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DEVELOPMENT IN INDIRECT INFRA-RED DETERMINATION OF MILK ACETONE

Oto Hanuš[1], Petr Roubal[1], Jan Říha[1], Marcela Vyletělová Klimešová[1], Eva Samková[2], Radoslava Jedelská[1], Jaroslav Kopecký[1]

[1] Dairy Research Institute, Ltd., Prague, Ke Dvoru 12a, 160 00 Prague 6-Vokovice, Czech Republic
[2] Faculty of Agriculture, University of South Bohemia České Budějovice, Branišovská 1, 370 05 České Budějovice, Czech Republic

Abstract

HANUŠ OTO, ROUBAL PETR, ŘÍHA JAN, VYLETĚLOVÁ KLIMEŠOVÁ MARCELA, SAMKOVÁ EVA, JEDELSKÁ RADOSLAVA, KOPECKÝ JAROSLAV. 2014. Development in Indirect Infra-red Determination of Milk Acetone. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis,* 62(5): 919–927.

Milk acetone (AC) is an indicator of energy metabolism of cows and ketosis occurrence. AC result inerpretation is essential for prevention and treatment in dairy cow herds. There is necessary an effective method with reliable results. The goal was to evaluate the mid infrared method MIR–FT in terms of calibration for AC. Microdiffusion photometric (485 nm) method with salicylaldehyde as reference (RE) and MIR–FT (Lactoscope FT–IR, Delta (D); MilkoScan FT 6000 (F); Bentley (Bentley Instruments (B)) as indirect method were used. Selected (from high yielding dairy cows in early lactation) individual milk samples (MSs; n = 89) were used for MIR–FT calibration development and evaluation. Log AC correlation (r) between RE and indirect MIR–FT (D) was low (0.22, $P < 0.05$). The same parameter between RE and MIR–FT (F) was closer (0.589, $P < 0.001$; 0.632, $P < 0.001$ for n = 64. The artificial AC addition to milk samples had no visible effect on AC recovery by MIR–FT instruments. The AC values increased from 4.91 and 5.23 to 45.22 mg.l^{-1} by RE. There is no possibility to prepare the AC reference samples using artificial addition for MIR–FT calibration. In dependence on possible AC evaporation (a risk of AC result reduction) during storage conditions a knowlege about AC stability in sample is important. The similar AC results were obtained after milk sampling and after 48 hours of storage under cold conditions. This is new information for analytical work.

Keywords: raw cow milk, acetone, ketosis, photometry, infrared spectroscopy, calibration

INTRODUCTION

Ketosis and Milk Ketones (Acetone) Importance

Ketosis as metabolic disorder (Reist *et al.*, 2002; Siebert and Pallauf, 2010; Manzenreiter *et al.*, 2013) is connected with losses on milk yield (Gasteiner, 2000; Heuer *et al.*, 2001; Hana *et al.*, 2007), worse reproduction performance (Říha and Hanuš, 1999), abomasum displacement (Geishauser *et al.*, 1997), shorter longevity and sometimes also on dairy cow life as fatal end. Its occurrence is also associated with higher ketone levels in all body liquids (Steen *et al.*, 1996; Enjalbert *et al.*, 2001; Beran *et al.*, 2012; Januš and Borkowska, 2013), mostly of acetone (AC) and beta–hydroxybutyrate (BHB) as products of fat catabolism when an animal solves its energy deficiency by destruction of body fat reserves (Manzenreiter *et al.*, 2013) along body condition score and reproduction performance losses (Beran *et al.*, 2012). The AC values highly correlate among these body liquids (Enjalbert *et al.*, 2001). Also genetic, lactation and milk yield impacts on milk acetone content and linked energy balance of dairy cows or other animals were evaluated (Miettinen, 1994; Heuer *et al.*, 2001; Janů *et al.*, 2007; Hanuš *et al.*, 2011 b, c). The interpretation procedures of ketones in blood, urine, milk and/or cervical mucus were described in previous papers (Gustafsson and Emanuelson, 1996; Enjalbert *et al.*, 2001; Heuer *et al.*, 2001; Hanuš *et al.*, 2001, 2011 a, b, c; Mottram *et al.*, 2002; Knegsel *et al.*, 2010; Beran *et al.*, 2012).

Practical result interpretation should flow into prevention and treatment measurements in dairy herds (Miettinen, 1995; Green *et al.*, 1999; Gasteiner, 2003; Tedesco *et al.*, 2004). This depends closely on speed and effectiveness of investigative methods for practical purposes of animal health solution.

Ketosis as production disorder deteriorates also milk quality (Hanuš *et al.*, 1993, where r = –0.21 between milk AC and its fermentation, $P < 0.05$) for processing and consumption. This is possible to select mastitis milk individually in the herds by stable tests and operative somatic cell count determination in suspect dairy cows (Ticháček *et al.*, 2007) and thus eliminate it from deliveries to dairy plants. This is not possible at ketosis occurrence up to now. Milk deliveries can be damaged by this effect. Therefore this is desirable to do the ketosis diagnosis more efficient for support of dairy cow health, milk quality and consequently milk products as well.

Milk Ketone Analytical Methods and Tests

Milk sample investigation has advantage as non-invasive monitoring (Hanuš *et al.*, 1999; 2001; Mottram *et al.*, 2002) while investigation of other body fluids is invasive monitoring which can be associated with sure disadvantages and risks. Milk samples can be attend fast, regularly and cheaply. That is reason why already previously the various methods with different efectiveness, advantages and disadvantages of ketone determination in milk has been developed (Mottram *et al.*, 2002), from stable tests (relatively cheaper (Geishauser *et al.*, 1997; Hanuš *et al.*, 1999; Carrier *et al.*, 2004)) to direct (colorimetric with salicylaldehyde, with vanillin, flow injection analysis with hydroxylamine and gas chromatography, relatively more expensive (O'Moore, 1949; Majewska and Rybczyńska, 1975; Vojtíšek, 1986; Hansen, 1999; Mottram *et al.*, 2002; Heuer *et al.*, 2001; Baticz *et al.*, 2002; Roos *et al.*, 2007; Beran *et al.*, 2012)) and indirect (infrared spectrometry, relatively cheap (Hansen, 1999; Heuer *et al.*, 2000; Roos *et al.*, 2007; Knegsel *et al.*, 2010; Hanuš *et al.*, 2011 a; Drift *et al.*, 2012)) laboratory analytical methods. Whole raw of semiquantitative stable tests exists for ketones in urine (for instance Ketophan (Hanuš *et al.*, 2001)) for quick diagnosis but there are only three (Carrier *et al.*, 2004) good usable tests for work with milk. However, these are relatively expensive (Ketocheck and Ketolac (Geishauser *et al.*, 1997)). Therefore fourth cheap milk test (Ketotest (Hanuš *et al.*, 1999)) has been developed in the Czech Republic. At this development a serious professional doubtfulnis existed if classical nitropruside reaction can be realized effectively in milk environment for its colour obscurant effect. Previous experiments have not been successful. It was shown that proposed construction of reaction mixture (Jílek, 1999, cited in Hanuš *et al.*, 1999), where the colour reaction was been taken on firm phase of the tets filler after lactoprotein precipitation and good visualized in this way, is capable. This was confirmed by testing in milk and also against urine ketones. The results were good (Hanuš *et al.*, 2001; r = 0.87; $P < 0.001$) and permited the practically usable differentiation of milk ketone concentrations.

The next way for more effective diagnosis of ketosis was beside existence of more expensive (because of labour costs) direct methods for ketone determination in body liquids (O'Moore, 1949; Vojtíšek, 1986; Baticz *et al.*, 2002; Beran *et al.*, 2012) also looking for ways of using of indirect methods (Hansen, 1999). The replacement of direct methods by tests or indirect methods could save the labour costs and open possibilities how to do the regular diagnosis of ketosis more frequent in practice and contribute positively to dairy cow health. The main variant is modern infra-red spectrometry of whole IR spectrum with Fourier's transformation (MIR–FT (Heuer *et al.*, 2000 a; Knegsel *et al.*, 2010; Drift *et al.*, 2012)). This was shown sometimes with usable results for ketosis diagnosis (Roos *et al.*, 2007) but it was not always the rule for more exact determination of real ketone concentration (Hanuš *et al.*, 2011 a; Drift *et al.*, 2012).

This development enables to construct a hypothesis that use of suitable procedure steps could lead also to improvement of estimation of milk ketone concentration using of effective indirect methods and by this also to improve the diagnostical possibilities and contribute positively to control of dairy cow health and milk quality as well.

Therefore, aim of this paper was to: verify possibilities of MIR–FT method in terms of its calibration to milk AC (ketones) determination; develop a practically usable method for preparation of relevant reference (calibration) standard samples; describe and evaluate aspects of calibration and result reliability; attest possibilities of proficiency testing at mentioned matter determination.

MATERIAL AND METHODS

Reference and Indirect Milk Ketone Investigations

AC concentration was investigated by spectrophotometry measurement (wavelength 485 nm) using Spekol 11 (Carl Zeiss Jena, Germany). AC was absorbed into KOH solution with salicylaldehyde (O'Moore, 1949; Vojtíšek, 1986) due to 24 hours microdiffusion in darkness at temperature 25 °C. This method determined the AC reference (RE) values. The MIR–FT method as Lactoscope FT–IR (Delta Instruments, The Netherlands (D)), MilkoScan FT 6000 ((F) Foss Electric, Denmark) and Bentley (Bentley Instruments, USA (B)) was used as indirect method (Hansen, 1999; Heuer *et al.*, 2000; Roos *et al.*, 2007; Knegsel *et al.*, 2010). This was calibrated and controlled by RE results (D). There were original calibrations as well (F and B).

Reference and Control Milk Sample Sets for Acetone Analytic Method Comparison

Individual cow milk samples (MSs; n = 89) were collected in high yielding (over 8 500 kg of milk per standard lactation) dairy herds (Holstein and Czech Fleckvieh). Only animals from 10 to 100 days in milk along all lactations were sampled. The presupposed probability for ketosis occurrence in the sample set was maximalized (Hanuš *et al.*, 2001) in this way although such presupposition was not confirmed by all papers (Janů *et al.*, 2007). Nevertheless, cows were sampled over whole lactation in these works. The goal was to obtain higher ratio of high AC values, as much as possible for good character of distribution of RE values in calibration set. MSs were transported to laboratory under refrigerator conditions. Selected MSs were analysed using RE, D and F methods on AC, log AC and log BHB (Tab. I) and their mutual relationships were calculated and evaluated (Figs. 1–7). Reference raw cow individual MSs were also characterized in terms of their composition using the MIR–FT method (Lactoscope FT–IR, Delta Instruments, The Netherlands (D)) with relevant calibrations. Milk components were investigated according to Tab. II.

Control bulk milk sample with normal composition was modified with goal to increase AC content for reaching of upper value of calibration line. AC was increased (Hanuš *et al.*, 2011 a) by artificial addition similarly as in urea reference samples for MIR–FT calibration (Hering *et al.*, 2008) using reference standards (AC water solutions, Tab. III) for RE method calibration line. Basic AC solution was prepared as follows: 250 mg of AC (0.316 ml) was added to 250 ml by destilled water. Increase in milk was performed by 10 as minimal and 40 mg.l^{-1} as maximal AC addition. 10 milk subsamples were obtained by modification. Original milk had approximately 5 mg.l^{-1} of basic AC content. Modified samples were analysed using RE and MIR–FT (D, F and B) method.

Shelf-life of reference MSs was tested in terms of stability of AC level during presupposed storage (transport) period under cold temperature conditions (at 5 °C) using RE and MIR–FT (D) method. There was used one current native milk sample and also the same milk sample with two (I and II) higher artificial AC additions (Tab. IV). Two measurements of each subsample were carried out immediately after its sampling and modification and next two measurements after 48 hours of cold storage. MSs were stored in normally closed (plastic cap with screw) little plastic sample bottles (100 ml volume/90 ml of milk).

Statistic Evaluation of AC Calibrations

AC concentrations were used in mg.l^{-1} and also after their logarithmic transformation ($\log_{10}$; Janů *et al.*, 2007; Roos *et al.*, 2007). This was done because of usually no normal frequency AC data distribution. Beside arithmetic means also geometric means and medians were used. Regression analyse was used for calibration evaluation. The evaluation of different forms of AC results was done to obtain maximal value of determination coefficient by Microsoft Excel programme.

RESULTS AND DISCUSSION

The basic statistical characteristics of AC reference sample set (n = 89) are shown in Tab. I. RE method for milk AC showed arithmetic mean and standard deviation 7.22 ± 10.69 mg.l^{-1} and geometric mean 5.12 mg.l^{-1}. Variability of values in the set was 148% which can be usable for calibration purposes in terms of suitable calibration equation range. This variability is typical for defined lactation period of dairy cow sampling which is characterized by higher subclinical and clinical ketosis occurrence. Also composition of reference MSs and its variability (Tab. II) is typical for mentioned lactation period. Especially variability in fat and urea content (42.4 and 45.0%) is high in dependence on presupposed (Steen *et al.*, 1996; Gasteiner, 2000, 2003; Heuer *et al.*,

I: *The basic statistic characteristics of reference (calibration) milk sample set and results of indirect measurement for acetone and beta–hydroxybutyrate (n = 89)*

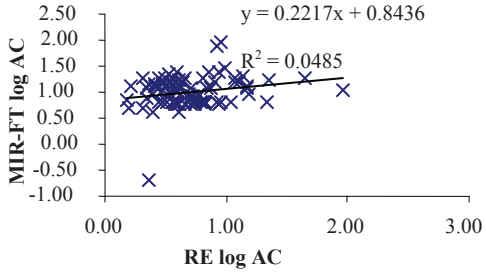
Indicator	RE AC	RE log AC	D AC	D log AC	F log AC	F log BHB
Unit	mg.l^{-1}	from mg.l^{-1}	mg.l^{-1}	from mg.l^{-1}	–	–
Stat. par.						
x	7.22	0.7094	5.49	0.7251	–0.9065	–1.8579
sd	10.69	0.3032	1.56	0.1135	0.3427	0.4046
xg	5.12		5.31			
m	4.59	0.6613	5.14	0.711	–0.9635	–1.899
vx (%)	148.0		28.3		37.8	21.8
min.	1.51	0.179	1.82	0.2601	–1.5085	–2.614
max.	91.8	1.9628	15.2	1.1818	0.206	0.218

Stat. par. = statistical parameter; x = arithmetic mean; sd = standard deviation; xg = geometric mean; m = median; vx = variation coefficient (%); min. = minimum; max. = maximum; log = $\log_{10}$ RE = reference (direct measurement); D and F = MIR–FT method; AC = acetone; BHB = beta–hydroxybutyrate.

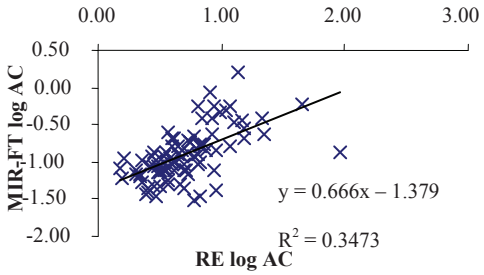
2001; Reist *et al.*, 2002; Siebert and Pallauf, 2010; Knegsel *et al.*, 2010; Hanuš *et al.*, 2011 c; Drift *et al.*, 2012; Manzenreiter *et al.*, 2013) high variability in level of energy metabolism of sampled dairy cows. The RE AC variability (Tab. I; 148.0%) is markedly higher as variability in indirect measurements (D and F, 28.3 and 37.8%). It means that dependency of AC indirect methods on RE values was lower in this evaluation.

The mean differences between RE and indirect method results (Tab. I) and their standard deviations were not calculated and tested because of specific expression of indirect F results (MIR–FT method, log AC and log BHB) as the correlation of MIR–FT to RE results is more important for MIR–FT calibration evaluation and development than their mean difference. In case of necessity this difference can be easily statistically compensated for instance by a linear regression equation. In general, also the pair-test of mean difference is not relevant procedure for analytical method result reliability evaluation from known reasons.

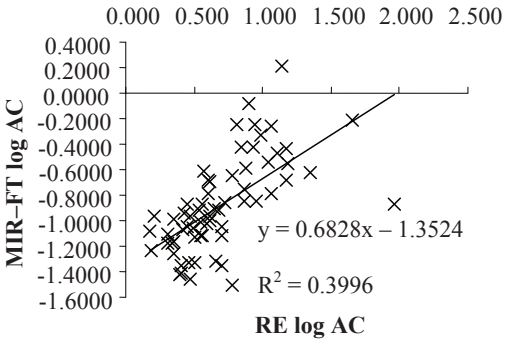
Only interesting and purpose representative regression relationships were chosen (Figs. 1–7) for result interpretation. The correlation coefficient (r) between RE and indirect MIR–FT (D) log AC results was quite low (Fig. 1; 0.22, $P < 0.05$). On the other hand, the same parameter between RE and MIR–FT (F) log AC results was quite close (Fig. 2; 0.589, $P < 0.001$). It could be acceptable for relevant analytical screening use. As only first experiment day RE sample set (n = 64) was used (Fig. 3) for calibration quality evaluation so the same value (for F) was 0.632 ($P < 0.001$). This fact means that 40% of variability in MIR–FT log AC results could be explainable due to variations in RE log AC results. The last our highest, good (promissing in terms of calibration usability) and comparable value was 0.804 ($P < 0.001$; Hanuš *et al.*, 2011 a) with relevant variability explanation by 64.8%. Further, the correlation coefficient (r) between both indirect MIR–FT measurements (D and F) of log AC was lower 0.315 (Fig. 4; $P < 0.01$) because of first



1: *The relationship between RE and indirect MIR–FT (D) log AC results*
n = 89; r = 0.22, $P < 0.05$; n = number of cases; r = correlation coefficient; P = probability level; AC = acetone; RE = reference; MIR–FT = infrared spectroscopy



2: *The relationship between RE and MIR–FT (F) log AC results*
n = 89; r = 0.589, $P < 0.001$



3: *The relationship between RE and MIR–FT (F) log AC results*
n = 64; r = 0.632, $P < 0.001$

II: *The characteristics of composition and properties of milk reference samples (n = 89)*

Indicator	F	L	SNF	CP	CAS	U	FPD
Unit	%	%	%	%	%	mg.100 ml^{-1}	°C
Stat. par.							
x	3.38	4.84	8.45	3.01	2.31	27.72	–0.5287
sd	1.436	0.245	0.48	0.429	0.375	12.48	0.0097
m	3.34	4.87	8.47	2.99	2.27	26.96	–0.5293
vx (%)	42.4	5.1	5.7	14.2	16.3	45.0	1.7
min.	0.49	4.25	7.36	2.07	1.63	5.66	–0.5519
max.	7.35	5.39	9.50	4.3	3.55	107.6	–0.509

F = milk fat content (g.100 g^{-1}; %); L = lactose content (monohydrate; g.100 g^{-1}; %); SNF = solids non fat content (g.100 g^{-1}; %); CP = crude protein (total N × 6.38; g.100 gl^{-1}; %); CAS = casein (casein N × 6.38; g.100 g^{-1}; %); U = urea concentration (mg.100 g^{-1}); FPD = milk freezing point depression equivalent (°C).

III: *The results of AC additions to original milk using reference (RE) and MIR–FT (D, F and B) method (n = 10)*

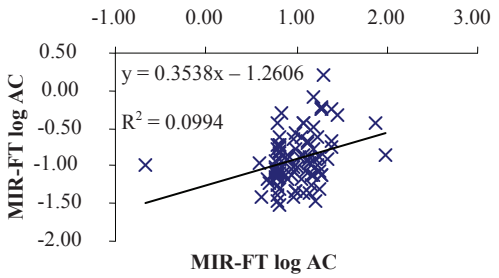
Method		RE	MIR–FT D	MIR–FT B	MIR–FT F	MIR–FT F
		mg.l^{-1}	mg.l^{-1}	mg.l^{-1}	log AC	log BHB
Subsample	Addition					
1	0.5 ml AS	10.23	5.84	5.55	–0.877	–1.677
2	0.75 ml AS	12.73	5.67	5.55	–0.966	–1.74
3	1 ml AS	15.22	5.83	5.53	–0.91	–1.7
4	2 ml AS	25.22	5.88	5.47	–0.875	–1.68
5	3 ml DW	4.91	5.81	5.40	–0.958	–1.735
6	2.5 ml AS	30.22	5.93	5.44	–0.848	–1.651
7	2.75 ml AS	32.72	5.51	5.43	–0.825	–1.669
8	3 ml AS	35.22	5.5	5.41	–0.867	–1.675
9	4 ml AS	45.22	5.99	5.36	–0.873	–1.719
10	4 ml DW	5.23	5.33	5.35	–0.952	–1.725

AC additions were performed to 100 ml of milk. Figures are means of two or three measurements. Composition and properties of native milk: F = 3.78%; L = 4.80%; SNF = 8.60%; CP = 3.19%; CAS = 2.42%; U = 47.9 mg.100 ml^{-1}; free fatty acids = 0.62 mmol.100g^{-1} of milk fat; FPD = –0.522 °C; somatic cell count = 327 10^3.ml^{-1}. DW = distilled water; AS = acetone solution.

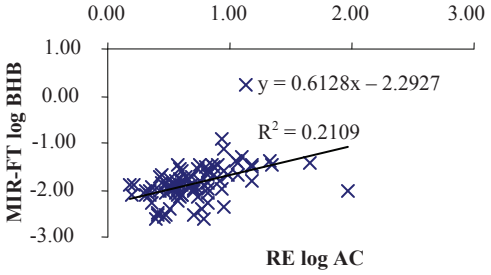
IV: *The results of shelf-life test of reference MSs with three acetone levels (in mg.l^{-1}) during presupposed storage period*

Method	RE	RE	MIR–FT (D)	MIR–FT (D)
Storage time	0	48 hours	0	48 hours
Subsample				
Native milk	5.90	8.23	4.53	5.91
Milk with AC I	23.10	26.34	4.45	6.11
Milk with AC II	29.91	35.44	4.55	6.41

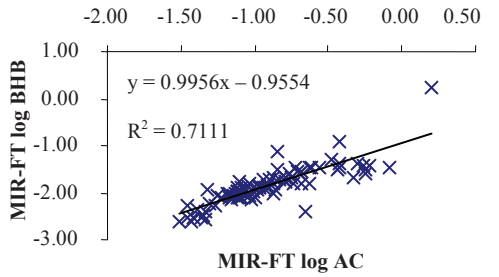
One native bulk milk sample with typical composition, two AC additions (I and II), figures are means of two measurements



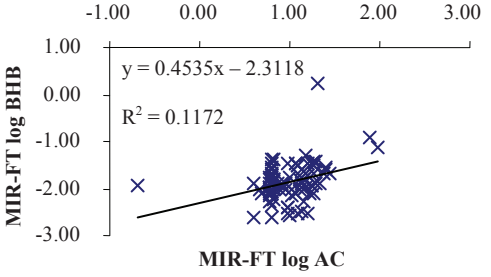
4: *The relationship between indirect MIR–FT measurements (D and F) of log AC*
n = 89; r = 0.315, $P < 0.01$



5: *The relationship between RE log AC and MIR–FT (F) log BHB*
n = 89; r = 0.459, $P < 0.001$; BHB = beta–hydroxybutyrate



6: *The relationship between MIR–FT (F) log AC and MIR–FT (F) log BHB*
n = 89; r = 0.843, $P < 0.001$



7: *The relationship between MIR–FT (D) log AC and MIR–FT (F) log BHB*
n = 89; r = 0.342, $P < 0.01$

mentioned lower relationship (Fig. 1) with 9.9% of mutual variability explanation which is too low of course.

Correlations between log AC and log BHB values were 0.459, 0.843 (both $P < 0.001$; Figs. 5, 6) and 0.342 ($P < 0.01$; Fig. 7). It means that 21.1 and 71.1% of variability in MIR–FT log BHB (F) values could be caused by variability in RE log AC and MIR–FT (F) log AC results. In last investigation (Hanuš *et al.*, 2011 a) this was from 25.2 to 33.7% in first and from 75.9 to 81.2% in second case. This similarity could be given by instrument (MIR–FT (F)) software design solution. On the other hand the MIR–FT D (Fig. 7) was lower with 11.7% of explanation probably also because of first mentioned lower relationship in Fig. 1. It all is logical especiall relationship RE log AC and log BHB (Fig. 5) and could be in accordance with ketosis pathogenesis.

As AC addition test results showed (Tab. III), the artificial AC addition to milk samples had no visible effect on AC recovery by MIR–FT instruments (D, F and B) although AC additions to native milk AC levels were relatively quite high. In contrast to this fact the AC values were increased from 4.91 and 5.23 (subsample 5 and 10, native milk) to 45.22 mg.l^{-1} (supplemented subsample 9) by RE method. These results confirmed our last conclusion (Hanuš *et al.*, 2011 a) where also no AC recovery was noted but only for MIR–FT D. This fact means that there is no possibility to prepare the AC reference milk samples using artificial AC addition for MIR–FT calibration. In contrast to this rule the additions to reference samples are possible at milk urea (Hering *et al.*, 2008) and citric acid MIR–FT calibrations according to our previous results. As also Hansen (1999) mentions, only samples with a naturally increased acetone content could be used, in the calibration step as samples containing added acetone do not produce an acceptable calibration equation.

The results of milk sample shelf-life test (Tab. IV) were similar in trends as results in Tab. III in terms of AC addition (native milk, AC I and AC II) as no recovery using MIR–FT (D) method was confirmed as well. On the other hand the relevant AC result increase was noticed (Tab. IV) by RE method (from 5.9 to 29.91 and from 8.23 to 35.44 mg.l^{-1}). This is not necessary to comment it any more in this sense. However, in dependence on possible AC evaporation during storage conditions (as volatile matter) there is a risk of real AC result value reduction at measurement. Therefore a knowlege about AC stability in milk sample is important for reliable result from practical point of view of analytical technology. For direct RE and indirect MIR–FT (D) method, the similar AC results were shown immediately after milk sampling and after 48 hours of storage under cold conditions (Tab. IV) for all AC concentration levels (native milk, AC I and AC II). The acceptable small (in terms of AC value practice interpretation to ketosis degree) AC differences (Tab. IV) during experimental period can be caused due to analytical effects such as methodical and instrumental time calibration variation (its repeatability) and so on. There was noticed no AC evaporation under mentioned conditions. This is new support information for purposes of analytical work and transport of milk samples in milk recording. There are no relevant results for comparison in mentioned sense in the literature sources.

Heuer *et al.* (2000 b) carried out the evaluation of prediction precision at multiple regression model for estimation of energy balance of high yielding dairy herd from second to twelfth lactation week. The control of milk yield, dairy cow body condition score, ketone test, fat, protein and lactose content from test day of milk recording and fat/ptotein ratio were included into this model. The information from milk recording test day without ketone level test and body condition score is sufficient for estimation of herd mean energy balance, but herd size limits the precision of prediction, as it was concluded by these authors.

While current calibrations of physical instruments for milk composition analyses (urea, acetone) as for instance spectrophotometry use first of all chemical aspects for reference sample preparation at current filter infrared spectrometry also the biological angles (selection of samples according to biological aspects) are applied beside chemical aspects (modifications as increase or decrease of component content by its addition or removing). Here in the case of MIR–FT calibration for selected minority milk components as for instance ketones it is possible to mention that for reference sample set creation this is necessary to take into account also physiological or pathological aspects at selection of suitable milk samples (dairy cows) which are not further modified in terms of chemical composition and the importance of their relevant matrix is growing up in this way. Not only according to results of this paper but also other works (Hansen, 1999; Heuer *et al.*, 2000; Roos *et al.*, 2006; Knegsel *et al.*, 2010) this is shown that improved calibration procedures of indirect methods otherwise will reach higher reliability of MIR–FT results at measurement of milk ketones but these possibilities are still limited (Hanuš *et al.*, 2011 a). According to here reached information the milk ketone results using indirect MIR–FT method can be marked only as orientation in terms of their reliability. The MIR–FT method for ketone (acetone) determination can not seem to understand as precise measurement method in the analytical sense but it is certainly appropriate screening tool of investigation of energy or health status respectively in dairy cows in early lactation. Therefore, the identification of animal hyperketonemie will be always pretty unsure (Knegsel *et al.*, 2010; Drift *et al.*, 2012). Further, for an improvement of ketosis diagnosis according to these MIR–FT values there will be necessary to design the interpretation models in combination with other lactation indicators such as lactation stage,

milk yield and contents of fat, proteins and lactose and milk ketosis (energy) quotients (Knegsel *et al.*, 2010; Hanuš *et al.*, 2011 b, c; Drift *et al.*, 2012; Manzenreiter *et al.*, 2013).

CONCLUSION

It is possible to conclude that probably usable diagnostical reliability of MIR–FT (Roos *et al.*, 2006) could be caused by its ability to evaluate all changes in milk which could be linked with ketosis (milk structure on whole molecular level) more than ability to measure real ketone concentrations as showed Hanuš *et al.* (2011 a) and also results of this paper. Also Knegsel *et al.* (2010) and Drift *et al.* (2012) mentioned concerns for practical applicability to hyperketonemia detection because of high proportion of false-positive tests (from 17 to 18%). The necessity to continue in the development of indirect methods of milk ketone determination and their calibrations for diagnostical purposes and support of animal health and milk quality follows from results of this paper as well. AC MIR–FT calibrations have to be based on choice of native milk reference samples with suitable AC variation range and not on AC addition to reference samples. This is obvious that quality and usability of MIR–FT AC calibration reference set depends closely on suitability of animal and native milk sample selection (on method of relevant selection) and consequently on variation of AC values in relevant variation range in every case.

Therefore, there should be important a procedure with animal and sample selection according to presupposition for high probability of subclinical ketosis occurrence or direct ketosis identification beside normal random milk sampling. One of the variants of biosensoric analytical methods development could be specific realization of measurable effect of Clark's bond as well.

SUMMARY

Milk acetone (AC) is an indicator of energy metabolism of cows and ketosis occurrence. AC result inerpretation is essential for prevention and treatment in dairy cow herds. There is necessary an effective method with reliable results. The goal was to evaluate the mid infrared method MIR–FT in terms of calibration for AC. Microdiffusion photometric (485 nm) method with salicylaldehyde as reference (RE) and MIR–FT (Lactoscope FT–IR, Delta (D); MilkoScan FT 6000 (F); Bentley (Bentley Instruments (B)) as indirect method were used. Selected (from high yielding dairy cows in early lactation) individual milk samples (MSs; n = 89) were used for MIR–FT calibration development and evaluation. The reference AC set has to have an acceptable statistics for good MIR–FT calibration. It was 7.22 ± 10.69 mg.l^{-1} and geometric mean 5.12 mg.l^{-1}, variation range from 1.51 to 91.8 mg.l^{-1}. Log AC correlation (r) between RE and indirect MIR–FT (D) was low (0.22, $P < 0.05$). The same parameter between RE and MIR–FT (F) was closer (0.589, $P < 0.001$). It could be acceptable for practical use. As only lower n (= 64) was used so the same value (for F) was 0.632 ($P < 0.001$). This fact means that 40% of variability in MIR–FT log AC results could be explainable due to variations in RE log AC results. The r between both MIR–FT measurements (D and F) of log AC was lower 0.315 ($P < 0.01$). The correlations between log AC and log BHB values were 0.459, 0.843 ($P < 0.001$) and 0.342 ($P < 0.01$). 21.1 and 71.1% of variability in MIR–FT log BHB (F) values could be caused by variability in RE log AC and MIR–FT (F) log AC results. The artificial AC addition to milk samples had no visible effect on AC recovery by MIR–FT instruments. The AC values increased from 4.91 and 5.23 to 45.22 mg.l^{-1} by RE. There is no possibility to prepare the AC reference milk samples using artificial AC addition for MIR–FT calibration. The results of milk sample shelf-life test were similar in trends in terms of AC addition as no recovery using MIR–FT was confirmed. In contrast, the relevant AC result increased from 5.9 to 29.91 and from 8.23 to 35.44 mg.l^{-1} by RE. In dependence on possible AC evaporation (a risk of real AC result value reduction at measurement) during storage conditions a knowlege about AC stability in milk sample is important for reliable result. For RE and MIR–FT method, the similar AC results were obtained after milk sampling and after 48 hours of storage under cold conditions. There was no AC evaporation under mentioned conditions. This is new information for analytical work and transport of samples in milk recording. AC MIR–FT calibrations have to be based on selected native milk reference samples with suitable AC variation range and not on artificial AC addition to reference samples because of no AC recovery by MIR–FT in this case.

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Contact information

Oto Hanuš: hanus.oto@seznam.cz
Petr Roubal: roubal@milcom-as.cz
Jan Říha: jan@bentleyczech.cz
Marcela Vyletělová - Klimešová: marcela.vyletelova@seznam.cz
Radoslava Jedelská: radka.jedelska@seznam.cz
Jaroslav Kopecký: jaroslav.kopecky@email.cz
Eva Samková: samkova@zf.jcu.cz