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MILK UREA ANALYTICAL RESULT RELIABILITY AND ITS METHODICAL POSSIBILITIES IN THE CZECH REPUBLIC

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Abstract

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Control of milk urea concentration (MUC) can be used in diagnosis of the energy–nitrogen metabolism of cows. There are more analytical methods for MUC estimation and there are also discussions about their result reliability. Aim of this work was to obtain information for MUC result reliability improvement. MUC and MUN (milk urea nitrogen) were investigated in 5 milk sample sets and in 7 calibration/comparison experiments. The positions of reference and indirect methods were changed in experiments. There were following analytical methods for MUC or MUN (in mg.100 ml^{-1}): – photometric method (PH, as reference) based on paradimethylaminobenzaldehyde reaction; – method Ureakvant (UK, as reference) based on difference measurement of the electrical conductivity change during ureolysis; – method Chemspec (CH) based on photometrical measurement of ammonia concentration after ureolysis (as reference); – spectroscopic method in mid infrared range of spectrum (FT–MIR; indirect routine method). In all methodical combinations the correlation coefficients (r) varied from 0.8803 to 0.9943 ($P < 0.001$). In this way all relationships were relevant. The limits of accuracy and precision of FT–MIR are depend on reference method. Therefore, to pay attention to reference methods results is recommended. Both UK and PH could be calibrated to each other with similar parameters. The MUC reference methods (UK and PH) had better values (lower standard deviations) of accuracy (from 1.14 to 1.81 mg.100 ml^{-1}) and comparable values of repeatability (from 0.65 to 1.83 mg.100 ml^{-1}) as compared to FT–MIR MUC or MUN methods (from 1.39 to 5.6 and from 0.76 to 1.92 mg.100 ml^{-1}) in performed experiments.

cow, enzyme, spectrophotometry, infrared spectroscopy, conductivity, repeatability, reproducibility, precision, accuracy

Control of variability in milk urea concentration (MUC) can be used in diagnosis of the energy–nitrogen metabolism of cows (Erbersdobler *et al.*, 1980; Oltner and Wiktorsson, 1983; Baker *et al.*, 1985; Jílek *et al.*, 2006; Zhai *et al.*, 2006). MUC is sometimes linked also with production and reproduction performance and longevity of dairy cows (Butler *et al.*, 1996; Johnson and Young, 2003; Hojman *et al.*, 2004; Řehák *et al.*, 2009). Prediction of nutrition state of dairy cows according to MUC is practically useable and important for prevention of their metabolic troubles (Kirchgessner *et al.*, 1986; Hanuš *et al.*, 1993; Hojman *et al.*, 2004). However, MUC varies during day in dependence on feeding and sampling time (Gustafsson and Palmquist, 1993; Carlsson and Bergström, 1994). Therefore reliability of results of used analytical methods and methods of sampling are important for good practical interpretation of MUC values.

There are more analytical methods for MUC estimation (Patton and Crouch, 1977; Wolfschoon–Pombo *et al.*, 1981; Oltner and Sjaunja, 1982;

Rajamäki and Rauramaa, 1984; Oltner *et al.*, 1985; Hanuš *et al.*, 1995 a, b, 2001, 2008; Ficnar, 1997; Broutin, 2000, 2006; Peterson *et al.*, 2004). From time to time there are discussions about their result reliability in professional milk laboratory staff community (Herre, 1998; Klopčič *et al.*, 1999; Hanuš *et al.*, 2001). Today we have similar situation once again. International Dairy Federation has not defined one reference method for MUC determination up to now. In general, specific enzymatic methods (for instance AFNOR) with various measurement principles can be seen as reference procedures (Hanuš *et al.*, 1995 b, 2008; Lefier, 1998; Hering *et al.*, 2008).

This work is focused on evaluation of analytical methods for MUC determination and reliability their results under different laboratory conditions. The aim was to develop support possibilities and to obtain information for MUC result reliability improvement.

MATERIAL AND METHODS

Principles of used analytical methods for MUC determination

Most of used analytical principles for MUC were explained in our previous papers (Hanuš *et al.*, 1995 a, b, 1997, 2001, 2008; Hering *et al.*, 2008). The other principles are mentioned also in papers of following authors: Patton and Crouch, 1977; Wolfschoon–Pombo *et al.*, 1981; Oltner and Sjaunja, 1982; Rajamäki and Rauramaa, 1984; Oltner *et al.*, 1985; Ficnar, 1997; Herre, 1998; Lefier, 1998; Klopčič *et al.*, 1999; Broutin, 2000, 2006; Peterson *et al.*, 2004. Used MUC method principles were as follows:

- photometric method with Ehrlich solution is based on the change of colour by means of reaction of paradimethylaminobenzaldehyde measured at 420 nm (Spekol 11, Carl Zeiss, Jena, Germany) and it was calibrated on the five degree scale of standard water urea samples from 6 to 60 mg.100 ml^{-1}. The wide–spreaded result uncertainty of measurement (1.96 times the combined uncertainty as standard deviation with probability level 95%) was ± 3.25 mg.100 ml^{-1}, it means ± 9.28%;
- method Ureakvant is based on difference measurement of the change in the electrical conductivity during ureolytical hydrolysis of the urea by urease which is fixed on the inside surfaces of biosensor and this was calibrated on a five point scale of milk based urea standards from 12 to 60 mg.100 ml^{-1} (Ficnar, 1997; Hanuš *et al.*, 1997, 2001, 2008; Hering *et al.*, 2008). This is the direct specific enzymatic method with reference ambition. The wide–spreaded result uncertainty of measurement was ± 2.91 mg.100 ml^{-1}, it means ± 8.31%;
- method Chemspec (Bentley Instruments, USA) is based on photometrical measurement of ammonia concentration (Broutin, 2000, 2006) after enzymatic (urease) splitting of urea in milk as Berthelot's reaction (Patton and Crouch, 1977). Chemspec was calibrated using milk urea standards with known concentration. This is the direct specific enzymatic method with reference ambition;
- spectroscopic method in mid infrared range of spectrum (FT–MIR) with instruments Bentley FTS Combi (Bentley Instruments, USA), Foss 6000 (Foss Electric, Denmark) were calibrated mostly on the ten point scale of samples of native milk with different MUC (Hanuš *et al.*, 2008, 2011; Hering *et al.*, 2008) according to the results of specific reference method (mostly using Ureakvant or by specific retrospective calibration procedure (Hanuš *et al.*, 2011) in the Czech Republic). FT–MIR is indirect physical method. The wide–spreaded result uncertainty of measurement for FT–MIR was estimated as follows ± 4.451 mg.100 ml^{-1}, it means ± 15.9% (Hanuš *et al.*, 2009).

Design of experiments

Following table contains full experimental design described in this paper.

Description of experimental MUC data sets

Data set 1 – The data set was created by 1567 individual mik samples. All of samples were analyzed by using FTS1 instrument with usage of manufacturer proposed spectral calibration for MUN (Milk Urea Nitrogen content, in mg.100 ml^{-1}). All of samples were measured once on FTS1 under routine operating laboratory conditions. Then, Ureakvant UK1 was used as a reference method for all of samples (one measurement). This dataset was inspected in experiment 1.

Data set 2 – 797 herd milk samples were measured by using FTS1 instrument to determine MUN by FT–MIR method. Manufacturer MUN spectral calibration was used for this purpose. As a reference method, UK1 was used as well. Obtained data were evaluated in experiment No. 2.

Data set 3 – The third data set was obtained based on measurement of 32 individual milk samples by using Bentley Instruments Chemspec – instrument CH (1 measurement), Ureakvant UK1 (one measurement) and FTS1 (2 measurements under repeatability conditions). Experiment 3 brings analysis of this dataset.

Data set 4 – Data set consited from 13 different samples of milk (6 herd samples, 3 individual samples). Beside native bulk and individual cow milk samples the control set included also modified bulk milk sample variants with urea artificial addition (3 samples – plus 10, 20 and 30 mg.100 ml^{-1} to normal MUC basis, according to Hanuš *et al.*, 2011) and water diluted sample (1:7) (1 sample). Samples were processed three times under repeatability conditions on Ureakvants UK1, UK2, UK3). They were analyzed two times using photometrical method (means of 2 following measurements under repeatability conditions were used) performed by

I: *Description of experimental design and used abbreviations*

Experiment	No. of samples	Dataset	FT– MIR instruments	Reference method
1	1567	1	1	1
2	797	2	1	1
3	32	3	1	1, 2
4	13	4	-	1, 3, 4, 5*
5	13	4	-	1*, 3*, 4*, 5
6	13	4	1, 2, 3, 4	mean values of 1, 3, 4, 5
7	10	5	1, 2, 3, 4	mean values of 1, 3, 4

* Mean values were used as reference method against the others.

ID	Abbrev.	Instrument
1	FTS1	Bentley Instrruments FTS Combi – LRM (laboratory) Tuřany
2	FTS2	Bentley Instrruments FTS Combi – LRM Buštěhrad
3	FOSS1	Foss 6000 1. – LRM Buštěhrad
4	FOSS2	Foss 6000 2. – LRM Buštěhrad

ID	Abbrev.	Instrument/Method
1	UK1	Ureakvant – LRM Tuřany
2	CH	Bentley Instruments Chemspec - Gödölö, Hungary
3	UK2	Ureakvant – LRM Tuřany
4	UK3	Ureakvant – LRM Buštěhrad
5	PH	Photometry

the National Reference Laboratory for Raw Milk Quality in Rapotín.

All of samples were analyzed by all of included FT–MIR instruments as well (FTS1, FTS2, FOSS1, FOSS2). Obtained data set was used to evaluate 3 experiments:

- In experiment No. 4, performance and precision of all of three Ureakvants (UK1, UK2, UK3) were evaluated against mean values of two photometric measurements (PH) used as reference values.
- In experiment No. 5, 2 measurements done by PH were evaluated against mean values of UK1, UK2, UK3 as reference values.
- In experiment No. 6, FTS1, FTS2, FOSS1 and FOSS2 were evaluated against grand mean of PH, UK1, UK2, UK3 as reference values.

Data set 5 – The last dataset is represented by 10 individual milk samples analyzed double on FTS1, FTS2, FOSS1 and FOSS2. Ureakvants' (UK1, UK2, UK3) mean values were used as reference. Data were evaluated in experiment 7.

A sequence among experiments ensued from occurred and practically defined result discrepancies. Use of one reference method is always necessary for calibration or check procedure at evaluation of results of indirect methods but use of more reference methods for validation can improve the reliability of reference results. Therefore also this variant was tested. The similar situation is with measurement repetition. For instance in experiments with high number of samples was worked only with one measurement but in experiments with low number of samples the repeated measurements were used.

Statistical evaluation of data sets

Basic evaluation was then performed with all of three datasets by using following equations (ČSN ISO 8196 – 1, 2; CNIEL, 2010; Cecalait, 2008; ICAR 2002):

n .....number of observations,
sample .....value of MUC or MUN measurement determined by non-reference method within single measurement,
$\bar{x}$ .....mean value of measurements of *x*,
$\hat{x}$ .....predicted value of *x*,
reference .....value of MUC determined by reference method,
Min – Max ..max and min values of measurement,
min – max ....max and min values of reference method.

Repeatability

$$S_r = \sqrt{\frac{\sum_{i=1}^{n}\left(sample_i - \overline{sample_i}\right)^2}{(n-1)}}, \quad (1)$$

where $\overline{sample_i}$ indicates all of different measurements of the same milk sample under repeatability conditions.

Standard deviation of reference method and measurements

$$S_Y = \sqrt{\frac{\sum_{i=1}^{n}\left(ref_i - \overline{ref}\right)^2}{n-1}}, \quad S_X = \sqrt{\frac{\sum_{i=1}^{n}\left(sample_i - \overline{sample}\right)^2}{n-1}} \tag{2}$$

Mean error of prediction

$$d = \frac{\sum_{i=1}^{n}\left(\widehat{sample_i} - reference_i\right)}{n} = \overline{\widehat{sample}} - \overline{reference} \tag{3}$$

Standard deviation of mean prediction error

$$S_d = \sqrt{\frac{\sum_{i=1}^{n}\left(\left(\widehat{sample_i} - reference_i\right) - d\right)^2}{n-1}} \tag{4}$$

Residual error of regression

$$S_{y,x} = \sqrt{\frac{\sum_{i=1}^{n}\left(reference_i - \widehat{reference_i}\right)^2}{n-2}} \tag{5}$$

Covariance

$$S_{xy} = \operatorname{cov}(x,y) = \frac{\sum_{i=1}^{n}\left(sample_i - \overline{sample}\right)\left(reference_i - \overline{reference}\right)}{n-1} \tag{6}$$

Regression coefficient

$$r = \frac{S_{xy}}{S_x S_y} \tag{7}$$

Regression equation parameters

$$b = r\frac{s_y}{s_x} \tag{8}$$

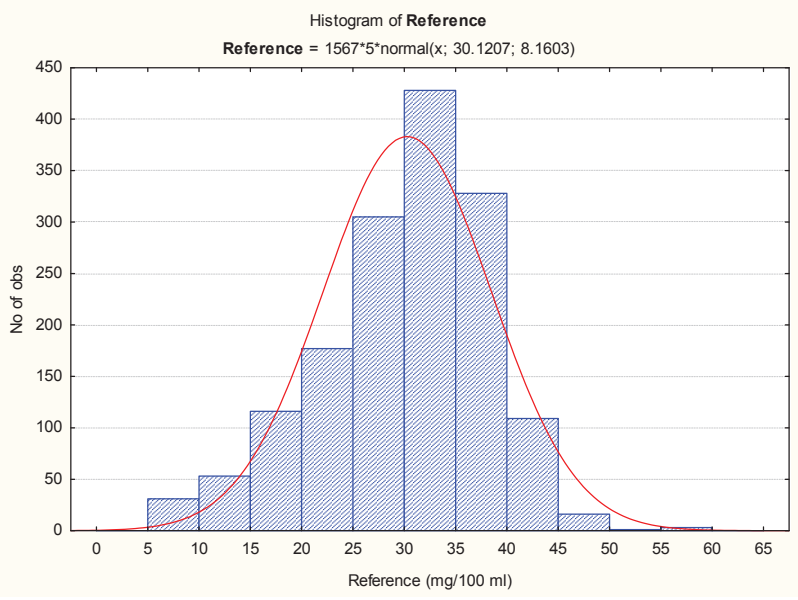
$$a = \overline{y} - b\overline{x} \tag{9}$$

RESULTS AND DISCUSSION

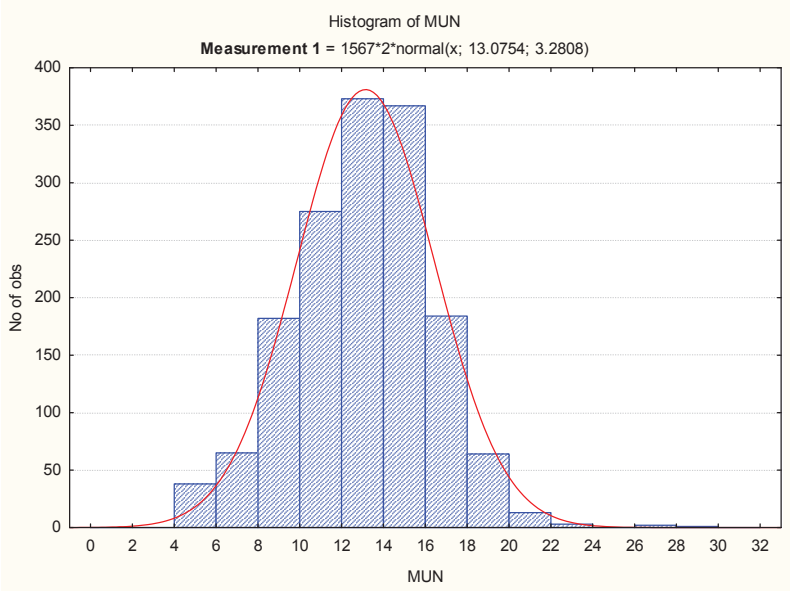
Experiment 1

In this experiment, 1567 individual milk samples were collected and analyzed under the normal operational conditions in LRM (laboratory) Tuřany in 2.5 month. All of samples were determined for urea in mg.100 ml^{-1} of milk with usage of Ureakvant UK1. All of samples were measured on FTS1 instrument with manufacturers spectral calibration for MUN.

Fluctuating quality of individual milk samples and randomly selected calibration samples (10–30)



1: *Histogram of reference values in individual samples (data set 1)*



2: *Histogram of FTS1 measurements for MUN in individual samples data set (data set 1)*

do not allow to cover the whole needed calibration interval properly.

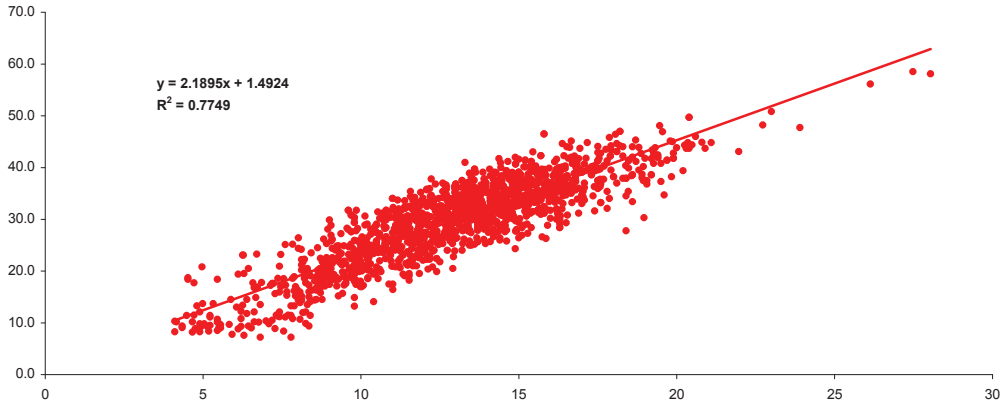
The aim of the experiment was to inspect daily measurements and try to find the most robust slope and bias calibration for MUN with using FTS Combi which can cover the whole interval of MUN which is normally processed in laboratory. Fig. 1 displays histogram of MUC measurements on included samples done with usage of UK1.

Values determined by reference method ranged from 7.20–58.50 mg.100 ml^{-1} of milk with arithmetic mean equaled to 30.12 mg.100 ml^{-1} of milk. As the best covered intervals in data set, 25–40 mg.100 ml^{-1} can be pointed. Fig. 2 displays histogram data for MUN (mg.100 ml^{-1}) measurement done with manufacturer spectral calibration on FTS1 (slope 1, bias 0). Values in data set ranged from 4.11–28.05 with mean value 13.08.

Linear regression was used to find proper slope and bias calibration (equation 6, 7, 8, 9). Then all of

II: *Summary results of experiment 1*

n = 1567	UK1	FTS1
Mean	30.121	13.075
Min	7.2	4.107
Max	58.5	28.047
Statistical evaluation		
Sr	-	
Sy	8.1603	
Sx	3.2808	
Sxy	23.5664	
R	0.8803	
R^2	0.7749	
B	2.1895	
A	1.4924	
D	–17.0453	
Sd	5.4973	
Syx	3.8732	



3: *Relationship between FT–MIR (MUN, FTS1) and Ureakvant (urea in mg.100 ml^{-1}) measurements in individual milk samples data set (experiment 1)*

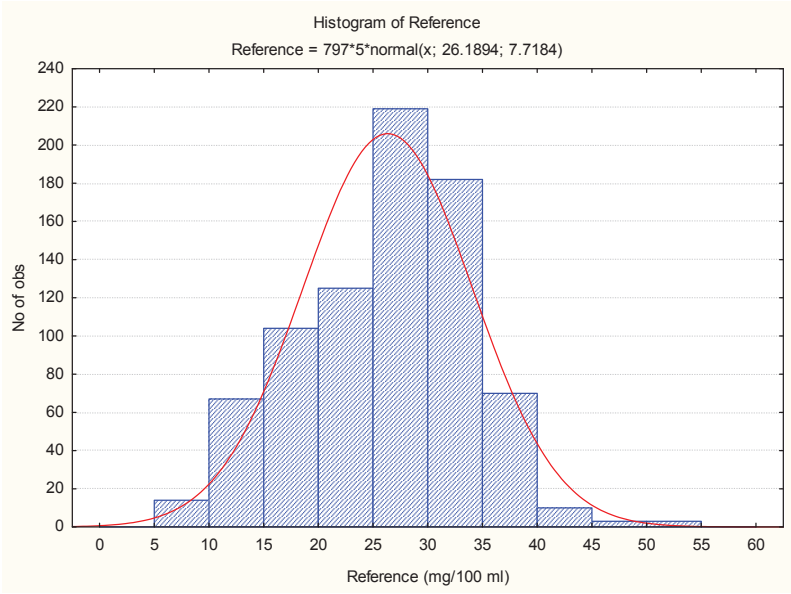
statistical values were calculated except repeatability (only 1 measurement for both of methods were available). Results of calibration are shown in Tab. II and Fig. 3.

According to suggested range of urea measurement (ICAR, 2002), in this experiment range covers values from 7.2 to 58.5 mg.100 ml^{-1}. As Fig. 1, 2 and 3 show, values of individual samples with urea content above 45 mg.100 ml^{-1} are very rare in normal laboratory testing as well as samples with values < 20 mg.100 ml^{-1}. This fact caused long term random sampling during calibration building to avoid failings in slope and bias calibration with not covered range of minimal and maximal values. Anyway, we should recommend another incremental steps aimed especially to samples with urea content above 50 mg.100 ml^{-1} which should lead to more robust calibration model.

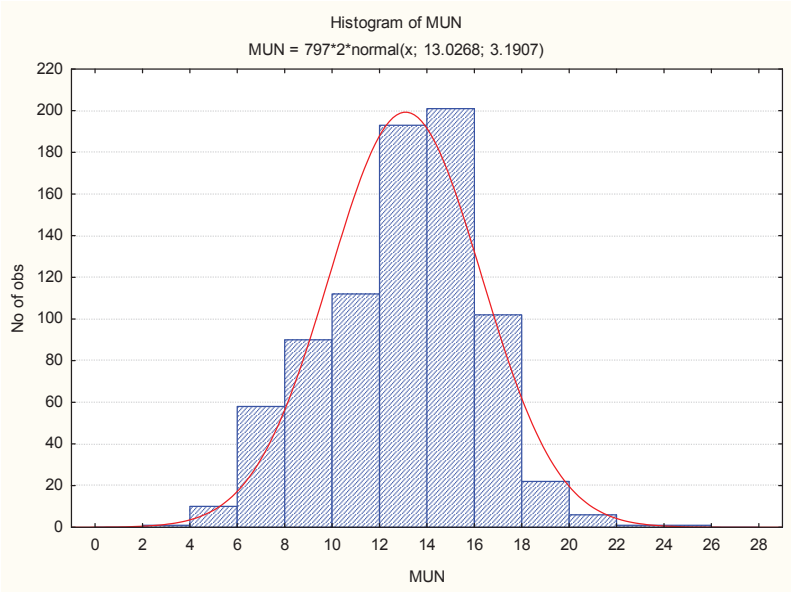
Regarding precision results of calibration model, Sy,x = 3.783 (Sy,x < 6.0 is recommended by ICAR, 2002 for individual samples) was reached for described dataset.

Experiment 2

In experiment 2, 797 herd samples were analyzed on UK1 and FTS1 to find out proper and robust slope and bias calibration which can improve and fits with routine laboratory samples. Distribution of reference values is displayed on Fig. 4.



4: *Distribution of reference values of urea content (UK1) in herd samples*



5: *Distribution of FTS1 determind values of MUN in herd samples*

III: *Summary results of experiment 2*

n = 797	UK1	FTS1
Mean	26.189	13.027
Min	6.6	3.827
Max	53.8	24.127
Statistical evaluation		
Sr	-	
Sy	7.7184	
Sx	3.1907	
Sxy	22.8551	
R	0.9280	
R^2	0.8613	
B	2.245	
A	–3.0554	
D	–13.1626	
Sd	4.9035	
Syx	2.8767	

Reference values ranged from 6.6–53.8 mg.100 ml^{-1}, arithmetic mean was estimated as 26.19 mg.100 ml^{-1}. Fig. 5. shows distribution of FTS1 measurement on the same data set. FTS1 values ranged from 3.83–24.13 MUN (mg.100 ml^{-1}) with arithmetic mean equals 13.03.

Results of slope and bias calibration performed between UK1 and FTS1 are summarized in Tab. III and Fig. 6.

Results obtained for long-term slope and bias calibration for herd milk samples show, however, better parameters reached for calibration model in comparison with individual samples. Correlation coefficient obtained for herd sample data set equals 0.861 in comparison with 0.775 in individual samples. Also, precision parameters like Sy,x = 2.877 are better (Sy,x = 3.783 in individual samples) and they fulfill ICAR specification for IR measurements for herd samples as well (recommended Sy,x = 4.000, (ICAR, 2002)).

On the other hand, we are missing samples with urea content higher than 40 mg.100 ml^{-1} in dataset, so recommendation for future work is clearly establish: to increment number of mentioned samples and calculate more robust calibration model then.

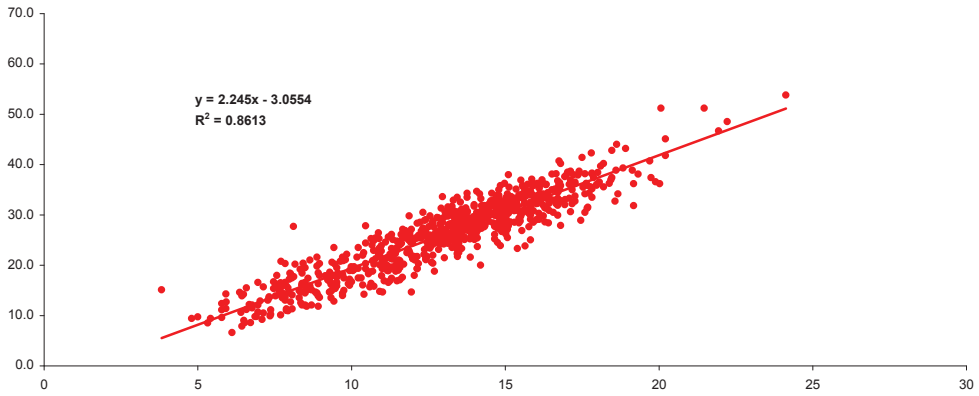
Experiment 3

Comparison with Chemspec instrument (CH) and Ureakvant UK1, both used as reference method with FTS1 measurement abilities was done in this eperiment on 32 randomly selected individual milk samples. The basic aim was to explore what kind of reference method is more usable and fits better with FT–MIR principles of MUN estimation. Manufacturer spectral calibration was used for FTS1. Samples were measured 2 times on FTS1, so repeatability results are available. Results are displayed in Tab. IV, V and on Fig. 7, 8.

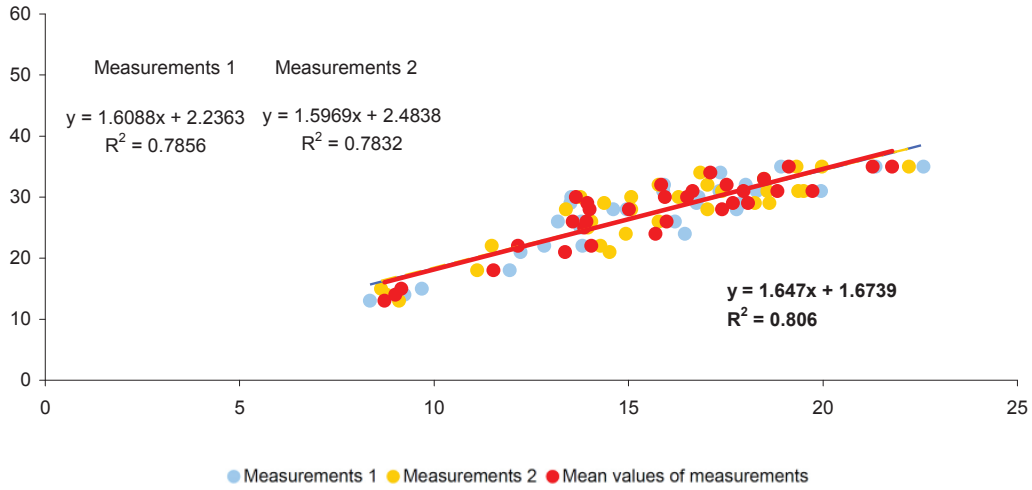
Possibilities of FT–MIR calibration against two reference methods are examined in this experiment. Samples in range 13.0–35.0 mg.100 ml^{-1} (CH) and 19.0–46.7 mg.100 ml^{-1} (UK1) were analyzed 2 times

IV: *Slope and bias calibration performance between CH and FTS1 measurements on 32 individual milk samples*

n = 32	CH	FTS1
Means	27.281	15.548
Min	13.0	8.354
Max	35.0	22.587
Statistical evaluation		
Sr	0.7584	
Sy	6.0174	
Sx	3.2802	
Sxy	17.7207	
R	0.8978	
R^2	0.806	
B	1.647	
A	1.6739	
D	–11.7332	
Sd	3.3951	
Syx	2.6939	



6: *Relationship between FT–MIR (MUN, FTS1) and Ureakvant (urea in mg.100 ml^{-1}) measurements in herd milk samples data set (experiment 2)*



7: *Relationship between two measurements using FTS1 and reference values measured on Chemspec*

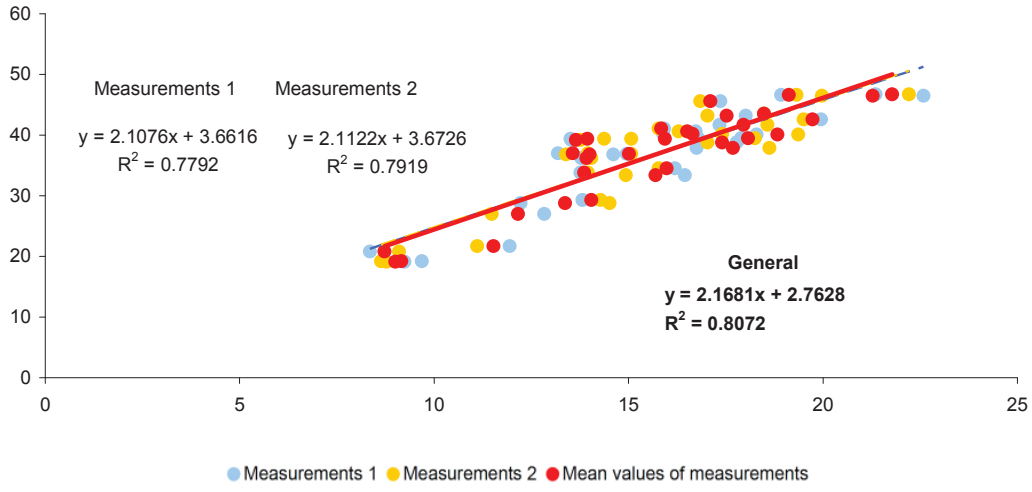
V: *Slope and bias calibration performance between UK1 and FTS1 measurements on 32 individual milk samples*

n = 32	UK1	FTS1
Means	36.472	15.548
Min	19.1	8.354
Max	46.7	22.587
Statistical evaluation		
Sr	0.7584	
Sy	7.9157	
Sx	3.2802	
Sxy	23.3272	
R	0.8984	
R^2	0.8072	
B	2.1681	
A	2.7628	
D	–20.9238	
Sd	5.1733	
Syx	3.5336	

on FTS Combi (FTS1) with repeatability Sr = 0.758. These results are in accordance with d values: d = –11.733 for CH, d = –20.924 for UK1. We also want to point on fact of these differences between reference methods, so their common calibration is more than highly recommended. Standard deviation of mean prediction error – Sd – was 3.395 for CH in comparison with 5.173 for UK1. Similar correlation parameters were reached for CH (r = 0.898) and for UK1 (0.898), so the outcome from these results is that both of methods are usable for FT–MIR slope and bias calibration purposes. When we compare precision results, Sy,x = 2.694 was reached for CH; Sy,x = 3.534 for UK1.

Experiment 4

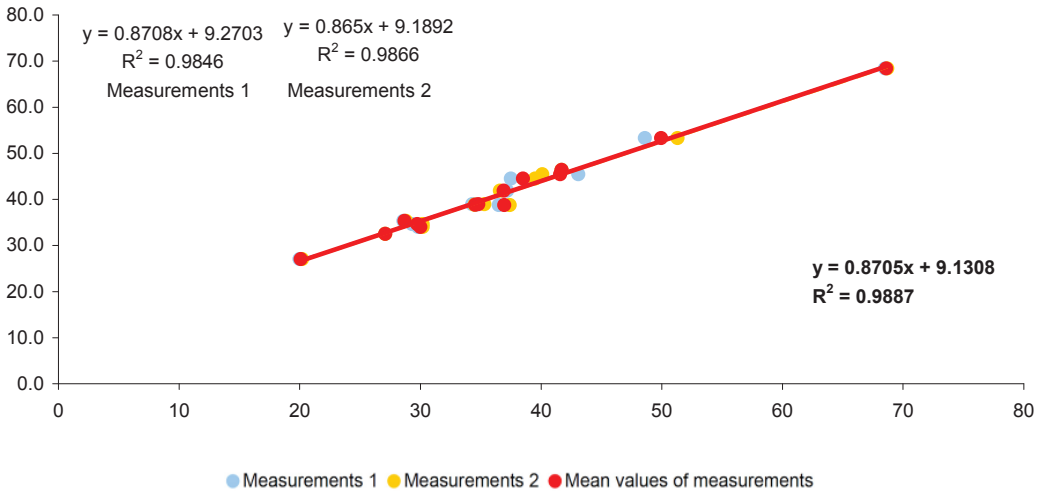
Experiments 4 and 5 are aimed to display how two reference methods (Ureakvant, photometry) fit each other. In experiment 4, means of two following measurements of photometrical method are used as reference values ones and 13 mixed samples



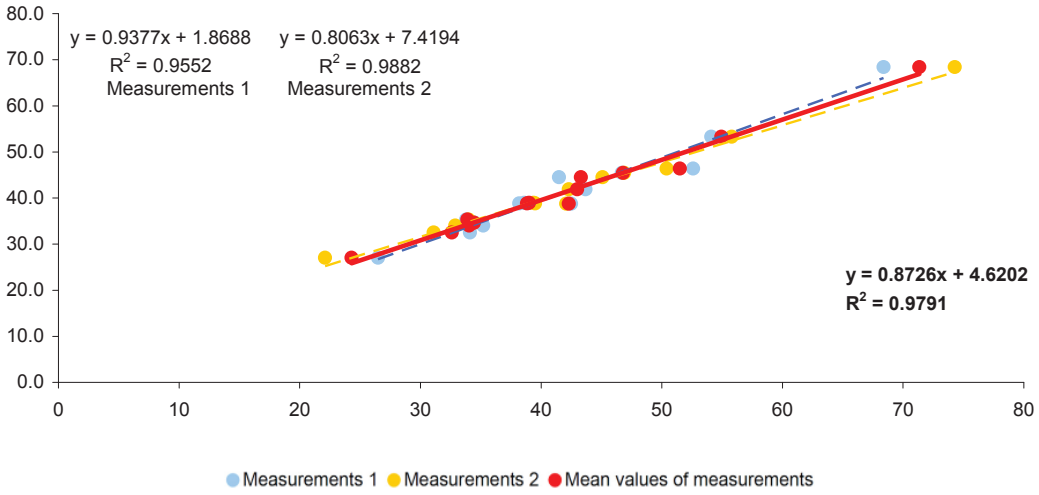
8: *Relationship between two measurements using FTS1 and reference values measured on UK1*

VI: *Comparison and calibration parameters of UK1, UK2, UK3 against means of photometric method as reference*

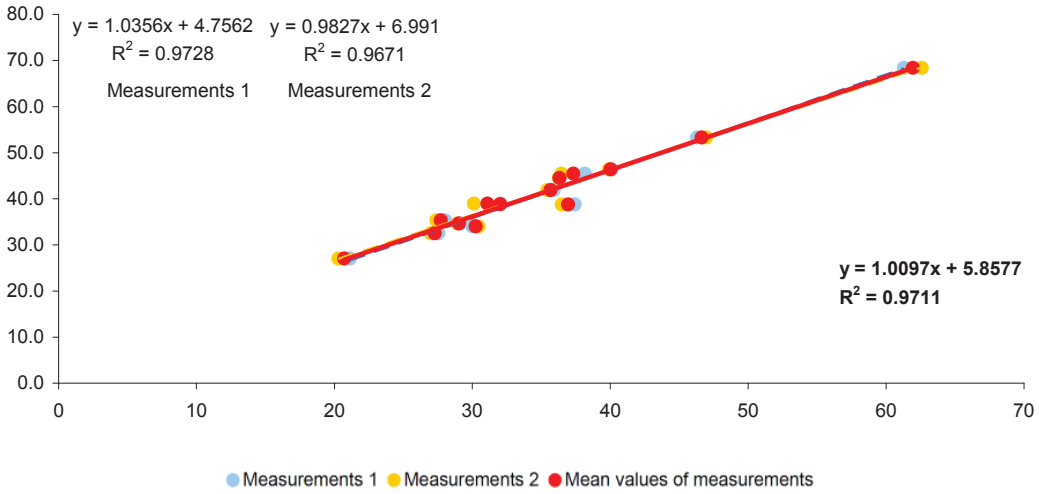
n = 13	Photometry	UK1	UK2	UK3
Means	41.415	37.086	42.168	35.215
Min	27.015	20.0	22.1	20.3
Max	68.380	68.7	74.3	62.61
Statistical evaluation				
Sr		0.9173	1.8292	0.6455
Sy		10.2349	10.2349	10.2349
Sx		11.6902	11.6063	9.9889
Sxy		118.9668	117.5423	100.7468
R		0.9943	0.9895	0.9854
R^2		0.9887	0.9791	0.9711
B		0.8705	0.8726	1.0097
A		9.1308	4.6202	5.8577
D		–4.3293	0.7529	–6.1995
Sd		1.8654	2.0916	1.7429
Syx		1.1348	1.5395	1.8112



9: *Relationship between two following measurements of UK1 and reference photometric method*



10: *Relationship between two following measurements of UK2 and reference photometric method*



11: *Relationship between two following measurements of UK3 and reference photometric method*

(dataset 4) are used to show relationship of both methods for all of three Ureakvants (UK1, UK2, UK3) separately. Tab. VI summarizes results of these comparisons as well as Fig. 9–11.

All of three Ureakvants show differences in minimum and maximum values of samples analyzed as well as in mean values of analyzed intervals in comparison with photometric method used as reference – see Tab. VI. Mainly UK1 and UK3 instruments seem to measure lower values on the whole sample range. Repeatability of each instrument ranges from 0.646 (UK3) to 1.829 (UK2) what represents more than double value of repeatability estimated on same samples.

Standard deviation of measurements is comparable for UK1, UK2 (11.690, 11.606) and lower for UK2 (9.989). Best correlation parameters for slope and bias calibration were reached for UK1 (r = 0.994). All of instruments showed high value of bias coefficient (e.g. for UK1, a = 9.131). The best values for mean error of prediction were obtained for UK2 (d = 0.753), the worst for UK3 (d = –6.200). Standard deviation of error results show the opposite trend – Tab. VI.

The most precise calibration (Sy,x = 1.135) was obtained for UK1, however it measures with worse values of d, Sd, Sx. The most „real" measuring UK2 (d = 0.753) reached Sy,x = 1.540 and the worst results of calibration precision were reached for UK3 (Sy,x = 1.811).

Based on mentioned results, we can point that UK1 is able to reach the best calibration with photometric method, however now, it is calibrated the differently as the rest of Ureakvants.

The summary of the experiment should be recommendation for common calibration of Ureakvants and photometric method as well as detailed experiments with Ureakvants themselves.

Experiment 5

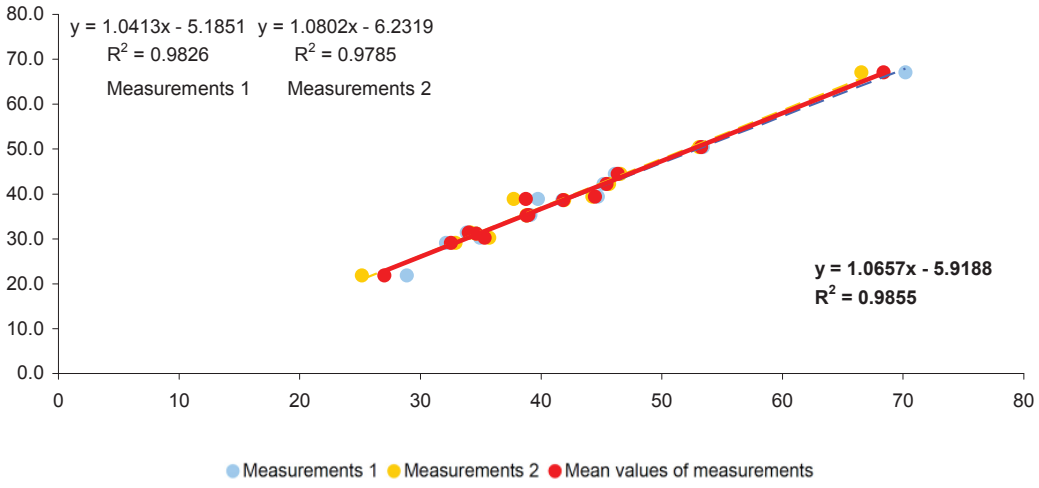
On the contrary of experiment 4, mean values of 3 Ureakvants UK1, UK2 and UK3 are used as a reference method against two photometric measurements done under repeatability conditions. Tab. VII and Fig. 12 summarize obtained results.

Photometric method reached Sr = 1.0911, what fits interval of Ureakvants repeatability in Experiment 4. Standard deviation of measurements Sx = 10.235 is comparable to Ureakvants mean standard deviation Sy = 10.9876 and is simmilar to independent Ureakvant instruments (Experiment 4). Also, correlation coefficient reached in this experiment (r = 0.993) is highly comparable to situation when reference and controlled methods were changed for each other.

When mean values of UK1, UK2, UK3 measurements are used as reference, we can see

VII: *Evaluation of relationship between 2 photometric measurements and mean values of UK1, UK2, UK3 used as reference values*

n = 13	UK1, UK2, UK3	Photometry
Means	38.218	41.415
Min	21.841	25.15
Max	67.086	70.2
Statistical evaluation		
Sr	1.0911	
Sy	10.9876	
Sx	10.2349	
Sxy	111.6364	
R	0.9927	
R^2	0.9855	
B	1.0657	
A	–5.9188	
D	3.1973	
Sd	1.4859	
Syx	1.3791	



12: *Relationship between 2 photometric measurements and mean values of UK1, UK2, UK3 used as reference values*

that photometric method measures with mean error of prediction (d = 3.197) – compare with Tab. VI. Value of Sd = 1.379 is lower than any value reach in Experiment 3, so photometric method can be pointed like less operating depend than Ureakvants in this meaning (on the whole range of samples).

Precisision of calibration was Sy,x = 1.379. Based on all of results of experiments 3 and 4, it can be easily seen, that both of methods could be calibrated to each other with similar parameters independent on real values (Sr, Sx, Sy, r, a, b, Sy,x), so they are closely equivalent with calibration, but with exception of real values measured in both experiments.

Again, the best recommendation is to perform common calibrations of both reference methods, lets say in kind of ring test.

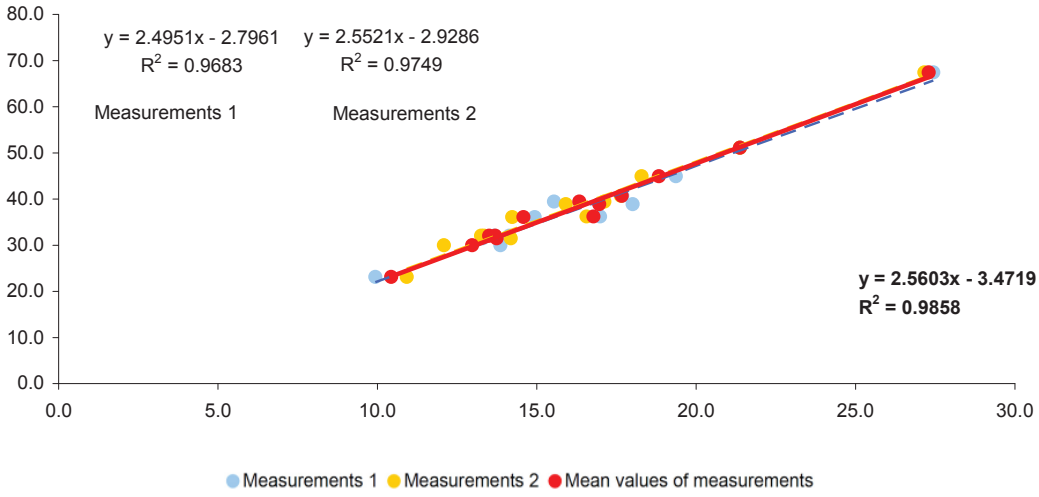
Experiment 6

With usage of set 4 data, four evaluations of FT–MIR instruments (FTS1, FTS2, FOSS1, FOSS2) double measurements under repeatability conditions were performed against a grand mean of two photometric measurements and UK1, UK2, UK3 double measurements used as a reference value. These values are represented the most robust reference values we were reached in this paper, so results can show effectively what performance and precision can be reached by different FT–MIR measurements. Following figures and table show results reached in this experiment.

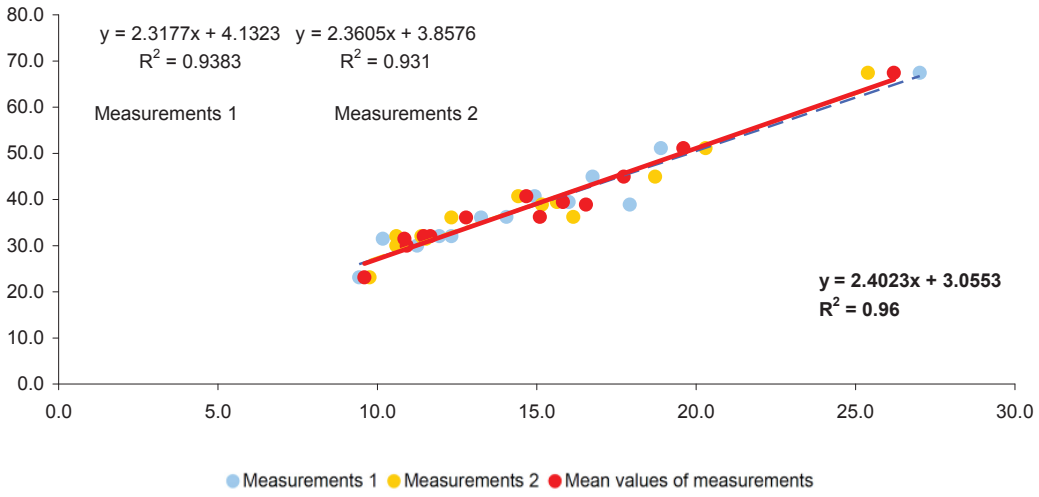
First of all, we need to mention again fact that sample set used for this experiment is normally used for round test purposes and contains samples with urea addition wich are not supposed for FT–MIR measurement as well as the whole set (individual

VIII: *Results about relationship between grand mean of UK1, UK2, UK3, photometry and FTS1, FTS2, FOSS1, FOSS2 measurements*

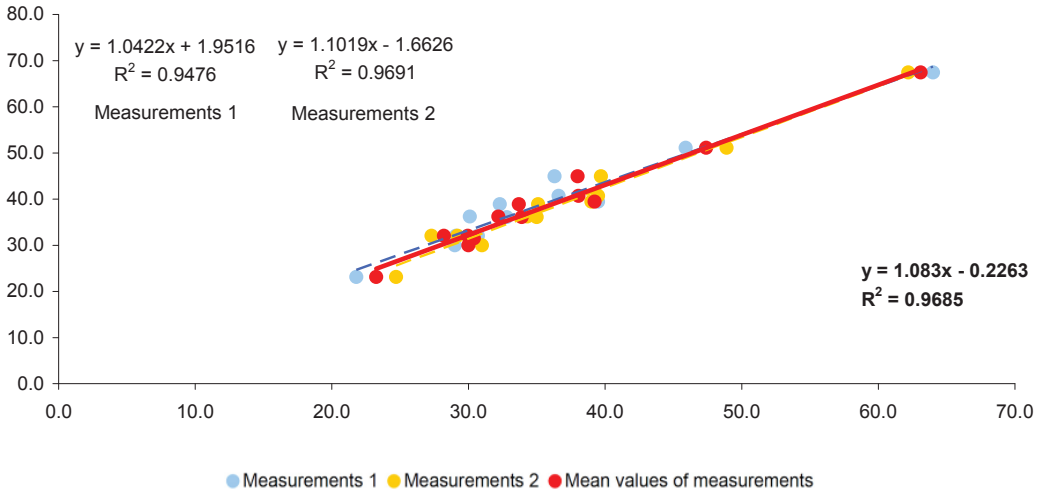
n = 13	UK1, UK2, UK3, Photometry	FTS1	FTS2	FOSS1	FOSS2
		MUN	*MUN*	*mg.100 ml^{-1}*	*mg.100 ml^{-1}*
Means	38.711	16.476	14.842	35.954	35.715
Min	23.135	9.94	9.427	21.8	24.6
Max	67.409	27.441	27.021	64.0	61.4
Statistical evaluation					
Sr		0.7497	1.0265	1.7616	1.274
Sy		11.162	11.162	11.162	11.162
Sx		4.3287	4.5525	10.1432	9.4923
Sxy		47.9734	49.7883	111.4205	104.4628
R		0.9929	0.9798	0.9841	0.9859
R^2		0.9858	0.96	0.9685	0.9721
B		2.5603	2.4023	1.0830	1.1594
A		–3.4719	3.0553	–0.2263	–2.6968
D		–22.235	–23.8684	–2.7568	–2.9952
Sd		6.8833	6.763	2.1524	2.4015
Syx		1.3868	2.3316	2.0692	1.948



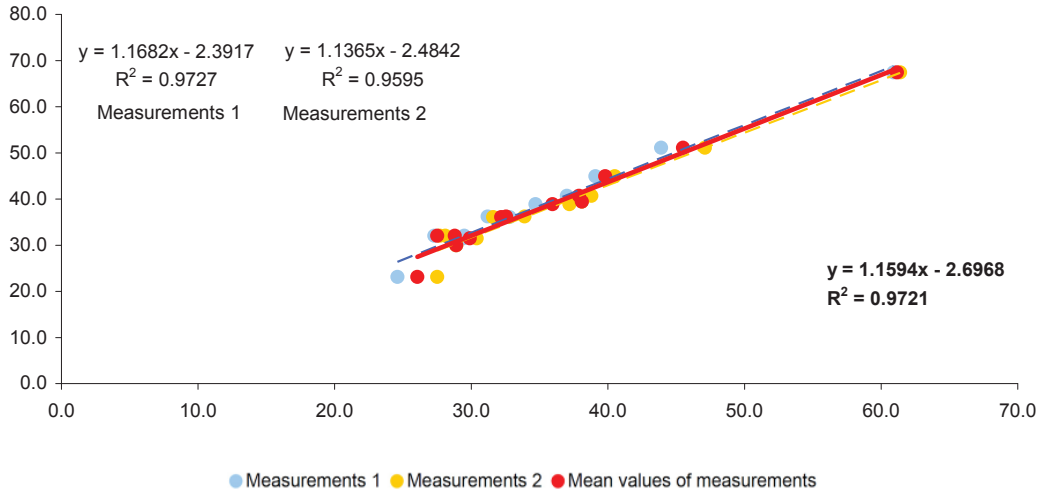
13: *Relationship between grand mean of UK1, UK2, UK3, photometry and FTS1 measurement of MUN*



14: *Relationship between grand mean of UK1, UK2, UK3, photometry and FTS2 measurement of MUN*



15: *Relationship between grand mean of UK1, UK2, UK3, photometry and FOSS1 measurement*



16: *Relationship between grand mean of UK1, UK2, UK3, photometry and FOSS2 measurement*

samples mixed with herd samples, diluted and added samples). On the other hand, advantages of the sample set are clear – they cover the whole range of possible values of MUN in milk (23.135–67.409 mg.100 ml^{-1}) and the final results can effectively summarize performance of FT–MIR method for the whole range of sample types.

The best repeatability was reached for instrument FTS1 (Sr = 0.750), followed by FTS2 (Sr = 1.027) in MUN. When slope and bias correlations were built for all of measurements, the best correlation coeficient was evaluated between FTS1 measurements and reference as r = 0.9929. As well, for other instruments, values of correlation coefficients are also acceptable – see Tab. VII. As this experiment is aimed directly to prove calibration abilities of each instrument on the best data set (mean of all examined reference methods) which we could obtain, Sy,x as the value of obtained calibration precision should be discussed here: for FTS1 Sy,x = 1.387 was obtained. This value is highly comparable with results showed in experiments 4 and 5 where two reference methods were compared against each other. Also, values observed for FOSS2, FOSS1 and FTS2 (Sy,x = 1.948, resp. 2.069, resp. 2.332) meet ICAR specification for individual samples as well as for herd samples (ICAR, 2002). All of these results show (plus results of other experiments in this paper), that limits of accuracy and precision of FT–MIR methods are highly depend on used reference method as well as on the other factors described. When the most „liable“ values (in the meaning of 4 reference repeated methods) are used, FT–MIR calibration can succesfuly fits these results as the another reference method.

When slope and bias coefficients are compared for the best performing instrument in experiment – FTS1 (a = –3.4719, b = 2.5603) and calibration obtained in experiment 2 for 797 herd samples (a = –3.0554, b = 2.2450), plus if we mention again results described in previous paragraph and samples used in this experiment data set, we can assume that routine calibration of FT–MIR instrument for herd samples can be done with similar sample set instead of long–term random sampling. But, as was previously mentioned as well, absolutely liable values of reference method must be use then for this purposes (or at least values comes from different reference methods).

Experiment 7

Four FT–MIR instruments (FTS1, FTS2, FOSS1, FOSS2) are used in this experiment to explore performance of calibration possibilities with usage of grand mean of three Ureakvants (UK1, UK2, UK3) double measurements as the most common reference method used in daily laboratory routine in LRM (laboratory) Tuřany and LRM Buštěhrad – Tab. IX, Fig. 17–20.

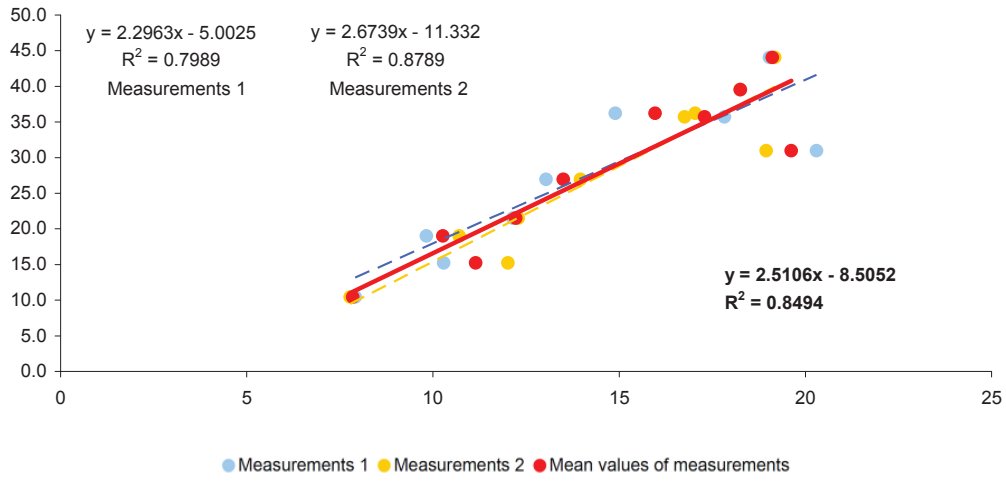
All of repeatability values for FT–MIR instruments obtained in this experiment (FTS1 = 0.782) are highly comparable with results described above. Randomly selected samples from routine laboratory sampling caused probably higher values of measurements standard deviations as well as worse correlation parameters. Also, slope and bias values are not suitable for enough robust calibration purposes. Values of this „checking“ data set for Sy,x meet closely ICAR specification anyway (3.461 for FTS1, 5.597 for FOSS2 as the best and the worst obtained results).

The main outcome of this experiment is suggested as the uncertainty (compare with Tab. VI. in experiment 4) of the reference method used (mean values of 3 Ureakvants) does not allow to obtain better results of calibration – compare with experiment 6. Again, based on these results we highly recommend to pay attention to reference methods results, testing and establishing to output common results produced by each.

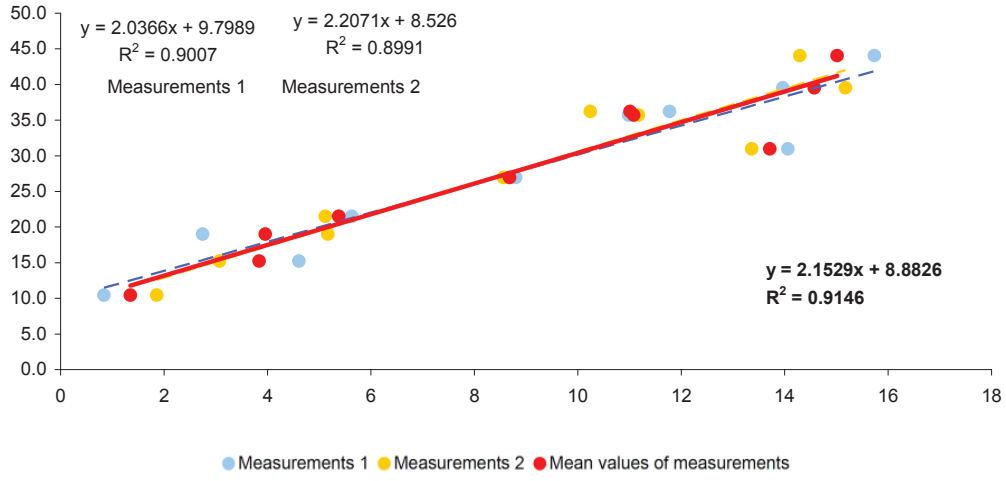
Most of calibration results comparisons is done to official ICAR limits in this paper. However, in

IX: *Statistical evaluation of precision reached between different FT–MIR instruments and grand mean of three Ureakvants (UK1, UK2, UK3)*

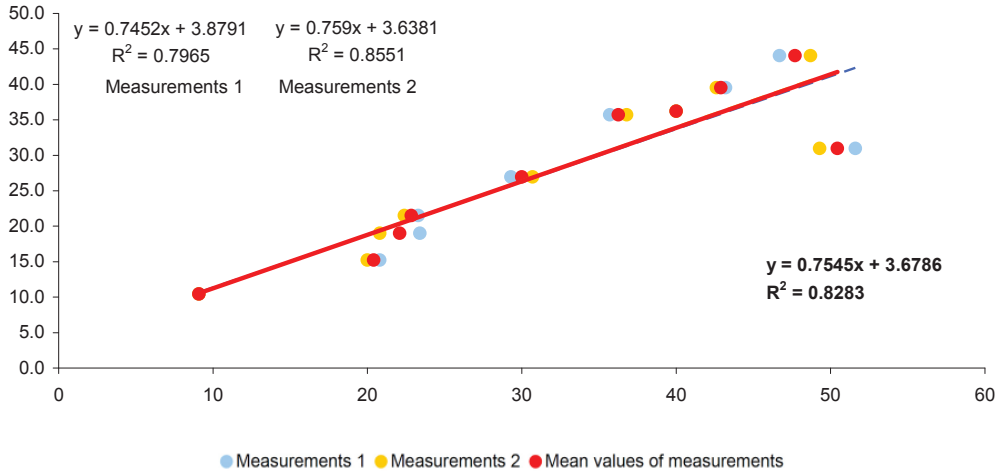
n = 10	UK1, UK2, UK3	FTS1	FTS2	FOSS1	FOSS2
		MUN	*MUN*	*mg.100 ml⁻¹*	*mg.100 ml⁻¹*
Means	27.956	14.523	8.859	32.175	30.485
Min	10.432	7.775	0.838	9.1	8.8
Max	44.055	20.298	15.736	51.6	49.1
Statistical evaluation					
Sr		0.782	0.8949	1.0254	0.7921
Sy		11.1646	11.1646	11.1646	11.1646
Sx		4.0984	4.9594	13.4667	12.3104
Sxy		42.17	52.9524	136.836	121.1214
R		0.9216	0.9563	0.9101	0.8813
R^2		0.8494	0.9146	0.8283	0.7766
B		2.5106	2.1529	0.7545	0.7992
A		–8.5052	8.8826	3.6786	3.5909
D		–13.433	–19.0964	4.2192	2.5292
Sd		7.5568	6.5832	5.6857	5.8267
Syx		4.5959	3.461	4.9067	5.5967



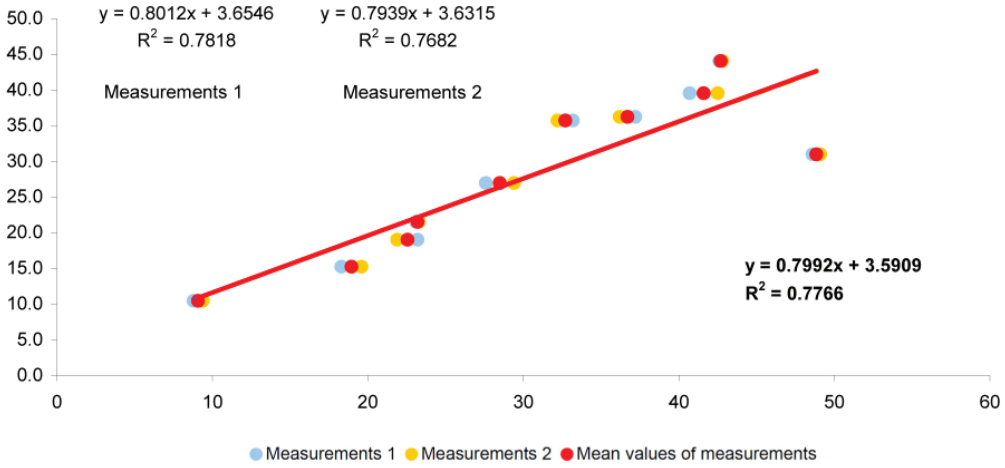
17: *Relationship between grand mean of UK1, UK2, UK3 and FTS1 measurements*



18: *Relationship between grand mean of UK1, UK2, UK3 and FTS2 measurements*



19: *Relationship between grand mean of UK1, UK2, UK3 and FOSS1 measurements*



20: *Relationship between grand mean of UK1, UK2, UK3 and FOSS2 measurements*

X: *Summary results of acuracy and repeatability for all measurements*

Instrument	Sample set	Reference	n	Accuracy Sy,x	Repeatability Sr
FTS1	1	Ureakvant – UK1	1567	3.87	–
FTS1	2	Ureakvant – UK1	797	2.88	–
FTS1	3	Chemspec – CH	32	2.69	0.76*
FTS1	3	Ureakvant – UK1	32	3.53	0.76*
UK1	4	Photometric – PH	13	1.14	0.92**
UK2	4	Photometric – PH	13	1.54	1.83**
UK3	4	Photometric – PH	13	1.81	0.65**
PH	4	UK1, UK2, UK3	13	1.38	1.09**
FTS1	4	UK1, UK2, UK3, PH	13	1.39	1.61*
FTS2	4	UK1, UK2, UK3, PH	13	2.33	2.2*
FOSS1	4	UK1, UK2, UK3, PH	13	2.07	1.76**
FOSS2	4	UK1, UK2, UK3, PH	13	1.95	1.27**
FTS1	5	UK1, UK2, UK3	10	4.56	1.68*
FTS2	5	UK1, UK2, UK3	10	3.46	1.92*
FOSS1	5	UK1, UK2, UK3	10	4.91	1.03**
FOSS2	5	UK1, UK2, UK3	10	5.6	0.79**

*Repeatability for MUN (manufacturer's calibration). ** Repeatability for MUC (manufacturer's calibration).

general, obtained results have quite comparable character as our previous results and results of other authors (Wolfschoon–Pombo *et al.*, 1981; Oltner and Sjaunja, 1982; Rajamäki and Rauramaa, 1984; Oltner *et al.*, 1985; Hanuš *et al.*, 1995 b, 1997, 2001, 2008; Ficnar, 1997; Lefier, 1998; Klopčič *et al.*, 1999; Broutin, 2000, 2006; Peterson *et al.*, 2004; Hering *et al.*, 2008) in terms of calibration quality parameters, accuracy or repeatability of measurements.

CONCLUSION

As this is shown, the MUC reference methods (UK and PH) had better values (lower standard deviations) of accuracy (from 1.14 to 1.81 mg.100 ml^{-1}) and comparable values of repeatability (from 0.65 to 1.83 mg.100 ml^{-1}) as compared to FT–MIR MUC or MUN methods (from 1.39 to 5.6 and from 0.76 to 1.92 mg.100 ml^{-1}) in performed experiments (Tab. X). In all methodical comparison combinations the correlation coefficients (r) varied from 0.8803 to 0.9943 ($P < 0.001$). It shows that all experimental methodical result relationships could be seen as relevant. As professional contribution of this paper, this tis possible to compare general reached results from Tab. X to results obtained under specific combination of laboratory environmental conditions anywhere as ruler. All of used direct methods (PH, UK, CH) showed suitable properties for recommendation as reference procedures.

SUMMARY

Control of variability in milk urea concentration (MUC) can be used in diagnosis of the energy–nitrogen metabolism of cows. MUC is sometimes linked also with production and reproduction performance and longevity of dairy cows. Prediction of nutrition state of dairy cows according to MUC is practically useable and important for prevention of their metabolic troubles. There are more analytical methods for MUC estimation. There are discussions about their result reliability in professional milk laboratory staff community. Aim of this work was to develop support possibilities and to obtain information for MUC result reliability improvement. MUC and MUN (milk urea nitrogen) were investigated in 5 milk sample sets and in 7 calibration or comparison experiments. The positions of reference and indirect methods, numbers and types of analyzers and numbers and types of milk samples were changed in experiments. There were used following analytical methods for MUC or MUN (in mg.100 ml^{-1}) determination: – photometric method with Ehrlich solution (PH, as reference) based on paradimethylaminobenzaldehyde reaction (420 nm); – method Ureakvant (UK, as reference) based on difference measurement of the electrical conductivity change during enzymatic urea hydrolysis; – method Chemspec (CH) based on photometrical measurement of ammonia concentration after enzymatic urea hydrolysis (as reference); – spectroscopic method in mid infrared range of spectrum (FT–MIR; indirect routine method). In all methodical comparison combinations the correlation coefficients (r) varied from 0.8803 to 0.9943 ($P < 0.001$). It shows that all experimental methodical result relationships could be seen as relevant. The limits of accuracy and precision of FT–MIR methods are highly depend on used reference method. We highly recommend to pay attention to reference methods results. Both of methods UK and PH could be calibrated to each other with similar parameters independent on real values. Most of calibration results comparisons is done to official ICAR limits in this paper. However, in general, obtained results have quite comparable character as our previous results and results of other authors in terms of calibration quality parameters, accuracy or repeatability of measurements. The MUC reference methods (UK and PH) had better values (lower standard deviations) of accuracy (from 1.14 to 1.81 mg.100 ml^{-1}) and comparable values of repeatability (from 0.65 to 1.83 mg.100 ml^{-1}) as compared to FT–MIR MUC or MUN methods (from 1.39 to 5.6 and from 0.76 to 1.92 mg.100 ml^{-1}) in performed experiments. This is possible to compare general reached results to results obtained under specific combination of laboratory environmental conditions anywhere as ruler. All of used direct methods (PH, UK, CH) showed suitable properties for recommendation as reference procedures.

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