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CHOSEN BIOTIC FACTORS INFLUENCING RAW COW MILK FREEZING POINT

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Abstract

HANUŠ, O., ZHANG, Y., BJELKA, M., KUČERA, J., ROUBAL, P., JEDELSKÁ, R.: *Chosen biotic factors influencing raw cow milk freezing point.* Acta univ. agric. et silvic. Mendel. Brun., 2011, LIX, No. 5, pp. 65–82

The milk freezing point depression (FPD) is important physical property. FPD is influenced by milk composition especially by components with osmotic pressure activity and by other physiological factors. There is possible to indicate a foreign (extraneous) water addition into milk by FPD. This is necessary to have a good estimated legislative FPD discrimination limit (FPD–L) for purpose of milk quality control. This paper was aimed at obtaining information to improve such estimation. Impacts factors as season variations, estimated state of dairy cow nutrition and some milk components and properties on milk FPD and their relations to FPD were quantified (n 11 540 – 72 607 bulk raw cow milk samples). The highest FPD was in Spring (–0.52097 ± 0.004877 °C), the lowest in Autumn (–0.52516 ± 0.005725 °C; $P < 0.001$). Correlation between FPD and lactose was 0.35 ($P < 0.001$). 12% and 5.4% of FPD variability is explainable by lactose and casein variability. Relationship between FPD and urea (U) was 0.26 ($P < 0.001$) in March. The worst FPD was in group with presupposed (according to milk urea and protein combination) nitrogen matter (NM) and energy (E) insufficiency (–0.51855 ± 0.007288 °C). The best FPD was in group with presupposed NM and E surplus in feeding ration (–0.52536 ± 0.004785 °C; $P < 0.001$). The FPD was worse in suspicion on E deficiency (on the basis of fat/crude protein ratio) as compared to presumption for balanced E nourishment of dairy herds (–0.52105 ± 0.006436 °C > –0.52244 ± 0.005367 °C; $P < 0.001$). Results can improve the estimation of objective FPD–L.

dairy cow, bulk milk sample, freezing point depression, fat, protein, casein, energy metabolism estimation, lactose, solids non fat, somatic cell count, urea, free fatty acids

In general, the milk freezing point depression (FPD) is an important physical milk indicator (as it was published by Freeman and Bucy, 1967; Demott, 1969; Eisses and Zee, 1980; Brouwer, 1981; Walstra and Jenness, 1984; Koops *et al.*, 1989; Rohm *et al.*, 1991; Buchberger, 1990 a, b, 1991, 1994, 1997; Wiedemann *et al.*, 1993; Bauch *et al.*, 1993; Buchberger and Klostermeyer, 1995; Crombrugge, 2003) with typically very low variability under natural conditions. This fact is valid both for individual and especially for bulk milk samples. Under sure conditions FPD can be used as one of possible indicators for detection of addition of foreign (extraneous) water into raw or pasteurized milk (Eisses and Zee, 1980; Walstra and Jenness, 1984; Rohm *et al.*, 1991; Buchberger, 1991 and 1997; MPR Bayern, 1993, 1994 and 1995; Hanuš *et al.*, 2003 a; Roubal *et al.*, 2004; Rasmussen and Bjerring, 2005). Such extraneous water addition can be caused by unintentional mistake during milking or milk processing or by poor technological discipline at the milking or further processing procedure. It means for instance by an unexpected failure on milking or processing equipment or by poor milking equipment and poor procedure including bad intention of an operator during milking or technological milk treatment. The last mentioned reason means milk falsification in the food chain which is unacceptable generally on the first hand. On the other hand, there is necessary a clear specification about FPD in national food legislation in terms of objectivity at the mentioned control using of milk FPD value because it has significant influence on raw milk payment according to its quality very often. An acceptable solution supposes a good definition of FPD discrimination limit value and its use conditions for standard milk quality confirmation or refusing. It means the objectivity towards the milk producer and processor as well. It has been not fulfiled every time in the Czech Republic. An imbalance in this state is probably the main reason (Hanuš *et al.*, 2003 a) why the problem of the milk FPD evaluation shows so called boomerang effect in terms of FPD importance for incidental strong discussions between relevant business partners on the milk market board. Of course, FPD as important quality indicator is also marker of general dairy food chain quality.

Effects on milk FPD

Proofs and reasoning about different aspects of milk FPD problem were shown as follows: – FPD essence and its relationships to milk composition and its osmotic pressure (Freeman and Bucy, 1967; Demott, 1969; Brouwer, 1981; Walstra and Jenness, 1984; Buchberger, 1990 a, b, 1991, 1994, 1997; Wiedemann *et al.*, 1993; Hanuš *et al.*, 2003 b; Crombrugge, 2003; Kirchnerová and Foltys, 2005; Kovářová *et al.*, 2005; Chládek and Čejna, 2005; Hanuš *et al.*, 2005 b, 2010, 2011 a; Macek *et al.*, 2008); – principles of various FPD analytical measurements (Koops *et al.*, 1989; Bauch *et al.*, 1993; Buchberger, 1994; Buchberger and Klostermeyer, 1995; Crombrugge, 2003); – influences of mammal species, cattle breed and height of the dairy cow milk yield on FPD value (Buchberger, 1990 and 1997; Hanuš *et al.*, 2003, 2009, 2010; Macek *et al.*, 2008; Genčurová *et al.*, 2008; Janštová *et al.*, 2007); – impacts of the nitrogen matter/energy and mineral element balance in the dairy cow nutrition on the FPD (Buchberger, 1990, 1991 and 1997; Hanuš *et al.*, 1998, 2010; Kološta, 2003); – effects of production disorder occurrence including milk secretion disorders and SCC (mastitis) on FPD variation (Eisses and Zee, 1980; Buchberger, 1990 and 1997; Hanuš *et al.*, 2003; Chládek and Čejna, 2005; Macek *et al.*, 2008); – technological impacts (farm and processing) and foreign water addition into milk on FPD levels (Eisses and Zee, 1980; Rohm *et al.*, 1991; Buchberger, 1991 and 1997; Hanuš *et al.*, 2003 a; Roubal *et al.*, 2004; Rasmussen and Bjerring, 2005). For instance there was stated that (MPR Bayern, 1993, 1994 and 1995) beside the foreign water addition reason (which occurred from 34.5 to 41.2% of incidents of studied cases only) the FPD values had been deteritorated mostly by the poor nutrition of dairy cows (from 50.2 to 58.8% of cases) in Bavaria. The knowledge about most of incidental influence factors on FPD under actual circumstances, which are on good level, create very important basis for the future correct FPD discrimination limit derivation.

Necessity for correct revision of legislative milk FPD discrimination limit

FPD discrimination limits, which are used officially and/or conventionally for standard milk quality confirmation differ mutually in dependence on historical and geographical points of view, namely from ≤–0.530 °C (in the CR at the lucidly lower milk yield level of dairy cows as compared to today state; Klíčník, 1978) to ≤–0.505 °C and ≤–0.500 °C (valid in the Netherlands and Norway; Bossuyt, 2003 and Tomáška *et al.*, 2005). However, the mostly used actual FPD discrimination limits vary between ≤–0.520 °C (EEC 92/46 and Regulation 853; for instance in Denmark, Great Britain, Sweden and so on) and ≤–0.515 °C (for instance in Germany (Bavaria), Austria and Switzerland; Buchberger, 1990 and 1997; Rohm *et al.*, 1991; Hanuš *et al.*, 2003 a). For example now, there is used the FPD discrimination limit with the value ≤–0.510 °C in Belgium (Bossuyt, 2003). Also there are used other exceptional limits as well. For instance ≤–0.512 °C (Cyprus) and ≤–0.500 °C (Norway), as it was summarized by Tomáška *et al.* (2005). Today we are using alternatively ≤–0.520 °C or ≤–0.515 °C (ČSN 57 0529; the Regulations 203/2003 Coll. about veterinary demands on milk and milk products and 638/2004 and 1234/2007; Hanuš *et al.*, 2003 a, b; Roubal *et al.*, 2004) for raw and pasteurized milk in the Czech Republic. There is prescribed for mean of drinking milk FPD the value according to raw milk under local conditions of collecting area in 1234/2007. Nevertheless, the value ≤–0.520 °C is not in the reasonable accordance with the fact state (Hanuš *et al.*, 2003 a), as the country average values were determined –0.5231, –0.5236 °C and –0.5271 (2003, 2004 and 2009) in the bulk milk samples (Roubal, Kopunecz, Pešinová *et al.*, 2004 and 2005, Kopunecz, 2010). It means, that 28.6% (2002), 23.5% (2003), 20.1% (2004) and 8.7% (2009) of raw cow milk deliveries into dairy plants were not in the accordance with the FPD discrimination limit ≤–0.520 °C and 5.1%, 5.1%, 4.4% and 2.5% with the limit ≤–0.515 °C. There are too high percentages about the first limit and still higher in the second case of the limit in order to be really all caused by the foreign watter additions into milk. There were the full 38% of unsatisfactory cases at the limit ≤–0.520 °C for raw cow milk in Slovakia (2004) as it was stated by Tomáška *et al.* (2005), too. Further, recently it was also stated by the screening that 37% of collected pasteurized drinking milk samples did not meet the FPD discrimination limit ≤–0.515 °C and also 65% of them did not meet the limit ≤–0.520 °C in the Czech Republic (Roubal *et al.*, 2004). In consideration of above mentioned facts, it is quite clear that a new correct revision of FPD discrimination limit is necessary in the Czech Republic. This is possible to do it only on the basis of exact scientific evaluation in terms of today fact situation and incidental impacts of different interferential factors and effects. Also for an incidental application on European Commission to reach an exception with regard to officially valid FPD discrimination limit it is necessary to support such material by the relevant actual scientific proofs and warrant.

Aim of the paper in terms of milk FPD interpretation

In general, it is well known that in less favourable areas (LFA, in terms of agriculture land exploitation), there could be problems with the dairy cow energy supplementation by their feeding rations on the basis of local feed sources. In this consequences there could be more difficult to meet the legislative limit of milk FPD for standard milk quality reaching in some of dairy herds. Such are reasons why it is important to define this FPD legislative discrimination limit in the right way under country specific conditions. Therefore the aim of this paper was to analyse the relationships between FPD and energy metabolism markers of dairy cows in their milk (such as milk citric acid content, milk F/CP ratio or combination classes of milk urea and protein contents). The second reason is to contribute to correct legislation FPD discrimination limit definition for standard milk quality under the Czech Republic conditions.

Regarding above mentioned facts this is quite clear that more actual studies are necessary for the explanation of relationships between FPD values and milk composition, milk markers for health state and nutrition of dairy cows and farmer factors under domestic conditions.

MATERIAL AND METHODS

Evaluated set of bulk milk samples

Bulk milk samples of large data set were obtained regularly (once or more times per month) from commercial dairy herds for milk quality determination (mostly according to standard ČSN 57 0529) in the framework of official milk payment system during twelve calendar months. Samples were treated by low temperature about 6 °C and immediately transported into accredited milk laboratory. Generally, the milk samples were analysed in accordance with relevant standard operation procedure manuals of laboratory. Milk samples came from both milked populations of dairy cows in the country, Holstein and Czech Fleckvieh cattle. There were investigated different numbers of milk samples for various indicators. Maximal sample number was for FPD and some other milk indicators (n = 72 607) and minimal for free fatty acids (FFA; n = 11 540).

Investigated milk indicators with their abbreviations and units

Measured and calculated tested milk indicators were as follows on the list of abbreviations and used units: FPD = milk freezing point depression (°C); F = milk fat content (g.100 ml^{-1}; %); L = lactose content (monohydrate; g.100 ml^{-1}; %); SNF = solids non fat content (g.100 ml^{-1}; %); DM = dry matter (g.100 ml^{-1}; %); CP = crude protein (total N × 6.38; g.100 ml^{-1}; %); CAS = casein (casein N × 6.38; g.100 ml^{-1}; %); SCC = somatic cell count (ths.ml^{-1}); F/CP = ratio between fat and crude protein; U = urea concentration (mmol. l^{-1}); FFA = concentration of free fatty acids (mmol.100 g^{-1}) in milk fat.

Used dairy analytical methods

FPDs were measured by two analytical methods. The first was carried out with MilkoScan 6000 system (Foss Electric, Denmark). This was adjusted according to reference cryoscopic method results in regular intervals. Such procedure was described for example by Crombrugge (2003) and Tomáška *et al.* (2005) as alternative measurement of milk FPD equivalent. The second measurement procedure was performed by the own cryoscopic method (ČSN 57 0538, ISO 5764:2002(E)), which was instrument Cryo–Star automatic Funke-Gerber (Germany). It was realized at this part of analysed milk samples, which afforded suspicious values by the first indirect measurement method. The selected measurement mood was reference Plateau Search in this case. The used instrument was under regular calibration by the standard NaCl solutions and took part in the national proficiency testing with successful results regularly. The incidental interferential effects were controlled (in accordance with Bauch *et al.*, 1993; Koops *et al.*, 1989; Buchberger and Klostermeyer, 1995; Crombrugge, 2003).

The other investigated milk parameters as the F, L, CP, CAS, DM, SNF, U, FFA were measured by the instrument MilkoScan 6000 (Foss Electric, Denmark; MIR-FT = mid infra red spectrophotometric apparatus with the mathematics evaluation of whole IR spectrum by the Fourier's transformations), which was regularly calibrated according to the reference method results (standard ČSN 57 0536 by the Gerber's method for fat content, Kjeldahl's method for crude protein content (with previous experience by Hanuš *et al.*, 1995) and polarimetric and gravimetric methods for lactose and SNF contents, according to standard ČSN 57 0530; for U and FFA according to direct ureolytic, photometrical and titration method results). The SCC was determined by the Fossomatic instrument (Foss Electric, Denmark) according to the standard ČSN EN ISO 13366-3. Both the previously mentioned instruments took part in the relevant national proficiency testing with the good results regularly.

Performed statistical procedures

The validation of the large data set was carried out by the determination of discrimination limits for all milk indicators. These limit values were derived from mean values and variability measure as x ± 1.96 or 2.58 × sd, which included 95 or 99% of likelihood, that values belong into data set. If it was not possible, for example due to expressive deviation of data distribution from normal frequency distribution, another procedure was chosen, including the application of a qualified estimation. The valid data set (I) was applied for most kinds of evaluations. Further, the data set was adjusted (II) for presupposition of no water addition according to our recent results (Hanuš *et al.*, 2003 a). This set was used for another kinds of evaluation in the framework of this paper. For these purposes all the milk samples with FPDs higher as –0.513 °C were excluded out of some statistical evaluation.

The main statistical characteristics, such as arithmetical (x) and geometrical mean (xg), standard deviation (sd) and coefficient of variation (vx), were calculated in the month data sets. If necessary, the milk indicator (SCC in this case) data were logarithmically transformed before statistical evaluation of the main statistical characteristics and mutual relationships because of no presumption of normal data frequency distribution (Meloun and Militký, 1992 and 1994; Kupka, 1997; Hanuš *et al.*, 2001).

The FPD value frequency distribution of whole data sets (I and II) were tested in terms of the normality by the Q and Q–Q graphs as part of the exploratory analyse (Meloun and Militký, 1992 and 1994; Kupka, 1997). It was carried out in the month data sets. The third and fourth (tercial and quarter) central statistical moments of FPD data file, it means the obliqueness and acuteness (excess) were tested as well. The linear and non linear (logarithmical, exponential, quadratical and polynomical in terms of second or third degree) regressions were used at the testing and rendering of the relationships between the FPD (in m°C×(–1)) and the other milk indicators in the month data sets. It was expressed by the relevant regression equations (with highest statistical recovery in terms of concrete relationship explanation – determination coefficient) and coefficients or indexes of correlation.

The FPD data were rendered in milk urea and protein combination classes (as diagnostic parameter of dairy cow nutrition in terms of their nitrogen matter and energy maintenance (overloading or malnutrition), it means N/E nutrition balance, according to works of Kirchgessner *et al.*, 1985 and 1986 or Illek and Pechová, 1997) by the box graph. The same strategy was used at the evaluation of influence of presupposed dairy cow energy metabolism on FPD according to F/CP ratio, too. The box graph with statistical testing was used for the seasonal effect evaluation as well. In the relevant box graphs: the median as the middle point value is demonstrated by central line; the upper and lower margins of the box are expressing the upper margin of the first and third quartiles, it means 50% central quantile; the abscissa is expressing the variation range as the difference between maximal and minimal value. The statistical significance of impacts on FPD was tested by the Student's t-test criterion.

RESULTS AND DISCUSSION

General description of bulk milk sample data set

The main statistical parameters of month data sets of all evaluated milk indicators of I data file are shown in Tab. I. The largest variability was observed at milk U and SCC, usually over 30 and 50%. The other variation coefficients were mostly below 10%. The all average values are in accordance with good raw milk quality. All FPD month data sets of II data file and most of I data file were deviated ($P \leq 0.05$) as compared to the normal standard frequency distribution. It means from conventional model. It was caused due to obliqueness and acuteness as well. Only at three month data sets (June, July and August) of I data file (with a possible foreign watter addition) no significant ($P > 0.05$) deviations from normality were discovered at obliqueness (difference from zero). The biggest difference from normality ($P \leq 0.05$) was observed in January in terms of obliqueness, which is more important for statistical testing as compared to acuteness. During whole year the obliqueness varied around zero as central position. The mentioned June and January month data sets (file I) were selected as the best and worst result examples in terms of recovery of obliqueness normality for rendering by the Q and Q–Q graphs (Fig. 1 and Fig. 2). The deviations from standard curve (in the case of Q graph) and diagonal line (in the case of Q–Q graph) are expressing the differences from normality (considering obliqueness and acuteness) and their character in the figures. In spite of that fact, that most cases differ significantly from normal frequency distributin. This is valid in particular for less important acuteness. The deviations for more important obliqueness are practically irrelevant. That is reason, why it is possible to conclude, that any FPD value transformations are not necessary for next classical statistical testing evaluation and arithmetical mean and standard deviation use as main data set representatives is suitable.

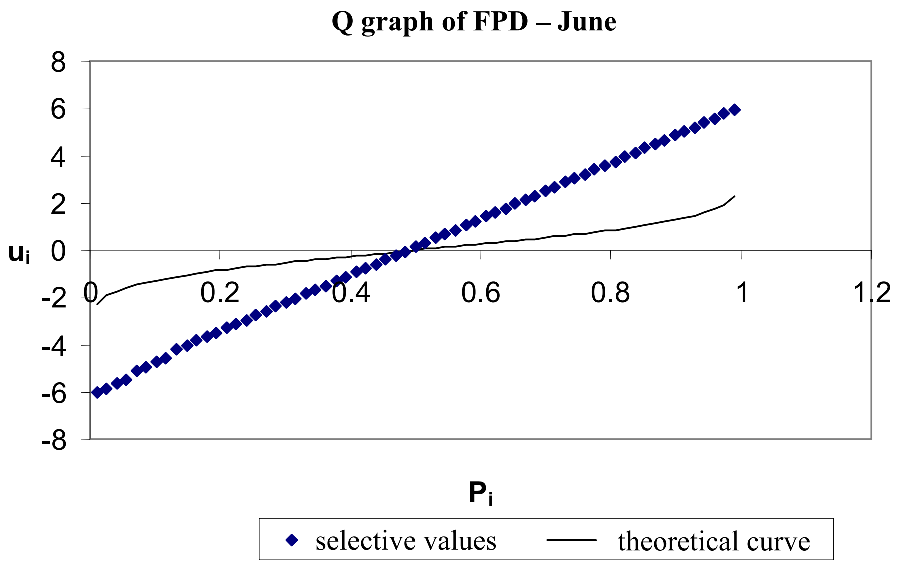
Milk freezing point regarding seasonal effect

The seasonal effect on FPD is rendered in Fig. 3 and 4. The highest (the worst) average FPD was noted in Spring (–0.52097 ± 0.004877 °C; variation coefficient = 0.9%; n = 12 524). The lowest (the best) FPD was investigated in Autumn (–0.52516 ± 0.005725 °C; variation coefficient = 1.1%; n = 12 054). The difference in FPD between these two seasonal periods was statistically significant ($t = 61.9$ and $P < 0.001$). There are six pair combinations of differences between seasonal periods. All these differences were statistically tested. In particular because of the high number of cases in the individual seasonal groups all the differences were statistically significant ($P < 0.001$). In general, the seasonal effect on FPD was confirmed as highly significant, similarly to the results, which were reported by Roubal *et al.* (2004, 2005), Hanuš *et al.* (2005) and Kopunecz (2010).

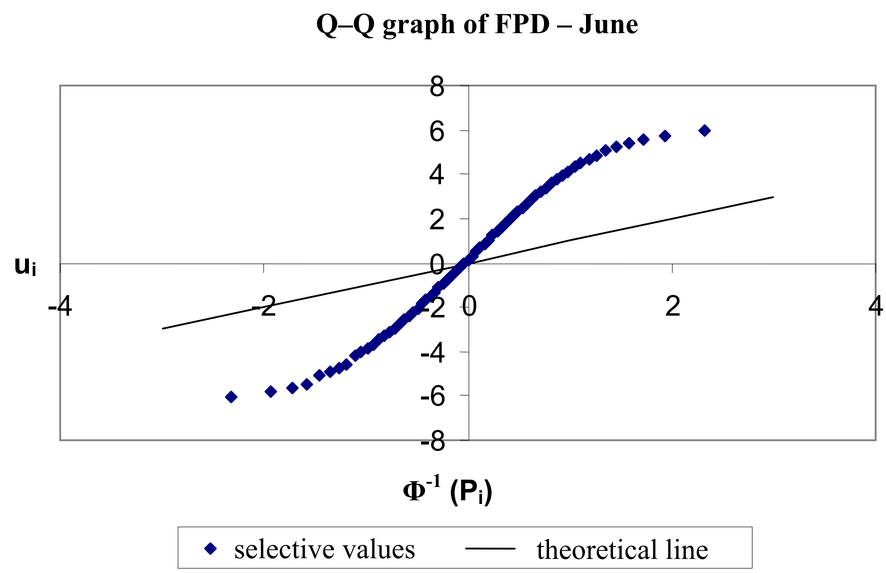
I: *Main statistical parameters of raw cow bulk milk sample indicators in months during year in validated data file (I, whole set)*

		FPD	F	L	SNF	DM	CP	CAS	U	FFA	SCC	log SCC	F/CP
		m°C×(−1)	g.100 ml⁻¹	g.100 g⁻¹	g.100 g⁻¹	g.100 g⁻¹	g.100 g⁻¹	g.100 g⁻¹	mmol.l⁻¹	mmol.100 g⁻¹	ths.ml⁻¹		
January	n	4937	6464	6481	6482	6388	6479	6481	6325		6302	6302	6427
	x	522	4.17	4.93	8.91	13.15	3.42	2.51	3.22		228	2.2986	1.22
	xg											199	
	sx	5.771	0.385	0.093	0.239	0.453	0.185	0.151	1.306		125.736	0.2342	0.115
	vx	1.1	9.2	1.9	2.7	3.4	5.4	6.0	40.6		55.1	10.2	9.4
February	n	6477	6469	6490	6502	6390	6506	6504	6321		6386	6386	6438
	x	522	4.13	4.96	8.93	13.14	3.41	2.48	3.81		246	2.3323	1.21
	xg											215	
	sx	4.733	0.376	0.091	0.237	0.450	0.184	0.150	1.306		133.842	0.2331	0.114
	vx	0.9	9.1	1.8	2.7	3.4	5.4	6.0	34.3		54.4	10.0	9.4
March	n	5760	5769	5791	5797	5709	5803	5397	5783		6097	6097	5744
	x	523	4.06	4.99	8.90	13.07	3.38	2.47	4.16		245	2.3345	1.20
	xg											216	
	sx	5.159	0.357	0.092	0.239	0.437	0.185	0.149	1.564		124.741	0.2268	0.107
	vx	1.0	8.8	1.8	2.7	3.3	5.5	6.0	37.6		50.9	9.7	8.9
April	n	6223	6241	6245	6271	6163	6272	6275	6048		6202	6202	6215
	x	521	4.03	5.00	8.86	13.00	3.35	2.44	4.16		255.03	2.3525	1.20
	xg											225	
	sx	4.907	0.354	0.085	0.245	0.429	0.184	0.148	1.370		129.702	0.2248	0.111
	vx	0.9	8.8	1.7	2.8	3.3	5.5	6.1	32.9		50.9	9.6	9.2
May	n	6301	6316	6338	6339	6242	6337	6341	6152		6279	6279	6278
	x	521	3.91	4.99	8.80	12.83	3.30	2.41	4.08		261	2.3583	1.18
	xg											229	
	sx	4.847	0.351	0.082	0.217	0.409	0.161	0.131	1.454		139.386	0.2330	0.110
	vx	0.9	9.0	1.6	2.5	3.2	4.9	5.4	35.6		53.4	9.9	9.3
June	n	6204	6401	6419	6375	6331	6422	6430	6228		6114	6114	6365
	x	520	3.83	4.99	8.72	12.69	3.24	2.36	3.62		288	2.3993	1.18
	xg											251	
	sx	5.516	0.336	0.082	0.206	0.397	0.155	0.125	1.274		154.560	0.2364	0.107
	vx	1.1	8.8	1.6	2.4	3.1	4.8	5.3	35.2		53.7	9.9	9.0
July	n	6425	6432	6444	6431	6354	6447	6455	6211		6342	6342	6391
	x	520	3.85	4.98	8.73	12.72	3.27	2.37	4.15		271	2.3684	1.18
	xg											234	
	sx	5.133	0.321	0.086	0.219	0.389	0.156	0.127	1.259		153.185	0.2463	0.102
	vx	1.0	8.4	1.7	2.5	3.1	4.8	5.3	30.4		56.4	10.4	8.6
August	n	6292	6293	6310	6268	6213	6324	6325	6087		6181	6181	6260
	x	521	3.86	4.96	8.70	12.71	3.27	2.35	4.17		303	2.4230	1.18
	xg											265	
	sx	4.994	0.338	0.093	0.225	0.407	0.160	0.130	1.295		157.637	0.2361	0.106
	vx	1.0	8.8	1.9	2.6	3.2	4.9	5.5	31.1		52.1	9.7	9.0
September	n	6234	6208	6239	6186	6135	6239	6257	6048		6203	6203	6171
	x	523	3.99	4.90	8.83	12.91	3.40	2.55	4.00		280	2.3926	1.18
	xg											247	
	sx	5.546	0.343	0.105	0.239	0.417	0.176	0.145	1.448		147.094	0.2262	0.104
	vx	1.1	8.6	2.1	2.7	3.2	5.2	5.7	36.2		52.5	9.5	8.9
October	n	6143	6150	6177	6144	6104	6165	6166	5906		6129	6129	6121
	x	524	4.15	4.91	9.05	13.19	3.51	2.67	4.41		253	2.3494	1.18
	xg											224	
	sx	5.779	0.374	0.112	0.255	0.448	0.183	0.117	1.393		132.856	0.2221	0.110
	vx	1.1	9.0	2.3	2.8	3.4	5.2	4.4	31.6		52.5	9.5	9.3
November	n	5911	5926	5932	5852	5852	5924	5930	5811	5840	5874	5874	5887
	x	526	4.25	4.95	9.17	13.35	3.52	2.71	3.32	0.614	256	2.3519	1.21
	xg											225	
	sx	5.581	0.378	0.105	0.246	0.455	0.193	0.122	1.234	0.458	138.006	0.2257	0.105
	vx	1.1	8.9	2.1	2.7	3.4	5.5	4.5	37.2	74.7	54.0	9.6	8.7
December	n	5700	5692	5707	5669	5632	5709	5710	5733	5746	5658	5658	5671
	x	523	4.14	4.88	9.03	13.10	3.45	2.66	3.62	0.835	254	2.3506	1.20
	xg											224	
	sx	5.9150	0.359	0.114	0.247	0.439	0.193	0.122	1.400	0.491	133.789	0.2240	0.107
	vx	1.1	8.7	2.3	2.7	3.4	5.6	4.6	38.7	58.8	52.6	9.5	8.9

n = number of cases; x = arithmetic mean; xg = geometric mean; sd = standard deviation; vx = coefficient of variability (in %)



1: *The quantile graph of the real data set of the milk freezing point value distribution (file I, June)*
u_i = standard quantils of original data; $P_{(i)}$ = order probability

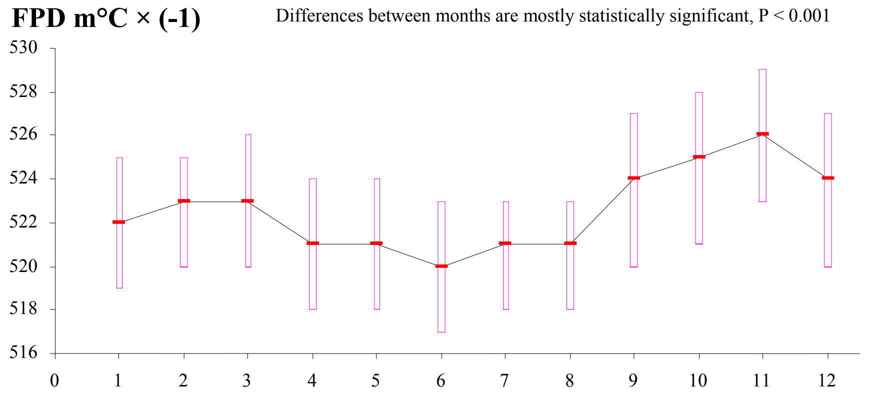


2: *The quantil-quantile graph of the distribution normality test for the data set of the milk freezing point values (file I, June)*
u_i = standard quantils of original data; $\Phi^{-1}(P)$ = theoretical quantils of standard normal distribution

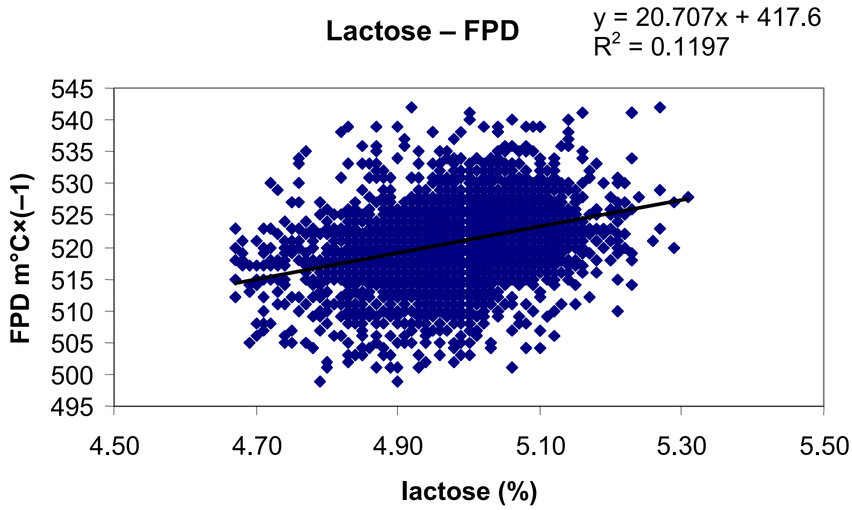
It could be probably influenced by nutrition and temperature effect variations, despite using of total mixed ration on the basis of preserved fodder feedstuffs for nourishment of dairy cow herds during whole year.

Description and interpretation of relationships between FPD and other health and milk indicators of dairy cow herds

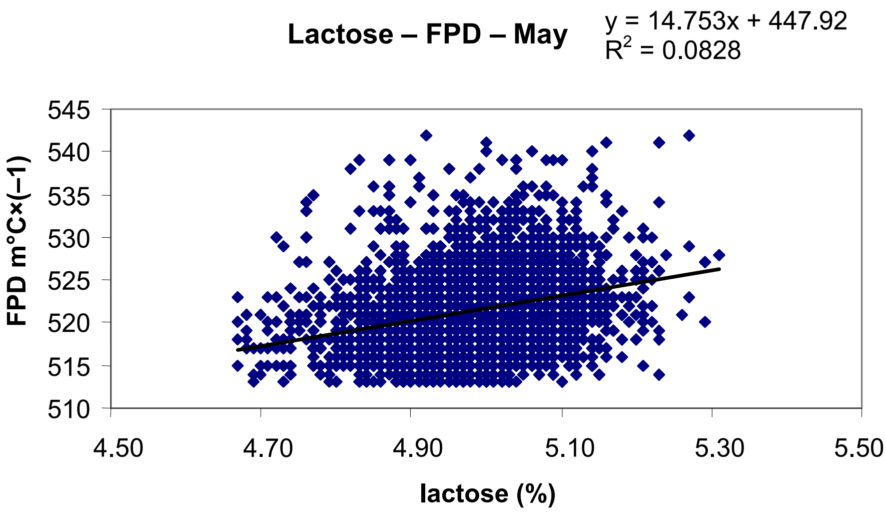
In general, the limitation of data file I range towards file II decreased all relationships between FPD and other milk quality indicators in terms of their tightness. The month relationships between FPD and fat content (file I) were very weak and coefficient of determination (R^2) varied from zero up to 0.013 ($r = -0.11$; $P < 0.01$; Tab. II) in July. However, the indicators were practically almost independent. The more relevant relationships (R^2) were stated between FPDs and crude protein contents, where varied from 0.0059 in August to 0.0408 in January. This second is equal to coefficient of correlation 0.20 ($P < 0.001$; Tab. II). It means slow consistent improvement



3: *Calendar month effect on raw cow milk freezing point depression (FPD) in the Czech Republic (bulk milk samples, n = 72 607)*
Month differences in sets are rendered by box graphs for good expression of data frequency distribution with median (central line) and 50% central quantile (the box).



r = 0.35***



r = 0.29***

4: *The relationship between FPD and lactose content in whole (I, validated data set) and adjusted (II, validated data set, FPD ≦ –0.513 °C) set in May*

II: *Selected month regression relationships between milk freezing point depression and sure milk quality indicators in the bulk milk samples*

Relationship	Data file	Month	Type of relationship	Equation	R^2	r	Significance
FPD × F	I	July	linear	y = –0.5673x + 522.69	0.0130	–0.11	**
FPD × CP	I	January	linear	y = 6.1696x + 500.87	0.0408	0.20	**
FPD × L	I	May	poweral	$y = 378.79x^{0.1982}$	0.1200	0.35	**
FPD × DM	I	January	linear	y = 1.6271x + 500.61	0.0167	0.13	**
FPD × DM	I	January	poweral	$y = 468.51x^{0.042}$	0.0174	0.13	**
FPD × F/CP	I	January	linear	y = –6.4255x + 529.83	0.0170	–0.13	**
FPD × F/CP	I	January	polynomical 2th st.	$y = -6.6196x^2 + 10.491x + 519.13$	0.0179	0.13	**
FPD × F/CP	I	July	linear	y = –7.346x + 529.16	0.0215	–0.15	**
FPD × F/CP	I	July	exponential	$y = 529.26e^{-0.0142x}$	0.0218	0.15	**
FPD × FFA	I	November	linear	y = 1.1865x + 525.21	0.0095	0.10	**
FPD × FFA	I	November	polynomical 2th st.	$y = 0.5618x^2 + 0.1705x + 525.45$	0.0141	0.12	**
FPD × FFA	I	December	linear	y = 1.3606x + 522.29	0.0127	0.11	**
FPD × FFA	I	December	polynomical 2th st.	$y = 0.2155x^2 + 0.8468x + 522.51$	0.0135	0.12	**
FPD × SCC	I	December	linear	y = –0.0036x + 524.31	0.0066	–0.08	**

r = coefficient or index of correlation; R^2 = coefficient of determination; ** = P < 0.01

III: *Results of month regression analyse between FPD (y) and lactose content (x) in year data file (I)*

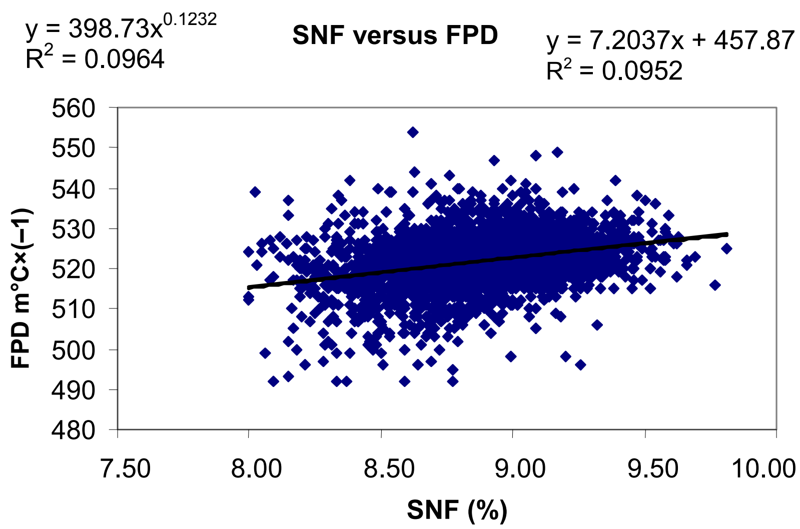
Month	Equation	Coefficient of determination (R^2)	Coefficient or index of correlation	Significance
January	y = 17.35x + 436.48	0.0799	0.2827	***
	$y = 401.78x^{0.1641}$	0.0809	0.2844	***
February	y = 15.83x + 443.9	0.0900	0.3000	***
March	y = 15.363x + 446.22	0.0749	0.2737	***
April	y = 18.951x + 426.25	0.1068	0.3268	***
May	y = 20.707x + 417.6	0.1197	0.3460	***
	$y = 378.79x^{0.1982}$	0.1201	0.3466	***
June	y = 19.422x + 423.3	0.0849	0.2914	***
July	y = 16.07x + 440.46	0.0728	0.2698	***
August	y = 14.068x + 450.88	0.0688	0.2623	***
September	y = 14.603x + 451.99	0.0757	0.2751	***
October	y = 15.58x + 448.03	0.0897	0.2995	***
November	y = 14.621x + 453.52	0.0761	0.2759	***
December	y = 11.555x + 467.02	0.0500	0.2236	**

ns = no significant P > 0.05; *, ** and *** = statistically significant P ≤ 0.05; P≤ 0.01 and P < 0.001

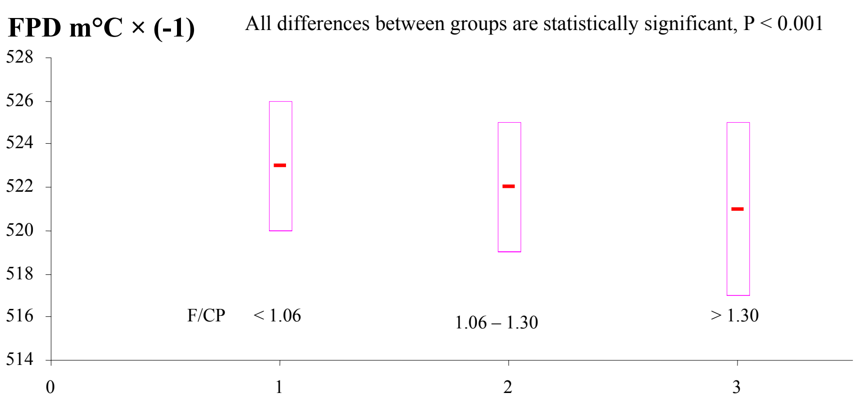
of FPD along crude protein increasing. The more efficient situation was observed logically at the relationships between the FPDs and the lactose contents (Tab. II and III; Fig. 5). Those swaied (R^2) from 0.0500 in December to 0.1197 in May, which is equal to 0.35 (P < 0.001) as the correlation coefficient (r). It means that 12% of the FPD variability is explainable by lactose content variability. The non linear (poweral) relationship was still a little closer (Tab. II and III with correlation index 0.35; P < 0.001). The FPDs decrease (are improved) regularly with higher lactose contents. Surprisingly, the poor relationships were calculated between month FPDs and total dry matter contents. Those increased from 0.0057 in September up to 0.0167 in January (R^2; r = 0.13; P < 0.01, Tab. II; the poweral relationship was a little closer only, Tab. II). Despite this result the quite closer month relationships were received between the FPDs and the solid non fat contents. Those ranged from 0.0449 (r = 0.21; P < 0.001) in October to 0.0952 in January (Tab. IV and Fig. 6). The last result is equal to the correlation coefficient 0.31 (P < 0.001). It means the consistent apparent improvement of the FPDs along the SNF contents increasing, according to the expectation. Our findings about the relationships between the FPD values and the lactose and SNF contents are comparable to our previous results (Macek *et al.*, 2005) and to the results which were published by some other authors (Demott, 1969; Brouwer, 1981; Walstra and Jenness, 1984; Koops *et al.*, 1989; Buchberger and Klostermeyer, 1995; Buchberger, 1994; 1997; Crombrugge, 2003; Chládek and Čejna, 2005), as well. On the contrary, the relationships

IV: *Results of he month regression analyse between FPD (y) and SNF content (x) in year data file (I)*

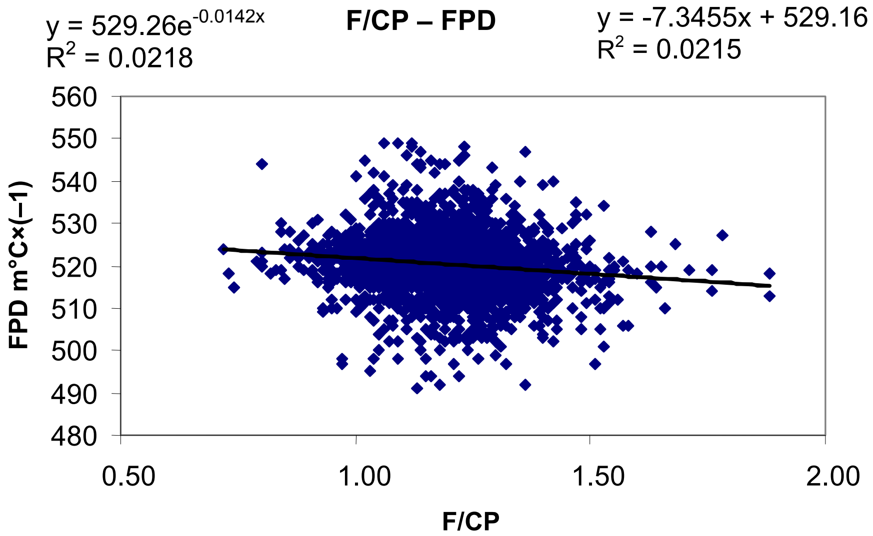
Month	Equation	Coefficient of determination (R^2)	Coefficient or index of correlation	Significance
January	$y = 7.2037x + 457.87$	0.0952	0.3085	***
	$y = 398.73x^{0.1232}$	0.0964	0.3105	***
February	$y = 5.4036x + 474.22$	0.0726	0.2694	***
March	$y = 5.8035x + 471.23$	0.7170	0.8468	***
April	$y = 6.1564x + 466.35$	0.0936	0.3059	***
May	$y = 6.5944x + 462.93$	0.0864	0.2939	***
June	$y = 7.9249x + 451.15$	0.0935	0.3058	***
July	$y = 6.6524x + 462.38$	0.0812	0.2850	***
August	$y = 5.7384x + 470.69$	0.0685	0.2617	***
September	$y = 6.16x + 469.09$	0.0716	0.2676	***
October	$y = 4.8065x + 480.97$	0.0449	0.2119	**
November	$y = 5.0558x + 479.54$	0.0518	0.2276	**
December	$y = 5.8861x + 470.27$	0.0613	0.2476	***



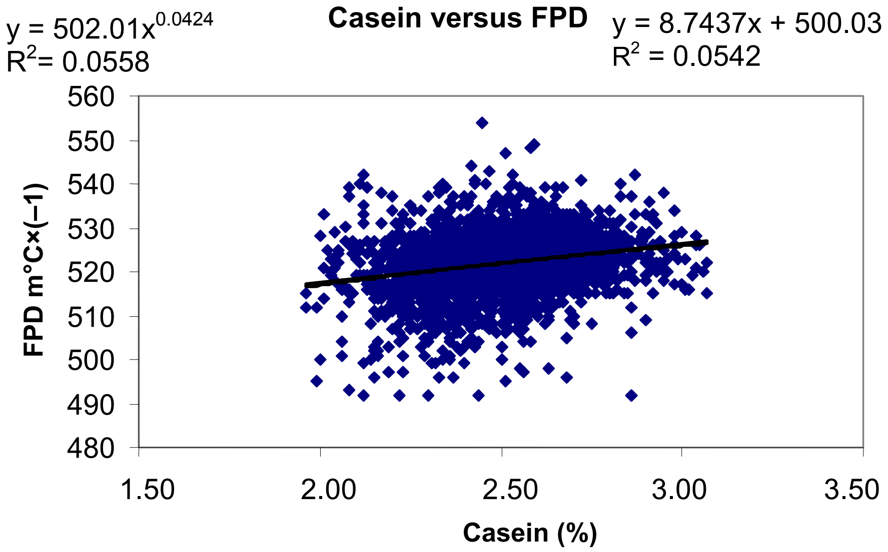
5: *The relationship between FPD and SNF in whole (I, validated data set) set in January*
linear r = 0.31***



6: *The box graph about the influence of milk F/CP ratio as an indicator of the energy metabolism of the dairy cow herds on the raw cow milk freezing point depression (FPD) in the bulk samples*
Ranked by presupposed energy metabolism of the dairy cows where the F/CP groups: < 1.06 = the shortage of the structural fiber in the feeding ration; 1.06–1.30 = in order; > 1.30 = the shortage of dairy herd energy maintenance, a risk of ketosis occurrence.

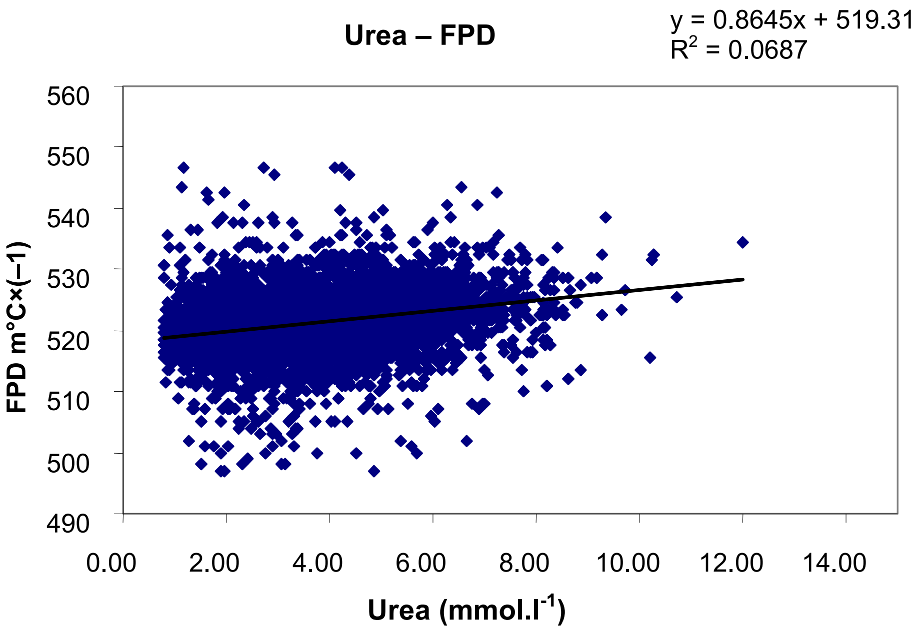


7: *The relationship between FPD and F/CP ratio in whole validated data set (I) in July*
Set I: July
linear r = –0.15*



8: *The relationship between FPD and casein content in whole (I, validated data set) set in January*
linear r = 0.23**

between the FPDs and F/CP ratios were poor. Determination coefficients ranged from 0.0059 in October up to 0.0215 in July, which is equal to –0.15 (r; $P < 0.01$; linear equation, Tab. II, Fig. 7). The non linear relationships show (as for instance in January, Tab. II, Fig. 7) the higher (worse) FPDs for low and high F/CP ratios. It could cohere with poorer or imbalanced nourishments of dairy cow herds. The problem will be discussed below once more. On the contrary again, the month relationships between the FPDs and the casein contents were closer. Those varied from 0.0157 (r = 0.13; $P < 0.01$) in October to 0.0542 (r = 0.23; $P < 0.001$; Fig. 8) in January and this fact indicates, that 5.4% of variation in the FPD is explained by variation in the casein content as maximum. The casein content increasing influences the FPD towards its improvement. The non linear regression equation (exponential) of this relationship was $y = 502.01x^{0.0424}$ (r = 0.24; $P < 0.001$; Fig. 8). The dependence of the FPD on urea concentration was a little closer and ranked between 0.0241 (r = 0.16; $P < 0.001$) in October and 0.0687 (r = 0.26; $P < 0.001$; Tab. V and Fig. 9) in March. The milk urea concentration enhancement improves the FPD value according to expectation. These our new results are in accordance with our previous find (Macek *et al.*, 2005) in terms of tendency and with discoveries and opinions of other authors (Walstra



9: *The relationship between FPD and milk urea concentration in whole (I, validated data set) set in March* r = 0.26**

V: *Results of month regression analyse between FPD (y) and milk urea concentration (x) in year data file (I)*

Month	Equation	Coefficient of determination (R^2)	Coefficient or index of correlation	Significance
January	y = 1.1074x + 518.35	0.0651	0.2551	***
February	y = 0.8297x + 519.27	0.0527	0.2296	**
March	y = 0.8645x + 519.31	0.0687	0.2621	***
April	y = 0.8516x + 517.34	0.0576	0.2400	***
May	y = 0.6009x + 518.51	0.0324	0.1800	**
June	y = 0.8808x + 516.97	0.0430	0.2074	**
July	y = 0.6772x + 517.66	0.0283	0.1682	**
August	y = 0.6851x + 517.74	0.0319	0.1786	**
September	y = 0.9062x + 519.78	0.0570	0.2387	***
October	y = 0.6412x + 521.62	0.0241	0.1552	**
November	y = 0.871x + 522.92	0.0369	0.1921	**
December	y = 0.8962x + 520.17	0.0451	0.2124	**

and Jenness, 1984; Buchberger, 1997; Kirchnerová and Foltys, 2005; Chládek and Čejna, 2005; Kirchnerová *et al.*, 2009), too. The dependence of the FPD on free fatty acid concentration in milk was quite low and varied from 0.0095 ($r = 0.10$; $P < 0.01$) in November up to 0.0127 ($r = 0.11$; $P < 0.01$; Tab. II) in December. The closes relationship was noted with the non linear regression equation ($r = 0.12$; $P < 0.01$; Tab. II). The FPD was very slowly reduced (improved) due to higher FFA concentration. The relationship between FPD and SCC was very poor and ranked between zero in June includig log transformed values of SCC and 0.0066 ($r = -0.08$; $P < 0.01$; Tab. II) only in December, although SCC is correlated usually with lactose content (Hanuš *et al.*, 1992), which is normally correlated to FPD. This find is a little different from our previous result (Macek *et al.*, 2005) or from the result of authors Chládek and Čejna (2005). Nevertheless the influence of the SCC on the FPD was noted as very poor in the bulk milk samples under the raw milk quality conditions in the Czech Republic.

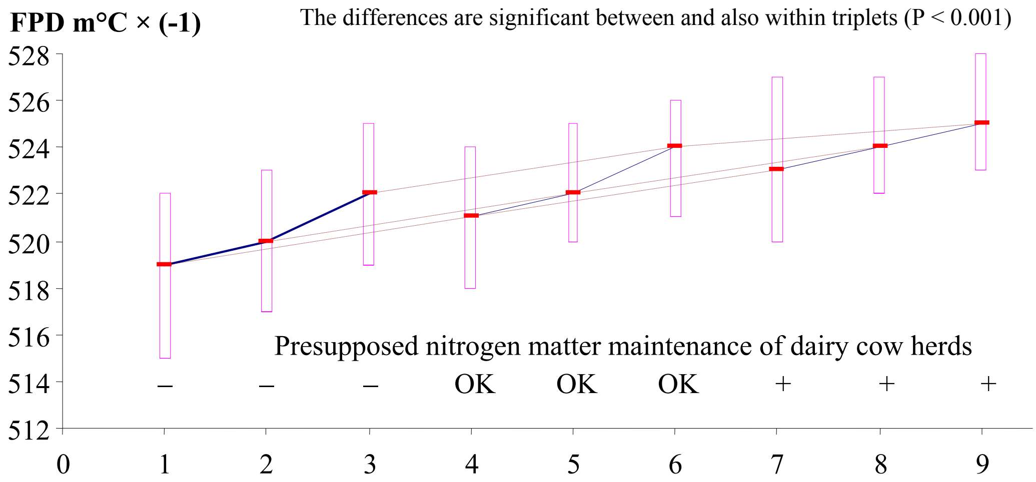
Milk freezing point and balance in presupposed state of dairy cow herd nutrition in terms of nitrogen matter/energy ratio

The presupposed nutrition state of dairy cow herds (bulk milk samples) in terms of nitrogen matters/energy balance in relationship to their milk yield was estimated according to combinations of urea and crude protein contents in milk. This was classified according to Tab. VI. Nine nutritional

VI: *The grouping of milk samples in accordance with presupposed dairy cow nutrition balance (nitrogen matters/energy, N/E) according to milk protein and urea contents (system is adjusted according to Kirchgessner et al., 1985, 1986)*

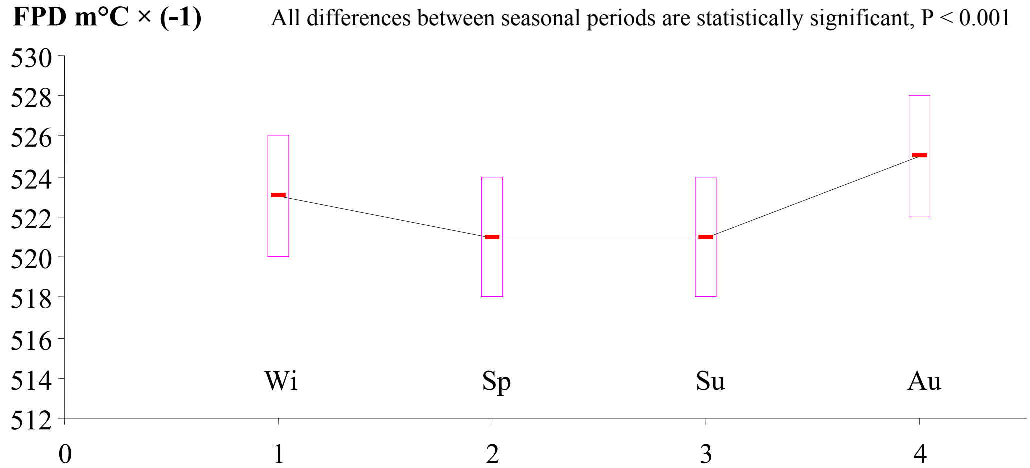
Milk		**Protein %**		
		< 3.10	**3.10–3.50**	**> 3.50**
Urea mmol.l^{-1}	**< 3.33**	1 N – E – n = 2 696 FPD –0.51855 ± 0.007288 vx = 1.4%	2 N – E 0 n = 2 242 FPD –0.51978 ± 0.006137 vx = 1.2%	3 N – E + n = 349 FPD –0.52140 ± 0.005674 vx = 1.1%
	3.33–5.80	4 N 0 E – n = 16 083 FPD –0.52090 ± 0.005874 vx = 1.1%	5 N 0 E 0 n = 26 867 FPD –0.52228 ± 0.004840 vx = 0.9%	6 N 0 E + n = 4 621 FPD –0.52363 ± 0.004818 vx = 0.9%
	> 5.80	7 N + E – n = 5 540 FPD –0.52326 ± 0.005833 vx = 1.1%	8 N + E 0 n = 9 407 FPD –0.52432 ± 0.004876 vx = 0.9%	9 N + E + n = 1 743 FPD –0.52536 ± 0.004785 vx = 0.9%

n = number of cases; vx = variation coefficient; milk freezing point depression (FPD) in °C; N = nitrogen matter dairy cow (herd) maintenance; E = energy maintenance; + = surplus; 0 = balanced; – = insufficiency



10: *The box graph about influence of combinations of milk urea and protein contents as indicators of nitrogen and energy nutrition balance of dairy herds (according to Tab. VI) on the raw cow milk freezing point depression (FPD) in bulk samples*
Regarding main viewpoint the triplets of class boxes (–, OK, +) are ranked according to presupposed nitrogen matter loading of dairy cow metabolism, it means in accordance with class numbers in Tab. VI (n = 69 548): – = insufficiency; OK = right (in physiological range); + = overloading. Identical nitrogen nutrition boxes are linked by solid line according to increase of enegy nutrition level. The boxes are ranked within the triplets (–, OK, +) along the presupposed increasing of energy maintenance of dairy cow herds as well. Identical energy nutrition boxes are connected by dashed line according to increase of nitrogen nutrition level.

groups of milk samples were obtained. The milk urea and protein physiological ranges were adjusted according to mentioned literature references. The average EPDs, which were relevant to created classes, are shown in the table as well. FPD values of this groups were tested in consideration of significance of their mutual differences by the t–test. Results are shown by the box graphs in Fig. 10. These are grouped according to nitrogen matter maintenance in the first viewpoint (–, OK, +). The highest (the worst) FPD average was stated in the group 1 with presupposed nitrogen matter and energy insufficiency (–0.51855 ± 0.007288 °C; Tab. VI). The lowest (the best) FPD was foud in the group 9 with presupposed nitrogen matter and energy surplus in the feeding ration (–0.52536 ± 0.004785 °C). The FPD difference between these two groups was statistically significant (t = 34.5 and P < 0.001). It is clear that this estimated phenomenon is linked directly with protein and urea contents in milk.



11: *The effect of year season on the raw cow milk freezing point (bulk samples)*
Winter, Wi = from December to March; Spring, Sp = April and May; Summer, Su = from June to September; Autumn, Au = October and November. The ranking was carried out in accordance with the country feeding and seasonal temperature conditions.

Nevertheless, this shows indirectly on relevancy of dairy cow nourishment in terms of occurrence of FPD defects and their prevention, too. There are 36 pair combinations differences between nine nutrition groups. All these differences were tested. Most of them were significant (35 = 97.2%; $P < 0.001$). Only one difference was no significant (3 – 4; $P > 0.05$). The FPD values were significantly lower (better) in the case of higher likelihood for higher nitrogen matter loading of dairy cows due to their nourishment (Fig. 10 and Tab. VI; $P < 0.001$). Within all three triplets (1, 2, 3; 4, 5, 6; 7, 8, 9) the same trend was reinforced by increased probability for higher energy maintenance of dairy herds ($P < 0.001$). The mentioned tendencies are linked with higher milk urea concentrations, as it has been already introduced above. This second tendency is a little different from our results in another paper (Macek *et al.*, 2005) where such trend was not observed. The nutrition class effect of dairy herds in terms of probability for nitrogen matter/energy balance of dairy cows on FPD was exhibited as highly significant ($P < 0.001$) according to our results. These our presented results are similar to those which were described previously by Buchberger (1997) in the main trends. Also according to the results of MP Bayern (1993, 1994 and 1995) a conclusion was estimated that FPD defects had been caused due to poor dairy cow nourishment in 50.2 and 58.8% of cases while due to extraneous water addition it was caused only by 34.5 and 41.2% of cases in the practice.

Milk freezing point and presupposed energy nourishment of dairy cow herds

Milk fat and F/CP ratio are good indicators of dairy cow energy balance during the lactation, especially in its first third (Agabriel *et al.*, 1991; Bíro *et al.*, 1992; Geishauser and Ziebell, 1995; Schulz, 1997; Illek and Pechová, 1997; Pechová *et al.*, 2000; Gasteiner, 2000, 2003). A higher F/CP value demonstrates an energy deficiency (ketosis risk) and lower value shows on deficiency of structural fibre in dairy cow nourishment. F/CP ratio, which ranked according to Tab. II was related to FPD. This is demonstrated in Fig. 11. The FPDs were significantly higher (worse) in the case of a suspicion on the energy deficiency and the risk of ketosis as compared to the presumption for normal balanced nourishment of dairy herds (Fig. 11; -0.52105 ± 0.006436 °C $> -0.52244 \pm 0.005367$ °C; $t = 22.3$; $P < 0.001$). On the other hand surprisingly, the FPDs were lower (better) in the case of a suspicion on the the shortage of the structural fiber in the feeding ration in comparison to the presumption for normal balanced nourishment of the dairy cow herds (Fig. 11; -0.52291 ± 0.005469 °C $< -0.52244 \pm 0.005367$ °C; $t = 5.9$; $P < 0.001$). Nevertheless, the difference and t–value are evidently lower than in the previous case. The FPD was improved by normal or lower value of F/CP ratio which is different result as compared to our previous finding (Macek *et al.*, 2005). However, this result is more probable in terms of the right interpretation of the possible mistakes in the dairy cow herd nourishment because of larger data set.

CONCLUSION

This is very well known that there are sure problems to meet the legislative discrimination limit value of raw milk freezing point (≤–0.520 °C) for part of dairy herds, especially with high milk yield or in less favourable areas of the country where the sources for adequate dairy cow nutrition are poorer or too expensive. It means not for extraneous water addition as technological lack of discipline but for other physiological or sometimes pathological reasons. That is reason why this is important to define a new real FPD legislative discrimination limit in right way for specific country conditions. This is important to explain all effects on FPD as far as possible. Results published in this paper about FPD variability sources are interesting for improvement of mentioned explanations and estimations.

SUMMARY

The milk freezing point depression (FPD) is very important physical property. FPD is influenced by milk composition, especially by components, which are connected with osmotic pressure and by other physiological factors as well. Under sure conditions this is possible to indicate a foreign (extraneous) water addition into milk according to FPD value. Therefore FPD is used to raw milk quality control in the framework of milk food chain quality assurance generally. This is necessary to have a good estimated legislative FPD discrimination limit for such purpose of milk quality control. This paper was aimed at obtaining information for possibility to improve mentioned estimation with taking of real biotic FPD variability sources in the country into account. There is necessary at derivation of milk FPD discrimination limit to take various factors into account as (in groups): 1) season variations; estimated state of dairy cow nutrition (in terms of the nitrogen/energy balance by milk urea and protein content combinations or by fat/crude protein ratio); cattle breed ratio; milk yield of dairy cows; 2) possible foreign (extraneous) water addition (technologically unavoidable water – for instance condensing water – as technical effect of machine milking); pasteurization; carbon dioxide and water steam evaporation under technological conditions; technological fat content manipulation (Hanuš *et al.*, 2010, 2011 a, b); 3) some milk components and properties (such as true and whey protein, somatic cell count, urea and citric acid content or value of titration acidity); 4) other country conditions. Impacts some of mentioned factors on milk FPD and their relations to FPD are quantified in this paper. A large data set was evaluated (n = from 11 540 to 72 607 bulk raw cow milk samples). Althouhg the frequency distributions of FPD month data sets were different from normal model (but first of all for high number of cases) the differences were lower in more important obliqueness. Therefore conventional statistic was suitable method for evaluation. Regarding seasonal effect on FPD the highest (the worst) value was in Spring (–0.52097 ± 0.004877 °C; variation 0.9%). The lowest (the best) FPD was in Autumn (–0.52516 ± 0.005725 °C; 1.1%). This difference was significant ($P < 0.001$). It could be probably influenced by nutrition and temperature effect variations, despite using of total mixed ration on the basis of preserved fodder feedstuffs for nourishment of dairy cow herds during whole year. The month relationships between FPD and fat content were practically almost independent. More relevant relationships (determination R^2) were stated between FPDs and crude protein contents, 0.0408 in January (correlation 0.20; $P < 0.001$). It means slow consistent improvement of FPD along crude protein increasing. More efficient situation was observed between FPDs and the lactose contents. R^2 was 0.1197 in May (r 0.35; $P < 0.001$). It means that 12% of FPD variability is explainable by lactose content variability. FPDs decrease (are improved) regularly with higher lactose contents. Relationship between FPD and solid non fat content increased to r 0.31 ($P < 0.001$). Relationship between FPD and F/CP (fat/crude protein) ratio was poorer. R^2 increased to 0.0215 in July (r –0.15; $P < 0.01$). Month relationships between FPDs and casein contents were closer (r increased to 0.23 ($P < 0.001$) in January). 5.4% of FPD variations are explained by variation in casein content. The dependence of FPD on urea concentration (U) was also closer and ranked to r 0.26 ($P < 0.001$) in March. The milk U enhancement improves FPD value. The dependence of FPD on free fatty acid concentration in milk was quite low. The relationship between FPD and somatic cell count was poor. The presupposed nutrition state of dairy herds in terms of nitrogen matters/energy balance was estimated according to combinations of urea and crude protein contents in milk. The worst FPD average was in group with presupposed nitrogen matter and energy insufficiency (–0.51855 ± 0.007288 °C). The best FPD was in group with presupposed nitrogen matter and energy surplus in feeding ration (–0.52536 ± 0.004785 °C). Difference was significant ($P < 0.001$). FPDs were significantly worse in the case of a suspicion on the energy deficiency and the risk of ketosis (on the basis of fat/crude protein ratio) as compared to the presumption for normal balanced energy nourishment of dairy herds (–0.52105 ± 0.006436 °C > –0.52244 ± 0.005367 °C; $P < 0.001$). This is important to estimate a new real FPD legislative discrimination limit and its inherent variation according to biotic factors in right way for specific country conditions to possibility of better added water identification. Results published in this paper about FPD variability sources are interesting for improvement of such estimation.

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