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Doctoral dissertation

Study on migration of hydrocarbons contaminants from food contact materials in food

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SUMMARY

Food packaging is a rapidly evolving field. Packaging keep food safe and retains its nutritional properties and sensory characteristics; furthermore, it provides additional features that are important for consumers. Packaging system, as well as other food contact materials (FCM) such as adhesives and printing inks, are the main sources of chemicals in food products and beverages. Human exposure to chemicals from food contact materials may occur as a result of migration, a mass transfer phenomenon resulting from a tendency to balance all chemical potential within a system from the packaging into foodstuff. There are several parameters affecting migration, which are related to food, to packaging and to chemicals that are involved. The food packaging legislation establishes an overall migration limit (OML), which measures the inertness of the materials and that regards all chemicals in a packaging, and a specific migration limit (SML) for specific substances that may be dangerous for human health. An important tool to evaluate the compliance of FCM with the limits are the migration tests. Food simulants as well as temperature, time and contact conditions for migration testing are regulated for plastic materials, but not for paperboard material, so that contact condition are derived at least partially from plastic legislation.

A very important class of contaminants involved in migration from packaging into food are hydrocarbons, in particular mineral oil hydrocarbons (MOHs) consisting of MOSH (mineral oil saturated hydrocarbons) and MOAH (mineral oil aromatic hydrocarbons), and polycyclic aromatic hydrocarbons (PAHs). The MOSH fraction may include polyolefin oligomeric saturated hydrocarbons (POSH), oligomers of polyolefin, which can migrate from plastic bags, heat-sealable layers and other laminates as well as adhesives and plasticizers. Different studies have demonstrated that MOHs, POSH and PAHs migrate from packaging into food and contaminate it with negative effects for human health.

In the first part of this PhD work a rapid migration test, using simple and cheap commercially glass weighing bottles was developed to assess the migration of mineral oil from cardboard. At the end of the test, mineral oil was extracted from cardboard and from Tenax® and the entity of migration was calculated both in terms of direct migration (amount of MOHs found in simulant) and in terms of indirect migration (contamination lost by cardboard). The two measures were in good agreement demonstrating the mass balance in the cell. The migration test performed was subsequently used to evaluate the efficiency of a new sequential polymer treatment of paperboard to develop a barrier against the migration of mineral oil. The material consisted of two layer deposited on the cardboard. The first, poly (methyl methacrylate) protective layer, gave hydrophobicity to cardboard, while the second, cyclic olefin copolymer, filled the open pores of the material surface and reduced the mineral oil hydrocarbon migration.

Subsequently, the developed migration tests was also used to study the migration of MOHs from recycled cardboard into dry semolina and egg pasta under accelerated condition (40°C 10days) to determine the influence of some parameters on migration process. Amount of pasta, type of contact pasta/cardboard, food size and head space volume, were studied parameter that showed a great effect on MOHs migration from cardboard. They influenced the entity of the migration, but also the molecular weight range of hydrocarbons that can migrate into the pasta. The study of these parameters pointed out that the migration tests suggested to evaluate the compliance of the packaging material, do not always reflect the real mechanisms involved in migration in real food. Semolina and egg pasta behaved very differently: egg pasta tends to have a higher absorbing effect than semolina pasta,

especially when low amount of pasta are in contact with the cardboard. This is not only attributable to the fat content but also at structure and porosity of the matrix. A correlation with data obtained from migration test with Tenax® was finally carried out. Tenax® tended to overestimate the migration in semolina pasta and, at the opposite, to underestimate the migration in egg pasta.

Another part of this work regarded the investigation on the presence of selected hydrocarbon contaminants (mineral oil and polycyclic aromatic hydrocarbons) in pizza boxes collected in different areas of Italy. From the analysis of cardboards, three sources of contamination were clearly identified: illegal presence of recycled paper, printing ink containing alkylbenzenes, and refined paraffin (*food-grade* mineral oil used in the waterproofing of paper). All of the cardboard boxes showed low level of PAHs. The potential of these contaminants to migrate from cardboard into the pizza was demonstrated carring out migration tests using Tenax® as simulant. The per capita daily intake from "take away" pizza was calculated both for MOHs and for PAH8, showing a negligible (0.005%) global diet intake contribution for PAHs, but not for mineral oils, for which, the average contribution ranged between 7.3% and 12.8%.

Regarding plastic materials, a study on the presence of POHs in ready-to-eat vegetable soup, packaged in polypropylene container, was conduct. The analysis of food before and after microwave heating demonstrated that a part of POHs contamination was from the production of the soup and another part migrated from PP container. The migration depends on fat content of the soup, on original amount of POHs in the container and on heating conditions. Different simulant were tested to assess the migration of the POH from containers and their performance was compared with those in real food. Vegetable olive oil as simulant tends to overestimate the contamination, also when fat reduction factor was applied. Instead, the ethanol 95% seems to be the best choice for the migration test.

Finally, the last part of the work a simple method based on a liquid–liquid partition step to eliminate the bulk of the saturated hydrocarbons, followed by DI-SPME using a Carbopack Z/PDMS fiber, was employed for selective uptake and concentration of BaP (Benzo(a)pyrene) in the analysis of microcrystalline waxes. A preliminary step using liquid-liquid partition was necessary to remove the bulk of saturated hydrocarbons. Wax sample was dissolved in cyclohexane, and BaP was extracted with dimethyl formammide (DMF)/water 9/1 (*v/v*). A mixture of water and hexane were added to the residual DMF/water in order to change the coefficient partition of BaP and extract it in hexane. The latter was the most suitable solvent to carry out the final enrichment step by direct-immersion solid phase microextraction (SPME). The method showed good performances of repeatability and recovery. Characterization of gas chromatographic profile of waxes was also evaluated. They show different profiles of distribution ranging from *n*-C_{20} to *n*-C_{60}. All of the samples presented less than 5% of hydrocarbons below *n*-C_{25} In addition, artificial saliva from chewing tests was collected and analyzed in order to evaluate possible release of hydrocarbon particularly BaP from waxes, gum bases and chewing gums during their contact with human saliva in the mouth. No detectable amount of contaminants were found in samples.

INTRODUCTION

1. FOOD CONTACT MATERIALS

1.1. General aspects

Food contact materials (FCM) can be defined as all types of materials that are intended to come in contact with food. This wide group of materials includes packaging and containers, kitchen equipment, cutlery and dishes. Processing equipment or production machinery as well as containers used for transport are also considered FCM.
Packaging represents the most popular FCM, it is important both for the food industry and for the distribution and consumers. The food industry would like a suitable and inexpensive packaging. The industry of distribution asks the package to extend the shelf life of the product and to make easy the transport and the handling of it. Finally, the consumers want the packaging ensures the quality of the food and that it is cheap and easy to carry away; furthermore they are interested in the preservation of the environment.
Due to all these needs, this area of production is characterized, as a few other, to a great innovation and dynamicity.
The packaging has a great variety of functions, however all of them can be included in five main functions:

- *Protection*: packaging is the interface between the product and the external environment, therefore it is a barrier against spoilage by external agents such as pests, microorganisms, odours, oxygen and light. The package is also used to prevent shock damage caused from vibration, snagging, friction and impact of packaged food and to avoid fraud manipulation.

- *Containment*: This is the most ancient and original function. In some cases as for liquid and powder products, the container is crucial to provide a movement of the food. As opposed to solids, the "free flowing" products have a greatest need of containment in each phase of their production. Since ancient time all types of materials and all type of packaging have been used to make containers with an unmatched variety, in order to facilitate the consumers but also the transport of the products from the factory to place of sale, by train cargo or, eventually, by ship.

- *Information*: Packaging conveys necessary information for the consumer. The common information provided by the package includes general features of the product such as weight, nutritional information, ingredients, name of the manufacturers and expiration date. In special products, packaging can report information about preparation, heating, recipes and compliance with regulation.

- *Utility of use*: Convenience is the most recent function but has become of great importance due to its ability to meet the consumer's need. The re-sealable, easy to open and easy to dispensing packaging are just few examples of convenience.

- *Promotion*: Packaging has been defined "the silent seller". Companies use shape, color and aspect of package to promote the product and to influence a consumer's purchase decision.[1]

Packaging industry includes many different areas such as chemical and metallurgical industry, paper and board manufactory, production of machines etc. The global packaging market is valued approximately US$ 400b. Europe produces about a third of the packaging used in the world for a value around 130m euro and the demand is expected to growth both due to an increasing demand of the emergent areas and the need for more sophisticated packaging.
The raw packaging material consumption is very similar worldwide. In 2012 plastic (rigid and flexible) was the largest category of packaging materials (37% of the market) followed by paper and board with a 34% share [2] (Figure 1).

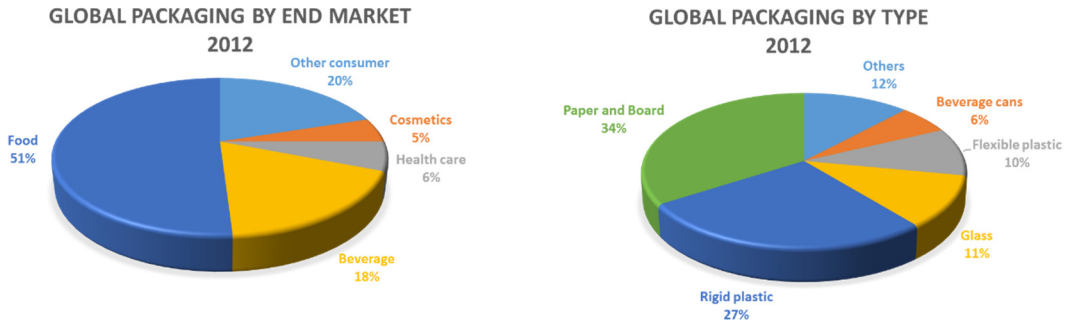

Figure 1. *Global packaging distribution by end market and by type in the world - Total market size US$ 400b (adapted from ref.*[2] *).*

1.2. Legislation

Until the mid of 70's FCM legally rules were adopted only at national levels. In those years, the European authorities perceived that national rules were impeding the free movement of food contact materials on the common market. Therefore, in 1976 the first rules about FCM were adopted to harmonize FCM law at Community level. Nowadays at the European level, food contact materials are regulated under the EU framework regulation EC 1935/2004 [3]. The framework covers all food contact materials and puts general safety requirements for the manufacturing, procession and distribution of all possible FCMs. Annex I of the regulation reports a list of seventeen possible materials which could be used to make FCMs and which could be regulated. However only four FCMs are subject to such "specific measures" and to detailed harmonization at EU level:

Plastics (including recycled plastics)
Ceramics
Regenerated cellulose film
Active and intelligent materials.

Based on the regulation, FCMs have to be produced in compliance with the good manufacturing practices, reported in the regulation EC 2023/2006, so that, under normal and foreseeable condition of use, they do not transfer constituents in food, at level which could: a) being dangerous for human health; b) lead to an unacceptable change to the composition of the food; c) or bring a deterioration of their organoleptic characteristics. The regulation 2003/2006 introduces, in a very concise way, the Good Manufactures Practices (GMP) as compulsory for packaging production to ensure quality and safety during every step of packaging manufactory[4]. It is introduced after the "ITX scandal" in 2006 when baby milk has been contaminated by the photoinitiator ITX(2-isopropylthioxanthone) which

was migrated from the surface through the packaging in the food. The problem was caused by the non-efficient UV fixation system during production of Tetra Pack®. In the light of the facts it clear that food packaging productions requires the same care given to food production in order to avoid hazard to human health.

In addition to these principles, materials included in the list of the framework regulation must be produced with raw materials and recognised ingredients that are considered safe by the law and are included in positive lists.

An increasing part of packaging is made also by combination of different materials (e.g. a multilayer plastic and board with an adhesive and printing) and pass through a complex chain of manufacturers. Therefore, every stage of production must respect the good manufacturing practices and each material involved in a final FCM should have a declaration of compliance (DoC) which explains potential migration of substances and gives a toxicological evaluation.

The lack of harmonized laws, for some FCMs included in Annex I of framework Regulation, should be filled by EU member states with specific national measures.

The legislation about FCM has appeared in Italy in 1962 with law n. 283 dated 30/04/1962, it was subsequently substituted by art.3 of the D.P.R 777/1982 and then by art. 3 of the D.Lgs. 25 January 1992 n. 108 in order to implement European Directive 89/109/CEE.

In accordance with the rules above, the D.M. 21 March 1973[5] gives specific requirements about the following materials:

- Plastic
- Rubber
- Regenerated cellulose
- Paper and paperboard
- Glass
- Stainless steel

The legislation is based on a "positive list" of substances that can be used in the production of these materials with some limitations and restrictions, as well as on how to control the suitability for food contact. The D.M. 21/03/1973 has been modified several times to be conformed to the European law and to update it regarding new materials. However, as concerns some materials such as paper and paperboard, this decree has not yet been modified.

2. PAPER AND PAPERBOARD PACKAGING

2.1. Paper and board production

Currently a large number of paper and board are present on the market and they were widely used in the food packaging industry. Paper and board can be used as primary as well as secondary and tertiary packaging based to their weight, thickness, chemical properties, composition and appearance. The most important source involved in the production of paper and board materials are cellulose fibers from conifer wood and deciduous plants (virgin fibers) and paper materials from recycling process (recovery fibers). The "non-fiber substances" e.g. fillers, gluing agents, and additives have a fundamental rule during production of paper and board.

The fillers comprise a variety of substances from different origin, which allow to obtain a closed and flat surface, furthermore they improve some characteristics of fibrous mixture such as ink receptivity, smoothing and opacity. Using gluing agents paper and board become impermeable to liquids and to inks and they can be printed. The additives are used to improve the working conditions and to have a product with improved performances. They can be divided into two main categories: functional additives and processing aids [6].

- *Functional additives*: including sizing agents, wet and dry strength resins, softeners, dyes, and pigments are able to modify the properties of the paper and to be transferred to the final product;
- *Processing aids*: the group consist of some substances used to improve the paper making processes, which are, only in traces, transferred into the final product. This group includes biocides, deposit control agents, defoamers, and felt cleaner.

In the first part of the paper and board production, wood fibers are grinded and mixed with water in order to remove impurities and lignin thereby achieving the paper pulp. Instead, in the recycled paper production the recovery fiber are directly sent to the production. Mechanical and chemical processes are the main kind of production, however, to meet the needs of paper industry semi-chemical or chemi-thermomechanical approaches are largely used to improve the performance of the process. Chemical pulping involves a full chemical treatment to remove non-cellulose components of wood leaving intact the cellulose fibers. This process provides a paste with excellent characteristics in terms of mechanical resistance, purity degree and optical qualities. On the other hand, the chemi-termomechanical technique, which starts with a mechanical treatment followed by a chemical one, allows to obtain a greater yield than other pulping techniques, and the pulp, called "high-yield past", can be used in the production of all types of paper and board.

The pulp obtained at the end of process is washed, refined, cleaned and sometimes bleached. Pigments and dyes, coatings and other additives are mixed in, and the pulp is pumped onto a moving wire screen. As the pulp travels down the screen, water is drained away and recycled. The resulting crude paper sheet, or web, is squeezed between large rollers to remove most of the remaining water and to ensure smoothness and uniform thickness. The semidry web is then run through heated dryer rollers to remove the remaining water. The finished paper is ready to use, to print and to form in packaging.

The manufacture of recycled paper involves different raw materials such as newspapers, magazines and leaflets, which originally are not produced with the purpose to be reused for food contact. A wide variety of non-chemical and chemical substances (inks, waxes, plasticizers) can be present in the

recycled fiber thus a step of purification is important in the production of paper. During the first step of purification pulp mix passes through a system of centrifugal cleaning equipment and some screens. This is done to remove large contaminants like wood, plastic, stones, glass and paper clips, as well as small contaminants such as string, glue and sticky materials. In some case pulp is subjected to a de-inking process, which involves a flotation of inks using soapy chemicals and a bleaching step after which the pulp grey and dirty comes out whiter and cleaner. The resulted recycled pulp is right to be made into paper. The finally process is, substantially, the same used for the production of virgin paper [6].

2.2. Paper and board legislation

2.2.1. European legislation

As already mentioned paper and board materials that come in contact with food are regulated, at European level, by framework Regulation 1935/2004. However, this regulation does not provide specific requests for this kind of materials. Some country of European Union have enacted national requirements about cellulosic packaging (France, Italy, Finland and Holland). In 2002 Resolution ResAP (2002)1 [7], on paper and board materials and articles intended to come into contact with foodstuffs, was adopted by the Council of Europe (CoE), a no binding document unless it is transposed in national laws. This CoE Resolution comprises four technical documents, which indicate characteristics that paper and board must comply:

- a list of substances to be used in the manufacture (technical document 1);
- guidelines on test conditions and methods of analysis (technical document 2);
- guidelines for recycled fibers (technical document3);
- Confederation of European Paper Industries (CEPI) guide for good manufacturing practice (technical document 4);
- a practical guide (technical document 5).

In 2012, the “Industry guideline for the compliance of paper and board materials and articles for food contact” is developed by the CEPI, the International Confederation of Paper and Board Converters in Europe (CITPA), suppliers of chemicals (CIFPI) and paper and board multilayer manufacturer (FPE). This document is an operation guide for industry to comply the Regulation 1935/2004. CEPI guideline reports indications about paper and board production, transformation and traceability. It also reports methods to evaluate the compliance for food contact and a list of limits for substances may be present in the materials. (Table 1).

Table 1. *Purity requirements indicated in CEPI guidelines (from ref.* [8]*).*

SUBSTANCE	LIMIT IN FOOD	TESTED IN PAPER & BOARD	REMARK
	SML (mg/Kg food)	Limit	
Cadmium	-	0.5 mg/kg	#
Lead	-	3.0 mg/kg	#
Mercury	-	0.3 mg/kg	#
Pentachlorophenol	-	0.15 mg/kg	
Antimicrobial Substances	-	No release of substances in quantities which have an antimicrobial effect.	
4,4'-bis (dimethylamino)-benzophenone (Michler's ketone)	0.01 mg/kg (non-detectable)	0.0016 mg/dm^2	# *
4,4'-bis (diethylamino) benzophenone (DEAB)	0.01 mg/kg (non-detectable)	0.0016 mg/dm^2	# *
Azo colourants4	-	0.1 mg/kg as aromatic amine (non-detectable)	#
Dyes and colourants6	-	no bleeding	#
Fluorescent Whitening Agents (FWAs)6	-	no bleeding	#
Polycyclic Aromatic Hydrocarbons (PAHs)	0.01 mg/kg (non-detectable)	0.0016 mg/dm^2	*
Di-n-butylphthalate (DBP)	0.3 mg/kg	0.05 mg/dm^2	*
Diethylhexylphthalate (DEHP)	1.5 mg/kg	0.25 mg/dm^2	*
Diisobutylphthalate (DiBP)	0.3 mg/kg	0.05 mg/ dm^2	*
SUM DBP + DiBP	0.3 mg/kg	0.05 mg/dm^2	*
Benzylbutylphthalate (BBP)	30 mg/kg	5 mg/dm^2	*
Diisononylphthalate (DINP)	9 mg/kg	1.5 mg/dm^2	*
Diisodecylphthalate (DIDP)	9 mg/kg	1.5 mg/dm^2	*
Benzophenone	0.6 mg/kg	0.1 mg/dm^2	*
SUM benzophenone+ hydroxy-benzophenone+ 4-methylbenzophenone	0.6 mg/kg	0.1 mg/dm^2	
Diisopropylnapthalenes (DIPN)	-	As low as technically possible	*
Bisphenol A	0.6 mg/kg	0.1 mg/dm^2	# *

The document lists raw materials and substances with their operating limits allowed in paper and packaging materials. Furthermore, it gives specific indications, about good manufacture and transformation practices and requires, to fabricate board to secondary and tertiary packaging. Moreover, the guideline, in the annex 2, explains requirements for recycled paper and paperboard, for their raw materials and it specifies technological processes that can be used to remove physical and chemical substances from recycled fiber used in the paper production.[8]

A few years later in 2015 in Germany, the Federal Institute for Risk assessment (BfR) published the Recommendation XXXVI about "Paper and board intended for food contact" which is widely recognized as a reference in the Countries where there are no national laws [9]. In the U.S., paper and paperboard components are regulated as indirect food additives under the Code of Federal Regulation (*21 CFR 176*). Alternatively, food contact substances used in paper and board may also be acknowledged by an effective *Food Contact Substance Notification* (FCN). Substances that have been

affirmed as *Generally Recognized as Safe* (GRAS) for use in food packaging, subject of the *Threshold of Regulation* (ToR), or *sanctioned prior* to 1958 are exempted from Regulation.

2.2.2. Italian legislation

In Italy the Administrative Order 21/03/1973 [5] sets, as already explain, the hygienic characteristics for packaging, utensils and container intended to come in contact with food. In a specific section, decree reports requirements of composition and purity for paper and board materials. The analytical control must be performed on fibrous fibers, charge substances and auxiliary substances and their limits take in account the kind of food in contact. Paper and board can be used in contact with wet and fat foods (such as pizza, meet, fried food and cheeses), for which migration tests are foreseen, if they are made of paper containing at least 75% of fibrous materials, with maximum 10% and 15% of, respectively, charge substances and auxiliary substances. For dry foods, for which migration tests are not required, paper and board packaging have to been made with at least 60% of fibrous materials, with maximum 25% of charge substances and maximum 15% of auxiliary substances.
As regards purity requirements, they are referred to all types of food and concern: lead, which has to be less than 3µg/dm^2, PCB (≤ 2 mg/Kg) (D.M. n°267 del 30 Maggio 2001 [10]) and optical whitening (absent). A multilayer cardboard of 200 g/m^2, consisting at least of three layers, can be used in contact with dry cereals (as they are or 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. The provisions of the D.M. 21 March 1973 and subsequent amendments shall be applied, as regards the lead, only for the layer intended for direct contact with the food, called "rear." The contact layer must have a minimum basis weight of 35 g / m^2. (D.M. 21/03/73 [5]). In Italy, recycled fibers can be used in contact only with particular dry foods, which are considered no-extractable as dry pasta, rice, salt, sugar and all fruit and vegetables where no migration tests are set.

3. PLASTIC PACKAGING

3.1. Production of plastic

Humankind worked hard from the earliest times to develop materials, which would offer benefits not found in natural products. The development of plastic materials started with the use of natural materials with plastic properties (e.g., chewing gum, shellac) then evolved with the development of chemically modified natural materials (e.g., rubber, nitrocellulose, collagen, galalite) and finally the wide range of completely synthetic material that we would recognize as modern plastics started to be developed around 100 years ago. Perhaps the earliest example of completely synthetic plastic material was invented by Alexander Parkes in 1855. We know it today as celluloid, but he named it Parkesine. Polyvinyl chloride (PVC) was first polymerized between 1838-1872 and a key breakthrough came in 1907 when Leo Baekeland created Bakelite, the first real synthetic, mass-produced plastic (https://plastics.americanchemistry.com/How-Plastics-Are-Made/). First industrial production of resins dates back to 1930-1940. However, in about half century they have been used in a great variety of application due to their low cost and due to a combination of flexibility (from film to rigid

applications), strength, lightness, stability, impermeability and ease of sterilization. Plastic materials are completely or partially synthetic organic substances. Their principal components are high molecular weight compounds (polymers) consisting by a large number of fundamental units, called monomers, which are linked mainly by covalent chemical bonds. This kind of materials can be formed into a hundreds and hundreds useful products although the food industry uses only a few of them. Monomers, such as ethylene, propylene or styrene come from natural gas, coal or fractional distillation of crude oil. Monomers are then chemically bonded into chains to form polymers. There are two basic mechanisms for polymerization: addition and condensation reaction.

Addition reaction: this mechanism is used when monomer have an insaturation. Using a special catalyst or particular temperature and pressure conditions, the unsaturated bond is opened to link the next monomer and that to the next and so on. The reaction can be carried out in gaseous phase dispersed in liquids. Addition polymerization is used to form the most common materials intended for food packaging i.e. polyolefins. Polymerization of ethylene to form polyethylene can be schematize as follows:

$$n CH_2=CH_2 \rightarrow (CH_2-CH_2)_n$$

Condensation reaction: In the condensation mechanism, a catalyst is used to help reaction of all monomer with others monomers to form dimer plus a low molecule weight byproduct (typically water). The dimer reacts with others to form tetramer and so on. Formed byproduct must be removed and can be used to produce useful products. Condensation is done in a mass of molten polymer. Polyamide and polyester are the main examples of condensation polymer. The polyester polymerization involves a di-acid which reacts with a di-alcol to form the monomer:

$$n R_i(OH)_2 + n R_0(COOH)_2 \rightarrow n H_2O + \ldots -R_0-\overset{\overset{O}{\|}}{C}-O-R_i-O-\overset{\overset{O}{\|}}{C}-O- \ldots$$

The next step of plastic manufacturing is the compounding where, using extrusion, several compounds such as additives, dyes and/or charges are added to polymer in order to obtain the pellet which is used in the production of plastic final products [11] [12].

3.1.1. Additives of plastic materials

Additives are chemical substances, which are included in the plastic materials composition at low percentage (typically about 1%) to give it specific functionalities. Additives have medium or low molecular weight and are linked to the polymer with low energy bonds, secondary interactions but almost never with high-energy chemical bonds. Several kind of material properties are improved using additives: mechanical, surface, thermal properties and aesthetic.

- Mechanical properties: plasticizers, blowing agents, strengthening agents, additives to give rubberiness.

- Surface properties: antistatic and slip agents

- Thermal properties: heat stabilizers, inhibiting agents of the decomposition and depolymerization

- Resistance to the environmental factors: antioxidant and anti UV agents

- Aesthetic: pigments and charges.

Generally this components are included in the final step of plastic manufacturing in the form of master batch pellet with high concentration of additive in order to make easier their dosage and incorporation. A positive list of monomers and additives approved for use in plastic intended for food contact is present in the Regulation 10/2011. In Italy the use of additives in plastic material is regulated in depth by D.M. 4 May 2006, n. 227 (an update of DM 21 March 1973 transposing the directives 2004/1/CEE, 2004/13/CE and 2004/19/CE).

3.2. Polymers used in food packaging

In the food packaging two or more films and plastic materials can be combined through blending, co-extrusion, lamination and coating to improve mechanical and physic-chemical characteristics of package such as its heat sealability, flexibility, stretchability, gas and moisture barrier properties, UV and visible light transmission and lower glass transition. Table 2 reports main characteristics of the most common polymers used in food industry.

Table 2. *Properties of most common polymers used in FCM (adapted from ref.* [11]).

	LDPE	***HDPE***	***PP***	***PVC plast.***	***PS***	***PET***	***PA6***
Density (g cm^{-3})	0,91-0,94	0,94-0,96	0,88-0,91	1,2-1,4	1,05-1,2	1,34-1,39	1,12
Tm (C°)	110	137	176	150	–	265	210
Tg (C°)	– 25	– 125	– 20	40-50	94	69	50
Tensile strenght MPa	8-30	22-30	30-40	20-40	36-57	50-60	55-75
Elongation at fracture (%)	100-950	10	100	100	1	50	30-100
Oxygen transmission rate OTR on 25µm (cm^3 $24h^{-1}$ m^{-2} bar^{-1})	7.000	2.800	2.300	6.000	3.800	45	20-40
Moisture vapor transmission rate WVTR (g m^{-2} $24h^{-1}$) on 25µm at 38°C and 90% RH	15-25	5	4-10	70-450	100-155	15-20	150-300

3.2.1. Polyolefins

This type of polymer are some of the most used polymer in the food packaging. The two main and common type of polyolefin are polyethylene and polypropylene. According to the condition of temperature and pressure during the manufacture, the polyethylene can be used to produce low-density polyethylene (LDPE) and high-density polyethylene (HDPE). The first one is produced by polymerization of ethylene with high temperature and high pressure (1500-3500 bar) using free

radical polymerization process. LDPE is a branch-chain structured with several short lateral chain resulting in a relatively low molecular weight (0.91-0.94 g cm^{-3}). It presents a low degree of crystallinity (40%). Sometimes the definition of "low-density polyethylene" is also attributed to different resins obtained by polymerization of others α-olefins (such as 1-butene, 1-exene, 1-octane) or no-olefinic comonomers. Of a certain diffusion is the linear low-density polyethylene (LLDPE) in which the polymer structure composed by α olefinic comonomers (up to 10%) has no long-chain branches and a linear orientation of molecules resulting in a stiffer and more crystallinity structure [13].

Figure 2. *Microstructure of low density polyethylene (LDPE), high density polyethilene (HDPE) and linear low-density polyethylene (adapted from ref.*[11]).

LLDPE and HDPE are synthetized with low temperature and low pressure thanks to the use of specific catalysts (Ziegler-Natta, Phillips, metallocene or late-transition metal catalysts). The introduction of the coordination catalysts in the '50s allows to predict microstructure of polyolefins and to modify their characteristics thus leading to a great application development of this type of polymer[14]. Accordingly with its linear long chain structure with a low presence of branches, HDPE has high degree of crystallinity (up to 60%) and high density (0.941-0.965 g cm^{-3}). As polypropylene (PP) is an asymmetrical monomer, it can be produced with different stereochemical configurations, however in FCM isotactic form of PP is mainly used. It has the lowest density among the most common polymers and a high degree of crystallinity but lacks in transparency. PP is rather rigid and resistant, and it is used, as the HDPE, for rigid plastic production. Different types of PP are produced in order to obtain the diversification of products. Due to its bidirectional orientation, oriented polypropylene (OPP) is used in some applications such as snack food packaging, beverage bottles and soup wrappers; a type of PP with improved transparency is produced using the 1,7% of comonomer ethylene which is in great demand for packaging industry. Polyolefins, in particular HDPE and PP have temperature of melting and softening above 100 °C so they are used as sealing layers or when packaging is intended to high temperature treatments (sterilization or pasteurization).

3.2.2. Polystyrene

Polystyrene (PS) is a substituted olefin originated by addition polymerization of styrene. PS is rigid and very brittle, transparent and brilliant. To improve its mechanical resistance PS is formed by incorporating synthetic rubber to produce high-impact polystyrene (HIPS). Another form is PS expanded polystyrene (EPS) widely used to produce container for dairy products and white trays for meat, cheese and eggs. PS is generally suitable for food products with short shelf life due to its permeability to gases and vapors [12].

3.2.3. Polyvinylchloride

Polyvinylchloride is an addition polymer originated from vinyl chloride, a monomer with similar structure of PE, but one of the hydrogen attached to each other carbon atom is substituted with a chlorine. This material was one of the first polymers used in food packaging application to replace traditional material such as glass and paper, nowadays it is the second widely used synthetic polymer in the packaging industry. A wide range of applications is achieved by formulation of based PVC polymer with additives, plasticizers and stabilizers. It has lower gas permeability than olefins and for this reason it is used for packaging of wine, beer and fatty food, but on the other hand, PVC film has lower water vapor permeability than olefins and it can not be used in the package of dehydrated or dry food.
In the past PVC has been a subject of concern about possible migration of its vinyl chloride monomer (VCM) and dangerous additives (plasticizers, phthalates and thermal stabilizers containing heavy metals) in food.

3.2.4. Polyesters

The most important polyester used in food packaging is the polyethylene terephthalate (PET) produced by esterification of terephthalic acid with ethylene glycol (with release of water) or by transesterification of ethylene glycol and dimethyl terephthalate. Linear saturated PET is hard, semicrystalline, and transparent. PET has low water and oxygen permeability, excellent tensile strength and chemical resistance. It is not heat sealable and for this reason, its use in the flexible packaging is limited. On the opposite, the polymer is widely used to produce bottles, also for carbonated drink thanks to its high resistance to gases and moisture permeation. PET is the most recycled plastic worldwide and after use it can also be reverted back to the original monomers by methanolysis process and then repolymerized.

In several packaging designs the before mentioned polymers are combined with each other and/or with different kind of materials such as paper or paperboard obtaining multilayer materials. In this way, the properties of every single material improve the final product. Tetra pack® is the most common example of how the combination of printed paper layer with plastic layer inside (and an aluminum foil between them) allows the use of paperboard in contact with liquid food. The chemical nature and thickness of plastic bag and layers allow the use of these polymers also to protect food against the penetration of oxygen and moisture, but also against the external contaminants. Nevertheless plastic materials are themselves source of contaminants of different nature[15].

3.3. Plastics legislation

Plastic materials, like all food contact materials and articles, are subjected to Framework Regulation (EC) n. 1935/2004, referred to materials and articles intended in contact with food, and to Good Manufactured Practices listed in the Regulation (EC) n. 2023/2006.
In addition to the general legislation, European Union leads specific requirement for plastic materials used to produce food packages. Commission Regulation (EU) No. 10/2011[16] comes into effect on 1st May 2011 replacing the Plastic Directive 2002/72/EC.

The Regulation 10/2011 regulates all plastic manufactured, that are intended for food contact such as mono and multilayer plastic articles, as well as coatings of plastic and gaskets of glass jar closures. Plastic materials must be produced only with substances (monomers, starting substances and additives) included in a positive list, reported in the Annex I of the Regulation, which is called "Union list". In some cases specific requirements, for example about additives, inks and monomers are present also at national level in Europe. Regulation gives *specific migration limits* (SML) for some substances and an *overall migration limit* (OML) of 60 mg/kg of food. There are rules for assessing compliance with the above limits. The regulation reports a list of food simulants and testing conditions (time and temperature) to evaluate migration from plastic materials. In multilayer materials where a functional barrier separates plastic from food, plastic may be manufactured with unlisted monomers and additives only if the latter are not classified as carcinogenic, mutagenic or toxic to reproduction, the limit of vinyl chloride is respected and if they are not in nanoform. Furthermore only for plastic multilayer the migration of unlisted substances is *not detectable* with a detection limit of 0.01 mg/kg. An important point of the regulation is the Annex IV where the rules to write the Declaration of Compliance (DoC) are specified in some details. DoC is a supporting documentation that the materials complies with the rules, available to the authorities on request. Recently, on August 25th, 2016 the European Commission published the Regulation (EU) 2016/1416 [17] in the Official Journal of the European Union, amending the Regulation (EU) No 10/2011 which introduces some changes. Following a scientific opinion of EFSA, new substances have been added to the "Union list" and can be used in the manufacturing of FCMs. The newly authorized substances are, for example, polymer production aid, nanomaterials used as additives and monomers. In addition, new regulation gives some modifications about migration test conditions and food simulants, in particular, simulant E is referred, in the migration tests, to fresh fruits and vegetables.
A separate regulation (Regulation (EC) n.282/2008) [18] was enacted to regulate the use and production of recycled plastic in FCMs. Recycled plastic shall only be produced from food contact grade plastic and manufactory process shall be authorized in the EU following a safety assessment performed by EFSA. The Regulation lists the conditions to an authorized process and provides a Community Register of authorized process for Member State auditing.
The use of authorized recycling process as well as the register number must be indicated in the DoC.

4. MIGRATION FROM FOOD PACKAGING MATERIALS

A great amount of chemicals may enter in the food supply through intentional or unintentional addition at different steps of the food chain. Packaging system as well as other food contact materials such as adhesives and printing inks are the main source of chemicals in food products and beverage. Human exposure to chemicals from FCM may occur as a result of migration from the packaging into foodstuff involving different compounds according on the nature of material. The classes of migrants may be:

- Substances used in the manufacture of the packaging material, such as monomers, adjuvants or additives. This latter class includes antioxidants, antistatic substances, antifogging agents, slip additives, plasticizers, heat stabilizers, dyes and pigments;
- Substances used in the conversion of the material, such as printing inks, laminating adhesives or solvents;

- Non-intentionally added substances (NIAS), like degradation products of allowed molecules, impurities and unknown substances resulting from bad practices.

Some studies were conducted to evaluate the migration of compounds from plastics materials in food such as phthalates or bisphenol etc. [15][19]. In addition, other elements of plastic packaging, such as inks, varnishes, adhesives can release undesirable substances into the food.
However, nowadays an increasing number of food crisis are originated from non-plastic materials. Few years ago Member State authorities notified through the Rapid Alert System for Food and Feed (RASFF) the occurrence, at concern levels, of two different substances, the photoinitiator isopropylthioxanthone ITX (in 2005) and 4-methylbenzophenone (in 2009) in liquid milk for babies and in breakfast cereals respectively. Paper and board are often used as primary, secondary and tertiary (transport) packaging and an increasing number of 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 [1][20]. Migration from paper and board is completely different from the migration that occurs from plastic. In the polymer, indeed, the migrants are homogeneously distributed into the matrix. At the opposite, in cellulosic materials, contaminants can be absorbed from the fibers that form an open and porous structure. For this reason the mobility from the paper and board is both a diffusion process and a desorption/evaporation and adsorption/condensation process.
During the last years health concerns have been raised over the use of recycled paper and board for food contact. Although recycling sounds both economic and ecological, recovered paper should not be used in all paper grades because there are health safety issues connected to its use in contact with food. The step of purification of recycled fibers is usually not able to remove some residues of contaminants, which may be present in the final paper as potential migrants into food [21]. For example: Diisopropylnaphtalenes (DIPNs), are commonly used in the preparation of special papers, such as carbonless and thermal copy paper; phthalates, such as diisopropyl, diisobutyl, di-2-ethylhexyl phthalate are impurities from adhesives; photoinitiator, such as benzophenone, is an impurity from printing inks; mineral oils come especially from printing inks [22] [23][24] [25] [26].

5. MIGRATION

5.1. Interaction phenomenon

The term "interaction" can be used to define wide and different range of mechanisms such as volatile compound adsorption, gas and vapors transmission, reaction on interface (corrosion), mass transfer (migrations and contaminations) and biological phenomena like mold growth or pest attacks. Each of these phenomena:

- take place on the interface of two distinct phases;
- lead to an exchange of matter;
- modify the integrity and quality of food components.

Food, packaging, environment, can interact with each other to give a large variety of mass transfer.
Interactions between food/environment are considered the most dangerous, both to the food hygiene and to the maintenance of food organoleptic characteristics. However recently, interaction between food and packaging has been of particular concern to consumers, lawmakers and food and packaging industries, due to increased use of synthetic materials and new chemical substances used in manufacturing of packaging. Anyhow "migration" can be defined as the mass transfer phenomenon which occurs from FCMs to food and *vice versa* [27].

5.2. Migration mechanism

According to some authors migration should be a diffusion process affected both by kinetic and thermodynamic control and can be described by diffusion mathematics derived from Fick's law [28] [29]. A potential migrating constituent of the packaging is gradually transferred to the food causing its concentration to gradually decrease in the packaging and increase in the food. Eventually, a point of equilibrium is reached when the concentration of the constituent stays constant in food and packaging [30]. The migration is described as function of different factors such as time, temperature, thickness of materials, partition and distribution coefficients of the chemical substance amount.
The extent of the diffusion process is defined by kinetic dimension, instead, the thermodynamic dimension dictates how extensively the transfer of substances will be at the end of the process (when the system is at equilibrium) [30].
Based on the properties of chemicals, the nature of matrix and the contact surface, migration can occur in three different modes:

- *Migration via gas phase*: in this kind of migration, the contact food/packaging is not necessary. It is the typical migration of volatile compounds which can migrate both from the packaging into food and from packaging in the environment.
- *Migration by contact of diffusive substances*: the contact food/packaging is essential. This type of migration is always fast, due to the high diffusion coefficient of compounds, which are present on the interface.
- *Migration by contact of no diffusive substances*: in order to allow the migration of no-diffusive chemicals, a component of food (such as lipid phase) must penetrate in the packaging and

swell it, leading to enhance the rate of diffusion of packaging material and increased migration [31].

Migration is affected by several parameters that can be referred to the packaging, to the type of food in contact with packaging material and to the conditions of contact food/packaging. Each parameter has direct influence on migration extent and rate.

Both extrinsic and intrinsic characteristics of packaging materials have a great effect on the level of migration. The latter includes the nature and the molecular structure of materials such as polarity and crystallinity. The extrinsic characteristics are referred to the geometry of the package such as thickness, weight, and shape. It has been proved that thinner packages allow higher migration rate [32]. Migration is a superficial phenomenon and for this reason, if the packaging surface increases, the migration increases too. Particularly relevant on the migration is the packaging surface/volume of food because with equal surface of packaging the amount of substance migrated depends on the amount of food. Therefore, in the small packages or in case of film, where a high surface of packaging is in contact with a low amount of food, the ratio packaging surface/volume of food is high and consequently the migration is higher [33][20].

The characteristics of the migrant have a great impact on the migration. The transfer of the compounds usually refers to the molecular weight and shape of the potential migrants and to their affinity for the food or packaging materials as well as their amount. If the chemical substance is not present in the packaging, it cannot migrate in the food. On the contrary it has been shown that, in some cases, higher concentration of migrants lead to higher levels of the substance in food [34]. Some studies demonstrate that chemical structures of a migrant can influence the partition coefficient of migration.

The quantity of the constituent in the food, at the point of equilibrium, depends on the physical affinity of the constituent with the packaging and food. For example, alcohols and short chain esters have higher affinity with the low density polyethylene when they are in an oil solution rather than in water solution. In addition, due to their polarity, short-chained esters are lost more from oil than from aqueous solution. Furthermore, aldehydes dissolved in oil have less solubility in polymer on account to their long carbon non-polar chain [35]. Moreover, low molecular weight substances migrate to a greater extent than high molecular weight ingredients [23].

Some studies investigated how the nature of the food affects the migration, focusing on the interaction of food with packaging materials and their ability to dissolve migrants. The extraction capacity of food is affected by physical (shape) and chemical (pH, percentage of water, fat amount) characteristics. Food can be divided in five categories: aqueous, acid, alcoholic, fatty and dry. On the base of these categories three main types of migration can be characterized, considering the type of chemicals that have high affinity for food and that tend to migrate more easily (Table 3) [29].

Table 3. *Classification of affinities between food and chemicals migrants* (adapted from ref.[29])

Nature of the food in contact	**Nature of chemicals most likely to migrate**
Acidic foods, aqueous foods and low alchol beverage	Polar organic chemicals, salts, metals
Fatty foods, distilled spirits	Non-polar, lipophilic (“fat-loving”) organic substances
Dry foods	Low molecular weight, volatile substances

The packaging conditions that most affect migration are the temperature and the time of contact. Temperature has a direct influence on rate and extent of migration and it seems that the migration is faster when the temperature rises, thus achieving a rapid establishment of equilibrium [36]. Packaging materials are subjected to a wide range of temperature conditions during the transport, shelf life and storage. Clearly not all materials are suitable to all temperatures. Furthermore, materials suitable for short duration contact may not be suitable for longer shelf life. The concentration of the migrating substances is directly proportional to the square root of the time of contact.
The extent of migration is correlated to the specific type of food-packaging contact which is influenced by the physical properties of food (solid foods make only limited contact, whereas liquids make more extensive contact) and by the shape of the packaging [29].

5.3. Assessment of migration

The food packaging legislation, as reported in paragraph 1.2, establishes the overall migration limit (OMG) and the specific migration limit (SML). The OMG is a limit to control the potential transfer of substances from packaging into food. It regards all chemicals in the material and it is a measure for the inertness of the material. The SML is established for a specific substance, which may be dangerous for the consumer health. European authorities assume that an ideal cubic container (with side of $1dm^2$) can lead to a maximum amount of 10mg, from each side, to food contained in it ($1dm^3$ or 1L with a density of 1 that is 1kg). Therefore the limit is expressed in mg/kg of food in contact with food packaging, or converted in mg/dm^2 (mg/kg x 6 = mg/dm^2) when the limit refers to the non-filled object or to the object with a capacity greater than 10 L or less than 500 mL [27].
Migration data represents an important information for exposure and safety assessment and these data should give a realistic statement of the migration into foodstuff. There are four different approaches to assess the migration: residual content, migration modelling, migration tests using simulants and direct measuring in real foods.

5.3.1. Residual content

The approach is the first and simplest method to establish compliance of a packaging material with the legislation. It is calculated without considering dynamics of migration over the time. In this type of approach, the migration was calculated as if the total amount of considered substance in FCM migrated in the food. The obtained data (potential migration) is a borderline case, hardly achievable in the real food but it allows to estimate the risk to overcoming the legal limits.
To carry out the residual content approach is important to know the amount of substance present in the packaging material before the contact with food, the superficial area of the final packaging and the quantity of food that will be in contact with the packaging.

5.3.2. Predictive mathematical models

Mathematical models, which describe physical processes of practical interest, can bring a big advantage as replacement for, or as auxiliaries to the experimental studies. A model to describe the mass transfer of contaminants from food contact materials in food represents a useful tool for industry, for legislative and control authorities and in consumer exposure estimation.

Mathematical models may follow different approaches as stochastic, probabilistic, empirical, deterministic, including uncertainty and variability.
The deterministic models consider that the variables assume a single and constant value leading to a single value of migration, in this type of approach the accidental component is not evaluated. Considering the migration from packaging in food, most of the studies employ the deterministic approach, which allows to obtain an overestimation of possible migration (*worst case* approach). These models are based on the assumption that mass transfer from packaging in food is a diffusional process that can be described by Fick's law:

$$\frac{\partial C^P}{\partial t} = D^P \frac{\partial^2 C^P}{\partial x^2}$$

where C^P represents the concentration of the migrating species in the packaging material, t represents the time, x the linear dimension of migration and D^P is the diffusion coefficient of the migrant in the packaging material. Solution of equation is different according to boundary conditions of packaging materials and foods. In terms of initial conditions, it is considered that:
- the initial concentration of migrant in food is zero;
- the migrant is homogenously distributed in the packaging material matrix.
The latter condition depends on the package production and on the migrant losses during storage of the material, but in some cases it is a good approximation even if the migration may be overestimated. In absence of evaporation or reaction there is a mass balance: the total amount of migrant is the sum of the amount that migrates into the food after time t ($m_{F,t}$), plus the amount remaining in the packaging. If the migration is complete:

$$m_{P,0} = m_{F,t}$$

$m_{P,0}$ is the initial amount of migrant in the packaging.
At equilibrium, K_{PF}, the partition coefficient may be defined by:

$$K_{PF} = C_{P,eq}/C_{F,eq} = c_{P,eq}\,\rho_P\,/c_{F,eq}\,\rho_{PF}$$

Where $C_{P,eq}$ and $C_{F,eq}$ are the concentration of migrant in packaging and in the food (mass/volume), $c_{P,eq}$ and $c_{F,eq}$ are the concentration of migrant in packaging and in the food (mass/mass) and ρ_F and ρ_P are the density of food and material of packaging [37]. Diffusion and partition coefficients have a great importance in the migration regulation. The diffusion coefficient (D) can regulate the rate of migration, at the opposite the partition coefficient ($K_{P,F}$) regulates the amount of migrant from packaging into food. D and $K_{P,F}$ must be measured with expensive and time consuming experiments. One of the most important parameters that influence the diffusion mechanism is the storage temperature. The fundamental equation correlating diffusion coefficient and temperature is the Arrhenius equation:

$$D_P = D_0\, e^{-\frac{E}{RT}}$$

Where D_0 is the pre-exponential factor, E is the activation energy for diffusive molecules, R is the gas constant, and T is the absolute temperature of the system. Several approaches have been carried out to develop a new model for predicting diffusion of compounds. The European Union introduced

modelling potential migration, using generally recognised migration models, as a novel compliance and quality assurance tool in Directive 2001/62/EC, and in the EU Directive 2002/72/EC. In addition it published guidelines in support to the users of the described model to predict conservative, upper bound migration values [38].

5.3.3. Migration tests

Standard migration tests were recognized as important tool to evaluate compliance of food packaging with the migration limits at European level as well as at national level. The tests are conducted on packaging material using indicated food simulants, selected according to the food to be packaged. The selection of time and temperature conditions of tests is based on the intended uses of packaging and food. The six food simulants, reported in European Commission Regulation n.10/2011 and listed in table 4, are either liquid or solid substances that have a simple and known composition. They have similar properties to those of one or more categories of food. Generally, food simulants A, B and C are assigned for hydrophilic foods. In particular, simulant B is for food which has a pH below 4.5, while simulant C shall be used for food with amount of alcohol up to 20% or foods containing a significant amount of organic ingredients that made them more lipophilic. Simulant D1 and D2 are intended for foodstuff that have lipophilic character and that are able to extract lipophilic substances. The D1 food simulant is used for alcoholic beverages with an alcohol content of more than 20% and for emulsions of the oil type in water. The simulant D2 is used for foods containing free fats in the surface. Finally, the simulant E is used for migration tests with dry foods [16].

Table 4. *Food simulants suggested for migration tests by Reg. 10/2011 (From ref.*[16].

Food simulant	Abbreviation
Ethanol 10 % (*v/v*)	Simulant A
Acetic acid 3% (*w/v*)	Simulant B
Ethanol 20 % (*v/v*)	Simulant C
Ethanol 50 % (*v/v*)	Simulant D1
Any vegetable oil containing less than 1 % unsaponifiable matter	Simulant D2
poly(2,6-diphenyl-p-phenylene oxide), particle size 60-80 mesh, pore size 200 nm	Simulant E

After the simulant and the time and temperature conditions are chosen, based on the exanimated food, a specimen of packaging material comes in contact with simulant. The UNI-EN 1186:2003 provides guidance to select appropriate conditions to determine global migration in food simulants. Furthermore, the standard indicates differentiated proofs for fillable packaging and materials that are in thin films or sheets. The methods proposed are:

- Diving method (for specimens);
- Fill method
- Single side method;
- Special cases (capsules, gaskets, caps etc.)

The *diving method* provides that the surfaces of the test material are to come fixed to a metal support and then immersed in a filled vessel of the simulant chosen for the test. Regarding the fillable object the migration test shall be conducted with *fill method* under similar condition to real use of package. *Single side* method is expected for materials where only one side comes in contact with food. Two kind of equipment are suggested: a completely hermetic steel cell or closable Petri dishes.

5.3.3.1. Simulant E: Tenax®

Tenax® is the commercial name to indicate the poly 2,6-diphenyl-p-phenylene oxide. Tenax® is a high adsorbent solid composed of small granules. The density of this material is about 0.25 g/cm^3 which roughly means that about 75% of the Tenax® is air. The particle size is about 60-80 mesh with a molecular weight of 500000-1000000 Da. Tenax® is stable up to 350 °C. Two types of this simulant are available on the market, Tenax TA and Tenax GR, the latter presents different density and specific surface than Tenax® TA, and it is constituted also by 30% of graphite. This simulant is chemically inert against organic substances, it presents thermal stability and it is easy to manipulate. On the other hand, Tenax® is more expensive but it can be reused several times after solvent washing.
The migration limits fixed by legislation are referred to real food, but the rules on migration tests use the term "food" and "food simulant" without discriminations.
The migration behavior of compounds from paper and paperboard in food simulants and real food are compared in some studies. In 1999, Aurela et al. compared migration of some phthalates (DiBP, DBP and bis(2-ethylhexyl)phthalate (DEHP) and DEHA from cardboard into sugar and into Tenax® demonstrating that levels of contaminants in Tenax® were higher, but corresponded well with the real-life migration into sugar [39]. Subsequently, Suciu et al. 2013 confirmed the suitability of Tenax® as a simulant for the migration of BPA, DEHP and nonylphenols from recycled paperboard in sugar and salt [40]. The Tenax® seems to be a good simulant also for fatty dry foods such as semolina, instant baby cream and infant whole milk powder, however the high fat content of the foodstuffs contributed to a more pronounced migration tendency [21][32]. At the opposite, some authors observed large overestimation of migration using the food simulant. Tenax® overestimated the real migration ratio in cereals by 70% (for 4-(dimethylamino)benzophenone) up to 92% (for 2 ethylanthraquinone) [41]. Furthermore, overestimation by at least a factor of 10, for the migration of ten different contaminants, have been reported with Tenax® in comparison with fresh fruits and vegetable in contact with paper [42]. Finally, Zurfluh et al. 2013 affirmed that Tenax® is too much strong adsorbent for mineral oil hydrocarbons to be comparable to dry foodstuffs. Authors also indicated the conditions of the test (60°C of temperature) as inadequate due to the tendency of the indicated temperature to increase migration of some hydrocarbon (that do not significantly migrate at room temperature) and to lose polypropylene barrier properties [43].

5.3.3.2. Alternative adsorbents

Even though, Tenax® is the only official material to simulate migration into dry food, in the last years, the performance of other possible simulants have been investigated. Poropack™ is a porous polymer available on the market. The behavior of this substance in comparison with Tenax® has been widely studied. In 2007, Nerin et al. investigated performance of Poropack™ and Tenax® in migration of phthalates and DiPNs from paper and board, demonstrating no substantial differences in

the behavior of the two simulants [32]. The performance at high temperature showed contrasting results. Migration study involved alkylbenzenes, 1,4 Dichlorobenzene, styrene and 1-octene from plastic materials and demonstrated that the Poropack™ was the better simulant up to 150°C [44]. On the opposite Lin et al. in 2011 found Tenax® the more suitable simulant for assessing the migration at high temperature of isothiazolinone biocide from paper packaging because the Poropack tended to lose the migrant increasing temperature [25]. The performance of the simulant is strongly dependent on the nature of the compounds investigated.

On the contrary, some studies, investigated the use of real food such as powder milk, rice or polenta as food simulants. Tests in real samples and tests with accelerated conditions were compared in order to understand if the accelerated migration tests is able to assess the real migration from packaging into food. The rice, showed lower migration rate compared to Tenax® in order to evaluate the migration of two antioxidants [45]. Polenta (corn semolina) showed more realistic migration values with the real food conditions, compared with Tenax®.

The use of alternative simulants can be advantageous thanks to the relative low cost of material, but in some cases, the adsorption properties of the various alternative simulants are influenced by the nature of migrant.

To avoid the use of Tenax and other type of simulants Fiselier and Grob [46] developed a migration test with alternative design to evaluate the barrier effect of some polymers against the migration of mineral oil from paper and board materials. The test involved a donor paper, spiked with a mix of migrants of interest, and a polyethylene foil, used as receptor (instead of simulant), divided from the barrier material. The system was packed in aluminum foil and put to the chosen condition of time and temperature. At the end of the test, the acceptor foil was simply extracted. In a subsequent study, the polyethylene acceptor foil was replaced by a silicone paper in order to improve the range of compounds that could be adsorbed [47] (Figure 3).

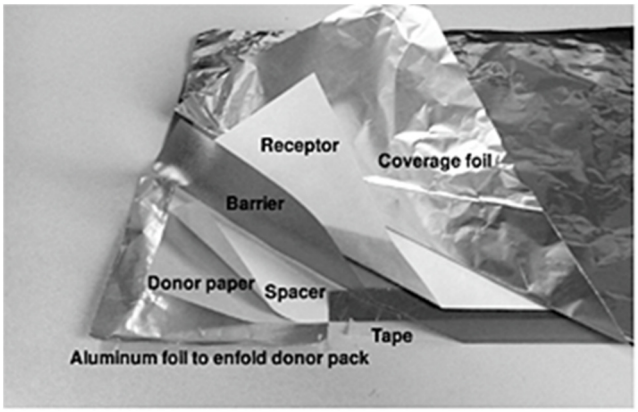

***Figure 3**. Test pack using for migration tests by Biedermann-Brem & Grob 2014 (from ref.*[47]).

Recently, a Tenax® film instead of powder was proposed to evaluate migration. The film can be constructed by first dissolving Tenax® in a chlorinated solvent, such as chloroform, followed by spreading a known volume on a solid support (e.g. a glass Petri dish), and finally by evaporation under air. Several migration experiments were carried out by authors to evaluate the performance of the new simulant in comparison with the Tenax® powder. They observed that, especially for the rough cardboard sample, the migration of most contaminants into film was found to be lower than into powder, likely due to the possibility for a less intense direct contact when film is used [48].

5.3.4 Direct measurement in foods

When simulation tests are not possible or not reliable, direct analyses in food are needed. They allow having the most realistic data but interpretation of their results is very tricky due to the high amount of contamination sources and great variety of substance-related compounds, such as NIAS, which may be present in food. The food should be selected to ensure that it represents all food or categories of food intended for the FCM application. Furthermore, the conditions determining migration have to be respected, such as the mobility and solubility of migrant, as well as time and temperature conditions, and the conditions of time and temperature used to process and store the packed foods.

6. POLYCYCLIC AROMATIC HYDROCARBONS

6.1 General aspects

The term polycyclic aromatic hydrocarbons (PAHs) indicates a large class of organic compounds that are present in environment. PAHs are formed by 2-6 fused aromatic rings (with linear, cluster, or angular arrangement) and can be present substituting groups such as alkyl, aryl, nitro, amino or heterocyclic rings [49]. Figure 4 shows the structure and abbreviation of the main PAHs considered by European legislation.

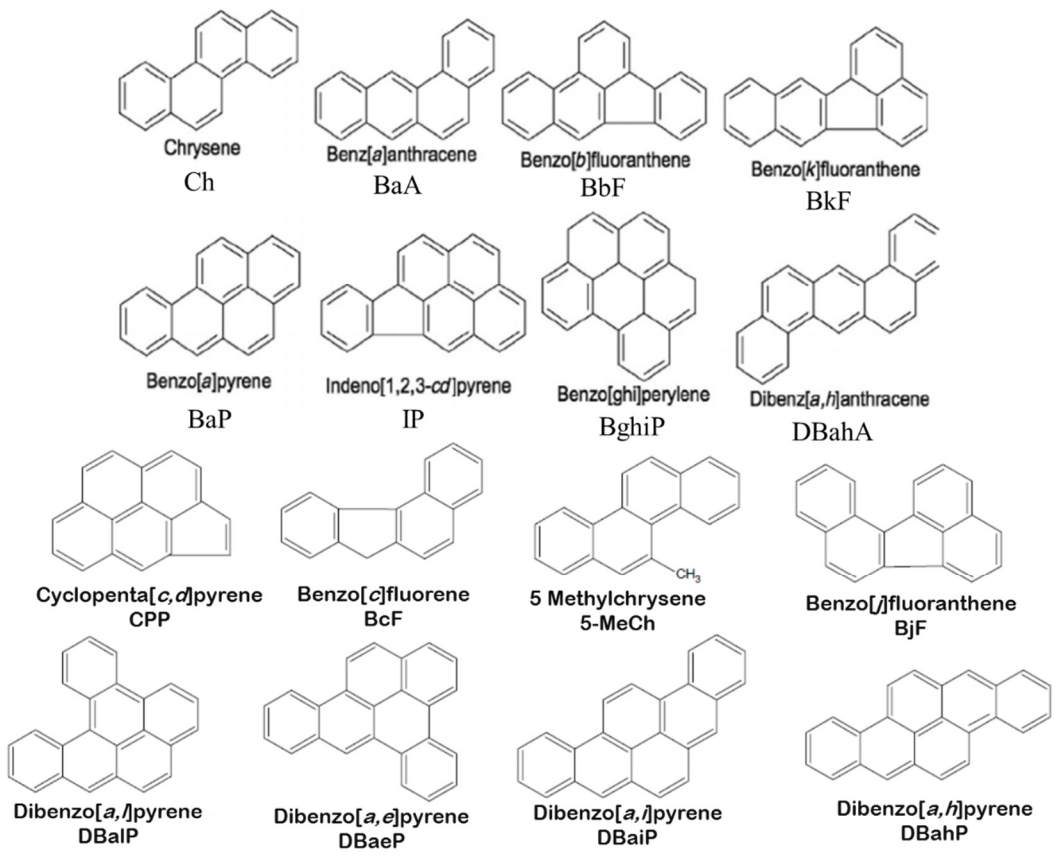

Figure 4. *Chemical structure and abbreviation of 16 priority EU PAHs.*

Molecular weight of PAHs affects their chemical and physical characteristics. An increase in the molecular weight leads to a decrease in the solubility in water and in the resistance to chemical degradation. PAHs (non alkylated aromatic hydrocarbons) originate at high temperature (500-700

°C) from incomplete combustion or pyrolysis of organic matter. They can be formed also at low temperature (100-150°C) but it requires a period on the geological time scale. The latter process originates a large amount of alkylated PAHs. Thus, the ratio between alkylated and non-alkylated PAHs can be an indication of the source of contamination. Natural source of PAHs emission are forest fire, volcanoes, hydrothermal processes and carbonization [50][51]. Nevertheless, they are present in the environment mainly due to anthropogenic sources: the combustion of fossil fuels and the direct release of oil and oil products or lack of manufacturing practices during food production, such as technological processing involving high temperature (grilling, smoking). These are also the major route of contamination of food. Scientific Committee of Food (SFC) in 2002 [52], and EFSA in 2007 [53] affirmed that the main human intake is attributable to vegetables oils and fats, either directly or indirectly such as by their incorporation into food preparations. Some studies reported high level of PAHs contamination in dried fruits (transported in jute bags or contaminated directly by contact with combustion products) and in smoked fish and meat products (related to the smoking methods)[54]. The toxicity of PAHs is well known. Humans are exposed to PAHs contamination both by inhalation of environmental particles and by ingestion. PAHs are no toxic when they are absorbed in the organism, but they become toxic during the attempt to eliminate them through faeces and urine. In fact they are rapidly absorbed and metabolized and the formed metabolites are very active and can form the adducts with the proteins of DNA, thus starting the carcinogenic process. The most harmful are the PAHs that present 4-6 condensated rings, in particular BaP which can cause infertility and development disorders. However, not all the PAHs congeners have the same activity, SFC reported that 15 out of 33 considered PAHs showed clear evidence of both genotoxicity and carcinogenicity; some were genotoxic but not carcinogenic, other ones were non-carcinogenic, but may act as synergists [52]. The BaP is the most studied PAHs compound, and it is analysed in several environmental and food matrices. For a long time BaP was taken as *marker* of PAHs contamination and carcinogenicity. Member States PAHs legislation were harmonized thanks to the EC Regulation 188/2006. In 2005 European Authorities recommended that all the Member States should investigate the levels of the 15 PAHs pointed out by the SFC and one (BcF) highlighted by the JECFA[55] in order to review the limits already set. In 2007, EFSA led to the conclusion that BaP was no a suitable marker and suggested to use the sum of 8 PAHs (BaA, Ch, BbF, BkF, BaP, BBahA, BghiP and IP), or a subgroup of 4 PAHs (BaA, Ch, BbF and BaP) [54]. In 2011 a new Regulation was published (Reg 835/2011) [56] which fixes the new limits for BaP and PAH4 for several food classes. The limits for PAHs in the paper and cardboard intended for food contact, reported by CEPI Guidelines, is of 0.0016 mg/dm^2 referred to their sum. The value is calculated from specific migration limits (0.001 mg/kg of food) and is expressed as permissible maximum amount admitted in paper or paperboard, if the total migration occurs.

6.2 Sample preparation

No specific analytical method is required to evaluate PAHs, but Regulation 836/2011[57] sets performance criteria about limit of detection (LOD), limit of quantification (LOQ), precision and recovery that the used method has to comply. PAHs analysis in different food and packaging materials requires particular attention according to the matrix in question.

Traditional method for PAHs determination in fats and oil involves a saponification followed by liquid liquid extraction of the unsaponifiable matter and purification on a packed column or an SPE

cartridge. LLE step with a mix of ACN/acetone is also used in the ISO method for the quantification of EPA priority PAHs. However, these type of methods are long and solvent consuming. Alternative types of techniques were proposed over the years to simplify the PAHs analysis. SPE is probably the most employed technique because it allows extraction and purification in a unique step, and it is applicable on several matrices. Different solvent phases such as Silica, C18/Florisil, polystyrene-divinylbenzene (PS/DVB) are employed according to the sample preparation objective (retention pf fat, eluting analytes, retention of analytes of interest etc.). Both head space solid phase microextraction (SPME) and direct immersion SPME were largely applied techniques to extract PAHs from different kind of samples. The HS-SPME mode is worthy of attention since good repeatability and LOD values without sample manipulation. Extraction techniques used in specific application for determination of PAHs in food are the pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), microwave assisted extraction (MAE), as well as some miniaturized techniques such as membrane-assisted solvent extraction (MASE) or dispersive liquid liquid microextraction (DLLME) [58]. Usually the ultrasound-assisted extraction is used in order to extract PAHs from paper and paperboard. Different solvents are involved, dichloromethane and acetonitrile are the most selected ones.

6.3 Analytical determination

The most used analytical techniques for determination of PAHs are the gas chromatography coupled with a mass spectrometry and the liquid chromatography with spectrofluorometric detector (FLD). The latter technique allows to obtain high sensitivity and selectivity and high resolution. The high sensitivity reached with FLD detector allows to minimize the sample preparation and to inject the partially purified samples. Furthermore, HPLC technique is used instead of GC, thanks to its ability to analyze the heavy PAHs, which due to degradation at high temperature are not detected with GC. However, the spreading and improvement of the MS technology in the LC field have led to an increase of LC-GC methods. A specially designed C_{18} phase is usually employed for a proper resolution of 16 PAH. The mobile phase used in the analysis is an acetonitrile (ACN) or methanol (MeOH) gradient in water [59]. Methods using comprehensive two-dimensional chromatography, such as LC-GC, LC-LC-GC, or LC-LC, are proposed by few studies in order to reduce sample manipulation and risk of cross contamination, especially for fat and oil analysis. For these types of food an LC silica column for sample pretreatment is necessary to remove the triglycerides and eventually high amount of polyolefins. Multidimensional comprehensive techniques, such as GCxGC, have been also applied, mainly on soil, petroleum fraction and tobacco but few works are reported for food analysis. This technique enables to isolate the matrix interferences requiring less clean up step [58].

6.4 PAHs contamination from FCM

Although the major source of human exposure is food, some works reported the presence of PAHs in the packaging materials. Due to the excess of high temperature and chemical reagents some PAHs can be formed during the paper production and be released in the atmosphere [60]. Furthermore, PAHs are present in printing inks used in the printing process of food contact materials. In 2011, Parigoridi and co-workers evaluated the presence of 13 PAHs in commercially available recycled cardboards used for food packaging applications. However, only Pa presented level higher than

0.0011 mg/dm^2. Subsequently Vavrouš *et al.* (2016) found levels up to 0.080 mg/kg of PAHs in cellulosic packaging used for food contact [61].

7 MINERAL OIL

7.1 Chemical characteristics and source of contamination

Wide range of products, called “mineral oil”, are made during the distillation of crude oil and subsequent refining treatments such as extraction, crystallization, acid purification or dehydrogenation, which crude oil undergoes. These products are the result of final blending operations of different intermediate products in the refinery, which are controlled by on-line measurements of some principal physical properties other than composition. Moreover, since crude sources for the refinery may change and the process parameters may be adjusted to market demands, the composition may change accordingly, so, the composition of different products is only known in general terms or not known at all [62] [63].

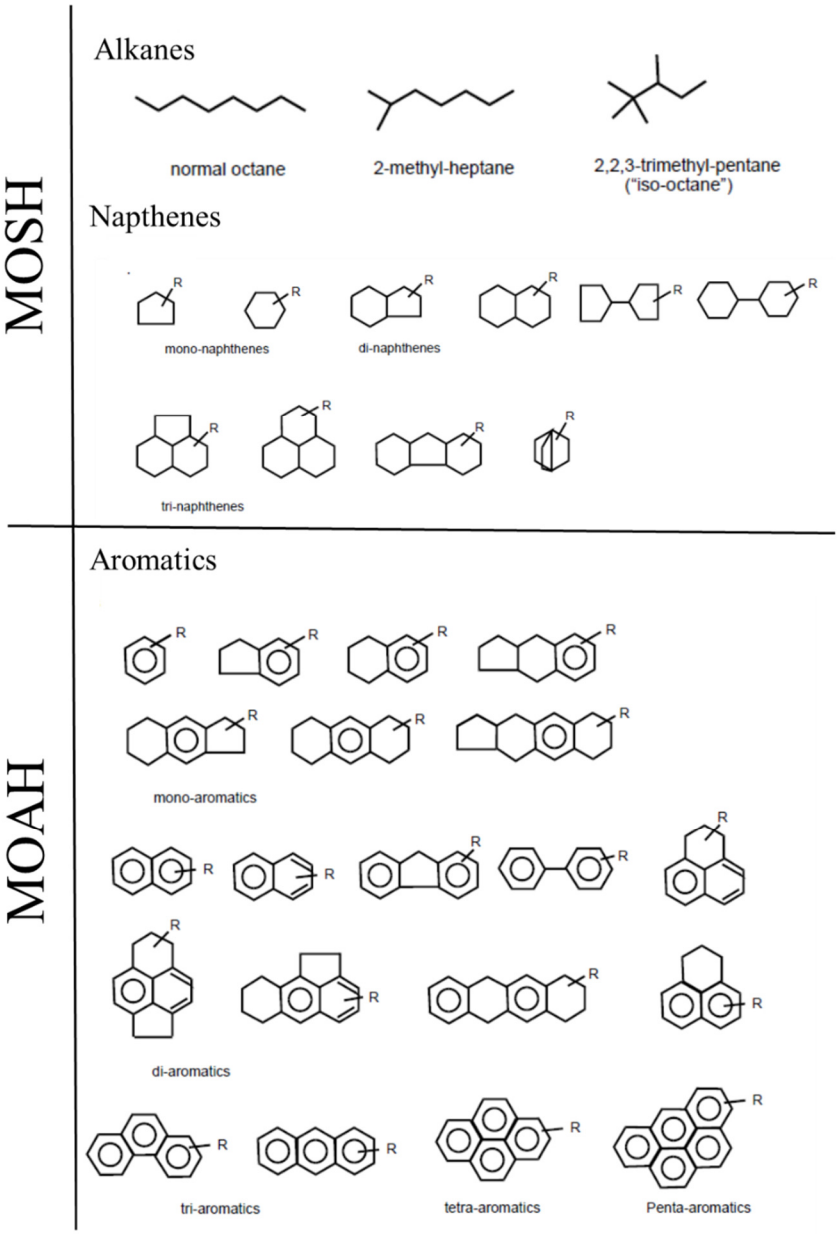

Figure 5. *Chemical structure of MOSH and MOAH (from ref.*[64]*).*

The main constituents of mineral oil are hydrocarbons and compounds that contain heteroatoms. Petroleum hydrocarbons or mineral oil hydrocarbons (MOHs) are a very complex mixture of isomers mainly related to three different classes:

Paraffins: this group consists of aliphatic or alkane hydrocarbons (compounds constituted by linear and branched chains of carbon and hydrogen respectively named *n*-alcanes and isoalcanes;

Naphtenes: cyclic compounds with one or more rings namely cycloalcanes, mainly cyclopenthane and cyclohexane, alkylated and non-alkylated;

The term MOSH (mineral oil saturated hydrocarbons) involves paraffins and naphtenes.

Aromatics: mono- di- and higher ring systems, including alkylsubstituted. Alkylated benzene rings are predominant on non-alkylated ones [64]. The term MOAH (mineral oil aromatic hydrocarbons) indicates mineral oil aromatic hydrocarbons (Figure 5).

Mineral oil is distributed in all refined and distillated products of crude oil based on their range of volatility (Table 5). These petroleum products are widely used in the industry either with their original forms or as additives, increasing their spread (and spread of mineral hydrocarbons) as contaminants in all level of the environment. Mineral oil released in the air from exhausted gases of means of transport or domestic heating, lubricating oil and road tar debris contributes to create a background environmental contamination which can be found in plant materials and food plants [65][66]. Food products are subject to mineral oil contamination also during their production. In food industry, food grade mineral oils have a great variety of uses and should be made fit for direct contact with food by removing the aromatic fraction. They are used as lubricants, release agents, dust suppressants for grain and animal seed, protective coatings for raw fruit and vegetables and as additives (microcrystalline waxes are used in confectionery products). Food contact materials are another important source of contamination. In 1991 amounts in the range of 10-100 mg/kg of mineral oil from jute and sisal bags were found to be an important source of contamination for foodstuff, such as coffee, rice, cocoa etc. stored in these bags [67]. During the years, the introduction of new packaging materials as well as the new manufactory processes and use of chemicals for improving performances of the products have led to an increase in the presence of mineral oils in the packaging. Data on migration of this type of contaminants from paperboard and recycled paperboard boxes through the gas phase and from printing inks was published in [68][69][70]. Mineral oil are also used as lubricants and in the formulation of adhesives and plastic materials [64]. For many foods the presence of MOSH and MOAH contamination comes from different sources and gives an overlapping distribution of molecular weights making the analysis and the interpretation of the results very difficult.

Table 5. *Characteristics and main uses of petroleum distillated products (from ref.* [71]*).*

Boiling range (°C)	**Composition (No. of carbon atoms per molecule)**	**Name**	**Main uses**
<20	C_1 to C_4	Natural gas (gaseous compounds)	Fuel chemical synthesis(i.e. raw material for chemical industry)
0-30		Natural gas (Zymogene and Rhigolene)	Liquid zymogene is used in the manufacture of ice. Rhigolene is used in medicine as local anesthesia
20-90	C_5 to C_7	Light petroleum (petroleum ether)	Solvent
30-150	C_5 to C_{14}	Crude naphtha	Solvent
70-90	C_6 to C_{18}	Gasoline of petrol	Motor fuel, solvent in dry cleaning
70-200	C_6 to C_{10}	Petrol (gasoline)	Motor fuel
90-120	C_7 to C_8	Ligroin (high b.p. petroleum ether or light petroleum)	Solvent in dry cleaning
100-200	C_5 to C_{10}	Fuel	Automobiles
120-160	C_5 to C_{10}	Benzene	Solvent (in dry cleaning and in oil and paint industry
150-300	C_{10} to C_{38}	Kerosene	Fuel, illuminant, making oil gas
175-300	C_{10} to C_{18}	Kerosene (paraffin)	Fuel for jet engines or central heating systems
200-300	C_{12} to C_{18}	Kerosene (paraffin)	Fuels (lamps and stoves)
>275	C_{12} to C_{20}	Gas oil	Fuel for diesel engines
>300	C_{18} to C_{38}	Gas oil or heavy oil	
300-400	C_{15} to C_{25}	Diesel oil	Fuel (locomotive)
	C_{20} to C_{24}	Lubricating oil	Lubricant (making candle, shoe polish)
>400	C_{21} to C_{30}	Paraffin wax	Various
Non-volatile oil	> C_{30}	Lubricating oil, waxes etc.	Lubricant candles
Solid residue	> C_{40}	Asphalt bitumen Vaseline Pitch Petroleum coke (on predistillation of tar)	Road surfaces, roofing Lubricant In toilet goods and ointments In paints and varnish. As fuel.

7.2 Toxicity, exposure and legislation

The major routes of exposure of the population to mineral oil are ingestion, inhalation and dermal contact. However there are also several occupational environments, which lead to constant or repeated exposition to hydrocarbon contaminants, such as occupations include metalworking, printing-press operating, and cotton- and jute-spinning. Nowadays the dietary exposure to MOSH in the European population is from 0.03 to 0.3 mg/kg body weight (BW), and EFSA considers it to be of potential concern based on low margin of exposure. The toxicity of mineral oils has been studied for several years and is still an open topic. The danger of these oils for human health depends on the distribution of molecular weight of hydrocarbons and from presence of MOAH, which represent the most toxic fraction. For this reason the mineral oil contained in non-refined petroleum products are considered carcinogenic [72].

The toxicity of the MOSH is under study and controversial but their tendency to accumulate in the animal tissue is well known. In 2002, the Joint and FAO/WHO expert Committee on Food Additives (JECFA) re-evaluated the white mineral oil and waxes setting acceptable daily intake (ADI), based on their viscosity and molecular weight. The ADI were established according to chronic experiments performed in Fisher-344 rats, which demonstrate the accumulation of mineral oil in the tissue of

animals [73]. Several studies were conducted on this topic demonstrating the onset of various pathologies in animals after feeding a diet contain mineral oil. Highly refined mineral oil seems to be non-carcinogenic for human, but depending on the type of mineral oil and its viscosity, an amount of 0.01-20 mg/kg body weight (b.w.) cause undesirable effects, in particular in *Fisher 344* rats, such as formation of granulomas in liver and inflammations of mesenteric lymph nodes [74] [75]. Focal histiocytosis, increase weight of liver, granulomas and microgranulomas of the liver, and damage of the mild are other effects observed in the animals during the toxicological tests on mineral oils. The analysis of tissue shows that MOSHs tend to accumulate in amounts that are inversely related to the length of their carbon chain [76]. The use of toxicological data for human safe assessment is uncertain. Animal tests last for far less time than human lives and the weight of animals is very different from the average human body weight. The number of studies on human tissues is limited with respect to that on animals. Analyzing human milk Noti et al. found levels from 95 mg/kg to 1300 mg/kg [77] of n-C_{14}-C_{45}. MOHs concentrations were also evaluated in fat tissues of women during their Casearian section that were from 15 to 360 mg/kg fat. Milk fat showed an initial contamination up to 355 mg/kg that decreased during the lactation. The composition of the MOSH was identically in all fat tissues and milk samples. However, the unique legal limit established for the content of mineral oils, both in packaging and in food, was the limit of 50 mg/kg for the mineral paraffins in sunflower oil from Ukraine, applied by the European Commission [78] after a case of contamination of Ukrainian sunflower oil with mineral oil at concentration above 1000 mg/kg. The limit was withdrawn in 2014[79]. In 2009, the EFSA established a new ADI of 12 mg/kg b.w. per day for high viscosity white mineral oils based on a 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 2011, the German Federal Institute for Risk Assessment (BfR), after conducting toxicological studies in rats Sprague Dawley, claimed that saturated hydrocarbons, with a carbon chain comprise from n-C_{10}-C_{16}, are not accumulated in tissue. Assuming the consumption of 1 kg of contaminated food by a person weighing 60 kg, it concluded that the transfer of these substances into food should not exceed 12 mg/kg. In 2012, the EFSA Opinion about mineral oil, considered the acceptable daily intake values previously stated by SFC (1985) and by JECFA (2002 and 2009) as valid [64]. Furthermore, EFSA introduced the *Reference Point* (RP) used 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. Regarding MOSH two different levels of RP were individuated:

- 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.

EFSA affirmed that due to their carcinogenicity, the presence of MOAHs 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 [80].

After the publishing of the first draft in 2011, in 2013, the German Ministry for Nutrition, Agriculture and Consumer Protection (BMEL) published a draft proposal regulating mineral oil contaminants in paper and board intended as food contact materials, specifying that migration of MOHs from n-C_{10} to n-C_{25} from paper and board into foods may not occur. In May 2014, another draft was published about regulation of mineral oil contamination in packaging of paper and board made by recycled fibre and food in contact with them. It established a maximum limit of 24 mg/kg for MOSH and 6 mg/Kg

for MOAH, that involved carbon chain in the range from *n*-C_{16}-C_{25} for contact with dry foods, and in the range from *n*-C_{16}-C_{35} when packaging is in contact with wet foods. When these limits exceed, migration tests are requested and the packaging can be placed on market if migration value for food does not exceed 2 mg/kg for MOSH *n*-C_{20}-C_{35} and 0.5 mg/kg for MOAH *n*-C_{16}-C_{35}. A very new version of the draft ordinance, on May 2017, removed the 2 mg/kg limit for MOSH and recommends the introduction of functional barriers to reduce the migration of chemicals from recycled paper and board used in contact with food [81].
Recently, new studies on the accumulation and toxicity of MOSH in animals (female Fischer 344 rats) were published [82][83][84] and the results were compared with human biomonitoring data from an earlier study [85][86]. The authors investigated the MOSH dose effect as well as the effect of molecular range, the structure and the viscosity on accumulation in rats tissue up to 120 days of feed control diet. The analysis show that in the animals, the extent of MOSH accumulation occurred, is higher in the liver than in adipose tissue and spleen, while in human samples amounts of contaminants were similar in adipose tissue, liver and spleen. This behaviour is probably due to chronic exposure (humans) vs subchronic exposure (animal studies). MOSH concentrations in the rats tissues do not increase proportionally with the administered doses. The authors thus deem linear extrapolation from high to low doses questionable, because low dose tissue concentrations would consequently be underestimated. The most volatile part of the administered mixture is accumulated in adipose tissue whereas in the liver, the most volatile and the highest boiling part of the mixture were nearly absent. A high decrease of MOSH concentration was observed in the liver, but not in adipose tissue, after exposure was stopped. Furthermore, MOSH exposure resulted in a significant increase in liver and spleen weights.
On January 2017 the European Commission (EC) adopted Recommendation (EU) 2017/84 that requires the union member states a monitoring of mineral oil hydrocarbons in food, in materials and in articles intended to come into contact with food, to be performed in 2017- 2018 [87].

7.3 Analytical methods

Mineral oils consist, as already mentioned, of paraffinic (MOSH) and aromatic (MOAH) components which are a complex mixture of isomers that cannot be individually separated as single peaks using classic capillary GC. Their presence is easily recognizable, in chromatographic trace, by one or more “humps” consisting in a great number of unresolved peaks with a balanced *n*-alkane distribution between odd and even carbon numbers hydrocarbons. However, sometimes the MOSH determination is made more complicated by the presence of natural paraffins (present in all type of food and packaging made from natural materials) which is characterized by the pattern where odd *n*-alkanes prevail on even ones. To quantify the MOSH, endogenous *n*-alkanes areas have to be detracted from the total area of hump. Since MOSH and MOAH form one or more humps of unresolved peaks with the same range of volatility, a preliminary step of separation in order to quantify the two fractions separately is necessary.

7.3.1 Sample preparation

The analysis of total food contamination must be preceded by appropriate sample preparation to isolate the analytes of interest and eliminate any interfererence. Furthermore, a great attention has to

be posed to avoid the potential cross-contamination of the blank, during all the analytical steps. Solvents must be purified, and inert accurately cleaned materials, such as glass, aluminum or stainless steel, have to be used in contact with samples.
The sample preparation involves four steps:

- Sampling
- Extraction
- Purification
- Concentration or dilution

Extraction and purification are "key" steps in the process to allow quantitative analysis.
Even if the GC coupled with a mass spectrometer (MS) has been proposed in mineral oil analysis, this technique is not suitable for petroleum hydrocarbon quantification because it gives very different responses for two different hydrocarbon compounds of the same mass. For this reason flame ionization detector (FID), which provides a virtually equal response per unit of mass for all hydrocarbons, is the most utilized detector in mineral oil analysis. However, FID requires a great sample preparation and a preseparation step must guarantee that only MOSH and MOAH enter the detector [88]. Some methods based on off-line solid phase extraction SPE-GC-FID [89][90][91]; and on-line liquid chromatography LC-GC-FID [92][93][94] are usually used for determination and quantification of mineral oil in foods and packaging. The reduction of sample manipulation and solvent consumption as well as saving time are the main advantages of these techniques. However, in case of fatty foods or foods with a complex matrix, a step of purification and fat removal is crucial to avoid exceeding the column retention limit and to obtain lower detection limits. Saponification was the first approach used to eliminate fat before GC analysis, traditional saponification followed by unsaponifiable extraction has been previously applied for determination of mineral oil or endogenous *n*-alkanes in different food samples [95]. Recently a rapid, microwave assisted saponification was developed by Moret et al. [96] as alternative to a time and solvent consuming traditional method of saponification. A second approach is the use of fat retainers such as activated silica gel and aluminum oxide. MOAH analysis in vegetable oils and fats is complicated by the presence of high amounts of olefins, such as squalene and its isomerization products, sterenes and carotenoids, which co-elute with MOAH. Bromination by Wagner et al. [65] was the first method proposed to solve the problem, currently the most used method to remove olefins is the epoxidation introduced by Biedermann et al. which involves derivatization to increase the olefins polarity and hence their retention, beyond that of MOAH [92]. Very recently, Nestola and co-workers proposed some modification in the reaction conditions of epoxidation, and they developed an automated method to simplify the manual operations and to have higher control of the reaction [97].
For liquid samples, such as water, wine or beverages, liquid-liquid extraction (LLE) with hexane is the most used method followed or replaced by solid phase extraction (SPE)[98][99]. In wet samples, water is an almost perfect barrier against the extraction of MOHs from particles using an apolar solvent, for this reason a dehydration is required [100]. Solvent extraction with *n*-hexane was also applied to extract superficial contamination of dry foods, such as that migrated from the packaging[69]. In order to reach a complete extraction of contamination, an alternative pressurized liquid extraction (PLE) has been proposed by Moret and co-workers [101], who applied the PLE extraction also to extract mineral oil from paper and cardboard intended for food contact [102]. The most common method to extract MOHs from paper and board packaging was proposed by Lorenzini et al. in 2010 [103]. It provides an extraction using a mixture of *n*-hexane/ethanol 1:1 (v/v). Due to

its ability to swell the fibers, the ethanol improved extraction of the high molecular weight hydrocarbons. Comparable results were obtained with the PLE technique using n-hexane, high temperatures (60°C), and high pressure (100bar) for 30 minutes [102]. Furthermore, in 2013, Gaudreault et al. proposed a method to characterized the level of MOSH and MOAH in newspaper, paperboards. The extraction was performed with the Accelerated Solvent Extractor (ASE) using *n*-hexane/dichloromethane 50:50 mix (v/v) at high temperature (150°C) and high pressure (1500 psi). MOSH and MOAH were fractionated by silica/alumina column and injected in a GC-FID system.

7.3.2 Analytical determination

MOSH and MOAH separation can be performed both *on-line* and *off-line*. *Off-line* fractionation can be carried out on LC column, a solid phase extraction-SPE-cartridge, or on a glass column filled with a suitable sorbent. At the end of the procedure, the collected fractions are injected in a GC system. Two *off-line* methods are reported in literature both based on the use of silver silica SPE cartridge, which compared on activated silica, allows for better separation of MOSH and MOAH fraction and a better retention of olefins [90]. The *on-line* separation using LC-GC method is the most suitable technique for mineral oil determination and is widely used for routine analysis. In 2009, Biedermann et al. [92] proposed a rapid on-line LC-GC-FID method to separate MOSH and MOAH on 250 x 2 mm i.d. silica gel column able to retain 20 mg fat. LC-GC transfer was performed by the retention gap technique and the partially concurrent eluent evaporation through the Y interface developed by the same authors. The recovery of a particular mix of standards is required to evaluate the performances of the system, in terms of recovery and efficient MOSH/MOAH separation. In particular, the standard solution involves *n*-$C_{11,}$ to control the loss of volatiles, *n*-C_{13}, being eluted closely to the cyclohexyl cyclohexane (CyCy) which is used for the quantification. Cholestane (Cho) serves to control the end of the HPLC elution window for the MOSH. In addition, as standards for the MOAH, *n*-pentyl benzene (5B) is used to control the loss of volatiles, the closely eluted pair of 1- and 2-methylnaphtalene (MN) were selected to identify possible coelution with a sample components. Finally tert-butyl benzene (TBB) and perylene (Per) keep the beginning and the end, respectively, of the MOAH fraction under control [104]. In 2013 Barp et al. [93] proposed an optimization of this method increasing a throughput sample and reducing the consumption of solvent. The use of an additional LC column on the system was studied by Zoccali and co-workers to analyze the MOHs in edible oils. The first silica column, retains the bulk of fat and allows the MOSH and MOAH separation, and the second silver-ion column retains interfering olefins. Simultaneous quantification of the contamination and evaluation of the presence of markers (i.e. hopanes), to confirm the petrogenic origin of contaminants, is possible due to the presence of dual detection (FID and MS) [105]. The most powerful technique, for a detailed characterization of mineral oil hydrocarbons, is the comprehensive two dimensional GC (GCxGC) [106][107][108]. It offers an outstanding separation power and a systematic displaying of the components in the plots. Furthermore, the high sensitivity is reached thanks to re-concentration at the end of the first separation column. However, in some cases LC pre separation is required for MOAH quantification. In fact, GCxGC does not enable a complete separation of MOSH and MOAH, therefore naphtenic hydrocarbons (cyclic saturates) are coeluted with highly alkylated aromatic components. In particular, four- and five-ring saturated hydrocarbons, such as sterenes, hopanes, and bicyclic sesquiterpenes, coelute with the highly alkylated two- and three- ring aromatics [109][110]. GCxGC is often used

with a non-polar stationary phase in the first dimension (separation by "volatility") followed by a polar one in the second dimension. A reverse arrangement was more effective for some applications such as MOSH resolution.

8. OLIGOMERS

Oligomers consist in a few monomer units. They usually contain between two and 40 repeating units depending on the chemical composition of the building block. Molecules with weight < 1000 Da are usually considered as potential migrants [111]. Incomplete polymerization and hydrolytic or thermal degradation of polymer chain during the polymerization process are the main sources of production of oligomers. Other ways of oligomer formation are the degradation of polymer by thermal or irradiation energy. These types of degradation are involved during main process of food treatment, such as sterilization of packed food with high temperature, as well as during novel food processing, such as gamma or beta irradiation, microwave, high pressure, UV-light or ozone [112]. Further degradation can happen during the transport or the storage phase by thermo-mechanical process or photo oxidation by sunlight. The resulted oligomer mix can be very complex and it may have different migration properties and toxicological profiles. However, little is known about oligomer gastro-intestinal degradation.
The oligomer patterns are strictly related to the polymerization process and give information about a polymer type. They are often comprised in the non-intentionally added substances (NIAS) group when referring to components present in FCM [113]. No specific rules about oligomers are present in Regulation 10/2011 due to the many different chemical classes, and to their different analytical detectability and response factors.

8.1 Oligomers from polyolefins

Polyolefins such as polyethylene and polypropylene homo polymers and copolymers contain olefins and polyolefins oligomeric saturated hydrocarbons (POSHs), which are a mix of linear and branched alkanes and alkenes (no aromatics). The specific composition of this oligomeric fraction depends not only on the monomer and comonomers used, but also on the type of catalyst and the polymerisation process and conditions.
During analysis of mineral oil contamination in foods, oligomers from polyolefins may interfere with MOSH determination because they are eluted from HPLC in the same retention times, and form humps in the same region of chromatograms. Biedermann et al. [114] analyzed different packaging materials from the market. They present chromatograms with typical elution pattern of POSH, correlating them with the polymer from which they are originated. Furthermore, authors demonstrated the migration of these substances in food packaged in polymers, including powdered infant formula contaminated from heat sealable layers at detection limits of 0.1-1 mg/kg of food. Nonetheless, when the POSH are analyzed in food or in a non-plastic material it is not possible to distinguish them from the MOSH using silica gel column. The unique distinctive feature of POSH from MOSH is the presence of substantial amount of monounsaturated hydrocarbons. The quantification of unsaturated components in mixture with saturates may be achieved by derivatization, such as bromination (petrol chemistry) or addition of iodine (fatty acids). An on-line LC-LC(Ag^+)-GC method, able to separate polyolefin oligomeric hydrocarbons (POH) into POSH and polyolefin monounsaturated

hydrocarbons (POHM), was developed very recently by Lommatzch et al. [115]. The method was performed by a silver-impregnated HPLC column installed after the silica gel column. The fractions eluted were subsequently transferred at GCxGC-FID and GCxGC-MS to obtain additional information about the composition of POH from different polyolefin samples. Furthermore, using a phenyl methyl polysiloxane column in the first dimension and a dimethyl polysiloxane column in the second dimension, Biedermann and Grob were able to produce plots distinguishing MOSHs from POSHs and characterizing the degree of raffination of a mineral oil [116].

9. MINERAL OIL FROM FOOD CONTACT MATERIALS

During the last 20 years data of concern have emerged about migration of mineral oil from food contact materials, in particular recycled paper and paperboard, into food. Mineral oil in recycled paper coming from several routes such as newspaper and other printed paper that enter in the recycled process, adhesive and solvent used as carriers for binders and additives, and waxes which are added to improve water resistance. Offset printing inks, used in the newspaper, are the important source of MOSH < *n*-C_{24} in the recycled paper. Furthermore, thanks to their volatility these hydrocarbons may transfer into the food also by gas phase [103][117]. Already in 1997 Droz and Grob demonstrated that migration of mineral oil from recycled paperboard into dry food exceeded 100 mg/kg of food. Within a survey required by Federal Ministry of Food and Agriculture (BMEL) 120 dry food samples intended for long storage at ambient temperature were analyzed to evaluate the levels of mineral oil. The average of MOSH concentration in 60 products packed in recycled paperboard was 10.9 mg/kg of food and in products packed in virgin fiber was 6.2 mg/kg. MOAH rapresented the 10-20 % of the contamination [69]. With the same purpose in the 2011, the UK Food Standard Agency (FSA) organized a survey on migration of selected ink components from printed packaging materials and screening of printed packaging for the presence of mineral oils. MOSH was detected in all 51 samples with average of 160 mg/kg and 31 mg/kg for MOSH and MOAH respectively[118]. Similar results were reported after an Austrian survey that included 38 food samples mainly packaged in recycled paperboard [64].
Recently, the non-profit organization *Foodwatch* published an evaluation of mineral oil residues in food measured by German official food control authorities, making this topic a public domain. 446 products were tested for mineral oil contamination between 2011 and 2015. Results show that 375 products contained MOSH and 128 products contained MOAH. The contaminated are mostly dry foods packaged in paper or paperboard [119].

9.1 Strategies to prevent the migration of mineral oil from FCM

In order to reduce the levels of mineral oil in the paper and paperboard food packaging, many paper and ink manufacturing industries as well as food industries mobilized to find solutions to the problem. Many strategies have been adopted through the packaging value chain, such as limit the percentage of recycled fibres in the production of packaging intended for food contact, replace the recycled fibres with virgin ones, select high quality paper to recycling process, use of mineral oil free inks and use of functional barriers. The use of functional barriers was suggested also by BMEL in the new draft proposal regulating mineral oil contaminants in paper and board intended as food contact materials [81]. In 2017, the French Agency for Food, Environmental and Occupational Health & Safety

(ANSES) published an opinion on "Migration of mineral oil compounds into food from recycled paper and cardboard packaging." ANSES prepared the opinion upon formal request by the French Directorate General for Competition, Consumer Affairs and Fraud Control (DGCCRF). About the high content of MOH in recycled paper and board used as packaging, ANSES recommends the use of MOAH-free printing inks, glues, additives, and processing aids in the printing sector and the use of the functional barriers to limit migration of MOH from paper and board packaging into food.
Functional barriers are components of the packaging materials used in order to prevent migration of contaminants, which can be dangerous for public health, as may be mineral oils. These components are integrated into the packaging in the form of a film or a foil through which migration takes place at very low levels. Functional barriers have two main functions:

- Extension of the migration lag phase: in the first contact period the mineral oil does not reach the food-oriented side because it is prevented from the barrier and migration does not occur;
- Decrease of the speed of migration after the lag phase: the speed of migration and time to reach balance is lengthened.

Thanks to these two phenomena, the lag phase can have the same length of the shelf life and migration can result at low levels.
Polymer internal bags as well as aluminum foils, with barrier properties, are largely used in food packaging and it are diffused in the market. Nonetheless, few of the materials available on the market allow acceptable protection of food from migration [120].
Several test methods to study barrier properties of packaging materials against mineral oil compounds have been applied. They can divided in three groups: migration experiments, permeation experiments and lag time experiment. Migration experiment allows to evaluate realistic concentration of potential migrants in packaging and in food in a specific time, usually at the end of the shelf life. The permeation experiments investigated how much mineral oil contaminants are permeated through the barrier independently from the packed food. Finally, in the lag time experiment a spiked gas stream of contaminants are used instead of donor materials. The most important information from lag time experiments are the diffusion coefficient and the partition coefficient [121].
Biedermann-Brem performed a test using a sandwich structure as already described in the paragraph 5.3.3.2 . The test was applied on various barrier materials in order to evaluate the selectivity of barrier for different types of contaminants but also for varied molecular mass/volatility. In 2013, a different quick procedure was developed to evaluate the migration of MOH, through the functional barrier coated on a paperboard surface, based on the gravimetric determination of the hexane and /or heptane vapour transmission rate (HVTR). The determination of HVTR was performed in a permeability cup (evaporation chamber) with a sealable closure. The closure had an open surface area, which was sealed with barrier material. A volume of solvent was put into a sponge and the weight of vapours, that pass the surface of the material, were expressed in g/m^2 of the surface area per day[122].
Migration test for functional barrier was also developed by Laine et al.. They used Tenax® or sugar as food simulants put on the bottom of petri dishes. The board samples were cut in circular specimens and were put on the top of the cups. The cups were placed in the desiccator. Five surrogate compounds (*n*-decane, isobutylbenzene, 1-cyclohexylbutane, 1-cyclohexylheptane and 1-cyclohexyldecane) were posed in a small petri dishes into the desiccator and they acted as donor in the migration tests [123].

Recently, an innovative approach to prevent the migration of MOH from recycled paper and paperboard into food, without an additional layer or functional barrier, was proposed by Buscaroli et al. [124]. The authors proposed the use of an organo-functionalized mesoporous silica (provide in form of pellet/monolites or powder) for fast adsorption of MOH from water-based paper production step. After the selection of the organo-modified powder silica MCM-41-Si $(CH_3)_3$ as sorbent (it showed the best combination between adsorption capacity and thermal stability), it was added to waste paper (containing a relevant amount of MOH) for recycled paper production (at laboratory-scale plant) according to the washing-based process. The amount of MOH in the sorbent-enriched pulp/paper sample increased in each production step. A low MOH migration (8%) to the flower from sheet produced during the experiment was evaluated, and it showed a good performance of the method.

Functional adsorbents have been also studied in order to their introduction in the matrix of paper and paperboard to decrease the tendency of mineral oil, presented in the packaging, to migrate into the food[125] [126].

AIM OF THE WORK

The first aim of this work was study migration of mineral oil from cardboard into dry food. To this purpose, a rapid and economic single side migration test was developed and used to evaluate the main parameters affecting migration in order to understand the differences of the mechanism characterizing the accelerated test and the real condition of use. Furthermore, a new barrier material against MOHs migration was tested.

Another scope of this thesis was to feature the hydrocarbon profile of different food contact materials used in real food package, presented on the market, in particular take away pizza boxes and polypropylene containers used in ready to eat products. For each material, tests were carried out at the scope to evaluate the migration entity of the hydrocarbons and to understand the main parameters that affect the transfer of these substances into food.

The last scope of this thesis was to characterize also the hydrocarbon profile of microcrystalline waxes, a group of substances that can be used as additives in food or as components in the structure of food contact materials. In addition a method to evaluate the presence and the level of BaP in these waxes was developed. Finally, a test was performed in order to assess the release of PAHs and MOHs from waxes and confectionery products into human artificial saliva

PHD EXPERIMENTAL WORK

1. DEVELOPMENT OF A RAPID METHOD TO EVALUATE MOSH AND MOAH MIGRATION FROM CARDBOARD AND ITS APPLICATION TO TEST THE BARRIER PERFORMANCE OF SEQUENTIAL POLYMER TREATMENT

A part of this chapter has already been published in: Paul, U.C., D. Fragouli, I.S. Bayer, E. Mele, C. Conchione, R. Cingolani, S. Moret, and A. Athanassiou. 2017. "Mineral Oil Barrier Sequential Polymer Treatment for Recycled Paper Products in Food Packaging." *Materials Research Express* 4 (1).

1.1. Introduction

Recycled paper and paperboard are mainly composed of a network of cellulosic units (fibrils and fibers) arranged in multiple levels, obtained from different waste of sources such as newspaper, magazines catalogs, packing papers. In food industry, recycled paper is typically used to produce boxes for packaging dry food and for transport and storage. The use of recycled instead of virgin paper has environmental and economical benefits, such as sustainability, control of deforestation reduction of energy and water consumption and decreases packaging costs [127]. Despite this positive aspects, a significant drawback of recycled paperboard is the amount of non-desirable contaminants present in it [61], [128]. Among these, the amount of mineral oil hydrocarbons is currently regarded as one of the most important limitations of using this type of paper in food packaging. In fact, different studies have demonstrated that mineral oil hydrocarbons (MOHs) can migrate from the package to the dry foodstuff and contaminate it, with negative effect for human health [64]. This, in combination with the highly hygroscopic properties of the paperboard [129], which result in the deterioration of its mechanical and physical properties, makes the use of this material for food packaging applications quite restrictive. On 2011, Vollmer et al. [69], reported high concentration of MOHs in foods that are packaged in recycled paper boxes with inner bags made of paper, polyethylene (PE) or polypropylene (PP). On the other hand bags of aluminum, cellulose propionate (CP), polyethylene terephthalate (PET) or acrylate coated polypropylene (PPacr) provide a good barrier against MOSH migration. An efficient and environmental sustainable internal bag barrier should be characterized by reduced MOH migration rate with breakthrough period longer than the shelf life of the food, it should be environmental friendly, of low cost, biodegradable and recyclable. Metal foils, like aluminum, offer a complete barrier but pose limits in terms of biodegradability, recyclability and costs [46], [69]. It is important to note that also food packaging, made of virgin paperboard, can be affected by the migration of MOHs from secondary packaging materials[128], [130][131] In order to avoid the internal bags, several studies have been focused on the development of coatings for paperboard with barrier properties against mineral oil. For example, starch-based coatings were developed on paperboards which significantly reduce the migration of MOSHs by a factor of 5–20, while the treated paper remains water absorbing [132]. A hydrophilic or water absorbing packaging system is undesirable since when exposed to humid environments, the contained food can be easily spoiled due to the penetrated moisture. A waterproof cardboard with mineral oil barrier properties is an ideal

material for both primary and secondary packaging applications [120][133], since it can be used to store food even in high humidity environments, and can also be used for transport of fresh fruits and vegetables. To increase the hydrophobicity, the combination of starch-based coatings with latex is effective, but it results in the deterioration of the mineral oil barrier properties [134]. Other studies deal with the introduction of active carbon adsorbents in the paperboard in order to reduce effectively the migration of mineral oil hydrocarbons and to prevent the release of other contaminants into the food. However, such adsorbents risk to be eventually saturated by the contaminants, compromising their performance[120][125]. Some pure polymer based approaches have also been used recently in order to reduce the migration of different contaminants including MOHs from paperboard to the food, such as Ecovio® PS 1606 (polylactic acid and polyester from 1,4-butanediol, adipic and terephthalic acid) and Epotal® A 816 (acrylate copolymer) coatings developed by BASF [12][13]. Trademarked coated paperboards (selectively recycled) also appeared in the market recently, like Foodboard® developed by Mayr-Melnhof Karton, and Unifood® by Weig Karton that effectively reduce the migration of mineral oils and other contaminants towards the food.
The aim of the present work was to develop a rapid migration test, using simple and cheap commercially available glassware, to assess the migration of mineral oil from cardboard. The developed test was subsequently used to evaluate the performance of proper functionalization of the recycled paperboard with polymer treatments against the mineral oil migration from recycled paper in food.

1.2. Materials and methods

1.2.1 Reagents and standards

Acetone, ethanol, acetonitrile, pentane of HPLC grade were purchase from Sigma Aldrich (Milan), dichloromethane, *n*-hexane (Sigma Aldrich, Milan) were distilled before used. The water was purified with a Mill-Q System (Millipore, Bedford, MA, USA). The C_{10}-C_{40} (all even) standard mixture of *n*-alkane was from Fluka Analytica, Sigma Aldrich (Switzerland). The working standard solution used to check the LC-GC performance, the efficient MOSH and MOAH separation and to quantify the contamination of sample, as described by Biedermann and Grob (2012) [137], was prepared by mixing: 5-α-cholestane (Cho, 0.6 mg/mL), n-C11 (0.3 mg/mL), n-C13 (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.
For migration tests, simulant Tenax® (MPPO, 60-80 mesh) was purchased from Supelco Analytical (Pennsylvania). The simulant was introduced in an Erlenmeyer flask and washed in an ultrasonic bath with redistilled acetone and then with *n*-hexane until obtaining a clean blank. At the end, the Tenax® was dried using a vacuum pump and heated at 160°C overnight. The blank test of the simulant was carried out periodically before starting migration tests.
Glassware and other materials were rinsed with distilled acetone and *n*-hexane just before use.

1.2.2 Instrumentation and chromatography conditions

The microwave extractor (MARS 5MD7797) was from CEM (Bergamo, Italy) and used Teflon-lined vessels (GreenChem). To to concentrate the sample extract before the injection, a centrifuge (UNIVAPO 100) connected with a vacuum pump (Buchi Vacuum Pump V-700) was used.

1.2.2.1 GC-FID system

GC-FID analysis was carried out using a GC 7890A system (Agilent Technologies), with a 7693 autosampler and an on-column inlet. 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 separation column of 0.15 µm of film thickness, 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. H2, 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.

1.2.2.2 LC-GC-FID system

LC-GC-FID analysis was performed with an on line coupled LC-GC9000 system (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 transfer of the fraction of interest was carried out through the Y-interface using the retention gap technique [92]. The MOSH fraction was eluted from 2.0 to 3.5 min and transferred to the GC operating at a hydrogen constant pressure mode. The fraction comprising the MOAH ranged from 4.0 to 5.5 min. The MOSH and the MOAH fractions were eluted (at 300 µL/min) using a gradient, starting with 100 % *n*-hexane (0.1 min) and reaching 30 % of dichloromethane in 0.5 min. At the end of the LC-GC transfer, 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 velocity of 4 mL/min. The oven temperature was programmed at 40 °C/min from 50 °C to 350 °C. 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).

1.2.3 Samples

Unprinted recycled paperboard and printed virgin paperboard used to optimize the migration test were kindly furnished by the end-user. The offset printing ink used to print the virgin paperboard contained 35% of a mineral oil based solvent.
The study on the barrier sequential polymer treatment involved unprinted recycled paperboard (weight density 350 g/m^2) provided by Reno de Medici Spa (R_DM®, Italy) treated with poly methyl methacrylate (PMMA) and cyclic olefin copolymer (COC) Topas® at Smart Material, Istituto Italiano di Tecnologia. Paperboard samples are better described in table 6.

Table 6. *Paperboard samples*

Paperboard without barrier			
RN	Unprinted recycled paperboard		
VS	Printed virgin paperboard		
Paperboard with barrier			
	PMMA	COC	
Untreated	-	-	
P5	5% (w/v) solution	-	Dip coating
T20	-	20% (w/v) solution	Rod coating both faces 1 pass
P5/T20	5% (w/v) solution	20% (w/v) solution	Treatment P5 + T20

1.2.4 Migration tests

Migration tests were carried out in glass cells (weighing bottles, low form with ground-in stopper in borosilicate glass) of 4.6 cm of i.d. and 3 cm of height (figure 6). The recycled paperboard to test was cut in circles of the same diameter of the glass cell, placed at the bottom of the migration cell and covered with 0.66g of Tenax® (poly(2,6-diphenyl-p-phenyllene oxide) (4g per dm^2), previously washed as reported in paragraph 1.2.1. The glass weighing bottles were closed with the cap and sailed with aluminum foil and parafilm stripes and put in the oven 10 days at 40°C. Different time and temperature conditions were also tested.-

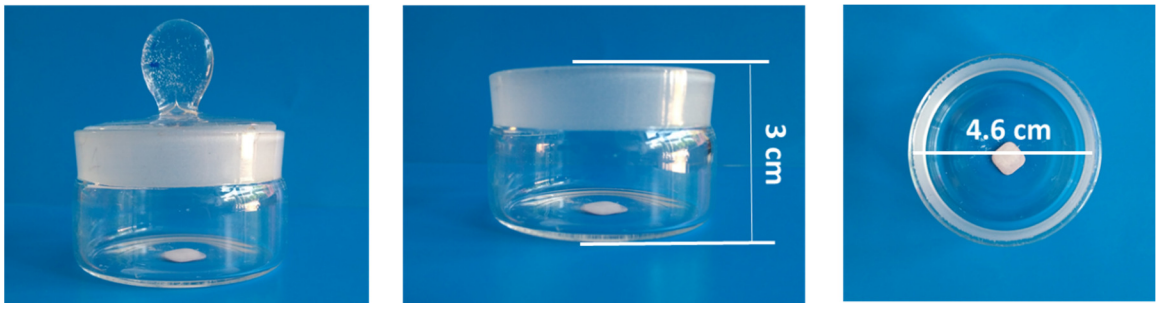

Figure 6*. Glass weighing bottled used for migration tests.*

After the contact, the glass weighing bottles were transferred in the freezer at -20°C for 15 min. At the end, cardboard and simulant were separated and stored in aluminum foils in the freezer until the extraction.
All of the tests were performed in duplicate

1.2.5. Extraction, purification and separation of MOSH and MOAH fraction

1.2.5.1 Cardboard extraction
All paperboards were extracted following the method described by Lorenzini et co-workers [103]. Briefly, 1 g of cardboard was cut in small pieces about 0.5 x 0.5 cm. The pieces were put into the 20 mL vials with 10 mL of hexane/ethanol solution 1:1 (*v/v*) and 20 µL of internal standard (Cho, CyCy, n-C_{11}, *n*-C_{13}, 5B, 1-MN, 2MN, TBB, Per). The extraction was lasted 120 min, of which, the first and the last 5 minutes, under magnetic agitation. After that 5mL of *n*-hexane were transferred in a vial and added with 10 mL of millQ water to separate the ethanol from the hexane. Finally, an aliquot of extract was directly used for the analysis or concentrated before the analysis.

1.2.5.2. Tenax Extraction
The simulant Tenax ® used in the migration tests was quantitatively recovered and introduced in the tubes of the microwave extractor with 20 mL of *n*-hexane/acetone 1:1 (*v/v*) and 20 uL of the standard mixture. The sealed tubes were underwent microwave-assisted extraction for 20 min at 120°C. After extraction, the sample was left to cool down before opening the vessel. After cooling, 20 mL of milliQ water was added into the vessel and part of the extract was transferred into an autosampler vialfor the injection.

1.2.5.3 MOSH and MOAH fractionation

First migration tests on paperboards without barrier were carried out using the off-line SPE-GC-FID approach proposed by [90]. MOSH and MOAH were fractionated on a silvered silica gel cartridge (Ag SPE) prepared in the laboratory. Silica gel previously activated (400 °C overnight) was added with a silver nitrate solution (0.75 g/mL) in Milli-Q water. The silica was blended for about 30 min and then 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 discarded, 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 discarded, and the MOAH fraction was eluted with further 7 mL of *n*-hexane/dichloromethane (50:50 *v/v*). The eluted fractions were concentrated to 1 mL, prior to GC-FID analysis.
Analyses to verify the barrier properties of the treated paperboards were performed using an on-line LC-GC approach. After extraction, the solution was eventually concentrated and directly injected in the system.
As reported elsewhere off-line and on-line methods gave comparable results.
The MOSH area was determined by the integration of the total hump of largely unresolved peaks and by subtraction of internal standards, while for the MOAH all the peaks on the top of the hump were

subtracted from the whole area. The position of the baseline was determined by repeated solvent injections. Quantification was performed using internal standards.

1.3. Results and discussion

1.3.1. Migration tests

The first part of the work concerned the development of a protocol for migration testing using the single side method of contact, where only one side of the packaging material comes in contact with the simulant. As reported in UNI EN 14338:2004, single side migration test on cardboard should be conducted in a specific steel migration cell, hermetically sealed, or in Petri dishes where sealing can be difficult to obtain. Due to the high cost and difficulty to find still cell, an alternative migration tests was developed It makes use of glass weighing bottles which are easily available in different size and wich can be closed with cap.

1.3.1.1 Tenax®Extraction

At the end of migration test, the glass weighing bottles were transferred in the freezer at -20°C for 15 min to help absorption of vapors eventually present in the head space of the cell which might be lost during opening of the system. The next step was the extraction of the MOHs from the simulant. The UNI EN 14338:2004 method suggests to perform three extractions using increasing volume of diethyl ether. Alternative method used extraction with ethanol or methanol under magnetic stirring, or ultrasonic extraction with ethanol [25] The proposed alternative extraction method involved microwave assisted extraction (MAE) with an *n*-hexane/acetone 1:1 (*v/v*) mixture. The use of high temperature (in our case 120°C) and high pressure allows to obtain quantitative yield in a very short time (20 min). Furthermore, in the microwave extractor up to fourteen samples can be processed at the same time with reduced solvent consumption (20 mL per sample) and low costs. Figure 7 shows an overlay of the MOSH chromatograms of two consecutive extractions on the same sample. The amount of MOSH in the second extract was less than 2% of the total amount demonstrating as a single extraction is able to obtain practically quantitative yield.

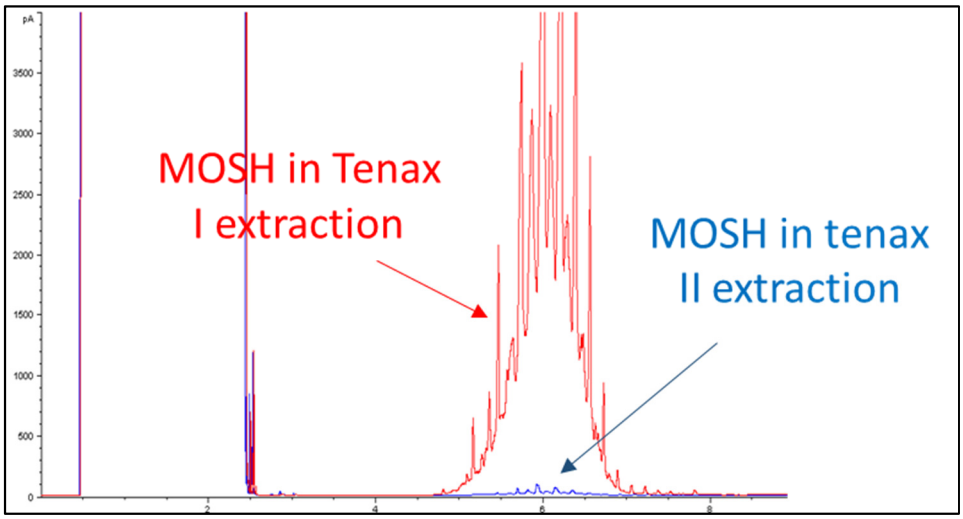

Figure 7. *Overlay of two consecutive extractions of the Tenax after the migration test.*

1.3.1.2 Repeatability of migration test and verification of cell closure.

Migration tests were always carried out in duplicate for each sample and the two replicates showed results in good agreement. In figure 8 MOSH chromatograms of four replicates of a cardboard (RN) put in contact with Tenax® for 3h at 70°C are reported. The complete overlapping of the chromatograms indicates the high repeatability of the migration test (coefficient of variation, CV, 2.44 %).

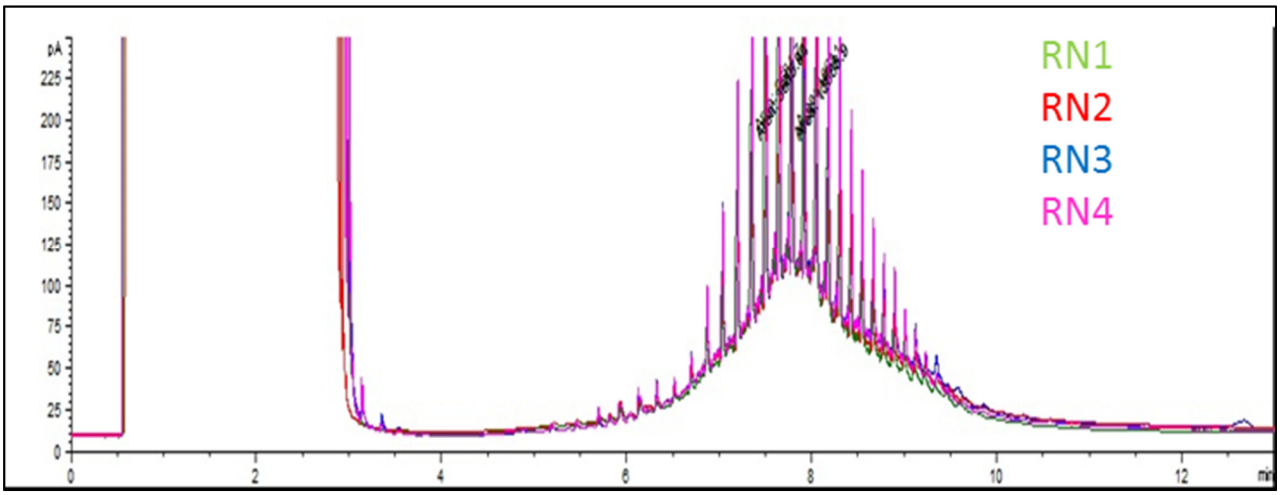

***Figure 8**. Overlapping of four replicates of RN after migration test*

Since a mass balance occurs in a closed system (such as the cell used for migration testing), the possibility of calculating the amount of contaminants migrated both with direct and indirect method was verified. The "direct method" measured the contamination found in the simulant, while the "indirect method" measured the contamination lost by the paperboard during contact with the simulant (it was calculated subtracting the contamination content of the paperboard after the contact to that of the same material before the exposure). The direct and indirect migration data were in good agreement confirming the accuracy of the direct measurement, and demonstrating as indirect migration which has the advantage to avoid the analysis of food simulant or food (which is more demanding) can be used for more rapid migration testing . In table 7 the results of migration tests performed using two different kinds of cardboard and two different contact conditions are reported as an example.

***Table 7**. Direct and indirect migration data in RN and VS cardboard at difference time and temperature condition.*

	% MOSH			
	70°C 3h		40°C 10 days	
	Lost by cardboard (indirect migration)	Migrate in Tenax (direct migration)	Lost by cardboard (indirect migration)	Migrate in Tenax (direct migration)
RN1	56.9	46.1	79.8	72.4
RN2	58.4	37.7	79.5	87.8
Average	**57.6**	**41.91**	**80.0**	**79.64**
VS1	87.4	88.6	96.1	94.3
VS2	89.4	99.4	95.9	81.9
Average	**88.0**	**94.43**	**96.5**	**88.14**

The perfect closure of the cell, proved by the good mass balance found between hydrocarbon lost by the paperboard and hydrocarbon found in the simulant, can be easily demonstrated also by overlapping the chromatograms of paperboard before and after the contact, with the chromatogram of the respective Tenax®. Figure 9 shows an example of the chromatograms of Tenax® and cardboard involved in the migration test. Graphically, the area of the contamination in the Tenax® (blue), which measures direct migration, corresponds to the area of the contamination lost by the paperboard after the contact with the simulant, which corresponds to the difference of pre-contact (red) and post-contact (green) areas.

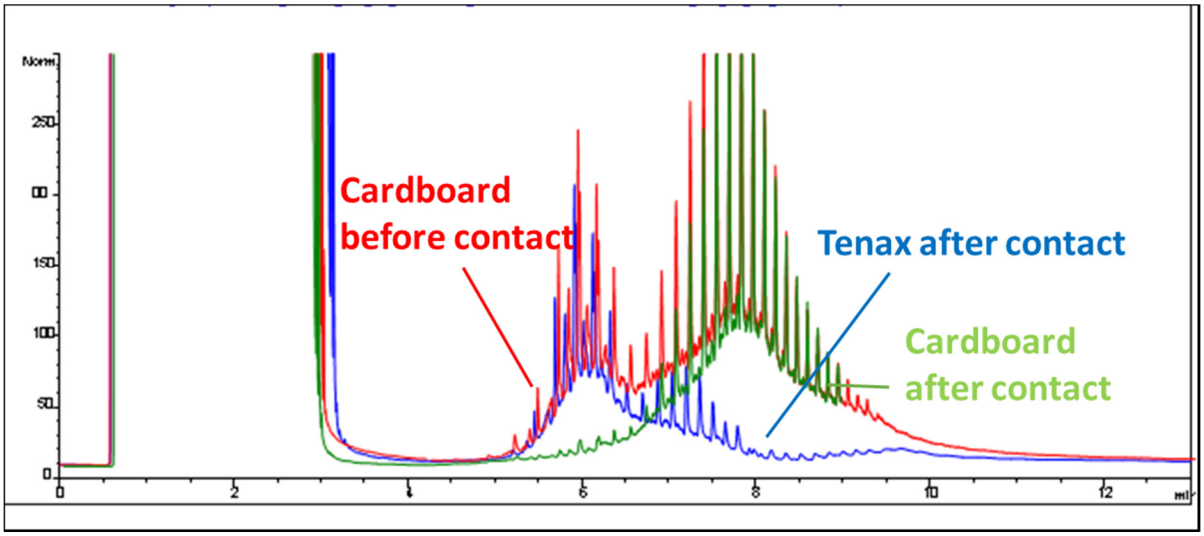

***Figure 9**. Chromatograms of migration test performed on a recycled paperboard without barrier.*

1.3.2. Migration tests to study the barrier effect

The cardboard involved in this study was an unprinted paperboard (weight density 350 g/m^2) formed by different layers, bonded together by starch. The outermost layers were of recycled paper coming from cellulose paper: the external layer was coated with calcium carbonate to make the final product white and printable. The intermediate layers were of recycled paper collected from urban waste, including newspapers and magazines, and the food contact layer was made of recycled paper coming from selected cellulose paper. The cardboard was modified in order to reduce drastically the migration

of mineral oil from recycled cardboard and to render it waterproof and grease resistant making it an ideal material for food contact.
The first poly (methyl methacrylate) treatment, applied by dip-coating, penetrated the paper network and created a protective layer around each fiber, permitting thus the transformation of the paperboard to a hydrophobic material throughout its thickness. Subsequently, the second layer, cyclic olefin copolymer (COC), was deposited on the cardboard in the layer of 6.4 µm of thickness filling the open pores of the surface. The COC is a biocompatible cyclic olefin amorphous copolymer , derived from polymerization of ethylene and norbornene. It is transparent, water-repellent, heat resistant, and has high moisture, gas and aroma barrier properties[138]. The modified cardboard was subjected to some analysis to evaluate its properties. Morphological analysis was carried out using scanning electron microscopy (SIM) to observe the surface and cross section of material before and after treatments, furthermore wettability measurements and grease resistance test were applied. The mechanical properties of material were also evaluated as well as its biodegradability.
In order to evaluate the effect of the PMMA and COC treatments on the MOSH and MOAH migration from the recycled paperboards towards food, mineral oil migration tests were carried out using Tenax as a dry food simulant and analysis was performed by on-line LC-GC-FID. Concerning the choice of conditions for migration tests, EU Directive 82/711EEC for plastic materials, provided that migration at ambient temperature of unlimited durations could be simulated during 10 d at 40 °C. EU Regulation 10/2011 [16] revised this rule for specific migration testing and provided that acceleration should be achieved to cover the real situation by temperature increase estimated on the basis of the Arrhenius equation (using a worst case activation energy). Nevertheless, a maximum of 10 d at 60 °C, was established, which corresponds to an acceleration by a factor of about 30 (when compared to the regulatory RT of 25 °C). According to Zurfluh et al. [43], conventional migration testing with Tenax® at 40 °C and 60 °C hardly reflects real migration in food, often resulting in an overestimation of the maximum migration by several times (especially at 60 °C). The higher the temperature, the higher is the risk to overestimate migration. At high temperature (60 °C) barrier properties can be lost, migration can occur beyond $C_{24,}$ as observed at ambient temperature. Regulation 10/2011 also indicates that for storage at RT, testing time can be reduced to 10 d at 40 °C, if there is scientific evidence that migration has reached the equilibrium under these test conditions. Taking into account what above reported, and after verifying that equilibrium was reached during 10 d at 40 °C, it was chosen to test our specimens under this condition.

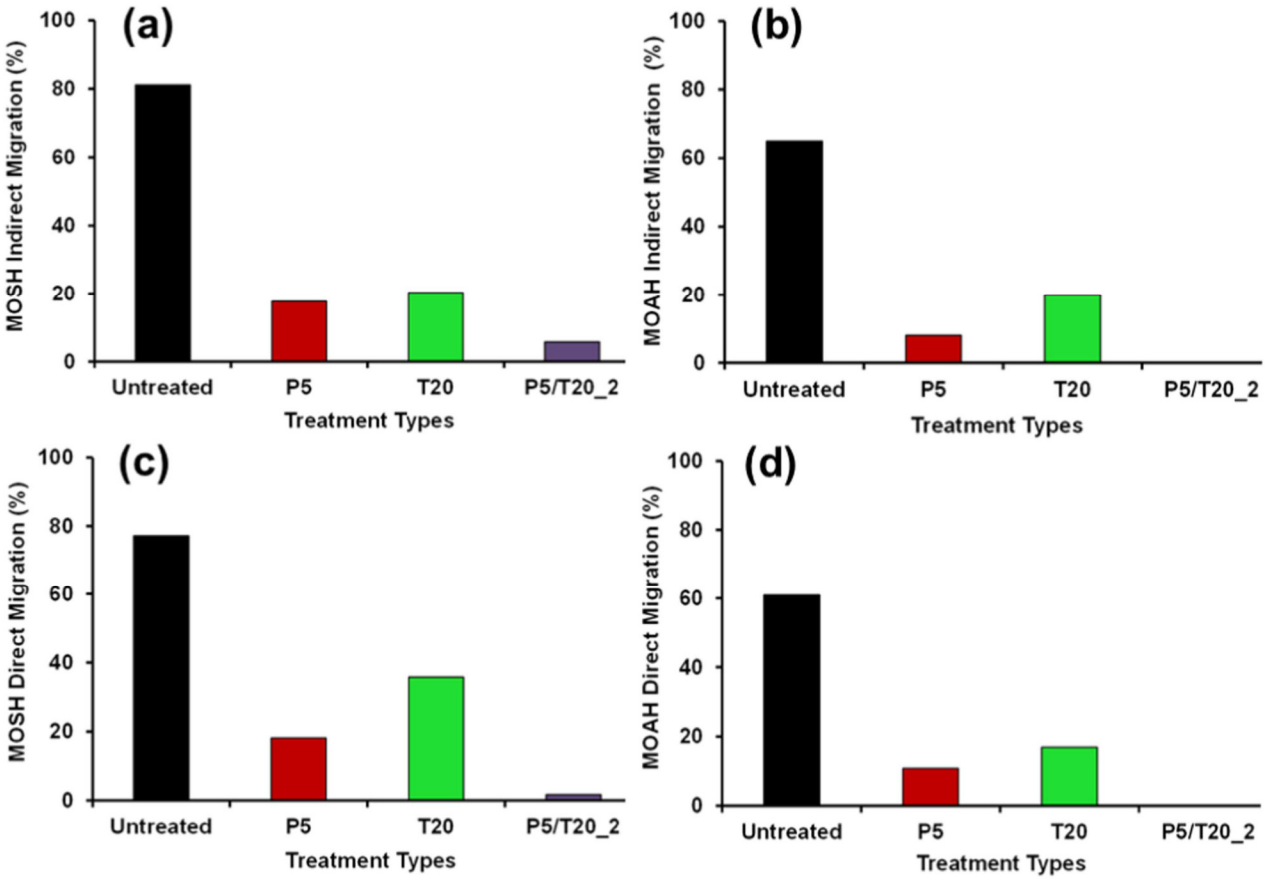

***Figure 10**. Indirect/overall migration of (a) MOSH and (B) MOAH from the different types of recycled paperboards (untreated and treated), and direct migration analysis of (c) MOSH and (d) MOAH from the recycled paperboards (untreated and treated) to Tenax.*

Figure 10 reports both 'direct migration' evaluated by measuring the MOH migrated into the Tenax®, and 'indirect migration' calculated by determining the MOHs lost by the paperboard during the contact with the simulant. All data are expressed as percentage of the amount in the paperboard (before the contact with the simulant).

As shown in the histograms of figures 10 (a) and (b), 'indirect migration' data, although prone to higher uncertainty especially when difference between paperboard before and after the contact are low (they derive from a difference between two measurements), were in good agreement with those obtained by 'direct migration', figures 10 (c) and (d). According to direct migration analysis, 77% of the MOSH and 61% of the MOAH migrated from the untreated sample. The application of the polymer treatments effectively decreased the MOHs migration, imposing a barrier of 82% for MOSH and 89% for MOAH when the paperboard was treated with PMMA (P5), and of 65% and 83% for MOSH and MOAH respectively when COC was used (T20). Remarkably, when both polymers were combined (sample P5/T20_2), the barrier efficiencies reached 98% for MOSH (migration 4.0 mg/Kg) and 100% for MOAH (migration non detectable). As evidenced, still 4 mg/kg of total MOSH (C_{10}-C_{35}) present in the recycled paperboard (177 mg/kg) migrated into the Tenax® in the test conditions. Of these 4mg/kg of total MOSH (C_{10}-C_{35}), about 1 mg/kg were in the range C_{20}–C_{35}. The remaining MOSH were in the range C_{10}–C_{16} (1 mg/Kg) and C_{16}–C_{20} (2 mg/kg), for which the BfR suggested a SML of 12 and 4 mg /kg of food, respectively. So, it can be stated that the P5/T20_2 treatments can prevent MOAH migration and reduce MOSH migration at levels below those recommended by BfR and proposed by the BMEL [81]. Since Tenax® is a far stronger adsorbent than food; migration in real food can be significantly lower. All treated samples were analyzed after a 2.5-month storage at

ambient temperature. We cannot exclude that some migration might occur during storage of the treated paperboard (before being used).

In all cases described, the PMMA treated paperboard provides a higher overall barrier for MOHs in comparison to the COC treatment. This is probably due to the fact that PMMA forms a more effective layer thanks to its capacity to penetrate inside the paper network and to coat each individual recycled cellulose fiber but also due to the higher polarity of PMMA, which can reduce the migration of non-polar mineral oil hydrocarbons enhancing the barrier properties [132][121]. Nevertheless, a single polymeric treatment was inadequate to completely stop the migration of MOHs. The superficial layer of COC on the P5 samples is necessary for the optimal performance. This layer should be thick enough so as to inhibit the arrival of the mineral oils to the food simulant. In fact, when a single layer of COC is applied, the thickness of the superficial film reaches 16.8 ± 6.2 µm and the migration barrier is good but not optimal like in the case of the 2 COC layers where the thickness of the film is 34.8 ± 13.9 µm. Figure 11 reports, for both treated (P5/T20_2) and untreated sample, the overlay of MOSH and MOAH LC-GC traces of cardboard before and after contact (used to calculate indirect migration) and of the Tenax® (used to calculate direct migration) at the end of migration test. Is evident how, using untreated material, a part of MOSH and MOAH initially present in the cardboard before contact (purple trace), left the cardboard to migrate in the simulant (blue trace). In fact, the chromatogram of Tenax® presents a hump at the same retention time. On the other hand, after migration test performed using P5/T20_2 cardboard, chromatogram of Tenax® had a flat base line, and the LC GC traces of cardboard, before and after contact, are completely overlapped demonstrating that no MOHs migration occurred from the packaging material into the simulant.

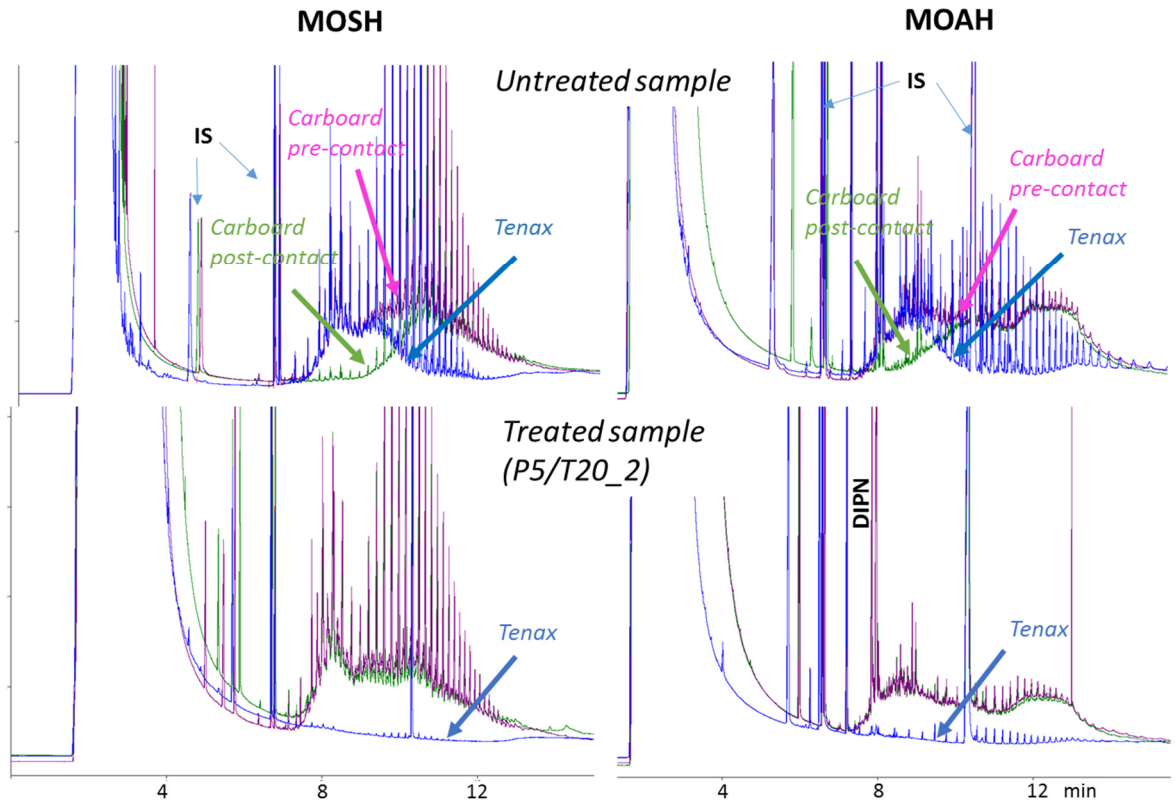

Figure 11. *Overlay of chromatograms resulting at the end of migration tests with untreated (on the top) and P5/T20_2 treated (on the bottom) paperboard. Cardboard before contact (purple),cardboard after contact (green) and Tenax® (blue)*

As described above, the PMMA treatment does not form an overall superficial film, but rather covers each fiber of the paperboard throughout its volume. In this way, the MOHs migration is sufficiently reduced and the paper adopts water resistance properties not only on the surface but also in its internal layers. The additional COC treatment increases the MOHs barrier properties even further since it decreases the porosity of the recycled paperboard's surface.

1.4. Conclusions

A rapid test to evaluate the migration from cardboard using Tenax® as simulant was developed. The method uses commercially available glass cell and low amount of simulant. A rapid and efficient method using microwave assisted extraction, exploiting the higher extraction power of a solvent taken at high temperature and high pressure, was used to obtain a quantitative extraction of mineral oil, with minimal solvent volumes, in short time (20 min). Furthermore until fourteen samples can be processed at the same time in the microwave apparatus

In the second part of the work, the testing method previously optimized was used to test the efficiency of a new sequential polymer treatment of paperboard to develop a barrier against the migration of mineral oil.

The deposition of PMMA and COC treatment does not form an overall superficial film, but rather covers each individual fiber of the paperboard throughout its volume. In this way, MOHs migration is sufficiently reduced at levels below those proposed by the BMEL. The present novel barrier treatment opens up new avenues toward the use of recycled paperboard in food industry for packaging application.

2. A STUDY OF PARAMETERS AFFECTING MIGRATION OF MINERAL OIL FROM CARDBOARD TO DRY FOODS.

2.1. Introduction

Packaging is essential to ensure protection, preservation, and to contain the food through the supply chain. Paper and paperboard are widely used as food packaging also in direct contact with food. In the last years the use of recycled paper for food contact has raised an increased concern due to the presence of different substances that can migrate into food [64]. Recycled cardboard contains mineral oil (MOH) from different routes such as newspaper and other printed paper entering the recycling process, adhesives, solvents, waxes and additives used in the production process as well as printing inks and sealants used in the final product [117].

Migration is a mass transfer phenomenon of chemical compounds from packaging materials into the food. Several factors can influence the transfer of chemicals into food, such as characteristics of the packaging materials, type of contact, chemical nature and initial amount of the migrants as well as the type of food in contact.

Migration from paper and paperboard packaging can occur: from the matrix to surrounding environment, through the different layers of the matrix and from the fiber matrix into food, both by direct contact and by indirect contact, via gas phase.

The structure of cellulosic materials has a great influence on migration of substances. The paper and paperboard material consist in an open and porous structure of fiber and air pores and, for this reason, depending on the raw fiber utilized in the pulp formation, the migration might be different in the different areas of the material [137]. The mobility of mineral oil and other contaminants through the paper comprises a combination of adsorption and desorption mechanism on the fiber, transfer mechanism across the fibers and diffusion of migrants in the air pores [20]. Subsequently, migrants can be transferred, into food both by the gas phase and by diffusion according to their volatility. The partition coefficient between paper and air is another essential parameter in the migration process, it depends on the boiling point of the migrant, on its chemical nature and on the type of paper [23]. Typically, cellulose fibers have an overall negative charge due to the carboxyl group from the carbohydrates and the hydroxyl group of the lignin. Thus, retention from the fiber of more polar substances is higher than retention of non polar substances which tend to be repelled by the fibers [36]. Some characteristic, especially content of fat, humidity, and pH influence the extraction capacity of food and are directly related to migration. For example, higher amount of fat increase the migration of non-polar compounds which have an high affinity for the lipid phase [21].

MOSH and MOAH, present in paper and paperboard material, cover a wide range of molecular weight and volatility. The main transfer of these substances occurs by evaporation from the paperboard and recondensation on the food surface. This type of migration involves only the volatile part of the contamination. Nonetheless, in some dry foods (such as powder), wetting contact is relevant and significant transfer of hydrocarbons of a molecular mass over *n*-C_{24} has been found [114]. Considering products stored for a long time, such as many dry foods contained in the recycled paper or paperboard, migration tests with Tenax® as simulant was suggested. The packaging material shall be placed in contact with the simulant using condition of time and temperature that reproduce the worst-case scenario (overestimation) of real condition of use in order to protect the consumer, but excessive overestimation can lead to unjustly penalize the FCM. The D.M. 21/03/1973 indicates to

perform accelerated migration test at maximum of 10 gg at 40°C [5]. The Regulation 10/2011 suggest that acceleration of migration obtained with tests should be achieved increasing temperature, which are estimated on the basis of Arrhenius equation using a worst case activation energy of 80 kJ/mol. For plastic materials it sets a maximum of 10gg at 60°C [16]. For products stored for a long period of time, the acceleration used in the migration tests allows to obtain data of migration in a short period. However, some data on food analysis showed a number of cases for which simulation underestimated migration in food [139]. On the other hand the migration behavior of chemicals from paper in Tenax® have been compared to the migration into real dry food such as salt, sugar, rice, flour etc. Contrasting results were obtained, some studies showed an overestimation of migration using Tenax® [39][34][32], but in other cases, in particular at high temperature, results obtained with Tenax® were lower than results in real food [20]. In 2013, Zurfluh and co-workers questioned the suitability of simulation to assess the real migration. The authors compared results obtained from the migration in real food packaged in paper and stored during 9 months and results obtained using migration test with Tenax® at 40°C and 60°C. The simulant overestimated the migration of saturated hydrocarbons in the real pack by 46 and 73 % respectively [43].
The aim of this work was to study the migration of mineral oil hydrocarbons from recycled cardboard into dry food (pasta) using accelerated migration test in the glass cell at 40°C for 10 days. The study was conducted to evaluate the differences in the behavior of semolina and egg pasta and to analyze the effect of different parameters on migration. Type of contact cardboard /food, head space volume, particle size of pasta, different type of cardboard, humidity of cardboard were the parameter taken into account and investigated. Furthermore, the work was carried out to understand how the accelerated migration tests surface-to-mass ratio differ from migration carried out using surface-to-mass ratio in actual contact conditions. Finally the migration results were compared with migration data obtained from tests using Tenax® as simulant.

2.2. Materials and methods

2.2.1 Reagents and standards

Acetone, ethanol, *n*-hexane, dichloromethane of HPLC grade were purchase from Sigma Aldrich (Milan). The water was purified with a Mill-Q System (Millipore, Bedford, MA, USA). The C_{10}-C_{40} (all even) standard mixture of *n*-alkane was from Fluka Analytica, Sigma Aldrich (Switzerland). The working standard solution used to quantify the contamination of sample, to verify the LC-GC performance and to check the MOSH and MOAH separation as described in [137], was prepared by mixing: 5-α-cholestane (Cho, 0.6 mg/mL), n-C11 (0.3 mg/mL), n-C13 (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. Simulant Tenax® (MPPO, 60-80 mesh) was purchased from Supelco Analytical (Pennsylvania). Before the first use the simulant was introduced in an Erlenmeyer flask and washed in an ultrasonic bath with redistilled acetone and then with *n*-hexane until obtaining a clean blank. At the end, the Tenax® was dried using a vacuum pump and heated at 160°C overnight. The blank test of the simulant was carried out periodically before starting migration tests. Glassware and other materials were rinsed with distilled acetone and *n*-hexane just before use.

2.2.2 Samples

Pasta and cardboard used in migration tests were provided directly by producers. Rice-shaped small size dry semolina and egg pasta (risoni) (1.5% and 4% fat, respectively) were used. A part of semolina and egg pasta was ground with mill (A11 basic analytical mill, IKA) to reduce particle size of pasta and to obtain a powder. In order to remove fat about 50g of egg pasta was put in the SpeedExtractor E-916 (Büchi, Flawil, Switzerland) equipped with six 10-mL stainless steel extraction cells and washed using *n*-hexane at 180°C for 2h. Different kind of recycled paperboard were involved in migration tests. C1 was printed and had a grammage of 330 g/m^2, while C2 was not printed and had a grammage of 480 g/m^2. Cardboard and pasta samples were previously extracted and analysed to quantify the initial mineral oil contamination.

2.2.3 Migration conditions

A rapid migration test developed in a previous part of the study was used. The migration cells consisted of a glass weighing bottles with an internal diameter of 46 mm and a height of 30 mm. The internal volume was 37.7 cm^3. Circle-cut paper specimens with surface area of about 0.166 dm^2 were used in the tests.
Cardboard and pasta were put in glass weighing bottles with different configurations providing the combination of 4 parameters: amount of pasta in contact with the paperboard speciment, head space volume in the glass cell, type of contact (direct or indirect), and pasta particle size. Table 8 illustrates the organization of the migration tests.

***Table 8**. Resume of different parameter tested*

Studied parameter	**Tested conditions**				
Weight-to-surface ratio (g/dm^2)	4	12	24	48	96
Head space volume	16.6 cm^3 V1	37.4 cm^3 V2	45.7 cm^3 V3		
Contact type	Direct contact	Gas-phase contact			
Pasta size	Ground pasta	Whole pasta			
Cardboard type and initial contamination level	Level 1	Leve1 2			
Cardboard humidity	Dry cardboard	Wet cardboard			

To study the weight-to-surface effect five different amounts of pasta (0.66, 2, 4, 8 and 16 g) were put in contact with the paperboard specimen (0.166 dm^2) to obtain the weight-to-surface ratio reported in the table.
The effect of different head space volumes: 16.6 cm^3 (V1), 37.4 cm^3 (V2) and 45.7 cm^3 (V3) were studied. Steel disks (with 35 mm diameter and 1 mm thickness) were used to modify the internal head space volume of the glass cell.
In “direct contact” (“touching contact”) tests, the pasta was put over the cardboard (placed at the

bottom of the bottle), while, in "indirect contact" (via gas-phase), the cardboard specimen was put at top of the bottles.
Furthermore, two different kinds of recycled paper and pasta samples (one with a pre-existent contamination) were also tested. To increase the cardboard humidity two circle-cut paper tester were introduced in a glass chamber previously saturated with vapour (from a gauze impregnated with water), and left to equilibrate overnight. After weighting, to measure the humidity increase, the paperboard specimens were immediately used for migration test. Migration tests were carried out to 40°C for 10 days. The glass cells were closed with aluminum foil and plastic film (Parafilm®) to avoid any escape. By gently shaking, the pasta was evenly distributed over the surface to be tested. The cells were placed into an oven at 40 °C The temperature was controlled by using a thermostat (Incucenter IC240, SalvisLab). Each test was performed in twice or more replicates.

2.2.4 Extraction of mineral oil from samples

At the end of migration tests the pasta was completely recovered and whole pasta was grinded. 2g of semolina pasta was extracted with 10mL of *n*-hexane during overnight at RT, since this simple procedure allows to obtain comparable results with respect to microwave assisted saponification. The resulted extract, previously centrifuged, was directly injected into the LC-GC-FID system. Egg pasta was extracted by microwave assisted saponification (120°C for 20 min) according to [96]. Two grams of egg pasta were placed in microwave vessel with 10mL of saturated potassium hydroxide solution in methanol and 10mL of *n*-hexane. After microwave extraction, 20mL of water was added to sample and vessel stayed in a freezer for 20 min to improve the separation phase. After that, the *n*-hexane surnatant was taken, concentrated and injected into the LG-GC-FID system. Mineral oil was extracted from cardboard as described in [103] using a mixture of *n*-hexane and ethanol (1:1 *v/v*) with liquid partition for 2 hours. Then the ethanol was removed by addition of water and the hexane extract was used for the analytical determination.
The standard mixture was added to each sample before extraction. Standards were used to quantify mineral oil contamination as well as to verify the performances of the LC-GC-FID system.

2.2.5 Analytical determination and quantification

The extracts were analyzed using an LC-GC-FID system (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 x 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 transfer of the fraction of interest was carried out through the Y-interface using the retention gap technique [92]. The MOSH fraction was eluted from 2.0 to 3.5 min and transferred to the GC operating at a hydrogen constant pressure mode. The fraction comprising the MOAH ranged from 4.0 to 5.5 min. The MOSH and the MOAH fractions were eluted (at 300 µL/min) using a gradient, starting with 100 % *n*-hexane (0.1 min) and reaching 30 % of dichloromethane in 0.5 min. At the end of the LC-GC transfer, the LC column was backflushed with dichloromethane. A 10 m x 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 x 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 velocity of 4 mL/min. The oven temperature was programmed at 40 °C/min from 50 °C to 350 °C. 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 chromatograms were processed by Exachrome and MOSH and MOAH were quantified by standard addition. Both MOH migrated in Tenax and MOH lost by the paperboard were evaluated. The potential of migration was calculated, assuming that the total amount of MOHs present into the packaging material migrated into the food.

2.3. Result and discussion

Accelerated migration tests are usually carried out in hermetic glass or steel cells respecting standardized conditions. The conditions used in these tests, in particular for some products, are very different from real contact conditions of foodstuff. They differ not only in terms of time and temperature, but also because of the use of a simulant instead of real food. Furthermore, there are several parameters affecting the results. The study of these different parameters, and how they can influence the outcomes, is useful to better understand migration, to explain migration test results and to give a more realistic assessment of the migration.
One of the most important parameter influencing migration is the temperature. Several works studied the temperature effect. As reported in the introduction, Regulation 10/2011 established that the use of 10 days at 60°C as condition in the migration test for dry foods reflect long storage condition at 25°C, such as in the case of shelf life of 2-3 years. However, as reported in several studies the higher the temperature, the higher is the risk to overestimate migration. Regulation 10/2011 affirms that for storage at RT (room temperature) testing time can be reduced to 10d at 40°C if there a scientific evidence that migration has reached the equilibrium under these test condition.
A preliminary study on migration kinetics under accelerated condition at 40 °C, was conducted by Barp et al. in 2014[140]. In the work by these authors, migration of MOSH and MOAH from recycled cardboard into dry semolina and egg pasta finely ground was monitored up to 30 days. The migration tests were performed with the same condition of weight-to surface ratio (g pasta/dm^2 paperboard packaging) proposed in the UNI EN 14338:2004 for Tenax®. The results obtained for pasta were compared with those obtained using the simulant at the same accelerated condition. The data obtained showed a similar migration behavior and involved involved similar molecular masses ranges, but higher levels of MOHs migrated in the simulant Tenax® in comparison with pasta. As expected, egg pasta showed higher migration of MOHs with respect to semolina pasta. In addition, after 10-12 days at 40°C a steady state was reached for both semolina and egg pasta. For this reason, the condition chosen to perform on parameters affecting migration were 10 days to 40°C.Considered the great differences observed on migration behavior of egg and semolina pasta, migration tests were conducted using both type of pasta.

2.3.1. Initial matrices

No detectable contamination of MOSH was found in semolina pasta before exposure while egg pasta contained about 1.2 mg/Kg MOSH up to *n*-C_{25}. This value, as well as the contribution due to

endogenous *n*-alkanes (giving sharp peaks standing up the MOSH hump), was subtracted from the total contamination of pasta after the migration test. No MOAH were detected in both samples. Cardboards were analyzed before and after the contact with the pasta in order to evaluate the initial contamination and to estimate the "indirect migration", assuming that the MOH lost by the paperboard are completely transferred to the pasta. Table 9 reports the contamination presents in the cardboard analyzed. The contamination presented a molecular weight distribution ranging between *n*-C_{13} and *n*-C_{55}, splitted in two partially co-eluted humps. Mineral oil in the first part of MOSH fraction derived from printing applied on packaging and printing inks from printed material that were involved in the recycling process. The second hump (centered about *n*-C_{27}-C_{28}) derived from the waxes used in the paper processing.

Table 9 *Amount of MOSH and MOAH in cardboards considered in the study*

	MOSH mg/kg		**MOAH mg/kg**	
	< *n*-C_{25}	***n*-C_{25}-C_{35}**	**< n-C_{25}**	***n*-C_{25}-C_{35}**
Cardboard 1(printed)	370	560	78	60
Cardboard 2 (non-printed)	333	214	68	46

2.3.2. Migration test configurations

The investigation on parameters affecting migration was performed considering the four different scenarios illustrated in figure 12. In general, migration of mineral oil from cardboard into pasta occurs in two main ways: by gas phase and by diffusion. The scenarios considered are characterized by these two ways of migration or by a combination of them. Due to the small size of particles and their tendency to organize in a compact layer, when the ground pasta is put in direct contact with cardboard (Figure 12, scenario1), mineral oil in the closed system can migrate almost totally by diffusion. At the opposite, the whole paste is made up of larger sized particles that tend to settle in disorderly, leaving many empty spaces. As a consequence, there are few contact points between the paperboard and the pasta, which limits the migration of mineral oil by diffusion only through the contact points. For these reasons, when whole pasta is put in contact with the cardboard (Figure 12, scenario 3) the migration of mineral oil is a combination of migration by gas phase and diffusion by contact. In scenarios 2 and 4 instead the pasta is not in direct contact with cardboard and the migration must occur only via gas phase. To compare the effect of different parameters on migration, different scenarios must be taken into account in order to critically evaluate results of migration testing.

PARTICLE SIZE OF PASTA	TYPE OF CONTACT	
	GROUND PASTA IN **DIRECT** CONTACT WITH CARDBOARD **Scenario 1**	**GROUND** PASTA IN **INDIRECT** CONTACT WITH CARDBOARD **Scenario 2**
	WHOLE PASTA IN **DIRECT** CONTACT WITH CARDBOARD **Scenario 3**	**WHOLE** PASTA IN **INDIRECT** CONTACT WITH CARDBOARD **Scenario 4**

Figure 12. *Scenarios in the migration system configuration*

2.3.3. Effect of pasta amount

To assess migration from food contact materials, Reg. EC 10/2011 suggested tests using food simulants. As already mentioned the simulant selected for dry food is the Tenax®. According to the European Standard EN 14338:2003, the packaging material must be covered with 4 g/dm^2 of simulant. The specimens of cardboard considered in this study had a superficial area of 16.6 cm^2, which means that amount of Tenax® to be used was 0.66 g .

The surface-to-volume (or mass) ratio in actual contact condition can greatly differ from that used in migration testing. In the specific case, the actual contact conditions, were 500 g of pasta in contact with an area of packaging of about 461 cm^2 thus, the ratio g pasta/area of packaging is about 1.1 g/cm^2.

When reporting the final migration test results to verify the compliance of a FCM, the results of the migration test (expressed in mg/kg) must be corrected for the surface-to-mass (or volume) ratio, from experimental to actual contact. This correction takes into account that the same amount of migrants released by the FCM is "diluted" in a different volume of food (the smaller the amount of food in contact with a given surface of material, the higher the concentration of the migrant in the simulant/food). Nevertheless, a deviation from the expected behavior can be observed when reaching the saturation of the migrants into food. Rapid saturation can be easily reached if the migrants remain on the surface of the food (if there is adsorption instead of absorption). In this case measured migration rate can be affected by the amount of simulant/food used in the migration test.

To study this effect, a first set of migration tests was carried out using different amount of pasta (comprised between 0.66 g to 16.0 g) in contact with the cardboard specimen. The smallest amount considered was equal to the one indicated for test with Tenax® (4g/dm^2 = 4g/100cm^2 = 0.66g/16.6 cm^2), while the highest one (16g) corresponded approximately to the actual contact condition in the real packed food.

Maintaining constant the head space volume (V1) and increasing the amount of pasta in the system, the effect of different quantities of pasta (0.6, 2, 4, 8, and 16 g) on migration rate was evaluated for both semolina and egg pasta. Figure 13 shows migration data expressed as concentration (mg/kg of pasta).

As expected, by increasing the amount of pasta in contact with the paperboard specimen, the concentration of the migrants decreases due to the dilution effect. Nevertheless, the observed decrease

is lower than the theoretical one obtained by extrapolating the data obtained using standardized testing condition (0.6 g di food) and applying the correction for the different surface-to-mass ratio.

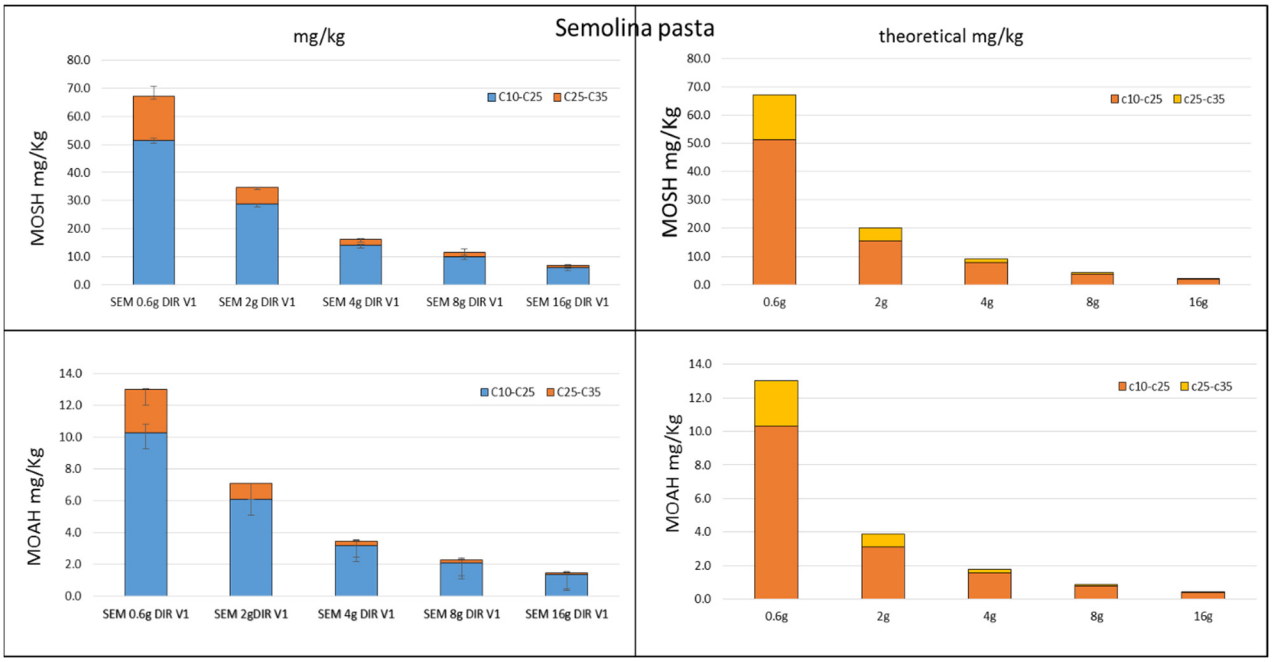

***Figure 13**. (Left) MOSH and MOAH (reported in mg/kg) migrated in different amounts of ground* **semolina** *pasta in direct contact with cardboard with V1 head space.(Right) theoretical amounts (mg/kg) of MOSH calculated using standardized testing conditions(0.6g) and corrected for the different surface-to-mass ratio.*

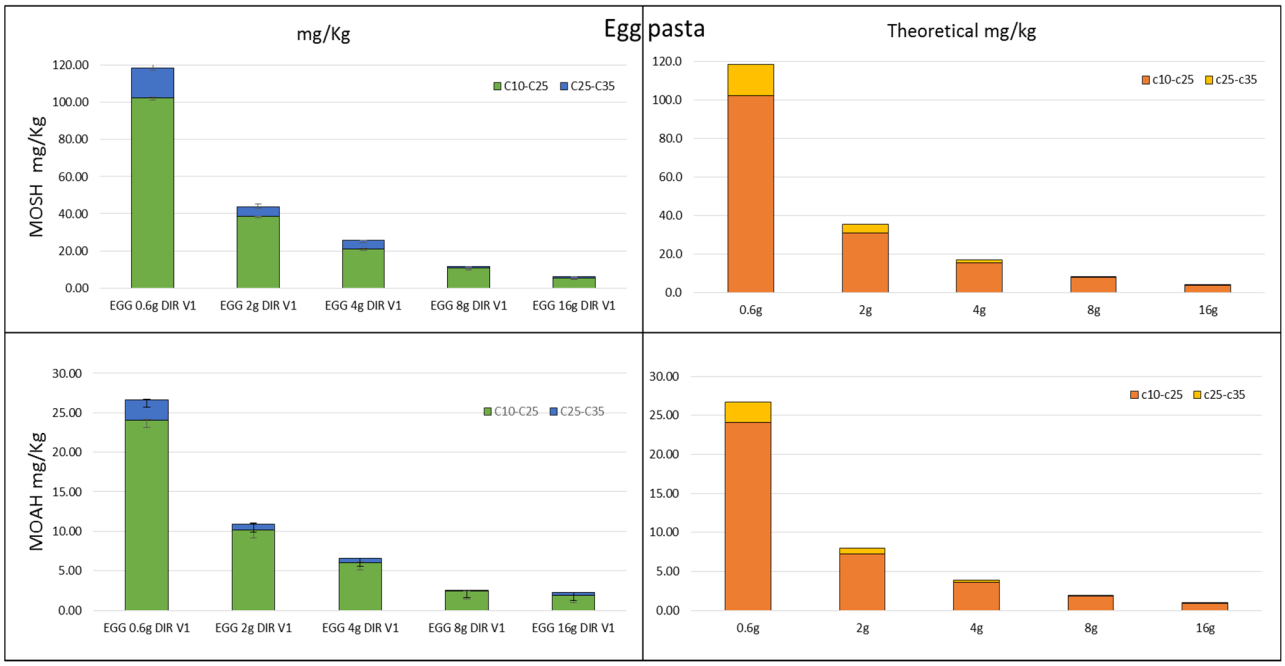

Figure 14.** (Left) MOSH and MOAH (reported in mg/kg) migrated in different amount of ground* ***egg *pasta in direct contact with cardboard with V1 head space. (Right) thoretical amounts (mg/kg) of MOSH and MOAH calculated using standardized testing conditions (0.6g) and corrected for the differents surface-to-mass ratio.*

Observing the figure 13 and 14 is evident that the theoretical migration extrapolated from the migration test tends to underestimate the real migration into food because the theoretical data takes into account only the dilution effect due to the increased amount of pasta and not the absorption effect

that pasta has on contaminants present into the cardboard. The effect is evident observing figure 15. Increasing the amount of pasta from 0.6 g to 16g the amount of MOSH and MOAH lost by cardboard and migrated into pasta increased from 45 µg to 106 µg and from 11 µg and 23 µg respectively for semolina pasta in direct contact with cardboard. The effect is evident also in the chromatograms of cardboard analyzed after migration test (figure 16).

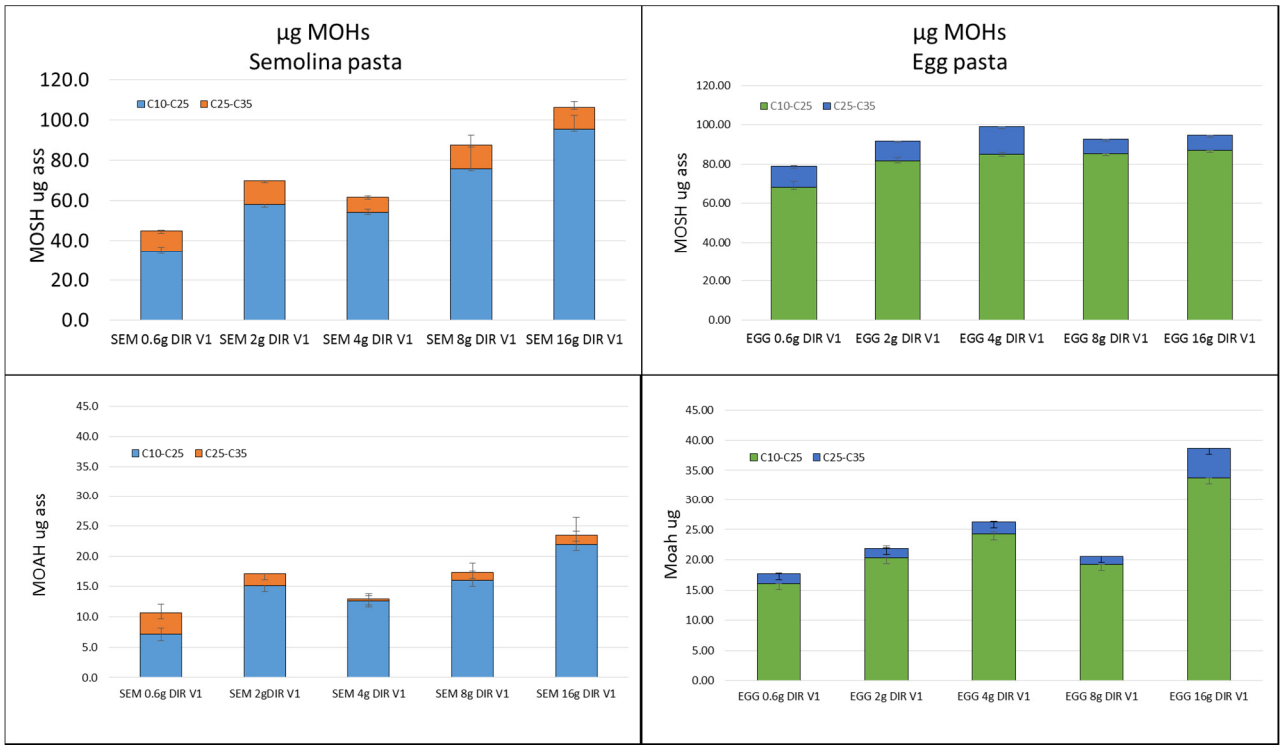

***Figure 15**. Absolute amounts of MOSH and MOAH migrated in different amount of semolina and egg ground pasta after 10days at 40°C.*

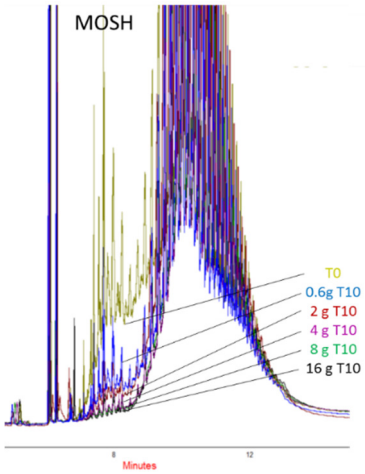

***Figure 16**. Overlay of MOSH chromatograms obtained from analysis of cardboard after migration tests*

Egg pasta, instead, showed a different behaviour, the increase of MOSH and MOAH migration is much lower in this type of pasta than in semolina pasta. It seems that the amount of MOHs migrated into egg pasta is almost independent from the grams of pasta present in contact with the packaging material.

In conclusion, it was proven that depending on the dry food type (semolina or egg pasta), the use of the surface-to-weight ratio indicated in UNI EN ISO 1186 (4g/dm^2) to assess the migration, tends to underestimated the real migration. High quantity of pasta in the system tend to extract higher amount of mineral oil from the cardboard but, at the opposite, the high quantity have a dilution effect on the contamination and the net amount of MOSH and MOAH migrated in pasta decreases when amount of pasta increases.
Comparing data in figure13 and 14 it is possible to notice that, at the same configuration of migration test (with head space V1 and direct contact pasta/cardboard), after 10gg at 40°C, contamination migrated in egg pasta is about 1.5 time higher than the amount of contamination found in semolina pasta.

2.3.4 Type of contact

According to some authors, migration of mineral oil is primarly due to a process of evaporation from the cardboard and recondensation into the food. It means that only volatile compounds could migrate. Nonetheless, in some cases, the direct contact food/cardboard can lead to a non negligible migration also for the non-volatile substances [141]. Maintaining constant the amount of pasta and the head space volume, direct and indirect contact between pasta and cardboard was studied.

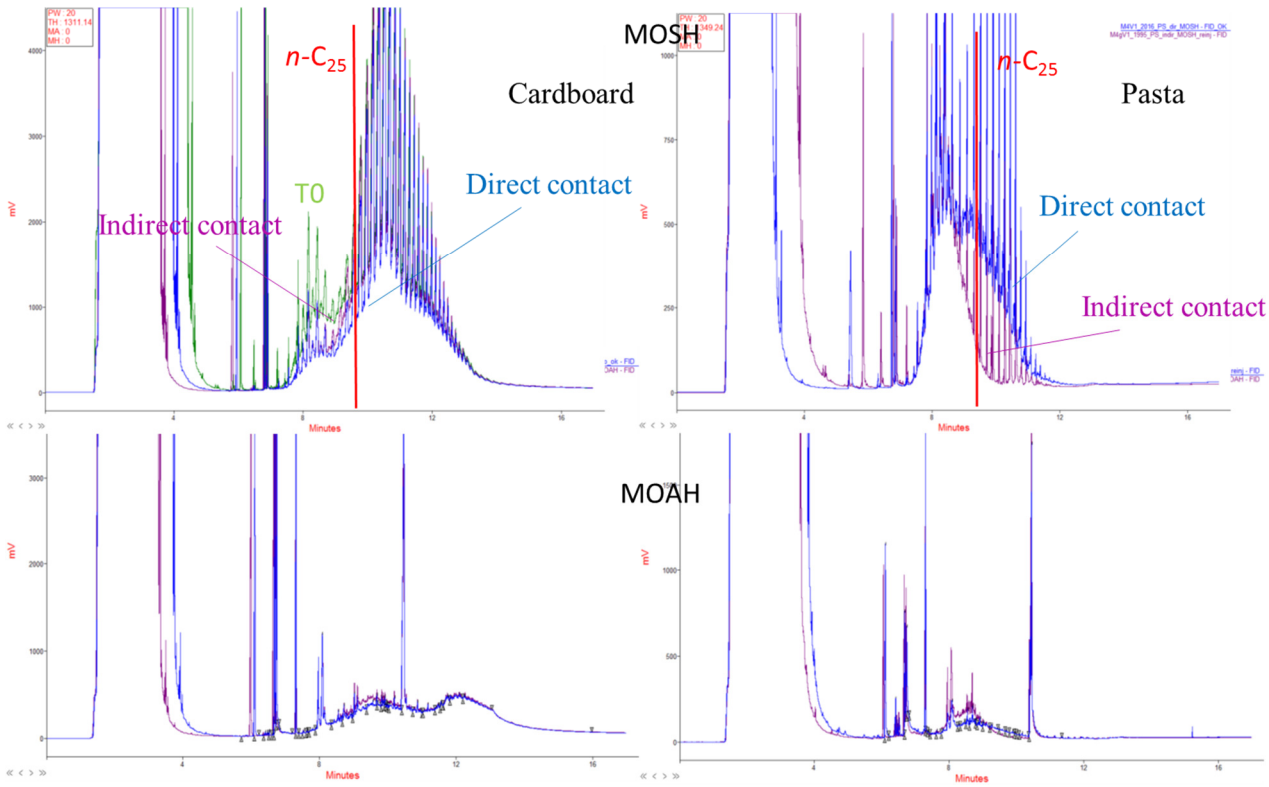

Figure 17. *Comparison between MOSH and MOAH LC-GC traces migrated into ground pasta and lost by cardboard with direct and indirect contact.*

Observing figure 17 the ground pasta after 10 days of contact with the cardboard showed a different profile with respect to the pasta put in indirect contact with the cardboard (via gas-phase contact). The latter presents an LC-GC trace with a hump of contamination that involves mostly molecular weight up to *n*-C_{25}. Negligible amount of MOSH > *n*-C_{25} were migrated into pasta in indirect contact

with cardboard. Profile of pasta in direct contact shows, a similar hump of contamination, with pasta in indirect contact, in the range of *n*-C_{13}-C_{20} and an additional contamination, given by higher molecular weight hydrocarbons, up to n-C_{35},the latter contamination resulting from a migration by touching contact. The difference between migration by gas phase and by direct contact was evident not only in the ground pasta but also in the whole pasta (the scenario that simulate the real contact condition of pasta and cardboard). Whole pasta in direct and in indirect contact presented a similar percentage on potential migration for MOSH in the range of n-C_{10}-C_{25} (22.4% and 19.8% respectively), but differed for hydrocarbons of higher molecular weight (1% for direct contact and 0.3% for indirect contact) which can not be transferred by gas phase (Table 10).

***Table 10**. Percentage on potential migration of MOSH in ground and whole semolina pasta in two different type of contact with cardboard.*

	Direct contact		Indirect contact	
	C10-C25	C25-C35	C10-C25	C25-C35
16 g Ground semolina pasta	53%	5%	36%	0.2%
16 g Whole semolina pasta	22%	1%	20%	0.3%

Observing the behaviour of ground and whole pasta is evident how the different particle size influences the migration of mineral oil from cardboard.

2.3.5. Size effect

The simulant suggested to carry out migration test on FCM intended to come in contact with dry foods such as pasta, is Tenax®. As mention in the general introduction of this thesis, Tenax® has a particle size of 60-80 Mesh, which correspond to 0.25- 0.177 mm, even lower than particle size of wheat flour (0.200-0.400 mm). It is reasonable to assume that particle size can affect the migration rate. Indeed, migration measured in the food simulant can be different from that measured in the real food also related to the different food size. This is particularly true when migrants tend to remain on the surface of the food (i.e. there is adsorption instead of absorption). Eicher and co-workers [141] reported that particle size of food have a great influence on migration rate in particular when direct contact occurs. An increase in the size of particles leads to a decrease in the density of contact point food/cardboard and to a decrease of migration rate, mostly for non-volatile compounds. Furthermore, the authors affirmed that migration by gas phase is not influenced, by the size of particles.
To evaluate the difference in the behaviour of migration related to the pasta particle size, a part of whole pasta was reduced to a fine particulate by grinding. Keeping constant the amount of pasta, head space volume and type of contact, the differences obtained with migration test using whole pasta and ground pasta were evaluated. As expected, the size of particle in contact with cardboard had a great effect to migration of MOH. In figure 18 a comparison of the behaviour of ground and whole pasta is reported. Both in direct and in indirect contact, ground pasta lead to higher migration rate than whole pasta, due to its higher superficial area exposed to contaminants. In direct contact the percentage on potential migration in 4g of whole pasta was 12% for MOSH in the range of *n*-C_{10}-C_{25} and 0.8% for *n*-C_{25}-C_{35} (similar percentages were found also for the MOAH). In ground pasta the

percentage of MOSH migrated on potential migration was 34% and 4 % for n-C_{10}-C_{25} and n-C_{25}-C_{35} respectively, proving that pasta size influences also the molecular weight range of the migrant. However, these results may be affected not only by particles size but also by the direct contact paperboard/pasta. For this reason, a more representative result of the particles size effect on migration can be obtained from the comparison of ground and whole pasta in indirect contact with cardboard, that are the situation in scenario 2 and 4 where only migration via gas phase occurs. 4g of ground pasta in indirect contact with cardboard showed a percentage of about 18% and 0.1% for MOSH (and 17% and 0.1 % for MOAH) in the range of *n*-C_{10}-C_{25} and *n*-C_{25}-C_{35}, while whole pasta reported a percentage about half of those reported by ground pasta (8% for MOSH and 9% of MOAH).

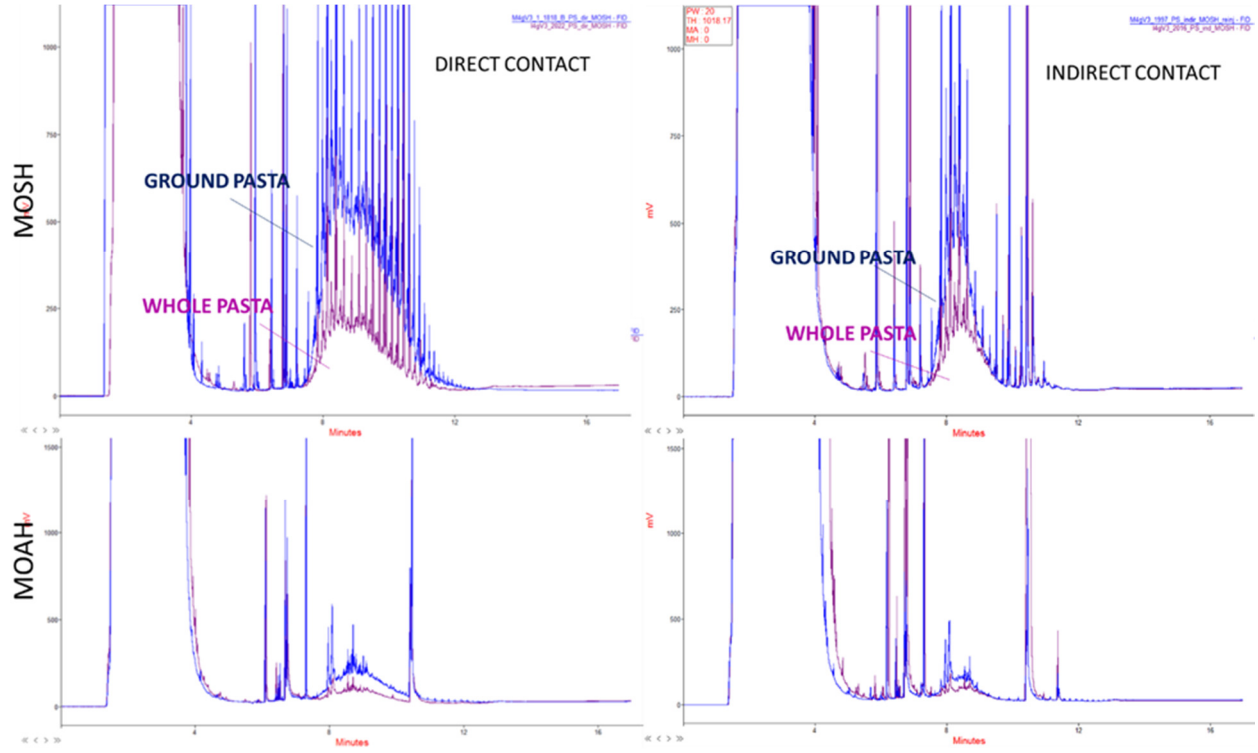

Figure 18 Comparison between MOSH (top) and MOAH (at bottom) migrated into ground and whole pasta in contact and in indirect contact with cardboard after 10 days at 40 °C.

A different behaviour was observed comparing semolina and egg pasta. Both for ground and whole pasta, as already observed, egg pasta showed a greater absorption of MOHs than semolina pasta. When 16 g of ground pasta was in direct contact with cardboard, egg pasta showed an absorption capacity almost 1.5 times higher than semolina pasta. Similar ratios were found both for MOSH and MOAH in the range of *n*-C_{10}-C_{25} and in the range of *n*-C_{25}-C_{35}. Furthermore, comparing percentage resulted from migration test with 16g of whole pasta is evident that the difference from egg and semolina pasta was much larger, egg pasta lead a percentage on potential migration 3.8 times higher than semolina pasta for MOSH and MOAH about *n*-C_{10}-C_{25} and 5 times higher for less volatile ones. From this results seems that semolina and egg pasta have a similar behaviour when the particles are small but a great difference in the behaviour when the whole pasta is considered (that is the real condition of use).

***Table 11**. MOSH and MOAH Percentage of the potential migration in egg and semolina pasta*

MOSH % on potential migration						
	EGG PASTA		SEMOLINA PASTA		**EGG PASTA/SEMOLINA PASTA**	
	C_{10}-C_{25}	C_{25}-C_{35}	C_{10}-C_{25}	C_{25}-C_{35}	C_{10}-C_{25}	C_{25}-C_{35}
16 g ground direct contact	97	22	65	12	**1.5**	**1.8**
16 g whole direct contact	85	10	22	2	**3.8**	**5**
MOAH % on potential migration						
	EGG PASTA		SEMOLINA PASTA		**EGG PASTA/SEMOLINA PASTA**	
	C_{10}-C_{25}	C_{25}-C_{35}	C_{10}-C_{25}	C_{25}-C_{35}	C_{10}-C_{25}	C_{25}-C_{35}
16 g ground direct contact	103	11	64	7	**1.6**	**1.6**
16 g whole direct contact	86	5	25	1	**3.4**	**5**

2.3.6. Head space volume

Another parameter tested during this study was the volume had space present in the migration cells. Three different head space volumes were tested in migration tests. Volumes tested were of about 16.6 cm^3 (V1), 37.4 cm^3(V2), and 45.7 cm^3(V3). Volume steel disk (with thickness of 0.1cm) were put in the cell to obtain the chosen volume to test. The volume V3 was the biggest and it was obtained putting the pasta in the cell without steel disks (figure 19.).

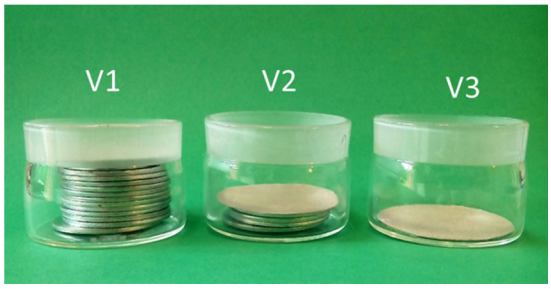

***Figure 19**. Three different volumes studied in the migration cell organization.*

Little but appreciable differences in migration were evaluated with different head space volumes in the system when ground pasta was in indirect contact with the cardboard, but not when it was in direct contact with the cardboard. These differences are visible in the LC-GC traces of pasta and cardboard displayed in figure 20. The chromatograms of pasta in indirect contact with V1 and V3 head space volumes do not show a complete overlap, while those resulting from direct contact overlap perfectly. Table 12 reports migration data expressed as percentage on the potential. In this way it is possible to compare data obtained from different trials, using different contact conditions. The influence of the head space volume on migration is well evident, as already mentioned, in ground pasta not in contact

with cardboard, in fact the V1/V3 ratio is 1.4 with 4 g of pasta sample. The higher is the volume in the cell, the lower is the MOH percentage on potential migration. This effect is not evident for the whole pasta, for which a ratio V1/V3 of about 1.0 was found. This effect is probably attributable to the fact that the whole pasta has a higher free space in between the particles and the volume head space is summed with the space between the particles, resulting in an equivalent total head space both for pasta in contact and pasta not in contact. About ground pasta, on the contrary, the space volume is only space over the pasta and when pasta is more far to the cardboard (in indirect contact) the MOH have probably more difficult to reach pasta and to condensate into it. With V1 the pasta is closed to the cardboard and the migration can carry out more easily.

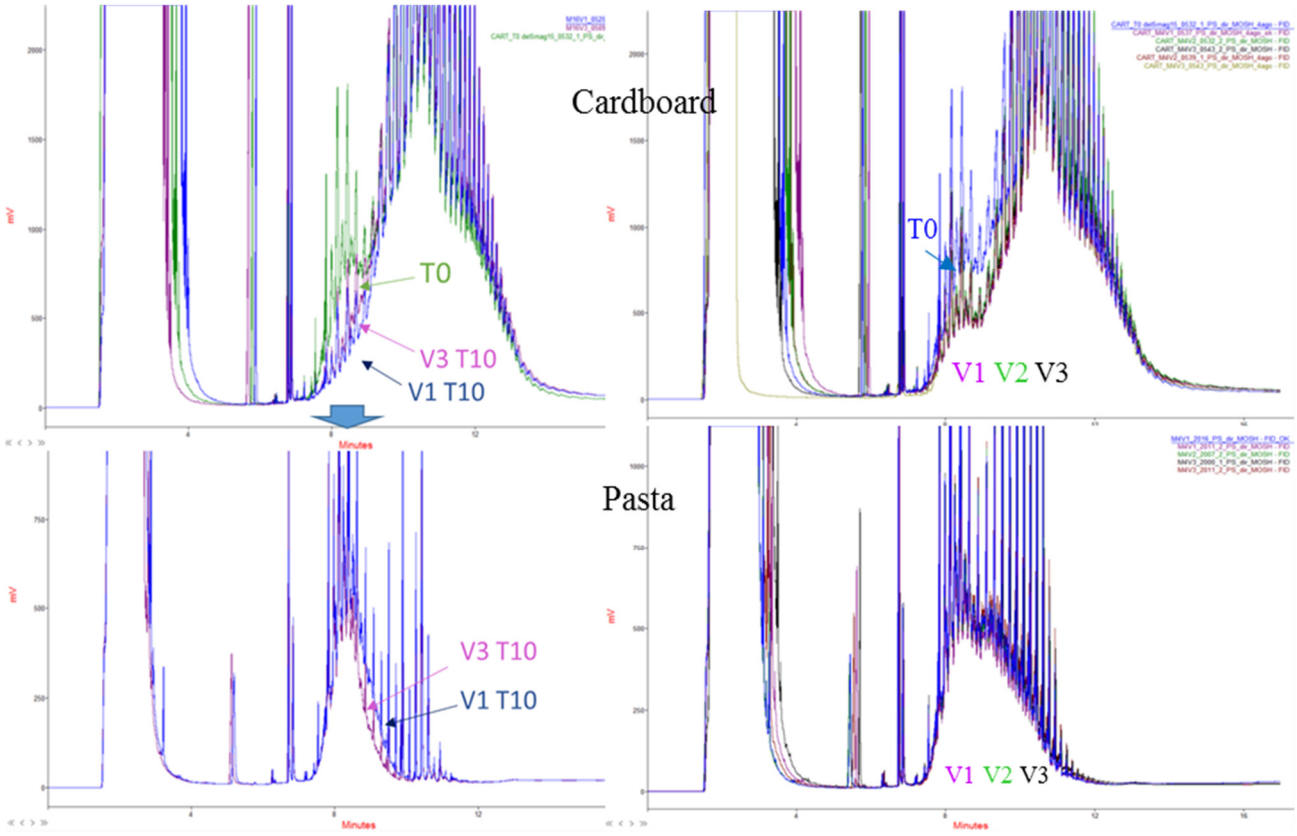

Figure 20. *Overlaps of chromatograms resulted from analysis of paperboard (top) and pasta (bottom) put in the migration system with different head space volumes.*

***Table 12**. Percentage of MOH on potential migration for ground and whole pasta in direct and indirect contact with cardboard.*

	MOSH % on potential migration			
	ground pasta		**whole pasta**	
	direct contact	indirect contact	direct contact	indirect contact
4 V1	31.7	26.0	10.8	7.9
4 V3	33.3	18.8	12.7	8.0
V1/V3	**1.0**	**1.4**	**0.9**	**1.0**

2.3.7. Cardboard type effect

The influence of cardboard characteristics on migration of contaminants into food was studied by some authors. The three principal characteristics taken into account are the grammage, the thickness of the materials and the percentage of recycled pulp in the fibers of cardboard. The effect of the latter characteristic is not very clear. In 2007, Nerin et al. found that the rate of migration was not influenced by the presence of recycled fiber in the cardboard [32]. The same authors reported that, instead, the thickness and grammage (cardboard tested were 128, 178 and 406 g/m^2) of the cardboard in contact with Poropack© at different temperature, affected the rate of migration of DIPNs. In particular, low grammage resulted in faster migration rate.

In this study two different cardboards were used in the migration tests. The materials were completely made by recycled fibres. C1 was a final product, printed on one side, ready to use to package the pasta. Cardboard C2 was a not completely ready product. It was not printed with a smooth white side prepared to be printed.

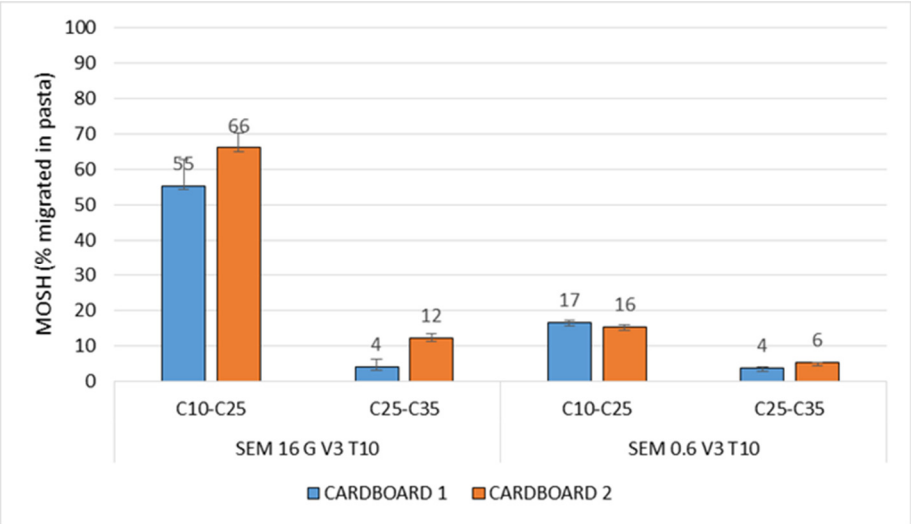

***Figure 21.** Percentage of MOSH, on potential migration, for semolina pasta in contact with C1 and C2 cardboard after 10days at 40°C.*

Figure 21 illustrates levels of MOSH, separated in two different molecular weight, migrated from C1 and C2 cardboards into two different amount of semolina pasta after contact at 40°C for 10 days. In the same condition of contact, a slight difference in percentage of migration is possible to notice

between the two types of cardboard. Pasta in contact with C2 shows higher percentage of MOSH migrated, therefore, migration of MOSH is more favourite from C2 (480 g/m^2 grammage) than from C1 (330 g/m^2 grammage) both for MOSH in the *n*-C_{10}-C_{25} range and for *n*-C_{25}-C_{35} range. Probably the influence on migration is not only give by the grammage. Differences in the state of production of cardboard (in this case the presence or not of a printed surface) may also have an impact on the greater or lesser retention or release of MOSH and MOAH from cardboard. On the other hand, the differences in the grammage and in the thickness as well as in the level of MOSH and MOAH of two cardboard was not so high to give a great effect on the migration rate.
Subsequently, the effect of the humidity of cardboard on migration of mineral oil into the semolina and egg pasta was also studied for C2. In literature, the effect of humidity was evaluated referred at the humidity of food or the humidity of environmental where migration tests are performed.
In order to increase the humidity of cardboard a paper filled with water was posed on the bottom of a closed glass box. The recycled cardboard was put in the box (not in contact with paper) overnight. Subsequently the cardboard was used to migration tests. The final percentage of water in the cardboard was 12%. There were no differences in the percentage of MOSH and MOAH migrated from cardboard into the pasta referred to potential migration. Figure 22 reports overlap of chromatogram resulted from analysis of wet and dry cardboard and relative pasta in contact with them. Is evident the complete overlapping of traces LC-GC that highlight the absence of effect by moisture on migration both for MOSH and for MOAH.

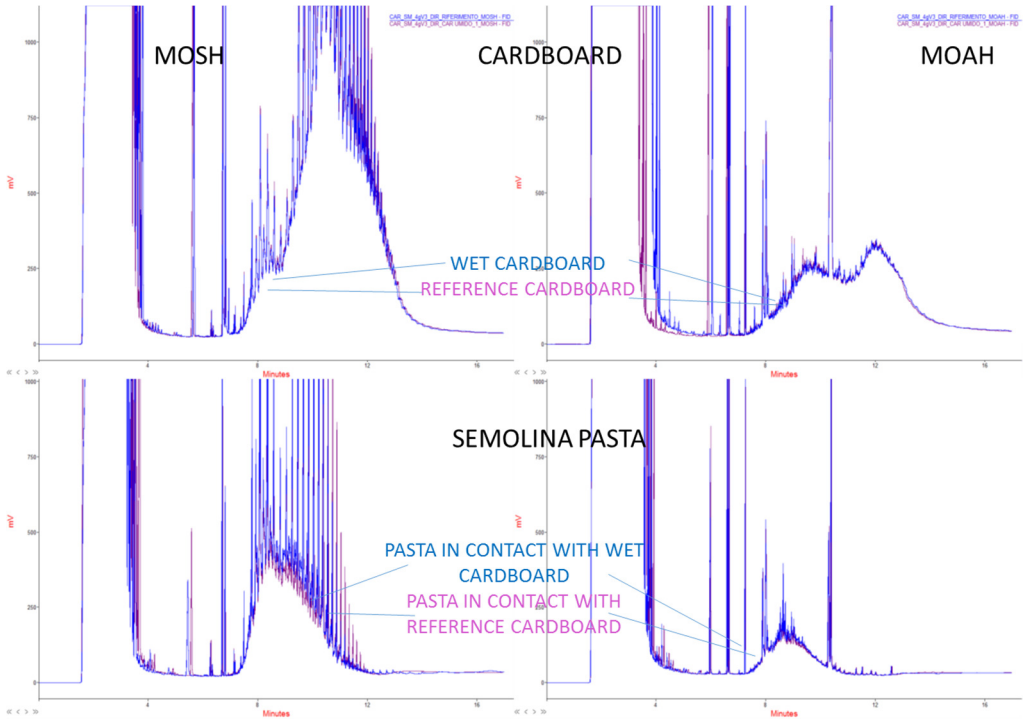

***Figure 22**. Overlap of chromatograms relatives to reference cardboard and wet cardboard and pasta after contact with them.*

2.3.8 Semolina pasta VS egg pasta

Recent studies about migration of mineral oil from recycled paperboard was demonstrated showed that in real stored conditions the behavior of the semolina pasta and the egg pasta is very different. In the semolina pasta the contamination tends to remain on the surface of the pasta while in the egg pasta

a not negligible part of contamination can penetrate deep in the matrix [131]. Is evident how, the nature of the food in contact with recycled paper, is the main parameter that influences the migration. For this reason, in this work, semolina pasta and egg pasta were considered, and their different behavior was studied in through and in a systematic way, performing comparison tests for each parameter considered.

The first parameter studied was the amount of pasta into the migration cell. The results showed that two different effect were involved by varying the volume-to-mass ratio in the test: a dilution effect and an absorption effect. The semolina and egg pasta showed a different behavior towards migrated MOH. In fact, by increasing the amount of pasta in the glass cell, while maintaining constant the cardboard specimen surface, the absolute amount of contaminants migrated increased proportionally in the semolina pasta, while in egg pasta the increase was lower. It seems that the amount of MOHs migrated into egg pasta is almost independent from the grams of pasta present in contact with packaging material.

When comparing results obtained by different migration tests (using different amount of pasta), in all cases egg pasta showed higher capacity to absorb migrated MOH. Another interested difference between the two types of pasta was the behaviour of ground and whole pasta. Table 13 reports the ratio between the percentage of MOSH and MOAH on potential migration for the two types of pasta. When ground pasta was considered, the egg pasta showed 1.5 times higher contamination than semolina pasta, instead, in whole pasta the ratio was higher (about 3.8). These data seems to suggest again, that the contamination in whole egg pasta rapidly penetrates into the whole particle volume (absorption), while remain on the surface of semolina pasta (adsorption). In the light of these results, it is possible to affirm that semolina pasta has only an adsorbent effect, and, on the contrary the egg pasta behaves like a liquid, showing a great absorption against the mineral oil.

As suggested also by [131] the different behaviour of these two matrices can not be attributed only to their different fat content. To demonstrate this concept a migration test using defatted pasta was performed. Egg pasta was defatted in order to obtain a pasta with negligible fat content (0.05% in the specific case). The contamination found in defatted pasta, after contact with cardboard, seemed to depend on both the fat content and the pasta composition. In fact, egg pasta, which presented the highest fat content, resulted the most contaminated. On the other hand, observing contamination found in semolina and defatted pasta after contact with cardboard, there is no linear correspondence between the fat percentage and the amount of MOSH and MOAH migrated. Even though, semolina pasta has higher fat content than defatted pasta, it presented the lowest percentage of contamination and potential migration (Tab 13). Probably, after the process of defatting, the egg pasta loses the fat but maintains its porosity and structure that allow it to have higher absorption capacity with respect to semolina pasta

Table 13. *Percentage of potential migration in egg, semolina pasta and defatted pasta.*

	MOSH		MOAH	
	% potential migration		**% potential migration**	
	C_{10}-C_{25}	C_{25}-C_{35}	C_{10}-C_{25}	C_{25}-C_{35}
Ground pasta				
EGG 4 g DIR	53	4	45	4
SEM 4g DIR	34	4	27	4
EGG 0% FAT 4g DIR	**49**	**11**	**45**	**11**
EGG 4g IND	33	0	25	0
SEM 4g IND	19	0	16	0
EGG 0% FAT 4g IND	**25**	**2**	**23**	**0**
Whole pasta				
EGG 4g DIR	56	3	71	6
SEM 4g DIR	12	1	13	1
EGG 0% FAT 4g DIR	**35**	**2**	**40**	**5**

2.3.9. Correlation with Tenax®

The most realistic scenario in the migration test used in this study was those realized with 16 g of pasta in direct contact with cardboard and the V3 head space volume, which used volume-to-mass ratio similar to that of the packaged pasta present on the market.

As already mentioned, in the work conducted by Barp in 2015 the same recycled paperboard considered in this work, was used. The author performed kinetic migration using Tenax® in contact with cardboard in the migration cell up to 30 days. In order to compare the behaviour of pasta with Tenax®, the data obtained after migration tests for 10 days at 40°C was extrapoled and normalized using a the surface-to-mass ratio of 27 (calculated as in the table).

The table reported the comparison of data in Tenax® and data obtained after migration test with 16g of whole pasta in contact with cardboard.

It is possible to notice that simulant in cell migration tests tends to overestimate the MOSH migration into semolina pasta and to underestimate the migration in egg pasta demonstrating how the matrix can influence the migration

***Table 14**. Data comparison between Tenax® and semolina pasta and equation to obtain the pasta/paperboard exposed ratio.*

Real condition in packaged pasta → 500 (g)/ 461 (cm^2) = 1.08 g/ cm^2 Glass cell condition for Tenax® → 0.664 (g) /16.6 (cm^2) = 0.04 g/ cm^2 **Pasta /paperboard exposed → 1.08 (g/ cm^2) / 0.04 (g/ cm^2) = 27**			
	Tenax® normalized	Semolina pasta (16g whole T10)	Egg pasta (16 g whole T10)
MOSH (mg/kg)	8.10	2.7	14.1
MOAH (mg/kg)	1.18	0.7	2.3

3.8 Conclusion

The migration of contaminants, in particular, of mineral oil hydrocarbons from recycled paper into dry food is a complex topic due to the complexity of the food and cardboard matrix, as well as the wide variety of chemical compounds involved. In order to evaluate the compliance of packaging materials with the law, migration tests using simulant instead of food have been proposed by authorities. Many studies compared the behavior of food simulants with real foods and results showed, as not always the simulant reflects the real behavior of the food in contact with the packaging. In this part of the thesis, the influence of some parameters on migration of mineral oil from recycled cardboard into semolina pasta and egg pasta was studied. For this scope, migration tests using glass weighing bottles with a previously developed procedure was used.

Pasta amount in contact with cardboard (surface-to-mass ratio), the type of contact pasta/cardboard, the head space volume in the migration cell, and the pasta size were all parameters that had an effect on migration.

The surface-to-mass ratio in the accelerated migration test can greatly differ from that present in the actual contact condition (real condition foodstuff). For this reason, the migration data resulted from migration test are corrected for the surface-to-mass ratio to actual condition. Nonetheless, the migration test conducted varying the amount of pasta in the system showed that the correction proposed tends to over-estimate the real migration because takes into account only the dilution effect of the higher amount of pasta and not the effect of saturation that the pasta presents in the real conditions.

The type of contact influences the range of the molecular weight of hydrocarbons that can migrate into the pasta. Only volatile compounds can migrate via gas phase, however when pasta is put in contact with cardboard also hydrocarbons over the *n*-C_{25} molecular weight were into pasta. The particle size of pasta in contact with cardboard influences the total entity of the migration. Due to the higher superficial area exposed to contaminants higher amount of MOHs were found into ground pasta than in the whole pasta. The head space volume influenced the migration only when ground pasta was put in indirect contact with the cardboard. Two types of cardboard (different grammage and differential contamination) were tested. No differences in the migration was observed using the two materials. Furthermore, also the humidity of the cardboard seemd to have negligible effect on migration of mineral oil from cardboard into pasta

Finally, analyzing the effect of the parameters on migration, it was evident the different behavior of egg and semolina pasta. Egg pasta tends to absorb MOH in all the mass, while semolina pasta adsorb MOH. This is not only attributable to the fat content but also at structure and porosity of the matrix.

A correlation with data obtain from migration test with Tenax® was finally carried out. Even though the data of Tenax® was normalized to the surface-to-mass ratio of the actual contact condition, it tended to overestimate the migration in semolina pasta and, at the opposite, to underestimate the migration in egg pasta.

These results demonstrated that migration tests sometimes fails in giving the right simulation of the migration in real food.

This study contributed to better understand migration of lipophilic contaminants (such as mineral oil) in dry food in direct contact with recycled paperboard. Results obtained could be used as a starting point to elaborate correction factors that, taking into account the complexity the migration phenomenon, could help in obtaining migration results which better reproduce real migration in food.

3. PIZZA BOXES TAKE AWAY: survey on the organic contaminants and assessment of their migration in Tenax®

3.1. Introduction

According to the Consumer Union, about 5 millions of pizza are baked in Italy every day. This important food is consumed in the restaurant but in the last few years, the habit to take pizza at home for consumption is getting more and more popular. The pizza is usually consumed at home after being transported in particular paperboard boxes. The contact with the transport box occurs at temperature around 60-65 °C for a time generally shorter than 30 minutes, but often, the pizza boxes are used to warm the pizza in the domestic oven, as well as a plate for food consumption. Pizza boxes are made of corrugated cardboard consisting of two flat surface paper, called covers (havana-tobacco or white color), which contain internal paper undulation (wave), that set the characteristics of stability and strength of the cardboard. It typically, consists in a single wave center layer enclosed between internal and external paper covers sealed with corn or potatoes starch adhesive. The covers can be mono or two layered. According to D.M. 21/03/1973, the pizza, a wet and fat food, can be brought into contact only with pure cellulose packing, the recovered paper is forbidden for the production of "boxes for take away pizza". Nevertheless, a survey conducted in Italy in 2008 revealed that about 90% manufacturers of pizza boxes produce board with a percentage of recycled fiber not less than 20% (www.consumatori.it). The non-observance of the specific law regulating the compulsory use of virgin cellulose, rather than recycled cellulose, has been demonstrated by the identification of diisobutylphthalate (DIBP) in the headspace of the boxes. This substance, indeed, is used as solvent in the recycled paper production and, as well as Pb, it easily migrates from packaging into food [142]. Recycled paperboard is mainly used in direct contact with dry food like flour, grain, salt, rice and pasta. In this type of material a large number of chemical components are involved in the recycling process such as bleach, paper strengthening agents and inks. In the last years, many authors reported studies on migration of these substances from packaging in food. In particular, in a study of 2013, Bisphenol A (BPA) and nonylphenol di-ethoxilate (NDP) have been detected in pizza boxes at level respectively of 0.87 mg/kg and 0.27 mg/kg [40]. In the same year, the German authorities notified through the Rapid Alert System for Food and Feed (RASFF) a migration of MOSH and MOAH at level of about 411 mg/kg and 77 mg/kg, respectively, from "carton box for pizza from Italy". Different fast food packaging, such as pizza boxes, packaging for burger, fries and wraps were analyzed in a study conducted in 2015. Levels ranged from 19 to 682 mg/kg of MOSH, and from 9 to 92 mg/kg of MOAH were found in samples and, in particular, highest amount of contamination were shown by pizza boxes. Chromatographic patterns of samples demonstrated the usage of recycled fibers [143].

The aim of this work was to investigate on the presence of selected hydrocarbon contaminants (mineral oil and polycyclic aromatic hydrocarbons) in pizza boxes collected in different areas in Italy, the possible sources of contamination (use of recycled paperboard, printing ink), and to evaluate their migration potential into Tenax®, used as food simulant.

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3.2. Materials and methods

3.2.1 Samples

Thirty-eight samples of pizza boxes cardboard were directly taken from pizza restaurants or take away pizzeria located in 4 different areas in the North and the South of Italy. MOSH and MOAH analysis were carried out on all samples. Migration tests using Tenax® as simulant were performed only on selected samples. Twenty-seven samples were also subjected to the determination of PAHs. Migration tests were performed using spiked samples produced in laboratory.

3.2.3. Reagents and standards

Acetone, ethanol, acetonitrile, pentane of HPLC grade were purchase from Sigma Aldrich (Milan), dichloromethane, *n*-hexane (Sigma Aldrich, Milan) were distilled before used. The water was purified with a Mill-Q System (Millipore, Bedford, MA, USA). The C_{10}-C_{40} (all even) standard mixture of *n*-alkane was from Fluka Analytica, Sigma Aldrich (Switzerland). The working standard solution used to check the LC-GC performance and the efficient MOSH and MOAH separation, and to quantify the contamination of samples, as described by Biedermann and Grob (2012) [137], was prepared by mixing: 5-α-cholestane (Cho, 0.6 mg/mL), n-C11 (0.3 mg/mL), n-C13 (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 standard PAHs mixture (EPA 610 PA mix, Sigma-Aldrich, Germany) consisted of: naphthalene (Na, 1000 µg/mL), acenaphthene (Ac, 2000 µg/mL), fluorene (F, 200 µg/mL), fluorene (F, 200 µg/mL), phenanthrene (Pa, 100 µg/mL), anthracene (A, 100 µg/mL), fluoranthene (Fl, 200 µg/mL), pyrene (P, 100 µg/mL), benzo[*a*]anthracene (BaA, 100 µg/mL), crysene (Ch, 100 µg/mL), benzo[*b*]fluoranthene (BbF, 200 µg/mL), benzo[*k*]fluoranthene (BkF, µg/mL), benzo[*a*]pyrene (BaP, 100 µg/mL), dibenzo[*a,h*]anthracene (DBahA, 100 µg/mL), benzo[*g,h,i*]perylene (BghiP, 200 µg/mL), indeno[1,2,3-*c,d*]pyrene (IP,100 µg/mL). A diluted (1:20000) standard solution was obtained with acetonitrile and spiking standard solution (1:1000) with *n*-hexane. For migration tests simulant Tenax® (MPPO, 60-80 mesh) was purchased from Supelco Analytical (Pennsylvania).The simulant has been subjected to a purification process by repeated washings with solvent (distilled acetone and hexane) until obtaining a clean blank. At the end, the Tenax® was dried using a vacuum pump and finally was heated at 160°C overnight.

3.2.4. Instrumentation and chromatography conditions

Glassware and other materials were rinsed with distilled acetone and *n*-hexane just before use. The microwave assisted extractor (MARS 5MD7797) was from CEM (Bergamo, Italy). To evaporate the solvents, a centrifuge (UNIVAPO 100 H) connected with a vacuum pump (Buchi Vacuum Pump V-700), was used.

3.2.4.1. LC-GC-FID

Analytical determination of mineral oil was performed using an HPLC-GC system (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 valves) 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 transfer of the fraction of interest was carried out through the Y-interface using the retention gap technique [92]. The MOSH fraction was eluted from 2.0 to 3.5 min and transferred to the GC operating at a hydrogen constant pressure mode. The fraction comprising the MOAH ranged from 4.0 to 5.5 min. 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, 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 velocity of 4 mL/min. The oven temperature was programmed at 40 °C/min to 50°C from 350°C. FID (sampling frequency 50 Hz) and the SVE were heated at 360 °C and 140 °C, respectively. Data was acquired and processed by the ExaChrom software (Brechbühler, Switzerland). The MOSH area was determined by the integration of the total hump of largely unresolved peaks and by subtraction of internal standards.

3.2.4.2. GC-MS

The GC-MS system was used to confirm the presence of alkylbenzenes. The system consisted of a GC2010 gas chromatograph and a QP2010 quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The column was a SLB-5ms 30 m × 0.25 mm id. × 0.25 µm of film thickness. The GC oven temperature program was: 50°C (hold 2 min) to 340°C (hold 20 min) at 20°C/min. Helium as the carrier gas was supplied at 1 mL/min (constant linear velocity). The injection temperature was 330°C, the injection mode was splitless for 1.5 min. Interface and ion source temperatures were 330°C and 200°C respectively. The MS ionization mode was electron ionization (EI) at 70eV.

3.2.4.3 HPLC system

A Varian model PS-210 HPLC gradient pump (Varian, Palo Alto, CA, USA) was used for analytical determination of PAHs. The mobile phase consisted of acetonitrile and water (flow rate of 1 mL/min). The gradient elution programm started with 40% acetonitrile (isocratic for 5 min), moving linearly to 100% acetonitrile (40 min total run time). The HPLC was equipped with a C_{18} column (Supelcosil LC-PAH, Supelco), 250 x 3mm I.D., 5 µm particle size, thermostated at 38°C. The detector was a programmable spectrofluoremeter (Jasco, model FP-1525, Cremella, Como, Italy); wavelength setting is reported in table 15. Quantification was carried out by the external standard method.

Table 15. *Wavelenght settin used for spectrofluorimetric detection*

	λ ex (nm)	Λ em (nm)
Na, Ac, F	276	330
Pa	250	366
A	250	402
Fl	270	470
P	240	386
BaA, Ch	270	390
BbF	260	430
BkF, BaP	256	410
DBahA, BghiP	290	410
IP	290	484

3.2.5. Migration tests and sample preparation

3.2.5.1 Mineral oil

3.2.5.1.1 Migration tests

The migration tests were carried out on selected samples, using glass weighing bottles of 4.6 cm of i.d.. Circular pieces of cardboard (with the same diameter of the glass weighing bottle) were cut from pizza boxes. The paperboard disks were laid on the bottom of the glass and covered with 0.66g of simulant forming a uniform layer (4g Tenax per 1 dm^2 in accordance with UNI EN 14338:2004). The glass weighting bottles were closed with the cap and sailed with aluminum and parafilm stripes. The samples were put in the oven at selected time and temperature conditions (30min at 70°C; 10 days at 40°C.

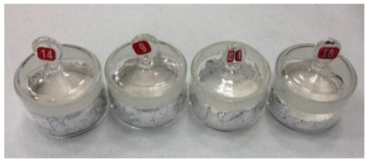

Figure 23*. Glass weighing bottles used for migration tests.*

After contact, the glass weighing bottles were transferred in the freezer at -20°C for 15 min. At the end cardboard and simulant were separated and stocked in aluminum foils in the freezer until the extraction

All migration tests were performed in duplicate

3.2.5.1.2 Mineral oil extraction from paperboard and Tenax

Extraction method for mineral oil in cardboard has been conducted as reported in [103]. Briefly, 1 g of cardboard was cut in small pieces of about 0.5 x 0.5 cm. The pieces were put into the 20 mL vials with 10 mL of *n*-hexane/ethanol solution 1:1 (*v/v*) and 20 µL of internal standard (Cho, CyCy, n-C_{11}, n-C_{13}, 5B, 1-MN, 2MN, TBB, Per). The extraction was carried out for 120 min, of which, the first and the last 5 minutes, under magnetic agitation. After that, 5mL of hexane were transferred in a vial and added with 10 mL of millQ water. Finally, an aliquot of the hexane extract was injected into the LC-GC-FID system. The same extraction method was used to assess the mineral content of the cardboard after migration test and the results obtained used to calculate the MOH lost by the

paperboard during the contact.
The simulant Tenax ® used in the migration tests was quantitatively transferred in a Teflon lined extraction vessel (GreenChem plus, CEM Corporation) and extracted in the microwave extractor after the addition of 20 mL of a mix *n*-hexane/acetone 1:1 (*v/v*) and 20 uL of the internal standard mixture. The sealed tubes were subjected to microwave assisted extraction for 20 min at 120°C. After cooling, an aliquot mL of milliQ water was added to 8 mL of extract and 2.5 mL of the *n*-hexane phase were concentrated at 500uL. An aliquot of 100uL of the sample was injected in the on line coupled LC-GC system to assess MOH migrated in the simulant.
To identify the sharp peak evidenced in the GC-FID trace of some cardboard samples (MOAH fraction), an extract of one of these samples was fractionated off-line on the LC column of the LC-GC system and the MOAH fraction was collected, concentrated and dissolved in 25 uL of hexane and injected into the GC-MS system.

3.2.5.2. Polycyclic aromatic hydrocarbons (PAHs)

3.2.5.2.1. Migration tests
Migration tests of PAHs were performed on a spiked sample as described in paragraph 2.5.1.1. To this purpose a circular specimen (i.d. 4.6 cm) of a clean cardboard (sample n.6) was spiked with 50 ng/g of BaP, Ch, BkF, DBahA, IP and 100 ng/g of BbFand BghiP and left to rest overnight in the dark. Migration tests were performed as described in the paragraph. Time and temperature conditions used to study migration of PAHs were 30 min at 70°C and 2 h at 70°C.

3.2.5.2.2 PAHs extraction from paperboard and Tenax®
To extract PAHs from cardboard, samples were cut in stripes and introduced in vial with 10 mL of acetonitrile. The vials were put in the ultrasonic bath for 30 min at 30°C. After extraction, 5 mL of the acetonitrile extract were transferred in a conical tube and concentrated at 1 mL. At the end 20 uL of the sample were injected into the HPLC system.
At the end of migration tests, the simulant Tenax® was transferred in a vial, added with 10 mL of acetonitrile and placed in the ultrasonic bath for 10 min at 30°C. Then the extract was concentrated 5:1 and injected into the HPLC.

3.2.5.3 Migration assessment

Migration from packaging into Tenax® was calculated directly in the simulant (“direct migration”), but also with an indirect method (“indirect migration”) by measuring the migrants lost by the packaging (comparing the contamination content of the paperboard before and after the contact) and assuming that all the migrants lost in the paperboard specimen migrated into the simulant). Migration data were expressed as percentage of the potential. Potential migration was calculated considering that all the migrants present in the cardboard before exposure migrated into the simulant.

3.3. Results and discussion

3.3.1 Mineral oil in "take away" pizza boxes

Based on their visual appearance and capacity to adsorb water, samples were separated into 2 groups, one including boxes apparently made of virgin paperboard (light brown or white color), the other including samples suspected to contain recycled fiber (with at least one grey or dark brown layer). The layer suspected to contain recycled fibers was the center wave, or the external cover (the inner layer which supports the printable layer).

As known, recycled and virgin paper have different water absorptivity. To test the capacity to absorb water, a drop of water was put in contact with the paper surface for some seconds observing its capacity to penetrate or not the fibers. It is well know that water penetration is more rapid in the presence of recycled fibers. The "drop of water test" confirmed classification made by visual observation. Of the 38 samples analyzed, 12 were classified in group II among samples suspected to contain recycled fibers, while the others in group I (virgin paper).

***Table 16**. MOSH and MOAH C_{10}-C_{35} and DIPNs concentration (mg/kg) in the samples.*

n.	Group	MOSH < C_{35}	MOAH <C_{35}	DIPNs	n.	Group	MOSH < C_{35}	MOAH <C_{35}	DIPNs
1	I	25.4	4.2	< 0.2	**20**	I	9.8	4.7	< 0.2
2	I	67.7	<2.0	< 0.2	**21**	I	7.9	3.6	< 0.2
3	I	13.0	40.1	< 0.2	**22**	II	386.8	29.5	2.9
4	I	5.6	9.0	< 0.2	**23**	II	596.9	33.8	0.2
5	I	14.5	26.8	< 0.2	**24**	I	44.6	< 2.0	< 0.2
6	I	6.4	< 2.0	< 0.2	**25**	II	337.3	39.4	7.3
7	I	5.5	6.6	< 0.2	**26**	II	317.0	51.4	7.4
8	I	2.6	< 2.0	< 0.2	**27**	II	294.3	70.3	3.2
9	I	3.7	< 2.0	< 0.2	**28**	I	5.2	< 2.0	< 0.2
10	I	6.4	8.6	< 0.2	**29**	II	370.4	58.8	11.2
11	II	675.3	28.8	3.4	**30**	I	6.0	3.3	< 0.2
12	II	368.3	21.0	1.5	**31**	I	10.3	< 2.0	< 0.2
13	I	7.9	5.6	< 0.2	**32**	I	19.0	3.3	< 0.2
14	I	23.0	15.8	< 0.2	**33**	I	3.2	4.1	< 0.2
15	I	44.8	4.7	< 0.2	**34**	I	13.4	4.2	< 0.2
16	I	24.3	18.3	< 0.2	**35**	II	210.2	20.2	1.7
17	I	20.1	12.9	< 0.2	**36**	II	123.6	10.4	0.3
18	I	8.7	3.7	< 0.2	**37**	II	135.1	9.8	0.3
19	I	17.4	5.4	< 0.2	**38**	II	203.2	26.7	0.9

Table 16 reports the results obtained by on-line LC-GC-FID analysis. The samples showed very different amount of MOSH C_{10}-C_{35} (from 2.6 to 675.3 mg/kg) and MOAH C_{10}-C_{35} (from <LOQ to 70.3 mg/kg).

The lowest MOSH and MOAH amounts were found in samples of group I. At the opposite samples of the group II, which presented at least one dark brown or grey layer, had the highest mineral oil contamination and contained detectable amount of DIPNs. Concerning the MOAH, some samples had high contamination with the typical distribution showed in fig 24.

Different samples were characterized by different chromatographic profiles. Figure 24 shows representative chromatograms of the analysed samples. Except for chromatogram of fig. 24*a* and 24*b* corresponding to an unprinted virgin paperboard box, respectively, which did not present detectable contamination, other traces showed different examples of contamination which will be discussed in the next paragraphs.

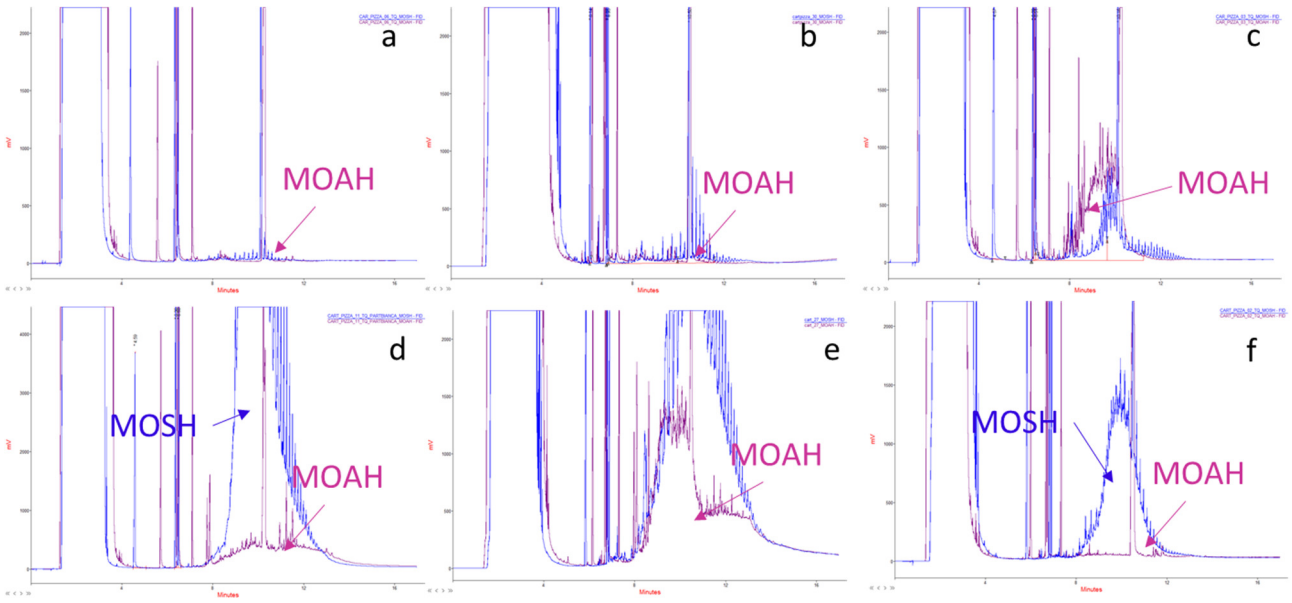

Figure 24. *LC-GC-FID profiles of different cardboard boxes: a) unprinted virgin paperboard (sample 6); b) printed virgin paperboard 1 (sample 30); c) printed virgin paperboard 2 (sample 3); d) printed recycled paperboard 1 (sample 11); e) printed recycled paperboard 2 (sample 27); f) paperboard containing paraffins from waterproofing treatment (sample2).*

3.3.1.1. Source of contamination

3.3.1.1.1. Recycled paper

Samples included in group II showed high levels of both saturated and aromatic hydrocarbons (MOAH C_{10}-C_{35} were from 4.3% and 19.3%) and had the typical chromatographic profile of recycled paperboard as shown in figure 24*d*. In these samples the profile of MOAH was characterized, by the presence of the DIPNs peaks. Even though there is no direct correlation between the amount of DIPNs and the amount of MOH, DIPNs are a good markers of recycled fiber used in the production of paper [137]. According to the XXXVI BfR Recommendation, this group of substances, originated from carbonless copy paper, should be present in the paper at the lowest technically possible concentration [9]. Cardboard analyzed contained level of DIPNs from 0.2 mg/kg to 11.2 m/kg. Similar concentration were reported by Zhang et *al*. (2008) (0.09-18.0 mg/kg) [144] and Triantafyllou et *al*. (2002) (12.5-15.6 mg/kg) [21] in paper and paperboard used as food packaging.

To confirm the presence of recycled fiber, as revealed by visual observation, 3 of the samples included in group I (samples 11, 22 and 37) were divided into the different layers, each one of which was

analyzed separately to evaluate the distribution of the mineral oil. As presented in the figure 25, samples 11 and 22 showed a Gaussian distribution of the contamination in the different layers. In fact, the corrugated wave center, showed the highest concentration of hydrocarbons (434.4 mg/kg and 184 mg/kg of MOSH C_{10}-C_{35} and 23.1 mg/kg and 14.3 mg/kg of MOAH C_{10}-C_{35}, respectively). Based on the Gaussian distribution of the contamination in the different layers, it was concluded that, in case of sample 11 and 12, the source of the contamination was the center wave, and that migration took place from the wave to the outer layers which were less contaminated. Differently, in case of sample 37, the most contaminated was the internal layer of the external cover (66.2 mg/kg of MOSH C_{10}-C_{35} and 4.5 mg/kg of MOAH C_{10}-C_{35}), while the internal cover, intended to come in contact with pizza, had the lowest amount of MOH.

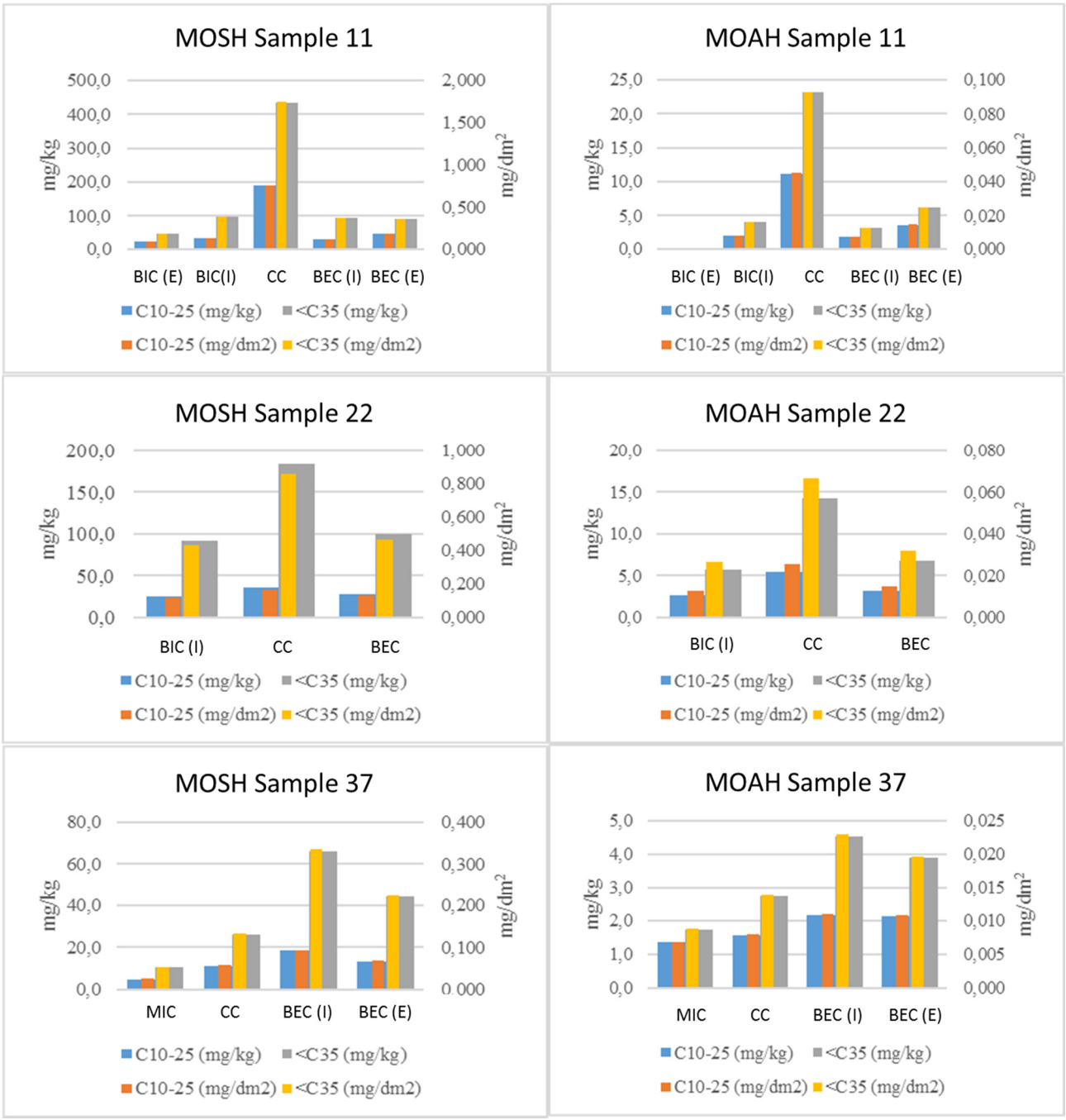

***Figure 25**. Concentration of MOHs (C_{10}-C_{25} and $<C_{35}$) in different layers of sample 11, 22, 37.*

3.3.1.1.2. Waterproofing treatments

Another source of contamination was the refined paraffin used in the production of some cardboard for waterproofing treatments (fig. 24*f*). These substances are highly refined petroleum products suitable for food contact because of the removal of the aromatic fraction. This type of contamination was observed only in 2 samples (samples 2 and 24 with 67.7 and 44.6 mg/kg of MOSH in the rage from *n*-C_{17} to *n*-C_{35}, respectively). MOAH were absent (figure 24f).

3.3.1.1.3. Printing inks

In addition to the contamination given by the presence of recycled fibers and waterproofing treatments, some of the pizza boxes analyzed presented a contamination characterized by the GC hydrocarbon profile showed in figure 24*c*, with MOAH higher than MOSH. Looking at the MOAH chromatograms, we can see a hump of partially resolved peaks with a range of volatility comprised between *n*-C_{16} and *n*-C_{28}. This profile was later related to the printing ink used in some of the samples. This contamination was found in 10 of the 38 samples and was more evident in samples 3, 4, 5, 10, 14, 17, independently on the presence of recycled fibers. Trace 24*c* refers to a sample included in group I (no presence of recycled fibers), while trace 3d refers to a sample classified in group II which was also contaminated with MOH.

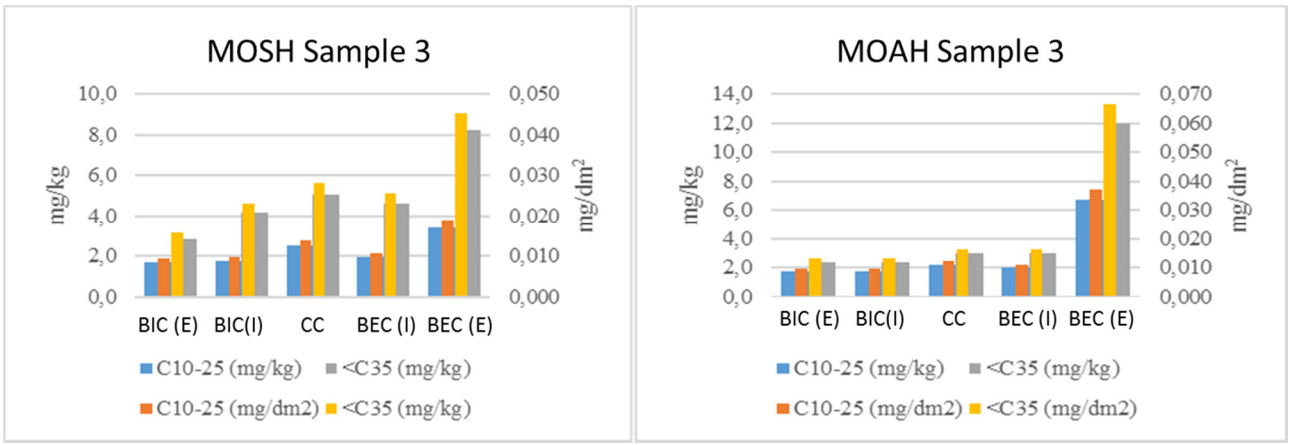

***Figure 26**. Amount of mineral oil C_{10}-C_{25} and <C_{35} in layers of sample 3.*

To confirm the origin of the contamination, specimens with different printed area were cut from sample 3 and 11. Different from sample 11 (printed recycled paperboard) where a homogeneous contamination was observed, independently on the amount of the printing ink, less intensely printed area of sample 3 had lower MOAH than more intensely printed area taken from the same paperboard. On the other hand, fig. 26 shows MOH distribution in the different layers, which confirmed that the contamination comes from the printing ink.

Finally, a GC-MS analysis was applied to better elucidate the nature of this contamination. The MOAH fraction eluted from the HPLC column of the on-line LC-GC system was concentrated off-line under a nitrogen stream and injected into the GC-MS. In fig. 27 the total ion chromatogram (TIC) and the extracted ion chromatograms at *m/z* 91 and 105, which are characteristic of the fragmentation pattern of linear alkylbenzenes (LABs), are reported. The GC-MS analysis evidenced the presence of two group of peaks that were assigned respectively to the C13-LABs and C14-LABs by comparison of the mass spectra with the instrument library and literature data [145]. The main compound in both

groups was the 2-phenyl isomer and in particular 2-phenyltridecane (C13-LAB-2) for the C13-LABS and 2-phenyltetradecane (C14-LAB-2) for the C14-Labs. The mass spectra of these compounds (fig. 28) are characterized by the base peak at m/z 105.

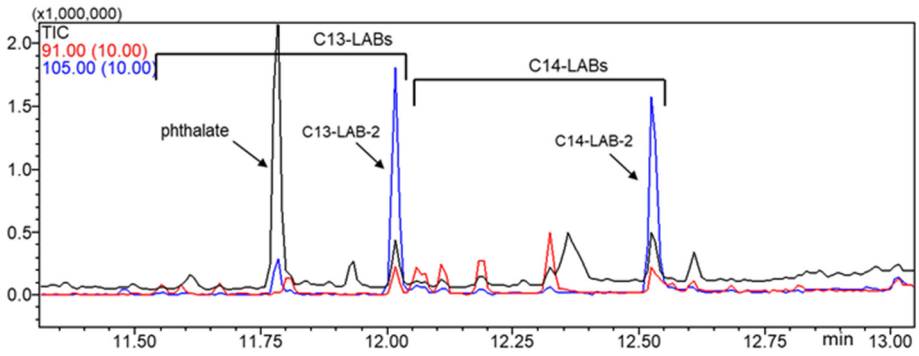

Figure 27*.GC-MS chromatogram of sample 3 characterized by the presence of C13-LAB-2 C14-LAB-2.*

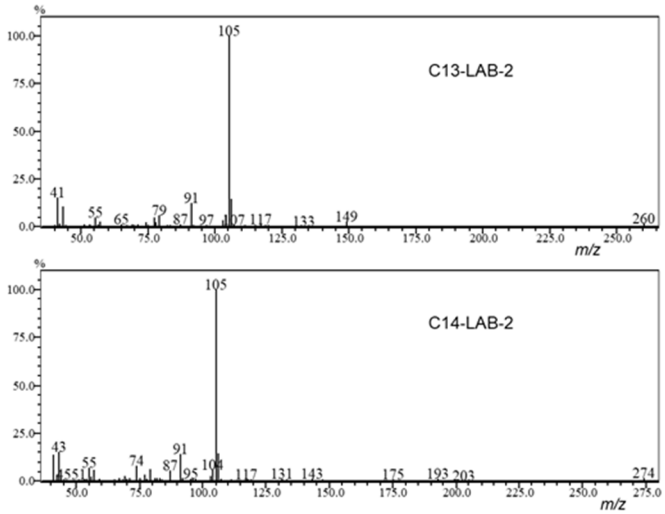

Figure 28. *MOAH fraction mass spectra of sample C13-LAB-2 C14-LAB-2.*

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Migration of alkylbenzenes, used as solvent components in certain printing inks, from offset printed packaging samples has been reported by [146].

3.3.1.2. Migration test for mineral oil

Mineral oils are largely transferred via gas-phase through evaporation from cardboard and recondensation in the dry food. Considering that pizza is a wet matrix, part of mineral oil in cardboard can migrate in pizza also through direct contact. Migration tests on pizza boxes were conducted referring to UNI EN 14338: 2004, using contact conditions reported in paragraph 3.2.5.1.1, chosen according to Reg. 10/2011[16]. Since some consumers has the habit to leave the pizza in the box and to consume it the day after, or to warm the pizza in the oven before consumption, a more severe migration test at 40°C for 10 days was also carried out. The test and subsequent quantification were carried out as reported in paragraph 3.2.5.1.1. The migration test was performed on sample 11, both on the whole cardboard and on the same cardboard after removing the internal cover.

Table 17*. Migration percentages about C10-C25 fraction of sample 11.*

	30 min 70°C		30 min 70°C without internal cover		10 days 40°C	
	% potential migration MOSH	% potential migration MOAH	% potential migration MOSH	% potential migration MOAH	% potential migration MOSH	% potential migration MOAH
Direct migration	17.9	29.9	20.4	30.3	93.7	92.6
Indirect migration	12.4	25.2	20.1	27.0	80.9	76.2

Direct and indirect percentage of migration were in good agreement. As can be seen after 30 minutes at 70°C, the percentage of migration was about 20% for the MOSH and 30% for the MOAH and these value increased with the contact time. As expected, the sample without the internal cover gave migration rate comparable to that of the whole sample. Such migration values, if applied to food, leads to overcome by far the migration limit of 2.0 mg/kg food for MOSH and 0.5 mg/kg food for MOAH, established in the 3rd mineral oil BMEL Ordinance.

3.3.2. Polycyclic aromatic hydrocarbons in “take away” pizza boxes

Despite the fact that polycyclic aromatic hydrocarbons are ubiquitous environmental contaminants, low amount of them can be found also in food packaging. Low amount of PAHs can be formed both in the manufacturing process of paper and board, and in the recycling process of cellulosic materials. Even though the Industry Guideline for the Compliance of Paper & Board Materials and Articles for Food Contact (2012)[8] established a limit for the maximum PAH content in cellulosic packaging (expressed as a sum of un unspecified compounds), literature data on the presence of these ubiquitous contaminants and their extraction and analytical determination in paperboard packaging, are very limited.

3.3.2.1. Optimization of PAHs extraction method

Generally, PAHs extraction from paper and paperboard consists in an ultrasonic assisted liquid extraction using methylene chloride or acetonitrile as indicated by Parigordi et *al.*(2011) [147] and by Vavrouš et *al.* in 2016 [61].
Due to its lower toxicity, acetonitrile was chosen as extraction solvent. Figure 29 reports recoveries obtained from a cardboard sample with a low contamination spiked at 3 different fortification level and extracted with acetonitrile in an ultrasonic bath for 30 min, as described in paragraph 3.2.5.2.2. Good recoveries were obtained for the 8 heavy PAHs (from 67 to 101.3%) except for BaP and BghiP at lowest level of fortification (34.1 and 54.4 % respectively). The light PAHs showed low and variable recovery levels probably due to their volatility. The HPLC chromatograms of spiked sample are shown in figure 30.

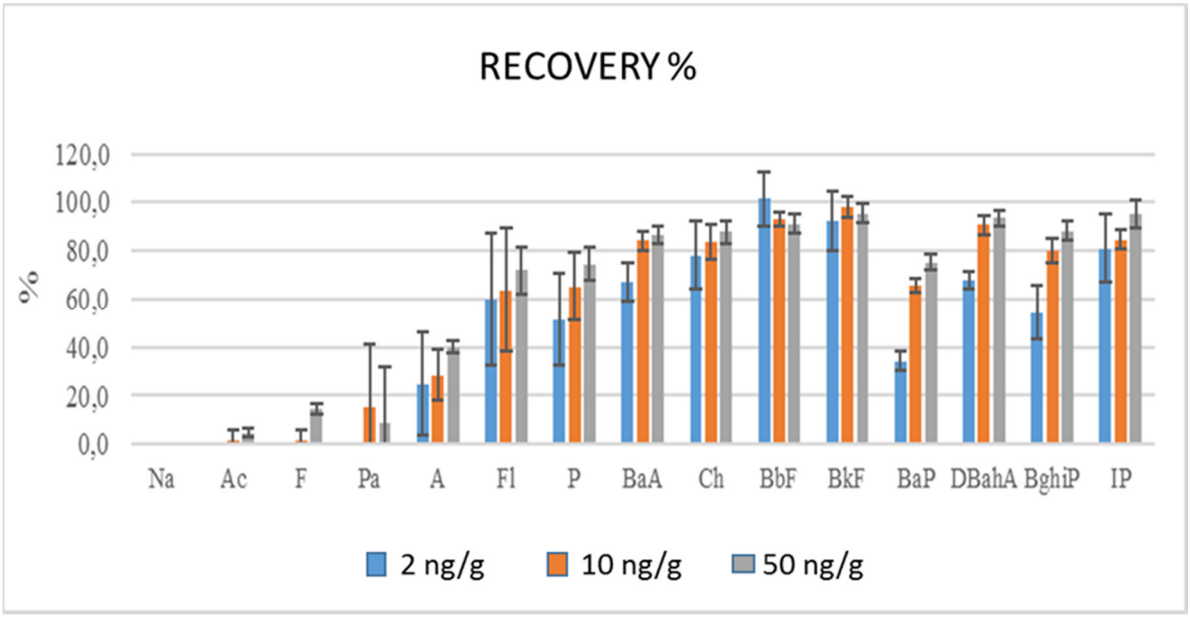

Figure 29. *Recovery of 8 PAH using optimized extraction method.*

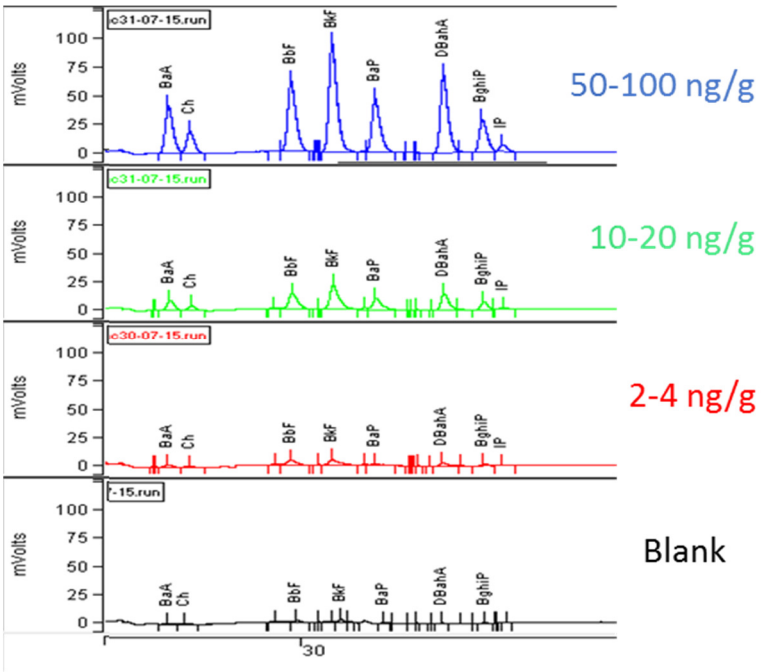

Figure 30. *HPLC traces relative to blanks and fortified samples at 2, 10 and 50 ng/g for BaA, Ch,BkF, BaP, DBahA, IP and at 4, 20 and 100 ng/g for BbF and BghiP.*

3.3.2.2. Results on real samples

PAHs content was evaluated in 27 cardboard pizza boxes (1-27) and in two recycled paparboard (printed and not printed), intended for food contact. Table 18 reports the data expressed in mg/dm^2. Light PAHs were present at higher concentration with respect to the heavy PAH. Phenanthrene was the predominant PAH and was present in each of the analysed sample at concentrations ranging from 30.2 to 868.8 ng/dm^2 (corresponding to 8.5 and 218.7 ng/kg). These data were lower than those obtained by Parigordi et *al*. 2011 for recycled food board [147] (400-1100 ng/dm^2), but similar to those found by Vavrouš et *al.* in 2016 [61] (comprised between 7.4 and 240 ng/g). Among high

molecular weight PAHs (BaA, Ch, BbF, BkF, DBahA, BghiP and IP) the most present was Ch (0.0-146.5 ng/dm^2 corresponding to 0.0-37.5 ng/kg) and BghiP (0.0-126.9 ng/dm^2 corresponding to 0.0-32.6 ng/kg). BbF and BkF were not detectable in many samples.

Table 18. *Sample concentrations of PAHs expressed in ng/dm^2.*

car	group	Na	Ac	F	Pa	A	Fl	P	BaA	Ch	BbF	BkF	BaP	DBahA	BghiP	IP	PAH 8
1	I	123.1	374.8	6.3	868.8	33.5	336.5	323.7	1.8	14.2	5.6	2.5	11.7	0.0	121.6	10.9	168.3
2	I	34.9	287.1	11.9	609.4	5.2	322.0	89.1	0.7	1.5	1.1	0.4	1.1	0.0	2.6	0.0	7.4
3	I	38.1	263.0	5.3	468.7	8.9	256.5	345.5	1.4	10.9	8.1	3.4	17.3	0.3	126.9	21.3	189.6
4	I	41.7	168.5	5.7	105.9	5.3	51.4	26.9	2.8	0.8	1.0	2.0	1.3	0.4	6.9	0.0	15.1
5	I	86.8	180.2	8.6	335.1	9.0	189.4	300.2	1.1	6.7	9.5	4.1	12.0	0.9	92.1	21.9	148.3
6	I	63.1	208.3	8.3	171.1	6.8	108.0	37.1	1.8	4.0	7.6	4.0	1.8	0.9	13.4	3.5	36.9
7	I	73.7	254.7	12.5	264.8	3.7	111.4	35.8	9.5	2.2	1.2	10.0	0.0	0.0	18.4	6.5	47.9
8	I	0.0	40.6	11.3	98.1	0.8	52.4	12.9	0.0	0.0	0.3	0.3	0.0	0.0	0.0	0.0	0.6
9	I	41.5	162.1	11.4	96.9	3.5	32.7	22.1	1.3	2.9	2.3	1.5	2.2	0.5	11.3	1.5	23.4
10	I	77.4	189.2	4.8	49.1	3.2	61.3	53.7	3.8	5.6	9.7	3.6	4.1	1.5	17.9	1.6	47.8
11	II	386.5	125.9	4.1	182.0	21.9	89.1	78.4	8.9	37.0	15.7	2.4	1.6	0.4	17.3	6.7	90.0
12	II	276.5	188.1	2.9	215.4	23.7	154.0	85.8	9.4	18.5	16.4	2.6	1.7	0.0	11.6	5.2	65.4
13	I	125.4	191.9	3.0	66.3	2.5	51.3	6.8	44.1	9.0	0.0	0.0	1.4	1.2	16.5	1.7	73.8
14	I	60.5	166.5	5.0	160.1	1.7	90.0	53.2	10.7	6.9	6.4	0.0	1.1	1.9	17.1	5.7	49.7
15	I	190.7	123.2	20.4	47.9	1.2	55.4	110.9	0.8	4.8	6.5	0.0	1.1	0.4	35.7	4.6	54.0
16	I	164.0	318.2	11.6	409.6	55.3	222.6	217.7	5.1	9.1	3.9	0.0	7.7	0.0	69.2	7.7	102.6
17	I	154.2	163.1	3.9	147.0	21.0	124.1	65.6	3.5	4.0	0.0	0.0	1.7	2.6	8.0	0.0	19.8
18	I	107.3	114.5	2.3	37.6	1.0	52.5	103.0	0.9	11.7	4.7	0.9	4.7	2.8	54.7	3.4	83.8
19	I	140.5	223.7	8.6	472.3	14.0	303.7	82.1	3.1	3.3	1.0	0.0	0.7	2.1	2.2	0.0	12.4
20	I	65.3	113.8	3.5	189.1	0.8	119.4	6.9	0.0	0.0	0.7	0.0	0.0	0.0	5.0	0.0	5.8
21	I	78.9	131.2	3.7	94.6	4.6	41.8	31.7	1.4	5.8	3.0	1.1	3.3	0.9	11.7	0.0	27.3
22	II	231.1	60.1	5.6	100.5	36.9	51.4	87.2	11.1	0.0	15.9	0.0	2.0	0.0	11.9	7.2	48.1
23	II	114.3	46.5	2.9	61.0	17.2	35.0	54.8	8.0	0.0	12.0	2.2	2.0	0.0	10.3	4.8	39.2
24	I	6.6	36.6	3.0	30.2	1.2	26.8	9.7	0.0	1.4	2.0	0.1	0.4	0.3	0.5	0.0	4.8
25	II	399.5	51.6	6.9	117.5	41.9	64.8	72.0	0.0	77.4	11.2	2.2	4.3	1.3	22.9	21.4	140.7
26	II	679.1	79.1	9.4	182.8	54.0	78.3	101.8	40.7	146.5	17.2	2.9	4.9	1.1	25.6	23.7	262.8
27	II	183.9	111.0	5.0	180.4	32.8	70.7	103.6	24.4	51.7	10.7	1.8	3.0	0.0	20.5	17.9	130.0
R		874.6	364.7	22.6	476.3	42.8	86.2	292.9	125.8	174.8	30.9	0.9	12.8	3.0	63.2	21.6	433.0
RS		592.3	291.7	29.1	611.4	42.5	145.9	254.0	106.6	250.0	34.7	1.9	12.1	3.0	66.4	25.9	500.6

Higher amount of all PAHs were observed in the two recycled paperboard samples but always with a predominance of the light terms. Before 2008 to monitoring these contaminants in the food only BaP was evaluated because it was considered as a marker of carcinogenic and genotoxic PAHs. Afterwards, the sum of 8 heavy PAHs (PAH8: BaA, Ch, BbF, BaP, BkF, DBahA, BghiP and IP) or a subgroup of 4 (PAH4: BaA, Ch, BbF, and BaP) were taken as reference. In 2011 the new EC Regulation [56] fixed limits for BaP and PAH4 for several food classes. The "Industry Guidelines" reported a limit of 1600 ng/dm^2 for the non-specified sum of PAHs. However, all analyzed samples

had contamination levels lower than the proposed limit, with PAH8 amounts from 0.3 to 256.4 ng/dm^2, while the two recycled paperboard showed PAH8 levels of 433 and 500.7 ng/dm^2 .
By observing the PAH profiles of different samples, it was evidenced that the relative abundance of some heavy PAH (particularly Ch, BbF and BghiP) allows to distinguish samples contaminated with mineral oils (recycled paperboard), as well as those printed with ink containing alkylbenzenes (figure 31).

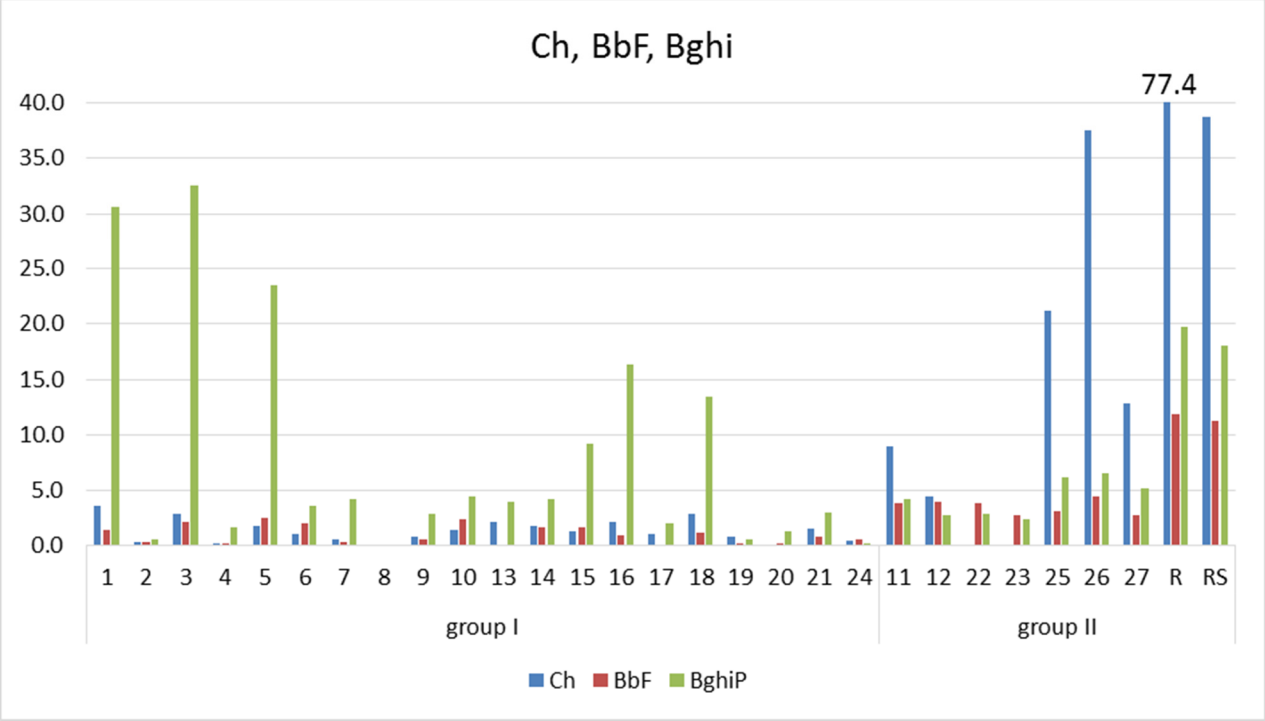

***Figure 31**. Ch, BbF and Bghi levels in samples of different groups.*

Particularly, in recycled paperboards and pizza boxes containing recycled fibres, Ch was present at higher concentrations than other PAHs, e.g. 37.5 ng/g (146.5 ng/dm2) in sample 26. The amounts obtained were very similar to those found by Vavrouš and co-workers [61]. In pizza boxes contaminated with the printing ink (alkylbenzenes), the prevailing PAH was BghiP. It is interesting to observe that the highest concentration of this hydrocarbon was found in samples in which the contamination with printing ink was more evident; in particular, BghiP reached 32.6 ng/g (126.9 ng/dm^2) in sample 3.

3.3.2.3. PAHs migration

3.3.2.3.1. PAHs extraction from Tenax®

Before studying and evaluating the extent of PAHs migration, extraction of hydrocarbons from simulant Tenax® was optimized. To assess the recovery the simulant was spiked with PAHs at 10-20 ng/g (each). Two different extraction techniques were compared: ultrasound assisted extraction and microwave assisted extraction.
No significant differences were observed when changing the extraction time from 10 to 30 min, demonstrating that a quantitative recovery of PAH8 can be reached already in 10 min of extraction. Ultrasound extraction gave extraction yield only slighter lower than microwave-assisted extraction at

120°C for 20 min, and cleaner extracts. Many interfering substances remained in the extract after microwave extraction, both in the blank and in the samples, so the integration of peaks was more difficult. Ultrasound technique was also chosen for PAHs extraction from Tenax® because it proved to be faster and less laborious than microwave extraction.

3.3.2.3.2. Potential migration

Migration studies were conducted on spiked samples using the developed migration tests reported in paragraph 3.2.5.1.1.

The migration process was evident for all PAHs. Data obtained for the cardboard after and before contact, illustrated in figure 32, demonstrate that some migration occurs already after 30 min of contact (from 1.7 to 29.8 ng/g) and increases after 2 hours (1.8 to 40.2 ng/g). Migration of these compounds accounted for 14% of the potential.

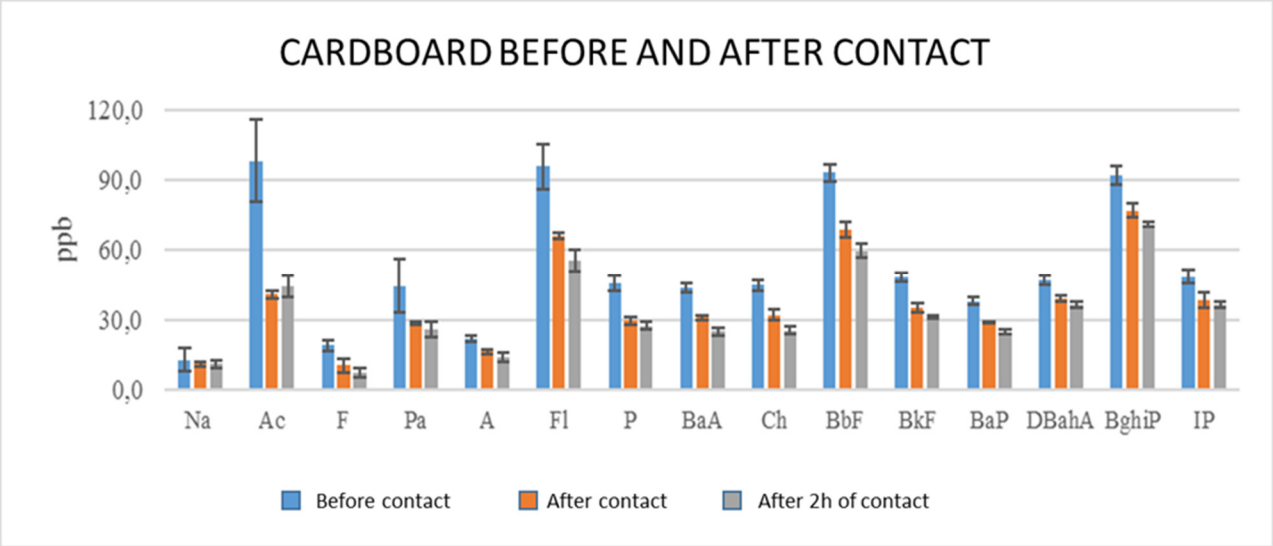

Figure 32. *Levels of PAHs in cardboard before and after contact at two different different time of contact conditions (30 min and 2hours) at 70°C*

Figure 33 reports migration data expressed as percentage of the potential. As expected, this percentage increased with the contact time. In particular, indirect migration raised from 0.1 to 13.1 % of the potential, while migration in Tenax® increased from 0.2 to 14.5 % of the potential. Amount of contaminants calculated using direct and indirect methods presented slight differences, but percentage values were very similar. About PAH8 it was observed that the percentage of migration decreased for PAHs with higher molecular weight. After 30 minutes of contact, indirect migration was 26.8% of the potential for BaP and 15% for IP. The difference was constant after two hour (38.8 % and 20.9 % respectively for BaP and IP). The high molecular weight compounds are not very volatile and for this reason, they migrate from the cardboard with more difficulties.

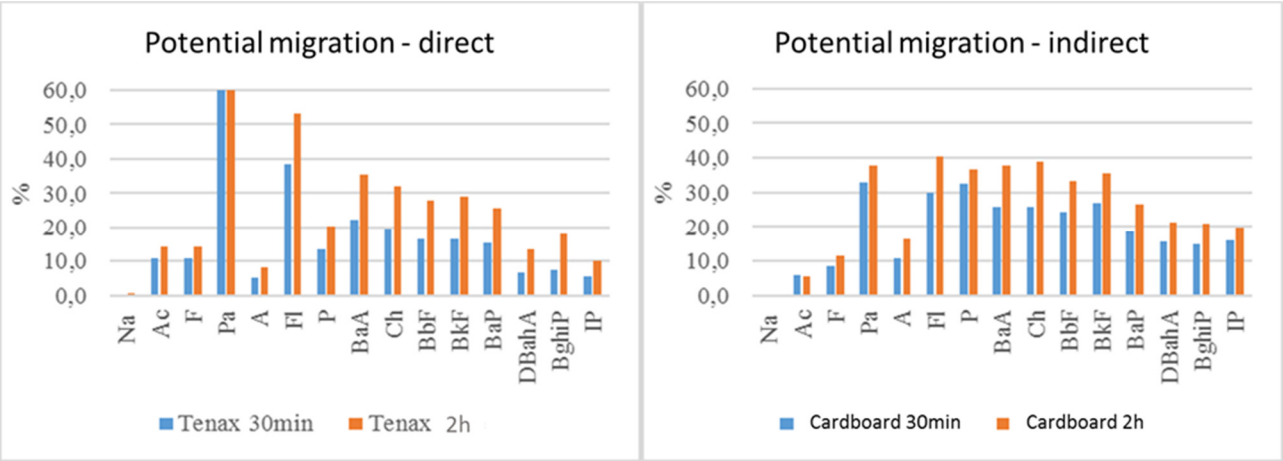

***Figure 33**. Percentage on potential migration*

3.3.3. PAHs and MOHs intake through consumption of "take away" pizza

Considering the average per capita pizza consumption (16 kg per years), the amount of mineral oil and PAHs in the analyzed pizza boxes, and the observed migration (expressed as percentage of the potential), the per capita daily intake of these packaging contaminants, was assessed. Considering a migration of 20% of the potential (for MOSH C_{10}-C_{35} contained in pizza boxes), the average daily intake resulted of 0.0050 mg/kg b.w. Compared to the average total daily intake reported by EFSA in 2012 (0.039-0.068 mg/kg b.w.), it was calculated that the contribution due to pizza ranges between 7.3% and 12.8% of the total. Concerning PAH8, the calculated average daily intake from pizza boxes resulted of 0.0015 ng/kg b.w. (migration of these compounds accounts for 14% of the potential), with a maximum of 0.0067 ng/kg b.w for the most contaminated sample, while the average daily intake reported by EFSA for the total diet was of 28.8 ng/kg b.w. Comparing these results, it can be concluded that the contribution to the global dietary intake of PAHs, as result of the migration from food packaging is negligible (0.005 %). Since migration tests have been carried with simulant under condition that can overestimate migration, migration tests using real food sample should be carried out to confirm these data.

3.4. Conclusions

This study allowed to evidence a great heterogeneity concerning the quality of the paperboard boxes used for pizza transport. Three source of contamination were clearly identified: illegal presence of recycled paper, printing ink containing alkylbenzenes, and refined paraffin (*food-grade* mineral oil used in the waterproofing of paper). Boxes containing recycled paperboard can be easily distinguished based on visual observation. The presence of recycled paper (banned by law in cardboard intended for contact with wet foods) was confirmed by the high levels of MOSH and MOAH and by the identification of DIPNs. The most contaminated samples reached 675.3 mg/kg of MOSH and 70.3 mg/kg of MOAH C_{10}-C_{35}.

All the cardboard boxes contained amount of PAHs below the limit proposed by the Industry Guideline (0.0016 mg/dm^2). Cardboard made with recycled fiber showed high levels of Ch, while higher amount of BghiP were observed in the boxes contained alkylbenzenes.

Even if no legal limits was established for these substances, these levels of contamination might be dangerous to consumer health .
The migration tests, conducted with Tenax® as simulant, under different contact conditions, have demonstrated the potential of these contaminants to migrate from cardboard into the pizza.
The per capita daily intake from “take away” pizza was calculated both for MOHs (0.0050 mg/kg bw per day) and for PAH8 (0.0015 ng/kg b.w. per day). PAHs contribution to global diet intake is negligible (0.005%), while for mineral oils the average contribution ranges between 7.3% and 12.8%.

4. MIGRATION OF POLYPROPYLENE OLIGOMERS INTO READY TO EAT VEGETABLE SOUPS

4.1. Introduction

Polyolefins represent the food packaging material most widely used in contact with food in most cases as primary packaging in direct contact with the food.

Material migrating from polyolefins into food mostly includes oligomers, here named polyolefin oligomeric hydrocarbons (POH), which comprise a major fraction consisting of saturated hydrocarbons (POSH), and variable amounts of monounsaturated hydrocarbons (POMH). POH originate by incomplete polymerization during the polymer production and are present in variable amounts in polyethylene (PE) and polypropylene (PP) packaging from which they can easily migrate into food. Some reaction products (i.e. oxidation products), have been also identified in the polymers. Toxicological information on polyolefin oligomers are lacking, but due to their similarity to mineral oil saturated hydrocarbons (MOSH), their presence in food is of concern. Similarly to MOSH, POSH mainly consist of linear and branched alkanes; the presence of linear and branched cyclopentane and cyclohexane has been observed in PE but not in PP [64].

Concerning POSH safety, it should be evaluated as in the case of the MOSH, considering their bioaccumulation potential in human tissues [85]. Migration of POSH into infant formula exceeded the temporary acceptable daily intake (*t*ADI) established by the JECFA in 2002 for MOSH of the same molecular mass range [73]. Due to insufficient data to support this *t*ADI, it was later withdrawn [80]. New toxicity data are expected to establish updated ADI values.

On-line HPLC-GC, according to the method developed by [104] for mineral oil determination, allows for a rapid determination of POH, but did not allows to distinguish hydrocarbons of mineral origin (MOSH) from those of synthetic origin (POH). Furthermore, the HPLC-GC method did not pre-separate POSH and POMH, which co-elute with the MOSH fraction. The presence of POMH can be determined indirectly with a second analysis after derivatization (bromuration or epoxidation) of the double bonds. Very recently Lommatzsch, et al. [115] used a rather complex HPLC-HPLC-GC-FID system with a first silica gel column to separate MOSH and MOAH, and a second silver-impregnated HPLC column to achieve POSH and POMH separation. After the removal of the olefins, mass deficiency (2 Da per ring) also enabled to distinguish between open chain POSH (POSHoc) and the cyclic POSH (POSHcy). The combination of on-line HPLC-GC-FID with GC×GC, which represents the most powerful technique to characterize complex hydrocarbon mixtures, allowed to distinguish between MOSH and POH [116].

Even though under favorable conditions POH may migrate in high amount from the packaging into the food, till now relatively few works investigated migration of POH into foods. High fat content and high contact temperatures are expected to accelerate migration.

In 2012, Biedermann et al. [114] determined the migration of the polyolefin oligomeric hydrocarbons (POH) into various dry foods, including infant formula, using on-line HPLC-GC-FID without POSH and POMH separation [137]. POH coeluted with MOSH and were distinguished from MOSH by their typical chromatographic pattern. In a similar manner, [131] analysed POH in egg pasta packaged in PP film, finding migration up to 1.7 mg/kg .

The food industry offers a wide range of ready-to-eat products, among these vegetable soups (and other products), designed to be stored at refrigeration temperature for several weeks, or for longer time at ambient temperature, and to be heated in the packaging container before use, mostly by applying microwave heating.
The aim of the present work was to evaluate, for the first time, POSH residues in PP containers used in contact with vegetable soups, and to evaluate their migration into the food product, both during storage in the container and after microwave heating, according with the instruction reported on the label.
On-line HPLC-GC followed by flame ionization detection (FID), according to [93], was applied for POH analysis in a number of ready-to-eat vegetable soups packaged in PP containers, all intended to be heated in a microwave oven for a few minutes. Migration tests in a clean extra virgin olive oil, used as food simulant, were carried under different conditions: at refrigeration temperature (to simulate storage in the fridge) and at different temperatures using both microwave and conventional heating. Others parameters affecting migration such as duration of microwave heating, as well as fat content, were studied.

4.2. Materials and methods

4.2.1. Reagents and standards

All solvents and reagents were from Sigma–Aldrich (Milan, Italy). Hexane and dichloromethane were distilled before use. Ethanol was of HPLC grade. Water was purified with a Milli-Q System (Millipore, Bedford, MA).
The *n*-alkane C_{10}-C_{40} standard mixture (50 mg L^{-1} each) was purchased from Sigma–Aldrich. Internal standards were from Supelco (Milan, Italy). The working standard contained: 5-α-cholestane (Cho, 0.6 mg mL^{-1}), n-C_{11} (0.3 mg mL^{-1}), n-C_{13} (0.15 mg mL^{-1}), cyclohexylcyclohexane (C_yC_y, 0.3 mg mL^{-1}), *n*-pentylbenzene (5B, 0.30 mg mL^{-1}), 1-methylnaphthalene (1-MN, 0.30 mg mL^{-1}), 2-ethylnaphthalene (2-MN, 0.30 mg mL^{-1}), tritert- butylbenzene (TBB, 0.3 mg mL^{-1}) and perylene (Per, 0.6 mg mL^{-1}) in toluene.
Before use, all the glassware was carefully washed and rinsed with distilled acetone and hexane.

4.2.2. Instrumentation

The microwave extractor used for POH extraction from food products was a Mars-X (CEM Corporation, Matthews, NC) able to process up to 14 samples simultaneously.
The on-line HPLC–GC instrument was an LC–GC 9000 from Brechbühler (Zurich, Switzerland) and consisted of a Phoenix 40 with three syringe LC pumps and four switching valves and a UV/Vis detector (UV-2070 Plus; Jasco, Tokyo, Japan). The GC was a Trace GC Ultra from Thermo Scientific (Milan, Italy). The autosampler was a PAL LHS2-xt Combi PAL (CTC, Zwingen, Switzerland).
The concentration unit consisted of a centrifuge (Univapo 100 H, Uniequip System; Martinsrieder, Munich, Germany), connected to a vacuum pump (Buchi, Flawil, Switzerland).

4.2.3. Samples

Twelve vegetable soup samples plus one potato puree (6 different brands) and respective PP containers, were analysed for their POH content, the latter both before and after microwave heating. Heating conditions were those reported on the label. Most of the products contained only vegetables (V), while others contained also legumes (L) and/or cereals (C). Samples included also one puree (PU). All samples were directly purchased from the supermarket. To evaluate variability due to lot production in one case 3 samples of the same product (V4), but different production lot were purchased, while in another case two samples of the same product (VLC) and same lot. Table 19 reports a description of the samples and a classification of the containers into 7 groups; based on their visual appearance (color, internal surface, thickness, etc.) container with similar characteristics were classified in the same group. Instruction on the label indicated to drill the protective film (or to lift it before heating). Only in one case it was indicated to remove the protective layer before heating. Suggested heating conditions were in all cases, except one, 4 min (in one case 3 min) at a power ranging from 750 to 1000W. Container were all in PP.

Table 19 *Overview of the sample analysed*

Sample code	Ingredients	Brand	Plastic type	Container weight (g)	Food weight (g)
V1	Mixed vegetables	A	I	26	620
V2	Mixed vegetables	C	III	26	600
V3	Mixed vegetables	D	V	26	350
V4a	Pumpkin and carrot	B	IV	26	620
V4b	Pumpkin and carrot	B	IV	26	620
V4c	Pumpkin and carrot	B	IV	26	620
V5	Cauliflower and cabbage	G	VII	29	600
V6	Porridge and potatoes	G	VII	29	600
LC	Legumes and cereals	E	VI	32	620
VLCa	Vegetables, legumes and cereals	F	VI	23	600
VLCb	Vegetables, legumes and cereals	F	VI	23	600
VC	Vegetables, arley and spelled	H	III	26	620
LP	Pasta and beans	B	IV	26	600
PU	Potato puree	B	II	26	450

4.2.4. Extraction

Oligomer extraction from the plastic container was performed according to the methods proposed by [114]. Since the container before the contact with the food was not available, a strip of the plastic material, taken half a centimeter below the upper edge of the container (part usually not in contact with the food) was cut into little pieces. About 300 mg were exactly weighted into an extraction vial, added with 10 mL of hexane and extracted overnight under magnetic stirring. As an alternative, the same sample amount was directly weighted into a Teflon lined vessel (Green Chem plus, CEM

Corporation), added with 10 mL of a mixture hexane/ethanol 1:1 (v/v) and subjected to microwave assisted extraction (80 °C for 30 min). After cooling and water addition to separate the ethanol from the hexane phase, the latter was directly used for the HPLC-GC analysis.
Food samples were extracted according to the method described by [96]: 5 g of sample, previously homogenized (if necessary), were directly weighted into a Teflon-lined vessel, added with 5 µL of internal standard solution, 10 mL of a saturated methanolic potassium hydroxide (KOH), and 10 mL of *n*-hexane. Microwave-assisted saponification and simultaneous unsaponifiable extraction was carried out at 120 °C for 20 min. After cooling the vessels were opened and added with about 20 mL of water and 3-4 mL of methanol (without mixing), and left to rest for about 20 min at -20 °C to facilitate phase separation avoiding the formation of emulsion. After concentration (5 mL were concentrated to 1 mL), the hexane extract underwent on-line HPLC-GC analysis. Limit of quantification of 0.1 mg/kg were reached by applying this concentration factor. Recovery tests were performed on an home made zucchini soup spiked with a known amount of POH previously extracted from a PP container.
For migration test carried out using different food simulants, the oil simulant (a clean extra virgin olive oil) was diluted with hexane and analysed directly by on-line HPLC-GC. Ethanol 95% was added with hexane, mixed and the added with water to obtain and hexane extract free from ethanol. After concentration, the hexane extract was injected into the HPLC-GC apparatus.

4.2.5. Chromatographic conditions for HPLC-GC analysis

The HPLC column was a 25 cm x 2.1 mm i.d Lichrospher Si 60, 5 lm (DGB, Schlossboeckelheim, Germany). The GC was a Trace GC Ultra from Thermo Scientific (Milan, Italy).
A gradient, starting with hexane 100% and reaching 30% of dichloromethane after 0.5 min at a flow rate of 300 µL/min was used to separate the MOSH (fraction from 2.0 to 3.5 min) and the MOAH (fraction from 3.8 to 5.3 min). HPLC-GC transfer occurred through the Y-interface based on the retention gap technique and partially concurrent eluent evaporation [104]. A 10 m x 0.53 mm i.d. uncoated, deactivated precolumn was connected by a steel T-piece union to the solvent vapour exit (SVE) and a 15 m x 0.25 mm i.d. separation column coated with a 0.15 µm film of PS-255 (1% vinyl, 99% methylpolysiloxane) (Mega, Legnano, Italy). A rapid oven gradient (40 °C min^{-1}) starting from 55 up to 350 °C was used for GC analysis [93].
The FID and the SVE were heated at 360 and 140 °C, respectively. After the transfer, the LC column was backflushed with dichloromethane) and reconditioned prior to the subsequent injection. The data were acquired and processed by the ExaChrom software (Brechbühler, Switzerland). Data were processed using the Exachrom Software. Quantification was based on internal standards (mostly in the case of food) or, on external standard, when internal standard peaks coeluted with the POSH. Endogenous *n*-alkanes peaks were detracted from total contamination when analyzing food and fat simulant.

4.3. Results and discussions

4.3.1. POH extraction

The aim of this first part of the work was to quantify POH in the plastic containers. To achieve this goal, extraction conditions were optimized in order to obtain complete recovery of hydrocarbons up to C_{35} (upper molecular weight limit of interest by a toxicological point of view), limiting the extraction of higher molecular weight hydrocarbons, which tend to accumulate in the retention gap and in the first part of the capillary column, determining a rapid decrease of the GC performance.
To check the completeness of POH extraction from PP containers, some preliminary trials were carried out on different aliquots of the same plastic material, which was in part reduced into squares of about 0.5 cm^2, and in part cut into particles of about 1 mm of diameter. Different aliquots (300 mg) of the same sample were extracted at ambient temperature for different times (overnight for 16 h, and for 3 days) with 10 mL of *n*-hexane. No differences were found between the samples cut into squares and those reduces in particles, when extracted overnight with hexane, as well as between those extracted with hexane at 60 °C for 30 min and for 3 days at ambient temperature (figure 34).

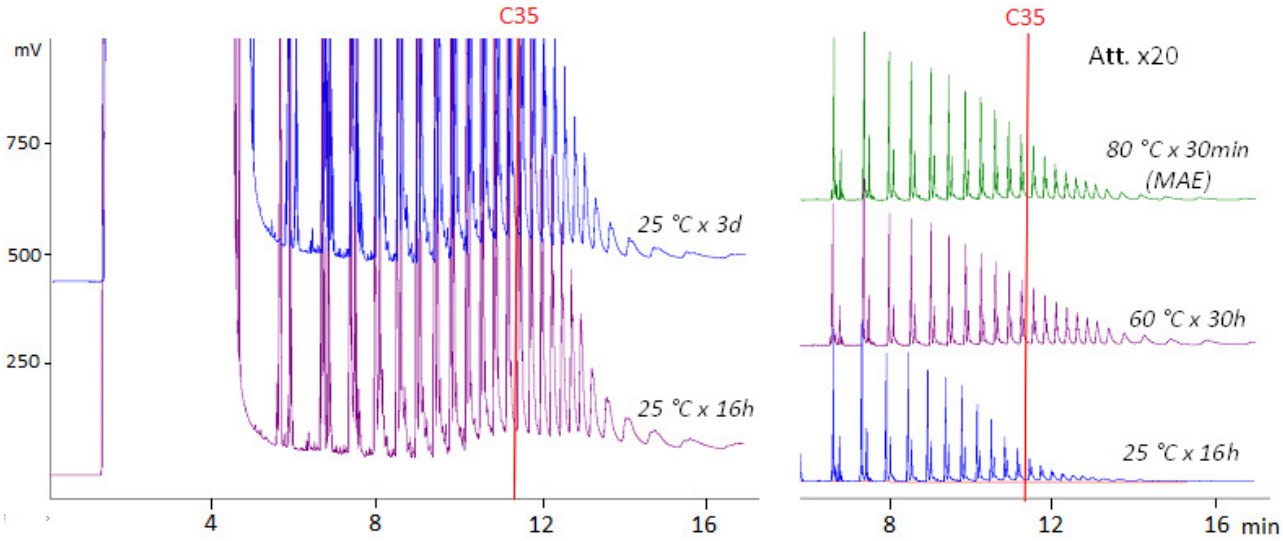

Figure 34. *Optimization of POH extraction from PP containers.*

By increasing the extraction temperature, oligomers of higher molecular weight were extracted in higher amount. With respect to extraction at ambient temperature, extraction at 60 °C for 30 h allowed to obtain comparable extraction of hydrocarbons up to *n*-C_{21}, and slightly higher yield of high molecular weight hydrocarbons (fig.34) in the range up to *n*-C_{35}. Particularly, recovery of POH in the range *n*-C_{16}-C_{35} at ambient temperature were 94% of those obtained at 60 °C (30h). Extraction at higher temperature (80 °C for 30 min) using both traditional and microwave heating, gave complete extraction for POH up to *n*-C_{35}, with no differences depending on the heating mode (traditional or microwave). Completeness of the extraction was further demonstrated with a second extraction at higher temperature (140 °C). This temperature determined the release of high molecular weight oligomers, which got dirty the retention gap and the GC column, making necessary strong column washing and replacement of the retention gap.

In conclusion, extraction at 80 °C for 30 min with *n*-hexane allowed to obtain practically quantitative extraction of POH up to *n*-C_{35} and can be advantageously used to process simultaneous 14 samples speeding up sample extraction. Overnight extraction with *n*-hexane can be also used. It gave only slightly lower extraction yield in the range *n*-C_{20}-C_{35} (yield over 93%). Repeatability trials (6 replicates of the same sample) gave coefficient of variations lower than 4%, for overnight extraction, and lower than 6% for microwave assisted extraction at 80 °C for 30 min.
Extraction of POH from food was carried out by applying microwave assisted saponification as described by [96]. In order to validate the use of this procedure on this matrix, recovery tests (6 replicates) were performed on a vegetable soup sample, with very low contamination, which was spiked with an extract of POH obtained from a PP container. Practically quantitative recovery and low coefficient of variations were found (<10%).

4.3.2 POH in PP containers

Figure 35 reports POH amounts (mg/kg) found in the plastic containers, which for total POH *n*-C_{10}-C_{35} ranged from 1096 to 4679 mg/kg, and the LC-GC traces of two sample with low and high POH content.
By considering the weight of the plastic container and the weight of the food in contact with the container, potential migration calculated by assuming that all the POH up to *n*-C_{35} present in the packaging migrated into the food during microwave heating, ranged from 53 to 242 mg/kg.

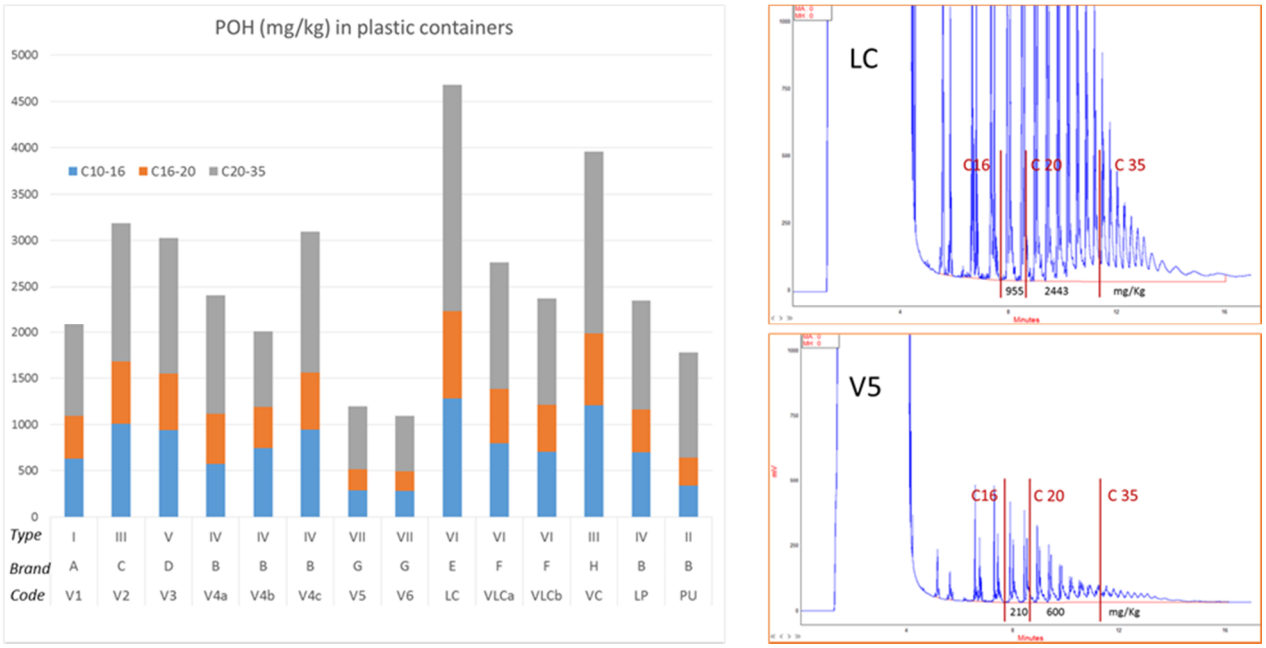

Figure 35. *POH content (mg/kg) of plastic containers (on the left) and HPLC-GC traces of two samples (V5 and LC) with different contamination levels (on the right)*

All samples had peak clusters showing humps of oligomers built of C_3-unit, which are typical of PP. Interesting to observe that the two samples with lower POH content were of the same plastic type, indicating as it is possible to obtain product with lower residual POH.
Except for these samples, other samples showed relatively limited variability: maximum of a factor of two. Three samples of the same product type (V4), but different lot, showed higher variability (2011-3093 mg/kg), than the two samples of the same product type and same lot (VLCa, 2375 mg/kg;

VLCb 2763 mg/kg). Some authors demonstrated that POH are only formed during polymerization, not during processing (film extrusion, corona treatment, blow molding) and this could explain the relatively low variability observed in most of the PP containers.

4.3.3 POH/MOSH in food products before and after microwave heating

Table 20 *POH/MOSH content (mg/kg) of vegetable soup samples before and after microwave heating*

Sample code	Fat (%)	POH/MOSH (mg /kg) BEFORE MW					POH/MOSH (mg/kg) AFTER MW				
		C_{10-16}	C_{16-20}	C_{20-35}	$>C_{35}$	C_{10-35}	C_{10-16}	C_{16-20}	C_{20-35}	$>C_{35}$	C_{10-35}
V1	1.8	0,3	0,3	0,5	0,4	1,1	0,4	0,5	3,1	0,5	4,1
V2	0.6	0,7	0,6	1,4	0,2	2,7	2,4	2,2	4,3	1,8	8,9
V3	1.0	0,4	0,6	2,1	1,5	3,0	0,3	0,6	1,8	2,2	2,8
V4	2.6	0,1	0,1	0,8	0,3	0,9	1,2	1,3	4,1	2,0	6,6
V5	1.7	0,1	0,1	0,5	0,3	0,6	0,1	0,2	0,8	0,9	1,0
V6	1.9	0,1	0,1	0,5	0,2	0,6	0,1	0,2	0,4	0,5	0,7
LC	1.0	0,3	0,3	0,4	0,1	1,0	0,3	0,5	0,9	0,3	1,7
VLC	3.1	0,3	0,3	1,0	0,2	1,6	0,8	0,7	1,4	0,7	3,0
VC	0.5	0,3	0,2	0,3	0,1	0,8	0,6	0,7	1,2	0,6	2,4
LP	1.1	0,5	0,4	1,3	0,3	2,3	0,9	0,8	1,6	0,6	3,4
PU	2.8	0,2	0,6	0,2	0,1	1,0	0,5	0,1	0,4	0,3	0,9

Most of the products contained detectable POH already before microwave heating. Some samples were clearly contaminated with MOSH. Since POSH cannot be distinguished from MOSH by on-line HPLC-GC, results reported in table 20 are expressed as POH/MOSH.
Figure 36 shows the HPLC-GC traces of a selection of samples with different contamination profiles.

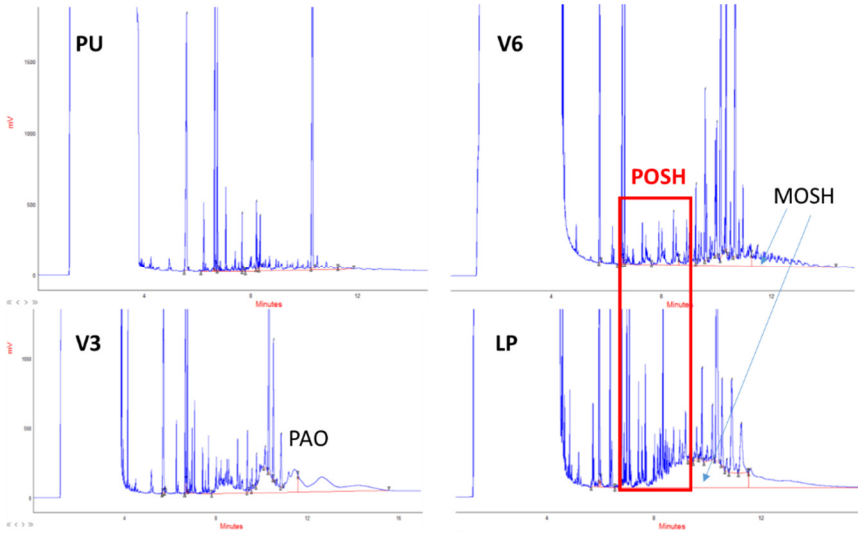

Figure 36 *HPLC-GC traces of vegetable soup/puree samples before microwave heating*

Sample PU had very little contamination. Sample V3 had the typical profile of a sample contaminated with polyalpha olefins (PAO) from adhesives. Further investigations on this sample demonstrated that the contamination was not from the PP container, or adhesive applied on it. It was concluded that it was already present before packaging and probably derived from one contaminated ingredient.

Samples V6 and LP had a profile characterized by the presence of POH (well visible in the first part of the trace) and MOSH (forming a hump) in the second part of the trace.
Contamination levels of samples before microwave heating ranged from 0.6 to 3.1 mg/kg (on average 1.4 mg), while after microwave heating ranged from 0.7 to 8.9 mg/kg (on average 3.3 mg/kg).
The presence of an evident contamination with POH already before the microwave heating, seems to indicate that some migration also occurred during the product shelf life, or during hot filling at the production plant.
To evaluate if some migration can also occurs during storage at refrigeration temperature (such product have a shelf life of about 2 months) during the product shelf life, two vegetable soup samples were purchased in double (2 samples of the same lot) and analyzed before the microwave heating. One was analyzed after storage at 4 °C for 6 weeks (it was opened at the expiry date), and another 6 weeks before. Figure 37A shows the LC-GC-FID traces obtained. Results showed as migration at refrigeration temperature occurs, but very slowly. Also migration tests performed at 4 °C using vegetable oil as simulant (using the same packaging to food contact condition as in the real condition; the specimen was completely dipped in the oil) confirmed low migration values (figure 37B). It was concluded that the high contamination found in some vegetable soups is mainly due to migration occurring during hot filling at the production plant.

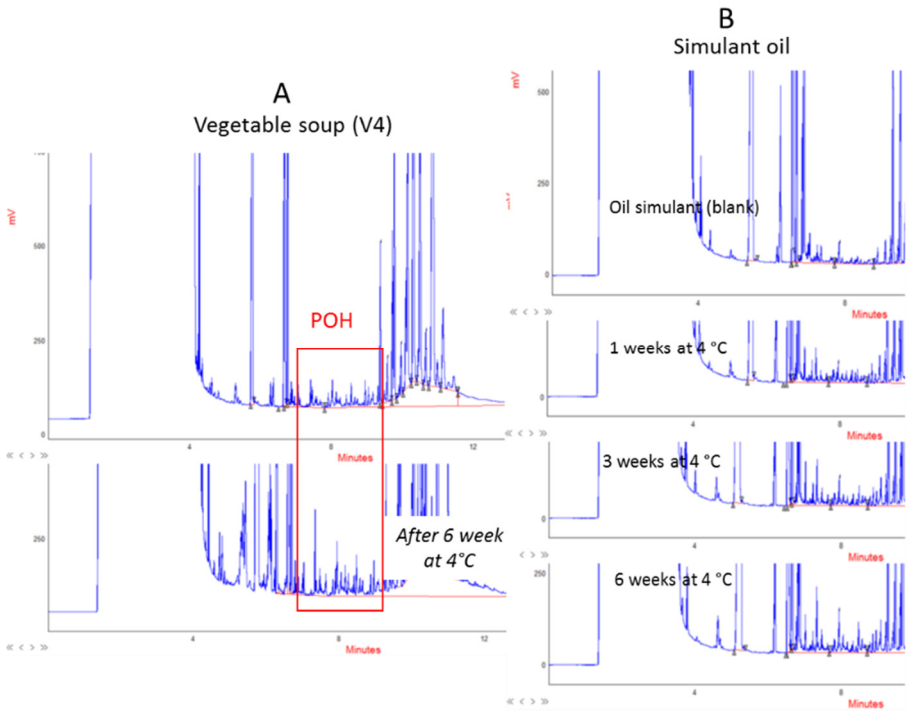

Figure 37. *POH migration at 4°C: A) in real sample, B) in oil simulant*

In general, POH content increased considerably after microwave heating. Nevertheless, the increase was more or less evident depending on the sample. Figure 38 shows two examples where such increase is well evident.

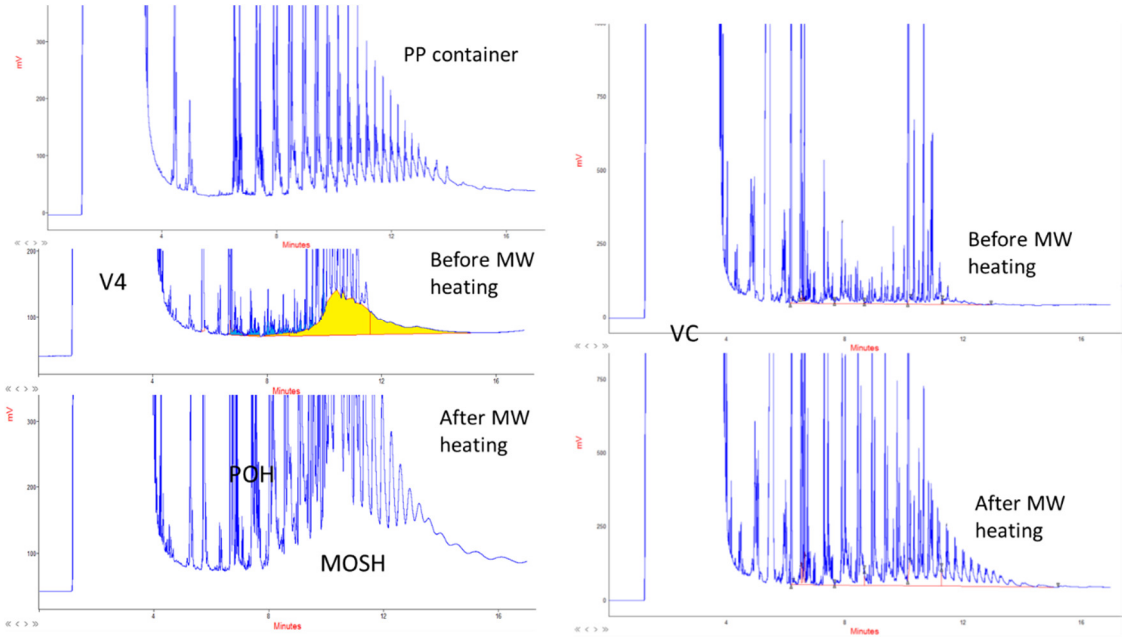

Figure 38. *Effect of microwave heating*

Sample V4 is a fresh vegetable soup with a little MOSH contamination before microwave heating, which increased its POSH content after 3 min at 750 Watt by a factor of 8.

To calculate net migrated POH, results obtained for soup samples before microwave heating were detracted from those obtained after the heating treatment. Figure 39 reports data regarding net POH migration (expressed in mg/kg for POH *n*-C_{10}-C_{35}) and migration rate expressed as percentage of the potential migration.

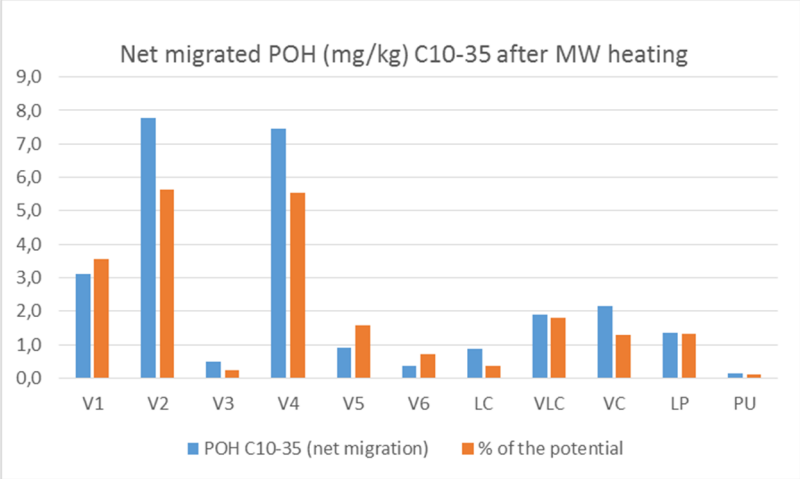

Figure 39. *Net POH migration as a consequence of microwave heating*

As we can see, POH migrated in the soup represent only a little part of those which could potentially migrate from the PP container. This probably depend on the low fat content of these food products. Fat content can certainly influence the amount of POH migrated, but no clear correlation was found between the fat content and the amount migrated. Migration rate is probably influenced also by the original amount present in the PP container (in fact low migration was found in sample V5 and V6 packaged in containers with the lowest POH concentration), sample composition and heating conditions. To better understand parameters affecting migration, a study has been undertaken in food and food simulants

4.3.4. Migration behavior in food and food simulants

According to Regulation EC 10/2011, the compliance of a plastic material intended to be placed in contact with food should be proved with migration tests carried out under standardized conditions, using contact conditions which are at least as severe as the real contact conditions. Annex III of the Regulation indicates simulants designed for different food groups. Simulants designed for "processed vegetables in the form of purée" (reference number 04.05 C) include aqueous simulants (acetic acid 3% and ethanol 20%). Nevertheless, vegetable soups, which are mainly aqueous foods not prone to extract lipophilic contaminants such as oligomers of polyolefins, also contain same fat, which can favor migration of such contaminants. For this reason, simulants for fatty foods such as simulant D2 (vegetable oil), or fat substitute simulant should be also used in migration tests. Such substitutive simulant can be, for example, applied when there is an interference due to the oil matrix.
To investigate the effect of fat amount on migration rate of POH, a fresh pureed zucchini (prepared at home), was added with different amounts (1, 2 and 6%) of a clean extra virgin olive oil, and homogenized with a Politron until obtaining a homogeneous emulsion. Aliquots (5 g each) of the pureed zucchini used as food simulant, were directly weighed in Teflon-lined extraction tubes, put in contact with 200 mg of plastic material and heated in a domestic microwave oven at 700 W for a constant time (1 minute). Longer time were avoided because they determined water evaporation and sample concentration. Results obtained clearly demonstrated that the fat content has an important impact, not only on the amount of POH migrated (the migration rate increased with increasing the oil content), but also on the molecular weight of the migrants. The higher is the fat content, the higher is the amount of high molecular weight hydrocarbons, which migrated into the food. Fig 40 shows the effect of different fat amounts on POSH migration.

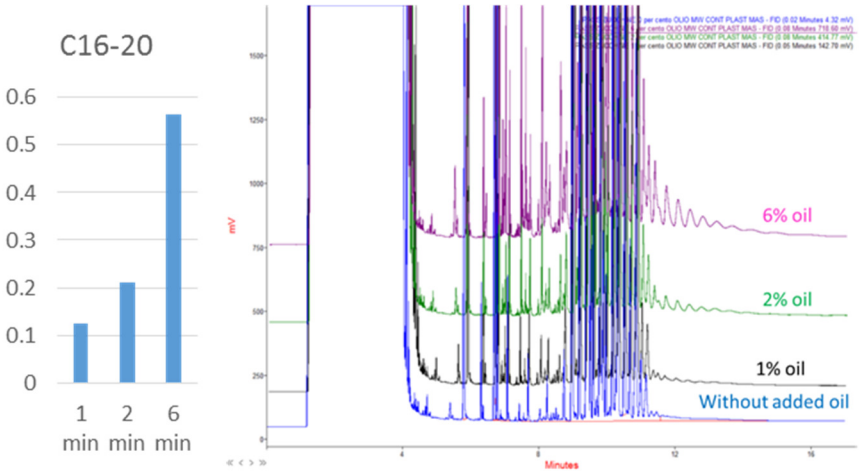

Figure 40. *Effect of different fat amount on POH migration*

Migration tests using oil as simulant were then performed to test temperature and time effect as well as to compare traditional oven heating with microwave heating. Figure 41 show the results obtained testing temperature and time effect on extra virgin olive oil used as simulant at 80°C for 30 min. Even though in the extraction with *n*-hexane there were no differences among traditional and microwave heating, comparing results obtained in the case of simulant oil with microwave heating the migration of POH was favorited and presented a value about twice higher than those obtained during traditional heating (figure 41 left). Probably, when using oil as a simulant, compared to traditional heating,

microwave heating causes a faster swelling of the plastic that facilitates a rapid release of entrapped oligomers. The same behavior is not evident when using hexane as extraction solvent, because in this case the swelling of the plastic takes place quickly in both conventional and microwave heating. On the other hand, the time led an increase comparable for microwave and traditional heating. Furthermore, the temperature had great effect on the extraction. As reported in figure 41 (right) temperature of 60°C allowed a migration of about 10 mg/kg of POH, while increasing the temperature at 100°C the migration increase at level of about 30 mg/kg.

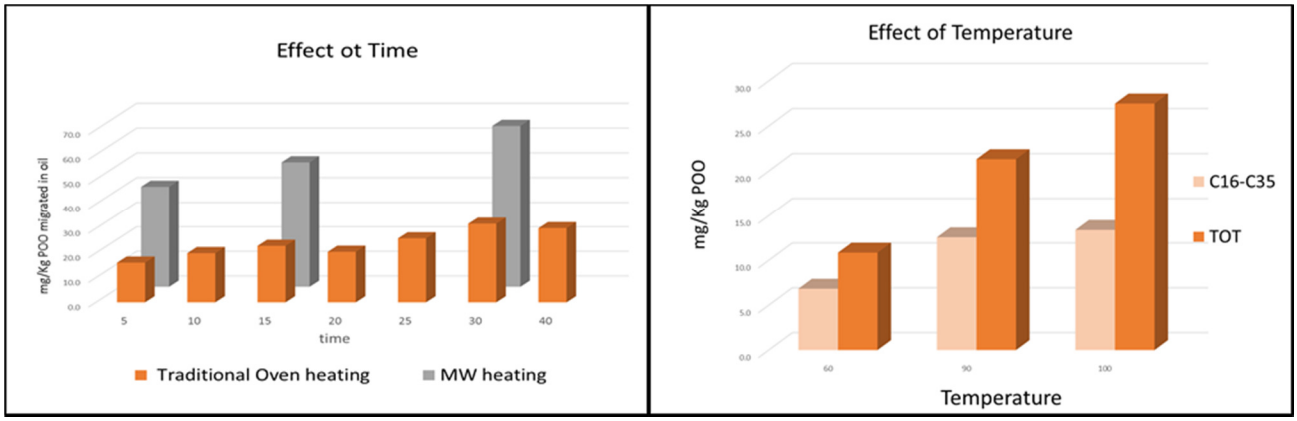

Figure 41. *Effect of time and temperature on POH migration in extra virgin olive oil simulant.*

4.3.5. Migration testing and food simulants

Use of food simulants allows to simplify analytical determination of migrant. POH analysis in vegetable oil used as fat simulant is simpler and more rapid than in the food and only requires oil dilution followed by injection into the LC-GC system.

Nevertheless, vegetable oil used as simulant contains, as all vegetable oils, endogenous *n*-alkanes in the range *n*-C_{21}-C_{35}, which can complicate quantitative determination of oligomers co-eluting with these hydrocarbons, forcing the analyst to analyse the oil before and after the contact and to subtract the contribution due to the oil.

In the presence of unavoidable interferences from the food simulant D2, use of substitute simulant (ethanol 95%) is also admitted. Fat reduction factors (FRF), comprised between 2 and 5 (depending on the fat content of the real food), are then used to account for the higher extraction power of such simulants.

Table 21 compares migration data obtained with real food (home-made pureed zucchini, added and not added with 6% of a clean extra virgin olive oil) with those obtained with simulant ethanol 20%, 50% and 95%, as well as with vegetable oil. Migration tests were performed at 80°C for 30 min using microwave assisted extraction. These temperature/time conditions were chosen to cover domestic heating condition where the product can reach temperature higher than 80°C and may remain in contact with the hot food for longer time during food consumption. Five grams of food/simulants were weighted into the extraction vessel and put in contact with about 200 mg of the plastic material (to approximately maintaining the same food to container mass ratio as in the real situation).

Table 21. *Comparison of different simulants on migration of POH at 80°C for 30 min.*

	POH (mg/kg)		
	C10-16	C16-35	>C35
VS (no oil)	0,6	3,2	0,2
VS (6% oil)	3,4	6,0	2,5
EtOH (20%)	0,0	0,0	0,0
EtOH (50%)	5,3	0,9	0,0
EtOH (95%)	7,1	16,2	4,2
EVOO	32,3	87,0	16,7
EVOO (FRF=5)	6,5	17,4	3,3

VS, home-made vegetable zucchini puree;
EtOH , Ethanol;
EVOO, extra virgin olive oil

As we can see from the table, extra virgin olive oil overestimate (by a factor of 2) migration found in vegetable soup added with 6% of oil, also when applying a FRF of 5 (as should be made in this case). Migration assessed in ethanol 95% gave an extraction yield 5 time lower than in the oil and underestimated migration over C_{35}. Since the focus was on the POH up to C_{35}, such simulant seems to be the best choice for migration testing for compliance assessment, but the use of a FRF should be evaluated carefully.

4.4. Conclusions

PP containers widely used to store and heat ready to use foods can release considerable amounts of POSH. Fat content and microwave duration are important parameters affecting the amount migrated. In the vegetable soups the level of POSH/MOSH before the microwave heating were up to 3 mg/kg. However after migration test at the refrigerated temperature the amount of MOSH/POSH migrated in the soup confirming that the pre-existing contamination of the soup is probably due to the hot filling during the production.

In general POHs content increased considerably after microwave heating but respect the potential migration only a part of POHs was transferred to food depending from original amount of PP containers, fat content of food and heating conditions.

Different simulant were tested to assess the migration of the POH and their performance was compared with those in real food. Vegetable oil demonstrated to be a good simulant and, when applying a FRF of 5, overestimated real migration of a factor of 2 (in the case of the vegetable soup added with 6% of oil). Nevertheless, it give interference problem due to the presence of endogenous *n*-alkane. For this reason use of ethanol 95%, as substitute simulant seems to be the best choice for the migration test, taking into account that in this case the use of the FRF is not advisable since it could lead to underestimate the real migration.

5. DEVELOPMENT OF METHODS TO DETECT THE PRESENCE OF HYDROCARBON CONTAMINANTS IN MYCROCRYSTALLINE WAXES AND THEIR RELEASE IN ARTIFICIAL SALIVA

A part of this chapter has already been published in**:** Conchione, C., G. Purcaro, L.S. Conte, and S. Moret. 2015. "Solid-Phase Microextraction with Gas Chromatography and Mass Spectrometry Determination of Benzo(a)pyrene in Microcrystalline Waxes Used as Food Additives." *Journal of Separation Science* 38 (10).

5.1. Introduction

Microcrystalline waxes are refined mixtures of saturated hydrocarbons, mainly branched paraffins, and cyclic compounds obtained from petroleum [148], they are elastic and often "sticky" or "tacky". This is because of the high proportion of multi-branched hydrocarbon chains (iso-paraffins) as opposed to straight-branched chains (normals). While normal paraffins form large crystals, iso-normal paraffins form micro-crystals. These small crystals give an amorphous structure to the product making it very flexible in contrast to the brittle nature of normal paraffins. Microcrystalline waxes contain long carbon chains over *n*-C_{60}. They are characterized by a kinematic viscosity of 11 mm^2/s at 100 °C, an average molecular weight of 500 g/mol and a carbon number 25 at 5% distillation point [73]. Microcrystalline waxes (E905) are authorized *quantum satis* as surface treatment agents on non-chocolate confectionery chewing gum and decorations, coatings and fillings, except fruit based fillings. They are also permitted as a surface treatment of melons, papaya, mango, and avocado [149]. In chewing gum, microcrystalline waxes are used to modify the properties of the chewing gum base. The wide range of properties available help chewing gum base manufacturers to formulate a broad variety of chewing gum, ranging from the traditional hard stick gum to the softer bubble gum. Furthermore, microcrystalline waxes are used in wax paper production and plastic in contact with food [16]. In fact, hydrocarbon paraffin and microcrystalline waxes are used by themselves or in combination with other additives (e.g. high molecular weight polyethylene and ethylene vinyl acetate copolymers) to improve the performance of paper packaging and flexible packaging [150]. Despite being highly refined, the residue of toxic compounds, in microcrystalline waxes needs to be kept under control. Even if some products are not intended for ingestion (chewing gum, plastic or paper), the microcrystalline waxes included in their composition may get in direct contact with human mouth and saliva and interact with the latter. Migration tests using artificial saliva are important tools to evaluate release of contaminants. Artificial saliva was used in some studies to evaluate migration of phthalates from plastic toys [151][152]. Furthermore European commission indicates artificial saliva as simulant in the determination of n-nitrosamines and n-nitrosatable substances released from elastomer or rubber teats [153]. Polycyclic aromatic hydrocarbons (PAH) in microcrystalline content is considered in the chemical specification for microcrystalline waxes by the Joint Expert Committee on Food Additives (JECFA) [154] which suggest, a determination of the extractable polycyclic aromatic hydrocarbons (PAHs) using UV spectrometry in reference to a standard solution of naphthalene. This approach is amended by Commission Regulation (EU) No 231/2012 [155] that established a maximum limit of 50 µg/kg for benzo(a)pyrene (BaP) without defining any analytical method and excluding any other PAHs from the analysis. However, in its opinion of 2013, concerning

medium-viscosity oils, EFSA stressed the need to supplement this measurement of benzo[a]pyrene by characterising three other PAHs[149]. Due to matrix interference and the low solubility of microcrystalline waxes in organic solvent commonly used for PAH extraction, the analytical determination of BaP in petrolatum and petroleum waxes represents a difficult task. In 1960, a complex method based on a combination of adsorption chromatography on magnesia Celite and subsequent paper chromatography using different solvent systems was developed by Lijinsky [156] for determination of BaP and three other PAHs (dibenz[*a,h*]anthracene, benz[*a*]anthracene, and chrysene) in petroleum waxes. In the mid-1960s, the Food and Drug Administration (FDA) undertook an extensive research program to develop a method for PAHs determination in petrolatum and petroleum waxes. This method, reported in the US Federal Register, was later applied by Howard et al. [157]. In 2000, a similar reference method to evaluate PAHs in microcrystalline waxes was reported by the JECFA[154]. Briefly, the sample, dissolved in a heated sulfoxide-phosphoric acid mixture and *iso*-octane, undergoes several liquid–liquid partition steps requiring large consumption of organic solvents (about 150 mL of dimethylsulfoxide/phosphoric acid mixture and 200 mL of isooctane per sample). After the partition, samples should meet the UV absorbance limits posed by the JECFA. If these limits are exceeded, UV absorbance must be measured again after adsorption chromatography on a mixture of magnesium oxide/Celite 545 (PAHs are eluted with 300 mL a mixture of acetone/benzene/water). If UV absorbance still exceeds the limits, the sample is rejected. Based on the work of Grimmer and Böhnke [158], who selectively isolated PAHs from triglycerides of vegetable oils and lipid extracts using liquid–liquid partition between cyclohexane and a mixture of DMF/H2O 9:1 v/v, Mandalakis et al. [159] isolated PAHs from complex mixtures of aliphatichydrocarbons. Briefly, PAHs are extracted into the DMF phase while triglycerides and/or aliphatic hydrocarbons remain in the cyclohexane and are discarded. After water addition PAHs can be back-extracted into cyclohexane or hexane. Mandalakis et al. [159] tested biphasic systems of DMF with different aliphatic solvents (cyclohexane, *n*-pentane, *n*-hexane, *n*-heptane, *iso*-octane) and different water percentages. They found that the type of aliphatic solvent affected partition ratios of individual PAHs, which achieved the highest values with *iso*-octane and *n*-pentane (and the lowest value with cyclohexane). They also found that the enrichment factor of PAHs against *n*-pentane (selected as the extraction solvent, due to its lower boiling point with respect to *iso*-octane) gradually increased with increasing the water content (from 0 to 5%), and decreased for higher proportions of water. In recent years, environmentally friendly techniques, able to reduce time and solvent consumption [59][160][161][162], have gained particular attention. Among these, SPME, using a particular fiber coating, namely, Carbopack Z/polydimethylsiloxane (PDMS) has given interesting results for PAH extraction from vegetable oils and lipid extracts [163][164][165]. This fiber, which presents a high affinity for planar compounds, was used for the first time to analyze dioxins from organic solvents [166]. The primary mechanism of action is related to the $\pi - \pi$ interaction between the carbon surface and planar compounds, when the fiber is dipped in a non-polar solvent.

The aim of the present work was to develop and validate a method using an easy liquid–liquid partition method followed by SPME with Carbopack Z/PDMS for BaP determination in microcrystalline waxes, reducing sample manipulation, analysis time, and solvent consumption with respect to the JECFA method for total PAHs [154]. Characterization of gas chromatographic profile of waxes was also evaluated. In addition, artificial saliva from chewing tests was collected and analyzed in order to evaluate possible release of hydrocarbon and among these particularly of BaP from waxes, gum bases and chewing gums during their contact with human saliva in the mouth.

5.2. Materials and method

5.2.1. Reagents and standard

Hexane, cyclohexane, and DMF were of HPLC grade (Sigma - Aldrich, St. Louis, MO, USA). Before use, all the glassware was carefully washed and rinsed with acetone and HPLC grade *n*-hexane. Water was purified using a Milli-Q System (Millipore, Bedford, MA, USA). Stock solutions of BaP and [$^{2}H_{12}$] benzo[*a*]pyrene (*d12*-BaP) were obtained from Dr. Ehrenstorfer (Augsburg, Germany).

5.2.2. Sample preparation

5.2.2.1. Waxes

5.2.2.1.1. Dissolution of waxes
To obtain a homogeneous sample, some grams of the microcrystalline waxes were melted and poured on a pre-cleaned aluminum foil. After solidification in a thin layer, the sample was reduced into little pieces. About 5-25 mg of the sample (depending on the solubility of the waxes) was added with an internal standard (*d12*-BaP) and diluted with 20 mL of cyclohexane (100 mL in the case of low solubility microcrystalline waxes)

5.2.2.1.2. Sample purification

5.2.2.1.2.1 BaP analysis
20 mL of the wax dissolved in cyclohexane were partitioned with 20 mL of DMF/H2O (9:1 v/v) in a separatory funnel. After phase separation, the DMF/H2O phase was transferred in a second separatory funnel and washed with 20 mL of cyclohexane. The DMF/H2O phase, transferred in a third separatory funnel, was then added with 20 mL of MilliQ H2O and extracted with 20 mL of *n*-hexane. After phase separation, 10–20 mL of the *n*-hexane extract was concentrated to 1.5 mL for SPME under magnetic stirring. The fiber, a Carbopack Z/PDMS (15 m film thickness), kindly provided by Supelco (Bellefonte, PA, USA), was exposed for 15 min at room temperature.

5.2.2.1.2.2. Mineral oil analysis
For hydrocarbon profiling, 400uL of wax solution were diluted at 2 mL with *n*-hexane and directly injected in the GC-FID system.
For MOSH and MOAH analysis, 250 µL of the MCW solution, dissolved in cyclohexane, were purified on silica gel activated at 400 °C for 1 night (1g). The silica gel was weighted on a glass cartridge (6 mL) with a frit at its bottom, packed with hexane, and after sample loading, MOHs were eluted with a mixture of *n*-hexane/dichloromethane (70/30). The first mL (dead volume) was discarded, and the following 4 mL were collected, concentrated and then fractionated on silver silica gel according to the method described in [90]. MOSH and MOAH fractions, as well as the intermediate fraction, collected to check the separation between MOSH and MOAH, were concentrated (to 250 µL) and injected separately (50 µL) into the GC-FID.

5.2.2.2. Dissolution in artificial saliva

Artificial saliva was kindly prepared in an external lab and furnished ready to use. It is a solution of water and different salts such as sodium bicarbonate, sodium chloride and potassium carbonate.
Briefly, two samples of waxes, two samples of gum bases and two samples of final products (chewing gum) were powdered using a mortar and liquid nitrogen. The final granulometry was checked with sieve, resulting in less than 425 µm for all samples. 1.5 g of powdered sample were weighted for each test and dissolved into 75 ml of artificial saliva. Solutions were processed through the Varian VK7025 dissolution apparatus using the following settings: temperature 37 °C, agitation 100 RPM, stirring time 20 minutes. At the end of the analysis, the samples were filtered through Whatman GF/A. A blank of saliva was generated for this method, processed as samples, and called as "blank dissolution"

5.2.2.2.1. BaP and mineral oil analysis
20 mL of artificial saliva were extracted twice with 3 mL of cyclohexane (vortex 2 min); combined extracts were gently taken to dryness and dissolved with *n*-hexane (1.5 mL for BaP analysis and 2 mL for mineral oil analysis). A 1.5 mL of extract were used for SPME, using a Carbopack Z/PDMS fiber, while 2mL extract were collected and subsequently injected in the LC-GC-FID system. Deuterated BaP was used as internal standard.

5.2.3. Analytical determination

5.2.3.1. GC- MS conditions for BaP analysis

Analyses were carried out with a system consisting of a GC2010 gas chromatograph and a QP2010 Ultra quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The column was a BPX50 9 m x 0.10 mm id. × 0.10 µm *d*f column (50% phenyl polysilphenylene-siloxane). The GC oven temperature program: 80 (hold 2 min) to 170 °C at 70 °C/min to 350 °C at 15 °C/min. Helium as the carrier gas was supplied at an initial pressure of 474.4 kPa (constant linear velocity). The injection temperature was 350 °C and the
injection mode was splitless for 10 min. MS parameters: The samples were analyzed in the fullscan mode with a scan speed of 10 000 amu/s and a mass range of 50–350 *m/z* and simultaneously in the single ion monitoring mode, considering the 252 *m/z* ion for BaP and 264 *m/z* ion for *d12*-BaP. Spectra generation frequency: 20Hz. Interface and ion source temperatures were 250 and 200 °C, respectively. The MS ionization mode was electron ionization (EI).

5.2.3.2 LC-GC method for BaP analysis

The automated on-line LC-GC instrument was from Brechbühler (Zurich, Switzerland). The LC column was a 25 cm x 2.1 mm i.d. packed with Lichrospher Si 60, 5 µm (DGB, Schlossboeckelheim, Germany). The MOSH fraction was eluted from 2.0 to 3.5 min and transferred to the GC operating at a hydrogen constant pressure mode. The fraction comprising the MOAH ranged from 4.0 to 5.5 min. 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, the LC column was backflushed with dichloromethane. The transfer of the LC fraction into the GC was carried out through the *Y*-interface using the retention gap technique [104]. A 10 m x 0.53 mm i.d. uncoated, deactivated precolumn was followed by a steel T-piece union connected to the solvent vapor exit and a 10 m x 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 velocity of 4 mL/min. The oven temperature was programmed at 40 °C/min to 50°C from 350°C. FID (sampling frequency 50 Hz) and the SVE were heated at 360 °C and 140 °C, respectively. The saturated hydrocarbon fraction was eluted from 2.0 to 3.5 min, while the aromatic fraction including BaP was eluted from 4.3 to 5.8 min. Data was acquired and processed by the ExaChrom software (Brechbühler, Switzerland).

5.2.3.3. GC- FID conditions to analyze the microcrystalline waxes

The analytical determination of mineral oil was performed using a GC 7890A (Agilent Technologies) equipped with an autosampler 7693 and a FID.
The sample extract was injected into a 5 m x 0.53 mm i.d. retention gap attached by a press-fit connector to a 15 m x 0.32 mm i.d. separation column coated with cross linked Select Mineral Oil (0.15 µm; Varian, Milan, Italy). The oven temperature was programmed at 50 °C/min, from 65°C (4 min; solvent evaporation) to 380°C (maintained until the end of analysis). The carrier gas (helium) flow rate was set at 6 mL/min. The FID detector was thermostatted at 380°C. Data were acquired and processed by the ChemStation software.

5.3. Results and discussion

5.3.1. Determination of BaP in microcrystalline waxes

5.3.1.1. Optimization of sample preparation

The starting point for method optimization was an SPME-GC-MS method previously set up for BaP determination in vegetable oils and lipid extracts[163][164][165].
As previously highlighted [163], the fiber maximized its performance when dipped directly in *n*-hexane, therefore the first trials were performed by dissolving the microcrystalline waxes in such a solvent. Sensitivity problems, due to the low solubility of microcrystalline waxes in *n*-hexane, and an excessive uptake of aliphatic alkanes, which hindered the low BaP signal, were observed. To overcome these problems, the samples were solubilized in cyclohexane (solubility of microcrystalline waxes was about 10/15 times higher in cyclohexane and in *iso*-octane than in *n*-hexane), and a purification step to eliminate the bulk of hydrocarbons, present in very high concentrations with respect to BaP, was introduced. Sample purification was developed starting from the works of Grimmer and Böhnke [158] and Mandalakis et al. [159] who used similar partition procedures to isolate PAHs from triglycerides or complex mixtures of aliphatic hydrocarbons, respectively. Some minor modifications were introduced to limit analysis time and solvent consumption, and to obtain optimal purification.

Due to the interaction with the unshared electrons of the oxygen atoms of DMF molecules, PAHs dissolved in cyclohexane (or another aliphatic solvent) are easily extracted into the DMF/H_2O 9:1 *v/v* phase, while triglycerides and/or aliphatic hydrocarbons are retained in the cyclohexane phase, which is discarded. After the addition of an appropriate amount of water (able to form hydrogen bonds with the DMF, decreasing its ability to interact with aromatic compounds), partition coefficients of PAHs.

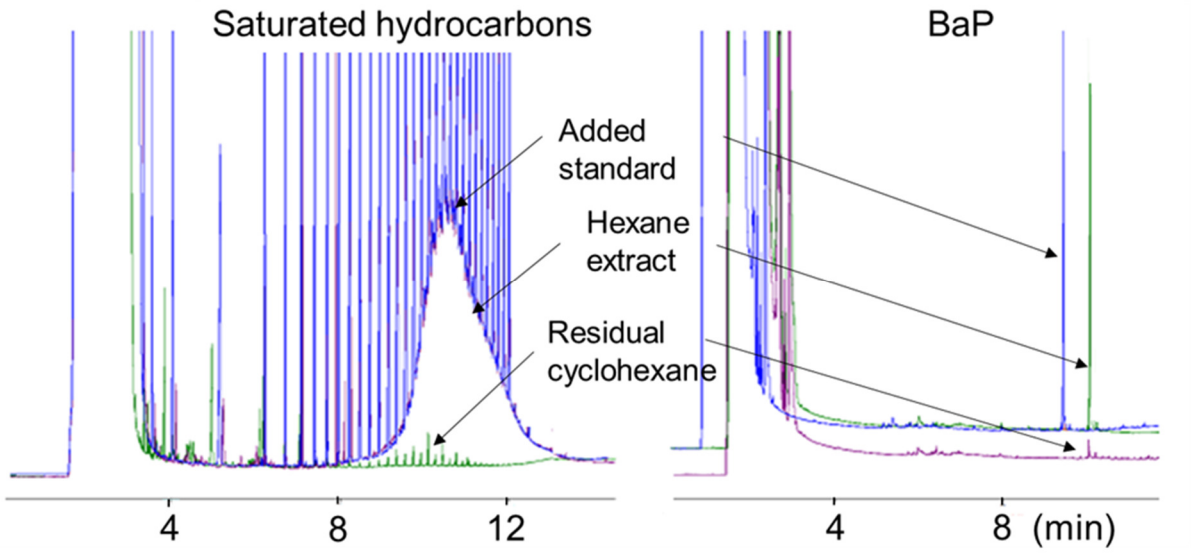

Figure 42. *On line LC-GC-FID traces showing the effect of partition on saturated hydrocarbons and BaP.*

Since according to Mandalakis et al. [159] higher enrichment factors can be obtained when using *iso*octane instead of cyclohexane, some preliminary tests to compare cyclohexane and *iso*-octane performance were performed. To select the optimal number of partitions and to assess efficient removal of saturated hydrocarbons and absolute recovery of the BaP, a mixture of saturated *n*-alkanes (C_7–C_{40}, 125 ng each), paraffin oil (comprising cycloalkanes and isoalkanes in the C_{18}–C_{48} range, 5 µg), and BaP (52 ng), was used. The standards, added into a 10 mL vial, were dissolved with 3 mL of the aliphatic solvent and extracted with 3 mL of the DMF/H_2O 9:1 *v/v* (vortex 3 min). To evaluate BaP recovery in the DMF phase (first partition) and efficient removal of saturated hydrocarbons, 2 mL of the lower phase (cyclohexane) was concentrated, then dissolved in 200 µL of hexane, injected into the LC–GC apparatus (75 µL), and the chromatographic areas obtained were compared with that obtained by direct injection of an equal amount of standard. To assess final BaP recovery, an equal volume of water was added to the residual DMF phase and, after rapid mixing, extracted with an equal amount of the aliphatic solvent (vortex for 3 min followed by centrifugation to accelerate phase separation) and analyzed after concentration. Again, for recovery calculation, the chromatographic area of BaP was compared with the area of the reference standard.

Figure 42 shows the LC–GC–FID traces obtained for both the saturated hydrocarbon and the BaP fractions. All the saturated hydrocarbons remained in the cyclohexane phase (only little traces are visible in the hexane extract), while BaP was almost quantitatively extracted into the DMF phase and then into the hexane. These tests indicated that >6% of the BaP remained in the cyclohexane (recovery of the first partition step was around 94%). Similar recovery was obtained when extracting standards dissolved in 5 mL of cyclohexane with 1mL of DMF/H_2O. Total extraction yields around 80% of the initial amount were obtained with one back-extraction into hexane after water addition). Comparable recovery was observed when using *iso*-octane instead of cyclohexane or when decreasing the amount of water in DMF from 10 to 5%. These preliminary trials, and particularly a

trial carried out on a microcrystalline wax sample fortified with 1 mg/kg of BaP also demonstrated that, when preceded by liquid–liquid partition to eliminate the bulk of saturated hydrocarbons and to allow for sample preconcentration, on-line LC-GC could be an interesting option for rapid BaP determination in complex mixtures of saturated hydrocarbons. Nonetheless, FID sensitivity is not sufficient to reach adequate sensitivity (at least 10 times lower than the legal limit), which could easily be achieved with a mass spectrometer (MS).

Based on the results from the preliminary tests, the optimized partition was applied to microcrystalline wax samples dissolved in 20 mL of cyclohexane and partitioned in separatory funnels (maintaining the same volume ratios). For lower solubility samples, a higher sample volume (100 mL) was extracted with 20 mL of DMF/H_2O. Processing samples with high amounts of saturated hydrocarbons, caused small amounts of these to remain in the DMF/H_2O phase, giving interference problems in the final hexane extract; therefore, an additional wash of the DMF/H_2O phase with cyclohexane was introduced. Figure 43 shows the GC–MS chromatograms obtained with and without the additional wash. Then H_2O was added to change the partition coefficients, and the BaP was back-extracted into hexane, which differently from cyclohexane, allowed for selective uptake and concentration of BaP by DI-SPME, employing the Carbopack Z/PDMS fiber. A further extraction of the residual DMF/H_2O confirmed that a single partition with hexane is sufficient to ensure good recovery with limited solvent consumption.

Finally, the hexane extract was concentrated to 1.5 mL and BaP was selectively extracted by using a Carbopack Z/PDMS fiber. An enrichment factor of about 70 was estimated using the fiber rather than performing a liquid injection.

As shown in table 22, the proposed method allows to significantly reduce the solvent consumption, in specific about 100 times compared to the method proposed by Lijinsky [156] and ten times compared to the method proposed by Howard et al. [157]. Furthermore, the use of a highly toxic solvent such as benzene is completely avoided. Finally, it is interesting to notice that comparable limits of quantification are obtained using all the three methods, but the starting amount of sample is rather different, in particular, the sample amount in the proposed method is 1000-20 000 times lower than the other two methods.

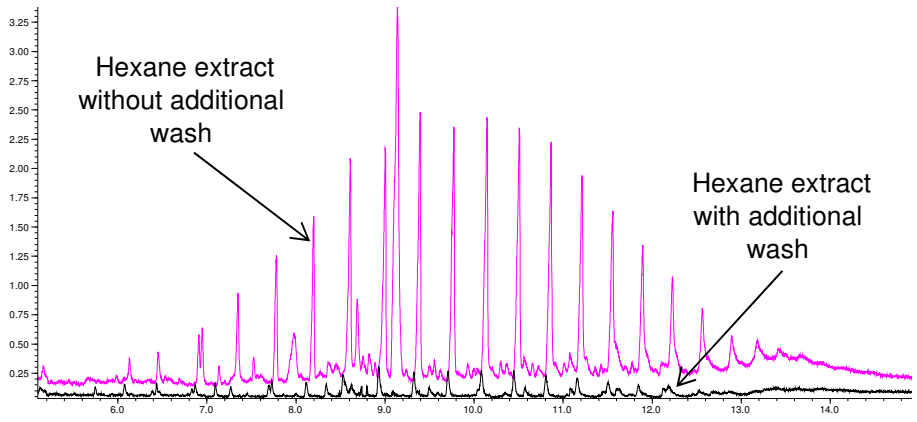

***Figure 43**. TIC chromatograms of the hexane extracts obtained performing and not additional washing of the DMF/H_2O phase with cyclohexane*

***Table 22**. Comparison among the proposed method and methods reported in literature*

	Proposed method	**Lijinsky, 1960** [156]	**Howard at al., 1965** [157] **and JECFA** [154]
Amount of sample	5-25 mg	100 g	25 g
Sample preparation	Liquid-liquid partitions followed by SPME	Adsorbtion chromatography on magnesia- Celite column	Liquid-liquid partitions. <u>*If absorbance limit is exceeded:*</u> additional adsorption chromatography on a mixture of magnesium oxide- Celite 545
Type/amount of solvents	Cyclohexane: about 40-120 mL DMF/H_2O 9:1 (*v/v*): 20mL Hexane: 20mL	Benzene: about 2500 mL Hexane: about 300 mL Acetone: about 4500 mL Ethyl alcohol: about 1000 mL Hydrocloridric acid: about 600 mL	Dimethyl sulphoxide/phosphoric acid 2:1 (*v/v*): about 150 mL Isooctane: about 350 mL <u>*Eventually:*</u> Isooctane: about 330 mL Benzene: about 155 mL Acetone: about 100 mL
Total solvent volume	*80-180 mL*	*about 8900 mL*	*about 1085 mL*
Analytical determination/detection	Gas chromatography/MS	Paper chromatography /Spectrophotometry	Spectrophotometry
Detected PAHs	Benzo[a]pyrene	Benzo[a]antracene Benzo[a]pyrene Chrysene Dibenzo[a,h]antracene	Sum of unspecified PAHs
LOQ	7 µg/kg	10 µg/kg	10 µg/kg
Time	about 40 min + 15 min GC	2 days	about 4h

5.3.1.2. Method validation

Isotope dilution internal standard calibration method, using deuterated BaP (*d12*-BaP), was used for BaP quantification. The occurrence of any matrix effect was assessed by performing a *t*-test on the slopes of the calibration curves obtained by the analysis of a BaP standard solution in *n*-hexane and by spiking a microcrystalline wax sample with BaP at four different concentration levels (25-200 ng/g range). The results proved no matrix effect up to 100 ng/g of BaP ($p < 0.05$). Therefore, a five-point calibration curve (Figure 44) in *n*-hexane (analyzed in triplicate) was constructed in the 5–100

ng/g range. The least squares method was applied to estimate the regression lines, obtaining regression coefficients (R^2) of 0.997.

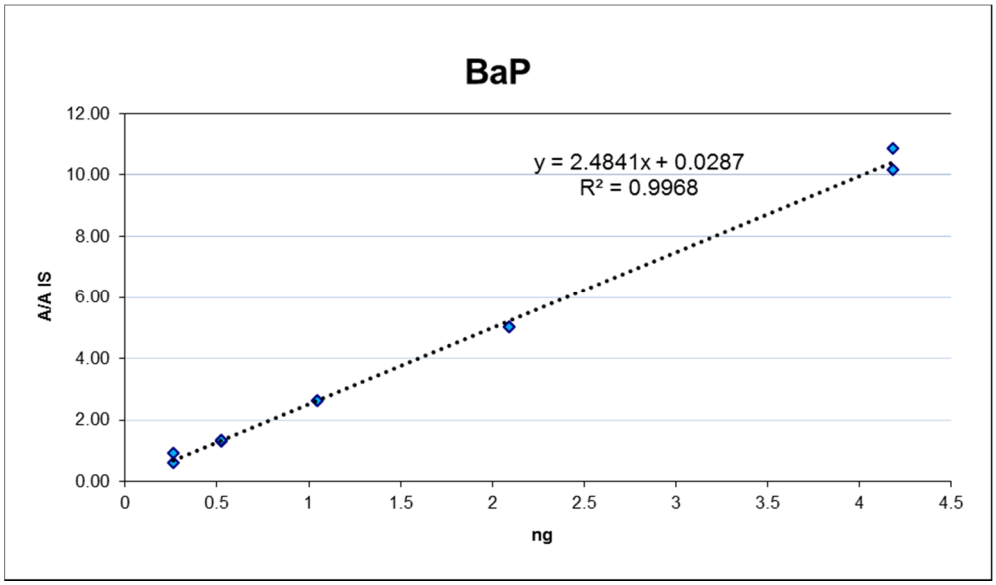

Figure 44. *Calibration curve GC-MS of BaP*

Linearity was assessed using Mandel's fitting test ($p < 0.05$). The LOD and LOQ were calculated according the following formulas:

LOD : $y_d = x_b + 3 * \sigma_b$ (1)

LOQ : $y_q = x_b + 10 * \sigma_b$ (2)

where y_d and y_q are the signal at the LOD and LOQ, respectively, x_b is the mean value of blank analyses and σ_b is the blank SD corrected according to blank correction method ($n = 3$), as indicated in the Eurachem Guidelines [167], using three replicates of blank. Finally, LOD and LOQ values were obtained by plotting y_d and y_q in the calibration line. They were equivalent to about 0.02 ng (LOD) and 0.04 ng (LOQ) in the final solution (1.5 mL), which corresponded to LOQ values in the samples ≤ 7 µg/kg. Repeatability was calculated analyzing a naturally contaminated microcrystalline wax sample (29.9 µg/kg of BaP) six times, obtaining a coefficient of variation (CV%) <6%. Accuracy was <3% ($n = 3$), determined as relative error deviation (A%) between the values observed in the spiked sample and the expected values. The presence of trace amounts of BaP in solvents has to be assessed for each batch before analyzing any samples.

5.3.2. Results on real samples

5.3.2.1. Hydrocarbons in microcrystalline waxes

Given the high molecular weight of the hydrocarbons present in microcrystalline waxes, the chromatographic conditions for hydrocarbon analysis (rapid programmed temperature and column utilization and thermostable pre-columns) have been optimized to allow the complete elution of heavy hydrocarbons. After dissolution in cyclohexane (different products had different solubility depending on the presence of high molecular weight hydrocarbons) the waxes were diluted with *n*-hexane to obtain a solution containing 20% of cyclohexane (which does not give problem to the injection) and directly injected into the GC-FID. Table 23 reports the percentage of hydrocarbons < *n*-C_{25} and < *n*-C_{35} found in the samples, while figure 45 shows the most representative GC profiles.

Table 23. *Total amount of MOHs in microcrystalline waxes*

	Samples of microcrystalline waxes								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
% hydrocarbons < C_{25}	4.5		0.6	1.4	1.6	2	0.5	0.3	3.9
% hydrocarbons < C_{35}	79.8	7.1	13	12.1	17.7	10.5	3.6	8.7	84.7

Sample 5 gave a GC trace characterized by the presence *n*-alkanes above a "hump" of unresolved peaks centred around *n*-C_{40}. The area above of n-alkane peaks is low and covers a wide range of molecular weights (from *n*-C_{25} to over *n*-C_{50}). Samples 2, 3, 4 and 6 presented the same GC profile and they show the same percentage of MOHs < *n*-C_{25} excluding sample 2 which, as mentioned above, had solubility problems. The GC profile of microcrystalline wax 7 (which is similar to that of sample 8) shows a high and narrow hump centred around *n*-C_{35}, with very little amounts of *n*-alkanes on the top. Both the samples had less than 1% of MOH below *n*-C_{25}. Samples 9 and 1 had high amount of *n*-alkanes from C_{21} to C_{40} (centred on *n*-C_{35}), and about 4% of MOH below *n*-C_{25}.

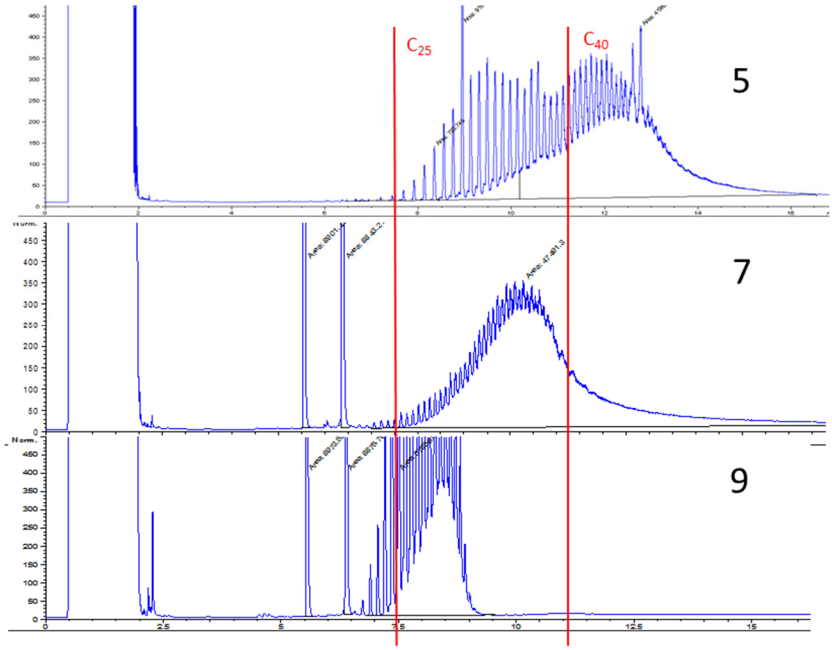

Figure 45. *Examples of MOHs profile of microcrystalline waxes*

The possibility to use a SPE to check for the presence of MOAH was also evaluated. The choice to use off-line SPE-GC instead of LC-GC analysis has the advantage to allow for loading high amount of sample reaching higher sensitivity. First trials were carried out using a double SPE cartridge: a first one of activated silica (1 g) for rapid sample purification and a second one of silver silica, for MOSH and MOAH fractionation according to the method developed by Moret et al. for cardboard and dry food[90]. Figure 46 reports the GC traces of a microcrystalline wax sample with no detectable MOAH.

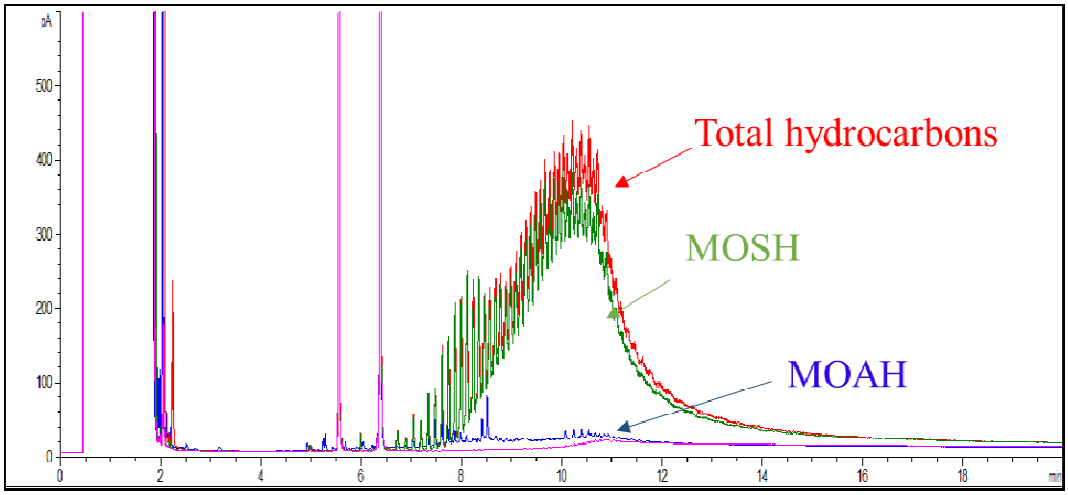

Figure 46*. GC traces of total hydrocarbons (red), MOSH (green) and MOAH (blu) in a microcrystalline wax sample.*

Later it was recognized that the first purification step can be avoided. The sample dissolved in cyclohexane is directly loaded into the cartridge (variable volume depending on the sample solubility and required sensitivity), saturated hydrocarbons are eluted with the dead volume while the aromatic compounds, if present, are retained and eluted later with a mixture of hexane/dichloromethane 1:1 (v/v). After SPE pre-separation the aromatic fraction can be reconcentrated allowing to detect trace amounts of aromatics in the presence of huge amount of saturated hydrocarbons.

5.3.2.2. BaP in microcrystalline waxes

Ten samples from different producers, all complying with the legal requirement for saturated hydrocarbons (less than 5% of hydrocarbons lower than C_{25} and average MW> 500 Da), were analyzed for their BaP content. As visible from the results reported in Table 24, most of the samples had BaP amounts <LOQ (7µg/kg).

Table 24. *BaP content on microcrystalline waxes*

Sample	BaP (µg/kg)
1	< LOQ
2	29.9
3	< LOQ
4	< LOQ
5	< LOQ
6	9.3
7	< LOQ
8	< LOQ
9	< LOQ
10	< LOQ

5.3.3. Analysis of hydrocarbons released from microcrystalline waxes in artificial saliva

In order to evaluate the potential migration of BaP and of hydrocarbons entering the composition of microcrystalline waxes, used in chewing gum formulation, a chewing test using artificial saliva as simulant was performed. Materials coming from three different steps of the production chain were testes: three type of raw microcrystalline waxes, three gum bases and three final chewing gums.

5.3.3.1. Extraction

PAHs and hydrocarbons entering the composition of microcrystalline waxes are organic compounds with very low water solubility.
An official method to evaluate the PAHs in water was established by the US EPA. The EPA method 610 is based on liquid-liquid extraction using dichloromethane, which is time and solvent consuming. Methods used on SPE have been also proposed [168][169]. A modified liquid-liquid extraction method, limiting solvent consumption, followed by SPME-GC-MS according to [164] was developed and used in the present study. Twenty millilitres of artificial saliva were extracted with 9 mL of cyclohexane, taken to dryness, and dissolved in *n*-hexane (1.5 mL). After that, the SPME fiber was directly exposed in the solvent. Recovery tests, performed in triplicate (on a blank artificial saliva) at 100 ng/g of BaP level, showed recovery of 70 % and 85 % after one and two extractions respectively (RSD < 9 %). Recovery around 60% was obtain at 20 ng/g level.
The same extraction procedure was used for hydrocarbon analysis. Recovery test was performed on a blank sample of artificial saliva spiked with a known amount of MCW. Comparing the area of the

fortified sample with that of the MCW used to spike the sample, a quantitative recovery was observed (>97%).
Most of the samples had BaP content between 0.2 and 0.4 ng/L (legal limit for water is 10 ng/L). No appreciable differences were observed among different types of chewed samples (raw microcrystalline waxes, gum bases and chewing gums). No detectable amount of hydrocarbons were found in artificial saliva samples (Figure 47).

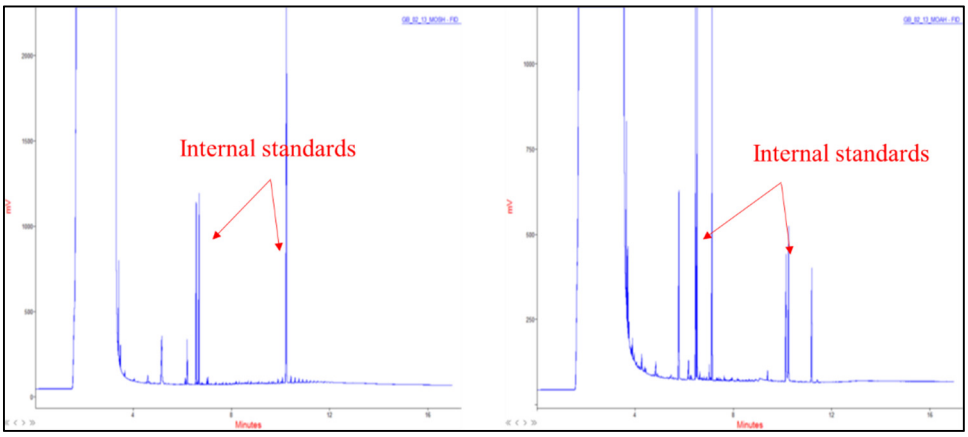

Figure 47. *Trace GC of hydrocarbons extracted from artificial saliva sample.*

5.4. Conclusions

A simple method based on a liquid–liquid partition step to eliminate the bulk of the saturated hydrocarbons, followed by DI-SPME using a Carbopack Z/PDMS fiber, was employed for selective uptake and concentration of BaP in the analysis of microcrystalline waxes. Although the SPME fiber used is highly selective for planar compounds, the bulk of hydrocarbons interfered with the analysis, requiring a previous cleanup step. The liquid–liquid partition method based on the Grimmer and Böhnke approach gave satisfactory cleaning results. The use of cyclohexane, rather than hexane (the most suitable solvent for SPME direct extraction), was necessary to increase sensitivity, due to the higher solubility of the microcrystalline waxes in cyclohexane rather than in hexane. Compared to previously reported methods, the newly developed method reduced significantly the amount of solvent employed (10–100 times lower); furthermore it presented good performances in terms of linearity, repeatability (<6%), accuracy (<3%), and LOQ (<10 ng/kg). All the analyzed samples complied with legal requirements.
LC-GC-FID analysis showed that microcrystalline waxes are characterized by different profile of molecular weight hydrocarbon distribution, ranging from *n*-C_{20} to *n*-C_{60}. All of the samples presented less than 5% of hydrocarbons below *n*-C_{25}. Migration tests using artificial saliva as simulant were performed on samples of gum bases and chewing gums as well as on the raw microcrystalline waxes that are included in their compositions. A rapid liquid-liquid method was developed to extract BaP and hydrocarbons possibly released into artificial saliva. The method demonstrated good performance. No amount of BaP were observed in chewed samples, demonstrating that chewing did not cause any release of detectable amount of BaP in artificial saliva.

Furthermore, artificial saliva resulted from migration test showed no detectable amount of hydrocarbons indicating that also in this case there was no detectable release of hydrocarbons from the products.

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