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Short communication

A simplified protocol for fatty acid profiling of milk fat without lipid extraction

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ABSTRACT

Determination of the fatty acid profile of milk fat generally involves a protocol that comprises total lipid extraction, transesterification and GC analysis. The lipid extraction step is time-consuming and often employs toxic solvents such as chloroform. A novel protocol is presented here that skips the lipid extraction step and allows the determination of fatty acid composition via direct methylation of milk fat isolated after centrifugation of raw milk. This new method is reliable for relative quantification of fatty acids in raw milk fat and offers a much higher throughput compared with the classical methods.

1. Introduction

Fatty acid (FA) composition is one of the most important indicators of nutritive quality and physicochemical properties of milk fat and can also be used to reflect the health status of cows (Chouinard, Girard, & Brisson, 1998; Liu, Rochfort, & Cocks, 2018). FA composition of bovine milk is known to be influenced by animal genetics, diets and stage of lactation (Palmquist, Beaulieu, & Barbano, 1993). As a result, analysis of FA composition is required in various research projects related to dairy research.

Generally, FA composition of milk fat is determined by gas chromatography-flame ionisation detector (GC-FID) or gas chromatography-mass spectrometry (GC–MS) as FA methyl esters (FAMEs) after acid- or alkaline-catalysed transesterification (Jensen, 2002; Kramer et al., 1997). Prior to the methylation step, milk lipids are usually extracted by chloroform-based solvent systems (Bligh & Dyer, 1955; Folch, Lees, & Stanley, 1957), and 2–3 cycles of extractions are often needed to achieve a good recovery of all lipid classes. Consequently, the lipid extraction step is not only hazardous and expensive, but also time-consuming, which contributes to a low throughput in FA profiling of milk samples.

With the aim to increase the throughput and reduce the cost of FA profiling of milk samples, in the first instance we have compared different methylation methods and validated a simple one-step protocol that combines the use of low concentration of KOH, a short reaction time and a mild reaction temperature (Liu, Ezernieks, Rochfort, & Cocks, 2018). The aim of this study was to evaluate the feasibility of performing methylation of milk fat isolated by a simple step of centrifugation of raw milk (i.e., skipping the lipid extraction step with organic solvents).

2. Materials and methods

2.1. Milk samples

Three raw milk samples were obtained from the Department of Economic Development, Jobs, Transport and Resources' Ellinbank centre in Victoria, Australia. These samples were aliquots from an afternoon milking of three individual cows (A, B and C); their total fat concentrations were 3.28%, 2.83% and 2.68%, respectively, as determined by infrared spectroscopy.

2.2. Chemicals and reagents

Solvents used for lipid extraction and GC–MS sample preparation were of chromatographic grade and were from Merck (Darmstadt, Germany) (methanol), Sigma–Aldrich (St. Louis, MO, USA) (chloroform) and Ajax Finechem (Scoresby, Victoria, Australia) (hexane). Potassium hydroxide used in methylation reaction, nonadecane (used as internal standard in GC analysis) as well as standards of FAMEs were purchased from Sigma–Aldrich.

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2.3. Lipid extraction and FAME preparation

Three methods of fat isolation from raw milk were compared in this study.

In Method 1 (Folch method), total lipids of raw milk (0.5 mL) were extracted twice by chloroform/methanol (2:1, v/v) and the organic phase was transferred to a 5-mL glass vial and evaporated to dryness under a stream of N_2 prior to methylation.

In Method 2 (direct methylation-1, DM-1), raw milk (14 mL) was centrifuged at 4 °C for 20 min (3000×*g*) and 25–30 mg of crude fat from the top fat layer weighed into a 5-mL glass vial and dried under a stream of N_2 for 60 min before methylation.

In Method 3 (direct methylation-2, DM-2), raw milk (14 mL) was centrifuged at 4 °C for 20 min (3000×*g*) and 25–30 mg of crude fat from the top fat layer weighed into a 5-mL glass vial and directly subjected to methylation. Three technical replicates were conducted for each method.

Alkaline-catalysed methylation was adopted for FAME preparation in this study. In all cases, 2.4 mL of derivatisation reagent (0.2 M KOH in methanol) was added to each vial that contained milk fat and the vial was tightly sealed with a Teflon lined cap; the methylation reaction was completed by incubation at 50 °C for 20 min with occasional shaking. After cooling to room temperature, 1 mL of water was added to each vial and FAMEs formed were extracted into 1 mL of hexane containing internal standard (nonadecane, 100 mg L^{-1}) and analysed directly by GC–MS.

2.4. GC–MS analysis of FAMEs

The separation of FAMEs was achieved by a Rt-2560 column (100 m × 0.25 mm ID, 0.20 μm film thickness, Restek (Bellefonte, PA, USA)) with a constant flow of 1.2 mL min^{-1} helium as carrier gas and the following oven temperature program: initial temperature of 100 °C and held for 4 min, increased by 6 °C min^{-1} to 170 °C, and then increased by 3 °C min^{-1} to 240 °C and held for 11 min. The injection inlet temperature was 240 °C and injection volume was 1 μL, with a split ratio of 20:1.

Detection was with an Agilent (Wilmington, DE, USA) 7000 GC/MS Triple Quadrupole with the following settings: scanning mass range of 40–500 amu, transfer line temperature of 240 °C, source temperature of 280 °C, quad temperature of 150 °C and a solvent delay of 11.8 min. A FAME standard mix (supplied by Sigma–Aldrich) containing 37 FAMEs was used to provide absolute quantification of each FA.

2.5. Statistical analysis of data

All FA analysis data were subjected to ANOVA (XLSTAT, Microsoft Excel); where significant differences were found between treatments, a Tukey's HSD test was conducted for pairwise comparisons.

3. Results and discussion

As the three milk samples gave very similar results throughout this study, only results obtained with one sample (sample B) will be presented and discussed.

3.1. Efficiency of fat isolation by centrifugation

The concentration of the most abundant FA in the raw milk sample was measured before (i.e., in full-fat milk) and after centrifugation (i.e., in skimmed milk). In both cases, lipids were extracted by the Folch method.

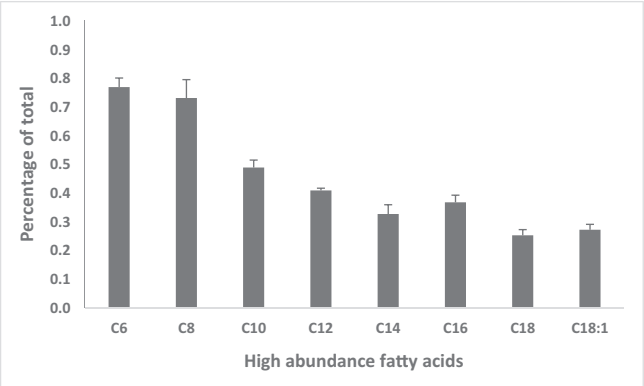

Fig. 1. Percentage of the high-abundance fatty acids found in skimmed milk after centrifugation. Error bars are standard deviation of three replicates.

Fig. 1 shows that after centrifugation conducted at 4 °C for 20 min (3000×*g*) and removal of the top fat layer, only a trace level of lipids can be found in the skimmed milk. Indeed, the high-abundance FA present in the skimmed milk are all present at <1% of their total amount found in raw milk (Fig. 1), while the low-abundance FA are not quantifiable in the skimmed milk. This implies that >99% of milk lipids are separated from the aqueous phase after centrifugation to form the top fat layer. The slightly higher content of short-chain FA in the skimmed milk may be related to their higher hydrophilicity.

It is important to mention here that, in contrast to raw milk, a single step of centrifugation does not allow formation of a firm and distinct fat layer in the case of homogenised milk. Consequently, this simple fat isolation method appears to be only applicable to raw milk, so the subsequent method validation was only limited to raw milk samples.

3.2. FAME profile comparison

The FA profile was examined across the three lipid isolation methods. For the Folch method, 0.5 mL of raw milk was used for lipid extraction, whereas about 27 mg of crude fat isolated by centrifugation was used in the case of DM-1 and DM-2. It is worth noting that, although quantitatively recovering all the fat in the top layer is very challenging, sampling 25–30 mg of fat from the entire fat layer of about 400 mg is easily achievable. The overall FA profile was found to be very similar for both high-abundance FA (Fig. 2A) and low-abundance FA (Fig. 2B) between solvent-extracted lipids (Folch) and the crude fat isolated by a simple centrifugation (DM-1 and DM-2). In addition, no visible difference in FA profile was observed whether the crude fat isolated by centrifugation was subjected directly to alkaline-catalysed methylation (DM-2) or after a 60-min drying step (DM-1) (Fig. 2A and B).

3.3. Comparison of FAME yield between DM-1 and DM-2

It was observed that milk fat isolated using centrifugation contains an appreciable amount of water (about 30%). Whether this could affect the transesterification efficiency needed to be verified. The yield of FAMEs prepared by alkaline-catalysed methylation of milk fat (25–30 mg) isolated by centrifugation was compared with and without a prior drying step.

When the FA yield is calculated based on the fresh weight of the crude fat used in the methylation reaction, there is no significant difference between fresh fat (i.e. containing water or DM-2) and dried fat (water free or DM-1) for all major FAs monitored (C4, C6, C8, C10, C12, C14, C14:1, C15, C16, C16:1, C17, C18, C18:1,

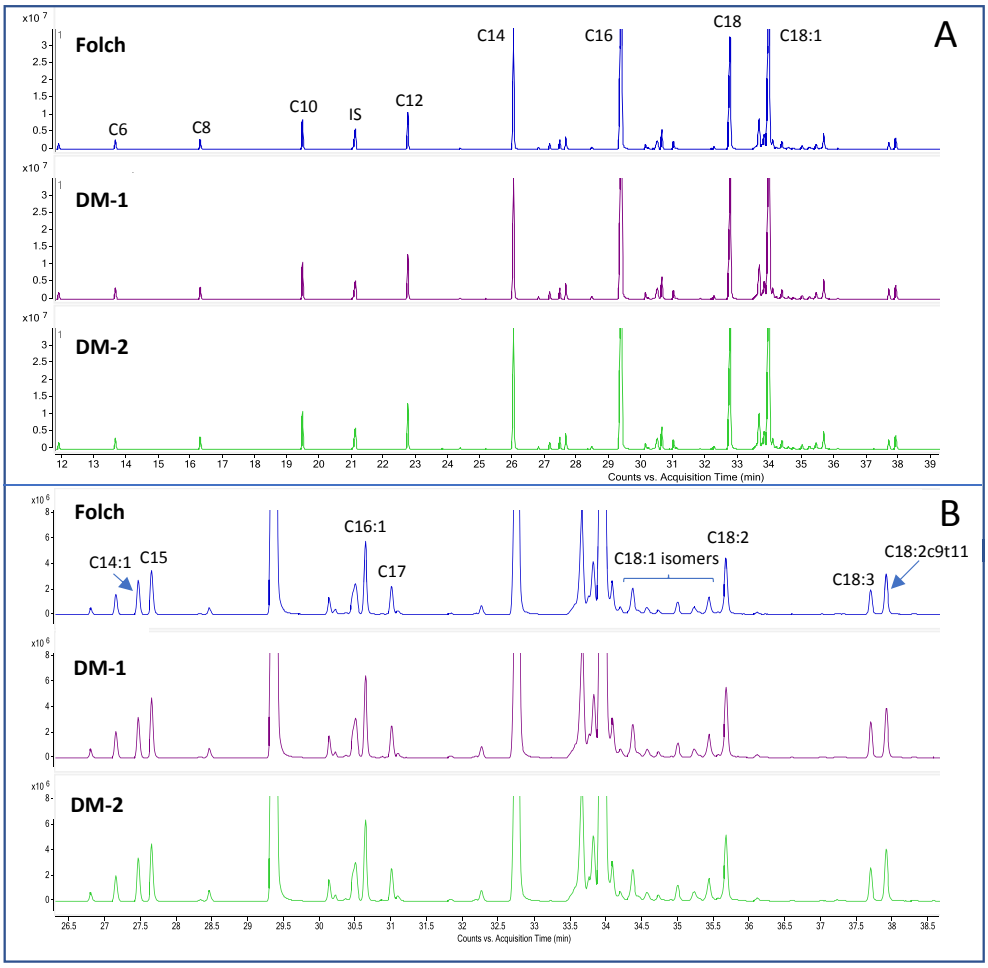

Fig. 2. GC–MS profile (total ion chromatogram) of milk fatty acid methyl esters with different lipid isolation methods: A, high-abundance fatty acids; B, low-abundance fatty acids. Abbreviations are: IS, internal standard; Folch, milk lipids extracted by the Folch method; DM-1, milk fat isolated by centrifugation and dried for 60 min; DM-2, milk fat isolated by centrifugation without drying.

C18:2 and C18:3; data not shown). This suggests that, although milk fat isolated by centrifugation contains a substantial amount of water, the actual water content in the reaction mixture is <0.5% with the current protocol (25–30 mg of fat in 2.4 mL of reagent), which does not appear to affect the methylation reaction. Ichihara and Fukubayashi (2010) also reported that 2.2% water in reaction mixture did not significantly affect the yield of FAMEs in acid-catalysed methylation. Consequently, an extra drying step for crude fat isolated by centrifugation is not necessary before methylation, which is preferred to boost the throughput in sample processing.

It should be pointed out, however, that, due to the presence of water and other components in the fat layer formed by centrifugation, direct methylation of an accurately weighed crude fat sample does not allow reliable calculation of the absolute content of individual FAs in milk or in milk fat (e.g., μg mL^{-1} milk or mg g^{-1} fat). For example, the theoretical FA yield of >900 μg mg^{-1} fat was reported for bovine milk (Moate, Chalupa, Boston, & Lean, 2007), but the total content of the major FAs amounts only to about 500 μg mg^{-1} crude fat in this study (detailed data not shown). This low FA content is by no means an indicator of a low recovery of the method, but instead results largely from the high moisture content of the crude fat, which distorts the calculation. Indeed, the total content of the major FAs rises to around 750 μg mg^{-1} based on the dry weight of the crude fat (determined for DM-1). To obtain absolute concentration of FAs in milk fat, both water content and the content of other impurities in the fat layer needs to be accurately determined in each sample and taken into consideration in calculation. Consequently, this protocol is not well suited to absolute quantification of FAs in milk fat.

3.4. Comparison of fatty acid profiling results across the three methods

Regardless of the purity of the crude fat isolated by centrifugation, when the proportion (or %) of different FAs was calculated and compared across the three fat isolation methods, no significant difference was found for any of the major FAs (Fig. 3). When the Folch method was used as a benchmark, the accuracy of the two direct methylation methods (DM-1 and DM-2) ranged between 96 and 106% across the major FAs. In addition, DM-1 and DM-2 displayed a satisfactory reproducibility in FA profiling (RSD < 7% for all the major FAs).

This indicates that using the crude fat isolated by a simple centrifugation generates similar results in relative proportion of each individual FA as compared with the classical protocols requiring lipid extraction with organic solvents. Given that the total fat percentage of milk samples is usually required in the first instance and can be readily determined by infrared-based methods, information on relative distribution of each FA determined using the current method is expected to fulfil the requirements for most studies.

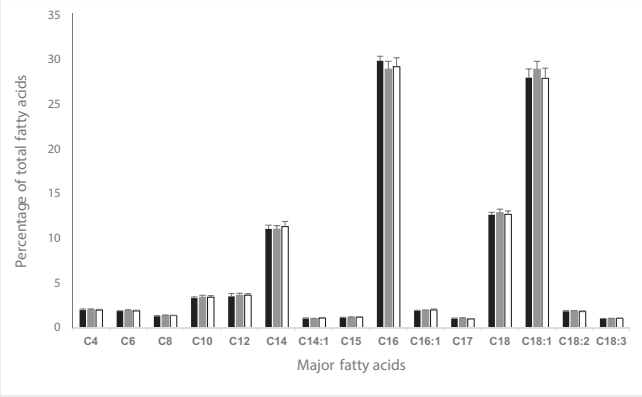

Fig. 3. Comparison of the relative proportion of the major fatty acids across three fat isolation methods: milk fat extracted by the Folch method (■), milk fat isolated by centrifugation and dried for 60 min (■), and milk fat isolated by centrifugation without drying (□). Error bars are standard deviation of three replicates.

4. Conclusions

Crude fat layer isolated by centrifugation of raw milk can be used reliably to perform relative quantification of FAs in milk fat. By omitting the step of lipid extraction with organic solvents, this new protocol is simpler and safer, and is expected to increase significantly the throughput of FA profiling of raw milk samples.

Acknowledgements

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