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Isomerisation of cis-9 trans-11 conjugated linoleic acid (CLA) to trans-9 trans-11 CLA during acidic methylation can be avoided by a rapid base catalysed methylation of milk fat

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This study investigated the evolution of trans-9 trans-11 conjugated linoleic acid (CLA) from cis-9 trans-11 CLA during methylation and its avoidance through a rapid base methylation of milk fat. The study examined three conditions shown to result in loss of cis-9 trans-11 CLA during methylation namely: temperature, methylation time, water contamination in old reagents and acidic conditions. Three techniques currently used for the conversion of milk fat into fatty acid methyl esters for analysis of CLA content by gas liquid chromatography and a fourth procedure designed to eliminate acidic conditions and to limit methylation temperature and time were used. The four methods were: (i) acidic methylation (AM); (ii) acidic and basic bimethylation with fresh reagents (FBM); (iii) acidic and basic bimethylation with pre-prepared reagents (PBM) and (iv) basic methylation (BM). Each regime was carried out on six milk samples over two periods and methylated 1 ml freeze-dried milk (n=12 per regime). Total CLA was not different across methylation regimes (0·30 mg/ml). Isomer cis-9 trans-11 was higher (P<0·01) with BM than the other regimes and lowest with AM: 21·2, 17·8, 18·8 and 14·7 mg/100 ml for BM, FBM, PBM and AM, respectively. The inverse relationship was shown for trans-9 trans-11 with higher (P<0·001) amounts with AM than the other regimes and lowest with BM: 0·57, 2·55, 2·36 and 3·69 mg/100 ml for BM, FBM, PBM and AM, respectively. The trans-10 cis-12 isomer was also shown to alter with methylation procedure being higher (P<0·001) with AM than the other regimes: 0·43, 0·47, 0·29 and 1·20 mg/100 ml for BM, FBM, PBM and AM, respectively. Validation with known CLA free fatty acid and triacylglycerol standards confirmed that AM resulted in conversion of cis-9 trans-11 to trans-9 trans-11, and also elevated trans-10 cis-12 whilst BM of triacylglycerol CLA did not isomerise cis-9 trans-11 and was comparable to FBM.

Keywords: Conjugated linoleic acid, milk fat, base methylation, trans-9 trans-11 CLA isomer, cis-9 trans-11 CLA isomer.

Accurate measurement of the predominant milk conjugated linoleic acid (CLA) isomer, cis-9 trans-11 is an important requirement for validating feeding regimes to increase the beneficial fatty acid profile for human consumption. High methylation temperatures (60 °C +), old reagents (water contamination) and acidic conditions have been shown to cause loss of this particular CLA isomer (Christie, 1993; Kramer & Zhou, 2001; Nuernberg et al. 2007). This study investigated the evolution of the artefact isomer trans-9 trans-11 from the cis-9 trans-11 isomer under four methylation regimes and how it could be avoided by a rapid base methylation of milk fat. This will help increase the accuracy of CLA measurements in milk.

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The four techniques used for the conversion of milk fat into fatty acid methyl esters (FAME) for analysis of CLA content by gas liquid chromatography were either: (i) acidic methylation (AM); (ii) acidic and basic bimethylation with fresh reagents (FBM); (iii) acidic and basic bimethylation with pre-prepared reagents (PBM) and (iv) basic methylation (BM).

Materials and Methods

Six high CLA milk samples were supplied by the organic milk suppliers co-operative (OMSCo, Worle, Somerset, UK). Each methylation regime was chosen as a commonly used procedure to methylate milk fat and were carried out on each milk sample twice over two periods (n=12 per

Table 1. $C_{18:1}$ and CLA isomers in milk fat (mg/100 ml) methylated by the four regimes

	Base (BM)	Bimethylation (FBM)	Bimethylation (PBM)	Acidic (AM)	S.E.D	*P*
$C_{18:1}$ cis						
9	670·1	683·3	663·2	643·1	49·91	NS
11	13·9	14·2	13·9	13·7	2·30	NS
12	4·53	4·61	4·53	4·45	0·690	NS
13	2·96	3·01	2·98	2·91	0·364	NS
15	3·23	3·19	3·14	3·13	0·216	NS
$C_{18:1}$ trans						
6+7+8	6·29	6·34	6·52	5·92	0·105	NS
9	5·11	5·33	4·54	5·38	0·809	NS
10	5·57	5·76	4·45	4·09	0·839	NS
11	39·1	39·4	39·9	39·0	4·22	NS
12	7·03	7·02	5·86	7·31	0·868	NS
15	5·83	5·68	5·69	5·87	0·534	NS
$C_{18:2}$ CLA						
cis 9 trans 11	21·2^{c}	17·8^{b}	18·8^{b}	14·7^{a}	1·64	**
cis 9 cis 11	5·49	6·02	5·73	5·35	0·448	NS
trans 9 trans 11	0·57^{a}	2·55^{b}	2·46^{b}	3·69^{c}	0·243	***
trans 11 trans 13	2·09	1·99	2·00	1·61	0·211	NS
trans 10 cis 12	0·43^{a}	0·47^{a}	0·29^{a}	1·20^{b}	0·076	***

^{abc} Figures with different superscript within rows are significantly different; S.E.D., standard error of the difference; NS, not significant; $^{**}P<0·01$; $^{***}P<0·001$

methylation regime): (i) acidic methylation (AM) based on the procedure of Sukhija & Palmquist (1988) – 1 ml freeze-dried milk resuspended in 2 ml toluene containing 0·4 mg $C_{23:0}$/ml as an internal standard. The methylation mixture and conditions consisted of 3 ml of a mixture of freshly made acetyl chloride to ten parts methanol at 70 °C for 2 h; (ii) fresh bimethylation (FBM) based on the procedure of Kramer & Zhou (2001) – 1 ml freeze-dried milk re-suspended in 1 ml n-heptane containing 1 mg $C_{23:0}$. The methylation mixture and conditions consisted of 4 ml NaOH in methanol (0·5 mol/l, 15 min) followed by 4 ml HCl in methanol (1·4 mol/l, 1 h) both incubated at 50 °C with all reagents freshly made; (iii) pre-prepared bimethylation (PBM) – same as FBM but with all reagents made up 14 d before and kept at room temperature in a glass stoppered bottle; (iv) base methylation (BM) 1 ml freeze-dried milk resuspended in 1 ml n-heptane containing 1 mg $C_{23:0}$. The methylation mixture and conditions consisted 4 ml NaOH in methanol (0·5 mol/l, 15 min) at 50 °C.

To confirm the effects of methylation on CLA isomers, two known certified standards were methylated in triplicate using the AM, BM and FBM procedures (PBM was shown to act the same as FBM and so is not reported): (i) free fatty acid (FFA) CLA standard (10-1823-90, CLA 9c, 11tr 90%, FFA, Larodan Fine Chemicals, Malmo, Sweden); (ii) triacylglycerol (TAG) CLA standard (33-1823-90, CLA 9c, 11tr-Triglyceride, 90%, Larodan Fine Chemicals, Malmo, Sweden).

Fatty acid methyl esters (FAME) were analysed by gas liquid chromatography on a CP-Select chemically bonded for FAME column (100 m × 0·25 mm ID, Varian Inc, California, USA.) with split injection (30 : 1) and He as the carrier gas. Detection temperature was set at 255 °C and injection temperature at 250 °C. The temperature profile of the oven was 70 °C for 1 min which then increased by 5 deg C/min to 100 °C this was held for 2 min and then increased by 10 deg C/min to 175 °C held for 34 min and then increased by 4 deg C/min to 225 °C and held for 29 min (Lee et al. 2005). Line pressure was maintained at 40 psi for the first 52·5 min of the run and then increased by 0·5 psi/min to 45 psi and then held for the remainder of the run (29·5 min). Identification of CLA isomers was performed using a commercially available CLA methyl ester standard (O-5632, Sigma-Aldrich, St Louis, Missouri, USA) and quantified using the internal standard.

Period was found not to be significant and so data was analysed using a general ANOVA with methylation regime as the treatment effect and blocking according to period (Payne et al. 2002).

Results and Discussion

Total fatty acids (35·5 mg/ml) and all individual fatty acids other than CLA were not significantly different between methylation regimes (data not shown). Concentrations of $C_{18:1}$ and CLA isomers in the milk samples methylated by the four regimes are shown in Table 1. Total CLA was not different across methylation regimes (0·30 mg/ml). Age of reagents and hence propensity for water contamination did not seem to affect loss of cis-9 trans-11 in milk fat (FBM *v.* PBM). CLA cis-9 trans-11 was higher ($P<0·01$) with the

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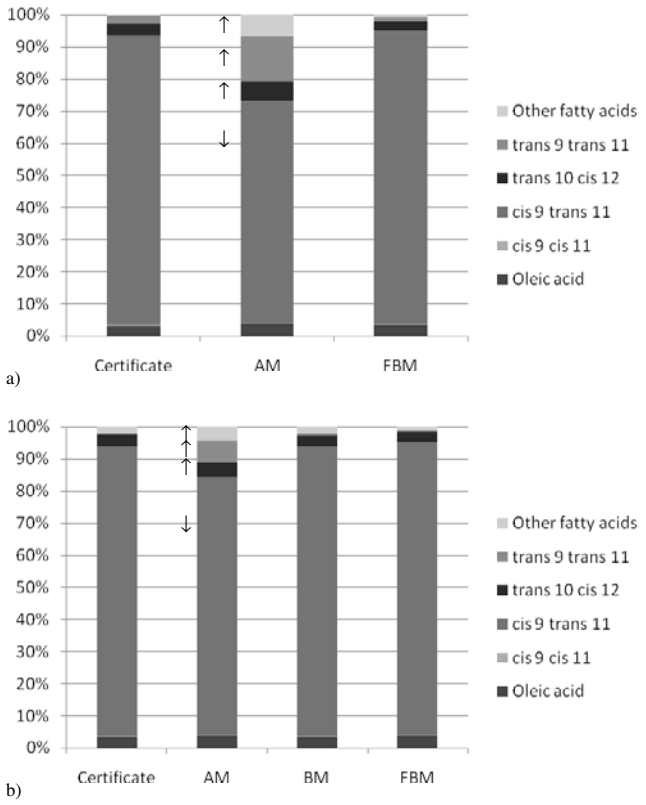

Fig. 1. Comparison of methylation procedures (AM, acidic methylation; BM, base methylation; FBM, fresh bimethylation) against the certified content of two standards: (a) free fatty acid CLA standard; (b) triacylglycerol CLA standard. ↑ increase of isomer compared with certificate ($P<0{\cdot}001$); ↓ decrease of isomer compared with certificate ($P<0{\cdot}001$). Other fatty acids refers to a mixture of $C_{16:0}$ (<0·1%, FFA and TAG) $C_{18:0}$ (<0·1%, FFA and TAG) and unidentified CLA (<0·1 and 1·4%, for FFA and TAG, respectively) in the certified standards and only the quantified fatty acids in the standards are reported for each methylation with all other fatty acids reported collectively.

BM methylation than the other regimes and lowest with AM with both bimethylations intermediate. The inverse relationship was shown with trans-9 trans-11, with higher ($P<0{\cdot}001$) amounts with the AM methylation than the other regimes, intermediate amounts with the bimethylations and lowest with BM as also reported by Nuernberg et al. (2007) with muscle lipid. AM also resulted in higher ($P<0{\cdot}001$) amounts of trans-10 cis-12 than the other methylation regimes.

Methylations of the FFA and TAG CLA standards compared with the certified content of each standard are shown in Fig. 1. For the FFA standard BM was shown, as previously reported (Christie, 1993), to be unable to methylate FFA and is not reported. For the other two methylation regimes with the FFA standard, FBM compared favourably to the certified content, whereas AM reduced ($P<0{\cdot}05$) cis-9 trans-11 content (0·77×) and increased ($P<0{\cdot}05$) trans-9 trans-11 (5·76×), trans-10 cis-12 (1·55×) and the other fatty acids group. For the TAG standard, BM and FBM both compared favourably with the certified content but again AM showed a reduced ($P<0{\cdot}05$) cis-9 trans-11 content (0·89×) and increased trans-9 trans-11 (6·72×), trans-10 cis-12 (1·15×) and the other fatty acids group but to a lesser numerical extent than with the FFA standard.

The results indicate that AM resulted in a partial conversion of cis-9 trans-11 to trans-9 trans-11 but also elevated trans-10 cis-12 and the other fatty acids group. Methylation of the TAG CLA standard by BM and FBM produced similar CLA profiles whilst FBM with milk fat resulted in higher levels of trans-9 trans-11 and lower levels of cis-9 trans-11 than BM. This difference between methylation of TAG standards and milk fat is difficult to explain but may be related to the low levels (<2%) of FFA in the milk fat. The existence of low levels of FFA in milk fat may also explain the greater increase of trans-10 cis-12 by AM when methylating the FFA standard and milk fat than when methylating the TAG standard. This effect on trans-10 cis-12 and the greater numerical elevation of trans-9 trans-11 in the FFA standard over the TAG standard suggests that CLA as FFA are more susceptible to acidic isomerisation than esterified CLA. Although BM has been shown to be ineffective in the methylation of FFA (Christie, 1993) the triacylglycerol nature (>98%, Gunstone, 1999) of milk fat results in a rapid base methylation being as effective as an acidic methylation or bimethylation for total fatty acid recovery of milk fat whilst avoiding conversion of cis-9 trans-11 to trans-9 trans-11 CLA and also the appearance of trans-10 cis-12 and other fatty acids (CLA isomers) potentially as isomerisation artefacts from cis-9 trans-11 or other fatty acids during acidic methylation.

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