JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 10793–10797 10793
DOI:10.1021/jf901769w

Effect of a Purification Step and the Type of Internal Standard Used on Fatty Acid Determination of Grass and Maize Silages

Susana P. Alves,[†,‡] Ana R. J. Cabrita,[§] António J. M. Fonseca,[‡] and Rui J. B. Bessa*[,†,∥]

[†]INRB - Instituto Nacional dos Recursos Biológicos, Unidade de Produção Animal, Fonte-Boa, 2005-048 Vale de Santarém, Portugal, [‡]REQUIMTE, ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua Padre Armando Quintas, 4485-661 Vairão VC, Portugal, [§]REQUIMTE, SAECA, Faculdade de Ciências, Universidade do Porto, Rua Padre Armando Quintas, 4485-661 Vairão VC, Portugal, and [∥]Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, CIISA, Pólo Universitário do Alto da Ajuda, Av. da Universidade Técnica, 1300-477 Lisboa, Portugal

The fatty acid (FA) analysis of grass and maize silages was studied by application of a direct transesterification method (DT) followed by purification by solid-phase extraction (SPE). The choice of the internal standard (IS) for quantification of FA by gas−liquid chromatography (GLC) was also studied. The acidic DT method applied to grass silage samples produced a high amount of non-fatty acid methyl ester compounds (non-FAME) compared with those formed in maize silages. The application of the SPE cleanup step reduced significantly the amount of non-FAME compounds in both samples. Five FAs were tested as IS; among them, 3 were naturally present in all silages, however their use as IS did not affect quantification of total FA composition. Nevertheless, some minor FAs present in silages were significantly affected by the IS used. Additionally, application of corrections to the GLC peak areas did not significantly influence quantification of total FA composition of silages.

KEYWORDS: Silage; fatty acid methyl esters; internal standard; gas−liquid chromatography; solid-phase extraction

INTRODUCTION

Herbages are important dietary sources of polyunsaturated fatty acids (PUFAs), mainly α-linolenic and linoleic acids, for ruminants. However, in confined animals or during pasture shortage, conserved forages (mainly silages and hays) constitute important sources of nutrients.

The analysis of FA composition in silages is commonly performed by gas−liquid chromatography (GLC) of fatty acid methyl ester (FAME) derivatives. These FAME derivatives may be prepared by a two-way procedure or by direct transesterification (DT) methods. In the first ones, lipids are first extracted using organic solvents and finally esterified into FAMEs. In DT methods, lipids are simultaneously extracted and derivatized by using basic or acid-catalyzed reactions (*1*).

As modification of lipid composition during ensiling involves the increase of free FAs due either to plant-mediated (*2*) and microbial-mediated lipolysis (*3*), acidic transesterification conditions should be used to prepare FAMEs, to ensure methylation of free FA present in silages.

The acidic DT procedure developed by Sukhija and Palmquist (*4*) has been widely used for the analysis of FAs in forages, feeds and rumen bacteria. However, we had recently described (*5*) that this acidic catalyst may cause the appearance of high amounts of non-FAME compounds in forage samples. Thus, we had developed a purification step using solid-phase extraction (SPE), that we had reported to be efficient in reducing these unnecessary compounds from silage samples (*5*).

Quantification of FAMEs by GLC is commonly performed by the internal standard (IS) method. The 15:0, 17:0, 19:0, 20:0 and 21:0 FA have been used as IS. However, both 15:0 and 17:0 may be produced by bacteria (*6*), thus might occur in silage samples; 19:0 may coelute with 18:1 and 18:2 isomers; and 21:0 may coelute with conjugated linoleic acid isomers (although not frequently present in silages). Schreiner (*7*) had reported that precision and accuracy of FA quantification might be dependent on the selection of the IS. Therefore, quantitative aspects of FA analysis of silage samples by GLC were studied and considered by testing different IS.

Furthermore, this study aims to improve the current methods of FA analysis in freeze-dried silage samples by application of the DT procedure followed by purification by SPE. Non-FAME compounds produced by the DT procedure of grass and maize silages will also be identified and quantified.

MATERIAL AND METHODS

Chemicals. All reagents and solvents were analytical and chromatographic grade, and were obtained from Sigma-Aldrich (St. Louis, MO). Prepacked silica gel cartridges (500 mg/ 3 mL) were purchased from Merck Biosciences (Darmstadt, Germany). The standards used for the internal

*Corresponding author. Tel: (+351) 213652871. Fax: (+351) 213652884. E-mail: rjbbessa@fmv.utl.pt.

Published on Web 10/28/2009

standard study were obtained from Sigma-Aldrich (St. Louis, MO) and were all of highest purity available and dissolved in *n*-hexane (GC grade >99%) from Sigma-Aldrich (St. Louis, MO).

Sample Collection. Samples of grass silages of ryegrass (R; *Lolium multiflorum Lam.*), consociations of ryegrass and triticale (RT; *Lolium multiflorum Lam.* and *Triticosecale* Wittm.) and ryegrass, triticale and vetch (RTV; *Lolium multiflorum Lam., Triticosecale* Wittm. and *Vicia sativa* L.), and two maize silages (MP; Pioneer; *Zea mays* L.) and (MD; Dekalb; *Zea mays* L.) were collected from commercial dairy farms (Vila do Conde, Portugal) in October (R and RT), January (RTV), April (MD) and May (MP) of 2007. All grass silages were from a first-cut and were ensiled after three days of field wilting. Commercial inoculants were used in the ensiling of RT and RTV (Pioneer G22) and maize silages (Pioneer G33).

Fatty Acid Analysis. Silage samples were freeze-dried, ground and kept at −80 °C until FA extraction.

Direct Transesterification Method. Fatty acids from silages were extracted by using the direct transesterification method adapted from Sukhija and Palmquist (*4*) and described in Alves et al. (*5*). Briefly, 1 mL of internal standard and 1 mL of toluene were added to 250 mg of sample, followed by the addition of 3 mL of 5% HCl solution in methanol (prepared by the addition of acetyl chloride to the methanol). After homogenization on vortex at slow speed, samples were maintained for 2 h at 70 °C in a water bath. After that, the solution was left to cool at room temperature and subsequently neutralized with 5 mL of 6% K_2CO_3. FAMES were extracted with 2 mL of hexane, and 1 g of Na_2SO_4 and 1 g of activated carbon were added. Finally, samples were centrifuged for 5 min at 2500 rpm, the supernatant was transferred to new tubes and the solvent was removed under nitrogen at 37 °C. The final residue was dissolved in 1 mL of hexane and stored until GC analysis and purification by SPE.

Purification by Solid-Phase Extraction. After direct transesterification, samples were submitted to SPE using dichloromethane as solvent, as described by Alves et al. (*5*). Briefly, the final residue was evaporated and suspended in 100 μL of dichloromethane which was introduced into the prepacked silica gel cartridge (500 mg/3 mL) after equilibration with dichloromethane. A volume of 0.5 mL of dichloromethane was added to remove phytadienes followed by the addition of 2 mL to remove FAMEs from the SPE column. Finally, 2 mL of methanol was added to remove other non-FAME compounds (organic acids, levulinic acid and others unidentified) that we observed to elute after FAMEs. The final residue was evaporated under nitrogen and stored at −4 °C until GLC analysis.

Internal Standard Study. Five internal standards (15:0, 17:0, 19:0, 20:0 and 21:0) at concentration of 1 mg/mL were tested in triplicate complete analysis for each silage sample. Additionally, calibration curves were prepared at concentrations ranging from 0.05 mg/mL to 1 mg/mL by application of the direct transesterification method to the 5 FA standards (>99% purity) obtained from Sigma-Aldrich (St. Louis, MO) and with the addition of 16:0 FAME at concentration of 0.5 mg/mL.

GLC and GC−MS analysis. A GC system from Agilent HP5890 (Agilent Techn. Inc., Palo Alto, CA) equipped with a flame ionization detector and a CP-Sil 88 capillary column (100 m, 0.25 mm I.D., 0.20 μm film thickness; Chrompack, Varian Inc., Walnut Creek, CA) was used for quantification. The chromatographic conditions were as follows: injector temperature, 250 °C; detector temperature, 280 °C; helium was used as carrier gas, and the split ratio was 1:50. The oven temperature program was 50 °C (maintained for 4 min), followed by a 13 °C/min ramp to 175 °C (maintained for 27 min), then increased at 4 °C/min to 215 °C (maintained for 60 min). Additionally, structural elucidation of FA and non-FA compounds was accomplished by using a GC−MS system from Varian Saturn 2200 (Varian Inc.,Walnut Creek, CA) equipped with an ion trap mass detector and a CP-Sil 88 capillary column (100 m, 0.25 mm i.d., 0.20 μm film thickness; Chrompack, Varian Inc., Walnut Creek, CA). The column temperature of 100 °C was held for 15 min, increased to 150 °C at a rate of 10 °C/min and held for 5 min, then increased to 158 at 1 °C/min and held for 30 min, and finally increased to 200 °C at a rate of 1 °C/min (maintained for 40 min). Helium was used as carrier gas, and the injector temperature was 250 °C. The ion trap parameters used in the presented analyses are similar to those described by Alves and Bessa (*8*).

Statistical Analysis. The effect of SPE cleanup on the two types of silage was evaluated by analysis of variance, considering the silage, the SPE, and the interaction between type of silage and SPE as fixed effects. The effect of correcting the GLC areas, using theoretical relative FID response factors and correcting the amount of the naturally present IS in the sample, on the total FA content (mg/g DM) of silages, was compared by analysis of variance, considering the silage, the correction method and the interaction between silage and correction as fixed effects. Finally, the effect of the IS on total FA content and FA composition (mg/g DM) of the two types of silage was evaluated by analysis of variance considering the IS as fixed effect. All the computations were conducted using the GLM procedure of SAS (SAS Inst. Inc., 2002, Cary, NC).

For the calibration assays, linear regression analysis was conducted by plotting response area vs concentration. Three replicates were made to obtain residual standard deviations (RSD), slope, intercept and coefficient of determination (R^2).

Table 1. Composition of Peaks on Chromatograms after Direct Transesterification of Silage[a] Samples (% of Area on Total GLC Area ± Standard Deviation) (n = 3)

	R	RT	RTV	MP	MD
total FAME	63.8 ± 0.5	77.3 ± 0.2	79.6 ± 0.3	92.2 ± 0.2	90.1 ± 0.1
total phytadienes	6.9 ± 0.1	6.4 ± 0.3	6.9 ± 0.1	1.4 ± 0.1	1.2 ± 0.1
organic acids					
succinic acid	15.7 ± 0.4	2.6 ± 0.0	2.1 ± 0.1	1.2 ± 0.0	1.9 ± 0.0
citric acid	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
azelaic acid	0.5 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
sugar derivative					
levulinic acid	4.1 ± 0.2	5.4 ± 0.2	3.4 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
fumonisin B derivative					
TCA[b]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.3 ± 0.1	5.8 ± 0.1
others	8.0 ± 0.3	6.8 ± 0.1	6.5 ± 0.5	0.9 ± 0.1	0.8 ± 0.1
unidentified					

[a] Silages of ryegrass (R); ryegrass + triticale (RT); ryegrass + triticale + vetch (RTV); maize Pioneer (MP); and maize Dekalb (MD). [b] TCA: tricarballylic acid, trimethyl ester.

RESULTS AND DISCUSSION

Application of the Direct Transesterification Method. Table 1 shows the composition of the five silages studied after DT of freeze-dried samples in order to produce FAMEs for quantification by GLC. Grass silages (R, RT and RTV) showed lower percentages of FAME in total chromatogram area compared with maize silages (MP and MD). The R silage showed only 63.8% of FAME, followed by 77.3% and 79.6% for RT and RTV, respectively. Conversely, percentages of total FAME in maize silages were 92.2% and 90.1% for MP and MD, respectively.

The R silage showed the highest percentage of non-FAME compounds, the organic acids being the most abundant, and the succinic acid the most prominent of them. However, phytadienes, sugar derivative products and other unidentified compounds were also present in R silage. RT and RTV silages had percentages of non-FAME compounds around 20% of total chromatogram area. In these two silages, phytadienes showed the highest percentage of identified non-FAME compounds, and organic acids the lowest percentage of non-FAME compounds. The levulinic acid, a sugar derivative product, was not detectable in MP and MD maize silages, but its percentage in total chromatogram area ranged between 5.4% and 3.4% for RT and RTV, respectively. A derivative product of fumonisin B, the TCA (tricarballylic acid, trimethyl ester), was identified in maize silages ranging from 4.3% for MP and 5.8% for MD. The fumonisin B is a *mycotoxin* mainly produced by *Fusarium moniliforme*, which is a common fungal contaminant associated with maize products (*9*−*11*). The fumonisin B under DT conditions undergoes acid hydrolysis to cleave the ester bonds and the subsequent production of the TCA derivative (*12*).

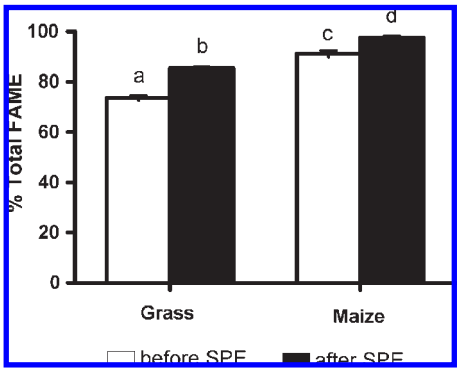

Figure 1. Effect of the SPE cleanup step on the percentage of total FAME in total GLC area of grass and maize silage samples prepared by direct transesterification (DT). Values with different letters (a, b, c, d) are significantly different ($P < 0.05$).

Recently, we had reported the presence of several non-FAME compounds in herbage after DT of freeze-dried samples (*5*). In fresh ryegrass were detected several phytadienes, which are derived from degradation of chlorophyll, and a levulinic acid derived from sugars in plant tissues. As expected, these products also occurred in silages containing ryegrass. Other compounds identified in herbage were products of esterification of organic acids, such as fumaric, malonic, succinic, malic and citric acids. From these, only succinic and citric acids were identified in silages. It is recognized that plant acids decrease considerably during fermentation, with a subsequent increase in succinic and lactic acids (*13*), although lactic acid was not detectable after DT of freeze-dried silage samples.

Development of a SPE Purification Step. Current methods of FA analysis of silage samples do not use a purification step. As discussed above, the elimination of non-FAME contaminants from silage samples may be important in order to prevent the occurrence of interferents in the GLC analysis. We recently proposed a method based on SPE to remove non-FAME compounds from freeze-dried herbage samples (*5*). We tested this method on the current silages in order to produce clean samples for FA analysis. The recovering experiments of the DT-SPE procedure, previously reported (*5*), have shown its accuracy and repeatability. The percentages of total FAME (**Figure 1**) in total chromatogram area after SPE cleanup increased about 11.9% for grass and 6.5% for maize silages, which showed to be significant. Due to the high percentage of FAME compounds on maize silages, the SPE cleanup step could be eventually dispensable. In our conditions, TCA derivative does not coelute with any FA, however, in other chromatographic conditions, if TCA derivatives are suspected to coelute with FAMEs, then the SPE cleanup step could be considered. In grass silages, the use of the SPE cleanup step is clearly justified to reduce the amount of interfering compounds, even because organic acids and levulic acid have been reported to coelute with FAMEs during GLC analysis (*5*).

Quantitative Studies. Quantification of total FA composition (mg/g dry matter (DM)) is commonly performed by GLC with a flame ionization detector (FID), using the method of IS. Several IS are described in the literature for the analysis of FA composition. We selected the five FA (15:0, 17:0, 19.0, 20:0 and 21:0) mostly used as IS in order to identify the most appropriate one. Linearity response of each IS on detector system and utilization of theoretical relative FID response factors were also evaluated and described below.

Natural Occurrence of the Each IS in Silage. In order to detect the presence of some selected IS in silages, samples of R, RT, RTV, MP and MD were prepared and injected without the addition of any IS. **Table 2** shows the percentage of IS (in total FAME) naturally present on silages. As expected, 15:0 and 17:0 were present on silages due to their predominant microbial origin (*6*), 20:0 being detected in percentages ranging from 0.85% for R silage and 0.37% for MD silage. However, 19:0 and 21:0 were not detected in the five silages studied. Indeed, Dewhurst and King (*14*) reported the presence of the 15:0 in perennial ryegrass silage, and Lee (*15*, *16*) and Shingfield (*17*) found arachidic acid (20:0) in grass and legume silages. Additionally, the detection of 15:0, 17:0, 19:0 and 20:0 was described by Vanhatalo (*18*), in silages prepared from early and late cuts of grass and red clover. Despite the natural presence of these FAs in silage samples, we tested their use as IS in order to check if the error in the quantification of FAs will be relevant.

Table 2. Percentage of FA (± Standard Deviation) Naturally Present on Silages, Obtained by Injecting Samples Prepared without Addition of IS ($n = 12$)

silages[a]	15:0	17:0	20:0
R	0.41 ± 0.04	0.31 ± 0.07	0.85 ± 0.03
RT	0.27 ± 0.09	0.27 ± 0.09	0.77 ± 0.10
RTV	0.32 ± 0.04	0.34 ± 0.06	0.74 ± 0.07
MP	0.09 ± 0.02	0.24 ± 0.03	0.54 ± 0.03
MD	0.07 ± 0.01	0.20 ± 0.02	0.37 ± 0.03

[a] Silages: ryegrass (R); ryegrass + triticale (RT); ryegrass + triticale + vetch (RTV); maize Pionner (MP); and maize Dekalb (MD).

Response Linearity of Each IS. The response linearity of all IS in the FID detector (area of peak vs concentration) was performed by using calibration curves. The calibration of triplicate analysis was carried out in the concentration range between 0.05 mg/mL and 1.0 mg/mL. The calibration curves were linear, having coefficients of determination ranging from 0.997 for 15:0, to 0.999 for 17:0, 19:0, 20:0 and 21:0. The residual standard deviation (RSD) for the 15:0, 17:0, 19:0, 20:0 and 21:0 FA was 8.3, 5.8, 3.3, 2.8 and 1.9, respectively. These RSD decreased with increasing of carbon chain length, which suggests that FAs with a long carbon chain length will be more precise as IS.

Correction of GLC Peak Areas. Considering that FID responds to ions generated by the combustion of the C−H components of the molecule, but does not to the C═O component, Ackman and Sipos (*19*) have proposed the use of theoretical relative FID response factors (TRF) to correct individual FAME areas. We tested the effect of correcting the IS concentration of silages, according to the amounts determined in **Table 2**, and also the effect of correcting individual GLC areas using TRF, determined by Ackman and Sipos (*19*), on the total FA composition (mg/g DM) of silages. **Table 3** shows that total FA composition is not affected by any of the corrections of the GLC areas. However, the lowest amount was determined when FID TRF was used (21.93 mg/g DM), followed by 21.98 mg/g DM when correction of the amount of IS concentration present on silages plus correction of individual FA areas with TRF (PSTRF) was used. The total FA composition of silages without any area correction (WOC) was 22.05 mg/g DM. These results suggest that the error of using GLC areas without any correction does not affect silage total FA composition.

Quantification of FAs Using Different IS. Five IS—15:0, 17:0, 19:0, 20:0 and 21:0—were tested on grass and maize silage samples. The effect of the IS in the FA composition was analyzed individually for each species and is given in **Table 4**.

In grass silages, total FA composition is not affected by the IS used, although, in maize silages, total FAME concentration did not differ when the 15:0, 17:0, 19:0 or 20:0 were used, but significantly differed when the 21:0 was used as IS.

Table 3. Effect of Corrections (WOC, PS, PSTRF, TRF)[a] on Total FA Composition (mg/g DM) of Grass ($n = 45$) and Maize ($n = 30$) Silages

	total FAME					significance[b]		
silage	WOC	PS	PSTRF	TRF	SEM	silage	corrn	silage × corrn
grass	18.30	18.37	18.23	18.18	0.5	***	ns	ns
maize	27.67	27.72	27.61	27.56	0.61			

[a] WOC: without GLC area correction. PS: correcting the internal standard concentration taking in account the amount of each FA present on silage (according to **Table 2**). PSTRF: correcting the amount of each FA present on silages and correcting all GLC area of FAs using theoretical relative FID response corrections (TRFs). TRF: correcting all GLC area of FAs using the theoretical relative FID response corrections. SEM, standard error of mean. [b] Significance: ns, $P > 0.05$. *** $P < 0.001$.

Table 4. Effects of the IS Used in the FA Composition (mg/g DM) of Grass and Maize Silages[a] ($n = 3$)

	FAME						significance[b]
	15:0	17:0	19:0	20:0	21:0	SEM	
Grass Silages							
12:0	0.09	0.07	0.08	0.08	0.08	0.01	ns
14:0	0.15	0.15	0.15	0.17	0.15	0.03	ns
16:0	3.71	3.60	3.64	3.82	3.96	0.20	ns
16:1*trans*-3	0.27	0.27	0.27	0.29	0.29	0.03	ns
16:1*cis*-9	0.08	0.07	0.07	0.08	0.08	0.01	ns
18:0	0.37	0.37	0.37	0.44	0.41	0.04	ns
18:1*cis*-9	0.53	0.51	0.54	0.56	0.62	0.08	ns
18:1*cis*-11	0.19 a	0.18 a	0.18 a	0.20 a	0.24 b	0.01	*
18:2n-6	2.88	2.84	3.11	3.02	3.17	0.16	ns
18:3n-3	8.37	8.50	8.57	9.07	9.22	0.44	ns
22:0	0.25	0.26	0.26	0.28	0.29	0.04	ns
23:0	0.06	0.05	0.06	0.08	0.07	0.01	ns
24:0	0.21	0.21	0.22	0.23	0.23	0.02	ns
26:0	0.08	0.07	0.07	0.10	0.08	0.01	ns
28:0	0.14	0.15	0.13	0.21	0.16	0.03	ns
total FAME	17.64	17.63	18.03	18.82	19.40	0.71	ns
Maize Silages							
12:0	0.04	0.04	0.05	0.04	0.07	0.01	ns
14:0	0.18	0.14	0.16	0.12	0.15	0.04	ns
16:0	4.19 a	4.13 a	4.49 a	4.65 a	5.23 b	0.18	**
16:1*trans*-3	0.05	0.05	0.05	0.06	0.06	0.01	ns
16:1*cis*-9	0.04	0.04	0.05	0.05	0.06	0.00	ns
18:0	0.47 a	0.48 a	0.52 ab	0.57 b	0.61 c	0.02	***
18:1*cis*-9	4.70	4.65	5.11	5.09	6.02	0.35	ns
18:1*cis*-11	0.26 a	0.27 a	0.27 a	0.29 a	0.37 b	0.01	***
18:2n-6	12.88	12.66	13.95	13.81	16.36	0.98	ns
18:3n-3	2.31	2.20	2.43	2.57	2.81	0.15	ns
22:0	0.01	0.10	0.11	0.12	0.12	0.01	ns
23:0	0.03	0.03	0.04	0.04	0.04	0.00	ns
24:0	0.12	0.13	0.15	0.15	0.16	0.01	ns
26:0	0.02	0.01	0.02	0.01	0.02	0.01	ns
28:0	0.00	0.01	0.00	0.01	0.02	0.01	ns
total FAME	25.60 a	25.10 a	27.63 a	27.68 a	32.36 b	1.57	*

[a] Grass silage includes ryegrass (R), ryegrass + triticale (RT) and ryegrass + triticale + vetch (RTV); maize silage includes maize Pioneer (MP) and maize DeKalb (MD). SEM, standard error of the mean. Different letters indicate differences among IS. [b] Significance: ns, $P > 0.05$. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

Moreover, the IS used only affected the quantification of 18:1*cis*-11 ($P < 0.05$) in grass silages, however, in maize silages it affected the quantification of 16:0 ($P < 0.01$), 18:0 ($P < 0.001$) and 18:1*cis*-11 ($P < 0.001$). Otherwise, FA quantification was similar ($P < 0.05$) when 15:0, 17:0, 19:0 and 20:0 were used as IS, but higher ($P < 0.05$) when 21:0 was used as IS. These results suggest that 21:0, apart from its absence on silages, is not a good choice as IS. One of the reasons for its inadequacy may be the high retention time compared with the majority of FAs present on samples. According to Grob and Biedermann (*20*), the main source of error in quantitative GLC is the injection technique, especially in vaporizing injectors, although in optimized systems this error must be regarded as a systematic error. Eder (*1*) reported that precision decreased with increasing difference of carbon chain length of the determined component and IS, which he compensates by the use of several IS. Otherwise, Schreiner (*7*) selected the 19:0 as IS for the quantification of unsaturated C20 FAMEs, and the 21:0 for quantification of unsaturated C22 FAMEs to improve both accuracy and precision of PUFA analysis. However, the use of more than one IS involves more costs and probabilities of coelutions. As IS with high carbon chain length might overestimate FAMEs and IS of short carbon chain length might underestimate FAMEs, when split vaporizing injectors are used (*7*), the 19:0 might be a good choice. Indeed, in our chromatographic conditions, it did not coelute with any other FAME, and eluted on the middle of the majority of the FAMEs, thus reducing the error of differences of volatilities between FAMEs.

The 15:0, 17:0 and 20:0 may also be useful as IS, because our results showed that, in spite of their natural occurrence, their use did not overestimate the quantification of FAs as compared to 19:0. However, its utilization might limit information about their natural concentration in silages, which might be important depending on the objective of the study.

Table 5 shows FAME composition (mg/g DM) of the silages studied using the 19:0 as IS. Nineteen FAs were identified in grass based silages (R, RT and RTV), and, as expected, the main FAME in these silages was the 18:3n-3, reaching concentrations of 7.74, 8.89, and 9.08 mg/g DM for R, RT and RTV, respectively. Moreover, the main FA on maize silages was the 18:2n-6, which reached concentrations of 11.3 and 16.6 mg/g DM for MP and MD, respectively. Variation of lipid contents and concentration of FAs can be attributed to plant variety, to stage of

Table 5. FA Composition (mg/g DM ± Standard Deviation) of Silages,[a] after SPE Cleanup, Using 19:0 as IS ($n = 3$)

FAME	R	RT	RTV	MP	MD
12:0	0.11 ± 0.02	0.05 ± 0.02	0.07 ± 0.01	0.03 ± 0.00	0.07 ± 0.01
14:0	0.27 ± 0.02	0.12 ± 0.00	0.07 ± 0.01	0.06 ± 0.02	0.27 ± 0.04
15:0	0.08 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
16:0	4.40 ± 0.08	3.31 ± 0.06	3.20 ± 0.10	4.29 ± 0.07	4.68 ± 0.02
16:1 *trans*-3	0.21 ± 0.02	0.29 ± 0.01	0.32 ± 0.02	0.06 ± 0.01	0.05 ± 0.01
16:1 *cis*-9	0.11 ± 0.01	0.05 ± 0.01	0.07 ± 0.02	0.05 ± 0.00	0.06 ± 0.00
17:0	0.05 ± 0.00	0.04 ± 0.01	0.06 ± 0.00	0.06 ± 0.00	0.07 ± 0.01
18:0	0.51 ± 0.03	0.28 ± 0.02	0.32 ± 0.01	0.50 ± 0.01	0.55 ± 0.01
18:1 *cis*-9	0.83 ± 0.02	0.38 ± 0.02	0.41 ± 0.03	4.16 ± 0.05	6.05 ± 0.05
18:1 *cis*-11	0.23 ± 0.02	0.15 ± 0.01	0.17 ± 0.02	0.27 ± 0.01	0.27 ± 0.02
18:2 n-6	3.31 ± 0.06	3.39 ± 0.97	2.62 ± 0.13	11.3 ± 0.12	16.6 ± 0.16
20:0	0.17 ± 0.00	0.12 ± 0.00	0.14 ± 0.00	0.31 ± 0.00	0.11 ± 0.00
18:3	0.05 ± 0.02	0.06 ± 0.00	0.07 ± 0.01	0.03 ± 0.00	0.04 ± 0.02
18:3 n-3	7.74 ± 0.17	8.89 ± 0.29	9.08 ± 0.28	2.39 ± 0.05	2.47 ± 0.03
22:0	0.40 ± 0.02	0.17 ± 0.00	0.21 ± 0.01	0.13 ± 0.01	0.09 ± 0.01
23:0	0.06 ± 0.00	0.08 ± 0.05	0.06 ± 0.01	0.04 ± 0.00	0.03 ± 0.00
24:0	0.31 ± 0.03	0.16 ± 0.00	0.18 ± 0.01	0.16 ± 0.01	0.13 ± 0.02
26:0	0.10 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.00 ± 0.00	0.04 ± 0.00
28:0	0.20 ± 0.06	0.09 ± 0.02	0.10 ± 0.02	0.00 ± 0.00	0.00 ± 0.00

[a] Silages of ryegrass (R); ryegrass + triticale (RT); ryegrass + triticale + vetch (RTV); maize Pioneer (MP); and maize Dekalb (MD).

growth (*21*) and in the case of silages to field manipulations prior to ensiling (*14*).

In conclusion, the DT method with the SPE cleanup step seems a good choice for the analysis of FA composition of freeze-dried maize and grass silage samples to reduce the amount of non-FAME compounds, and subsequently to produce clean samples for GLC analysis. Our results suggest that using 19:0 as IS might be the better choice, because it is not naturally present in silage samples, does not coelute with other FAs, and elutes close to the majority of the FAMEs, minimizing the effects of different volatilities on the injection system. Moreover, the correction of individual GLC areas using theoretical relative FID response factors or the correction of the natural content of the IS in the silages did not affect total FA content.

LITERATURE CITED

(1) Eder, K. Gas chromatographic analysis of fatty acid methyl esters. *J. Chromatogr. B: Biomed. Sci. Appl.* **1995**, *671*, 113–131.

(2) Lee, M. R. F.; Winters, A. L.; Scollan, N. D.; Dewhurst, R. J.; Theodorou, M. K.; Minchin, F. R. Plant-mediated lipolysis and proteolysis in red clover with different polyphenol oxidase activities. *J. Sci. Food Agric.* **2004**, *84*, 1639–1645.

(3) Kaneda, T. Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. *Microbiol. Mol. Biol. Rev.* **1991**, *55*, 288–302.

(4) Sukhija, P. S.; Palmquist, D. L. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. *J. Agric. Food Chem.* **1988**, *36*, 1202–1206.

(5) Alves, S. P.; Cabrita, A. R. J.; Fonseca, A.n.J.M.; Bessa, R. J. B. Improved method for fatty acid analysis in herbage based on direct transesterification followed by solid-phase extraction. *J. Chromatogr. A* **2008**, *1209*, 212–219.

(6) Vlaeminck, B.; Fievez, V.; Cabrita, A. R. J.; Fonseca, A. J. M.; Dewhurst, R. J. Factors affecting odd- and branched-chain fatty acids in milk: A review. *Anim. Feed Sci. Technol.* **2006**, *131*, 389–417.

(7) Schreiner, M. Quantification of long chain polyunsaturated fatty acids by gas chromatography—Evaluation of factors affecting accuracy. *J. Chromatogr. A* **2005**, *1095*, 126–130.

(8) Alves, S. P.; Bessa, R. J. B. Identification of cis-12,cis-15 octadecadienoic acid and other minor polyenoic fatty acids in ruminant fat. *Eur. J. Lipid Sci. Technol.* **2007**, *109*, 879–883.

(9) Sydenham, E. W.; Shephard, G. S.; Stockenstrom, S.; Rheeder, J. P.; Marasas, W. F. O.; vanderMerwe, M. J. Production of fumonisin B analogues and related compounds by Fusarium globosum, a newly described species from corn. *J. Agric. Food Chem.* **1997**, *45*, 4004–4010.

(10) Sydenham, E. W.; Stockenstrom, S.; Thiel, P. G.; Shephard, G. S.; Koch, K. R.; Marasas, W. F. O. Potential of Alkaline-Hydrolysis for the Removal of Fumonisins from Contaminated Corn. *J. Agric. Food Chem.* **1995**, *43*, 1198–1201.

(11) Sydenham, E. W.; Shephard, G. S.; Thiel, P. G.; Marasas, W. F. O.; Stockenstrom, S. Fumonisin Contamination of Commercial Corn-Based Human Foodstuffs. *J. Agric. Food Chem.* **1991**, *39*, 2014–2018.

(12) Sydenham, E. W.; Gelderblom, W. C. A.; Thiel, P. G.; Marasas, W. F. O. Evidence for the Natural Occurrence of Fumonisin-B1, A Mycotoxin Produced by Fusarium-Moniliforme, in Corn. *J. Agric. Food Chem.* **1990**, *38*, 285–290.

(13) Rumsey, T. S.; Noller, C. H.; Rhykerd, C. L.; Burns, J. C. Measurement of Certain Metabolic Organic Acids in Forage, Silage, and Ruminal Fluid by Gas-Liquid Chromatography. *J. Dairy Sci.* **1967**, *50*, 214–220.

(14) Dewhurst, R. J.; King, P. J. Effects of extended wilting, shading and chemical additives on the fatty acids in laboratory grass silages. *Grass Forage Sci.* **1998**, *53*, 219–224.

(15) Lee, M. R. F.; Connelly, P. L.; Tweed, J. K. S.; Dewhurst, R. J.; Merry, R. J.; Scollan, N. D. Effects of high-sugar ryegrass silage and mixtures with red clover silage on ruminant digestion. 2. Lipids. *J. Anim. Sci.* **2006**, *84*, 3061–3070.

(16) Lee, M. R. F.; Harris, L. J.; Dewhurst, R. J.; Merry, R. J.; Scollan, N. D. The effect of clover silages on long chain fatty acid rumen transformations and digestion in beef steers. *Anim. Sci.* **2003**, *76*, 491–501.

(17) Shingfield, K. J.; Salo-Väänänen, P.; Pahkala, E.; Toivonen, V.; Jaakkola, S.; Piironen, V.; Huhtanen, P. Effect of forage conservation method, concentrate level and propylene glycol on the fatty acid composition and vitamin content of cows' milk. *J. Dairy Res.* **2005**, *72*, 349–361.

(18) Vanhatalo, A.; Kuoppala, K.; Toivonen, V.; Shingfield, K. J. Effects of forage species and stage of maturity on bovine milk fatty acid composition. *Eur. J. Lipid Sci. Technol.* **2007**, *109*, 856–867.

(19) Ackman, R. G.; Sipos, J. C. Application of Specific Response Factors in Gas Chromatographic Analysis of Methyl Esters of Fatty Acids with Flame Ionization Detectors. *JAOCS* **1964**, *41*, 377–&.

(20) Grob, K.; Biedermann, M. The Two Options for Sample Evaporation in Hot GC Injectors: Thermospray and Band Formation. Optimization of Conditions and Injector Design. *Anal. Chem.* **2002**, *74*, 10–16.

(21) Hawke, J. C. Lipids. In *Chemistry and biochemistry of herbage*; Butler, G. W., Bailey, R. W., Eds.; Academic Press: London, 1973.

Received for review May 25, 2009. Revised manuscript received October 6, 2009. Accepted October 15, 2009. Individual PhD grant (SFRH/BD/37793/2007) to S.P. Alves from Fundação para a Ciência e a Tecnologia (FCT), Ministério da Ciência e Ensino Superior, Portugal, is greatly acknowledged.