Journal of Chromatography A, 1218 (2011) 316–323

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Direct analysis of fatty acid profile from milk by thermochemolysis–gas chromatography–mass spectrometry

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ARTICLE INFO

Article history:
Received 18 September 2010
Received in revised form 31 October 2010
Accepted 8 November 2010
Available online 16 November 2010

Keywords:
Milk
Fatty acid composition
Thermochemolysis
Trimethylsulfonium hydroxide
Thermodesorption
GC–MS

ABSTRACT

The fatty acid composition of milk is of considerable interest due to their nutritional and functional properties. Although rapid milk fat separation and transesterification procedures have been developed, the overall procedure remains time consuming, specially, for the analysis of a large number of samples. In this work, a fast and simple method for direct profiling of fatty acids from milk using thermochemolysis has been developed. This method has the capability of directly analyse fatty acids from one drop of milk without fat extraction or cleanup. Our approach for thermochemolysis is based on thermal desorption integrated with a cold trap inlet. The optimized method does not present isomerisation/degradation of polyunsaturated fatty acid and shows milk fatty acid profiles comparable to the conventional method based on fat extraction and alkaline transesterification. Overall, this method has demonstrated significant potential for high throughput analysis of fatty acids in milk.

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1. Introduction

Milk fat confers taste, smell and texture to dairy products [1] and has been associated with positive or negative factors that affect the health of consumers [2]. For these reasons the ability to modulate the milk fatty acid composition has been claimed as a feasible way to transform milk into a high-value product [3]. The modulation of fatty acid composition is achievable on-farm since in ruminant milk the fatty acid composition is related to intrinsic factors (such as animal species, breed, genotype, pregnancy and stage-of-lactation) and extrinsic (environmental) factors [4]. The presence of genetically linked components in fatty acid composition has been considered as a strategy to enhance the modulation of fatty acid composition on-farm and has motivated phenotypic investigations of dairy cows based on fatty acid composition [3,4]. Technologies that allow identification of special individuals with traits associated with the production of premium milk (i.e. rich in high-value components such as conjugate linoleic acid 'CLA') have become increasingly important [5]. Phenotypic investigations involve the analysis of large number of samples [5], thus an ideal technology would have high throughput; relatively low cost per analysis; be easily automated; be robust; produce highly informative data; and allow detection of unknown traits without bias. Gas chromatographic (GC) analysis is a robust and well established technology that meets most of these requirements, however, procedures for GC analysis of milk fatty acid profiles remain time consuming [6–11], specially for large number of samples such as phenotypic investigations [5].

Over recent years, the use of thermochemolysis has gained importance in analytical chemistry because it provides high sensitivity using a minimal amounts of sample and has been successfully applied for characterization of different materials [12–16]. Thermochemolysis is a thermally assisted derivatisation [16,17] of polar groups using methyl donors such as Tetramethylammonium hydroxide (TMAH) [18], Trimethylsulfonium hydroxide (TMSH) [19], Tetramethylammonium acetate (TMAAc) [20], and Trimethylphenylammonium hydroxide (TMPAH) [21].

Thermochemolysis has been implemented using pyrolysis devices such as the pyrolysis microfurnace [22], Curie-point [23], heated filament [24,25], by direct thermal desorption interface [26], programmed temperature vaporization injection [27], and also off line [28,29]. However, limitations have been pointed out during the analysis of lipids. The strong alkalinity of methyl donors causes undesirable isomerisation and/or degradation of polyunsaturated fatty acid (PUFA) components. Different strategies have been adopted for the detection of PUFA components without appreciable side reactions by replacing strongly alkaline conditions by lower reagents concentration or using other reagents such as TMAAc and TMSH [21,30,31].

This work presents a new analytical methodology for the rapid characterization of milk fat without the need for fat extraction and uses direct transesterification by thermochemolysis

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doi:10.1016/j.chroma.2010.11.011

Table 1
Linearity of 17 triglycerides commercially available and evaluated under optimized conditions for milk thermochemolysis.

FAME	Ions (m/z) used	Range ($\mu g\,mL^{-1}$)	Equation $n=2$	r^2	RSD (%) $n=4$
6:0	74	19–600	$y=9588.6x+109{,}718$	0.9854	5.162
8:0	74	19–600	$y=21{,}532x-148{,}633$	0.9900	5.78
10:0	74	19–600	$y=28{,}275x-531{,}30$	0.9904	6.30
12:0	74	19–600	$y=30{,}874x-200{,}373$	0.9908	6.15
14:0	74	19–1000	$y=25{,}003x+110{,}425$	0.9899	6.48
16:0	74	19–2000	$y=20{,}104x+1E+06$	0.9851	7.13
16:1	55	19–600	$y=35{,}667x-309{,}188$	0.9893	6.15
18:0	74	19–1000	$y=23{,}359x-33{,}358$	0.9846	8.13
18:1	55	19–1000	$y=29{,}705x+521{,}320$	0.9878	6.27
18:2	67	19–600	$y=16{,}296x-238{,}137$	0.9901	6.13
18:3	79	19–600	$y=27{,}164x-350{,}261$	0.9927	12.30
18:2 (9*c*,11*t*)	67	19–600	$y=31{,}459x-642{,}043$	0.9847	6.41
18:2 (10*t*,12*c*)	67	19–600	$y=31{,}466x-562{,}969$	0.9890	6.78
20:4	79	19–600	$y=26{,}908x-390{,}584$	0.9983	16.96
20:5*n*−3	79	19–600	$y=27{,}378x-486{,}717$	0.9961	20.34
22:6*n*−3	79	19–600	$y=25{,}254x-753{,}793$	0.9907	16.69

Determination coefficients (r^2) of calibration curve and relative standard deviation (RSD). n is the number of replicates.

within a thermodesorption–gas chromatography/mass spectrometry (TDS–GC–MS) platform. Thermochemolysis for qualitative milk fat determination was primarily applied on milk fat extract [32] but not for analysis of milk. We propose thermochemolysis for direct analysis of milk using thermal desorption integrated with a cold trap inlet aiming to: reduce the size of the milk sample needed for analysis (~1 μL); facilitate faster chromatographic analysis and obtain better peak shapes [33] by pre-focussing analytes into a cryo trap prior injection into the column; avoid disposal issues by using only the sample required for injection into GC; protect analytical column integrity by trapping low-volatile compounds in a replaceable inlet liner and avoid the transference of the thermochemolysis reagent to the analytical column.

Thus the proposed method can allow the reduction of the cost per analysis (in terms of time of analysis, use of chemicals and long term integrity of the system), be easily automated (extraction of the fat is not required) and suitable for GC high throughput fatty acid profiling of milk.

2. Materials and methods

2.1. Materials

Tetramethyl ammonium acetate (TMAAc), Trimethylphenylammonium hydroxide solution 0.5 M in methanol (TMPAH), Tetramethylammonium hydroxide solution 25 M in methanol (TMAH), trimethylsulfonium hydroxide solution 0.25 M (TMSH) and adsorbent 10% SP™ 2330 on 100/120 Chromosorb® were obtained from Aldrich (Sigma–Aldrich, New South Wales, Australia). Most of the standards (Table 1) used in the method optimization were purchased from Sigma–Aldrich Chemie (Sigma–Aldrich, New South Wales, Australia), except the triarachidonin (5,8,11,14-all cis), 10*t*, 12*c*-octadecanoyl triglyceride (CLA 10*t*, 12*c*), 9*c*, 11*t*-octadecanoyl triglyceride (CLA 9*c*, 11*t*), trieicosapentaenoin (5, 8, 11, 14, 17 all cis) triglyceride and tridocosahexanoin (4, 7, 10, 13, 16, 19 all cis) triglyceride standards, which were purchased from Larodan Fine Chemicals (Malmö, Sweden). Standard FAME 37 mixture and *cis/trans* mixture were obtained from Restek (Shimadzu –Auckland, New Zealand). The stock solution used to evaluate the derivatisation efficiencies were prepared in dichloromethane (tricaprylin, tributyrin, tripalmitolein, triolein, triarachidonin, trieicosapentaenoin, tridocosahexanoin), chloroform (tricaprin, trilaurin, trimyristin, tripalmitin, and tristearin), and hexane (trilinolein, CLA 10*t*, 12*c* and CLA 9*c*, 11*t* triglycerides). Stock solutions of the mixture of various components were prepared in dichloromethane. Milk samples were collected from the Ruakura No. 1 Dairy farm located at the Ruakura Research Centre, Hamilton, NZ in April 2009 and homogenized milk from local commercial supplier.

2.2. Instrumentation

The thermochemolysis reaction was performed with a Gerstel automated thermal desorption system (TDS) with programmable cooled injection system (CIS) coupled to a Shimadzu QP2010 GC–MS equipped with a fused-silica RT-2330 capillary column (10 m × 0.18 nm I.D., 0.1 μm film thickness; Restek) except, in the investigation of the reagents stability (see Section 2.6) and in the analysis of homogenized milk samples. In these cases, a fused-silica RT-2330 capillary column (20 m × 0.18 nm I.D., 0.1 μm film thickness; Restek) was used. The TDS platform was used as the injector in the splitless/split mode. The temperature of the TDS was 30 °C during the insertion of the TDS tube, and increased to 250 °C at rate of 720 °C/min and held at 250 °C for 0.40 min and then increased to 255 °C/min held for 0.40 min in the splitless mode. Analytes were cold trapped (−30 °C) in the CIS inlet packed with approximately 6 mg of 10% SP™ 2330 on 100/120 Chromosorb®. The CIS was ramped to 250 °C at 12 °C/s with hold of 2 min in a split mode (50:1). The analytes were transferred to the column of GC–MS system. The Mass spectrometry detection system was operating at 70 eV full scan m/z 40 to 400 a.m.u. Oven temperature programming was 50 °C isotherm for 1 min, increased to 175 at 50 °C/min, then increased to 195 °C at 8 °C/min and then to 250 °C at 150 °C/min and isotherm for 0.5 min (total run time 6.9 min). When using fused-silica RT-2330 capillary column (20 m × 0.18 nm I.D., 0.1 μm film thickness; Restek), oven temperature programming was 50 °C isotherm for 1 min, increased to 175 °C at 50 °C/min, then increased to 195 °C at 8 °C/min and then to 250 °C at 150 °C/min and held isothermally for 4 min (total run time 10.37 min). The carrier gas (He) flow was maintained at a constant velocity of 74 cm/s.

2.3. Method optimization

The derivatisation and TDS–GC–MS analysis were optimized using a representative set of test compounds including food standard mixture, methyl ester of CLA, triglycerides (long and short chain, saturated, monounsaturated and polyunsaturated fatty acids) and milk samples. Several parameters were optimized, i.e.: GC–MS parameters to achieve a rapid run with reduced effect on peak resolution; methylation reagent (TMAH, TMSH, TMAAc, TMPAH), reagent concentration (TMAH 10, 5, 2, 0.25, 0.1 M; TMSH 5, 0.25 and 0.05 M); reagent volume (1 μL and 2 μL); temperature

Table 2
Selected FAMEs mean relative abundance (RA) identified in milk using thermochemolysis and conventional derivatisation techniques. For each method, relative standard deviation (RSD) is shown.

FAME	Ions (m/z) used	Thermochemolysis (n = 6)		Conventional derivatisation (n = 4)	
		RA (%)	RSD (%)	RA (%)	RSD (%)
4:0[a]	74	1.42 ± 0.14	10.06	1.92 ± 0.18	9.17
6:0	74	2.40 ± 0.06	2.64	2.23 ± 0.20	9.02
8:0	74	1.78 ± 0.04	2.03	1.61 ± 0.12	7.70
10:0	74	4.00 ± 0.07	1.85	3.93 ± 0.19	5.00
12:0	74	4.90 ± 0.07	1.43	4.77 ± 0.14	3.04
14:0	74	17.09 ± 0.12	0.75	16.60 ± 0.12	0.70
14:1	55	1.06 ± 0.03	2.69	1.20 ± 0.26	2.22
15:0	74	1.71 ± 0.02	0.94	1.74 ± 0.13	0.75
16:0	74	42.28 ± 0.33	0.79	41.09 ± 0.67	1.67
16:1	55	1.28 ± 0.01	0.78	1.98 ± 0.03	1.46
17:0	74	0.63 ± 0.02	2.61	0.70 ± 0.01	2.12
18:0	74	8.93 ± 0.17	1.99	9.52 ± 0.24	2.57
18:1	55	10.22 ± 0.19	1.84	10.33 ± 0.18	1.71
18:2	67	0.81 ± 0.06	7.06	0.79 ± 0.02	2.69
18:3	79	0.33 ± 0.01	4.18	0.36 ± 0.01	0.49
18:2 (9*c*,11*t*)	67	0.87 ± 0.03	3.82	0.98 ± 0.04	3.97
20:3	79	0.03 ± 0.004	13.02	0.03 ± 0.003	19.09
20:4	79	0.03 ± 0.002	14.27	0.03 ± 0.001	17.42
20:5	79	0.03 ± 0.002	9.10	0.04 ± 0.003	7.44
22:5	79	0.05 ± 0.004	12.96	0.07 ± 0.007	11.19

[a] C4:0 is significantly different at 95% of confidence using Anova. n is the number of replicates.

at TDS platform (250, 270, 290, 310, 330 and 350 °C), and sorbent used in the CIS (Tenax TA, glass wool and 10% SP™ 2330 on 100/120 Chromosorb®).

2.4. Transesterification – thermochemolysis

Standard solution: an aliquot of 1 μL of the standard solution was added into the bottom of TDS micro vial using a pipette (1–10 μL) followed by addition of 2 μL (via 5 μL Hamilton syringe) of TMSH (0.5 M in methanol). A thorough contact between reagent and sample must occur in this procedure. Milk: raw milk samples were warmed to 37 °C to disperse any cream and then 0.125 mL of milk was diluted with 0.875 mL of water. From this solution, kept at 37 °C, 1 μL was added into the TDS micro vial, followed by 2 μL of TMSH (0.5 M in methanol). In the analysis of standards, the reagent was added to the solution just before the start of the analysis in the TDS–GC–MS to avoid isomerisation and/or degradation of polyunsaturated acids. For the analysis of milk, the samples were prepared consecutively, placed in the auto sampling system and kept at 15 °C until the start of the analysis in the TDS–GC–MS where the thermochemolysis reaction took place.

2.5. Analytical procedure for linearity and repeatability evaluations

The linearity of the method was evaluated using triglyceride standards with concentrations ranging from 19 to 2000 μg/mL in dichloromethane, obtained by dilution from a stock solution of the triglyceride mixtures (Table 1). All standards were freshly prepared on the day of use, transferred to 2 mL vials and stored at 4 °C in darkness. The linearity was evaluated from calibration curves obtained by linear least-squares regression analysis of the peak area versus analyte concentration using six concentration levels in duplicate. The repeatability was assessed as the relative standard deviation (RSD) of 4 replicates of standard mixture at level of 300 mg/L for each compound. In all the cases where the standard mixtures were used, the TMSH solution (0.5 M) was added just before the start of the analysis in the TDS–GC–MS to avoid isomerisation and/or degradation of polyunsaturated acids. In the case of milk samples, the repeatability of the thermochemolysis derivatisation was assessed by performing six thermochemolysis procedures using the same solution of raw milk (Table 2). In this case, the TMSH solution (0.5 M) was added to all replicates consecutively, and then these mixtures were placed in the auto sampling system and kept at 15 °C until the start of the analysis in the TDS–GC–MS. The same procedure was used to analyse homogenized milk (Table 3).

2.6. Sample stability

The raw milk samples, prepared with TMSH (0.5 M) as described in Section 2.4, were kept in the auto sampling system at 15 °C and analysed at 0, 15, 24, 40 and 48 h after preparation.

2.7. Transesterification – conventional method

The fat separation was carried out according to the Röse–Gottlieb procedure [34]. Preparation of the fatty acid methyl esters (FAMEs): 30 mg of fat was dissolved in 2 mL of hexane followed by addition of 20 μL of 2 M potassium hydroxide in methanol and then vortexed for 30 s. The resulting mixture was neutralized with 25 μL of 2 M hydrochloric acid and then centrifuged at 3000 rpm for 2 min. The upper hexane layer was analysed by GC–MS. The repeatability of the conventional derivatisation was assessed by performing 4 derivatisation procedures using the same milk fat extract (Table 2).

2.8. Statistical

Statistics were carried out using the R version 2.6.0 [35]. Statistical significance between the two obtained datasets (i.e. Thermochemolysis and conventional derivatisation of milk samples) was determined using a two-side Student's t-test or Anova (Table 4).

3. Results and discussion

3.1. Method optimization

Four different reagents were investigated for thermally assisted methylation of milk samples, i.e. TMPAH, TMAAc, TMAH and TMSH. TMAAc has been described as soft reagent for thermochemolys

Table 3
Selected FAMEs total peak area identified in homogenized milk using thermochemolysis techniques with their respective standard deviation (RSD).

FAME	Ions (m/z) used	Thermochemolisys reaction triplicate – homogenized milk Peak area			
		Replicate 1	Replicate 2	Replicate 3	RSD (%)
4:0	74	74,080	76,069	65,135	8.11
6:0	74	696,117	728,343	743,765	3.36
8:0	74	516,182	527,037	545,023	2.75
10:0	74	1,069,384	1,115,214	1,164,655	4.27
12:0	74	1,312,668	1,355,791	1,398,039	3.15
14:0	74	3,351,235	3,532,471	3,603,514	3.72
14:1	55	226,694	201,006	201,242	7.04
15:0	74	351,128	380,346	379,051	4.45
16:0	74	7,312,051	7,848,518	7,879,631	4.15
16:1	55	170,981	173,261	173,658	0.84
17:0	74	135,360	152,155	144,712	5.84
18:0	74	1,860,383	2,050,316	1,998,710	4.98
18:1 (11t)	55	261,242	262,517	261,938	0.24
18:1 (9c)	55	1,385,607	1,395,972	1,394,487	0.40
18:2	67	135,120	125,146	124,777	4.57
18:3	79	102,706	101,448	105,806	2.17
18:2 (9c,11t)	67	152,291	152,586	148,112	1.66
20:4	79	28,344	23,541	27,354	9.60
20:5	79	13,131	10,635	12,582	10.83
22:5	79	15,806	15,310	15,131	2.27

analysis and has been indicated for selective derivatisation of free fatty acids [20], therefore low milk FAMEs yield was observed as expected, since most of fatty acids in the milk are esterified as triglycerides. The TMAH at 0.5 M and the TMPAH at 0.5 M showed similar results, but with the advantage that the peaks due to TMPAH related products, which co-eluted with the milk FAMEs, were absent for TMAH. Therefore, different concentrations of TMAH (10, 5, 2, 0.6, 0.55, 0.4, 0.25 and 0.1 M) were investigated and the best results were achieved with TMAH at 0.55 M. In this case, it was observed that low concentrations of products from CLA isomerisation, good repeatability, and high stability for most compounds, including CLA, up to 24 h after sample preparation (milk plus reagent at 15 °C) occurred. However, the polyunsaturated fatty acids C20:3, C20:4, C20:5 and C22:5 were not detected (Fig. 1).

The reagent TMSH at 0.25 M resulted in low transmethylation for milk samples. The concentration of TMSH was then increased to ca. 0.5 M resulting in good yields for every fatty acid component, including those of polyunsaturated fatty acids analysed at 250 °C, and were comparable to the conventional method (Fig. 2). In contrast, the standard mixture of polyunsaturated triglycerides showed isomerisation and/or degradation of polyunsaturated fatty acid after 5 min of sample preparation (standard mixture plus 0.05 M TMSH solution), while polyunsaturated fatty acids from milk appeared to be stable up to 24 h after addition of TMSH solution (Fig. 3). This result might be attributed to the milk fat globule membrane or other milk component protecting the polyunsaturated fatty acid derivatives from decomposition and/or isomerisation.

The analysis of milk without dilution was outside the linear detection range of the MS detector. Therefore, milk samples were diluted with water prior to derivatisation to adjust concentrations to within the linear detection range of MS detector. Problems caused by water were not observed. Indeed, the thermochemolysis

Table 4
Comparison of relative abundance between the thermochemolysis (THM) and conventional (CON) derivatisation techniques, for three raw milk samples, in terms of statistical significance (SS).

	Milk 446			Milk 502			Milk 512		
	CON($n=3$)	THM ($n=2$)	SS[b]	CON($n=3$)	THM($n=3$)	SS[b]	CON($n=3$)	THM($n=3$)	SS[b]
4:0[a]	2.374 ± 0.353	1.432 ± 0.264	*	2.391 ± 0.211	1.362 ± 0.098	**	2.222 ± 0.126	1.11 ± 0.172	**
6:0	2.453 ± 0.4	2.267 ± 0.153	NS	2.548 ± 0.024	2.449 ± 0.007	*	2.624 ± 0.167	2.426 ± 0.118	NS
8:0	1.623 ± 0.305	1.554 ± 0.077	NS	1.779 ± 0.033	1.797 ± 0.015	NS	2.047 ± 0.122	2.029 ± 0.09	NS
10:0	3.701 ± 0.666	3.274 ± 0.111	NS	4.375 ± 0.107	3.991 ± 0.061	*	5.431 ± 0.319	5.314 ± 0.249	NS
12:0	4.342 ± 0.548	3.966 ± 0.166	NS	5.209 ± 0.201	4.771 ± 0.032	NS	6.675 ± 0.364	6.389 ± 0.016	NS
14:0	16.066 ± 0.715	15.879 ± 0.346	NS	17.272 ± 0.589	16.208 ± 0.038	NS	18.484 ± 0.288	18.273 ± 0.132	NS
14:1	0.97 ± 0.041	0.879 ± 0.017	*	0.921 ± 0.119	0.758 ± 0.002	NS	0.988 ± 0.031	0.934 ± 0.031	NS
15:0	1.929 ± 0.061	1.874 ± 0.002	NS	1.539 ± 0.003	1.474 ± 0.038	NS	1.845 ± 0.007	1.898 ± 0.031	NS
16:0	38.601 ± 0.63	41.324 ± 0.556	*	41.57 ± 0.462	43.563 ± 0.268	**	37.83 ± 0.55	39.495 ± 0.184	*
16:1	1.579 ± 0.067	1.629 ± 0.048	NS	1.413 ± 0.042	1.718 ± 0.309	NS	1.16 ± 0.022	1.174 ± 0.016	NS
17:0	0.833 ± 0.071	0.666 ± 0.023	*	0.605 ± 0.03	0.474 ± 0.007	*	0.675 ± 0.081	0.6 ± 0.004	NS
18:0	12.994 ± 1.173	12.119 ± 0.072	NS	9.969 ± 0.624	10.186 ± 0.007	NS	9.392 ± 0.339	9.318 ± 0.218	NS
18:1	10.499 ± 0.639	10.88 ± 0.108	NS	8.947 ± 0.431	9.538 ± 0.099	NS	8.892 ± 0.288	9.124 ± 0.124	NS
18:2	0.718 ± 0.057	0.765 ± 0.002	NS	0.585 ± 0.035	0.746 ± 0.049	*	0.708 ± 0.018	0.842 ± 0.06	*
18:3	0.287 ± 0.021	0.281 ± 0.011	NS	0.305 ± 0.024	0.329 ± 0.015	NS	0.385 ± 0.019	0.383 ± 0.015	NS
18:2 (9c,11t)	0.889 ± 0.1	0.85 ± 0.007	NS	0.46 ± 0.037	0.476 ± 0.01	NS	0.468 ± 0.018	0.466 ± 0.014	NS
20:3	0.031 ± 0.006	0.037 ± 0 .005	NS	0.024 ± 0.001	0.036 ± 0.005	*	0.04 ± 0.006	0.057 ± 0.003	*
20:4	0.022 ± 0.005	0.026 ± 0.005	NS	0.015 ± 0.003	0.034 ± 0.011	NS	0.021 ± 0.004	0.045 ± 0.01	*
20:5	0.035 ± 0.006	0.03 ± 0.002	NS	0.028 ± 0.005	0.039 ± 0.014	NS	0.048 ± 0.004	0.052 ± 0.009	NS
22:5	0.056 ± 0.008	0.048 ± 0.003	NS	0.045 ± 0.006	0.053 ± 0.005	NS	0.068 ± 0.008	0.071 ± 0.009	NS

[a] C4:0 is significantly different at 95% of confidence using Anova.
[b] NS = not significantly different .Significantly different at: *$p<0.05$; **$p<0.01$; ***$p<0.001$.
'n' is the number of replicates.

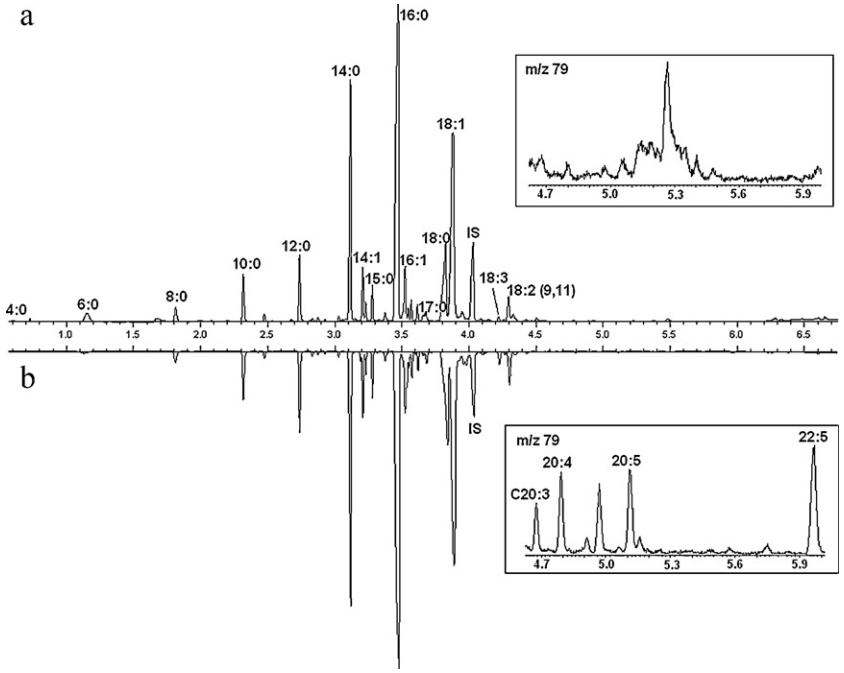

Fig. 1. GC–MS total ion chromatogram of FAMEs from milk by: themochemolysis 0.55 M TMAH and (b) conventional method. (IS – C19:0). Observation: short chain fatty acid methyl esters from extract obtained from the conventional method were lost by volatilization prior TDS–GC–MS analysis. The ion *m/z* 79 was selected to perform reconstructed ion chromatogram of polyunsaturated fatty acids region.

reaction has been described using aqueous solution of reagent [36]. The mechanism proposed for this reaction involves initially the hydrolysis of the triacyglycerol by the base followed by the transference of one methyl group from trimethylsulfonium ion to the analyte, producing methyl ester, $(CH_3)_2S$ and water [16,37]. As the transference of methyl group from $(CH_3)_3S^+$ is thermally assisted, methyl esters produced are immediately transferred to cold trap avoiding the hydrolysis of the ester by water as is observed in conventional transmethylation process (base or acid catalyzed) in the presence of water.

Three different sorbents were investigated to pack the cold trap: 1 – glass wool; 2 – Tenax TA and 3 – SP^{TM} 2330. Glass wool showed significant loss of short chain fatty acid methyl esters, while Tenax TA showed low desorption efficiency for long chain fatty acid methyl esters. The best performance was achieved using SP^{TM} 2330.

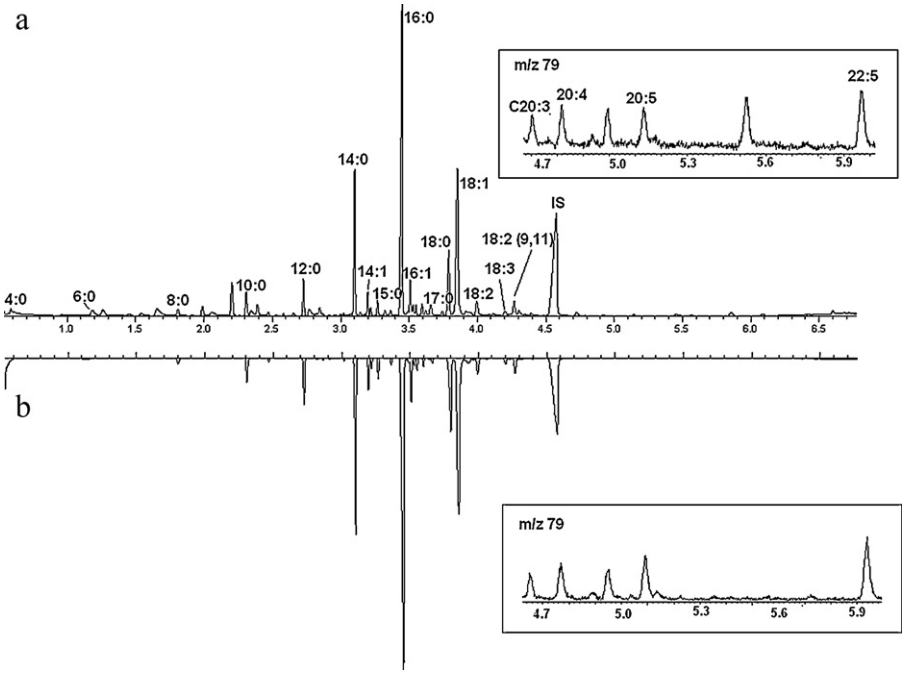

Fig. 2. GC–MS total ion chromatogram of FAMEs from milk by: (a) themochemolysis 0.5 M TMSH and (b) conventional method. (IS – C21:0). Observation: short chain fatty acid methyl esters from extract obtained from the conventional method were lost by volatilization prior TDS–GC–MS analysis. The ion *m/z* 79 was selected to perform reconstructed ion chromatogram of polyunsaturated fatty acids region.

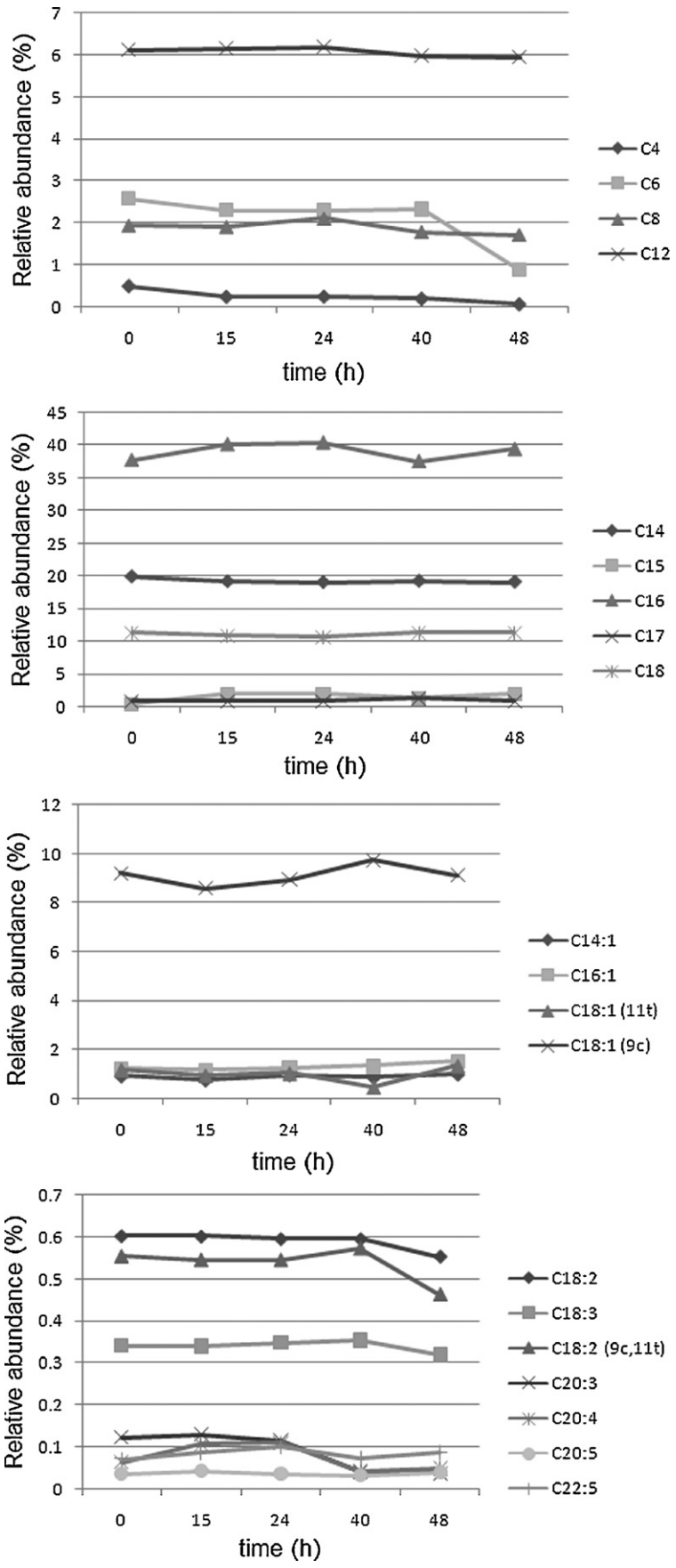

Fig. 3. Selected FAMEs relative abundance (RA), identified in milk using thermochemolysis as a function of time after sample preparation.

Fig. 2 shows the comparison between the milk fat profiles determined by thermochemolysis using 0.5 M TMSH and by the conventional method using the Röse–Gottlieb procedure to extract milk fat followed by transesterification using potassium hydroxide in methanol. The fatty acid compositional profile showed a similar profile for both methodologies. The most significant observed difference is due to the loss of the most volatile FAMEs (4:0 and 6:0) in the conventional method, which may have occurred prior to GC–MS analysis, since the milk FAME extract was introduced into the GC–MS through the TDS–CIS inlet and these compounds are very volatile.

The parameters of the final method were chosen on the basis of the derivatisation efficiency of the tested compounds and milk samples. Also, high recovery of polyunsaturated fatty acid derivative was taken into account, to insure its applicability for milk fatty acid profiling. The final method involves the combination of TMSH (ca. 0.5 M), TDS platform and 10% SP^{TM} 2330 on 100/120 chromosorb as a sorbent in CIS. These conditions resulted in the most satisfactory results with respect to derivatisation efficiency of milk samples comparable with the conventional method of derivatisation.

Milk is a complex mixture rich in protein, sugar and other components that can be retained in the column inlet if injected directly. The excess of thermochemolysis reagent is also described as corrosive and able to damage the column [38]. Thus the consistency of the system was monitored by analysing a food standard mixture daily, which allowed the assessment of the retention time stability of the column after this series of thermochemolysis reactions. Over five hundred analysis were performed in this period and no significant alteration was observed in the column performance, indicating that the TDS system was efficient in trapping excess thermochemolysis reagent and non-volatile compounds and therefore avoiding the need to cut off pieces of the column as described before [24,25].

3.2. Linearity on the response

The linearity of the optimized conditions was evaluated using seventeen triglycerides commercially available (Table 1). The mixtures of triglycerides and reagents were prepared immediately before each analysis to avoid the degradation and/or isomerisation of polyunsaturated fatty acids. Six calibration levels were used at amounts between 19 and 2000 μg/mL. Calibration curves showed good linearity and their determination coefficients (r^2) were above 0.9846 for all compounds.

3.3. Repeatability

The repeatability of the derivatisation for the seventeen triglyceride standards, covering different fatty acid characteristics (Table 1), was determinated using four replicates of the triglyceride standard mixture (300 μg L^{-1}). The relative standard deviations (RSDs) of the response were below 9%, except for polyunsaturated derivatives (C:18:3, C:20:4, C20:5 and C22:6), which presented RSDs between 12 and 20%. These results for polyunsaturated fatty acids are consistent with the previous results and indicate degradation/or isomerisation [21]. It was also observed that the absence of a thorough contact between reagent and sample resulted in a low yield and poor repeatability, as described by Klingberg et al. [39].

3.4. Repeatability for milk sample

The repeatability of the derivatisation for milk was evaluated using six replicates of the same milk sample. Results showed that thermochemolysis analysis of the milk generated reproducible profiles, with RSDs below 7%, except for methyl butanoate and polyunsaturated derivates, which showed RSD values between 10 and 14%. The results for the conventional method showed similar variation in the RSD (Table 2). In this case, the extract obtained from the conventional method was introduced through the split–splitless inlet of the GC–MS to avoid the loss of volatile fatty acids. When raw milk (non-homogenized) was used low repeatability was observed for the area of individual peaks by thermochemolysis (data not shown). One possible explanation for this result is that one drop (1 μL) of non-homogenized milk may present variation in the total fat content. Homogenized milk, with smaller milk fat droplet that prevent separation of the cream, showed good repeatability for the area of individual peaks (Table 3).

3.5. Stability

The samples of milk were kept in the auto sampler after TMSH (0.5 M) had been added for up to 48 h and the results showed that the mixture between reagent and milk was stable up to 24 h (Fig. 3).

3.6. Application in milk fatty acid profile

The feasibility of the new method for fast analysis of milk fat was evaluated by comparing the thermochemolysis method with the conventional method using fat extraction followed by transmethylation. Milk samples, collected from three Friesian cows were then analysed by both methodologies. Once more, the loss of volatile fatty acid was avoided by injecting the extract obtained from the conventional method through the split–splitless inlet. Significant differences were detected only for some FAMEs (e.g. methyl butanoate), but for most FAMEs monitored no significant difference was observed (Table 4). These results indicate that this method can be used for rapid milk fat characterization in a high throughput system.

4. Conclusion

The practicality of thermochemolysis for the analysis of milk fat has been significantly improved with the use of TDS–GC–MS under optimized conditions showed in this work. The thermochemolysis was found to meet the general requirements for non-regulated fatty acid characterization of milk samples. With some compromises in the analysis of most volatile FAMEs (i.e. 4:0 and 6:0), the system is capable of direct analyse of milk without fat extraction or cleanup. The optimized method for thermochemolysis produced milk fat profiles comparable to the official reference method using the Röse–Gottlieb procedure for extraction of milk fat followed by trasmethylation using potassium hydroxide in methanol. The thermochemolysis derivatisation is potentially an effective tool for high-throughput characterization of milk fat, requiring only a drop of milk, with the benefit of atom economy and using a cost effective system. The optimized method combined with fast or ultra fast GC [40,41] allow a high throughput platform for milk screening.

Acknowledgment

This work was supported by AgResearch Ltd. (AR&C).

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