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Title: Microwave assisted saponification (MAS) followed by on-line liquid chromatography (LC)-gas chromatography (GC) for high-throughput and high-sensitivity determination of mineral oil in different cereal-based foodstuffs

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Keywords: Microwave assisted saponification (MAS); mineral oil saturated hydrocarbons (MOSH); mineral oil aromatic hydrocarbons (MOAH); cereal-based products; food contamination; on-line LC-GC.

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Abstract: A high throughput, high-sensitivity procedure, involving simultaneous microwave assisted extraction (MAS) and unsaponifiable extraction, followed by on-line liquid chromatography (LC)-gas chromatography (GC), has been optimised for rapid and efficient extraction and analytical determination of MOSH and MOAH in cereal-based products of different composition. MAS has the advantage of eliminating fat before LC-GC analysis, allowing to increase the amount of sample extract injected, and hence its sensitivity. The proposed method gave practically quantitative recoveries and good repeatability (relative standard deviation lower than 10). Among the different cereal-based products analysed (dry semolina and egg pasta, bread, biscuits, and cakes), egg pasta packed in direct contact with recycled paperboard had on average the highest total MOSH level (15.9 mg kg-1), followed by cakes (10.4 mg kg-1) and bread (7.5 mg kg-1). About 50% of the pasta and bread samples and 20% of the biscuits and cake samples had detectable MOAH amounts. The highest concentrations were found in an egg pasta in direct contact with recycled paperboard (3.6 mg kg-1) and in a milk bread (3.6 mg kg-1).

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Editorial Office

Food Chemistry

Dear Editor,

here enclosed the revised manuscript: "Microwave assisted saponification (MAS) followed by on-line liquid chromatography (LC)- gas chromatography (GC) for high-throughput and high-sensitivity determination of mineral oil in different cereal-based foodstuffs" by Sabrina Moret, Marianna Scolaro, Laura Barp, Giorgia Purcaro, Lanfranco S. Conte. The paper has been revised following reviewer suggestions.

The authors confirm that the paper is unpublished and has not been submitted for publication elsewhere. Also, the authors confirm that the institutions where they work agree to the submission of this paper to the journal.

Udine, August 31, 2015

Sincerely,

Sabrina Moret

Replies to reviewers' comments:

Reviewer #1: The study proposes a new method to determine mineral oil in some types of foods allowing to achieve lower quantification and detection limits, making use of microwave assisted saponification, never used before to this purpose. The optimization and validation of the method have been performed and explained clearly in the manuscript. In my opinion there are only some minor changes and amendments to be done to make the manuscript acceptable for publication.

1) One of the strength points of the proposed method regards the improved sensitivity allowed, thus it could be interesting to show to the readers a comparison (and a related discussion) with values of LOD and LOD obtained in other methods proposed in literature for the determination of mineral oil;

Information about LOD and LOQ values reported by other authors by applying direct on-line LC-GChave been added in the introduction. As already reported, and now better evidenced in the discussion, the sensitivity improvement depends on the sample fat content.MAS eliminates the fat so higher concentration factors can be applied.

2) Terms use for explaining RSD are not homogeneous: e.g. in the abstract (line 32) it is used "…relative standard deviation (RSD)…" while in the text (e.g. line 291),"…residual standard deviation (RSD)…". Please check and correct in all the text and tables.
"Residual standard deviation" has been corrected in "relative standard deviation"

3) Line 324: The paragraph number should be 3.3. This paragraph discusses results on comparison between methods; the discussed data should be reported in a table or in a chart (figure 3 only shows chromatograms).
The paragraph number has been corrected.A comment on data obtained for unspiked bread sample of figure 3, extracted using both overnight extraction with hexane and MAS, has been added in the text. Other results concerning method comparison are commented.

4) Lines 337: please substitute "weekend" with the number of hours (or insert it after "weekend" in brackets).
The number of hours has been added in the text for both "over-weekend" and "overnight"

5) Lines 370-373: please reformulate the sentence (Currently it is: "Figure 4 shows…while figure 4 shows…").
The sentence has been reformulated.

6) Line 375: The paragraph number should be 3.4.
The paragraph number has been corrected.

7) Lines 440-441: please cite the reference for the mentioned ordinance (even if previously cited).
The reference to the German ordinance has been inserted.

8) Please use italics "n" in "n-Cx" (when indicating linear alkanes) also in figures, figure legends and tables.

"n-Cx" has been corrected in "n-Cx" also in figures and tables.

9) In the legend of Figure 5 the explanation for "RVP" is not reported.
The explanation of "RPV" has been added in the legend of figure 5.

10) Table 1: There are some lacks of homogeneity: in the decimal data sometimes is used the dot, sometimes the comma; please uniform it with dot (also in the y-axis of figure 5).
Wrong decimal data have been corrected replacing the comma with the dot.

11) Fig.2 is not so clear: maybe instead of reporting overlaid chromatograms it could be better to report them aligned.
We tried to remake the figure by reporting the 5chromatograms aligned but we personally prefer to leave it as it is. The enlargement on the right site of the figurehelp the reader to see that for each fortification levels there are 2overlapped traces: oneconcerning the added standard,and the other one concerning the fortified sample.To be more clear, some labels on the figure have been modified.

Reviewer #2: A well described and improved method for the determination of MOSH and MOAH. You may wish to consider the following comments:

It would be useful to include the MOAH data in the abstract as well as the MOSH.
A paragraph,which reassumes MOAH results,has been added at the end of the abstract.

For the samples section it would be useful to indicate here how long the products have been in the packaging.
The products purchased from the supermarket hadvariable lifetime, not exceeding their shelf life: on average 6 months for pasta, 1-2 days for fresh bread and 2-6 months for biscuits and bakery products. This information has been added in the text.

How was the homogeneity of the samples demonstrated? Line 152 says the samples were "accurately homogenised".
The homogeneity of the samples was demonstrated by replicate analyses (n= 4-6) of the same sample, which, as reported in the text (paragraph 3.2 on "Method performance"), gave always RSD<10%

In line 250 it is said that loss of CyCy occurs, could you explain why only this substance is lost
We have no solidexplanation for this behavior, so we did not comment it in the text. Probably some matrices contain compounds possibly reacting with CyCy. We know from literature that similar reactions have been observed for other analytes in the presence of the matrix, when using microwave extraction.

Looking at Figure 5 many of the samples contained MOSH at levels below the spiking concentrations used in the validation. What confidence do you have that the method performance was equally satisfactory at the concentrations measured. How have you accounted for the uncertainty in the integration of the "hump" and removal of the areas of the interferences and their effect on the uncertainty.

If we consider both MOSH and MOAH,spiking concentrations used for method validation range from 0.36 to 25 mg/kg. Some of the sampleshave contamination levels below the spiking level, but most of them have concentrations close to the fortification levels.
Due to the particularity of this analysis (we measure the area of a hump and not of a single peak), it is difficult to evaluate the confidence of the method at low concentration, because it strongly depends on the sample type, sources of contamination (enlarged humps give more problem), presence of interfering compounds. Of course the lower the measured concentration, the higher the uncertainty.

Did you investigate in-house reproducibility of the method, i.e. using a second analyst? If so what was the performance?
We investigated repeatability using the same apparatus and the same analysts. Anyways, based on our experience on other samples, no appreciable differenceshave been noted when changing the analyst.

It would also be useful to add whether or not the levels found in the samples are toxicologically significant in terms of exposure related to food consumption data as well as comparison with the BFR limits.
A comment on the toxicological relevance of reported data has been added in the text (section 3.4)

Highlights

- MOSH and MOAH determination in cereal-based products.
- Microwave assisted saponification and extraction followed by on-line LC-GC.
- Method performance and recovery tests on different matrices.
- Comparison with other extraction methods.
- Applications to dry pasta, bread, biscuits and cakes.

Microwave assisted saponification (MAS) followed by on-line liquid chromatography (LC)-gas chromatography (GC) for high-throughput and high-sensitivity determination of mineral oil in different cereal-based foodstuffs

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Abstract

A high throughput, high-sensitivity procedure, involving simultaneous microwave assisted extraction (MAS) and unsaponifiable extraction, followed by on-line liquid chromatography (LC)-gas chromatography (GC), has been optimised for rapid and efficient extraction and analytical determination of MOSH and MOAH in cereal-based products of different composition. MAS has the advantage of eliminating fat before LC-GC analysis, allowing to increase the amount of sample extract injected, and hence its sensitivity. The proposed method gave practically quantitative recoveries and good repeatability (relative standard deviation lower than 10). Among the different cereal-based products analysed (dry semolina and egg pasta, bread, biscuits, and cakes), egg pasta packed in direct contact with recycled paperboard had on average the highest total MOSH level (15.9 mg kg^{-1}), followed by cakes (10.4 mg kg^{-1}) and bread (7.5 mg kg^{-1}). About 50% of the pasta and bread samples and 20% of the biscuits and cake samples had detectable MOAH amounts. The highest concentrations were found in an egg pasta in direct contact with recycled paperboard (3.6 mg kg^{-1}) and in a milk bread (3.6 mg kg^{-1}).

Keywords: Microwave assisted saponification (MAS), mineral oil saturated hydrocarbons (MOSH), mineral oil aromatic hydrocarbons (MOAH), cereal-based products, food contamination, on-line LC-GC.

1. Introduction

Mineral oils are complex mixtures of hydrocarbons of petrogenic origin comprising two major classes of compounds of different toxicological relevance: mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH).

MOAH comprise carcinogenic compounds for whom it is not possible to establish an acceptable daily intake (ADI) and, therefore, should be not present in foods (EFSA, 2012). The toxicity of MOSH has not been fully elucidated and data supporting the establishment of ADIs covering the different molecular weight (MW) ranges of MOSH possibly contaminating food is still lacking. Based on bioaccumulation data on animals, the German Federal Institute for Risk Assessment (BfR) recommended temporary limits of 12 mg kg^{-1} food for the hydrocarbons C10-C16 and of 4 mg kg^{-1} for the adjacent fraction *n*-C17-C20 (BfR, 2013). The German Federal Ministry of Food, Agriculture and Consumers has recently presented a new draft ordinance (22^{nd} ordinance amending the food contact material regulation for paperboard made of recycled fibres) establishing a specific migration limit of 2 mg kg^{-1} for MOSH from *n*-C20 up to *n*-C25 (or *n*-C35 in the case of wet contact), and a limit of 0.5 mg kg^{-1} for MOAH *n*-C16-C35. Barp *et al.* (2014) have recently demonstrated how the use of animal data may underestimate accumulation in humans, and they found strong accumulation in different human tissues, mainly in the range above *n*-C21.

Mineral oils occur in food at various concentrations depending on the food nature and the source of contamination. Environmental contamination (Neukom, Grob, Biedermann and Noti, 2002; Moret, Populin, Conte, Grob, and Neukom, 2003), food grade mineral oils widely used for different purposes in many processed foods (Tennant, 2004), and food contact materials (Moret, Grob and Conte, 1997; Droz and Grob, 1997; Biedermann and Grob, 2012) represent important sources of contamination. Special attention has been paid over the last years on contamination from printing inks and packaging made of recycled fibres, responsible for the high contamination levels found in

many dry foods packaged in direct contact or with an inner barrier bag (Vollmer *et al.*, 2011; EFSA, 2012).

According to the EFSA Opinion (EFSA, 2012), cereal-based products such as bread, rolls and fine bakery wares, are among the most important contributors to dietary MOSH exposure , mainly due to the use of food grade mineral oils as release or spraying agents.

Different methods based on off-line solid phase extraction (SPE)- gas chromatography (GC)- flame ionisation detector (FID) (Moret, Barp, Grob and Conte, 2011; Moret, Barp, Purcaro and Conte, 2012; Fiselier *et al*, 2013) and on-line liquid chromatography (LC)-GC-FID (Biedermann, Fieseler and Grob, 2009; Tranchida *et al.*, 2011; Barp, Purcaro, Moret and Conte, 2013) are currently available for mineral oil determination in foods. Direct on-line LC-GC has the advantage to reduce sample manipulation and solvent consumption thus enhancing reproducibility of the method, but has the disadvantage to not allow for fat removal before GC analysis, which is mandatory to lower the detection limit. Detection (LOD) and quantification (LOQ) limits around 3 and 8 mg kg^{-1} were reported for oil samples by employing direct on-line LC-GC (Biedermann, Fiselier and Grob, 2009). Lower detection limits were obtained for other food items, depending on their fat content, by concentrating the sample without exceeding the capacity of the LC-column (20 mg of fat). A slight sensitivity increase was later also obtained by Barp, Purcaro, Moret and Conte (2013) by applying a rapid temperature increase during GC analysis.

Depending on the food composition, different extraction procedures, mainly based on classical solvent extraction (EFSA, 2012), have been applied in mineral oils extraction from cereal and cereal-based products. In the case of dry foods with a low fat content, solvent extraction with hexane was first applied in extraction of superficial contamination, such as that migrated from the packaging (Vollmer *et al.*, 2011). In the case of wet samples, solvent extraction (with hexane overnight) was preceded by a dehydration step (preferably carried out with anhydrous sodium sulphate). Later, it was recognized that overnight extraction with hexane did not allow for complete extraction of the mineral oil, also in some dry foods. In 2011, Biedermann-Brem and Grob

described a solvent-based approach for exhaustive extraction of mineral oil from wet samples. It involved sample equilibration (1 h) with ethanol (added in amount at least 10-fold that of the water present in the sample), followed by overnight extraction with hexane after ethanol removal. As the ethanol extract contains some mineral hydrocarbons, it was then recombined with the hexane extract and added with water (twice the hexane volume) to separate the hexane from the ethanol-water mixture.

The same method, preceded by soaking in hot water to make the matrix swell, was also applied in exhaustive extraction of mineral oil from dry pasta samples. As an alternative, pressurized liquid extraction (PLE) has been recently proposed for complete extraction of mineral oil from dry pasta and grain cereals (Moret, Scolaro, Barp, Purcaro, Sander and Conte, 2014), and later applied for MOH determination in dry pasta stored in different packaging material over the shelf life (Barp, Suman, Lambertini and Moret, 2015a; Barp, Suman, Lambertini and Moret, 2015b).

When processing high-fat content foods with non polar solvents, mineral oils are co-extracted with the fat. Since the LC column has a limited capacity to retain fat (20 mg), an additional purification step aiming at reducing or eliminating fat (passage through a bed of activated silica) is therefore required to reach higher sensitivity. As an alternative, to eliminate high amounts of fat before LC-GC analysis, the sample can be saponified. Traditional saponification followed by unsaponifiable extraction has been previously applied for determination of mineral oil or endogenous *n*-alkanes in different food samples (Castle, Kelly and Gilbert, 1993; Koprivniak, Procida and Favretto, 1997). Traditional saponification has the advantage that it can be applied to all food types, avoiding the need to remove water before the extraction step, but it has the disadvantage of being solvent- and time-consuming.

Microwave assisted saponification (MAS), applied by different authors for extraction of polycyclic aromatic hydrocarbons (PAHs) from different food matrices (García Falcón, Simal Gándara, Carril Gonzáles and Barros 2000; Hernández-Borges, Rodríguez-Delgado and Garcia- Montelongo., 2006; Pena, Pensado, Casais, Mejuto, Phan-Tan-Luu and Cela., 2006, Akpambang, Purcaro, Lajide,

Amoo, Conte and Moret., 2009, Moret Purcaro and Conte, 2010) represents an interesting alternative to conventional saponification. It allows for rapid extractions on a large number of samples, depending on the kind of apparatus.

The aim of this work was to explore the applicability of the MAS for high-throughput and high-sensitivity determination of mineral oil in cereal-based products with different fat and water content. Performance characteristics of the optimized procedure have been evaluated and the obtained results on selected samples were compared to those obtained by applying solvent-based extraction procedures. Finally, the optimised method was applied on a wide number of different cereal based products from the Italian market.

2. Materials and methods

2.1. Reagents and standards

All the solvents were purchased from Sigma-Aldrich (Milan, Italy). Hexane, acetone and dichloromethane were distilled before use. Ethanol was of HPLC grade. Water was purified with a Milli-Q System (Millipore, Bedford, MA, USA).

Internal standards were purchased from Supelco (Milan, Italy). The working standard solution was prepared by mixing 5-α-cholestane (Cho, 0.6 mg mL^{-1}), *n*-C11 (0.3 mg mL^{-1}), *n*-C13 (0.15 mg mL^{-1}), cyclohexyl cyclohexane (CyCy, 0.3 mg mL^{-1}), *n*-pentyl benzene (5B, 0.30 mg mL^{-1}), 1-methyl naphthalene (1-MN, 0.30 mg mL^{-1}), 2-methylnaphthalene (2-MN, 0.30 mg mL^{-1}), tri-tert-butyl benzene (TBB, 0.3 mg mL^{-1}) and perylene (Per, 0.6 mg mL^{-1}) in toluene.

The standard mixture of *n*-alkane C10-C40 (50 mg L^{-1} each) and the paraffin oil used for recovery tests were purchased from Sigma-Aldrich (Milan, Italy), while the printing ink solvent containing 9% of MOAH was kindly provided by a producer.

All the glassware was carefully washed and rinsed with distilled solvents (acetone and hexane) before use.

2.2. Samples

Different cereal-based foodstuffs were selected and analyzed with the proposed method: dry semolina, egg pasta, and various bakery products with different fat and moisture content (bread, biscuits and cakes). The products purchased from the supermarket had variable life time (not exceeding their shelf life): on average 6 months for pasta, 1-2 days for fresh bread and 2-6 months for biscuits and bakery products.

2.3. Microwave assisted saponification (MAS)

A microwave extractor (Mars, CEM Corporation, Matthews, NC, USA) able to process up to 14 samples simultaneously, was used to extract mineral oil from different cereal-based products.

The samples were accurately homogenized and finely ground with a laboratory mill (IKA A10 analytical mill). The extraction was carried out by applying the method described for PAH extraction from fish tissue (Akpambang et al., 2009) and propolis samples (Moret et al. 2010), with few modifications. Briefly, 5 g of the sample were weighed in a Teflon-lined vessel (Green Chem plus, CEM Corporation), added with 5 µL of the internal standard solution, 10 mL of saturated methanolic potassium hydroxide (KOH), and 10 ml of n-hexane. For high-fat content samples (more than 25% of fat), the amount of saturated methanolic KOH was increased to 20 mL. Microwave assisted saponification and simultaneous extraction was carried out at 120 °C for 20 min. Since microwave heating is sample dependent and the microwave instrument allowed pressure and temperature control only in a pilot vessel, to ensure uniform extraction conditions, only samples of the same type were processed together within each extraction cycle.

Depending on the food type, a sample post-treatment was sometimes required after MAS. The pasta and bread samples did not require any sample post-treatment and were injected directly into the LC-

GC system (after reconcentration of the hexane extract). On the other hand, the biscuits and cakes required a sample post-treatment and, after MAS, the sample extract was transferred into a separatory funnel, washed with successive aliquots of water and little amounts of methanol (avoiding agitation of the sample during the first wash in order to prevent formation of stable emulsions) until obtaining clear extracts and good phase separation. As an alternative, once cooled, 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. An aliquot of the hexane extract was then washed with a double volume of water in a screw-cap vial (vortex 1 min). The hexane extract was directly used for LC-GC injection or an aliquot (5 mL) was concentrated to 1 mL before injection, using an evaporation system consisting of a centrifuge (Univapo 100 H, Uniequip System; Martinsrieder, Munich, Germany) and a vacuum pump.

To remove interfering olefins (eluting in the MOAH fraction) some biscuits and cake samples were epoxidized prior to LC-GC determination. After MAS and wash with water, an aliquot of the sample was added with 100 mg of a clean vegetable oil and it underwent epoxidation according to the procedure described by Biedermann *et al.* in 2009.

2.4. Standard addition for recovery tests

A printing ink solvent (containing 91% of MOSH and 9% of MOAH in the *n*-C14-C20 range) and/or a paraffin oil (100% MOSH in the *n*-C24-C40 range) were used for recovery tests. The recoveries were calculated by comparing the chromatographic area of the spiked sample subtracted from the area obtained for the unspiked sample (with no detectable contamination or with low contamination levels), with that of the same amount of standard used to spike the sample.

The pasta samples as well as biscuits and plum-cakes were finely ground, weighed directly into the extraction vessel (5 g), added with mineral oil standards dissolved in 5-10 mL of pentane, and gently stirred for 30 min to uniformly distribute the added mineral oil (the added solvent evaporated during stirring) and was left to age for 3 days before MAS.

The bread samples for recovery tests were prepared using a bread machine (Severin, mod. BM 3981) to knead 400 g of flour type "0", 280 g of tap water, 16 g of extra virgin olive oil and 4 g of yeast. The mineral oil standard was added to the extra virgin olive oil used to make the bread. After kneading and leavening, the dough was baked according to the machine program. Two bread samples were prepared: one prepared with the unspiked extra virgin olive oil and the other with the same oil spiked with a known amount of printing ink solvent and paraffin oil.

2.5. LC-GC determination

The on-line LC-GC instrument (LC-GC 9000, Brechbühler, Zurich, Switzerland) consisted of a Phoenix 40 with three syringe LC pumps and four switching valves and an UV/VIS detector (UV-2070 Plus, Jasco, Japan). The autosampler was a PAL LHS2-xt Combi PAL (Zwingen, Switzerland). The LC column was a 25 cm × 2.1 mm i.d Lichrospher Si 60, 5 μm (DGB, Schlossboeckelheim, Germany). The GC was a Trace GC Ultra from Thermo Scientific (Milan, Italy).

A gradient, starting with hexane (0.1 min) and reaching 30% of dichloromethane (at 300 μL/min) in 0.5 min, was used to elute the MOSH (from 2.0 to 3.5 min) and the MOAH (from 4 to 5.5 min) as described by Biedermann *et al.* (2009).

LC-GC transfer occurred through the Y-interface based on the retention gap technique and partially concurrent eluent evaporation. A 10 m × 0.53 mm i.d. uncoated, deactivated precolumn was connected by a steel T-piece union 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). A rapid oven gradient (40 °C min^{-1}) starting from 55 °C up to 350 °C was used for GC analysis (Barp *et al.*, 2013). The FID and the SVE were heated at 360 °C and 140 °C, respectively. After the transfer, the LC column was backflushed (dichloromethane) and reconditioned prior to the subsequent injection.

231 The data were acquired and processed by the ExaChrom software (Brechbühler, Switzerland). The
232 MOSH area was determined by the integration of the whole hump of largely unresolved peaks,
233 subtracted from the endogenous *n*-alkanes. All sharp peaks standing at the top of the MOAH hump
234 were subtracted from the total area. Quantification was based on internal standards or on external
235 standard, when the presence on sample components co-eluting with the standards was suspected.
236
237 **3. Results and discussion**
238
239 *3.1. MAS procedure*
240 Simultaneous microwave assisted saponification and unsaponifiable extraction was first applied to
241 pasta samples and later to other cereal-based products with different moisture and fat content.
242 With respect to the conditions used by Akpambang *et al.* (2009) for PAH extraction from fish and
243 meat tissues (400 mg of lyophilized sample added with 1.6 mL of water, 8 mL of saturated
244 methanolic KOH and 20 mL of *n*-hexane), a higher amount of sample (5 g) was processed in order
245 to achieve higher sensitivity. The amount of saturated methanolic KOH was slightly increased to 10
246 mL, while the amount of *n*-hexane used was reduced from 20 to 10 mL to limit organic solvent
247 consumption and to obtain a more concentrated extract. Since an excess of KOH is required to
248 ensure complete fat hydrolysis (Pena *et al.* 2006), when processing samples with more than 25% of
249 fat, the volume of the saturated methanolic KOH solution was increased to 20 mL. Under these
250 conditions fat hydrolysis was complete (no fat traces were found in the residue obtained after
251 evaporation of the hexane extract), and an excess of KOH remained in the aqueous extract.
252 Comparative trials carried out in triplicate on different aliquots of the same dry sample (semolina
253 pasta) added and not with water (2 mL) before the MAS, demonstrated that the water content of the
254 sample does not significantly affect the results. Nevertheless, to obtain similar water content and
255 uniform extraction conditions, dry foods, such as pasta and biscuits, were added with 2 mL of
256 water. For what concerns extraction temperature and time, the same conditions (120 °C for 20 min)

previously used for PAH determination in fish and meat tissue (Akpambang *et al.*, 2009) and later for propolis samples (Moret *et al.*, 2010) were utilised.

The effect of the MAS treatment was preliminary investigated on C10-40 *n*-alkanes mixture, internal standard mixture, paraffin oil and printing ink solvent, which were analysed in duplicate. No appreciable differences in the chromatographic areas were observed with respect to the same standards which did not underwent the MAS procedure, demonstrating that MAS does not determine analyte losses or artefacts formation in absence of the matrix. The same results were obtained in the presence of the matrix, with the exception of CyCy (the internal standard usually used for MOSH quantification), for which some losses were sometimes noticed, especially when processing pasta samples, indicating a possible interaction with some food components. For this reason, quantification of the MOSH for these samples was based on C13 and/or on the external standard.

The pasta and bread samples did not require any sample post-treatment before LC-GC injection (except for reconcentration, depending on the sensitivity required). The alkalinity of the sample extract so obtained did not seem to affect column performance even after a number of analyses. Nevertheless, a rapid wash with water, which takes less than 4 min per sample, was performed during routine analysis to eliminate residual alkalinity (6 mL of the extract were washed with 12 mL of water). For the samples such as biscuits and cakes, a washing step was mandatory to avoid formation of emulsion and gels. It was performed in a separatory funnel as described in 2.3, but later it was found that the sample can be more conveniently added with water directly in the MAS extraction tube and left at -20 °C for 20 min, before concentration and LC-GC analysis.

3.2. Method performance

The method performance was assessed by checking linearity in a matrix-matched calibration curve, by evaluating LOD and LOQ, as well as selectivity, accuracy and precision.

Linearity of the analytical method, previously verified by Barp *et al.* (2013) for a mixture of paraffin oil and offset printing ink in *n*-hexane (in the range 1-50 µg mL^{-1} each), was confirmed also in the presence of the food matrix. To this purpose a blank pasta sample (with no detectable mineral oil) was spiked with increasing amounts of printing ink solvent (9% of MOAH) in the range between 0.20 and 25.0 mg kg^{-1} food (corresponding to 0.18-22.75 mg kg^{-1} of MOSH and 0.02-2.25 mg kg^{-1} of MOAH). The analyses were performed in duplicate at 4 different fortification levels. The slopes of the calibration curves built using the printing ink standard in *n*-hexane and the one built analyzing the fortified pasta sample were compared running a *t*-test at the 5% significance level to confirm the absence of any matrix effect ($p>0.05$). The least squares method was used to estimate the regression line and linearity and the goodness of the curve was confirmed using lack-of-fit and Mandel's fitting tests (Fcalc < Ftab) (Draper and Smith, 1981).

The matrix-matched calibration curve was linear within the concentration range tested ($R^2>0.997$

Although detection and quantification limits in mineral oil analysis are closely related to the molecular weight distribution of the contamination (width of the “hump”), an approximate estimation of these limits was made by considering 3 and 10 times the signal to noise ratio, respectively. A LOD of 0.03 mg kg^{-1} and a LOQ of 0.1 mg kg^{-1} of food sample was obtained when processing 5 g of sample with 10 mL of hexane and concentrating 5 mL of the extract to 1 mL before injection (100 µL).

Method selectivity was verified by analysing uncontaminated samples with MAS and verifying the absence of artefacts with respect to the traces obtained for the same sample by applying classical solvent extraction or PLE.

Method repeatability for different food types was tested by replicate analyses of the same spiked or unspiked sample (n = 4-6). Good repeatability with relative standard deviation (RSD) always lower than 9 was found for all the product types.

Since no certified reference material (with known amount of mineral oil) is commercially available, method accuracy was assessed with recovery tests performed on 4 different cereal-based foodstuffs

of different composition (dry pasta, bread, biscuits and plum cake), using the standard addition procedure described in 2.4.

Table 1 reports the amounts of MOSH and MOAH added, average MOSH and MOAH recoveries, and RSD. The average recoveries ranged from 89 to 104 for the MOSH and from 85 to 108% for the MOAH with RSD lower than 10.

More in detail, for the semolina pasta recoveries obtained (4 replicate analyses) for MOSH and MOAH at 2 different fortification levels were practically quantitative, with RSD lower than 8.

Concerning the bread samples, the standard addition was performed on the extra virgin olive oil used to prepare the bread as described in 2.4. The amount added is expressed on the whole product (before cooking). For recovery calculation, the loss of water occurred during cooking was also considered. The sample prepared with the unspiked oil presented a little contamination coming from the flour which, for recovery calculation (3 replicates), was subtracted from the contamination of the spiked sample. Extraction yields over 90% were obtained also in this case.

The biscuit sample used for recovery test was a baby food product containing 8% of fat (extra virgin olive oil). Figure 1 reports MOSH and MOAH traces of the unspiked and spiked samples, and of the printing ink solvent and paraffin oil standards used for the fortification. As is visible from the figure, the unspiked sample had no detectable contamination (natural *n*-alkanes are visible in the trace). The MOAH trace evidenced the presence of squalene as typical for extra virgin olive oil. Average recoveries, calculated on 5 replicate analyses, ranged from 97 to 103% with RSD $\leq$ 5.

Figure 2 shows MOSH and MOAH overlays of the LC-GC-FID traces obtained for the plum-cake sample before and after fortification with the printing ink solvent standard (two different fortification levels) and for the added printing ink standard directly injected. Since the unspiked sample was contaminated with MOSH in the same molecular mass range of the paraffin oil, it was not fortified with this kind of standard for the recovery test. As is visible from the MOAH traces, the presence of olefins from palm oil interfered slightly with MOAH detection in the fortified samples, but did not prevent recovery calculation.

Method accuracy was also confirmed comparing the LC-GC traces obtained for some selected samples by applying other extraction methods, as described in 3.3.

3.3. Comparison with other extraction methods

Concerning semolina pasta, previous tests demonstrated how overnight extraction with *n*-hexane allows for complete extraction of superficial contamination such as that migrated from the packaging, but does not lead to a complete extraction of deep contamination from different sources, which, instead, can be achieved using PLE as described by Moret et al. (2014) or the procedure described by Biedermann-Brem and Grob (2011). Complete extraction was also achieved when applying MAS on selected samples with different contamination profiles, as confirmed by the perfect overlay of the LC-GC traces with those obtained by applying the procedure described by Biedermann-Brem and Grob (2011) or PLE.

In the case of egg pasta, mineral oil migrated from the packaging rapidly diffuses into the whole matrix (Barp et al., 2015), and "overnight" extraction (16 h) with hexane allows for an almost complete mineral oil extraction, independently of the contamination sources. Complete extraction, comparable to that obtained using MAS, was also obtained by prolonging solvent extraction over the "weekend" (62 h) or using PLE and extracting the unground sample with hexane at 100 °C for 1 hour. LC-GC-FID traces obtained for different samples under these different extraction conditions overlaid perfectly with those obtained with MAS. When applying solvent extraction, mineral oil was co-extracted with the fat, which in the case of dry egg pasta required relatively long time to diffuse from the whole product into the solvent: lower extraction times gave lower fat extraction yields and hence incomplete mineral oil extraction. MAS has also the advantage of avoiding fat co-extraction, which, although present in little amount (egg pasta contains approximately 4% of fat) can rapidly overload the LC column, negatively affecting its performance and making frequent washing necessary to restore it.

The results obtained for bread samples evidenced how overnight extraction with *n*-hexane often underestimates the total mineral oil contamination present in the product (also when adding anhydrous sodium sulphate to remove the water before extraction). Particularly, it was found that after cooking, part of the contamination present in the ingredients becomes less easily extractable with *n*-hexane, but can be quantitatively extracted with MAS. Figure 3 displays an overlay of the LC-GC traces obtained by applying MAS and overnight extraction with *n*-hexane in both the spiked and the unspiked bread. Concerning the unspiked sample, overnight extraction with hexane enabled extraction of 40% of the MOSH extracted with MAS (1.0 mg kg^{-1}). This behaviour, already observed in Melba toast (unpublished data), seems to indicate that after cooking, part of the contamination remains firmly enclosed into the product and cannot be easily extracted with *n*-hexane overnight, but can be completely extracted after MAS.

Differently from semolina pasta and bread, complete extraction of mineral oil from the biscuit samples proved to be less difficult and was achieved also when using overnight extraction. During routine analysis a large number of biscuits with different fat content was analysed in duplicate by applying both MAS and overnight extraction with *n*-hexane (not all data are reported in this work). In all cases the results obtained were comparable, indicating that quantitative mineral oil extraction does not represent a difficult task for this type of samples characterized by a high fat content (typically ranging from 8 up to 25%) and a low moisture content (1-5%).

With respect to solvent extraction which co-extracts also the fat, MAS allows to eliminate the fat before LC-GC analysis, with the advantage that higher sensitivity can be reached because the sample extract can be concentrated before injection without exceeding the capacity of the LC column (20 mg). The higher the fat content of the sample, the higher the sensitivity increase reached by using MAS prior to on-line LC-GC. Furthermore, co-extracted fat injected into the LC column is not completely eliminated during the backflush with dichloromethane and slowly accumulates into the column decreasing progressively its capacity. For this reason, after a number of fat injections, an

intense wash of the LC column with isopropanol and/or methyl tert buthyl ether is required, which can be performed less frequently when using MAS.

Figure 4 shows the LC-GC trace of a biscuit sample (5 g) with 25% of fat, extracted overnight with hexane (10 mL) and directly injected (100 µL), without previous reconcentration to not exceed the column capacity. It also displays the trace of the same biscuit sample processed with MAS and injected after a 5-fold reconcentration to reach higher sensitivity (LOQ around 0.1 mg kg^{-1}).

3.4. Mineral oil content in the analysed samples

Figure 5 summarises the total mineral oil contamination found in a number of cereal-based products from the Italian market, such as dry egg and semolina pasta, and different bakery products (bread, biscuits, cakes) with different fat, sugar and moisture content. MOSH data were divided into three ranges of volatility: MOSH from *n*-C10 to *n*-C16, from *n*-C16 to *n*-C25, and from *n*-C25 to *n*-C35, while MOAH were quantified in the range *n*-C10-C35. MOSH from *n*-C16 to *n*-C35 are the most relevant from a toxicological point of view since they accumulate in human tissues, while MOSH and MOAH up to *n*-C25 are often an index of the presence of mineral oil migrated via gas-phase from recycled paperboard packaging (EFSA, 2012).

The dry semolina and egg pasta in direct contact with recycled paperboard presented always a MOSH contamination in the range *n*-C10-C25, generally centred on *n*-C17-18 and accompanied by 10-25% of MOAH in the same range of volatility, as is typical for products contaminated with mineral oils (from printing ink residues present in recycled fibres) and migrated to food via gas-phase. The highest contamination levels were found in an egg pasta packed in direct contact with recycled paperboard (23.4 and 3.6 mg kg^{-1} of MOSH and MOAH up to *n*-C25, respectively) and in an egg pasta packed in a paperboard tray made of recycled fibres covered with a thin layer of virgin paper and wrapped with a plastic film (14.6 and 2.0 mg kg^{-1} of MOSH and MOAH up to *n*-C25, respectively), while the lowest concentrations were found in semolina pasta packed in plastic film:

0.1 - 2.8 mg kg^{-1} of saturated hydrocarbons up to *n*-C25, mainly due to migration of polyolefin oligomeric saturated hydrocarbons (POSH) from the plastic packaging. The egg pasta packed in virgin paperboard boxes or in trays made of virgin paperboard and wrapped in a plastic film had intermediate contamination levels (MOSH up to *n*-C25 comprised between 1.0 and 3.0 mg kg^{-1}). As previously reported by Barp, Suman, Lambertini and Moret (2015), due to its lower fat content, semolina pasta is less subject to hydrocarbons migration and hence had maximum MOSH amounts around 4.0 mg kg^{-1} even when packed in direct contact with recycled paperboard. The GC profile of some of the egg and semolina pasta revealed the presence of further contamination sources giving "humps" centred on higher molecular weights, generally not accompanied by the presence of MOAH. Contamination in the range from *n*-C25 to C35 varied from 0.1 to 2.7 mg kg^{-1}.

Bread samples were purchased from different supermarkets with their own bakery and were packaged mainly in paperboard bags (sometimes with a plastic window) or in plastic film. The analysed samples presented variable contamination levels (MOSH up to *n*-C35 ranged from 0.7 to 26.4 mg kg^{-1}) from different sources, as confirmed by the LC-GC traces, characterized by the presence of one or more humps comprising hydrocarbons of different molecular weight, centred on *n*-C17-C19, *n*-C28, *n*-C30 and *n*-C33. Particularly, the most contaminated was a sample whose LC-GC profile evidenced a POSH contamination up to *n*-C25 (5.7 mg kg^{-1}) and a MOSH contamination in the range from about *n*-C25 to about *n*-C45, centred on *n*-C33 (20.7 mg kg^{-1} of MOSH from *n*-C25 to C35) with no detectable MOAH. About half of the samples contained detectable amounts of MOAH in the same range of hump volatility centred around *n*-C18 as typical for mineral oil migrated from packaging made with recycled fibres. The most contaminated one was a sample of milk bread with 2.2 mg kg^{-1} di MOAH in the range *n*-C10-35.

Most of the biscuit samples were packed in contact with plastic film or an aluminium layer. The analysed samples had contamination levels ranging from 0.9 to 10.5 mg kg^{-1} of MOSH up to *n*-C35 (on average 3.6 mg kg^{-1}), but only the most contaminated sample (packed in plastic film with a secondary packaging made of recycled paperboard) had detectable amounts of MOAH (0.3 mg kg^{-}

[1]). The LC-GC traces of many biscuits revealed the presence of palm oil used as an ingredient, which was probably responsible for most of the MOSH contamination observed in these samples, characterized by a large hump centred on *n*-C36, without MOAH.

The cake samples comprised plum cakes, brioches and typical Italian Christmas cakes (Panettone and Pandoro) having 15-30% of moisture and a fat content comprised between 10 and 25%. With the exception of traditional Christmas cakes, packed in plastic film and recycled paperboard (secondary packaging), the other products were packed in plastic film only. MOSH contamination up to *n*-C35 ranged from 1.0 to 19.9 mg kg^{-1} (on average 10.4 mg kg^{-1}) and was mainly centred on *n*-C33 and *n*-C36.

About 50% of the pasta and bread samples had MOSH and MOAH levels above 2 and 0.5 mg kg^{-1}, respectively (limits recently proposed in the 22nd ordinance amending the food contact material regulation for paperboard made of recycled fibres). Considering food consumption data, these results clearly indicate a toxicological concern towards cereal based products, particularly pasta packaged in direct contact with recycled paperboard and bread.

All the samples in direct contact with a plastic packaging presented a POSH contamination, which was higher in samples with higher fat content (biscuits and cakes).

4. Conclusions

A MAS procedure allowing for rapid extraction of mineral oil contamination present in cereal-based products of different water and fat content, has been optimized and validated. The proposed method allows to perform simultaneous saponification and extraction of up to 14 samples, with minimal sample manipulation and solvent consumption. Since MAS eliminates the fat, it is particularly advantageous for high-fat-content samples for which sensitivity is limited by the maximum amount of fat than can be injected into the LC column. Furthermore, it allows to avoid frequent washing of the LC column. Cereal-based products analysed with MAS showed relatively high contamination levels, often exceeding the limits proposed in the 22nd ordinance amending the

food contact material regulation for paperboard made of recycled fibres for MOH migrated from packaging.

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Figure 1. LC-GC traces of a biscuit sample used for recovery calculation. The figure displays MOSH and MOAH traces before and after fortification with a printing ink solvent and a paraffin oil.

Figure 2. LC-GC traces of a plum-cake sample used for recovery calculation. The figure displays MOSH and MOAH traces before (unspiked plum-cake) and after (spiked plum-cake) fortification with a printing ink solvent (added standard).

Figure 3. Comparison between MAS and overnight extraction. The figure displays MOSH traces of a sample prepared with unspiked extra virgin olive oil (control bread) and of a sample prepared with the same extra virgin olive oil spiked with printing ink solvent.

Figure 4. Comparison between MOSH traces of a biscuit sample, containing 25% of fat, analysed after overnight extraction (injecting into the LC column an amount of extract corresponding to 50 mg of sample) or after MAS and a 5-fold concentration (injecting an amount of extract corresponding to 250 mg of sample containing more than 70 mg of fat).

Figure 5. MOSH and MOAH content (mg kg^{-1}) of cereal-based products analysed with MAS. MOSH data are divided into 3 ranges of volatility (*n*-C10-C16, *n*-C16-25, *n*-C25-35). The type of packaging in contact with the food is also indicated: Pl, plastic; VP, virgin paperboard; RP, recycled paperboard; RVP, recycled/virgin paper; Al, aluminium.

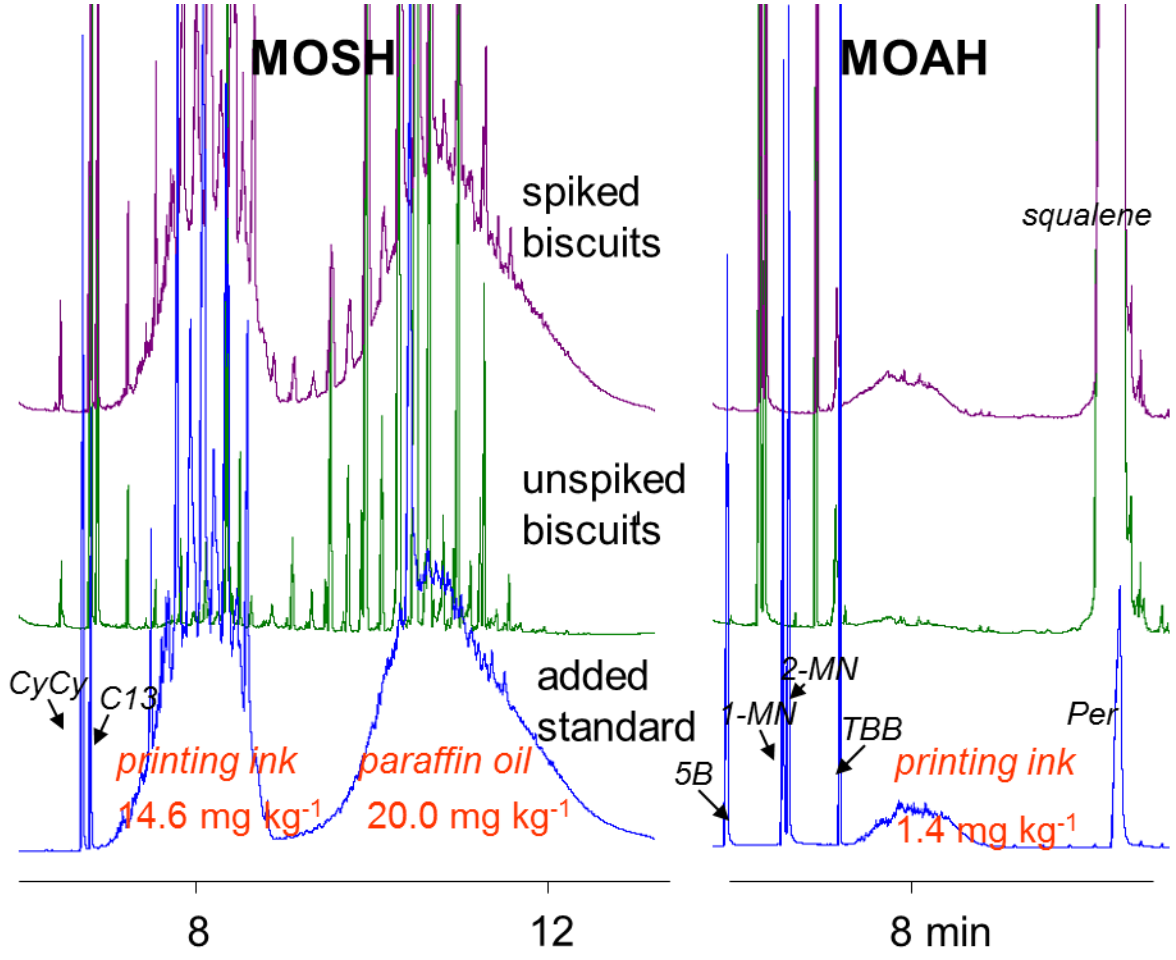
Table(s)

Table 1.Recovery tests:amounts of MOSH and MOAH added, average MOSH and MOAH recoveries, and residual standard deviation (RSD)

Sample	Number of replicates	Added MOSH from paraffin oil (mg kg^{-1})	% Recovery (mean)	RSD	Added MOSH from printing ink solvent (mg kg^{-1})	% Recovery (mean)	RSD	Added MOAH from printing ink solvent (mg kg^{-1})	% Recovery (mean)	RSD
Dry pasta	4	5.0-25	94-103	5.2	3.6-18.2	93-104	6.7-4.9	0.36-1.8	92-108	7.7-4.3
Bread	3	20	90	8.5	14.6	94	9.3	1.4	89	7.9
Biscuit	5	20	99	5.0	14.6	103	4.6	1.4	97	3.8
Plum-cake	4				3.6-18.2	93-101	6.2-7.2	0.36-1.8	95	8.7-5.6

Figure(s)

Fig.1



Figure(s)

Fig. 2

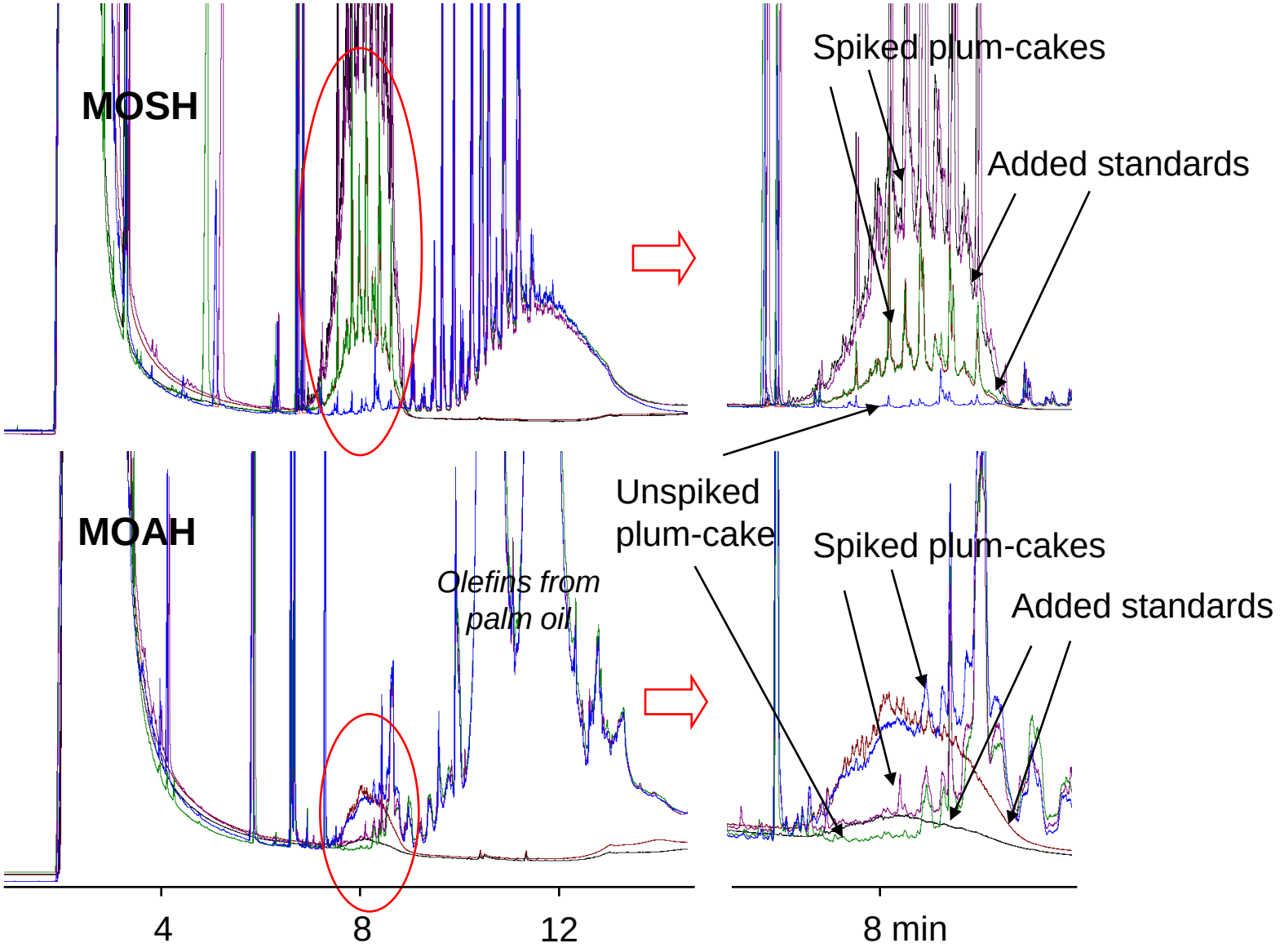


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Fig. 3

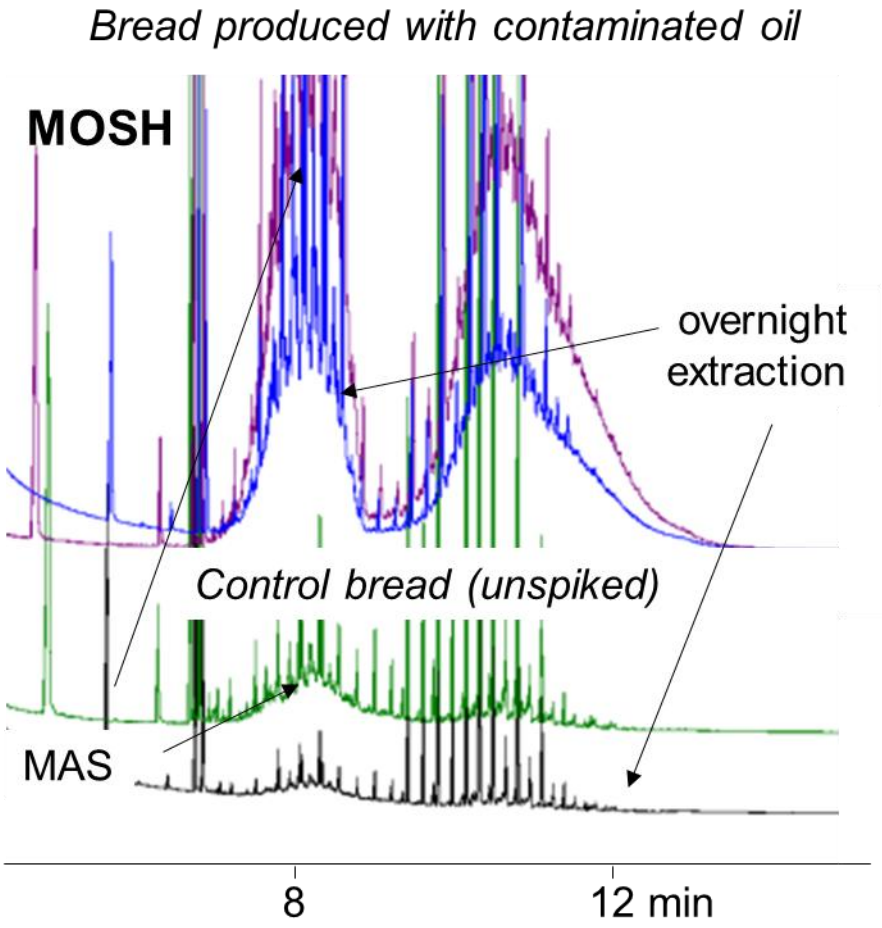

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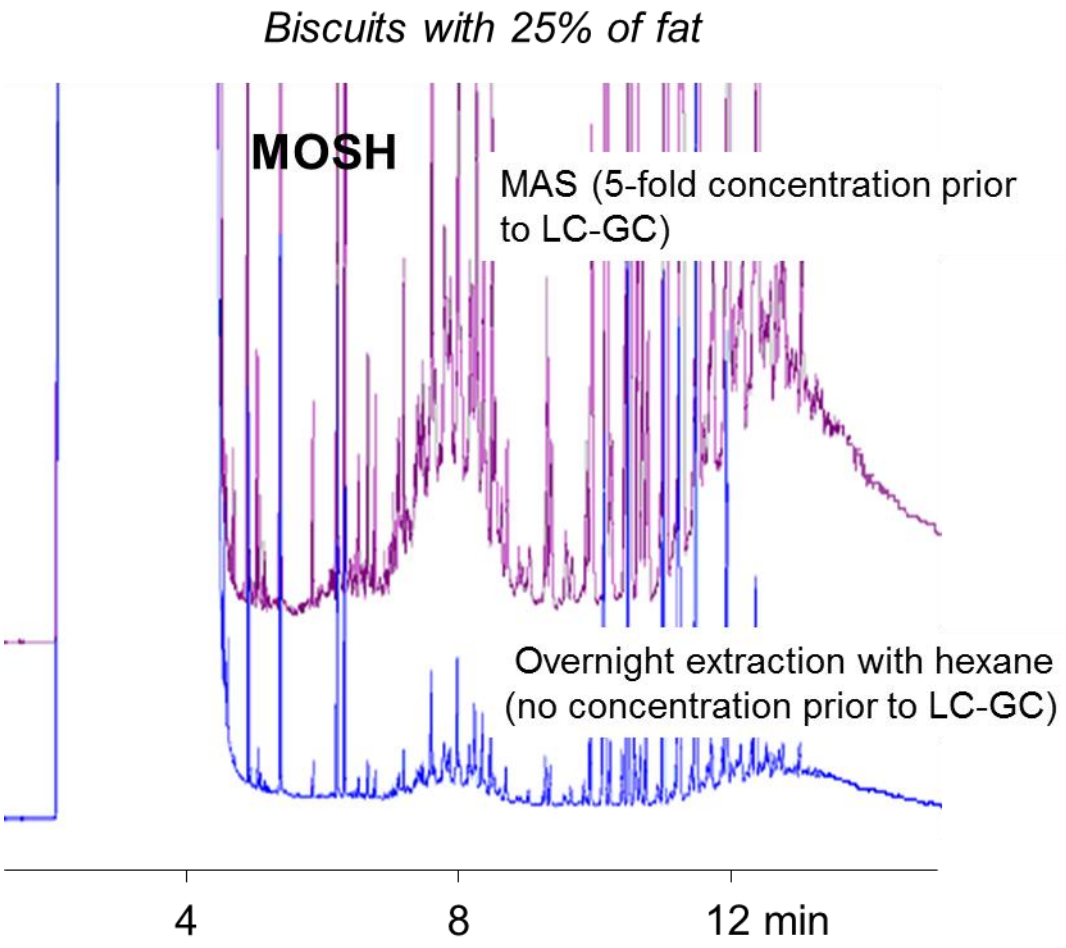
Fig. 4



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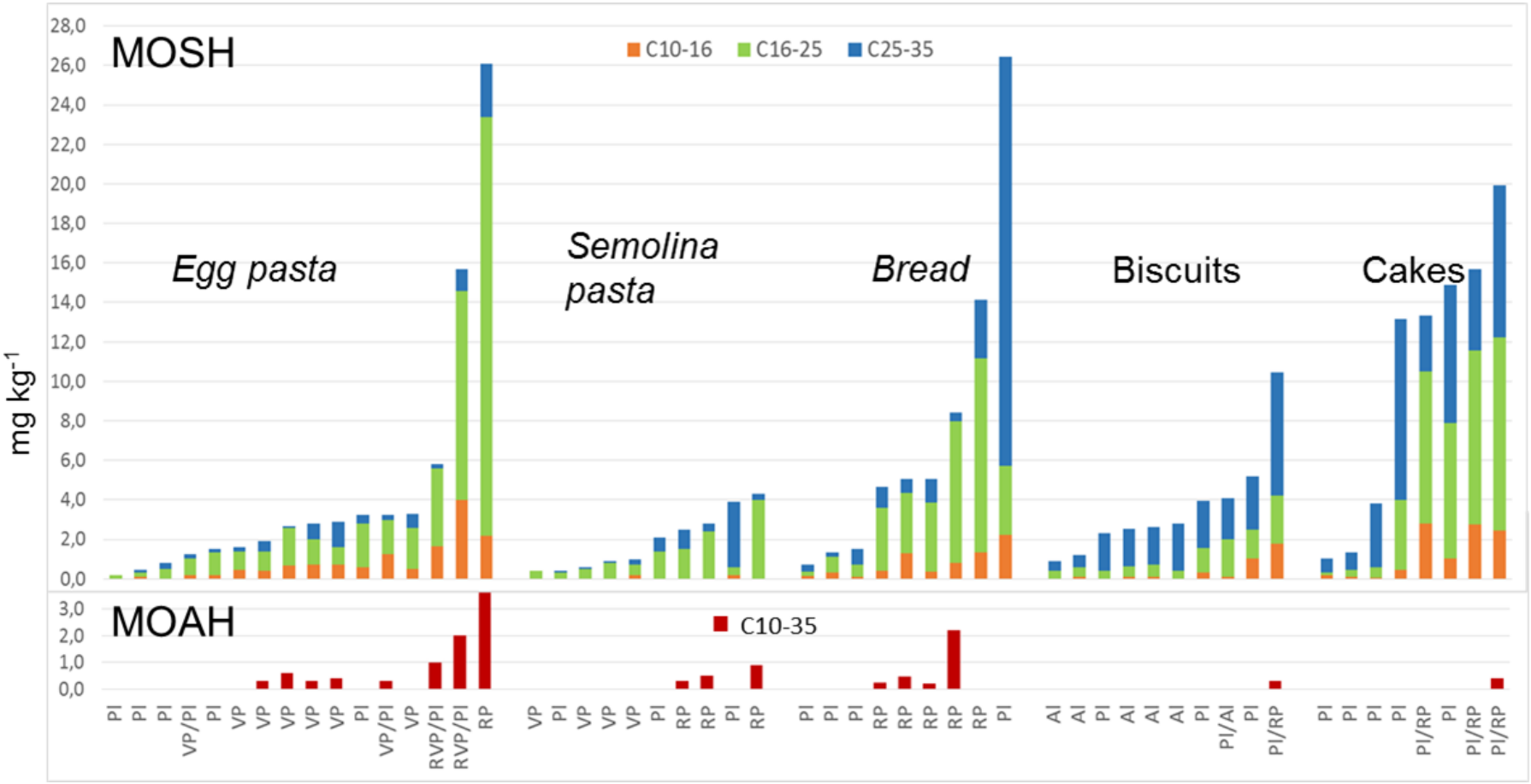
MOSH
C10-16
C16-25
C25-35
mg kg-1
28,0
26,0
24,0
22,0
20,0
18,0
16,0
14,0
12,0
10,0
8,0
6,0
4,0
2,0
0,0
Egg pasta
Semolina pasta
Bread
Biscuits
Cakes
MOAH
C10-35
3,0
2,0
1,0
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VP/PI
PI
VP
VP
VP
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