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Comparative researches on two direct transmethylation without prior extraction methods for fatty acids analysis in vegetal matrix with low fat content

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Abstract

Background: The aim of our work was to compare two methods, both based on direct transmethylation with different reagents, BF_3/MeOH (boron trifluoride in methanol) or HCl/MeOH (hydrochloride acid in methanol), in acid catalysis, without prior extraction, to find the fast, non-expensive but enough precise method for 9 principal fatty acids (lauric, myristic, palmitic, stearic, oleic, linoleic, linolenic, arahidic and behenic acids) analysis in vegetal matrix with low fat content (forage from grassland), for nutrition and agrochemical studies.

Results: Comparatively, between the average values obtained for all analysed fatty acids by the two methods based on direct transmethylation without prior extraction no significantly difference was identified ($p > 0.05$). The results of fatty acids for the same forage sample were more closely to their average value, being more homogenous for BF_3/MeOH than HCl/MeOH, because of the better accuracy and repeatability of this method. Method that uses BF_3/MeOH reagent produces small amounts of interfering compounds than the method using HCl/MeOH reagent, results reflected by the better statistical parameters.

Conclusion: The fast and non-expensive BF_3/methanol method was applied with good accuracy and sensitivity for the determination of free or combined fatty acids (saturated and unsaturated) in forage matrix with low fat content from grassland. Also, the final extract obtained by this method, poorer in interfering compounds, is safer to protect the injector and column from contamination with heavy or non-volatile compounds formed by transmethylation reactions.

Background

Lipids play diverse and important roles in nutrition and health and many lipids are absolutely essential for life. For instance, humans have dietary requirements for certain essential fatty acids (e.g., linoleic acid and α-linolenic acid), because they cannot be synthesized from simple precursors in our diet [1]. For humans lipids source can be the foods with animal origin and oleaginous seeds but for herbivores the lipids source is only natural forage or concentrates. Many studies were made about the influence of nutrition types on lipids composition of foods from poultry (meat and eggs), pork (meat) and herbivores (meat and milk) [2-6]. It is widely accepted that ruminants grazing or feeding with natural forage are beneficial to produce meat and dairy foods with healthier lipid composition than those fed with concentrates [7,8]. Hence, the increased interest to obtain vegetal matrix from grasslands with higher production, both qualitative and quantitative.

Conventional techniques for the extraction of fatty acids (FAs) in animal feed require Soxhlet extraction (with large volume of organic solvent), purification, hydrolysis, and transmethylation procedures that are both lengthy and cumbersome. Attempts to bypass extraction and purification steps have met with varying degrees of success. Many reports propose different techniques that exclude most of the preparative steps and consist of a one-step procedure. In this procedure FAs

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are simultaneously extracted and methylated by using a basic or acid catalyst. Fatty acids composition of plasma, feces, bile and liver was analysed by 1 hour direct transmethylation procedure carried out in methanol-benzene 4:1 with acetyl chloride [9]. The method was applicable for analysis of both simple (triglycerides) and complex lipids (cholesteryl-esters, phospholipids; and sphingomyelin) [9,10]. In application of nutritional and epidemiological studies, where the knowledge of fatty acids status is necessary, was successfully developed a rapid micromethod by direct transmethylation (3N HCl/MeOH) in a drop of human blood [11]. Different vegetal materials like peanuts [12], herbage [13], yeast [14], *Artemia* shrimp [15] were successfully investigated for fatty acids contents by direct transmethylation in one-step procedure, using as direct transmethyltion reagents: BF_3/MeOH, 5% HCl-MeOH/Toluene, 2% H_2SO_4/MeOH and HCl-MeOH/Benzene. They lead to more complete recoveries of all classes of lipids which, during the transmethylation procedure, are free from biological specimens.

Our goal was to identify a fast and enough precise method to determine the fatty acids in forage from permanent grassland in order to monitor the changes in their status by applying different types of fertilization. Data provided by the literature recommended direct transmethylation of feed samples using acid catalysis based on HCl/MeOH or BF_3/MeOH reagents [13,16]. In a critical study done by Weston et al. [16] shows that both methods have similar qualities but encourage all to use analytical reagent HCl/MeOH mainly due to its lower cost. But due to the large amounts of by-product (esters of organic acids present in plants, compounds of decomposition of sugars), Alves et al. [13,17] introduce their separation by SPE (solid phase extraction). Analytical results obtained by using SPE separation are superior but introduce a further step in the analytical process, which makes it less productive and more expensive. In this context we have reviewed the two methods of direct transmethylation without prior extraction to see which one can exclude SPE separation, without sacrifice the quality of the results.

Results and discussion

Quantification of FAs using BF_3/MeOH vs. HCl/MeOH method

Table 1 presents principal FAs composition of the forage from the 10 trials, with different types of fertilization, processed using BF_3/MeOH and HCl/MeOH methods. Statistical treatment was made with ANOVA: two factors with replication. Fixed factor A were the two methods (BF_3/MeOH and HCl/MeOH) and factor B: fatty acids compositions.

In forage from trials with organic fertilizer (D2-D4) the sum of total fatty acids increased considerably comparatively with the control (D1), from 4549.4 to 9114.4 mg/kg DM. This increase was observed particularly in the case of unsaturated fatty acids. The most important FAs identified in forage samples were the linoleic acid (18:2n6), ranged from 2909 - 3252 mg/kg DM for trial with high dose of organic fertilizer (D4). For the same trial, palmitic acid (16:0) ranged from 2414 to 2623 mg/kg DM, linolenic acid (18:3n6) ranged from 1808 to 1988 mg/kg DM and oleic acid (18:1n9) from 852 to 971 mg/kg DM. Lauric (12:0), myristic (14:0), stearic (18:0), arahidic (20:0) and behenic (22:0) fatty acids are the minor components of the forage's lipids, ranged from 15 to 210 mg/kg DM [18]. For the forage from trials with only mineral fertilization (D8-D10) a significant decrease of total fatty acids contents was observed comparatively with the control (D1), from 4549.4 to 3245.0 mg/kg DM. Also in these cases the most obvious decrease was observed for unsaturated fatty acids. For forage from trials with mix fertilization (D5-D7) the decrease is less pronounced comparatively with the control, from 4549.4 to 4053.9 mg/kg DM. From these data we conclude that the type of fertilization has an important impact on fatty acids composition of forage matrix from grassland.

Even significant differences ($p < 0.001$) were identified between FAs composition of forage from the same permanent grassland, depending by the type of fertilization, between the two tested methods no significant variations were identified for all analysed fatty acids ($p > 0.05$).

Although no significant differences between the contents in FAs were determined by the two methods, the statistical parameters standard deviation (SDV) and relative standard deviation (RSDV) were higher for HCl/MeOH method. This can be explained by the existence of strong interferences in the case of HCl/MeOH method. The strong acid medium and the high temperature of this method may cause the appearance of a great quantity of interfering compounds, and many of them can interfere in the GC analysis [13,17]. The more accentuated presence of interfering compounds in final extract of HCl/MeOH method can be observed also visually. The colour of hexane extracts of HCl/MeOH method are more dark (green-brown) than the hexane extracts obtained by BF_3 method (see Additional file 1). Alves et al [13,18] improved this direct transmethylation method (HCl/MeOH) using SPE step for removing the major interfering compounds. They also identified the most important interfering compounds by this GCMS analysis. These compounds are formed mainly from phytadienes (the presence of fragment ions at m/z 81, 95 and 123 in mass spectrum of the chromatographic peak), methyl levulinate and different methyl esters of non-volatile organic acids (oxalic, succinic, malonic and quinic acids) [13,17]. Phytadienes compounds may be

Table 1 Fatty acids composition of the forage from the 10 trials with different fertilization, using BF_3/MeOH and HCl/MeOH methods (average contents of 3 different samples for each trial; significance: ns = p > 0.05, *** = p < 0.001)

	Fatty acids, mg kg $^{-1}$										Significance	
FA/Fertilized trials	**Lauric (12:0)**	**Myristic (14:0)**	**Palmitic (16:0)**	**Stearic (18:0)**	**Oleic (18:1n9)**	**Linoleic (18:2n6)**	**Linolenic (18:3n3)**	**Arahidic (20:0)**	**Behenic (22:0)**	**FAs Sum**	**A (methods)**	**B (FAs)**
BF_3/MeOH method												
D1	33.4	73.7	1644.4	237.1	465.8	1167.9	665.7	132.2	129.2	4549.4	ns	***
D2	19.1	55.2	1876.3	239.9	564.7	1712.3	1605.9	127.0	103.9	6304.3	ns	***
D3	23.3	79.0	2257.6	424.1	736.8	2681.0	1546.9	169.4	154.6	8072.7	ns	***
D4	18.6	65.0	2413.7	509.8	851.8	2909.5	1988.1	207.5	150.5	9114.4	ns	***
D5	15.6	47.8	1729.9	295.8	565.0	1929.3	757.0	202.1	122.2	5664.7	ns	***
D6	15.3	51.4	1764.1	282.9	513.5	1471.4	749.4	125.1	76.3	5049.5	ns	***
D7	24.0	59.2	1474.4	200.3	423.2	1027.3	615.4	124.4	105.8	4053.9	ns	***
D8	18.4	55.9	1535.4	189.4	441.7	1069.6	608.2	106.8	96.0	4121.4	ns	***
D9	20.6	52.8	1531.5	179.0	352.2	721.6	509.7	93.2	77.5	3538.1	ns	***
D10	18.4	46.2	1378.7	156.4	298.4	673.4	475.0	104.2	94.2	3245.0	ns	***
Average SDV	1.9	3.2	61.4	14.7	15.0	10.7	7.9	6.6	13.3	131.4		
Average RSDV %	25.5	9.9	4.3	7.8	6.2	1.9	1.4	7.4	12.2	4.0		
HCl/MeOH method												
D1	14.2	43.1	1642.0	236.7	577.5	1349.6	523.8	142.0	70.5	4599.4	ns	***
D2	8.8	17.6	1805.7	232.3	620.9	1673.7	1310.5	115.1	77.1	5861.6	ns	***
D3	2.8	13.5	2208.5	518.3	974.6	2781.7	1223.9	131.0	156.3	8010.7	ns	***
D4	4.8	44.2	2622.6	542.2	970.9	3252.0	1808.1	230.5	238.9	9714.2	ns	***
D5	9.6	12.4	1876.8	341.9	782.5	2175.4	728.3	252.8	160.1	6339.9	ns	***
D6	4.1	26.9	1731.4	271.3	534.3	1388.7	612.0	136.7	118.1	4823.6	ns	***
D7	5.4	24.1	1449.8	178.2	438.1	1146.9	489.1	108.5	105.9	3946.0	ns	***
D8	12.3	42.5	1674.6	223.7	499.4	1241.3	536.2	124.6	135.9	4490.6	ns	***
D9	15.1	38.8	1401.4	188.6	328.8	721.2	423.6	67.9	108.9	3294.3	ns	***
D10	8.2	49.3	1467.6	186.9	444.7	805.2	402.6	100.7	119.1	3584.4	ns	***
Average SDV	4.9	4.2	218.1	72.2	189.1	115.9	68.6	6.7	13.5	669.4		
Average RSDV%	130.6	18.0	13.2	29.5	54.3	17.9	11.6	7.7	13.9	18.1		

derived from phytol, a degradation product of chlorophyll phytyl chain [19], methyl levulinate and other methyl esters are formed by hydrolysis of fructose, respectively other sugars present in herbages, in acid-catalysed reactions [20,21]. To evaluate the losses during the two tested methods, recovering test was employed by spiking 6 HRMs (homemade reference materials) samples with the same concentration (1 mg/mL) of margaric fatty acids (C17:0) standard. GCMS analysis of extracts obtained from these HRMs, by the two methods, highlights the lower contents of such interfering compounds in the extracts obtained by BF_3/MeOH method (Figures 1 and 2). Partial overlapping chromatograms of several organic acids and phytadienes are presented in Figure 2.

All interfering compounds were identified by analysis of GCMS chromatograms using equipment's MS library (NIST) and literature information about mass spectrum [13]. Quantitative analysis of interfering compounds were made using margaric (C17:0) fatty acid as internal standard.

Recovering results

Table 2 shows the recovering results of the 6 HRMs, 3 samples (HRMs 1-3) assessed by margaric fatty acid content by BF_3/MeOH method and 3 samples (HRMs 4-6) by HCl/MeOH method.

The average recovery for HRMs spiked at concentration level of 1.0 mg/mL was 95.75% for BF_3/MeOH method and 87.53% for HCl/MeOH method. SDV and RSDV for two methods were 0.028, 2.98%, and respectively 0.059, 6.80%. The recovery percentage and statistical parameters for method BF_3/MeOH were better than the same parameters for HCl/MeOH method. These differences can be associated with interfering substances that are higher in case of HCl/MeOH method.

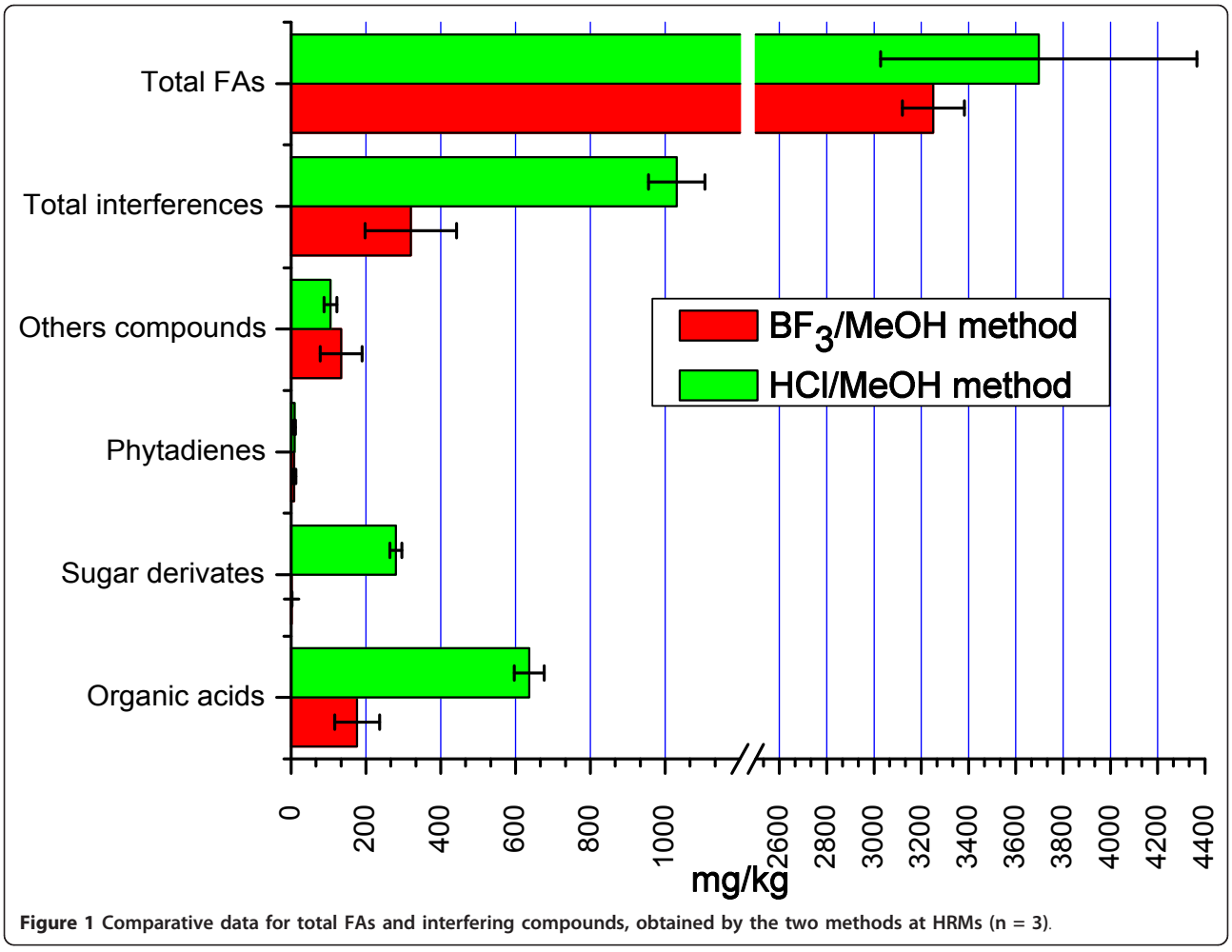

Figure 1 Comparative data for total FAs and interfering compounds, obtained by the two methods at HRMs (n = 3).

The repeatability of the two methods was determined by the coefficient of variation (CV %) of three consecutive analyses. This variation was around 0.1% for BF_3/MeOH method and around 0.4% for HCl/MeOH method, indicating that both assayed methods provide a good accuracy and repeatability range.

Experimental

Experimental site

The experimental site (permanent grassland) was situated near Gradinari village (45.151 °N/21.538 °E) altitude 200 m, a hill area in Banat County, Romania. The agrochemical experiment begins in 2003, when permanent grassland was divided in ten trials with five replications for each of them: D1-unfertilized trial; D2, D3, D4 - fermented sheep manure (20 to 60 t/ha), D5, D6, D7 - organic and mineral fertilizers (20 t/ha fermented sheep manure and different combination of 50 kg/ha of P_2O_5, K_2O, N); D8, D9, D10 - only mineral fertilizers (constant doses of 50 kg/ha P_2O_5 + 50 kg/ha K_2O and different N doses: 100, 150, and respectively 100 + 100 kg/ha). The mineral fertilizers were applied yearly, while the fermented sheep manure at each two years in late winter. The trials were arranged in randomized plots, in multiple stage blocks. The soil of permanent grassland was Calcic Luvisol. The annual average temperature in this region was around 10.4°C.

Samples collection and preparation

In plants fatty acids can be found in scarce amounts in free form, but generally they are combined in more complex molecules through ester bonds. The analysis of total fatty acids from biological materials is a complex task and precautions should be taken at all times to prevent or minimize the effects of degradation/oxidation by long time and high temperature manipulation of samples. Fresh samples of forage were collected in middle of June from permanent grassland of the BUASVM (Banat's University of Agricultural Sciences and Veterinary Medicine from Timisoara) experimental field, Gradinari village. For laboratory samples, herbages from 1 m^2 were cut 5 cm above soil, kept in plastic bags at 4°C and rapidly taken to the laboratory and dried at 70°C

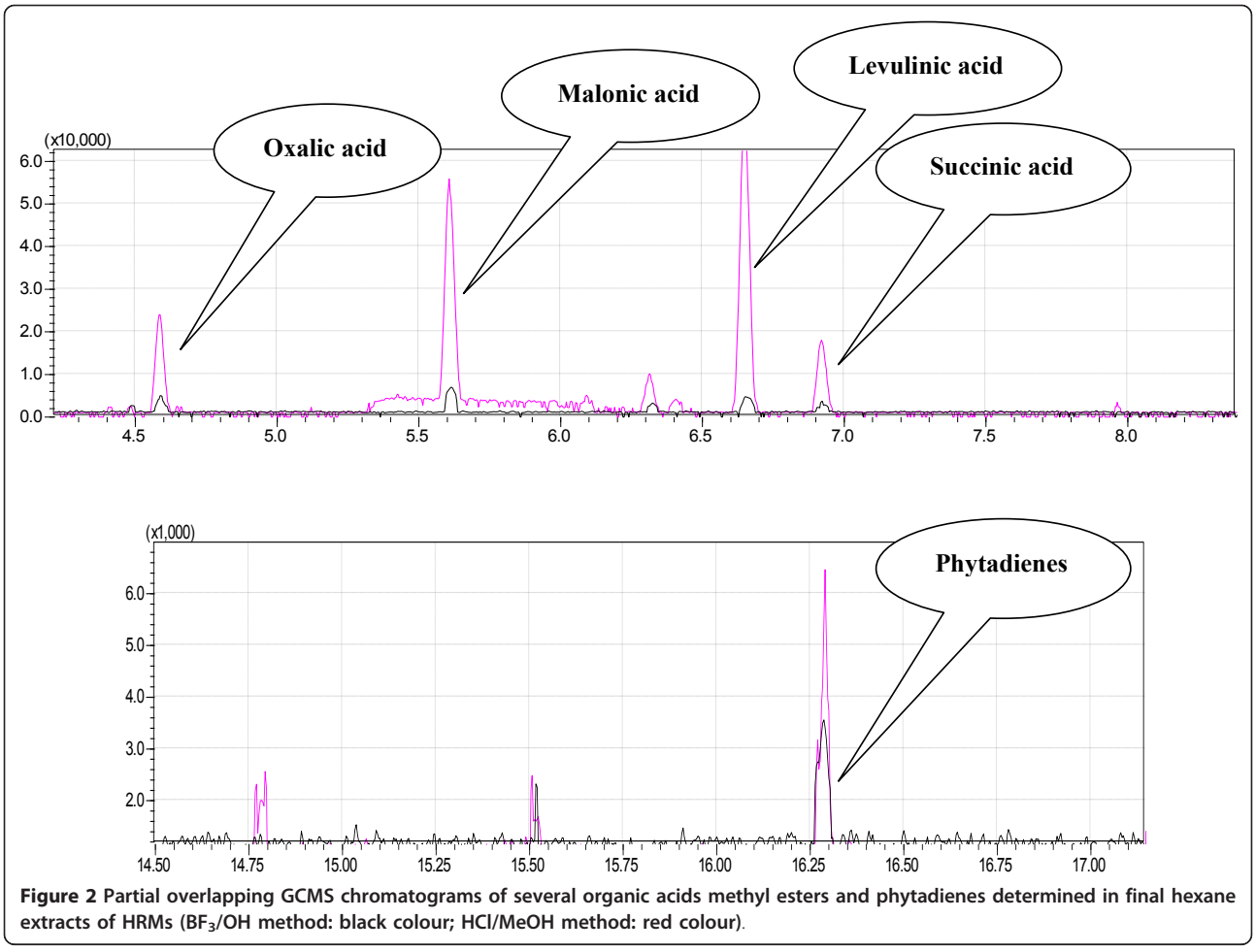

Figure 2 Partial overlapping GCMS chromatograms of several organic acids methyl esters and phytadienes determined in final hexane extracts of HRMs (BF_3/OH method: black colour; HCl/MeOH method: red colour).

Table 2 Recovery results of margaric fatty acid standard (C17:0) added to HRMs processed with direct transmethylation by the two methods (n = 3)

HRMs samples	Recovery (mg)	Recovery (%)
	BF_3/MeOH method	
HRM1	0.989	98.94
HRM 2	0.949	94.88
HRM 3	0.934	93.42
Average	**0.968**	**95.75**
SDV	0.028	2.86
RSDV %		2.98
CV %		0.083
	HCl/MeOH method	
HRM 4	0.809	80.93
HRM 5	0.924	92.36
HRM 6	0.893	89.30
Average	**0.875**	**87.53**
SDV	0.059	5.91
RSDV %		6.80
CV %		0.404

for short time (10 min) for stopped the enzymatic activity and after that, samples were dried at 45°C in ventilated oven (to constant mass, around 24 h), ground to pass a 0.5 mm screen and kept in dark and dry atmosphere at room temperature until analysis. 30 forage samples from 10 agrochemical experimental trials with different type of fertilization were analysed by the two BF_3/MeOH and HCl/MeOH methods for common 9 fatty acids (6 saturated and 3 unsaturated), predominantly found in forage [18]. The floristic matrix of forage (gravimetrically) consist in: *Festuca rupicola* (16 - 52%), *Calamagrostis epigejos* (5 - 13%), *Poa pratensis* (around 5%), *Alopecurus pratensis* (under 2%), *Trifolium repens* (7 - 38%), *Trifolium medium* (under 3%), *Lathyrus pratensis* (under 6%), *Medicago falcata* (under 2%), *Rosa canina* (7 - 18%), *Filipendula vulgaris* (3 - 9%), *Inula britanica* (under 5%), *Galium verum* (under 7%) and *Plantago lanceolata* (under 3%). The grasses were dominant in trials fertilized with mineral nitrogen, while the leguminous prevail in forage from trials fertilized with fermented sheep manure.

Methods

BF_3/MeOH method

The working method was adapted after Weston et al. [16]. 50 μL of internal standard (C17:0, 20 mg/mL) and 2.5 mL of 20% boron trifluoride-methanol reagent were added to weighed amounts of sample (0.3-0.5 grams dry matter) in a 20 mL centrifuge tube provided with a Teflon-lined screw cap under the nitrogen. The tube was closed, heated at 70°C for 30 min (ultrasound bad), then cooled and 1 mL of 10% NaCl aqueous solution were added. FAMEs were extracted with 2 mL of hexane, and 1 g of both Na_2SO_4 and activated carbon were added. Finally, samples were centrifuged for 5 min at 2500 rpm. 1.5 mL of the supernatant was transferred to auto sampler vial for GCMS analysis.

HCl/MeOH method

The working method was adapted after Alves et al. [13]: 50 μL of internal standard (C17:0, 20 mg/mL) 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 ultrasound water bath. After that, the solution was placed in a cool place 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 both Na_2SO_4 and activated carbon were added. Finally, samples were centrifuged for 5 min at 2500 rpm. 1.5 mL of the supernatant was transferred to auto sampler vial for GCMS analysis.

GCMS quantification method

The device used was GCMS QP 2010 (Shimadzu). This GCMS system equipped with a split/split less injector (set at 10:1) was used to analyse the derivatives of fatty acids. Separations were achieved using a fused silica Zebron ZB-FFAP capillary column (60 m × 0.25 mm ID, 0.25 μm film thickness). Helium was used as the carrier gas at flow rates of 1.99 mL/min. Temperature of injector was held constant at 250°C. The oven temperature was programmed as following: 140°C initially hold 10 min, and then increased to 250°C at 7°C/min, with the final hold for 10 min, with 35.71 min total time of GCMS analysis. LabSolution software was used to control the operation of GCMS, obtain the chromatograms, and perform data calculations.

MS parameters: ion source temperature: 210°C; interface temperature: 255°C; solvent cut time: 3 min; ionization mode: SEI; acquisition mode: scan; event time: 0.20; scan speed: 2500; start m/z: 40; end m/z: 500.

Quantification of FAs was made by external standard method. For the calibration assays, linear regression analysis was conducted by plotting response area vs. concentration. Three replicates were made to obtain relative standard deviations (RSD, ranged from 4 to 13%), slope and coefficient of determination (R^2, ranged from 0.9869 to 0.9931).

Chemicals, reagents, and materials

All reagents and solvents were analytical and chromatographic grade, and were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Hohenbrunn, Germany). FAMEs standard mixture (C12 - C22) was prepared from single standard purchased from Grace (USA) and Supelco Inc. (Bellefonte, PA, USA).

Conclusions

The two methods (BF_3/MeOH and HCl/MeOH) for fatty acids analysis of forage with complex matrix and low lipid content from grassland, based on direct transmethylation, without prior extraction, give the same qualitative and quantitative results. Both methods produced considerable amount of interfering compounds, but smaller in BF_3/MeOH method case. The protection of injector and column against the contamination is better when the quantities of interfering compounds in the final hexane extract are smaller. Also the statistical parameters of BF_3/MeOH method are superior to HCl/MeOH method. This simple, non-expensive and fast method, using small amounts of samples and small amounts of environmentally unfriendly reagents (BF_3/methanol as derivatization reagent and hexane as FAMEs extractor reagent) was applied with good accuracy and sensitivity for the determination of free or combined fatty acids (C12-C22, saturated and unsaturated) in complex forage matrix from grassland. Future studies are needed to elucidate whether the method requires the introduction of an additional SPE clean-up step, like in HCl/MeOH method case.

Additional material

Additional file 1: Supplementary figure. Figure of vial colours.

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Authors' contributions

MH study paper design develops the forage sampling and the comparative researches, analyses and performs the statistical interpretation of results.

Competing interests
The author declares that they have no competing interests.

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