Raman spectroscopy as a modern tool for lactose determination

MARTINA BUCKOVA[1], HANA VASKOVA[2], ZUZANA BUBELOVA[1]
[1]Department of Food Technology, [2]Department of Electronics and Measurements
Tomas Bata University in Zlin
Nam. T. G. Masaryka 5555, 760 01 Zlin
CZECH REPUBLIC
vaskova@fai.utb.

Abstract: - Digestion of lactose, the milk disaccharide, makes problem to a large number of the population. Therefore, the lactose intolerant individuals are limited in the intake of milk and dairy products. The lactose – free products offers a solution for the diet. Raman spectroscopic analyses were performed for a purpose of rapid assessment of lactose content in bovine milk, lactose-free milk, mixtures of bovine milk and lactose-free milk, and bovine milk with additions of lactose. Based on characteristic vibrations, Raman spectra of lactose, glucose and galactose exhibit enough differences to distinguish the content of lactose in milk. C-O-H bending mode at 1087 cm^{-1} is used for lactose quantification. Two different substances – phenylalanine contained generally in the milk and crystal violet used as an internal standard, were studied for the evaluation of the spectroscopic data. The content of phenylalanine in samples was verified by IEC. Better accuracy exhibits the phenylalanine normalization. The contents of lactose and more easily digestible monosaccharides - glucose and galactose from the mixtures of milk and lactose-free milk were analysed by HPLC, Raman spectroscopy provided corresponding results. The study shows that Raman spectroscopy is an effective method for the feedback control of lactose.

Key-Words: - Lactose intolerance, lactose content, lactose-free milk, Raman spectroscopy, milk, phenylalanine, crystal violet, quantitative analysis

1 Introduction

Lactose determination in milk becomes more and more important for milk producers due to quite high number of lactose intolerant people in population. However, milk and dairy products are commonly considered as an important part of human diet, for many people is intake of milk followed with digestive problems caused by high lactose content. Removing all milk and dairy products from diet can solve problems in lactose-intolerant persons, on the other hand very good nutritive source is lost because milk and dairy product are generally considered as excellent source of proteins, calcium, phosphorus, magnesium, and other crucial macro- and micronutrients.

The simple disaccharide lactose represents about 5% of the milk content. In a human organism is decomposed by an enzyme lactase to monosaccharides. Different extent of lactase deficiency is described in most of the world's population. Nowadays, approximately 70% of adult population worldwide suffer from lactose intolerance. In most cases the intolerance is gained during the lifetime as genetically programmed decrease of the amount of lactase; however, in

a small content can be inborn. Many people should control lactose intake in their diet. A number of nutritional specialists warn of complete exclusion of dairy products from the diet because of the increased risk of inadequate intake of calcium and other minerals, resulting in risk of osteoporosis, etc.

To avoid elimination milk and dairy product from diet, producers have to manufacture lactose-free products. For meeting this requirement it is necessary to handle appropriate equipment for fast, simple and real-time determination of lactose content in milk or dairy products.

Raman spectroscopy is one of rapidly developing modern spectroscopic methods expanding into many areas such as biochemistry [1], material science [2], medicine [3], pharmaceutical industry [4] material/food quality control [5], etc. Raman spectroscopy as a vibrational spectroscopic method reflects chemical composition, structure and chemical bonding of materials and allows qualitative and quantitative analyses. Raman spectroscopy provides very specific chemical „fingerprint" of every single chemical substance on the basis of inelastic scattering.

Raman spectroscopy brings advantages over conventional techniques. In particular, rapidity,

no need of sample preparation and of chemical reagents can be highlighted for the lactose analyses. However, Raman spectroscopy brings many other benefits as the method is non-destructive, contactless, usable for measuring through transparent glass or polymeric covering layers or containers, applicable to all states of matter and different forms, usable as *in situ* analysis and does not interfere with water [6]. 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. Considering the advantages of the method, Raman spectroscopy becomes popular and valuable part of laboratories around the world in recent years.

2 Importance of lactose detection

Lactose assessment is necessary in terms of food technology and analyses of food products in connection with nutritional value and also lactose intolerance. The development and use of modern methods offers fast experimental procedures independent of a number of chemical reagents that serve for an effective feedback control of quality /content. Using Raman spectroscopy for quantification requires signal of any constant part of sample, and a calibration set of samples. In this paper two procedures are presented - the constant share of protein (detected as amino acid phenylalanine in Raman spectra) as a natural part of milk; and addition of crystal violet to the samples as an internal standard. Furthermore, the lactose and glucose content is studied in the mixtures of bovine milk and lactose-free milk.

3 Milk and lactose

Milk and dairy products are important part of human nutrition. Mammalian milk is first food for infants, and milk consumption lasts to adulthood in many parts of population on the world. In European and American population cow milk is the most frequently consumed, but sheep and goat milk is consumed also. The chemical composition of milk is influenced by animal species, environmental conditions, nutrition of animals, their lactation state and others. Main characteristics of milk are described in Table 1. Bovine milk is composed of 87% water, 4-5% lactose, 3-4% fat, 3% proteins, 0,8% minerals and 0,1% vitamins, on average [7].
As was mentioned above, lactose content can be individually important for many people. Lactose

Table 1. Average composition of goat, sheep, bovine and human milk [7].

	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

is the main carbohydrate present in milk. It is a disaccharide molecule composed by glucose and galactose. In human digestion there is enzyme called lactase (connected to small intestine membrane) that hydrolyses lactose to glucose and galactose. Chemical structure is shown in Fig. 1. This lactase activity decreases significantly after weaning, but it is individual and it does not happen at the same grade [7].
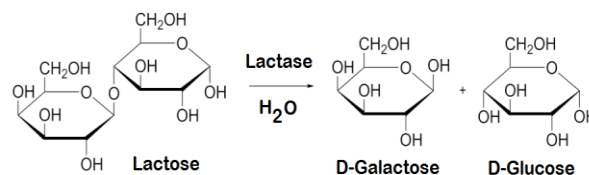

Fig. 1. Chemical structure of lactose, glucose and galactose.

It is estimated approximately 70% adult population worldwide suffer from lactose intolerance. This is strongly influenced by human origin. In an adult population in Europe and USA it is approximately 7 - 20 %, in some Asian countries it is close to 100% [8]. Similar situation is in African countries. Lactose intolerance causes problems in gastrointestinal tract due to non-hydrolysed lactose, which is fermented by microorganisms in the colon. The main symptoms are flatulence, abdominal cramps and bloating, diarrhoea, nausea and others [7].
Some years ago, it was necessary to avoid all products with lactose content. Today, milk producers offer many kinds of milk and dairy products with low lactose content. Cheese, curd cheese and fermented dairy products content low amount of lactose due to their technological origin.

On the other hand, products with high lactose content are milk, dried milk and cream. It is possible to found some lactose-free milk on the market.

3.1 Determination of lactose

Common methods for lactose determination in milk include gravimetric analysis, gas chromatography and most often high-performance liquid chromatography (HPLC). These methods are time consuming, need reagents and they are difficult for on-line analysis [9]. This is the reason for novel techniques development. For this purpose modern spectroscopy methods are often used. Near-infrared spectroscopy is one of those popular methods in dairy industry. However, it is necessary to adjust the determination due to water absorption interferences. Other new methods were developed using enzyme biosensors, which could be utilized for real-time lactose quantification in milk and dairy products [10]. Raman spectroscopy brings a significant contribution to substance, element and material identification.

4 Experimental part

4.1 Samples and chemicals

Samples were prepared from commonly sold milk (containing lactose) (LM) and lactose-free milk (LFM). Lactose, glucose and galactose from Sigma Aldrich were used as standards for HPLC calibration and to obtain Raman spectra of pure saccharides.

One set of samples was prepared from a common milk with additions of lactose to final amounts from 5,5 g/ 100 ml to 9,5 g/ 100 ml. Another set of samples contained also crystal violet (CV) as an internal standard [9].

Another set of samples was prepared as mixtures of LM and LFM in these ratios: 0-100, 20-80, 40-60, 60-40, 80-20 and 100-0.

4.1.1 Sugar content in milk by HPLC

The accurate contents of individual mono-saccharides and disaccharide were determined by HPLC. The data for common milk (LM), lactose-free milk (LFM) and the mixtures are listed in Table 2. The Fig. 2 reflects the share of lactose, glucose and galactose in mixtures.

Table 2 Content of lactose (LAC), glucose (GLU) and galactose (GAL) in mixtures of milk (LM) and lactose-free milk (LFM). NS = not specified.

LM:LFM [%]	LAC [g/100 ml]	GLU [g/100 ml]	GAL [g/100 ml]
LM~100:0	5,46 ± 0,12	NS	NS
80:20	4,17 ± 0,09	0,46 ± 0,01	0,48 ± 0,01
60:40	2,97 ± 0,07	0,94 ± 0,03	0,85 ± 0,02
40:60	1,92 ± 0,05	1,47 ± 0,04	1,48 ± 0,04
20:80	1,04 ± 0,03	1,93 ± 0,05	2,06 ± 0,06
LFM~0:100	NS	2,34 ± 0,06	2,39 ± 0,06

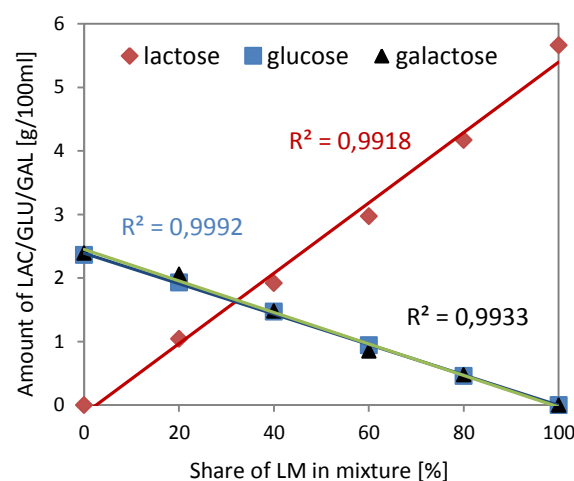

Fig. 2. Content of lactose, glucose and galactose in mixtures, HPLC results.

4.1.2 Phenylalanine content in milk by IEC

Phenylalanine (Phe) is one of essential amino acids. It occurs in all organisms, especially as a part of protein and must be supplied from a diet of animal of vegetable origin. Naturally it is found in a breast milk of mammals.

Amount of phenylalanine and 14 other amino acids in milk samples obtained after acid hydrolysis was assessed using ion-exchange liquid chromatography (IEC) as described in [11]. Phenylalanine was determined in common milk, lactose-free milk and also in all tested samples. The content was considered as constant (0,93 ± 0,04) g/kg. Under this condition, it is possible to use peak corresponding to phenylalanine for spectra normalization as is shown below in the result section.

4.2 Instrumentation

4.2.1 Raman microscope

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.

The acquisitions were collected with 5 second exposure time and 20 accumulations for milk and 1 second exposure time and 10 accumulations for carbohydrates. 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 then reduced to area from ca 300 to 1700 cm^{-1}.

4.2.2 HPLC-RI

Determination of lactose content in milk samples was carried on HPLC chromatograph Shimadzu LC-20AD Prominence with diferencial refractometric detector RID-20A, autosampler SIL 20AC (all Shimadzu Scientific Instruments) and Agilent Zorbax NH$_2$ column. Solution acetonitril: water in ratio 80:20 was used as mobile phase (acetonitil for HPLC, Sigma Aldrich).

4.2.3 IEC

Amount of phenylalanine in milk samples was determined by ion-exchange liquid chromatography (IEC). Amino Acid Analyzer AAA400 (Ingos, Prague, Czech Republic) was used for this analysis [11].

5 Results

All spectroscopic measurements were performed on dried milk droplets. After experiences with measurements of milk samples to evaluate the fat content in milk [12] and the appearing significant luminescence of liquid milk samples (see Fig. 3), the form of dried milk droplets on aluminium plates was considered for the measurements and evaluation.

Firstly lactose, glucose and galactose were measured. Raman spectra are shown in Fig. 4.

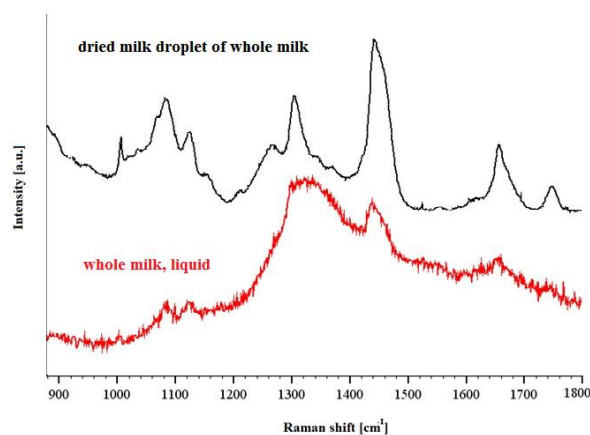Secondly Raman spectra of milk (LM), lactose-free milk (LFM) and mixtures of LM and LFM were

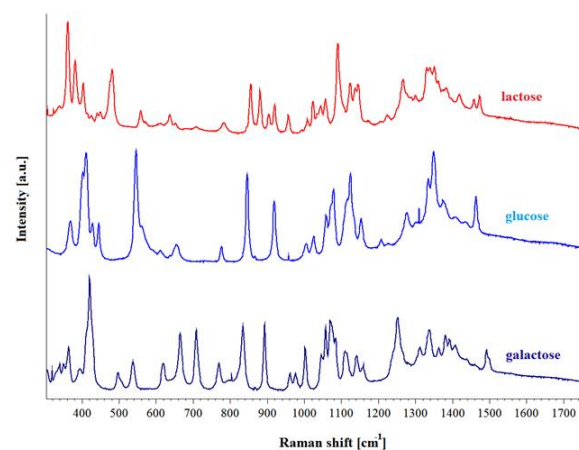Fig. 3. Raman spectra of dried milk droplet and the liquid milk (from the top).

Fig. 4. Raman spectra of lactose, galactose and glucose (from the top).

measured. Spectra are displayed in Fig. 5 (top). The Raman peaks of milk with the assignments are listed in [12]. The main differences of the lactose and lactose-free milk can be observed in region 400 cm^{-1} – 600 cm^{-1}, that is the area for endocyclic and exocyclic deformation bands [13], band 918 cm^{-1} corresponding to C-C stretching of glucose (see Fig. 5 - lower left), and mainly in the area around 1070 cm^{-1} – 1090 cm^{-1}. Clearly visible is intense band 1087 cm^{-1} corresponding to lactose vibrations C-O-H bending mode (see Fig.5 lower right). The band 1087 cm^{-1} is used for lactose content evaluation in the sample sets.

Acquisition of Raman spectra of the samples with lactose additions was the next step.

The cubic spline interpolation was used for the baseline correction to remove background luminescence. Removal of the luminescence allows better identification of the most relevant peaks.
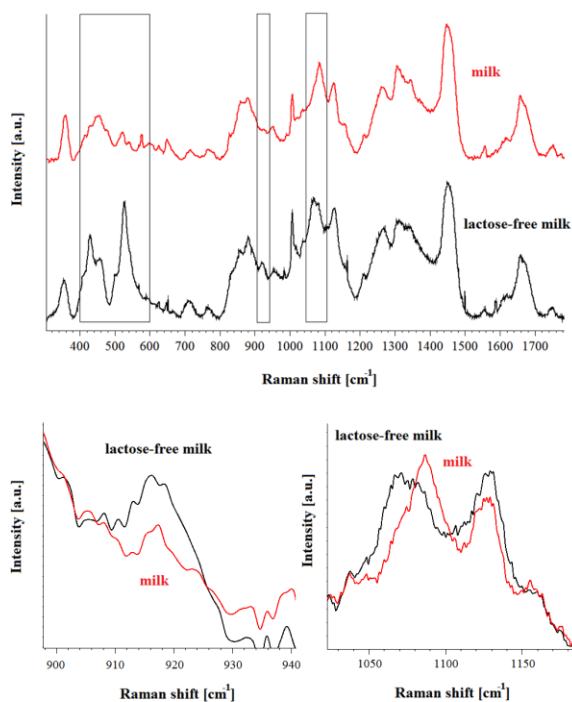
Fig. 5. Raman spectra of milk and lactose-free milk (at the top). Raman band 918 cm^{-1} for glucose (lower left) and the band 1087 cm^{-1} for lactose (lower right).

Two methods were used for assessment of the lactose content. First method based on constant content of phenylalanine as was verified by IEC. Second one uses crystal violet (CV) as an internal standard. The Raman spectra of LM, LM with addition of lactose and CV are displayed in Fig. 6. Furthermore, the contents of lactose in mixtures (LM-LFM) were analyzed from Raman spectral data and amounts of individual saccharides in the samples were verified by HPLC.
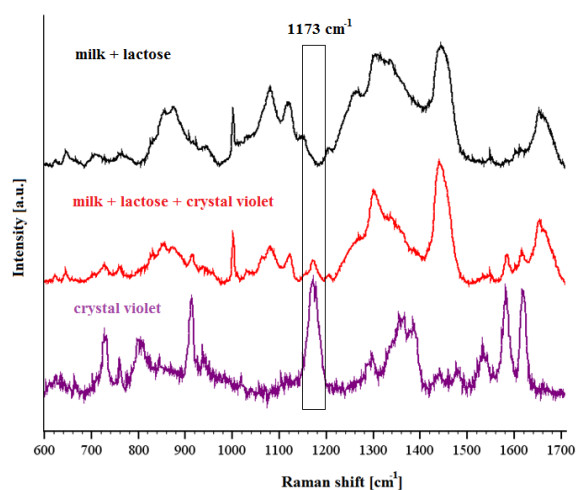

Fig. 6. Raman spectra of milk, milk with lactose additions and crystal violet.

5.1 Phenylalanine normalization

Raman spectra were normalized according to 1003 cm^{-1} band, which is characteristic for phenylalanine ring breathing band [14]. C-C twisting mode of phenylalanine can be found at 648 cm^{-1}.

Phenylalanine is indicative of protein content in the sample. The dependence of the ratio of Raman intensities from bands 1087 cm^{-1} and 1003 cm^{-1} and the content of the lactose exhibits a steady increase, with quite well correlation as can be seen from Fig. 7.
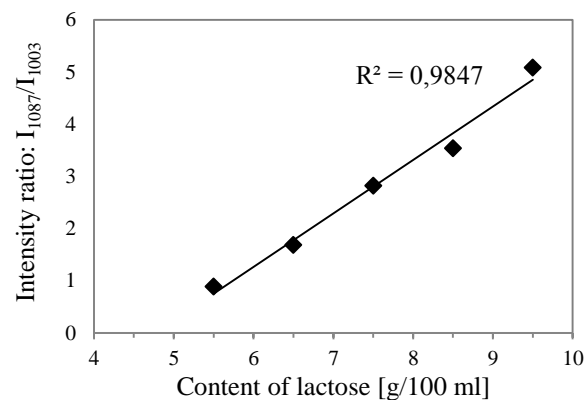

Fig. 7. The increase of Raman intensity ratio I_{1087}/I_{1003} corresponding to content of lactose in milk using phenylalanine as the standard.

5.2 Crystal violet normalization

Crystal violet was used as an internal standard and band 1173 cm^{-1} was taken for normalization as the most intense band in spectrum of CV, with a solitary position. Results acquired from Raman spectral data by linear regression shows (in Fig. 8.) not so precise linear behavior ($R^2 = 0.9122$) in comparison with the phenylalanine normalization ($R^2 = 0.9847$). However, the linearly rising trend is observed also when using CV.
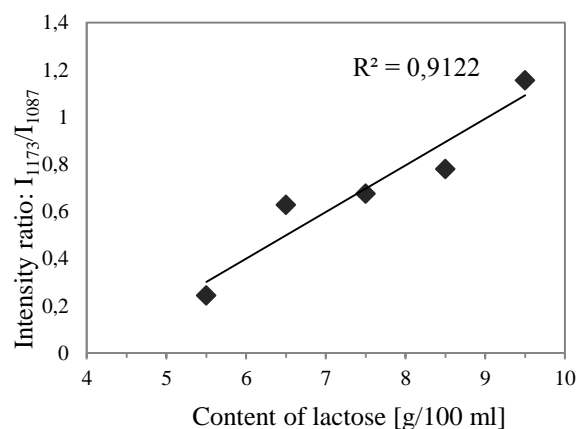

Fig. 8. The increase of Raman intensity ratio I_{1173}/I_{1087} corresponding to content of lactose in milk using CV as an internal standard.

5.3 Assessment of lactose in mixtures

The lactose content was studied in six LM-LFM mixtures. The aim was to obtain samples of milk with decreasing amounts of lactose. Motivation was the lactose-free milk production using enzymatic cleavage of lactose to glucose and galactose and also the possible utilization of Raman spectroscopic measurement for a fast control during the technology process or for output control.

Raman spectroscopic measurement confirmed the changing lactose content with the quite high accuracy of linear dependence, what is illustrated in Fig. 9 by the ratio of Raman intensities from bands 1087 cm^{-1} for lactose and 918 cm^{-1} for glucose.
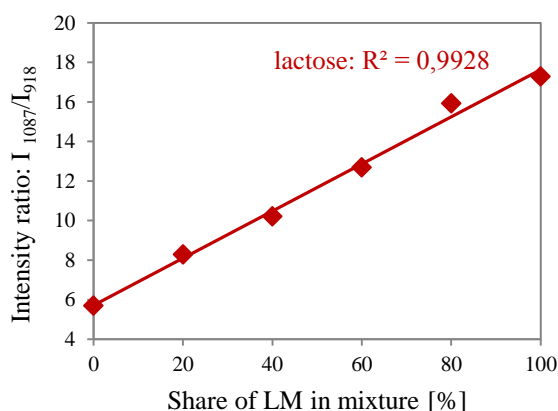

Fig. 9. Raman intensity ratio I_{1087}/I_{918} corresponding to increasing lactose content in LM-LFM mixtures.

6 Conclusion

Raman spectroscopy was used as an innovative method for measuring the lactose content in milk. Measurements were performed on dried milk droplets in order to obtain more precise spectral response. Acquired spectral data show the possibility to distinguish different lactose concentrations on the basis of characteristic bands for lactose, phenylalanine or crystal violet. Normalization using phenylalanine exhibits better accuracy and also easier procedure without adding other chemical reagents. Another confirmation of lactose assessment was presented on mixtures of milk and lactose-free milk.

Raman spectroscopic evaluation brings advantages mainly in terms of simplicity, rapidity, no use of chemical reagents with the only demand to prepare the milk droplets and can serve as an effective tool for lactose assessments.

References:
[1] E. Smith, and G. Dent, *Modern Raman Spectroscopy: a Practical Approach,* John Wiley & Sons, 2013.
[2] W. H. Weber, and R. Merlin, *Raman Scattering in Materials Science,* Springer Science & Business Media, 2013.
[3] K. Kong et al., Raman Spectroscopy for Medical Diagnostics—From In-vitro Biofluid Assays to In-vivo Cancer Detection, *Advanced Drug Delivery Reviews*, Vol. 89, 2015, pp. 121-134.
[4] Y.S. Li, and J. S. Church, Raman Spectroscopy in the Analysis of Food and Pharmaceutical Nanomaterials, *Journal of Food and Drug Analysis*, Vol. 22, No. 1, 2014, pp. 29-48.
[5] H. Vaskova, and M. Buckova. Thermal Degradation of Vegetable Oils: Spectroscopic Measurement and Analysis, *Procedia Engineering*, Vol. 100, 2015, pp. 630-635.
[6] J. M. Chalmers, G. H. Howell, and M. D. Hargreaves, *Infrared and Raman Spectroscopy in Forensic Science,* Chichester, West Sussex, UK: Wiley, 2012.
[7] P.C. Pereira, Milk Nutritional Composition and Its Role in Human Health, *Nutrition,* Vol. 30, No. 6, 2014, pp. 619-627.
[8] S.B. Matthews, J.P. Waud, A.G. Roberts, A.K. Campbell, Systemic Lactose Intolerance: A New Perspective on an Old Problem. *Postgraduate Medical Journal*, Vol. 81, No. 953, 2005, pp. 167-173.
[9] M. Li, J. Chen, J. Xu, S. Fu, H. Gong, Determination of Lactose in Milk by Raman Spectroscopy, *Analytical Letters*, Vol. 48, No. 8, 2015, pp. 1333-1340.
[10] K. Kaniyamattam, A. De Vries, Agreement between Milk Fat, Protein, and Lactose Observations Collected from the Dairy Herd Improvement Association (DHIA) and a Real-time Milk Analyzer, *Journal of Dairy Science,* Vol. 97, No. 5, 2014, pp. 896-2908.
[11] F. Buňka, O. Kříž, A. Veličková, L. Buňková, S. Kráčmar, Effect of Acid Hydrolysis Time on Amino Acid Determination in Casein and Processed Cheeses with Different Fat Content, *Journal of Food Composition and Analysis*, Vol. 22, No. 3, 2009, pp. 224-232.

[12] H. Vaskova, M. Buckova, L. Zalesakova, Spectroscopic analysis of milk fat and its mathematical evaluation, *International Journal of Biology and Biomedical Engineering*, Vol. 10, 2016, pp. 168-175.

[13] J. De Gelder, K. De Gussem, P. Vandenabeele, L. Moens, Reference Database of Raman Spectra of Biological Molecules, *Journal of Raman Spectroscopy,* Vol. 38, 2007, pp. 1133-1147.

[14] C.M. McGoverin, A. S. S. Clark, S. E. Holroyd, K. C. Gordon, Raman Spectroscopic Quantification of Milk Powder Constituents, *Analytica Chimica Acta*, Vol. 673, No. 1, 2010, pp. 26-32.