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Nota Técnica

VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF FATTY ACIDS IN SHEEP BLOOD SERUM SAMPLES

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Low-density dispersive liquid-liquid microextraction with a subsequent esterification step was proposed in this study for the determination of linoleic acid and stearic acid in sheep blood serum samples. The method developed aimed to quickly and efficiently extract and esterify both fatty acids with subsequent analysis by gas chromatography/flame ionization detection. The extraction method was optimized with Scheffé's polynomial model for three-component mixtures. The method was validated according to the parameters established by Eurachem Guide (2014). The optimal extraction conditions for LD-DLLME were 1400 µL of dispersion medium ($MgCl_2$ 0.017%), 400 µL of extractor solvent (toluene) and 1200 µL of dispersion solvent (methanol). The method performance showed adequate selectivity, sensitivity and precision to be applied to real samples, with an average recovery of 98.54% for linoleic acid and 103.83% for stearic acid. LD-DLLME was superior to the traditional method of analysis, which has been used until now for the determination of fatty acids in blood serum samples from ruminants. The analysis of real samples showed that the developed method is efficient for monitoring these substances in ruminants.

Keywords: Linoleic acid; Stearic acid; chemometrics; miniaturized extraction technique.

INTRODUCTION

Fatty acids are fundamental substances supplemented in ruminant diets as they improve the metabolism, energy supply and reproductive performance of these animals, especially during lactation.[1–4] These lipids are supplemented through cereal and oilseed forage, which are rich in polyunsaturated fatty acids (PUFAs).[5,6]

The supply of PUFAs in ruminant diets modulates the synthesis of prostaglandins, which are responsible for improving the uterine environment for the development of oocytes and embryos.[7–9] A low supply of PUFAs to these animals causes growth inhibition and health and ovulation problems. This situation can be reversed by supplying linoleic acid (LA, C18:2, cis-9 cis-12) and α-linolenic acid (ALA, C18:3, cis-9, cis-12, cis-15) because mammals do not synthesize these fatty acids naturally.[8]

Due to the biohydrogenation of LA, consecutive isomerization reactions occur in the rumen of ruminants, with stearic acid (SA, C18:0) as the major product.[5,10,11] Thus, LA supplementation is an important process since this substance is not absorbed in adequate amounts by these animals.[8] However, monitoring and evaluating the conversion of LA in the body of ruminants is complicated and expensive, thus driving the development of alternative analysis methods.[12]

The method of Folch *et al*.[13] (FO) is still the most commonly used in the extraction and purification of fatty acids in biological samples. The FO method is a liquid-liquid extraction method that features high consumption of potentially toxic solvents, low analyte transfer to the extraction phase and high time consumption.[14,15]

In recent years, the development of miniaturized methods has grown significantly because they meet green chemistry requirements. Among the miniaturized techniques developed, dispersive liquid-liquid micro-extraction (DLLME) has spread rapidly due to its operational simplicity, good cost-benefit ratio, high pre-concentration factor, high extraction efficiency and low consumption of organic solvents and samples.[16]

Throughout its development and consolidation, the DLLME method underwent extensive changes in the types of extraction solvent, dispersion solvent and extraction devices used.[16] An example of the results of these changes is low-density dispersive liquid-liquid micro-extraction (LD-DLLME), which uses solvents less dense than water to adapt to the characteristics of the analyte and study matrix.[17]

The objective of this study was to optimize and validate an extraction method using LD-DLLME with a subsequent esterification step for the determination of LA and SA in sheep blood serum samples by gas chromatography/flame ionization detection (GC-FID). The efficiency of the method in the extraction of both fatty acids in blood serum samples from sheep fed a traditional diet supplemented with lipids from roasted, cracked and ground soybean was evaluated. To confirm the validity of the developed method, the obtained results were compared to the results of the FO method, which is traditionally used in this type of analysis.

MATERIALS AND METHODS

Sample preparation and informed consent

Blood samples were obtained by puncture of the sheep's jugular vein of the Santa Inês breed in the prepubertal and pubertal period and collected in anticoagulant-free tubes (New Jersey, USA). After collection, the samples were centrifuged at 3000 rpm for 15 min in a 2.0 mL Eppendorf tube. Blood serum was collected and stored at –20 °C in a freezer (Bosch Intelligent Freezer 32 - Fast Freezing) until analysis.

The samples were provided by the Animal Reproduction Laboratory, Department of Animal Science, Federal University of Lavras (Universidade Federal de Lavras - UFLA), located in

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Lavras, Brazil. The study was previously approved by the UFLA ethics committee under the project *"Food restriction and lipid supplementation: effects on age at puberty and ovarian follicular development in Santa Inês sheep"* under protocol CEUA/UFLA 085/13.

Reagents

The following reagents were used: HPLC-grade toluene (Merck, Darmstadt, Germany); HPLC-grade methanol (MeOH), 99.5% chloroform, 99% potassium carbonate (K_2CO_3) and 99% magnesium chloride ($MgCl_2$) (Sigma Aldrich, Missouri, USA); and 99.9% acetyl chloride (Acros, New Jersey, USA). LA (99.9%), SA (99.9%), methyl linoleate (LM - 99.9%) and methyl stearate (MST - 99.9%) standards were acquired from Sigma Aldrich (USA). Type I ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, USA).

Standard solutions

Stock solutions of the fatty acids and fatty acid esters used in LD-DLLME were prepared in toluene at a concentration of 1.0 mmol L^{-1}. Working solutions were prepared by dilution of the stock solution in toluene to obtain a concentration of 0.1 mmol L^{-1}.

The stock solutions of fatty acids and fatty acid esters used in the FO method were prepared with a 2:1 chloroform: methanol (v/v) solution at a concentration of 1.0 mmol L^{-1}. Working solutions were prepared by dilution in a 2:1 chloroform: methanol (v/v) solution to obtain a concentration of 0.1 mmol L^{-1}.

All prepared solutions were stored in amber flasks at –20 °C.

LD-DLLME method

A total of 100 µL of sheep blood serum was added to a glass tube, followed by 1400 µL of 0.017% (w/v) magnesium chloride saline solution, dissolving the sample A mixture of 1200 µL of methanol and 400 µL of toluene were simultaneously added with a syringe, and the resulting phases were separated by centrifugation (5 min, 3000 rpm). The upper phase extract was transferred to a 15 mL glass flask and esterified.

Folch method

As a comparative extraction method, the FO method was applied to blood serum samples, as described by Pelegrino *et al.*[18] The extractions were performed in 5.0 mL Eppendorf tubes using 100 µL of sheep blood serum. A 2000 µL volume of a 2:1 chloroform: methanol (v/v) solution was added to the tube containing the serum. The samples were vortexed (30 s) and incubated for 1 h at room temperature. After incubation, 400 µL of NaCl (0.9%) was added, and the samples were vortexed (30 s). The solvent mixture was separated by centrifugation (5 min, 3000 rpm), and the upper phase was discarded. The clean-up step of the lower phase was performed by adding 400 µL of a 48:47:3 0.9% NaCl:MeOH:$CHCl_3$ (v/v/v) solution. The solvent mixture was centrifuged (5 min, 3000 rpm), and the upper phase was discarded. The clean-up step was performed twice. From the extract obtained, 200 µL was evaporated under a N_2 flow and esterified. The esterified extract was stored at -20 °C until analysis by GC-FID.

Esterification method

The esterification method used was adapted from Rodrígues-Palmero *el al.*[19] To the extract, 100 µL of methanol and 100 µL of acetyl chloride were added. The flask was properly sealed and placed in a water bath for 30 min at 60 ºC. After the esterification time, the solution (pH 1.5) was neutralized by the addition of 1.0 mL of 6.0% potassium carbonate (w/v). The container was centrifuged (5 min, 3000 rpm), and the organic phase was transferred to a vial with a glass insert. The esterified extract was stored at –20 °C until analysis by GC-FID.

The volume of the esterifying agent was adjusted in advance according to the mean mass of the obtained extracts.

Instrumentation

The chromatographic analyses were performed in an Agilent 7890A GC-FID instrument (Wilmington, USA) equipped with an HP-5 analytical column (Santa Clara, USA) measuring 30 m x 0.32 mm ID x 0.25 mm and a stationary phase composed of 5% diphenyl-polydimethylsiloxane. The samples were injected by a split/splitless injector in split mode using a split ratio of 1:20 (v/v) with an injector temperature of 250 °C and detector temperature of 275 °C. The injection volume was 1.0 µL. The column temperature was set at 80 °C and increased by 10 °C min^{-1} until reaching 250 °C. This temperature was maintained for 1 min and then increased by 7 °C min^{-1} until reaching 350 °C, totaling 29 min of operation. The carrier gas used was helium (He) at a constant flow rate of 1.1 mL min^{-1}.

Analytical performance parameters for method validation

The Eurachem validation guide (2014) was used to evaluate the performance of the developed analytical method.[20] The evaluated analytical performance parameters were selectivity, linearity, analytical sensitivity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ). The entire validation process was performed with the same pool of blood serum from ruminants with a low concentration of analytes since it was not possible to obtain a matrix free of the analytes.

The selectivity of the method was evaluated according to the retention time (RT) of both analytes and the matrix effect (ME). Linearity was evaluated using the analytical curve constructed by the standard addition method. The matrix extracts were spiked with a working solution of ML and MST to six concentration levels (0.0, 2.0 x 10^{-3}, 4.0 x 10^{-3}, 6.0 x 10^{-3}, 8.0 x 10^{-3} and 1.0 x 10^{-2} mmol L^{-1}) in triplicate for both evaluated fatty acids. The concentration of 0.0 mmol L^{-1} corresponds to the extract with a low analyte concentration without the addition of a standard.

Regression was performed by the ordinary least squares (OLS) method, and the coefficient of determination (R^2) and the linear interval for the fatty acids studied were determined.

The ME for each of the fatty acids was determined in order to evaluate the influence of matrix co-extractives that cause an increase or decrease in the analytical response. The ME was determined by comparing the matrix-matched analytical curve and the standard analytical curve in solvent presented in Equation 1:

$$ME = \frac{b_m - b_s}{b_s} \quad (1)$$

where b_m is the slope of the matrix-matched analytical curve and b_s is the slope of the standard analytical curve in solvent.

The validity of the developed method was evaluated by analyzing samples using the proposed method (LD-DLLME) and the reference method (FO). Analyte recovery by both methods was determined at three spike levels with working solutions of LA and SA (2.0 x 10^{-3},

6.0 x 10^{-3}, 1.0 x 10^{-2} mmol L^{-1}) in sextuplicate according to Equation 2.

$$R(\%) = \frac{x_{mean}}{x_{ref}} \times 100 \quad (2)$$

where x_{mean} is the mean value obtained and x_{ref} is the reference value for that particular concentration in the standard addition analytical curve.

Method precision was evaluated according to repeatability and intermediate precision. The repeatability test was performed in a single day by two different analysts, in which samples containing a small concentration of the analyte were spiked with LA and SA working solutions at three concentration levels (n = 3) and subjected to extraction and esterification. The tests were performed in sextuplicate, and the spike levels used in the analytical curve corresponded to 2.0 x 10^{-3}, 6.0 x 10^{-3} and 1.0 x 10^{-2} mmol L^{-1}. The intermediate precision was evaluated on three consecutive days of analysis performed by the same analyst at the same concentration levels as those used in the repeatability test. The data obtained were evaluated by analysis of variance (ANOVA) according to the specifications of the Eurachem guide (2014).

The LOD and LOQ were calculated from the gross standard deviation (s'_0), calculated according to Equations 3 and 4, of ten replicate analyses of serum samples with low analyte concentrations. For LOD, 3 x s'_0 was adopted, and for LOQ, 10 x s'_0 was adopted.

$$s_0 = \sqrt{\frac{\sum(xi - x)^2}{n-1}} \quad (3)$$

$$s'_0 = \frac{s_0}{\sqrt{n}} \quad (4)$$

Analysis of real samples

Five sheep blood serum samples were analyzed; two of the sample subjects did not undergo lipid supplementation, and three were subjected to supplementation with roasted and ground soybean. The fatty acids were extracted by LD-DLLME. The standard addition method was used to construct analytical curves for each of the samples at three concentration levels (n = 3) (2.0 x 10^{-3}, 6.0 x 10^{-3} and 1.0 x 10^{-2} mmol L^{-1}). The curves were extrapolated to determine the actual concentrations of LA and SA in each of the analyzed samples.

RESULTS AND DISCUSSION

Selection of solvent extractor, disperser and extraction medium

The types of solvent extractor, disperser and dispersion medium (in which the sample may be dissolved) are variables that can significantly affect the extraction efficiency of the micro-extraction procedure.[21] Thus, toluene, methanol and magnesium chloride solution (0.017% $MgCl_2$) were selected as the extractor solvent, disperser and sample dispersion medium, respectively. These solvents were selected according to the study conducted by Bazzana *et al*.[22]

Effect of solvent extractor, disperser and extraction medium volumes

The effect of the volume of the solvents used in LD-DLLME was evaluated by Scheffé's mixture design according to the method proposed by Bazzana *et al*.[22] The experimental variables were the proportions of the dispersion medium (z_1: $MgCl_2$ solution 0.017%, v/v), extraction solvent (z_2: toluene) and dispersion solvent (z_3: methanol). In the extraction optimization process, the proportions of z_1, z_2 and z_3 were varied from 80-57%, 48-10% and 33-10%, respectively, in a set of ten experiments (chemical space).[22] To facilitate the interpretation of the experimental data in the chemical space, z_1, z_2 and z_3 were coded as x_1, x_2 and x_3, which corresponds to a pseudomixture space. In the pseudomixture space, the proportions of the components varied from 0-1, and the results were analyzed in simplex space.

The experiments were performed by evaluating only the analytical response for LA in two pools of ruminant blood serum samples. The first pool of samples analyzed was from animals that did not receive dietary lipid supplementation, and the second pool was from animals that received supplementation. Figure 1 shows the surfaces obtained for each of the sample pools.

When analyzing the response surfaces shown in Figure 1, differences in the topology of the response surface of Scheffé's supersaturated (SS) model were observed for the sample pools without lipid supplementation Figure 1(a) and with lipid supplementation Figure 1(b).

The surface corresponding to the serum samples without lipid supplementation shown in Figure 1(a) describes a well-defined maximum region within the evaluated experimental domain. The surface in Figure 1(b) describes a maximum region close to the limits of the studied experimental domain. This result is due to possible

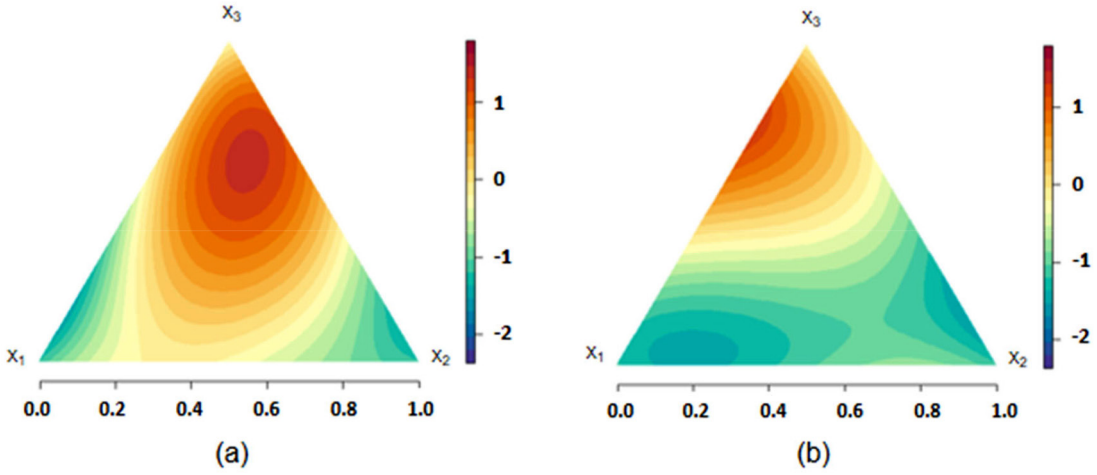

Figure 1. *Response surface for Scheffé's SS model: (a) pooled blood serum samples from ruminants without lipid supplementation; (b) pooled blood serum samples from ruminants with lipid supplementation. X_1: dispersion medium; X_2: extraction solvent and X_3: dispersion solvent*

co-extractives in the matrix of the lipid supplement that the study animals received.

To obtain a representative result for both sample pool types, the response surfaces shown in Figure 1 were measured. The mean Scheffé's SS model is shown in Figure 2.

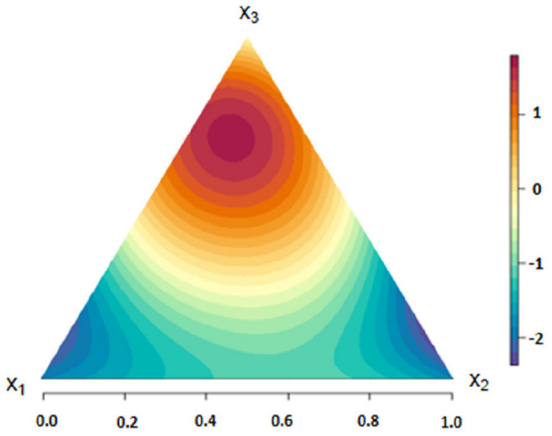

Figure 2. *Mean response surface for the Scheffé's SS models shown in Figure 1. X_1: dispersion medium; X_2: extraction solvent and X_3: dispersion solvent*

In the response surface shown in Figure 2, the color with the highest intensity, which represents the maximum point, is at the top of the simplex. The results for the pseudomixture system indicate that component x_3 (methanol) exerted the greatest influence on LA extraction from serum samples. Since methanol acts as a dispersion solvent, extraction solvent microdroplets with large surface areas were formed due to its rapid interaction with the dispersion medium. The formation of extractor solvent microdroplets with a large surface area reduces the extraction time of LD-DLLME and increases the enrichment factor.

The analysis of the response surface also shows that higher proportions of component x_2 (toluene) decrease the extraction efficiency. With an increase in the volume of toluene, which corresponds to the extraction solvent, the enrichment factor decreases.[23,24]

To determine the best proportion of each of the solvents used in LD-DLLME, the pseudomixture system (x_1, x_2, and x_3) was reconverted into the chemical system (z_1, z_2 and z_3) following Neto *et al.*[25]

The maximum point found in the pseudomixture system (Figure 2) corresponds to proportions of 46.70% dispersion medium, 13.30% extractor solvent and 40.00% dispersion solvent in the chemical mixture system. These proportions correspond to volumes of 1400 µL of $MgCl_2$ solution, 400 µL of toluene and 1200 µL of methanol.

The solvent volumes found for the proposed extraction method corroborate other studies that use DLLME and its variations in the determination of compounds in biological samples (blood, plasma, serum, urine, etc.).[26–32] Although the volumes found here are very close to the solvent volumes used in the FO method, the proposed method has an advantage because it is performed with fewer steps and shows operational simplicity and easy phase separation.[33]

The same analysis conditions were adopted for SA extraction since the structure and configuration of SA in the animal body are similar to those of LA.

Method performance

Table 1 shows the analytical performance parameters (ME, R^2, LOD and LOQ) obtained for the developed analytical method. For both fatty acids, determination coefficient (R^2) were greater than 0.990 in the evaluated working interval.

The ME may indicate the amplification or suppression of the analytical signal due to co-extractives in the matrix in which the analyte is found. The ME can be classified as (a) weak, when the values obtained are between -20% and +20%; (b) medium, when the values are between -50% and -20% and between +20% and +50%; and (c) strong, when the values are below -50% or above +50%.[34,35]

According to the values shown in Table 1, both analytes showed an amplification effect of the analytical signal. In both cases, the ME was classified as strong (considering a range between -50% and +50%). In this sense, all quantitative experiments were evaluated by the matrix-matched analytical curve.

The estimated LOD and LOQ values for each of the analytical curves (Table 1) have low values but could be determined since the approach involved the replication of ten analyses of diluted extract with a low concentration of analytes.

The slope obtained for each of the analytical curves shows that the developed method is more sensitive for SA than for LA. The same spike levels were used in both curves. Thus, the slope values shown in Table 1 indicate that a smaller variation in SA concentration results in a greater increase in the analytical response when compared to the result for LA, which has a lower slope value.

The precision and accuracy tests were performed and evaluated by ANOVA. The results of the repeatability tests are shown in Table 2.

Table 1. Analytical performance parameters of the evaluated method

Analyte	ME (%)	Levels	R^2	LOD (mmol L^{-1})	LOQ (mmol L^{-1})
LA (C18:2)	50.21	6	0.998	$8.30x10^{-5} \pm 3.16x10^{-6}$	$2.76x10^{-4} \pm 1.01x10^{-5}$
SA (C18:0)	60.55	6	0.999	$5.78x\ 10^{-6} \pm 1.41x10^{-7}$	$1.93x10^{-5} \pm 1.05x10^{-6}$

(ME): Matrix effect. (R^2): Coefficient of determination. (LOD): Limit of detection. (LOQ): Limit of quantification.

Table 2. Analysis of variance (ANOVA) results for the repeatability experiments

Variation Source	LA (C18:2)			SA (C18:0)		
	df	MS	p-value	df	MS	p-value
Analyst	1	$4.12x10^{-7}$	0.303	1	$9.44x10^{-8}$	0.767
Concentration	2	$1.43x10^{-4}$	$4.84x10^{-22}$	2	$2.37x10^{-4}$	$8.56x10^{-19}$
Interaction	2	$1.09x10^{-7}$	0.750	2	$6.47x10^{-7}$	0.547
Waste	30	$3.76x10^{-7}$		30	1.05	

(df): Degrees of freedom. (MS): Mean square. (p-value): Probability of significance.

Table 3. Analysis of variance (ANOVA) results for the intermediate precision experiments

Variation Source	LA (C18:2)			SA (C18:0)		
	df	MS	p-value	df	MS	p-value
Days	2	$3.36x10^{-7}$	0.375	2	$2.17x10^{-7}$	0.767
Concentration	2	$2.07x10^{-4}$	$1.91x10^{-33}$	2	$3.74x10^{-4}$	$8.56E^{-19}$
Interaction	4	$1.14x10^{-7}$	0.849	4	$5.58x10^{-7}$	0.547
Waste	45	$3.36x10^{-7}$		45	$1.38x10^{-6}$	

(df): Degrees of freedom. (MS): Mean square. (p-value): Probability of significance.

According to the results presented in Table 2 for both fatty acids, for the analyses performed on the same day by different analysts, the p-value for the interaction is greater than the adopted significance level ($p > 0.05$). This result indicates that the interaction of the analysts with the analyzed spiked concentrations did not show significant variations.

The analyses of the three concentrations showed a p-value lower than the adopted significance level ($p < 0.05$) for both analytes. This result was expected because the concentrations of each of the spike levels were not the same. Thus, this factor does not compromise the data analysis because this variation is intrinsic to the set of analyzed data.

The p-value comparing the data for each analyst was greater than the adopted significance level ($p > 0.05$), indicating that there was no significant variation in the data from the two analysts. This result implies that the developed analytical method is reproducible in a short period of time, even when the analysts change.

Since changing analysts did not result in significant variations, an ANOVA for intermediate precision was performed considering only the days of analysis and the spiked concentrations at three levels as variables. The results are shown in Table 3.

The results presented in Table 3 indicate that for both analytes, the interaction showed a p-value greater than the adopted significance value ($p > 0.05$). Thus, the interaction of the days of analysis with the three spiked concentrations did not show significant variations. Similar to the repeatability results, the spiked concentrations were different; therefore, the p-value lower than the adopted significance level ($p < 0.05$) indicates that there was variation among the data obtained at the different concentrations, which is an intrinsic condition of the analyzed data set.

The analysis of the data obtained on each day showed a p-value greater than the adopted significance level ($p > 0.05$). Thus, there were no significant variations among the data obtained on the three different days of analysis. This finding indicates that the method developed for the extraction of both acids is reproducible on different days of analysis. Table S1 (supplementary material) shows the calculated recovery values for the validation experiments for LA and SA at three concentration levels in the LD-DLLME and FO methods.

A comparison of the results obtained for each of the methods at the three concentration levels shown in Table S1 reveals that the highest mean recovery was obtained for LD-DLLME in the analysis of LA and SA, with values of 98.54% and 103.83%, respectively. At all concentrations evaluated by LD-DLLME, the recovery of both fatty acids was higher than 92.08% and lower than 108.82%, meeting the requirements stipulated by Eurachem guide (2014), which establishes that recovery values should range from 80-120%.

The recovery values determined by the FO method were lower than those determined by LD-DLLME at all evaluated concentrations. The values were higher than 82.18% and lower than 108.02% and thus also met the requirements stipulated by Eurachem guide (2014).

The obtained results can be partially explained as follows: ruminant blood serum is a very complex matrix with a high protein content; in the FO method, this matrix creates a thick layer between the lower extraction phase and the aqueous phase, which makes phase separation difficult and contributes to lower recovery.[18] In LD-DLLME, the extractor solvent, of lower density, is in the upper phase and can be easily collected.

Similar results were found by Pellegrino *et al*. for the FO method in human blood serum samples, with recoveries ranging from 70.5-106.2% in the extraction of lipids of various classes.[18]

A second determining factor is the water solubility of the extractor solvents used in the methods, since chloroform has a solubility of 8.0 g L^{-1} at 20 °C, while toluene has a solubility of 0.52 g L^{-1} at 20 °C. Thus, the extraction efficiency of the FO method may be lower than that of LD-DLLME in the extraction of more nonpolar compounds, corroborating the results obtained.

A comparison of the obtained recovery values shows that the developed method (LD-DLLME) is efficient and comparable to the standard method (FO) of lipid extraction in biological matrices and can be applied for the proposed purpose.

Real samples

The analytical results for the real samples are shown in Table S2 (supplementary material). Samples (a) and (b) are from animals that did not receive lipid supplementation, and samples (c), (d) and (e) are from animals that did receive lipid supplementation. Chromatograms of each type of samples are shown in Figure S1 (supplementary material).

Evaluation of the actual concentrations obtained for each sample (Table S2) shows that the animals that did not receive lipid supplementation had lower LA and SA concentrations than the animals that did receive it. Thus, the developed method (LD-DLLME) was efficient for both analyzed sample types. The coefficient of determination obtained in all analytical curves was greater than 0.99 ($R^2 > 0.99$), showing good linearity.

The concentration values found for LA and SA indicate that both fatty acids increased simultaneously. Thus, it can be inferred that ruminal biohydrogenation occurs extensively but that the provided supplementation was efficient in increasing the availability of LA in the bodies of these animals.

CONCLUSION

The developed method involving LD-DLLME with a subsequent esterification step allowed efficient determination of LA and SA in sheep blood serum samples by GC-FID. The optimized volumes used in the extraction process showed values consistent with the principles of extraction, which aims to reduce the volume of potentially toxic organic solvents. The method performance had adequate selectivity, sensitivity and precision to be applied to real samples. The linearity was satisfactory because the matrix-matched curves had linear behavior for both compounds. The proposed method has potential for application in the monitoring of LA and its intermediate (SA) resulting from biohydrogenation.

SUPPLEMENTARY MATERIAL

Supplementary material is available at http://quimicanova.sbq.org.br in pdf format, with free access.

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REFERENCES

1. Jenkins, T. C.; Harvatine, K. J.; *Vet. Clin. North Am.: Large Anim. Pract.* **2014**, *30*, 623. [Crossref]
2. Palmquist, D. L.; Jenkins, T. C.; Joyner, A. E. J.; *J. Dairy Sci.* **1986**, *69*, 1020. [Crossref]
3. Staples, C. R.; Burke, J. M.; Thatcher, W. W.; *J. Dairy Sci.* **1998**, *81*, 856. [Crossref]
4. Yanting, C.; Ma, G.; Harrison, J. H.; Block, E.; *Journal of Animal Science* **2019**, *97*, 4647. [Crossref]
5. Savoini, G.; Zorini, F. O.; Farina, G.; Agazzi, A.; Cattaneo, D.; Invernizzi, G.; *Animals* **2019**, *9*, 917. [Crossref]
6. Clapham, W. M.; Foster, J. G.; Neel, J. P. S.; Fedders, J. M.; *J. Agric. Food Chem.* **2005**, *53*, 10068. [Crossref]
7. Dutra, P. A.; Pinto, L. F. B.; Cardoso Neto, B. M.; Gobikrushanth, M.; Barbosa, A. M.; Barbosa, L. P.; *Theriogenology* **2019**, *127*, 26. [Crossref]
8. Santos, J. E. P.; Bilby, T. R.; Thatcher, W. W.; Staples, C. R.; Silvestre, F. T.; *Reprod. Domest. Anim.* **2008**, *43*, 23. [Crossref]
9. Childs, S.; Hennessy, A. A.; Sreenan, J. M.; Wathes, D. C.; Cheng, Z.; Stanton, C.; Diskin, M. G.; Kenny, D. A.; *Theriogenology* **2008**, *70*, 595. [Crossref]
10. Buccioni, A.; Decandia, M.; Minieri, S.; Molle, G.; Cabiddu, A.; *Anim. Feed Sci. Technol.* **2012**, *174*, 1. [Crossref]
11. Bauman, D. E.; Lock, A. L. In *Concepts in lipid digestion and metabolism in dairy cows*, 1st ed.; Eastridge, M. L., ed.; Ohio State University: Ohio, USA, 2006.
12. Delgado, B.; Bach, A.; Guasch, I.; González, C.; Elcoso, G.; Pryce, J. E.; Gonzalez-Recio, O.; *Sci. Rep.* **2019**, *9*, 1. [Crossref]
13. Folch, J.; Lees, M.; Stanley, G. H. S.; *J. Biol. Chem.* **1957**, *226*, 497. [Crossref] acessado em 29/11/2022.
14. Faraji, M.; Yamini, Y.; Gholami, M.; *Chromatographia* **2019**, *82*, 1207. [Crossref]
15. Wen, Y.; Chen, L.; Li, J.; Liu, D.; Chen, L.; *TrAC, Trends Anal. Chem.* **2014**, *59*, 26. [Crossref]
16. Li, G.; Row, K. H.; *TrAC, Trends Anal. Chem.* **2019**, *120*, 115651. [Crossref]
17. Bedassa, T.; Gure, A.; Megersa, N.; *J. Anal. Chem.* **2015**, *70*, 1199. [Crossref]
18. Pellegrino, R. M.; Di Veroli, A.; Valeri, A.; Goracci, L.; Cruciani, G.; *Anal. Bioanal. Chem.* **2014**, *406*, 7937. [Crossref]
19. Rodríguez-Palmero, M.; Lopez-Sabater, M. C.; Castellote-Bargallo, A. I.; la Torre-Boronat, M. C.; Rivero-Urgell, M.; *J. Chromatogr. A* **1998**, *793*, 420. [Crossref]
20. Magnusson, B.; Örnemark, U. In *Eurachem Guide: The Fitness for Purpose of Analytical Methods - A Laboratory Guide to Method Validation and Related Topics,* 2nd ed.; Magnusson, B., Örnemark, U., eds.; 2014.
21. Kamankesh, M.; Shahdoostkhany, M.; Mohammadi, A.; Mollahosseini, A.; *Anal. Methods* **2018**, *10*, 975. [Crossref]
22. Bazzana, M. J. F.; Saczk, A. A.; Faria, L. R.; Campos, J. P.; Alves, N. G.; Borges, C. N.; *J. Chemom.* **2020**, *34*, e3275. [Crossref]
23. Rezaee, M.; Yamini, Y.; Faraji, M.; *J. Chromatogr. A* **2010**, *1217*, 2342. [Crossref]
24. Rezaee, M.; Assadi, Y.; Hosseini, M. R. M.; Aghaee, E.; Ahmadi, F.; Berijani, S.; *J. Chromatogr. A* **2006**, *1116*, 1. [Crossref]
25. Barros Neto, B.; Scarminio, I. S.; Bruns, R. E.; *Como fazer experimentos - Pesquisa e Desenvolvimento na Ciencia e na Indústria*, 4ª ed.; Editora da Unicamp: Campinas, 2001.
26. Fernández, P.; González, C.; Pena, M. T.; Carro, A. M.; Lorenzo, R. A.; *Anal. Chim. Acta* **2013**, *767*, 88. [Crossref]
27. Behbahani, M.; Najafi, F.; Bagheri, S.; Bojdi, M. K.; Salarian, M.; Bagheri, A.; *J. Chromatogr. A* **2013**, *1308*, 25. [Crossref]
28. Vela-Soria, F.; Ballesteros, O.; Zafra-Gómez, A.; Ballesteros, L.; Navalón, A.; *Talanta* **2014**, *121*, 97. [Crossref]
29. Mudiam, M. K. R.; Chauhan, A.; Jain, R.; Dhuriya, Y. K.; Saxena, P. N.; Khanna, V. K.; *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2014**, *945*, 23. [Crossref]
30. Khodadoust, S.; Ghaedi, M.; *J. Sep. Sci.* **2013**, *36*, 1734. [Crossref]
31. Nabil, A. A. A.; Nouri, N.; Farajzadeh, M. A.; *Biomed. Chromatogr.* **2015**, *29*, 1094. [Crossref]
32. Rahimi, A.; Hashemi, P.; *J. Anal. Chem.* **2014**, *69*, 352. [Crossref]
33. Wang, Q.; Li, L.; Long, C. L.; Luo, L.; Yang, Y.; Yang, Z. G.; Zhou, Y.; *Environ. Technol.* **2020**, *41*, 1015. [Crossref]
34. Hu, Z.; Qi, P.; Wang, N.; Zhou, Q. Q.; Lin, Z. H.; Chen, Y. Z.; Mao, X. W.; Jiang, J. J.; Li, C.; *Food Chem.* **2020**, *309*, 125745. [Crossref]
35. Besil, N.; Cesio, V.; Heinzen, H.; Alba, A. R. F.; *J. Agric. Food Chem.* **2017**, *65*, 4819. [Crossref]