AN OVERVIEW OF DETERMINATION OF MILK FAT: DEVELOPMENT, QUALITY CONTROL MEASURES, AND APPLICATION

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Abstract

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Milk fat content is an important indicator of milk quality because of nutritional and technological aspects of dairying. In this sense the milk fat determination is important practice procedure. The work goal was to do an effective overview and comparison of reference and routine methods of fat determination during their development. Nowadays, there exist a number of methods for determining milk fat content. Reference methods require accurate analysis in compliance with the International Standard ISO, whereas routine methods perform analysis using routine instrumental techniques for faster and cheaper results with acceptable accuracy. Quality control measures have a significant role for result determination reliability and they include internal quality controls, external quality controls, precision of evaluation, and blank samples. In conclusion, due to continuous development and improvement, routine methods will be used more often.

Keywords: milk fat, raw cow's milk, reference methods, routine methods

INTRODUCTION

Milk quality is an important factor in the dairy industry. One of the major factors of milk quality is composition, which affects the properties of the milk. Milk fat plays a significant role not only in the nutritional, physical, and chemical properties of milk, but also in the purchasability of milk (Upadhyay *et al.*, 2018). Therefore, specific minimum standards of must be met in order for payment to be received by milk producers in the Czech Republic. These minimum standard values are, in accordance with standard CSN 57 0529, fat (33 g · l⁻¹), protein (28 g · l⁻¹), and solid non-fat content (8.5%) are required (CNI-Czech Normalization Institute, 1993).

Milk fat is the most variable component of ruminant milk (Samková *et al.*, 2012). The fat content can vary from about 3% to 6%, but typically in the range 3.5% to 4.7% (Lock and Bauman, 2011). Milk fat is composed of triacylglycerols (TAGs), diacylglycerols, phospholipids, free fatty acids, and cholesterol (Jensen and Newburg, 1995). The TAGs are the main component and represent about 97–98% of the total milk fat content (Vieitez *et al.*, 2016).

The aim of our work is to describe and compare reference and routine methods used for analysis of milk fat. Furthermore, this overview describes quality control measures and applications for the mentioned methods.

Development of measurement methods

Quality requirements in the food industry stimulate the development of analytical techniques capable of precise component quantification at a reasonable price of analysis (Kessler, 2013). Therefore, several analytical methods are reported for milk fat determination and quantification. In general, the efficiency of reference method results are usually more reliable, and also more expensive, than routine method results. For this reason, more routine methods for milk fat determination have been developed during the last hundred years.

The Butyrometric method is an operating method which can be used as a reference method for routine analytical procedures. The method was developed by the Swiss chemist and dairy-owner Niklaus Gerber (Gerber, 1891). In principle, proteins (mainly phospholipid envelopes of milk fat globules) are dissolved by sulfuric acid. Then, the addition of amyl-alcohol results in a sharp interface. Subsequently, the fat is quantitatively released and separated by centrifugation. Fat volume is read on the butyrometer scale which is calibrated to indicate the fat content in percentage by weight (ISO-International Standards Office, 1976). Under the conditions in the Czech Republic, this method is regulated by the COSMT-Czech Office for Standards, Metrology and Testing (2001) – CSN ISO 2446:2001. This method can be applied to raw milk, drinking milk (whole, semi-skimmed and skimmed milk), milk powder, cream and dairy products (cheese, yoghurt). In the USA and Canada the Babcock method is used with the same analytical principle as a modification of the Butyrometric method (CAES, 1894).

The Folch method (1957) is another method for fat content determination. In principle, lipid extraction is carried out with chloroform and methanol (e.g. 2:1). Then, the homogenate is filtered. The procedure enables extraction of lipid classes for subsequent fatty acid analysis (Budge *et al.*, 2006). After extraction, transesterification (formation of fatty acid methyl esters) is carried out. This method is commonly used for marine products (Ofstedal *et al.*, 2014). However, the Folch method was used for milk fat determination of Weddell seal (*Leptonychotes weddellii*) (Wheatley *et al.*, 2008) and bovine milk (Liu *et al.*, 2016).

Similarly, the Bligh and Dyer method (1959) is used for extraction of fat. In principle, this method is also based on extraction using chloroform and methanol, but it was developed for lipid extraction of lean fish tissue (Iverson *et al.*, 2001). After extraction, the homogenate is filtered and filtrate is transferred to a graduated cylinder. After allowing a few minutes for complete separation and clarification, the volume of the chloroform layer is recorded and the alcoholic layer removed by suction. Nevertheless, this method was used

by Nascimento *et al.* (2017) for determination of fat content in commercial dried dairy products.

Maxwell *et al.* (1986) used a “dry-column method” for determination of fat. Firstly, the sample is dried by anhydrous sodium sulphate and diatomaceous earth (e.g. product Celite® 545 by Supelco–Sigma-Aldrich Corp., USA). Subsequently, extraction is accomplished using a mixture of dichloromethane and methanol. The lipids may be isolated and simultaneously separated into neutral and polar fractions by a sequential elution procedure. Neutral lipids (without polar lipids) are eluted first with dichloromethane, followed by elution of polar lipids with the dichloromethane/methanol (9:1) mixture (Marmer and Maxwell, 1981). This method can be applied to raw milk, drinking milk (whole, semi-skimmed and skimmed milk) and buttermilk.

Supercritical fluid extraction (SFE) was investigated by Eller and King (1996) as an alternative to solvent-based extraction methods (Manganiello *et al.*, 2000). The sample is weighed to the extraction thimble, previously loaded with 1 g of diatomaceous earth. a temperature of 100 °C and sc-CO₂ (supercritical carbon dioxide) fluid density of 0.60 mg·ml⁻¹ are used for extraction. Every fluid is characterized by a critical point, which is defined in terms of the critical temperature and critical pressure, thus CO₂ is supercritical above 31.1 °C and 7.38 MPa (Sahena *et al.*, 2009). Sc-CO₂ is removed exclusively nonpolar lipid material. Wolf *et al.* (2003) used SFE for analysis of milk infant formula powder whereas Astaire *et al.* (2003) applied method on buttermilk powders (with using microfiltration).

The Weibull-Berntrop gravimetric method involves Soxhlet extraction (García-Ayuso *et al.*, 1999) and it is used for special dairy products–infant formula (ISO–Part 1, 2005), ice creams (ISO–Part 2, 2005) or special cases (ISO–Part 3, 2005). In principle, the method include hydrolysis (by hydrochloric acid) and extraction (by *n*-hexane). Next, the solvent was released in a rotary evaporator, followed by cooling in a dessicator and weighing. Tab. I summarizes individual methods.

QUALITY CONTROL MEASURES

The most important factors of correct analysis are sampling accuracy, sample manipulation, and quality control measures. Fat content is sensitive to sampling accuracy and may be influenced by this factor (Hanus *et al.*, 2008). Therefore, bulk or individual milk sampling is done according to the standards for sampling CSN EN ISO 707 (COSMT, 2009) to ensure accurate results.

The quality control measures serve as a mechanism for verifying the reliability of the results. Aside from compliance with the standard operating procedure (SOP), other measures include internal quality control (with quality control material), supervision of the working processes with audits (Glanzmann *et al.*, 2017), external quality

control–proficiency testing (Kaarls *et al.*, 2017), two different methods for the same sample, precision of evaluation–repeatability and reproducibility (Verrezen *et al.*, 2017), and blank samples. Basic statistical principles as well as rules of proficiency testing for reference and routine methods of raw milk fat determination were followed. These principles and rules are valid at the national and international level (milk laboratory networks) as many authors emphasized (Grappin, 1987, 1993; Hanuš *et al.*, 1998; Leray, 2009a, 2009b, 2010).

Quality control material based on known composition is valuable in internal quality controls (Gargis *et al.*, 2012). This material is comprised of control milk and positive and negative controls for different components (fat, protein, and others). Preparation of control milk starts with analysis of raw milk (primary and fast detection of composition and quality evaluation). Then, milk is properly mixed with preservatives (bronopol or sodium azide) and is dosed into plastic vials. Subsequently, milk can be refrigerated after dosing.

Under the Czech Republic conditions (Hanus *et al.*, 2014), the proficiency testing system is based on relevant standards—CSN 57 0530 (CNI, 1973), CSN 57 0536 (CNI, 1999) and CSN EN ISO/IEC 17025 (CNI, 2005). Proficiency testing not only reveals any errors in the results, but also provides the possibility for comparison with other participants who can identify potential 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).

Before each analysis, samples are prepared by heating in a water bath, gently mixing, and then cooling to the room temperature. This procedure

applies to particular samples designated in the calibration. To verify the accuracy of the results, it is also important to prepare a blank sample.

REFERENCE METHODS

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 methods are linked to the primary methods of measurement defined as “methods having the highest metrological properties, whose operation can be completely described and understood, for which a 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).

The ultimate precision method for determination of milk fat content is the Röse-Gottlieb method (Bogomolov *et al.*, 2017), which replaced the Butyrometric method described above. The Mojonnier analytical method, which is essentially the same as the Röse-Gottlieb method with its similar reference extraction (Choi *et al.*, 2015), is used in the USA and Canada for much the same reasons that the Röse-Gottlieb method is used over the Butyrometric method (Kleyn *et al.*, 1988). In short, approximately 10 g (with precision of 0.0001 g) of cow’s milk is hydrolysed by ammonia. Subsequently, ethanol is added. Then fat is extracted with diethyl ether and petroleum ether (repeated liquid-liquid extraction, LLE). Fat content is weighed after evaporating the solvents (e.g. device SMT-Moplant series by Flohr Instrumenten and

I: Comparison of individual methods.

Method	Principle	Use	Reference
Butyrometric	a dissolving of protein (particularly phospholipid envelopes of milk fat globules) by sulfuric acid	milk and dairy products	COSMT (2001)
Folch	a two-phase partition of the lipid fraction in the organic (chloroform) phase (chloroform-methanol-water mix to create a two-phase system)	milk	Liu <i>et al.</i> (2016)
Bligh and Dyer	a two-phase partition of the lipid fraction in the organic (chloroform) phase (chloroform-methanol-water mix to create a two-phase system)	milk and dairy products (dried dairy products)	Nascimento <i>et al.</i> (2017)
Dry column	an extraction of lipid by solvent elution (with using a dry column composed of an anhydrous sodium sulphate and diatomaceous earth)	milk and dairy products	Maxwell <i>et al.</i> (1986)
Supercritical fluid extraction	a separation technology that uses supercritical fluid as the solvent (sc-CO ₂)	infant formula powder; buttermilk powder	Wolf <i>et al.</i> (2003); Astaire <i>et al.</i> (2003)
Weibull-Berntrop	a gravimetric method including hydrolysis by hydrochloric acid and extraction by <i>n</i> -hexane	infant formula, ice cream and special products	ISO–Part 1-3 (2005)

Sc-CO₂—supercritical CO₂

Apparatenbouw, Netherlands). Under the Czech Republic conditions, this method is regulated by CSN EN ISO 1211:2011 (COSMT, 2011). This method can be applied to raw milk and drinking milk (whole, semi-skimmed and skimmed milk).

In conclusion, reference methods (Tab. II) perform accurate analyses, which comply with the internal SOP according to the ISO International Standards. By contrast, reference methods have several disadvantages—time-consuming process of analyse and fewer analysed samples.

ROUTINE METHODS

Efficient routine analysis of milk fat is an important part of dairy production (Zhu *et al.*, 2015). Nowadays, the aim is to develop fast and cost effective analytical methods that enable the identification of components with various physico-chemical properties in a single analytical run (Tankiewicz and Biziuk, 2018). Next, routine analyses are joined with routine implementation of performance-related tests for milk payment purposes under the Czech Republic conditions (Hanuš *et al.*, 1998).

The main routine milk fat measurement principle from 1950 to 1980 for milk payment and milk recording laboratories in Europe was turbidimetry (nephelometry), (e.g. Electronic Milko Tester by Foss Electric, Denmark). Turbidimetric analysis was based on the observed correlation between fat content and the detected elimination of light dispersed by a milk sample at specific wavelengths (Ashworth, 1969). At present, this method is obsolete. Furthermore, the turbidimetric method is sensitive to the size variability of milk particles (e.g.

milk fat globule), and, the testing of raw milk samples without prior treatment (by homogenization) is not very appropriate (Kucheryavskiy *et al.*, 2014). This method can be primarily applied to drinking milk (whole, semi-skimmed and skimmed milk). On the other hand, Hanuš *et al.* (2014) described good results also with raw milk samples. The next routine method for determination of milk fat and other milk components is the ultrasound analytical procedure (Perlín, 2003).

Optical spectroscopy methods have the potential to replace or at least to complement some of the classical laboratory methods (El-Abassy *et al.*, 2011). Spectroscopic methods of determination of fat are mainly based on the components absorption. The precondition is minimal to no scatter (Rinnan *et al.*, 2009). The spectral ranges (Fig. 1) that are commonly used in spectrometry include: visible (VIS), short-wave infrared (SWIR), near-infrared (NIR), mid-infrared (MIR), long-wave infrared (LWIR), or thermal infrared (TIR) light (van der Meer, 2018).

The VIS light region (360–780 nm), where the scatter strongly dominates, is rarely used in quantitative milk analysis (Crofcheck *et al.*, 2000). Cabassi *et al.* (2013) reported, that particularly in the region below 1100 nm, the scatter strongly dominates over weak absorption. Nevertheless, Bogomolov *et al.* (2013) describes scatter-based quantitative analysis of fat in the raw milk using VIS and SWIR spectroscopy. According to these authors, difference of individual spectral elements of scatter (e.g. wavelength dependence) at different-sized milk fat globules are said to be sufficient for their quantitative analysis using formal multivariate modelling, e.g. partial least-squares (PLS) regression.

II: Comparison of individual reference methods.

Method	Principle	Use	Reference
Mojonnier	a gravimetric method → separation the fat fraction (limited to the lipophilic ether phase) from the rest of the milk sample	milk	Choi <i>et al.</i> (2015)
Röse-Gottlieb	a gravimetric method including hydrolysis by ammonia and extraction by diethyl ether and petroleum ether (repeated LLE)	milk	COSMT (2011)

LLE—liquid-liquid extraction

1: Electromagnetic spectrum.

The NIR spectroscopic quantification of milk components makes use of the main region from 1100 to 2500 nm and relies on the component absorption overtones and combination bands (Kalinin *et al.*, 2008; Aernouts *et al.*, 2011). Upadhyay *et al.* (2018) showed that the spectral range for NIR region is from 750 to 2 500 (13 400–4 000 cm^{-1}), while for MIR it is between 2 500 and 25 000 nm (4 000–400 cm^{-1}). Tsenkova *et al.* (1999) describes the statistical accuracy (in terms of root mean-square error–RMSE) on the MIR spectroscopic analysis for fat content at very high complexity of PLS regression calibration models. NIR and MIR (Bogomolov and Melenteva, 2013; Feng *et al.*, 2013) are widespread for determination milk parameters, respectively milk fat (Bogomolov *et al.*, 2017). For example, a study by Mlček *et al.* (2016) reveals the determination of fat in milk using NIR which is calibrated by two routine methods (Gerber and Röse-Gottlieb). Nowadays, apparatuses based on MIR spectroscopy are the most used in industrial laboratories (e.g. MilkoScan series by Foss Electric, Denmark).

Fourier transform infrared (FTIR) spectroscopy (e.g. apparatus CombiScope FTIR series by Delta Instruments B.V., Netherlands) is rapid, easy to

apply and useful for routine analysis (Wolff *et al.*, 1998). In principal, FTIR is a biochemical fingerprinting technique (Nicolaou *et al.*, 2010). During measurement, IR radiation is absorbed as it passes through the sample, which leads to changes in the rotational-vibrational energy states of the molecule depending on variations of the dipole moment of the molecule. This method can be applied to raw milk, drinking milk (whole, semi-skimmed and skimmed milk), cream and dairy products (yoghurt).

Also, Raman scattering, especially Fourier transform Raman (FT-Raman) spectroscopy, gives access to the vibrational fingerprints of molecules (El-Abassy *et al.*, 2011) and similarly to FTIR, it has been demonstrated to be successful for the determination of nutritional parameters, e.g. in conjugated linoleic acids in cow's milk fat (Meurens *et al.*, 2005).

FTIR and attenuated total reflectance (ATR) have been used by van de Voort *et al.* (1992) for determination of fat and moisture contents in butter. Inon *et al.* (2004) used FTIR and ATR techniques with chemometric analyses (multivariate statistics) to classify drinking milks. Next, diffuse reflectance

III: Comparison of individual routine methods.

Method	Principle	Use	Reference
Turbidimetric analysis	a measurement of intensity light passing through the sample in the original direction	milk	Ashworth (1969)
Ultrasound procedure	a measurement of high-frequency ultrasound radiation passing through the sample	milk	Perlín (2003)
MW irradiation	a part of the electromagnetic wave spectrum and have wavelengths ranging from 1 mm to 1 m	milk	García-Ayuso <i>et al.</i> (1999)
NMR	based on the absorption of radiofrequency electromagnetic radiation of the nuclei of some atoms (e.g. ^1H -, ^{13}C -) in the molecules of the analyzed samples located in the magnetic field	milk and dairy products	Belloque and Ramos (1999)
TD-NMR	a method consist in a combined relaxation analysis (the magnetization at a certain time is determined by both the longitudinal (T1) and the transverse (T2) NMR relaxation processes)	milk and dairy products	Nascimento <i>et al.</i> (2017)

IR spectroscopy	a measurement of infrared irradiation (in near, mid etc. spectrum) absorbed or reflected by sample; occur to changes in the rotational-vibrational energy states of the molecule depending on variations of the dipole moment of the molecule		
FTIR	vibrational fingerprints of molecules with integral transformation (conversion of the signal from time domain to frequency domain)	milk and dairy products	Nicolaou <i>et al.</i> (2010)
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	Meurens <i>et al.</i> (2005)
ATR-FTIR	a reflective technique (occur to total reflection of the IR beam from modified prism–diamond, germanium etc.)	milk; butter	Inon <i>et al.</i> (2004); Van de Voort <i>et al.</i> (1992)
DRIFTS	a reflective technique (diffuse reflection consist in focusing the IR beam on a sample, mostly in a powder form)	milk	Pappas <i>et al.</i> (2008)

MW–microwave; NMR–nuclear magnetic resonance; TD–time-domain; IR–infrared; FT–Fourier transform; ATR–attenuated total reflectance; DRIFTS–diffuse reflectance Fourier transform infrared spectroscopy;

Fourier transform infrared spectroscopy (DRIFTS) has been applied to distinguish sheep milk from goat milk (Pappas *et al.* 2008). In principle, diffuse reflection consist in focusing the IR beam on a sample. The diffuse scattered irradiation is converted by a suitable optical device to the spectroscope detector (Machovič and Novák, 1998).

In general and according to the results of other authors (Tsenkova *et al.*, 1999; Kukačková *et al.*, 2000; Jankovská and Šustová, 2003; Hanuš *et al.*, 2009, 2014; Mlček *et al.*, 2016), the reliability of fat contents in raw milk (in terms of the gradual decreasing of individual differences (variability) in standard deviation value between routine and reference method results) by routine methods (in practical use under the conditions of their today technical design) is growing in the following order: IR spectroscopy with filter technology and in mid-range of spectrum = IR spectroscopy with whole spectrum and FT in mid-range of spectrum > IR spectroscopy in the near-range spectrum $\geq$ turbidimetric method > ultrasound method. The reliability order is also given by a growing correlation between coefficients of routine and reference results of milk fat in the same sources.

Microwave (MW) irradiation is the next method used for determination of fat. This method is used to accelerate hydrolysis of the matrix and extraction step of milk lipids (García-Ayuso *et al.*, 1999). MW irradiation have been developed as an alternative to conventional Soxhlet extraction (García-Ayuso

and de Castro, 1999). Despite this, the method has quantitative results similar to the Weibull-Berntrop extraction procedure but with lesser chemical transformation of triglycerides. However, MW irradiation is not used much for fat determination. Garber and Thole (2015) used this method for buffered dilute non-fat milk sample.

Nuclear magnetic resonance (NMR) spectroscopy provides unique qualitative and quantitative information regarding the physical properties (e.g. crystallization, thermal properties) of milk fat (Belloque and Ramos, 1999). Also, the structural characteristics (e.g. binding, spatial distance) can be determined using NMR spectroscopy. The method can be applied to raw milk, drinking milk (whole, semi-skimmed and skimmed milk), cheese, and ice cream.

Time-domain (TD)-NMR spectroscopy has been an alternative technique for fat analysis (Castell-Palou *et al.*, 2013; Pereira *et al.*, 2015). Compared with lipid extraction or NIR spectroscopy methods, TD-NMR has certain benefits. TD-NMR is rapid, non-invasive and provides analysis of the bulk milk composition (Nascimento *et al.*, 2017).

In conclusion, routine methods (Tab. III) perform analyses using routine instrumental techniques for faster and less accurate results. Utilizing routine methods, a large of number of samples can be analysed. However, the disadvantages are the high acquisition cost of the apparatus equipment and the necessity of verification.

CONCLUSION

Nowadays, routine methods are an integral part for checking the quality of milk and dairy products. However, reference methods are also very important in terms of detailed description and verification. Reference method results are used regularly in individual milk laboratories and laboratory network, not only for routine method calibration purposes but also for estimation of combined enlarged uncertainties of measurement results by routine methods. Compared to routine methods, reference methods have the disadvantage of being limited in the number of determined samples. In the future, due to continuous development and improvement, routine methods will be used more often.

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