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A simplified enzymatic method for total cholesterol determination in milk

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ABSTRACT

Methods for determination of milk cholesterol concentration were compared, with particular emphasis on an enzymatic method (ME). Bovine and human milk samples (n = 40) were pre-treated and analysed by three different protocols: (i) IDF Standard with gas chromatography (GC); (ii) direct saponification (DS) and GC; (iii) DS and ME with spectrophotometric determination. A simplified method (DS + ME) was developed with an acceptable coefficient of variation. The cholesterol concentration values assessed by DS + ME were higher compared with DS + GC. There were no statistically significant differences between the results obtained by the enzymatic and IDF method ($P = 0.09$). The mean cholesterol concentrations in bovine and human milk were 16.71 ± 4.21 mg dL^{-1} and 2.80 ± 0.65 mg dL^{-1}, respectively. The new method developed was less time-consuming and more economical, and could be suitable for large-scale studies. High recovery (95%) obtained by ME indicates that the results are reliable and could be the basis for routine analysis.

1. Introduction

Cholesterol is one of the components of biological membranes (Raguz, Mainali, Widomska, & Subczynski, 2011). Cholesterol is also the precursor of vitamin D (Tieu et al., 2012), steroid hormones and bile acids (Satoh, Uchida, Takase, Nomura, & Takeuchi, 1996). Lipoproteins (made up of proteins and lipids) contain both free cholesterol (polar coating) and cholesterol esters (in combination with long-chain fatty acids, with a hydrophobic core) (Smith, Pownall, & Gotto, 1978).

The concentration of cholesterol (ChC) in milk depends on the lactation stage (Pikul & Wójtowski, 2008; Wolanciuk, Barłowska, Pastuszka, & Topyła, 2013), consecutive days of collection (Kamelska, Pietrzak-Fiećko, & Bryl, 2012), or feeding, and is different during the day and at night (Kamelska & Bryl, 2012). The difference between morning and afternoon milkings was observed also in the case of dairy cows (Lakic, Sjaunja, Norell, Dernfalk, & Östensson, 2011). Moreover, the fat content was higher at the evening milking than in the morning milking. The cholesterol content was also increased in the former (Chladek et al., 2011). In other studies, cholesterol content in bovine milk was reported to be significantly lower ($P < 0.05$) in the morning milking compared with the evening milking (5.04 ± 4.41 and 12.64 ± 11.0 mg 100 g^{-1}, respectively; Faye, Bengoumi, Al-Masaud, & Konuspayeva, 2015).

Cholesterol in milk of different species is also affected by the daily endogenous production, e.g., in the mammary gland (Botham, 1991) and the level of expression of genes responsible for the formation of proteins that transport molecules across cell membranes, such as ABCA1, ABCG5 and ABCG8 (ATP-binding cassette family; Mani et al., 2011).

For the determination of total cholesterol in blood serum, as well as in food samples, an enzymatic method is used. Cholesterol esters are hydrolysed to cholesterol and fatty acids by the enzyme cholesterol esterase (CE). Free cholesterol is oxidised to cholestenone and hydrogen peroxide by cholesterol oxidase (CO). Conversion of 4-aminoantipyrine (in the reagent for the enzymatic determination) to the quinone imine (a dye compound that can be measured spectrophotometrically) is mediated by the oxygen released from the hydrogen peroxide, in the presence of peroxidase (PO) and phenol.

Studies on the enzymatic methods for the determination of cholesterol were initiated by Flegg (1973) and Allain (1974). Bachorik et al. (1988) compared the enzymatic determination of cholesterol in the serum with standard chemical procedures (the standardised chemical method used in the Lipid Research Clinic's (LRC) program) (Burchard, 1890; Liebermann, 1885); a statistically

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significant correlation between enzymatic method and LRC for both total cholesterol ($r > 0.97$) and high-density lipoprotein (HDL) cholesterol ($r > 0.93$) was observed. Viturro, Meyer, Gissel, and Kaske (2010), used initial chemical pre-treatment using direct saponification and enzymatic kits for the determination of cholesterol concentration in bovine milk. In previous research, the use of attenuated total reflectance Fourier transform infrared spectroscopy for the determination of ChC in human milk has been presented (Kamelska et al., 2012). The same was done in the case of ChC in bovine milk, dairy products and infants formulae (Kamelska, Pietrzak-Fiećko, & Bryl, 2013; Paradkar & Irudayaraj, 2002). Larsen (2012) used enzymatic–fluorometric quantification of cholesterol in bovine milk and this method was able to discriminate between esterified and free cholesterol.

Research on biological functions and chemical properties of cholesterol has been the starting point for the development and application of analytical methods for determination of ChC. The development and evaluation of new or modified analytical methods is usually associated with a validation process. The aim of this is to ensure compliance with the defined requirements posed for this method, and allow assessment of its usefulness. It also aims to determine whether the analysis process is performed in a fair and accurate way and if it gives reliable results. When developing a new analytical method, using some method modifications or when the method parameters are changed, validation studies are generally performed. It is also important in the case of research done in another laboratory, or by other analyst using different analytical equipment (Maleszka, 2010; Namieśnik & Konieczka, 2007).

Many of the conventional analytical methods suffer from several shortcomings. In the case of milk and dairy products, extraction of fat is used as a preliminary step. However, these procedures are cumbersome. To avoid monotonous and cost- and time-consuming pre-treatment and extraction procedures, some authors extracted cholesterol from milk and milk products using direct saponification (Bragagnolo, Saldanha, & Mazalli, 2004; Fletouris, Botsoglou, Psomas, & Mantis, 1998).

Cholesterol concentration can be measured using colorimetric methods (Bachman, Lin, & Wilcox, 1976), gas chromatography (GC) (Faye et al., 2015; Naviglio et al., 2012), high-performance liquid chromatography (HPLC) (Ahn et al., 2012; Hojo, Hakamata, & Kusu, 2011), Fourier transform infrared spectroscopy (FTIR) (Paradkar & Irudayaraj, 2002) and attenuated total reflectance Fourier transform infrared spectroscopy (FTIR–ATR) (Arsov & Quaroni, 2008; Kamelska et al., 2013).

In the case of blood analysis, enzymatic methods, GC and HPLC are most commonly used. However, detection by GC–flame ionisation detection (FID) and mass spectrometry (MS) involves silyl- or methyl-derivatisation, which is cumbersome; the derivatising agents are highly volatile, which affects the accuracy and repeatability of GC quantification, and cholesterol can be thermally decomposed in the GC column (Yang & Choong, 2001). However, methods that were based on enzymes, coupled with spectrophotometry for analysis of cholesterol from blood, were unsuitable for food applications (Jiang, Fenton, & Sim, 1991). Moreover, the recovery of total cholesterol fraction from milk fat globules is difficult. Enzymatic colorimetric methods may overestimate the cholesterol content and are less precise (Ulberth & Reich, 1992).

Because of this, the optimisation and development of rapid, economical, simplified and direct methods for the routine evaluation of cholesterol in milk is crucial.

The aim of this study was to validate methods for determination of the bovine and human milk cholesterol concentration, with particular emphasis on enzymatic methods. In this study, three analytical procedures were compared and a recovery study was performed to estimate systematic error of methods and “the losses” of cholesterol in different chemical procedures during sample preparation. Different mammals' samples were chosen on purpose, so as to test the methods with samples with a wide range of cholesterol concentrations.

2. Materials and methods

2.1. Milk samples and lyophilised control serum

One bovine milk standard sample (BMS) was obtained from Reference Laboratory Z/S of National Animal Breeding Centre in Parzniew, Poland. Cholesterol concentration was determined in 40 samples of human milk, marked as A(1–10), B(1–10), C(1–10), D(1–3) and E(1–7), collected from women aged from 23 to 34 years (from first to fourth month of lactation). Women breastfed exclusively and showed good health as evaluated by a public health nurse. The exclusion criterion was smoking. The women were living in the Warmia, Mazury and Podlasie regions of Poland. Human serum was purchased from Pointe Scientific Inc. (Canton, MI, USA). The reference value of cholesterol concentration in lyophilised control serum was 139 ± 14 mg dL^{-1}.

2.2. Experimental design and research methods

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee (Ethics Committee of the Medical University of Bialystok No. R-I-003/22/2000). The experiment was conducted with the understanding of each participant. All lactating mothers gave written informed consent to participate in this study.

Raw bovine milk samples were pooled from fifteen cows. All the animals were kept in identical environmental conditions. The cows were milked twice a day with the use of a pipeline milking machine. Milk was stirred with cooling in a farm in Krosna-Parcela. The material was then homogenised and preserved with the solution of bronopol (0.2–0.3 g L^{-1}) and stored in a refrigerator (0–4 °C). The composition of milk analysis was performed within 48 hours in the Reference Laboratory (Milk Assessment Laboratory of National Animal Breeding Centre in Parzniew, Poland) with ICAR (International Committee for Animal Recording) certificate, ACTALIA Cecalait member (France, BP 70129 – 39802 POLIGNY CEDEX). The fat content was determined by gravimetric Röse–Gottlieb method and was 39.11 ± 0.46 g kg^{-1}. Protein and lactose contents were 34.03 ± 0.46 g kg^{-1} and 47.18 ± 0.96 g kg^{-1}, respectively. Milk after the determination of composition was sent on ice to the University of Warmia and Mazury in Olsztyn, Poland.

Samples of milk were collected at each lactating mother's home using a breast pump immediately after feeding the baby (hind milk, mature milk), from one breast at the same time of the day, during consecutive days (two samples each day). The human milk was obtained in 2011–2012 year. All samples were stored in sealed, sterile containers at −20 °C for no longer than 48 h.

Peripheral blood was collected from donors into tubes coated with an anti-coagulant. The blood samples were taken postprandially. Donors were screened for HBsAg (surface antigen of the hepatitis B virus – HBV) and Anti-HCV (antibodies of the hepatitis C virus) and Anti-HIV (antibodies of the *human immunodeficiency virus*). Serum lyophilisate was supplemented with additives: non-protein ingredients, enzymes, other proteins, and bacteriostats. Using a volumetric pipette, all was reconstituted with 5 mL of distilled water and left for 10 min at 20–25 °C. Then, it was swirled gently to mix contents until the contents were completely dissolved. The serum was then stored at 2–8 °C until analysis.