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Effect of milking method and season on phthalate content in cow milk from organic production

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ABSTRACT

Dibutyl phthalate (DBP) and diethylhexyl phthalate (DEHP) content was determined by the high-performance liquid chromatography in the milk samples either mechanically or manually taken from 10 dairy cows in January, April, July and October (altogether 344 milk samples were collected). Milking method and season accounted for 23 and 77%, and 33 and 36% of the explained variability of DBP and DEHP content, respectively. Content of DBP in mechanically collected samples was higher in comparison with manually collected milk: 10.0 vs. 6.7 mg kg^{-1} milk ($P < 0.05$). Irrespective of the milking method, DBP content differed significantly ($P < 0.05$) between samples collected in January (6.6 mg kg^{-1}), July (4.2 mg kg^{-1}) and October (20.0 mg kg^{-1}). In the case of DEHP, only differences between April and October milk were found out (0.03 vs. 0.96 mg kg^{-1}; $P < 0.05$). Concentration of DBP in both mechanically and manually collected milk increased according to a polynomial function from spring to autumn ($P < 0.01$). Thirty times higher average DBP content than DEHP content was contrary to expectation. A migration of DBP in milk apart from the milking equipment (probably by contaminated silage) was more important in comparison with DEHP.

1. Introduction

Phthalic acid esters (PAEs) are synthetic substances obtained by esterification of phthalic acid with different alcohols (Ventrice et al., 2013). They are classified in two groups: PAEs with high molecular weight (7 – 13 C atoms) and PAEs with low molecular weight (3 – 6 C atoms; Luís et al., 2021).

They are used especially as plasticizers for lamination of polyvinylchloride or are added to polyethylene terephthalate, polyvinyl acetate and polyethylene. Quantitatively most important PAE, diethylhexyl phthalate (DEHP), represents half of the PAEs global annual production (da Costa et al., 2023). It is the most commonly used plasticizer worldwide (Feng et al., 2005).

Because PAEs do not form stable bonds with the polymeric structures, they easily migrate to their lipophilic environments after prolonged contact with them (Ventrice et al., 2013). Humans are steadily exposed to PAEs by ingestion, inhalation and dermal absorption (Luís et al., 2021). The main route of human exposure is the ingestion of contaminated food (Bogdanović et al., 2019).

Phthalates can enter food within the whole production chain, including storage in plastic materials (da Costa et al., 2023). Cow raw milk, in particular, can be contaminated by PAEs during the mechanical milking process; migration from the milking equipment was verified for DEHP by Feng et al., 2005 and Fierens et al., 2012. Fierens et al., 2012 also mention possible PAEs transfer from contaminated feed, which can be one reason for different PAEs content in the summer and winter milk.

As far as toxicity is concerned, DEHP and *di-n*-butyl phthalate (DBP) are classified as very dangerous (Ventrice et al., 2013). As agonists of PPARα (peroxisome proliferator-activated receptor α), they are able to induce hepatic adenoma/carcinoma leading to the liver tumors in rodents (Ito et al., 2007). Mankidy et al. (2013) demonstrated that all tested phthalates, including DEHP and DBP, exhibited potency as agonists of AhR (aryl hydrocarbon receptor), which can increase cell proliferation, cell differentiation and tumorigenesis (Schlezinger et al., 2006). Exposure to DEHP/MEHP (mono-ethylhexyl phthalate) increases (via activating progesterone receptor signaling) risks to develop breast

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cancer (Crobeddu et al., 2019). Phthalates are also endocrine disruptors due to their anti-androgen effects (Zhang et al., 2021). Accumulation of DBP in macrophages belongs to risk factor for atherosclerosis (Wang et al., 2020). Childhood exposure to DEHP may contribute to the development of attention-deficit hyperactivity disorder (Ku et al., 2020).

Due to the above-mentioned health concerns established European Food Safety Authority (EFSA) Tolerable Daily Intake (TDI) levels for DBP of 0.01 mg kg^{-1} body weight (EFSA, 2005a) and for DBHP of 0.05 mg kg^{-1} body weight (EFSA, 2005b). In accordance with the above-mentioned concerns, phthalate content is permanently monitored in foods (Baker et al., 2024; Isci and Dagdemir, 2024), including human phthalate exposure from foods (Fu et al., 2023). As far as milk is concerned, several studies evaluated DBP and DEHP content in the raw milk samples obtained from farms both within Europe (Fierens et al., 2012; Castle et al., 1990; Sorensen, 2006) and elsewhere (Feng et al., 2005; Sharman et al., 1994).

The present study aimed at obtaining more recent data regarding DBP and DEHP content in raw milk, specifically from the organic production. The objective was to test the following hypotheses. DEHP is present in raw milk in higher concentration than DBP. In spite of organic production, mechanically collected raw milk contains higher DEHP/DBP levels in comparison with manually obtained samples. Variations in DEHP/DBP content in organically produced milk during different seasons are small.

2. Material and methods

2.1. Sampling schedule

The milk samples were taken from ten cows (Czech pied cattle breed) in a breeding facility employing organic production. The facility is located in the region of the Czech-Moravian Highlands at an elevation of 600 m above sea level, with average yearly temperature of 7 °C and average yearly precipitation of 850 mm. The feeding regimen within the lactation period was a seasonal one, combining pasture during summer with indoor feeding based on hay and corn-grass-legume silage supplemented with concentrates during winter.

The following three factors that can presumably affect PAEs content in milk samples were considered: milking method, part of the lactation period and a daily period. Machine-collected and manually-collected samples taken at 5 a.m. and 6 p.m. in January, April, July and October, respectively, were evaluated. Mobile one-bucket milking apparatus Bauer-Agromilk (Forst Agro Ltd., Pelhřimov, Czech Republic) was used for the machine milking.

A 250 mL sample of milk was taken from each of the five machine-milked dairy cows and five manually-milked dairy cows at each morning and evening milking, respectively, for consecutive five days within each of the four seasons. Altogether 25 manually-milked and 25 machine-milked morning and evening samples, respectively, were taken within each season. Milk samples were taken to glass jars with lid equipped with polytetrafluoroethylene (PTFE) sealing, cooled immediately to 6–8 °C, transported to the laboratory within 2 hours and stored at –18 °C until the analyses.

2.2. PAEs determination

Concentration of two PAEs were evaluated: di-*n*-butyl phthalate and di-ethylhexyl phthalate. The following chemicals were used for the PAEs analysis. DBP and DEHP analytical standards (Supelco; Bellefonte, PA, USA), purity ≥ 99.9%; acetonitrile, HPLC purity (Merck, Prague, Czech Republic); *n*-hexane, cyclohexane, dichloromethane, acetone (all p.a.; Penta, Prague, Czech Republic); sulfuric acid, p. a. (Lach-Ner, Neratovice, Czech Republic); deionized water, purified using Mili-Q-patron (Milipore, Burlington, Mass., USA).

The milk samples were homogenized (vortex MS2; IKA-Werke, Staufen in Breisgau, Germany), weighed (400–600 g) to an aluminum bow (200 mL; P-LAB, Prague, Czech Republic), frozen to –18 °C in a freezer (Electrolux, Örebro, Sweden) and gradually lyophilized (Alpha 1–2 LD+; Christ, Osterode am Harz, Germany). Milk fat was extracted from the lyophilized milk sample with the *n*-hexane (3 ×80 mL; 60, 30, 30 min) using a laboratory shaker (GFL 3005; Gesellschaft für Labortechnik, Burgwedel, Germany). The *n*-hexane was evaporated from the combined filtered extracts at 40 °C using a vacuum rotary evaporator (RVO 05-ST; IKA-Werke).

PAEs were separated from the extracted fat by the gel permeation chromatography (ECOM, Chrastany u Prahy, Czech Republic) using isocratic pump LCP 400, spectrophotometric detector LCD 2083 and MAG 3 column (10 ×500 mm; Bio-Beads S-Xš sorbent, 200–400 mesh; mobile phase dichlormethane/cyclohexane 1: 1, V/V). Extracted fat (0.5 g) was weighed to a vial (volume of 5 mL) and the mobile phase was added to a final volume of 2 mL. The mixture was vortexed (MS2; IKA-Werke) and 1 mL of the sample was injected by a syringe to the column and eluted with the flow of 1 mL min^{-1}. A fraction containing DEHP and DBP was collected to a heart-shaped flask, evaporated to dryness at 40 °C on a vacuum rotary evaporator (RVO 05-ST; IKA-Werke) and transferred quantitatively by the *n*-hexane to a vial.

The eluate was purified using a procedure with sulfuric acid. The *n*-hexane was evaporated from the vial by nitrogen to a volume of 1 mL, and 1 mL of concentrated H_2SO_4 (> 95%; Lach-Ner, Neratovice, Czech Republic) was added. The vial was shaken for 10 min and then centrifuged (Hettich-Zentrifugen D-78532, Tuttlingen-universal 32 R, Tuttlingen, Germany) for 10 minutes at 112 x g at 4 °C. The upper hexane fraction (presumably containing polychlorinated biphenyls) was discarded using a Hamilton syringe. Then 2 mL of 65% H_2SO_4 and 1 mL of *n*-hexane were added, the vial was shaken for 10 minutes and centrifuged for another 10 minutes at 112 x g at 4 °C. This time the upper hexane fraction (with PAEs) was transferred to another vial (11 mL) and the same step with 65% H_2SO_4 and *n*-hexane was repeated twice more. The final extract containing PAEs was dissolved in 1 mL of acetonitrile.

Dibutyl phthalate and diethylhexyl phthalate were determined by high performance liquid chromatography (HPLC) using chromatograph Agilent Technologies LC/MSD VL (Agilent Technologies 1100 Series; Santa Clara, USA) and column Zorbax Eclipse XDB-C8 (150 ×4.6 mm, granulation of 5 μm; Agilent Technologies). Ratio of the mobile phase of acetonitrile/water changed with the elution time as follows: 0–3 min 80/20, 3–9 min 95/5, 9–12 min 100/0, 13–18 min 80/20. PAEs were detected by an UV detector (Agilent Technologies) at 224 nm and identified according to the retention times. Pertinent standards were injected in between analyzed samples in order to eliminate possible shifts of retention times. Data were evaluated using the program Agilent Chemstation (Agilent Technologies). Amount of DBP and DEHP in milk samples were expressed in mg per kg of the fresh milk. HPLC chromatogram of a typical milk sample, including a blank sample is presented in Fig. 1.

Limit of detection (LOD) was calculated based on measurements of 10 independent blanks as average blank concentration + three times the standard deviation of procedural blank. Limit of quantification (LOQ) was calculated based on measurements of 10 independent blanks as average blank concentration + ten times the standard deviation of procedural blank. LOD was 0.19 and 0.13 mg kg^{-1} milk, and LOQ was 0.57 and 0.39 mg kg^{-1} milk for DBP and DEHP, respectively. The values of recovery were 96.7% for DBP and 95.6% for DEHP. Corresponding repeatability values, expressed as relative standard deviations, were 3.3% and 6.5%, respectively.

2.3. Statistical evaluation

Based on the Shapiro-Wilk test, the data distribution was log-normal and normal regarding DBP and DEHP content in milk samples, respectively. So, the differences in DBP content between the milking methods or between daily periods were evaluated by Kolmogorov-Smirnov test, the differences between the seasons of the year by Kruskal-Wallis test.

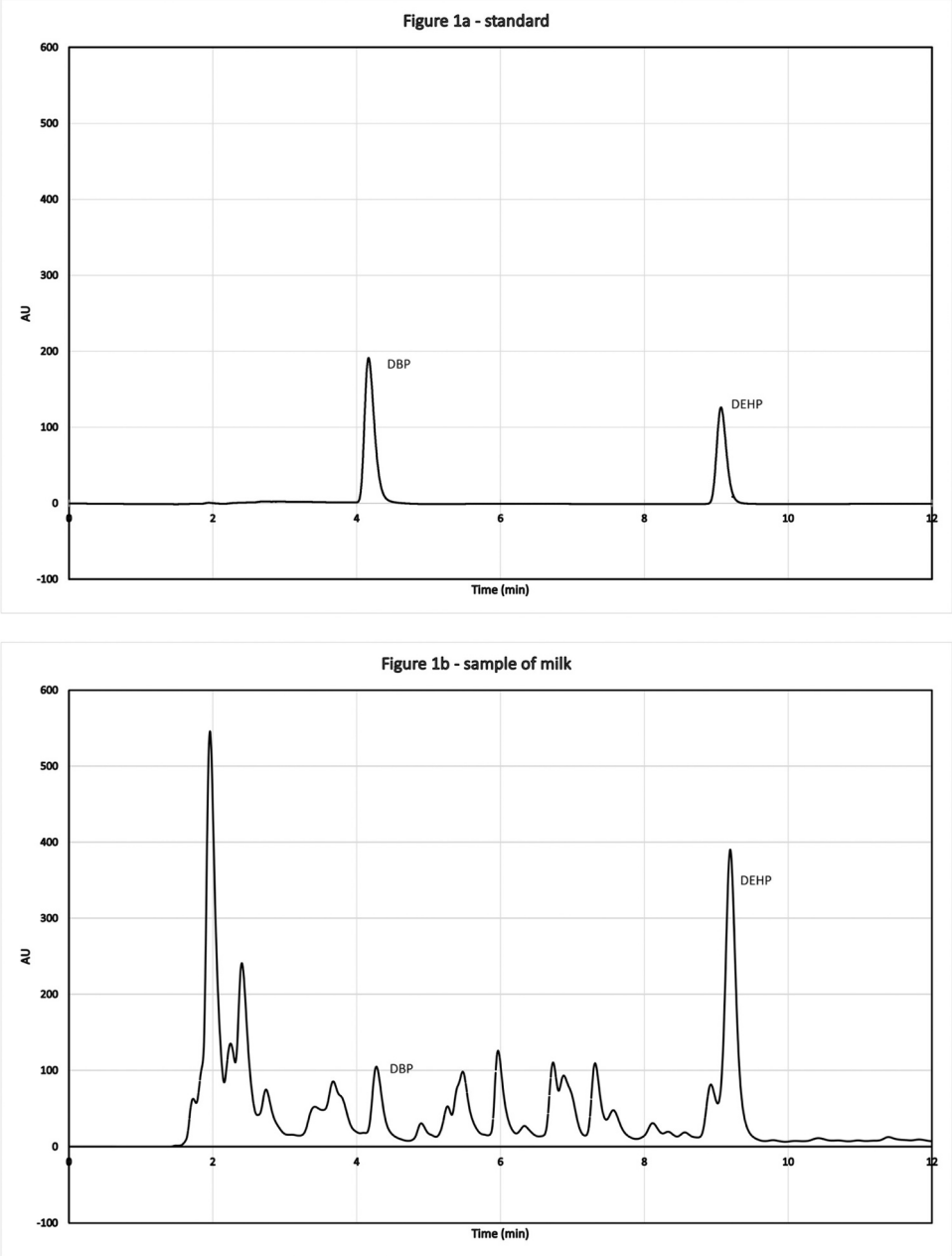

Fig. 1. High-performance liquid chromatography chromatogram of the separation of di-*n*-butyl phthalate (DBP) and di-ethylhexyl phthalate (DEHP): (a) standard; (b) sample of milk; AU – signal of the detector in arbitrary units.

Corresponding differences in DEHP content were assessed by one-way ANOVA with the *post-hoc* Tukey's test.

General linear model was used for an estimate of the proportion of the variance in the dependent (response) variables (DBP and DEHP content in milk, respectively) that can be explained by the independent (predictor) variables: milking method; season of the year; daily period. Moreover, contributions of the predictor variables on the total variance in the dependent variables was also tested in order to estimate a proportion of a variance due to factors not controlled in the present experiment. Testing the interactions of milking method x season and milking method x season x daily period was also included in the model. Polynomial regression was used for evaluation of the changes of DBP and DEHP content, respectively, during the lactation period (from January to October). Statistica 14 (Tibco Software Inc., Palo Alto, CA, USA) was used for the evaluation.

3. Results

Effects of the tested independent factors (milking method, season of the year, daily period) on the variability of the content of DBP and DEHP in the raw milk are shown in Table 1. Season and milking method accounted for more than two thirds and nearly 23%, respectively, of the explained variability of DBP content ($P < 0.001$). Effect of the morning/evening milking was negligible ($P > 0.05$). Insignificant was also interaction between milking method and season ($P > 0.05$). However, it is also apparent from Table 1 that the factors tested in the present experiment explained only small fraction (15%) of the total variability of DBP content in milk.

The results regarding DEHP were different. Nearly 60% of the total variability were explained by the factors tested in the present experiment. And each of the factors of milking method, season and their interaction accounted for approximately one third of the explained

Table 1
Effects of the tested factors (milking method; season of the year; daily period) on the total and explained variability of dibutyl phthalate (DBP) and diethylhexyl phthalate (DEHP) content in milk (General linear model, n = 171).

Independent variable	DBP				DEHP			
	F-value	Variability (%)		P-value	F-value	Variability (%)		P-value
		total	explained			total	explained	
Milking method	75.3	3.4	22.9	< 0.01	2.73	19.4	33.4	0.09
Season	251.5	11.3	76.6	< 0.01	2.93	20.9	35.9	0.03
Daily period	0.5	0.0	0.2	0.45	0.03	0.2	0.4	0.21
Method x season	0.6	0.0	0.2	0.58	2.47	17.6	30.2	0.06
Method x season x period	0.3	0.0	0.1	0.94	0.01	0.1	0.1	0.99

variability of DEHP content in milk, though only effect of the season was significant ($P < 0.05$). Though the interaction of milking method x season was not significant, there was a strong tendency ($P = 0.06$; Table 1), indicating a possibility that either the dependence of DEHP content in milk on the milking method could be affected by the season or its dependence on the season could be affected by the milking method. However, these two possibilities were not further tested in detail.

When the milking method was evaluated irrespective of the season of the year and the daily period, DBP content in the mechanically obtained milk samples was higher in comparison with the manually obtained counterparts ($P < 0.05$; Fig. 2). On the other hand, when the milking method and the daily period were not taken into consideration, DBP content differed in the milk samples obtained in different seasons of the year ($P < 0.05$). The highest ($P < 0.05$) DBP content was detected in milk obtained in October. DBP content in milk collected in July was lower in comparison with both the October milk and the January milk ($P < 0.05$; Fig. 2).

It is apparent from comparison of Fig. 2 and Fig. 3 that contents of DEHP in the milk samples were by one order of magnitude lower in comparison with DBP. Moreover, the variance of the DEHP values measured within the particular sets of the milk samples was relatively high with the consequence of the overlapped differences between the sets. So, the DEHP content in the mechanically obtained milk only tended to be higher ($P = 0.07$) in comparison with the manually milked samples. As the comparison of the seasons of the year is concerned, the only significant difference was found out between April and October samples ($P < 0.05$; Fig. 3).

The dependence of the DBP content in either mechanically or manually obtained milk samples on the season of the year is shown in Fig. 4 (an order of a given month within the year was used as an independent variable). The relationships were described by polynomial regression significantly better than by the linear regression ($P < 0.01$). Content of DBP in both mechanically and manually obtained milk significantly increased from spring to autumn ($P < 0.01$; Fig. 4). The corresponding dependences of DEHP content in mechanically and manually collected milk samples are not shown in Fig. 4 due to a very low coefficient of determination ($R^2 = 0.03$, $P < 0.05$) and insignificance ($P > 0.05$), respectively.

4. Discussion

The farm where the milk samples were collected in the present study practices organic production. However, it was not intended to compare PAEs content in milk produced organically and conventionally. One of the objectives was only to prove that mechanically collected milk, though organically produced, can still contain not negligible content of DBP/DEHP.

4.1. Average DBP and DEHP content

Based on our previous results regarding screening of PAEs in different raw materials (Jarošová et al., 2010), we focused on DBP and DEHP in the present study. This selection is substantiated based on the data of Fierens et al. (2012), who monitored content of dimethyl phthalate (DMP), diethyl phthalate (DEP), DBP, diisobutyl phthalate (DnBP), benzylbutyl phthalate (BBP), DEHP, dicyclohexyl phthalate (DCHP) and *di-n*-octyl phthalate (DnOP) in the raw milk produced from 10 Belgian farms. The authors were able to quantify DEHP in milk samples from most tested farms and DBP in one farm, but detected no DMP, DEP, DCHP or DnOP.

Nearly 30 times lower average DEHP concentration (0.29 mg kg^{-1} of milk) than the DBP concentration (8.36 mg kg^{-1} milk) was found out in

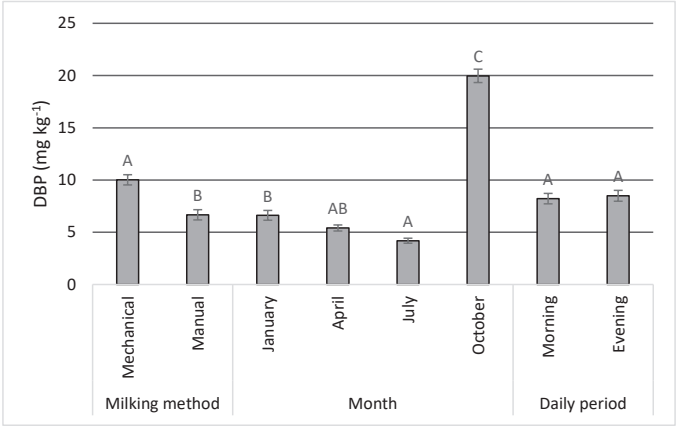

Fig. 2. Content of di-n-butyl phthalate (DBP) in the milk samples as affected by the three independent factors: differences between milk samples obtained either mechanically (n = 173) or manually (n = 171; Kolmogorov-Smirnov test); between milk samples obtained in four different seasons of the year (January, n = 88; April, n = 96; July, n = 80; October, n = 80; Kruskal-Wallis test); and between samples milked either in the morning (n = 171) or in the evening (n = 173; Kolmogorov-Smirnov test); the differences due to a given factor were evaluated irrespective of the other factors (e.g. the set of mechanically milked samples comprised all samples obtained both in the morning and in the evening within all four tested months).

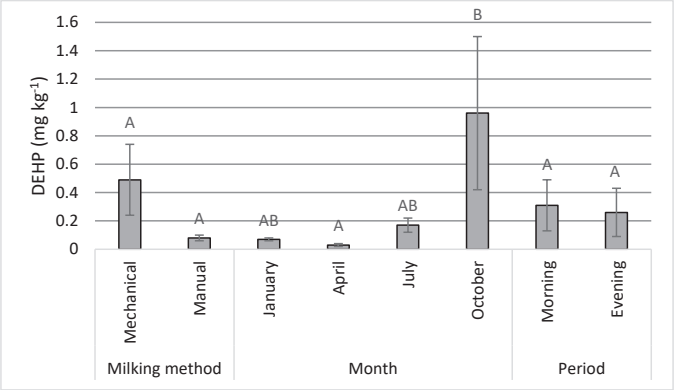

Fig. 3. Content of diethylhexyl phthalate (DEHP) in the milk samples as affected by the three independent factors: differences between milk samples obtained either mechanically (n = 173) or manually (n = 171; independent groups t-test); between milk samples obtained in four different seasons of the year (January, n = 88; April, n = 96; July, n = 80; October, n = 80; one-way ANOVA with *post-hoc* Tukey's test); and between samples milked either in the morning (n = 171) or in the evening (n = 173; independent groups t-test); the differences due to a given factor were evaluated irrespective of the other factors (e.g. the set of mechanically milked samples comprised all samples obtained both in the morning and in the evening within all four tested months).

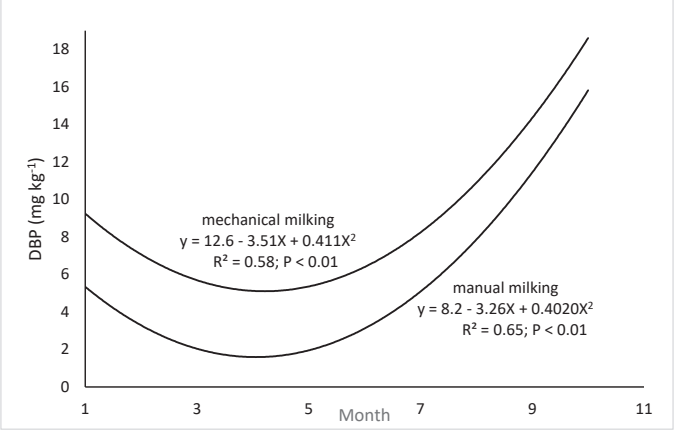

Fig. 4. Dependence of di-*n*-butyl phthalate content (DBP) in the raw milk (mg kg^{-1}; Y) on the order of the month within the year (X); polynomial regression (n = 344).

the present experiment. This ratio was very different from the study of Fierens et al. (2012). The quoted authors reported more than 20 times higher average DEHP concentration (in the range from 298.3 to 400.1 μg kg^{-1} fat) than DBP concentration (15.3 μg kg^{-1} of fat).

Irrespective of the variable fat content in the milk samples and assuming a standard fat content of 4%, the data of Fierens et al. (2012) regarding DEHP can be expressed as 0.012 – 0.016 mg kg^{-1} of milk. This is one order of magnitude lower than DEHP content found out in our study. DEHP content in the raw milk in the present experiment was also higher in comparison with the studies of Castle et al., 1990 (<0.005 – 0.05 mg kg^{-1} of milk) or Kim et al., 2009 (0.057 mg kg^{-1} of milk). Sorensen (2006) even found out no DEHP in the raw milk samples of the Denmark provenance. On the other hand, our results are comparable with the data of Feng et al., 2005 (0.008 – 0.28 mg kg^{-1} of milk) and Sharman et al., 1994 (0.12 – 0.28 mg kg^{-1} of milk), respectively.

From the results of the above-mentioned European studies (Fierens et al., 2012; Castle et al., 1990; Sorensen, 2006) follows that – at least in Europe – DEHP has already for a long time been continually substituted by different plasticizers. However, relatively high DEHP concentrations in the raw milk samples established in the present study are contrary to this trend. As far as DBP content is concerned, it was even by one order of magnitude higher (in average 8.36 mg kg^{-1} of milk) than DEHP content in the present experiment. Moreover, this value is several orders of magnitude higher in comparison with the data of Feng et al. (2005), who reported DBP content in the teat milk in the range of 0.004 – 0.01 mg kg^{-1} of milk.

Two trends can possibly be recognized based on the comparison of PAEs content established in the present experiment and in similar European studies. Firstly, higher PAEs content was found out in the Czech milk tested in the present experiment in comparison with e.g. Norway (Castle et al., 1990), Denmark (Sorensen, 2006) or Belgium (Fierens et al., 2012). And secondly, a converse ratio between contents of the two toxicologically important PAEs, DEHP and DBP, was established in the present study and the quoted European data.

There are two caveats regarding this comparison. The milk samples were taken from only one farm within the Czech Republic in the present study, so the results cannot be generalized. And moreover, there are substantial time differences between publishing of the studies being compared.

Taking into account tolerable daily intake (TDI) values established by EFSA (EFSA 2005a; EFSA 2005b) for DBP and DEHP (0.01 and 0.05 mg kg^{-1} body weight, respectively), ingestion of one liter of milk tested in the present study by an adult (70 kg) would represent 8% and 1200% of TDI for DEHP and DBP, respectively. TDI value represents an amount of a substance whose daily intake over a lifetime should not be exceeded. So, the data regarding the difference between DBP and DEHP content can be interesting from the viewpoint of food safety, but an occasional ingestion of milk tested in the present study would not constitute a serious health problem.

4.2. Comparison of mechanical and manual milking method

Irrespective of the season of the year, both DBP content (Fig. 2) and DEHP content (Fig. 3) in milk was increased when mechanical milking was applied in comparison with manual milking in the present study. However, the difference between the milking methods was more conspicuous in the case of DEHP. In comparison with the mechanically-milked samples, DEHP content in the manually obtained milk represented less than one fifth of that amount (0.08 vs. 0.49 mg kg^{-1} of milk; 16%), but in the case of DBP it were two thirds (6.67 vs. 10.02 mg kg^{-1} of milk; 67%).

Much stronger tendency of DBP than DEHP to migrate to milk outside the milking equipment found out also Feng et al. (2005), who reported the same amount of DBP in the "teat" milk as in the "line" milk, but more than ten times lower DEHP content in the teat milk in comparison with the line milk. The authors suggested potential leaching of DEHP, but not of DBP, from PVC tubing into row cow milk. And despite the fact that Fierens et al. (2012) quantified DBP only in a few manually obtained milk samples in their study, the differences in DBP content between hand milking and machine milking were practically nonexistent. On the other hand, DEHP amount in the machine-milked samples was substantially higher in comparison with the hand-collected samples.

4.3. Effect of season on DBP and DEHP content in the raw milk

Despite the differences between DBP and DEHP regarding effects of the tested variability factors, a season accounted for most of the explained variability in amounts of both these PAEs in milk in the present study (Table 1). Similarly, Fierens et al. (2012) found out substantial differences between summer and winter milk samples obtained from Belgian farms as far as both DBP and DEHP content is concerned. However, in a more detailed view, the results of Fierens et al. (2012) and the present experiment are different. In the Belgian study, an average DEHP content in milk was higher in summer than in winter: more than 400 mg kg^{-1} and less than 300 mg kg^{-1} of milk, respectively. Moreover, the authors detected DBP only in one summer sample (15.3 mg kg^{-1} of milk) and in no winter sample.

Contrary to these data, milk taken in the present study in July contained less DBP than the January milk (4.19 mg kg^{-1} vs. 6.22 mg kg^{-1}; Fig. 2). And DEHP content in milk did not differ between January and July (Fig. 3). Nevertheless, the highest DBP content within the present study was found out in the autumn (October) milk; the differences did not reach statistical significance in the case of DEHP due to a very high variability between particular samples.

Despite the above-mentioned significant effect of the season on the PAEs content in milk, this variability factor still accounted for only 11–21% of the total variability in the present experiment (Table 1). Due to a lipophilic character of PAEs, one factor that could possibly contribute to the seasonal differences in DBP/DEHP content in milk was a varying milk fat content during a year. From the results of an experiment carried out on 4852 dairy cows of the same breed that was used in the present study (Czech pied cattle) follows an increase of the fat content in milk (Y; %) with an increasing order of the day of lactation (X; days): $Y = 4.16 + 0.00075X$ (Hanuš et al., 2018). However, despite the fact that the dependence was highly significant, an increase in the milk fat content during the whole lactation period would be only 0.23%, which is insufficient for explaining the seasonal differences in the PAEs content found out in the present study.

Much more probable contributor to the contamination of milk with PAEs seems to be an environmental pollution (Luís et al., 2021; da Costa et al., 2023), including a possible migration of PAEs into animal feed (Fierens et al., 2013). PAEs-contaminated silage, pasture, concentrate and soil were identified as important contributors to the PAEs content in milk in the study of Fierens et al. (2012).

As mentioned above, DBP was quantitatively much more important than DEHP in the present study. Moreover, the course of an increase of DBP content in milk from the spring and summer months to the autumn and winter months was highly significant and very similar between manually and mechanically obtained samples (Fig. 4). On the other hand, though the dependence of DEHP content in the mechanically collected milk on the season was significant, the coefficient of determination of the regression was very low ($R^2 = 0.03$). And the corresponding dependence of the DEHP content on the season in the manually obtained milk was insignificant ($P > 0.05$).

Therefore, a probable contamination of milk from a feed source fed predominantly in autumn and winter (silage) can be inferred from the above-mentioned data. Fierens et al. (2012) reported DBP and DEHP content in silage in the range of 7.6 – 18.6 and 15.2 – 32.9 μg kg^{-1} fresh weight, respectively. We did not measure PAEs content in silage in the present experiment, but a previous study demonstrated PAEs migration from the plastic tanks to the plant oils used for production of the feeding mixtures (Harazim et al., 2008). At any rate, the feed-borne contamination was from at least three reasons more likely in the case of DBP than DEHP in the present study. Firstly, DBP content in milk was by one order of magnitude higher than DEHP content (compare Figs. 2 and 3). Secondly, much smaller difference in DBP content than in DEHP content between mechanically and manually collected milk was established. And thirdly, the tested factors explained less of total variability of DBP content in milk in comparison with DEHP content (Table 1).

5. Conclusions

Content of DBP in milk was thirty-times higher than DEHP content in the present experiment, which is contrary to the tested hypothesis and also contrary to the results of the most similar studies. Still, DEHP content in milk was by an order of magnitude higher in the present experiment in comparison with corresponding European studies (Belgium, The Netherlands, Denmark). So, a European trend of substituting DEHP in milking equipment by different plasticizers was not confirmed in the present study (with a caveat that only one farm was analyzed and therefore the results are not possible to generalize).

The hypothesis that mechanical milking increases PAEs content in the raw cow milk in spite of organic production was confirmed. Still, milking method affected DEHP content in milk much more than DBP content. A significant migration of DBP to milk from other sources are therefore likely (which is also true for DEHP, but in a much smaller extent). Because DBP content in milk significantly increased from summer to autumn and winter, a possible source of contamination was probably not pasture, but more likely silage stored in the plastic wraps. In any case, the hypothesis regarding only small changes in DEHP/DBP content in organically produced milk within a course of a year was unproved.

Author Statement

All authors of manuscript entitled "Effect of milking method and season on phthalate content in cow milk from organic production" disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. The authors disclose in this manuscript the use of AI and AI-assisted technologies in the writing process.The authors disclose the use of generative AI and AI-assisted technologies in the writing process of this manuscript.The authors state that the work entitled "Effect of milking method and season on phthalate content in cow milk from organic production" has not been published, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and by the responsible authorities of the Mendel University in Brno (where the work was carried out), and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

CRediT authorship contribution statement

Alžbeta Jarošová: Writing – original draft, Methodology, Investigation, Conceptualization. **Tomáš Gregor:** Formal analysis. **Olga Cwiková:** Formal analysis. **Magdaléna Krejčíková:** Investigation. **Soňa Bogdanovičová:** Investigation. **Tomáš Komprda:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis.

Declaration of Competing Interest

none.

Data availability

Data will be made available on request.

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