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Variation of the cholesterol content in breast milk during 10 days collection at early stages of lactation

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More and more research is done concerning nutritional programming. Human milk nutrients which are consumed by infants can influence their health in later life. High level of cholesterol in human milk paradoxically lowers the cholesterol concentration in blood in adults. During the course of human lactation the cholesterol concentration decreases from 31 mg/100cm³ (colostrum) to 16 mg/100 cm³ (mature milk). According to Scopesi *et al*., 2002, *Clin Nutr* 21: 379–384, cholesterol concentration in mature milk ranged from 6.5 to 18.4 mg/100 cm³. The aim of the study was to assess the variations in breast milk cholesterol content during 10 day collection at early lactation. 48 samples of human milk were analyzed. Mean age of women was 31 years. Women were collecting samples during 10 days of an early lactation stage (1–3 months after delivery). An Attenuated Total Reflectance Fourier Transformed Infrared (FTIR-ATR) method for easy and rapid determination of cholesterol in human milk was elaborated. Cholesterol content assessed by the FTIR method ranged from 3.36 to 12.98 mg/100 cm³. Results indicate that milk cholesterol concentration during 10 consecutive days of early lactation is highly variable. Cholesterol content depends on an individual. Therefore it is suggested that not only the period of lactation but also mother's diet, age, season and place of residence are important factors determining cholesterol content.

Key words: cholesterol variations, human milk, FTIR-ATR spectroscopy

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INTRODUCTION

The human milk is considered as being the gold standard of infant nutrition (Kowalewska-Kantecka, 2007). Its composition depends mainly on a growing infant's needs and also on mother's metabolism. Breast-feeding positively influences the child's proper development by stimulating the immune system, digestive system and others. The components contained in milk such as growth factors, cytokines, immunoglobulins, lactoferrin, immunocompetent cells have a beneficial effect on the infant's body (Koletzko *et al*., 2005). The metabolic events at a critical pre- and postnatal time largely affect health in later life (Koletzko, 2005; Koletzko *et al*., 2005). This is called nutritional programming.

Currently, a lot of research has been done on cholesterol, which is perceived to be an important factor in cardiovascular diseases (Castelli *et al*.., 1986; Castelli *et al*., 1992). On the other hand, cholesterol is also important in proper development of nervous system, hormone and vitamin synthesis in a growing infant (Adams & Hollis, 2002; Harvey *et al*., 2005; Dietschy & Turley, 2004).

The influence of infant plasma cholesterol levels on atherosclerosis development is still unknown (Agostini & Riva, 1998). According to studies by Reiser and Sidelman (Reiser & Sidelman, 1972) an inverse correlation between maternal milk cholesterol content and cholesterol in the blood plasma in the adult rat has been demonstrated. These studies became the starting point for attempts to determine the effect of the concentration of cholesterol delivered through milk on its metabolism in later life. Owen *et al.* (2008) showed that a high level of cholesterol in human milk paradoxically lowers the cholesterol concentration in blood in later life. Feeding infants breast milk containing high cholesterol affects normal metabolism of this compound and prevents the occurrence of hypercholesterolemia in later life.

A proper methodology to measure cholesterol concentration has not been developed yet. The authors use different methods and obtain divergent results. It is therefore necessary to calibrate methods used to assess the content of cholesterol. Cholesterol concentration in human milk is lower than in cow's milk (10.20–19.68 mg/dl) (Talpur *et al*., 2006) and higher than in infant formulas. Cholesterol concentration in infant formulas (0.93-5.45 mg/100 cm³) (Kamelska *et al*., 2011) is relatively low as compared to breast milk. Mean cholesterol level in breast milk assessed by Hamosh (1998) was 15 mg/dL. During the course of human lactation the cholesterol concentration decreases from 31 mg/100 cm³ (colostrum) to 16 mg/100 cm³ (mature milk) (Emmett & Rogers, 1997). Other authors assessed cholesterol concentration in mature milk ranging between 10–18 mg/100 cm³ (Kalio *et al*., 1989). According to Scopesi *et al.* (2002) cholesterol content in breast milk was in the range of 6.5–18.4 mg/100 cm³. Cholesterol concentration may increase during lactation. Breast milk also contains sterols and other metabolites of cholesterol biosynthesis (Kalio *et al*., 1989).

Cholesterol concentration in dairy products can be determined by various methods such as: colorimetry (Bachman *et al*., 1976), gas chromatography (Alonso *et al*., 1995; Fletouris *et al*., 1998), Fourier Transformed Infrared spectroscopy FTIR (Paradkar & Irudayaraj, 2002) and Attenuated Total Reflectance Fourier Transformed

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Abbreviations: AUC, area under curve; CV, coefficient of variation; FTIR-ATR, attenuated total reflectance fourier transformed infrared spectroscopy; GC, gas chromatography; S.D., standard deviation.

Infrared spectroscopy FTIR-ATR (Arsov & Quaroni, 2008). But none of them is suitable for human milk cholesterol content determination. Finding a cheap and rapid method for human milk is essential. Infrared spectroscopy FTIR-ATR has been previously adopted by us in order to determine cholesterol content in infant formulas (Kamelska *et al.*, 2010).

The aim of the study was to assess the variations in breast milk cholesterol content during a 10 day collection at early lactation (1–3 months after delivery) using the FTIR-ATR method.

MATERIALS AND METHODS

Materials. Human milk samples from lactating mothers with no parental history of hypercholesterolemia and/or hypertriglyceridemia were colected from June 2007 to August 2008. All women were living in the Warmia and Mazury region of Poland. All mothers were non smokers. 48 samples from 1 to 3 months postpartum were collected. This period was assigned by the authors as an "early stage of lactation". Samples have been collected immediately after infant breastfeeding once per day at the same time. The milk was taken from the breast not used for child feeding. All milk samples were frozen in plastic containers and stored immediately at –20°C until analysis. The milk remaining after cholesterol analysis was used for determination of other components.

Women at the age of 24-34 years (mean ±S.D. = 31±4.5 years) were collecting samples during 10 consecutive days. Body mass index (BMI) was calculated as weight (in kg) ÷ height^2 (in m^2). Table 1 shows characteristics of the coded population studied.

This research was approved by the Ethics Committee of the Medical University of Bialystok No. R-I-003/22/2000 and performed with the mothers who agreed to take part in this study.

Table 1. Characteristics of the population studied

Characteristic Factor	Specification	Number of samples
Place of living	Village	0
	City	49
Smoking	Yes	0
	No	49
Education	Elementary	0
	Secondary	10
	Higher	39
Lactation (month)	1st	29
	2nd	0
	3rd–4th	20
Mothers' age	24	10
(years)	29	10
	34	29
Mean mothers' height (cm)	167.8	
Mean mothers' weight (kg)	63.4	
Mean BMI (±S.D.)	22.52 ± 3.22	
BMI MIN	19.47	
BMI MAX	27.28	

Reagents. Pure cholesterol standard was obtained from Sigma Aldrich Company. Hexane was obtained from Merck KGaA. Ethanol (96%) was purchased from Eurochem BGD Tarnow Company (Poland). Chloroform was supplied by POCH.

Isolation of cholesterol from human milk. The method of cholesterol determination in dairy products e.g. yogurt, butter was used by Paradkar and Irudayaray (2002). The authors of this study adopted this method for human milk. Stock solution was prepared by dissolving pure cholesterol powder in chloroform (2 g per 100 cm^3). The appropriate dilutions of cholesterol concentration from 2 to 14 mg/cm^3 were prepared from the stock solution. The cholesterol content range was consistent with the cholesterol content of the samples analyzed.

Human milk samples, 2 cm^3, were placed in a test tube. 9 cm^3 of ethanol and 1 cm^3 of potassium hydroxide solution (50 g per 100 cm^3) were added and all was vortex mixed for 20 s. The capped test tube was then placed in a water bath at 60°C, saponified and stirred continuously at 200 r.p.m for 1 h. After cooling to room temperature, 5 cm^3 of deionized water and 10 cm^3 of hexane were added and vortex mixed for approximately 2 min. The sample was then centrifuged for 3 min at 2000 r.p.m. and the upper hexane layer was transferred with a pipette into a clean test tube. Another 10 cm^3 portion of hexane was added to the water phase. The extraction and the centrifugation steps were repeated. The combined hexane extract was then used for cholesterol determination. The hexane extract was evaporated to dryness and the sample was re-dissolved in 5 cm^3 of chloroform and used for FTIR analysis (Paradkar & Irudayaraj, 2002).

Analysis of human milk samples by FTIR-ATR method. A 7000e Digilab spectrometer equipped with a deuterated triglycine sulphate detector was used for FTIR analysis. The sampling station was equipped with an ATR accessory, comprising transfer optics within the chamber through which infrared radiation is directed to

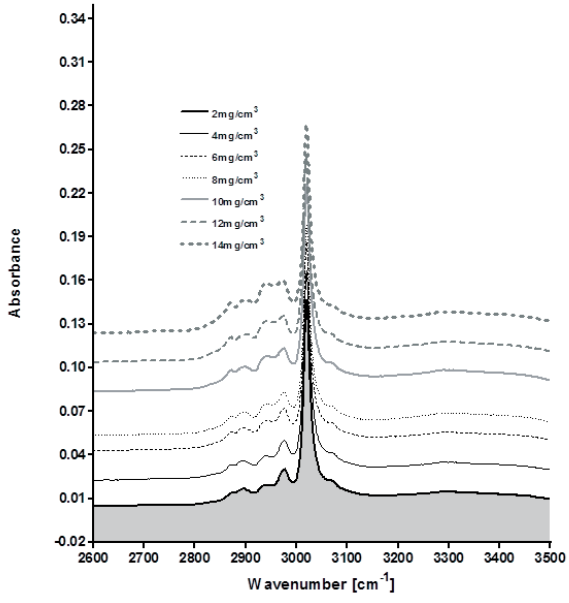

Figure 1. FTIR-ATR spectra of cholesterol standards at different concentration ranging from 2 mg/cm^3 to 14 mg/cm^3 at intervals of 2 mg/cm^3.
Spectra are normalized to the same value of absorbance at 4000 cm^{-1}. For the sake of clarity, spectra are parallel shifted.

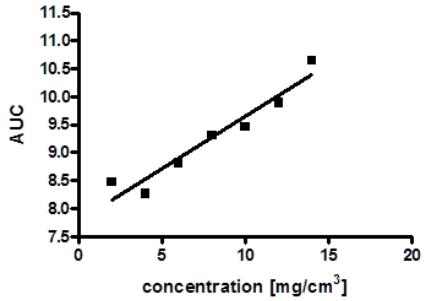

Figure 2. FTIR-ATR calibration plot for cholesterol using the spectral region between 2800 and 3200 cm^{-1}: AUC (area under curve).
Coefficient of determination r^2=0.9303 (adopted from Kamelska *et al.,* 2010).

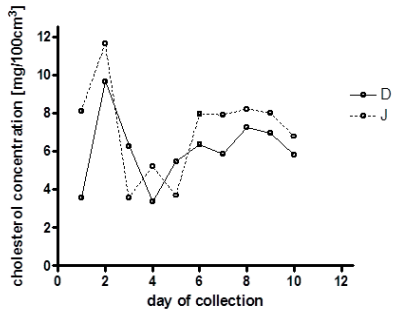

Figure 3. Cholesterol concentration variations during 10 days of collection in the first month of lactation (samples D and J).

a detachable ATR zinc selenide crystal (Pike Technologies) mounted in a shallow trough for sample containment.

The spectrum of a clean ATR crystal was used as the background and cholesterol extract from human milk samples was dissolved in chloroform and used for FTIR analysis. FTIR-ATR spectra were obtained during 16 scans from 400–4000 cm^{-1}. The ATR crystal was carefully cleaned with pure chloroform after each measurement.

The data were analyzed and prepared in the GraphPad Prism program.

Statistical analysis. The human milk samples were homogenously selected so as to find differences in human milk cholesterol fluctuations depending only on the stage of lactation. Data for the human milk are presented as mean and standard deviation. One-way ANOVA was applied to find differences between cholesterol concentration in groups from different months of lactation.

The correlation between other factors (weight and BMI) and cholesterol concentration in human milk was shown as Pearson's correlation coefficient. All statistical analyses were done using STATISTICA 10.0.

RESULTS

Calibration of the method

Normalized FTIR-ATR spectra of the cholesterol standard at concentrations from 2 to 14 mg/cm^3 with the intervals of 2 mg/cm^3 in the spectral range from 4000 to 2500 cm^{-1} are presented in Fig. 1. The absorbance of the prepared cholesterol standards was assessed according to the peak height or the extended area of the spectral region between 2800 and 3000 cm^{-1}. This region is correlated with symmetric and asymmetric vibrations of CH_2 and CH_3 groups and is diagnostic for cholesterol content determination (Paradkar & Irudayaraj, 2002). However, these vibrations are characteristic for different compounds identified in oil (Lai, 1995), animal fat, cake and chocolate adulterations (Syahariza, 2005). Therefore, when analyzing one constituent of multivariate mixture one needs to prepare a precise calibration curve. The calibration curve based on cholesterol standards was prepared taking into account the correlation between Area Under Curve (AUC) and concentration (Fig. 2).

In order to perform quantitative analysis of the results it was necessary to validate FTIR-ATR method.

Validation of the method and determination of cholesterol content in human milk samples

The aim of the study was to assess the variations in breast milk cholesterol content during 10 days of early lactation using an easy, rapid and cheap method for human milk cholesterol content determination. FTIR-ATR can be used in this case, but the method should be validated. Raw milk samples can't be analyzed directly in relation to their various constituents. Thus specific sample preparation was needed.

The Region between 2800 cm^{-1} and 3200 cm^{-1} was used for identification of cholesterol bands. This region is responsible for C–H stretching vibrations of methyl groups and vibrations of cyclic hydrocarbons, which are characteristic of cholesterol.

Cholesterol content assessed by the FTIR-ATR method ranged from 3.36 to 12.98 mg/100 cm^3 (Table 2). There were no statistically significant differences between cholesterol concentration in samples from different months of lactation (p=0.16). The minimal and maximal

Table 2. Cholesterol concentration (mg/100 cm^3) in samples of human milk from different stages of lactation.
D, J, W, AG, F — sample code

Sample code	Day of collection									
	Cholesterol concentration (mg/100 cm^3)									
	1	2	3	4	5	6	7	8	9	10
D	3.57	9.66	6.27	3.36	5.45	6.35	5.86	7.24	6.96	5.78
J	8.11	11.65	3.54	5.19	3.68	7.96	7.90	8.22	7.98	6.77
W	11.95	4.26	5.91	6.75	7.96	4.47	4.43	12.51	12.98	5.93
AG	5.12	9.05	8.23	8.47	8.99	10.38	7.48	7.15	8.15	–
F	8.07	10.74	5.80	7.65	6.62	6.70	8.06	8.41	8.11	–

Table 3. Mean, minimal, maximal, standard deviation (S.D.) and coefficient of variation (CV) of samples studied.
D, J, W, AG, F — sample code

Sample code	Month of lactation	X	MIN	MAX	SD	CV
D	1	6.05	3.36	9.66	1.80	29.81
J	1	7.10	3.54	11.65	2.43	34.30
W	2	7.71	4.26	12.98	3.49	45.21
AG	2	8.11	5.12	10.38	1.47	18.10
F	3	7.80	5.80	10.74	1.41	18.05

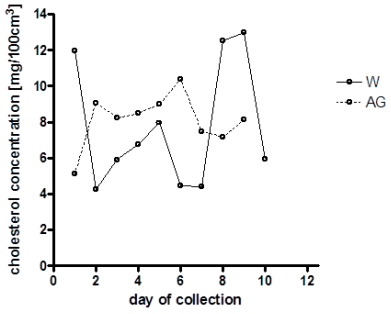

Figure 4. Cholesterol concentration variations in the second month of lactation (samples W and AG).

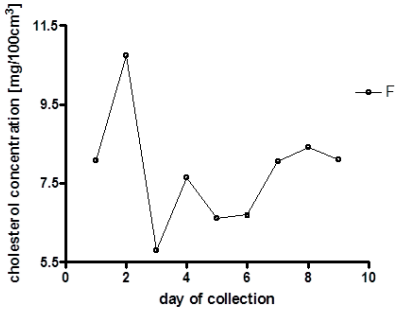

Figure 5. Cholesterol concentration variations during 9 days of collection in the third month of lactation (sample F).

value of cholesterol concentration in the first month of lactation was 3.36 and 11.65 mg/100 cm^3, respectively. In the second month of lactation these values were 4.26 and 12.98 mg/100 cm^3 and for the third month of lactation 5.8 and 10.74 mg/100 cm^3 (Table 3). Cholesterol content variations in samples from the first, second, and third month of lactation are shown in Figs. 3–5.

Empirical data clearly indicate a high negative correlation between mothers' weight and mean cholesterol concentration (r=–0.8072). There was also a high negative correlation between mothers' weight and the minimal value of cholesterol concentration (r=–0.9907). There was a very weak correlation between mothers' weight and the maximal value of cholesterol concentration (r=–0.1667).

DISCUSSION

There are not many scientific publications about cholesterol content determination in human milk. According to Scopesi (Scopesi *et al.,* 2002) the cholesterol content in human milk is in the range of 6.5–18.4 mg/100 cm^3. However these authors did not analyze cholesterol concentration during consecutive days of lactation. According to our findings the cholesterol content assessed by the FTIR-ATR method ranged from 3.36 to 12.98 mg/100 cm^3. This range is very similar to that described by Scopesi.

We analyzed cholesterol content variations during 10 days of collection. The mean cholesterol concentration was comparable in the first, second and third month of lactation and did not decrease during 10 consecutive days of collection. Therefore other factors, such as influence of diet, should be analyzed.

According to our findings it is assumed that the FT-IR-ATR method is very accurate for cholesterol content determination. It is an easy, powerful, cheap and non-destructive method. Cholesterol content determination in human milk is essential not only for mothers but also for the health of a newborn in his/her adult life.

Preparations of samples for FTIR investigations were less destructive and simpler than those for other methods e.g. chromatography. The latter method requires 0.5 g of fat from a sample of human milk. It is very difficult to collect a sufficient amount of milk from mothers who are feeding their babies.

The region between 2800 cm^{-1}–3200 cm^{-1} is accurate for cholesterol content determination. However, there is a need to find new diagnostic peaks for cholesterol determination in human milk. Attempts to use absorption maxima at 2400 cm^{-1} and 2450 cm^{-1} have been undertaken. This region may correspond to the absorption maximum of pure cholesterol in chloroform (Channa *et al.*, 2008). A very strong absorption band of CO_2 is observed in this region so the analysis is very difficult. The great advantage of FTIR spectroscopy is the ability to simultaneously record a registration signal (absorbance) over very broad spectral range.

CONCLUSIONS

Cholesterol concentrations differ significantly between individual days of collection but do not differ significantly in different months of lactation**.** There was a negative correlation between mothers' weight and cholesterol concentration in human milk. It can be concluded that not only the stage of lactation, but also individual predispositions and mothers' diet can influence cholesterol concentration in human milk, which should be analyzed in later studies.

FTIR-ATR spectroscopy was successfully used for rapid determination of cholesterol in human milk. The calibration model using area under curve in the 2800–3200 cm^{-1} frequency band was found to be the most accurate in this study. The method used was rapid and less expensive than the conventional methods. FTIR-ATR spectroscopy results can be obtained in less than 5 min.

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