Relationship between freezing point and raw ewes' milk components as a possible tool for estimation of milk adulteration with added water

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Summary

Milk adulteration with added water is controlled by freezing point depression (FPD) determination. The aim of this study was to evaluate the suitability of an alternative method for FPD equivalent (FPD-E) measurement and to estimate the basis for FPD cut-off value (to identify milk adulteration with added water) for ewes' milk. Raw bulk ewes' milk samples ($n = 811$) from Slovakia were analysed for FPD, FPD-E and composition in 2014. Influence of season and lactation was significant for all milk indicators. The mean FPD of bulk ewes' milk samples was –0.559 °C ± 0.029 °C. The FPD cut-off value can be in the range close to –0.511 °C (mean + standard deviation × 1.64, one-sided 95% confidence interval). An extent of 89.4% of the variability in FPD-E could be explained by variations in FPD. The total correlation coefficient was 0.945 ($p < 0.001$) and high correlation was also for FPD greater than –0.511 °C (0.992, $p < 0.001$). This could be explained by high correlations between FPD and milk components: –0.228 ($p < 0.01$) for fat, –0.231 ($p < 0.001$) for proteins, –0.219 ($p < 0.001$) for lactose, –0.497 ($p < 0.001$) for non-fat solids and –0.341 ($p < 0.001$) for total solids. Determination of FPD-E in ewes' milk can be an effective tool for estimation of milk adulteration with added water.

Keywords

raw ewes' milk; freezing point; mid-infrared analysis with Fourier transformation; cryoscopic method; composition; adulteration

Several traditional specialities guaranteed (TSG) like "Ovčí hrudkový syr" (lump cheese, typically produced in chalets) [1] and Slovakian bryndza (Protected Geographical Indication – PGI) as the final product of its processing [2] are produced form raw ewes' milk in Slovakia. GÁLIK [3] estimated at the end of 2014 the number of sheep was 396 000, of which 267 000 were ewes. In 2014 he predicted the production of raw ewes' milk to be 10 250 t. In 2013, the average price of raw ewes' milk was 0.93 EUR (in May–June) to 1.22 EUR (in November) per litre.

The quality of raw ewes' milk in Slovakia is regularly controlled, especially for the total number of microorganisms and antibiotic residues [4]. Dairies and farmers are also interested in its composition. However, freezing point depression (FPD) of the milk is not measured at all. It is because no cut-off is legislatively established for FPD of raw ewes' milk, unlike for raw cows' milk, where the values of –0.515 °C or –0.520 °C were established. KERESTEŠ [5] only estimated the mean FPD for raw ewes' milk in Slovakia to be from –0.560 °C to –0.610 °C. In the Czech Republic, a few studies were conducted on raw ewes' milk. JANŠTOVÁ et al. [6] determined the average value of FPD as –0.617 °C ± 0.052 °C. However, it was only measured in single sheep farm. MACEK et al. [7] reported the value –0.605 °C, which was obtained only for samples from a single herd. Much

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more attention is focused on this issue in the countries of south-eastern Europe and Mediterranean countries, where sheep breeding has a long tradition [8–11].

The reference method for measuring of FPD of milk is based on the use of a thermistor cryoscope with seeking of the plateau [12]. However, an alternative method based on mid-infrared spectroscopy with Fourier transformation (FTIR) is used more often at routine quality control of raw milk. Here, FPD is measured indirectly and, therefore, it is called the equivalent of FPD (FPD-E). FPD depends on the water content of milk, but also depends on the concentrations of all water-soluble components (e. g. lactose, salts and urea), and on its certain characteristics, e. g. acidity, or conductivity. Relationship between composition together with properties of raw cows' milk and FPD were reported by several studies [13–16]. However, ewes' milk has a different composition and properties, which vary significantly during the season [17].

The aim of this work was to analyse the relationship between FPD, its equivalent and raw ewes' milk components in a large and representative set of samples obtained during the traditional season of raw ewes' milk processing (March 2014 to October 2014) in Slovakia. Based on the results, we were able to formulate recommendations for measuring of FPD-E by FTIR, and for the use of the determined FPD for the assessment of raw ewes' milk quality. At present, any adulteration of raw ewes' milk with added water is very difficult to detect, which represents a risk of economic losses for its producers.

MATERIALS AND METHODS

Unpreserved bulk ewes' milk samples were used for testing in this study (n = 811). The database included bulk ewes' milk samples from March 2014 to October 2014 (March = 18, April = 140, May = 115, June = 126, July = 156, August = 123, September = 128 and October = 5). They came from 4 important Slovak ewes' milk processing dairies (D1 = 600, D2 = 199, D3 = 7 and D4 = 5) and mostly from 5 breeds (Improved Walachian sheep, Tsigai, Lacaune, East Friesian sheep and Slovak milk sheep [3]). The samples were taken by a qualified sampler and were stored refrigerated (from 1 °C to 8 °C). Then they were delivered to the testing laboratory Examinala (Dairy Research Institute, Žilina, Slovakia), which is designated as a central laboratory for testing of raw milk in Slovakia.

Inbox samples were tested in the laboratory within 48 h after sampling by the two different methods, from two prepared sub-samples. The first sub-sample was tested for FPD by the reference thermistor cryoscope method [12] using the instrument CryoStar Automatic (Funke-Gerber, Berlin, Germany). The device was calibrated daily by reference calibration standards (Funke-Gerber) with FPD of –0.408 °C and –0.557 °C. The accuracy was checked periodically by measuring the reference sample with FPD of –0.512 °C (Funke-Gerber) and by performing repeatability tests.

The second sub-sample was tested for the amount of fat, protein, lactose, non-fat solids, total solids and FPD-E (equivalent) by FTIR spectroscopy on MilkoScan FT 6000 (Foss Electric, Hillerød, Denmark), according to the recommendation of the equipment manual. Slope and intercept for these parameters (excluding FPD-E) were adjusted (3 times) by introducing of the local reference samples based on raw ewes' milk (Dairy Research Institute, Prague, Czech Republic). The accuracy was evaluated by internal pilot samples and by performing several quality tests (evaluation of repeatability, control of blank-zero sample and carry-over check). The laboratory also regularly participated in the international interlaboratory comparisons (organized by Dairy Research Institute, Prague, Czech Republic) regarding both methods.

The results were evaluated using MS Excel (Microsoft, Redmond, Washington, USA). The basic statistical characteristics were calculated for data, such as mean, standard deviation, variation coefficient and median. The differences between means were tested by Student's t-test. The linear regression analysis with calculation of correlation and determination coefficients of the relationships between FPD, FPD-E and other milk indicators was performed to explain mutual dependencies.

RESULTS AND DISCUSSION

The basic statistical parameters of the sample set of raw bulk ewes' milk are shown in Tab. 1. The mean results and variability of milk indicators did not show obvious anomalies. The mean of freezing points of ewes' milk (–0.559 °C by the reference method and –0.554 °C by FPD-E) was clearly lower than usual for cows' milk (–0.532 °C [13] or –0.534 °C [16]). Another published freezing point value of –0.515 °C [18] was less relevant, as that was determined for heat-treated drinking milk and it is well known that heat treatment affects FPD [6, 15, 19]. The mean FPD of ewes'

Tab. 1. Main statistical parameters of ewes' milk indicators.

Indicator	FPD [°C]	FPD-E [°C]	Fat [g·kg^{-1}]	Protein [g·kg^{-1}]	Lactose [g·kg^{-1}]	Non-fat solids [g·kg^{-1}]	Total solids [g·kg^{-1}]
Arithmetic mean	–0.559	–0.554	74.4	59.8	45.6	113.5	186.7
Standard deviation	0.029	0.023	11.5	7.9	4.3	5.4	13.9
Median	–0.563	–0.559	72.8	57.9	46.3	113.4	185.2
Minimum	–0.733	–0.644	24.8	35.8	23.0	90.5	144.1
Maximum	–0.360	–0.369	124.0	87.8	86.9	129.8	242.1
Variation coefficient	5.1%	4.1%	15.5%	13.2%	9.4%	4.7%	7.5%

n = 811. FPD – freezing point depression, FPD-E – FPD equivalent.

milk determined in this study was more similar to goats' milk: –0.5533 °C and –0.5513 °C [19–21]; –0.5544 °C [14].

The variability of ewes' milk's FPD was considerably higher (from 4.1% to 5.1%) than it is usual in cows' milk (0.9% [13]). In the scientific literature, not much information is available regarding FPD of ewes' milk and in particular about its quality control (adulteration with water) by this indicator. The FPD values which were reported by other authors [5–7] were similar to our results (–0.559 °C; Tab. 1). In other regions of Europe, FPD values from –0.578 °C to –0.581 °C and from –0.550 °C to –0.580 °C were introduced [9–11]. Also these values correspond approximately with Tab. 1.

The median and mean for FPD, FPD-E and other milk indicators were comparatively close values. This indicates approximately normal frequency distribution of values. Other indicators, such as contents of milk components, showed mean values typical for ewes' milk [22]. Variability was higher in components than that indicated for cows' milk [13]. However, it was comparable to the other earlier set of ewes' milk samples [22]. The reason for the greater variability of milk indicators of ewes' milk may be the stronger genetic variability of the milking animals (more breeds) and simultaneously acting effects of diet changes (season – vegetation) and changes in the lactation course (hormones).

Simultaneous influence of season and lactation was statistically significant for all milk indicators (FPD and FPD-E, $p < 0.01$, Fig. 1 and Fig. 2; fat, protein, lactose, non-fat solids and total solids, $p < 0.001$). FPD values were slightly lower in the beginning and higher in the end of the season and of the lactation progress. However, this deterioration in the end of the season (lactation) could be due to a smaller number of samples in October. Otherwise, the observed trend was fully consistent with the results published by Pavić et al.

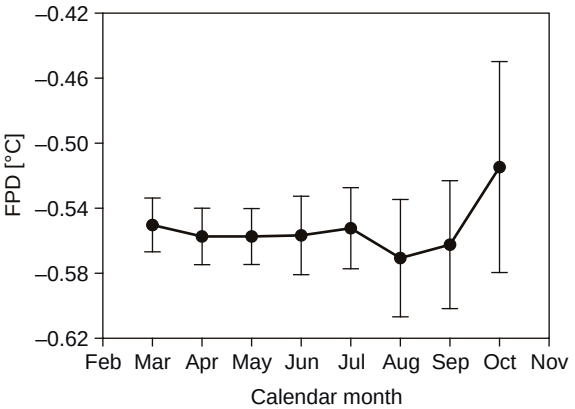

Fig. 1. Variations in the FPD along the calendar year and lactation progress in raw ewes' milk.

Values represent average ± standard deviation. Student's test criterion of the difference between month averages $t = 3.23$; $p < 0.05$ (for difference between October and August). FPD – freezing point depression.

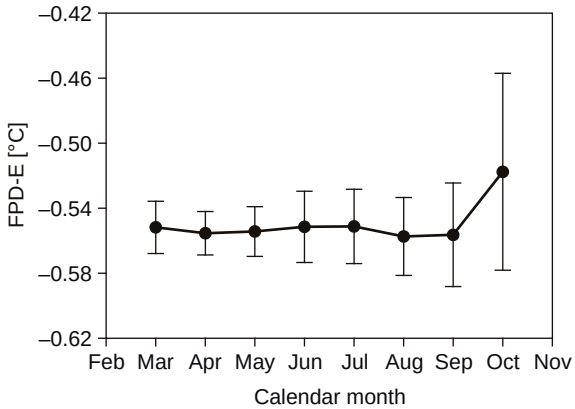

Fig. 2. Variations in FDP-E along the calendar year and lactation progress in raw ewes' milk.

Values represent average ± standard deviation. Student's test criterion of the difference between month averages $t = 3.28$; $p < 0.05$ (for difference between October and August). FPD-E – freezing point depression equivalent.

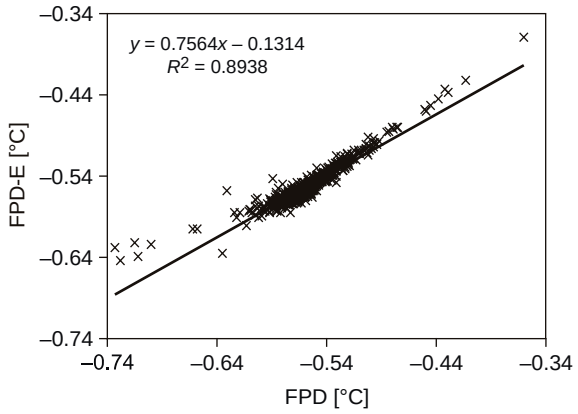

Fig. 3. Methodical linear regression relationship and correlation coefficient between FPD and FPD-E .

n = 811, correlation coefficient r = 0.945 (p < 0.001).
FPD – freezing point depression, FPD-E – freezing point depression equivalent.

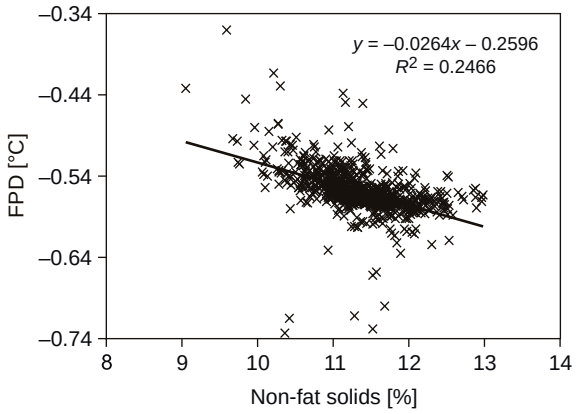

Fig. 4. Physiological linear regression relationship and correlation coefficient between non-fat solids and FPD.

n = 811, correlation coefficient r = -0.497 (p < 0.001).
FPD – freezing point depression.

[8]. The amounts of fat, protein, non-fat solids and total solids were increased continuously during the season and lactation: fat from 59.4 g·kg^{-1} to 88.7 g·kg^{-1}; protein from 48.9 g·kg^{-1} to 80.2 g·kg^{-1}; non-fat solids from 105.8 g·kg^{-1} to 120.9 g·kg^{-1}, and total solids from 165.2 g·kg^{-1} to 205.6 g·kg^{-1}. On the contrary, the lactose content continuously decreased from 50.5 g·kg^{-1} to 31.5 g·kg^{-1}. These trends are usual in lactating domesticated animals [8, 9, 16].

The methodical difference between FPD and FPD-E was relatively small (0.005 °C; Tab. 1), but not negligible in terms of identification of possible foreign water addition into milk. However, this difference is easily reducible by any calibration or simple calculation, as it is only a shift of a calibration regression line, being managable on the software level. The principle of the FPD-E method was designed and tested previously on cows' milk in the Netherlands and in Germany [23–25]. It was studied also in Slovakia in cows' milk [26]. Nevertheless, the relevant scientific research results for ewes' milk were still missing. According to the results of methodical regression analysis (Fig. 3), 89.4% of the variability in the FPD-E values (indirect identification) was explainable by variations in the reference cryoscopic determination (FPD). The correlation cofficient was 0.945 (p < 0.001), which suggests that the relationship was highly significant. This correlation was obviously stronger than the same relationship in cows' milk (0.43; p < 0.01; [27]). This indicates that the FPD-E method can be more successfully used for ewes' milk quality control (milk adulteration with water) than for cows' milk. There was only a portion of 18.7% of the variations in FPD-E values, which could be explained by FPD variability in cows' milk. However, Tomáška et al. [27] used in their study the methodology that is not common for sample preparation in all cases, as samples for FPD determination were un-preserved and samples for FPD-E were preserved by Acidiol (Merck, Darmstadt, Germany). Further regarding this fact, markedly larger variability of ewes' milk basic composition was determined, which was essentially included in FPD-E value estimation by FTIR technology, as compared to cows' milk as the main reason. So, in this sense, the basic statistical principle was also playing an important role in the discussed case. That fact mentioned for ewes' milk may be also partly explained by simultaneous detection of significant physiological correlations between FPD, FPD-E and other major milk components. These were in FPD –0.228, –0.231, –0.219 (p < 0.01), –0.497 (Fig. 4) and –0.341 (p < 0.001) for the fat, protein, lactose, non-fat solids and total solids (Tab. 2). The corresponding values in FPD-E (Tab. 2) were –0.232, –0.230 (p < 0.01), –0.334, –0.554 and –0.363 (p < 0.001). It is clear that both types of correlations were similar. The relevant correlations in bovine milk (maximum values) were –0.46 (p < 0.01), –0.31, –0.35, –0.33 (p < 0.05) and –0.50 (p < 0.01) and these were similar in this case as well, but only at their highest values. The relevant relations were significantly less tight [13, 27] for other similar data files on cows' milk.

At physical-chemical analysis of cows' milk, a portion of 53.8% of FPD can be ascribed to lactose, 30.4% to inorganic ions and organic salts

Tab. 2. Physiological relationships and correlations between FPD, FPD-E and other ewes' milk components.

Indicator	FPD [°C]			FPD-E [°C]		
	Equation	*r*	Significance	Equation	*r*	Significance
Fat	$y = -0.0057x - 0.5166$	–0.228	$p < 0.01$	$y = -0.0046x - 0.5198$	–0.232	$p < 0.01$
Protein	$y = -0.0084x - 0.5086$	–0.231	$p < 0.01$	$y = -0.0067x - 0.5140$	–0.230	$p < 0.01$
Lactose	$y = -0.0145x - 0.4925$	–0.219	$p < 0.01$	$y = -0.0178x - 0.4730$	–0.334	$p < 0.001$
Non-fat solids	$y = -0.0264x - 0.2596$	–0.497	$p < 0.001$	$y = -0.0235x - 0.2868$	–0.554	$p < 0.001$
Total Solids	$y = -0.0070x - 0.4283$	–0.341	$p < 0.001$	$y = -0.0059x - 0.4429$	–0.363	$p < 0.001$

$n = 811$. FPD – freezing point depression, FPD-E – FPD equivalent, *r* – correlation coefficient.

(K^+, Na^+, Cl^-), 3.3% to citrates, 1.9% to urea and 6.9% to residual bonds (fat, protein) [28]. This is the total influence of the components on the FPD formation but not their share on FPD variability, which is about 1% in cows' milk and 4% in ewes' milk (Tab. 1, FPD, bulk milk). Then in this file, there was 5.2%, 5.4%, 4.8%, 24.6% and 11.6% of the FPD variability explainable by variations in fat, protein, lactose, non-fat solids and total solids values, respectively.

These findings indicate that FPD (cryoscopic) determination is probably relatively less dependent on the milk conductivity (on the concentration of ions and osmotic pressure), and a greater proportion of FPD variability is influenced by the major milk components in ewes' milk as compared to cows' milk. However, in connection with this fact, one of the main factors may be the much higher variability of FPD values and major milk components in bulk samples of ewes' milk compared to cows' milk.

The interval correlations were calculated on the basis of relevant data classification according to the FPD values (Tab. 3). High and the highest correlation coefficients can be expected in the boundary intervals (0.996; $p < 0.001$). These results partly confirmed the importance of higher variability of FPD and components in ewes' milk, for a closer relationship between FPD and FPD-E, than in cows' milk.

Furthermore, the relations among milk indicators were tracked in the FPD interval > –0.511 °C

Tab. 3. Methodical linear regression relationships and correlation coefficients between FPD and FPD-E according to FPD intervals.

FPD interval [°C]	Equation	Correlation coefficient *r*	Significance	*n*
> –0.500	$y = 0.9488x - 0.0288$	0.996	$p < 0.001$	21
–0.500 to –0.537	$y = 0.8936x - 0.0558$	0.877	$p < 0.001$	111
–0.538 to –0.575	$y = 0.8365x - 0.0886$	0.866	$p < 0.001$	554
< –0.575	$y = 0.4503x - 0.3086$	0.854	$p < 0.001$	125

FPD – freezing point depression, FPD-E – FPD equivalent,

Tab. 4. Physiological linear regression relationships and correlation coefficients between FPD, FPD-E and other ewes' milk constituents in FPD interval > –0.511 °C.

Indicator	FPD [°C]			FPD-E [°C]		
	Equation	*r*	Significance	Equation	*r*	Significance
Fat	$y = -0.0038x - 0.4560$	–0.146	$p > 0.05$	$y = -0.004x - 0.4580$	–0.164	$p > 0.05$
Protein	$y = 0.0061x - 0.5170$	0.154	$p > 0.05$	$y = 0.0044x - 0.5109$	0.118	$p > 0.05$
Lactose	$y = -0.0439x - 0.3149$	–0.764	$p < 0.001$	$y = -0.0409x - 0.3301$	–0.753	$p < 0.001$
Non-fat solids	$y = -0.0182x - 0.2905$	–0.324	$p > 0.05$	$y = -0.0194x - 0.2820$	–0.364	$p < 0.05$
Total Solids	$y = -0.0053x - 0.3895$	–0.233	$p > 0.05$	$y = -0.0056x - 0.3876$	–0.262	$p > 0.05$

$n = 36$. FPD – freezing point depression, FPD-E – FPD equivalent, *r* – correlation coefficient.

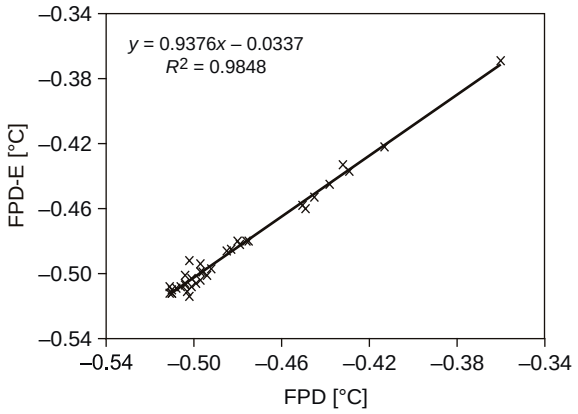

Fig. 5. Methodical linear regression relationship and correlation coefficient between FPD and FPD-E in FPD interval > –0.511 °C.

n = 36, correlation coefficient r = 0.992 (p < 0.001).
FPD – freezing point depression, FPD-E – freezing point depression equivalent.

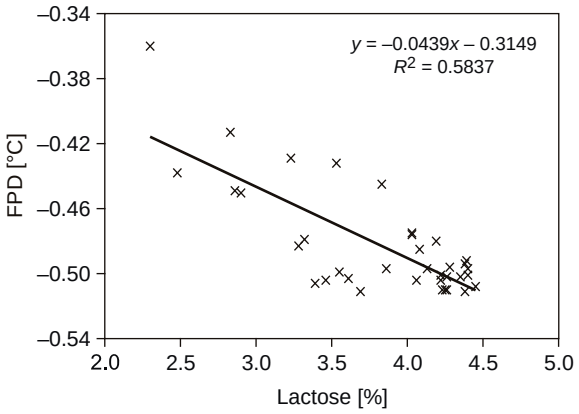

Fig. 6. Physiological linear regression relationship and correlation coefficient between lactose content and FPD in FPD interval > –0.511 °C.

n = 36, correlation coefficient r = –0.764 (p < 0.001).
FPD – freezing point depression.

(Tab. 4, Fig. 5). This interval corresponds to the range (mean + standard deviation·1.64 °C, one-sided 95% confidence interval as theoretically probable pre-supposition for adulteration) for possible searching of cut-off values for suspicion of milk adulteration with added water in raw bulk ewes' milk according to conventional statistic estimation. Methodical correlation (Fig. 5) was significantly high, 0.992 (p < 0.001). A portion of 98.5% of the variations in FPD-E was determined as being due to variability in FPD reference values. This result is very promising for the methodical use of FPD-E in routine way to detect any ewes' milk adulteration with added water.

Physiological FPD relationships to other milk indicators (Tab. 4) are slightly different from the overall evaluation (Tab. 2) and also less significant. However, this is not surprising. There is a relationship with protein content, which is less logical and not consistent with the total evaluation (Tab. 2). The correlation to lactose content (Fig. 6) was significantly tight, –0.764 (p < 0.001). Yet, 58.4% of the variations in FPD is explainable by changes in the lactose content in the peripheral range of FPD analysis. Likewise, relatively tight though insignificant (with respect to the lower number of samples) correlations of FPD values to the non-fat solids and total solids were recorded, –0.324 and –0.233 (p > 0.05), respectively. Relevant relations for FPD-E (Tab. 4) were similar as follows: –0.753 to lactose (p < 0.001); –0.364 to non-fat solids (p < 0.05); –0.262 to total solids (p > 0.05). These facts reinforce the potential of reliable identification of possible ewes' milk adulteration with water by FPD (reference cryoscopy) and by FPD-E (indirect method).

CONCLUSIONS

It is important to find a suitable FPD cut-off value for identification of ewes' milk adulteration with water for countries where ewes' milk is processed in dairies, for raw material quality control. The results of this study indicated better applicability of FPD indicator (reference) and also routine FPD-E method (indirect) to identify potential ewes' milk adulteration with water, as compared to cows' milk. It was estimated that a standard FPD cut-off value for identification of raw bulk ewes' milk adulteration with added water can be in a range of values close to –0.511 °C. Besides conventional statistical evaluation, for reliable determination of FPD threshold it is necessary to carry out a detailed assessment of the impacts of the season (lactation) on ewes' milk indicators, and to quantify the impacts of artificial modification of ewes' milk composition on FPD.

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