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EVALUATION OF THE LEVEL OF CONTAMINATION WITH PHTHALATES IN DAIRY PRODUCTS FOUND ON THE ROMANIAN MARKET

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ABSTRACT. Human exposure to phthalates (artificial phthalic acid esters) is ubiquitous because of the widespread use of these chemicals in consumer and industrial products. The aim of this study was to determine the presence and amount of the phthalates in milk and dairy products collected in Romania. Samples were gathered at several stages during the production, primary production (farm), milk collection center and retail level. Six types of phthalates were assessed by gas chromatography with mass spectrum detection (GC-MS, of which only 4 were detected in the samples - di-n-butyl phthalate (DBP), di-2-ethylhexyl phthalate (DEHP), di-n-octyl phthalate (DOP) and dibutylbenzyl phthalate (BBP). The obtained concentrations ranged between 0.060 – 0.298 mg/kg for DEHP, in raw milk samples and 0.038 - 0.173 mg/kg respectively, in commercial milk. DMP and DEP were not detected in any sample. The lowest quantity of phthalates was detected in yogurt with 0.1% fat – 0.042 mg/kg, and the largest amount was recorded in butter with 75% fat – 0.683 mg/kg. The level of total phthalates in all samples analysed did not exceed the maximum permitted limit of 60 mg/kg.

Keywords: *phthalates, milk, dairy products, GC-MS*

INTRODUCTION

Phthalates (PAEs) are esters of phthalic acid which are a class of chemicals with a wide use, since 1930. These substances are commonly used as plasticizers - additions to polymers (plastics, rubber, paints) designed to

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impart polymers (PVC) plasticity, extensibility and tear resistance. They are also used as solvents, lacquers, resins, or surfactants, alcohol denaturants in cosmetics, perfumes, pesticides, etc. [4, 8, 35].

Phthalates are compounds synthesized by double esterification of 1.2-benzenedicarboxylic acid (phthalic acid) with linear or branched alcohols [40].

These substances are classified into two groups: low-molecular-weight phthalates such as di-n-butyl phthalate (DBP) or benzyl butyl phthalate (BBP), and high-molecular-weight phthalates such as diisodecyl phthalate (DIDP) or diisononyl phthalate (DINP) [37].

Depending on the molecular weight, they can be used in various industrial applications. Low molecular weight phthalates, such as diethyl phthalate (DEP) and dibutyl phthalate (DBP), benzyl butyl phthalate (BBP) are used in personal care and hygiene products (as solvents and fixation agents in perfumes, in the preparation of shampoos, soaps, lotions, cosmetics, and softeners, or added as plasticizers of cellulose acetate), in the process industry (e.g., production of lacquers, paints, lubricating oils, adhesives, inks, waxes, insecticides) and also in the pharmaceutical industry (in some drugs, it is used to regulate the release speed) [38, 40].

On the other hand, high molecular weight phthalates, such as bis(2-ethylhexyl) phthalate (DEHP), diisononyl phthalate (DINP), and di-n-octyl phthalate (DnOP) are mainly used as plasticizers in the production of vinyl, which is often used in products such as flooring and wall covering or another construction materials, clothing and furnishings, toys, food packing, and medical devices [39, 40]. The plasticizing phthalates, which also include diisodecyl phthalate (DIDP), dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), and benzyl-butyl phthalate (BBP), are used as intermolecular lubricants conferring hardness, flexibility, malleability, and elasticity [3, 25, 31, 35, 40].

At room temperature, phthalates are oily liquids with low volatility and varying miscibility with polymers. Their addition reduces intermolecular interactions and increases the mobility of polymer chains [37, 40, 42]. Their solubility in water decreases with an increase in the length of the carbon chain or molecular weight [37].

The chemical and physical properties vary with the structure, with the length of the chain and the branches. They are generally colorless, odorless and lipophilic, they have a weak solubility in water and a satisfactory solubility in most organic solvents. Also, these substances show a high boiling point and a low vapor pressure, both parameters influencing their high stability and presence in the environment [37, 40, 42].

Humans can be exposed to phthalates after ingestion of food or water (orally), from the air (inhalation - indoor/outdoor air, hair/paint sprays), through

dermal contact (personal care products, toys, textiles, gloves, paints/adhesives, and dust particles) or parenteral application [7, 43]. Phthalates have been detected in environmental samples like rainwater, water, soil and sediments, indoor air or dust and aquatic systems, being considered ubiquitous substances in the environment [2, 3, 51].

The highest human exposure to phthalates comes from food, especially by those with high fat content which accumulates phthalates [18, 21, 30]. Due to their lipophilic nature, phthalates may also lead to accumulation from the feed and the environment in animal tissues, muscle, fat, and also the phthalates may pass through the digestive tract in the milk, which leads to another potential threat chain of the consumer [18, 23].

From the environmental point of view, phthalates have a duration of several hours in the atmosphere and of months in the soil, whereas they can persist for years in sediments [46]. They can bioaccumulate in invertebrates, fish, and plants, whereas in complex animals they are efficiently metabolized and excreted. This last consideration is very important because the possible presence of phthalates in tissues [47] indicates a very recent exposure/ contamination [23, 41, 47].

Once they enter the body, phthalates undergo a series of phase I hydrolysis and phase II conjugation reactions and are subsequently excreted in feces and urine [44, 48, 49]. In the first step, diester phthalate are hydrolysed to monoester in a process catalysed by lipases and esterases in the intestines or other tissues. While this step for xenobiotic in most of the cases bring to a detoxification, in such a case leads phthalates to become more bioactive as monoester. The second phase of metabolism, conjugation, is often catalysed by enzyme UDP-glucuronosyl-transferase to form the hydrophilic glucuronide conjugate, and the conjugates are easily excreted into urine [45].

Because phthalates have a short half-life in human bodies and are excreted quickly in urine as monoester metabolites, the metabolites are suitable biomarkers for human exposure to parent compounds. The half-life of phthalates in human bodies (in plasma and urine) is less than 24 h, and following metabolism, monoesters of phthalates are conjugated with glucuronide or sulfate and excreted in urine [50].

Phthalates do not form covalent bonds with the polymers they are mixed with. Therefore, they can freely migrate to the surface of products and further into food and beverages in contact with these surfaces. As a result, they can easily escape and spread to the environment during the production and the utilization of products that contain them [42].

Also, phthalate esters may migrate into foods during food processing and storage in plastic packing materials [11, 20, 35]. For instance, bottled milk, as well as milk products, can be contaminated with phthalates in several

ways: through water, air or soil, also during the bottling process or migration from packing material to milk or milk products, absorption from PVC tubing to raw milk during milking on dairy farms, etc. [1, 12, 21].

Moreover, due to their low vapor tension, phthalates are easily evaporated and diffuse into the atmosphere; they are trapped in aerosols and, through rainfall, end up in receiving water bodies and soil. As a result, the phthalates have been detected in samples taken from sewage, air, surface water, soil, and aquatic organisms [2, 3, 51].

Consequently, this has led to their widespread dispersion into the environment, providing an easy source of human exposure by inhalation, ingestion, dermal absorption, or even intravenous route [42].

In a literature review, Cao Xu-Liang [3], identified various sources of phthalate food contamination, including PVC tubing used in food production, food packaging films (also known as wrapping or cling films), PVC gaskets in jars, printer ink on labels, and other sources.

Many plasticizers and additives are listed as suspected endocrine disrupters or mutagens, which can have adverse effects on human health even at low levels. Several phthalates are able to act as anti-androgens, estrogens, anti-estrogens or inhibitors of steroidogenic enzymes and are able to act with thyroid hormones and their receptors through interaction, as well as within the brain and the immune system [20,15].

Due to the chemical composition (vitamins, minerals, carbohydrates, lipids, and proteins) milk is considered one of the most nutritionally complete natural foods [17, 28]. Also, many specialized milk products such as cheese, yogurt, butter, cream or ice cream are popular in diets worldwide. However, most commercial milk and milk products are packaged in plastic or other polymer materials. Therefore, it is extremely important for human health protection to evaluate and monitor phthalates in this type of products [20].

To guarantee human health, the European Union established limits for many compounds used in packaging and established regulations, specifying migration tests using food simulants to determine their probable migration into food. The EU fixed Specific Migration Limits (SMLs) for single contaminants or group of contaminants in Regulation 10/2011. These values are in particular 0.3 mg/kg food simulant (fs) for DBP, 30 mg/kg fs for BBP, 1.5 mg/kg fs for DEHP. For compounds for which there are not SML, a restriction value of 60 mg/kg of food product is applied. For containers and other articles, for sheets and films in contact with less than 500 mL or g or more than 10 L and for materials and articles for which it is not possible to estimate the relationship between the surface area of such materials and the quantity of food in contact, the SML are expressed in mg/kg applying a surface to volume ratio of 6 dm^2 per kg of food. Overall, the plastic packaging

must not be released to food simulants more than 10 mg of all compounds in one dm^2 of contact surface between food and packaging (Overall Migration Limit or OML) [6].

For infants and small children which have a higher consumption of food per kilogram bodyweight than adults and do not yet have a diversified nutrition, special provisions should be set in order to limit the intake of substances migrating from food contact materials.

Based on these findings, a careful analysis of these endocrine disrupters is mandatory, in order to respect the food safety legislation [34].

There are two main reasons to study the contamination of milk with phthalates. Firstly, especially for children, milk is a significant consumer product. In order to quantify the phthalate amount that humans are exposed to by means of their dietary, it is important to know the phthalate content of such food products.

Secondly, phthalates are likely to be concentrated in the lipid phase of the foods due to their lipophilic characters. Since dairy products like milk and other products can be classified as high-fat foods, they have higher tendency to be contaminated by phthalates than low-fat content foods.

In Romania, phthalate esters assessment was performed mainly on bottled water [9, 26, 29]. Regarding our knowledge, no literature is available dealing with the contamination levels of dairy products packaged in plastic containers. There is only a single study performed on milk by Miclean *et al.* (2012), where determined phthalates esters were DBP and DEHP.

Given the lack of knowledge regarding the level of phtalates in dairy products produced in Romania and the importance of this subject, our aim was to investigate a particular milk production chain and to evaluate the extent of this chemical hazard.

RESULTS AND DISCUSSION

The levels of phthalates detected in milk samples are shown in Table 1. These were calculated as the average means of concentrations obtained for the same analyzed milk samples. Only 4 out of the six phthalates taken into study were present in the samples.

In the case of commercial milk, the highest concentration of phthalates (DOP), respectively 0.3152±0.2441 mg/kg, was found in a milk sample with 3.5% fat content and the lowest, of 0.0201±0.0349 mg/kg (DBP) was determined in the case of milk samples with 1.5% fat content (Table 1).

Based on statistical analyses, significant differences regarding the level of phthalates were calculated only in the case of raw milk 4% fat content, when compared BBP and DEHP ($p=0.008$) (Table 1). Higher total

phthalates levels were observed in the case of raw milk with 4%, followed by pasteurized milk with 3.5%, raw milk with 3.5%, and pasteurized milk with 1.5% fat content. Significant differences were noticed only when compared raw milk with 4% versus pasteurized milk with 1.5% (p=0.01). Thus, between the level of total phthalates and fat content there is direct correlation (Table 1).

Table 1. Phthalate esters level (mean ±SD, mg/kg) in raw and pasteurized milk samples (n=5)

Milk samples	BBP	DEHP	DOP	DBP	DMP	DEP	Total phthalates
Control sample	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Raw milk 3.5%	0.104± 0.018	0.1454±0 1057	0.1005± 0.0222	0.497± 0.06	<LOD	<LOD	0.3996± 0.085
Raw milk 4%	0.1115± 0.0155	0.1685±0 0773*	0.2172± 0.2284	0.0573± 0.0632	<LOD	<LOD	0.5678± 0.2687
Pasteurized milk 1.5%	0.0352± 0.0601	0.1277±0 0485	0.099± 0.0992	0.0201± 0.0349	<LOD	<LOD	0.2818± 0.1756
Pasteurized milk 3.5%	0.0794± 0.0701	0.1075±0 0605	0.3152± 0.2441	0.0224± 0.0388	<LOD	<LOD	0.5245± 0.3301

* Significant differences between BBP, DEHP, DOP and DBP ($p<0.05$)

For milk products, analyses were made from the following ingredients: sour cream, yogurt, different types of cheese (fresh, salted, ripened, cream), cream, ice cream and butter. The results of phthalates from the investigated milk products are presented in Table 2.

In the case of sour cream, the highest concentration of phthalates (DMP) respectively 0.0831±0.0214 mg/kg, was found in a sample with 20% fat. By comparison of the values obtained for sour cream samples, significant differences were observed for BBP (p=0.035) and DOP (p=0.0089). In the yogurt samples, the only significant difference was observed by comparing the total amount of phthalates (p=0.001) (Table 2).

For fresh fat cheese, based on statistical analyses, significant differences regarding the level of phthalates were calculated when compared BBP and DEHP (p=0.002), BBP and DOP (p=0.00074), respectively BBP and DBP (p=0.006). Higher total phthalates levels were observed in the case of maturated cheese (0.5905±0.0175 mg/kg), followed by burduf cheese (kneaded cheese), cream cheese, fresh fat cheese and telemea cheese. When comparing telemea cheese with cream cheese and burduf cheese, significant differences were noticed only for total phthalates (p=0.00001) (Table 2).

Following analyses, the lowest quantity of total phthalates was detected in yogurt with 0.1% fat - 0.042±0.0052 mg/kg, and the highest concentration was recorded in butter with 85% fat - 0.683±0.0072 mg/kg (Table 2).

Table 2. Phthalate esters level (mean ±SD, mg/kg) in different dairy products (n=5)

Dairy product	BBP	DEHP	DOP	DBP	DMP	DEP	Total phthalates
Sour cream 20%	0.0315±0.0027	0.0672±.0007*	0.062±0.0031*	0.0831±0.0214*	<LOD	<LOD	0.244±0.0225
Sour cream 33%	0.0541±0.0122	0.0729±.0058	0.0224±.0139*	0.0819±0.0177	<LOD	<LOD	0.231±0.0251
Yogurt 0.1%	<LOD	0.0185±.0038	<LOD	0.0237±0.0015	<LOD	<LOD	0.042±0.0052
Yogurt 2%	0.0135±0.0042	0.0180±.0062	0.0279±.0137	0.0431±0.0154*	<LOD	<LOD	0.102±0.012
“Telemea” cheese	0.0224±0.0048	0.0052±.0001*	0.034±0.0062	0.0568±0.0044*	<LOD	<LOD	0.118±0.0031
Cream cheese	0.0873±0.0147	0.1106±.0123	0.1620±.0147*	0.1164±0.0105*	<LOD	<LOD	0.4764±0.0228
Maturated cheese	0.1301±0.0122	0.1324±.0043	0.1707±.0215*	0.1573±0.0225	<LOD	<LOD	0.5905±0.0175
Fresh fat cheese	0.0642±0.0068	0.1079±.0088*	0.1322±.0101*	0.1407±0.024*	<LOD	<LOD	0.4451±0.029
Burduf cheese	0.0887±0.0225	0.1562±.0179*	0.1446±.0106*	0.1216±0.0049	<LOD	<LOD	0.5112±0.0216
Raw cream	0.1399±0.0127	0.2321±.0278*	0.1796±.023	0.1173±0.021	<LOD	<LOD	0.669±0.0426
Ice cream	<LOD	0.0431±.0096	0.0713±.0065*	0.0611±0.013	<LOD	<LOD	0.1755±0.0099
Butter	0.1455±0.0174	0.1854±.0182	0.2122±.0147*	0.139±0.023	<LOD	<LOD	0.683±0.0072

* Significant differences between BBP, DEHP, DOP and DBP ($p<0.05$) for each dairy product analysed. Among the six phthalates examined, DEHP was the most commonly detected phthalate compound, followed by DOP, BBP and DBP. Two phthalates, DMP and DEP, were not detected in any sample.

For the last three decades, various researchers have reported phthalate levels in milk and dairy products. Fierens *et al.* (2013), for example, mentioned in their review more studies conducted between 1985 and 2012, to investigate the presence of phthalates in milk and dairy products. Other researchers also continued analyzing phthalates in these types of food [2, 3, 10, 16, 19, 33].

In most of the studies, phthalate levels were determined in retail milk and dairy products while only a few surveys reported phthalate concentrations in raw milk and/or in samples collected from dairy factories. However, in

order to reduce the risk of phthalate contamination of milk or dairy products, it is important to know how these compounds can enter into milk during the technological stages of milk processing. Therefore, phthalates should not only be investigated at the retail level, but also at other stages of milk processing. [13].

Studies also demonstrated that milk and dairy products are contaminated with phthalates (especially DEHP) during collection due to the use of flexible milking tubes [5, 12, 27].

In a subsequent study, Fierens *et al.* (2013), evaluated how the quantity of phthalates increases with the milk processing and packaging. The authors revealed that during pasteurization, the DEHP content in milk increased from 364 μg/kg^{-1} fat (mean level) in raw milk to 426 μg/kg^{-1} fat and the reason of this increase was most likely due to DEHP containing food contact materials (tubings and sealants). The DEHP migration might have been facilitated by increasing temperature during pasteurization. After packaging, the level further increased to 630 μg/kg^{-1} fat in cans and to 523 μg/kg^{-1} fat in plastic pouches. DBP was detected only at the point just before packaging (32 μg/kg^{-1} fat) and after packaging the concentrations increased to 52 and 60 μg/kg^{-1} fat, respectively, when packaged in cans and in pouches. BBP was detected only in milk after packaging at 12 μg/kg^{-1} fat in cans and 53 μg/kg^{-1} fat in pouches. The possible sources of the contamination were labelled as mechanical milking process and intake of the feed by the cattle [14].

In a comprehensive study conducted by Wendi *et al.* (2009) in 3 European countries (UK, Norway and Spain), DEHP and total phthalate esters were determined in milk obtained during different stages of collection, transportation and packaging and also cream, butter and cheese samples. The author reported that after processing the milk contaminated with DEHP, the dairy products obtained lead to high concentrations of DEHP, while low-fat milk while maintaining lower concentrations of phthalates. The same authors added that the total levels of contamination in the raw milk were between 0.12 – 0.28 mg/kg. They suggested that on processing the DEHP phthalate is concentrated in the cream at levels up to 1.93 mg/ kg, whereas low fat milk contained from 0.01 to 0.07 mg/kg. In the cheese samples collected from the UK, the maximum allowed limit for DEHP was exceeded, obtaining 17 mg/kg, respectively for total phthalates - 114 mg/kg [24, 32].

Also, our results are in accordance with those published by Miclean *et al.* (2012), with the concentrations of DBP and DEHP in the range of 2.85-6.28 ng/g and 36.84-112.3 ng/g.

According to the study conducted by MeeKyung *et al*., 15 out of 30 raw bovine milk samples monitored in their study contained DEHP, the concentrations in raw milk ranging from non-detectable under the LOD level

to 154 μg/kg^{-1}, and the mean concentration was 57 μg/kg^{-1}. DBP was observed at concentration from non-detectable to 99 μg/kg^{-1} in twenty samples and the mean concentration was 30 μg/kg^{-1} [36].

J. Lin *et al.* (2015), conducted a study to determinate the concentrations of phthalates from commercial whole milk products packaged in plastic materials, metal or glass containers. The concentrations of phthalates (DEHP) in milk samples packaged in plastic materials (79.3±2.6 ng/g) were much higher than those in metal (8.8±0.7 ng/g) or glass containers (6.6±0.5ng/g). The amounts of phthalates were much higher in milk samples packaged in plastic containers compared with glass or metal containers, indicating that plastic packaging materials are the likeliest source of phthalate contamination in commercial whole milk products. These findings are in accordance with our results, because in the case of samples collected directly from the cattle, in glass bottles, the phthalates were not detected. In contrast, all milk and dairy product samples that came into contact with plastic packaging recorded different levels of phthalates. The phthalates are more soluble in fat, higher level of these residues is usually recorded in the case of products rich in fat, like ripened cheeses and butter [18, 21, 30].

The differences between phthalates levels in dairy products, could be represented by different types of packaging materials, with different levels of PAEs in the plastic material. Even if the Commission Regulation No. 10/2011, the maximum accepted concentration (MAC) of phthalates were established, ranged from 0.3 mg/kg in the case of DBP to 30 mg/kg for BBP, the manufactures of packaging materials must follow those rules. Also, the following phthalates, DEHP, DBP and BBP are not allowed to be used in the manufacture of packaging materials for food products containing fats [6,34].

Recent studies observed that phthalates were found in all environmental (air, water, soil and sediment) at different concentrations in many countries which reflect the widespread usage of plastic products [24].

CONCLUSIONS

Even the total phthalates levels in all the samples analyzed do not exceed the maximum limit (60 mg/kg), rich fat milk and dairy products could be considered as a high-risk food category, being a potential source of human exposure to phthalates. Analyses should be performed at a more extent level to accurately evaluate the extent of this risk for human health.

The protocol used in this research for the detection of the phthalates can be applied for further implementation in food agencies laboratories for a more thorough investigation. Promoting the substitution of phthalates with other non-toxic substances could be a way to reduce the risk of food contamination with these types of compounds that are dangerous to human health.

EXPERIMENTAL SECTION

Sample collection

For this study, samples of milk and dairy products were collected randomly, at several stages in the milk chain, from farm, milk collection center and retail level. From each dairy product, seven samples were collected.

Milk samples were collected from several specific sources: milk obtained by manual milking, milk obtained by mechanically milking - collected from the bulk tank and commercial milk.

The control milk sample was collected from a dairy farm in Maramures County, by manual milking into a glass container, avoiding any contact with plastic materials. This milk was not pasteurized before analysis.

The milk samples collected from farm and milk collection center were packed in plastic (polyethylene terephthalate, PET) bottles. For each milk sample, the fat content was also determined, using standard Gerber method. The samples collected represent raw milk with 3.5% fat and 4% fat, respectively. The shelf life of milk is 10 days after packaging and the milk was used within this period.

To investigate phthalate contamination at retail level, samples of milk with different quantity of fat were purchased from supermarket, with 1.5% fat and 3.5% fat. All milk samples (1.5% fat and 3.5% fat), were packed in plastic cans and were from the same brand.

The dairy products were bought from supermarket and were represented by sour cream with 20% fat and 33% fat, yoghurt with 0.1% and 2% fat content, butter with 75% fat, raw cream and ice cream, all with varied validity period. All samples were packed in various plastic containers.

In order to investigate phthalates in cheese samples, 5 different variety were collected, depending on the amount of fat contained, the shelf life and the production method. The types of cheese collected were fresh cheese with 25% fat, telemea cheese (fresh salted cheese maturated in brine) with 50% fat content, maturated cheese with 45% fat, cream cheese with 60% fat in dry matter and burduf cheese. Burduf cheese is a traditional Romanian cheese made from sheep's milk. The name burduf cheese means kneaded cheese, referring to the method of production in which a traditional sweet cheese called *caș* is cut, salted, and then kneaded in a large wooden bowl. The mixture is then placed in a sheep's stomach or skin that has been cleaned and sawed on the edges. Alternatively, it can be stored in a tube made of pine bark. Burduf cheese is a salty and fermented cheese, quite compact but pasty, with 45% FDM (fat in dry matter).

All samples were transported to the laboratory in a cool box and were stored at −18 °C prior to analysis.

Reagents and standards

The standard solutions of individual phthalates consisted of di-n-butyl phthalate (DBP), di-2-ethylhexyl phthalate (DEHP), di-n-octyl phthalate (DOP), benzyl butyl phthalate (BBP), dimethyl-phthalate (DMP) and diethyl-phthalate (DEP), all dissolved in methanol, each at a concentration of 1 $mg \cdot L^{-1}$ were purchased as Pestanal®, analytical standards from Merk (Darmstadt, Germany).

All reagents and water used for the analyses were checked for contamination with phthalates. The solvents used had an analytical purity (suitable for GC) > 99 %.

To avoid phthalate contamination, all the employed laboratory dishes were made of glass, previously washed with water, rinsing with hexane and dried at 60 °C for 2 hours.

Milk samples liquid-liquid extraction

For phthalates analysis from milk samples, 50 mL of sample was placed in separatory funnel with 15 mL methanol – n-hexane solvent (1:2 v/v) and subjected for shake (LaboShake, Gerhardt Analytical System, Germany) for 30 min at 130 rpm. Phase equilibration was allowed for 10 min. Emulsion was removed with 2.5 mL salt solution (15 % NaCl). The extraction was repeated once again in the same manner and the obtained supernatant was combined with the previously obtained. Na_2SO_4 was added at this extract to remove potential remained water. In finally, the extract was transferred at an Erlenmeyer flask and subjected for fully evaporation at 40°C with a rotary evaporator (Laborota 4010, Heidolph Instruments, Germany). Finally, samples were redissolved in 1 mL n-hexane and analyzed by GC-MS.

Dairy products ultrasound assisted extraction

In case of each studied dairy products 15 g of samples was weight in a 150 mL volume Erlenmeyer flask. Extraction solvent was a mixture of 30 mL methanol – n-hexane (1:2 v/v). In the following ultrasound assisted extraction was performed using a Sonorex ultrasound bath (Bandelin, Germany). The extraction was allowed for 1 h at 35 kHz ultrasonic frequency. Resulted extract was filtered on Whatman filter paper and cleaned on a high purity grade silica column with average pore size 60 A (52-73A) and 70-230

mesh. Resulted extract was fully evaporated with a rotary evaporator. After this procedure, the samples were redissolved in 1 mL n-hexane and analyzed by GC-MS.

GC-MS analysis of phthalates

Phthalate analysis was performed on gas chromatograph with mass spectrometer system (6890 series Agilent GC system, 5975 series Agilent MS detector). From each extract 1 µL was injected in SSL injector used in splitless mode. The mass spectrometer was operated at the electron impact mode with 70 eV. Phthalates were separated on a HP-5MS, 5 % diphenyl 95 % dimethyl polysiloxane capillary column (Agilent Technologies) with the following characteristics: 30 m x 0.25 mm i.d. x 0.25 µm film thickness. The compounds were separated using the following oven program: 100ºC, increased at 8ºC/min up to 260ºC, increased at 35ºC/min up to 310ºC and held for 10 min and the running time being 31.43 min. The MSD transfer line heater, ion source and quadrupole analyzer temperatures were set at 320, 230 and 150 ºC, respectively.

The qualitative and quantitative analyses were performed by comparison with the external standards. Full scan mode with the mass/charge ratio ranging from 100 to 550 m/z was applied. In Figure 1 is presented a TIC (Total Ion Chromatogram) of a blank sample and a cheese sample.

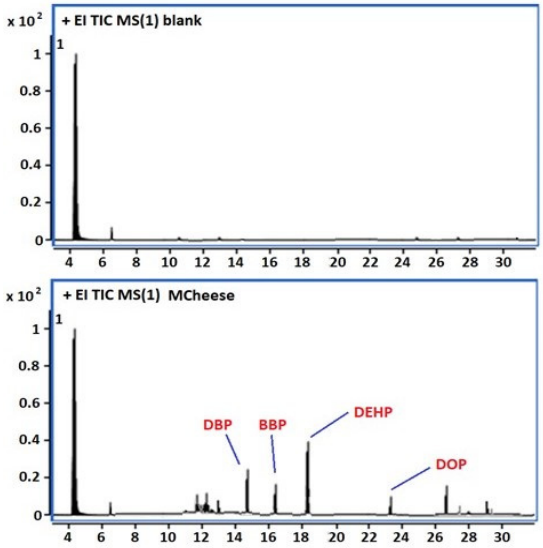

Figure 1. GC-MS analysis of phthalates from a blank and a cheese sample

Methods performance

Efficiency of applied analytical method was evaluated considering parameters as linearity, limit of detection (LOD), limit of quantitation (LOQ), and recovery. Stock solutions of individual phthalate standards (10 mg of each phthalate in part as BBP, DEHP, DOP, DBP, DMP and DEP) were weighted and dissolved in 10 mL of methanol. Working standard mixture was obtained after combining the six stock solutions and dilution until to reach 1 $mg \cdot L^{-1}$. Series of five calibration standards within range of 0.01 – 500 $\mu g \cdot g^{-1}$ were obtained after serial dilution of working standard solution. Obtained linear regression curves were used for quantification of phthalates. LOD and LOQ were determined based on standard error of the calibration curve at y-intercept multiplied three and ten times, respectively (see Table 3).

Table 3. Calibration curve correlation coefficient, LOD and LOQ of applied analytical method

Parameter	BBP	DEHP	DOP	DBP	DMP	DEP
R^2	0.9925	0.9954	0.9961	0.9938	0.9971	0.9962
LOD ($\mu g \cdot g^{-1}$)	0.0052	0.0034	0.0061	0.0042	0.0035	0.0029
LOQ ($\mu g \cdot g^{-1}$)	0.0173	0.0113	0.0203	0.014	0.0117	0.0097

Method recovery was established by spiking each sample matrix with 100 $\mu g \cdot g^{-1}$, respectively. Obtained values for each phthalate are presented in Table 4.

Table 4. Method recovery (%) for milk and dairy products matrices

Sample matrix	BBP	DEHP	DOP	DBP	DMP	DEP
Milk	75.9	85.5	74.8	105.5	88.6	84.5
Sour cream	88.2	95.6	86.9	115.3	76.9	90.5
Yogurt	87.2	75.9	84.6	91.2	77.7	103.5
Cheese	91.8	95.6	102.5	93.5	84.4	95.6
Butter	77.6	84.2	68.9	105.2	102.3	84.6

Statistics

The statistical analyses were realized with Origin 8.5 software (OriginLab Corporation, Northampton, MA 01060, USA). Mean differences between dairy products were analyzed using analysis of variance ANOVA.

The results were expressed according to the standard deviation (SD), with significance level established at $P < 0.05$. Post-hoc test comparation using Bonferoni, Tukey's and Scheffe's was performed.

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