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EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers and Reference Materials (Geel)
Food and Feed Compliance

Standard Operating Procedure (SOP)

Method for official control of mineral oil content in infant formulas

Foreword

Following the RASFF notification message 2019.3734 (dated 25/10/2019) [1] and the Foodwatch findings [2] related to mineral oil aromatic hydrocarbons (MOAH) in infant formula and follow-on formula (IF), the Directorate General for Health and Food Safety (DG SANTE) of the European Commission requested the Joint Research Centre (JRC) to organise a Roundtable Workshop on the determination of MOAH in IF [3]. The meeting held in Brussels on December 5, 2019, was attended by various stakeholders (e.g. official control laboratories, industry and NGOs), DG SANTE and EFSA. The comparability and reliability of the analytical procedures applied by laboratories to monitor the MOAH content in IF was thoroughly discussed. A broad variety of experimental procedures were reviewed. Participants agreed to simplify the experimental protocols and identified the need for a harmonised method to be validated and further standardised.

JRC committed (i) to coordinate the work; (ii) to collect the available standard operating procedures used by the experienced laboratories; (iii) to draft a harmonised SOP to be reviewed; and (iv) to organise a ring-trial exercise for the validation of the harmonised protocol.

While the data collected during the Method Validation Study (MVS) are currently being evaluated, this Note (Edition 1) presents the Standard Operating Procedure applied by the participating laboratories during the MVS. The performance characteristics derived will be included in the second edition of this Note.

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Contents

1 Scope

The Standard Operating Procedure (SOP) presented in this Note specifies the method to be used by the official control laboratories (OCLs) for the determination of mineral oil aromatic hydrocarbons (MOAH) in infant formulas (IF). This method may also apply for the determination of the mineral oil saturated hydrocarbons (MOSH) in the same matrices.

This method was successfully validated through an interlaboratory comparison (ring trial or collaborative study), attended by 26 experienced laboratories that analysed naturally contaminated and spiked infant formula samples containing 0.5 to 5 mg/kg total MOAH and 5 to 12 mg/kg total MOSH, in three different matrices based on rapeseed oil, palm oil or sunflower oil.

2 Principle

An aliquot of IF powder is reconstituted in hot water and saponified with a potassium hydroxide (KOH) solution. The mineral oils (MO) are extracted with hexane two times. The extracts are then combined, concentrated and cleaned-up over a column filled with activated silica gel eluted with 30 % dichloromethane (DCM) in hexane. After re-concentration the interfering substances (e.g. olefins like squalene) are epoxidised with meta-chloroperoxybenzoic acid (mCPBA) and the reaction is stopped with an aqueous sodium thiosulfate solution followed by the addition of an aqueous sodium carbonate solution. The upper organic phase is separated and re-concentrated, if needed, before its injection into the on-line LC-GC-FID system where MOAH is separated from MOSH.

An additional column is required when a manual MOSH/MOAH separation is performed. After re-concentration of the eluate (30 % DCM in hexane), MOSH are separated from MOAH over a column filled with silica gel covered with silver nitrate. MOAHs are then eluted from the column with a hexane/DCM/toluene mixture, re-concentrated to 0.4 ml and transferred to a vial for injection into the GC-FID.

The total MOAH (C10-C50) mass fraction is expressed as mass fraction (mg MOAH /kg IF).

3 Materials and reagents

Use only reagents recognized as analytical grade at least unless otherwise stated.

Usual laboratory glassware and materials and in particular different sizes of volumetric and conical flasks, beakers, glass Pasteur pipettes, centrifuge glass vials with PTFE sealed screw caps, glass columns with glass frits, glass syringes, spatula etc. All glassware should be rinsed before analysis with acetone followed by hexane to avoid contaminations.

3.1 Chemicals and reagents

- MOSH/MOAH internal standard solution (ISTD): Toluene solution containing n-undecane (C11), cyclohexylcyclohexane (CyCy), 1-methylnaphthalene (1-MN), 2-methylnaphthalene (2-MN), n-pentylbenzene (5B) and 1,3,5-tri-tert-butylbenzene (TBB) at 300 µg/ml, cholestane (Cho) and perylene (Per) at 600 µg/ml and n-tridecane (C13) at 150 µg/ml, as from Restek Corporation (Bellefonte, PA, USA) or equivalent.

Note 1: The verification of the start of the MOAH fraction based on TBB may result in losses of higher alkylated benzenes and naphthalenes present in the sample. Such losses may indicate that the chromatographic performance of the HPLC column is deteriorated. In such cases di(2 ethylhexyl)benzene (DEHB) should be added as verification standard and the fractionation should be adapted.

- QC10 (JRC) solution of Shell SN500* in hexane (2.024 g/L).
- n-Alkane standard solution C10-C50 – (RT calibration mix).
- n-Hexane for gas chromatography, minimum purity 99 %.
- Dichloromethane (DCM) for gas chromatography, minimum purity 99.8 %.
- Ethanol, puriss. p.a., minimum purity 99.9 %.
- Toluene.
- Potassium hydroxide (KOH) pellets for analysis EMSURE®, minimum purity 90 % w/w.
- Water, HPLC grade or equivalent (e.g. milli-Q purified water).
- Sodium sulfate anhydrous, minimum purity 99 %.
- 3-chloroperbenzoic acid (mCPBA), ≤ 77 %.
- Sodium thiosulfate, minimum purity 99 %.
- Sodium carbonate, minimum purity 99.5 %.
- Silica gel 60, size range: 0.063-0.200 mm (70-230 mesh ASTM), activated at 400 °C for at least 16h.
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Note 2: Materials and chemicals listed above and used for this analysis should be checked for the absence of mineral oils by preparing a reagent blank.

3.2 Solutions and preparations

- Saponification solution: 0.5 g/ml KOH in water. Weigh 50 g of KOH pellets (corrected for purity if necessary) in a beaker and dissolve in a small amount of water. Transfer quantitatively the solution into a 100 ml volumetric flask. Wait until cooling down to room temperature and dilute up to the mark with water.
- Epoxidation reagent solution: 0.1 g/ml of 3-chloroperbenzoic acid in ethanol. 1.0 g of mCPBA is purified three times with 10 ml of hexane in a conical flask by vigorous manual shaking for 1-2 minutes followed by decanting the hexane (after a rest of 1-2 min for the particles to settle down) in a waste collection container. Centrifugation for 1 min at 250 rcf is an option as well. Let the hexane evaporate and dissolve the purified acid in 10 ml of ethanol. Prepare the reagent daily.

Note 3: Do not dry at elevated temperatures, mCPBA may then become unstable – **DANGER of explosion!**

- Eluent solution 1: hexane/DCM (7:3) - Mix 70 ml of hexane with 30 ml DCM.
- Ethanol/water solution (1:1) - Mix equal volumes of ethanol and water
- Sodium thiosulfate solution in water – 100 mg/ml e.g dissolve 10g of sodium thiosulfate in 100 ml distilled water and mix thoroughly.
- Sodium carbonate solution in water – 100 mg/ml e.g dissolve 10g of sodium carbonate in 100 ml distilled water and mix thoroughly.
- manual MOSH/MOAH separation - Eluent solution 2: hexane/DCM/toluene (75:20:5, v:v:v). Add 25 ml of DCM and 5 ml of toluene into a 100 ml volumetric flask and fill to the mark with n-hexane.
- manual MOSH/MOAH separation: Silver nitrate - silica gel mix: Silica gel 60 activated containing 0.3 % of silver nitrate . Weigh 29 g ± 0.1 g of activated silica gel 60 into a 250 ml flask. Add 1.0 g ± 0.1 g of silver nitrate on silica gel. Mix thoroughly the mixture by repeated shaking and inverting the flask for at least one minute to ensure a good homogenisation.

Note 4: This mixture should be freshly prepared before filling the glass columns. Prepare only the needed quantity. 3 g of this silica gel mixture are sufficient to prepare one glass column.

4 Apparatus

4.1 General

- N_2 flow evaporator or Rotary evaporator;
- Analytical Balance, readability 0.0001 g, weighing accuracy 0.001 g;
- Centrifuge min 250 rcf (min 1000 rpm) and centrifuge tubes;
- Muffle furnace (min 450°C);
- Test tube shaker with temperature control and agitation (min 500 rpm) (water bath or similar).

4.2 On-line coupled LC-GC-FID system or GC-FID

In order to achieve proper on-line separation of MOSH and MOAH using an on-line LC-GC-FID system the working conditions specified in the manufacturer guidance and/or EN16995:2017 [4] must be strictly applied. Additional guidance is provided hereafter.

4.2.1 GC

The GC separation columns, fused silica or metal capillary, should be stable up to at least 370 °C and should produce low column bleed at high temperature. The instrumental parameter settings for the GC should provide well-shaped chromatogram with a hump attributed to MOSH/MOAH easy to integrate. A fast ramp for the oven temperature is recommended up to 370 °C for 5-6 min. The detector temperature should be above 350 °C in compliance with the guidance of the LC-GC provider.

Note 5: High detector temperatures can lead to a shortened lifetime of the temperature sensor. On the other hand, lower oven and detector temperatures could result in a higher baseline drift at the end of the chromatograms, lack of discrimination of the high boiling point n-alkanes and FID nozzle may require frequent cleaning.

4.2.2 Criteria for the GC performance

Laboratories should use a temperature programme and GC columns and pre-columns that

- Produce symmetrical individual peaks.
- Separate as much as possible the 1-MN and 2-MN peaks.
- Separate completely C10 from the solvent.
- Allow the determination of the mineral oil of up to C50 without significant column bleeding**.**
  The baseline should be straight with as limited positive drift as possible
  The offset of the baseline in the area of the MOSH/MOAH integration should not exceed 1/3 of the height of the hump to be integrated.
- Provide a response ratio for the signals of C50/C20, and C10/C20 - measured in the n-alkane standard solution C10-C50 - between 0.85 and 1.15 in order to guarantee the absence of discrimination against low- or high-boiling point substances. The n-alkane standard solution is also used to determine the retention times (RT) of the n-alkanes. The absence of discrimination check and the RT determination of the n-alkanes should be performed for the two channels by injecting the RT calibration mix of n-alkanes (C10-C50) in both channels.

- Ensure consistent performance of the LC-GC-FID system by providing percent recoveries (of % MOSH + % MOAH in Shell SN 500*) ranging from 90 to 100 % for a QC10-4 solution containing 10.1 mg/l of SHELL 500* in hexane (see JRC report [5]).

5 Procedure

5.1 Sample preparation

Samples should be stored at room temperature.

Note 6: The personnel performing sampling, extraction and analysis should take all necessary precautions to avoid contamination of the sample. For example, the use of cosmetics such as hand cream should be avoided. Each new batch of sample containers and reagents should be checked for mineral oil contamination by running reagent blank sample.

5.1.1 Reconstitution, saponification and extraction of the IF powder

Weigh 5.0 g of IF powder in a 60 ml vial, add 10 µl of ISTD working solution, add 10 ml of water (pre-heated to approximately 35 °C) and shake vigorously (by hand or Vortex) immediately after until no clumps are observed.

Note 7: Caution should be paid when performing the addition of the ISTD as the volume added has a direct impact on the quantification of MOSH and MOAH. Using a 10-µl syringe is recommended. As an alternative, perform a 1:5 dilution of the ISTD solution with hexane and add 50 µl of the diluted ISTD solution obtained.

Add 10 ml of KOH solution (0.5 g/ml) and 5 ml of ethanol and immediately shake manually even before placing the vial into the test tubes shaker with temperature control. Heat the vials at 60 °C for 30 min under intense shaking (>200 rpm). Meanwhile, after the first 15 min, shake vigorously the vial manually for a few seconds. After 30 min remove the vial from the shaker and wait for about one minute. Visually check for the presence of a second fatty layer above the KOH solution (see Annex 1), which would indicate an incomplete saponification. If a layer is observed, proceed with another 30 min of saponification (or until the fat layer disappears) at 60 °C, under intense shaking (> 200 rpm), and applying vigorous manual shaking each 15 min.

Note 8: The time for full saponification depends mainly on the sample composition, on the intensity of the shaking and on the area of contact between the two phases. Horizontal shaking in a water bath is not sufficient to ensure adequate contact. Hence, intense manual shaking each 15 min is required. Alternatively, more efficient shakers could be used. Ensure to avoid the second oily layer above the KOH solution as 3 g of silica gel may then not be sufficient for the column clean-up.

Cool down the solution for about two minutes and extract with 15 ml of hexane while shaking manually for 2 min. After extraction, add 2.5 ml of ethanol to improve the hexane recovery. If needed, centrifuge for better phase separation. Transfer the hexane phase into a 60 ml vial. Add another 15 ml of hexane and perform a second extraction by manual shaking for 2 min. Let the phases separate and centrifuge if needed. Combine the hexane extracts. Wash the extract with 15 ml ethanol/water (1:1 v:v) while shaking gently for 2 min. After the phase separation, evaporate the hexane extract to ca. 2 ml under a gentle stream of nitrogen.

Note 9: Be careful not to transfer even a droplet of KOH solution in the hexane phase. If that happens one washing only will not be enough to obtain a neutral sample extract.

Note 10: Losses of internal standards during concentration of the extract may be problematic. A keeper could be needed, such as MOH free vegetable oil, or 2-3 drops of any higher boiling point plasticizer (e.g. bis(2-ethylhexyl terephthalate or bis(2-ethylhexyl maleate)). However, it may introduce some impurities. Such a keeper may be added at the beginning of the sample preparation (just after addition of the ISTD) to allow correct quantification of the reagent blank.

Note 11: Concentration by an evaporation system such as TurboVap® (Biotage) or Syncore® (Büchi) requires careful optimisation, because losses up to 20 % of the volatile standards including CyCy and MNs, may occur.

5.1.2 Silica gel column purification and re-concentration

This step removes any remaining lipids after the saponification and ensures proper epoxidation.

Fill 3 g of activated silica gel into a glass column. Add 1 g of sodium sulfate on the top. Rinse the column with 10 ml of 30 % DMC in hexane solution. When the entire rinsing solution enters the column, load the concentrated extract onto the column. Wash the vial with 1 ml of eluent 1 and transfer it onto the column as well. Start collecting the eluate in a 40 ml vial from the moment when the entire sample (2 ml) and 1 ml of the washing solution enter the column. Elute by adding 14 ml of the DCM solution in hexane (see above) onto the column. Evaporate the eluate to ca. 1 ml.

Note 12: In this step, the DCM should be completely removed to ensure reliable fractionation in the LC-GC system. Also refer to note 10.

Note 13: If full saponification is not reached after a prolonged time, and a fat layer is still visible above the KOH aqueous phase before the extraction with hexane, then use a 12 g silica column for clean-up. The elution from such a column should be performed with 45 ml DCM solution (see above).

5.1.3 Epoxidation

Add 1.0 ml of the 0.1 g/ml mCPBA solution in ethanol to the concentrated eluate (1 ml) and manually shake briefly. Carry out the epoxidation for 15 min at 40 °C while shaking intensely (>120 rpm). Stop the reaction by adding 2 ml of the 100 mg/ml $Na_2S_2O_3$, solution in water and shake manually. Add 2 ml of the 100 mg/ml Na_2CO_3 solution in water, 0.5 ml of ethanol and 0.5 ml of hexane. Shake the sample for approximately one minute. Remove the aqueous phase (lower phase, sometimes opalescent), and discard it. Wash with 2 ml of ethanol:water (1:1 v:v). Manually shake vigorously and discard the aqueous phase. Dry with a spatula tip of sodium sulfate. Transfer the organic (upper) phase into a 2 ml vial, concentrate to 300 - 500 µl under a gentle stream of nitrogen and inject 50 µl onto the on-line LC-GC-FID system or proceed to 5.2.

Note 14: Similar results could be obtained by following the epoxidation recommended by the DGF [6] procedure for edible oil and fats, a procedure tailored for automation. In some cases, when adding thiosulfate and carbonate in the same solution, crystals were observed in the vials after evaporation. As such it would not compromise the analysis itself, but may be unfavourable for HPLC columns.

5.1.4 Manual MOSH and MOAH separation (when required)

Prepare a silver nitrate-silica column by filling the glass column with 3 g of the 0.3 % silver nitrate on silica gel mixture. Rinse the column with 10 ml of hexane before sample loading. Load the hexane extract (5.1.3) onto the glass column and allow this volume to pass through. Wash the vial with 2 ml of hexane and add it to the column. Discard the eluate.

Elute with 5 ml of hexane. Collect the eluate into a 40 ml vial containing 300 µl of isooctane. Add 1 ml of the hexane/dichloromethane/toluene (75:20:5, v:v:v) mix. Collect the eluate containing the MOSH fraction in the same vial.

Take a new 40 ml vial, and add 10 ml of the hexane/dichloromethane/toluene (75:20:5, v:v:v) mix. Wait until complete elution of the MOAH fraction (until the eluent has completely run out from the column). Evaporate the MOAH eluate under a N_2 flow to 0.3 ml. Inject 50 µl into the GC-FID.

5.2 Verification and quantification of total MOAH

5.2.1 Verification of the SOP's performance and the GC performance criteria

In order to quantify properly the content of MOAH in the sample, the following ratios between the signals of the ISTDs should be verified:

- The 1-MN/2-MN signal ratio should be close to 1.0 ± 0.1; if not, check for interferences on one of the peaks (usually 1-MN).
- The TBB/2-MN signal ratio should be close to 1.0 ± 0.1.

Note 15: This is difficult to achieve after saponification, due to the different distribution ratio of MNs (double ring aromatics) and TBB (single ring aromatic) between aqueous and hexane phase. Even a second extraction is not sufficient to bring the ratio closer to 1. Consequently, a TBB/2MN ratio less than 1.2 is acceptable.

- The TBB/CyCy signal ratio in the sample should be close (within ± 10 %) to the same ratio in the ISTD mix injected directly into the on-line system (to guarantee that there are no losses of TBB during the sample preparation). No losses of TBB should occur due to the deterioration of the HPLC column.

Note 16: The signals for TBB and for CyCy come from different detectors and the response factors might not be similar, consequently the ratio could be different from 1.

- Reagent blank areas should be smaller than 1/3 of the area of the MOAH hump at the level of the limit of quantification. At least one reagent blank sample should be analysed with every batch of samples.

5.2.2 Integration guidance

The parameter "total MOAH content" has to be determined by integration of the whole signal interval in the chromatogram, starting at the retention time of the peak start of C10 and ending at the retention time of the peak end of C50 (C10-C50), after the trimming of the identified riding peaks above the hump(s) and the correction/subtraction of the reagent blank. The shape of the signal has to be identified unambiguously as a smooth hump.

The presence of any superimposed interfering humps should be avoided as much as possible. This can be checked by comparing the MOSH and MOAH chromatograms. When clearly defined and when they not contribute to more than 1/3 of the total area, such superimposed humps have to be subtracted and not included in the area attributed to MOAH (Annex 3, example 1).

When 1-MN and 2-MN are not fully resolved, these peaks must be separated by a vertical line until the baseline and then integrated. Integration valley-to-valley does not represent the entire area of the ISTD and compromises the results.

Positioning of the baseline should follow the baseline of the reagent blank, but sometimes the background in the sample is shifted from the background of the blank chromatogram (signal offset). Consequently, to account for such a shift the baseline of the blank should be adjusted to the background of the sample (see example 2 of Annex 3).

Detailed Guidance on integration and quantifications are given in the JRC Report [7].

5.2.3 Quantification

Total MOAH (C10-C50) should be reported referring to the TBB internal standard, and calculated as specified in Section 4.4 of the JRC Guidance [8]:

$$w_{MOAH} = \frac{A_i \times m_{TBB} \times 1000}{A_{TBB} \times m}$$

where:

A_i is the signal area attributed to MOAH (C10-C50) after the elimination of the identified sharp peaks/overexposed humps above the MOAH hump and if possible, elimination of POH and/or POA signals;

A_{TBB} is the peak area of the internal standard (TBB);

m_{TBB} is the mass of the internal standard (TBB) added to the sample in mg;

m is the mass of the test portion, in g.

Note 17: Quantification of the MOSH fraction (C10-C50) by using CyCy as internal standard proved feasible for IF containing rapeseed oil, palm oil and sunflower oil, from the extract prepared following this SOP, without additional ALOX clean up. More information about MOSH analysis, verification and quantification can be found in the European standard EN16995:2017 [4] (revision in progress).

References

[1] RASFF notification Nr. 408917, dated 25/10/2019. https://bit.ly/3gc0Lad (accessed on 14/10/2022)

[2] "foodwatch Laboratory Tests: Suspected Carcinogenic Mineral Oil Residues in Baby Milk" News, dated 24/10/2019. https://bit.ly/3CXXJyZ (accessed on 14/10/2022).

[3] Report of the "Roundtable Workshop on the Determination of MOAH in Infant Formula." https://europa.eu/!DhbrT3 (accessed on 14/10/2022).

[4] EN 16995:2017 - Foodstuffs — Vegetable oils and foodstuff on basis of vegetable oils — Determination of mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH) with on-line HPLC-GC-FID analysis. European Committee for Standardization, Brussels. https://bit.ly/3VxfrAW

[5] Bratinova S., Robouch P., Goncalves C., Karasek L Beldi G., Senaldi C.,., Valzacchi S., Hoekstra E., H. Determination of MOSH/MOAH in Shell SN500 mineral oil; JRC IF 2021-03 - The 3rd interlaboratory comparison, European Commission, Geel, 2021. https://bit.ly/3VsuvQ7 (accessed on 14/10/2022).

[6] German standard method DGF C-VI 22 (20) in the German collection of standard methods "Deutsche Einheitsmethoden" http://www.dgfett.de/methods/

[7] Robouch, P., Bratinova, S., Oliveira Goncalves, C., Karasek, L., Beldi, G., Senaldi, C., Valzacchi, S. and Hoekstra, E., Mineral oil in infant formulas - guidelines for integrating chromatograms, EUR 31101 EN, Publications Office of the European Union, Luxembourg, 2022, ISBN 978-92-76-53278-1, doi:10.2760/904003, JRC129603. https://bit.ly/3T4eApo (accessed on 14/10/2022).

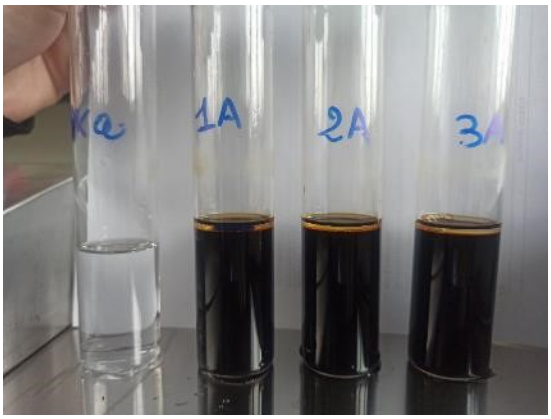
[8] Bratinova S., E. Hoekstra (Editors) Guidance on sampling, analysis and data reporting for the monitoring of mineral oil hydrocarbons in food and food contact materials, Luxembourg: Publications Office of the European Union, 2019 ISBN 978-92-76-00172-0, doi:10.2760/208879, JRC115694. https://bit.ly/3MuJTrf (accessed on 14/10/2022).

Annexes

Annex 1. Flow chart of the procedure in pictures.

Annex 2. Pictures from some difficult samples

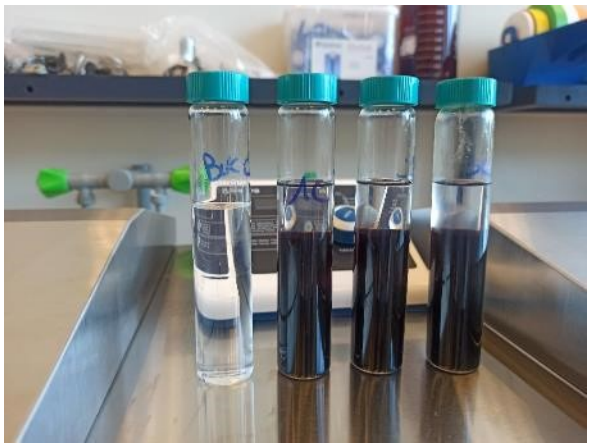
Sample A - Example for non-sufficient saponification after 30 min at 60 °C



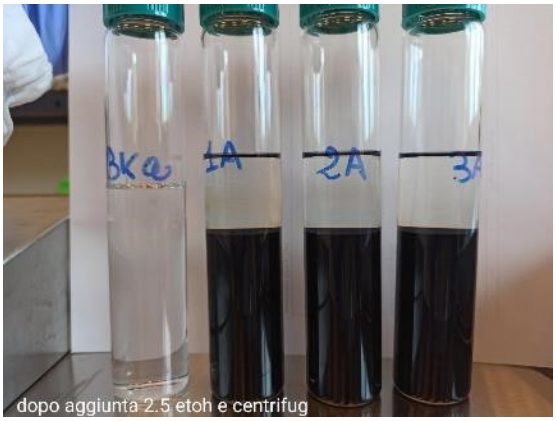
Sample C - After extraction with 15 ml hexane before addition of 2.5 ml ethanol

Sample A - The same samples after 75 min of saponification

Sample C - Same samples as above after addition of 2.5 ml ethanol (better phase separation)



Sample A - After extraction with 15 ml hexane and addition of 2.5 ml ethanol for better phase separation



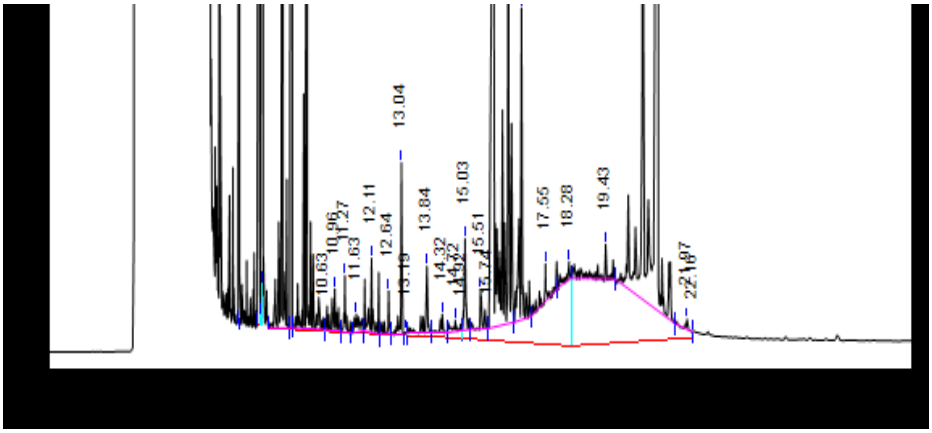
Additional examples – impact of time & shaking on the efficiency of the saponification

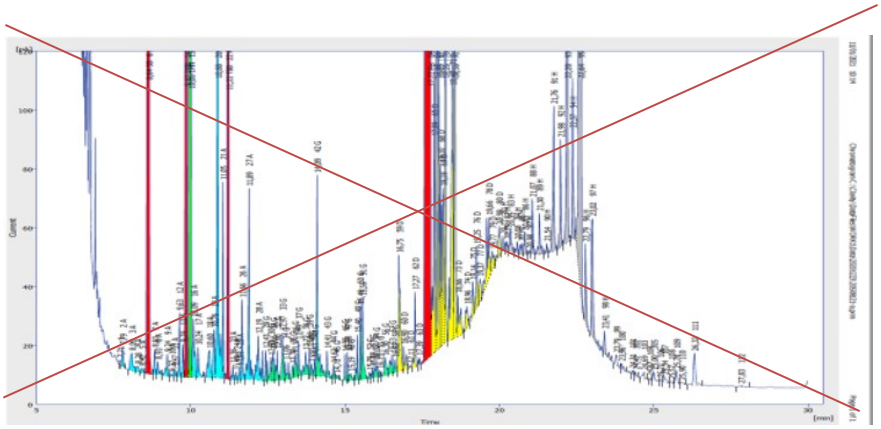
a)- after 30 min at 60 °C – manual shaking at 15 min

b) after 60 min at 60 °C – manual shaking each 15 min

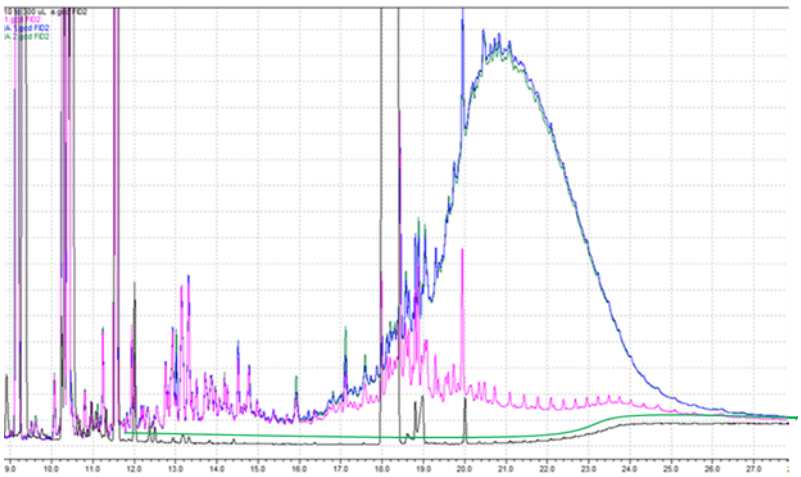
Annex 3. Peak trimming and baseline correction

Example 1 Correct and not correct integration of the interferences to be subtracted





Example 2. Compensation for the baseline offset



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