МЕТОДОЛОГІЧНІ АСПЕКТИ ОЦІНЮВАННЯ ЯКОСТІ ТОВАРІВ

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EXPRESS METHOD OF QUANTITATIVE DETERMINATION OF UREA IN MILK

The proposed methodical approach in the variant of using the method of additives (MA) expects quantitive determination of urea in milk samples by spectrophotometric method without the need to build a calibration curve and long calculations. This allows significantly speed up the analysis process, reduce material and time costs and at the same time to obtain correct and accurate results, which is confirmed by the validation. The procedure is effective in the range of 0.14–0.7 mg/mL, the detection limit is 0.05 mg/mL. The uncertainty forecast was 7.33 %, which does not exceed the maximum allowable uncertainty of the technique (10 %).

Keywords: method of additives, milk, urea, spectrophotometry.

Жукова Я., Петров Ф., Клименко Л. Экспресс-метод количественного определения мочевины в молоке. *Предложенный методический подход в варианте использования метода добавок (МД) предполагает количественное определение мочевины в образцах молока спектрофотометрическим методом без необходимости постройки калибровочной кривой и длительных расчетов. Это позволяет значительно ускорить процесс анализа, сократить материальные и временные затраты, и при этом получить правильные и точные результаты, что подтверждается проведенной валидацией. Методика эффективна в диапазоне 0.14–0.7 мг/мл, предел обнаружения составляет 0.05 мг/мл. Прогноз неопределенности составил 7.33 %, что не превышает максимально допустимую неопределенность методики (10 %).*

Ключевые слова: метод добавок, молоко, мочевина, спеткрофотометрия.

Background. Urea is the final metabolite of nitrogen-containing compounds of ruminants, which is formed in liver from ammonia as a result of deamination reaction of amino acids. Typically, the urea content in milk varies in the range of 0.15–0.70 mg/mL [1].

Cattle diet and feeding mode are the most significant factors influencing the milk urea content (MUC). Consumption of feed affects the daily variation of rumen ammonia, urea nitrogen plasma and milk urea nitrogen concentrations. A separate feeding of forage and concentrated feed, as opposed to the use of total mixed ration on farms leads to divide intake of protein and carbohydrate feed to rumen. This causes rapid degradation of proteins in rumen and consequently increases concentration of rumen ammonia and urea nitrogen plasma, as evidenced by relevant experiments [2; 3].

High level of urea concentration in milk effects on its technological properties: it can decrease the acidity level, increases the rennet coagulation duration, inhibits the activity of starter cultures during fermentation. The introduction of excessive amount of nitrogen-containing fertilizers in pasture, the addition of carbamide in the ration of cows under the conditions of minimum hay content, as well as increasing the carbamide content more than 30 % of feed protein digested by the normal content of hay in the diet, can lead to a decrease in the content of alpha-casein, which worsens the cheese suitability of milk. The property of urea to increase the thermostability of milk causes cases of falsification of milk by this compound in summer.

Thus, determination of MUC values is important for different fields: veterinary, cattle feeding, dairy processing, food quality control etc.

Today there is a wide range of methods for determining urea, which are divided into direct and indirect. Direct methods include direct determination of urea (without its preliminary cleavage) by means of physico-chemical methods of analysis (for example, absorption spectrophotometry in the UV, IR and visible spectral regions, voltammetry or high performance liquid chromatography). Studies are carried out both before and after preliminary derivatization of urea by performing special chemical reactions that occur with formation of compounds that absorb light with a given wavelength [4–6]. Indirect methods (or enzymatic) involve enzymatic cleavage of urea before measurements.

However, for the listed direct methods, complex and expensive equipment is required [4], they are limited to the quantification limit of urea [5] or the need for additional studies and trials [6], and in the case of enzymatic methods the use of enzymes and reagents with short-term use is required.

The *purpose* of our work is to develop and conduct a phased validation of the method of quantitative determination of urea content in milk by method of absorption spectrophotometry in the visible spectrum region in variant of additive method use (MA), that will accelerate the analysis process, reduce material and time costs and at the same time get correct and accurate results.

Material and methods. Reagents and chemicals. Hydrochloric acid (≥ 37 %, puriss. p.a., ACS reagent, fuming), *p*-dimethylaminobenzal-

dehyde (*p*-DMAB), urea were purchased from Sigma-Aldrich Co. LLC (USA). Potassium dihydrogen phosphate anhydrous (KH_2PO_4) and potassium hydrogen phosphate anhydrous (K_2HPO_4) were purchased from Prayon S.A. (Belgium). Trichloroacetic acid (TCA) was purchased from PanReac AppliChem (Germany). All other reagents were of analytical grade.

p-DMAB reagent: 1.6 g of *p*-DMAB was dissolved in 10 mL of concentrated hydrochloric acid and the solution was diluted to 100.0 mL with ethanol.

Phosphate buffer solution (pH 7): a) 3.403 g of KH_2PO_4 was dissolved in distilled water and the solution was diluted to 100.0 mL with the same solvent; b) 4.355 g of K_2HPO_4 was dissolved in distilled water and the solution was diluted to 100.0 mL with the same solvent; the solutions *a)* and *b)* were mixed and diluted to 1 L with distilled water.

TCA solution: 24.0 g of trichloroacetic acid was dissolved in distilled water and the solution was diluted to 100.0 mL with the same solvent.

Equipment. All spectrophotometric measurements were carried out using a single beam VIS-spectrophotometer UNICO S2100 (UNICO, USA) with wavelength scanned from 1000 to 325 nm. The spectral band width was 5 nm. The pair of quartz square cells S90-309Q (UNICO, USA) with 10 mm pathlength and wavelength range from 200 to 1200 nm was used throughout the whole experiment.

Weighing was carried out using digital analytical balance AN100 (AXIS, Ukraine) with $d = 0.0001$ g.

Glassware satisfied ISO 648:2008 "Laboratory glassware – Single-volume pipettes", ISO 1042:1998 "Laboratory glassware – One-mark volumetric flasks", ISO 4788:2005 "Laboratory glassware – Graduated measuring cylinders", ISO 385:2005 "Laboratory glassware – Burettes" and calibrated according to ISO 4787:2010 "Laboratory glassware – Volumetric instruments – Methods for testing of capacity and for use" and "Guidelines for calibration in analytical chemistry" [7] was used throughout this study.

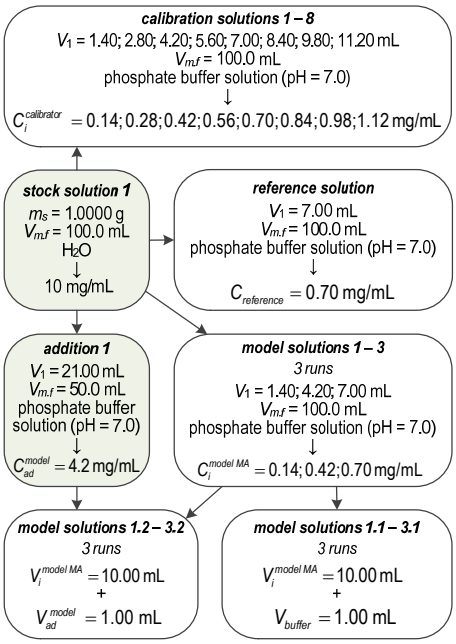
Solutions and samples (*Schemes 1* and *2*). The stock solutions 1 and 2 (10 mg/mL) were prepared by dissolving m_s = 1.0000 g of urea in the measuring flask ($V_{m.f}$ = 100.0 mL) in distilled water and the solutions were diluted to 100.0 mL with the same solvent.

The reference solution ($C_{reference}$ = 0.70 mg/mL) was prepared by diluting 7.00 mL of stock solution 1 to 100.0 mL with phosphate buffer solution (pH 7).

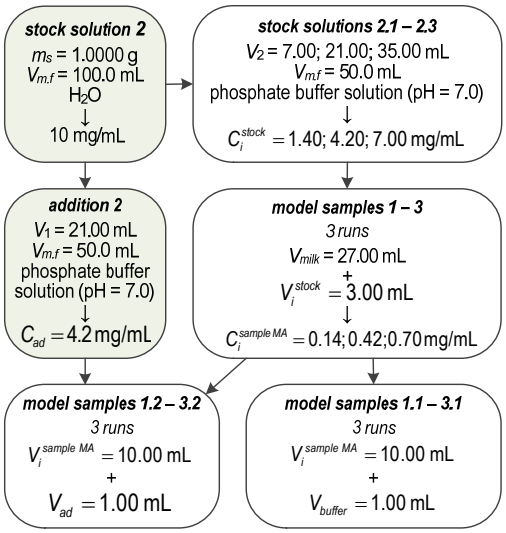
To prepare the calibration solutions 1–8 (having concentrations $C_i^{calibrator}$ of 0.14; 0.28; 0.42; 0.56; 0.70; 0.84; 0.98 and 1.12 mg/mL respectively) the stock solution 1 was diluted with phosphate buffer solution (pH 7) to 100.0 mL.

The addition 1 (C_{ad}^{model} = 4.2 mg/mL) and 2 (C_{ad} = 4.2 mg/mL) were prepared by diluting 21.00 mL of the stock solution 1 and 2 respectively to 50.0 mL with phosphate buffer solution (pH 7).

To prepare the model solutions 1–3 (having concentrations $C_i^{model\ MA}$ of 0.14; 0.42; 0.70 mg/mL respectively) the stock solution 1 was diluted with phosphate buffer solution (pH 7) to 100.0 mL.



Scheme 1. The preparation procedure for reference and model solutions of urea



**MA* – the parameter is attributed to the method of additions

Scheme 2. The preparation procedure for model milk samples with urea

To prepare the model solutions 1.1–3.1, 10.00 ml of the respective model solutions 1–3 were mixed with 1.00 mL of phosphate buffer solution (pH 7).

To prepare the model solutions 1.2–3.2, 10.00 ml of the respective model solutions 1–3 were mixed with 1.00 mL of the addition 1.

To prepare the stock solutions 2.1–2.3 (having concentrations C_i^{stock} of 1.40; 4.20; 7.00 mg/mL respectively) the stock solution 2 was diluted with phosphate buffer solution (pH 7) to 50.0 mL.

Three batches (in 3 samples each) of respective matrix (milk) obtained from three different sources were used to prepare the model samples.

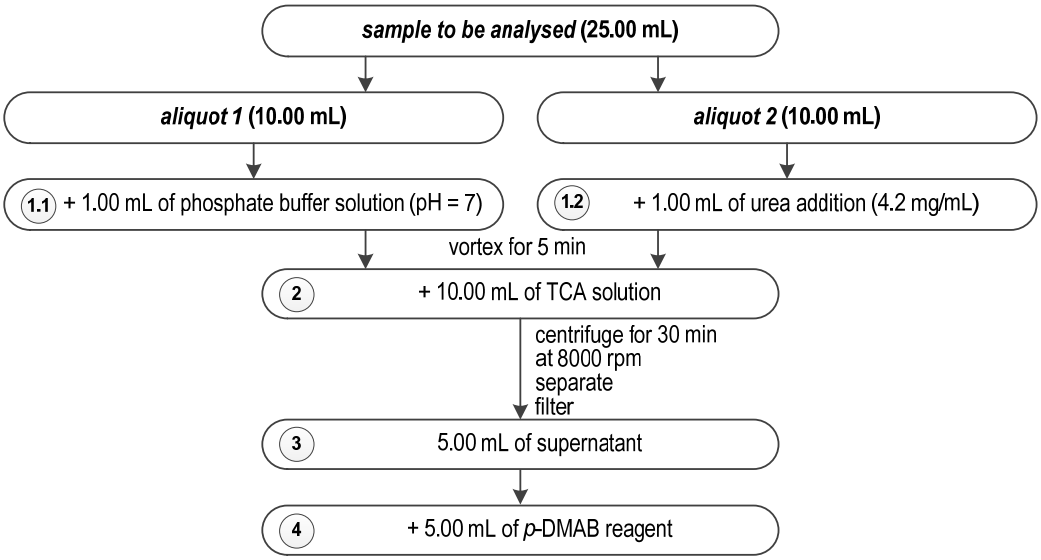
The model samples 1–3 (having concentrations $C_i^{sample\ MA}$ of 0.14; 0.42; 0.70 mg/mL respectively) were prepared by mixing 27.00 mL of milk and 3.0 mL of the stock solutions 2.1–2.3 respectively.

To prepare the model samples 1.1–3.1 in 10.00 ml of the respective model samples 1–3 were mixed with 1.00 mL of phosphate buffer solution (pH 7).

To prepare the model samples 1.2–3.2 in 10.00 ml of the respective model samples 1–3 were mixed with 1.00 mL of the addition 2.

Blank-samples were prepared by mixing 3 samples (27.00 mL) of respective matrix (milk) obtained from three different sources with 3.00 mL of phosphate buffer solution (pH 7).

Analytical sample preparation (*Scheme 3*).



Scheme 3. The main stages of analytical sample preparation for urea quantification

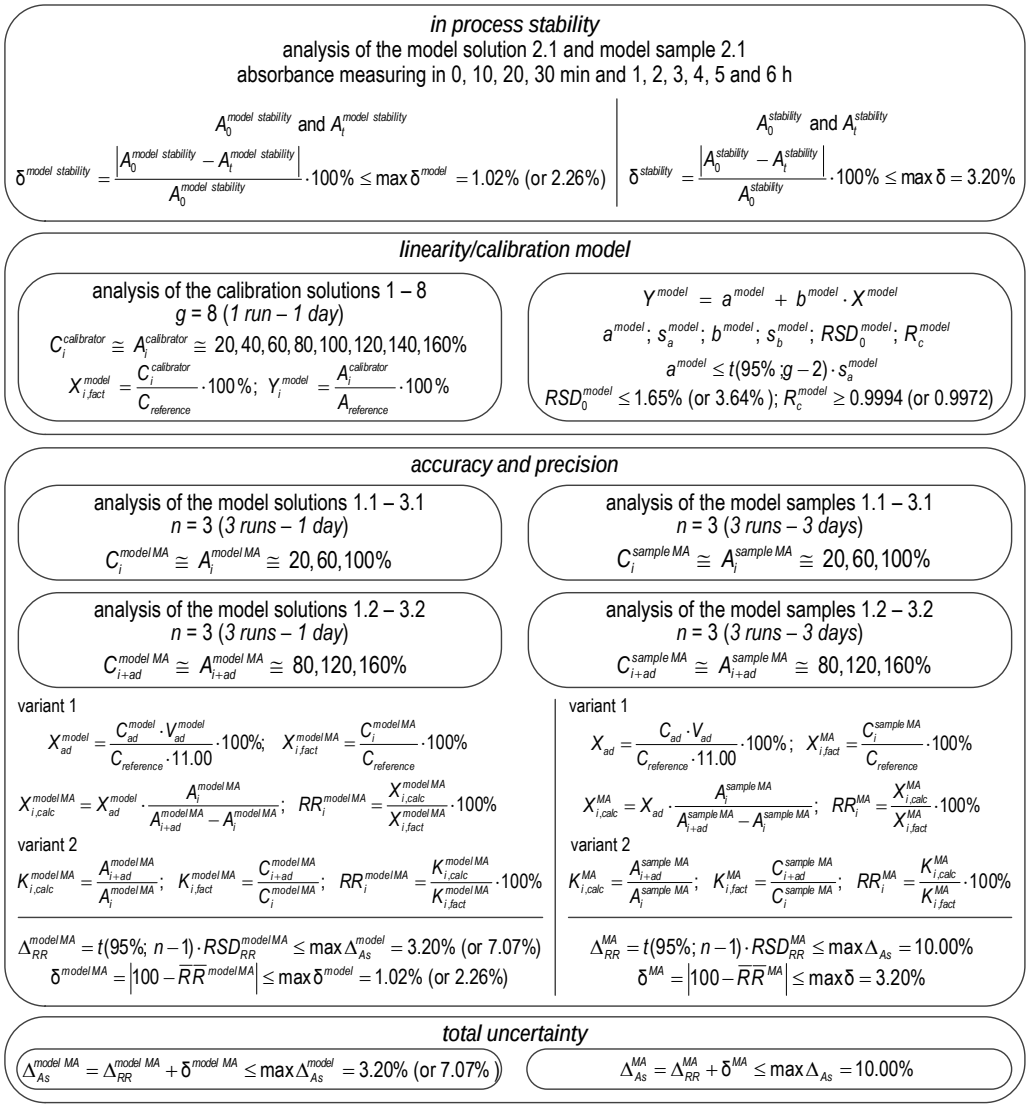
Analysis is carried out in two stages: 2 aliquots of milk (in 10.00 mL each) are taken from the sample to be analysed. 1.00 mL of phosphate buffer solution (pH 7) is added to the first aliquot and 1.00 mL of urea addition (4.2 mg/mL) is added to the second aliquot (stages 1.1 and 1.2). The following stages are the same for both aliquots. The mixture is vortexed for 5 min and processed with 10.00 mL of TCA solution (stage 2), then centrifuged for 30 min at 8000 rpm. The supernatant is separated and filtered. 5.00 mL of *p*-DMBA reagent are added to 5.00 mL of the obtained supernatant (stages 3 and 4) and the solution to be analysed is ready.

The absorbance of the solutions to be analysed is measured 3 times (λ_{max} = 420 nm) with randomization of cell position. The mixture of the phosphate buffer solution (pH 7), TCA solution and *p*-DMBA reagent (11:10:20) is used as a compensation solution.

Method validation *(Scheme 4).* Validation of the developed procedure has been carried out in variants of method of additives (Klimenko, 2015).

For *in process stability* verification the model solution 2.1 and model milk sample 2.1 with urea concentration of 0.42 mg/mL were processed according to the procedure. The absorbance measurements for the final solutions were carried out immediately ($A_0^{model\ stability}$ and $A_0^{stability}$ respectively)

and for the subsequent 6 hours (in 10, 20, 30 min, 1, 2, 3, 4, 5 and 6 h) after its preparation ($A_t^{model\ stability}$ and $A_t^{stability}$ respectively), and the systematic errors $\delta^{model\ stability}$ and $\delta^{stability}$ respectively were calculated and assessed.



*MA – the parameter is attributed to the method of additions
*model – the parameter is determined using model solutions

Scheme 4. The validation stages of spectrophotometric procedure for urea determination

To determine *linearity/calibration model* the calibration solutions 1–8 were analysed within 1 run. The values of concentrations and analytical responses $A_i^{calibrator}$ were normalized and processed by the method of least squares [8]; correlation coefficient R_c^{model}, rest standard deviation RSD_0^{model}, slope b^{model} and its standard deviation s_b^{model}, and also absolute term a^{model} and its standard deviation s_a^{model} were calculated [9; 10] and assessed.

To estimate *precision (repeatability) and accuracy:*

- the model solutions 1.1–3.1 and 1.2–3.2 were analysed within 3 runs; the model solutions 1.1–3.1 concentrations were recalculated and the values "found/given" $RR_i^{mode\ MA}$ were used to determine the confidence interval $\Delta_{RR}^{mode\ MA}$ and the systematic error $\delta^{mode\ MA}$ respectively;
- the model samples 1.1–3.1 and 1.2–3.2 were analysed within 3 runs; the model samples 1.1–3.1 concentrations were calculated and the values "found/given" RR_i^{MA} were used to determine the confidence interval Δ_{RR}^{MA} and the systematic error δ^{MA} respectively.

The values of confidence intervals and systematic errors were compared with the respective acceptability criteria.

Results and discussion.

Analysis and validation scheme justification. Our research is based on the method for the determination of urea in milk by method of absorption spectrophotometry in the visible region of spectrum, which requires the photometric reaction of Schiff base formation during the interaction of urea with *p*-DMAB [1].

This technique involves working in variant of calibration graph method, constructed from the response data of series of urea aqueous solutions. In such a situation, the matrix influence on the analysis results can be significant (both in large and lower side), and the study results may be irregular [11–13]. Besides that, this technique implementation requires the construction of calibration graph for each analytical sequence, which substantially loads the laboratory work. The use of such an analytical technique as the method of additives allows to solve this problem to some extent.

The use of method of additives supposes the following: two samples of the same volume are taken from the sample received for the analysis; one of them is injected with a certain amount of a solution-addition of the target analyte (in our case, urea); then both samples are subjected to the analysis procedure in accordance with the technique and the values of the responses are got (analytical signals), A_i and A_{i+ad} respectively.

The classical variant of using the method of additives consists in calculating the analyte concentration in the analyzed sample C_i from the relation:

$$\frac{A_i}{A_{i+ad}} = \frac{C_i}{C_i + C_{ad}} \quad \Rightarrow \quad C_i = C_{ad} \cdot \frac{A_i}{A_{i+ad} - A_i}\ , \qquad (1)$$

where A_i – analytical signal for a sample without an additive;

A_{i+ad} – analytical signal for a sample with an additive;

C_i – analyte concentration in sample without additive;

C_{ad} – increase in analyte concentration in sample due to addition of an additive.

A simplified version of the use is also possible – rationing of A_i and A_{i+ad} responses ratio, i. e. coefficient K^{MA} calculation. The second option is applied in quality control sphere – when the true value of analyte content in sample is not important, but only the fact of exceeding or not exceeding the specified critical parameter [9].

To study the possibility of applying both variants of this analytical approach to monitoring the quality of milk according to urea content, the described complex studies were done.

The development of the technique was carried out by its step-by-step validation by such validation parameters as range, in process stability, linearity/calibration model, accuracy and precision, limit of detection and limit of quantification, specificity/selectivity, and also total uncertainty.

The validation provides application of the normalized coordinates:

$$X_i = \frac{C_i}{C_{st}} \cdot 100\,\%; \qquad Y_i = \frac{A_i}{A_{st}} \cdot 100\,\%. \tag{2}$$

i. e. transition from the equation $A_i = b_1 \cdot C_i + a_1$ to the equation $Y_i = b_2 \cdot X_i + a_2$, that allows to calculate the validation characteristics, which do not depend on the analyte and features of the method of analysis.

The urea concentration in the model solution for the point of 100 % in the normalized coordinates $C_{100\%}^{model}$ has been chosen as the concentration provided the absorbance at the level of 0.3–0.5.

For normalization of the obtained experimental data the reference solution with the analyte concentration of $C_{reference} = C_{100\%}^{model}$ is used.

Acceptability criteria for validation parameters have been formed according to the recommendations [9; 10], proceeding from the approximate requirements of *Codex Alimentarius* to the extreme uncertainty of analytical procedures Δ_{As} (±10 %) [14], on the basis of systematic application of "insignificance concept" at the conventional level p = 95 % [9]:

$$\delta \le 0.32 \cdot \Delta_{As} = 3.20\,\%. \tag{3}$$

When working with the model solutions two following approaches were taken into account [15]:

Approach 1: uncertainty of analytical procedure proper Δ_{As}^{model} is equal to uncertainty of pre-analytical sample preparation:

$$\max \Delta_{As}^{model} = \frac{\max \Delta_{As}}{\sqrt{2}} = 0.707 \cdot \max \Delta_{As} = 0.707 \cdot 10.00\,\% = 7.07\,\%;$$
$$\max \delta^{model} = 0.32 \cdot \max \Delta_{As}^{model} = 2.26\,\%. \tag{4}$$

Approach 2: uncertainty of analytical procedure proper Δ_{As}^{model} is practically insignificant as compared with total uncertainty Δ_{As}:

$$\max \Delta_{As}^{model} = 0.32 \cdot \max \Delta_{As} = 0.32 \cdot 10.00\ \% = 3.20\ \%;$$
$$\max \delta^{model} = 0.32 \cdot \max \Delta_{As}^{model} = 1.02\ \%. \quad (5)$$

Validation results.

Range. According to our preliminary studies [16] and foreign authors publications [3] the normal urea content in milk is in the range of 0.1 to 0.3 mg/mL; the maximum fixed value was 0.7 mg/mL, and this value was taken by us as nominal (i. e. 100 %). We proposed a threshold content of 60 % of the maximum, i. e. 0.42 mg/mL.

Taking into account the obtained values of urea concentration in milk, the range of application of the developed method was proposed as 20–100 %. According to [10; 11] the additive quantity should, first, be close to the limit value determined, and secondly, be approximately halfway between the upper and lower points of the technique application range. Thus, in our case, the optimal additive will correspond to 60 % level.

In process stability. The results of stability studies are given in *tables 1* and *2* (using one analytical sequence as an example).

Table 1

The results of stability studies for the spectrophotometric technique for the quantitative determination of urea (model solutions)

a) in relation to initial time

Parameter		Values									
		0	10 min	20 min	30 min	1 h	2 h	3 h	4 h	5 h	6 h
$A^{model\ stability}$		0.259	0.262	0.263	0.264	0.266	0.259	0.251	0.247	0.241	0.237
$\left\|A_0^{model\ stability} - A_t^{model\ stability}\right\|$		–	0.004	0.005	0.005	0.008	0.000	0.008	0.011	0.017	0.021
$\delta^{model\ stability}, \% \le \max \delta^{model}$		–	1.42	1.80	1.93	2.96	0.00	3.09	4.38	6.70	8.25
Approach 1	$\le 2.26\ \%$		+	+	+	–	+	–	–	–	–
Approach 2	$\le 1.02\ \%$		–	–	–	–	+	–	–	–	–

б) in relation to optimal time

Parameter		Values								
		10 min	20 min	30 min	1 h	2 h	3 h	4 h	5 h	6 h
$A^{model\ stability}$		0.262	0.263	0.264	0.266	0.259	0.251	0.247	0.241	0.237
$\left\|A_{10\ min}^{model\ stab\ ility} - A_t^{model\ stab\ ility}\right\|$		–	0.001	0.001	0.004	0.004	0.012	0.015	0.021	0.025
$\delta^{model\ stability}, \% \le \max \delta^{model}$		–	0.38	0.51	1.52	1.40	4.45	5.72	8.01	9.53
Approach 1	$\le 2.26\ \%$		+	+	+	+	–	–	–	–
Approach 2	$\le 1.02\ \%$		+	+	–	–	–	–	–	–

Table 2

The results of stability studies for the spectrophotometric technique for the quantitative determination of urea (model solutions)

a) in relation to initial time

Parameter	Values									
	0	10 min	20 min	30 min	1 h	2 h	3 h	4 h	5 h	6 h
$A^{stability}$	0.412	0.423	0.428	0.431	0.438	0.447	0.461	0.474	0.481	0.495
$\left\|A_0^{stability} - A_t^{stability}\right\|$	–	0.011	0.017	0.020	0.027	0.036	0.049	0.063	0.070	0.084
$\delta^{stability}$, %	–	2.67	4.05	4.78	6.48	8.66	11.98	15.22	16.92	20.32
$\delta^{stability}$, % ≤ 3.20 %		+	–	–	–	–	–	–	–	–

б) in relation to optimal time

Parameter	Values								
	10 min	20 min	30 min	1 h	2 h	3 h	4 h	5 h	6 h
$A^{stability}$	0.423	0.428	0.431	0.438	0.447	0.461	0.474	0.481	0.495
$A_{10\ min}^{stability} - A_t^{stability}$	–	0.006	0.009	0.016	0.025	0.038	0.052	0.059	0.073
$\delta^{stability}$, %	–	1.34	2.05	3.71	5.84	9.07	12.22	13.88	17.19
$\delta^{stability}$, % ≤ 3.20 %		+	+	–	–	–	–	–	–

Thus, it is optimal to measure the optical density of spectrophotometric solutions not earlier than 10 min, and no later than 30 min after their preparation, which was taken into account in determining the main validation parameters of the procedure.

Linearity/calibration model. The explored method is planned to be applied in variant of method of additives, which requires the presence of a directly proportional relationship between analyte content and analytical signal within the specified range. Thus, it is necessary to confirm not only an acceptable level of technique linearity, but also to demonstrate the insignificance of free part in a linear dependence of the form $Y = b \cdot X + a$ [8; 9].

The validation parameter "linearity / calibration model" was determined using calibration solutions, the range of linearity of the method is from 20 to 100 % + additive, i. e. 20–160 %.

In accordance with [14], the number of concentration levels (g) in the linearity range should be at least 6, and they should be evenly distributed [10]. Preliminary calculations showed that with an amount of concentration levels greater than 8, an acceptable uncertainty of the technique (10 %) can be achieved with an allowable value of the correlation coefficient (0.99), so the following scheme was proposed, %: 20 – 40 – 60 – 80 – 100 – 120 – 140 – 160, i. e., g = 8.

The results of the linearity check are given in *table 3*.

Table 3

The results of testing the linearity of the spectrophotometric technique for urea quantitation

Parameter	Values	Acceptability criteria			
		Approach 1		*Approach 2*	
b^{model}	1.022	–	–	–	–
s_b^{model}	0.020	–	–	–	–
a^{model}	–1.253	$a^{model} \le t(95\,\%; g-2) \cdot s_a^{model}$		$a^{model} \le t(95\,\%; g-2) \cdot s_a^{model}$	
s_a^{model}	2.012	**satisfied**		**satisfied**	
RSD_0^{model}	2.583	≤ 3.64 %	**satisfied**	≤ 1.65 %	**unsatisfied**
R_c^{model}	0.9989	≥ 0.9972	**satisfied**	≥ 0.9994	**unsatisfied**

Thus, the technique is characterized by statistical insignificance of a^{model} coefficient, regardless of the approach used to assess the acceptability, a sufficient degree of linearity is provided only within the framework of softer *Approach* 1.

Accuracy and precision. These parameters were evaluated in two stages – using model urea solutions and using model milk samples.

According to the recommendations [9; 10; 14] the accuracy and precision of the procedure were evaluated at low (20 %), medium (60 %), and high (100 %) concentration levels within three analytical sequences.

The results of the studies are given in *table 4* for two variants of using the method of additives and show that the contribution of the actual analytical procedure to the overall error of the technique can not be considered insignificant.

In this case, the technique correctness (i. e., systematic error) is characterized by satisfactory indicators with sufficient margin of safety.

At the second stage, the validation was carried out using model samples of milk. Milk samples of different fat content, % (0.5, 3.2, 0) from three different manufacturers for each type (i. e. 9 samples) were taken, according to preliminary studies with a low urea content.

Three aliquots were taken from each test sample and urea (as standard solutions) was introduced into them at various concentration levels. Then two aliquots were taken from the samples, one was fed with a standard fixed urea additive, and the second one – the same volume of phosphate buffer solution (pH 7), and two aliquots were analyzed in accordance with the procedure. As a compensatory solution, a solution obtained by processing the appropriate blank sample (a native milk sample) was used in accordance with the procedure.

The validation results are presented in *table 5* for two variants of using the methods of additives.

Table 4

The results of checking the correctness and precision of the spectrophotometric technique for the quantitative determination of urea (model solutions) in variant of the method of additives

Factual concentration of urea in model solution ($C_{reference}$ = 0.70 mg/mL)		Absorbance		Calculated concentration of urea in model solution	$RR_i^{model\ MA}$, %	$K_{i,fact}^{model\ MA}$	$K_{i,calc}^{model\ MA}$	$RR_i^{model\ MA}$, %
$C_i^{model\ MA}$, mg/mL	$X_{i,fact}^{model\ MA}$, %	$A_i^{model\ MA}$	$A_{i+ad}^{model\ MA}$	$X_{i,calc}^{model\ MA}$, %				
0.14	20	0.081	0.331	19.44	97.20	4.000	4.086	102.16
0.14	20	0.082	0.323	20.41	102.07	4.000	3.939	98.48
0.14	20	0.080	0.323	19.75	98.77	4.000	4.038	100.94
0.42	60	0.250	0.491	62.24	103.73	2.000	1.964	98.20
0.42	60	0.267	0.534	60.00	100.00	2.000	2.000	100.00
0.42	60	0.249	0.493	61.23	102.05	2.000	1.980	99.00
0.70	100	0.413	0.656	101.98	101.98	1.600	1.588	99.27
0.70	100	0.423	0.675	100.71	100.71	1.600	1.596	99.73
0.70	100	0.417	0.665	100.89	100.89	1.600	1.595	99.67
				$\overline{RR}^{model\ MA}$, %	100.82			99.72
				$\delta^{model\ MA},\% = \left\|100 - \overline{RR}^{model\ MA}\right\| \le \max\ \delta^{model}$	0.82			0.28
		Approach 1		≤ 2.26 %	**satisfied**			**satisfied**
		Approach 2		≤ 1.02 %	**satisfied**			**satisfied**
				$RSD_{RR}^{model\ MA}$, %	1.97			1.23
				$\Delta_{RR}^{model\ MA} = t(95\%;\ n-1) \cdot RSD_{RR}^{model\ MA} \le \max\ \Delta_{As}^{model}$	3.73			2.33
		Approach 1		≤ 7.07 %	**satisfied**			**satisfied**
		Approach 2		≤ 3.20 %	**unsatisfied**			**satisfied**

Table 5

The results of checking the accuracy and precision of the spectrophotometric technique for the quantitative determination of urea (model milk samples) in variant of method of additives

Factual concentration of urea in model sample		Absorbance		Calculated concentration of urea in model sample $X_{i,calc}^{MA}$, %	RR_i^{MA}, %	$K_{i,fact}^{MA}$	$K_{i,calc}^{MA}$	RR_i^{MA}, %
$C_i^{sample\ MA}$, mg/mL	$X_{i,fact}^{MA}$, %	$A_i^{sample\ MA}$	$A_{i+ad}^{sample\ MA}$					
Milk, 0.5% fat								
0.14	20	0.080	0.318	20.17	100.84	4.000	3.975	99.38
0.14	20	0.085	0.337	20.24	101.19	4.000	3.965	99.12
0.14	20	0.090	0.352	20.61	103.05	4.000	3.911	97.78
0.42	60	0.245	0.483	61.76	102.94	2.000	1.971	98.57
0.42	60	0.258	0.525	57.98	96.63	2.000	2.035	101.74
0.42	60	0.268	0.528	61.85	103.08	2.000	1.970	98.51
0.70	100	0.403	0.647	99.10	99.10	1.600	1.605	100.34
0.70	100	0.424	0.676	100.95	100.95	1.600	1.594	99.65
0.70	100	0.439	0.688	105.78	105.78	1.600	1.567	97.95
				$\overline{RR}^{MA}$, %	101.51			99.23
				δ^{MA},% $= \lvert 100 - \overline{RR}^{MA} \rvert \le \max \delta = 3.20$ %	1.51			0.77
					satisfied			**satisfied**
				RSD_{RR}^{MA}, %	2.64			1.25
				$\Delta_{RR}^{MA} = t(95\ \%;\ n-1) \cdot RSD_{RR}^{MA} \le \max \Delta_{As}^{MA} = 10.00$ %	5.00			2.37
					satisfied			**satisfied**
Milk, 3.2 % fat								
0.14	20	0.090	0.347	21.01	105.06	4.000	3.856	96.39
0.14	20	0.096	0.375	20.65	103.23	4.000	3.906	97.66
0.14	20	0.089	0.354	20.15	100.75	4.000	3.978	99.44
0.42	60	0.254	0.501	61.70	102.83	2.000	1.972	98.62
0.42	60	0.291	0.588	58.79	97.98	2.000	2.021	101.03
0.42	60	0.265	0.517	63.10	105.16	2.000	1.951	97.55
0.70	100	0.423	0.678	99.53	99.53	1.600	1.603	100.18

Table 5

Factual concentration of urea in model sample		Absorbance		Calculated concentration of urea in model sample $X_{i,calc}^{MA}$, %	RR_i^{MA}, %	$K_{i,fact}^{MA}$	$K_{i,calc}^{MA}$	RR_i^{MA}, %
$C_i^{sample\ MA}$, mg/mL	$X_{i,fact}^{MA}$, %	$A_i^{sample\ MA}$	$A_{i+ad}^{sample\ MA}$					
0.70	100	0.457	0.723	103.08	103.08	1.600	1.582	98.88
0.70	100	0.446	0.705	103.32	103.32	1.600	1.581	98.79
				$\overline{RR}^{MA}$, %	102.33			98.73
				$\delta^{MA}, \% = \lvert 100 - \overline{RR}^{MA} \rvert \le \max\ \delta = 3.20\ \%$	2.33			1.27
					satisfied			**satisfied**
				RSD_{RR}^{MA}, %	2.43			1.41
				$\Delta_{RR}^{MA} = t(95\ \%;\ n-1) \cdot RSD_{RR}^{MA} \le \max\ \Delta_{As}^{MA} = 10.00\ \%$	4.61			2.68
					satisfied			**satisfied**
Milk, reconstituted and fatless								
0.14	20	0.078	0.319	19.42	97.10	4.000	4.090	102.24
0.14	20	0.081	0.322	20.17	100.83	4.000	3.975	99.38
0.14	20	0.082	0.324	20.33	101.65	4.000	3.951	98.78
0.42	60	0.235	0.475	58.75	97.92	2.000	2.021	101.06
0.42	60	0.239	0.478	60.00	100.00	2.000	2.000	100.00
0.42	60	0.246	0.478	63.62	106.03	2.000	1.943	97.15
0.70	100	0.399	0.636	101.01	101.01	1.600	1.594	99.62
0.70	100	0.398	0.639	99.09	99.09	1.600	1.606	100.35
0.70	100	0.405	0.646	100.83	100.83	1.600	1.595	99.69
				$\overline{RR}^{MA}$, %	100.50			99.81
				$\delta^{MA}, \% = \lvert 100 - \overline{RR}^{MA} \rvert \le \max\ \delta = 3.20\ \%$	0.50			0.19
					satisfied			**satisfied**
				RSD_{RR}^{MA}, %	2.57			1.42
				$\Delta_{RR}^{MA} = t(95\ \%;\ n-1) \cdot RSD_{RR}^{MA} \le \max\ \Delta_{As}^{MA} = 10.00\ \%$	4.87			2.69
					satisfied			**satisfied**

Thus, it was found that the precision of using the second variant of the method of additive (according to the calculation of the coefficient $K^{MA} = A_{i+ad} / A_i$) is relatively slightly higher. At the same time, both approaches are characterized by the correctness indicators at the same level.

Limit of detection and limit of quantification. The technique's limit of quantification (LOQ) is set as the lower limit of application range [10], i. e. 0.14 mg/mL. The limit of detection (LOD) is calculated as 0.33 [17], i. e. it is 0.05 mg/mL.

Specificity/selectivity. In the framework of this technique, it is not possible to evaluate its specificity with respect to the matrix components by a direct method because it is impossible to simulate a blank sample. Therefore, we proposed to determine the specificity/selectivity of the technique by comparing the analytical signals $A_i^{sample\ MA}$ obtained during the validity and precision testing for samples with urea content at quantitative limit level (model samples 1.1), with the corresponding values of the analytical signals $A_i^{model\ MA}$ obtained for model solutions 1.1. The ratio of these values R_i^{MA} should not differ from the nominal value (100 %) by more than the quantification limit value, i. e. 20 %.

The results of this study are shown in *table 6* and show the acceptable specificity of the explored method.

Table 6

The results of testing the specificity/selectivity of the spectrophotometric procedure for urea quantitation

Factual concentration of urea in model sample		Absorbance		$R^{MA} = \left\|1 - \frac{A_i^{sample\ MA}}{A_i^{model\ MA}}\right\| \cdot 100\ \%$	Acceptability criterion $R^{MA} \le 20\ \%$
$C_i^{sample\ MA}$, mg/mL	$X_{i,fact}^{MA}$, %	$A_i^{sample\ MA}$	$A_i^{model\ MA}$		
Milk, 0.5 % fat					
0.14	20	0.080	0.081	1.23	satisfied
0.14	20	0.085	0.082	3.66	satisfied
0.14	20	0.090	0.080	12.50	satisfied
Milk, 3.2 % fat					
0.14	20	0.090	0.081	11.11	satisfied
0.14	20	0.096	0.082	17.07	satisfied
0.14	20	0.089	0.080	11.25	satisfied
Milk, reconstituted and fatless					
0.14	20	0.078	0.081	3.70	satisfied
0.14	20	0.081	0.082	1.22	satisfied
0.14	20	0.082	0.080	2.50	satisfied

Total uncertainty. The forecast of the technique uncertainty was performed using the method of total allowable error [18] and recommendations [10] and represents the maximum possible error of the technique

with the worst combination of conditions for its implementation; this value was 7.33 %, which does not exceed the maximum permissible uncertainty of the technique (10 %).

Results of urea determination in milk samples. Using the proposed technique, a quantitative determination of urea in commercially available milk samples was done.

Three types of milk (0.5 % fat, 3.2 % fat, reconstituted and fatless) were selected for the analysis, from one producer; for each type of milk, 4 samples were taken from three different packages. An appropriate additive (phosphate buffer solution and urea solution at level of 20, 60, 100 % of nominal content) was introduced into each milk sample and analyzed in accordance with the procedure. According to the obtained optical density data, the urea content in milk was calculated for different concentrations of the additive.

The analysis results are presented in *table 7*.

Table 7

The results of quantitative determination of urea in milk samples in variant of method of additives

Parameter	Milk, 0.5 % fat			Milk, 3.2 % fat			Milk, reconstituted and fatless		
A_i^{MA}	0.129	0.128	0.139	0.179	0.181	0.183	0.154	0.155	0.180
A_{i+ad}^{MA} (20 %)	0.221	0.216	0.222	0.270	0.276	0.273	0.237	0.233	0.277
A_{i+ad}^{MA} (60 %)	0.388	0.384	0.400	0.439	0.45	0.443	0.401	0.400	0.452
A_{i+ad}^{MA} (100 %)	0.556	0.545	0.586	0.608	0.633	0.628	0.564	0.574	0.633
$X_{i,calc}^{MA}$, %	28.04	29.09	33.49	39.34	38.11	40.67	37.11	39.74	37.11
	29.88	30.00	31.95	41.31	40.37	42.23	37.41	37.96	39.71
	30.21	30.70	31.10	41.72	40.04	41.12	37.56	36.99	39.74
$\overline{X}_{calc}^{MA}$, %	29.38	29.93	32.18	40.79	39.51	41.34	37.36	38.23	38.85
C_{calc}, %	0.21	0.21	0.23	0.29	0.28	0.29	0.26	0.27	0.27
RSD_X^k, %	3.98	2.69	3.78	3.12	3.10	1.95	0.62	3.65	3.87
RSD_X^{total}, %	3.53			2.78			3.09		
$\Delta_{X,r}^{total}$, %	6.56			5.16			5.75		

Thus, we can see that the total relative uncertainty of the average result obtained does not exceed the maximum allowable value (10 %) and the forecast value calculated by us during the validation (7.33 %).

The urea content in the analyzed commercially available milk samples is in the range of 0.21–0.29 mg/mL, which corresponds to the

preliminary monitoring data (not more than 0.3 mg/mL), does not exceed the suggested standard value (0.42 mg/mL).

Thus, the method of additives is based on measuring the optical density of two aliquots of milk sample, one with the addition of urea of known concentration, and the other without it, and further incorporating the results into formula. This method allows significantly speed up the analysis of urea content in milk.

In connection with the importance of measuring this parameter in the field of analysis of dairy raw materials quality, production of dairy products, veterinary medicine, it seems reasonable to introduce requirements for the permissible content of urea in milk. In this work the validation of fast and convenient analysis technique for this purpose is conducted.

Conclusion. As a result of the work, a step-by-step validation of the method for the quantitative determination of urea in milk by method of absorption spectrophotometry in visible region of spectrum was carried out in variant of using the method of additives (MA). It was found that the measurement of optical density of spectrophotometric solutions is optimal not earlier than 10 minutes, and no later than 30 minutes after their preparation; the acceptable uncertainty of the technique (10 %) with an allowable value of correlation coefficient (0.99) can be achieved with a number of concentration levels more than 8. The correctness of the technique is characterized by satisfactory indicators. The procedure is effective in the range of 0.14–0.7 mg/mL, the detection limit is 0.05 mg/mL. The uncertainty forecast was 7.33 %, which does not exceed the maximum permissible uncertainty of the technique (10 %).

The use of the proposed technique allows quantifying the urea content in milk samples without the need to build a calibration curve and long calculations, which significantly speeded up the analysis process, reduced material and time costs, and at the same time to obtain correct and accurate results.

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Articles submitted to editors office of 06.09.2017.

Жукова Я., Петров П., Кліменко Л. Експрес-метод кількісного визначення сечовини в молоці.

Постановка проблеми. Раціон великої рогатої худоби та спосіб годівлі є найважливішими факторами, що обумовлюють вміст сечовини в молоці. Споживання корму впливає на щоденний вміст аміаку в кишечнику, сечовини в плазмі крові та молоці, що важливо для працівників ферм, технологів молокопереробної галузі, спеціалістів лабораторій контролю якості.

Мета роботи – розробка і проведення поетапної валідації методики кількісного визначення вмісту сечовини в молоці методом абсорбційної спектрофотометрії у варіанті використання методу добавок. Цей метод застосовують при аналізі складних розчинів, оскільки він дозволяє автоматично врахувати вплив "третіх" компонентів.

Матеріали і методи. Використання методу добавок передбачав відбір двох проб однакового об'єму зразка, який надходить на аналіз; до однієї вводили певну кількість розчину-добавки цільового аналізу (сечовини). Потім обидві проби аналізували відповідно до методики і отримували значення оптичної щільності на спектрофотометрі та проводили розрахунки вмісту сечовини за формулою. Розробку методики проведено поетапною валідацією за такими параметрами: стабільність, діапазон застосування, лінійність, правильність, прецизійність, межа виявлення, кількісне визначення, специфічність, робастність.

Результати дослідження. Встановлено, що проведення вимірювання оптичної щільності розчинів оптимально не раніше, ніж через 10 хв, і не пізніше, ніж через 30 хв після їх приготування; прийнятна невизначеність методики (10 %) при допустимому значенні коефіцієнта кореляції (0.99) може бути досягнута при кількості концентраційних рівнів, більше за 8. Правильність методики характеризується задовільними показниками. Методика ефективна в діапазоні 0.4–0.7 мг/мл, межа виявлення становить 0.05 мг/мл. Прогноз невизначеності – 7.33 %, що не перевищує максимально допустиму невизначеність методики (10 %).

Висновки. Виконано кількісне визначення сечовини в зразках молока без необхідності побудови калібрувальної кривої і тривалих розрахунків, що дозволило значно прискорити процес аналізу, скоротити матеріальні й витрати часу, і при цьому отримати правильні й точні результати, завдяки проведеній валідації методики.

Ключові слова: метод добавок, молоко, сечовина, спеткрофотометрія.