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Analysis of the main conjugated linoleic acid (CLA) precursors (C18:2 n-6 and C18:3 n-3) in *Brachiaria ruzizienses* by capillary zone electrophoresis

Research Article

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Abstract: An alternative method for extraction optimization of C18:2 n-6 and C18:3 n-3, the main precursors for the synthesis of conjugated linoleic acid (CLA), in *Brachiaria ruzizienses* forages was proposed. Three methods of lipid extraction were tested: 1. Hara & Radin, 2. Micro Folch and 3. Bligh & Dyer. The preliminary test showed the Hara & Radin method as the most promising procedure. Then, a 3^3 Box Behnken design with triplicate in the central point was applied in Hara & Radin method in order to optimize the extraction procedure. The optimization extraction was monitored by quantification of C18:2 n-6 and C18:3 n-3 through capillary zone electrophoresis (CZE). The results obtained by CZE were compared to gas chromatography (AOCS official method) in real samples using the paired t-test. No significant difference between methods was found within a 95% confidence interval (p-value= 0.937). The alternative CZE method for *Brachiaria ruzizienses* forages analysis has some advantages in comparison with official GC method such as, short analysis time (10 min), no derivatization step for sample preparation, absence of specific separation columns, lower analytical cost and high throughput.

Keywords: *Factorial Design • Forage • CLA Precursor • Extraction method*

1. Introduction

Forages are the main source of energy and nutrients for the synthesis of ruminant-derived products such as milk and meat. Among the *Brachiaria* species grown in Brazil, *Brachiaria ruzizienses* is the only diploid species which has sexual reproduction, allowing selection and recombination of superior genotypes. This forage species has received growing amounts of attention due to its use in integrated crop-livestock farming systems.

Forages are also an important source of essential fatty acids such as linoleic (C18:2 n-6) and α-linolenic (C18:3 n-3), which are the main precursors for the synthesis of CLA in ruminants (Fig. 1) [1-3]. CLA is a collective term describing a mixture of positional and geometric isomers of linoleic acid, with conjugated double bonds. Ruminant milk fat is the main source of CLA in human diet, with C18:2 cis-9, trans-11 representing the main CLA isomer found in dairy products (85-90% of total CLA). Due to its health-promoting properties, an increasing number

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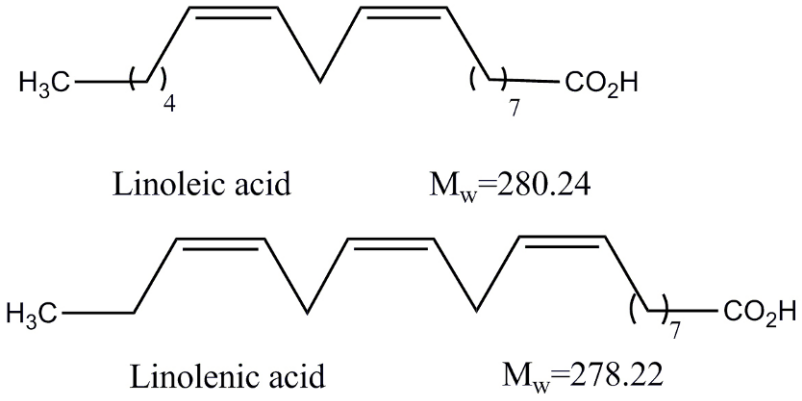

Figure 1. Fatty acid chemical structures (CLA precursors).

of CLA-related studies have been conducted over the past two decades. In particular, the quantification of C18:2 n-6 and C18:3 n-3 fatty acids (FA) in forages has been considered as an indicator of their potential for increasing the milk CLA content [4,5].

The official method for FA quantification is gas chromatography (GC) and the sample preparation involves lipid extraction and derivatization to form fatty acid methyl esters (FAME), which is time-consuming and cumbersome [6-9].

However, since 1990, capillary electrophoresis (CE) has gained ground in scientific community as a promising alternative technique for the determination of FA composition in food and biological samples [10-14]. The most common electrolyte systems are composed of buffers, chromophore agents p-anisato [15], sodium dodecyl benzenesulfonate (SDBS), organic solvents such as: methanol (MeOH), acetonitrile (ACN), 1-octanol, surfactants (sodium dodecyl sulfate (SDS) and polyoxyethylene 23 lauryl ether (Brij 35®) [13,15]. However, to the best of our knowledge, no studies involving FA analysis by CE in forage samples have been reported in the literature. Within this context, the aim of the work was a systematic study based on the most traditional lipid extraction procedures such as Hara and Radin [16], Micro Folch [17], and Bligh and Dyer [18] to optimize the lipid extraction using CZE methodology to CLA precursor analysis.

2. Experimental procedure

2.1. Chemicals and materials

All reagents were of analytical grade and the water was purified by deionization (Milli-Q system; Millipore, Bedford, MA, USA). The solvents MeOH (Vetec, Rio de Janeiro, Brazil), ACN (Merck, Rio de Janeiro, Brazil), 1-hexano, petroleum ether, isopropanol (Merck, Rio de Janeiro) and 1-octanol (Merck, Rio de Janeiro, Brazil) were chromatographic grade. The Brij 35 and SDBS were obtained from Sigma-Aldrich (St. Louis, MO, USA).

FA standards of C16:0, C18:2cc, C18:3ccc and C13:0 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Individual FA stock solution at concentration of 30.0 mmol L^{-1} was prepared by dissolving appropriate amounts of the selected standards in MeOH; they were then stored in a freezer until analysis. A mixture of all of the standards was prepared at concentration of 0.5 mmol L^{-1} by the appropriate dilution in MeOH.

Aqueous Brij 35 stock solution was prepared by weighing and dissolving an amount corresponding to 50.0 mmol L^{-1} in a 100.0 mL volumetric flask. A mass of NaOH corresponding to 0.5 mol L^{-1} was weighed and dissolved in a 100.0 mL volumetric flask and the volume was made up with MeOH. Aqueous SDBS stock solution was prepared by weighing and dissolving a mass corresponding to 100.0 mmol L^{-1} in a 100.0 mL volumetric flask.

The aqueous phosphate buffer stock solution (100.0 mmol L^{-1}) at pH = 6.8 ± 0.2 was prepared in a 250.0 mL volumetric flask by weighing and dissolving adequate mass of sodium and disodium phosphate salts. Phosphate buffer and the Brij 35 stock solutions were kept at approximately 4°C to prevent mold formation. The run electrolyte solution was prepared fresh through the appropriate dilution of stocks and the incorporation of solvents.

2.2. Process for drying the sample

Brachiaria samples were collected in the experimental Embrapa dairy cattle field located in Coronel Pacheco city, Minas Gerais state, Brazil. The drying process is very important to keep unmodified long chain and polyunsaturated FA, which are easily susceptible to oxidation processes. The selected process used was

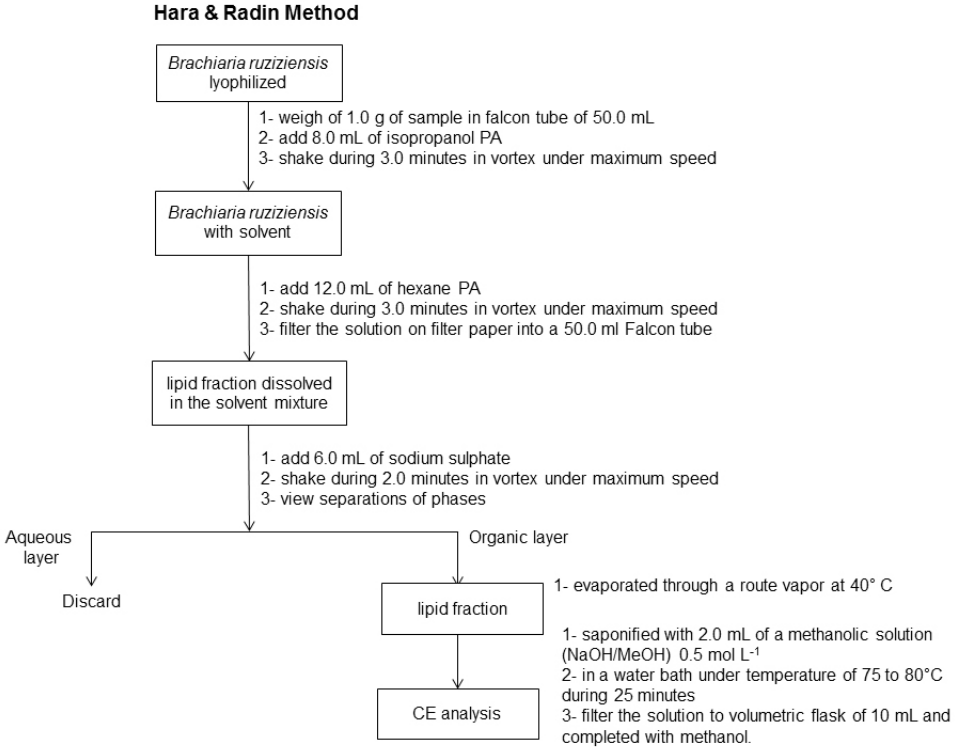

Figure 2. Hara and Radin extraction methods flow charts.

lyophilization because the chemical properties of the samples remain unaltered. However, before beginning the process, the samples must be maintained under temperature of -80°C for 24 hours and in a vacuum. After lyophilization, the samples were milled (knife mill fitted with sifters with holes of 1 mm) and stored in the freezer at -20°C.

2.3. Extraction methods

2.3.1. Hara and Radin method

The procedure consisted of weighing of 1.0 g of sample in a 50.0 mL falcon tube, after which 8.0 mL of isopropanol PA were added and shaken for 3.0 minutes in a vortex under maximum speed. 12.0 mL of hexane PA were added and the solution was shaken again for 3.0 minutes under maximum velocity. Then, the solution was filtered through filter paper to another 50.0 mL falcon tube and a solution of 3:2 hexane/isopropanol was used to wash the filter paper in order to remove any possible residues. Afterward, 6.0 mL of Sodium Sulphate solution (1.0 g of Na_2SO_4/ 15.0 mL of distilled water) were added, shaken for 2.0 minutes in a vortex and then maintained at rest until the phase separation was achieved. The organic phase was then removed and evaporated through a route vapor at 40°C until only the lipid phase remained in the flask (Fig. 2).

2.3.2 Micro Folch method

The procedure consisted in weighing 0.1 g of sample in a 15.0 mL falcon tube, 1.2 mL of a 2:1 chloroform/ MeOH solution was added and shaken in a vortex for 3.0 minutes. Next, 0.4 mL of methanol was added and shaken again for 3.0 minutes in vortex. The solution was filtered through filter paper to another 15.0 mL falcon tube. Then 0.8 mL of chloroform and 1.90 mL of NaCl solution 0.73% were added and shaken for 2.0 minutes and the solution was then maintained at rest until the phase separation was achieved. The organic phase was then removed and evaporated through a route vapor at 40°C until only the lipid phase remained in the flask (Fig. 3).

2.3.3 Bligh and Dyer method

The procedure consisted in weighing 1.0 g of sample in 50.0 mL falcon tube. 1.2 mL of a 2:1 chloroform / MeOH solution was added and shaken in a vortex under maximum velocity for 10.0 minutes. 5.0 mL of chloroform and 5.0 mL of sodium sulphate solution (1.0 g of Na_2SO_4 / 15.0 mL of distilled water) were added to the solution and shaken in vortex for 2.0 minutes. Then, it was maintained at rest until the phase separation was achieved. The organic phase was filtered through a filter paper containing anhydrous

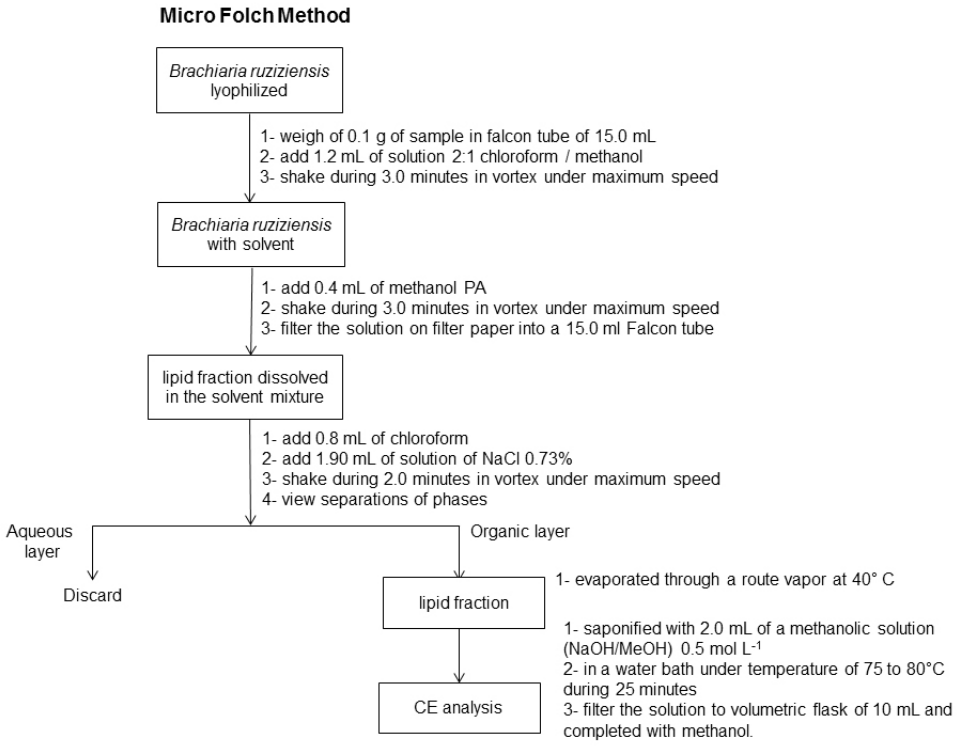

Figure 3. Micro Folch extraction methods flow charts.

sodium sulphate. Finally, the organic phase was removed and evaporated through a route vapor at 40°C until only the lipid phase remained in the flask (Fig. 4).

2.4. Instrumentation

2.4.1. Capillary electrophoresis system

Separation optimization experiments were conducted using a CE system (HP3d CE, Agilent Technologies, Palo Alto, California, USA) equipped with a Diode-Array Detector (DAD), indirect detection at 224 nm, temperature control device (set at 25°C), and software of acquisition and treatment data (HP ChemStation, rev A.06.01). Samples were hydrodynamically injected (12.5 mbar for 4 s) and the electrophoretic system was operated under normal polarity and constant voltage (+19 kV). For all experiments, a fused-silica capillary tube with Fluoro-polymer (TSH) external coating was used (Polymicro Technologies, Phoenix, AZ, USA) 48.5 cm total length (40 cm effective length) 75 µm internal diameter (ID) and 375 mm outside (OD). The TSH capillary is more abrasion resistant and offers unique solvent resistance properties. The TSH capillary was used as it avoids irreversible deleterious adsorption into internal capillary wall, which causes poor separation performance, as demonstrated by Balesteros *et al*. [19].

2.4.2. Gas chromatography

FA analysis was performed in a Shimadzu Gas Chromatograph equipment (GC17A model), with a flame ionization detector (FID), using a capillary fused silica column with a cyano propyl polysiloxane stationary phase (CP-7420TM, 60 m × 0.25 mm id, 0.25 µm film thickness, Varian, USA). The chromatographic conditions were those established in the AOCS Ce 1h-05 (AOCS): isothermal column temperature at 200ºC, injector and detector temperature at 250ºC, the carrier gas was hydrogen, and pressure column at 170 kPa. The compounds were identified by standard co-injection and relative retention time to FAME 13:0 (internal standard). Appropriate response factors were employed to convert area percent of FAME into true weight percentage. The correct response for each FAMEs was calculated theoretically and expressed in terms of the methyl palmitate response. Fatty acids were determined by FAME 13:0 addition as internal standard and expressed in g per 100g of sample. The method precision was evaluated by relative standard deviation (%RSD) [20].

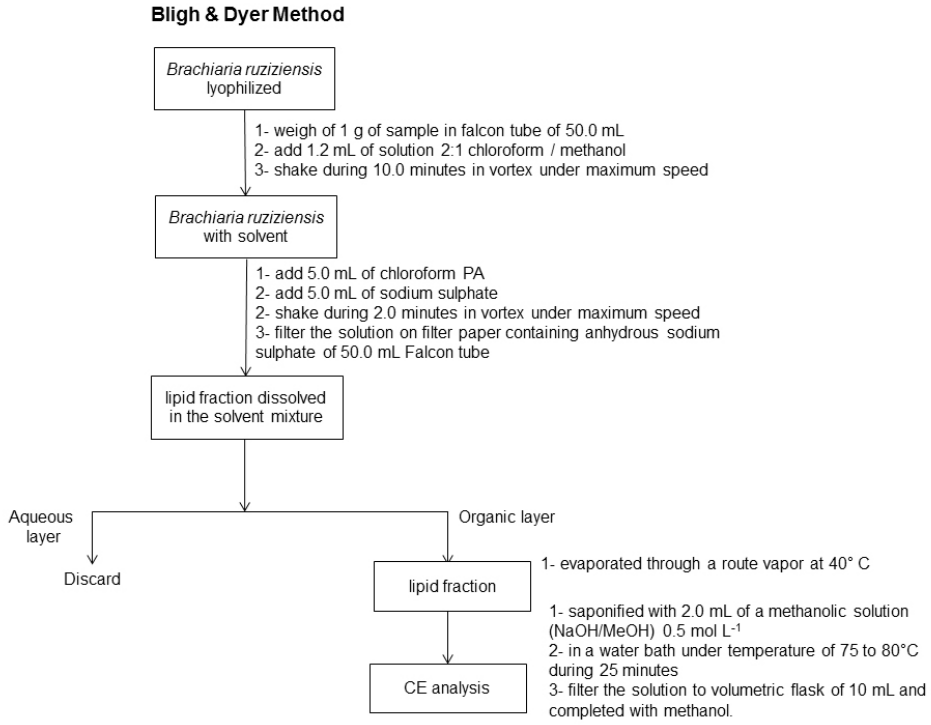

Figure 4. Bligh and Dyer extraction methods flow charts.

2.5. Sample preparation

2.5.1. Capillary electrophoresis

Brachiaria ruziziensis samples were extracted according to Hara and Radin [16], optimized method. Then lipid fraction was saponified with 2.0 mL of a methanolic solution (NaOH/MeOH) 0.5 mol L^{-1} in water heated bath (75 - 80°C) for 25 minutes. After the saponification step, the samples were transferred to 10 mL volumetric flask of and made up with methanol. When solid residue remained into the saponification flask, the mass of the residue was subtracted from original sample mass weighed, before the final calculation. Before injection in CE equipment the samples were diluted with methanol in proportion of 1:10.

2.5.2. Gas Chromatography official method

The extraction process was the same performed in CE analysis. However, after the extraction step, the samples were esterified according to Metcalfe, Schmitz, & Pelk and Hartman and Lago [21-22].

To the esterification method was added 2.5 mL of KOH 0.50 mol L^{-1} in methanol. Then the flask, on a heating mantle, is connected to a condenser. After the water begins to reflux the flask is left for 4 minutes. After 4.0 minutes, the system is left cooling in the presence of the condenser (approximately 3 minutes). 7.5 mL of the esterification reagent (prepared from a mixture of 2.0 g of ammonia chloride, 60.0 mL of methanol, and 3.0 mL of concentrated sulfuric acid for ca. 15 min) was then added to the solution and allowed to reflux for 3 minutes after viewing the condensing of the solvent. The mantle was turned off and the solution cooled in the presence of condenser. Then it was transferred to a separation funnel along with 250.0 mL of ethyl ether P.A and 25.0 mL of deionized water. After agitation and phase separation, the aqueous phase was discarded. 25.0 mL of deionized water was added to the organic phase and then it was agitated. After phase separation, the aqueous phase was discarded and the procedure was repeated. The ether phase was filtered through sodium sulfate (or magnesium) anhydrous PA into a 50 mL round bottom flask. The separating funnel and filter paper was washed with ethyl ether, total recovery of methyl esters. The organic phase was collected, the solvent was evaporated in a rotavapor apparatus and the residue was removed under nitrogen flow. The methyl esters were solubilized in dichloromethane PA before injection into the gas chromatographer.

2.6. Statistical analysis

The statistical tests such as normality, homoscedasticity and independence were performed in SPSS 8.0 for windows software. The lack of fit analysis was performed in Microsoft Office® Excel software.

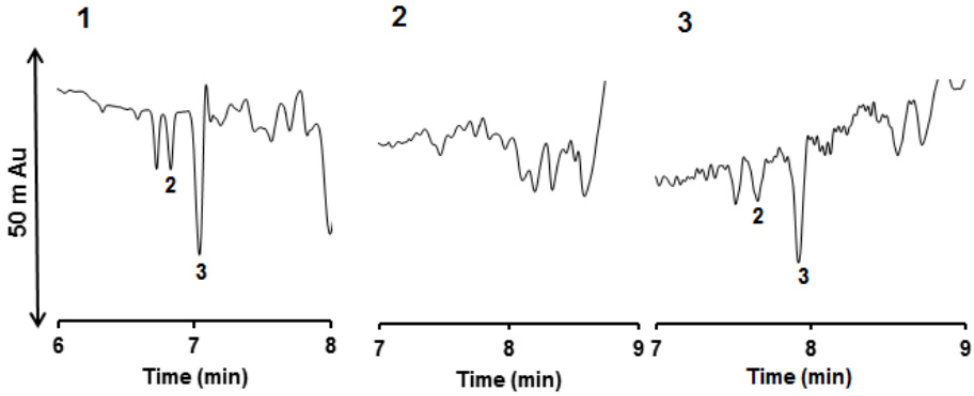

Figure 5. Electropherograms obtained from analysis of the three extraction methods, respectively, Hara & Radin, Micro Folch and Bligh & Dyer methods. Peaks: 1-C16:0, 2-C18:2 and 3-C18:3. Operational conditions: injection 4 s 12.5 mbar, voltage +19 kV, indirect detection at 224 nm and 25°C temperature inside the cartridge, TSH capillary with 48.5 cm long (40 cm effective length) 75 μm I.D and 375 mm O.D.

2.7. Analytical procedures

Before use, new capillaries were conditioned by pressure flushing with 1.0 mol L^{-1} NaOH (30 min), deionized water (5 min) and electrolyte solution (10 min). In between runs, capillaries were regenerated by washing with 0.2 mol L^{-1} NaOH (2 min), deionized water (2 min) and fresh electrolyte solution (3 min, pressure flush). The conditioning procedure was found to be critical for ensuring peak area and migration time repeatability, and for preventing deleterious solute adsorption to the capillary wall.

3. Results and discussion

3.1. Background electrolyte (BGE)

Traditionally, the analysis of fatty acid (FA) by CE takes into account the BGE characteristics: use of organic solvent such as ACN in order to avoid micelle formation among FA; pH must be higher than 7.0 to promote the carboxyl group dissociation (FA has pKa about 5.0) and to make it possible to analyze FA in anionic form under counter electrosmotic flow (EOF) and catodic EOF; use of chromophore agent such as SDBS to promote indirect detection of saturated FA as generally they present low molar absorptivity in the UV range. In this work the FA targets were linoleic (C18:2 n-6) and linolenic acid (C18:3 n-3). However, in the sample it is common to have a considerable amount of palmitic acid (C16:0) present which presents similar electrophoretic mobility to linoleic (C18:2 n-6). The BGE used was based on a paper recently published by our research group [23], taking into account optimization of FA separation by using factorial design associate to principal component analysis (PCA) and TSH capillary which is more abrasion resistant and offers unique solvent resistance properties. Thus, the variables evaluated were: NaH_2PO_4/Na_2HPO_4 buffer pH = 6.8 ± 0.2, Brij 35, ACN, 1-octanol and SDBS. Other variables such as voltage, capillary dimensions, wavelength, cartridge temperature, standard mixture concentration, buffer concentration and SDBS were maintained constants. The optimum BGE condition considered to CLA precursor separation was: 15.0 mmol L^{-1} of buffer NaH2PO4 / Na2HPO4 pH = 6.8 ± 0.2, 10.0 mmol L^{-1} Brij 35, 2.2% of 1-octanol, 43.5% of ACN and 4.0 mmol L^{-1} of SDBS.

3.2 Extraction optimization

According to literature, brachiaria contain of 2.0 to 3.0% of total lipids and from this total 40 to 50% are formed by FA. Thus, on the total dry mass of lipids present in sample lyophilized there is 1.0 to 1.5% of FA. So, it is necessary to make the lipid fraction extraction of the brachiaria by a method optimized to analysis of main CLA precursors. Within this context, a preliminary test with traditional extraction methods such as Hara and Radin; Micro Folch and Bligh and Dyer was performed according to 2.3.1 to 2.3.3. The BGE described in item 3.1 was applied to different extractions methodologies according to shown in Fig. 5. By qualitative profile of the electropherograms obtained, that is base line stability, peak separation and signal noise behavior, was possible to conclude that among the methods tested the Hara and Radin was the one that presented positive results, along with use of a less toxic solvent, in comparison with the others (Micro Folch and Bligh and Dyer).

Then, in order to achieve the best extraction performance for Hara and Radin procedure, a 3^3 Box Behnken experimental design with triplicate in central point has been done taking into account the factors: sample mass, shake time and hexane and isopropanol proportions, according to described in Table 1. Among the

experiment carried out, the trial number 12 was selected as the superior performance because it presented the best electrophoretic baseline stability profile, and higher resolution for the C16:0/C18:2cc critical pair. The best condition was performed in real sample according to shown in Fig. 6.

3.3. CLA precursor quantification in Brachiarias samples by CZE

Once the extraction procedure was optimized, the next step was to perform analysis in real samples. Thus, the approach to FA quantification was based on a statistical study which involved the response factor (R_f) calculation by using C13:0 as internal standard (IS) [10]. In order to calculate R_f two calibration curves were developed for each FA. Then, a random experiment in genuine replicates using solutions of C18:2cc and C18:3ccc standards with varying concentrations at 0.03, 0.05, 0.07, 0.09 and 0.11 mmol L^{-1} for the first range of the analytical curve and concentrations at 0.2, 0.3, 0.4 and 0.5 for the second range of the analytical curve, for both curves the C13:0 was fixed at 0.5 mmol L^{-1}, according to shown in Table 2.

Due to the variation of C16:0, C18:2cc and 18:3ccc in Brachiarias samples, it was necessary to perform calibration curves in different range for each FA in order to avoid deviations from linearity. Thus, the regression models were fitted through the linear least-square regression using internal standard approach. The homoscedasticity was verified by Levene (different numbers of replicates into the same level) or Cochran's test (same numbers of replicates into the same level). In the present study, since analytical curve in the 2nd range C18:3ccc presented heteroscedasticity behavior, so the use of weighted least-square regression was necessary [24]. After regression implementation it was necessary to verify lack of fit into the model through a priori test hypothesis (ANOVA) [25,26]. This test consists of comparing the deviations of the means from the calibration line the residual standard deviation (s_{yx}) with the y values from their means (s_y) by using Eq. 2, where m_i is the number of measurement, p is the calibration points and m is the product between p and m_i. The test is carried out by the comparison between $F_{calculated}$ and $F_{critical}$; $f_{1= p-2;\ f2=m-p}$ ($F_{critical}$). If $F_{calculated} \geq F_{critical}$, the linear model cannot be applied. In the present case, the regression model diagnosis was satisfactory since no lack of fit was presented, because the value of $F_{calculated}$ was lower than $F_{critical}$ for all fatty acids in 95%

Table 1. 3^3 Box-Behnken experimental design coded matrix containing levels and factors.

Experiment	X_1	X_2	X_3
1	-1	-1	0
2	1	-1	0
3	-1	1	0
4	1	1	0
5	-1	0	-1
6	1	0	-1
7	-1	0	1
8	1	0	1
9	0	-1	-1
10	0	1	-1
11	0	-1	1
12	0	1	1
13	0	0	0
14	0	0	0
15	0	0	0

X_1= mass (g): (-1) 0.5 g; (0) 1.0 g ; (1) 1.5 g
X_2= time (min): (-1) 1.0 ; (0) 2.0; (1) 3.0
X_3= solvent (hexane/isopropanol) (ml): (-1) 10.0/8.0; (0) 12.0/8.0 ; (1) 14.0/8.0

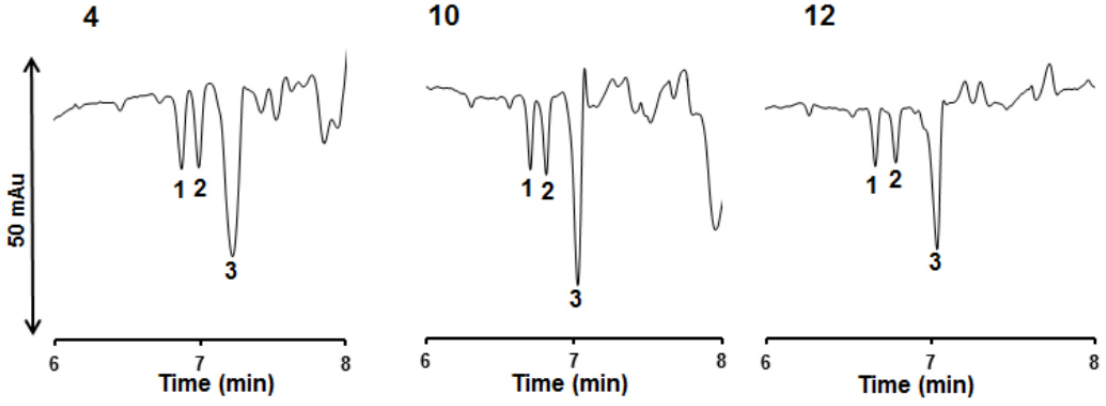

Figure 6. The three best results obtained from analysis of the 3^3 Box Behnken experimental design by Hara & Radin extraction method. Peaks: 1-C16:0, 2-C18:2 and 3-C18:3. Operational conditions: injection 4 s 12.5 mbar, voltage +19 kV, indirect detection at 224 nm and 25°C temperature inside the cartridge, TSH capillary with 48.5 cm long (40 cm effective length) 75 μm I.D and 375 mm O.D.

Table 2. Values used to regression model implementation with genuine replicates.

FA	[C_{FA}]/ [C13:0]	1ª Replicate	2ª Replicate	3ª Replicate
C18:2 1° range	0.06	----	0.069	0.074
	0.10	0.238	----	0.146
	0.14	----	0.295	0.225
	0.18	----	----	----
	0.22	0.389	----	0.345
C18:2 2° range	0.40	0.308	----	0.379
	0.60	----	0.378	0.480
	0.80	----	0.518	0.530
	1.00	---	0.618	0.599
C18:3 1° range	0.06	----	0.103	0.093
	0.10	0.154	0.179	----
	0.14	0.176	----	0.280
	0.18	0.214	----	0.332
	0.22	----	0.383	0.416
C18:3 2° range	0.20	----	----	----
	0.40	0.294	0.299	0.305
	0.60	0.372	---	0.622
	0.80	0.555	----	0.721
	1.00	0.725	0.793	----

$C_{FA}/C_{[C13:0]}$: mmol L^{-1} ;[C13:0] fixed in 0.50 mmol L^{-1}

of confidence interval. The values used to regression model implementation were summarized in Table 3.

$$F_{calc} = \frac{S^2_{y,x}}{S^2_y} = \frac{\sum_{i=1}^{p} m_i(\bar{y}_i - \hat{y}_i)^2 \,/\, (p-2)}{\sum_{i=1}^{p}\sum_{j=h}^{m_i}(y_j - \bar{y}_i)^2 \,/\, (m-p)} \quad (1)$$

The quantification procedure involved the calculation of R_f as described by the following mathematical expression:

$$\frac{A_{FA}}{[FA]} = R_f \frac{A_{C13:0}}{[C13:0]} \quad (2)$$

Where: A_{FA} is the area for each fatty acid, $A_{C13:0}$ (internal standard area): tridecanoic acid area, [FA] is the concentration in mmol L^{-1} for each fatty acid and [C13:0] is the tridecanoic acid concentration fixed in 0.5 mmol L^{-1}.

Whereas the regression model was found to be satisfactory, the slope can be used as the response factor (R_f) in Eq. 2, as long as the internal standard C13:0 at 0.5 mmol L^{-1} was used (the FA concentration, that is, [FA] remains unknown) [26]. The percentage of FA in the sample was determined through Eq. 3, which was obtained by rearranging Eq. 2:

$$\%FA = \frac{A_{FA}.[C13:0].V.MW_{FA}}{R_f.A_{C13:0}.m}.100 \quad (3)$$

Where: A_{FA} is the area for each fatty acid, $A_{C13:0}$ tridecanoic acid area, [C13:0] is the tridecanoic acid concentration fixed in 0.5 mmol L^{-1}, V is the volume in liters, m is the sample mass in milligrams, R_f is the response factor (fitted model slope), and MW_{FA} is the molecular weight for each fatty acid.

3.4 Comparison between CE and GC methods

In order to apply the optimized method in real sample, five genuine forage samples from *Brachiaria ruziziensis* were analyzed by CE methodology and results were compared to those obtained using the official AOCS GC method. Table 4 shows statistical

Table 3. Response Factor calculated for each FA.

FA	Slope	Intercept	r	R_f	F_{calc}	F_{crl}
C 18:2cc (1º range)	1.644 (± 0,235)	0.004 (± 0,036)	0.93	1.64	1.12	$F_{2,4} = 6.94$
C 18:3ccc (1º range)	1.773 (± 0.255)	-0.015 (± 0.038)	0.93	1.77	0.42	$F_{3,5} = 5.41$
C 18:2cc (2º range)	0.445 (±0.058)	0.165 (±0.043)	0.95	0.44	0.01	$F_{2,6} = 5.14$
C 18:3ccc (2º range)	0.773 (±0.054)	-0.010 (±0.019)	0.98	0.77	0.40	$F_{2,7} = 4.74$

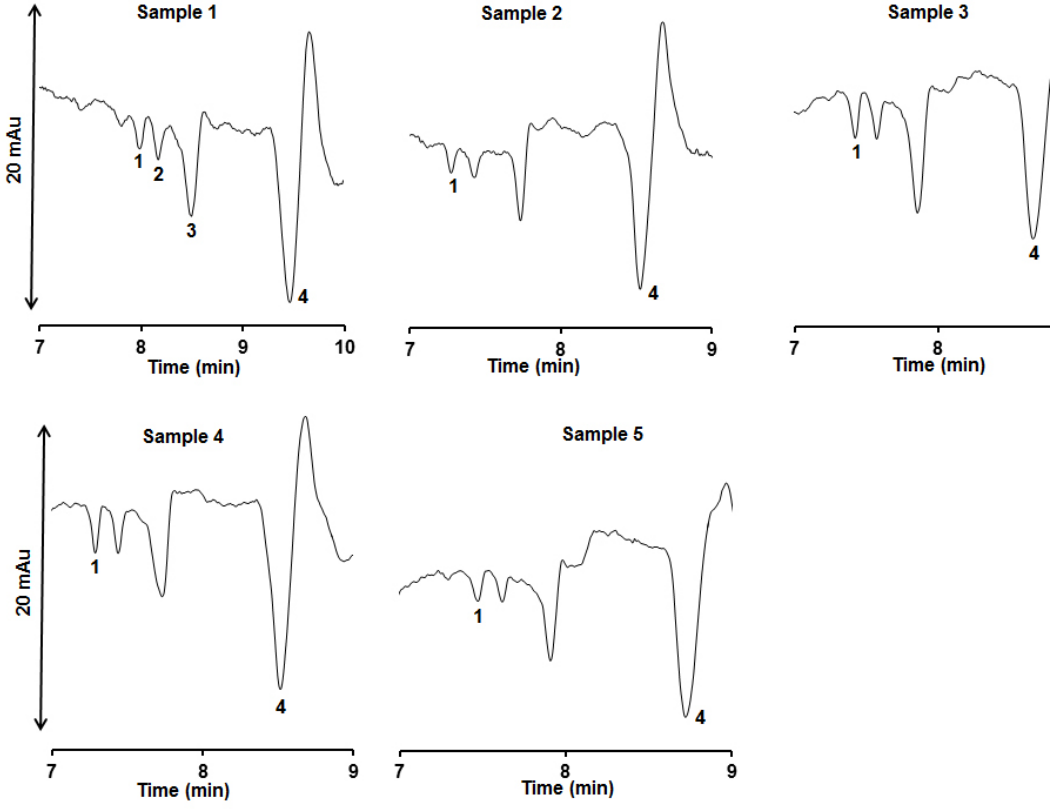

Figure 7. Electropherograms obtained through the best condition by 3^3 Box Behnken experimental design by Hara & Radin extraction method of five genuine replicates of forage. Peaks: 1-C16:0, 2-C18:2, 3-C18:3 and 4-C13:0. Operational conditions: injection 4 s 12.5 mbar, voltage +19 kV, indirect detection at 224 nm and 25°C temperature inside the cartridge, TSH capillary with 48.5 cm long (40 cm effective length) 75 μm I.D and 375 mm O.D.

comparative results (Shapiro-Wilk normality test and paired sample t test) for CE and GC: no evidence of significant difference between the two methodologies was observed at the 95% of confidence interval (p-value > 0.05) and the limit of detection (LOD) and limit of quantification (LOQ) to CE method. Fig. 7 shows electropherograms obtained through the best condition by 3^3 Box Behnken experimental design by Hara & Radin extraction method of five genuine replicates of forage.

A comparative scheme was built as shown in Fig. 8 to make clear the advantages of CE developed methodology in comparison with Official Gas Chromatography in respect to analysis time, amount of chemical reagents and analytical throughput. Then for CE is possible to perform 6 samples analysis within 145 minutes whereas by GC Official Method needs around 5 hours for each sample.

4. Conclusions

An alternative, lipidic extraction methodology to CLA precursor (C18:2cc and C18:3ccc) analysis in forage was optimized. The method proposed in comparison with the classical methodology by soxleht presented

Table 4. Analysis of different forages by CE in comparison with official method by GC.

Samples	C18:2		C18:3	
	CE(%m/m)	GC(%m/m)	CE(%m/m)	GC(%m/m)
1	4.13	4.38	19.99	18.88
2	4.06	4.51	21.29	18.81
3	4.88	5.27	17.80	21.20
4	5.93	4.05	21.85	17.38
5	4.83	5.19	17.41	21.82
Mean	4.77	4.68	19.67	19.62
sd	0.75	0.53	2.01	1.84
LOD (mmol L^{-1})	$3.20\ 10^{-3}$	---	$1.94\ 10^{-6}$	---
LOQ (mmol L^{-1})	$1.57\ 10^{-2}$	---	$1.74\ 10^{-5}$	---

Shapiro-Wilk test: p-value equal to 0.684
Paired sample test: p-value equal to: 0.937
LOD: limit of detection
LOQ: limit of quantification

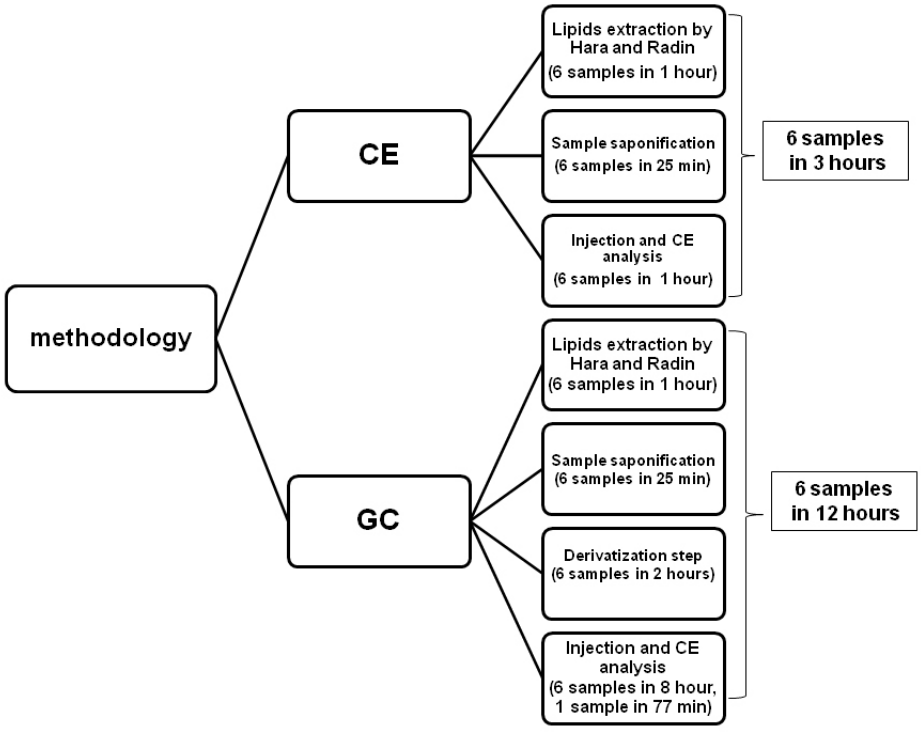

Figure 8. Analytical throughput comparative scheme between CE and GC methods.

the advantages of - shorter analysis time, simplicity, use of a less toxic solvent and low consumption of organic solvent. The CZE method applied could be used in routine analysis because of its efficiency, speed, absence of derivatization steps in sample preparation, absence of specific columns, simple BGE, low consumption of reagents and chemicals and low cost of analysis. The CE methodology compared to gas chromatography (AOCS official method) presented no significant difference within a 95% confidence interval for analysis of real samples. Thus, the present methodology has been successful applied to study involving brachiaria improvement, taking into account CLA precursor monitoring.

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