Fast, simple and reliable triglyceride composition analysis of milk fat for discrimination of cheese origin and adulteration detection

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Abstract. Optimization of a novel high performance liquid chromatography with refractive index detection method, together with accelerated solvent extraction for determining the triglyceride composition of lipids to enable rapid discrimination of goat and cow cheeses is presented. The method was applied to determine adulteration of goat and cow cheeses with vegetable oils/fats, to distinguish cheese analogues from natural cheeses, and to determine the amounts of palm fat added to cheese. Principal component analysis of triglycerides grouped by equivalent carbon number was used for confirmation of cheeses’ animal origins. A total of 130 cheeses or cheese analogues were included in this study. The method was calibrated in the range of 0 to 50 % palm fat in milk fat, with recovery values being from 46.50 % to 108.48 %. The content of palm fat found in adulterated cheese was from 10 to 16 %, and 5 to 10 % in cow and goat cheeses, respectively. Due to the rapidity of the method, employment of relatively inexpensive equipment and the reliability of the results, the method proved to be suitable for analytical laboratories that are required to process large numbers of cheese samples in a short time.

1. Introduction
Cheese is an important source of essential substances and fundamental ingredients in diets that prevent nutritional deficiencies [1]. Among other cheese constituents, milk fat (MF) is one of the major components and can be present in amounts up to 40 % [2]. The main lipid constituents of MF are triacylglycerols (triglycerides, TGs), as approximately 98 % of MF consists of TGs [3]. Some fatty acids (FA) in the milk TGs are only found in ruminant MF [4,5].

The trends of substituting goat cheese with cow or natural cheeses with cheese analogues are substantially and clearly economically motivated. This kind of food fraud has not only a financial effect but also has impact on human health and nutrition. Lactic acid bacteria present in natural cheeses produce numerous beneficial effects in consumers [6], but cheese analogues lack these microorganisms. Long-term deprivation of nutrients normally provided by natural cheeses could have adverse effects on consumers’ health if cheese analogues are unwittingly consumed [1,6].

Efforts to detect the animal origin of cheese or cheese adulteration and fraud have resulted in development of numerous analytical methods. Techniques like real-time polymerase chain reaction (PCR), electrophoresis, gas and liquid chromatography were employed for milk and cheese authenticity determination [7-10]. Since the cheese adulteration and frauds include partial or whole milk fat substitution with foreign fat, emphasis could be on development of reliable methods for
analysis of TGs and FAs found in MF. Determination of the lipid fraction of MF constituents included, for the most part, gas and liquid chromatography techniques [9-11].

The most common methods for MF extraction are traditional methods such as Rose-Gotlieb or other liquid-liquid extraction methods. Recently, pressurized (PSE, PLE) or accelerated (ASE) solvent (liquid) extraction methods are commonly used in analyzing TGs and FAs from MF. PSE and ASE methods have several advantages over the traditional methods, like lower consumption of organic solvents, higher efficiency due to high pressure and temperature during the extraction process, automation of extraction, etc.

The most commonly applied methods for detection of animal origin and food fraud related to cheese composition are time consuming or required expensive and specialized instrumentation. This study had the main goal of developing a fast and simple HPLC-based method for TG analysis, retaining satisfactory reliability, and employing low cost and common analytical instrumentation.

2. Materials and methods

2.1. Chemicals and standards

All HPLC-grade solvents were obtained from Merck (Darmstadt, Germany) and Sigma Aldrich (Germany). Palm fat (PF), bleached deodorized (Santa, Abidjan, Ivory Cost), and anhydrous milk fat (Vitusa, New Jersey, USA) were used in method development. Petroleum ether b.p. 40-60 °C for extraction, n-hexane p.a. and acetone p.a. for extract dilution were from Sigma Aldrich (Germany).

2.2. Cheese samples

The total of 130 cheese samples (23 goat cheeses, 98 cow cheeses and 9 cheese analogues) were obtained from across the whole region of the Serbian market. All cheeses were stored in cold storage prior to analysis. Fresh cheeses were predominant, and cheeses in various stages of ripening constituted a minor part of the total cheeses.

2.3. ASE extraction

Lipids were extracted from cheese by ASE (ASE 200, Dionex, Sunnyvale, CA) with petroleum ether (b.p. 40-60 °C) at 125 °C and nitrogen pressure of 10.3 MPa in two static cycles of 5 min. Cheese (3-5 g) was weighed, mixed with approximately twice the amount of diatomaceous earth and transferred to 33 mL Dionex extraction cell with glass fiber filters 19.8 mm (Dionex). The extracts were collected in 60 mL glass vials. Solvent was removed under a stream of nitrogen (Dionex Solvent evaporator 500) at 50 °C until dryness. The fat extracts were kept in a desiccator.

2.4. Chromatography

Analysis of the TG composition of fats and oils was based on a high performance liquid chromatography (HPLC) method with refractive index detection commonly used for determination of TGs [12]. The chromatographic system consisted of an isocratic pump 1515, autosampler 717, column heater with temperature control module, and refractive index detector 2414 (Waters, Milford, USA). The mobile phase composition was acetone:acetonitrile 64:36 v/v with flow rate of 1 ml/min. Separation of TGs was achieved on two serially coupled Luna C18 columns. Column and detector temperatures were set to 40 °C. Fat extracts were diluted with n-hexane or acetone and filtered through filter discs, pore size 0.22 µm. Sample injection volume was 10 µl.

2.5. Statistical analysis

Data preparation for statistical evaluation of results was performed in Microsoft Excel from MS Office. Data for principal component analysis (PCA) was verified by Bartlett’s test of sphericity and Kaiser-Mayer-Olkin’s test of sample adequacy. For PCA and graphical expression of results, JMP 10 Statistical Discovery (SAS Institute, Cary, USA) software was used.
3. Results and discussion

According to our hypothesis that sophisticated, exotic or expensive equipment is not necessary for such analysis, an older HPLC system was chosen with a refractive index detector and the most common C18 reverse phase (RP) column. The developed method is designed for reliable determination of cheese’s animal origin and possible adulteration by analyzing the quantity of TGs using equivalent carbon number (ECN) groups, not for precise insight in quantifying individual TGs in MF. Considering this, the employed instrumentation was sufficient to achieve this goal.

3.1. Setting of method parameters

In order to develop a fast and reliable method for determination of TGs in milk and vegetable fat, it was necessary to optimize extraction and chromatographic conditions. Because MF is thermally stable [13] and heating has a great influence on both extraction efficacy [14] and retention time [15], higher temperatures were chosen for MF extraction and chromatographic analysis. Thus, according to optimization results, extraction of all cheese samples was performed at 125 °C in two static cycles of 5 minutes each, and 80 % of each extract volume was flushed into an extraction vial between cycles. The total time consumption for ASE extraction is less than 25 minutes per sample.

To achieve satisfactory separation of TGs within 20 minutes’ runtime, after optimization of temperature, mobile phase composition, and flow rate, and after selection of the most suitable chromatographic column, the following optimal conditions were established: column and detector temperatures were 40 °C, mobile phase composition was acetone:acetonitrile 64:36 v/v, flow rate 1 ml/min and two serial coupled C18 columns were used for chromatographic separation of TGs.

3.2. Analysis of cow and goat milk fat extracts

Our optimized method was applied to determine the TG composition of MF. In order to verify that the developed extraction procedure was generally applicable, cheeses with various MF contents were examined. Figure 1 clearly renders the characteristic differences in the constitution of cow and goat MF with regard to TGs. Results confirmed that the greatest difference was in TGs with ECN 38-42, due to differences in the short chain FA content of goat and cow MF [4].

![Figure 1. Chromatograms of cow (left) and goat (right) milk fat](image-url)
3.3. Cheese analogues’ TG profile

In the next step, the developed method was tested for its ability to discriminate between natural cheeses and cheese analogues. Results of method as applied to determine the TG profile of cheese analogues showed that PF was utilized to manufacture these products. For illustration and confirmation, commercial PF was analyzed under the same conditions with same method, and its typical chromatogram is shown in Figure 2.

Analysis of cheeses obtained from the Serbian market showed that in a number of our cheese samples, MF was partially replaced with PF. A typical chromatogram of such a cheese is shown in Figure 2. Partial replacement is unequivocally an indicator that economically motivated adulteration (EMA) of cheeses is present on the Serbian market, but until now, it could not be detected or proved. The importance of discovering EMA and its impact on consumers was, in the greatest part, described in the last decade [16].

3.4. PCA of chromatographic results

PCA on correlations of the chromatographic data produced the first two components that explained over 4/5 total data variance, and corresponding eigenvalues were 5.94 and 1.56. PCA results are shown in Figure 3. Density ellipses cover confidence intervals of 95%. The Kaiser-Meier-Ol森 value was 0.91 and Bartlett’s test of sphericity had high significance, with a probability value of $p < 0.0001$.

Cheeses were grouped into three clearly separated clusters, based on the TG content of their fat extract as determined by developed method (Figure 3). Chromatographic peak areas of each group of TGs with the same ECNs were integrated. Integrated area data were used as variables in PCA. Data for PF and anhydrous cow MF standard substances are shown as a black triangle and black circle, respectively, in Figure 3. As can be seen, these data could be regarded as central points for corresponding groups of data.

The content of TGs with ECNs from 46 to 50 in the fat extracted from the cheeses was responsible for separating and grouping the cheese analogues, because they predominantly contained PF. TGs with lower ECNs were characteristic for PCA grouping of natural cheeses, in this case TGs with ECNs 34 and 36, and ECNs 40 and 42 for cow and goat cheeses, respectively. TGs with ECNs 38 and 44 were neutral for these groups but of great significance for separating the natural cheeses from the cheese analogues.

PCA results revealed the existence of mislabeled cheeses with respect to animal origin; of these, six cow cheeses were labeled as goat, and one goat cheese was labeled as made from cow milk.
3.5. Method calibration and determination of added fat

A set of MF and PF mixtures with six concentration levels were used for calibration – pure MF, 1 %, 5 %, 10 %, 20 % and 50 % of PF in MF. As shown by PCA and in Figure 2, PF has a significant amount of TGs with ECN from 46 to 50, while MF is richer in TGs with lower ECN. On analyzing chromatograms in our calibration set, the greatest variability in peak area values was obtained for ECN 36 and 48. Quantification of added PF was performed by plotting logarithmic value of ECN 48 to ECN 36 ratio vs. percent of added PF to MF. The linear regression equation is:

$$PF (\%) = 0.0175 \log_{10} \left( \frac{ECN_{48}}{ECN_{36}} \right) - 0.1187 \quad (1)$$

Table 1. Calibration data for estimating the amount of palm fat in milk fat

| Palm fat (%) | ECN48/ECN36 | $\log_{10}(ECN48/ECN36)$ | Palm Fat calculated (%) | Palm fat recovered (%) |
|--------------|-------------|---------------------------|-------------------------|-----------------------|
| 0            | 0.72        | -0.14                     | -1.29                   | -                     |
| 1            | 0.78        | -0.11                     | 0.46                    | 46.50                 |
| 5            | 0.95        | -0.02                     | 5.42                    | 108.48                |
| 10           | 1.17        | 0.07                      | 10.67                   | 106.73                |
| 20           | 1.81        | 0.26                      | 21.50                   | 107.49                |
| 50           | 5.53        | 0.74                      | 49.23                   | 98.47                 |

A very low recovery value was obtained for 1 % PF in MF, i.e., only 46.5 %. However, it is very unlikely that addition of such a small amount of PF would actually occur in EMA, and therefore, this concentration is of minor significance to the relevance of this developed method.

Summarized results of the amount of PF estimated as having been added to the cheeses are given in
Table 2. About 28% (27 of 98) of cow and 35% (8 of 23) of goat cheeses included in this investigation were positive for the presence of added PF. Almost double the amounts of PF were detected in cow cheeses than the amounts in goat cheeses.

Table 2. Palm fat amounts estimated in fat extracted from cheeses and cheese analogues

| Samples     | Total | Positive for PF<sup>1</sup> | Lowest % of PF detected | Highest % of PF detected |
|-------------|-------|-----------------------------|--------------------------|--------------------------|
| Cow         | 98    | 27                          | 10                       | 16                       |
| Goat        | 23    | 8                           | 5                        | 8                        |
| Vegetable fat | 9     | -                           | 71                       | 135                      |

<sup>1</sup>PF – palm fat

Vegetable fats extracted from nine different cheese analogues were used as a control for evaluation of method precision and overall accuracy. PF was detected in 97% of these cheese analogue extracts, with low and high limits given in Table 2.

4. Conclusion

Cheese adulteration, as well as mislabeling of cheese’s composition and origin, is present on the Serbian market. Considering the price difference in favor of goat cheese for example, attempts to increase profits by mislabeling are, unfortunately, becoming increasingly common.

The TG composition of goat and cow MF differs sufficiently so that the animal origin of the cheese from which it is extracted can be undoubtedly confirmed based on the results of this developed method of chromatographic analysis. Also, the composition of vegetable fat used for manufacturing cheese analogues has a completely different TG profile, and this can be distinguished from the TG profiles of natural cheeses, both goat and cow. For these reasons, and particularly in conjunction with PCA, this novel, optimized HPLC RID method can be successfully applied to discriminate the goat vs. cow animal origins of cheeses and cheese analogues. The added quantity of vegetable fat can be estimated using the ratio of the content of TGs with ECN 48 and 36 in the extracted cheese fat.

The importance of such rapid and simple food authenticity method development is in providing clear evidence of food composition and origin. This information is of invaluable significance for detecting food fraud and adulteration because, in recent years, such phenomena have become more frequent and have great impact on the public health and economy.

The rapidness of analysis, employment of relatively inexpensive and common equipment and the reliability of the results, compared with other methods used for same purposes, make the developed method suitable for analytical laboratories with limited resources that are required to process large numbers of cheese samples in a short time.

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