SUPPLEMENTARY MATERIALS
Methods to determine the quality of acid oils and fatty acid distillates used in animal feeding

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INTRODUCTION

This compendium of methods is the result of applying different methods to 92 samples, 79 acid oils (AO) and 13 fatty acid distillates (FAD), intended for animal feeding (Table 1). These methods are based on methods described in the literature for crude or refined fats and oils. However, there is little information about their application to by-products of edible oil refining, such as AO and FAD. As these samples are very heterogeneous, the application of these methods rises several drawbacks that must be overcome in order to obtain reliable results for these samples. The objective of this compendium is to give a detailed description of the methods, including how to overcome the drawbacks. This compendium will contribute to improve the quality control of these samples.

Table 1

Samples classified according to the refining process and the botanical origin.

Refining process	Botanical origin	Subgroup: different mixtures	n	Total
Chemical refining (Acid oils, AO)	Blends of AO from seed oils, cocoa butter and palm oils[a]	Cocoa butter, rapeseed, soybean and palm oils (40/30/20/10)	2	12
		Cocoa butter, palm and seed oils	10	
	Blends of AO from seed and palm oils[a]	Soybean, rapeseed and palm oils (40/40/20)	2	5
		Sunflower, soybean, palm, corn and rapeseed oils	3	
	AO from olive pomace oil and blends of AO from olive pomace and olive oils	Olive pomace oil	1	18
			3	
		Olive pomace and olive oils (90/10)	5	
	Blends of AO from seed oils[ab]	Sunflower (80-90), rapeseed (20-10) and traces of palm and palm kernel oils and palm stearin	1	9
		Sunflower, corn and grapeseed oils (40/30/30)	3	
		Sunflower, soybean and corn oils	3	
		Sunflower, high oleic sunflower, soybean, corn and olive pomace oils	2	
	AO from sunflower oil	Sunflower oil	18	18
	Blends of AO from sunflower and soybean oils[a]	Sunflower and soybean oils	4	15
		Sunflower and soybean oils (10/90)	7	
		Sunflower and soybean oils (80/20)	2	
		Sunflower and soybean oils (90/10)	2	
	AO from soybean oil	Soybean oil	2	2
Physical refining (Fatty acid distillates, FAD)	FAD from coconut oil and blends of FAD from coconut and palm kernel oils (lauric FAD)[a]	Coconut and palm kernel oils	3	5
		Coconut oil	2	
	FAD from palm oil	Palm oil	6	6
	FAD from olive pomace and olive oils	Olive pomace oil	1	2
		Olive oil	1	

[a] For some blends the proportions were unknown.
[b] Some blends have traces of fruit oils.

METHOD S1- PREPARATION OF THE TEST SAMPLE

1. SUBJECT

This protocol specifies the procedures for the preparation of the samples before the analysis. Applicable to vegetable acids oils from the chemical refining (AO) and fatty acid distillates from physical refining (FAD) of vegetable oils and fats.

2. PRINCIPLE

A representative sample of about 1 L of AO or FAD was obtained from fat and oil producers or feed manufacturers. This sample is homogenized and aliquoted in glass vials of different volumes according to the amount of sample necessary for the subsequent analyses.

3. PREPARATION OF TEST SAMPLE

For samples that are liquid at room temperature, normally those coming from seed oils, the first step consists of shaking vigorously the container containing the representative sample. If complete homogenization is achieved, the sample is immediately aliquoted into vials. If on the contrary, just stirring is not enough for a complete homogenization, it is necessary to heat the sample. In this case, the sample is heated in a water bath to the minimum temperature necessary for a complete melting while it is vigorously shacked every 5 minutes to achieve homogenization (Table S1.1). Once sample is homogeneous it is poured into 1 L glass beaker, making sure that the entire sample falls into it. The beaker is heated in a plate to the same temperature (Table S1.1, controlled by a probe immersed in the oil) with constant agitation (magnetic stirring). Finally, the sample is aliquoted into vials as fast as possible.

In the case of samples that are solid at room temperature, generally FAD from palm, coconut and palm kernel oil, it is necessary to heat the samples to homogenize them. The FAD sample is heated in a water bath (Table S1.1), vigorously shaken at intervals of 5 min during warming to achieve complete homogenization and transferred completely into a 1 L glass beaker. The beaker is heated in a plate to the same temperature (Table S1.1, controlled by a probe immersed in the oil) with constant agitation (magnetic stirring). Finally, the sample is aliquoted into vials as fast as possible.

Sample vials are filled with N_2 in their head-space, capped and stored at -20 ºC until analysis. Before weighing the sample for analysis, the vial is thawed by immersing it in a water bath according to the temperatures established in Table S1.1. To achieve the complete homogenization the vial is shaken vigorously from time to time. As each determination is carried out in duplicate, it should be checked that the sample in the vial remains homogeneous throughout the weighing operations.

The heating temperatures and times are always the minimum necessary to avoid any alteration of the samples by oxidation or polymerization.

Table S1.1

Heating temperatures for the samples according to refining process and botanical origin of the oils.

Refining process	Botanical origin	Heating temperature[a]
CHEMICAL REFINING (Acid oils, AO)	Blends of AO from seed oils, cocoa butter and palm oil	65 ºC
	Blends of AO from seed and palm oils	65 ºC
	AO from olive pomace oils and blends of AO from olive pomace and olive oils	From RT[b] to 35/40 ºC
	Blends of AO from seed oils	If traces of palm, 65 ºC, if not, from RT to 35/40 ºC
	AO from sunflower oil	
	Blends of AO from sunflower and soybean oils	From RT to 35/40 ºC
	AO from soybean oil	
PHYSICAL REFINING (Fatty acid distillates, FAD)	FAD from coconut oil and blends of FAD from coconut and palm kernel oils (lauric FAD)	45 ºC
	FAD from palm oil	65 ºC
	FAD from olive pomace and olive oils	From RT to 35/40 ºC

[a] Heating temperature is applied the minimum time needed to melt and homogenize the sample.

[b] Room temperature.

METHOD S2- DETERMINATION OF FATTY ACID COMPOSITION

1. SUBJECT

This method describes a procedure for the determination of the fatty acid profile (expressed as %) by gas chromatography (GC) in vegetable acid oils (AO) and fatty acid distillates (FAD) of vegetable oils and fats. The method described below is an adaptation of the method described by Guardiola et al. [1].

2. PRINCIPLE

To determine fatty acid composition, a double methylation in methanolic medium is carried out, first with sodium methoxide and later with boron-trifluoride, to ensure that free fatty acids are completely methylated. Then, fatty methyl esters (FAME) are separated by GC-FID and identified by means of comparison of retention times with those of a standard mixture.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.

3.2 Test tubes with screw caps, 10 mL.

3.3 Positive displacement micropipettes suitable to dispense exact volumes of volatile solvents or solutions (MICROMAN®, 100 and 1000 µL from Gilson, Middleton, USA).

3.4 Water bath B-491 from Buchi (Flawil, Switzerland).

3.5 Volumetric pipettes, 3 mL.

3.6 Graduated pipettes, 10 mL.

3.7 Gas chromatograph 4890D from Agilent Technologies (Santa Clara, USA) equipped with flame ionization detector (FID) and split/splitless injector, set at 300 and 270 ºC, respectively. The split ratio is 1:30. Chromatographic separation is performed on a fused-silica capillary column SP-2380 (Merck, Darmstadt, Germany) (60 m × 0.25 mm i.d.) coated with 0.2 µm of a stationary phase of 90% biscyanopropyl- plus 10% cyanopropylphenyl-polysiloxane. Hydrogen, at 25 psi, is used as a carrier gas. The program of the oven is: firstly 1 min at 150 ºC, then increases the temperature by 1.5 ºC/min to 180 ºC at which point it is held for 0.5 min; then by 14.5 ºC/min to 220 ºC

at which point it is held for 3 min, and then finally the temperature is increased by 9.9 ºC/min to 250 ºC and held for 9 min and 30 s. The sample volume injected is 1 µL.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Diethyl ether free from peroxides for analysis, ACS, ISO grade, stabilized with approx. 7 ppm of 2,6-Di-tert-butyl-4-methylphenol (BHT) from Scharlau (Sentmenat, Spain).

5.2 Sodium methoxide 0.5 M solution in methanol, ACS grade, from Sigma-Aldrich (St. Louis, USA).

5.3 Boron trifluoride-methanol complex (20% in methanol), for synthesis, from Merck (Darmstadt, Germany).

5.4 Methanol for analysis, ACS, ISO grade, from Scharlau (Sentmenat, Spain).

5.5 Phenolphthalein indicator, ACS grade, from Scharlau (Sentmenat, Spain).

5.5.1 Phenolphtalein indicator solution, 1% in methanol.

5.6 n-Hexane EMSURE® for analysis, ACS grade, from Merck (Darmstadt, Germany).

5.7 Sodium chloride, Pharmpur®, Ph Eur, BP, USP from Scharlau (Sentmenat, Spain).

5.8 Sodium sulphate anhydrous, powder, for analysis, ACS, ISO grade from Scharlau (Sentmenat, Spain).

5.9 Standard mixture “Supelco 37 component FAME Mix” from Merck (Darmstadt, Germany).

6. PROCEDURE

6.1 Approximately weigh 50 mg of test sample (AO or FAD) into a 10 mL test tube.

6.2 Add 100 µL of diethyl ether using a positive displacement micropipette, to help the dissolution of fat and to favor the subsequent extraction of FAME with n-hexane. Diethyl ether remains until the end of the procedure when FAME are extracted with

n-hexane. At this point the diethyl ether increases the polarity of the n-hexane fraction facilitating the extraction of the more polar FAME coming from short chain FA.

6.3 Add 1.25 mL of sodium methoxide 0.5 M using a positive displacement micropipette. Mark the level of the solvent mixture on the tube.

6.4 Put the tube in a boiling water bath for 20 min. Mix the tube gently after 5 min heating. Check that there is no evaporation of the solvents. If it is the case, add methoxide again until reaching the expected volume.

6.5 Cold the tube to room temperature before uncapping them by placing them in tap water.

6.6 Add 2-3 drops of phenolphthalein 1% in methanol. The solution must become pink.

6.7 Add 1.5 mL of boron trifluoride-methanol complex (20%) in methanol. The tube will become colorless. Mark the level of the solvent mixture on the tube.

6.8 Cap the tube and put into the boiling water bath again for 15 min. Check that there is no evaporation of the solvents during heating.

6.9 Cool down the tube by placing it in tap water. The tube needs to be colorless. If otherwise it is pink, more boron trifluoride-methanol must be added, and the tube needs to be placed in the boiling water bath again for 15 min.

6.10 Add 3 mL of n-hexane EMSURE®. Shake the tube turning upside down for 10 s.

6.11 Add 2 mL of sodium chloride saturated solution and turn the tube upside down for 10 s.

6.12 Wait until the two phases have been separated.

6.13 Transfer the hexane upper phase to another tube containing sodium sulphate anhydrous.

6.14 After 1 h, transfer the hexane to a GC vial and inject into GC.

7. CALCULATIONS

Fatty acid methyl esters are quantified by peak area normalization (peak area percentage):

$$\% \text{ FA} = \frac{\text{FA peak area}}{\text{Total peak areas}} \times 100$$

Where:

FA peak area: is the area of each FAME peak identified.

Total peak areas: is the sum of areas of all the identified FAME peaks.

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

8. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the results obtained for an AO were: for C16:0, 11.66% (RSD = 0.84%); for C18:0, 4.45% (RSD = 0.35%); for C18:1 n-9, 30.46% (RSD = 0.17%); for C18:2 n-6, 46.58% (RSD = 0.13%) and for C18:3 n-3, 1.89% (RSD = 0.25%). For a FAD the results were: for C16:0, 47.53% (RSD = 1.31%); for C18:0, 4.28 % (RSD = 0.98%); for C18:1 n-9, 35.74% (RSD = 0.73%); for C18:2 n-6, 8.99% (RSD = 0.79%); and for C18:3 n-3, 0.35% (RSD = 1.03%).

9. REFERENCES

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METHOD S3- DETERMINATION OF TOCOPHEROL AND TOCOTRIENOL CONTENT

1. SUBJECT

This method describes a procedure for the determination of the amounts of tocopherols (T) and tocotrienols (T3) as measured by HPLC and expressed in mg/kg. This method assesses the eight homologs of vitamin E: α-, β-, γ-, and δ-T and their corresponding T3 in vegetable acid oils from chemical refining (AO) and fatty acids distillates from physical refining (FAD) of vegetable oils and fats. The method described below is a slight adaptation of the methods described by Aleman et al. [1] and Hewavitharana et al. [2].

2. PRINCIPLE

The sample is subjected to saponification. The unsaponifiable matter is extracted with petroleum ether, filtered, evaporated, and dissolved in n-hexane prior to injection in a high performance liquid chromatograph equipped with a silica column and a fluorescence detector.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.

3.2 Volumetric flasks, 50 mL.

3.3 Beaker, 250 mL.

3.4 Glass centrifuge tubes with screw caps, 50 mL.

3.5 Graduated pipettes, 5 and 10 mL.

3.6 Positive displacement micropipettes suitable to dispense exact volumes of volatile solvents or solutions (MICROMAN®, 100, 250 and 1000 µL from Gilson, Middleton, USA).

3.7 Vortex mixer.

3.8 Graduated cylinder, 20 mL.

3.9 Centrifuge Meditronic-BL-S from J.P. Selecta®, with a rotor radius of 10 cm (Abrera, Spain).

3.10 Syringe filter PTFE membrane, 25 mm diameter and 0.45 μm of pore from VWR™ International (Llinars del Vallés, Spain).

3.11 Round bottom flasks, 200 mL.

3.12 Glass test tubes with screw caps, 10 mL capacity.

3.13 Water bath B-491 from Buchi (Flawil, Switzerland).

3.14 Rotary evaporator Rotavapor® R-210 from Buchi (Flawil, Switzerland).

3.15 Dry block heater Tembloc from J.P. Selecta® (Abrera, Spain).

3.16 HPLC system 1100 series from Agilent Technologies (Santa Clara, USA) with the following characteristics:

Mobile phase: n-Hexane/ Dioxane (95/5, v/v)

HPLC isocratic pump, pulseless, with a flow of 1.0 mL/min

Injection valve with a 20 μL loop

Precolumn: security guard system with silica cartridge of 3.0 mm I.D. x 4 mm.

Phenomenex Luna Silica (2) column, 4.6 mm I.D. x 150 mm, 3 μm diameter of the particles, pore size: 100 Å (Phenomenex, Torrance, CA).

Detector: fluorescence (FLD) with excitation wavelength set at 290 nm and emission wavelength set at 320 nm.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Butylated hydroxytoluene (BHT), ACS grade, from Sigma-Aldrich (St. Louis, USA).

5.2 Anhydrous citric acid, ACS grade, from Scharlau (Sentmenat, Spain).

5.3 Pyrogallol, ACS grade, from Sigma-Aldrich (St. Louis, USA).

5.4 Potassium hydroxide (KOH) 85%, ACS grade, from Acros Organic (Geel, Belgium).

5.5 Ethanol absolute, ACS grade, from Panreac (Castellar del Vallés, Spain).

5.6 Methanol, ACS, ISO grade, from Scharlau (Sentmenat, Spain).

5.7 α-, β-, γ-, δ- Tocopherol standards from Calbiochem® from Merck (Darmstadt, Germany).

5.8 1,4-Dioxane, HPLC grade, stabilized with BHT from Carlo Erba Reagents (Val de Reuil, France).

5.9 n-Hexane 99%, HPLC grade, from Scharlau (Sentmenat, Spain).

5.10 Preparation of the antioxidant solution: weigh 0.006 ± 0.0005 g of BHT (*Reagent 5.1*), 0.20 g ± 0.05 g of citric acid (*Reagent 5.2*) and 0.50 g ± 0.05 g of pyrogallol (*Reagent 5.3*), transfer to a 50 mL volumetric flask and dissolve with absolute ethanol. This solution must be prepared extemporaneously.

5.11 Preparation of the potassium hydroxide 1.5 M (approximately) solution: weigh 26.5 g of KOH (*Reagent 5.4*) and transfer it to a 250 mL beaker using methanol. This solution can be used for 1 week.

5.12 Petroleum ether, boiling point 40-60 ºC, ACS grade, from Scharlau (Sentmenat, Spain).

6. PROCEDURE

All the process must be carried out under attenuated lighting conditions (warm white LED light with nil emission at UV region is adequate).

6.1 Accurately weigh 0.20-0.25 g (± 0.0001g) of AO or FAD into a 50 mL centrifuge tube, immediately add 5 mL of the antioxidant solution using a graduated pipette. Mix gently and put the tube in an ice bath. Weigh the next sample and add immediately the antioxidant solution.

6.2 Once every sample is weighted and added the antioxidant solution, add 10 mL of methanolic KOH 1.5 M using a graduated pipette.

6.3 Mix with a vortex mixer for 1 min.

6.4 Put the tubes in a water bath at 70 ºC under gentle agitation during 30 min. Every 10 min mix the tubes manually and put them back in the water bath.

6.5 After exactly 30 min take the tubes from the water bath and put them in an ice bath for 7 min.

6.6 Once the tubes are cold, add 15 mL of distilled water with a graduated cylinder.

6.7 Add 10 mL of petroleum ether with a graduated cylinder.

6.8 Mix the tubes with a vortex mixer for 1 min.

6.9 Centrifuge at 591.42 g for 7 min.

6.10 Take the upper layer with a Pasteur pipette and transfer it to a round bottom flask.

6.11 Add another 10 mL of petroleum ether to the centrifuge tube and repeat steps 6.8-6.10 separating the upper layer using the same Pasteur pipette as before. Repeat this step once more until the total volume of petroleum ether used for the extraction is 30 mL.

6.12 After the three extractions are done, rinse the Pasteur pipette with petroleum ether and collect it in the round bottom flask.

6.13 Using a rotary vacuum evaporator evaporate the solvent at 30 ºC near to dryness.

6.14 Dissolve the dry residue in the round bottom flask with petroleum ether (5 mL) and filter it through a PTFE syringe filter (0.45 μm). Collect the filtrate in a screw-capped test tube.

6.15 Rinse the round bottom flask and the syringe + filter various times with 1-2 mL of petroleum ether each time, collecting the filtrate in the same tube, until approximately 10 mL are collected. To help the filtration process, connect the manifold to the vacuum pump until the whole volume has been filtered and collected in the test tubes.

6.16 Transfer the test tubes to a dry block heater at 30 ºC and evaporate under a nitrogen stream until dryness. Close the tubes and keep them under refrigeration until injection.

6.17 Re-dissolve the final extract in exactly 1 mL of n-hexane and inject in the HPLC system.

6.18 Dilute or concentrate the extract according to the total T concentration (see Table S3.1) and re-inject to do the final quantification.

Table S3.1.

Dilution or concentration of the final extract according to the total concentration of tocopherols (sum of the four tocopherol homologs) in the sample.

Volume of n-hexane added to dissolve the final extract (V) (step 6.17)	Dilution/ concentration factor (f) (step 6.18)[a]	mg/kg of ∑T
1 mL	0.5 or 0.33	2000-8500
1 mL	1	500-2000
1 mL	2, 3, 4 or 5	100-500

Abbreviations: ∑T, sum of the four tocopherol homologs (α-, β-, γ- and δ-tocopherol).

[a] For example, a dilution factor of 0.5 was carried out by taking 200 µL of sample plus 200 µL of n-hexane, and a concentration factor of 2 was carried out by taking 400 µL of sample, evaporating it and re-dissolving it in 200 µL of n-hexane.

7. CALCULATIONS

When T and T3 are identified, the areas are passed through calibration curves previously built with the α-, β-, γ- and δ-T standards. The calibration curves are constructed by plotting values of peak areas versus known concentrations of tocopherols in the standard solutions. Each T3 is quantified using the calibration curve for the corresponding T analogue. Then, the concentrations of T and T3 (expressed as mg/kg) are calculated considering the sample weight and the final dilution of the injected extract, using the following formula for each homolog:

$$\text{Tocol content } \left(\frac{\text{mg}}{\text{kg}}\right) = \frac{(\text{A} - \text{b}) \times (\text{V/f})}{\text{a} \times \text{m}}$$

Where:

A = is the peak area.

a, b = are the coefficients of the calibration curve: y = ax + b (x: µg tocopherol/mL of hexane; y: peak area).

V = is the volume of n-hexane used to re-dissolve the unsaponifiable fraction in mL. It is always 1 mL.

f = is the dilution/concentration factor, if necessary.

m= sample weight, in grams.

Last, to obtain the real T and T3 concentrations in samples, recovery percentages must be applied to the concentrations of T and T3 (see below).

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

8. INTRALABORATORY REPEATIBILITY

Eight determinations of two samples, one AO and one FAD, were carried out to study repeatability of the method, by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions, for AO the RSD were 4.5, 8.4, 4.7 and 6.1% for α-, β-, γ- and δ-T (means = 188.03, 6.72, 158.43 and 5.80 mg/kg respectively, sample weight = 0.20 g) and for FAD were 10.6, 11.0, 13.5 and 7.8% for α-, β-, γ- and δ-T (means = 6.05, 6.02, 5.06 and 18.68 mg/kg respectively, sample weight = 0.20 g).

9. RECOVERY OF THE METHOD

Recovery was evaluated by analyzing six times the same two samples used for repeatability spiked with a mixture of the four tocopherol standards, by the same analyst within a day, using the same reagents, equipment and instruments. The recovery results obtained (mean % ± SD) were for AO 87 ± 0.02%, 82 ± 0.02%, 78 ± 0.03% and 36 ± 0.01% for α-, β-, γ- and δ-T, respectively, and in case of FAD were 96 ± 0.05%, 93 ± 0.04%, 93 ± 0.04% and 63 ± 0.03% for α-, β-, γ- and δ-T, respectively.

10. SENSITIVITY OF THE METHOD

The limits of detection and quantification of the method were respectively: for α-T, 0.09 and 0.30 mg/kg; for β-T, 0.12 and 0.38 mg/kg; for γ-T, 0.12 and 0.39 mg/kg; and for δ-T, 0.15 and 0.50 mg/kg. Both limits were calculated as three and ten times the standard deviation of the base line noise, respectively [3]. Even the recoveries were lower than 100%, the limits were not corrected by the recoveries because the recoveries changed between samples.

11. REFERENCES

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METHOD S4- DETERMINATION OF MOISTURE AND VOLATILE MATTER

1. SUBJECT

This method determines the moisture and any other volatile material under the conditions of the test. Applicable to vegetable acids oils from chemical refining (AO) and fatty acid distillates from physical refining (FAD) of vegetable oils and fats. The method described below is a slight adaptation of the AOCS official method Ca 2d-25 [1] and AOAC official method 926.12 [2].

2. PRINCIPLE

The sample is subjected to heat and vacuum and the % loss in weight is reported as moisture and volatile matter.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.

3.2 Vacuum oven (VO200 from Memmert, Schwabach, Germany).

3.4 Desiccator, containing an efficient desiccant (silica gel with moisture indicator and phosphorus pentoxide, both used to capture the accumulated moisture inside the desiccator).

3.5 Aluminium moisture dishes (55 mm of internal diameter x 35 mm of internal height) from Humboldt (Elgin, USA).

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. PROCEDURE

5.1 Accurately (± 0.0001g) weigh about 5 g of test sample into a tared moisture dish, previously dried under the stablished conditions in a vacuum oven including its lid (see Fig. S4.1): pressure = 66 mbar (approximately 50 mm Hg); temperature = 58 ºC;

min/max temperature = 56-60 ºC. Always use powder-free gloves to handle the moisture dishes.

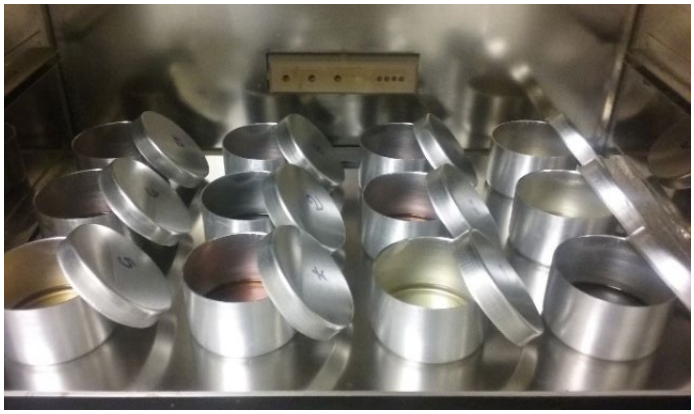


Fig. S4.1. Position of the dishes and lids inside the vacuum oven.

5.2 After 1 h of drying, the dish is lidded, cooled in the desiccator for 30 min and weighed.

5.3 Dry the sample to constant weight in a vacuum oven under the same conditions described above (drying period = 1 h).

5.4 Quickly lid the dish, remove it from the oven, cool to room temperature in a desiccator for 30 min and weigh.

5.5 Repeat the procedure (5.2 and 5.3) until constant weight is attained (when successive 1 h drying periods show additional loss of ≤ 0.05%).

NOTES:

- Some AO samples can give problems due to an excess of moisture, so they can explode producing splattering inside the vacuum oven if they are subjected to the stablished conditions. To avoid that, once the sample has been weighed into the tared dish, the uncovered dish is kept overnight (16 h) into the desiccator under a progressive vacuum until reaching 10 mm Hg to remove part of the moisture and volatiles from the sample (vacuum should be applied progressively to the desiccator to avoid violent boiling and splashing). After this operation, the determination follows the procedure described above (5.2 and subsequent steps).

- Lauric fats contain a significant amount of C6:0-C12:0 fatty acids. In the case of the lauric FAD coming from the physical refining of coconut or palm kernel oils most of the fatty acids (65-82%) are in free form. It has been reported that some of these free fatty acids, mainly C6:0 (boiling point at 50 mm Hg = 135 ºC), can be volatilized in the vacuum oven. This volatilization under our working conditions (constant weight achievement) conducts to an overestimation of the moisture and volatile content. This overestimation was proven in several lauric FAD samples by comparing the results obtained as described above and following the one-component reagent volumetric Karl Fischer method (ISO 8534:2017) [3]. As it can be observed in Table S4.1, if constant weight is attained, the overestimation is considerable; however, if only one 1 h-drying period is applied, the difference with the Karl Fischer results is minimum. These results agree to the fact that during the first drying period, water (boiling point at 50 mm Hg = 37.5 ºC) and the most volatile compounds are lost. Thus, for lauric FAD only one drying period of 1 h was applied to obtain the results of moisture and volatile compounds.

- Alternatively, moisture could be determined in lauric FAD by the Karl Fischer method because these samples are highly saturated and generally have a low content in interfering substances (e.g. peroxides, carbonyl compounds).

- The application to these samples of the hot plate methods, AOCS official methods Ca 2b-38 [4] and Tb 1a-64 [5] gives erroneous results due to the presence of a high amount of C6:0-C12:0 free fatty acids.

Table S4.1.

Determination of moisture (%) in lauric FAD samples by different methods (determinations in duplicate).

	Karl Fischer (ISO 8534:2017)	Vacuum oven	
		One drying period	Constant weight (5 drying periods)
Sample 1	0.28	0.32	0.47
Sample 2	0.16	0.14	0.36
Sample 3	0.18	0.21	0.57

6. CALCULATIONS

The percentage of moisture and volatile matter is given by the formula:

$$\text{Moisture and volatile matter, \%} = \frac{\text{loss in mass, g}}{\text{mass of test portion, g}} \times 100$$

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

7. INTRALABORATORY REPEATIBILITY

Eight determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the RSD obtained for an AO was 0.96% (mean = 0.98%, sample weight = 5 g), 12.80% for a non-lauric FAD (mean = 0.07%, sample weight = 5 g) and 8.43% for a lauric FAD (mean = 0.21%, sample weight = 5 g).

8. REFERENCES

[1] AOCS official method Ca 2d-25. Moisture and volatile matter, in fats and oils, vacuum oven method. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

[2] AOAC official method 926.12. Moisture and volatile matter in oils and fats, vacuum oven method. Official methods of analysis of AOAC International, 21st ed. AOAC International, Rockville, MD. 2019.

[3] International Standard ISO 8534:2017. Animal and vegetable fats and oils, determination of water content, Karl Fischer method (pyridine free). 3rd ed. 2017.

[4] AOCS official method Ca 2b-38. Moisture and volatile matter in butter, fats, margarines, and oils, hot plate method. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

[5] AOCS official method Tb 1a-64. Moisture and volatile matter in fatty acids, hot plate method. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

METHOD S5- DETERMINATION OF INSOLUBLE IMPURITIES

1. SUBJECT

This method describes a procedure for the determination of the content of insoluble impurities in vegetable acids oils from chemical refining (AO) and fatty acid distillates from physical refining (FAD) of vegetable oils and fats. The method determines dirt and other foreign substances insoluble in light petroleum ether. The method is based on the ISO 663:2017 method [1] and the AOCS official method Ca 3a-46 [2].

2. PRINCIPLE

A test portion is treated with an excess of petroleum ether, then the obtained solution is filtered. The filter and residue are washed with the same solvent, then dried at 103 ºC in an air oven and weighed to determine the percentage of insoluble impurities.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.

3.2 Erlenmeyer flask with ground glass stopper, 250 mL.

3.3 Desiccator, containing an efficient desiccant [silica gel with moisture indicator and phosphorus pentoxide, both used to capture the accumulated moisture inside the desiccator, and last, paraffin M.P. 51-53 ºC pellets from Panreac (Castellar del Vallés, Spain) that captures solvents inside the desiccator].

3.4 Filter crucible, ROBU® glass filter, Glasfilter-Geraete GmbH (Hattert, Germany). Capacity of filter crucible: 50 mL; filter plate diameter: 40 mm; and pore: 4, which is equivalent to P16 [10-16 µm of pore size].

3.5 Glass centrifuge tubes with screw caps, 50 mL.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2). In addition, AO and FAD must be dried to constant weight in a vacuum oven as described in this compendium of methods (Method S4, pages 14-17).

5. REAGENTS

5.1 Petroleum ether 40-60 ºC ROTIPURAN® for analysis, ACS, ISO grade from Carl Roth (Karlsruhe, Germany). Having a distillation range between 30 ºC and 60 ºC and having a bromine value of less than 1.

5.2 Celite® Hyflo Super Cel® filter aid (kieselguhr) from Carl Roth (Karlsruhe, Germany).

5.3 Cleaning reagents: isooctane for analysis and chromic acid solution both from Scharlau (Sentmenat, Spain).

6. PROCEDURE

6.1 Prepare the filter crucible: in a 100 mL glass beaker, prepare a slurry consisting of 2 g of kieselguhr and approximately 30 mL of petroleum ether 40-60 ºC. Pour the mixture into the filter crucible under reduced pressure to obtain a homogeneous layer of kieselguhr on the glass filter (according to safety instructions for ROBU® glassware do not exceed 735.56 mm Hg of vacuum). Let the crucible dry for 1 h in the oven at 103 ºC, and then allow it to cool in the desiccator (30 min) and weigh it (m1). Repeat the drying operation until constant weight (maximum difference between weights of 0.003 g), usually constant weight is obtained after the first drying operation). Always use powder-free gloves to handle the filter crucibles.

6.2 Accurately weigh (± 0.0001g) about 5 g for AO or FAD (m0, see notes below) into a tared moisture dish and determine the moisture and volatile matter.

6.3 Transfer the dried sample from the moisture dish to a 250 mL Erlenmeyer flask, using 10 mL of petroleum ether.

6.4 Stopper the flask and shake to assure the complete sample dissolution. Filter slowly through the filter crucible, using suction.

6.5 Repeat the 6.3 step 4 times, so that in total 50 mL of petroleum ether are used to transfer the sample.

6.6 Rinse the flask and wash thoroughly the filter crucible with five fractions of 5 mL of warm petroleum ether (warm the solvent to 50 ºC in a capped centrifuge tube during 10 minutes before to use it) to ensure that all impurities are washed into the crucible.

6.7 Allow most of the solvent remaining in the filter to evaporate in air under fume hood and complete the evaporation in the oven set at 103 ºC for 15 min. Then, remove from the oven, allow to cool in the desiccator (45 min) and weigh. Repeat procedure until constant weight (m2) (this is when the lost in weight does not exceed 0.004 g; normally two weights are needed to achieve constant weigh).

NOTES:

- AO samples with a high percentage of insoluble impurities may be difficult to filter during the analytical procedure. For such samples, 2 g can be weighed (m0) without significantly affecting the precision of the method. According to our results, in AO samples with impurities percentages higher than 10% it is necessary to reduce the sample weight to 2 g instead of 5 g, in order to carry out the method without any problem in the filtration step nor in transferring the entire sample from the dish to the filter. In the case that some remains do not dissolve in the ether and remain in the moisture dish, they must be weighed and this value must be added to the total weight for the calculation of the impurities. In addition, we observed an AO sample with a low percentage of insoluble impurities whose filtration was problematic due to the consistency of the sample itself. In this case the weight was reduced to 2.5 g.

7. CALCULATIONS

The insoluble impurities content, expressed as a percentage by mass, is given by the next formulas. The first one is expressed on wet weight and the other one on dry weight.

$$\text{Insoluble impurities, \% (on wet weight)} = \frac{\text{m2} - \text{m1}}{\text{m0}} \times 100$$

$$\text{Insoluble impurities, \% (on dry weight)} = \frac{\text{m2} - \text{m1}}{\text{m0} \times (\frac{100 - \text{moisture\%}}{100})} \times 100$$

Where:

m0: is the mass of the test portion, in grams.

m1: is the mass of the kieselguhr and the filter crucible, in grams.

m2: is the mass of the kieselguhr, the filter crucible and dry residue, in grams.

Moisture %: moisture and volatile matter content of the sample.

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

8. CLEANING FILTER CRUCIBLES

8.1 Remove all residues from the filter crucible (kieselguhr and insoluble impurities) and dispose of in the appropriate waste container.

8.2 Clean the filter using appropriate solvents under vacuum filtration: first isooctane and then distilled water.

8.3 Let the filter crucible dry and soak it overnight in chromic acid solution.

8.4 Wash the filter thoroughly with water and soap and then rinse with distilled water.

8.5 Dry the filter crucibles 1 h at 103 ºC.

9. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions, the RSD obtained for four different samples of AO were: RSD of 10.85% for the first (mean = 0.51% on wet weight, sample weight = 5 g), RSD of 11.97% for the second (mean = 3.65% on wet weight, sample weight = 5 g), RSD of 12.75% for the third (mean = 10.24% on wet weight, sample weight = 2 g) and RSD of 12.65% for the fourth (mean = 1.57% on wet weight, sample weight = 2 g). For one FAD sample the RSD was 7.72% (mean = 2.85% on wet weight, sample weight = 5 g).

10. REFERENCES

[1] International Standard ISO 663:2017. Animal and vegetable fats and oils, determination of insoluble impurities content. 5th ed. 2017.

[2] AOCS official method Ca 3a-46. Insoluble impurities in fats and oils. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

METHOD S6- DETERMINATION OF UNSAPONIFIABLE MATTER USING DIETHYL ETHER EXTRACTION

1. SUBJECT

This method describes a procedure for the determination of unsaponifiable matter in vegetable acid oils from chemical refining (AO) and fatty acids distillates from physical refining (FAD) of vegetable oils and fats. Unsaponifiable matter includes those substances frequently found dissolved in fats and oils that cannot be saponified by the usual caustic treatment and that are not volatile under the specified operating conditions, but that are soluble in diethyl ether. The method described below is a slight adaptation of the AOCS official method Ca 6b-53 [1], the AOAC Official Method 933.08 [2] and the ISO 3596:2000 method [3].

2. PRINCIPLE

A test portion is saponified with an ethanolic potassium hydroxide solution. The unsaponifiable matter is extracted from the soap solution by diethyl ether. The solvent is then evaporated, and the residue is weighed after drying.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.

3.2 Glass centrifuge tubes with screw caps, 50 mL.

3.3 Erlenmeyer flasks, 250 mL.

3.4 Glass separatory funnels, 250 mL and 500 mL.

3.5 Glass beakers, 500 mL.

3.6 Water bath B-491 from Buchi (Flawil, Switzerland).

3.7 Rotary evaporator Rotavapor® R-210 from Buchi (Flawil, Switzerland).

3.8 Graduated pipette, 10 mL.

3.9 Volumetric flask, 100 mL, to prepare the sodium hydroxide solution 0.01 N.

3.10 Burette, capacity 10 mL, graduated in 0.05 mL.

3.11 Desiccator, containing an efficient desiccant (silica gel with moisture indicator and phosphorus pentoxide, both used to capture the accumulated moisture inside the desiccator, and last, paraffin M.P. 51-53 ºC pellets from Panreac (Castellar del Vallés, Spain) that captures solvents inside the desiccator).

3.12 Air oven capable of operating at 105 ºC.

3.13 Vacuum oven VO200 from Memmert (Schwabach, Germany).

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Ethanol 96% reagent grade, from Panreac (Castellar del Vallés, Spain).

5.2 Potassium hydroxide (KOH) 85%, ACS grade, from Acros Organic (Geel, Belgium).

5.2.1 Potassium hydroxide, 1.5 M (approximately) ethanolic solution: prepared by dissolving 70.6 g of potassium hydroxide in 40 mL of water and dilute to 700 mL with 96% ethanol. The solution should be colorless or straw-yellow.

5.2.2 Potassium hydroxide aqueous solution, approximately 0.5 M: prepared by dissolving 35.3 g of potassium hydroxide in 1 L of distilled water.

5.3 Diethyl ether free from peroxides for analysis, ACS, ISO grade, stabilized with approx. 7 ppm of 2,6-Di-tert-butyl-4-methylphenol (BHT) from Scharlau (Sentmenat, Spain).

5.4 Phenolphthalein indicator, ACS grade, from Scharlau (Sentmenat, Spain)

5.4.1 Phenolphtalein indicator 1% (w/v) solution in ethanol 96%.

5.5 Extemporaneous preparation of a neutralized solvent mixture of ethanol (*Reagent 5.1*) and diethyl ether (*Reagent 5.3*) (20:4, v/v): add phenolphthalein as an indicator (0.3 mL of phenolphthalein by 100 mL of mixture) and neutralize it to the phenolphthalein endpoint.

5.6 Sodium hydroxide (NaOH) 0.1 M solution from Scharlau (Sentmenat, Spain), standardized volumetric solution, factor 0.999-1.001, checked by means of

potentiometric methods using Scharlau´s potassium hydrogen phthalate volumetric standard.

5.6.1 Extemporaneous preparation of the sodium hydroxide 0.01 M solution: pipet exactly 10 mL of sodium hydroxide solution 0.1 M into a volumetric flask of 100 mL and dilute to volume with distilled water. Freshly prepare this solution for each batch of samples.

5.7 Acetone reagent grade, from Scharlau (Sentmenat, Spain).

6. PROCEDURE

6.1 Accurately (± 0.0001g) weigh about 2.0 g of test sample into a 50 mL centrifuge tube. Add 26 mL of potassium hydroxide, approximately 1.5 M ethanolic solution, and heat in a water bath for 45 min at 85 ºC, with occasional swirling. No loss of ethanol should occur during saponification, thus, mark the level of the saponification medium in the tubes to check it.

6.2 Transfer the tube content while warm to the 250 mL separatory funnel using two fractions of 25 mL of water. Wash the centrifuge tube with two fractions of 25 mL of diethyl ether and add to the separatory funnel.

6.3 Insert the stopper and shake vigorously for 1 min and wait until both layers are clear. Draw off the lower aqueous layer into a 250 mL Erlenmeyer flask, including a small amount of diethyl ether layer as well. Transfer the upper diethyl ether layer to a 500 mL separatory funnel. Transfer the aqueous layer from the Erlenmeyer to the same 250 mL separatory funnel to continue the extraction.

6.4 Repeat the extraction three more times, using 50 mL portions of diethyl ether each time and shaking vigorously for 1 min. Combine all the diethyl ether extracts in the 500 mL separatory funnel. AO and FAD are by-products with a high content of unsaponifiable matter, which may require more than four extractions for complete extraction of the unsaponifiable matter. Test, if necessary, by making a further extraction and evaporating this extract. If unsaponifiable matter is found by gravimetry, dissolve the residue in two fractions of 5 mL of diethyl ether and add back to the combined extracts. Continue with the extractions until the extract has no unsaponifiable matter.

6.5 Gently rotate during 20 s the combined diethyl ether extracts with 20 mL of water. Avoid violent agitation at this step since it may result in emulsions that are difficult to separate. Allow the layers to separate completely and discard the lower aqueous layer into the 250 mL Erlenmeyer flask. Always let some aqueous phase stay in the separatory funnel containing the diethyl ether phase, instead it is otherwise indicated. Wash the diethyl ether layer two more times, using 20 mL of water each time.

6.6 Wash the diethyl ether extracts in the separatory funnel three times with 20 mL of KOH 0.5 M (aqueous) each time, shaking vigorously during 20 s and follow each alkali wash by a wash with 20 mL of water.

6.7 After the third wash with 0.5 M KOH, wash the diethyl ether extracts with successive 20 mL portions of water until the washes are no longer alkaline to phenolphthalein 1%. When the last wash is carried out, do the separation eliminating all water from the ether extract.

6.8 Transfer the diethyl ether to a 500 mL flat bottom flask (previously tared after heating at 105 ºC during 30 min and cooling in desiccator). Rinse the separatory funnel using two fractions of 20 and 10 mL of diethyl ether and add the rinses to the solution in the flask.

6.9 Evaporate up to about 1 mL with a rotary vacuum evaporator at 25 ºC and then, under a stream of nitrogen.

6.10 When almost all of diethyl ether has been evaporated, add 3 mL of acetone. The flask will remain in the desiccator all night.

6.11 Complete the drying to constant weight in a vacuum oven for 1 h at 58 ºC, 56/60 ºC min/max temperature and 66 mbar (approximately 50 mm Hg). Cool in a desiccator to room temperature (45 min) and weight. Usually three 1 h-drying steps are necessary to achieve the constant weigh. The result become “A” in the calculations.

6.12 After weighing the residue, dissolve it in 24 mL of a neutralized solvent mixture (ethanol/diethyl ether, 20:4, v/v, containing phenolphthalein) (*Reagent 5.5*).

6.13 To correct the residue for the free fatty acid content, titrate with 0.01 M NaOH solution to a faint pink color (although these by-products are rich in free fatty acids, many of these are washed away, so it is decided to use a 0.01 M NaOH solution and a 10 mL-capacity burette graduated in 0.05 mL). Correct the weight of the residue

for free fatty acid content, using the following relationship: 1 mL NaOH 0.01 M is equivalent to 0.00282 g oleic acid. The grams of fatty acid determined by this titration become “B” in the calculations. Carry out a blank titration with only 24 mL of the neutralized solvent mixture (*Reagent 5.5*) and correct, if necessary, the free fatty acid content (“B”).

6.14 Correct the weight of the residue (“A”) for any possible reagent residue doing a blank by conducting the whole unsaponifiable matter procedure without any fat or oil present. The blank determined by this procedure becomes “C” in the calculations. Using the described reagents, the blank was always zero, but if this residue exceeds 1.5 mg investigate the reagents and the procedure.

7. CALCULATIONS

The unsaponifiable matter, expressed as a percentage, is given by the formula:

$$\text{Unsaponifiable matter, \%} = \frac{A - (B + C)}{m} \times 100$$

Where:

A: is the mass of the residue, in grams.

B: is the mass of the free fatty acids, in grams.

C: is the mass of unsaponifiable blank, in grams.

m: is the mass of the test portion, in grams.

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

8. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the RSD obtained for an AO was 5.35% (mean = 5.02%, sample weight = 2 g) and for a FAD, 3.46% (mean 1.22%, sample weight = 2 g).

9. REFERENCES

[1] AOCS official method Ca 6b-53. Unsaponifiable matter, high level method. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

[2] AOAC official method 933.08. Unsaponifiable of oils and fats. Official methods of analysis of AOAC International, 21st ed. AOAC International, Rockville, MD. 2019.

[3] International Standard ISO 3596:2000. Animal and vegetable fat and oils, determination of unsaponifiable matter, method using diethyl ether extraction. 1st ed. 2000, reviewed and confirmed in 2016.

METHOD S7- DETERMINATION OF ACIDITY AND ACID VALUE

1. SUBJECT

This method describes a procedure for the determination of the content of free fatty acids in vegetable acid oils from chemical refining (AO) and fatty acid distillates from physical refining (FAD) of vegetable oils and fats. The method described below is a slight adaptation of the ISO 660:2009 method [1] and considering some aspects described in the AOCS official method Cd 3d-63 [2].

2. PRINCIPLE

The sample is dissolved in a suitable solvent mixture, and the acids present are titrated with an 0.1 M ethanolic solution of potassium hydroxide. While the acidity or free fatty acid content is expressed as mass percent of lauric, palmitic, erucic or oleic acids, according to the fatty acid composition of the sample (see Table S7.1), the acid value is expressed as the number of milligrams of potassium hydroxide required to neutralize the free fatty acids present in 1 g of fat.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.

3.2 Burette, capacity 25 mL, graduated in 0.05 mL, class A.

3.3 Erlenmeyer flasks with ground glass stopper, 250 mL.

3.4 Erlenmeyer flasks with ground glass stopper, 1 L, to prepare the solvent mixture.

3.5 Volumetric flask, 100 mL, to prepare the phenolphthalein indicator solution 1% in ethanol 96%.

3.6 Graduated cylinder, 100 mL.

3.7 Magnetic stirring device.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Solvent A: Ethanol 96 % (v/v) for analysis, ACS grade, from Panreac (Castellar del Vallés, Spain).

5.2 Solvent B: Toluene for analysis, ACS grade, from Scharlau (Sentmenat, Spain).

5.3 Solvent mixture: mix equal volumes of solvents A and B. Prepare the total quantity depending on the number of samples. Neutralize just before use, under constant agitation, by adding 0.1 M potassium hydroxide ethanolic solution (*Reagent 5.4*) in the presence of 0.3 mL of the phenolphthalein solution (*Reagent 5.5.1*) per 100 mL of solvent mixture. The endpoint of the neutralization consists in the appearance of a persistent faint pink color in the whole solvent mixture. The color change must be achieved by the addition of a single drop of the alkali solution. As some samples must be warmed to achieve a complete solution of the sample before titration, the mixture of ethanol-toluene was used for all samples. The mixture of ethanol-diethyl ether is only appropriate for samples that can be completely dissolved by shaking at room temperature.

5.4 Potassium hydroxide (KOH) 0.1 M solution in ethanol from Carl Roth (Karlsruhe, Germany), standardized volumetric solution, factor 0.996-1.004.

5.5 Phenolphthalein indicator, ACS grade from Scharlau (Sentmenat, Spain).

5.5.1 Phenolphtalein indicator 1% (w/v) solution in ethanol 96 %.

6. PROCEDURE

6.1 Accurately weigh (±0.0001 g) about 0.5 g of test sample into a 250 mL Erlenmeyer flask.

6.2 Add 75 mL of the neutralized solvent mixture (*Reagent 5.3*) by means of a graduated cylinder. Be sure that the sample is completely dissolved. Most samples are dissolved by shaking at room temperature, but some samples need gentle warming to achieve the complete dissolution. The samples more difficult to dissolve are the palm fatty acid distillates that need to be warmed under constant agitation in water bath to maximum of 60 ºC to achieve the complete dissolution.

6.3 Add approximately 1 mL of phenolphtalein indicator solution and titrate with constant magnetic stirring using standardized potassium hydroxide 0.1 M solution in

ethanol. The endpoint of the titration is reached when the addition of a single drop of the alkali solution produces a persistent global color change of the reaction medium. The pink color (or orange pink color for dark samples) must persist at least 30 s. To facilitate the observation of the endpoint, make to fall the drops of the titrating solution away from the eddy created by the magnetic stirring. In this way, it is easily observed when the titration approaches the endpoint due to the persistence of the pink color around the drop that has fallen in the reaction mixture. Remember that the titration endpoint is reached when the addition of a single drop of alkali produces a slight but clear color change affecting the whole reaction mixture and persisting for at least 30 s.

7. CALCULATIONS

The acidity or free fatty acid content expressed as a percentage mass fraction is equal to:

$$\text{FFA (\%)} = \frac{\text{V} \times \text{c} \times \text{M} \times 100}{1000 \times \text{m}}$$

Where:

V = is the volume, in milliliters, of the standardized volumetric potassium hydroxide solution.

c = is the concentration, in moles per liter, of the standardized volumetric potassium hydroxide solution.

M = is the molar mass, in grams per mole, of the fatty acid chosen to express the results according to the fatty acid composition of the fat type (Table S7.1).

m = sample weigh, in grams.

The FFA content (%) or acidity is expressed according to the length of the major fatty acids in the fat (Table S7.1). Thus, in the AO and FAD samples collected in this study, the acidity is expressed as lauric acid for the FAD coming from coconut and palm kernel oils, as palmitic acid for the palm FAD, and as oleic acid for the rest of samples where clearly predominate the fatty acids having 18 carbons.

Table S7.1

Fatty acids used to express acidity (ISO 660:2009).

Type of fat	Expressed as	Molar mass (g/mol)
Coconut oil, palm kernel oil and similar oils	Lauric acid	200
Palm oil	Palmitic acid	256
Oils from certain *Cruciferae*[a]	Erucic acid	338
All other fats	Oleic acid	282

[a] In the case of rapeseed oil having a maximum erucic acid content of 5%, the acidity shall be expressed as oleic acid.
NOTE: If the result is simply as "acidity" without further definition, this is, by convention, expressed as oleic acid.

The acid value expressed as mg of KOH/g of fat is equal to:

$$\text{AV (mg KOH/ g of fat)} = \frac{56.1 \times c \times V}{m}$$

Where:

c = is the concentration, in moles per liter, of the standardized volumetric potassium hydroxide solution.

V = is the volume, in milliliters, of the standardized volumetric potassium hydroxide solution used for the titration.

m = sample weigh, in grams.

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

8. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the RSD obtained for an AO was 4.04% (mean = 61.98 g oleic acid/100 g or 123.30 mg KOH/g of fat, sample weight = 0.5 g) and for a FAD, 1.06% (mean = 74.77 g lauric acid/100 g or 209.74 mg KOH/g of fat, sample weight = 0.5 g).

9. REFERENCES

[1] International Standard ISO 660:2009. Animal and vegetable fat and oils, determination of acid value and acidity. 3rd ed. 2009, reviewed and confirmed in 2014.

[2] AOCS official method Cd 3d-63. Acid value of fats and oils. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

METHOD S8- DETERMINATION OF LIPID CLASSES: POLYMERIC COMPOUNDS, TRIACYLGLYCEROLS, DIACYLGLYCEROLS, MONOACYLGLYCEROLS AND FREE FATTY ACIDS

1. SUBJECT

This method describes a procedure for the determination of the lipid classes (expressed as %) in vegetable acid oils from chemical refining (AO) and non-lauric fatty acid distillates from physical refining (FAD) of vegetable oils and fats by liquid chromatography (HPLC), according to the IUPAC 2508 method [1]. This method is not applicable to fats and oils rich in medium- and/or short-chain fatty acids, such as lauric fats.

2. PRINCIPLE

The triacylglycerols, diacylglycerols, monoacylglycerols, FFA and polymeric compounds in AO and FAD are separated by size exclusion chromatography by means of two HPLC columns connected in series and determined through a refractive index detector. The polymeric compounds are mainly formed when radicals of the main lipid classes (FFA and acylglycerols) react and form new intermolecular bonds.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.

3.2 Test tubes with screw caps, 10 mL.

3.3 Positive displacement micropipettes suitable to dispense exact volumes of volatile solvents or solutions (MICROMAN®, 250 and 1000 µL from Gilson, Middleton, USA).

3.4 Volumetric glass pipettes, 3mL.

3.5 Vortex mixer.

3.6 Nylon filter, 13 mm diameter and 0.45 µm of pore from Scharlau (Sentmenat, Spain).

3.7 Plastic syringe, 2.5 mL.

3.8 HPLC system was an Agilent 1100 series equipped with an isocratic pump, oven (35 ºC) and refractive index detector (RID) from Agilent Technologies (Santa Clara,

USA). Two HPLC columns (30 cm x 7.8 mm i.d.) filled with a highly cross-linked styrene-divinylbenzene copolymer of 5 µm particle size (Styragel HR 1 and Styragel HR 0.5) of 100 and 50 Å pore size, respectively, were used connected in series. Tetrahydrofuran is used both to dissolve samples and as mobile phase at 1 mL/min. The analysis time is about 20 min.

This method is only applicable to fats and oils that are basically composed of fatty acids with 16 and 18 carbons. The method is not applicable to fats and oils rich in medium- and/or short-chain fatty acids, such as lauric FAD as their lipid classes have a wide range of molecular weights, which disables the size exclusion columns from separating them. We noted this problem using several triacylglycerol, monoacylglycerol and free fatty acid standards with different molecular weights. For instance, monolaurin is included in the peak corresponding to C16 and C18 free fatty acids (Fig. S8.1). This is because the molecular weight of monolaurin is 274.4 g/mol and of palmitic and oleic acid, respectively, 256.4 and 282.5 g/mol. For the same reason, tricaprin elutes together with the diacylglycerols in peak 2 (Fig. S8.1). Therefore, we discarded this method for lauric FAD since the separation between lipid classes cannot be achieved as the ranges of molecular weights of different lipid classes are clearly overlapped.

On the contrary, the method separates well the lipid classes in fats and oils mainly composed of fatty acids with 16 and 18 carbons (Fig. S8.2). However, in some AO with low content of polymeric compounds, the peak corresponding to these compounds (peak 1, Fig. S8.2) appears like a shoulder prior the peak corresponding to the triacylglycerols (peak 2).

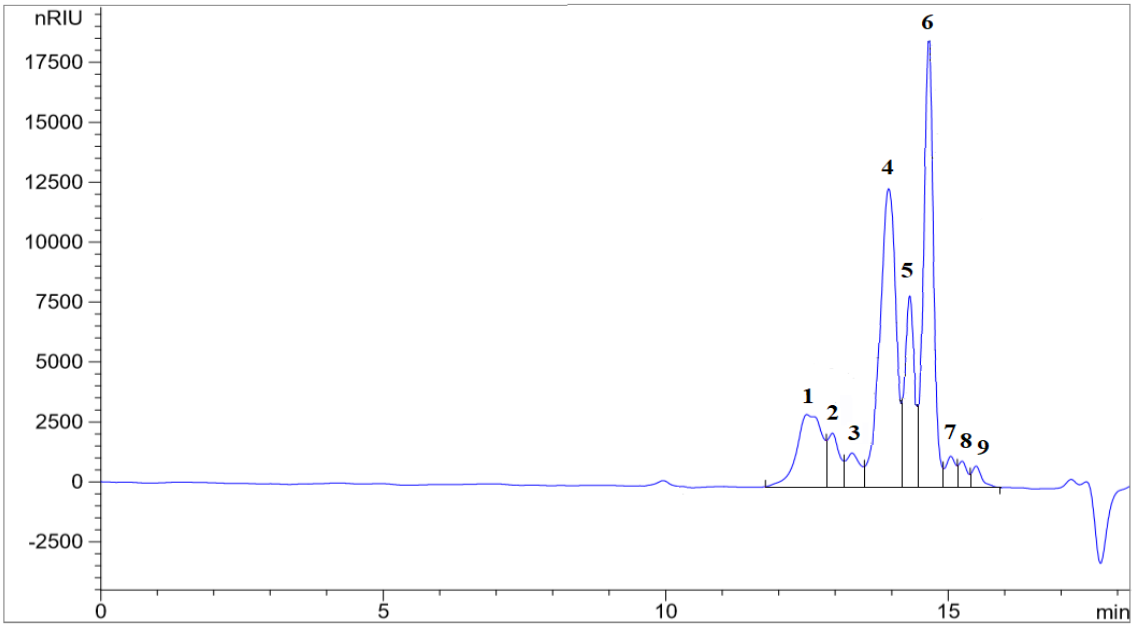


Fig. S8.1. Separation of the lipid classes from a lauric fatty acid distillate. Main compounds corresponding to peaks: 1, triacylglycerols; 2, diacylglycerols; 3, monoacylglycerols; 4, C16 and C18 free fatty acids; 5, myristic acid; 6, lauric acid; 7, capric acid; and 8 and 9, free fatty acids with less than 10 carbons.

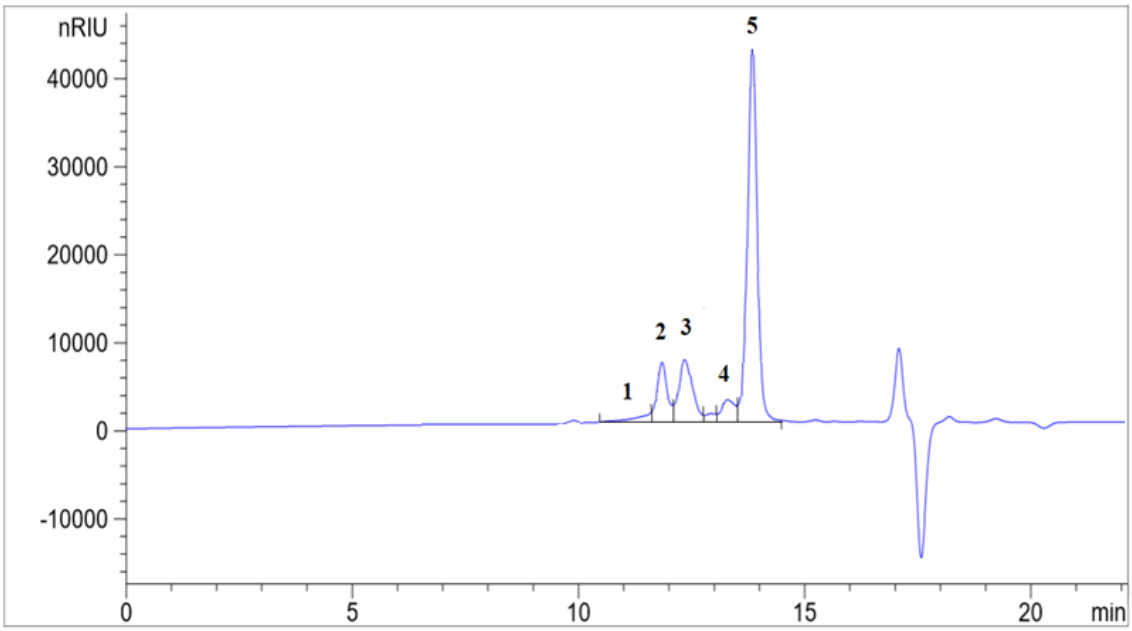


Fig. S8.2. Separation of the lipid classes from an acid oil coming from the refining of olive pomace oil. Main compounds corresponding to peaks: 1, polymeric compounds; 2, triacylglycerols; 3, diacylglycerols; 4, monoacylglycerols; and 5, C16 and C18 free fatty acids.

4. PREPARATION OF THE SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Tetrahydrofuran, HPLC grade, from Scharlau (Sentmenat, Spain).

6. PROCEDURE

6.1 Approximately weigh 150 mg of test sample (AO or FAD) into a 10 mL test tube.

6.2 Add 3 mL of tetrahydrofuran with a volumetric pipette.

6.3 Homogenize using the vortex for 30 s.

6.4 In an empty screw capped tube, add 800 µL of tetrahydrofuran using a positive displacement micropipette.

6.5 Transfer 200 µL of the sample solution prepared in point 6.3, using a positive displacement micropipette, into the tube prepared in point 6.4. Cap the tube and homogenize using the vortex for 10 s.

6.6 Remove the plunger from the plastic syringe and connect the syringe to a Nylon filter (0.45 µm).

6.7 Filter the solution (point 6.5) into a new glass tube or an HPLC vial, using the plunger if necessary.

6.8 Inject in the HPLC-RID system.

7. CALCULATIONS

Each lipid class (triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids or polymeric compounds) is quantified by peak area normalization (peak area percentage):

$$\% = \frac{\text{Peak area}}{\text{Total peak areas}} \times 100$$

Where:

Peak area: is the area of each lipid class peak.

Total peak areas: is the sum of areas of all peaks corresponding to the different lipid classes.

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

8. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the results obtained for an AO were: for polymeric compounds, 2.51% (RSD = 11.38%); for triacylglycerols, 21.39% (RSD = 0.79%); for diacylglycerols, 17.98% (RSD = 1.13%); for monoacylglycerols, 4.05% (RSD = 5.04%); and for free fatty acids 54.07% (RSD = 0.73%). For a non-lauric FAD the results were: for triacylglycerols, 6.03% (RSD = 6.79%); for diacylglycerols, 5.88% (RSD = 9.83%); for free fatty acids, 88.09% (RSD = 0.93%); the polymeric compounds and the monoacylglycerols were not detected.

9. REFERENCES

[1] International Union of Pure and Applied Chemistry (IUPAC). Determination of polymerized triglycerides in oils and fats by high performance liquid chromatography (method 2508). In Standard methods for the analysis of oils, fats and derivatives, 7th ed. IUPAC, Blackwell Scientific Publications, Oxford, UK. 1991.

METHOD S9- DETERMINATION OF PEROXIDE VALUE

1. SUBJECT

This method describes the determination of peroxide value in vegetable acid oils from chemical refining (AO) and fatty acid distillates from physical refining (FAD) of vegetable oils and fats. The method described below is a slight adaptation of the method adopted by the European Union for the analysis of olive oils [1].

2. PRINCIPLE

The peroxide value is the quantity of peroxides (expressed in milliequivalents of active oxygen per kg of fat) in the test sample. The test sample is dissolved in chloroform-acetic acid and treated with a solution of potassium iodide. The iodine liberated by oxidation of potassium iodide is titrated with a standard volumetric sodium thiosulfate solution. The oxidant substances under these conditions are generally assumed to be peroxides.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.

3.2 Erlenmeyer flasks with ground glass stopper and totally dry, 250 mL.

3.3 Volumetric flask, 1000 mL, to prepare the sodium thiosulfate solution.

3.4 Graduated pipettes, 10 mL.

3.5 Micropipette, 1000 µL (PIPETMAN™ from Gilson™ Middleton, USA).

3.6 Graduated cylinders, 25 mL and 100 mL.

3.7 Burette, capacity 25 mL, graduated in 0.1 mL.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Chloroform stabilized with ethanol for analysis, ACS, ISO grade from Scharlau (Sentmenat, Spain).

5.2 Glacial acetic acid for analysis, ACS, ISO grade from Scharlau (Sentmenat, Spain).

5.3 Sodium thiosulfate 0.1 N standardized volumetric solution from Scharlau (Sentmenat, Spain); factor 0.999- 1.001, checked by means of potentiometric methods using Scharlau's potassium iodate volumetric standard.

5.3.1 Extemporaneous preparation of the sodium thiosulfate 0.001 N solution: pipet exactly 10 mL of sodium thiosulfate 0.1 N solution into a volumetric flask of 1000 mL and dilute to volume with distilled water. Freshly prepare this solution each day.

5.4 Potassium iodide for analysis, ACS, ISO grade from Scharlau (Sentmenat, Spain).

5.4.1 Extemporaneous preparation of the saturated solution of potassium iodide free from iodine and iodates: in a small baker add a little amount of distilled water and add the necessary amount of potassium iodide (about 10 g KI in 6 mL of water). Shake with a spatula to favor its dissolution. It can be used when the solution remains saturated (undissolved KI crystals) and colorless. Freshly prepare the solution for each batch of samples (approximately each 30 minutes) and always store in the dark when not in use.

5.5 Starch solution 1% (w/v) from Scharlau (Sentmenat, Spain).

6. PROCEDURE

All the process must be carried out under attenuated lighting conditions (warm white LED light with nil emission at UV region is adequate).

6.1 Accurately (±0.0001 g) weigh by difference about 2.5 g of fat into a 250 mL Erlenmeyer flask. Since some samples are very dark and this makes it difficult the observation of the endpoint of the iodometric titration, no more than 2.5 g should be weighed, even if the expected peroxide value of the sample is much lower than 10 (the 92 samples analyzed had peroxide values lower than 10 and the 95% had peroxide values between 0 and 3).

6.2 Add 10 mL of chloroform using a graduated pipette. Dissolve by shaking the test portion.

6.3 Add 15 mL of glacial acetic acid using a graduated cylinder.

6.4 Pass a nitrogen stream inside the Erlenmeyer flask to remove the O_2 from the solution. Stopper the Erlenmeyer flask.

6.5 Add 1 mL of potassium iodide solution put the caps and shake during 1 min. Then, keep the Erlenmeyer flask in darkness during 5 min at room temperature (between 15-25 ºC). When several samples are analyzed, this step can be done by pairs of Erlenmeyer flasks allowing enough time (around 10 min between pairs) to do the volumetric titration. A maximum of 3 samples in duplicate plus the blank are carried in each batch of samples.

6.6 After the 5 minutes in the darkness, immediately add 75 mL of distilled water.

6.7 Add about 1 mL of starch indicator and titrate with constant and vigorous swirling using sodium thiosulfate 0.001 N solution until the deep blue color disappears.

As the samples showed very low peroxide values and therefore the amount of liberated iodine is low, in most cases, the indicator could be added at the beginning of the titration. If the amount of liberated iodine is high (samples with high peroxide values), start the titration and add the indicator when the iodine yellow color is light. In some cases, there is no apparent liberation of iodine and after adding the indicator no color change is observed at all, which indicates a peroxide value equal to zero.

In the case of FAD samples, the observation of the titration endpoint is clear. However, several AO samples possess very dark colors, hindering the observation of the endpoint. In these samples, the indicator should be added before starting the titration and the constant vigorous shaking should be periodically reduced to gentle shaking to clearly observe the endpoint. The slowdown of the agitation allows the concentration of the sample´s dark color into the chloroform phase and the clear observation of the change of the indicator's color in the aqueous phase.

To facilitate the visualization of the endpoint always use a white background. However, in these samples, remember the importance of the intermittent vigorous shaking during all the titration because the iodine has more affinity for the chloroform phase, but its reduction by thiosulfate to iodide takes place in the aqueous phase.

6.8 Carry out determinations in duplicate and simultaneously perform a blank test for each batch of samples. If the blank test result exceeds 0.1 mL of the sodium thiosulfate 0.001 N, replace the reagents and repeat the determination.

7. CALCULATIONS

The peroxide value is given by the formula:

$$PV = \frac{V \times N \times 1000}{m}$$

Where:

V: is the volume, in milliliters, of sodium thiosulfate solution used for the titration.

N: is the normality of sodium thiosulfate solution.

m: mass of sample, in grams.

All samples were determined in duplicate. Take as the result the arithmetic mean of the two replicates.

8. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the RSD obtained for a dark AO sample was 2.14% (mean = 2.63 milliequivalents of active oxygen per kg of fat, sample weight = 2.5 g) and for a non-dark FAD sample it was 3.05% (mean = 1.27 milliequivalents of active oxygen per kg of fat, sample weight = 2.5 g).

9. REFERENCES

[1] Commission regulation (EEC) 2568/91 of 11 July 1991 on the Characteristics of olive oil and olive-residue oil and on the relevant methods of analysis (and its amendments). Annex III - Determination of the peroxide value, Off. J. Eur. Communities L248 (1991) 1-83.

METHOD S10- DETERMINATION OF *p*-ANISIDINE VALUE

1. SUBJECT

This method describes a procedure for the determination of the amount of aldehydes (principally 2-alkenals and 2,4-dienals) in vegetable acid oils from chemical refining (AO) and fatty acids distillates from physical refining (FAD) of vegetable oils and fats. The method described below is an adaptation of the AOCS official method Cd 18-90 [1].

2. PRINCIPLE

The amount of aldehydes is determined by reaction in an isooctane/acetic acid solution of the aldehydic compounds present in the sample and the *p*-anisidine. The reaction products formed in this reaction absorb at 350 nm. The intensity of the absorption of the reaction products formed depends not only on the amount of aldehydic compounds but also on their structure. A double bond in the carbon chain conjugated with the carbonyl double bond increases the molar absorbance at 350 nm four to five times, so especially 2-alkenals and 2,4-dienals, will contribute substantially to the *p*-anisidine value.

The *p*-anisidine value is defined by convention as 100 times the optical density measured at 350 nm in a 1 cm cuvette of a solution containing 1 g of the sample in 100 mL of a mixture of solvents and *p*-anisidine reagent.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.

3.2 Pyrex® test tubes with Teflon®-lined screw caps, 10 mL.

3.3 Volumetric flasks of 25 mL, or of 50 mL of capacity in case of high absorbances.

3.4 Volumetric pipettes, 1 mL and 5 mL.

3.5 Spectrophotometer suitable for observation at 350 nm (Shimadzu UV-3600, Kyoto, Japan).

3.6 Glass cuvettes, 1.00 ± 0.01 cm.

3.7 Syringe filters ReZist® PTFE membrane, 30 mm diameter and 5 µm of pore from Whatman® (Cytiva, Little Chalfont, UK).

3.8 Syringe filters OlimPeak® CR (Cellulose regenerated) membrane, 13 mm diameter and 0.45 µm of pore from Teknokroma (Sant Cugat del Vallés, Spain).

3.9 Glass syringes, 30 mL.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Isooctane UV, IR, HPLC grade, from Panreac (Castellar del Vallés, Spain).

5.2 Glacial acetic acid for analysis, ACS grade from Scharlau (Barcelona, Spain).

5.3 *p*-Anisidine ≥ 99% from Sigma-Aldrich (Madrid, Spain). During storage, *p*-anisidine tends to darken because of oxidation. It should be stored at 0-4 ºC in a dark bottle.

5.3.1 Preparation of the p-anisidine solution: Weigh 0.25 g of *p*-anisidine. Transfer *p*-anisidine into a 100 mL volumetric flask. Dissolve and dilute to volume with glacial acetic acid. Measure the absorbance of *p*-anisidine at 350 nm (maximun absorbance 0.20).

6. PROCEDURE

All the process must be carried out under attenuated lighting conditions (warm white LED light with nil emission at UV region is adequate).

6.1 Accurately (±0.0001 g) weigh by difference 0.5-0.8 g for AO and 0.7-1.5 g for FAD into a 25 mL volumetric flask. Most often, the proper weight for AO is 0.8 g, however, when after point 6.2. the solution is very turbid it is necessary to reduce the weight to 0.5 g.

6.2 Dissolve and dilute to volume with isooctane (also, n-hexane was tested with these samples, but it was discarded since more turbid solutions were obtained for some AO).

6.3 Most AO samples show suspended particles after dissolution in isooctane and a filtration is necessary before measuring the absorbance of the solution. The filtration of 10-12 mL of the solution is usually made by a PTFE filter of 5 µm of pore (*Apparatus 3.7*). If after filtration the solution obtained is not limpid the procedure is repeated by weighing 0.8 g of sample and doing the filtration with a CR filter of 0.45 µm of pore (only necessary for 6 samples out of 79 AO samples analyzed) (*Apparatus 3.7*). If the filtrate is still turbid, weigh 0.5 g of sample and filter the solution with a CR filter of 0.45 µm of pore (only necessary for 1 sample out of 79 AO samples analyzed). Sometimes gravity is sufficient to filter at an adequate speed, but if not, gentle pressure is applied by the syringe plunger. Average filtration rate: 12.32 mL/min.

For FAD, limpid solutions are obtained and filtration is not necessary. For these samples usually the appropriate weight is 1.5 g, but when absorbance after the reaction with *p*-anisidine is higher than 3, weight must be reduced up to 0.7 g (see linearity section below).

6.4 Measure the absorbance (Ab) of the fat solutions at 350 nm using isooctane as a blank.

6.5 Pipet exactly 5 mL of isooctane into one Pyrex® tube of 10 mL (blank) and exactly 5 mL of the different fat solutions into Pyrex® tubes of 10 mL (samples). Add exactly 1 mL of the *p*-anisidine reagent to each tube and gently shake to complete the homogenization. If you analyze more than one sample in the same batch of analysis, wait 1-2 min between the addition of *p*-anisidine to each sample tube, in order to accurately control the reaction time before the measurement of the absorbance. A maximum of 3 samples were analyzed by duplicate in each batch (6 sample tubes).

6.6 After exactly 10 min of reaction, measure the absorbance at 350 nm (As) of each sample using as a blank the content of the blank tube (isooctane plus *p*-anisidine reagent).

7. CALCULATIONS

The *p*-anisidine value (*p*-AnV) is given by the formulas:

$$p - \mathrm{AnV} = \frac{25 \times (1.2\mathrm{As} - \mathrm{Ab})}{\mathrm{m}}$$

Where:

As: is the absorbance of the fat solution after reaction with the *p*-anisidine reagent.

Ab: is the absorbance of the fat solution.

m: sample weight, in grams.

All samples were determined in duplicate. Take as the result the arithmetic mean of the two replicates.

8. LINEARITY OF THE RESPONSE

To check the linearity of the response, the *p*-AnV of various AO and FAD samples was determined in duplicate using different weights from 0.5-1.5 g. The linearity of the response was confirmed up to an As value of 3.2, since until this As value the *p*-AnV of several samples was independent of the weight. Thus, in all cases the weight of the samples was adjusted in order to obtain As values equal or lower than 3.0. If when weighing 0.5 g of sample, the As is higher than 3.0, then weight the appropriate amount of sample (> 0.5 g) into a 50 mL volumetric flask and use the following formula (this is very unlike to happen; it did not happen in any of the 92 samples analyzed, but it was close to happen in a couple of samples).

$$p - \mathrm{AnV} = \frac{50 \times (1.2\mathrm{As} - \mathrm{Ab})}{\mathrm{m}}$$

9. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the RSD obtained for an AO was 2.96% (mean = 13.47, sample weight = 0.8 g) and for a FAD, 6.98% (mean = 12.62, sample weight = 1.5 g).

10. REFERENCES

[1] AOCS official method Cd 18-90. *p*-Anisidine value. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

METHOD S11- DETERMINATION OF OXIDATIVE STABILITY BY RANCIMAT INSTRUMENT

1. SUBJECT

This method describes a procedure for the determination of the oxidative stability measured by the Rancimat instrument from Metrohm (Herisau, Switzerland) in vegetable acid oils from chemical refining (AO) and fatty acid distillates from physical refining (FAD) of vegetable oils and fats. The method described below is a slight adaptation of the AOCS official method Cd 12b-92 [1] and ISO 6886:2016 method [2].

2. PRINCIPLE

In the Rancimat instrument, the sample is subjected to an air flow and a constant temperature (between 80-140 ºC depending on the sample oxidability) which produces a deterioration of the sample. The air stream sweeps the highly volatile oxidation products such as alcohols, aldehydes, ketones and carboxylic acids, among others, which are transferred into the measuring vessel containing Milli-Q® water and the electrode that measures the conductivity. The conductivity is continuously registered and increases slowly to a sudden jump, at which point the oxidation accelerates and becomes high very rapid. The time elapsed until this rapid acceleration of the oxidation is the measure of the resistance to oxidation and is referred to as the induction time or induction period, which is a good characteristic of the oxidative stability of the sample. The computer software determines the break point of the conductivity curve by using the maximum of the second derivative of the curve and gives the induction time expressed in hours. The longer this time, the greater the oxidative stability of the sample.

3. APPARATUS

3.1 Precision balance, with an accuracy of ± 0.01 g.

3.2 The 892 Professional Rancimat from Metrohm (Herisau, Switzerland) is equipped with two heating blocks, each one with 4 heating positions and the corresponding measuring positions. The 892 Professional Rancimat is controlled by the StabNet® computer software from Metrohm. The Rancimat instrument has different parts that must be assembled to do the determination (Fig. S11.1):

- Fluorinated ethylene propylene (FEP) tubing: for supplying air into the reaction vessel.

- Polyvinylidene fluoride (PVDF) tubing adapters

- Reaction tube cover.

- Disposable glass reaction tube: where sample is weighed.

- Disposable glass cannula to bubble air through the sample. To properly insert it in the reaction tube cover (3), and to tight it, it is inserted through a nitrile O-ring (which does not appear in Fig. S11.1) placed on the reaction tube cover and it is tighten to the tubing adapter.

- Foam barrier piece: used with highly foaming samples to avoid the foam to pass to the measuring vessel. This barrier is optional but, in our case, as some samples foam heavily, it was used for all the AO and FAD samples. The foam barrier can melt if it is introduced too deeply into the heating block. Thus, ensure that the foam barrier is at least 7 cm above the base of the reaction tube. In our case, it was placed approximately at 9.5 cm.

- Silicone tubing: for connecting the reaction (heating) tubes to the measuring vessel.

- Measuring vessel cover: contains an integrated conductivity electrode protected by a ring.

- Polytetrafluoroethylene (PTFE) cannula to bubble air through the water (measuring solution).

- Measuring vessel: filled with 60-80 mL of milli-Q® water.

- Oil separator: Used if samples have a high content of volatile compounds, to avoid that important amounts of sample are transferred to the measuring vessel in the vapor phase. The use or not of the oil separator was tested in AO and FAD, but finally no differences were observed, so it was decided not to use it.

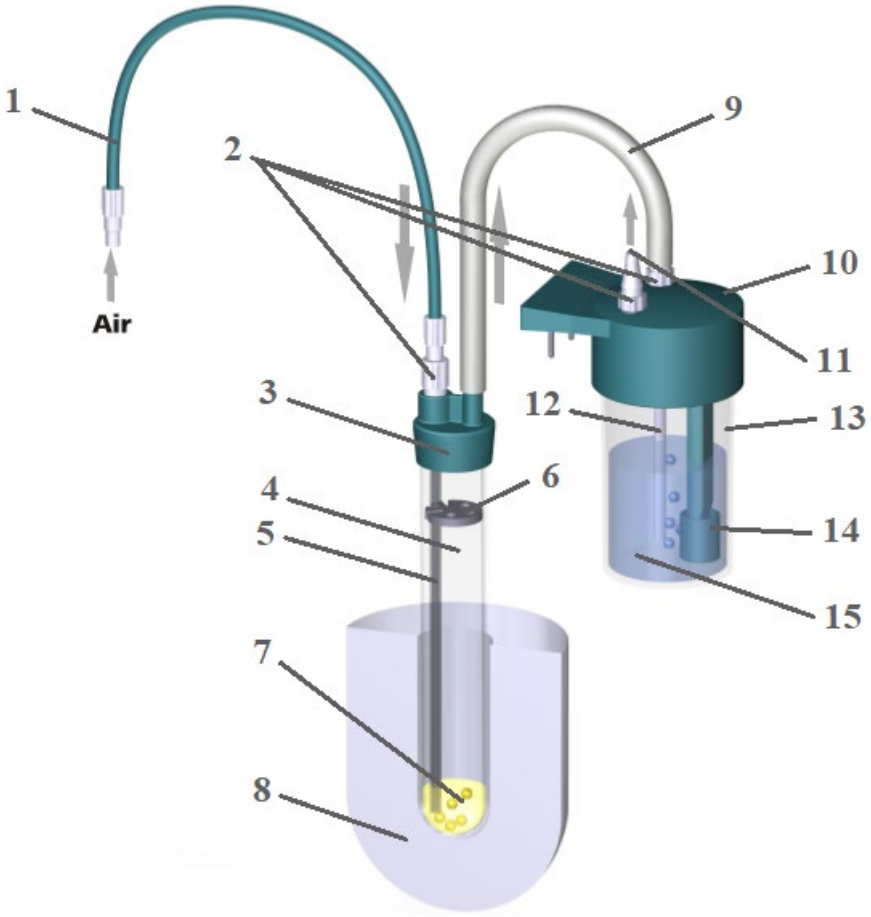


Fig. S11.1. Rancimat parts [3]: 1) Fluorinated ethylene propylene (FEP) tube; 2) Polyvinylidene fluoride (PVDF) tubing adapters; 3) Reaction tube cover made of thermoplastic elastomer-ether-ester (TEEE); 4) Glass reaction tube; 5) Glass cannula; 6) Foam barrier made of polypropylene (PP); 7) Sample; 8) Heating block; 9) Silicone tube; 10) Measuring vessel cover made of PP; 11) Air out hole; 12) Polytetrafluoroethylene (PTFE) cannula; 13) Measuring vessel made of polystyrene (PS); 14) Conductivity electrode protected by a ring; 15) Measuring solution.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. PROCEDURE

5.1 The Rancimat instrument is recognized by the StabNet computer program when both are turned on. Select a method already created or create a new one. The setting parameters of the method used in our case are:

- Air flow, 20 L/h.

- Heating temperature, 120 ºC.

- Temperature correction factor, 1.6 (indicates the deviation of the current sample temperature from the temperature of the heating block).

- Evaluation suppression, 1 h (prevents the evaluation of the conductivity curve for 1 h). This option is useful for samples such as some AO and FAD that contain per se a high content of volatile compounds that cause a very early increase in conductivity, which does not correspond to the endpoint (Fig. S11.3).

- Evaluation sensitivity, 1 (the higher is this value, the lower is the sensitivity to detect the endpoint). The default value is 1, but if a false endpoint is found due to oscillations in the conductivity curve, the curve can be reprocessed by increasing the sensitivity in order to find the true endpoint.

- Stop criterion, 300 h. We do not use other stop criteria (conductivity value, automatic stop once found the endpoint) and set the stop at a very long time (300 h) to be able to manually stop the determination once the sudden increase of conductivity is clearly observed in the screen of the computer. This was done in this way to prevent the determination from stopping before reaching the true endpoint, which forces to make a new determination in duplicate for the sample. The automatic stopping is useful when the samples are known and they behave in a similar manner, which was not our case.

5.2 Fill the measuring vessel with 80 mL of Milli-Q® water. The initial specific conductivity of the water in the measuring vessel should not exceed a value of 5 µS/cm. The amount of water must be between 60-80 mL, so mark both levels in the measuring vessel. During the determination, the air stream evaporates water (approximately 7 mL per day). Thus, for samples with long induction times it may be necessary to add Milli-Q® water to maintain the water level of 60 mL and to keep the electrode safely immersed. If more water needs to be added it must be done through the air out hole (Fig. S11.1). In our case, is done by a glass syringe equipped with a stainless-steel needle.

5.3 Weigh the sample material (3 or 3.5 ± 0.1 g) directly in the reaction tube. The usual weight for AO and FAD samples is 3 ± 0.1 g, but for some AO samples that have a very long induction time (> 80 h), 3.5 ± 0.1 g of sample is weighed for the glass cannula immerse deep enough into the sample throughout the determination.

5.4 The temperature defined in the method has to be reached before the reaction tube is inserted in the heating block (at this moment the Rancimat screen will go from red to green color). Connect the reaction tube to the air supply and to the measuring system as represented in Fig. S11.1 (first connect the FPE tube and then the silicone one), introduce the reaction tube into the heater and immediately press the start button to start the data recording. When mounting the system remember that the PTFE cannula must be positioned such as to avoid air bubbles directly reaching the conductivity electrode (Fig. S11.2). Air bubbles at the electrode produce noisy measuring curves that are difficult to evaluate.

5.5 Once the endpoint of the determination is reached, press stop in the StabNet software, disconnect the tubes from the system and remove them from the heating block. Then, the air stream can be turned off (never turn off the air stream before the tubes have been removed and disconnected from the system because the sample could be sucked in and contaminate the FPE tube and finally the equipment.

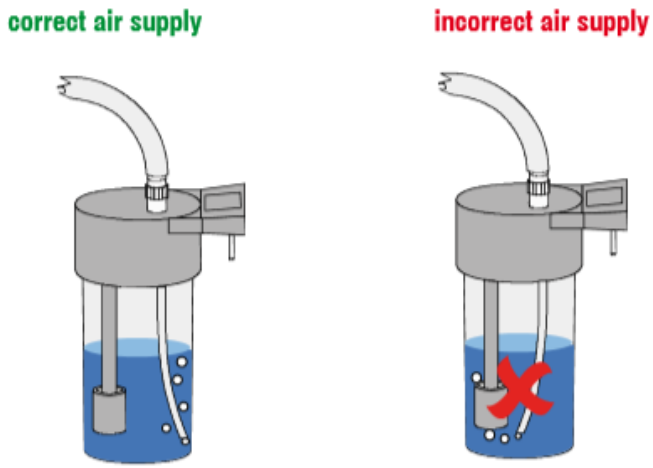


Fig. S11.2. Correct and incorrect position of the PTFE cannula with respect to the electrode [4].

6. CLEANING

Dispose used glass reaction tubes and glass cannulas and use new ones for the next determination. If there is no lipid polymerization during the determination that makes difficult the cleaning, the reaction tubes are cleaned and reused a maximum of two times.

The reaction tubes, measuring vessels and all the accessories represented in Fig. S11.1, except the fluorinated ethylene propylene (FEP) tube, are cleaned as follows:

- First remove the excess of dirt by hand cleaning with dishwashing detergent and suitable laboratory brushes, this is particularly important in the polyvinylidene fluoride (PVDF) tubing adapters, reaction cover tube, reaction tube, foam barrier, silicone tube and polytetrafluoroethylene (PTFE) cannula.
- Afterwards, all the material, except the O-ring and the measuring vessel cover, is cleaned at the dishwasher at 80 ºC. The O-ring and the measuring vessel cover with the incorporated electrode are thoroughly cleaned by hand with dishwashing detergent. To clean the electrode more easily, the protective ring can be removed (the protective ring is cleaned at the dishwasher).
- Afterwards, all the material, except the polystyrene measuring vessel, is rinsed with acetone and thoroughly with Milli-Q® water. The acetone cannot be used for cleaning the measuring vessel because it degrades the polystyrene.
- The following pieces must be dried in the oven at 80 ºC for 2 hours: PVDF tubing adapters, reaction tube cover, nitrile O-ring, glass reaction tube, foam barrier, silicone tube and PTFE cannula. The plastic pieces of the previous list can absorb reaction products during the measurement and to avoid carry over to the next measurement, it is important to dry these accessories at 80 ºC for 2 h.
- The measuring vessel cover with the incorporated electrode and the measuring vessel are dried at room temperature. The FEP tube should only be cleaned if it gets dirty by error (see point 5.5 in the procedure).

7. OPERATION AND MAINTENANCE

The air flow in the 892 Professional Rancimat is usually supplied using the internal air pump, which aspirates laboratory air. For air purification, the following accessories must be checked:

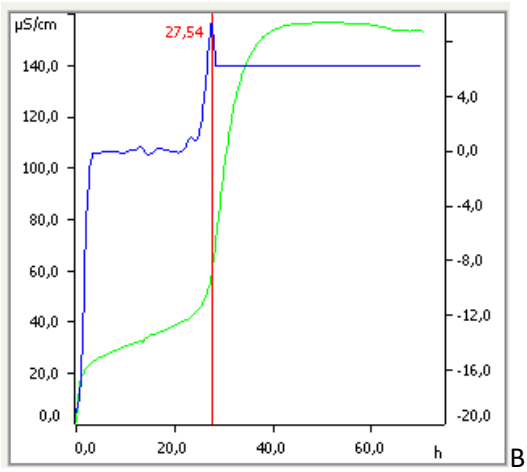
- The molecular sieve serves to adsorb interfering water from the aspirated air. Each 6-8 weeks it is necessary to change it. It is possible to regenerate it in the drying oven at 160 °C for 24 h.
- The dust filter serves to filter the air aspirated through the air pump and must be checked at periodic intervals and replaced in the case of intense contamination (the filter contamination level can be evaluated through the color of the filter in the air intake side).

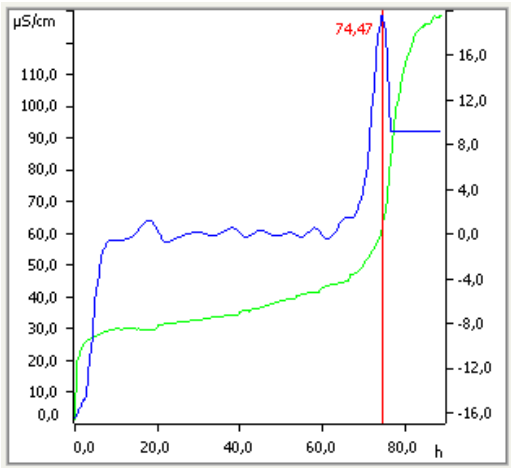
8. CALCULATIONS

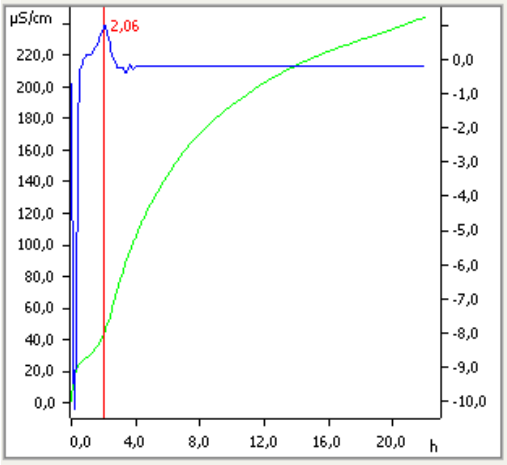
The computer software determines the break point of the conductivity curve by using the maximum of the second derivative of the curve and gives the induction time expressed in hours. Below some graphs corresponding to AO and FAD samples are shown (Figs. S11.3 and S11.4). The determination of the induction time has been attempted in a total of 92 samples (79 AO and 13 FAD samples), but in 10 AO samples the conductivity curve did not show a clear jump and the induction time could not be determined.

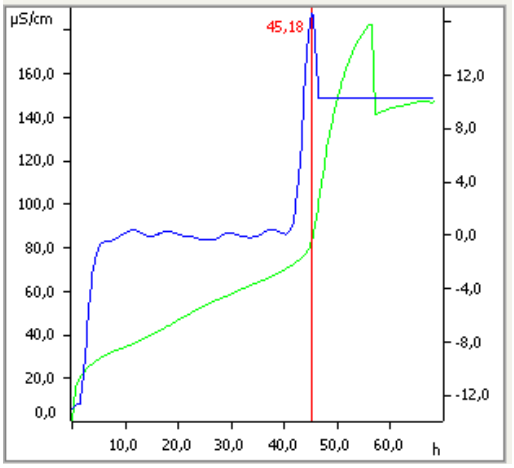
All samples were determined in duplicate. Take as the result the arithmetic mean of the two replicates.

The repeatability of the determination is good since the relative standard deviation (RSD%) was calculated for the duplicates of the 82 samples determined and the median of the RSD was 2.33%.

A) 

B) 

C) 

D) 

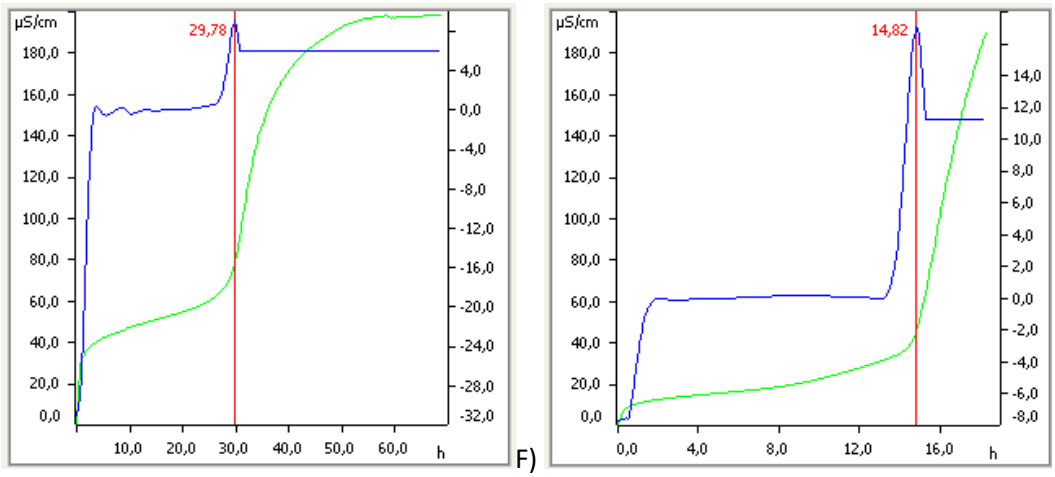


Fig. S11.3. Examples of the conductivity curve (green), its second derivative (blue) and its induction time (red) for some acid oil (AO) samples: A) Blend of AO from seed oils, cocoa butter and palm oil; B) Blend of AO from seed and palm oils; C) AO from olive pomace oil ; D) Blend of AO mainly from seed oils (sunflower, high oleic sunflower, soybean, corn and olive pomace oils); E) AO from sunflower oil; F) Blend of AO from sunflower and soybean oils.

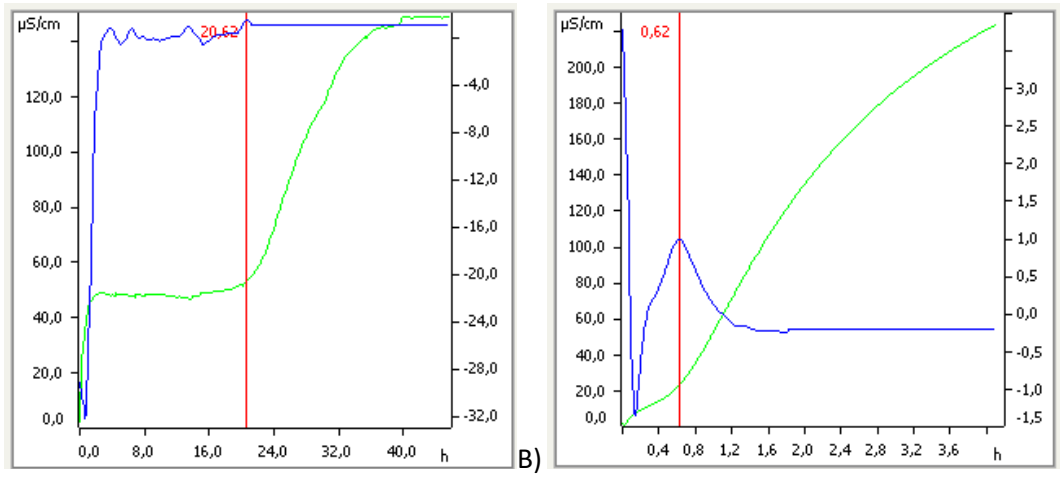


Fig. S11.4. Examples of the conductivity curve (green), its second derivative (blue) and its induction time (red) for some fatty acid distillate (FAD) samples: A) FAD from coconut and palm kernel oils; B) FAD from palm oil.

9. REFERENCES

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