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MILK PROTEIN ANALYSIS: AN OVERVIEW OF THE METHODS – DEVELOPMENT AND APPLICATION

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Abstract

Milk protein content is an important component of milk, especially from a nutritional point of view and also for payment purposes. The aim of work was to draw up an overview on reference and routine methods for protein determination. Reference methods perform accurate analyses comply according to the International Standard ISO whereas routine methods perform analyses using routine instrumental techniques for faster and cheaper results with acceptable accuracy and a large number of processed samples. In most of cases, using of routine indirect methods for milk protein analysis requires their specific calibrations according to biological kind of measured milk (cow's, goat's or sheep's milk) or specific conditions of milk technology treatment. Also, the quality control measures have a significant role for result determination reliability.

Keywords: dairy cow, goat, sheep, raw milk, nitrogen matters, crude and true milk proteins, reference methods, routine methods

INTRODUCTION

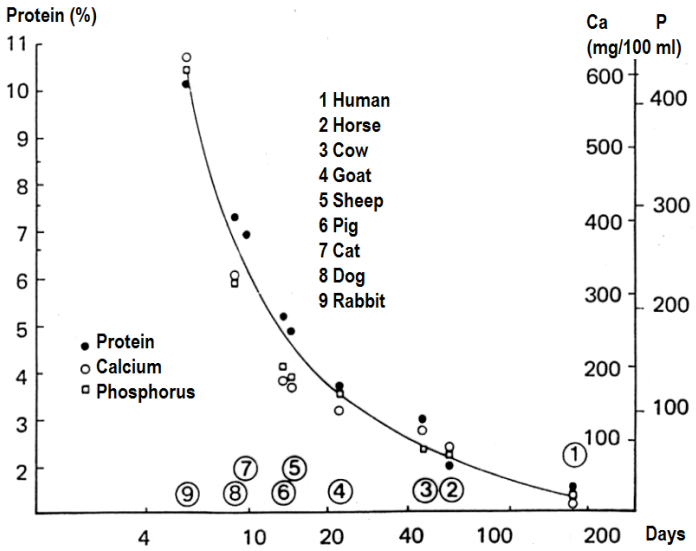
Milk is a complex food containing basic nutrients (e.g. proteins, lipids, vitamins) with positive health benefits (Haug *et al.*, 2007; Finete *et al.*, 2013). The proteins have a biological activities – acting as growth factors, hormones, enzymes, antibodies and immune stimulants (Korhonen *et al.*, 1998; Clare and Swaisgood, 2000).

Proteins are undoubtedly the most important organic substances. Their basic biochemical-physiological role in the life process is generally well known. In the case of milk proteins, their importance for selected mammals has been very well expressed graphically by Renner (1982) in the past century (Fig. 1) and this scheme has a generalizing character. The explanation deals with the composition of calcium phospho-caseinate and its relationship to the growth rate of youngsters of various mammalian species. Although the view of milk proteins from a nutritional point of view has also undergone critical opinions, mostly substantively

unfounded, especially in the field of human nutrition, this scheme has not been overcome so far in terms of its comprehensibility expressing the biological potential of proteins. Therefore, it is not surprising that chemical analysis of protein content, structure and properties is practically very important in the biological sciences.

Milk proteins are composed from protein, includes casein (about 80%) and whey proteins (about 20%) (Zhang *et al.*, 2016), and non-protein nitrogenous compounds (Walstra *et al.*, 2006). Tab. I describes individual fractions of milk protein. Lactoferrin, immunoglobulines and lysozyme are also contained in the whey fraction (Lönnerdal, 2004).

Content of milk proteins may be negatively influenced by high intake of dietary fat by the lactating cow (Jenkins and McGuire, 2006); however, it can be also positively influenced by high intake of other energy nutritional sources (Erbersdobler *et al.*, 1980; Kirchgessner *et al.*, 1985, 1986). The specific minimum standards must be met in order for payment to be received by milk producers in the Czech Republic. These minimum standard values are required, in accordance with standard CSN 57 0529, protein (28 g · l^{-1}), and solid



1: *Protein content (%; Y-axis, left), Ca and P concentration (mg • 100 ml^{-1}; Y-axis, right), and growth rate (time to double birth weight in days) of mammalian offspring.*
Reference: modified by Renner (1982)

I: *Individual fractions of cow's milk protein.*

Protein	Content (%)	Content (g · l^{-1})	Subtypes
Casein protein fractions			
α_s-casein	42	13.4	α_{S1}-casein, α_{S2}-casein
β-casein	25	8.0	
γ-casein	4	1.3	
κ-casein	9	2.9	
Whey protein fractions			
α-lactalbumin	4	1.3	
serum albumin	1	0.3	
β-lactoglobulin	9	2.9	
immunoglobulin	2	0.6	IG_G, IG_A, IG_M, IG_D, IG_E
polypeptides	4	1.3	proteoses, peptones

Reference: Velíšek and Hajšlová (2009)

non-fat content (8.5%) (CNI-Czech Normalization Institute, 1993).

Therefore, the aim of our work was to describe and compare reference and routine methods used for analysis of content of milk proteins. Furthermore, the overview describes quality control measures and applications of mentioned methods.

Development of measurement methods

Requirements of quality (Kessler, 2013) and animal genetic improvement (Hanuš *et al.*, 2006) in the food industry stimulate the development of analytical techniques capable of precise component quantification at a reasonable price of analysis (Kessler, 2013). In terms of this opinion, several analytical reference and routine methods are reported for milk protein determination and quantification. In general, the performance of reference method results is usually more reliable and also more expensive than routine method results. These protein results are important and necessary for milk quality assessment at its payment and dairy cow breeding improvement by milk recording in the historical and also present practice of dairying. For this reason, more routine methods for milk protein determination have been developed during last hundred years.

One of the way for quantitative and also direct, research and possible reference determination of nitrogen in milk is method by Dumas (Jakob *et al.*, 1995). This method was described by Jean-Baptiste Dumas in the early 19th century. In principle, the sample is burned (approximately 900 °C) in pure oxygen. This leads to release of carbon dioxide, water, and nitrogen oxides. The gases pass through special sorption columns that absorb carbon dioxide and water. Nitrogen is measured of the chemiluminescence reaction of the NO_2 or in the elemental form by thermal conductivity detection (e.g. device LECO FP-428 series by LECO Corporation, USA). Under the Czech Republic conditions, the method is regulated by CSN EN ISO 14891:2002 (COSMT-Czech Office for Standards, Metrology and Testing, 2002). The method can be applied to raw milk, drinking milk (whole, semi-skimmed and skimmed milk), cream and milk products (cheese, yoghurt).

Formol titration is the next fast, practical and technological method in the dairy system to determine milk protein. The method was described by Steinegger (1905) and modified by Pyne (1932), Cole (1969) and Peeples and Heath (1979). Moreover, Taylor (1957) developed two modifications of method – direct and indirect. In principle, free amino acids and protein-bound amino acids and peptides react with formaldehyde, producing methylene amino acid derivatives and changing the pK_a of these amino groups (Moore *et al.*, 2010). The value pK_a = –log 10 K_a is the co-logarithm of acid dissociation constant (acid dissociation constant K_a is a quantitative measure of the strength of an acid in solution), which measures the tendency for a group to give up a proton (Hu *et al.*, 2018). The method can be applied to raw milk, drinking milk (whole, semi-skimmed and skimmed milk), milk powder or ice cream.

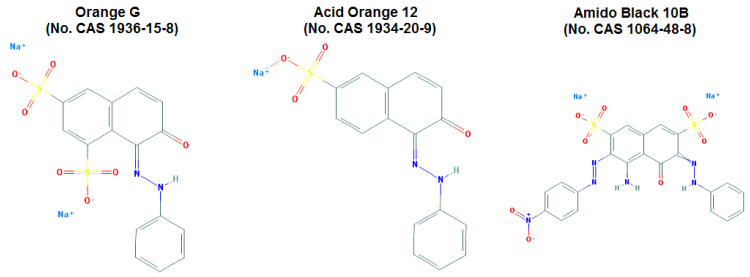
In thirties of 20th century, coulometry was described by Szebellédy and Somogyi (1938) as practical, direct, technological and also possible reference method. In principle, the method applies laws of electrolysis. The material for analysis is placed in an electrolytic cell and it is neutralized, oxidized, reduced and, eventually precipitated by a measured quantity of electricity (Taylor and Smith, 1959). Subsequently, the Faraday's laws are used to determine the amount of chemical substance. Coulometry is one of the most accurate techniques of chemical analysis (Lingane, 1958; Curran, 1996; Bard and Faulkner, 2001). In the dairy analyse, this method is based on measurement of mineralized original sample material after concrete type of mineralization similar to Kjeldahl method with careful selection of the catalyst (copper sulphate and potassium sulphate, 1 : 10) to sulphuric acid, since selenium and other used metals may disturb (in their oxidative forms) their own determination. Under our conditions, one variant of analytical measurement for raw milk was described by Brauner *et al.* (1981). They found no significant ($P > 0.05$) mutual differences between results of coulometry procedure and Kjeldahl. So, above mentioned time-demanding processing is the reason why it is not too much quick procedure in practice a point of view. Than, an ammonium ion is determined by coulometric titration and biamperic indication (Bořecký and Olbrecht, 1974). In this way, coulometric titration can successfully replace the classical distillation and titration used in Kjeldahl procedure. The method can be applied to raw milk and drinking milk (whole, semi-skimmed and skimmed milk).

Dye-binding methods are used to determine milk protein as indirect, routine procedures. Several authors (Fraenkel-Conrat and Cooper, 1944; Owusu-Apenten, 2002; Urh, 2008) described these methods. The most commonly used dyes are Orange G (no. CAS 1936-15-8), Acid Orange

12 (no. CAS 1934-20-9) and Amido Black 10B (no. CAS 1064-48-8) whose 2D chemical structure is illustrated in Fig. 2 (Ashworth, 1966; Sherbon, 1978; Aalaei *et al.*, 2016). The dyes belong to a group so called "azo dyes" which have sulfonic groups in their molecules. In acid-buffered solutions, negatively charged sulfonic groups in azo dyes are stoichiometrically connected with positively charged constituents in proteins to form an insoluble dye-protein complex (Moore *et al.*, 2010). Subsequently, the insoluble complex is separated and the unbound dye can be determined by spectrophotometer (e.g. Pro-Milk with using of Amido Black 10B by Foss Electric, Denmark). The apparatus was also used in automated version for routine protein determination in milk quality payment systems (bulk milk samples) and in regular milk recording laboratories (individual milk samples). Usually, it was calibrated according to results of Kjeldahl reference method on crude protein measurement. The correlation coefficients between milk protein results of these methods can be 0.887 ($P < 0.01$; Carboné *et al.*, 1976), 0.952 ($P < 0.001$; Hanuš and Ficnar, 1990) and 0.99 ($P < 0.001$; Renner and Ömeroglu, 1971). In general, this Amido Black method started routine milk protein analysis in dairying (Renner and Ömeroglu, 1971; Grappin, 1992). By modification of this Pro-Milk procedure, there is possible to determine not only crude and true milk protein but also casein and whey protein via relevant methods of instrument calibration and milk chemical fractionation. Tab. II summarizes individual methods.

Quality Control Measures

The most important conditions for proper analysis is sampling accuracy, samples manipulation and quality control measures. The protein content is not so much sensitive to milk sampling accuracy as fat content, nevertheless it may be influenced by this factor as well (Hanuš *et al.*, 2011). Therefore, bulk or individual milk sampling is provided according to the standards for sampling CSN EN ISO 707 (COSMT, 2009) to ensure proportionality and mediocrity of sample.



2: *2D standardized chemical structure of dyes used for dye-binding methods.*
Reference: NCBI – National Center for Biotechnology Information (2006, 2008, 2009)

II: *Comparison of individual methods.*

Method	Principle	Use	Reference
Dumas	a burning of sample in pure oxygen (a releasing of CO_2, water and NO_x and measuring of the chemiluminescence reaction by TCD)	milk and dairy products	COSMT (2002)
Formol titration	free amino acids and protein-bound amino acids and peptides react with formaldehyde, producing methylene amino acid derivatives and changing the pK_a of these amino groups	milk and dairy products	Moore *et al.* (2010)
Coulometry	a measuring of quantity of electricity (applying Faraday 's laws) after previous neutralization, oxidation, reduction, eventually precipitation (organic material has to be mineralized in mixture with concentrated sulphuric acid and catalyst under conditions of high temperature for longer time, similarly to Kjeldahl method)	raw milk	Brauner *et al.* (1981)
Dye-binding	a forming an insoluble dye-protein complex (a stoichiometrically connection of negatively charged sulfonic groups in azo dyes with positively charged constituents in proteins → separation of the insoluble complex and determination of the unbound dye by spectrophotometer)	milk and dairy products	Owusu-Apenten (2002)

NO_x – nitrogen oxides; TCD – thermal conductivity detection

The quality control measures serve as a mechanism for verifying the reliability of the results. In addition to compliances of standard operating procedure (SOP), it also includes internal quality controls (with quality control material), supervising working processes in audits from time to time (Glanzmann *et al.*, 2017), external quality control – proficiency testing (Grappin, 1993; Hanuš *et al.*, 1998; Leray, 2009a, 2009b, 2010; Kaarls *et al.*, 2017), two different methods for the same sample, precision of evaluation – repeatability, reproducibility (Verrezen *et al.*, 2017) and blank samples. The basic statistic principles and design rules of proficiency testing for reference and routine methods at raw milk protein and other components determination were proposed and given at national and international level by more authors (Grappin, 1987a; Leray, 2009a, 2009b, 2010). For these result reliability control reasons, the milk laboratory networks were proposed and organized according to scheme (Grappin, 1993; Fig. 3) as main progress system measure. It can be stated that this measure was a basic step at worldwide improvement of routine result reliability of milk analyses. This general scheme is based on a mutually effective link between the levels of reference and routine laboratories through control activities and the distribution of reference materials to the checking and calibration of indirect methods for reliable results.

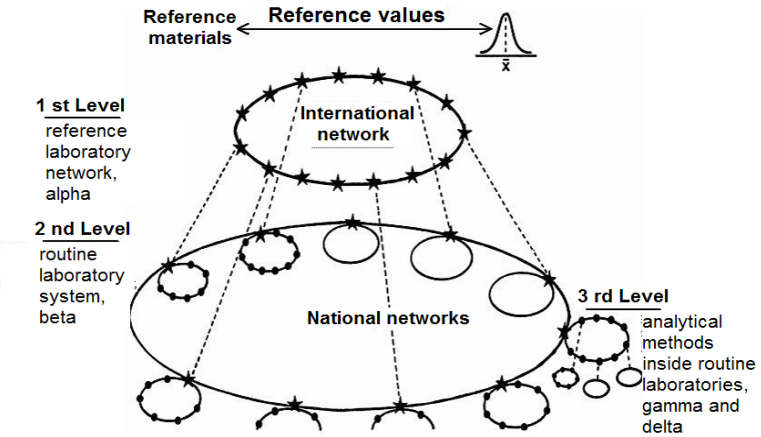
Quality control material of known concentration is valuable in internal quality controls (Gargis *et al.*, 2012) and it comprises control milk and positive and negative control for different types of analytes (components). Preparation of reference materials and control milk starts with analysis of raw milk for primary and fast composition and quality evaluation. Then, milk is properly mixed with preservatives (bronopol or sodium azide for composition analytes) addition and it is dosed into plastic vials. Subsequently, milk can be refrigerated after dosing.

The system of proficiency testing is based in the Czech Republic (Hanuš *et al.*, 1998, 2006, 2014) to relevant standards – CSN 57 0530 (CNI, 1973), CSN 57 0536 (CNI, 1999) and CSN EN ISO/IEC 17025 (CNI, 2005). Benefits of proficiency testing are not only demonstrating non-errors and errors of the results, but also for confirming competent performance by comparison with other participants which can identify possible problems, e.g. problems with calibration (Glanzmann *et al.*, 2017). The procedure of proficiency testing is described in "Statistical methods for use in proficiency testing by inter-laboratory comparison" (ISO, 2010).

Reference methods

The reference methods are thoroughly studied and defined measurement procedures. These methods are used to assess the reliability of other measurement procedures (Šprongl and Paulík, 2011).

Reference method is linked to the primary methods of measurement defined as "a method having the highest metrological properties, whose operation can be completely described and understood, for which the complete uncertainty statement can be written down in terms of SI units" (Milton and Quinn, 2001). Primary methods used for chemical measurements include coulometry, gravimetry, titrimetry and colligative methods (Quinn, 1997).



3: *Worldwide scheme for national and international milk laboratory network for carrying out of checking activities and proficiency testing and distribution of reference materials to reach improvement in reliability of analytical results.*
Reference: Grappin (1993)

The Kjeldahl method (Tab. III) is the most commonly used method in determination of protein nitrogen (Lima *et al.*, 1999). The method was described by Samuel J. Rowland (1938a, b) as the analytical procedure for fractionation, measurement, and classification of N-containing compounds in milk (Wojciechowski and Barbano, 2015). Briefly, approximately 5 g (with precision of 0.0001 g) of cow's milk is dissolved by sulphuric acid and catalyst tablets (copper sulphate). Tablets also contain potassium sulphate and titanium dioxide. The function of the potassium sulphate is to elevate the boiling point of the sulphuric acid and to provide a stronger oxidizing mixture for digestion. At this point, the organic nitrogen compounds are converted to ammonium sulphate, using block digestion unit (e.g. device Kjeldatherm® series by C. Gerhardt GmbH and Co. KG, Germany). Subsequently, sodium hydroxide to the cooled digest is added to liberate ammonia. Next, steam vapour distillation using distillation apparatus (e.g. device Kjeldatherm® Vapodest series by C. Gerhardt GmbH and Co. KG, Germany) is carried out into the excess boric acid solution. Then titration with hydrochloric acid standard volumetric solution is carried out. Under the Czech Republic conditions, the method is regulated by CSN EN ISO 8968-1:2014 and 8968-2:2014 (COSMT, 2014a, b). The method can be applied to raw milk and milk (whole, semi-skimmed and skimmed milk).

Milk protein analyses are historically very specific in their approach. According to the convention, crude protein is analysed in most countries (according to Kjeldahl's classic, mineralization-distillation-titration method: total nitrogen × 6.38). This approach is less laborious and may involve less manipulation errors during analysis; nevertheless it is burdened by a certain error (theoretical and practical as well) arising from inclusion the content of relatively variable non-protein nitrogen matters into proteins (Renner, 1980; Barbano and Dellavalle, 1987; Grappin, 1987a, 1992; Barbano and Lynch, 1990, 1992; Barbano *et al.*, 1991; Barbano and Clark, 1989; Grappin and Lefier, 1993; Hanuš *et al.*, 1995). In some countries (e.g. USA, France), the milk protein content is more accurately measured, calibrated and expressed in % as true protein (protein N × 6.38). These aspects need to be taken into account in the practical comparison of milk protein contents among countries.

Thus, reference methods perform accurate analyses, which comply with the internal SOP according to the ISO International Standards. In general, reference methods, when compared to routine, have better practical links to reliability and control but they also have some practical disadvantages which can be summarized as follows: – higher labour consumption; – higher time consumption; – usually higher material consumption; – higher analytical costs; – lower analytical efficiency with respect to the number of samples per unit of time.

Routine methods

Protein content is routinely monitored during the commercial production and in the final products (Bogomolov *et al.*, 2012).

An important routine indirect method for determination of milk proteins between 1960 and 1985 was the dye-binding spectroscopy method (Pro-Milk). In the world (e.g. Denmark, Germany, Netherlands), this method was used to determine proteins in the payment system (bulk milk samples) and to determine proteins in milk recording system for cattle genetic improvement (individual milk samples). In the Czech Republic, it was used only for research purposes. For practically routine analyses, dye-binding spectroscopy method was used formerly than the infrared (IR) spectroscopy. The development of the routine application of milk protein analysis methods has also been linked to the development of the proficiency testing system (Grappin, 1993; Hanuš *et al.*, 1998; Leray, 2009a, 2009b, 2010; Kaarls *et al.*, 2017).

Currently, the protein content of milk is analysed through automated flow analysers (Silveira *et al.*, 2004) which are based on the mid-infrared (MIR) spectroscopy (Biggs, 1978; Coleman and Moss, 1989; Botaro *et al.*, 2011). These analysers use optical filter technology (MIR) or whole infra-red spectrum scan in mid-range (MIR-FT, Fourier transform). Goulden (1964), Aernouts *et al.* (2010) and other authors

III: *Definition of individual reference method.*

Method	Principle	Use	Reference
Kjeldahl	a mineralization of sample (with concentrated H_2SO_4) by boiling → N present in the form of various functional groups is converted to NH_3, which remains bound in the form of $(NH_4)_2SO_4$ → N is liberated from the $(NH_4)_2SO_4$ by alkalization, and titrated	milk	COSMT (2014a, b)

showed that IR spectroscopy was introduced to determine the milk chemical composition fifty years ago. In principle, the electric field of incident radiation interacts with the molecular dipole and simultaneously the frequency of the radiation (~10^{13} • Hz) resonates with the molecular vibration, whereby absorption can occur, particularly if excitation of that vibration has an effect on the molecular dipole moment (Beden and Lamy, 1988; Stole *et al.*, 1991; Benziger, 1995). The energy changes involved in exciting vibrational modes in this way correspond to the IR spectral region (Bard and Faulkner, 2001).

Milk has pronounced light-scattering properties due to the presence of suspended casein micelles (Walstra *et al.*, 2006; Frisvad *et al.*, 2007). The next optically important elements in milk include vitamin B_2 and fat globules (Crofcheck *et al.*, 2002). Attaie and Richter (2000) showed that casein micelles only scatter light and the refractive index is η_{casein} = 1.503 in the visible range.

A complex size distribution of scattering particles significantly complicates the spectroscopic analysis of milk, especially in the region of visible (Vis) light (400–700 nm), where the scatter is essentially stronger than in near-infrared range (NIR) (Bogomolov *et al.*, 2012). It follows that use of the Vis region is not appropriate for protein determination. Bogomolov *et al.* (2012) suggested that there is no scatter-based method for quantitative determination of total protein in milk. However, a few published works exploiting the Vis region for milk analysis exist (e.g. Muñiz *et al.*, 2009). Next, the spectral ranges, which are used in spectrometry, include: short-wave infrared (SWIR), NIR, MIR, long-wave infrared (LWIR) or thermal infrared (TIR) (Van der Meer, 2018).

Biophysical methods including MIR-FT spectroscopy (e.g. device CombiScope FTIR series by Delta Instruments B.V., Netherlands) or Nuclear Magnetic Resonance (NMR) can be used to characterize protein and thus provide important information on conformational and secondary structure changes and their causes (Devi *et al.*, 2011). In principal, MIR-FT is a biochemical fingerprinting technique (Nicolaou *et al.*, 2010). The method can be applied to raw milk, drinking milk (whole, semi-skimmed and skimmed milk), cream and milk products (yoghurt).

Another well-known feature of reference analyses (Kjeldahl method) of milk proteins is the application of the conversion factor (6.38), which represents the average percentage (15.67%) of nitrogen in milk protein. However, this approach is still a theoretical application of practical experience and must be continually controlled in the light of specific circumstances. Karman and van Boekel (1986) have been extensively dealt with by Kjeldahl's factor theory. The following fractionated factors for the Kjeldahl method were given: – for milk proteins 6.34 rather than 6.38; – for casein 6.34; – for paracasein 6.29; – for the genetic variants of α_{S1}-casein from 6.32 to 6.39; – similarly for β-casein from 6.31 to 6.39; – similarly for γ-casein from 6.08 to 6.35; – for the genetic variants of κ-casein 6.37 and 6.38; – for whey protein 6.3; – for non-protein nitrogen matters 3.6. Grappin (1992) mentioned Kjeldahl's factors for casein, whey protein, and non-protein nitrogen (NPN) in the milk at 6.35, 6.38 and 3.6. Grappin (1992) at the same time cited higher accuracy in protein testing of true protein calibrated devices against crude protein calibrations with a high correlation coefficient (−0.8) between errors and a percentage of NPN in total nitrogen. All of these reasons also suggest the possibility of differences in factors among biological types of milk. Therefore, species-specific calibrations of indirect methods for the measurement of proteins in cow, sheep and goat milk as well as in other milk species are necessary (Grappin, 1987b; Hanuš *et al.*, 2009) but not only from this reason. The need for a species-specific calibration of the IR method according to the Kjeldahl method results and the possibility of sources of errors in its non-compliance may, besides the possible interspecies variability of the Kjeldahl factor, be different. E.g. for IR spectroscopy with optical filters (MIR) technology, the credibility of goat milk protein content values analysed at cow calibration of IR spectroscopy (Grappin, 1987b) is unsatisfactory. There exist also different interference effects between milk matrix of biological milk kinds, it means between cow and goat milk for protein, which is given by lower citrate concentration in goat milk as compared to cow. Under such conditions, goat milk absorbs less IR energy at the protein wavelength than cow milk (3 to 4% less) and requires a different calibration from cow and sheep milk. Part of this difference is the result of the lower citric acid content of goat milk than cow milk. Citric acid absorbs the wavelength at the protein by its R-COO^- groups and in this way goat milk is regularly overestimated by 0.134% of protein on average. Unfortunately, this does not have to be valid in such a determined degree at the present-day modern technology of IR analysis of the whole spectrum with Michelson interferometer and FT, as it can be inferred by the results of Lefier *et al.* (1996). Another practical reason for species-specific calibration of

indirect methods may be, for example, significant differences in protein content among biological types of milk (cow and goat *vs*. sheep).

For the analysis of milk protein content, there is also successfully used an indirect routine IR spectroscopy procedure in the NIR range often with Fourier transform in multi-purpose adjustment with almost limitless calibration possibilities for various components and materials from liquid milk over yoghurt to cheese (Tsenkova *et al.*, 1999, 2000; Kukačková *et al.*, 2000; Jankovská and Šustová, 2003; Šustová *et al.*, 2007). At solid materials there is used a mechanical spinner for homogenization; however, actual homogenization of the liquid milk sample to the correction of the optical scattering effects of the fat globules by their size unification is generally not performed. Therefore, it is possible to declare greater suitability (Lefier *et al.*, 1996; Hanuš *et al.*, 2009, 2014) as well as the effectiveness of flow analysers MIR (Voort *et al.*, 1987) and MIR-FT with homogenization of samples to analyse the content of liquid milk fat and proteins.

The next important routine method for milk composition determination is the ultrasound analytical procedure which has also to be calibrated according to reference method results (Perlín, 2003; Hanuš *et al.*, 2014). Also, the blue (opto-unit) and red (thermal unit) box method (BRB) is used for milk determination (Hanuš *et al.*, 2014). BRB is based on nephelometry (blue box, measurement by impedance or conductance) and thermo-analytical method (red box, two measurement temperature: 40, resp. 65 °C) (e.g. device Lactostar 3510® series by Funke-Dr. N. Gerber Labortechnik GmbH, Germany).

In general and according to results of more authors (Tsenkova *et al.*, 1999, 2000; Kukačková *et al.*, 2000; Jankovská and Šustová, 2003; Šustová *et al.*, 2007; Hanuš *et al.*, 2009, 2014), the reliability of raw milk protein results (in terms of gradual decreasing of individual difference (variability) standard deviation value between routine and reference method results (MDsd)) by routine methods in often practical use is growing up in the following order: IR spectroscopy with filter technology and in mid-range of spectrum (MDsd = 0.035 ± 0.01 %) = IR spectroscopy with whole spectrum and FT in mid-range of spectrum (0.033 ± 0.013 %) > IR spectroscopy in near-range of spectrum > analyse method by blue and red box (0.091 ± 0.03 %) > ultrasound method (0.127 ± 0.046 %). This reliability order is also given by growing up of correlation coefficients between routine and reference milk protein results in the same sources. From these figures also method specific relevant limits of calibration quality for indirect analytic procedures have statistically derived (Hanuš *et al.*, 2014). These can be important at practical laboratory work as reference values for relevant comparisons during calibration activities.

Raman spectroscopy is suitable for the analysis of solid and liquid samples. McGoverin *et al.* (2010) used Fourier transform Raman (FT-Raman) spectroscopy for quality control and quantitative analysis of milk constituents (protein, fat, lactose, dry matter) in powdered milk. FT-Raman spectroscopy is an efficient technique for the investigation of structural changes of protein molecules (Liang *et al.*, 2006). Eryilmaz *et al.* (2017) used surface-enhanced Raman spectroscopy (SERS) for analysis of skimmed milk. SERS gives enhanced Raman signal from an analyte molecule which was adsorbed on metal surfaces.

Bassbasi *et al.* (2014) used FTIR spectroscopy with attenuated total reflectance (ATR), coupled with chemometric methods (e.g. partial least squares regression, PLS). This has been applied to the fast and non-destructive quantitative determination of solid non-fat (SNF) content in raw milk. Previously, the method was applied by Moros *et al.* (2006) for determination of constituents (protein, fat and lactose content) of yoghurt samples.

Nuclear magnetic resonance (NMR) spectroscopy provides unique qualitative and quantitative information regarding the physical state of milk protein (Belloque and Ramos, 1999). Also the structural characteristics can be determined using NMR spectroscopy. The method can be applied to skimmed milk, cheese and ice cream.

Today's development of other routine indirect methods for protein content measuring based on real time analysis (RTA) is directed to permanent control of the composition of individual milk samples during each milking (Katz, 2007; Ishay *et al.*, 2011). Based on the development of relevant hardware and software, this process leads to ongoing animal health control and possibility of prevention of cow production disorders (mastitis, ketosis) and milk quality improvement as well as control of their reproduction performance (Katz and Pinsky, 2008; Arazi *et al.*, 2012; Durkin, 2012). The cases of marked improvement in milk quality and dairy herd health were mentioned in a certain period of time after the practical introduction of the RTA system of measurement and result evaluation into function. The RTA system works as a flow, stepper IR spectroscopy in the near area (e.g. device Lely Astronaut and AfiLab™ (NIR) series by S.A.E. Afikim, Israel). For example, this measurement may be combined with the outputs

of the milk electrical conductivity determination and other quantities for assessment of other indicators such as the somatic cell count. The RTA system in the milking parlor is calibrated monthly according to routine analyser reference values based on the results of regular milk recording. AfiLab method (as alternative) showed less close relationships as compared to the reference values as relationships between results of reference methods (MIR and MIR-FT). This was expected. However, these relationships as correlation coefficients were mostly significant, for protein from 0.284 to 0.787 ($P > 0.05$ and $P \leq 0.001$). It can explain up to 61.9% of variability in reference values (Hanuš *et al.*, 2016). The results of Lely Astronaut RTA reliability (as alternative) as coefficients of determination (R^2) and correlation (r) of results between methods (RTA and MIR reference) were 0.744 ($P < 0.001$) for milk protein determination. In this case R^2 explains 55.3% of the variability of RTA values by reference method (MIR and MIR-FT) variations (Pecová *et al.*, 2017). Representative milk component and property values for animals are calculated as averages from more consecutive milking cases. This procedure increases probability of result reliability. Therefore, the reliability of the results of RTA milk protein analyses has been found to be suitable for the above mentioned purposes of dairy cow herd management. Tab. IV summarizes individual routine methods.

IV: *Comparison of individual routine methods.*

Method	Principle	Use	Reference
Ultrasound procedure	a measurement of high-frequency ultrasound radiation passing through the sample material	milk	Perlín (2003); Hanuš *et al.* (2014)
BRB	an indirect method based on combination of thermo-optical process	milk	Lactostar (2005)
Pro-Milk	a dye-binding photocolorimetry	milk	Hanuš *et al.* (1998)
NMR	based on the absorption of radiofrequency electromagnetic radiation of the nuclei of some atoms (e.g. ^{1}H-, ^{13}C-) in the molecules of the analysed samples located in the magnetic field	milk and dairy products	Belloque and Ramos (1999)
IR spectroscopy	**a measurement of infrared irradiation (in near, mid etc. spectrum) absorbed or reflected by sample; it occurs changes in the rotational-vibrational energy states of the molecule depending on variations of the dipole moment of the molecule**		
MIR	IR in the mid-range using optical selective filters with the specific wavelength corresponding to the measured component	milk	Sjaunja (1984); Hill *et al.* (1991)
MIR-FT	Fourier transform of IR with using the Michelson interferometer (in whole spectrum); vibrational fingerprints of molecules with integral transformation (conversion of the signal from time domain to frequency domain)	milk and dairy products	Lefier *et al.* (1996); Nicolaou *et al.* (2010)
NIR-FT	IR in the near-range with Fourier transform	milk (raw)	Sato *et al.* (1985, 1987); Šustová *et al.* (2006)
NIR (RTA)	a flow, stepper IR spectroscopy in the near area	milk	Katz (2007); Pecová *et al.* (2017)
FT-Raman	an interaction between photons of incident light with rotational-vibrational energy states of the molecule (the scattered irradiation has a different wavelength than the incident irradiation)	milk and dairy products (dried)	McGoverin *et al.* (2010)
ATR-FTIR	a reflective technique (occur to total reflection of the IR beam from modified prism – diamond, Ge etc.)	milk; yoghurt	Bassbasi *et al.* (2014); Moros *et al.* (2006)
SERS	an amplified signal adsorbed on the surface of Ag, Au, and Cu (amplification depends on surface morphology, "rough surface" → nanoparticles)	milk (dried)	Eryilmaz *et al.* (2017)

BRB – blue and red box; NMR – nuclear magnetic resonance; IR – infrared; MIR – mid-infrared; NIR – near-infrared; RTA – real time analysis; FT – Fourier transform; ATR – attenuated total reflectance; SERS – surface-enhanced Raman spectroscopy

CONCLUSION

An overview showed that the reference method (Kjeldahl) is more demanding due to the preparing of milk samples and necessity of results recalculation. Using of routine indirect methods for milk protein analysis requires their specific calibrations according to biological kind of measured milk. There is necessary: – to take attention to correct calculation with nitrogen reference method factors and fraction of nitrogen matters when calibrate indirect protein analyse methods; – to keep in regard the stated limits of calibration quality indicators.

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