Method for determination of fatty acids in bovine colostrum using GC-FID

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ABSTRACT

Bovine colostrum is potentially valuable source of essential fatty acids (FAs), but so far only few studies have made the effort to estimate FA composition of this potential resource. The aim of current research was to fill this gap with selecting and validating an accurate procedure for the analysis of the composition of the FAs in bovine colostrum. We used colostrum samples of Holstein-Friesian cattle from Märja experimental farm as a test material. The validated method includes derivatization, in which FAs are sent through esterification with the acidic catalyst boron trifluoride. Formed methyl esters of fatty acids (FAMEs) were analysed using GC-FID. The obtained LOD and the LOQ of FAMEs were 0.11–0.68 and 0.37–2.27 ppm, respectively. The analysis of fortified samples showed very good and similar recoveries, indicating that the method proposed here can be routinely used for determination and investigation of the fatty acids in dairy products.

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1. Introduction

Large-scale milk production is accompanied by remarkable quantity of by-product known as colostrum. Colostrum is a secretion from the mammary gland formed in the first days after parturition (Blattler et al., 2001). Colostrum composition differs significantly from milk (Penchev Georgiev, 2008), containing higher levels of proteins, fatty acids (Kehoe, Jayarao, & Heinrichs, 2007), vitamins (Duplessis et al., 2015), minerals (Kehoe et al., 2007), immunoglobulins (Rivero, Valderrama, Haines, & Alomar, 2012), and antioxidants (Przybylska, Albera, & Kankofer, 2007).

Bovine colostrum can be an important source of fatty acids in human diet. However, it is crucial to know the content of fatty acids as it can have significant influence on health. A diet high in saturated fatty acids has been related with chronic diseases of the cardiovascular system while polyunsaturated fatty acids are linked with prevention of cancer (Baum et al., 2012). Despite of the high importance, only limited number of studies, so far examined the composition of fatty acids in bovine colostrum (Laakso, Manninen, Mäkinen, & Kallio, 1996; Parodi, 1983; Paszczyk, Zegarska, & Borejszo, 2005; Contarini et al., 2014; Santschi, Wettstein, Leiber, Witschi, & Kreuzer, 2009; Varga-Visi et al., 2011). In addition, the number of samples, used to analyse FA content in bovine colostrum is remarkably low, both in overall as well as per publication. Further, it is difficult to make general conclusions of the fatty acid content of bovine colostrum as existing studies measure and report different variables. The availability of one method, validated for the suitability of determining FA content in both, bovine colostrum and bulk milk samples, would enable to overcome these problems. Considering that the fatty acid composition of vegetable oils (Orsavova, Misurcova, Ambrozova, Vicha, & Micek, 2015) and animal products (Wei & Zeng, 2011) are rather well known. Thus, we foresee that the availability of validated method would advance the research in bovine colostrum as well as in dairy products in general.

Fatty acids in various types of samples can be measured using different equipment, such as high performance liquid chromatography (Dillona, Aponte, Tarozo, & Huang, 2013; Lima & Abdalla, 2002), gas chromatography-mass spectrometry (Boggia, Borgogni, Hysenaj, Leardi, & Zunin, 2014; Dayhuff & Wells, 2005; Ecker,

In gas chromatography (GC), fatty acid detection is usually facilitated by converting these compounds into their methyl ester derivatives (FAMEs) using various esterification methods. Conventionally, there are various methods to prepare FAMEs: base- or acid-catalyzed esterification; methylation with diazomethane in ethereal solution, boron trichloride or boron trifluoride (Siciliano et al., 2013). In addition, other methods, such as methylation with tetramethylammonium hydroxide or trimethylsulfoniumhydroxide, derivatization with tert-butylidimethylsilyl or dimethylloxazolidine, and also cyanomethyl derivatization are also used (Siciliano et al., 2013). To separate obtained FAMEs by GC, polar polymer-type capillary columns, such as cyanopropyl polysiloxane (Lachman et al., 2015; Mjøs, 2005; Petrović, Kezic, & Bolanc, 2010) or polyethylene glycol (Alves & Bessa, 2009; Ugoala, Ndukwe, & Audu, 2008; Wawrzyniak, Wasiak, & Frackowiak, 2005) are frequently used. The use of extremely polar column also allows a good separation of FAME cis- and trans-isomers present in dairy products (Delmonte et al., 2011).

The aim of current research was to establish and validate a simple and accurate procedure for the analysis of the composition of the FAs in bovine colostrum. The AOAC 969.33 method, which is widely used for FAME detection in oils was chosen as a base of our method. The method included fat extraction and derivatization, during which, the fatty acids went through esterification with the acidic catalyst boron trifluoride. Subsequently, the suitability of polar polyethylene glycol column was tested for the separation of FAMEs by GC-FID from bovine colostrum extract.

2. Materials and methods

2.1. Chemicals and standards

The standard reference material (SRM) 2377 FAMEs mixture was obtained from National Institute of Standards & Technology. This SRM was used in calibration of chromatographic instrumenta-
tion. Identification of the individual FAME was achieved by the use of FAME standards C8-C24 from Dr. Ehrenstorfer GmbH (Germany). Boron trifluoride – methanol complex solution (13–15%) was purchased from Sigma-Aldrich (Germany). Sodium hydroxide (purity 98%) and sodium chloride (purity 98%) were obtained from Reachim (Russia). Methanol (purity 99.8%) and heptane (purity 99%) were purchased from Riedel-de Hahn (Germany). Sodium sulfate (purity 99%) originated from Mikhailovsky Plant of Chemical Reagent (Russia). Lauric acid (C12:0), myristic acid (C14:0), pentadecylic acid (C15:0) and stearic acid (C18:0) were obtained from Reachim (Russia). These fatty acids were used to perform a spike-and-recovery experiment. Isopropanol was from Lachema (purity 98%).

2.2. Sample preparation

The reference procedure was performed according to Association of Analytical Communities 969.33 procedure (AOAC, 2000). Colostrum samples, used as a test material, were collected from Holstein-Friesian cattle from Märja experimental farm. All collected samples were stored in a 50 mL vial at −20 °C. FAMEs were prepared by adding twelve drops of the colostrum (approximately 500 mg) to 8 mL 0.5 M methanolic NaOH and heated at 80 °C for 7 min. Thereafter 9 mL methanolic boron trifluoride was added and the mixture was further heated at the same temperature for 2 min. Subsequently 4 mL of heptane was added through con-
denser and the mixture was boiled for 1 min. After boiling, the mixture was slightly cooled down by keeping it at room tempera-
ture for 2 min, after which 15 mL of saturated sodium chloride (NaCl) solution was added. Then the flask was stoppered and shaken vigorously for 15 s, after which saturated NaCl solution was added to the flask to trigger the floating of the heptane solution into neck of the flask. Next, ca 1 mL of upper heptane layer was transferred into test tube and anhydrous sodium sulfate was added to remove water. Obtained concentrate was transferred into a new test tube and dissolved in 2 mL of heptane. Finally, 1 µL of dilution was injected manually in GC for the analysis.

2.3. Gas chromatography of FAMEs

FAMEs were analysed on a Varian gas chromatography 3900 equipped with flame ionization detector. Fatty acids methyl esters are polar compounds in nature; therefore, a highly polar column, DB-FFAP from J and W Scientific: Agilent Technologies (Santa Clara, USA) was selected for the analysis. This column consists of a polyethylene glycol bonded nitroterephthalic acid phase. The dimensions of the column were 30 m × 0.53 mm i.d., 0.50 μm film thickness. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The temperature program was as follows: initial temperature 60 °C for 3 min, increased by 5 °C/min to final temperature 240 °C and held for a further 5 min. The detector temperature was 260 °C. The injector was set at 250 °C with split ratio of 20:1.

Star Chromatography Workstation Version 6.3 (Varian, USA) was used for data collection, and calculation of all parameters. 26 FAMEs were analysed from SRM 2377 mixture with a total run time of 44 min.

2.4. Method validation

The developed method was validated in terms of linearity, limit of detection (LOD), quantification (LOQ), and precision in accordance with Eurachem guideline (Magnusson & Örnemark, 2014). To evaluate linearity, six concentration levels of SRM 2377 mixture dissolved in heptane were used. Obtained analytical curves were linear from 0.2 up to 15.0 ppm. The linearity was thereafter assessed by the linear regression equation. LOD and LOQ were cal-
culated statistically through linear regression line from calibration curve, considering that the LOD was three times the baseline noise and the LOQ was ten times the baseline noise (Magnusson & Örnemark, 2014).

Method precision was evaluated at two levels: subsamples and injections repeatability. The subsamples repeatability was estab-
lished on the bases of independently prepared subsamples (n = 6) from same bovine colostrum sample using one experimental setup and one set of reagents on one day. Injection repeatability evalu-
ation was based on multiple injections of a single preparation of a sample on the same day.

Evaluation of accuracy of the method was based on the spike-and-recovery experiment. For this reason four fatty acids and the sample of skimmed cow milk with a low content of FAs (fat content <0.05%) was selected. Isopropanol was used as the diluent for these fatty acids due to their solubility. This part of validation was per-
formed by adding of FAs solution with approximate concentration of 10 ppm into cow milk sample.
3. Results and discussion

3.1. Method optimization

The results of the current study indicate that DB-FFAP column can also be used for analysing of FAMEs. During 44 min, most of the peaks of the FAMEs exhibited excellent peak symmetry (Fig. 1) and DB-FFAP column was selected for further study.

The tests of two inlet temperatures (220 and 250 °C), at the start of the optimization of this GC method indicated that at the inlet temperature of 220 °C, some FAMEs with high boiling points could not be eluted as the energy was too low to vaporize. Therefore at the inlet temperature of 250 °C, the recoveries of saturated fatty acids were increased as the peak areas were increased and no thermal degradation was observed. Hence, the inlet temperature of 250 °C was chosen for further optimization.

The separation of FAMEs was optimized by changing the initial temperature of the column oven and the rate of the temperature program, as also demonstrated by earlier studies (Ntsomboh-Ntsefong et al., 2014; Zhang et al., 2015). Slow temperature rate and lower initial temperature improved peak symmetry for basic FAMEs in tested method.

The results of the two tested inlet liners, open glass and single gooseneck injector inserts indicated that the peaks areas of FAMEs were higher in case of using open glass liner. Thus, this liner was chosen for further analyses. Relative retention times of methyl ester peaks of the colostrum sample were comparable with those of well-known FAME standards when estimated by the peak areas normalization method (Fig. 1).

Altogether, the results showed an adequate separation of basic FAME’s in bovine colostrum using DB-FFAP column. However, few compounds like cis-and trans-C18:1 coelute, cis-and trans-C18:2 coelute, C20:3n6 and C21:0 coelute, and C22:6 and C24:1 coelute were not separated. Therefore, the initial temperature was additionally lowered to 60 °C and the temperature rate of 5 °C/min was applied.

In general, method optimization demonstrated that the choice of polar column, inlet temperature and column temperature program influence the separation efficiency of short and medium-chain fatty acids from bovine colostrum sample. All these aspects have strong impact to the accuracy of FA determination but so far, they have received little or no attention in FA analyses (Contarini et al., 2014; Ntsomboh-Ntsefong et al., 2014; Varga-Visi et al., 2011).

Fig. 1. GC-FID chromatograms of FAMEs using Agilent J&W DB-FFAP 30 m × 0.53 mm × 0.50 μm GC column: (A) SRM 2377 mixture and (B) real sample extract (bovine colostrum).
3.2. Method validation

The determination coefficient for various FAMES, which is used to estimate the linearity of the model, ranged from 0.9990 to 0.9999 (Table 1). Various slope values of the regression lines indicated different detector calibration sensitivity for each analyte. Overall, obtained LOD values ranged from 0.11 to 0.68 ppm, while the range of LOQ was from 0.37 to 2.27 ppm for the target FAMES. These results of LOQ and LOD indicated adequate sensitivity of the tested method.

Coefficients of variation (CV) of FAMES, which were used to evaluate subsample repeatability ranged between 0.63 and 1.62% (Table 2). Such low variation of peak area values indicates good robustness of the tested method. In addition, the results showed also high injection repeatability, demonstrating constant signal and low CVs across 10 injections (Table 3). Recovery values of spiked FAs ranged between 100.6 and 103.3%, showing that the derivatization method used to determine fatty acids in dairy samples is appropriate (Table 4, Fig. 2).

The FA composition of colostrum differs significantly from bulk milk (Contarini et al., 2014; Varga-Visi et al., 2011). Since the period of colostrum production is short, lasting only for few days, the FA composition of colostrum changes rapidly towards that of bulk milk. This method has shown to give adequate evaluation for FA composition both in colostrum and spiked bulk milk, hence it can be successfully used to study FA composition dynamics during colostrum transitioning into bulk milk.

4. Conclusions

This study presents the first validation of the AOAC 969.33 method, commonly used for oil samples, for determining fatty acids composition of bovine colostrum. The results indicate that the DB-FFAP column, which has so far not been used in separating colostrum FAs, is in general suitable for separating short and medium chain fatty acids and other components of colostrum. This method has shown to give adequate evaluation for FA composition both in colostrum and spiked bulk milk, hence it can be successfully used to study FA composition dynamics during colostrum transitioning into bulk milk.
medium-chain fatty acids. The recovery results of the spiked milk sample were adequate, showing that the derivatization method with the acidic catalyst boron trifluoride used to determine fatty acids in dairy samples was appropriate. Overall it can be concluded that described method enables to estimate fatty acids in bovine colostrum with adequate precision, stability, linearity as well as respective detection and quantification limits. This validated method allows more profound examination of the fatty acid composition of bovine colostrum in future studies.

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