in the food industry up to the 1990s. A correlation with cosmetics use was observed as well, in particular related to dermal uptake (Concin et al., 2011).

2. Sources of contamination

2.1. Main sources of food contamination with mineral oil: a general overview

Due to their wide use, mineral oil hydrocarbons (MOH) can contaminate foodstuffs in several ways (Grob et al., 1991; Moret et al., 1997; Droz and Grob, 1997; Grob et al., 1997; Moret et al., 2003; Biedermann and Grob, 2012b). The contaminations vary in their mode of entering the food: those entering through the gas phase are restricted to hydrocarbons of sufficient volatility, whereas contamination by wetting contact is not influenced by volatility. Foodstuffs of vegetable origin present a background contamination reflecting environmental contamination (Neukom et al., 2002). Processed food can be contaminated with food grade mineral oils (treated to eliminate MOAH) largely used in food industry as lubricating, release agents, dust suppressants for grain or animal feed, protective coatings for raw fruits and vegetables and in some cases as ingredients (EFSA, 2012). Food contact materials (FCM) are another important

source of contamination and in particular recycled paperboard used as food packaging and mineral oil based printing inks. In these cases 15-35 % of the contamination is represented by MOAH (Biedermann and Grob, 2012b).
The main sources of contamination are summarized in Table 1: the predominant MOH distribution, the occurrence and, when present, regulations are reported for each type of contamination.
It is evident that for many foods MOH can derive from different sources, with different overlapping distributions. In Figure 4 is reported the example of an extreme case, such as rice, and the possible sources of contamination are indicated with an estimate of the proportion of rice samples containing MOH from the given sources. The estimated concentration range is reported on a logarithmic scale in the horizontal axis. It is well visible that almost all rice is contaminated at a low concentration (usually below 1 mg/kg) from the environment. Harvesting adds lubricating or fuel oil to about 50 % of samples at a concentration around 1 mg/kg. Jute bags contaminate rice, primarily Asian rice such as Basmati, in a typical range of 2-15 mg/kg. Most rice packed in paperboard boxes is contaminated at considerable levels from printing inks and recycled board. Data on real occurrence of mineral oil in antidusting agents are not available; occasionally rice is still sprayed with mineral oil to improve the shine, resulting in concentrations around 1000 mg/kg (EFSA, 2012).

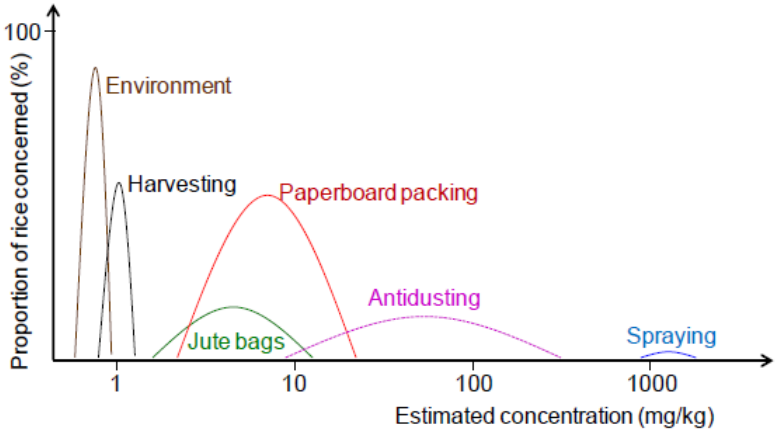

Figure 4. Various sources of MOH potential contamination in rice (EFSA, 2012).

Table 1. Examples of possible sources of food contamination with mineral oil products.

Type of contamination	Sources		MOH involved	Occurrence	Regulation
Naturally occurring in biota	Marine biota: marine algae, fish, phytoplankton	Marine organisms	C_{13}-C_{33} with odd chain predominance		
		Marine algae	C_{15}, C_{17} and C_{19} are the predominat *n*-alkanes		
		Zooplankton and fish	C_{27} or C_{29} are the predominant		
	Terrestral biota:bacteria and fungi, plants, insects	Bacteria	C_{14}-C_{20} and C_{26}-C_{33} with odd chain predominance for photosyntetic and non-photosyntetic bacteria, respectively		
		Fungal spores and mycelyum	C_{14}-C_{37} with C_{27}, C_{29} and C_{31} predominance		
		Plants	C_{21}-C_{37} with C_{29} and C_{31} predominance		
		Insects	C_{21}-C_{33} with odd chain predominance		
Environmental contamination	Atmosphere	Exhaust gases from vehicles, smoke from fuel oils, debris from tyres and road tar	C_{16}-C_{40} (centered on C_{25}-C_{26}) for diesel engines C_{20}-C_{36} (centered on C_{27}) for lubricating oils	Leaves from lettuce and beech tree (4 mg/kg MOSH) Sunflower oil (0.1 - 2.4 mg/kg MOSH)	

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Type of contamination	Sources		MOH involved	Occurrence	Regulation
	Marine and fresh water ecosystem	Accident or other oil spills	C_{17}-C_{28}	Sea fish (3-150 mg/kg MOSH)	
Food processing	Release agents	Bakery industry and industry working with sugar, such as candy manufacturers	Paraffin oils, typically centered on C_{23}.	Bread and biscuits (500-3000 mg/kg)	
	De-dusting agents	In transport and storage of grain or rice	Highly refined mineral oils	Grain and rice	The US Federal Drug administration (FDA) authorized the use for grain and rice with limits reaching 800 mg/kg in rice. This practice is not authorized in Europe.
	Machine oils	Harvest	C_8-C_{26} for diesel oil; C_{18}-C_{34} for lubricating oils	Sunflower seeds (3-5 mg/kg)	
		Machinery maintenance and cleaning	C_{24}-C_{30}, virtually free of MOAH	Edible oils (10-30 mg/kg); chocolate and chocolate products (10-100 mg/kg)	
	Coating of foods	Waxes for shining	Centered on C_{24}, virtually free of MOAH	Rice (1000-3000 mg/kg)	Up to 1990 it was common practice to spray refined rice with mineral oil in order to render it shiny. This practice has never been authorized, either in Switzerland or in the EU.

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Type of contamination	Sources		MOH involved	Occurrence	Regulation
Migration from food contact materials		Waxes for preservation and to improve the appearance	C_{41}-C_{51}	Fruits and vegetables	The Directive 95/2/ECC authorized the microcrystalline waxes (E905) for coating of melons, papayas, mangos, avocados.
	Jute and sisal bags	Batching oil	C_{13}-C_{25}, centered on C_{17}-C_{18}, with 20-25% MOAH	Hazelnuts, cocoa beans, coffee, rice from Asia, oil seeds (10-100 mg/kg)	In 1998, the International Jute Organization (IJO) adopted "special criteria for the manufacture of jute bags used in packaging of selected foods": the batching oil shall only contain non-toxic ingredients.
	Waxed packaging materials	Waxed paper	C_{25}-C_{40}, largely consist of *n*-alkanes	Meat products, cheese, bakery ware or candies	
	Wax coating applied directly on food	Wax layer on food		Cheese (1-27 mg/kg)	
	Plastic materials		POSH (polyolefins saturated hydrocarbons)	Migration into food from 1-110mg/kg up to the overall migration limit of 60 mg/kg, depending on the type of food, the polymer type, the nature of the contact	Regulation (EU) n° 10/2011 approve the use of specific grade of mineral oils as additives (internal lubricants)
	Lubricating oils for cans	Sheet lubricants			

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Type of contamination	Sources		MOH involved	Occurrence	Regulation
		Wax additives in can coatings (lacquers)			
		Components of can sealing compounds			
	Printing inks	Off-set printing inks (with 20-30% of mineral oil)	C_{13}-C_{21}, centered on C_{17}-C_{19}, with 1-50% MOAH	Dry foods in paperboard boxes sometimes exceed 100 mg/kg. A maximum of 210 mg/kg MOH was found in cocoa powder packed into a paper bag and printed virgin paperboard	
	Recycled board	Recycled paper and board	C_{13}-C_{50}: oils from printing inks, adhesives, solvents and waxes with 10-20% MOAH		
	Adhesives	Hot melts			According to the European legislation microcrystalline waxes are approved as food additives
Food Additives	Microcrystalline waxes	Glazing agents, protecting coatings or antifoaming agents.	High viscosity mineral oils (P100)		
Pesticides	Used as active compounds or as formulation aid			Data on residues in food and feed are not known	

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Type of contamination	Sources		MOH involved	Occurrence	Regulation
Feed	From edible oil refining	Removal of free fatty acids in the deodorisation step	MOH below about C_{27}	Mixed animal feed (200-3000 mg/kg MOSH)	
	Binders for additives	Facilitate the mixture of mineral and vitamins	MOH centered on C_{21}-C_{24}	Feeds (500-1000 mg/kg); egg yolk (30-80 mg/kg)	
	Motor oils and wastes	Spent edible oil		1-10% of mineral oil in spent edible oils used in animal feed.	In Europe, feeding of farmed animals with catering waste is prohibited under the EC Regulation 1774/2002

2.2. Paper and board packaging

Packaging carries out several important functions, such as preventing physical, chemical and biological modifications, avoiding a possible contact with external agents, facilitating transport, storage and consumption (Piergiovanni and Limbo, 2010a). According to the present legislation (DM. 21/03/73), only dry foods, such as cereals, flour, sugar, rice and pasta can be packaged in direct contact with recycled paperboard. In recent years, alarming data have been reported regarding mineral oil migration from recycled paper and board packaging into food.
Unprinted recycled board contained 300-1000 mg/kg mineral oil $<n\text{-}C_{28}$ (Biedermann and Grob, 2010). This contamination comes mostly from printing inks used in newspapers fed into the recycling process, from adhesives, solvents used as carriers for binders and additives, and from waxes used to improve water-resistance. Droz and Grob (1997) demonstrated that mineral oil contamination in dry foods packed in paperboard boxes can reach values of 100 mg/kg.
The main sources of mineral oil in paperboard are offset printing inks, either directly applied for decorating the food-packaging material or entering via recycling fibres contaminated by mineral oil-based inks. In this case, more intensively printed areas of a sheet have a high mineral oil content due to the high amount of printing ink applied (Lorenzini et al., 2010; Biedermann et al., 2011b).
Migration of mineral oil hydrocarbons from the packaging into dry foods exclusively occurs through the gas phase, by evaporation from the paperboard and recondensation on the food surface, and involves hydrocarbons with volatility up to about that of $n\text{-}C_{25}$ (Biedermann et al., 2011b).
A cumbersome topic is the use of recycled fibers in food packaging. The EU Directive 94/62 promoted the recycling of several materials in order to minimize the environmental impact (94/62/EC). However, some undesirable substances not suitable for food contact, such as additives and inks, feed into the recycling process and accumulate in the final product. Oils found in recycled board are of technical quality and contain 15-25 % of aromatic compounds (1-3 aromatic rings). Then, recycling is supported for the sustainable use of materials, but on the basis of present toxicological assessments the migration is often far beyond acceptable (Biedermann and Grob, 2010).
In August 2013, the European Recovered Paper Council (ERPC) announced an impressive, 71.7 %, paper recycling rate for Europe (http://www.erpa.info/). The current paper consumption in Europe is of 13 % (the same level of 1998), and the recycled amount of paper is 1.5 times higher than in 1998. Since 2000 the ERPC has worked consistently on improving the quantity and quality of paper available for recycling (http://www.cepi.org/). In September 2013, the European Commission proposal on End of Waste criteria for paper fails to address the objectives of increasing the quality and availability of paper for recycling (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2013:0502: FIN:EN:PDF).
In 2012 the Confederation of European Paper Industries (CEPI) estimated a total production of 77364 million of tons of paper and board (3.8 % less than in 2011) and about 46 % of this production is used for packaging (1.5 % less than in 2011) (CEPI, 2012).

2.2.1. Paper and board production

The raw materials for paper and board production involve cellulose fibers primarily coming from coniferous wood or from deciduous plants and paper materials coming from the recycling process. In addition, several chemical substances, additives and adjuvant are added to confer the adequate characteristics to paper and board (strength, water- and moisture-resistance, etc.) (Piergiovanni and Limbo, 2010a).
The first step of paper and board production involves changing wood into an aqueous pulp solution, generally by using a mechanical, or "semi-chemical process", a combination of both mechanical and chemical treatments. The mechanical approach involves a grinding up of wood. No purification or selection is achieved in mechanical pulping and the pulp has essentially the same composition of the wood from which it was made, except for a small percentage of water

soluble material. Chemical pulping involves a full chemical treatment in which non-cellulose wood components are removed leaving intact the cellulose fibers. In practice, only 45-55 % of the components is separated. Before pulp can be made into paper, it must undergo several steps called stock preparation. This amounts to a mechanical beading process that will make the fibers more readily suited to laying in a uniform web and for interconnecting with other fibers in order to make a strong sheet. The furnish (as it is now referred to) can also be treated with chemical additives. These include resins to improve the wet strength of the paper, dies and pigments to affect the color of the sheet, filler such as talc and clay to improve optical qualities, and sizing agents to control penetration of liquids and to improve printing properties. After stock preparation, the next step is to form the slurry (99.5 % water and 0.5% pulp fiber) into the desired type of paper at the wet end of the paper machine. Now an additional dewatering step occurs by pressing and drying the paper (http://www.westpak.com/whitepapers/05_paper_corrugated_paperboard_who_what_when_where.pdf; Piergiovanni and Limbo, 2010; Kirwan, 2011). The recycling process does not foresee the purification of recycled fibers from ink. Only 30 % of hydrocarbons $<n\text{-}C_{24}$ are evaporated during pulp manufacturing. Newspaper and leaflets printed by similar techniques are identified as the predominant sources of mineral oil, whereas office paper, books and corrugated board are the starting materials of lowest mineral oil content. Paperboard produced from the latter sources would contain about five times less mineral oil than average observed today, but the migration into food could still exceed the limit derived by the available ADI by a factor of up to 20. On average, European newspapers produced by offset printing ink, contained 4.1 mg/kg $<n\text{-}C_{24}$ mineral oil with 21 % aromatic hydrocarbons (Biedermann et al., 2011b).

2.3. Printing inks

Printing inks are complex mixtures manufactured from pigments (5-30 %), binders (15-60 %), solvents (20-70 %) and additives including plasticizers (1-10 %) (Pedersen et al., 2012).
Pigments used in printing inks include both inorganic pigments, such as carbon black and titanium dioxide, and organic pigments, which are frequently dyes rendered insoluble by complexing with a metal ion. Pigments produce colour by selective absorption of light, but, because they are solid, they also scatter light. Lead and other toxic pigments have been eliminated from inks from food packaging. Vehicles generally consist of a resin or polymer with a liquid dispersant, which can be a solvent, oil or monomer. The vehicle for a printing ink is chosen depending on the printing process, how the ink will be dried and the substrate on which the image is to be printed (Robertson, 2013).

2.3.1. Chemicals used in printing inks

Colorants include insoluble particles of pigments or solutions of dyes in a varnish or vehicle. The particles of pigments are usually 0.01-2 μm in size and dispersive agent must be added to ensure that they do not agglomerate. Pigments are inorganic (e.g. titanium dioxide and carbon black) or organic based. The latter are the most used for food packaging printing inks and one of the most abundant groups is the azo pigments (azo functional group -N≡N-).
Binders are polymeric resins and the film forming component of an ink in which the colouring material is finely dispersed or dissolved. The main function of the binder is to fix the ink film to the printed substrate. Binders can be derived from natural resins from trees, asphalt from crude oil, or fully synthetic resins such as acrylic, polyamide, epoxy and polyurethane resins.
Solvents keep the binders in soluble state until the application of the printing ink on the substrate. Solvents can present different boiling points and are divided into volatiles and non volatiles (mineral oils with boiling points from 210 and 300 °C).
Varnish is a mixture of binders and solvents and the composition depend on the printing process used.
Different additives for the achievement of different technical effects are added to the printing inks. They include catalysts, antioxidants, adhesion promoters, stabilizers, antifoam agents, biocides, dispersing agents, etc. (Aurela and Söderhjelm, 2007; Robertson, 2013).

2.3.2. Printing processes

The most used technologies for printing food packaging are flexography, lithography, gravure printing and ink jet printing. Specific inks are designed for each category (Robertson, 2013).
Depending upon the process, the printed image is transferred to the substrate either directly or indirectly. In direct printing the image is transferred directly from the image carrier to the substrate; examples of direct printing are gravure and flexography. Flexography is known as the liquid ink process, and is based on volatile solvents that evaporate readily at room temperatures. Typically, the evaporative process removes about 35-40 % of the delivered ink volume.
Flexography (Figure 5) is the most common process used to print packaging materials, such as corrugated containers, folding cartons, multiwall sacks, paper sacks, plastic bags, milk and beverage cartons, disposable cups and containers, labels, adhesive tapes, envelopes, newspapers, and wrappers (candy and food). In the typical flexo printing sequence, the substrate is fed into the press from a roll. The image is printed as the substrate is pulled through a series of stations, or print units. Each print unit is printing a single color. The various tones and shading are achieved by overlaying the 4 basic shades of ink, namely magenta, cyan, yellow and black (http://www.pneac.org/; Pederse et al., 2012).

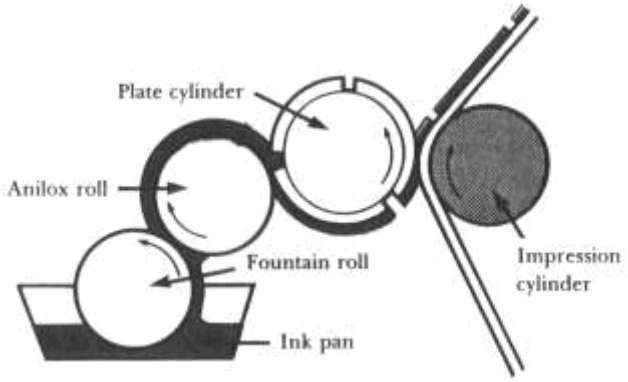

Figure 5. The flexographic printing process (http://www.pneac.org/).

In indirect, or offset, printing, the image is first transferred from the image carrier to the blanket cylinder and then to the substrate. Lithography, currently the dominant printing technology, is an indirect (offset) process (Figure 6) and uses paste inks, which are essentially non-volatile at room temperature (Robertson, 2013; http://www.pneac.org/).
Offset printing is the most used technique to print paper and cardboard (Richter et al., 2009). Inks for off-set printing are non volatile and are traditionally mineral or vegetable oils with boiling points from 210 to 300 °C (Pedersen et al., 2012). Currently new inks are being developed for paper and board with the use of novel fatty acid esters to substitute for mineral oils and reduce the risk of migration of hydrocarbons (Richter et al., 2009). Ink is applied to the printing plate to form the "image" (such as text or artwork to be printed) and then transferred or offset to a rubber "blanket". The image on the blanket is then transferred to the substrate (typically paper or paperboard) to produce the printed product. There is a total of 3 types of offset printing: non-heatset sheetfed, heatset, and non-heatset web offset. The difference between heatset and non-heatset is primarily dependent on the type of ink and how it is dried (http://www.pneac.org/).

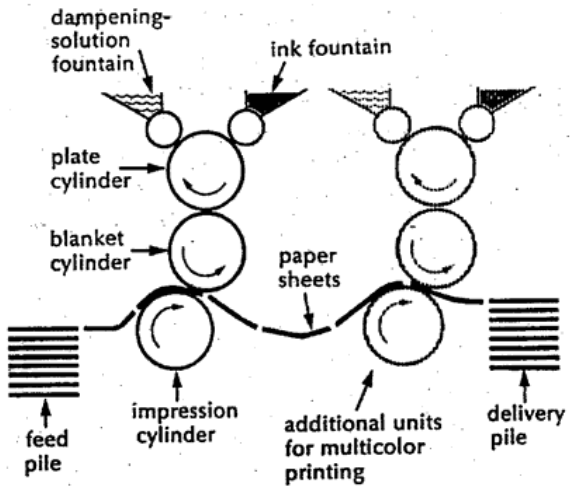

Figure 6. The lithographic printing process (http://www.pneac.org/).

Gravure printing is an older printing technique, characteristically used for high quality printing but is generally more expensive than flexographic and offset printing. The substrate can be paper or plastic. Gravure inks are fluid with a very low viscosity that allows them to be drawn into the engraved cells in the cylinder, and then transferred onto the substrate. Water based inks, especially used for packaging and product gravure, require a higher temperature and longer drier exposure time in order to drive off the water and lower vapor pressure constituents (Pedersen et al., 2012).
Ink jet printing is a technique whose use is growing in the manufacture of FCM, and has already become a common printing technology used in printing labels (Pedersen et al., 2012).

3. Legislation and Limits

3.1. General legislation on mineral oil

In 1989 the Scientific Committee on Food (SCF) reviewed the toxicity studies in *Fischer 344* rats in which abnormalities in various organs had been observed after feeding mixtures of mineral paraffins (SCF, 1998). It was concluded that "there was no toxicological justification for the continued use of mineral hydrocarbons as food additives". A temporary tolerable daily intake (TDI) of 0-0.005 mg/kg b.w. was established for oleum-treated mineral hydrocarbons (still containing some aromatics) and of 0-0.05 mg/kg b.w. for hydrogenated products.
In 1995 the SCF evaluated the safety of mineral and synthetic hydrocarbon oil and waxes for use as food additives, in food processing and in food packaging materials. SCF referred to refined mineral oils and waxes containing hydrocarbons with a mean molecular weight of no less than 480 and 500 Da, respectively, with a minimum carbon number of 25 at the 5 % and with a viscosity of no less than 8.5 and 11 mm^2/s at 100 °C, respectively (SCF, 1995).
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 assessments in 1995 (FAO/WHO, 1995) in the light of new studies and established acceptable daily intakes (ADIs). The recommendations are summarized in Table 2 (JECFA, 2002). For mixtures of highly refined paraffinic and naphthenic liquid hydrocarbons with boiling points above 350 °C (high viscosity) the ADI was 0-20 mg/kg b.w.. For medium and low viscosity mineral oil of class I (mixture of highly refined paraffinic and naphthenic liquid hydrocarbons with boiling point above 200 °C), a temporary ADI of 0-10 mg/kg b.w. was set. For medium and low viscosity mineral oil of class II and III (mixture of highly refined

paraffinic and naphthenic liquid hydrocarbons with boiling points above 200 °C) a temporary group ADI of 0-0.01 mg/kg b.w. was established (JECFA, 2002).
During winter/spring 2007/2008, in the Ukraine around 100,000 tons of sunflower oil were contaminated with mineral oil at concentrations often above 1000 mg/kg. To protect consumers, a broad analytical campaign was initiated throughout Europe and in June 2008 the European Commission decided to apply a legal limit of 50 mg/kg to the mineral paraffins in Ukrainian sunflower oil (Biedermann and Grob, 2009b).
An EFSA opinion in 2009 established a new ADI of 12 mg/kg b.w. per day for high viscosity white mineral oils, based on no-observed-adverse-effect level (NOAEL) of 1200 mg/kg b.w. per day. This value substituted 0-4 mg/kg b.w. 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 (EFSA, 2009). In 2013, following a request from the European Commission, the EFSA Panel on Food Additives and Nutrient Sources added to Food (NAS) was asked to deliver a scientific opinion on the safety of medium viscosity white mineral oil with a kinematic viscosity between 8.5 - 11 mm^2/s at 100°C as food additive. The Panel established a group ADI of 12 mg/kg b.w. per day for high and medium viscosity mineral oils by applying an uncertainty factor of 100 to a NOAEL of 1200 mg/kg b.w. per day, the highest dose level tested in a chronic toxicity and carcinogenicity study on *Fisher 344* rats (EFSA, 2013).
The German Federal Institute for Risk Assessment (BfR) studied the toxicity on *Sprague Dawley* rats and demonstrated that saturated hydrocarbons with a carbon chain from *n*-C_{10} to *n*-C_{16} are not accumulated in tissues. Assuming the consumption of 1 kg of contaminated food by a person weighing 60 kg, it was concluded that the transfer of these substances into food should not exceed 12 mg/kg (BfR, 2011). A limit of 4 mg/kg for MOSH from C_{16} to C_{20} is now under discussion.
The latest EFSA opinion of June 2012 considered the limits stated for mineral oils in 1985 by SCF, in 2002 and 2011 by JECFA as valid. In addition, a Reference Point (RP) value has been introduced to calculate the Margin of Exposure (MOE). MOE represents the amount of mineral oil to which humans are exposed during life due to an intake through food (EFSA, 2012).
For the MOSH fraction, two RP values were calculated for two different kinds of exposure:

- base exposure level: the RP value set was equal to a NOAEL value of 19 mg/kg b.w. per day;
- high exposure level: the RP value is equal to the Lowest Observed Adverse Value Effect Level (LOAEL) of 45 mg/kg b.w. per day, which corresponds to the minimum daily amount administered, causing adverse health effects.

Concerning the MOAH fraction, the EFSA declared that the RP value to calculate the MOE cannot be established. Moreover, due to the proved carcinogenicity of aromatic hydrocarbons, their presence in food is always of concern.
After the publication of the EFSA opinion, the JECFA withdrew the temporary ADI of 0.01 mg/kg b.w. for Class II and III mineral oils free of MOAH specified in 2002 (JECFA, 2002; JECFA, 2012), from which a limit of 0.6 mg/kg MOSH in food had been derived (Biedermann and Grob, 2010).
In May 2011, the German Ministry for Nutrition, Agriculture and Consumer Protection (BMELV) has drafted a mineral oil ordinance describing migration limits for MOSH and MOAH, but it was never applied. On May 16, 2013 the BMELV published another draft proposal regulating mineral oil contaminants in paper and board food contact materials. The draft amendment states that mineral oils with carbon numbers 10 to 25 may not migrate from paper and board into foods. The proposed amendment comes in response to a position statement by the German Federal Institute for Risk Assessment (BfR) on the risks arising from mineral oils December 9, 2012.

Table 2. Classification and assessment of highly refined mineral hydrocarbons intended for use in food (adapted from JECFA, 2002).

	ADI (mg/kg b.w.)	**Viscosity at 100 °C (mm^2/s)**	**Average relative molecular mass**	**Carbon number at 5 % distillation point**
Microcristalline wax High melting point wax	0-20	≥ 1	≥ 500	≥ 25
Low melting point wax	Withdrawn		No specification	
Low melting point wax		3.3	380	22
Mineral oil (high viscosity)	0-20	> 11	≥ 500	≥ 28
Mineral oil (medium and low viscosity) class I	0-10	8.5-11	480-500	≥ 25
Mineral oil (medium and low viscosity) class II	0-0.01*	7.0-8.5	400-480	≥ 22
Mineral oil (medium and low viscosity) class III	0-0.01*	3.0-7.0	300-400	≥ 17

* temporary ADI group

Table 3. Comparison of ADIs established by SCF (1995), JECFA (2002) and EFSA (2009) (adapted from EFSA, 2012).

	SCF (1995)				FAO/WHO (2002)				EFSA (2009)			
	ADI (mg/kg b.w. *per* day)	NOAEL (mg/kg b.w. *per* day)	Uncertainity factor	Comments	ADI (mg/kg b.w. *per* day)	NOAEL (mg/kg b.w. *per* day)	Uncertainity factor	Comments	ADI (mg/kg b.w. *per* day)	NOAEL (mg/kg b.w. *per* day)	Uncertainity factor	Comments
High viscosity	0-4[a]	1951	500	90-days NOAEL	0-20	1951	100	90-days NOAEL	12	1200	100	2-years NOAEL
Medium and low viscosity class I	0-4[a]	1951	500	90-days NOAEL	0-10	1200	100	2-years NOAEL	(12)[b]	(1200)	(100)	2-years NOAEL
Medium and low viscosity class II	no ADIs established				0-0.01[a]	2	200	90-days NOAEL	-	-	-	-
Medium and low viscosity class III	no ADIs established				0-0.01[a]	2	200	90-days NOAEL	-	-	-	-
Microcrystalline wax high melting point wax	0-20	1951	100	90-days NOAEL	0-20	1951	100	90-days NOAEL	-	-	-	-
Low melting point wax	no ADIs established				Withdrawn				-	-	-	-

a: temporary group ADI

b: EFSA concluded that the ADI established for high viscosity mineral oil could have been potentially applicable also to medium- and lo-viscosity mineral oil class I

3.1.1. Risk assessment

The risk assessment process consists of several steps including hazard identification, hazard characterisation, exposure assessment and risk characterisation.
The difficulties for risk assessment are caused by the complexity of mineral oil (it is not meaningful to base the hazard characterization on single compounds or indicator substances) and the lack of data. The tested mixtures cannot be related to the MOH mixtures to which humans are exposed. The critical effect considered for the selection of the NOAELs was the incidence of hepatic microgranulomas in rats following exposure to MOSH. However, several uncertainties regarding the extrapolation from data on experimental animals to human exist, in particular the relevance of these lesions for humans and the sensitivity of humans in comparison with the most sensitive species tested (Fischer 344 rats).
The MOE is a tool usually used to consider possible safety concerns arising from the presence in food and feed of substances which are both genotoxic (that is, which may damage DNA, the genetic material of cells) and carcinogenic. The MOE is a ratio of two factors: one (NOAEL) assesses the dose at which a small but measurable adverse effect is first observed for a given population and the other is the level of exposure to the substance considered (EHE: Estimate Human Exposure). Although, MOSH are neither mutagenic nor carcinogenic, the CONTAM Panel decided to use a MOE approach and considered the results from toxicity testing of a range of different MOSH mixtures to select an appropriate RP to this purpose. The CONTAM Panel selected the NOAEL for microgranulomas in the liver of 19 mg/kg b.w. per day for the most potent mixtures (low and intermediate melting point wax) from the 90-days study of Smith et al. (1996) for use as an RP in the calculation of MOEs for the human background MOSH exposure.
For average and high consumption the MOEs for toddlers and children were from 100 to 290 and from 59 to 140, respectively; for adolescents and adults they were from 200 to 680 and from 95 to 330, respectively, while for the elderly and very elderly they were from 320 to 610 and from 200 to 330, respectively (EFSA, 2012).
The range of MOSH mixtures involved in the high exposure scenario (MOSH used as release agents for bread and for spraying rice) was more restricted than that for background exposure. Therefore, the CONTAM Panel decided to use the highest NOAEL of 45 mg/kg b.w. per day from the study of Baldwin et al. (1992) for these grades of MOSH. Except for infants, the MOEs, calculated for the high exposure scenario, for the different age classes ranged from 16 to 55 for average consumption and in some cases were below 10 for high consumption of bread and rolls with high levels of MOSH. This exposure scenario was considered to be of particular concern. For the regular consumers of grains with high content of MOSH, the MOEs varied greatly between different age classes: between 35 (toddlers) and 1900 (other children) for average consumption and between 12 (other children) and 200 (elderly) for high consumption (EFSA, 2012).
In view of the lack of data on the compositional characterization of the toxicological effects, and on the human exposure to MOAH, the CONTAM Panel was not able to perform a meaningful risk assessment for this class. In particular, because no RP could be derived and no appropriate exposure data exist, it is not possible to characterize the risk associated with human exposure to MOAH via food. Of the MOH content present in food, about 20 % are MOAH, but this value can reach 30-35 % in vegetable oil and oil seeds. Since MOAH fraction could represent a carcinogenic risk, the CONTAM Panel considered the exposure to MOAH through food to be of potential concern (EFSA, 2012).

3.2. Legislation on paper and board

In Italy, the legislation on FCM started with Law n° 283 of 30/04/1962, which has subsequently been modified and reviewed. Regulation (EC) N° 1935/2004, emanated by the European Parliament and the Council in order to harmonize the national rules, establishes the principles for materials and articles intended to come into contact with food. These materials “shall be

manufactured in compliance with good manufacturing practices so that, under normal or foreseeable conditions of use, they do not transfer their constituents to food in quantities which could endanger human health, or bring about an unacceptable change in the composition of the food, or bring about deterioration in the organoleptic characteristics". Moreover, materials intended to come in contact with food shall be produced with raw materials and ingredients judged as safe according to the law and reported in the list of authorised substances (Reg. EC 1935/2004). There are no specific measures regarding mineral oils with the exception of products used as additives in plastic materials reported in the positive list reported in Regulation (EU) 10/2011 (EFSA, 2012).
Paper and paperboard are still not regulated in Europe, but in Italy there is the administrative order 21/03/73 that sets the hygienic characteristics for packaging, container and utensils intended to come in contact with food. Their suitability for food contact is based on their composition conformity and on the inertia of the material. The analytical control of paper and board composition is performed on raw materials and additives.
Paper and board, coupled together or with other materials, can be used in direct contact with food, for which migration tests are foreseen, if made following a good industrial technique and if they are constituted of at least 75 % of fibrous material, with a maximum 10 % of charge substances and maximum 15 % of auxiliary substances. The contact with food, for which migration tests are not foreseen, paper and board have to include at least 60 % of fibrous material, maximum 25 % charge substances and maximum 15 % of auxiliary substances. All the percentages are referred to the dry matter (Art. 27 of the D.M. 21/03/73).
Packagings made of multilayer paperboard with a minimum weight of 200 g/m^2 (constituted at least of three layers) can be used for dry cereals as they are and in the form of flour and semolina, dry pasta, dry bakery products without fat substances on the surface, dry or dehydrated pulses as they are or in the form of flour or powder, fresh pulses with pod, dry fruits with shell, fresh fruits with protective peel, solid sugars, salt, toasted cereals, chamomile, tea and grasses intended for infusion (Art. 27-bis of the D.M. 21/03/73).
The suitability of paper and board for food contact includes the control of technological aids and optical whitening (only for contact with foods where migration tests are supposed to be done) and specific migration tests for formaldehyde, phenols and cresols. In the administrative order 21/03/73, overall migration tests are not set for paper and board, with the exception for papers treated on the surface or coupled with plastic materials (D.M. 21/03/73).
The administrative order 18/06/79, which contains an update of the D.M. 21/03/73, allows the use of recycled fibres for all kinds of foods as long as paper and board are respected, in addition to the dispositions reported in the D.M. 21/03/73, also to the purity requisites that include PCB ($\leq$ 10 mg/kg), lead ($\leq$ 3 $\mu g/dm^2$) and optical whitening (absent) (D.M. 18/06/79). In a later update (D.M. 26/04/93 n° 220) the use of recycled fibres was allowed only for food for which migration tests were set (D.M. 220/93).

3.3. Legislation on printing inks

Printing inks are covered by the general requirements in Regulation (EC) 1935/2004 and in EU Regulation n. 2023/2006 on Good Manufacturing Practice (GMP).
Art. 3 of Regulation 1935/2004 ensure food safety stating that FCM shall, under normal and foreseeable conditions of use, not transfer their constituents into foodstuffs in quantities which could endanger human health, bring about an unacceptable change in the composition of foodstuffs or deterioration in their organoleptic characteristics.
Furthermore, Regulation (EC) 1935/2004, art. 16, declared that inks need a written declaration stating that they comply with the roles applicable to them. Moreover, an appropriate document shall be available to demonstrate such compliance (Reg. 1935/2004). Negative lists, as information for producers, are drafted by EuPIA (European Printing Ink Association) on chemicals which should not be used in printing inks.

Commission Regulation 2023/2006/EC on GMP for materials and articles intended to come in contact with foodstuffs stated the necessity to be sure that printing inks applied to the non-food contact side are not transferred into the food by set-off or transferred through the substrate (Reg. EC 2023/2006).
The Commission Directive 2007/42/EC, relating to materials and articles made of regenerated cellulose film intended to come into contact with foodstuffs, declared that "to protect the health of consumer, direct contact between foodstuffs and the printed surfaces of regenerated cellulose film should be avoided" (Dir. 2007/42/EC).
In 2010 Switzerland introduced a positive list (Swiss Ordinance on packaging inks SR 817.023.21) of all substances allowed in the manufacturing of printing inks for food contact packaging.

4. Mineral oil analysis

Mineral oils are composed primarily of paraffins (MOSH) and aromatics (MOAH). Paraffins are the main part of mineral oils and the simpler fraction to analyze. Therefore, MOSH are often use to evaluate the presence of mineral oil contamination in foods. To this purpose, it is necessary to distinguish between naturally occurring and petrogenic paraffins. The natural paraffins present a typical distribution in which hydrocarbons with an even carbon number prevail over those with an odd carbon number. In mineral oil paraffins, there is no discrimination between odd and even carbon number hydrocarbons, and a high number of isomers is present. These mixtures cause an unresolved gas chromatographic (GC) trace with one or more unresolved "humps". Although the analysis of the only MOSH fraction is the most performed due to its relative simplicity, is not sufficient to understand the total level of contamination. In fact, relevant amount of MOAH can be present and due to the concern for aromatics presence in food, it is important to quantify these fractions separately.
In order to reduce the risk of an external contamination, it is important to take some precautions during sample preparation, such as avoiding contact with plastic materials. All glassware must be accurately cleaned and the solvent used should be distilled or checked for the presence of impurities (Moret et al., 2012b).

4.1. Sample preparation

Sample preparation can be more or less demanding depending on the complexity of the matrix and it usually consists of an extraction step, followed by a further clean-up before the analytical determination. Usually, for liquid samples, such as wine, canned beverages, etc., liquid-liquid extraction (LLE) with hexane is the most used method. This extraction can be followed or replaced by solid phase extraction (SPE). For samples completely soluble in non-polar solvents, such as edible oils, saponification or a sample dilution is usually applied, before enrichment and purification. Solid samples are more demanding and an extraction step to transfer the mineral hydrocarbons from the food matrix to a suitable solvent is required. The sample extract, obtained with the more appropriate method, is further purified and analyzed using off-line or on-line techniques. Off-line purification is generally performed on SPE cartridges or high performance liquid chromatographic (HPLC) columns packed with silica gel or aluminum oxide. The silica gel allows the retention of fat and more polar compounds with the elution of hydrocarbons with the void volume (Moret et al., 2012b). Leaves, plant materials and certain foods may contain a high amount of natural *n*-alkanes (primarily odd numbered in the range of C_{23}-C_{33}), which prevents a correct MOSH quantification. This problem can be overcome by an on-line or off-line purification step on activated aluminum oxide, which strongly retains long chain *n*-alkanes (Fiselier et al., 2009a; Fiselier et al., 2009b; Moret et al., 2011, Biedermann and Grob, 2012a;). In Figure 7 is presented the effect of a purification step on aluminum oxide using off-line SPE for the MOSH fraction of an extra virgin olive oil spiked with 100 mg/kg of paraffin oil.

Observing that activated aluminum oxide (300-400 °C) retains *n*-alkanes with more than about 20 carbon atoms, whereas iso-alkanes pass largely non-retained, Fiselier and co-workers proposed a different HPLC-LC-gas chromatography (GC) method (Fiselier et al., 2009a). A first silica gel LC column (Spherisorb Si, 250 x 2 mm i.d., 5 µm) isolated the paraffins from the bulk of the sample (20 mg of oil or fat), and it was backflushed with dichloromethane. The second column (100 x 2 mm i.d.) was laboratory packed with activated aluminum oxide of standard particle size (63-200 µm), allowing the separation of the long chain *n*-alkanes, aromatics and olefins from the fraction of the iso-alkanes.

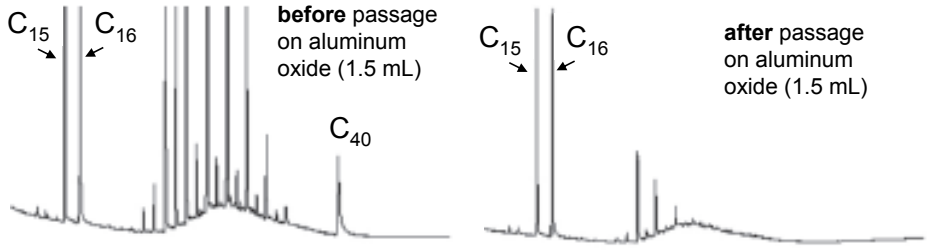

Figure 7. Effect of aluminum oxide on MOSH fraction of an extra virgin olive oil spiked with 100 mg/kg paraffin oil (adapted from Moret et al., 2011).

A similar problem may occur in the MOAH fraction, where the presence of high amounts of naturally occurring olefins may prevent a correct quantification. Olefins can be removed by applying bromination or epoxidation (Wagner et al., 2001; Biedermann et al., 2009; Biedermann and Grob, 2012a). Bromination, usually performed with a bromine solution in chloroform (Wagner et al., 2001), and epoxidation, performed with a peracid, are used to convert olefins to more polar derivatives, thus being more retained than MOAH (Grob et al., 2001; Biedermannet al., 2009; Biedermann and Grob, 2012a).

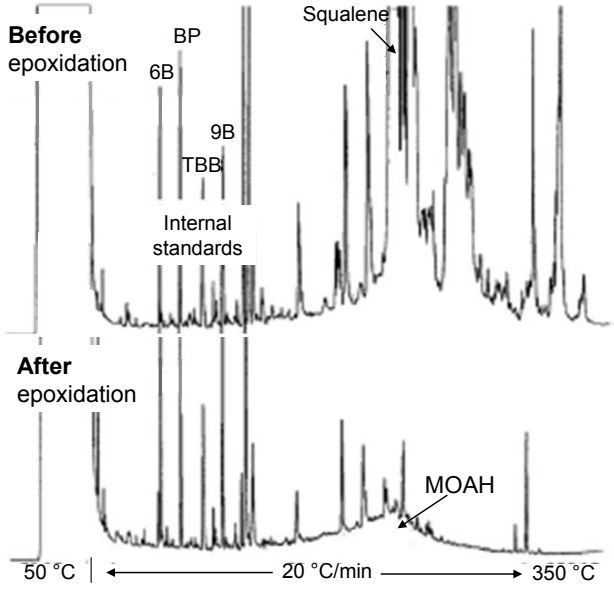

Figure 8. Effect of epoxidation on MOAH fraction of a rapeseed oil (adapted from Biedermann and Grob, 2012a).

Epoxidation can be performed on the raw extracts or on edible oils before the analytical determination. Selectivity is improved by cooling in an ice bath. In sample extracts with low or no fat content, the addition of vegetable oil is required to prevent that the excess of peracid attacks the aromatics, shortly after olefins (Biedermann and Grob, 2012a). However, depending on the mineral oil composition, 20-40 % of the MOAH is lost by this procedure (Biedermann et al., 2009). In figure 8 is shown the effect of epoxidation on MOAH fraction of a rapeseed oil. The use of two silica columns was also proposed to avoid the elution of olefins (Grob and Bronz, 1995; Grob et al., 2001). A first silica gel column of relatively low retention power (Spherisorb Si, 5 µm) retained the bulk of lipids (removed by backflush) and the breakthrough fraction (300 µL) containing hydrocarbons passed into a second column (Lichrosphere 60, 250 x 2 mm i.d., 5 µm) packed with higher retentive silica gel, thus maintaining a high activity for the retention of unsaturated components (olefins and aromatics).

4.2. Analytical determination

Capillary GC followed by Flame Ionization Detection (FID) is the most utilized approach for the analytical determination of MOH in foods.

Contamination with MOSH is easily recognizable by the presence in the GC trace of one or more "humps" of unresolved peaks and/or *n*-alkanes with a balanced distribution between odd and even carbon number hydrocarbons. Also, MOAH form one or more humps of unresolved peaks, with the same range of volatility of MOSH, indicating that they originated from the same mineral oil fraction obtained from petroleum distillation (Biedermann et al., 2009; Moret et al., 2012b; EFSA, 2012).

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 has 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 and is insensitive to its type (aromatic, olefin, *n*-alkane) (Weisman, 1998). However, FID is not selective and the pre-separation step must guarantee that only MOSH and MOAH enter the detector (Moret et al., 2012b).

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 (Wang and Fingas, 2003; Populin et al., 2004).

On column injection technique is mostly utilized in off-line GC-FID analysis, because it allows loss prevention of volatile hydrocarbons and discrimination effects. The sample is introduced as a liquid inside an uncoated, deactivated fused silica capillary (retention gap) connected to the separation column: up to 200 µL can be introduced by using a 10-15 m x 0.53 mm i.d. retention gap. Large volume injection can also be done by using the PTV (Programmed Temperature Vaporizer) injector. The sample is introduced in a (cold) liner packed with trapping material, which retains the analytes until the injector is heated-up, thus transferring the components to the analytical column. The settings, however, must be chosen carefully. If excessive packing is present, mineral oil components are retained for too long resulting in discrimination (De Zeeuw et al., 2006.).

The GC separation of mineral oil is usually carried out on non-polar columns (methyl silicone), usually of short length (e.g. 5-10 m x 0.25 mm i.d.) with a small amount of stationary phase in order to speed up the analysis and to minimize the baseline drift (Wagner et al., 2001).

MOSH and MOAH separation can be efficiently performed by using both off-line and on-line approaches. Several off-line processes based on preparative LC, or SPE techniques, have been reported over the years (McGill et al., 1993; Tan and Kuntom, 1993; Guinda et al., 1996; Koprivnjak et al., 1997; Wagner et al., 2001; Fiorini et al., 2010; Moret et al., 2011)

In particular, methods based on the use of SPE, with slightly different cartridge packing materials have been proposed. For the analysis of only the MOSH fraction activated silica gel, non-activated silica gel (Fiorini et al., 2010), or silver (Ag) silica gel (Moret et al., 2011) materials have been utilized in SPE glass cartridges as packing materials. More recently, off-

line approaches based on the use of silver silica gel were employed for the analysis of both MOSH and MOAH (Moret et al., 2012a; BfR, 2012). In the method proposed by Moret and co-workers (Moret et al., 2012a), the SPE cartridge was manually packed with 1 g of silver silica (10 %) before sample extract loading (250 µL). The sample was first eluted with 1 mL of *n*-hexane, which was discharged (dead volume), then the MOSH fraction was eluted with 1.5 mL of *n*-hexane, followed by 0.5 mL of *n*-hexane/dichloromethane (50:50 *v/v*). The following 0.5 mL *n*-hexane/dichloromethane fraction was discharged and the MOAH class was eluted with further 7 mL of *n*-hexane/dichloromethane (50:50 *v/v*). In the same year, the BfR (German Federal Institute for Risk Assessment) proposed a similar approach (BfR, 2012), based on the use of an SPE glass cartridge filled with 3 g of silver silica gel (3 %). After the dead volume (2 mL of *n*-hexane), the MOSH fraction was eluted with 4 mL of *n*-hexane, followed by 2 mL of *n*-hexane/dichloromethane/toluene (75:20:5 v/v). The MOAH class was immediately eluted with 12 mL of the *n*-hexane/dichloromethane/toluene (75:20:5 v/v). With respect to the method proposed by Moret and co-workers (Moret et al., 2011), the BfR method allows a better retention of was esters present in vegetable oils and fatty foods, but a worst retention of olefins.

Despite the flexibility guaranteed by off-line methods, the efficiency of an LC column to retain a relatively large amount of fat and other constituents has always been the best choice for such a task, both off-line (Tan and Kuntom, 1993; McGill et al., 1993) and on-line (Grob et al., 1991; Fiselier et al., 2009a-b; Biederemann et al., 2009). The loading capacity of a silica column is strictly related to the kind of solvent used as the mobile phase. For instance, a 10 cm × 2 mm i.d. column retains approximately 25 mg edible oil when hexane is used as the mobile phase, while its capacity is decreased at 15 mg if 20% of dichloromethane is used as the modifier, and at 5 mg with the addition of 1% of methyl-*tert*-butyl ether (MTBE) to hexane (Biedermann and Grob, 2012a).

In fact, among the numerous analytical methods proposed for mineral oil analysis, on-line heart-cutting LC-GC-FID is the most popular choice. Such a technique was introduced the first time by Majors in 1980 (Majors, 1980). Over the last three decades, several types of interfaces to link the LC part with the GC one, have been proposed (Biedermann and Grob, 2009; Purcaro et al., 2012). The latest is the so called Y-interface, which is a variant of the classical on-column interface with advantage of avoiding memory effects due to the slow transfer of the HPLC eluent. In particular, the use of the Y-connection avoided the presence of dead volumes in the inter-space between the transfer line and the retention gap wall, where some liquid is sucked due to capillary forces and then pushed back by the carrier gas, with a small proportion going back to the transfer line causing the memory effect (Biedermann and Grob, 2009). Partially concurrent eluent evaporation is the most suitable transfer technique, especially when samples contain hydrocarbons more volatile than *n*-C_{13} (as in the case of samples contaminated by mineral oil migrated into foods from printed cardboard). In particular, the HPLC eluent is introduced into the retention gap through the interface, at a speed that slightly exceeds the eluent evaporation rate. The size of the retention gap must allow retention of the flooding eluent to exploit solvent trapping and retention of the more volatile components: for the transfer of fractions of 200 – 1000 µL volume, typically a 5-10 m x 0.53 mm i.d. uncoated pre-column is used. High boiling solutes, which at the end of the transfer are spread along the flooded retention gap, are reconcentrated in the inlet of the separation column by the retention gap effect (Moret et al., 2012b; Biedermann and Grob, 2012b).

Recent investigations have reported the use of a syringe-based programmed temperature vaporizer (PTV) interface (Tranchida et al., 2011; Mondello et al., 2012). The syringe-type interface has been developed by David and co-workers (David et al., 1999) and presents several advantages: the packed liner retains more liquid per unit internal volume, and wettability of the packaging is not required (which means that it may be suitable also for reversed-phase LC coupling). Furthermore, the packing material is more stable than the retention gap and retains high boiling interferents. However, the syringe transfer device requires fine optimization to avoid discrimination of both the lighter and the heavier compounds, which may be lost during the solvent vent or retained in the packed liner, respectively.

Early LC-GC methods focused on MOSH determination. In 1997, Moret and co-workers (Moret et al., 1997) presented the first application focused on the determination of the MOAH fraction (totals concentration as well as grouped by ring number) in complex food samples. The system was based on a rather complicated LC-LC-GC system, with a first silica column (25 cm × 4.6 mm i.d.) and a second aminosilane column (10 cm × 4.6 mm i.d.) with a miniaturized solvent evaporator (SE) inserted on-line between the two columns (Moret et al, 1996).
More recently, Biedermann and co-workers (Biedermann et al., 2009) developed a more straightforward method. They employed a 25 cm × 2 mm i.d. silica column, which allows to retain 20 mg of fat with a dichloromethane gradient up to 30 %, leaving the bottom half of the column active for the isolation of the two fractions. In such a way, the limit of quantification (LOQ) is around 0.5 mg/kg for food containing up to 20 % of fat. For foods with higher amounts of fat, an enrichment tool may be necessary. The method was adapted according to the matrix considered, using two auxiliary tools, namely epoxidation to remove natural olefin and off-line enrichment on a packed silica gel (12 g) to increase sensitivity (Biedermann and Grob, 2012b).
Quantitative determination can be performed by comparing the area of the internal standard and the mineral oil hydrocarbons, or by external calibration with a mineral oil standard, especially when sample components interfere with the internal standard. An external standard can be integrated without interferences, but the sample must be worked up strictly reproducing the aliquot finally analyzed. Internal standards render sample preparation more flexible and immune to change of aliquots another factors influencing sensitivity of the analysis. However, in the case of mineral analysis, the choice of a suitable substance is difficult, since the MOSH and MOAH to be measured may cover virtually the whole chromatogram, leaving little opportunity to place an undisturbed internal standard. Biedermann and Grob (2012a) proposed a mixture of different compounds to verify the MOSH and MOAH separation and to perform the quantitative analysis. In particular, the standard solution for the 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) and being added only half of the amount, ensures that the correct peak is taken for the quantification (CyCy); finally cholestane (Cho) serves for the control of the end of the HPLC elution window for the MOSH. As standards for the MOAH analysis the closely eluted pair of 1- and 2-methylnaphtalene (MN) were selected to identify possible coelution with a sample components; *n*-pentyl benzene (5B) is use to control the loss of volatiles; tri-tert-butyl benzene (TBB) and perylene (Per) keep the beginning and the end, respectively, of the MOAH fraction under control.
Although integration/interpretation of MOSH and MOAH traces is a much debated topic, in this work the integration of traces has been performed on the basis of what is suggested by Biedermann and Grob (Biedermann and Grob, 2012b). Therefore, for quantification purpose, the position of the baseline needs to be assessed by blank runs performed on the same day. Furthermore, natural *n*-alkanes, when present on the top of the "hump" must be subtracted from the total MOSH area. Although not all peaks on the top of the MOAH "hump" have been identified, they have to be subtracted in order to obtain a correct quantification (Biedermann and Grob, 2012b). The detection limits depend obviously on the amount of sample processed and injected into the chromatographic system and the amount of interference from the matrix. Measurement uncertainty and limits of quantification cannot be derived from repeated analysis and signal to noise ratios. They depend on the chromatograms, but also the experience and care of the analyst interpreting the chromatograms, positioning the baseline and cutting extraneous components. In most cases uncertainty can be kept clearly below 20 % (Biedermann and Grob, 2012b).
Further resolution can be achieved by comprehensive two-dimensional GC (GCxGC) which allows also the characterization of the MOAH fraction by ring number and degree of alkylation after pre-separation on an LC column. (Biedermann and Grob, 2009).
The GCxGC system comprising a 1 m x 0.53 mm i.d. deactivated pre-column followed by a 20 m x 0.25 mm i.d. first-dimension column (PS-255, 0.12 μm film thickness) and a 1.5 m x 0.15

mm i.d. second-dimension column (50 % phenylpolysiloxane, 0.075 μm film thickness), was equipped with a cryogenic jet modulator, and connected alternatively to a quadrupole MS for identification and to a FID for routine analysis and quantification. In Figure 9 a typical GCxGC trace of MOAH fraction of different mineral oils is reported.

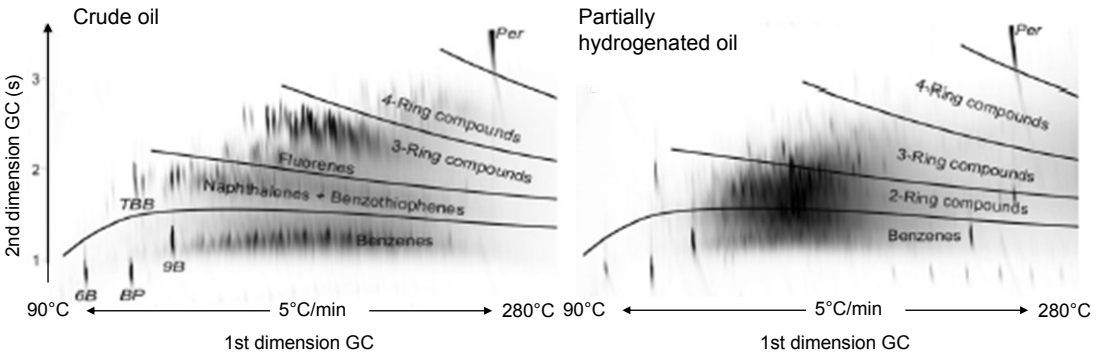

Figure 9. GCxGC plot of the MOAH fraction of a crude mineral oil and of a partially hydrogenated oil (adapted from Biedermann and Grob, 2010).

5. Migration from food packaging

5.1. Chemical migration and the main factors involved

Migration of chemical substances is a diffusion process subject to both kinetic and thermodynamic control, and can be described by diffusion mathematics derived from Fick's Law. Mathematically, diffusion is described as a function of time, temperature, thickness of the material, amount of chemical in the material, partition coefficient and distribution coefficient. The kinetic dimension of migration dictates how fast the process of migration occurs. The thermodynamic dimension dictates how extensively the transfer of substances will be when migration is finished, meaning when the system is at equilibrium (Brown and Williams, 2003; Castle, 2007; De Meulenaer, 2009).

First of all, migration depends on the identities and concentrations of the chemicals present in the packaging material. Other important parameters are the nature of the food along with the conditions of contact. Lastly, the intrinsic properties of the packaging material itself are important considerations (Castle, 2007; Piergiovanni and Limbo, 2010b). The main factors involved in migration are shown below.

The packaging material is the source of any chemical migration. The quantity of available potential migrants in the packaging material determines the extent of any migration.

The nature and extent of any contact between the packaging and the food primarily depends on the physical properties of the food (solid foods make only limited contact whereas liquids make more extensive contact) and the size and shape of the pack. The presence of a barrier layer may retard or prevent migration from occurring (Brown and Williams, 2003; Fiselier and Grob, 2012).

The intrinsic factors of a food greatly influence migration, since they determine the amount of migration that may occur. A potential migrating constituent of the packaging is gradually transferred to the food causing a decrease of its concentration in packaging and an increase in the food. Eventually, an equilibrium is reached when the concentration is constant for the packaging and the food. The amount of migrating constituent in the food at equilibrium depends on the physical affinity of the constituent for the packaging and the food (Brown and Williams, 2003; Castle, 2007; Piergiovanni and Limbo, 2010b). In Table 4 are reported different categories of food and the types of chemical migration that they are likely to be subjected to.

The migration is influenced by temperature, and usually accelerated by heat. Packaging materials are increasingly used under a very wide range of temperature conditions, ranging from

deep freezing, refrigerated and ambient temperature storage, to boiling, sterilization, microwaving and even baking in the pack.

Table 4. Classification of food and possible migrants (adapted from Castle, 2007).

Nature of food in contact	Nature of chemicals most likely to migrate
Acidic and aqueous foods, low alcohol beverages	Polar organic chemicals, salts, metals
Fatty foods, distilled spirits	Non-polar, lipophilic organic substances
Dry foods	Low molecular wieght, volatile substances

The time of contact for common packaging can vary enormously (from minutes to years). The kinetics of migration are, by a first approximation, first order in that the extent of migration increases according to the square-root of the time of contact (Castle, 2007).
The mobility of a chemical in the packaging material depends on the size and shape of the molecule and their interaction with the material.

5.2. Migration from plastic packaging

The concerns on migration from polymeric packaging materials generally focused on the levels of residual monomers and plastic additives, such as plasticizers and solvents. A typical plastic packaging material may comprise many different constituents, which all have the potential to result in safety and/or quality problems. The material itself is a polymer or copolymer manufactured from one or more types of monomers such as styrene, vinyl acetate, ethylene, propylene or acetonitrile. All polymers contain small quantities of residual monomers left unreacted from the polymerization reaction. Additives are used to aid the production of polymers and to modify the physical properties of the finished material or as processing aids: the most common are plasticizers, such as phthalates or adipates and mineral hydrocarbons (PhD Work: Part II). Furthermore, other elements of plastic packaging, such as inks, varnishes, adhesives can release undesirable substances into the food (Brown and Williams, 2003).

5.3. Migration from paper and board packaging

Paper and board are often used as primary, secondary and tertiary (transport) packaging. However, some substances present in the paper and board can migrate into food by direct contact or indirectly through the gas phase between the material surface and the food surface (Piergiovanni and Limbo, 2010b; Poças et al., 2011). Migration can occur not only from the primary packaging material, but also from the secondary system, typically a paperboard or a corrugated board box: in fact, volatile and semi-volatile substances may be transferred through the inner packaging into the food. The mechanism of migration from fibre-based materials is different from the one occurring in plastics, where the migrant is general homogeneously distributed in the polymeric matrix (Zülch and Piringer, 2010). Since paper and board materials are heterogeneous, open and porous structures consisting of cellulose fibres and air pores, migrants can be absorbed on the fibres or other constituents employed for paper and board production. Therefore, the mobility through the paper and board can be described as a repeated process of desorption/evaporation and adsorption/condensation until thermodynamic equilibrium is reached in the system. In order to be transferred through the gas phase, the migrants must exhibit a certain propensity to volatilize, which means that they must posses a

certain vapour pressure at the temperature of use (lower than its boiling point) (Zülch and Piringer, 2010; Piergiovanni and Limbo, 2010b; Poças et al., 2011).
Health concerns have been recently raised over the use of recycled paper and board for food contact because of the possible migration of different substances into food: diisopropylnaphtalenes (DIPNs), which are commonly used in the preparation of special papers, such as carbonless and thermal copy paper; phthalates, such as diisopropyl, diisobutyl, di-2-ethylhexyl phthalates, which are impurities not intentionally added in recycled fibres or impurity from adhesives; photoinitiator, such as benzophenone, which is an impurity from printing inks in recycled fibres; mineral oils, especially from inks fed into the recycling process (PhD Work: Part II) (Zülch and Piringer, 2010; Brown and Williams, 2003).

5.4. Mineral oil migration from packaging into food

Cardboard made with recycled fibers contains mineral oils from different routes such as newspaper and other printed paper entering the recycling process, adhesives and solvents used as carriers for binders and additives, and waxes added to improve water resistance (Biedermann et al., 2001b). Of particular concern are the offset printing inks used for newspapers, since they contain hydrocarbons of sufficient volatility ($<C_{24}$) to be transferred via gas-phase (Biedermann and Grob, 2010).
First data on migration of mineral oil from printing ink and recycled cardboard were published in 1997 (Droz and Grob, 1997), but the issue became public only after the German Federal Institute for Risk Assessment (BfR) released its opinion (expressed in December 2009) stating that the migration of mineral oil from boxes of recycled cardboard into dry foods should urgently be reduced (BfR, 2009).
In 2011, a survey on 120 dry food samples intended for long term storage at ambient temperature (shelf-life 1.5-3 years), from the German market revealed high contamination levels of MOSH$<C_{24}$ in packaging materials (paperboard and internal bag) and in foods (as a consequence of migration from the packaging). The mean and maximum concentrations calculated for boxes largely consisting of recycled fibres (n=107) were 433 and 1820 mg/kg, respectively; while in boxes made of virgin fibres (n=13) the corresponding concentrations were 175 and 402 mg/kg, respectively. The mean MOSH concentration in 60 products packed in recycled paperboard without an internal bag acting as a barrier was 10.9 mg/kg while in the 13 products packed in virgin fibres boxes it was 6.2 mg/kg. MOAH represented 10-20 % of the total mineral oil contamination and the maximum determined concentration was 6.1 mg/kg of food (Vollmer et al., 2011).
Similar results were reported for recycled and virgin printed packaging materials in the recent survey (51 samples) of the UK Food Standard Agency: on average 160 and 31 mg/kg of MOSH$<C_{24}$ and MOAH$<C_{24}$, respectively, were found (FSA, 2011).
Data from an Austrian survey included 38 food samples, mostly from packages with recycled fibres (n=34). The Analyses were performed after 6 months of storage of the samples wrapped in aluminum foil in order to simulate the situation closer to the end of the shelf-life. The mean concentration for all foods in packagings made of recycled fibres and without a MOH barrier migration was 7.0 and 2 mg/kg for MOSH and MOAH, respectively (EFSA, 2012).
Some of foods packed in paper or board packaging materials are consumed without further processing, like in the case of breakfast cereals and baked goods. Though other foods, such as rice, are cooked before consumption, cooking in boiling water removes only a minor part of the migrated MOH (Biedermann-Brem and Grob, 2011).

5.5. Migration tests

The objective of a food packaging legislation is to protect the consumer by controlling the contamination of the food by chemicals transferred from the packaging. There is an overall migration limit for all adulterants from the plastic and a specific migration limit (SML) for each monomer or additive, based on their hazardous properties. These limits can be brought into effect either by controlling migration from food packaging into food (SLM approach) or by

setting limits for the concentration of the substance of concern in the plastic (compositional limits). Standard migration tests are needed to estimate the extent of migration of substances into food. These are based on prescribed food simulants, chosen on the basis of the type of food to be packaged, as well as standard time and temperature conditions for the extract period, again based on the intended use (Piergiovanni and Limbo, 2010b).
The list of food simulants reported in the European Commission Regulation n. 10/2011 assigned food simulants A, B and C for foods that have a hydrophilic character, while food simulants D1 and D2 are assigned for food that have a lipophilic character. All the food simulants are reported in Tab.5. Food simulant B shall be used for those foods which have a pH below 4.5; food simulant C shall be used for alcoholic foods with an alcohol content of up to 20 %, and those foods which contain a relevant amount of organic ingredients that render the food more lipophilic. Food simulant D1 shall be used for alcoholic foods with an alcohol content of above 20 % and for oil in water emulsions. Finally, food simulant D2 shall be used for foods which contain free fats at the surface. Food simulant E is assigned for testing specific migration into dry foods (Reg. EC 10/2011). In UNI-EN 1186:2002, the official version in the Italian language, provides a guide for selecting appropriate conditions for migration tests and methods for testing the overall migration in food simulants. The European Standard EN 14338:2003 specifies a method to assess the transfer or migration of specific volatile and semivolatile substances from paper and board intended to come in contact with dry, non fatty foodstuffs and for baking purposes. Briefly, the surface of the article to be tested is covered with MPPO and held at the desired time-temperature test conditions. The exposure is followed by extraction of the adsorbent using an organic solvent depending on the used specific analytical method.
Several factors intrinsic to the food-packaging system, such as the vapour pressure and the coefficient of partition, and extrinsic, namely the food shelf life or presence of barriers between the packaging and the food, influence the migration mechanism, rendering the study rather complicated (Biedermann and Grob, 2012b). Furthermore, the legislation refers the limits to real food instead of food simulants, and roles on migration test use indiscriminately the term "food" and "food simulant", increasing the confusion regarding this topic.
Simulants for migration studies from paper and board must be solidly adsorbent to mimic contact with dry foods. Tenax® (modified polyphenylene oxide) is included in the European legislation (simulant E) and it is the most commonly referred simulant for testing migration at high temperatures simulating fatty foods. The migration behavior of components from paper in Tenax® has been compared to the migration into real dry foods: semolina pasta, sugar, flour, milk powder and rice (Aurela et al., 1999; Mariani e al., 1999; Nerín et al., 2007; Triantafyllou et al., 2007). Results from different studies suggest that equilibrium concentration in this simulant is often higher than the equilibrium concentration found in the real food, thus indicating that results obtained by using the simulant have a safe margin. However, in other cases, particularly in high temperature testing, results obtained with Tenax® can be lower than that obtained by using real food (Poças et al., 2011). This different behavior between food and food simulant sometimes leads to an overestimation of the real contamination in food. Thus, the problem is that consumers and producers are not protected in the same way (Grob, 2008).
Moreover, migration tests are expensive and time consuming, and the use of modelling migration studies can simplify the situation (Zülch and Piringer, 2010; Poças et al., 2011).

Table 5. Food simulants suggested for migration tests by Reg. 10/2011 (Reg. EC 10/2011).

Food simulant	**Abbreviation**
Ethanol 10 % (v/v)	Food Simulant A
Acetic acid 3 % (w/v)	Food Simulant B
Ethanol 20 % (v/v)	Food Simulant C
Ethanol 50 % (v/v)	Food Simulant D1
Vegetable oil (*)	Food Simulant D2
poly(2,6-diphenyl-p-phenylene oxide), particle size 60-80 mesh, pore size 200 nm	Food Simulant E

(*) This may be any vegetable oil with a fatty acid distribution of

N° of carbon atoms in fatty acid chain: n° of unsaturation	6-12	14	16	18:0	18:1	18:2	18:3
Range of fatty acid composition expressed % (w/w) of methyl esters by gas chromatography	< 1	< 1	1.5-20	< 7	15-85	5-70	< 1.5

PhD WORK PART 1: EXTRACTION METHODS AND OPTIMIZATION OF THE LC-GC SYSTEM.

1. A HIGH SAMPLE THROUGHPUT LIQUID-GAS CROMATOGRAPHIC METHOD FOR MINERAL OIL DETERMINATION[1].

1.1. Background

Until 2009, only MOSH were routinely analyzed, but since MOSH and MOAH have a different toxicological relevance, it is important to quantify them separately. In 2009 Biedermann and co-workers (Biedermann et al., 2009) proposed a straightforward method to quantify MOSH and MOAH using an on-line LC-GC system, which has the advantages of minimizing sample manipulation, preventing sample contamination and allowing for a complete transfer of the fraction of interest into the GC column. Generally, two separate runs are used to analyze the MOSH and MOAH fractions, allowing the change of the amount injected, maximizing the sensitivity for both fractions without overloading the column.
In this work, a fast LC-GC method for mineral oil determination has been developed speeding up both the GC and the LC step, with respect to that described by Biedermann and co-workers (Biedermann et al., 2009), and consequently increasing the sample throughput *per* day. The possibility of performing the analysis in the multi-transfer mode (by stopping the LC flow) was assessed, evaluating the occurrence of band broadening during the LC stop-flow time.

1.2. Experimental

1.2.1. Reagents and standards

Dichloromethane, acetone and *n*-hexane were purchased from Sigma-Aldrich (Milan, Italy) and distilled before use. The ethanol from Sigma-Aldrich (Milan, Italy) was of HPLC grade. The internal standards were all from Supelco (Milan, Italy). The working standard solution, used for quantification and to verify LC-GC performance and efficient MOSH and MOAH separation, as described by Biedermann and Grob (2012a), was obtained by mixing 5-α-cholestane (Cho, 0.6 mg/mL), *n*-C_{11} (0.3 mg/mL), *n*-C_{13} (0.15 mg/mL), cyclohexyl cyclohexane (CyCy, 0.3 mg/mL), *n*-pentyl benzene (5B, 0.30 mg/mL), 1-methyl naphthalene (1-MN, 0.30 mg/mL), 2-methylnaphthalene (2-MN, 0.30 mg/mL), tri-tert-butyl benzene (TBB, 0.3 mg/mL) and perylene (Per, 0.6 mg/mL) in toluene. The C_7-C_{40} standard mixture and the paraffin oil (code 18512) were from Supelco and Sigma-Aldrich (Milan, Italy). A sample of offset printing ink was kindly provided by a local supplier.

1.2.2. Samples and sample preparation

Three pasta samples, purchased from a supermarket in Udine (Italy), were ground and extracted overnight using *n*-hexane with a 1:2 food to solvent ratio. Then the extraction solvent was centrifuged and the collected supernatant (4 mL) was concentrated to 1 mL before the injection (100 μL) in the LC-GC system.
Three samples of recycled paperboard were chopped (1 g) and extracted at room temperature with 10 mL of hexane/ethanol 1/1 (*v/v*). The extract solution was washed with water to separate the ethanol (Lorenzini et al., 2010; Biedermann and Grob, 2012a) and the supernatant hexane was analyzed injecting 50 μL into the LC-GC system.

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[1] License Agreement with John Wiley and Sons number 3325230928353.

1.2.3. LC-GC analysis

The samples were analyzed using an LC-GC instrument (LC-GC 9000, Brechbühler, Zurich, Switzerland), consisting of a PAL LHS2-xt Combi PAL autosampler (Zwingen, Switzerland), a Phoenix 40 three syringe LC pump equipped with four switching valves (injection, backflush, transfer and additional valve) and a UV/VIS, UV-2070 Plus detector (Jasco, Japan). The LC column was a 25 cm × 2.1 mm i.d. packed with Lichrospher Si 60, 5 µm (DGB, Schlossboeckelheim, Germany). The GC was a Trace GC Ultra from Thermo Scientific (Milan, Italy).

The saturated hydrocarbons were eluted from 2.0 to 3.5 min and transferred to the GC operating at a carrier gas inlet pressure of 60 kPa (hydrogen in constant pressure mode). The fraction comprising the MOAH ranged from 4.0 to 5.5 min. The inlet pressure is increased to 110 kPa during the transfer. The MOSH and the MOAH fractions were eluted (at 300 µL/min) using a gradient, starting with 100 % hexane (0.1 min) and reaching 30 % of dichloromethane in 0.5 min. At the end of the LC-GC transfer, performed through the Y-interface using the retention gap technique (Biedermann et al., 2009), the LC column was backflushed with dichloromethane. A 10 m × 0.53 mm i.d. uncoated and deactivated pre-column was followed by a steel T-piece union connected to the solvent vapor exit (SVE) and a 15 m × 0.25 mm i.d. separation column coated with a 0.15 µm film of PS-255 (1% Vinyl, 99% Methyl Polysiloxane) (Mega, Italy). Hydrogen was used as carrier gas at a constant pressure of 60 kPa. The oven temperature started and was kept for 5.5 min at 55°C, then increased to 350°C (held 4 min) at 20°C/min (Biedermann et al, 2009). FID (sampling frequency 50 Hz) and the SVE were heated at 360 °C and 140 °C, respectively.

Data were acquired and processed by the ExaChrom software (Brechbühler, Switzerland). The MOSH area was determined by the integration of the whole hump of largely unresolved material and by subtraction of internal standards, and in the case of food samples, by subtraction of the peaks of the endogenous *n*-alkanes on the top of the hump. The position of the baseline was determined by repeated solvent injections. All sharp peaks standing on the top of the MOAH hump were subtracted from the total area.

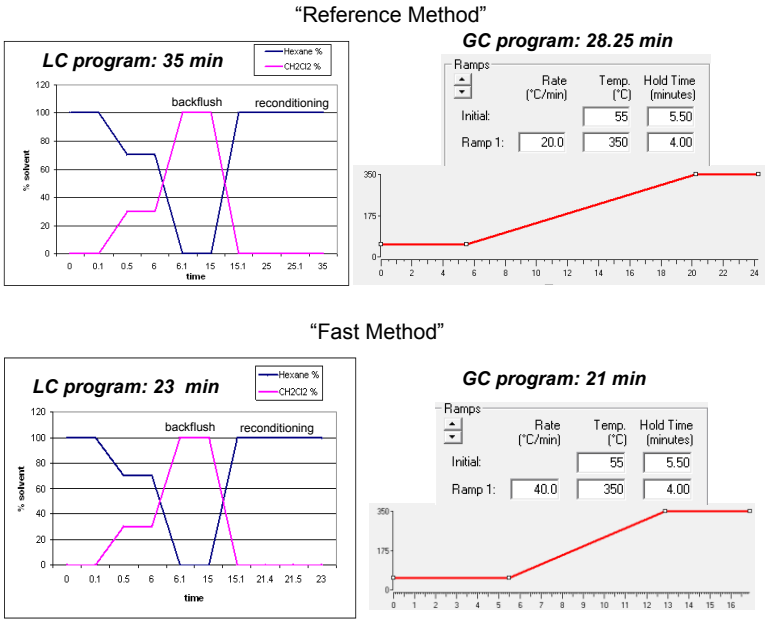


Figure 10. Scheme of LC and GC programs used in the "reference" and in the "fast method".

In particular, in the method developed by Biedermann and co-workers (hereafter defined as "reference method") (Biedermann and Grob, 2009) the sample was eluted at 300 µL/min using a gradient starting with hexane (0.1 min hold) and reaching 30 % of dichloromethane after 0.5 min. Six minutes after the injection, the column was backflushed with dichloromethane at 500 µL/min for 9 min and then reconditioned at 500 µL/min with hexane for 10 min and at 300 µL/min until the subsequent injection (for about 10 min). Using the "reference method", a total LC time of 35 min (6 min of elution, plus 9 min of backflush, plus 10 min of reconditioning at 500 µL/min and further 10 min at 300 µL/min) is spent. Within the LC time, the GC analysis lasts 28.25 min (oven program plus about 4 min of oven cooling).
In the method proposed (hereafter defined "fast method"), the GC run was sped up by increasing the oven program rate to 40 °C/min and using a carrier gas flow of 4 mL/min at a constant flow mode, while the LC chromatographic conditions were maintained as in the "reference method", except for the reconditioning step, in which a hexane flow at 700 µL/min for 6.5 min and then at 300 µL/min for 1.5 min was employed. In Figure 10, the LC and GC programs used in the "reference" and in the "fast method" are schematically reported.
The multi-transfer mode, which was carried out by stopping the LC flow during the GC analysis of the first fraction transferred allowing the transfer of several fractions to the GC within a single LC injection, was investigated using both methods. Furthermore, the band broadening occurring during the stop flow was evaluated by comparing the variances (σ^2_{off}) (n = 5) of the perylene (Per) peak width at half height, as suggested by Bedani and co-workers (Bedani et al., 2006).

1.3. Results and Discussion

Implementation of LC-GC methods, with particular attention to the reduction of time and solvent consumption, is quite suitable for obtaining an increase of the analysis throughput. Considering the careful optimization of the LC elution conditions carried out by Biedermann and co-workers (Biedermann and Grob, 2009) to effectively separate MOSH and MOAH fractions from the bulk of the matrix (mainly triglycerides), the only improvement margins were on the backflush and reconditioning steps, along with an optimization of the GC oven temperature rate.

1.3.1. Optimization of LC and GC parameters.

Different oven rates (40, 45 and 50 °C/min) were tested and the constant pressure mode applied in the "reference method" was replaced by the constant flow mode, minimizing broadening and distortion in the late eluted peaks (Figure 11).
In fact, applying 60 kPa over-pressure in the inlet (constant pressure mode), a flow of about 3.7 mL/min was generated in the analytical column at an initial temperature of 55 °C, while at about 300 °C the flow decreased to 1.5 mL/min. After a few tests, an oven temperature rate of 40 °C/min and a flow rate (constant flow mode) of 4 mL/min were selected (Figure 12). At these conditions the *n*-C_{35} is eluted at 50 °C below the maximum oven temperature (specifically at 295 °C) assuring that the delayed material (eluted clearly after *n*-C_{35}) did not interfere with the following analysis (Biedermann and Grob, 2012a). On the other hand, the resolution ($R_{s;}$ calculated between C_{39} and C_{40}) decreased from 2.5 to 1.6, causing almost complete co-elution of C_{28} with the Cho. Cho is used as an internal standard to verify the end of the MOSH fraction. Furthermore, since it is added in a concentration twice as high as CyCy (quite volatile internal standard used for quantification purposes), the ratio between these two compounds can be used to rule out a possible loss of CyCy. However, it was not considered a problem, since an accurate integration of Cho is very difficult in most samples due to partial co-elution and common overload of natural alkanes, as observed by Biedermann and Grob (2012b). Finally, no significant bleeding of the column was observed when applying higher oven temperature rates.

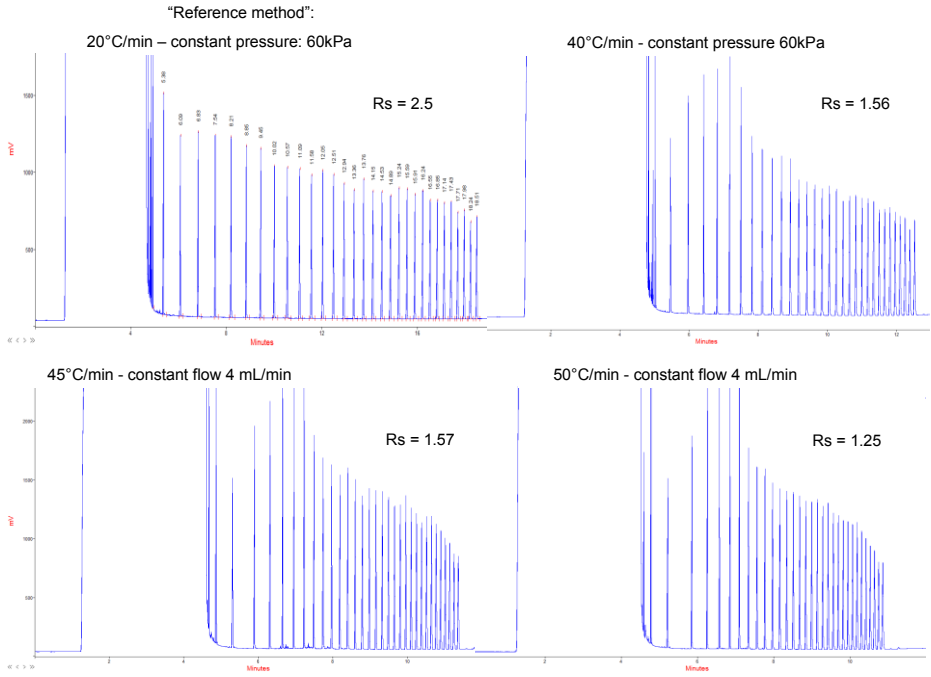


Figure 11. Chromatograms of n-alkanes C_{10}-C_{40} standard mixture obtained from testing different GC oven temperature rates. The resolution obtained (R_s) is also reported for each trial.

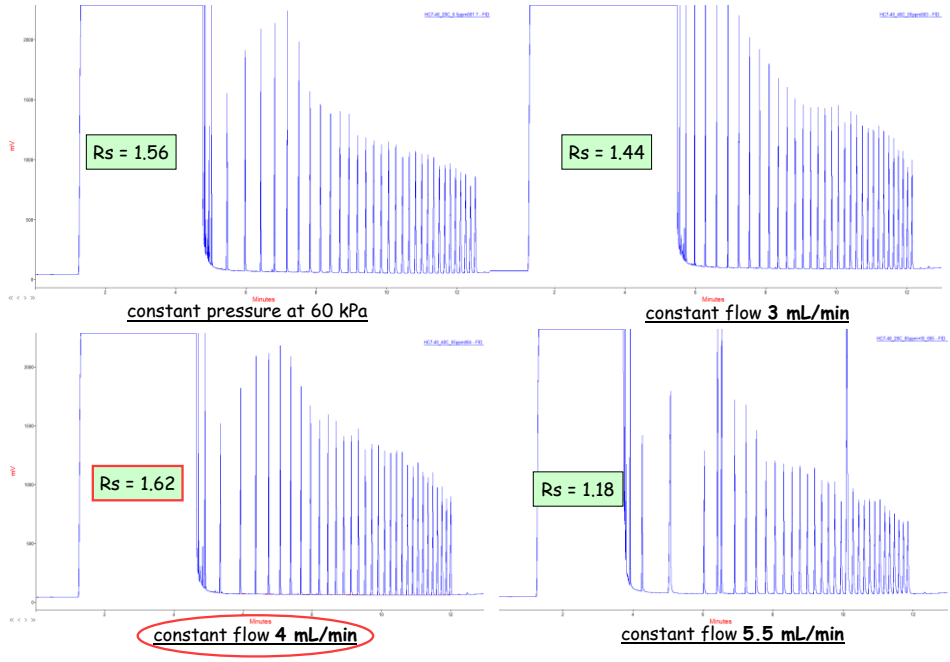


Figure 12. Chromatograms of n-alkanes C_{10}-C_{40} standard mixture obtained from testing different sets of carrier gas, using a GC oven temperature rate of 40 °C/min. Rs: resolution.

In the "fast method" the total GC run lasted about 23 min, considering the 2 min before the transfer of the MOSH fraction and the 20.8 min of the GC run (oven temperature program plus 4 min of oven cooling).
Elution of the fractions of interest lasted 6 min; therefore the goal was to modify the LC program to last as long as the GC run, in particular, reducing the remaining time (backflush and reconditioning time) from 29 min to 17 min. The backflush was maintained at a flow rate of 500 µL/min for 9 min, as in the "reference method" due to its importance for removing the bulk of the sample (mainly triglycerides), thus assuring the stability of the retention time and the life of the LC column. Then, the LC column was reconditioned in hexane at 700 µL/min for 6.5 min and further 1.5 min at 300 µL/min to reset the initial condition. The optimized method allowed to perform about 62 samples/24h compared to 40 samples/24h of the "reference method", saving 34 % of time (from 35 min to 23 min *per* run). Furthermore, a 23 % of the total hexane volume was saved during the reconditioning step.

1.3.2. Method performance

The figure-of-merits for both the "reference" and "fast method" were assessed and compared using a mixture of offset printing ink (hydrocarbon range between C_{13} and C_{21}) and paraffin oil (hydrocarbon range between C_{21} and C_{50}) (Figure 13).

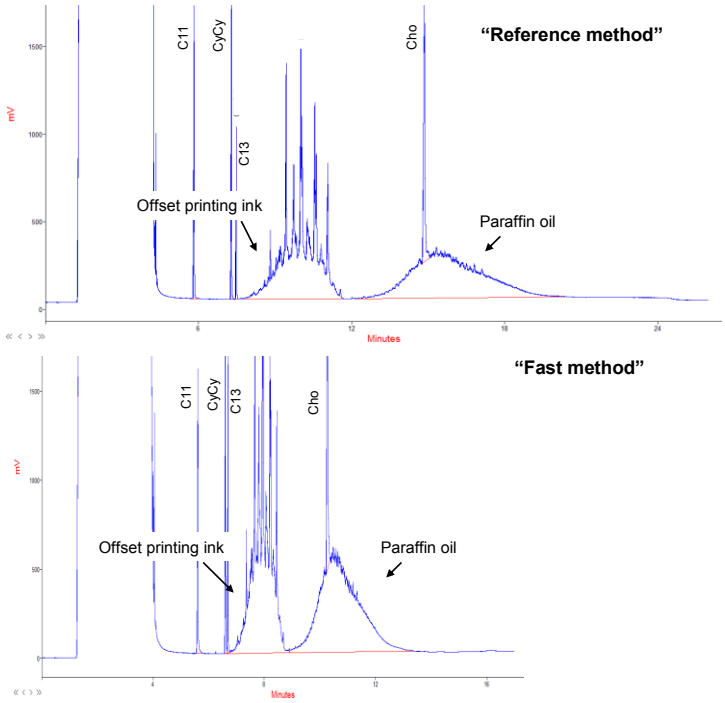


Figure 13. Chromatograms of a mixture of offset printing ink (hydrocarbon range between C_{13} and C_{21}) and paraffin oil (hydrocarbon range between C_{21} and C_{50}) analyzed using the "reference" and the "fast method". C_{11}, CyCy, C_{13} and Cho are internal standards.

The regression lines (constructed in the 1 - 50 mg/kg range) for both humps was estimated applying the least squares method; the linearity and the goodness of the curves were evaluated using Mandel's fitting test ($F_{calc} < F_{tab}$) (Draper and Smith, 1981). The MOSH and MOAH overlayed chromatograms, obtained from injecting the mixture of offset printing ink and

paraffin oil in the range 1 - 50 mg/kg, are reported in Figure 14. Aromatic hydrocarbons are present only in the offset printing ink (about 9 %). By running a *t*-test at the 5 % significance level, the intercepts did not prove significant. As expected, using FID, linearity was satisfactory for both methods, with no significant differences between the slopes of the calibration curves (running a *t*-test at the 5 % significance level). The coefficient of regression resulted above 0.999 for both methods, and both for paraffin oil and printing ink.
Repeatability of the "fast method" was calculated injecting 4 times a solution of paraffin oil and offset printing ink at 1 mg/kg, obtaining a coefficient of variation (*CV %*) of 2.3 % and 4.2 %, respectively.
An approximate estimation of the limit of detection (LOD) and quantification (LOQ) was made by considering 3 and 10 times the signal-to-noise ratio, respectively, although the evaluation of these limits in mineral oil determination is closely related to the molecular weight distribution of the contamination, thus to the width of the hump. Table 6 shows the LOD and LOQ values obtained by considering both the paraffin oil and the printing ink humps, which presented different alkanes distribution, using both methods. As expected, a slight increase in sensitivity was observed when a faster oven ramp was employed (Tranchida et al., 2011; Moret et al., 2012a).

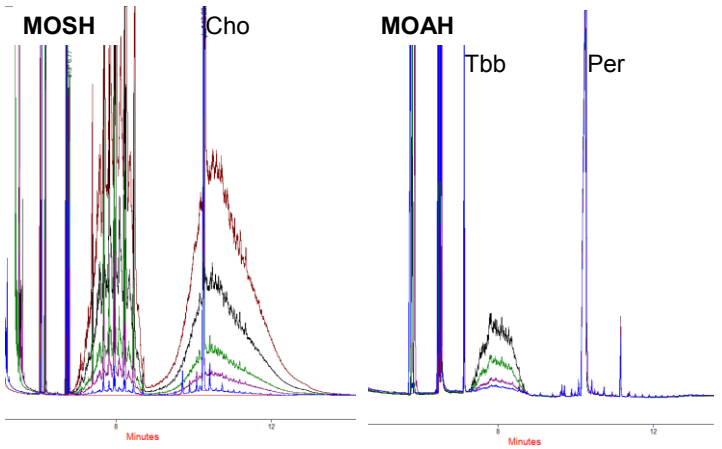


Figure 14. Overlay of MOSH and MOAH chromatograms obtained by injecting different amount of the paraffin oil and offset printing ink mixture, in the 1 - 50 mg/kg range, using the "fast method". 5-α-cholestane (Cho), tri-tert-butyl benzene (TBB) and perylene (Per) are internal standards.

1.3.3. Multi-transfer mode

The instrument allows to automatically perform multi-transfer by the LC stop-flow mode (Biedermann et al., 2009; Biedermann and Grob, 2012a). The transfer of several fractions within a single LC injection allows to use less sample extract, reduce solvent consumption and save time. However, this feature has been rarely used for mineral oil analysis, mainly due to the great difference in concentration between MOSH and MOAH, but when analyzing highly contaminated samples, such as recycled cardboard, the sensitivity for both MOSH and MOAH is more than satisfactory.
The multi-transfer with the LC stop-flow mode has been exploited few times in literature, and mainly for comprehensive LC-GC (LC×GC) applications (Janssen et al., 2003; De Koning et al., 2004a; De Koning et al., 2004b; De Koning et al., 2006; Janssen et al., 2006; Purcaro et al., 2012). An axial diffusion broadening of the compounds held in the column can be expected during the LC column stop-flow (Tran et al., 2006). Although De Koning and co-workers (De Koning et al., 2004a) proved that such a phenomenon did not affect the resolution in the LC

separation for triglycerides, it has never been proved for lower-molecular-weight compounds, such as MOAH. Perylene (Per) was used as a marker since it can be detected by UV, even though the diffusion coefficient of each MOAH may be different. The peak width obtained without stop-flow in both methods was very similar (p>0.05, running a t-test at 95 % of confidence), while the comparison of the peak width between the two modes (non-stop and multi-transfer) proved to be significantly different using both methods (Table 6).
By comparing the variances (σ^2_{off}) (n = 5) of the peak width at half height (Bedani et al., 2006), the band broadening occurring during the stop flow was evaluated in both the "reference" and the "fast method": a slightly higher broadening occurred in the "reference method" compared to the "fast" one (0.792 and 0.433, respectively), due to the longer stop time. As a counter-check, the effective diffusion coefficient (D_{eff}) of Per was calculated also using the Einstein diffusion equation ($\sigma^2=2D_{eff}t_{off}$), since the diffusion occurring in the column with the same chromatographic conditions is mainly related to the nature of the compound considered in the calculation. Comparable results in both the "reference" and the "fast methods" were obtained.

Table 6. Comparison of the "reference" and the "fast method" in terms of limit of detection (LOD) and limit of quantification (LOQ), peak widths ($w_{(s)}$), and time of stop-flow (t_{off}) (adapted from Barp et al., 2013).

	reference method		**fast method**	
	Printing ink	*Paraffin oil*	*Printing ink*	*Paraffin oil*
LOD (mg/kg)	0.10	0.11	0.08	0.08
LOQ (mg/kg)	0.33	0.35	0.25	0.27
$w_{(s)}$ (non-stop)	71.35 ± 1.15		72.30 ± 1.02	
$w_{(s)}$ (multi transfer)	80.92 ± 1.45		80.07 ± 1.22	
p	<0.05		<0.05	
t_{off} (s)	1695		1219	

Due to a not very high reproducibility of the stopping and re-starting of the pumps, a slight shift in the Per retention time was observed in the multi-transfer mode, as visible from UV traces reported in Figure 15.

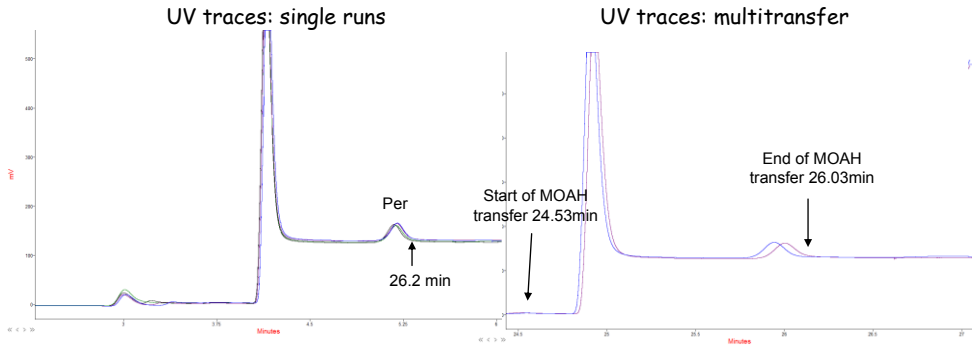


Figure 15. UV traces obtained using the "fast method" in non-stop flow (single runs) and in multi-transfer mode. The shift of the perylene (Per) peak is visible in the multi-transfer mode.

For this reason the quantification of the MOAH fraction of the printing ink, performed by the multi-transfer and non-stop modes, was compared to confirm the reliability of the fraction transferred (not significantly different running a *t*-test at 95 % of confidence). Although a relatively low time-saving is obtained by using the multi-transfer compared to the non-stop mode (8 and 1 min less in the "reference" and "fast method", respectively), half of the solvent amount is saved.

1.3.4. Real samples

In order to counter-check the reliability of all the approaches when carried out on real samples, a series of paperboards and pastas were analyzed using all the methods discussed ("reference" and "fast method", non-stop and multi-transfer). Quantification was carried out up to the retention time corresponding to the *n*-alkane C_{35}, important from the toxicological viewpoint (EFSA, 2012), and performing a cut at *n*-C_{25} for both kinds of sample, to highlight possible differences in the more volatile and heavy fractions among the methods. The results obtained along with reproducibility of the data acquired with the different methods expressed as a coefficient of variation (*CV %)* are reported in Table 7.
In figures 16 and 17 the MOSH and MOAH profiles of paperboard and pasta samples, respectively, obtained in all four conditions, namely the "reference method" and "fast method", non-stop and multi-transfer, are reported.

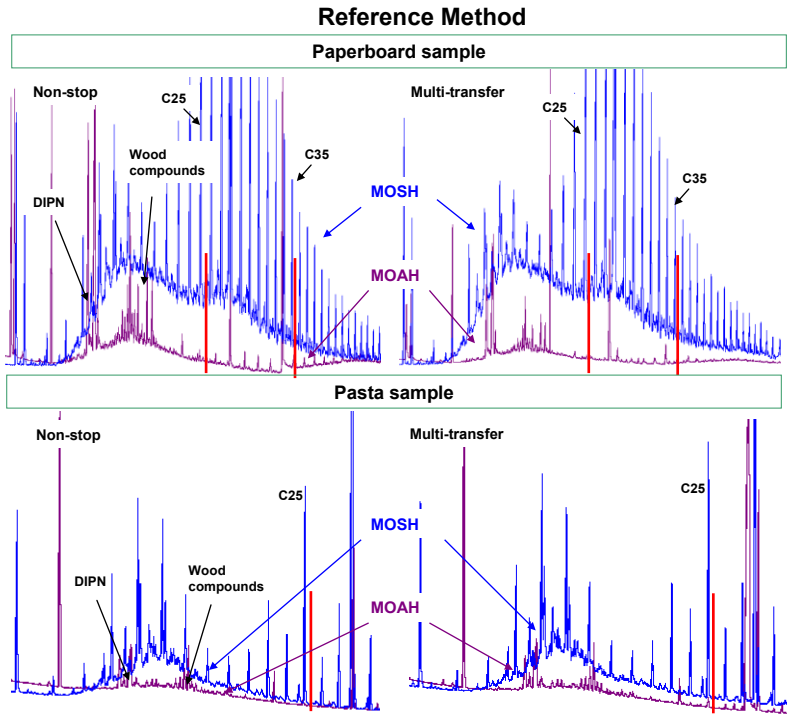


Figure 16. MOSH and MOAH profile of paperboard and pasta samples, obtained by using the "reference method" in the non-stop and multi-transfer mode. The red bars indicate the cut at C_{25} and C_{35}. DIPN: diisopropylnaphthalene (adapted from Barp et al., 2013).

Table 7. Quantification values (below C_{25} and in the C_{25}-C_{35} range) relative to the MOSH and the MOAH fractions, in paperboard and pasta samples using the "fast" and "reference method" in non-stop and multi-transfer (MTR) mode. Reproducibility among the four analyses, expressed as a coefficient of variation (CV %), is reported (adapted from Barp et al., 2013).

		MOSH mg/kg					**MOAH mg/kg**				
		Fast method		**Reference method**		**CV%**	**Fast method**		**Reference method**		**CV%**
		non-stop	**MTR**	**non-stop**	**MTR**		**non-stop**	**MTR**	**non-stop**	**MTR**	
Paperboard 1	< C_{25}	412.6	407.2	395.3	389.3	2.7	74.2	71.8	70.5	76.0	3.4
	C_{25}-C_{35}	281.6	278.2	266.1	267.5	2.8	13.2	13.7	10.4	13.8	12.5
Paperboard 2	< C_{25}	296.0	304.5	283.6	291.2	3.0	58.4	57.0	51.6	56.76	5.3
	C_{25}-C_{35}	216.4	221.6	203.6	213.6	3.5	7.0	7.3	5.7	6.13	11.8
Paperboard 3	< C_{25}	186.9	198.0	195.1	203.6	3.5	36.92	36.87	35.68	34.64	3.0
	C_{25}-C_{35}	411.0	434.6	429.0	439.0	2.9	35.17	34.35	30.67	29.39	8.6
Pasta 1	< C_{25}	3.3	3.4	3.2	3.5	4.8	0.5	0.6	0.6	0.5	10.8
	C_{25}-C_{35}	< LOQ	< LOQ	< LOQ	< LOQ	-	< LOQ	< LOQ	< LOQ	< LOQ	-
Pasta 2	< C_{25}	4.2	4.7	4.5	4.3	4.4	0.9	0.9	0.8	0.9	4.6
	C_{25}-C_{35}	< LOQ	< LOQ	< LOQ	< LOQ	-	< LOQ	< LOQ	< LOQ	< LOQ	-
Pasta 3	< C_{25}	2.8	2.8	2.7	2.8	2.2	0.2	0.2	0.2	0.3	5.9
	C_{25}-C_{35}	< LOQ	< LOQ	< LOQ	< LOQ	-	< LOQ	< LOQ	< LOQ	< LOQ	-

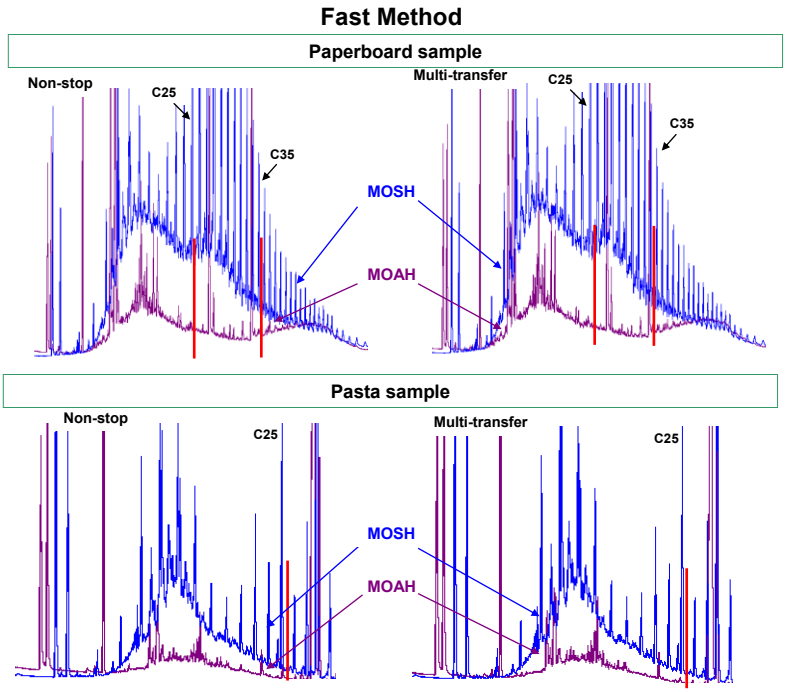


Figure 17. MOSH and MOAH profile of paperboard and pasta sample, obtained by using the "fast method" in the non-stop and multi-transfer mode. The red bar indicates the cut at C_{25} and C_{35}. DIPN: diisopropylnaphthalene (adapted from Barp et al., 2013).

The recycled paperboards proved to be highly contaminated, in the range of 480 - 700 mg/kg and 55 - 90 mg/kg for MOSH and MOAH up to C_{35}, respectively. Contamination presented a molecular weight distribution ranging between C_{13} and C_{55}, split in two humps and partially co-eluted. The first part of MOSH fraction mainly consisted of mineral oil deriving from printing inks, while the later eluted hump derived from waxes used in the paperboard manufacturing. The MOAH fraction presented the typical bunches of peaks of diisopropylnaphthalenes (DIPN) and wood compounds (derivatives of abietic and primaric acid). The main MOAH hump ranged between C_{13}-C_{24} indicating that it derives from printing ink. The later eluted hump (above C_{35}), particularly evident in the fast method, derived from petroleum resins used as binders in printing ink (Biedermann and Grob, 2012b) (figure 16). The pasta samples were contaminated in the 2.5 - 5.0 mg/kg range with MOSH and below 1 mg/kg for the MOAH fraction. The mineral oil hump was centered in the C_{17}-C_{19} range, which is typical of the migration from paperboard packaging. Furthermore, the typical marker of paperboard, namely DIPN and wood compounds, was present in the MOAH fraction (Figure 17). The natural alkanes (starting from C_{22}) present on the tail of the MOSH hump of pasta samples were subtracted from the total area for quantification.

The variability between the methods was very low for the MOSH fraction, both for the paperboard and pasta samples (below 5 %), while a slightly higher variability was observed for the MOAH fraction, mainly in the C_{25}-C_{35} range, related to the hump distribution. The variability was mainly determined by the difference between the data obtained with the "fast" and the "reference method" (independently

from the mode, non-stop or multi-transfer). In fact, the "reference method" had lower sensitivity due to the more broadened hump; thus affecting mainly the quantification of the lower part of the hump located in the C_{25}-C_{35} fraction. Furthermore, integration of the MOAH fraction was

more cumbersome since all the interfering peaks standing on top of the hump had to be subtracted from the total area, adding another source of error. The interpretation/integration of the chromatogram has been evaluated as the main source of uncertainty, reaching up to 20 %, when the work is not carried out carefully (Biedermann and Grob, 2012b; Purcaro et al., 2013b).

1.4. Conclusions

The application of a minor technical improvement on the LC-GC method proposed by Biedermann and co-workers (Biedermann et al., 2009), allowed to largely improve the data throughput (from 40 to 62 samples *per* day), saving 34 % of time and 23 % of solvent. Furthermore, a slight increase in sensitivity was obtained. The proposed "fast method" has been largely applied in routine analysis in our laboratory on a wide range of samples (both cardboards and different kind of food samples) proving the robustness of the method.
The occurrence of band broadening during LC-GC multi-transfer mode for aromatic compounds was verified for the first time. Although a band broadening effect occurred (proportional to the LC flow stop time), it did not affect the reliability of the fraction transferred. Furthermore, half of the solvent amount was saved using the multi-transfer mode.

2. A COMPARISON OF TWO DIFFERENT MULTIDIMENSIONAL LC-GC INTERFACES FOR THE DETERMINATION OF MOSH IN FOODSTUFFS[2].

2.1. Background

The uncertainty of an analytical determination is mainly related to the sample preparation procedure, the instrumental analysis (LC-GC in this case), and the interpretation/integration of the chromatographic profile. In the specific case of mineral oil determination, the latter contribution has been demonstrated to be the major one (Biedermann and Grob, 2012b). The contribution towards uncertainty of the sample preparation (extraction) process is very low if recovery is complete; the contribution of the instrumental analysis is also minimal, and can be estimated lower than 2 %, when replicate analyses are performed on the same instrument (specifically on the Y-interface) (Biedermann and Grob, 2012b). However, information on the influence of different instrumentation, on the analytical determination is lacking. Consequently, the aim of the present work was to compare the results obtained in two distinct laboratories (located in Udine and Messina, Italy), using the same extraction method, but different LC-GC instrumentation. In particular, the results obtained by using the Y interface, applying the method described by Biedermann and Grob (Biedermann et al., 2009; Biedermann and Grob, 2012b), and the PTV interface (Tranchida et al., 2011; Mondello et al., 2012), for the analysis of MOSH in different food samples, were compared.

2.2. Experimental

2.2.1. Samples and sample preparation

Two samples of pasta, two samples of rice, and an icing sugar were purchased from supermarkets located in Udine and Messina. A sample of extra-virgin olive oil was obtained from a local retailer in Messina.
All the dry samples were ground and extracted overnight in duplicate using hexane, with a food to solvent ratio of 1:2. Then, the extraction solvent was centrifuged and the collected supernatant (carefully measured) was concentrated to 1 mL before the LC-GC analysis (injected in triplicate). The oil sample was diluted 1:4 in hexane before the injection.

2.2.2. LC- GC analyses

The LC-GC systems used were set up as follow:
1) Y-interface LC-GC system (hereafter defined "Y/LC-GC"): the instrument and conditions have already been described (see "reference method" in the paragraph *1.2.3* of this section). The instrument and the scheme of the Y-interface are reported in Figure 18.
2) The syringe-based PTV LC-GC system (hereafter defined "PTV/LC-GC"): the LC part of the automated instrument consisted of a Shimadzu Prominence LC-20A system (Shimadzu, Kyoto, Japan), equipped with a CBM-20A communication bus module, two LC-20AD dual-plunger parallel-flow pumps, a DGU-20A online degasser, an SPD-20A photodiode array detector, a CTO-20A column oven, and an SIL-20AC autosampler. The data were acquired by the LCsolution software (Shimadzu). LC conditions: a 100 × 3 mm ID × 5 µm d_p silica column (SUPELCOSIL LC-Si, Supelco, Milan, Italy) was operated under isocratic conditions, using hexane as mobile phase (0.35 mL/min). Injection volume: 80 µL. At the end of the heart cut, the column was backflushed with dichloromethane.
A Shimadzu AOC-5000 autoinjector equipped with a dedicated dual side-port syringe enabled the chromatography band transfer, which was achieved in the stop-flow mode, through a

[2] License Agreement with Springer number 3325231486684.

modified 25-µL syringe, connected in the lower part to the LC detector exit and the waste, via two transfer lines. The movement of the syringe plunger determines the transfer of the LC eluent to the GC (when the plunger is located between the two transfer lines) or to the waste (when the plunger is located below both transfer lines) (Tranchida et al., 2001). From 0 to 1.5 min the LC effluent was directed to the waste, while from 1.5 to 2.0 min the LC effluent was directed to the GC. The GC part of the automated instrument consisted of a Shimadzu GC2010 Plus system, equipped with an Optic 3 injector (ATAS GC International, Eindhoven, The Netherlands). The Optic 3 injector was temperature-programmed as follows: 35 °C (1 min) to 360 °C at 250 °C/min. The whole system, the transfer device and the scheme of the mechanism of the transfer system are reported in Figure 19.

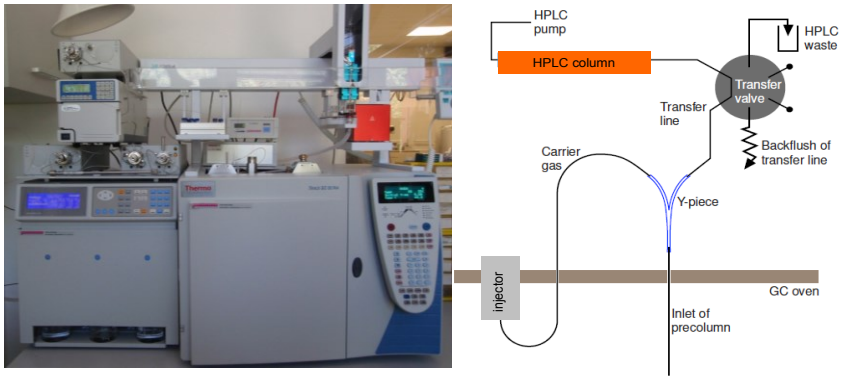


Figure 18. LC-GC 9000 instrument (Brechbühler) on the left and scheme of the Y-interface on the right.

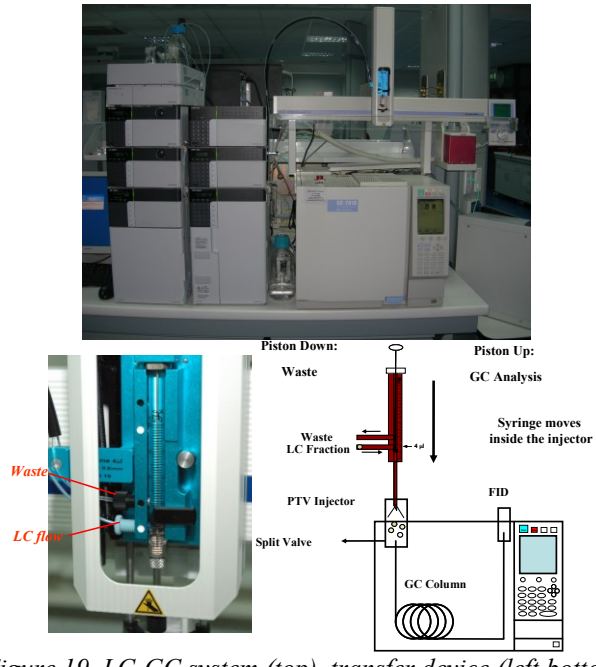


Figure 19. LC-GC system (top), transfer device (left bottom) and the transfer mechanism system scheme (right bottom).

The injection mode: split, at a ratio of 200:1 for 1 min during sample introduction and solvent vent, then splitless for 1.80 min during sample desorption and transfer, then 100:1 for the remaining analysis time to remove high boiling compounds from the liner. The data were acquired by the GCsolution software (Shimadzu).
GC conditions: an SLB-5ms [silphenylene polymer, virtually equivalent in polarity to poly(5 % diphenyl/95 % methylsiloxane)] 15 m × 0.10 mm ID × 0.10 µm d_f column (Supelco) was heated from 35 °C (3 min) to 360 °C (2 min) at 70 °C/min. The carrier gas, hydrogen, was supplied at an initial pressure of 529 kPa (constant linear velocity: 100 cm/sec). FID (360 °C) sampling frequency was 50 Hz. The transfer conditions of the PTV/LC-GC method were modified, compared to ref. 28, to optimize discrimination, in particular of lighter alkanes. The inlet temperature was reduced from 75 °C to 35 °C with the aid of an air flow, as well as the oven starting temperature, which was held at 35 °C for 3 min (previously, it was set at 50 °C for 1 min). In the former method the solvent vent was performed with the same split ratio and time period, while the split valve was then closed for only 1.11 min; after sample transfer, a split ratio of 50:1 was applied.

2.3. Results and Discussion

Two analytical methods were optimized for the determination of MOSH, and compared in terms of hydrocarbon discrimination, accuracy and general method performance. A series of relatively simple samples (from an interpretational point of view) were then subjected to analysis. For the purpose of optimization, both instrumental methods were slightly modified compared to the published reference methods.

2.3.1. Method validation

The performance of the transfer conditions was assessed by 10 injections of a mixture of *n*-alkanes (C_7-C_{40}). The repeatability (expressed as coefficient of variation – CV %) of each alkane (n = 10), considering the C_{10}-C_{40} range, was on average 3.0 % and 2.1 %, for Y/LC-GC and PTV/LC-GC, respectively. Figure 20 shows the comparison, between the alkane distributions, obtained with both instruments. The repeatability (CV %), of the average of the alkane areas from C_{10} to C_{40}, was 1.7 % and 1.6 % for the Y/LC-GC and PTV/LC-GC instruments, respectively. In the PTV/LC-GC instrument a slight discrimination above C_{35} can be observed; however, since results are usually expressed up to C_{35} for a toxicological evaluation, it was not considered as a relevant factor. Moreover, alkanes up to C_{12} are completely lost, but since MOSH contamination below C_{13} rarely occurs, and alkanes important from a toxicological viewpoint start from C_{16}, such a behavior did not affect the analytical results of the real samples.
Linearity was evaluated to verify that the LC band was always entirely transferred to the GC dimension. A calibration curve was constructed using the paraffin oil diluted in hexane, in the 0.5-250 mg/kg range (0.5, 1.25, 2.5, 6.25, 12.5, 25, 125, 250 mg/kg), with each level analyzed in triplicate (Figure 21). The external calibration curve was assessed by the least squares method to estimate the regression line; the linearity and goodness of the curve were confirmed using Mandel's fitting test (Draper and Smith, 1981). The significance of the intercept was established running a *t*-test. All the statistical tests were carried out at the 5 % significance level.
The results obtained with the two systems are reported in Table 8. Both calibration curves were linear in the range of the considered concentration, characterized by regression coefficients (R^2) of 0.9991 and 0.9989 for Y/LC-GC and PTV/LC-GC, respectively. The goodness of the curves was satisfactory for both of them ($F_{calc} < F_{tab}$), while the intercept was not significant in both cases ($p > 0.05$). The accuracy (expressed as % relative error related to the deviation from the amount added) and repeatability (expressed as CV %) of the two systems were evaluated by injecting ten-fold a mixture of paraffin oil (hydrocarbon range between C_{24} and C_{40}) and offset printing ink (hydrocarbon range between C_{14} and C_{20}), each diluted at the 5 mg/kg level. Repeatability was always below 5 % for both systems, and considering both humps. The perfect

overlay of the chromatograms, as shown in Figure 22, confirms the good repeatability. Using the PTV/LC-GC system accuracy was -12.7 % and -6.8 % for the printing ink and paraffin hump, respectively; while using the Y/LC-GC it was -5.0 % and -8.6 %, respectively.

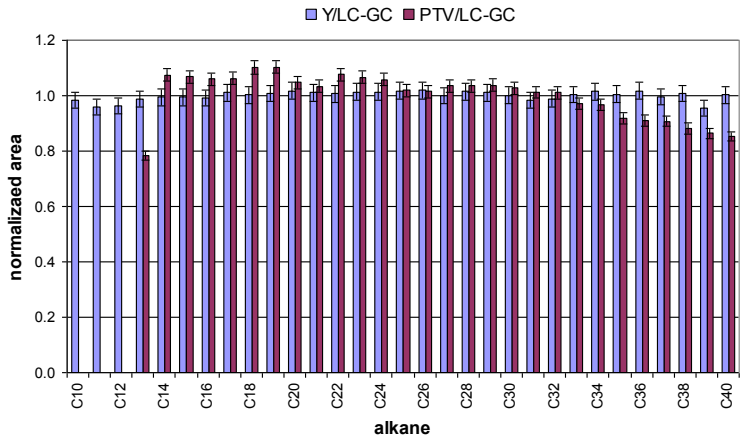


Figure 20. Comparison between n-alkane C_{10}-C_{40} distributions obtained by using both Y/LC-GC and PTV/LC-GC instruments (Purcaro et al., 2013b).

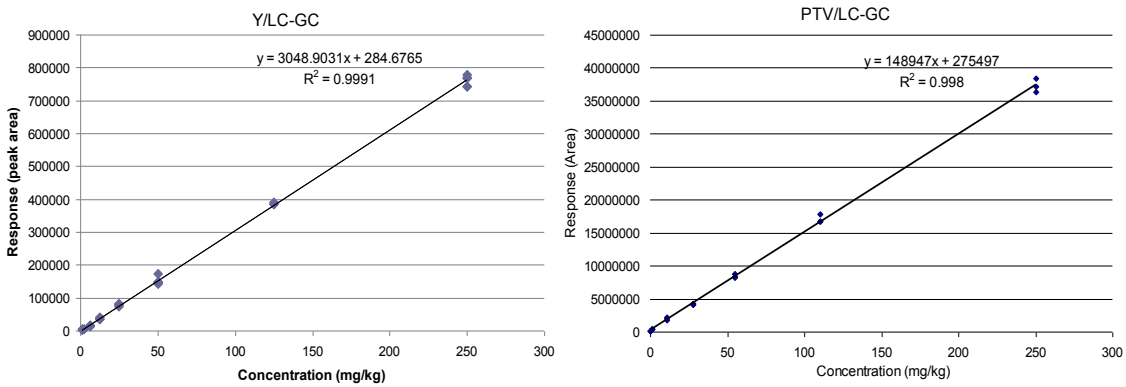


Figure 21. Calibration curve obtained by injecting different amounts of paraffin oil in the Y/LC-GC (left) and in the PTV/LC-GC (right) system.

An approximate estimation was made considering 3 and 10 times the signal to noise ratio (S/N) for the LOD and LOQ, respectively, and the values were extrapolated from the lowest point of the calibration curve performed with the paraffin oil (C_{24}-C_{40} range). The LOD and LOQ were evaluated as below 0.2 and 0.7 mg/kg, respectively, for both systems.

Table 8. Average quantification, standard deviation (SD), repeatability expressed as coefficient of variation), and accuracy obtained from analyzing ten times an offset printing ink and a paraffin mixture at a concentration of 5 mg/kg each (Purcaro et al., 2013b).

	PTV/LC-GC		Y/LC-GC	
	offset printing ink	***paraffin***	***offset printing ink***	***paraffin***
Average (µg/kg) n = 10	4.4	4.7	4.7	4.6
standard deviation (SD)	0.2	0.2	0.1	0.1
Repeatability (CV %)	3.8	4.5	2.1	2.1
Accuracy % (n = 10)	-12.7	-6.8	-5.0	-8.6

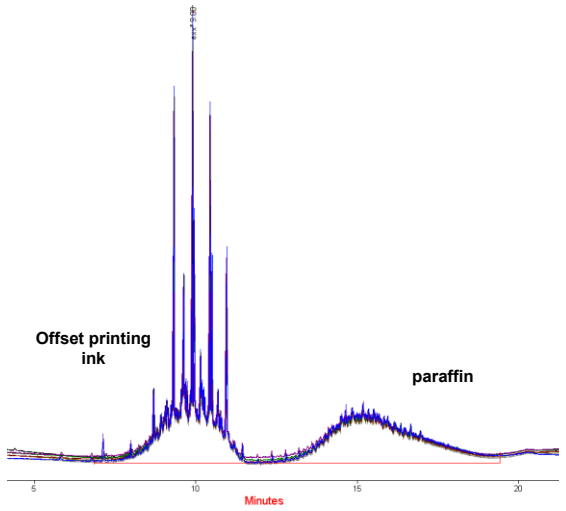


Figure 22. Ten overlaid chromatograms obtained using Y/LC-GC system injecting a mixture of paraffin oil and offset printing ink, each diluted at the 5 mg/kg level.

2.3.2. Food samples

The internal standard solution, previously described, was added to the samples prior to extraction. The analyses performed by using the Y-interface were quantified both with the internal (CyCy used as quantifier) and the external standard method, for comparative purposes, while external calibration was employed using the PTV/LC-GC system, since the loss of hydrocarbons up to C_{13} (including a significant percentage of CyCy) excluded the possibility to use internal standard calibration.

All the samples were extracted in duplicate and each extract was injected three times. The average results obtained from the duplicate extraction quantified using both internal and external standard methods were compared performing a *t*-test. No significant differences ($p>0.05$) were highlighted. The replicates were then considered together, to compare the two quantification methods applied, when using the Y/LC-GC system, and the two analytical systems employed. The data obtained by using the internal standard and the external standard methods were compared using a *t*-test, obtaining results not significantly different ($p>0.05$) (Table 9). This comparison demonstrated that, despite a much care needed to assure volume

control during the preparation procedure, in particular to avoid uncontrolled concentration of the extraction solvent, both internal and external standard methods gave comparable results.
The data obtained with external calibration, with the Y/LC-GC system, were then compared to those obtained by using the PTV/LC-GC instrument (Table 9). The results were not significantly different ($p>0.05$), except for the "pasta 2" sample; however, in this specific case the results derived were similar, namely Y/LC-GC *vs.* PTV/LC-GC was 1.3 *vs.* 1.6 mg/kg in the C_{10}-C_{25} range, and 1.3 *vs.* 1.1 mg/kg in the C_{25}-C_{35} range.
All the samples analyzed proved to be contaminated (except the extra-virgin olive oil), in the 2.5 to 70 mg/kg range. The MOSH hump was mainly centered in the C_{10}-C_{25} range, which can be related to the migration from the cardboard packaging. Figure 23 illustrates a direct comparison between the Y/LC-GC and PTV/LC-GC results for samples rice 1 and 2. As can be seen, the chromatogram profiles for both samples are very similar.

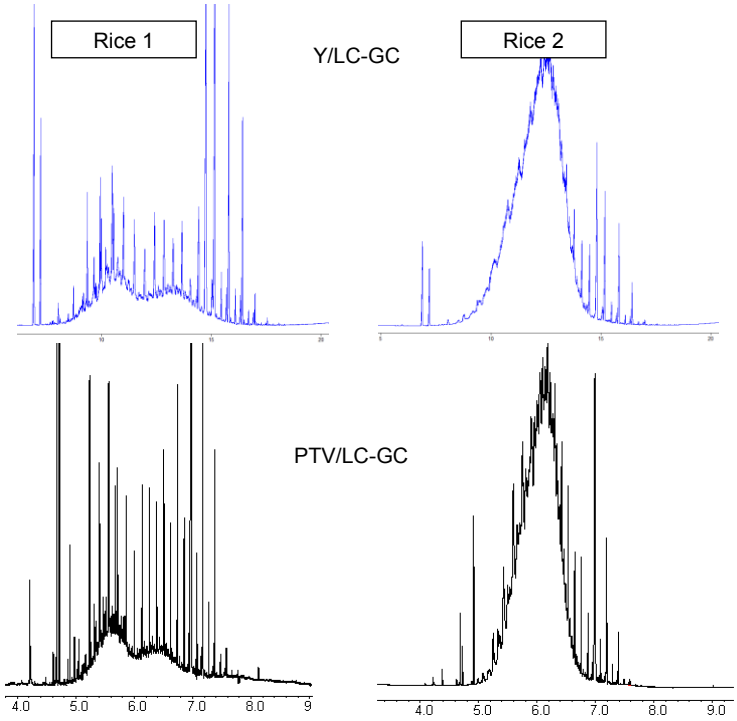


Figure 23. Direct comparison of the Y/LC–GC and the PTV/LC–GC results for samples rice 1 and 2 (Purcaro et al., 2013b).

The present investigation has demonstrated that both LC-GC interfaces provided a satisfactory and comparable performance for the determination of MOSH contamination in food products. In fact, even if some specific characteristic had to be considered to properly carry out the quantification procedure, the use of different instruments did not significantly interfere with the final analytical results.

Table 9. Data obtained from the analysis of 6 real samples analyzed using the Y/LC-GC and the PTV/LC-GC system. The data were quantified using internal and external standards with the Y/LC-GC interface, and the averages were compared running a t-test (p value). The data obtained with the PTV/LC-GC were quantified with the external standard and the results were compared running a t-test (p value) with the data obtained by using the Y/LC-GC system (external standard). All the extractions were performed in duplicate and injected in triplicate (Purcaro et al., 2013b).

		Y/LC-GC							**PTV/LC-GC**			
		External standard (ES)			**Internal standard (IS)**			***p***	**External standard (ES)**			***p***
		average (mg/kg)	*SD*	*CV %*	*average (mg/kg)*	*SD*	*CV %*	*ES vs IS*	*average (mg/kg)*	*SD*	*CV %*	*PTV vs Y*
Rice 1	MOSH C_{10}-C_{25}	**5.0**	0.3	6.6	**5.2**	0.4	7.2	0.323	**4.7**	0.3	6.0	0.118
	MOSH C_{25}-C_{35}	**1.1**	0.2	16.5	**1.1**	0.2	16.4	0.831	**0.9**	0.1	11.3	0.175
Rice 2	MOSH C_{10}-C_{25}	**65.2**	1.1	1.8	**64.7**	4.4	6.8	0.810	**66.4**	2.4	3.6	0.354
	MOSH C_{25}-C_{35}	**3.7**	0.2	4.5	**3.7**	0.3	7.4	0.830	**4.0**	0.1	2.8	0.057
Icing sugar	MOSH C_{10}-C_{25}	**8.5**	0.3	3.4	**9.0**	0.9	10.3	0.254	**8.4**	0.5	6.2	0.680
	MOSH C_{25}-C_{35}	**< LOQ**	-	-	**< LOQ**	-	-	-	**< LOQ**	-	-	-
Pasta 1	MOSH C_{10}-C_{25}	**2.6**	0.1	3.4	**2.6**	0.1	2.4	0.49	**2.7**	0.1	2.1	0.073
	MOSH C_{25}-C_{35}	**0.7**	0.1	12.0	**0.8**	0.1	13.6	0.63	**0.7**	0.0	6.6	0.162
Pasta 2	MOSH C_{10}-C_{25}	**1.3**	0.1	9.2	**1.3**	0.0	2.7	0.13	**1.6**	0.1	7.6	<0.05
	MOSH C_{25}-C_{35}	**1.3**	0.0	3.3	**1.3**	0.0	3.7	0.77	**1.1**	0.2	14.3	<0.05
Olive oil	MOSH C_{10}-C_{25}	**< LOQ**	-	-	**< LOQ**	-	-	-	**< LOQ**	-	-	-
	MOSH C_{25}-C_{35}	**< LOQ**	-	-	**< LOQ**	-	-	-	**< LOQ**	-	-	-

2.4. Conclusions

The present investigation has demonstrated that both LC-GC interfaces provided a satisfactory and comparable performance for the determination of MOSH contamination in food products. In fact, the use of different instruments did not significantly interfere with the end analytical results; even if some specific characteristic had to be considered to properly carry out the quantification procedure.

3. DETAILED ELUCIDATION OF HYDROCARBON CONTAMINATION IN FOOD PRODUCTS BY USING SPE AND COMPREHENSIVE GAS CHROMATOGRAPHY WITH DUAL (MS AND FID) DETECTION[3].

3.1. Background

Since MOSH and MOAH have a different toxicological relevance, it is important to quantify them separately. For instance, Biedermann and co-workers exploited the separation efficiency of an LC silica column (in an on-line LC-GC system), to separate the MOSH from the MOAH, and in turn, separate both from lipid interferents (Biedermann et al., 2009). Additionally, some off-line SPE methods, based on the use of a silver silica gel SPE cartridge, have been developed for MOSH and MOAH analysis (Moret et al., 2012a; BfR, 2012).
As already explained in the introduction, since FID provides virtually the same response *per* unit of mass of hydrocarbons, it is the best choice to reliably quantify the humps of unresolved complex mixtures (UCM) generated in mineral-oil applications. However, structural information is missing and detailed information on the mineral oil composition, in particular regarding the MOAH fraction, can give important toxicological information, and for the determination of the source of contamination. For this purpose, Biedermann and Grob used an MS detector, along with a comprehensive 2D GC (GC×GC) analysis (Biedermann and Grob, 2009). In the specific application, a pre-fractionation of the MOSH and MOAH classes, performed through off-line LC, was necessary to avoid the co-elution of steranes and hopanes (present in the MOSH fraction), with alkylated two- and three-ring aromatics (Biedermann and Grob, 2009). The GC×GC system was coupled alternatively with an MS system for identification purposes, and with an FID for quantification; thus, two runs were necessary to obtain both types of information. A GC×GC-MS system, after an off-line LC purification step, has also been employed by Mondello and co-workers to attain detailed information on MOSH contamination in baby foods (Mondello et al., 2012).
The aim of the present research was to simultaneously quantify and characterize mineral oil contamination in various foodstuffs, optimizing a GC×GC method with dual FID and MS detection. MOSH and MOAH pre-fractionation was carried out using the SPE method proposed by Moret and co-workers (Moret et al., 2012a). The quantitative results (both for the MOSH and the MOAH fractions) obtained were compared with those derived: I) by performing a large volume injection (LVI) GC-FID, after the same Ag-SPE fractionation step; II) through the LC-GC method described in ref. Biedermann et al., 2009.

3.2. Experimental

3.2.1. Sample extraction and purification

Samples of pasta, rice and icing sugar were purchased in a supermarket in Udine. The ground samples were extracted overnight with *n*-hexane, and then purified through SPE (Moret et al., 2012a). Briefly, a 1:2 food to solvent ratio was used to extract mineral oil from the samples. An aliquot of the extract was concentrated prior to the SPE clean-up on a 1 g silvered silica gel cartridge (Ag-SPE). Silver silica gel was prepared by adding a silver nitrate solution (0.75 g/mL) in Milli-Q water to a previously activated (400 °C overnight) silica gel, blended for about 30 min, and left to rest for 12 h; finally, the mixture was heated overnight at 75 °C to eliminate the residual water. The SPE glass cartridge was manually packed with 1 g of silvered silica before sample loading (250 µL). The sample was first eluted with 1 mL of *n*-hexane, which was discharged, then the MOSH group was eluted with 1.5 mL of *n*-hexane, followed by

[3] License Agreement with Elsevier number 3325231201013.

0.5 mL of *n*-hexane/dichloromethane (50:50 *v/v*). The following 0.5 mL *n*-hexane/dichloromethane fraction was discharged, and the MOAH class was eluted with further 7 mL of *n*-hexane/dichloromethane (50:50 *v/v*). The elution procedure is schematized in Figure 24. The eluted fractions were concentrated to 1 mL, prior to LVI-GC-FID and LC-GC analysis; with regards to GC×GC analysis, the fractions were reduced to a final volume of 100 µL to increase sensitivity, since LVI was not employed.

3.2.2. LC-GC analysis

The LC-GC instrument is shown in Figure 19, and the general characteristics are previously described (see the "reference method" in the paragraph *1.2.3* of this section).

3.2.3. LVI-GC-FID analysis

LVI-GC-FID analyses were carried out using a GC 7890A system (Agilent Technologies), equipped with a 7693 autosampler and an on-column inlet (Figure 24). Large volume injection was performed through the on-column/retention gap injection technique: 50 µL of the sample extract were injected onto a 5 m × 0.53 mm i.d. retention gap (uncoated pre-column), at a controlled injection rate (300 µL/min). The retention gap was connected to a 10 m × 0.25 mm i.d × 0.15 µm d_f separation column, coated with cross-linked PS-255 (1% vinyl, 99% methyl polysiloxane; MEGA, Milan, Italy). The oven temperature was programmed from 65 °C (4 min; solvent evaporation) to 330 °C (4 min) at 50 °C/min. The carrier gas (helium) flow rate was set at 8 mL/min (constant flow). The FID temperature was set at 350 °C. H_2, air and make up (He) flows were 35.0, 400.0 and 25.0 mL/min.
The data were acquired and processed by the ChemStation software. The MOSH area was determined through integration of the UCM hump up to *n*-alkane C_{25}, as suggested by the BfR (BfR, 2012), and then subtraction of the endogenous *n*-alkane peaks on the top of the hump was done. All sharp peaks located on top of the MOAH hump were subtracted. The position of the baseline was determined by repeated solvent injections. Quantification was performed using external calibration (Moret et al., 2012a).

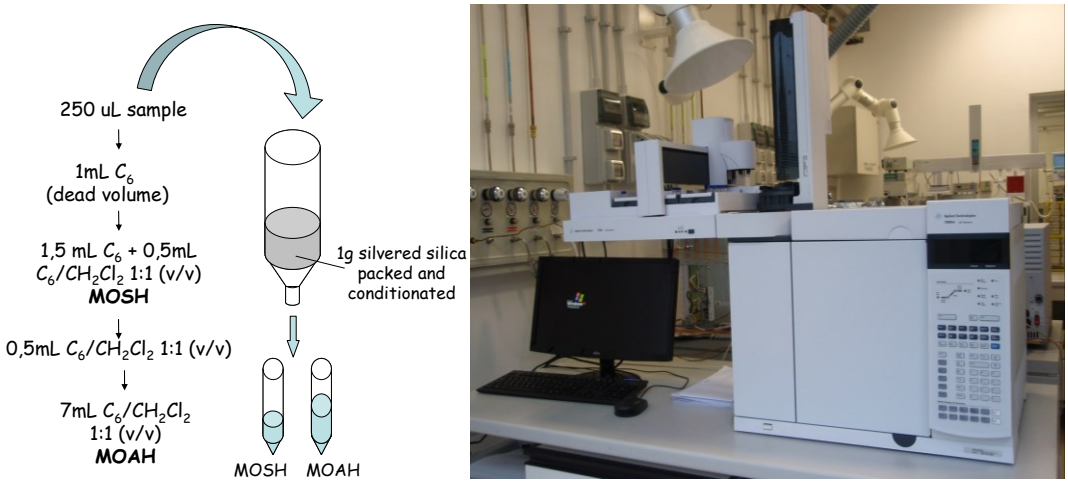


Figure 24. Scheme of MOSH and MOAH elution from a SPE cartridge packed with silvered silica (left) and GC-FID 7890A system (Agilent Technologies) used for a large volume injection (right).

3.2.4. GC×GC-FID/MS analysis

GC×GC applications were carried out on a system consisting of two GC2010 gas chromatographs, and a QP2010 Ultra quadrupole mass spectrometer (Shimadzu, Kyoto, Japan), shown in Figure 25.

The primary column was an SLB-5ms 30 m × 0.25 mm i.d. × 0.25 µm d_f column [silphenylene polymer, virtually equivalent in polarity to poly(5% diphenyl/95% methyl siloxane)], connected to an uncoated capillary segment (1.0 m × 0.25 mm i.d., used to create a double-loop), and to a 1.0 m × 0.10 mm i.d. × 0.10 µm d_f Supelcowax-10 (polyethylene glycol) segment (Supelco). The second column was connected through a capillary column splitter (SGE) to two uncoated capillaries, with these linked to the FID (0.5 m × 0.1 mm i.d.) and to the MS (0.25 m × 0.05 mm i.d.) systems.

Modulation was performed every 6 sec, by using a loop-type modulator (under license from Zoex Corporation, Houston, TX, USA). The duration of the hot pulse (350 °C) was 375 msec.

GC oven temperature program: 50 °C to 350 °C at 5 °C/min in the first oven, and 50 °C (hold 2 min) to 280 °C (hold 17 min) at 5 °C/min in the second oven. Carrier gas, helium, was supplied at an initial pressure of 243 kPa (constant linear velocity). The injection temperature was 360 °C. The injection mode and volume were: pulsed injection (300 kPa hold for 1 min) in the split mode (1:10); 6 µL. The FID was operated as follows: H_2 flow: 40.0 mL/min; air flow: 400.0 mL/min; make up (He): 30.0 mL/min.

The MS parameters: the samples were analyzed in the full scan mode with a scan speed of 20,000 amu/s and a mass range of 40-510 *m/z*; spectra generation frequency: 33 Hz; interface and ion source temperatures were 250 °C and 200 °C, respectively. MS ionization mode: electron ionization.

Bidimensional visualization was carried out using the ChromSquare v. 1.5 software (Shimadzu Europe, Duisburg, Germany). The MS libraries used for spectral matching were NIST05, FFNSC, and FAME library.

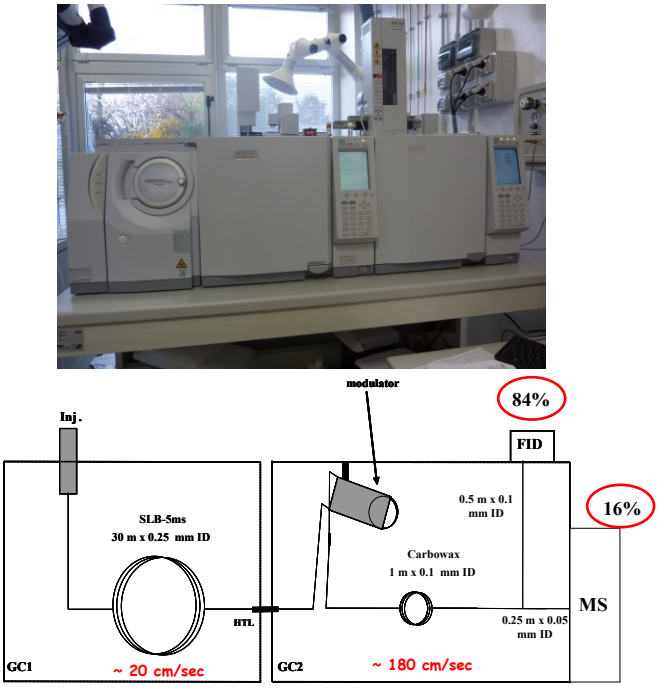


Figure 25. GC×GC-FID/MS system (Shimadzu) and scheme of the column set.

3.3. Results and discussion

3.3.1. GC×GC-FID/MS optimization and validation

Optimization of the GC×GC conditions was carried out by using a sample of offset printing ink, formed mainly of MOSH, and of less than 10 % of MOAH. Pre-fractionation on the Ag-SPE cartridge was necessary because, if the offset ink is injected as it is, the MOSH group would overload the system, while the MOAH constituents would be scarcely visible on the chromatogram (Figure 26).

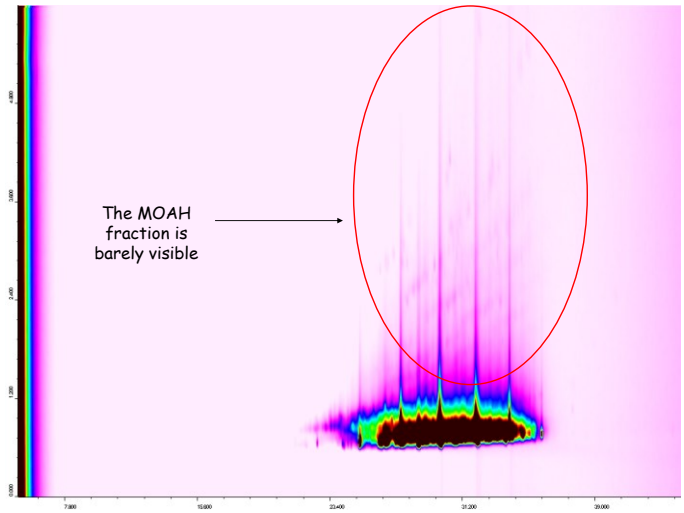


Figure 26. GCxGC-FID trace of the off-set printing ink injected as it is (6µL), without pre-fractioning.

To reach a satisfactory FID sensitivity, the split ratio between the FID and MS units was chosen. In particular, an MS-linked 0.25 m × 0.05 mm i.d. capillary, and a 0.5 m × 0.1 mm i.d. FID-connected one were used. Two branches with different diameters were employed, because the two detectors work under different pressure conditions. Thus, to divert the majority of the flow to the FID, an excessive length of «MS» column was needed to generate an adequate resistance.

Such a splitting configuration satisfied both the physical constraints of the second GC oven (MS transfer line: 20 cm; distance between the transfer line and the FID entrance), and the flow conditions, with about 84 % and 16 % of the effluent reaching the FID and MS at 50 °C, respectively. The split ratio changed slightly during the analysis time, with about 87 % and 13 % of the effluent diverted to the FID and MS at 300°C. Since the calibration curve was built under the same analytical conditions, the quantitative results were not affected.

The dual-detection GC×GC experimental conditions were optimized with the aim of maintaining the same chromatographic performance as that obtained by using an MS-only system, as illustrated in Figure 27. In the latter approach, using the same analytical columns, the head pressure (*circa* 150 kPa) was selected to generate about 20 cm/s and 210 cm/s in the first and second dimension, respectively. In the dual detector system, a 243-kPa inlet pressure generated the same linear velocity in the first dimension (to maintain approximately the same elution temperature), and a slightly lower one in the second (180 cm/sec).

A 6-point calibration curve was constructed through the FID trace, using diluted solutions of paraffin oil in *n*-hexane, in the 0.35-24 mg/kg range (Figure 28). Each point was derived through analysis in duplicate. The least squares method was applied to estimate the regression

line; the linearity and the goodness of the curve were evaluated through the coefficient of regression (0.9993), and a visual inspection of the residual plot, all confirmed using Mandel's fitting test ($F_{calc} < F_{tab}$) (Draper and Smith, 1981). The CV % of the slope was 1 %. The significance of the intercept (p = 0.03) was established running a *t*-test at the 5 % significance level. The repeatability of the analysis was less than 1 %, calculated at the lower point of the calibration curve ($n = 3$).

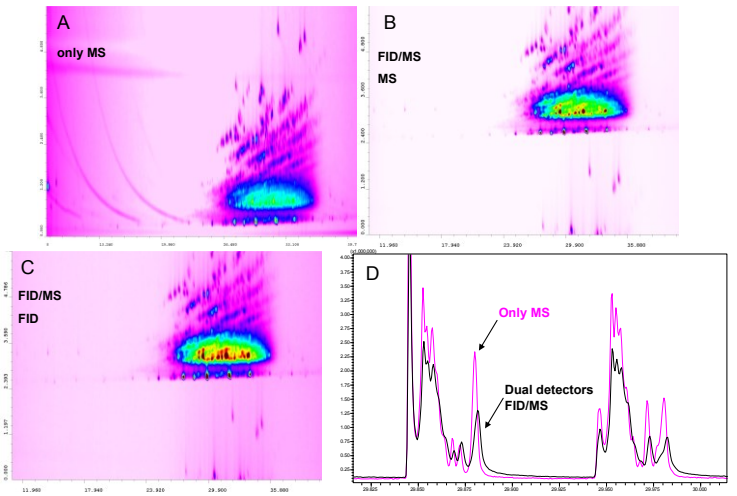


Figure 27. GCxGC traces obtained from the printing ink MOAH fraction analysis using only the MS (A) and the dual detector system MS/FID (B and C). D) Comparison of the raw TIC chromatogram expansions obtained (printing ink) using a GC×GC-MS system (pink line), and a GC×GC-FID/MS one (black line).

An approximate estimation of the LOQ was made by considering the standard deviation (n = 3) calculated at the lower point of the calibration curve and multiplied by 10. The LOQ was estimated to be approximately 1.2 mg/kg. The latter could be increased using an LVI method, such as the on-column, retention gap technique, or by equipping the system with a programmed temperature vaporizer injector.

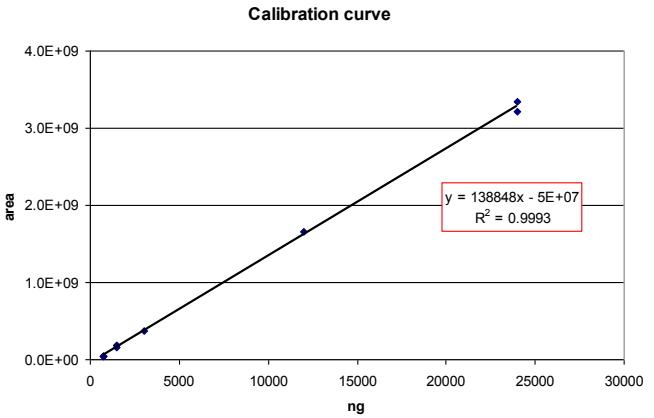


Figure 28. Calibration curve constructed through the FID trace, using diluted solutions of paraffin oil in n-hexane, in the 0.35-24 mg/kg range.

3.3.2. Food samples

For a more in-depth GC×GC investigation, few samples among those analyzed by using both Ag-SPE-LVI-GC-FID and LC-GC-FID approaches were chosen. These samples were characterized by a MOAH hump with sharp peaks on the top and a different chromatogram profile by using the two different techniques. As example, the Ag-SPE-LVI-GC-FID and LC-GC results, relative to a pasta sample, are illustrated in Figure 29, showing significantly different MOAH profiles. In the LC-GC chromatogram, the characteristic presence of groups of diisopropylnaphthalene (DIPN) and of "wood" compounds highlighted the typical paperboard contamination (Biedermann et al., 2012b). On the other hand, several high-intensity unidentified peaks sat on the top of the MOAH hump were present in the Ag-SPE-LVI-GC-FID chromatogram.
Since all the other samples considered in this work presented the same differences between the two approaches, it can be concluded that a series of compounds were removed by the silica LC column, but not by the Ag-SPE cartridge. In the present case, the potential information available through the use of a GC×GC-FID/MS system seemed to be necessary, more than advisable. In fact, the enhanced-resolution power of GC×GC appeared to be the best solution to separate the unknown peaks from the rest of the sample constituents, thus delivering fully-isolated peaks to the MS dimension.

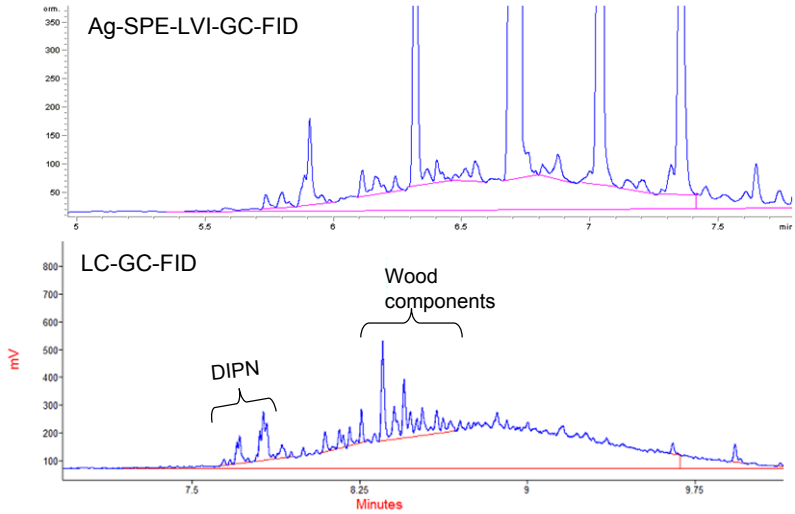


Figure 29. Chromatograms relative to a pasta sample analyzed through Ag-SPE-LVI-GC-FID and LC-GC methods. DIPN: diisopropylnaphthalene (adapted from Purcaro et al., 2013a).

Both the MOSH and the MOAH fractions, relative to the pasta, icing sugar and rice samples, were quantified up to *n*-C_{25} (BfR, 2012), after the analysis using the three techniques above mentioned. During integration all the natural alkanes were subtracted from the MOSH hump, as well as all the unknown peaks from the MOAH one. In the case of GC×GC-FID quantification, the "so-called" polygonal integration function was used, which enabled the definition of a polygonal area in which all the integrated peaks are automatically summed, and the data relative to each peak is saved as well. In this way, the undesired peaks can be easily selected, and then subtracted from the total area. To determine the MOAH amount, the area integrated was confined by a polygon as reported in Figures 30, 31 and 32, which show GC×GC-MS and LVI-GC-FID chromatograms for the pasta, rice and icing sugar samples, respectively. The polygonal area in the 2D plot, contains the peaks integrated with the automatic software function.

It did not include the aliphatic hydrocarbons, present as contaminant (deriving from the solvent), in such a fraction. The presence of such a low level of paraffins is not a problem since they can be easily separated from the fraction of interest. As shown in Table 10, the values obtained by applying the three techniques were very similar, both for the MOSH and the MOAH fractions, for all samples. In fact, peak subtraction from the humps enabled reliable quantification, despite the interfering compounds present in the GC analysis, using the Ag-SPE purification step.

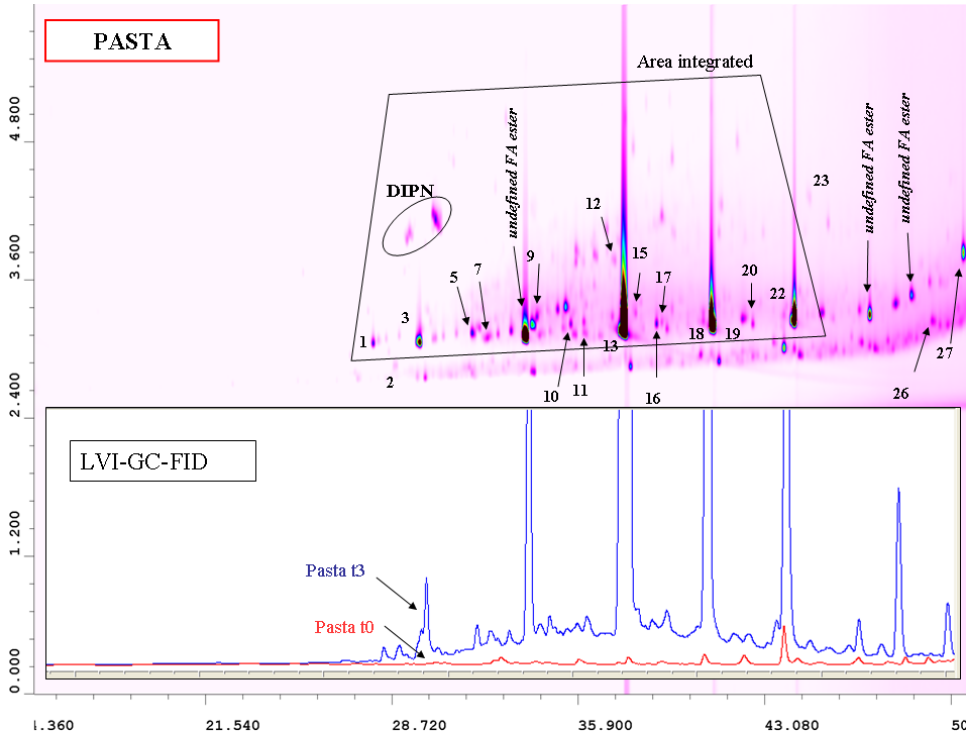


Figure 30. Upper trace: GC×GC-MS chromatogram, relative to the MOAH fractions of a pasta sample; lower traces: LVI-GC-FID chromatograms relative to the pasta sample, before packing (t0) and after three months of storage in a box made of recycled paperboard (t3). Identification as reported in Table 11. The polygonal area indicates the integrated area, before subtraction of the single peaks. FA: fatty acid; DINP: diisopropylnaphthalene (Purcaro et al., 2013a).

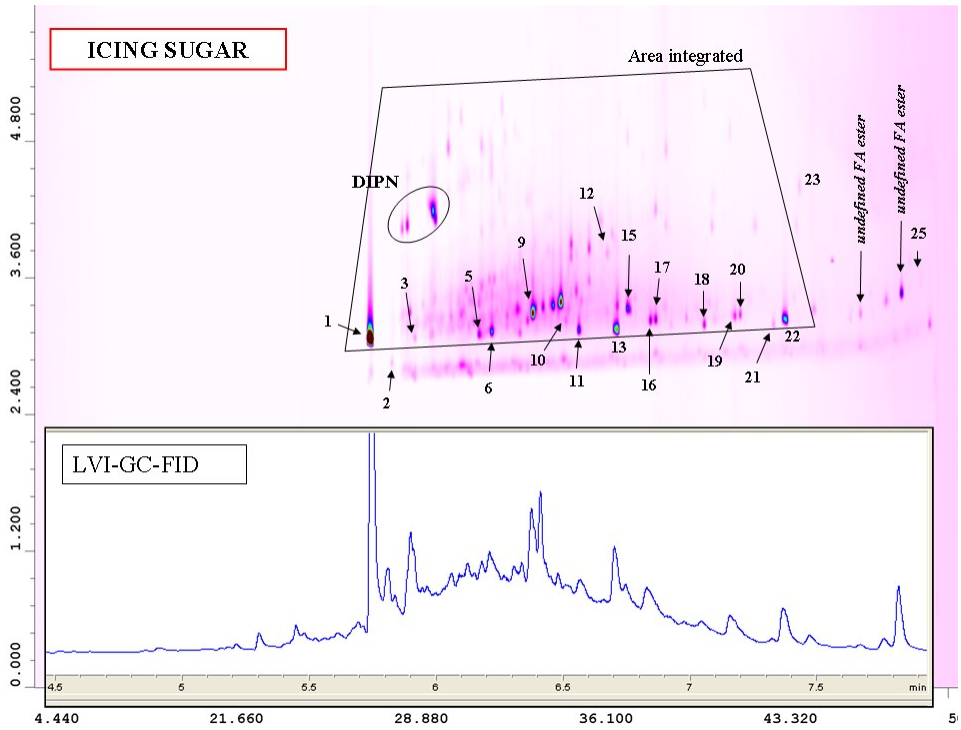


Figure 31. Upper trace: GC×GC-MS chromatogram, relative to the icing sugar MOAH fraction; lower trace: LVI-GC-FID chromatogram relative to the same compounds. Identification as reported in Table 11 (Purcaro et al., 2013a).

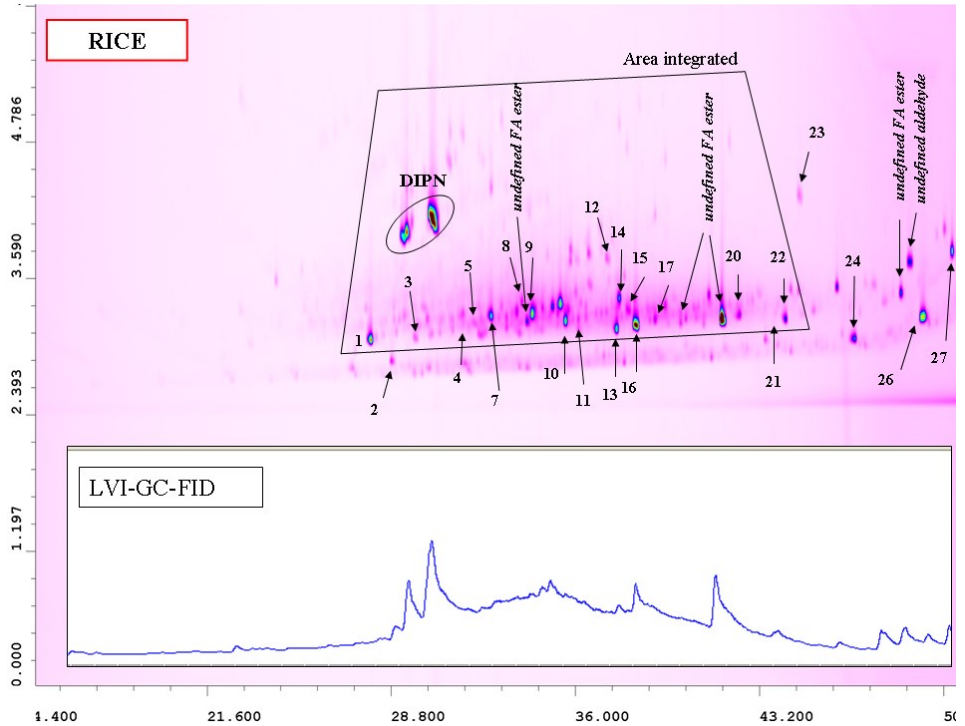


Figure 32. Upper trace: GC×GC-MS chromatogram, relative to the rice MOAH fraction; lower trace: LVI-GC-FID chromatogram relative to the same compounds. Identification as reported in Table 11 (Purcaro et al., 2013a).

Table 10. Quantification values relative to the MOSH and the MOAH fractions, in samples of pasta, icing sugar, and rice, using Ag-SPE-LVI-GC-FID, Ag-SPE-GC×GC-FID/MS, and LC-GC-FID (adapted from Purcaro et al., 2013a).

Sample	Technique	MOSH $<C_{25}$ (mg/kg)	MOAH$< C_{25}$ (mg/kg)
PASTA	Ag-SPE-GC-FID	3.8	2.1
	Ag-SPE-GC×GC-FID/MS	3.6	1.7
	LC-GC-FID	3.8	1.7
ICING SUGAR	Ag-SPE-GC-FID	8.6	1.2
	Ag-SPE-GC×GC-FID/MS	8.3	1.2
	LC-GC-FID	8.5	1.4
RICE	Ag-SPE-GC-FID	35.9	2.5
	Ag-SPE-GC×GC-FID/MS	33.8	2.0
	LC-GC-FID	34.2	2.4

3.3.3 GC×GC-MS elucidation of the MOAH profile

For the three samples, the peaks present in the GC×GC chromatograms were tentatively-identified on the basis of database match similarities (≥ 80 %) and in accordance with linear retention indices (LRI), contained in the MS database used (see section 3.2.4). Since a generally-accepted process for the calculation of GC×GC LRI values has not been defined, these retention data were calculated in a one-dimensional manner; furthermore, a rather wide LRI filter window (± 25 units) was applied (eliminating incorrect matches), to compensate for the retention effects of the second polar column. The tentatively identified peaks, along with experimental and database LRI, are shown in Table 11.

Two compounds were out of the LRI filter range, specifically, octyl dodecanoate and octyl tetradecanoate were characterized by a difference of +58 and +59 units, respectively. It must be added that, in these cases, the database LRI values, which were reported in the NIST web site (http://webbook.nist.gov/chemistry/), were attained using a methyl silicon capillary column [(Ultra-1) 25. m × 0.32 mm × 0.25 μm], while in the present work a 30 m × 0.25 mm ID × 0.25 μm silphenylene polymer phase column (see section 3.2.5) was employed. Since the similarity matches were acceptable, and the spatial position in the bidimensional chromatogram gave another idea on analyte structure, these analytes were given a name. The identification of the specific aromatic compounds, present in the MOAH "cloud", was outside the objective of the present research; however, even if desired, the identification of such constituents could not have been performed with sufficient reliability (only the chemical class can be confirmed), because of the low amounts present in the sample. However, it was possible to derive the MOAH quantities (FID trace) and patterns, which are highly important to determine the contamination source, as discussed by Biedermann and Grob (Biedermann and Grob, 2009). The unknown peaks located on the top of the MOAH humps were identified as esterified fatty acids (FA). Such esters were not present in the MOAH fraction analyzed using LC-GC-FID, since the FA esters were removed by purification on the silica column; on the other hand, silver silica is more effective in removing olefins compared to silica, while it resulted less selective for the esters. The presence of FA esters in the eluate of the SPE method can be avoided by introducing a previous SPE step on the silica gel. The use of a mixed sorbent bed may be an alternative.

However, as discussed in section 3.3.2, their presence did not affect reliable quantification as long as these compounds were subtracted from the hump area. The esterified FAs derived from the paperboard packaging. In fact, as shown in Figure 30, the sample of pasta analyzed prior to box packing showed no sign of MOAH contamination (t0), while after three months of storage (t3) it did. The possibility to use offset printing ink based on vegetable oils has been known for more than 15 years (Sabin et al., 1997a; Sabin et al., 1997b; Erhan and Bagby, 1995), though its use has become more frequent since contamination from paperboard packaging has become an issue of concern. A series of peaks in the GC×GC-MS chromatograms were defined as "undefined FA esters", since the MS spectrum was clearly that of a FA ester, although the database searches gave different possible "homologue" matches with good similarities, but not always reporting the LRI. Thus, it was not possible to assign these compounds with sufficient confidence, even though they were marked in the figures, as their FA ester nature was evident. It also could not be excluded that such compounds were not present in the MS database. For instance, in the pasta sample (Figure 30), of the four main peaks, only three were assigned, namely octyl dodecanoate, octyl tetradecanoate, and octyl hexadecanoate. However, it can be deduced from the GC×GC position that the "undefined FA ester" corresponded to octyl decanoate, even though such a compound was not present in the MS databases available. A good visual similarity was observed comparing the spectrum with that reported in the NIST web site, however no information on the LRI value was found, thus this compound remained unassigned. It is interesting to note that practically the same compounds were found in all the samples analyzed, though with a very different quantitative profile, probably due to a different ink formulation or/and to a different source of contamination. It can be hypothesized that the vegetable oil offset printing ink was directly used in the pasta packaging (highly contaminated),

while it was present, in different amounts, in the recycled fiber entering the packaging of the other two samples.

Table 11. Compounds identified in the "MOAH" GC×GC–MS analysis; database-derived (LRI Lib) and experimental LRI (defined as LRI) values, and spectral similarities (MS %) (adapted from Purcaro et al., 2013a).

	compounds	pasta MS %	icing sugar MS %	rice MS %	LRI	library LRI
1	Isopropyl dodecanoate	95	94	94	1623	1627
2	Dioctyl ether	93	93	95	1664	1688
3	2-Ethylhexyl octanoate	88	86	88	1705	1715
4	Ethyl tetradecanoate	-	-	80	1796	1795
5	Isopropyl tetradecanoate	94	94	94	1824	1828
6	Isoamyl dodecanoate	-	94	-	1843	1844
7	6,10,14-Trimethyl-2-pentadecanone	-	-	95	1843	1846
8	2-Heptadecanone	-	-	95	1891	1906
9	Methyl hexadecanoate	94	93	94	1927	1925
10	Ethyl hexadecanoate	94	80	92	1990	1993
11	Isopropyl hexadecanoate	92	90	90	2023	2024
12	Abietatriene	86	80	85	2084	2075
13	Octyl dodecanoate	85	84	84	2100	2158
14	2-Nonadecanone	-	-	90	2106	2106
15	Methyl octadecanoate	92	94	90	2128	2124
16	Dodecyl octanoate	93	92	-	2173	2177
17	n-Butyl hexadecanoate	92	95	90	2196	2188
18	octyl tetradecanoate	84	85	-	2300	2359
19	Tetradecyl octanoate	86	91	-	2375	2375
20	n-Butyl octadecanoate	84	91	89	2388	2388
21	Pentadecyl octanoate	-	83	81	2474	2475
22	octyl hexadecanoate	85	82	81	2500	2505
23	Di(ethylhexyl) phthalate	95	93	94	2542	2550
24	1-Tetracosanol	-	-	94	2693	2710
25	Squalene	96	96	-	2822	2847
26	1-Hexacosanol	95	-	95	2874	2877
27	Tetradecyl tetradecanoate	82	81	91	2970	2950

3.4. Conclusions

The need for on-line LC-GC instrumentation, for the determination of MOSH and MOAH, is not mandatory, since the same analytical outcome was obtained when using off-line Ag-SPE, for the sample-preparation step. Additionally, the use of a high-resolution separation method, such as GC×GC, can help greatly in attaining much more detailed information on the composition of unresolved complex mixtures (mineral oil "humps"). Furthermore, both MS and FID detectors are necessary, being the FID the most suitable for quantification purposes.

4. OPTIMIZATION OF PRESSURIZED LIQUID EXTRACTION (PLE) FOR MOSH AND MOAH DETERMINATION IN CARDBOARD AND PAPER INTENDED FOR FOOD CONTACT[4].

4.1. Background

Cardboard packaging represents an important source of food contamination with MOSH and MOAH, especially when recycled fibers or mineral oil based printing inks are used.
Rapid MOSH and MOAH determination in packaging material is a useful tool for monitoring purposes and migration studies.
The slowest stages of an analytical method often are represented by extraction and sample preparation. In 2010, Lorenzini e co-workers (Lorenzini et al., 2010) proposed a method for MOSH and MOAH extraction from cardboard, based on classical solvent extraction. Briefly, the cardboard is extracted with a mixture of hexane/ethanol 1:1 (*v*/*v*), able to insure good extraction yields and with a limited extraction of high-boiling material disturbing GC analysis. The ethanol is then separated by adding an appropriate amount of water, and the hexane phase is used for LC-GC analysis.
Pressurized Liquid Extraction (PLE), often used for extraction of environmental contaminants from waste samples and soils (Ezzell et al., 1995; Ramos et al., 2002), is based on the use of high temperatures and pressures for extraction from solid samples. The high pressure allows to maintain liquid the solvent at temperatures well above its atmospheric boiling point, and the high temperatures reduce the viscosity of the solvent, increasing its ability to wet the matrix and solubilize the analytes, and cause a higher diffusion and desorption rate (Richter, 2000; Richter and Raynie, 2012).
The purpose of this study was to optimize a PLE method for the extraction of MOSH and MOAH from cardboard and paper intended for food contact.

4.2. Experimental

4.2.1. Samples and extraction

Unprinted and printed cardboard samples of different thicknesses (sample 1: 210 g/m^2 and sample 2: 350 g/m^2), were directly taken from producers. Cardboard boxes used in direct contact with foods (printed recycled and virgin cardboard) and other paper samples (paper bags used for bread, baking paper, baking cups, etc.) employed for analysis and method comparison, were obtained from the market.
PLE was carried out using a SpeedExtractor model E-916 (Büchi, Flawil, Switzerland) equipped with six 10-mL stainless steel extraction cells (Figure 33). Cellulose filters were placed at the exit of the cells to prevent clogging of the metal frit. The cells are loaded with two 2-mL stainless steel bars (expansion elements) to fill part of the void volume and reduce solvent consumption and with the sample (1 g for cardboard, 0.2-0.5 g for paper samples) cut in strips (6 cm x 2-4 mm). Finally, 20 µL of the internal standard working solution were added into the cell for quantification and to verify LC-GC performance and efficient MOSH and MOAH separation as described by Biedermann and Grob (Biedermann and Grob, 2012a). For PLE optimization, different solvents or mixtures (hexane, ethanol, hexane/ethanol), extraction temperature (from 30 °C to 100 °C), extraction times (5, 10 min) and number of extraction cycles (1-3), were tested.

[4] License Agreement with Elsevier number 3325231049915.

Figure 33. Pressurized Liquid Extractor from Büchi; 10-mL stainless steel extraction cells with cellulose filters, metal frits and 2-mL expansion elements.

4.3. Results and discussion

4.3.1. Method optimization

For the extraction of MOSH and MOAH from paper and board using PLE, a rapid and simple sample preparation is required. In particular, the paperboard was cut in strips and inserted into a 10 mL cell containing two 2-mL expansion elements placed on the bottom to reduce solvent consumption (up to 3 expansion elements can be used per cell to further reduce solvent consumption). To prevent a possible external contamination, it is necessary to pre-clean, with acetone and hexane, all the parts in contact with the solvent, namely extraction cells, cellulose filters, metal frits and the expansion elements.
The definitive optimized extraction conditions, such as extraction solvent, number of extraction cycles, extraction temperature and extraction time, are reported in Table 12. A default value of 100 bar, which guaranteed that the solvent remained liquid, was selected for this application, since pressure adjustments have, in general, a little impact on recovery (Richter, 2000).

Table 12. Optimized extraction conditions for paper and paperboard

Speed Extractor E-916 (6 positions)	
Temperature	60 °C
Pressure	100 bar
Cells (volume)	10 mL
Solvent	hexane
Cycles (number)	2
- Heat up	1 min
- Hold	5 min
- Discharge	2 min
Flush with solvent	0 min
Flush with gas (N_2)	1 min
Total extraction time	30 min

4.3.1.1. Extraction solvent

One of the most important parameter to optimize for a PLE extraction is the choice of the solvent. Firstly, the solvent must be able to solubilize the target compounds limiting co-extraction of interferences. A mixture hexane/ethanol 1:1 (*v/v*) combines the advantages of the two solvents but complicates the sample post-treatment, since a washing step with water (to separate ethanol), followed by centrifugation, is required.
Figure 34-A shows MOSH and MOAH results, in terms of the minimum, maximum and average of 2 replicates, from C_{10} to C_{24} (relevant for migration) and from C_{10} to C_{35} (relevant from a toxicological point of view and which could migrate under wet-contact condition) for two different recycled cardboards using hexane, ethanol and the mixture ethanol/hexane 1:1 (*v/v*). The three different solvents have similar extraction power for both MOSH and MOAH. A slightly higher extraction yield of high boiling compounds was observed in one case for hexane. There were no differences even when testing the solvents on printed and unprinted paperboards, as shown by LC-GC chromatograms in figure 34-B. The unprinted cardboard (213 and 56 mg/kg of MOSH and MOAH up to C_{24}, respectively) shows the typical MOSH pattern of a recycled cardboard characterized by a first narrow hump ranging from C_{14} to C_{20} and centred on C_{17}, coming from newspaper inks present in recycled fibres and a second larger hump from about C_{15} to over C_{40} (in this case centred on C_{28}) with long-chain *n*-alkanes on the top (waxes) (Biedermann and Grob, 2012b). An increased MOSH and MOAH content (in the C_{13}-C_{23} range) is observed in the printed cardboard, due to the presence of mineral oil-based printing ink (1277 and 189 mg/kg of MOSH and MOAH up to C_{24}, respectively).

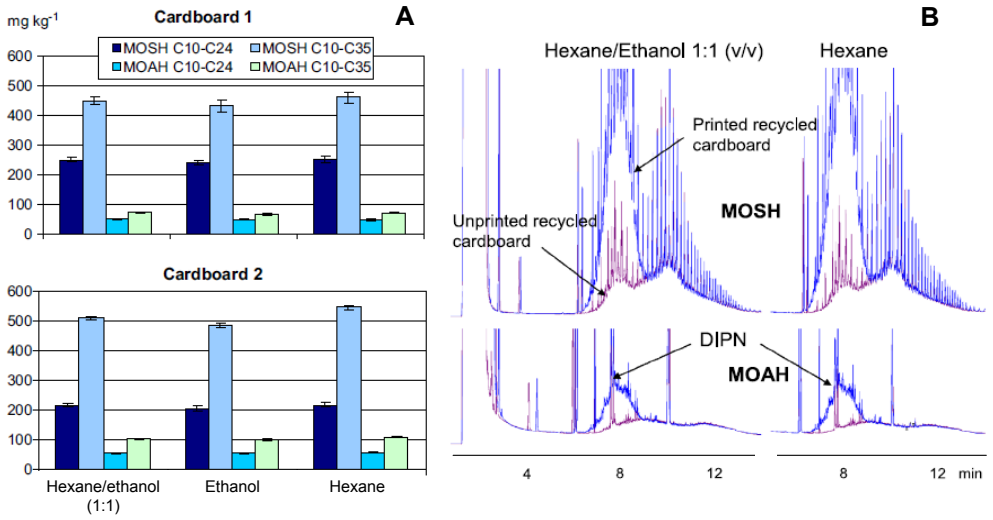


Figure 34. A) Comparison of PLE (1 cycle at 60 °C for 5 min) data obtained by using different extraction solvents, namely hexane/ethanol (1:1 v/v), ethanol and hexane. Results are expressed as an average of two replicates (bars), minimum and maximum values (vertical lines). B) Overlay of LC-GC-FID traces obtained from the analysis of printed (mineral oil printing inks) and unprinted recycled paperboards extracted with PLE using hexane/ethanol (1:1 v/v) and only hexane. DIPN: diisopropylnaphthalenes (Moret et al., 2013).

The lower extraction power towards low molecular weight hydrocarbons, observed for hexane at ambient conditions by Lorenzini and co-workers (Lorenzini et al., 2010) was not observed using the PLE technique. Thus, the hexane was chosen as the extraction solvent both for its efficient extraction power and for the advantage of avoiding any treatment of the extract before the injection in the LC-GC system. Solvent consumption depends on the volume of the cell and

the number of extraction cycles. By using two 2-mL expansion elements in 10 mL extraction cells, the solvent consumption is about 15 mL per sample.

4.3.1.2. Extraction temperature and time

Different extraction temperatures, namely 30, 50, 60, 70 and 100 °C, were tested in duplicate on an unprinted recycled cardboard performing a one-cycle extraction with hexane and using the extraction conditions reported in Table12. The temperature of 60 °C allows the extraction of 20 % more than that at 30 °C for MOSH and MOAH up to C_{35}, but no appreciable differences were observed when the temperature was further increased (Figure 35).
The extraction was performed for 5 and 10 minutes, obtaining the same results, which, otherwise, were not quantitative for all samples (as discussed in the next paragraph).

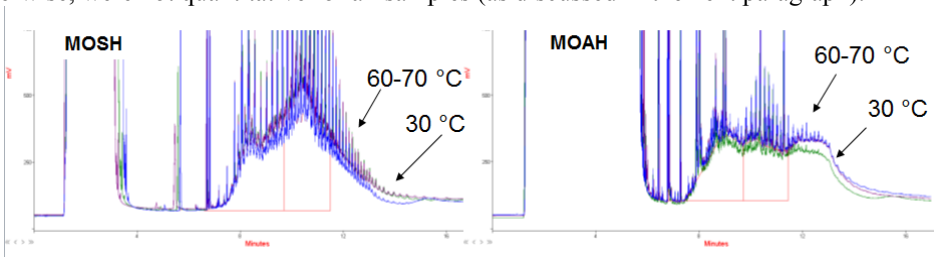


Figure 35. Overlay of MOSH and MOAH chromatograms of an unprinted paperboard extracted at different temperatures.

4.3.1.3. Number of cycles and extraction yield

One or more PLE cycles are usually necessary to obtain quantitative extraction yields, depending on the matrix and its interactions with the analytes (Richter, 2000; Richter and Raynie, 2012).
The efficiency of one- and 2-cycle extractions was tested: a 2-cycle extraction always gave a quantitative extraction (less than 2 % of the total contamination found in a third separate cycle), while 1-cycle extraction reached 76-100 % of recovery and was influenced by cardboard thickness, as visible in figure 36. The effect of a wash (with 5 mL of hexane) after a one-cycle extraction was also tested to verify if a second cycle could be avoided, but the extraction yields did not increase.

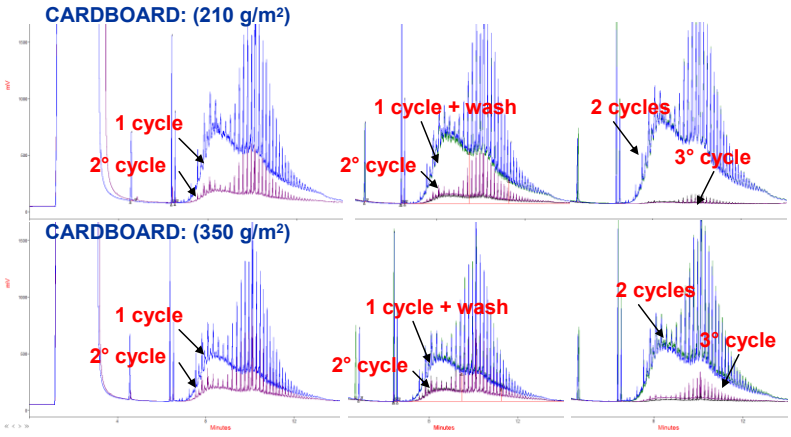


Figure 36. LC-GC-FID chromatograms (MOSH) of two unprinted cardboards of different thickness (210 and 350 mg/m^2), obtained performing a 1-cycle extraction along with a second cycle, a 1-cycle extraction plus a wash with 5 mL of hexane along with a second cycle and a 2-cycle extraction along with a third cycle.

4.3.1.4. Repeatability and recovery

Eight replicate extractions (in two different days), followed by on-line LC-GC analysis, on different aliquots of the same unprinted recycled cardboard, were performed to assess the repeatability of the PLE method. The MOSH and MOAH overlaid chromatograms are reported in Figure 37. A residual standard deviation lower than 5 % was obtained both for MOSH and MOAH.

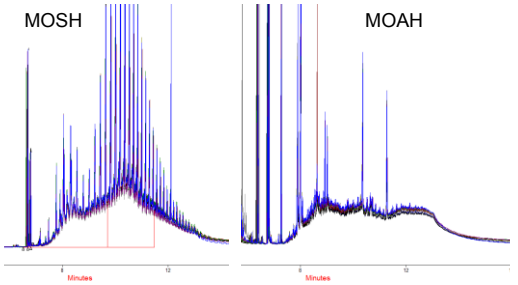


Figure 37. Overlay of eight LC-GC-FID chromatograms (MOSH and MOAH) obtained from the replicate analysis of an unprinted recycled cardboard extracted with the optimized PLE method.

Quantification of mineral oil, as well as performance verification of the LC-GC analysis, are based on volatile internal standards (C_{11}, CyCy, C_{13}, TBB, 1-MN, 2-MN). Recovery tests were carried out to assess possible losses of volatiles due to the purge with nitrogen (1 min) carried out at the end of the cycle to eliminate residual solvent.

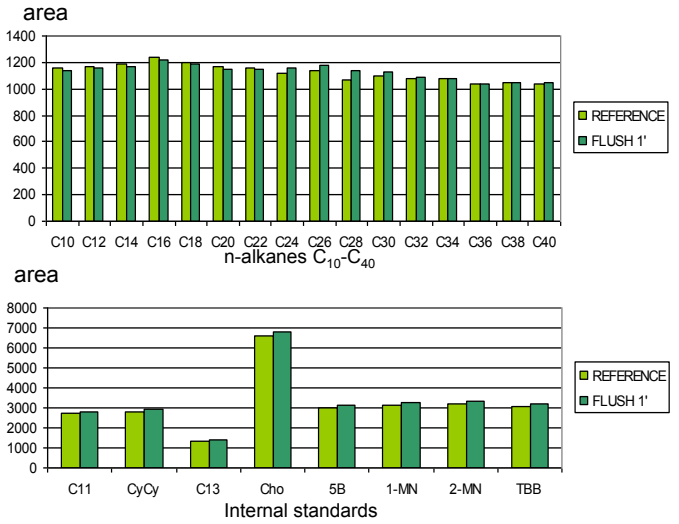
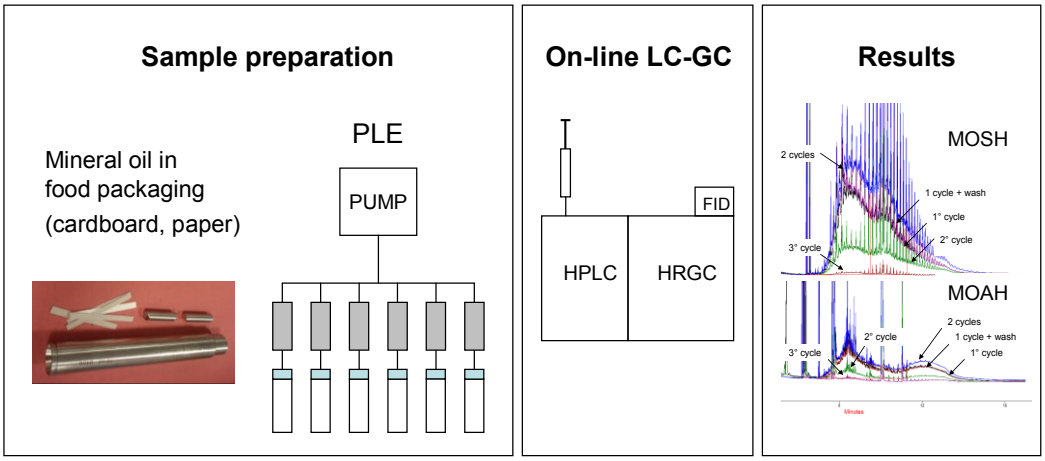


Figure 38. Comparison between areas of n-alkanes (top) and internal standards solutions (bottom) directly injected (reference) and injected after 1 min of nitrogen flush (flush 1').

For this purpose, after the addition of a known amount of internal standard solution (20 µL) and of *n*-alkanes C_{10}-C_{40} on the extraction cells, a single cycle with hexane (at 60 °C for 5 min) was performed, applying a 1-min nitrogen purge at the end. The areas (mean of two replicates) after the injection of the extracts into the LC-GC system (reported in Figure 38) were compared with that obtained from the direct injection of the same solutions of *n*-alkanes and internal standard.

With a nitrogen flush of 1 or 2 minutes, no losses were observed, also for the most volatile compounds.
This PLE method developed for MOSH and MOAH extraction from cardboard and paper materials delivered results similar to those obtained with the method based on classical solvent extraction developed by Lorenzini et al. (Lorenzini et al., 2010); furthermore a low sample manipulation and extraction time are required. The complete procedure for mineral oil analysis in paper and board using PLE and the LC-GC-FID system for the analytical determination is schematized in Figure 39.

Figure 39. Scheme of the optimized procedure for mineral oil analysis using PLE and the LC-GC-FID system.



4.3.2. Analysis of different cardboard and paper intended for food contact

The developed PLE method was then applied to commercial grade recycled and virgin cardboard boxes from the market as well as paper used or intended for food contact, such as baking paper, baking cups, paper bags, etc. In Table 13, the results obtained are reported.
As expected, recycled cardboard boxes presented higher contamination levels with respect to virgin ones. Probably, for recycled cardboards, a large part of the contamination below C_{24} had already migrated into the food before the analysis. Of the 6 printed cardboard packaging, all except one (cardboard box 4) were printed with mineral-oil-free or low-migration inks, confirmed also by no differences when comparing the contamination level of two different areas printed more or less intensely.
The two bread paper bags (one printed, the other not) showed similar LC-GC traces, shown in Figure 40, with a relatively low MOSH contamination (maximum 33 mg/kg of MOSH up to C_{35}).

Table 13. MOSH and MOAH content (mg/kg) of different cardboard packaging and paper materials intended for food contact.

Sample	Packaging		MOSH (mg/kg)		MOAH (mg/kg)	
			C_{10}-C_{24}	C_{10}-C_{35}	C_{10}-C_{24}	C_{10}-C_{35}
Cardboard box 1	virgin	printed	20	26	<2	<2
Cardboard box 2	virgin	printed	16	36	<2	<2
Cardboard box 3	recycled	printed	136	545	32	72
Cardboard box 4	recycled	printed	354	538	99	155
Cardboard pastry tray	virgin/recycled	unprinted	89	276	51	103
Cardboard cake box	virgin/recycled	printed	120	288	25	450
Cardboard egg box	recycled	printed	188	503	40	124
Pizza box	virgin	printed	2	7	<2	<2
Bread paper bag 1	recycled	printed	13	33	<2	<2
Bread paper bag 2	recycled	printed	8	19	<2	<2
Oven bag	virgin	printed	8	15	<2	<2
Baking cups 1	virgin	printed	76	854	<2	<2
Baking cups 2	virgin	unprinted	69	430	<2	<2
Baking cups 3	virgin	printed	39	46	5	<2
Oven paper 1	virgin	unprinted	22	25	2	<2
Oven paper 2	virgin	unprinted	18	22	<2	<2
Oven paper 3	virgin	printed	23	28	2	2

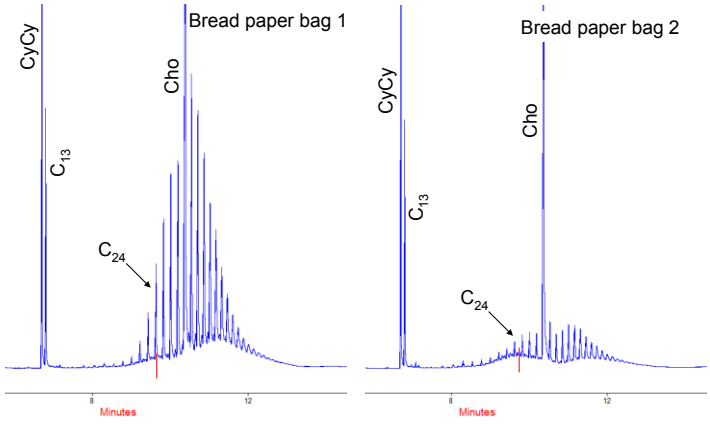


Figure 40. LC-GC-FID traces (MOSH) of the two samples of bread paper bag. The red line marks the n-C_{24}.

Figure 41 shows the LC-GC traces of some of the paper samples intended for food contact at high temperatures. High migration rate can be expected from the oven and baking paper into the

food, also for less volatile hydrocarbons (migration under wet-contact condition), considering the high temperature of use and the nature of the food usually cooked in contact with these materials (high fat content).

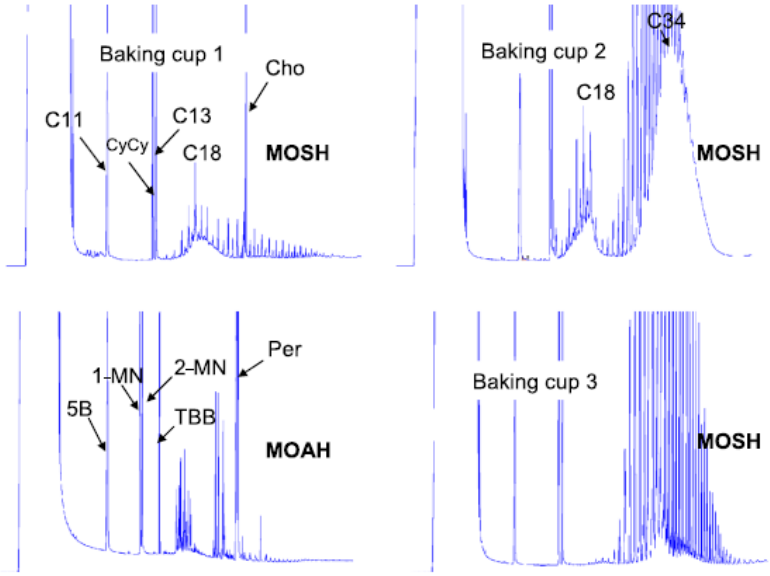


Figure 41. LC-GC-FID traces of some baking cup samples.

Baking paper (in roll) of different brands showed similar MOSH levels (22-25 mg/kg of MOSH up to C_{35}). Most of the contamination was in the range C_{10}-C_{24} with a maximum centred on C_{18}, as is typical of contamination transferred from recycled printed cardboard to food via gas-phase. Since the baking paper was packaged in recycled cardboard, it can not be ruled out that at least part of the contamination came from the packaging.

4.4. Conclusions

A PLE method for rapid and efficient extraction of MOSH and MOAH from cardboard and paper material has been set up. It delivered results similar to those obtained with the method based on classical solvent extraction developed by Lorenzini et al. (Lorenzini et al., 2010), with an advantage in terms of sample manipulation and time saving. Six samples can be processed in parallel in less than 30 min compared to a processing time of 3 h with classical solvent extraction. The extracts obtained do not require any treatment prior to injection.

5. PLE APPLIED TO LOW FAT CONTENT DRY FOODS FOR SELECTIVE EXTRACTION OF SUPERFICIAL AND TOTAL MINERAL OIL CONTAMINATION.

5.1. Background

A complete extraction of mineral oil from food can be more or less demanding, depending on the food composition and source of contamination. Mineral oil migrated from packaging into low fat dry foods, such as pasta or grain cereals, required an easy extraction step, in most cases represented by an overnight solvent extraction with hexane of unground (Moret et al., 2012c) or ground samples (Vollmer et al., 2011; Biedermann-Brem and Grob, 2011). In fact, this kind of contamination seems to remain on the surface of the food product.
Since the contaminants can be firmly included into the matrix, the extraction of pre-existing contamination from different sources is, in fact, more difficult. To purchase this, a somewhat laborious extraction method was proposed by Biedermann-Brem and co-workers in 2011, which allows to obtain complete MOSH and MOAH recoveries. Briefly, the ground sample has to be soaked in hot water (80 °C for 1 h), and extracted first with ethanol (1 h) in order to exchange the water in the particles by ethanol, and then with hexane overnight (EFSA, 2012; Biedermann-Brem and K. Grob, 2011). Another possibility for an efficient extraction of mineral oil from dry foods and more complex matrices (especially those with a high fat content) is represented by traditional saponification, followed by unsaponifiable extraction with an organic solvent and washing with water. However, it is particularly solvent- and time-consuming and could be advantageously replaced by microwave assisted saponification (Moret et al., 2012c).
The aim of the present work was to develop and optimize two rapid PLE methods: one for selective extraction of superficial MOSH and MOAH contamination, mainly coming from packaging, and another for quantitative extraction of total contamination coming from different sources.

5.2. Experimental

5.2.1. Samples and extraction

Dry food samples (pasta and rice) were purchased from the market. Some samples were part of the study on MOSH and MOAH migration from packaging into pasta during shelf-life, under different storage conditions (see PhD work: part 2). The paperboard samples used for migration tests were directly purchased from the producer or were provided by the final user.
PLE was carried out using a SpeedExtractor E-916 (Büchi, Flawil, Switzerland) equipped with six 10-mL stainless steel extraction cells. Pre-washed cellulose filters were placed at the exit of the cells to prevent clogging of the metal frit (Figure 33).

5.3. Results and discussion

5.3.1. Optimization of PLE method A (for contamination coming from the packaging)

The method has been optimized applying different extraction conditions (temperature, time, etc.) to small size pasta samples packed in direct contact with recycled paperboard. The food sample (about 8 g of small size pasta or rice) was directly loaded into a 10 mL PLE cell (without using any dispersing agent); large size pasta would need to be reduced to little particles. Prior to extraction, 8 µL of the internal standard working solution, previously described (see "General reagents and standards" at the beginning of PhD part 1), was added into the extraction cell for quantification. PLE optimization was carried out on pasta samples packaged for different lengths of time (1-9 months) in boxes of recycled cardboard at ambient temperature. Soon after packaging, the boxes were wrapped in aluminum to avoid exchange

with the external environment. Before packaging, the pasta sample had no detectable contamination (by applying PLE method A). Hexane was chosen as the extraction solvent since it was previously used when applying classical solvent extraction (Vollmer et al., 2011) and the extract was suitable for direct injection. Different extraction temperatures (80°C, 100°C and 120 °C), and extraction times (5 and 10 min) were tested for method optimization. The final extract, collected in a 60-mL glass vial, was centrifuged to separate little amounts of water, extracted from the sample, and directly injected into the LC-GC without pre-concentration. Final conditions are reported in Table 14.

Table 14. Optimized PLE conditions for method A (for contamination coming from the packaging).

PLE method A	
Temperature	100 °C
Pressure	100 bar
Cells (volume)	10 mL
Solvent	hexane
Cycles (number)	1
- Heat up	1 min
- Hold time	5 min
- Discharge	2 min
Flush with solvent	180" at 2 mL/min
Flush with gas (N_2)	1 min
Total solvent consumption	13 mL
Total extraction time	< 20 min

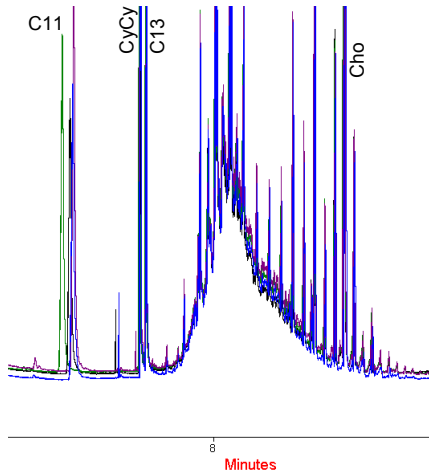


Figure 42. Overlay of LC-GC traces (MOSH) obtained from the analysis of a pasta sample at 80, 100 and 120 °C for 5 min and at 100 °C for 10 min (1 cycle).

No appreciable differences in extraction efficiencies were observed when increasing the temperature from 80 °C to 120 °C and the extraction time from 5 to 10 min. Figure 42 shows an overlay of the MOSH traces obtained at different temperatures and extraction times for 3 different aliquots of the same pasta sample stored for 3 months in a recycled paperboard box.

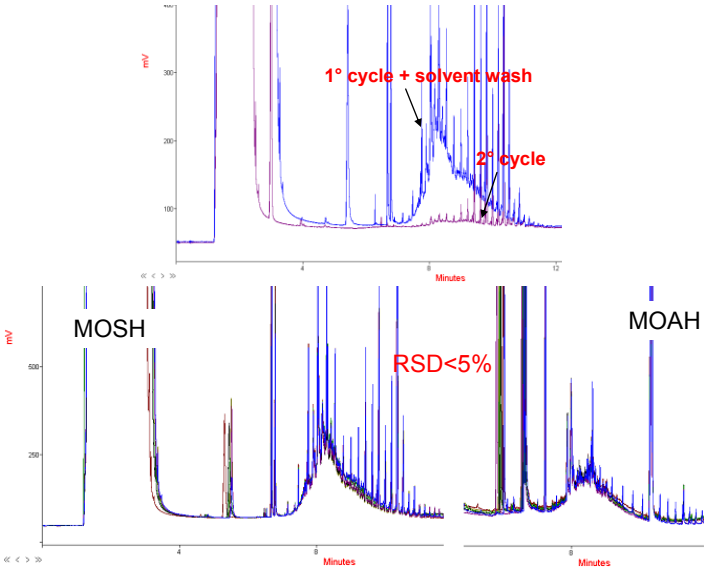


Figure 43. Top) LC-GC traces (MOSH) of a pasta sample packed for 3 months in recycled cardboard, obtained by performing a 1-cycle extraction with hexane (100 °C for 5 min) including a solvent wash (5 mL of hexane) and a second separate extraction cycle (100 bar, 100 °C, 5 min). Bottom) Six overlaid LC-GC traces (MOSH and MOAH) of a pasta sample stored in recycled paperboard for 9 months and extracted with the optimized method. The relative standard deviation (RSD) is reported.

Extraction yields with 1 cycle at 100 °C for 5 minutes were not quantitative, but a washing with hexane at 2 mL/min for 2.5 min (5 mL) performed at the end of the cycle allowed to reach practically quantitative recoveries. Figure 43 (top) shows the MOSH traces obtained with 1-cycle extraction (100 °C for 5 min) plus a washing with hexane (5 mL) followed by a second extraction cycle (collected separately) which contained less than 3 % of the total contamination. Repeatability was assessed with 6 replicate analyses carried out on a pasta sample stored for 9 months in recycled paperboard. All the overlaid MOSH and MOAH chromatograms are reported in Figure 43 (bottom). A relative standard deviation lower than 5 % was found for both MOSH and MOAH.

5.3.2. Optimization of PLE method B (for total contamination from different sources)

The extraction cell was filled, bottom to top, with a 5 g of fat free quartz sand (0.3-0.9 mm) (Büchi) to avoid cell clogging, 2.0 g of ground sample (IKA A10 analytical mill) mixed with 6 g of sand (which increases the superficial area exposed to the solvent), added with an internal standard (1 µL per g of sample) and sand to fill the void volume. Alternatively, the standard can be added directly in the collecting vial. To avoid blank problems, the sand was pre-washed with hexane before use at 100 °C, 100 bar for 5 min (washing can be easily carried out in 40 mL extraction cell). PLE optimization was carried out on pasta samples naturally contaminated with mineral oil of difficult extractability (not from packaging), selected among the samples previously analyzed by Moret and co-workers using both overnight extraction with hexane and microwave assisted extraction (Moret et al., 2012c).
Different extraction solvents, extraction temperature, extraction time, number of cycles, and wash with solvent, were tested in duplicate for method optimization. Optimized extraction conditions are reported in table 15.

Table 15. Optimized PLE conditions for method B (for total contamination coming from different sources).

PLE method B	
Temperature	100 °C
Pressure	100 bar
Cells (volume)	10 mL
Solvent	hexane/ethanol 1:1 (*v/v*)
Cycles (number)	2
- Heat up	1 min
- Hold time	5 min
- Discharge	2 min
Flush with solvent	no
Flush with gas (N2)	1 min
Total solvent consumption	15 mL
Total extraction time	28 min

Different extraction solvents, namely: hexane, acetone, hexane/acetone 1:1 (*v/v*) and hexane/ethanol 1:1 (*v/v*), were tested on the same pasta sample, contaminated with mineral oil (MOSH) probably from 2 different sources (two *humps*, the first one centred on C_{21}, the second one centred on C_{33}).

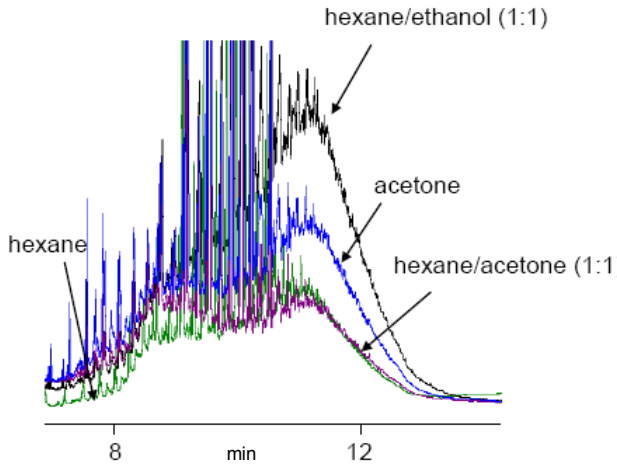


Figure 44. Overlay of the LC-GC traces (MOSH) of a pasta sample contaminated with mineral oil from different sources (not from the packaging), obtained by performing a 2-cycle extraction (100 bar, 100 °C, 5 min) with different extraction solvents or mixtures.

Figure 44 shows the LC-GC traces obtained by performing a two-cycle extraction with different solvents (100 °C, 5 minutes at 100 bar). As visible in the figure, hexane alone gave the worst result, while the mixture hexane/ethanol 1:1 (*v/v*) gave the best results; for this reason, the hexane/ethanol 1:1 (*v/v*) mixture was chosen as the extraction solvent: it well exploits the swelling power of the ethanol towards starch (favouring the release of physically entrapped hydrocarbons) and proteins (which, in its folded structure, could retain some fat and hence

lipophilic contaminants), as well as the extracting power of hexane towards hydrocarbons. At the end of the 2-cycle extraction, the extract (about 15 mL) was collected in 60-mL vial, added with water (about 30 mL) to separate the ethanol from the hexane. To facilitate phase separation avoiding formation of an emulsion, the extract was maintained at -20 °C for about 20 min. An aliquot of the hexane extract was used for LC-GC analysis.
To verify the number of extraction cycles needed to achieve satisfactory extraction recovery, the same pasta sample was processed by performing (in duplicate) 3 consecutives cycles (collected separately). Figure 45 shows an overlay of the GC traces obtained. This trial demonstrated that a two-cycle extraction is needed to obtain good recoveries.
Different extraction temperatures (100 °C, 120 °C and 150 °C) were then tested in triplicate on the same pasta sample. Comparable responses were obtained by increasing the extraction temperature from 100 °C to 120 °C, while a slight decrease along with some degree of browning was observed at 150 °C.

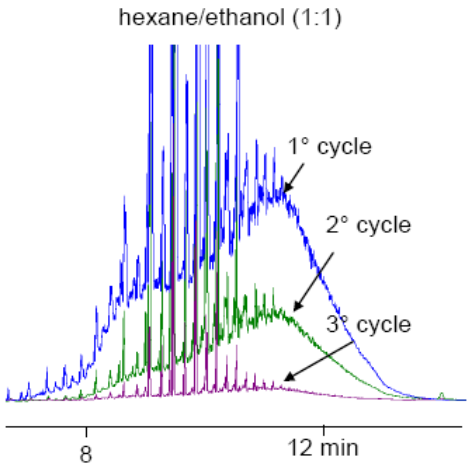


Figure 45. Overlay of LC-GC traces (MOSH) of a pasta sample contaminated with mineral oil from different sources (not from the packaging), obtained by performing three consecutives extraction cycles (100 bar, 100 °C, 5 min).

5.3.2.1. Method performances and comparison with other extraction methods.

With optimized conditions (Table 15) good repeatability (relative standard deviation lower than 6 %) was obtained for samples considered: low (0.8 mg/kg) and high (13.7 mg/kg) contamination level. Extraction recoveries were assessed (in duplicate) on three different samples contaminated with mineral oil from different sources (different LC-GC profiles), by comparing the response obtained with an additional extraction cycle and it was higher than 95 %.
Results obtained with the optimized method were compared with those obtained (in duplicate) by applying overnight extraction (on ground sample) and with the method proposed by Biedermann-Brem and Grob (2011), involving soaking in hot water followed by extraction with ethanol and overnight extraction with hexane. It was confirmed that, in contrast to overnight extraction with hexane, the optimized PLE method allows a complete extraction of mineral oil and gives results well in agreement with those obtained by applying the method proposed by Biedermann-Brem and Grob (2011), which is more laborious and solvent-consuming.
Furthermore, data obtained by using the PLE method B were compared with that obtained from microwave assisted saponification (MAS) and microwave assisted extraction (MAE) and very similar results were obtained, further confirming the extraction power of the method proposed, also in the case of mineral oil contamination firmly embedded into the matrix (Moret et al., 2012c).

5.3.3. Determination of pre-existing contamination in the presence of the one from packaging.

To evaluate pre-existing contamination in the presence of mineral oil migrated from the packaging, the sample which underwent PLE method A for packaging contamination, was recovered from the extraction cell, ground and, an aliquot (2 g) was re-extracted by using PLE method B.

The two PLE methods were applied in combination to a semolina pasta with a pre-existing contamination of 0.8 mg/kg, before and after a one- and a nine-month storage in recycled cardboard boxes wrapped with aluminum (to exclude exchange with the external ambient), in order to verify if discrimination between contamination migrated from the packaging and contamination from other sources was really possible. Before injection, the extracts obtained with the two methods were adjusted in order to inject a volume corresponding to the same sample amount (200 mg). Figure 46 shows an overlay of the MOSH and MOAH traces obtained with PLE method A. When applying this method of extraction, the pasta sample before packaging (T0) had no detectable contamination. After the first month of contact, most of the contamination from the packaging migrated (T1 had 3.4 and 0.5 mg/kg of MOSH and MOAH, respectively), and reached higher level after 9 months (T9 had 4.6 and 0.7 mg/kg of MOSH and MOAH, respectively).

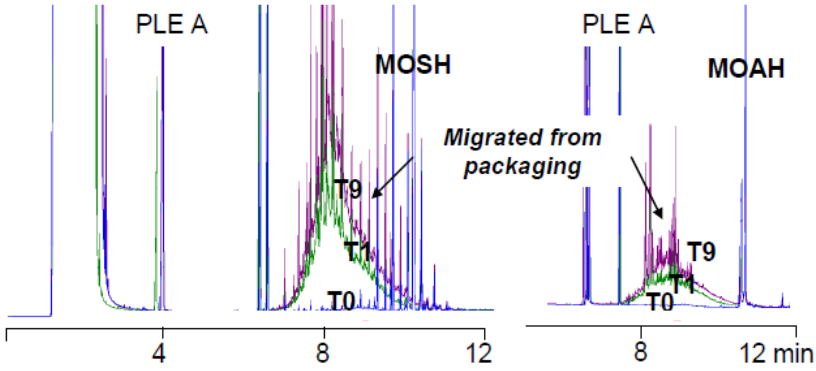


Figure 46. Overlay of MOSH and MOAH LC-GC-FID traces obtained from the analysis of pasta before packaging (T0) and after a 1- (T1) and a 9-month (T9) storage in recycled cardboard and extracted using PLE method A to investigate MOH migrated from packaging.

Figure 47a shows an overlay of MOSH traces of the same pasta samples extracted with PLE method B to assess the total MOH contamination. In sample T0, the presence of a pre-existing contamination, not extracted with PLE method A, was well evident (0.8 mg/kg of MOSH), as well as in sample T1 (4.2 mg/kg of MOSH) and T9 (5.3 mg/kg of MOSH). These results pointed out the selectivity of PLE method A towards mineral oil migrated from the packaging, while PLE method B allowed for extracting total contamination from different sources, such as pre-existing contamination as well as contamination from the packaging. It was possible to eliminate the contribution of mineral hydrocarbons migrated from the packaging through the quantification of the pre-existing contamination, by applying PLE method B on residual pasta which already underwent PLE method A (Figure 47b).

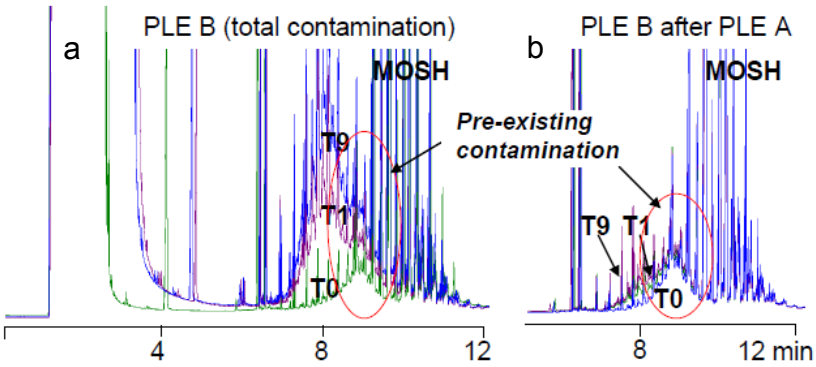


Figure 47. a) Overlay of MOSH LC-GC-FID traces obtained from the analysis of pasta before packaging (T0) and after a 1- (T1) and a 9-month (T9) storage in recycled cardboard and extracted using PLE method B to investigate total MOH contamination. b) Overlay of MOSH LC-GC-FID traces obtained from the analysis of pasta before packaging (T0) and after a 1- (T1) and a 9-month (T9) storage in recycled cardboard and extracted using PLE method B after PLE method A, to investigate pre-existing contamination.

Furthermore, it was found that a small amount of the contamination from the packaging remained in the sample after applying PLE method A. This amount slowly increased with storage time, indicating that only a negligible part of the contamination from the packaging penetrated deeply into the sample and/or interacted with sample components, becoming less available for the extraction with PLE method A. Nevertheless, it was possible to detect a pre-existing contamination also in the presence of a contamination from the packaging. As expected, the results obtained with PLE method B were in good agreement with those obtained from summing up the results acquired by applying PLE method A and PLE method B in succession on the same sample.

5.4. Conclusions

By properly exploiting the selectivity of different extraction solvents, and the advantage of operating at a high temperature under pressure, it was possible to develop rapid methods able to discriminate between superficial contamination, mostly coming from the packaging, pre-existing contamination, or total contamination from different sources.

PhD WORK PART 2: MINERAL OIL MIGRATION FROM PACKAGING INTO FOOD.

1. A CASE STUDY: DIFFERENT STORAGE CONDITIONS AFFECTING MINERAL OIL MIGRATION FROM PACKAGING INTO SEMOLINA AND EGG PASTA DURING SHELF-LIFE.

1.1. Background

Most foodstuffs are provided with a packaging, which has several important functions, such as preventing physical, chemical and biological modifications to avoid a possible contact with external agents, facilitating transport, storage and consumption (Piergiovanni and Limbo, 2010a). Nevertheless, the migration of undesirable packaging components, such as phthalates, photoinitiators, diisopropylnaphthalene (DIPN) and mineral oil hydrocarbons can occur in the food during shelf-life (Lorenzini et al., 2013; Biedermann et al., 2013). As already mentioned in the Introduction (Chapter 5), the extent to which migration occurs depends on several factors such as the physico-chemical properties of the migrant, the packaging material, the food (e.g. fat content), as well as storage conditions (time, temperature, size of the packaging in proportion to the foodstuff volume, etc.).

In the MOSH fraction of mineral oil polyolefin oligomeric saturated hydrocarbons (POSH) can possibly co-elute, which can migrate from plastic bags (mainly made of polyethylene or polypropylene), heat-sealable layers, adhesives and plasticizers, as well as polyalphaolefins (PAO) (Biedermann and Grob, 2012b). The latter are of synthetic origin, and are used as lubricants, or in adhesives and hot melts. They may contain olefins with a polarity that sometimes determines their elution also in the MOAH fraction, and their presence can be recognized because they form rather narrow humps of unresolved branched hydrocarbons with a regular distance between them (Biedermann and Grob, 2012b).

MOSH and MOAH migration from recycled paper and board packaging virtually exclusively occurs through the gas phase, which means by evaporation from the paperboard and recondensation on the food surface. For this reason, only the volatile part is relevant with a transfer rate decreasing with decreasing volatility (Biedermann and Grob, 2012b). It was demonstrated that migration is substantial up to n-C_{24} (Lorenzini et al., 2010), but it may reach *n*-C_{28} (Biedermann and Grob, 2010).

Pasta is the staple food of the Mediterranean diet and different types of dry pasta (maximum moisture of 12.5 %) are available on commerce. In particular, in this study semolina and egg pasta, which mainly differ for their fat content, were taken into consideration. Semolina pasta is usually sold packaged in plastic film bags or in direct contact with recycled or virgin paperboard, while egg pasta is mainly packed in virgin paperboard boxes, even though it is possible to find egg pasta in direct contact with recycled paperboard.

Very recently, Lorenzini and co-workers (Lorenzini et al., 2013) published a study on migration kinetics of mineral oil hydrocarbons from recycled paperboard packaging into dry foods. Two real cases, a müesli packed in recycled paperboard with an unprinted internal polyethylene (PE) bag, and an egg pasta in direct contact with an unprinted tray of recycled paperboard wrapped in a printed polypropylene (PP) plastic film, were monitored up to the end of their shelf-life (during one year). The influence of time, storage conditions, food packaging structure and temperatures were studied. As expected, this study confirmed that migration is strongly influenced by temperature. Furthermore, the migration into food (especially after prolonged storage) was the highest in packs kept in transport boxes, followed by shelved and free-standing ones.

In this work mineral oil migration from 3 different packaging materials namely printed recycled paperboard, printed virgin paperboard and plastic PP film, was been monitored at room temperature (up to 2 years) for both dry egg and semolina pasta. Tests were performed for all the food/packaging combinations. Three different storage conditions (packs wrapped in aluminum to prevent any influence of the surrounding environment, packs standing on shelves and packs stored in corrugated cardboard boxes) have been investigated. Furthermore, contribution to the total food contamination deriving from hot melt adhesives applied to seal paperboard packs, was also evaluated.

1.2. Experimental

1.2.1. Samples, storage conditions and sampling frequency

Two kinds of dry pasta (semolina and egg with 1.5 and 4 % fat, respectively) of the same small size (rice-shaped), three different types of packaging (PP plastic film, recycled and virgin paperboard) and two different storage conditions at unconditioned room temperature (boxes wrapped in aluminum foil, boxes on shelves) were combined for migration tests. Tables 16-A and 16-B report the properties of packaging, food, storage conditions and the sampling frequency used in migration tests, as well as data on DIPN, MOSH and MOAH content in the packaging before contact with food. The potential migration, calculated considering that all the contamination present on the packaging before the contact with food migrated into pasta, was also reported. The samples stored on shelves were left unwrapped to simulate the real ordinary storage conditions. The other packs were wrapped in aluminum foil in order to eliminate the external environment influence and to minimize a possible escape of a part of the contaminants, which are, therefore, forced to migrate into the food. In particular, the external environment includes the surrounding area to the sample, from and to which migration can goes. The semolina pasta in recycled paperboard and egg pasta in virgin paperboard followed the normal production line and were packed at the producer plant with adhesive deposition (hot melt) on the top and bottom side of the box. The other pasta-packaging combinations (egg pasta in recycled paperboard and semolina pasta in virgin paper, as well as pasta in plastic film) were prepared manually, closing the two sides of the box with staples. Since PAOs from hot melt adhesives, prevented a correct quantification of mineral oil migrating from the packaging, new samples were prepared manually later (without hot melts), also for semolina pasta in recycled paperboard and egg pasta in virgin paperboard stored up to 6 month wrapped in aluminum foil (see tables 16-A). Sampling frequency (starting from packaging time) was established for each type of pasta-packaging-storage combination, as reported in Tables 16-A and 16-B. All samples were collected in duplicate.
The contribution of migration deriving from a secondary packaging was tested storing semolina pasta, packed with the three different packagings cited above, into a transport box consisting of corrugated board. Two samples were collected for each type of packaging: one in contact with the board, and the other from the middle of the second packaging.
After sampling, pasta and paperboard were separated, wrapped in aluminum and stored in a freezer until analysis.

Table 16-A. Characteristics of semolina pasta and packaging samples and sampling frequency.

Semolina pasta				
Food size and shape	Small size, rice-shaped			
Food fat (%)	1.5			
	Recycled paperboard		***Virgin paperboard***	***Plastic film (PP)***
Exposed food weight (g)	500		250	500
Pack weight (g)	20		12	4
Pack size (cm2)	133.8		124.3	287.12
Pack volume (cm3)	561.8		373.0	
DIPN in paperboard (mg/kg)	12.41			
MOSH<C25 (mg/kg)	388		21.5	290
MOAH <C25 (mg/kg)	90			
Max MOSH <C25 potential migration (mg/kg food)	15.7		1	2.3
Max MOAH <C25 potential migration (mg/kg food)	3.7			
Hot melt adhesives	yes	no	no	no
	Storage conditions (sampling frequency in months)			
	wrapped in aluminum kept on shelves (1,2,3,4,5,6,9,12,15,18,21,24) in the transport box (3,6,12)	wrapped in aluminum (1,2,3,6)	wrapped in aluminum kept on shelves (1,2,3,4,5,6,9,12,15,18,21,24) in the transport box (3,6,12)	wrapped in aluminum kept on shelves (1,2,3,4,5,6,9,12,15,18,21,24) in the transport box (3,6,12)

Table 16-B. Characteristics of egg pasta and packaging samples and sampling frequency.

Egg pasta				
Food size and shape		Small size, rice-shaped		
Food fat (%)		4		
	Recycled paperboard	***Virgin paperboard***		***Plastic film (PP)***
Exposed food weight (g)	500	250		500
Pack weight (g)	20	12		4
Pack size (cm2)	133.8	124.3		287.12
Pack volume (cm3)	561.8	373.0		
DIPN in paperboard (mg/kg)	12.41			
MOSH<C25 (mg/kg)	388	21.5		290
MOAH <C25 (mg/kg)	90			
Max MOSH <C25 potential migration (mg/kg food)	15.7	1		2.3
Max MOAH <C25 potential migration (mg/kg food)	3.7			
Max DIPN potential migration (mg/kg food)	0.5			
Hot melt adhesives	no	yes	no	no
	Storage conditions (sampling frequency in months)			
	wrapped in aluminum kept on shelves (1,2,3,4,5,6,9,12,15,18,21,24)	wrapped in aluminum kept on shelves (3,6,12,18,24)	wrapped in aluminum (1,2,3,6)	wrapped in aluminum kept on shelves (3,6,12,18,24)

1.2.2. Migration assessment

Migration from packaging into food was evaluated in two ways: by measuring the mineral oil migrated into pasta samples after the contact with the packaging, and by determining the mineral oil amount lost by the paperboard during storage (the paperboard samples were analyzed before and after the contact with the food).
Before starting with the migration tests, all foods and packaging were analyzed to assess the base contamination. The results presented in this study were referred to real mineral oil migration only, since they were subtracted from the contamination at time zero.

1.2.3. Extraction

The semolina pasta samples were extracted using a SpeedExtractor model E-916 (Büchi, Flawil, Switzerland) according to the methods described in this PhD Work: Part 1 (Moret et al., submitted paper). Briefly, for the determination of contamination coming from packaging (PLE-A method), about 8 g of whole pasta were extracted with hexane (1cycle-extraction followed by a solvent manual wash) at 100 bar and 100 °C, held for 5 min, and an aliquot of the extract (100 µL) was directly injected into the LC-GC system. For the determination of the total (pre-existing) contamination, after a first extraction by using PLE-A method, the residual pasta was ground to a fine powder, mixed with a dispersive agent and extracted with hexane/ethanol 1/1 (*v/v*) (2 cycles) at 100 bar and 100 °C for 5 min (PLE-B method).The higher amount of fat in the egg pasta seemed to cause a homogeneous distribution of the mineral oil contamination in all the pasta grain, preventing the differentiation between superficial (coming from packaging) and deep contamination (pre-existing). Therefore, about 10 g of whole egg pasta were extracted overnight with 20 mL of hexane added with 10 µL of the internal standard working solution (described in the paragraph *1.2.1* in this PhD Work: Part 1). 50 µL of the extract were directly injected into the LC-GC system.
The corrugated board of the transport box, printed recycled and virgin paperboards were extracted with the method recently proposed by Moret and co-workers and reported in this PhD Work: Part 1 (chapter 4) (Moret et al., 2013). Briefly, 1 g of paperboard sample was cut in strips (6 cm x 2-4 mm) added with internal standards (20 µL), and extracted with hexane (2 cycles) at 100 bar and 60 °C for 5 min. The hexane phase was centrifuged and analyzed by on line LC-GC.
Printed plastic films were extracted using the method proposed by Biedermann and Grob (Biedermann and Grob, 2012a). Briefly, 300 mg film were immersed in 10 mL hexane overnight at ambient temperature. For the LC-GC analysis, 50 µL of the extract was injected in the system.

1.3. Results and discussion.

The semolina pasta before exposure had no detectable superficial contamination and there was a deeper contamination of 0.8 mg/kg of MOSH. The egg pasta presented a total contamination of 0.5 mg/kg of MOSH. No MOAH were detected, in either semolina or egg pasta samples.
The homogeneous distribution of the contamination in both virgin and recycled paperboard was assessed analyzing two different parts of a same box: one with printing ink of different colors and the other with only the white color. The analysis was performed in duplicate, sampling the same zones for each box. The perfect overlay of the MOSH and MOAH traces obtained from white and coloured parts (Figure 48) indicated that mineral oil free printing inks were used for decorating the boxes.

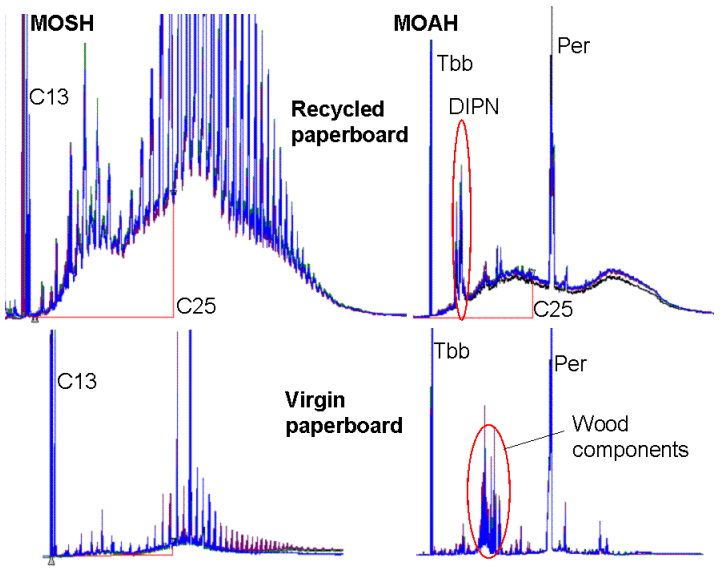


Figure 48. Overlay of 4 MOSH and MOAH traces (two from analysis of colored parts and two from white parts) of recycled (top) and virgin (bottom) paperboard before food contact. The red line marks the hump up to n-C_{25}. DIPN: diisopropylnaphtalenes; CyCy, C_{13}, Tbb and Per are used as internal standards.

1.3.1. Virgin paperboard packaging

The printed virgin cardboard before contact with food was contaminated with about 21.5 mg/kg of MOSH<C_{25} (total MOSH 71 mg/kg up to about *n*-C_{45}). The MOAH fraction of virgin paperboard presented the typical profile of wood components (derivatives of abietic and primaric acid) (Figure 48 bottom), and the epoxidation of the extract confirms that it was free of aromatic hydrocarbons.

Egg pasta

The virgin paperboard boxes containing egg pasta followed the normal production line, thus were closed by adhesive deposition. The egg pasta samples packed in these boxes presented a contamination centered on *n*-C_{18} that was not present in the same samples packed in laboratory using staples to close the top and the bottom sides of the boxes, as visible in Figure 49 A. In fact, the presence of the hot melt adhesives strongly increased the contamination due to the PAOs presence, which are non-homogenously distributed, as visible in Figure 49C from the overlay of the chromatograms obtained from the analysis of two pieces of paperboard at a different distance from the glue deposition. The MOSH trace of the adhesive reflects the PAO pattern, built of C_8 units (Figure 49 B).

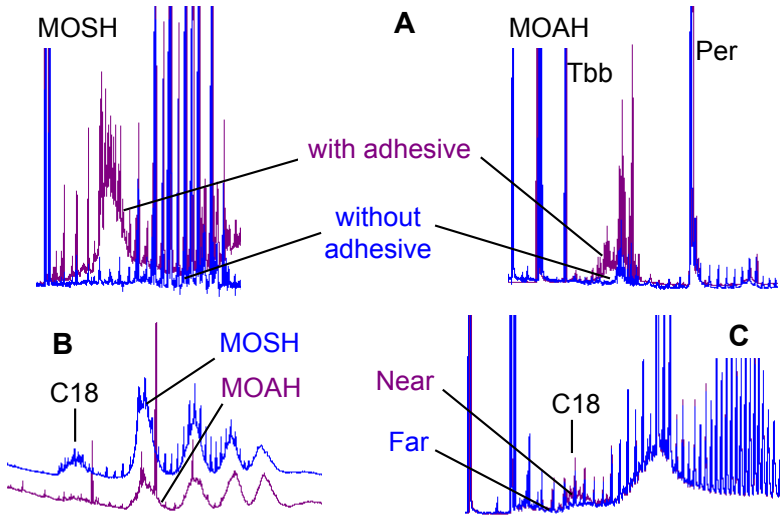


Figure 49. A) MOSH and MOAH fractions of 3-month storage egg pasta packed in recycled paperboard boxes with and without adhesive deposition B) MOSH and MOAH chromatograms of hot melt used for box closure. C) Overlay of MOSH fraction of two parts of a virgin paperboard box sampled at a different distance from the adhesive deposition.

The egg pasta in virgin paperboard boxes kept on shelves presented higher values of MOSH contamination than that found in packs wrapped in aluminum. All the MOSH contents in egg pasta packed in virgin paperboard with and without adhesive deposition and stored at different conditions are reported in Figure 50.

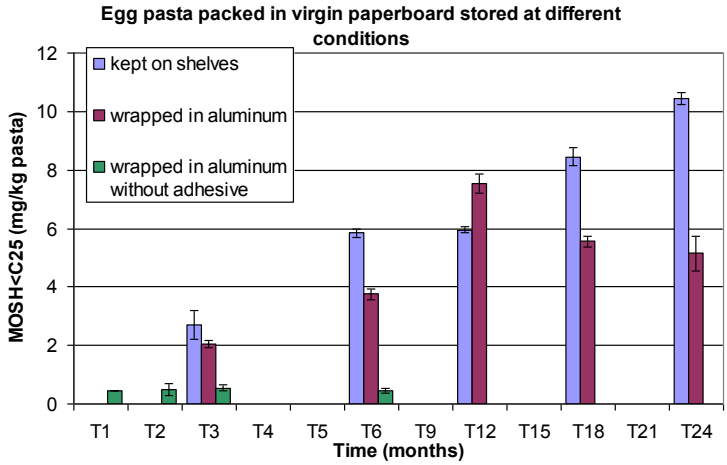


Figure 50. MOSH<C_{25} in egg pasta packed in virgin paperboard boxes with adhesive deposition wrapped in aluminum and kept on shelves and in boxes without adhesives wrapped in aluminum. Results are expressed as average of 4 replicates (bars) and standard deviations (vertical lines).

The adhesive and external environment contributions were more evident using virgin paperboard packaging, with respect to the recycled one, due to its low base contamination.
The external environment influence was well evident comparing the same kind of egg pasta in virgin paperboard boxes stored in different conditions. In fact, the maximum value of 10.9

mg/kg MOSH<C_{25} was reached in pasta after 24 months of storage on shelves, while the samples wrapped in aluminum, after the same time of exposure, reached about half of this level of contamination. The decrease over time of MOSH<C_{25} amount in samples wrapped in aluminum can be due to the non-homogeneous distribution of PAOs through the paperboard and thus to a non-homogeneous migration during time. Figure 51 shows an overlay of MOSH and MOAH chromatograms obtained from the analysis of the egg pasta packed in virgin paperboard boxes with adhesive stored for 6 months on shelves (violet line) and wrapped in aluminum (blue line). The hump obtained for samples kept on shelves presented an abundant amount of volatile hydrocarbons, up to about *n*-C_{17}, which is missing in the samples wrapped in aluminum. Furthermore, DIPNs, the typical markers of recycled paperboard contamination, are present only in the samples kept on shelves, indicating the presence of an external contamination due to the storage environment (see paragraph 3.4 of this chapter).

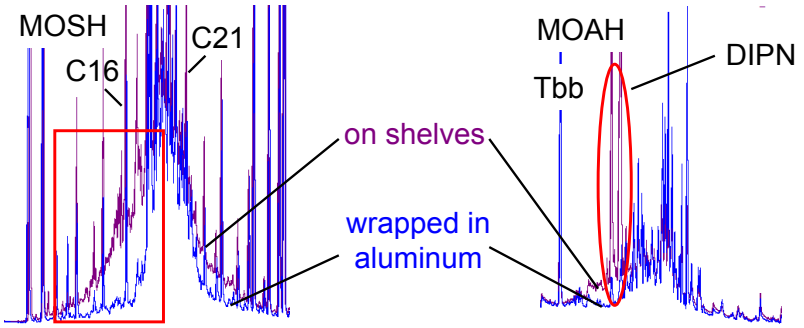


Figure 51. MOSH and MOAH fractions of T6-storage egg pasta packed in virgin paperboard (with adhesives) wrapped in aluminum and kept on shelves.

Semolina pasta

The semolina pasta packed in virgin paperboard (without adhesives) wrapped in aluminum and kept on shelves presented a maximum level of contamination around or below 0.6 mg/kg of MOSH, even after 24 months of storage. Slightly higher contamination levels were found in the samples stored in the transport box, with a maximum of about 0.8 mg/kg after 12 months. All the data are reported in Figure 52.

The influence of the external environment, evident for the boxes kept on shelves, caused, on average, an increase of MOSH contamination of about 0.2 mg/kg pasta, especially after 12 and 18 months of storage, with respect to the values found in the semolina pasta packs wrapped in aluminum. After this time, the contamination seems to decrease, maybe due to a migration towards the external environment. However, due to the limited sampling frequency, it was not possible to confirm this hypothesis.

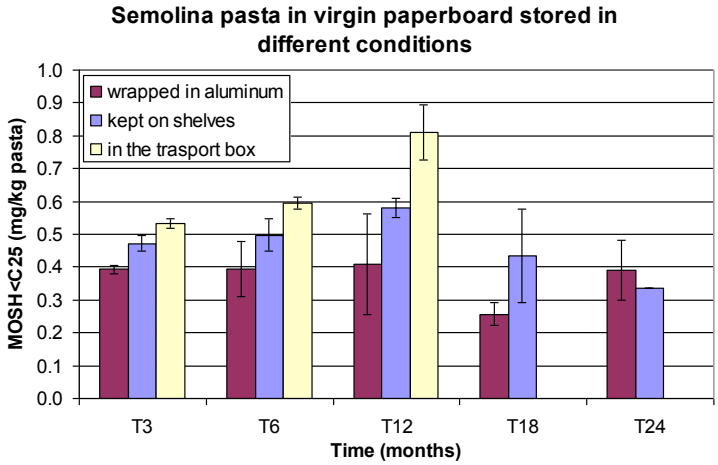


Figure 52. MOSH<C_{25} in semolina pasta packed in virgin paperboard boxes with adhesive wrapped in aluminum, kept on shelves and stored in the transport box. Results are expressed as average of 4 replicates (bars) and standard deviations (vertical lines).

Also the contribution of a secondary packaging made of corrugated board slightly increased the MOSH contamination: about 0.8 mg/kg of MOSH<C_{25} were found in pasta after 12 months of storage, which means, on average, 0.2 mg/kg more than for the pasta packs kept on shelves and 0.4 mg/kg more than the aluminum-wrapped samples. No evident differences were observed between samples stored in contact and in the centre of the transport box (maximum standard deviation of about 0.2 between the two packs sampled in contact and in the middle of the corrugated board).

1.3.2. Recycled paperboard packaging

The printed recycled paperboard before contact presented a mean value of 388 mg/kg and 90 mg/kg of MOSH<C_{25} and MOAH<C_{25}, respectively. The contamination presented a molecular weight distribution ranging between C_{13} and C_{55}, split in two partially co-eluted humps (Figure 48 top left). The first part of MOSH fraction mainly consisted of mineral oil deriving from printing inks originated from printed material fed into the recycling process, such as newspapers (centered on *n*-C_{16}-C_{17}), while the later eluted hump (centered on *n*-C_{27}-C_{28}) derived from waxes used in the paperboard manufacturing. The MOAH fraction of recycled paperboard presented the typical bunches of peaks of diisopropylnaphthalenes (DIPNs) and wood compounds (derivatives of abietic and primaric acid). DIPNs originate from carbonless copy paper and are used as markers for migration from recycled paper and board (Biedermann and Grob, 2012b). The main MOAH hump (Figure 48 top right) ranged between *n*-C_{13}-C_{24} indicating that it derives from the off-set printing ink. The later eluted hump (above C_{35}), derived from petroleum resins used as binders in printing ink and should be subtracted from the MOAH, since it does not represent mineral oil (Biedermann and Grob, 2012b).

Semolina pasta

The recycled paperboard boxes containing semolina pasta were closed with hot melt adhesives, thus not only mineral oil hydrocarbons but also PAO migrated into pasta, as already described above for the egg pasta in virgin paperboard. The MOSH contamination in the pasta samples ranged from *n*-C_{13} to about *n*-C_{32} (less than 4 % over *n*-C_{25}) and was centered on the *n*-C_{17}-C_{18}, which is typical of the migration from recycled paperboard packaging. Furthermore, the typical markers of recycled paperboard, namely DIPNs, were present in the MOAH fraction (Figure 53).

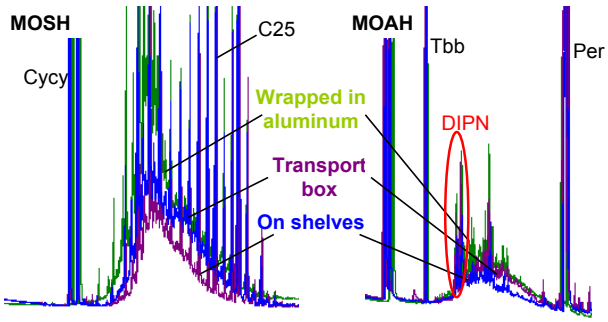


Figure 53. HPLC-GC-FID traces of MOSH and MOAH fractions obtained from the analysis of semolina pasta packed in recycled paperboard after 12 months of storage in different conditions. All the boxes were closed with hot melt adhesives.

After 1 month of storage in recycled paperboard wrapped in aluminum, the semolina pasta reached about 50 % of the total contamination found after 24 months (3.3 and 6.5 mg/kg MOSH; 0.6 and 1 mg/kg MOAH, after 1 and 24 months, respectively), while in the samples kept on shelves, the contamination reached about the maximum level (about 3 mg/kg of MOSH and 0.6 mg/kg of MOAH) after 1 month of storage and remained quite constant for up to 24 months, probably due to a migration also towards the external environment. An overlay of MOSH and MOAH chromatograms obtained from the analysis of the semolina pasta packed in recycled paperboard (with adhesive) and stored in different conditions for 12 months is reported in Figure 53. All the MOSH and MOAH values up to *n*-C_{25} are reported in Figure 54.
The volatile contaminants from corrugated paperboard can migrate into food (up to *n*-C_{25}) and the contribution from transport box resulted lower than migration from primary packaging made of recycled paperboard, according to Biedermann et al. (2011a). After 3 months of storage, the pasta packs stored in the transport box consisting of corrugated board (containing 308 and 42.3 mg/kg of MOSH and MOAH up to C_{25}, respectively, at time zero) reached intermediate values with respect to those found in the pasta whose packs were kept on shelves and wrapped in aluminum. Up to 12 months of storage the contamination remained quite constant; in particular, 3.9 and 0.7 mg/kg were found as maximum values for MOSH and MOAH, respectively. No relevant differences between the samples in contact and in the middle of the transport box were observed (standard deviation between the two packs sampled in contact and in the middle of the corrugated board was always below 0.5) (Figure 54).

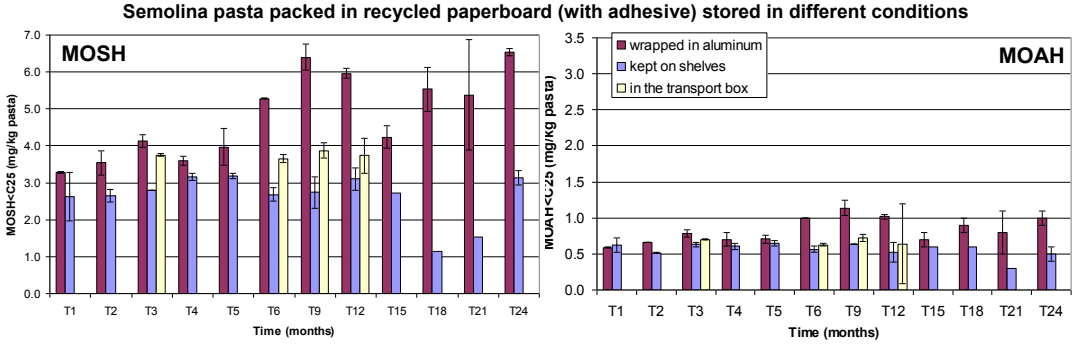


Figure 54. MOSH (scale 0 - 7 mg/kg of pasta) and MOAH (scale 0 - 3.5 mg/kg of pasta) up to n-C_{25} in semolina pasta packed in recycled paperboard stored in different conditions. Results are expressed as average of 4 replicates (bars) and standard deviations (vertical lines).

MOSH and MOAH presented very similar migration kinetics: the percentage of MOAH/MOH ranged from 14 to 17 % up to 24 months of storage
Comparing these results with those obtained from the pasta packed in manually prepared boxes, without any adhesive, it is well evident that the hot melts increased the contamination found in pasta, as already discussed for the egg pasta in virgin paperboard (Figure 55).
The presence of PAO in the MOSH fraction prevents a correct quantification leading to an overestimation. In fact, in semolina pasta the contamination due to MOSH<C_{25}, without the interference of PAO, was lower by about 1 mg/kg after 6 months of exposure: 5.3 and 4.2 mg/kg MOSH<C_{25} were found in the semolina pasta packed in recycled paperboard with and without adhesive, respectively. The values are reported in Figure 56. The presence of adhesives in the packaging increased the contamination found in the pasta by about 50 and 20 % after the first and the sixth month of storage (boxes wrapped in aluminum and kept at room temperature).

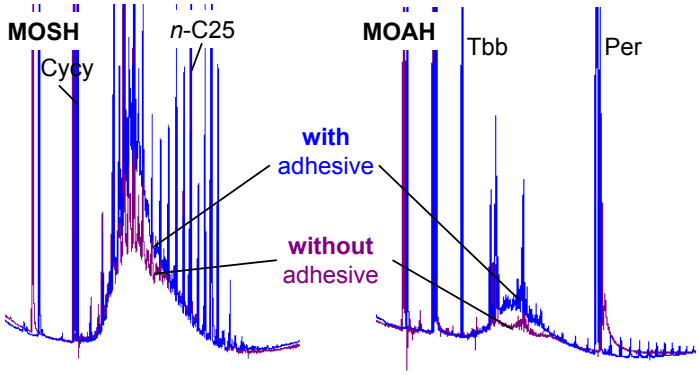


Figure 55. MOSH and MOAH fractions of 3-month storage semolina pasta packed in recycled paperboard boxes with (blue line) and without (violet line) adhesive deposition.

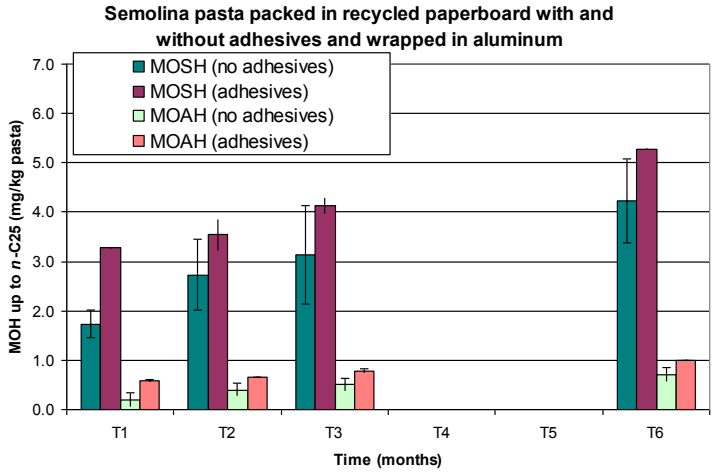


Figure 56. MOSH and MOAH <C_{25} (mg/kg of pasta) found in semolina pasta packed in recycled paperboard with and without adhesives and wrapped in aluminum for up to 6 months. Results are expressed as average of 4 replicates (bars) and standard deviations (vertical lines).

Egg pasta

The egg pasta packed in recycled paperboard boxes (without adhesives) kept on shelves presented a slow, but continuous increment of contamination during storage. In particular, 3.3 and 11.1 mg/kg of MOSH<C_{25}, 0.8 and 2 mg/kg of MOAH<C_{25} were found after 1 and 12 months of storage, respectively. From 12 to 24 months the contamination remained quite constant (the maximum value of 11.5 mg/kg MOSH<C_{25} was reached after 15 months of storage) with a minimal decrease in the last month of storage (8.5 mg/kg).

The mineral oil content in the egg pasta packed in virgin paperboard boxes wrapped in aluminum was 6.0 and 8.9 mg/kg for the MOSH<C_{25}, 0.8 and 1.5 mg/kg for the MOAH<C_{25}, after 1 and 24 months of storage, respectively. All values are reported in Figure 57. These values were generally lower than those found in the egg pasta whose packs were kept on shelves, indicating that the external environment had a great influence (confirmed also by the higher amount of DIPNs).

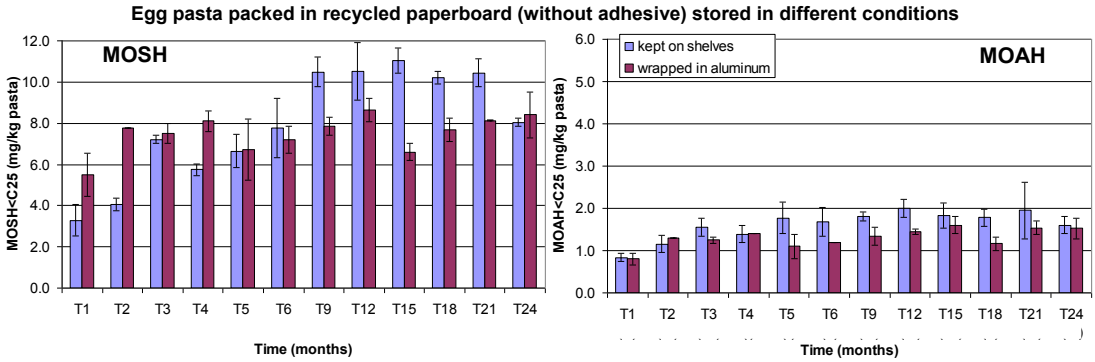


Figure 57. MOSH and MOAH up to n-C_{25} in egg pasta packed in recycled paperboard stored in different conditions. Results are expressed as average of 4 replicates (bars) and standard deviations (vertical lines).

The contribution to the total contamination deriving from the external environment was well evident considering the molecular mass of the hydrocarbons involved in the migration. In fact, in the egg pasta packed in recycled paperboard boxes wrapped in aluminum, the more volatile saturated hydrocarbons (up to *n*-C_{21}) were almost completely migrated after the first month of storage and a significant increase of the heavier hydrocarbons (the second hump centered on *n*-C_{23}) was observed during storage time. On the contrary, during shelf storage of the egg pasta packed in virgin paperboard boxes an evident increment of the contamination of volatile hydrocarbons was observed, up to about *n*-C_{21}, while the heavier components migrated more slowly, as confirmed by LC-GC traces reported in Figure 58.

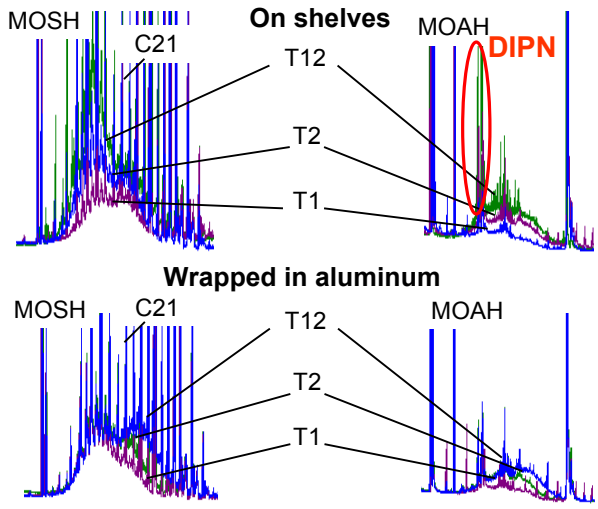


Figure 58. MOSH and MOAH fractions of egg pasta packed in recycled paperboard boxes (without adhesives) kept on shelves (top) and wrapped in aluminum (bottom) for 1, 2 and 12 months.

1.3.3. Plastic film packaging

POSH, polyolefin oligomeric saturated hydrocarbons, such as oligomers from polyethylene or polypropylene, can also migrate into foods and are easily mistaken for MOSH, since both largely consist of highly isomerized branched and possibly cyclic hydrocarbons forming humps of unresolved components in gas chromatography (Biedermann-Brem et al., 2012). The trace of the plastic film (saturated hydrocarbons fraction) presented a typical profile of polypropylene material with characteristic groups of peaks (Biedermann and Grob, 2012b), well recognizable in the pasta packed with the same material (Figure 59)

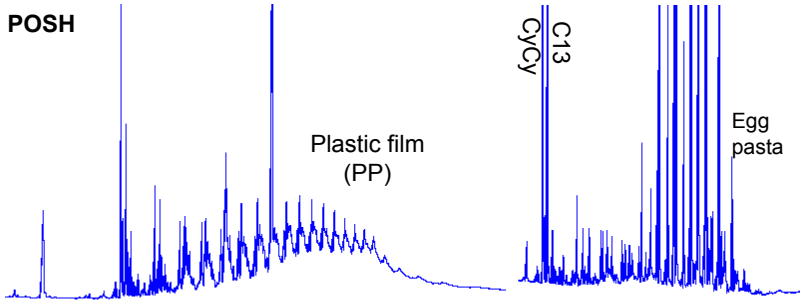


Figure 59. On the left: PP plastic film at time zero containing about 291 mg/kg of POSH<C_{25}. On the right: typical profile of contamination from PP film found in egg pasta after 12 months of exposure containing 1.2 mg/kg MOSH<C_{25}.

In the semolina pasta packed in printed polypropylene film, both kept on shelves and wrapped in aluminum, values lower than 0.8 mg/kg for POSH/MOSH were found. Higher values, up to about 1.2 mg/kg, were found in the egg pasta. All MOSH<C_{25} data are reported in Figure 60. No MOAH or DIPN were detected, even after 24 months of storage on shelves. The transport box had no visible influence on the total contamination found in the semolina pasta samples. As already demonstrated by Fiselier and Grob (2012), polyolefins adsorb significant amounts of mineral oil and PP has quite a high efficiency. In fact, the PP plastic film stored in the transport

box seems to act as a barrier which limits the MOSH migration from the corrugated board to the pasta. This effect is well evident up to 6 months of storage, but seems to disappear for longer storage times, according to Lorenzini et al. (2013).

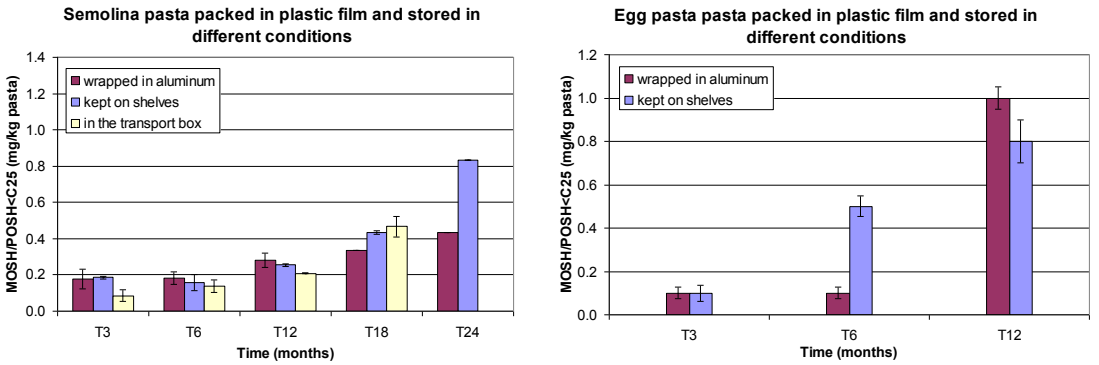


Figure 60. MOSH/POSH<C_{25} in semolina and egg pasta packed in plastic film wrapped in aluminum, kept on shelves and stored in the transport box. Results are expressed as average of 2 replicates (bars) and standard deviations (vertical lines).

1.3.4. DIPNs

DIPNs, originated from carbonless copy paper, are excellent markers for migration from recycled paper and board, since they have no other uses in the food sector (Biedermann and Grob, 2012b).
All the values of DIPN found in the semolina and egg pasta packed in recycled paperboard are reported in Figure 61.
DIPNs, found in the MOAH fractions of the semolina pasta packed in recycled paperboard, presented quite a constant value during the all the storage conditions tested (below 0.2 mg/kg). The DIPNs behavior is the same in recycled paperboard boxes with and without a hot melt adhesive.

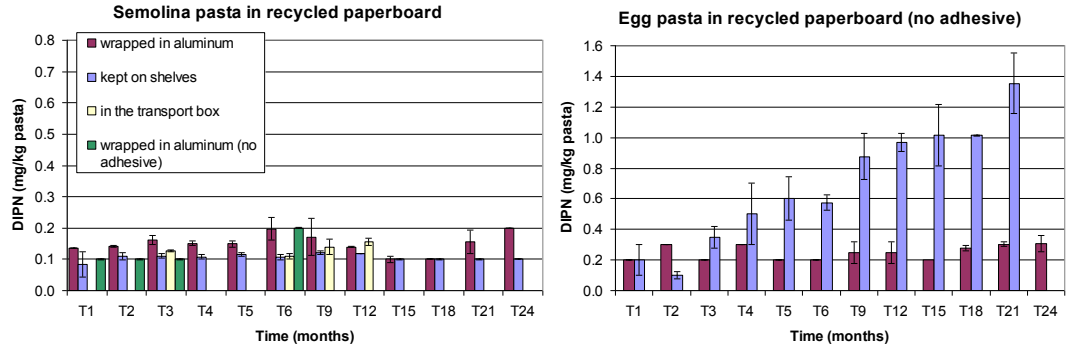


Figure 61. DIPN amount migrated in semolina (scale 0 – 0.8 mg/kg of pasta) and egg pasta (scale 0 – 1.6 mg/kg of pasta) packed in recycled paperboard. Results are expressed as average of 4 replicates (bars) and standard deviations (vertical lines).

DIPN found in the egg pasta packed in recycled paperboard (without hot melts) presented quite constant values in boxes wrapped in aluminum (0.2 – 0.3 mg/kg), while in the samples kept on shelves its value increased from 0.2 mg/kg (after 1 month) to about 1.4 mg/kg (after 21 months)

during storage. These values confirmed a higher affinity to the migration from egg pasta (as suggested by the MOSH and MOAH data), and for the samples kept on shelves, a contribution from the external environment.
As already touched on, DIPN detected in a fresh fiber paperboard is indicative either of admixture of some recycled fibers or a migration from recycled paperboard, such as a transport box (Biedermann and Grob, 2012b). In fact, the semolina pasta packed in virgin paperboard presented traces of DIPN in packs kept on shelves and in the transport box, while the wrapped in aluminum were completely free. This confirmed also the good barrier properties of aluminum toward MOH migration. In the egg pasta packed in virgin paperboard, the maximum DIPN value found was 0.3 mg/kg after 12 months of storage on shelves, and was certainly due to the external environment, since in packs wrapped in aluminum DIPN was not present.

1.3.5. Migration

For the migration study, MOSH and MOAH values up to *n*-C_{25} were considered, in accordance with Lorenzini and co-workers (Lorenzini et al., 2010), who demonstrated that evaporation at ambient temperature was significant for the hydrocarbons eluted from gas chromatography up to about the *n*-alkane C_{24}.

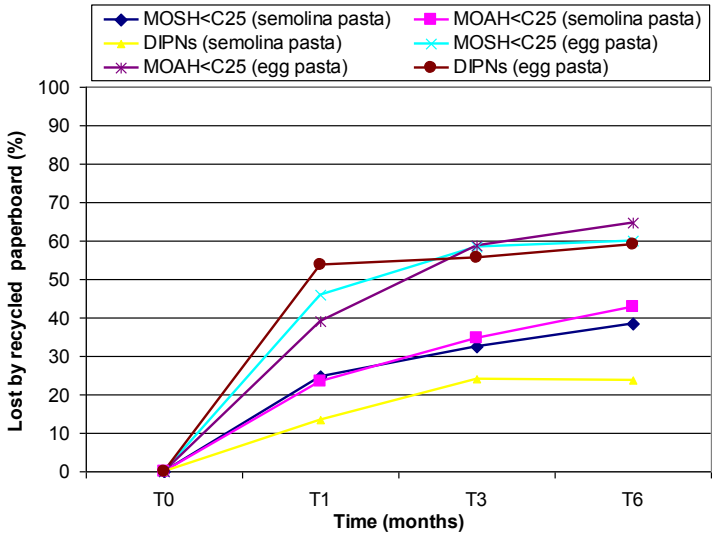


Figure 62. DIPNs, MOSH and MOAH <C_{25} lost by recycled paperboard boxes (expressed as percentage with respect to the amount before the contact with food), without adhesive, filled with semolina and egg pasta and wrapped in aluminum.

Figure 62 shows the results as a percentage of hydrocarbons lost by the recycled board after exposure. It highlights a faster loss of hydrocarbons if the paperboard is in contact with egg pasta (60 % of MOSH<C_{25} lost by paperboard after 6 months of exposure) with respect to the contact with semolina pasta (39 % of MOSH<C_{25} lost by paperboard after 6 months of exposure). It also pointed out that MOSH and MOAH have very similar migration kinetics. The semolina pasta data go up to 6 months of exposure, but for the egg pasta a loss of 82 % of MOSH<C_{25}, 88 % of MOAH<C_{25} and 56 % of DIPNs can be calculated after 24 months of exposure. After six months of contact between recycled paperboard and both semolina and egg pasta, the DIPN seemed to reach the equilibrium. Also MOSH and MOAH seemed to reach an equilibrium when the paperboard was in contact with the egg pasta. When the recycled paperboard was in contact with the semolina pasta 6 months of exposure seemed insufficient to

reach the equilibrium. However, due to the limited sampling frequency (only 1, 2, 3 and 6 months of storage) it was not possible to experimentally prove this.
The same type of graphic related to virgin paperboard is reported in Figure 63, where the different migration behavior during the contact with semolina and egg pasta is emphasized. After 6 months of exposure, only 7 % of MOSH<C_{25} was lost by the virgin paperboard in contact with the semolina pasta, while 71 % was lost if the paperboard was in contact with the egg pasta.
Considering the amount of pasta, the weight of an empty paperboard box (about 20 and 12 g for recycled and virgin paperboard, respectively) and the contamination at time zero, a potential migration can be calculated for each box. The complete data are reported in tables 16-A and 16-B. For recycled paperboard boxes, a potential migration of 15.7 and 3.7 mg/kg of pasta for MOSH and MOAH up to *n*-C_{25}, respectively, was calculated. The potential migration calculated for each pack of virgin paperboard was about 1 mg/kg of pasta for MOSH up to *n*-C_{25}.

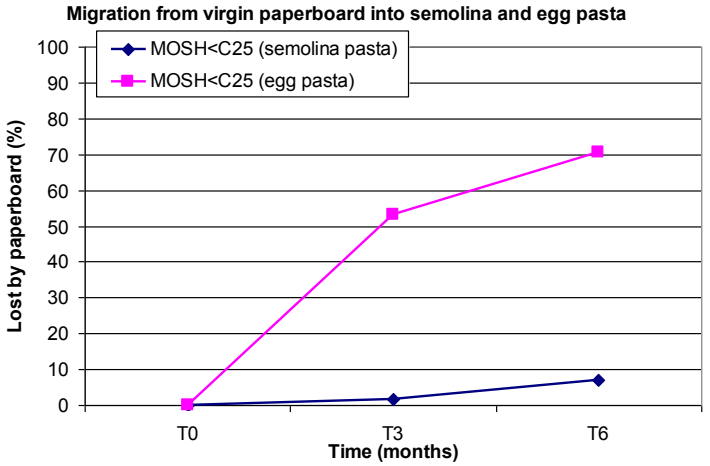


Figure 63. MOSH<C_{25} lost by virgin paperboard boxes (expressed as percentage with respect to the amount before the contact with food), without adhesive, filled with semolina and egg pasta and wrapped in aluminum.

From the difference between the mineral oil amount found in the paperboard pre- and post-contact, the real percentage of MOSH and MOAH up to *n*-C_{25} migrated from paperboard boxes was calculated. The migration was evaluated only for samples without adhesives, since the presence of PAO affected the data.
In Figure 64, MOSH<C_{25} (mg/kg pasta) lost by the paperboard and found in the pasta are reported. The amount lost by the paperboard was calculated as difference between the contamination before and after the exposure referred to the whole box, while the contamination found in the pasta was referred to the amount of MOSH<C_{25} found in the pasta after the exposure, opportunely subtracted by any contamination before the contact with packaging.
All the samples considered for these observations were packed without adhesives and wrapped in aluminum foils. It is well known that in a closed system, the mass balance should be respected: everything that evaporates from packaging should be found in food. The boxes wrapped in aluminum would simulate a closed system, but the data revealed that mass balance is not fully respected when considering only the packaging and the food. In fact, the amount of hydrocarbons lost by the paperboard was higher than the amount found in the pasta after exposure. Probably a part of the contamination escaped from the aluminum cover and/or part of the contamination remained in the vapor phase in the head space of the boxes without re-condensing into the food.

It is well evident considering the egg pasta that there was a better correspondence between the hydrocarbons lost by the paperboard and found in the pasta (78, 85, 75 % of MOSH found in pasta with respect to that lost by paperboard after 1, 3 and 6 months, respectively) The higher fat content in the egg pasta seems to promote the migration from packaging and the re-condensation on the food. In the semolina pasta packed in recycled paperboard a lower amount of saturated hydrocarbons with respect to that lost by packaging was found (46, 64 and 72 % after 1, 3 and 6 months, respectively). Perhaps parte of the contamination escaped from the aluminum foil or remained in the vapour phase in the head space without re-condensing on the pasta surface. In figure 65 the contamination found in the pasta after the contact with the packaging (real migration) and the potential migration (calculated considering that all the MOSH<C_{25} present in the box before the exposure migrated into the pasta contained in it) are reported. The percentages are related to the contamination found in the pasta with respect to the potential migration. Also after 24 months of exposure, the real migration never reached the 70 % empirically derived by Biedermann and Grob (Biedermann and Grob, 2010; Lorenzini et al., 2010; Biedermann and Grob, 2012b). In fact, 64 % was the maximum value found for the egg pasta packed in recycled paperboard for 12 and 24 months, but generally the real migration is below 50 % of the potential value.

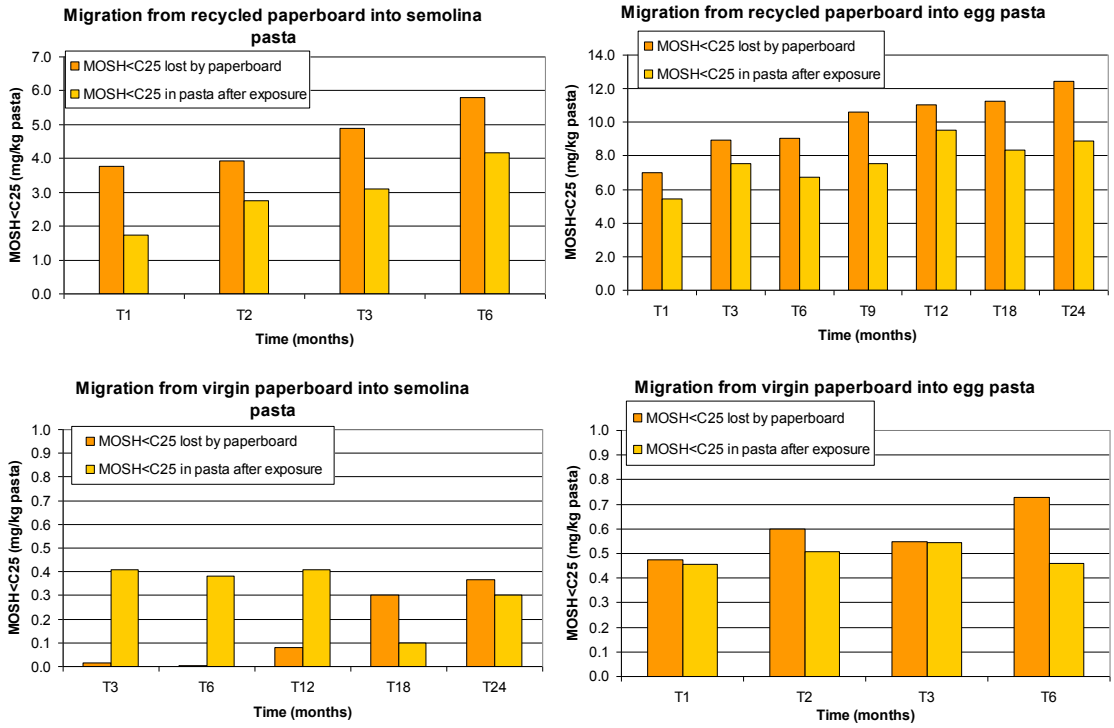


Figure 64. MOSH<C_{25} (mg/kg pasta) values lost by paperboard and migrated into pasta.

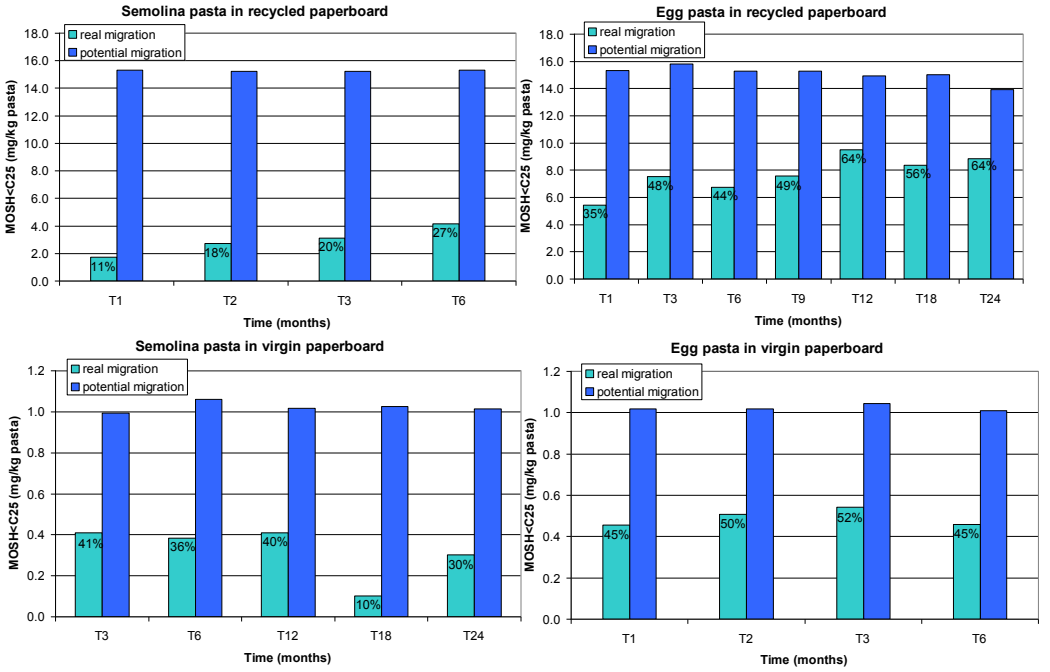


Figure 65.Comparison between real and potential migration of MOSH up to n-C_{25}.

1.3.6. Penetration inside the food product

The penetration of the contamination from the packaging inside the product was evaluated comparing the data obtained from the analysis of ground pasta, which revealed the total contamination, and the data from the superficial contamination, given by the packaging.
The data obtained from the analysis of semolina pasta packed in recycled paperboard with hot melts revealed that a negligible part of the contamination from the packaging penetrated deeply into the sample (and slowly increased with storage), becoming less available for a superficial extraction. After 12 months of storage (all conditions tested), the deep contamination reached levels of about 10 % of the contamination coming from the packaging. In Figure 66, three different chromatograms obtained from the analysis of semolina pasta before the contact (green line) and after 1 (violet line) and 12 months (blue line) of exposure in recycled paperboard boxes closed using adhesives are overlaid to highlight the increase of the contamination migrated from packing and penetrated deep into the pasta sample. The chromatograms are relative to ground semolina pasta extracted using PLE method B, after a first superficial extraction on the whole pasta using PLE method A (as described in *2.3 Extraction*).
The same procedure of analysis was applied in semolina pasta packed in recycled paperboard boxes without adhesive deposition and the data obtained up to 6 months of exposure indicated that mineral oil hydrocarbons migrated from the packaging did not penetrate deep into the pasta grain (the different chromatograms are perfectly overlaid). Thus, it was concluded that the PAOs presence increased not only the superficial value of contamination coming from the packaging, as discussed above, but also the level of contamination penetrated deep into the food.

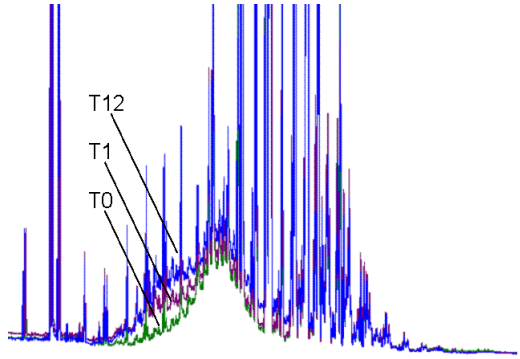


Figure 66. Semolina pasta at time zero and after 1 and 12 months of exposure with recycled paperboard boxes closed with adhesive: traces of MOSH fraction of ground pasta analyzed with PLE-B method, after a first superficial extraction with the PLE-A method.

1.4 Conclusions

Mineral oil migration from packaging to food is a rapid phenomenon and reaches considerable levels already after 1 month from packaging time, in particular if recycled paperboard was used. Semolina and egg pasta packed in virgin paperboard boxes presented MOSH contamination below 0.6 mg/kg, also after 24 months of storage.

A consistent contribution to food contamination coming from packaging can be due to adhesives applied to close the boxes. In particular, PAO presented a greater inclination to penetrate inside the pasta grain with respect to MOSH. Not only the packaging, but also the external environment contributes to the total food contamination.

Food characteristics seem to influence mineral oil migration from packaging: the egg pasta reached higher levels of contamination than the semolina pasta, due to its higher fat content.

2. MINERAL OIL MIGRATION FROM PACKAGING INTO DRY SEMOLINA AND EGG PASTA UNDER ACCELERATED CONDITIONS.

2.1. Background

As already mentioned in the introduction, the Regulation EC 10/2011 (Reg. EC 10/2011) suggested migration tests to assess food packaging using food simulants. Modified polyphenylene oxide (MPPO or Tenax®) is the simulant (E) assigned for testing specific migration into dry foods. The main aim of accelerated migration tests is to shorten the duration of the testing, in particular for products stored for long periods of times, such as dry pasta in paperboard boxes, with a typical shelf-life of 1-3 years. Simulation usually depictes the worst case for migration into food (overestimation), however, some data on food analysis have revealed a number of cases for which simulation underestimated migration into food. This way, consumers and producers are not equally protected: some producers complain about the over-restrictive legislation and testing with simulant grossly overestimating real migration, unnecessarily restricting their possibility (Grob, 2008).
In this work mineral oil migration kinetics was investigated performing accelerated tests (temperature controlled) on dry semolina and egg pasta packed in different materials (printed polypropylene film, virgin and recycled paperboard). These data were compared with those obtained from the storage in different conditions the same food-packaging combination at room temperature up to 2 years [see chapter 1 of this PhD work part 1].

2.2. Experimental

2.2.1. Samples and sampling frequency

Two kinds of dry pasta (semolina and egg with 1.5 and 4 % fat, respectively) of the same small size (rice-shaped) and three different types of packaging (PP plastic film, recycled and virgin paperboard) were combined for migration tests. All the pasta and packaging samples were provided directly by producers. All packs, manually prepared in the laboratory, closing the two sides of the boxes with staples, were wrapped in aluminum in order to eliminate the external environment influence and to minimize a possible escape of a part of the contamination, which is therefore forced to migrate into the food. Accelerated migration kinetics were studied at 40 °C (for virgin and recycled paperboard) and at 60 °C (for plastic film). The temperature was controlled by using a thermostat (Incucenter IC240, SalvisLab), reported in figure 67. Sampling frequency (starting from packaging time) was established for each type of pasta-packaging combination, as reported in Table 17. All the samples were collected in duplicate. After the sampling, pasta and paperboard were separated, wrapped in aluminum and stored in a freezer until analysis.

Figure 67. Thermostat (Incucenter IC240, SalvisLab) containing pasta packs wrapped in aluminum.

2.2.2. Migration assessment

Migration from packaging into dry food was calculated in two different ways: the direct migration was calculated from the mineral oil contamination found in the pasta samples (after subtracting of base contamination), while the indirect migration was calculated comparing the mineral oil content of the paperboard before and after the exposure.
Before starting with the migration tests, all the foods and packaging were analyzed to assess the base contamination (see chapter 1 of this section). The results presented in this study were referred only to real mineral oil migration, since they were subtracted from the contamination at time zero.

2.2.3. Extraction

The food and packaging samples were extracted as already described in chapter 1 of this section (*1.2.3*).

Table 17. Characteristics of pasta and packaging samples and sampling frequency.

	Semolina pasta			Egg pasta		
Food size and shape	Small size, rice-shaped			Small size, rice-shaped		
Food fat (%)	1.5			4		
	Recycled paperboard	***Virgin paperboard***	***Plastic film (PP)***	***Recycled paperboard***	***Virgin paperboard***	***Plastic film (PP)***
Exposed food weight (g)	500	250	500	500	250	500
Pack weight (g)	20	12	4	20	12	4
Pack size (cm2)	133.8	124.3	287.12	133.8	124.3	287.12
Pack volume (cm3)	561.8	373.0		561.8	373.0	
DIPN in paperboard (mg/kg)	12.41			12.41		
MOSH<C25 (mg/kg)	388	21.5	290	388	21.5	290
MOAH <C25 (mg/kg)	90			90		
Max MOSH <C25 potential migration (mg/kg food)	15.7	1	2.3	15.7	1	2.3
Max MOAH <C25 potential migration (mg/kg food)	3.7			3.7		
Temperature	40 °C	40 °C	60 °C	40 °C	40 °C	60 °C
Sampling frequency	1,3,5,7,10,12,15,20,25,30	5,10,15,20,25,30	5,10,15,20,25,30	5,10,15,20	5,10,15,20	5,10,15,20

2.3. Results and discussion.

2.3.1. Recycled paperboard packaging

The semolina pasta (about 500 g) was packed in recycled paperboard boxes, wrapped in aluminum and kept at 40 °C for 30 days. The mineral oil saturated hydrocarbon levels found in the pasta (up to *n*-C_{20}, *n*-C_{25} and the total contamination) after the exposure are reported in figure 68.

After 30 days at 40 °C the semolina pasta packed in recycled paperboard reached a contamination of about 3.1, 5.8 and 6.5 mg/kg of MOSH up to *n*-C_{20}, *n*-C_{25} and total (up to about *n*-C_{31}), respectively. The saturated hydrocarbons up to *n*-C_{20} migrated very rapidly, in fact after 3 days of exposure, 72 % of the maximum MOSH<C_{20} amount found had already migrated and the 92 % was reached after 10 days. From 10 to 30 days this value remained quite constant, while the hydrocarbons in the C_{20}-C_{25} range were still migrating. In particular, 3.2, 4.6 and 5.8 mg/kg of MOSH<C_{25} were found in the semolina pasta after 3, 10 and 30 days, respectively (54, 79 and 100% of the higher value found). A small amount of MOSH over *n*-C_{25}, up to about *n*-C_{31}, migrated during that time, reaching a maximum of 10 % of the total contamination after 30 days. MOAH up to *n*-C_{25} reached a maximum value of 0.4 mg/kg of pasta already after 7 days and remained constant for up to 30 days of exposure. Comparing the data obtained during the migration tests performed at room temperature, the value of MOSH<C_{25} of about 3 mg/kg in semolina pasta packed in recycled paperboard (without adhesive) and wrapped in aluminum was found after 3 months of exposure. The trend described is well evident also in the overlay of MOSH traces obtained by analyzing the semolina pasta in recycled paperboard wrapped in aluminum and kept at 40 °C for up to 30 days (figure 69).

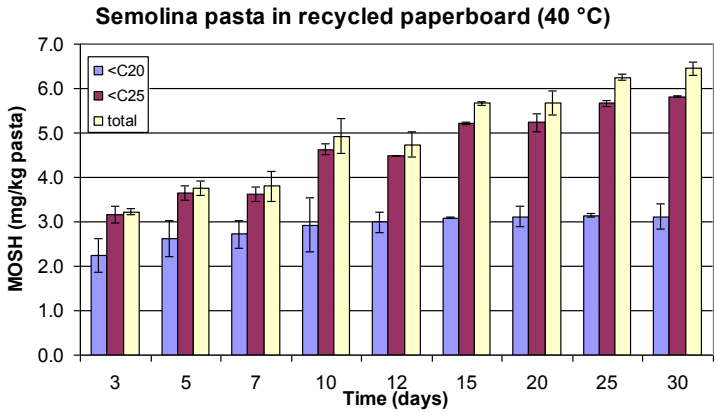


Figure 68. MOSH found in semolina pasta packed in recycled paperboard boxes wrapped in aluminum and kept at 40 °C up to 30 days. Results are expressed as average of 4 replicates (bars) and standard deviations (vertical lines).

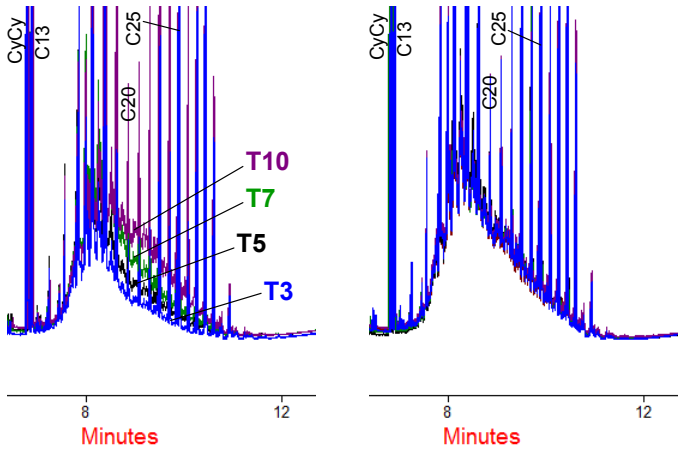


Figure 69. LC-GC-FID traces of MOSH fraction of semolina pasta in recycled paperboard wrapped in aluminum and kept at 40 °C for 3, 5, 7, 10 days (on the left)and for 12, 15, 20, 25, 30 days (on the right).

The egg pasta packed in recycled paperboard reached a maximum value of 4.7, 7.2 and 7.5 for MOSH up to C_{20}, C_{25} and total, respectively, after 30 days at 40 °C. All the data are reported in figure 70.
After 10 days at 40 °C the migration of all saturated hydrocarbons seems to be completed also for the heavier ones; in fact from 10 to 20 days their amounts remained quite constant. Probably, the higher fat content accelerated the migration and the equilibrium was reached quite fats.

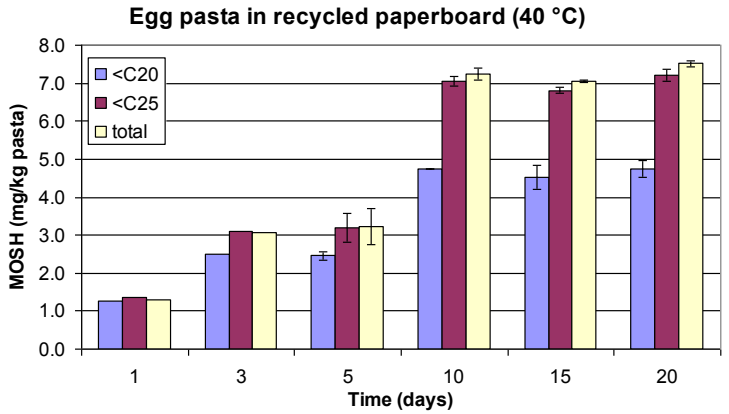


Figure 70. MOSH found in semolina pasta packed in virgin paperboard boxes wrapped in aluminum and kept at 40 °C up to 30 days. Results are expressed as average of 4 replicates (bars) and standard deviations (vertical lines).

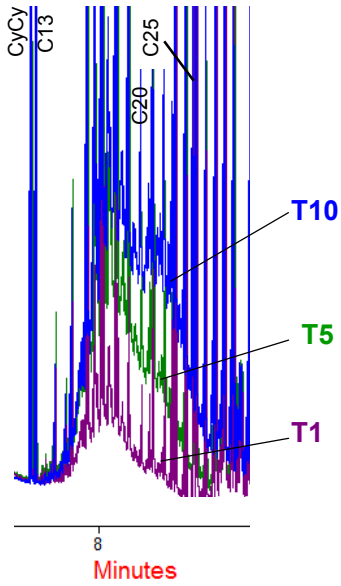


Figure 71. LC-GC-FID traces of MOSH fraction of egg pasta in recycled paperboard wrapped in aluminum and kept at 40 °C for 1, 5 and 10 days.

In figure 71, LC-GC-FID traces (MOSH) obtained from the analysis of the egg pasta packed in recycled paperboard and kept at 40 °C for different times are reported. It is well evident how from the time of 5 to 10 days another "hump" in the C_{20}-C_{25} range appeared. At room temperature the maximum value found in the egg pasta packed in recycled paperboard wrapped in aluminum was 8.5 mg/kg of MOSH up to *n*-C_{25}, after 24 months of exposure. This value was never reached under accelerated conditions, indicating that, perhaps, at high temperature, such as 40 °C, a greater amount of mineral oil hydrocarbons remains in the head space of the box in the vapour phase.

2.3.2. Virgin paperboard packaging

In the semolina and egg pasta packed in virgin paperboard and kept wrapped in aluminum at 40 °C, a maximum level of MOSH below 0.5 mg/kg was found already after 10 days of exposure. No MOAH were detected. The semolina and egg pasta packed in virgin paperboard, wrapped in aluminum and stored at room temperature reached a maximum MOSH level of 0.4 and 0.6 mg/kg, respectively, after 3 months of storage. Therefore, the higher temperature does not seem to influence the mineral oil migration from virgin paperboard packaging. Furthermore, no difference was observed between pastas with a different fat content. However, since the virgin paperboard before the contact with food presented low contamination levels, the values found in the pasta were close to the LOD, thus the data were affected by a rather high variability.

2.3.3. Plastic film packaging

The semolina and egg pasta (about 500 g) were packed in a PP packaging, wrapped in aluminum and kept at 60 °C up to 20 days (Reg. 10/2011 suggests accelerated tests at 60 °C for a maximum of 10 days for plastic materials used for contact time with food above 30 days at room temperature). After the exposure, a negligible amount of MOSH/POSH was found in the semolina pasta. However, in the egg pasta 1.3 mg/kg of MOSH/POSH up to *n*-C_{25} was found already after 5 days at 60 °C and remained constant up to 20 days. This value is very similar to that found in egg pasta packed in plastic film wrapped in aluminum and stored at room temperature for 12 months.

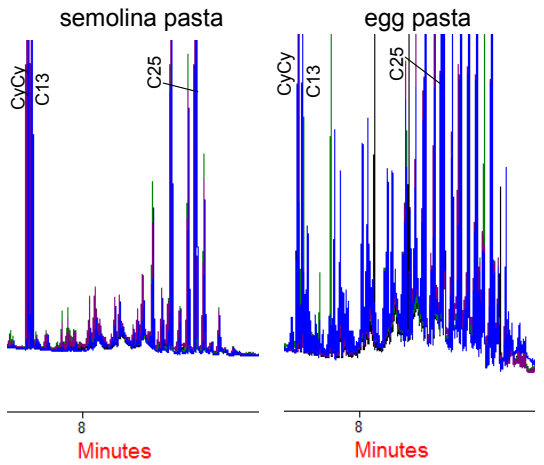


Figure 72. LC-GC-FID traces obtained from the analysis of semolina and egg pasta packed in PP plastic film, wrapped in aluminum and kept at 40 °C for 5, 10, 15 and 20 days. CyCy and C_{13} are internal standards.

2.3.4. Migration

Figure 73 shows the results as a percentage of hydrocarbons (MOSH<C_{25}) lost in the recycled board after exposure. It highlights a faster loss of hydrocarbons if the paperboard is in contact with egg pasta (61 % of MOSH<C_{25} lost by paperboard after 5 days at 40 °C) with respect to the contact with semolina pasta (31 % of MOSH<C_{25} lost by paperboard after 5 days at 40 °C).
A similar graphic referred to the virgin paperboard is reported in figure 74. In this case, as already mentioned, the migration seems to be independent of the amount of fat in the food packed in the boxes: the trend of evaporation of MOSH<C_{25} from paperboard is very similar for the virgin paperboard boxes containing both semolina and egg pasta. However, while the amount of MOSH<C_{25} lost by the virgin paperboard boxes containing semolina pasta and stored at room temperature reached 7 % after 6 months of exposure, using accelerated conditions, such as 40 °C, this amount reached 54 % after 5 days of exposure.

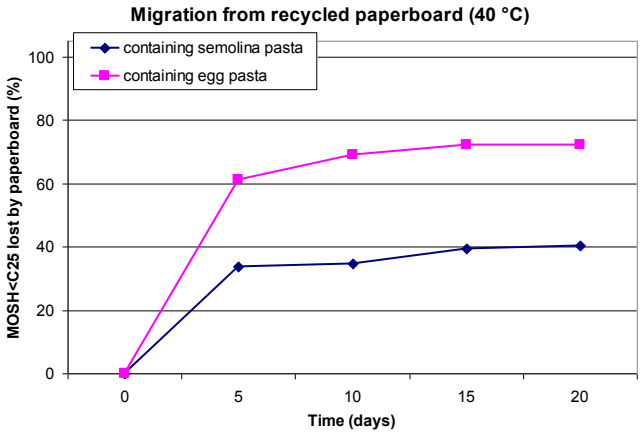


Figure 73. Evaporation of MOSH<C_{25} from recycled paperboard boxes, filled with semolina and egg pasta, wrapped in aluminum and kept at 40 °C up to 20 days.

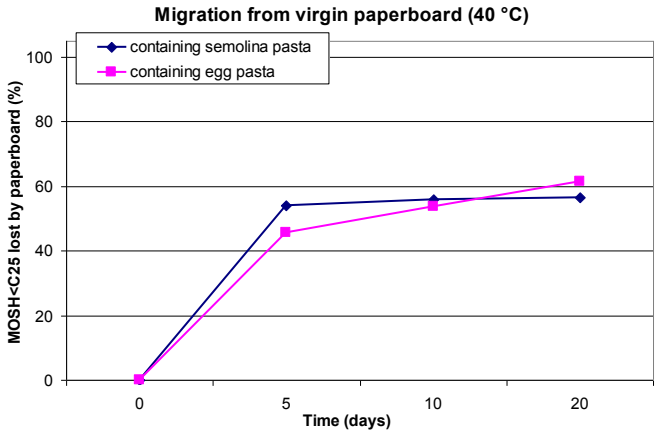


Figure 74. Evaporation of MOSH<C_{25} from virgin paperboard boxes, filled with semolina and egg pasta, wrapped in aluminum and kept at 40 °C up to 20 days.

As visible in figure 75, the amount of MOSH<C_{25} lost by the paperboard is always higher than the amount found in the pasta, especially when semolina pasta is packed in recycled paperboard, that is, the contrary of what happened during migration at room temperature (see paragraph *1.3.5* of this section). In particular, 70 and 85 % of MOSH were found in the semolina pasta with respect to that lost by paperboard after 5 and 10 days, respectively; while 33 and 66 % were found in the egg pasta.
Not all the hydrocarbons lost by paperboard were found in the pasta, as had been supposed during the migration tests at room temperature, because a part of the contamination was lost from the aluminum or remained in the head space in the vapor phase, also due to the high temperature applied. The same behavior was observed in the virgin paperboard boxes containing egg and semolina pasta: the amount of MOSH<C_{25} found in pasta were clearly lower than those lost by the packaging.
However, since the virgin paperboard had a low level of contamination, the values found in pasta were close to the LOD and thus affected by a rather high variability.

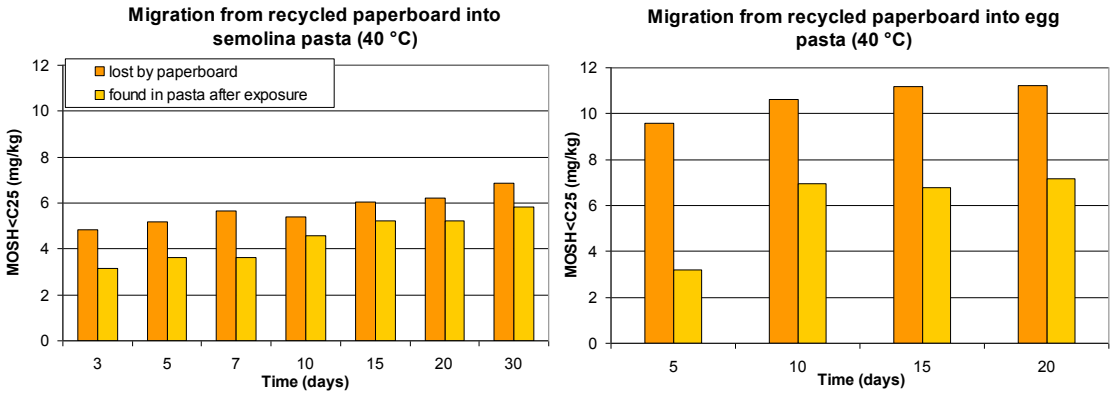


Figure 75. MOSH<C_{25} (mg/kg) values lost by paperboard and migrated into pasta.

In figure 76 the contamination found in the pasta after contact with the recycled paperboard (real migration) and the potential migration (calculated considering that all the MOSH<C_{25} present in the box before exposure migrated into the pasta contained in it) are reported. The percentages are related to the contamination found in the pasta with respect to the potential

migration. The migration was higher in the case of the egg pasta, like during migration at room temperature. However, the levels of real migration were lower than those reached during the tests at room temperature. Neither in this case the level of 70 % of migration supposed by Biedermann and Grob (Biedermann and Grob, 2010; Lorenzini et al., 2010; Biedermann and Grob, 2012b) was reached.

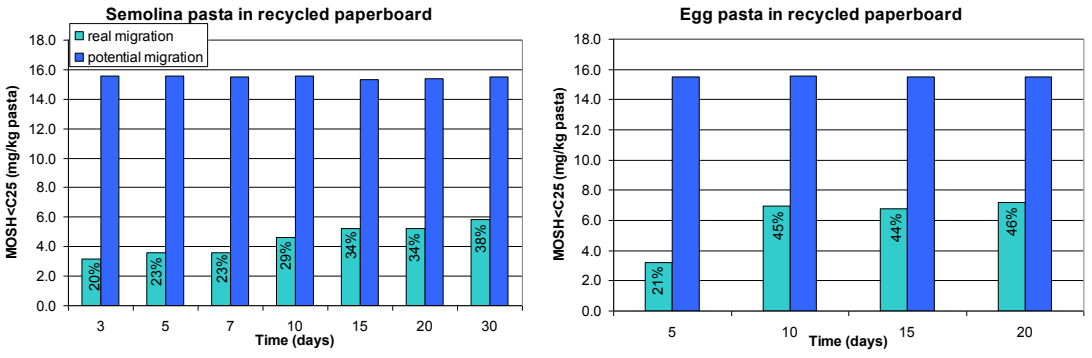


Figure 76. Comparison between real and potential migration of MOSH up to n-C_{25}.

Furthermore, the migration data obtained by using accelerated tests were completely comparable with those obtained at room temperature, not only in terms of concentration, but also in terms of molecular mass that migrated. In fact, under accelerated conditions, migration of MOHs from paperboard into the semolina and egg pasta in the C_{20}- C_{25} range was faster than at room temperature, and mostly involved the molecular mass up to about *n*-C_{25}, and also it happened at room temperature. These data are in disagreement with those reported by Lorenzini et al. (2013) for egg pasta and muesli, where a higher molecular mass ($<_n$-C_{29}) migrated significantly at higher temperatures (40 and 60 °C) and after prolonged storage.

2.4. Conclusions and future perspectives

The study of mineral oil migration from packaging into food under accelerated conditions was performed in order to find a correlation with the migration during the shelf-life of the product.
The migration kinetic is accelerated with respect to that at room temperature, in particular for the heavier hydrocarbons, but the trend is very similar. The migration of volatile hydrocarbons up to *n*-C_{20} was very fast and complete in a short time and the molecular masses involved (up to about *n*-C_{25}) were comparable to that migrated at room temperature.
Nevertheless, the data obtained from accelerated condition tests are difficult to translate to long-term migration at ambient temperature, and further tests are necessary to find a useful correlation with shelf-life conditions.
A further research objective will include a comparison between data here reported and data obtained from migration tests performed using Tenax®. It will be possible to study the suitability of Tenax® for simulate the behaviour of real dry food with different amount of fat, at different temperatures.

PhD WORK PART 3: MINERAL OIL IN HUMAN TISSUES.

1. MINERAL OIL IN HUMAN TISSUES: CONCENTRATIONS AND MOLECULAR MASS DISTRIBUTIONS.

1.1. Background

Transfer of animal data to humans faces uncertainties. Firstly, animal tests last for far less time than human lives and, therefore, accumulation is not adequately reflected. In none of the animal tests a steady state concentration in tissue was reached.
In abdominal fat from 144 women living in Austria collected during a Caesarean section, Concin et al. (2008) measured MOH concentrations from 15 to 360 mg/kg fat (average of 60.7 mg/kg). Milk fat from days 4 and 20 contained up to 355 mg/kg MOSH, with averages of 45 and 22 mg/kg on days 4 and 20, respectively. The composition of the MOSH was largely identical in all fat tissues and milk samples, in gas chromatographic retention times ranging from *n*-C_{17} to *n*-C_{32} and centered at *n*-$C_{23/C24}$. Since the mineral oil products humans are exposed to range from much smaller to much higher molecular mass, and may contain prominent *n*-alkanes, the MOSH in the fat tissue and milk must have been a residue from selective uptake, elimination by evaporation and metabolic degradation.
In order to identify the most relevant sources, the MOSH concentration in fat tissues were correlated with women's personal data, nutrition habits and cosmetics use. The age was the predominant predictor for accumulated MOSH, supporting the assumption that part of the MOSH are persistent for a long time, possibly accentuated by a wider use of mineral oils in the food industry up until the 1990s. There was also some association with cosmetics, which translates to dermal uptake (Concin et al., 2011).
Although limited information exists on toxicokinetics of MOAH, the available data indicate that these compounds are well absorbed and are rapidly distributed amongst all organs. The data also indicate that MOAH are extensively metabolised and do not bioaccumulate (EFSA, 2012).
In the present work, human tissues, namely spleen, liver, MLN, lung, fat tissues, brain, kidney and heart, were sampled during an autopsy. They were analyzed for MOH (mainly MOSH) in terms of concentration and molecular mass distribution. The work has been done at the Official Food Control Authority of Zurich, in collaboration with the Medical University of Innsbruck and of Vienna and with the Bacteriology Institute of Vienna.

1.2. Experimental

1.2.1. Sample collection

Between February and August 2013, tissue samples from liver, spleen, mesenteric lymph node (MLN), lung and abdominal fat tissues were collected from 37 patients (11 females and 26 males) during autopsy at the Clinical Institute of Pathology (Medical University) in Vienna. In addition, brain, heart and kidney samples were collected only from 14 patients. Samples were kept frozen at -20 C° until the analysis time. Some personal information, such as age, sex, weight, height, relevant diseases during life, occupation and cause of death, were available for each patient.

1.2.2. Sample preparation and extraction

In order to rule out infection problems, the thawed tissues were rinsed with 1 % hydrochloric acid before handling. Then, they were dried with absorbent paper to remove water and blood, immersed in ethanol in 1:1 (w/w) sample to solvent ratio and homogenized using a Polytron® homogenizer (PT 10-35 GT). The extraction of MOH was performed as described by

Biedermann and Grob (2012a) for wet matrices. Briefly, to 2 g of tissue/ethanol (w/w) homogenate weighted in a 10 mL centrifuge tube, 4 mL ethanol and 20 µL the internal standard solution were added. This mixture was intensively shaken and allowed to stand for at least 1 hour to allow ethanol to enter the particles. After centrifugation, ethanol was decanted into a 30 mL centrifuge tube. The tissue residue was accurately homogenized with 5 mL hexane and extracted overnight at ambient temperature. After the centrifugation of the tube, supernatant hexane was added to the previously collected ethanol and approximately 10 mL of water was admixed in order to separate the hexane from the rest of the mixture. The final hexane extract was injected (100uL) in the LC-GC system.

1.2.3 Chromatographic analysis

MOSH were analyzed by on-line normal phase HPLC-GC-FID (Biedermann et al., 2009; Biedermann and Grob, 2012a). Briefly, 100 µL extract in hexane was injected into a 25 cm × 2mm i.d. HPLC silica gel column (LiChrospher Si 60 5 µm). The MOSH fraction was eluted between 1.45 and 2.45 min using hexane at 300 µL/min as eluent. After the transfer of the MOSH fraction to the GC, the eluent pushed by the LC pump is forced to pass through the backflash loop, filled with 1 mL of dichloromethane by using a pressurized reserve. This way, the LC column is backflushed in order to eliminate the bulk of the sample. The transfer from the LC to the GC system was performed by using the retention gap technique with partially concurrent eluent evaporation. A 7 m × 0.53 mm i.d. uncoated, deactivated pre-column was connected to the solvent vapor exit and a 15 m × 0.25 mm i.d. separation column coated in the laboratory with a 0.13 µm film of PS-255, a dimethyl polysiloxane. GC oven temperature was programmed from 65 °C (5 min) to 350 °C at 15 °C/min. The area corresponding to the MOSH was determined by the integration of the whole hump of largely unresolved material above the baseline transferred from a blank run (obtained by injecting hexane). Internal standards and endogenous hydrocarbons were subtracted from the hump (Biedermann et al., 2009; Biedermann and Grob, 2012b).

1.3. Results and discussion

1.3.1. Validation of the extraction method

In order to validate the extraction method, cow liver containing less than 0.5 mg/kg of MOSH was spiked with 10, 50, 100 and 150 mg/kg of a white mineral oil used as a release agent in a candy factory. After 1 h immersion in ethanol, extraction with hexane was performed during 1 h or overnight. After 1 h, recoveries ranged from 59 to 93 %, while the overnight extraction gave recoveries above 90%.

The extraction yield was also determined on the human tissues (liver, lung, spleen, lymph nodes and fat tissue). After the 1 h equilibration with ethanol, the samples were extracted with hexane for 1 h. The residues were extracted another time overnight. The proof that 1 h was insufficient for a quantitative extraction of the MOSH was obtained from a comparison with the overnight extraction: overnight extract still contained 25 % MOSH for the lung and 0.5-9 % MOSH for the other tissues. The residues from the overnight extracts were also treated with hydrochloric acid at 80 °C for 30 min: less than 1 % MOSH compared to the previous extracts was found.

Monitoring the pyrene (polycyclic aromatic hydrocarbons with 4 benzoic rings used as a marker) by HPLC-UV detection (245 nm), the amount of water added to split the hexane/ethanol 1/1 (v/v) solution from the combined extracts was optimized. 82 % of the pyrene was recovered in the hexane phase, adding a volume of water equal to the ethanol, while recovery increased to 97 % when adding a doubled volume of water.

In order to avoid fat breakthrough of the LC column, 20 mg tissue was injected. As there was still some breakthrough for brain extracts, a programmed temperature vaporizing (PTV) injector was inserted between the transfer line and the uncoated GC pre-column, as described by Biedermann and Grob (2013a), with an upper injector temperature of 250 °C.

The coefficient of variation (CV %), calculated by performing 5 replicate extractions from the same human liver homogenate containing 18 mg/kg MOSH, was about 3 %.
A calibration curve was constructed using a mixture of the extract from a liver tissue containing 32 mg/kg MOSH and the extract from a cow liver without detectable MOSH in the 2.7 – 29.6 mg/kg range (2.7, 5.2, 7.9, 19.1 and 29.6 mg/kg, whose LC-GC traces are reported in figure 77), with each level analyzed in triplicate.
The LOD and LOQ were calculated as 3.3 and 10 times, respectively, the standard deviation at the lower calibration point (2.7 mg/kg), and this value was plotted on the calibration line. The resulting LOD and LOQ were 0.7 and 2 mg/kg, respectively.

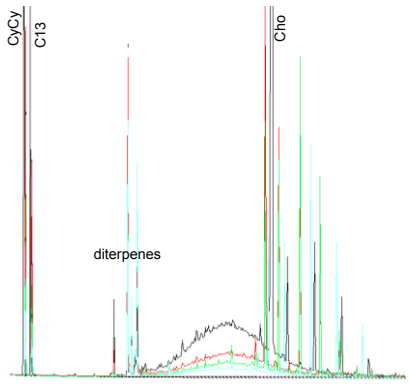


Figure 77. Overlay of MOSH traces obtained from injecting several concentrations (2.7, 5, 8, 20 and 30 mg/kg) of an extract from a human liver tissue (containing 32 mg/kg MOSH) mixed with the extract from a cow liver (without detectable MOSH). CyCy, C_{13}, Cho are internal standards.

1.3.2. Concentrations of mineral oils hydrocarbons in human tissues

MOSH concentrations (referred to fresh weight) in the liver, MLN, fat tissue, spleen and lung from 37 individuals sorted by increasing concentration are reported in figure 78. The highest concentrations, 1400 and 1390 mg/kg in the spleen and in the MLN, respectively, were from the same 87 years old male subject of 88 kg body weight. The highest value in liver, 900 mg/kg, was from a female subject of 65 years and 50 kg body weight.
The plots, all in the same scale, show similar MOSH concentrations in liver, MLN, fat tissue and spleen, but with different frequency distributions. Lung tissue contained far less MOSH than the other four tissues.
Ranges of concentrations, mean values and medians, separately for female and male subjects as well as for the complete set of samples, are listed in Table 18. MLN presented the highest mean and median concentrations (223 and 166 mg/kg, respectively, for the complete set of subjects). The liver was next in concentration, but only third in terms of the median, for which the fat tissue was second. The spleens contained 2.4 times less MOSH in terms of mean values and 5.9 times less in terms of the median. The brain, heart and kidney contained far less MOSH than the other tissues analyzed. The mean values and medians of MOSH contents were higher in females than in males for all the tissues, except for the lungs. This might indicate a higher dermal exposure (use of cosmetic products), however, higher concentrations were observed at equal oral exposure for female *Fisher 344* rats (Baldwin et al., 1992).

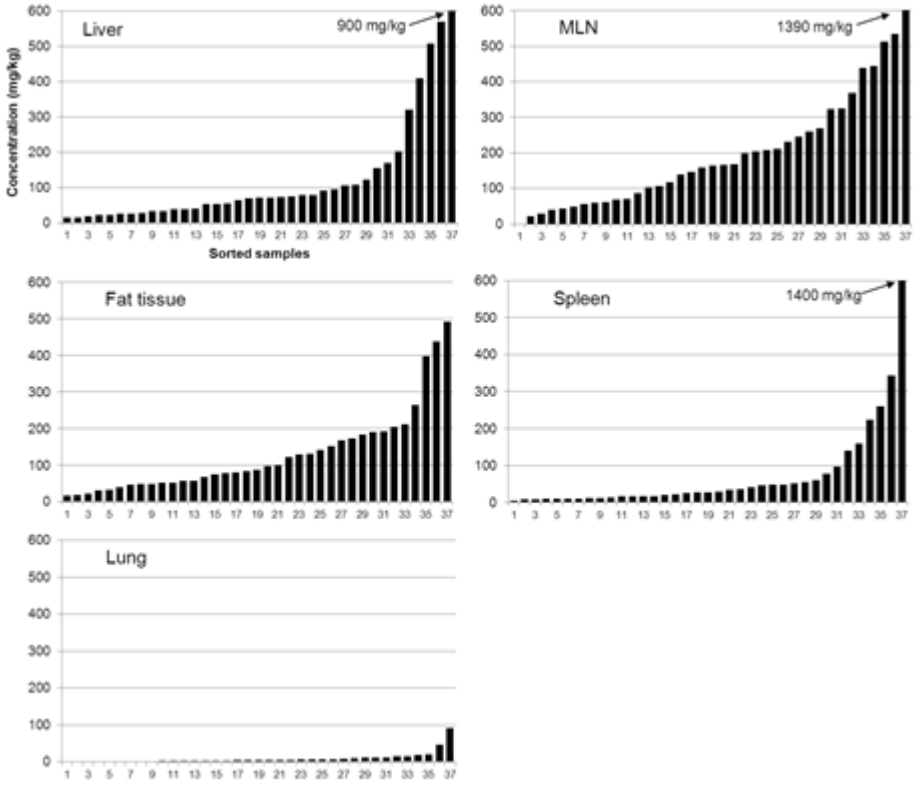


Figure 78. MOSH concentrations found in liver, spleen, lung, fat tissue and MLN sorted by increasing values.

Table 18. Minimum/maximum of MOSH concentrations (mg/kg fresh weight), mean values and medians listed for females, males and for the total of samples.

	Female (n=11)			Male (n=26)			Total (n=37)		
	Min - max values	Average	Median	Min - max values	Average	Median	Min - max values	Average	Median
MLN	71 - 512	261	232	21 - 1390	206	146	21 - 1390	223	166
Liver	38 - 901	197	122	14 - 571	103	54	14 - 901	131	71
Fat tissue	49 - 493	211	190	17 - 398	96	72	17 - 493	130	87
Spleen	12 - 343	103	61	6 - 1400	89	19	6 - 1400	93	28
Lung	< 2 - 21	7	5	< 2 - 91	11	5	< 2 - 91	12	7
	Female (n=5)			Male (n=4)			Total (n=14)		
	Min - max values	Average	Median	Min - max values	Average	Median	Min - max values	Average	Median
Kidney	< 2 - 11	5	6	< 2 - 12	4	3	< 2 - 12	6	6
Heart	2 - 41	12	5	< 2 - 14	5	3	< 2 - 41	9	6
Brain	< 2			< 2			< 2		

The data reported by Concin et al. (2008) relative to MOSH in abdominal fat of women obtained by Caesarean sections ranged from 15 to 360 mg/kg of fat, with an average and a median of 60.7 and 52.5 mg/kg of fat (figure 79). To compare these data (referred to the fat) with that obtained in this study (referred to the fresh weight), the concentrations reported by Concin et al. have to be reduced correspondingly (adipose tissue includes 5-30 % of water). Taking this into account, the mean concentration in fat tissues was roughly 2.5 times higher with respect to that found in the abdominal fat from the Caesarean sections. This difference can be in part ascribed to the higher age of most subjects (in the Concin's study the women had a mean age of 38 years). No mineral oil aromatic hydrocarbons (MOAH) were detectable.

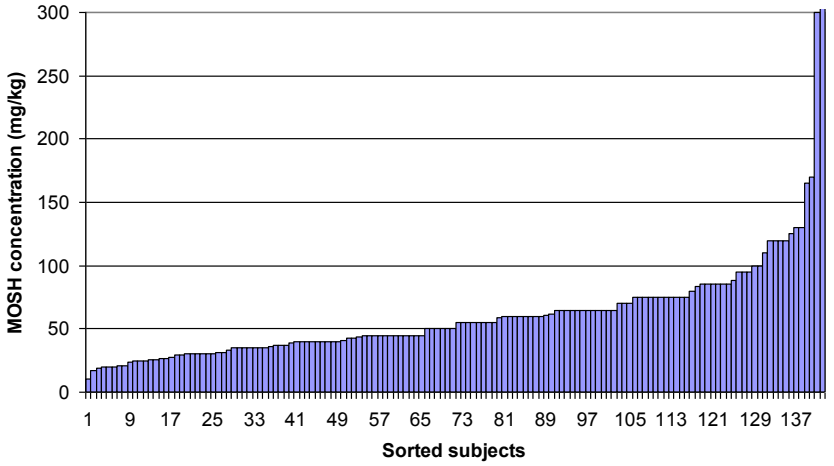


Figure 79. MOSH concentrations found in abdominal fat of women obtained by Caesarean section (Concin et al., 2008) sorted by increasing values.

1.3.3. Molecular mass distribution

Typical HPLC-GC-FID chromatograms from the various tissues of the same female subject of 78 years of age and 56 kg body weight are reported in figure 80. They allow to characterize the molecular mass distribution, inform about the presence of *n*-alkanes and show components of natural (mostly food) origin. The internal standards (C_{11}, CyCy, C_{13} and Cho) are marked in the top left chromatogram.

In the fat tissue (top left), the composition of the hydrocarbons corresponded to that shown by Concin et al. (2008). In particular, the molecular mass distribution is quite narrow, ranging from about *n*-C_{16} to *n*-C_{36} and centered near *n*-C_{23} (marked in the chromatogram). A bunch of hydrocarbons, with a retention time around *n*-C_{18}, were probably diterpenes as they occur in milk fat and most plant materials.

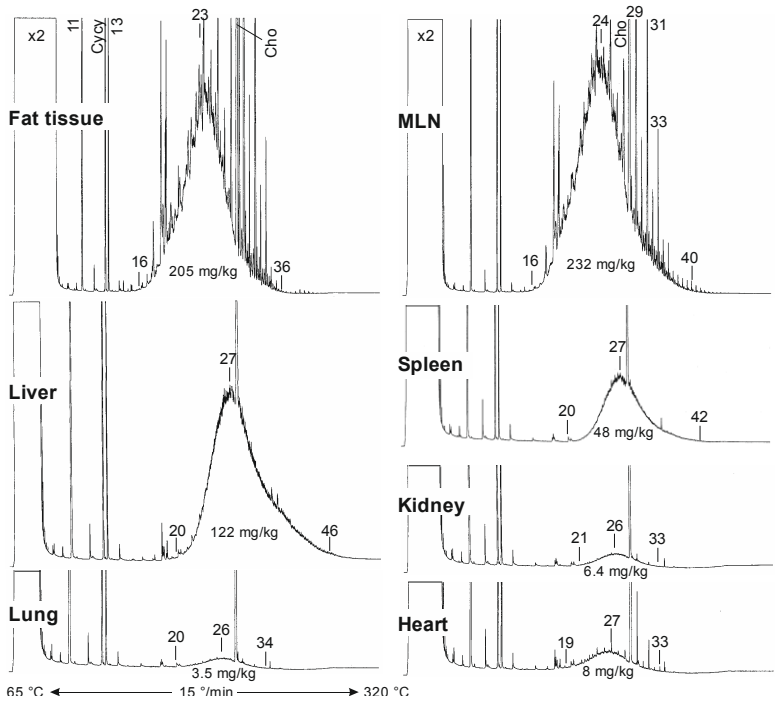


Figure 80. HPLC-GC-FID chromatograms from the various investigated tissues from the same subject.

Odd-numbered *n*-alkanes of plant origin, such as *n*-C_{29} to *n*-C_{33} are observed on the downslope. These components seem to be transient. In fact there is a steady state of intake with food and elimination (a single apple contains roughly 1 mg of these *n*-alkanes).

The hydrocarbons of the MLN (shown on the right) had virtually the same composition of that observed in the fat tissue. Both these chromatograms were recorded at double attenuation compared to the others.

The chromatogram obtained from the liver proved different from the previous two. In fact, it presented a higher molecular mass, ranging from *n*-C_{20} to about *n*-C_{46}, centered on *n*-C_{27}, and there are virtually no sharp signals on top of the hump, indicating the absence of hydrocarbons of natural origin. The hydrocarbons in the spleen were almost identical to that in liver. Those in the kidney, lung and heart were between those of the pairs fat/MLN and liver/spleen.

As noted by Concin et al. (2008), the composition of the hydrocarbons in the fat tissue varied little among the various subjects, pointing out that the composition is determined less by the mineral oils the individual is exposed to (nearly all of which are strongly different from that observed in the tissue), but more so by the selectivity of the uptake, evaporation (exhalation) and metabolic elimination. Now the same was noticed for the MLN which contained MOSH of a composition almost identical to that of the fat tissue.

This was not the case for liver and spleen, as shown in Figure 81. The pattern in liver 1 was most commonly observed: fairly symmetric humps with some tailing towards higher retention times, here centered early, namely on *n*-C_{25}, other times on *n*-C_{27}. The sharp signals from plant hydrocarbons observed in fat and MLN, were absent.

The pattern observed in the liver and the spleen corresponded in quite some detail for all the subjects. The MOSH in liver and spleen 2 were centered at the highest molecular mass of all samples (slightly beyond *n*-C_{27}) and there was a stronger tail ranging beyond *n*-C_{40}. Samples 3 and 4 were more exceptional, with an additional later eluted hump, maybe indicating two

different types of accumulated MOSH. This might suggest a different path of uptake, since there is no substantial exposure to a mineral oil of this molecular mass composition.

Figure 81. HPLC-GC-FID chromatograms of the MOSH in livers and spleens of the same subject, pointing out the range of variation.

Hydrocarbons in lung varied considerably (Figure 82). Most commonly they formed a hump without peaks on the top, centered on *n*-C_{25} or *n*-C_{26} (lung 1). The hump in lung 2 was topped by many small peaks, of unclear origin. Humps of unusual positions were also observed: an exceptionally early eluted, narrow hump in lung 3 and a broad one in lung 4. Both chromatograms include sharp peaks eluted after the standard Cho, the dominant ones corresponding to the *n*-alkanes C_{29}, C_{31}, C_{33} and C_{35} and smaller even- numbered *n*-alkanes in-between. The neophytadiene, recognized by using GCxGC analysis, is an important component in cigarette smoke (Guerin and Olerich, 1975).

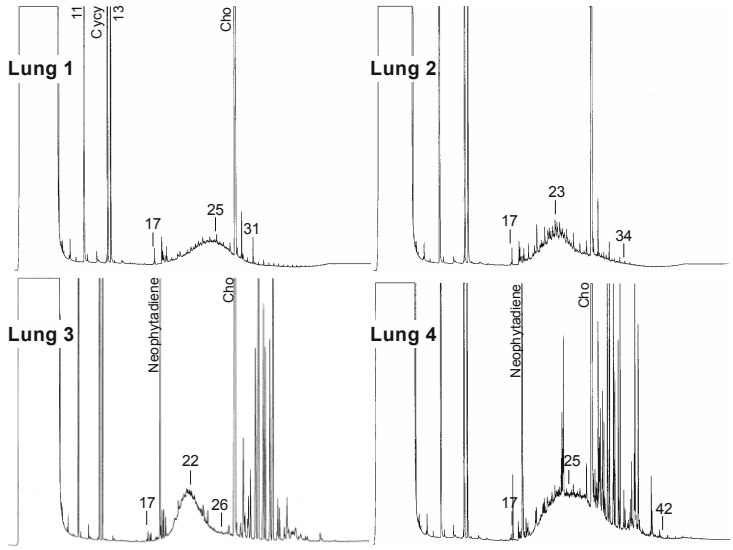


Figure 82. HPLC-GC-FID of the MOSH in lung tissues, pointing out a range of variation.

1.3.4. Statistical analysis

1.3.4.1. Correlation of MOSH concentrations in various tissues

It could be assumed that the high MOSH concentration in the fat tissue reflected a high exposure to MOH and that therefore, concentrations would be high in all the tissues of a given individual. In fact, the subject with the highest MOSH concentrations in spleen and MLN (tissues with different MOSH compositions) also had the highest concentration in the lungs. However, the concentration in the liver (410 mg/kg) was less than half of the maximum value found. On the contrary, the subject with the maximum value in liver had 4.1 and 5.2 times less MOSH in her spleen and MLN, respectively, with respect to the maximum concentrations found in these tissues.

Concentrations found in the different tissues for a given subject, were divided by that found in the fat tissue. The resulting value was converted to $\log_{10}$, so that concentrations higher in a tissue than in the fat tissue had positive values, while lower concentrations were negative. The concentrations ranged from 5.1 times above that in the fat tissue to 6.7 times less for the liver, and from 3.5 times more to 28 times less, for MLN. This strong variation indicates that the MOSH concentrations not only vary between individuals, but also between tissue types of a same individual. Table 19 reports correlation data (R^2) of MOSH concentrations in various tissues (straight lines forced through the origin). The strongest association is observed between the spleen and MLN (0.47), followed by the fat tissue and the spleen, as well as the fat tissue and MLN. The lowest R^2 are noted for liver with fat tissue and MLN.

Table 19. Correlation data (R^2) of MOSH concentrations in various tissues.

	Correlations (R^2)			
	Liver	Fat tissue	Spleen	MLN
Liver	1.00	-0.05	0.26	-0.01
Fat tissue	-0.05	1.00	0.31	0.31
Spleen	0.26	0.31	1.00	0.47
MLN	-0.01	0.31	0.47	1.00

1.3.4.2. Correlation with age

The age of the subjects varied between 25 and 91 years, with a mean age of 66 years, and with 68 % of the subjects over 60 years of age (Figure 83). Assuming that MOSH concentrations would increase with time, concentrations should increase constantly with age. However, although the 25-year-old male had clearly the lowest concentrations in all tissues, the second was the oldest male (91 years old).

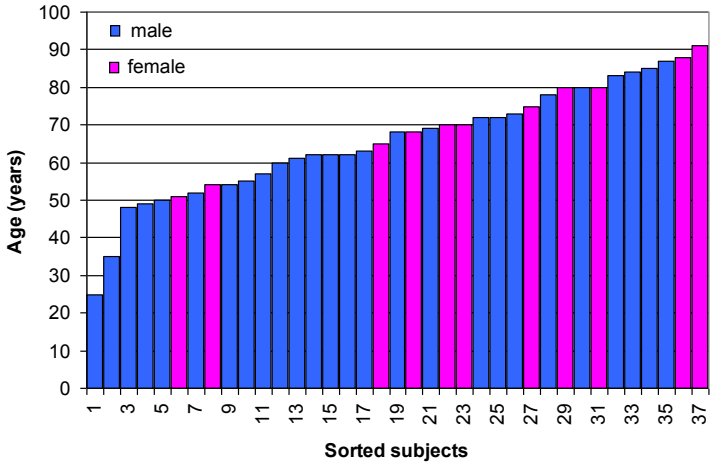


Figure 83. Subjects sorted by age, violet for females and blue for males.

In figure 84 the plots obtained by correlating MOSH concentrations in tissues and age of the subjects are reported, distinguishing between female and male. Furthermore, in figure 85 a similar plot of correlation is reported for the combined females and males.
In the plot representing the correlation between age and MOSH concentration in the fat tissue (upper left in figure 84) also the 144 data from abdominal fat from Caesareans, published by Concin et al. (2008), were reported reduced by 20 %, since they referred to isolated fat. Trends were assumed to be linear and to pass through the origin. The correlation coefficient (R^2) and the slope are reported in table 20. Considering the correlation between MOSH concentrations in fat tissue and age for the combined females and males, a slope of 2.04 is obtained with a R^2 of 0.17. This confirms the results obtained by Concin et al. (2008) for a larger dataset. However, correlation in the other main tissues was a little worse, indicating that other factors different from age are involved.

Table 20. R^2 values and slopes of the trend lines forced through zero for the correlation between MOSH concentrations in various tissues and age for female, males and both combined.

	female (n=11)		male (n=26)		combined (n=37)	
	R^2	slope	R^2	slope	R^2	slope
Fat tissue	0.10	2.97	0.20	1.56	0.17	2.04
MLN	0.32	3.79	0.09	3.27	0.12	3.45
Liver	-0.05	2.63	-0.02	1.55	-0.02	1.91
Spleen	-0.13	1.33	0.04	1.58	0.03	1.50

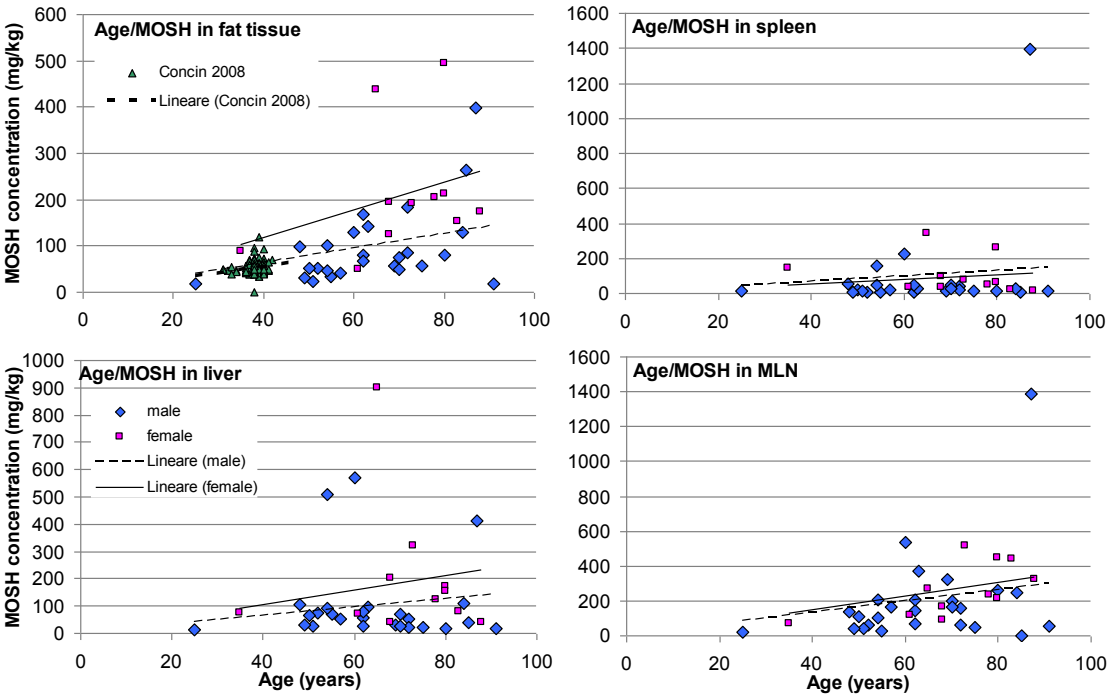


Figure 84. Correlation between age and MOSH concentration in fat, spleen, liver tissue and MLN divided for males and females. In the age/fat tissue plot, MOSH values determined in 114 samples from Concin et al. (2008) are also reported.

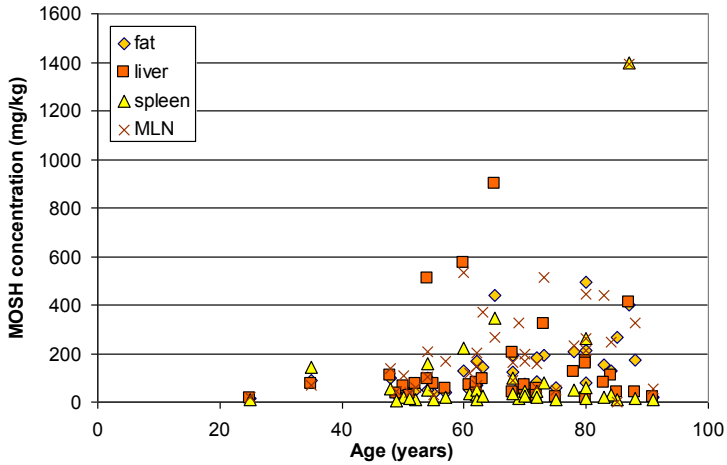


Figure85. Correlation between age and MOSH concentration in fat, spleen, liver tissue and MLN for the combined males and females.

1.3.4.3. Correlation with body weight

Body weights of subjects considered in this study ranged from 50 to 138 kg (mean value of 82 kg). In figure 86 the plots obtained by correlating MOSH concentrations in tissues and body weight of the subjects are reported distinguishing between females and males. Furthermore, in figure 87 a similar plot of correlation is reported for the combined female and male values. The correlation coefficient (R^2) and the slope data are listed in Table 21. Only for the females and the combined data, the weight is negatively correlated with the MOSH content in the fat tissue. This suggests that a similar exposure is distributed over more fat and agrees with Concin et al. (2008). Other correlations are contradicting for females and males.

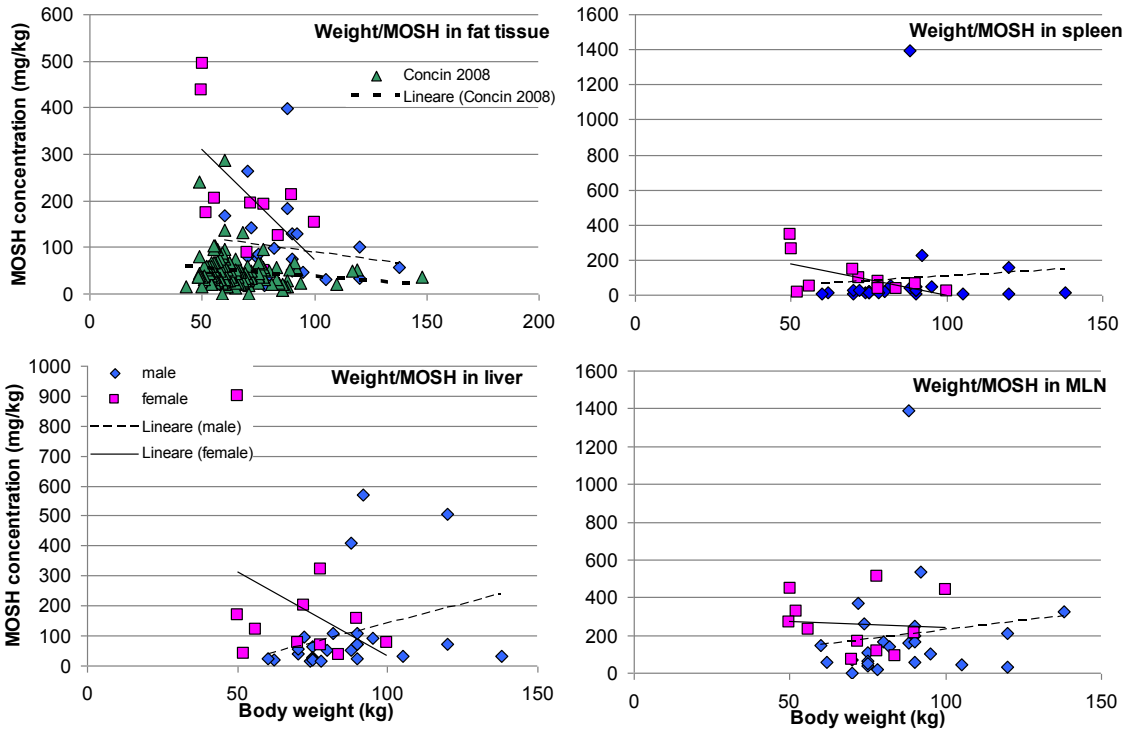


Figure 86. Correlation between body weight and MOSH concentration in fat, spleen, liver tissue and MLN divided for males and females. In the body weight/fat tissue plot, MOSH values determined in 114 samples from Concin et al. (2008) are also reported.

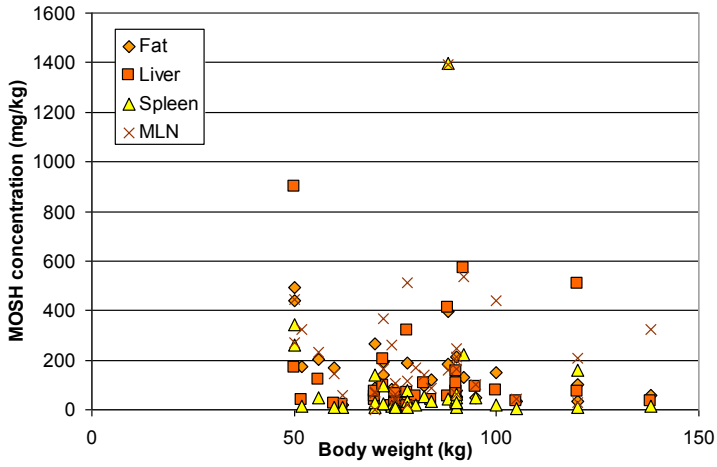


Figure 87. Correlation between body weight (kg) and MOSH concentration in fat, spleen, liver tissue and MLN for the combined males and females.

Table 21. R^2 values and slopes of the trend lines for the correlation between MOSH concentrations in various tissues and body weight age for females, males and both combined.

	female (n=11)		male (n=26)		combined (n=37)	
	R^2	slope	R^2	slope	R^2	slope
Liver	0.15	-5.56	0.10	2.59	0.00	-0.44
Fat tissue	0.35	-4.73	0.02	-0.62	0.17	-2.48
Spleen	0.33	-3.58	0.00	1.05	0.00	-0.26
MLN	0.02	1.92	0.01	-0.68	0.00	0.50

1.3.5. Amount of MOSH in the human body

The total amount of MOSH in the human body was calculated from the weight of each tissue multiplied by the MOSH concentration found in it. The data obtained are reported in Table 22, with a distribution shown in Figure 88.
For each subject, the mass of fat tissue was calculated multiplying the total body weight with the percentage of fat tissue obtained from the body mass index (BMI) according to Deurenberg et al. (1991): 1.20 x BMI + (0.23 x age) - (10.8 x gender) - 5.4, with male =1 and female =0). For the other tissues, mean masses were used (Molina and DiMaio, 2012a, 2012b). The largest amount of MOSH was found in the fat tissue (maximum value of 12.4 mg), due to its large mass (up to 66 kg of adipose tissue). The liver was second, with contents up to 1.4 g MOSH.
The maximum amount of MOSH in the whole body (13.3 g) was calculated for a male subject of 88 years of age and with 87 kg body weight (BMI 26.3). The second one was a female of 65 years and 50 kg body weight (BMI 19.5). For the heaviest three subjects (138, 120 and 120 kg), all males, only 3.8, 4.5 and 1.5 g MOSH were derived due to low MOSH concentrations.

Table 22. Minimum/maximum values, averages and medians of MOSH content (mg) calculated from mean mass of human tissue and the MOSH concentrations for each tissue.

	Mass of tissue	MOSH (mg)		
	(kg)	Min - max	Mean	Median
Fat tissue	12-66	325 – 12369	3390	2207
Liver	1.56	23 – 1407	205	110
Spleen	0.14	1 - 194	13	4
Lung	0.84	0 - 77	8	4
Heart	0.28	0 - 12	2	1
Kidney	0.30	0 - 4	1	1
Total		349 – 13282	3619	2322

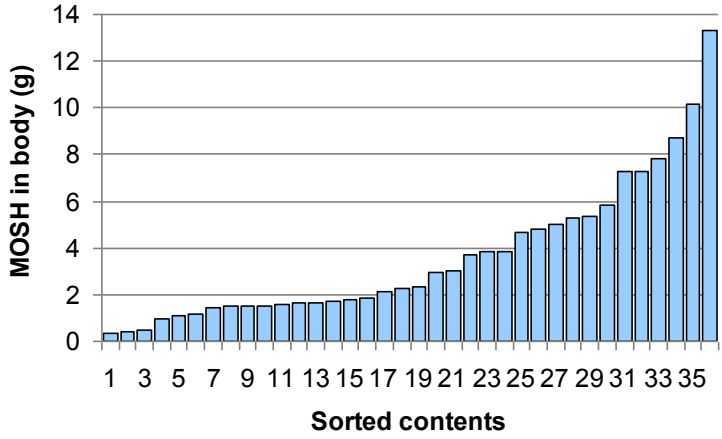


Figure 88. MOSH in human body (g) sorted by increasing values.

1.3.6. Comparison between human- and animal-study data.

The concentrations found in the human tissues are high if compared to values which could be extrapolated from rat experiments. The highest values were found in female *Fischer 344* rats: after feeding MOH at 20.000 mg/kg in the diet. Their livers contained roughly 5000 mg/kg MOSH. The 200 mg/kg found on average in the female human liver would then suggest a mean concentration in the human diet of 800 mg/kg, but the data reviewed by EFSA (EFSA, 2012) concluded on a daily oral exposure of 0.03-0.3 mg/kg b.w. (1.8-18 mg per a person of 60 kg) which is equivalent to 1.8-18 mg/kg in the diet if 1 kg food is consumed per day. Hence, compared to the exposure, the concentrations in the human tissue are roughly 100 times higher than predicted by animal experiments.

There are various factors potentially contributing to this large discrepancy: (i) absorption is not proportional to the concentration in the diet (Firriolo et al., 1995). (ii) Oral uptake is not the only source of MOSH in the human body. (iii) Human exposure was higher some decades ago. Furthermore, if humans accumulated MOSH over 90 years, a 365 times higher concentration would be reached than in the 90 days animal experiments.

The accumulation is influenced by the volatility and the structure of mineral oils humans are exposed to: a part can be evaporated after absorption (e.g. exhaled), and the linear and probably many branched and cyclic alkanes are metabolized and eliminated. Furthermore, high molecular mass hydrocarbons probably do not pass through membranes and are, therefore, not absorbed.

Animal experiments confirmed oral uptake of MOSH, but other ways of exposure had to be considered, namely dermal uptake, observed for breast salves in human milk (Noti et al., 2003) and inhalation, considered a minor route on the basis that even old cows usually have little MOSH in their body fat; hence the relative importance of oral and dermal uptake would be of main interest.

1.4. Conclusions and future perspectives

The concentrations measured confirm that the MOSH are the by far predominant contamination of the human body (Concin et al., 2008). The body of a quarter of the subjects contained more than 5 g MOSH, and the concentrations in active organs reached 1.4 g/kg (spleen and MLN). This is not necessarily indicative for a health problem, but calls for a particularly careful toxicological evaluation.

A further research objective will include a characterization of some samples by comprehensive two-dimensional gas chromatography (GCxGC). Using this technique it will be possible to reach more structural information of the MOSH fraction and obtain a partial separation between

paraffins (open chain) and naphthenes (cyclic compounds). Furthermore, the real source of this kind of contamination in human tissue has yet to be found. Data reached up to now seems to attribute the cause of contamination to an external source, such as dermal uptake, food or air intake, especially due to the ubiquity of MOSH. However, the less likely hypothesis of a natural phenomenon or reaction in the organs should not be excluded.

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