Spectroscopic analysis of milk fat and its mathematical evaluation

H. Vaskova, M. Buckova, and L. Zalesakova

***Abstract*—**The aim of this paper is to verify the applicability of Raman spectroscopy for measuring the content of fat in milk and dairy products. Accurate monitoring of milk nutritional compositions is essential for producers of milk and also for milk and dairy products quality control. Raman spectroscopy enables effective material identification and offers rapid, non-contact, nondestructive, reagent free measurements and possibility to insert devices for automatization. These are the main benefits of this method comparing to traditional time-consuming techniques. The statistical method Principal component analysis was performed for large spectral datasets evaluation. For specific spectral information was used linear regression. Liquid milk samples as well as dried milk droplets with fat concentration range 0,1 % to 3,5 % and dairy products with 10 % and 82 % fat concentration were used for analyses by Raman spectroscopy. Röse-Gottlieb gravimetric method and butyrometric method served as a standard control methods for correlation with experimental spectroscopic data for milk fat analysis. Methods accuracy is discussed in the paper. Quite high agreement is obtained for Raman spectroscopy.

***Keywords*—**Milk, fat, quality control, Raman spectroscopy, PCA, rapid determination.

I. Introduction

THROUGHOUT the human lifetime, milk plays an important role in a healthy and balanced diet. According to their proper composition milk and dairy products are considered to be complex and high quality nutrient source, mainly as a calcium and protein source and are well available in many countries. Moreover, current variability of produced forms and flavours of dairy products fulfils consumers' requirements in terms of different taste preferences and also demands for culinary use. Main components of milk comprise milk proteins, carbohydrates and fat. The reliable information about these main components of milk is crucial characteristic for adequate technology application. Additionally, this information has to be labelled on milk or dairy products to inform customers about their nutritional parameters and value. Nowadays many people are looking for milk and dairy products with special nutritional properties like lower fat content, lactose-free or vitamin D fortified products. That is why dairy industry has to adjust milk to these special consumers' demands.

Effective quality monitoring of raw milk composition and other ingredients as well as monitoring of quality confirmation of final milk products is essential especially for the producers. Common methods are based mostly on traditional laboratory techniques using usually sample pretreatment, application of many chemicals, several steps and time consuming analysis, skilled and experienced personnel. The most widely used methods include the Gerber method and the Rose-Gottlieb method, both are off-line of course, time consuming and chemicals and special lab equipment needed. Therefore, methods allowing fast, simple, ideally nondestructive and accurate analysis need to be developed and improved. Also, methods with possibility to be connected with automatic regulation unit or small portable measuring systems are required in technology processes. However, for any automation there is a necessity to perform measuring of required physical or chemical characteristics for further evaluation and continuous evaluation.

From this point of view, new spectrometry methods (UV-visible, infrared, luminescence or Raman spectroscopy) are promising techniques. Moreover, multivariate chemometric tools coupled with spectrometric methods can significantly increase their application (not only) in food analysis and may have value for solving problems in dairy science and technology [1], [2].

The popularity of usage Raman spectroscopy related to analysis of milk and dairy product has an increasing trend as show the number of publications released lately. In contrast to many other spectroscopic methods, Raman spectroscopy does not require optical purity of the samples. Method has been applied to study molecules in different forms: liquids, fibres, thin films, powders, precipitates, gels or crystals. Raman scattering is generally a weak effect, giving signal intensity in order of $1x10^{-9}$ to $1x10^{-6}$ of those of elastic Rayleigh scattering. To obtain sufficient response signal, fairly high concentrations of target analytes are required to be contained

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in samples. Raman spectroscopy, therefore represent a suitable tool for direct *in situ* analysis of the major constituents of food systems [3].

The main topic studied in dairy sciences is determination of protein, carbohydrate and fat content and their change during technology or storage conditions [3] - [6]. The most known case in food chemistry and forensic toxicology was the melamine adulteration causa. In this case, Raman spectroscopy provided a very rapid screening test for melamine-adulterated dried milk [4], [7]. Additionally a portable compact Raman spectrometric system suitable for on-line analysis was constructed to determine melamine adulteration in milk powder [4]. Quantification of whey protein, a cheap by-product of cheese production added to milk powder has also been investigated [8]. Focused on milk fat, analysis of Raman spectra in combination with chemometrics methods was used to detect, classify and quantify the adulteration of butter with margarine [9].

II. Scope of the Research

Milk fat assessment is necessary both in terms of food technology and in terms of maintaining the nutritional value. The development and use of modern methods offers fast experimental procedures independent of a number of chemical reagents. The objective was to study the potential of Raman spectroscopy that due to its advantages seems to be a promising choice for measuring the fat in milk. For the study of essential features - method sensitivity and accuracy the mathematical and statistical methods were used.

III. Theory – Milk Fat

Bovine milk is composed of approximately 87% water, 4-5% lactose, 3% protein, 3-4% fat, 0.8% minerals and 0.1% vitamins (see Fig. 1). Milk fat, its amount and composition, is responsible for physical and sensory properties of dairy products. 98% of the contained fat in milk is in form of triacylglycerol. Other lipids forms are represent only minor content: diacylglycerol (2%), cholesterol (lower than 0.5%), phospholipids (approx. 1%), and free fatty acids (0.1%). Trace amounts of hydrocarbons, fat soluble vitamins, flavour compounds and other ingredients are arising in milk during animal feeding [10]

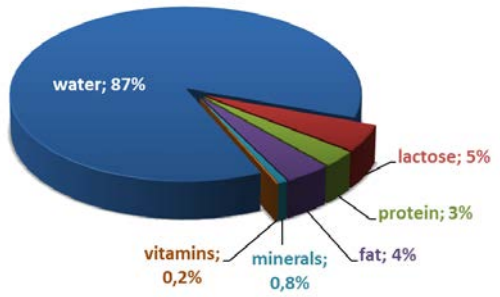

Fig. 1 Composition of bovine milk

Knowledge of the fatty acids composition in milk is important, because they can influence cardiovascular health. The main negative concern has been related to saturated fat content, which represents approximately 70% of total milk fat, only 30% of fat share is comprises unsaturated fatty acids [10]. Furthermore, milk products made from milk with more mono-unsaturated fatty acid composition are softer and have a satisfactory flavour [11]. Determination of milk fatty acids is considered to be quite difficult due to large number of fatty acids and their structural variety. Milk fat is composed of more than 400 different fatty acids. However, most of these acids are present at only trace concentrations. The average concentrations of the principal fatty acids in milk fats are following: 29% for oleic acid (C18:1), 26% for palmitic acid (C16:0) and 14% for stearic acid (C18:0) [12]. In the unsaturated fatty acids, also polyunsaturated fatty acids constitute only around 2,3% of total fatty acids, mainly linoleic and α-linolenic acids. Additionally, short-chain fatty acids (mainly butyric and caproic acids) and trans-fatty acids (like vaccenic acid and conjugated linoleic acid) can be also found [10]. Quantification of minor milk fatty acids using vibrational spectroscopy becomes especially difficult in raw milk, due to the presence of other milk components, which interfere with fatty acid specific signal [13]. For instance the fat soluble vitamin fraction can be included in the fat milk signal. The liposoluble milk vitamin profile includes mainly vitamin A, D and E. Their concentration in milk depend on milk fat content, therefore low-fat milk has lower amount of this vitamins [10]. Raman spectroscopic evaluation of fatty acids contained in vegetable oils can be found e.g. in [14], [15].

The comparison of the main components of goat, sheep, cow and human milk is listed in Table 1. The closest similarity is between cow and goat milk.

Table 1 Average composition of goat, sheep, bovine and human milk [10]

	Milk			
	Bovine	**Sheep**	**Goat**	**Human**
Fat [%]	3,6	7,9	3,8	4,0
Lactose [%]	4,7	4,9	4,1	6,9
Protein [%]	3,2	6,2	3,4	1,2
Calcium [mg/100 g]	122	193	134	33
Phosphorus [mg/100 g]	119	158	121	43
Vitamin A [IU]	126	146	185	190
Vitamin D [IU]	2,0	0,18	2,3	1,4
Energy [kcal/100 g]	69	105	70	68

IV. Materials and Methods

A. Samples

Samples were obtained as mixtures of commonly sold milks with 0,1%, 1,5 % a 3,5% of fat. The fat content in milk samples was calculated to 0,1 %, 0,8 %, 1,5 %, 2,0 %, 2,5 %,

3,0 % and 3,5 %. The indicated concentrations were verified for fat content using Röse-Gottlieb gravimetric method based on European Standard EN ISO 1211, using screening Gerber method and by Raman spectroscopy. In order to eliminate influence of proteins, all samples have constant value of protein. The content of protein was determined by automatic milk analyser MilkoScope Julie C5 instrument. When protein content is constant in all measured samples, the changes in Raman spectra can be contributed to the different fat content. The Milk samples were measured in two forms: directly in liquid form in opened aluminium dishes at a laboratory temperature and in form of dried milk droplets on aluminium plates.

Table 2 fat content in milk samples

Sample	Fat [%]		
	Calculated	Measured	
		Röse-Gottlieb	Butyrometry
1	0,1	0,074 ± 0,010	-
2	0,8	0,872 ± 0,031	0,83 ± 0,05
3	1,5	1,539 ± 0,009	1,55 ± 0,05
4	2	2,015 ± 0,029	2,08 ± 0,05
5	2,5	2,558 ± 0,016	2,63 ± 0,11
6	3	3,048 ± 0,037	3,07 ±0,06
7	3,5	3,506 ± 0,002	3,60 ±0,08

B. Chemical Methods for Milk Fat Determination

For determination of fat content in milk many laboratories are still using Gerber method, often named "butyrometric method", however this method was published in 1891. This method is often used because it is relative simple, fast, low-cost and suitable for a relatively high sample throughput. On the other hand, highly concentrated sulphuric acid is used, what involves a certain risk and potential environmental damage. Moreover, handling the butyrometer requires practical skills which can cause grater variability of results. [16] Brief description of procedure: 10 ml of Gerber sulfuric acid was placed into butyrometer tube, than 11 ml of well homogenized milk sample and 1 ml of amylalcohol was added. Butyrometric tube was locked by stopper, well shaken and centrifuged. The fat level was read from butyrometer scale under temperature 65 °C. Whole procedure was carried out according to IDF 105:2008. This method is used as a screening test.

Röse-Gottlieb method is based on extraction using a mixture of organic solvents and gravimetric determination of milk fat expressed as g of extracted fat per 100 g of milk.

Before extraction, all milk samples were heated to 38±1°C to ensure complete homogenization. 100 ml milk samples were digested by NH3 solution (25 % v/v) and mixed with ethanol (96 % v/v). The extraction was performed 3 times using the mixture of diethyl ether and petroleum ether (1:1). Finally the solvent phase was evaporated under vacuum and fat was weighted and calculated. This method is based on European Standard EN ISO 1211 and it is considered as reference method for milk fat determination [17].

C. Raman Spectroscopy

Raman spectroscopy is a vibrational spectroscopic method with a potential to answer a number of questions related to chemical details of molecular structure what makes this technique definitely proper for material identification [18], [19]. This method becomes a valuable part of laboratories around the world in recent years.

Raman spectroscopy provides very specific chemical „fingerprint" of every single chemical substance in the form of the Raman spectrum. The method is based on so called Raman scattering. Raman scattering is an inelastic scattering resulting from an interaction of a photon and a molecule. In inelastic scattering photons have slightly changed wavelengths that are characteristic for specific bonds in surveyed material. Since most photons are on molecules scattered elastically (Rayleigh scattering i.e. without changing the wavelength), it is necessary to filter out of the spectrum of the strongly present wavelength of laser. Diagram of the measurement using Raman spectroscopy is shown in Fig.2.

Although the fundamental phenomenon is known since thirties of the 20th century, its effective use in Raman spectroscopy occurs in about last decade. The rebirth of this method goes hand in hand with advances in a laser, detectors and computer technology. Raman spectroscopy brings many

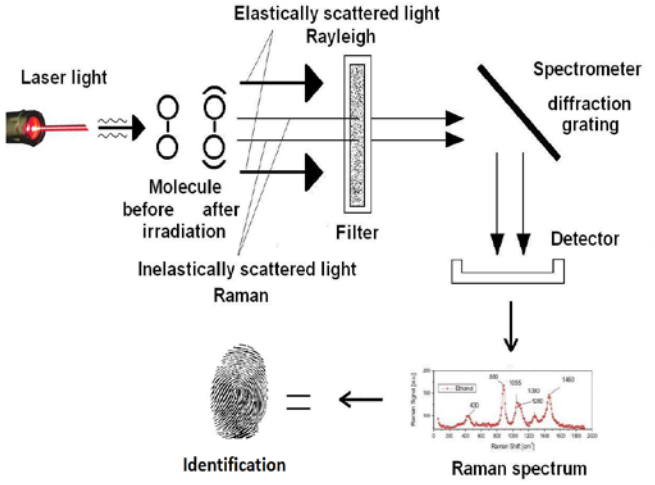

Fig. 2 laser irradiate the sample, molecules vibrate, filter eliminates intense Rayleigh scattering, the grating disperses the light onto a detector to generate a spectrum, which gives the information about molecule bonding and provides a chemical fingerprint utilizable for identification.

advantages as the method is:

- relatively rapid
- non-destructive
- contactless
- usable for measuring through transparent glass or

polymeric covering layers or containers

- applicable to all states of matter and different forms
- without special requirements for sample preparation
- usable as *in situ* analysis

The greatest drawback of the method is the fact that Raman scattering is a weak effect. Luminescence as much stronger quantum effect with bigger intensity can overlap Raman spectra and mask spectral information. Another disadvantage is eventual degradation of a sensitive sample when using intense laser beam [19].

Raman spectroscopy finds many applications in recent years in a number of scientific areas such as chemistry, biochemistry, material science, mineralogy, arts, medicine, also is used for pharmaceutical or forensic and security purposes.

InVia Basis Raman microscope (Renishaw) was used to measure Raman spectra of all samples. The Raman microscope uses two lasers as light sources: argon ion laser with the maximum power 20 mW and 785 nm NIR diode laser with maximum output power 300mW. Both were tested, however, more accurate and by luminescence less affected results were obtained using NIR laser. A Leica DM 2500 confocal microscope with the resolution 2μm was coupled to the Raman spectrometer.

All measurements were collected with 10 s exposure time and 3 accumulations. The samples were firstly scanned in common range 100 to 3200 cm^{-1} with 2 cm^{-1} spectral resolution. After determining the principle vibrational response the spectral range was reduced to area from 300 to 1800 cm^{-1}.

D. Spectral Data Fitting

For some known spectral information a spectral fitting was used for their selection from data sets. Coming out of the quantum theory, transitions between energy levels in molecules after absorbing or emitting energy and related lifetimes it is proper to use for fitting Raman spectral data different functions. Gaussian (1) is usually used for solids, Lorentzian (2) for gasses

$$f(x)=\frac{1}{\sigma\sqrt{2\pi}}e^{\frac{(x-\mu)^2}{2\sigma^2}} \quad (1)$$

$$L(x)=\frac{1}{\pi}\frac{\frac{1}{2}\Gamma}{(x-x_0)^2+(\frac{1}{2}\Gamma)^2} \quad (2)$$

Where x_0 represent the centre, parameter Γ specifies the width. In the case of milk – a liquid a combination of these functions the Gaussian-Lorentzian profile is an appropriate solution. Spectral line shapes has features of both Gaussian and Lorentzian character. The Gaussian-Lorentzian is represented

$$A*G+(1-A)*L \quad (3)$$

Where A is a variable parameter in the fit being the fraction of Gaussian character ($0 \le A \le 1$).

E. Principal Component Analysis

Principal Component Analysis (PCA) is useful and powerful statistical method for analysing data used to find and identify patterns in data of high dimension, to highlight hidden similarities and differences and extract relevant information.

Raman spectral data are multivariate, since they reflect the composition of material and its structure. Raman bands are assigned to the vibrations of chemical bonds in examined samples. PCA was used for monitoring composition similarities of fat in milk.

V. Results

A. Raman spectra of milk

Spectroscopic measurements were performed both on milk samples with 0,1 % – 3,5 % fat content and on dried milk droplets. Due to the appearing luminescence at milk samples as can be seen in Fig. 3 and Fig 4 spectra of dried milk droplets were considered for evaluation.

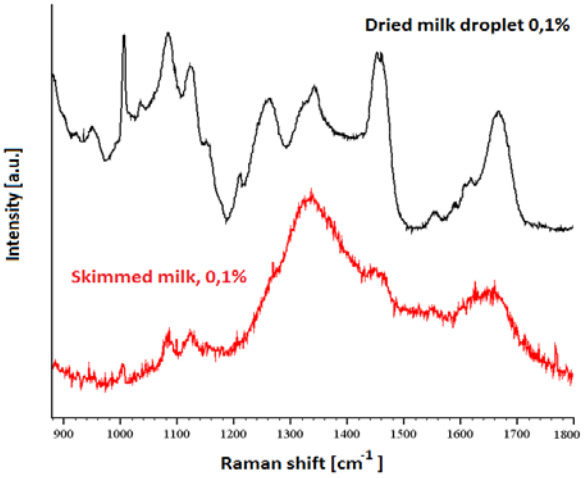

Fig. 3 Raman spectra of dried milk droplets and of liquid sample of 0,1% milk fat concentration.

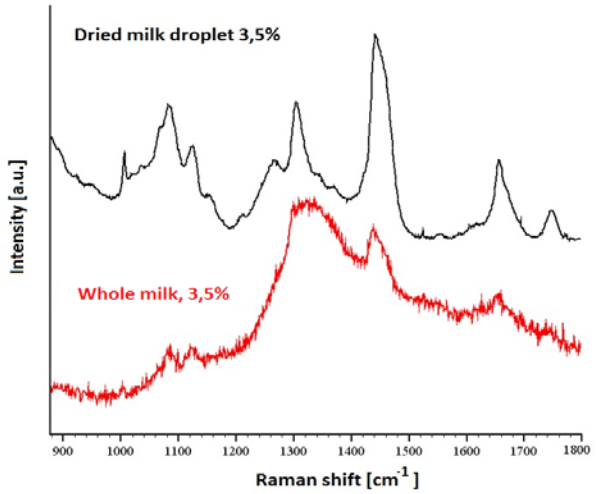

Fig. 4 Raman spectra of dried milk droplets and of liquid sample of 0,3,5% milk fat concentration.

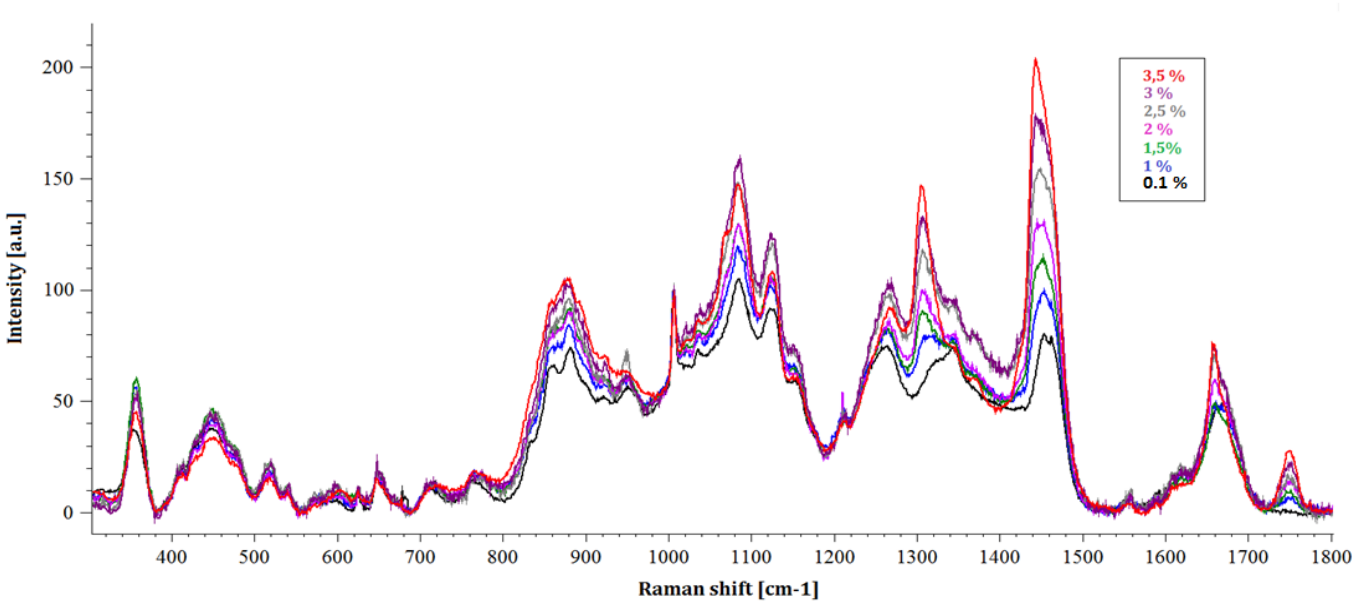

Fig. 5 Raman spectra of dried milk droplets with fat content 0,1 % – 3,5 %

Raman spectra of droplets of all samples are displayed in Fig. 5. Several peaks, that are discussed in the text show changes of intensities relating to the content of fat.

Milk fat in Raman spectra is represented by C=O stretching of the ester groups of triglycerides at 1748 cm^{-1}, whereas the 1005 cm^{-1} phenylalanine ring breathing band is indicative of protein [1]. The CH_2 deformation vibrations at 1303 cm^{-1} and 1443 cm^{-1} are specific to the saturated fatty acids, C=C at 1654 cm^{-1} for unsaturated fatty acids in *cis* configuration [6]. Raman bands of carotenoids can be found at 1008 cm^{-1}, 1150 cm^{-1}, 1525 cm^{-1} [12].

Amount of phenylalanine and 14 other amino acids in milk samples obtained after acid hydrolysis was determined by ion-exchange liquid chromatography (IEC) using Amino Acid Analyser AAA400 (Ingos, Prague, Czech Republic) [20]. The results listed in the Table 3 show the protein content does not nearly alter in the samples, therefore the peak for phenylalanine at 1005 cm^{-1} was taken as a standard to normalise intensity values.

For the evaluation of fat content in samples, the attention was directed to three significant bands: 1303 cm^{-1}, 1443 cm^{-1} and 1748 cm^{-1}. The baseline correction was applied on all spectra and the spectra were normalized. Details of the spectral response for listed bands are displayed in Fig. 6 and Fig. 9.

The linear dependence of the normalized intensities was revealed for all three examined bands, results are shown in Fig. 7 and Fig 10. In all cases the linearity exhibit quite high accuracy. Therefore based on a set of calibration data and specified procedure of data processing it is possible to determine the amount of fat in the samples. More proper for the evaluation and data processing seems to be the band 1748 cm^{-1} due to its solitary position in the spectra. However the other bands can serve for the measurement confirmation.

Table 3 Share of phenylalanine in source samples

	Fat in milk samples		
	0,1 %	**1,5 %**	**3,5 %**
Phenylalanine [g/kg]	1,44 ± 0,02	1,42 ± 0,02	1,45 ± 0,03
Total amount of aminoacids (after acid hydrolysis) [g/kg]	28,73	28,25	28,07

Table 4 Assignments of Raman bands

Raman peak [cm-1]	**Assignment**
1005	phenylalanine ring breathing
1008	Carotenoids
1150	Carotenoids
1267	=C-H symmetric rocking
1303	CH_2 in-plane twist
1442	CH_2 scissoring
1525	**C=C stretching of carotenoids**
1640	C=C trans, trans 2,4 decadienal
1654	**C=C cis double bond stretching**
1748	C=O ester-carbonyl stretching
2850	C-H symmetric stretching of methylene groups
2890	C-H asymmetric stretching of methylene groups
3015	=C-H stretch

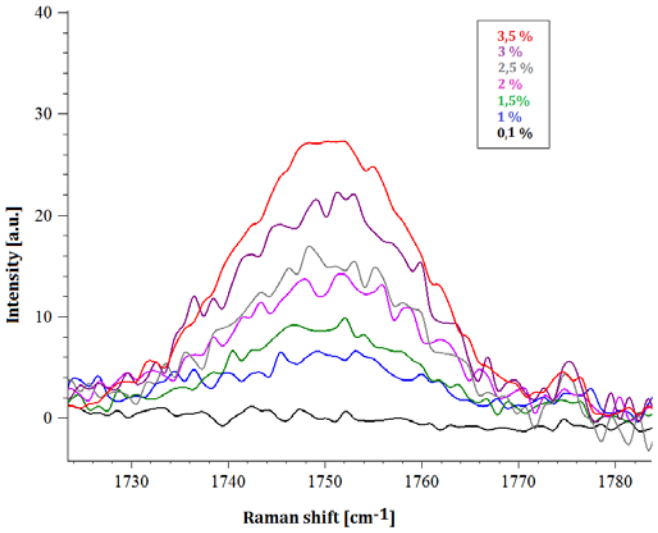

Fig. 6 Raman spectra of dried milk droplets - the increase of the normalised intensity at 1748 cm^{-1} with the content of fat

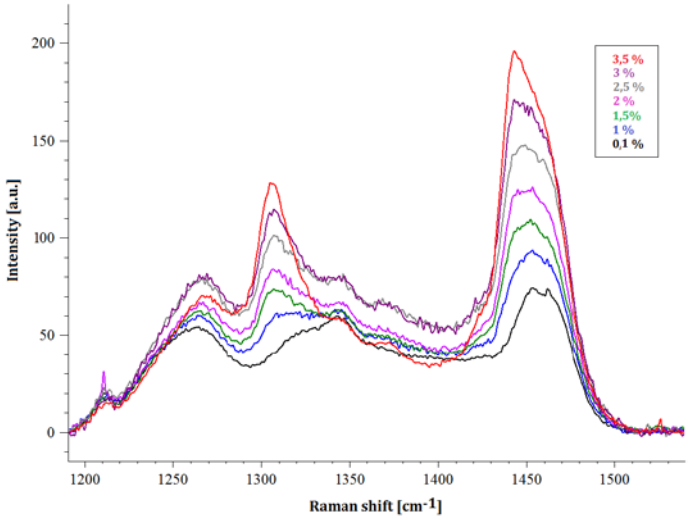

Fig. 9 Raman spectra of dried milk droplets - the increase of the normalised intensity at 1303 cm^{-1} and 1443 cm^{-1} with the content of fat

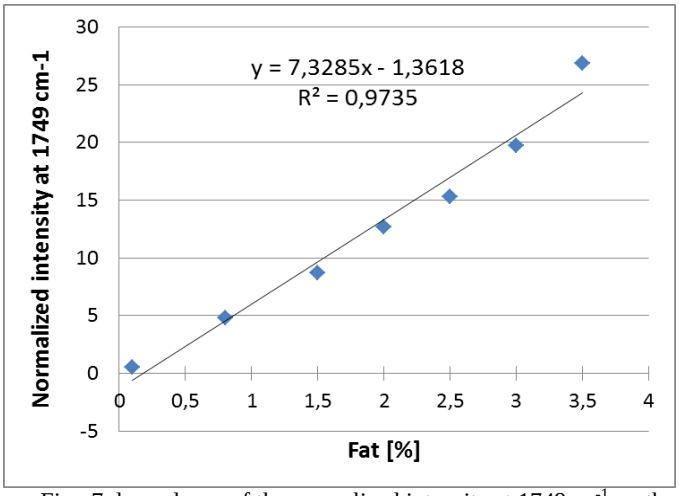

Fig. 7 dependence of the normalized intensity at 1749cm^{-1} on the fat content in dried milk droplets

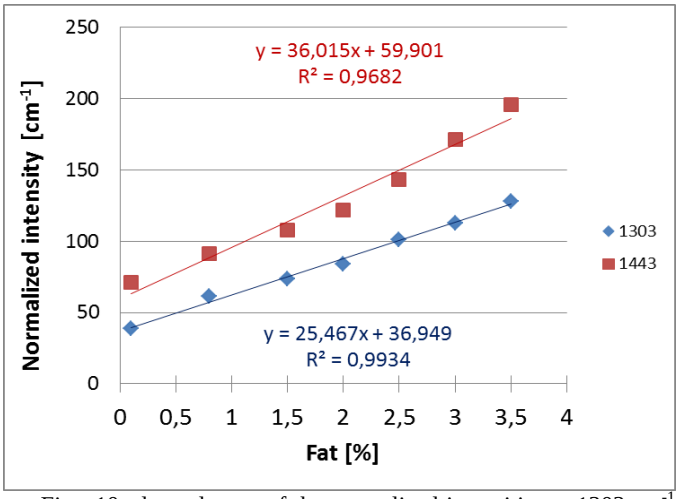

Fig. 10 dependences of the normalized intensities at 1303 cm^{-1} and 1443 cm^{-1} on the fat content in dried milk droplets

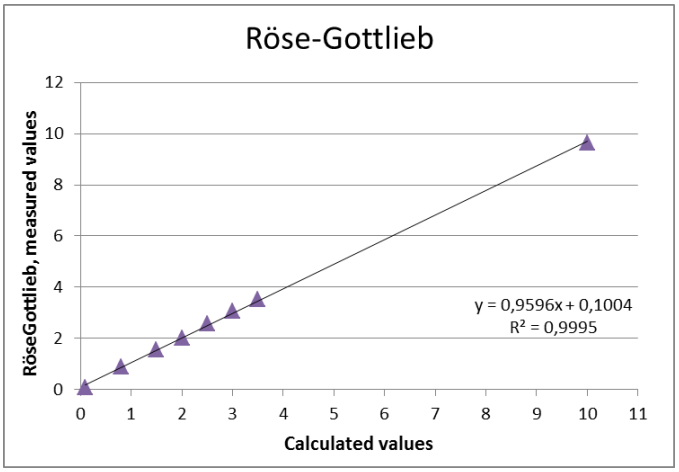

Fig. 8 measured vs. calculated values for Röse-Gottlieb method

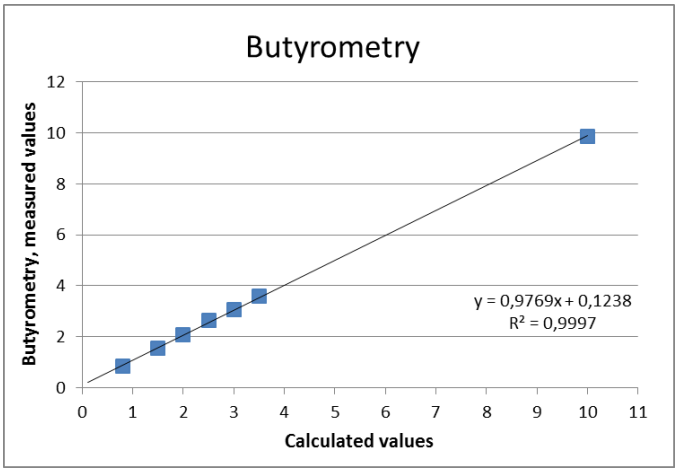

Fig. 11 measured vs. calculated values for butyrometric method

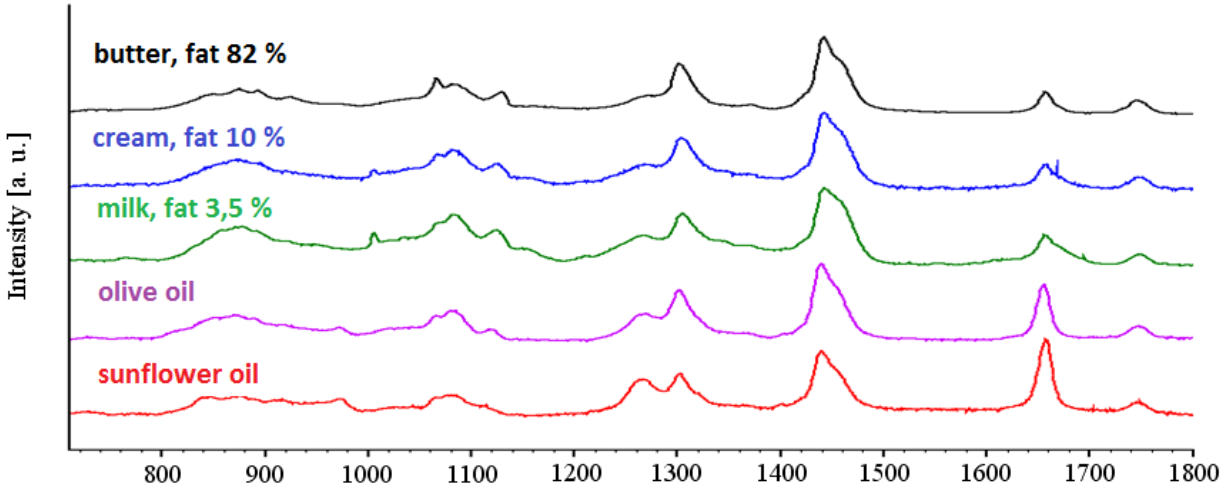

Fig. 12 Raman spectra of milk and dairy products - butter (82 %), cream (10 %), olive and sunflower oil.

B. *Methods accuracy*

Data obtained by measurements using conventional methods Röse-Gottlieb and butyrometric method were plotted against calculated values. The graphs in Fig. 8 and Fig. 11 show a good linearity with the coefficient of determination R^2 = 0,9995for Röse-Gottlieb and R^2 = 0,9997 for butyrometric method.

Results acquired from Raman spectral data by linear regression shows the best linear behavior R^2 = 0,9934 for the evaluated response from peak 1303 cm^{-1} corresponding to unsaturated bonds of fatty acids. Raman peak 1748 cm^{-1} exhibit R^2 = 0,9735 and the least accurate from the three investigated peaks is 1443 cm^{-1} with R^2 = 0,9682. Raman spectral data brings the possibility of relatively precise and simultaneously mainly fast and reagent free analyses.

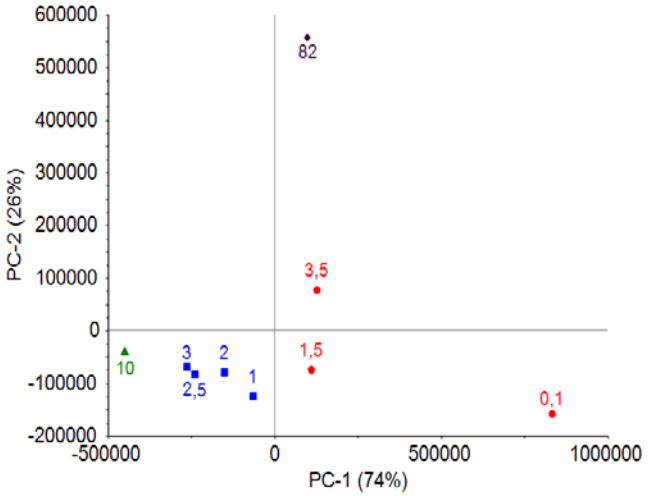

Fig. 13 PCA of the pure samples of milk, mixtures of milk, cream and butter (the label number indicate the content of fat in sample)

C. *Dairy products*

Two representatives of dairy products - cream with fat content 10 % and butter with fat content 82 % were also measured, apart from the milk samples. Their Raman spectra are depicted in Fig. 12 together with Raman spectra of sunflower oil and olive oil [13]. Areas representing saturated (1303 cm^{-1}) and unsaturated bonds in the fatty acids (1267 cm^{-1}) are clearly recognizable in the spectra and enable easily distinguish between the samples containing animal fats (butter, milk, cream, etc.) from vegetable oils.

The Principal Component Analysis applied to spectral data sets for pure milk samples (0,1 %, 1,5 %, 3,5% of fat), the prepared mixtures (1 %, 2 %, 2,5 %, 3 %) cream (10 %) and butter (82 %). Results in Fig. 13 show the similarity for samples mainly for mixtures and pure milks and their grouping. Also the diversity is evident mainly for the three extreme samples: skimmed milk with the lowest fat share and cream and butter with the highest fat share.

VI. Conclusion

Raman spectroscopy was used as an innovative method for measuring the fat contained in milk. To obtain more precise spectral response, the measurements were performed also on dried milk droplets and evaluated using PCA and regression method. The analysis of the content of amino acids including phenylalanine showed these amounts are not changing therefore the peak of phenylalanine was a suitable choice for normalizing the data. Results acquired in this study indicate that on the basis of characteristic bands for saturated fatty acids it is possible to distinguish different fat concentrations. Raman spectroscopic evaluation brings advantages over traditional methods mainly in sense of simplicity, rapidity and no use of chemical reagents with the only need to prepare the milk droplets. These aspects of measuring mean costs and time savings. Also the accuracy of this method is close to the conventional methods.

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