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Manual

of lipid extraction, methylation and gas chromatography, for the study of different tissues in ruminant research

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List of reagents and equipment

List of reagents and equipment

1. Chloroform HPLC 99.8%
2. Methanol HPLC 99.9%
3. Chloroform/methanol: 2:1, 1:1 (v/v)
4. Methyl ester of *n*-nonadecanoic acid minimum 98% GC
5. Calcium chloride dehydrate 99%: $CaCl_2$ x $2H_2O$ (0.02%)
6. Sodium sulfate anhydrous powder 99%
7. Potassium carbonate 99.5%
8. Toluol
9. $NaHCO_3$ solution: Sodium hydrogen carbonate solution: diluted in distilled water.
10. Boron trifluoride methanol solution 14%

11. $NaOCH_3$ solution: 2.71 g sodium methoxide in 100 mL MeOH
12. *n*-hexane 99%
13. *n*-heptane 98.5%
14. Isopropanol: 2-propanol min 99.7%
15. Na_2SO_4: anhydrous sodium sulfate
16. Methanolic HCl: 50 mL hydrochloric acid 32% p.a. in 1 liter of methanol
17. K_2CO_3: potassium carbonate 99%
18. K_2CO_3 solution 6%: 60 g of K_2CO_3 in 1 Lt H_2O
19. EDTA: ethylenediaminetetraacetic acid 99% anhydrous
20. BHT solution: butylated hydroxytoluene (7.5 g diluted in 100 mL methanol)
21. Multiboy
22. Ultra Turrax
23. Vortex
24. Centrifuge
25. Desiccator
26. Rotary evaporator
27. Water bath

1.1. C19:0 Internal standard

Methyl ester of nonadecanoic acid (C19:0) will be added as an internal standard. This compound is ideal as an internal standard because its traits are similar to other analytes of interest, but it is unlikely to be present in the natural fat sample since it has an odd number of carbon atoms on the backbone. When you extract the triglycerides (TG), diglycerides (DG), monoglycerides (MG), free fatty acids (FFA), phospholipids (PL), cholesterol (Cho), and cholesterol ester (ChE) from the fat sample, you will also extract the nonadecanoic acid standard. The amount of C19:0 ME (methyl ester) serves as the internal standard for the quantitative calculation of all the fatty acids in the extracted fat. Furthermore, using C19:0 ME compensates the loss during all the single extraction steps and increases the efficiency of the method.

In order to obtain the exact response from the detector, you should use different dilutions of a commercial FAME mix to get the calibration curve for each single fatty acid. The C19:0 ME is used only to quantify the single acid according to a known fatty acid. Both the calibration and the quantitative estimation are done by means of the software of gas chromatography (GC).

2 Introduction

Increased interest in functional foods has caused a corresponding increase in consumer demand for information on the health benefits of these foods. For this reason, during the last decade investigations have been focused on the fatty acid composition of different ruminant tissues in order to understand their metabolism and to increase beneficial fatty acids for human health.

It has been shown that appropriate analytical methods must be used to obtain reliable lipid composition data. Extraction of lipids with organic solvents, followed by transesterification (methylation) to form fatty acid methyl esters (FAME) and the subsequent separation by gas chromatography (GC), represents the classical method used for tissues and food fatty acid (FA) analysis.

The technique of GC has been an indispensable analytical technique allowing sensitive and reproducible fatty acid analysis, as well as the characterization of complex mixtures of geometric isomers when combined with other chromatographic separations and spectroscopic identification (Seppänen-Laakso et al. 2002).

This manual will present the protocols for gas chromatography and for previous steps (lipid extraction and methylation) that have been used, adapted, and evaluated in animal research by the Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany. The purpose of this manual is to contribute to the standardization of methods for education and research.

3

Lipid extraction and methylation

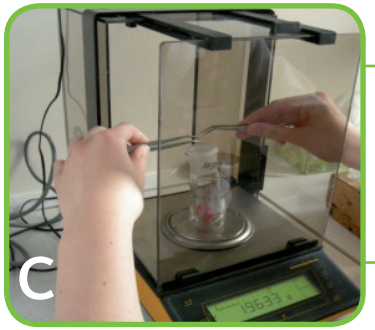
3.1. Beef lipid extraction

In this protocol, one of the most commonly used procedures for lipid extraction from animal tissues will be described; it was originally described by Folch (1957) using chloroform/methanol. For methylation, a method including the combination of a base-catalyzed method with sodium methoxide ($NaOCH_3$) will be explained, followed by an acid-catalyzed method with boron trifluoride methanol solution (BF_3/MeOH). All lipid fractions (TG, PL, free fatty acids, amides, and alk-1-enyl ethers) will be esterified by using this combined method (Nuernberg et al. 2007).


a


b

1. The samples must be stored at -20 ºC to avoid peroxidation and degradation of fat. Cut the muscle samples in small pieces or use powder samples or other homogenates (a). If samples are not homogenized, they should be homogenized using a homogenization device (b).


c

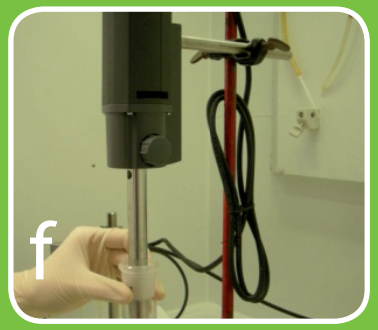
2. Weigh 2 g of the homogenized sample using two repetitions per sample (c).

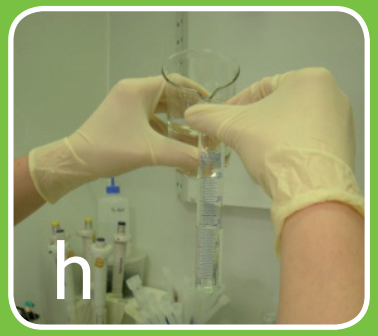
3. Add 50 µL of *n*-nonadecanoic acid ME as internal standard (1.5 g C19:0 ME; in 25 mL chloroform/methanol) if samples are going to be used for GC. Add 14 mL of chloroform/methanol 2:1 v/v (d).


d


e

4. Leave 30 minutes (minimum) in the refrigerator (e).





5. Homogenize with the Ultra Turrax device at 7000 rpm for 10 seconds followed by 20000 rpm for 30 seconds, rinsing at the end with chloroform/methanol 2:1 v/v (f).

6. Cover the tubes and keep the samples in the refrigerator overnight.

7. The next day, leave the samples at room temperature for 30 minutes.

8. Filter each sample, using both paper and glass filters, into a 15 mL tube (A) rinsing with chloroform/methanol 2:1 v/v (g).

9. Complete each graduated tube A until 15 mL with chloroform/methanol 2:1 v/v (h).

10. Shake slowly by hand 5 times.

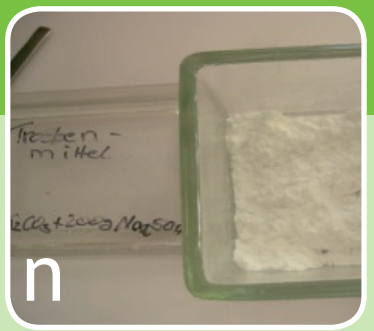
11. Transfer 7.5 mL of the content of tube A to another tube B (i). Keep tube A in the refrigerator as security.


j


k


l


m

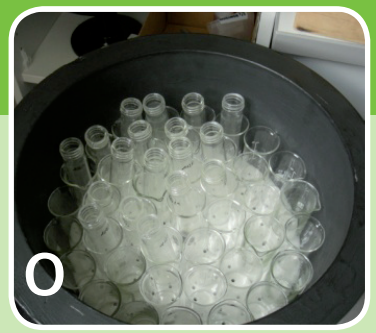

n

12. Add 1.9 mL of a wash solution of calcium chloride dehydrate ($CaCl_2$ x H_2O 0.02%) in the tube B in order to eliminate water soluble non-lipid compounds (j).

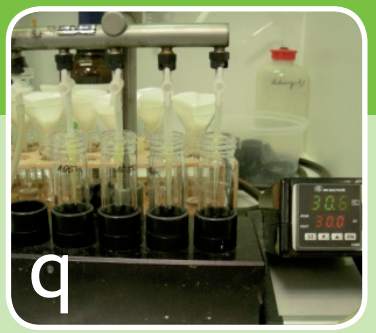
13. Vortex tube B for 5 seconds (k).

14. Centrifuge tube B at 2500 rpm for 2 minutes, 4ºC (l). Extract and throw away the upper layer from tube B (m).

15. Add a small spoon of dry agent composed of 20 g potassium carbonate (K_2CO_3) + 200 g anhydrous sodium sulfate (Na_2SO_4) in tube B to eliminate water (n).


o


p


q

16. Cover tubes B, vortex 5 seconds and leave at room temperature for 1 h. In parallel, dry Pyrex tubes C in a desiccator for 30 minutes (o), weigh and organize them for filtering.

17. Vortex 5 seconds, filter the content of tubes B into tubes C rinsing with chloroform/methanol 2:1 v/v. Allow the filter paper to dry for 3 minutes (p).

18. Evaporate solvents from tubes C under a stream of nitrogen (q).

19. Add chloroform/methanol and evaporate again under a stream of nitrogen (two times).

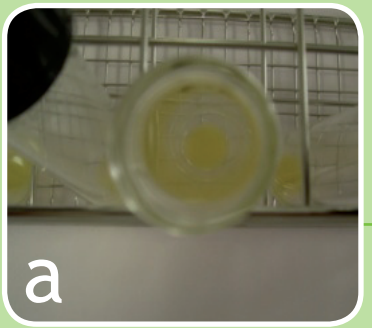
20. Repeat the last step (three times).

21. Keep tubes C in a desiccator for 30 minutes and weigh them (r). It is the total lipid content extracted.

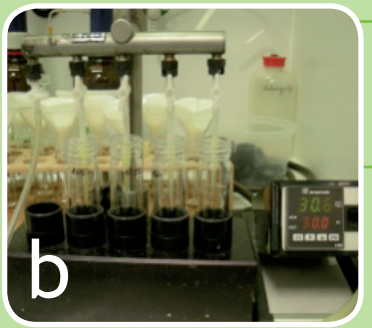
22. Add 500 µl chloroform/methanol 2:1 v/v and remove circa 25 mg of lipids (it has to be calculated for each sample set) and keep them into tubes C (s).

23. Keep the tubes C at -20 °C until methylation.

Beef lipid methylation



1. Defrost tubes C at room temperature (a).



2. Vortex tubes C for 10 seconds.

3. Evaporate solvents from tubes C under a stream of nitrogen (b).

4. Add 300 µl of toluol and 2 mL of sodium methoxide 0.5M solution ($NaOCH_3$) to tubes C (c).

5. Put tubes C in water bath at 60 °C, shaking for 10 minutes (d). Add 1 mL of BF_3/MeOH solution 14% to tubes C.

6. Put tubes C again in water bath at 60 °C, shaking for 10 minutes.


e


f


g

7. Add 2 mL of saturated anhydrous sodium sulfate ($NaHCO_3$) solution to tubes C, cover them, and put them at room temperature for 10 minutes.

8. Add 2 mL of *n*-hexane to tubes C (e), cover them, and vortex them for 10 seconds.

9. Extract and transfer the upper layer from tubes C to tubes D (g).

10. Add again 1.5 mL of *n*-hexane to tubes C (e).

11. Add one small spoonful of dry material to the new tubes D (f).

12. Vortex tubes C for 10 seconds, extract the upper layer and transfer it to tubes D (h). Cover tubes D.

13. Filter the content of tubes D to tubes E, using wool and glass filters, and rinsing with *n*-hexane (i).

14. Evaporate solvents from tubes E in the rotary evaporator at 30 ºC (j).

15. Add 1 mL of *n*-heptane if samples are going to be used for GC, or 500 µL of *n*-hexane for HPLC and TLC. Transfer the content of tubes E to vials (k). Freeze at -20 ºC.


h


i


j


k

3.2. Milk lipid extraction and methylation

As described by Hara and Radin (1978), a mixture of *n*-hexane and isopropanol was used, which is popular for milk lipid extraction prior to methylation and analysis of FA (Feng et al. 2004). The method of Kraft (2003), without centrifugation, was used by Duske et al. (2009) and Angulo et al. (2011), and will be described in this protocol. For methylation, it is recommended to use a method similar to the one used for beef lipids.

Milk lipid extraction

1. Mix the milk several times before taking the real sample for fatty acid analysis.

2. Take 10 mL of fresh milk sample in a 50 mL tube A.

3. Add 200 µL (12 mg) of *n*-nonadecanoic acid internal standard C19:0 ME (1.5 g C19:0/25 ml) if samples are going to be used for GC.

4. Add 20 mL of *n*-hexane:isopropanol v/v 3:2.

5. Homogenize with Ultra Turrax at 10000 rpm for 40 seconds.

6. Leave it for 1 hour at room temperature.

7. Transfer the upper layer (*n*-hexane + milk lipids) to another tube B.

8. Dry by addition of 500 mg of Na_2SO_4. Leave it for 30 minutes at room temperature.

9. Filter using wool and glass funnels to a new weighed tube C.

10. Evaporate under a stream of nitrogen. Weigh and the result is the total lipid extracted. Add 1000 µl *n*-hexane:isopropanol, remove circa 25 mg of milk fat (it has to be calculated for each sample set) in tube D, and freeze the samples until the next step. Keep the samples at -20ºC until methylation.

Milk lipid methylation

1. Defrost the samples at room temperature.

2. Evaporate solvents under N_2.

3. Add 300 µL toluol.

4. Add 2 mL of $NaOCH_3$.

5. Put the samples in a water bath at 60 ºC, shaking for 10 minutes.

6. Add 1mL of BF_3/MeOH (14%) and put the samples again in a water bath at 60 ºC, shaking moderately for 10 minutes.

7. Add 2 mL of $NaHCO_3$ and 2 mL of *n*-hexane, and shake the mixture vigorously by hand for 2 minutes.

8. Leave it 30 minutes at room temperature. Then, extract the upper layer and transfer it to a clean tube E.

9. Eliminate the water by adding a small spoonful of dry agent mix of (20g K_2CO_3 + 200g Na_2SO_4).

10. Leave it 1h at room temperature. Then, to a new tube F filter using wool and glass filter, rinsing with n-hexane. Evaporate solvents under a rotary evaporator at 30ºC.

11. Add 1mL of n-heptane if samples are going to be used for GC. Add 500µL of n-hexane if samples are going to be used for HPLC and 500µL of chloroform/methanol (2/1) if are going to use for TLC. Vortex approximately 10 seconds and transfer to a vial.

3.3. Subcutaneous fat lipid extraction and methylation

1. Remove the connective tissue from the sample.

2. Cut the sample and homogenize by using multiboy/mulinex. Weigh for each tube A 1 g of sample fatty tissue.

3. Add 100 µL C19:0 ME internal standard and 17 mL chloroform/methanol v/v 2:1 and put this for 30 minutes in the refrigerator.

4. Homogenize with the Ultra Turrax
10 sec. Speed 1 (6000 rpm/min.)
30 sec. Speed 4 (20000 rpm/min.)
10 sec. Speed 1 (6000 rpm/min.)

5. Rinse the Ultra Turrax with ½ Pasteur pipette of chloroform/methanol v/v 2:1. Put this overnight in the refrigerator.

6. The next day, filter the samples in graduated tubes B.

7. Fill the tubes with chloroform/methanol to 20 mL and shake the tubes.

8. Transfer an aliquot of 4 mL to another tube C.

9. Wash it with 1.0 ml $CaCl_2$ x 10 H_2O (0,02%).

10. Centrifuge (2 min, 2500 rpm/min, 4°C).

11. Discard the upper layer.

12. Add drying agent (K_2CO_3 + Na_2SO_4 1:10). Leave it 1 h at room temperature.

13. Filtrate in the previously weighed Pyrex tubes D and rinse the tubes twice with ½ Pasteur pipette of chloroform/methanol v/v 2:1.

14. Remove the solvents and concentrate the sample under a stream of nitrogen.

15. Put the Pyrex tubes into the desiccator for 30 minutes.

16. Weigh the Pyrex tubes and determine the weight of the fat. It is the total lipids extracted.

17. Add circa 500µl chloroform/methanol (2:1) and remove circa 25 mg of milk fat (it has to be calculated for each sample set) in tube D and freeze the samples until the methylation step in tube E.

Subcutaneous fat lipid methylation

1. Thaw the sample in the tubes at room temperature and evaporate solvents under a stream of N_2.

2. Add 300 µl toluol and 2 ml $NaOCH_3$. Close the tube and place it in a water bath at 60 °C for 10 minutes.

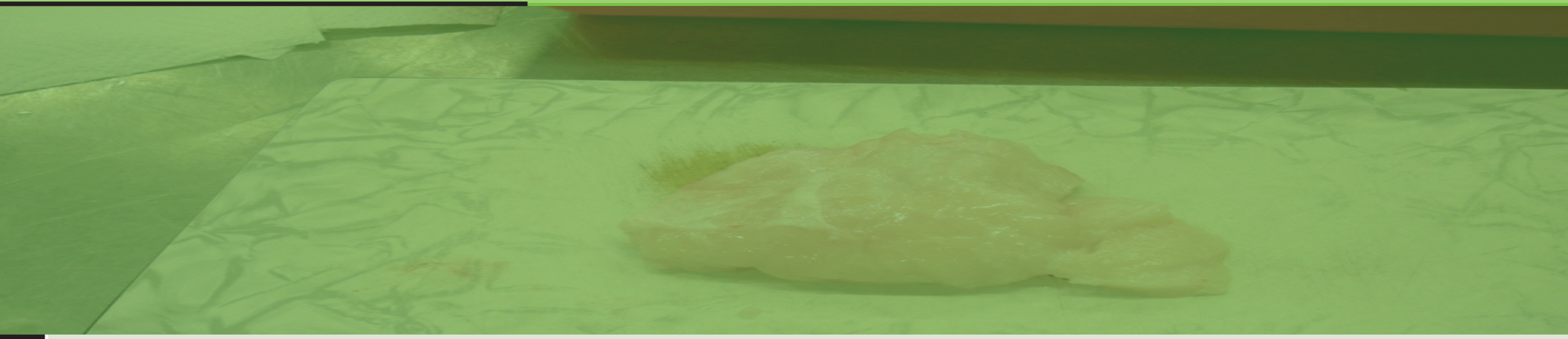
3. Add 1 ml BF_3/MeOH.

4. Close the tube and place it again in the water bath at 60 °C for 10 minutes and moderate shaking.

5. Cool down. Then add 2 ml saturated $NaHCO_3$ solution, shaking.

6. Add 2 ml *n*-hexane and shake this strongly for several minutes, wait to get two layers.

7. Carefully transfer the upper layer to a new tube F.



8. Add 1.5 mL *n*-hexane and shake again strongly.

9. Transfer the upper layer again to another tube G and add drying agent to this tube.

10. Leave it for 1 hour at room temperature.

11. After this hour, filter into a tube H for the rotary evaporator.

12. Evaporate by using the rotary evaporator.

13. Add 1 mL of *n*-heptane for GC. Add 500 µL of *n*-hexane or chloroform/methanol (2/1) for HPLC or TLC, respectively.

14. Vortex approximately 10 seconds and transfer to a vial I. Keep at -20 °C.

3.4. Rumen content lipid extraction and methylation

1. Weigh 1.5 g of freeze dried powder rumen content (representative mix of whole rumen content) in each tube A.

2. Add 1.5 mL toluol and 2.0 mL toluol standard (50 mg C19:0 ME in 100 ml toluol).

3. Add 5.25 mL of 10% methanolic HCL.




4. Vortex at the highest speed.

5. Put the tubes into a shaking water bath at 70 °C for 2 hours. During this time, take the tubes each 30 minutes and vortex them.

6. Cool down.

7. Add 8.75 mL (6%) K_2CO_3 solution and 3.5 mL *n*-hexane. Vortex again.

8. Centrifuge at 3500 rpm for 5 minutes.

9. Put the upper layer into tube B and add 1 g Na_2SO_4 plus activated charcoal to discolor. This must stay until the solution is colorless.




10. Centrifuge at 3000 rpm, for 2 minutes.

11. Filter over wool and glass filters. The tube C must be previously weighed and evaporated under a stream of nitrogen.

12. Put the tubes into the desiccator for 30 minutes.

13. Weigh, it is the total lipids extracted. Add *n*-heptane for GC according to the FAME content (15-24mg = 800µl; 10-15mg = 500µl; less 10mg = 100µl in microvials), add *n*-hexane for HPLC, respectively. After vortex, transfer it to vials.

14. Keep the tubes at -20 °C for GC or HPLC.

3.5. Plasma lipid extraction and methylation

The protocol for plasma lipid extraction also includes the traditional chloroform/methanol method, according to Folch et al. (1957).

1. Collect blood samples in EDTA-containing tubes A for routine analysis (centrifuge tubes). Centrifuge at 10000 rpm 4°C for 5 min, transfer plasma aliquots into 2 ml cryotubes and store at -20 °C until analysis.

2. For extraction, thaw the plasma, weigh the tube with plasma.

3. Put 8 mL of cold methanol/chloroform v/v 1:1 in a new tube C. Add 60 µL (1:40) of standard (C19:0 ME) and 30 µL of butylated hydroxytoluene (BHT). Vortex all the time, drop the plasma dropwise into the tube C. Let stand for 10 minutes.

4. Vortex and add at the same time 4 mL of chloroform. Store it overnight in the refrigerator.

5. The next day, filter into a new tube D through a paper filter, rinse the filter and the tube with 2 mL chloroform/methanol (2:1 v/v with BHT). Add 3.5 mL calcium chloride ($CaCl_2$ x H_2O). Shake this strongly, and centrifuge at 2500 rpm for 2 minutes at 4° C. Discard the upper layer as waste. Add drying agent into the tube, shake again strongly and wait 1 hour at room temperature.

6. Filtrate into weighed Pyrex tubes E. Evaporate the solvent under nitrogen, store it in a desiccator for 30 minutes and weigh. It is the total lipids extracted.

7. Use the same methylation method as for beef lipids. If the extracted fat is lower than 25 mg, use half the volume of methylation substrates.

8. After methylation, follow the same procedures: washing, FAME extraction, phase separation, drying procedure, evaporation

9. Add 500µl *n*-heptane and store at -20 °C for GC.

4

Gas chromatography

Gas chromatography is a technique that can be used to separate organic compound mixtures which can be vaporized without degradation. A gas chromatograph consists of a separation column containing the stationary phase, a detector, an injector, and a data recording system, and uses a flowing mobile phase.

The organic compounds are separated due to differences in their partitioning behavior between the mobile gas phase and the stationary phase in the column. The chain length and the number and geometric location of double bands induce different absorption and retention times.

The fatty acid composition of samples in FBN are determined by capillary gas chromatography using a CP SIL 88 CB column (100m x 0.25mm, 0.25µm, Chrompack-Varian, USA) installed in a Perkin Elmer gas chromatograph Autosys XL with a flame ionization detector and split injection (Perkin Elmer Instruments, Shelton, USA).

In all the cases, hydrogen is used as the carrier gas at a flow rate of 1 mL/min. The split ratio is normally 20:1, the injector is set at 260 °C and the detector at 280 °C.

For beef muscle, subcutaneous fat, and plasma, the following temperature program is recommended: initial oven temperature of 150 °C, held for 5 min, subsequently increased to 200 °C at a rate of 2 °C min^{-1}, held for 10 min, then to 220 °C at 15 °C min^{-1}, held for 35 min.

For milk and rumen content, the following temperature programs are recommended: initial oven temperature of 45°C, held for 4 min, subsequently increased to 150 °C at a rate of 13 °C min^{-1}, held for 47 min, then to 215 °C at 4°C min^{-1}, held for 35 min. It is possible to use another temperature program of 45°C, held for 4 min, subsequently increases to 175 °C at a rate of 13 °C min^{-1}, held for 27 min, then to 215 °C at 4°C min^{-1}, held for 35 min in order to separate the different C18:1 *trans* isomers.

Before running the samples in the GC, 5 dilutions of a standard should be prepared for calibration of the equipment. Each dilution is composed of a standard mix ('FAME mix' obtained from Supelco™, diluted in *n*-heptane), the internal standard C19:0, and *n*-heptane. For example, the dilution can be prepared in the following way:

Dilution 1: 100µL standard mix + 50µL internal standard C19:0 + 850 µL *n*-heptane

Dilution 2: 150+50+800, respectively

Dilution 3: 300+50+650, respectively

Dilution 4: 500+50+450, respectively

Dilution 5: 700+50+250, respectively

Then, it is necessary to build a table of calibration with the exact amount of each fatty acid included in the mixture, based on each dilution. After that, it is necessary to create and run a sequence with the 5 dilutions of the standard for calibration using duplicates. Finally, the sequence of the samples is run in the GC, and the chromatograms obtained are integrated for the final calculation. Based on these data, the software produces the calibration factors for each component.

The standard mix 'FAME mix' from Supelco™ does not include all the fatty acids necessary for the analysis. Therefore, it should be completed. For example: the standard mix of Supelco™ does not include *trans*-11 C18:1 methyl ester, *cis*-9, *trans*-11 CLA methyl ester, C22:5*n*-3 methyl ester, *cis*-11 C18:1 methyl ester, C18:4*n*-3 methyl ester, and C22:4*n*-6 methyl ester. All of them should be added for analysis of all the tissues.

After creating a method and running the samples, the amounts are calculated using the internal standard method of the Turbochrom workstation software (Nuernberg et al. 2002). It is necessary to control the correct peak integration for each fatty acid, and if there was no baseline separation, this should be done manually.

5

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