EVALUATION OF DEVELOPMENT IN INDIRECT DETERMINATION OF MILK FAT FREE FATTY ACIDS IN CZECH REPUBLIC

O. Hanuš, E. Samková, J. Říha, M. Vyletělová, P. Roubal

Determination and interpretation of free fatty acids (FFAs) in milk

Small portion of fatty acids in milk which are not esterified in triglycerides is freely diffused mostly in fat phase and a little bit in water phase and it is called as free fatty acids (FFAs). Current content of FFAs in milk fat lies between 0.5 and 1.2 mmol.100g^{-1} and maximal enabled is 13.0 mmol.kg^{-1} for method by churning and 32.0 mmol.kg^{-1} for method by extraction and titration according to CSN 57 0529 and CSN 57 0533 (Cvak *et al.*, 1992). Gerber´s acidobutyrometrical method for milk fat determination holds as many as 90% of FFA content into milk fat portion but on the contrary the extraction gravimetric method according to Röse-Gottlieb does not include FFAs into fat portion so reliably and thus 70% of them is lost in this way (Kerkhoff Mogot *et al.*, 1982).

Increase of FFAs means negative impacts as lipolysis usually from reason of metabolic problems of dairy cow (Fig. 1). Increased concentration of FFAs causes an aggravation of milk technological properties (Vyletělová *et al.*, 2000 a, b) but mostly deterioration of sensory milk properties as taste and flavour. After that it has lightly bitter smack as consequence and this can damage quality of dairy products. Fat destruction is phenomenon which is caused by native enzymes as lipases (Antonelli *et al.*, 2002; Deeth, 2006; Ferlay *et al.*, 2006) in milk or by lipases which are supplied by bacterial contamination of milk. Therefore lipolysis is spontaneous or induced (Fig. 1). Of course, lipases can be thermoresistent and thus in this way to influence milk also after its heat treatment by dairy product degradation. Wasteful milk handling as often pumping and ripple at manipulation (Sjaunja, 1984; Thomson *et al.*, 2005; Hanuš *et al.*, 2008 b; Genčurová *et al.*, 2009 and 2011) and its freezing on technology

surfaces also induce own lipolysis. Heat and mechanical energy which is added into multicomponent milk system destroys the membranes of fat globules and thus releases fatty acids from esteric linkage of triglycerides. Therefore, milk stream (Peterková, 2002) should not exceed the speed 1 till 1.5 $m.s^{-1}$. Poor hygiene of dairy cow stabling and milking as well as bad storage and treatment of raw milk can lead to propagation of undesirable psychrotrophic, thermoresistant and sporulating milk microflora (Vyletělová *et al.*, 1999 a, b; Cempírková and Thér, 2000; Cempírková, 2001, 2002, 2007; Dankow *et al.*, (2004); Hanuš *et al.*, 2004; Foltys and Kirchnerová, 2006 and 2010; Hantis–Zacharov and Halpern, 2007; Torkar and Teger, 2008; Cempírková and Mikulová, 2009; Cempírková *et al.*, 2009). The mentioned facts can increase the lipolysis intensity (Fig. 1).

Definition of FFAs and paper goal

FFAs are a mixture of fatty acids released from milk fat by lipolysis or such which overpassed from animal blood and body fat tissues. In terms of proportions this mixture is influenceable by animal nutrition and health state, season and other factors which means that this is very hardly seizable, expressible and interpretable from analytical and molar point of view respectively. Analytically this is result of alkaline titration which is not in constant ratio to molar concentrations of individual fatty acids. However, this way of expression corresponds very good to practical dairy purposes as conventional interpretation. Values of FFAs can serve to control the health of dairy cows or raw milk quality in consideration of quality and shelf-life of resulting milk products.

FFA analytical methods are relatively complicated in terms of reliability and expressin of units in spite of matter definition simplicity. Variable mixture of acids form broken triglycerides is namely instable in composition ratios. Authors of various articles were concerned with FFA analytical methods (Sjaunja, 1984; Koops *et al.*, 1990; Foss, 2001, 2004; Bijgaart, 2006; Hanuš *et al.*, 2008 a, 2009) and above mentioned methodical complications

ensued on these papers. Reference and routine FFA analytical methods can be so called extraction-titration method, churning-titration method, BDI and mid infrared spectroscopy (MIR) also in modification with Fourier´s transformations (MIR–FT; CSN 57 0533; Koops *et al*., 1990; IDF, 1991; Cvak *et al.*, 1992; Foss, 2001, 2004; Thomson *et al*., 2005; Bijgaart, 2006; Mikulová, 2011).

Therefore, aim of this paper was to verify possibilities of MIR–FT method in terms of its calibration to milk fat free fatty acids (FFA) determination, time stability of MIR–FT FFA calibration and calibration levelling in instrument working nets of dairy laboratories.

MATERIAL AND METHODS

Analytical and instrumental systems for determination of FFAs

Experimental and development methodical observation was carried out in CR reference and routine milk laboratory network as a case study. One reference laboratory (A) and two routine laboratories (B and C) with one reference method and five MIR–FT instruments (three types) were included in this paper. Calibration (reference) sets of milk samples (one set is equal to eight samples) were prepared according to previously tested procedures (Hanuš *et al.*, 2008 a, b, 2009; Genčurová *et al*., 2009, 2011) and in accordance with standard CSN 57 0533 using churning method in reference laboratory. The following MIR–FT instruments with indirect measurement principle were included in this analytical system and its evaluation: once LactoScope FTIR (DE; Delta Instruments, The Netherlands); two times Bentley FTS (BE; Bentley Instruments, USA); two times MilkoScan FT 6000 (FO; Foss, Denmark).

Experimental reference sample preparation, pilot calibration and between calibration intervals

Milk for preparation of calibration samples and for reference method was stored and transported in the same way. Before analyse the milk samples were in water bath only time which was necessary to reach 40° C for measurement. Whole sample mixing was made carefully. Reference method was carried out two times for calibration samples. Churning-titration method (with KOH titration, CSN 57 0533) was used as reference (RE) procedure for determination of FFAs and results were expressed in mmol.100g^{-1} of fat.

During one year three (I, II and III) pilot calibrations (two winter in February and November and one summer in August) of five MIR–FT instruments in three dairy laboratories, which were linked in network, were performed. Bulk milk samples came from four dairy cow herds, two with Czech Fleckvieh and two with Holstein breed. One Holstein herd had poorer energy nutrition and higher native content of FFAs in calibration sample set was established in this way. These four samples were used for calibration in native form and also were stressed by mechanical stirring (Hanuš *et al.*, 2008 a, b, 2009; Genčurová *et al*., 2009, 2011) for increase of FFAs. One litre of milk was in vessel (height 19 cm, diameter 13 cm), temperature from 18 to 22 °C, eccentric stirrer (stainless steel surface 7 × 7 cm) with perforation like three circle inlets (diameter 12 mm), 550 wheel revolutions per minute, stirring time from 15 to 20 minutes. It was used in first two calibrations (I and II). This kind of treatment can increase content of FFAs approximately by 100% (60 – 140%). Reduced milk mechanical stress (10 minutes) was used at last calibration (III). In this way the calibration set consisted regularly of eight milk samples.

One between calibration interval was checked monthly by proficiency testing (PT; Grappin, 1993; Leray, 1993, 2009 a, b, c, 2010). PT sample set consisted of ten milk samples for current commercial MIR–FT calibration of main milk components such as fat, protein, casein, lactose and solids non fat content. Five bulk samples were native milk (Czech Fleckvieh and

Holstein breed) and five samples had artificially modified fat content to lower (2, milk dilution) and higher (3, cream addition) values.

Calibration (reference) and also PT (control) samples were cooled, preserved (Broad Spectrum Microtabs II, DF Control Systems, England; 0.02%) and sent to laboratories for measurement in thermoboxes under controlled conditions (Sojková *et al.*, 2009). Calibration, analytical work and instrument maintenance were performed according to relevant producer operation manuals.

Statistical procedures

Evaluation of calibration results was performed in accordance with relevant system of statistical treatment (Grappin, 1987, 1993; Hanuš *et al.*, 2008 a, 2009; Leray, 2009 c, 2010; Genčurová *et al*., 2011) and proficiency testing was evaluated by Euclidian distance from origin (Leray, 1993, 2009 a, b) using Microsoft Excel programme on the basis of difference comparison or testing and regression analyse.

RESULTS AND DISCUSSION

Molecularly inhomogeneous mixture of milk FFAs can be a source of variations in calibration certainty and result reliability of MIR–FT method in dependence on various factors as animal feeding, hygiene and milking considerateness, milk storage and transport. Nevertheless, also the reference method is assessable to these factors in sure sense when in its principle (KOH titration) also grades mentioned FFA molecular variability. MIR–FT use for FFA determination and raw milk quality control is still at the beginning for these various theoretical and practical questions of MIR–FT application, especially in laboratory networks. The development of suitable MIR–FT calibration procedures and developing targeted

measures in laboratory networks could contribute to increase of reliability of instrumental FFA determination in milk laboratories.

In Tab. I, II and III there are shown the results of three calibrations of five MIR–FT instruments (BE1 and 2, FO1 and 2 and DE) for determination of milk FFAs. Means and standard deviations (x ± sd) are mentioned before (FM) and after (CM; as validation result of accepted calibration) calibration. As it is seen according to mutual agreement among reference and instrumental means (CM), the calibration results were the best in first calibration (Tab. I) and the worse in third calibration (Tab. III) in the laboratory network. Due to lower mechanical stress of chosen milk samples the RE mean of FFAs was lowest (1.152 mmol.100g^{-1}) in the third calibration (Tab. III) as compared to the first and second (Tab. I and II; 1.934 and 1.746 mmol.100g^{-1}). The real reasons for this fact are unknown. In the same sense, there is also lower variability of FFA values (Tab. III and II; 0.8102 < 1.2215 mmol.100g^{-1}). From this results (Tab. I, II and III), there is possible to derive an maximal value as statistical parameter of calibration quality for its validation under practical laboratory conditions. This is x (sd of d, mean standard deviation of difference means between MIR–FT and RE) plus 1.64 (one side limitation) multiple of relevant sd (on 95% level of certainty, according to Grappin, 1987), which is 1.0613 mmol.100g^{-1} (0.622474 + 0.2662 x 1.64). This is visibly higher value than estimated Genčurová *et al.* (2011) at smaller data file, 0.75 mmol.100g^{-1}.

The best relationships (correlation coefficients (r) 0.856, 0.945 and 0.837; $P < 0.001$) from three calibrations between MIR–FT instrumental determination (according to instruments DE, BE and FO) of FFAs and results of reference method (RE) after calibration (CM; validation correlation coefficients) are shown in Tab. IV and Fig. 2 as example. According to instruments their statistical parameters as x ± sd, minimum and maximum were (Tab. IV): for DE 0.822 ± 0.036, from 0.784 to 0.856; for BE 0.832 ± 0.095, from 0.708 to 0.945; for FO

0.764 ± 0.077, from 0.666 to 0.837. In terms of total statistic these were: 0.802 ± 0.082 (P < 0.001), from 0.666 to 0.945. It means that on the average 64.3% (89.3% as maximum) of variability in MIR–FT FFA results could be explained by variability in the real FFA results using reference method. From this results, there is possible to derive an minimal value as statistical parameter of calibration quality for its validation under practical laboratory conditions. This is x minus 1.64 multiple of sd (on 95% level of certainty, according to Grappin (1987), one side limitation), which is 0.668 (minimal acceptable r). This is lower value than estimated Genčurová *et al.* (2011) at smaller data file, 0.841. Foss professional materials (2001, 2004) show similar possibilities with r 0.897 as one regular example (n = 1,927, n = 22). General linear regression equation for all results of instruments (x axis) in the network in relation to RE values (y axis) in one calibration (I) is shown in Fig. 3 as example (r = 0.784; P < 0.01). Of course, this kind of evaluation could be probably useable as model for levelling of results of all instruments in good technical condition in laboratory network during following between calibration interval.

In Tab. V, there are shown the correlations (r) between instrumental calibration FFA results mutually. Only one result is insignificant (P > 0.05) and highest coefficient is 0.995 (P < 0.001; Fig. 4). In general, these coefficients are higher as compared to those in Tab. IV (MIR–FT to RE values) by 8.4% on the average (mean r 0.869 (P < 0.001) > 0.802) which is probably caused by higher methodical homogenity (Tab. V) in this file. Further, interesting thing is that these coefficients are usually higher between the same types of instruments (in total 0.894 ± 0.079, from 0.782 to 0.988 (BE x BE and FO x FO), n = 6, where BE 0.856 ± 0.088 and FO 0.938 ± 0.06) than between different instruments (0.861 ± 0.029, from 0.662 to 0.995 (DE x BE, DE x FO and BE x FO), n = 24). This fact shows logically on various solutions of infrared signal processing and its information recovery between used instrument types. However, from analytical point of view, this is possible also correlations between

different kinds of instruments to specify as suitable. Also, as it has been mentioned already these „hetero– and homo–methodical“ (Tab. IV (0.802, RE and MIR–FT) and V (0.869, MIR–FT)) correlation coefficients between FFA results (as minor milk component) are still lower than the same type of correlations at investigation of major milk componets such as fat, protein, lactose and solid non fat (RE x MIR–FT and inside MIR–FT) in accordance with other professional materials (Koops *et al*., 1990; Foss, 2004; Bijgaart, 2006; Hanuš *et al.*, 2008 a, 2009; Genčurová *et al*., 2011).

Example results from one PT about determination of FFAs are shown in Fig. 5 and 6 as example. These were carried out for ten milk calibration samples during current milk composition MIR–FT calibration (fat, protein, lactose, solid non fat) including fat content modified samples (Fig. 6). It means that no all samples have native composition and this test is not regular for PT od FFAs in this way. However, for the future, it could be probably more suitable to perform this PT evaluation only with native bulk milk samples (5 in this case and used system (Fig. 6)) because of result protection against possible interference impacts due to too high variability of main milk components. That is also reason why the PT result pattern can be a little bit different for the same PT trial (Fig. 6). The relationships in Fig. 5 are similar to those in Tab. V for 10 (native and modified) and also 5 (only native) samples by their character. The instrumental r were quite comparable for the same PT case (Fig. 6), 0.887 and 0.953 ($P < 0.001$ and $P < 0.05$; Fig. 5). Therefore that fact is very interesting that differences among instruments (MIR–FT) in inter–calibration interval are very often stable in terms of trend. It offers us possibility to construct a levelling programme for already calibrated instruments because also mutual correlations are usually close. So some generally calculated regression equation between derived PT reference and instrumental values could correct following MIR–FT results in the network for their better comparability. According to above

mentioned facts it could be probably carried out mostly in bias field of stated instrumental equations.

In other factors of mentioned problem Thomson *et al*. (2005) found an impact of timing (at sampling and 24 hours after sampling at cold storage) of milk sample preservation (0.1 ml of 0.2% hydrogen peroxide added to 30 ml of milk) on FFA value estimation using BDI method. In this context so called BDI method, which is often used for instance in the Netherlands, serves systematically lightly lower results as compared to reference churning method (CSN 57 0533) because certain portion of FFAs penetrates into water phase during this procedure (Cvak *et al.*, 1992), so there are principle reasons for such phenomenon. Concentration of FFAs (Thomson *et al.*, 2005) was increased by 21% at lengthening of interval from collection to analyse by 24 hours and by 9% at delayed milk collection from daily to every second day. However the between-farm variation remained reasonably consistent.

Because of preparation way (mechanical stress in some cases) the FFA reference milk samples (Hanuš *et al.*, 2008 a, b, 2009; Genčurová *et al.*, 2009, 2011) are very sensitive to way of treatment and transport. In consideration of these our results, this could be advantageous to reduce used time (approximately by 50%) of mechanic stress at preparation of relevant reference samples because of improvement of stability and shelf–life of these milk FFA calibration samples. That is also reason why it is necessary to use quick and cold it means controlled sample set transport in case of sending a delivery to routine laboratories to calibration in the working net, for instance under conditions which were experimentally validated by Sojková *et al.* (2009).

CONCLUSION

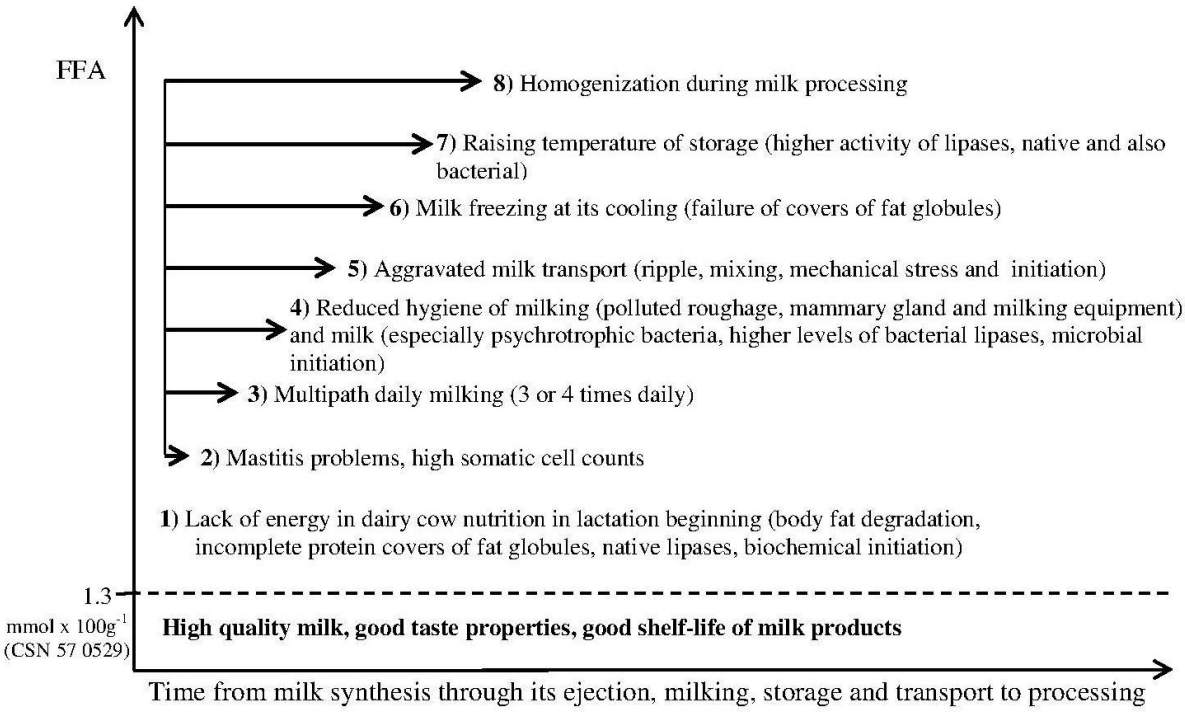
From analyse of calibration stability variations of MIR–FT method for FFA measurement it is possible to deduce as suitable to construct PC programme for calculation of levelling equations of individual instruments in laboratory working net for improvement of FFA result reliability: – current calibration of instruments on milk FFA measurement will be carried out in the working net; – first possibility is direct use of reference method values from reference sample set after performed calibration for next calculation (a); – second variant is that Grubbs test of remoteness (on level of probability 0.05%) will be used at measurement of reference sample set during actual calibration for insertion of instrumental sample results into calculation of reference sample value (b); – this value will be created by arithmetical mean of purged set of sample values according to individual instruments (laboratories); – sample values of reference set of each instrument will be related to relevant reference values of instrument set in working net (a or b); – after that the individual levelling equation will be calculated for each instrument; – this equation will be used during period from actual calibration to next calibration for FFA result transformation at relevant instrument on levelling results of working net.

SUMMARY

Content of free fatty acids (FFAs) in fat is important indicator of raw milk quality in terms of sensorial properties and its product shelf–life. FFAs are product of dairy cow energy metabolism and milk fat lipolysis induced by native or bacterial enzymes. Therefore, their content is influenceable by various factors as animal feeding, hygiene and milking considerateness, milk storage and transport. Such are the reasons why result reliability of analytical methods for series determination of FFAs is important. Therefore, aim of this paper was to verify possibilities of MIR–FT (mid infrared spectroscopy with Fourier´s

transformations) method in terms of its calibration to determination of milk fat FFAs, time stability of MIR–FT FFA calibration and calibration levelling in instrument networks of dairy laboratories. Reference (RE) milk samples (one set is equal to eight samples) were prepared according to previous procedures and CSN 57 0533 (FFAs in mmol.100g^{-1} of fat). The following MIR–FT instruments were included: 1 LactoScope FTIR (DE); 2 Bentley FTS (BE); 2 MilkoScan FT 6000 (FO). Three pilot calibrations (two winter in February and November and one summer in August) of five MIR–FT instruments in three dairy laboratories in network were performed. Bulk milk samples came from four dairy cow herds and two breeds. These four samples were used for calibration in native and modified form in terms of fat content. Modification increased FFAs approximately by 100%. Calibration set consisted of 8 milk samples. One between calibration interval was checked monthly by proficiency testing (PT). PT sample set consisted of 10 milk samples. Five bulk samples were native milk and five had artificially modified fat content to lower and higher values. Calibration, analytical work and instrument maintenance were performed according to relevant producer operation manuals. Statistical evaluation procedures of calibration results and PT were on the basis of regression analyse and difference comparison or testing. The calibration results were the best in first calibration and the worse in third calibration in the laboratory network. Due to lower mechanical stress of chosen milk samples the RE mean of FFAs was lowest (1.152 mmol.100g^{-1}) in the third calibration as compared to the first and second (1.934 and 1.746 mmol.100g^{-1}). Maximal value of difference variability as validation parameter of calibration quality is x (sd of difference MIR–FT and RE) plus 1.64 multiple of sd (on 95% level) which is 1.0613 mmol.100g^{-1}. Mean validation correlation coefficient (r) between MIR–FT and RE results was 0.802 ± 0.082 ($P < 0.001$), from 0.666 to 0.945. On the average 64.3% (89.3% as maximum) of variability in MIR–FT FFA results could be explained by variability in the RE results. Minimal value as validation parameter of calibration quality is x minus 1.64 multiple

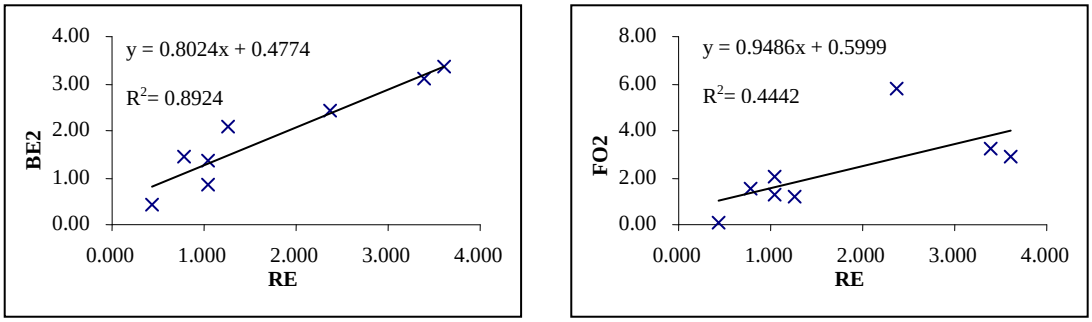
of sd which is 0.668. Correlations between MIR–FT results were higher by 8.4% (0.869 ($P <$ 0.001) > 0.802) as compared to validation r. Example PT with 10 (native and modified) and 5 (native bulk samples) milk samples had similar results of r 0.887 and 0.953 ($P < 0.001$ and $P < 0.05$). Differences among instruments (MIR–FT) in inter–calibration interval are very often stable. It offers the possibility to construct a levelling programme for calibrated instruments. So some generally calculated regression equation between derived PT reference and instrumental values could correct following MIR–FT results for their better comparability in the relevant network. According to above mentioned facts it could be probably carried out mostly in bias field of stated instrumental equations. According to the obtained results a philosophy of levelling programme was defined in steps as paper conclusion.



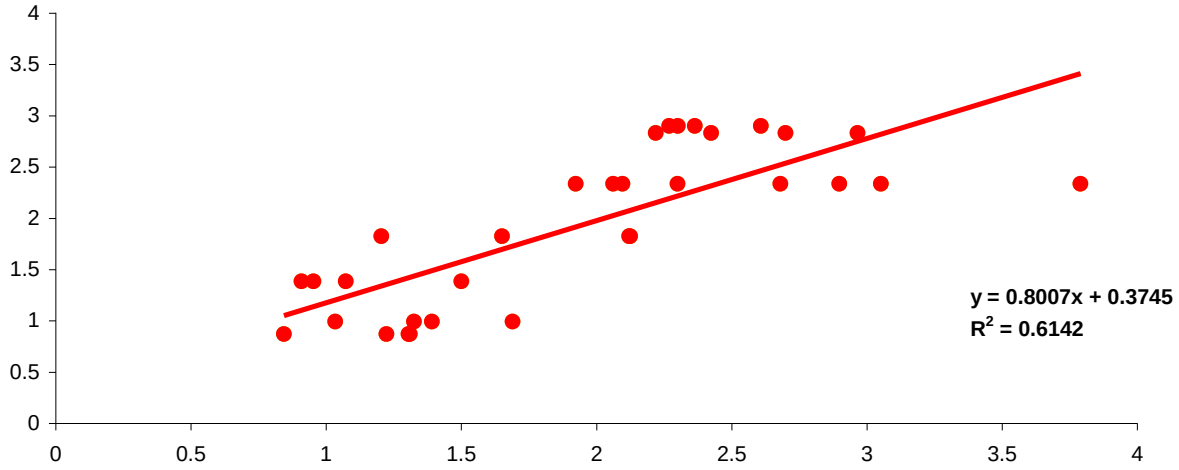
1: Lipolysis rise of fat in raw milk, content increase of free fatty acids (FFA), threat of milk and dairy product quality – factors and their combinations, related to animal and technology (modified according to Sjaunja (1984), Shelley *et al.* (1987), O´Brian *et al.* (1998), Vyletělová *et al.* (2000 a), Ma *et al.* (2000), Antonelli *et al.* (2002), Santos *et al.* (2003),

Wiking *et al.* (2003, 2006), Hanuš *et al.* (2004, 2008 b), Thomson *et al.* (2005), Ferlay *et al.* (2006), Genčurová *et al.* (2009 and 2011) and Mikulová (2011)).

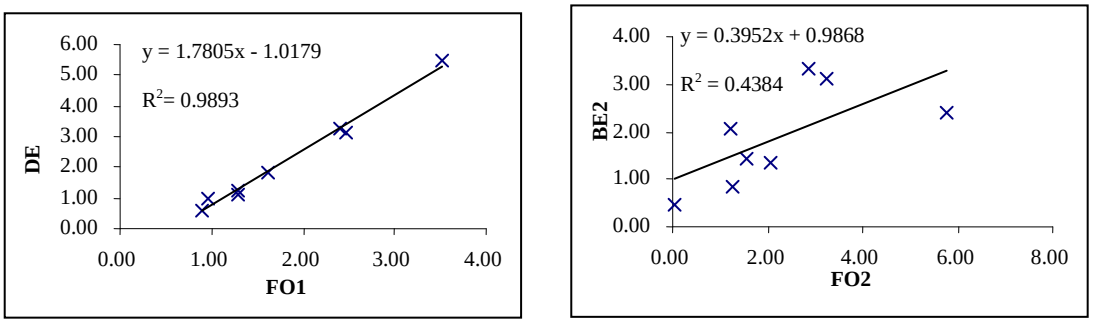
2: Closest and weakest regression relationship of determination of FFAs (in mmol.100g^{-1}) between RE (x) and MIR–FT (y) as calibration validation (r = 0.945 and 0.666, $P < 0.001$ and $P > 0.05$)



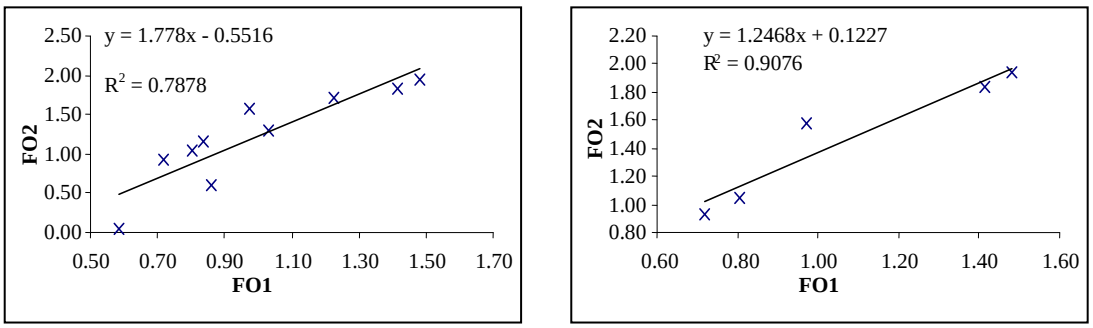
3: General regression of relationship of FFA results (in mmol.100g^{-1}) between MIR–FT (x, 5 instruments) and reference method (y) at calibration I (r = 0.784; $P < 0.01$)



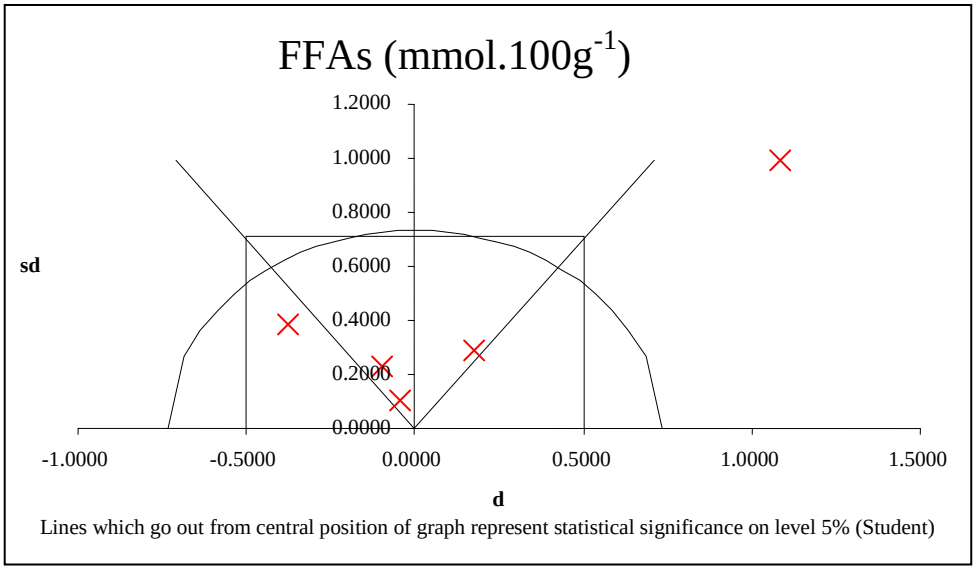
4: Closest and weakest regression relationship of determination of FFAs (in mmol.100g^{-1}) between MIR–FT mutually after carried out calibration (r = 0.995 and 0.662, $P < 0.001$ and $P > 0.05$)

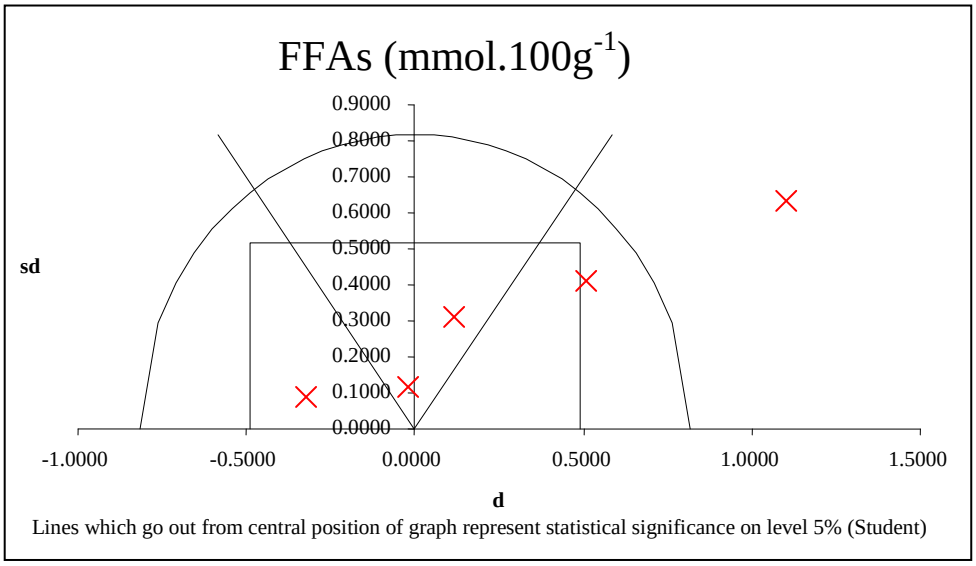


5: Example of regression relationship of determination of FFAs (in mmol.100g^{-1}) between MIR–FT mutually in proficiency tetsting for 10 (native and modified bulk) and 5 (native bulk) milk samples (r = 0.887 amd 0.953, $P < 0.001$ and $P < 0.05$)



6: Example of evaluation of Euclidian distance from origin for determination of FFAs in PT between MIR–FT for 10 and 5 milk samples





d = mean difference (indirect method – reference value); sd = standard deviation of individual differences along samples; lines which go out from central position of graph represent statistical significance on level 5% (pair t–test, Student´s distribution), points which are below lines are significantly different ($P \leq 0.05$), points over lines are insignificantly different ($P > 0.05$); bow incloses 90% of confidence interval of Euclidian distance and successful result in PT, box incloses very successful result.