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Development of a headspace solid-phase microextraction method combined with gas chromatography mass spectrometry for the determination of phthalate esters in cow milk

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Abstract

Phthalate esters released from plasticized polyvinyl chloride (PVC) tubing into raw cow milk during milking at dairy farms could be a potential source of contamination by phthalate esters in dairy products. A method was developed for the determination of these phthalate esters in raw cow milk samples using a headspace solid-phase microextraction (HS-SPME) technique. The milk samples were mixed with sodium chloride and extracted for 60 min at 90 °C. The phthalates collected on the SPME fibre were then desorbed in the GC injection port followed by GC-MS analysis in single ion monitoring (SIM) mode. The extraction efficiency of the SPME fibre was dependent on the fat content in the milk sample. Since the fat content in each cow milk sample was different, a combined standard addition and internal standard method was used for the quantification of the phthalate esters in milk samples. The recoveries at two spiking levels were over 90% except for dimethyl phthalate. The method detection limit for di-(2-ethylhexyl) phthalate (DEHP) was from 0.31 to 3.3 ng g^{-1} for samples containing up to 10.8% fat. This method was sufficiently sensitive to detect di-(2-ethylhexyl) phthalate, dibutyl phthalate (DBP) and diethyl phthalate (DEP) in two groups of raw cow milk samples (cow milk samples collected using and without using PVC tubing). While similar levels were found in both types of samples for DBP and DEP, the level of DEHP was much higher in samples collected using PVC tubing (215.36 ng g^{-1}) than once without (16.04 ng g^{-1}), indicating potential leaching of DEHP from PVC tubing into raw cow milk.

Keywords: Cow milk; di-(2-Ethylhexyl) phthalate; Fat content; Headspace solid-phase microextraction; Phthalate esters; Polyvinyl chloride tubing

1. Introduction

Phthalate esters are widely used as additives in the manufacturing of polyvinyl chloride (PVC) plastics to make them flexible. Extensive use of these chemicals resulted in their presence in various environmental matrices such as water, soil and food, including milk and other dairy products [1]. Phthalate esters have endocrine disrupting properties [2]. Studies have shown that phthalate esters are estrogenic [3,4] and have reproductive effects in mammals [5,6]. Because of the potential health impact on humans, the European Commission, for example, is proposing a ban on the use of phthalate esters in soft PVC materials for making baby toys [7].

Among all phthalate esters, di-(2-ethylhexyl) phthalate (DEHP) is the most commonly used plasticizer worldwide. DEHP is released to the environment through volatilization and leaching from plastics and other sources. Its widespread usage coupled with its persistence in the environment result in its ubiquitous presence in the environment and in biota including humans [1]. DEHP levels in Danish retail whole milk samples were found at up to 0.14 mg kg^{-1} [8]. A surveillance work on phthalate esters in Canadian dairy products and margarine has shown that the levels of DEHP and butylbenzyl phthalate (BBP) were up to 11.9 and 47.8 mg kg^{-1}, respectively [9]. Although Government of Canada does not

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have an permissible level of phthalates in milk or dairy products, it has recommended a tolerable daily intake (TDI) of 44 μg kg (body weight)$^{-1}$ day^{-1} for DEHP, in an assessment report in 1994 where the exposure of Canadians to DEHP from all sources was estimated at 5–19 μg kg (body weight)$^{-1}$ day^{-1} depending on their age [10].

Several analytical methods have been developed for determining phthalate esters in food samples [11,12]. The most widely used approaches in sample preparation were liquid–liquid solvent extraction (LLE) [11] and solid-phase extraction (SPE) [12]. One of the drawbacks of using solvent in the sample preparation is that residues of phthalate esters, especially DEHP, are also found in solvents. Furthermore, fat in dairy product samples such as milk could be co-extracted and require extra steps to remove them prior to phthalate analysis by instrument analysis [13].

Solid-phase microextraction (SPME) was introduced by Pawliszyn [14] and Pawliszyn and Arthur [15] in the early 1990's. In SPME, the extraction of target analytes from sample matrix to the fibre is conducted either directly, with the coated fibre directly immersed in the liquid sample (direct SPME), or in the headspace (HS-SPME), where the extracting fibre is suspended above the sample. Although suitable for the analysis of phthalate esters in water [16–19], sediments and sludge [20], the direct SPME method cannot be applied to complex matrices such as milk and other dairy products which often contain fat and other biological components.

The advantage of HS approach is the elimination of co-extraction of undissolved particles and non-volatile components in the sample matrix. Therefore, HS-SPME could be a potentially useful tool to selectively extract the phthalate esters, as the fibre is not directly submerged in the matrix. However, thus far, HS-SPME has only been tested in the determination of phthalate esters in water [21]. The objectives of this study were: (1) to develop a HS-SPME based method to eliminate the fat removing procedure in the cow milk sample preparation prior to phthalate esters analysis with GC-MS (single ion monitoring, SIM) and (2) to apply this method to measure the levels of phthalate ester in milk samples collected at a dairy farm.

2. Experimental

2.1. Chemicals and materials

A standard stock solution containing six phthalate esters, dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), butylbenzyl phthalate (BBP), di-(2-ethylhexyl) phthalate (DEHP) and di-*n*-octyl phthalate (DOP), in hexane at a level of 2000 ng μL^{-1} per compound was purchased from Supelco (Bellefonte, PA, USA). DBP-d_4 and DEHP-d_4 (100 ng μL^{-1}) in nonane, used as internal standards in this study, were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Dichloromethane (DCM) (GC Resolv Grade) was from Fisher Scientific (NJ, USA). Two secondary stock solutions (20 and 200 ng μL^{-1}) and calibration standard solutions (0.1, 0.4, 1, 5 and 10 ng μL^{-1}) were prepared by serial dilution in DCM using volumetric flasks. DBP-d_4 and DEPH-d_4 solution (20 ng μL^{-1}) was prepared in the similar manner. Calibration standard solutions were spiked with DBP-d_4 and DEHP-d_4 at a concentration level of 1 ng μL^{-1}.

SPME fibre holder and 10 different types of SPME fibres (see Table 1) were obtained from Supelco. Fifteen millilitres SPME clear vials with PTFE/Silicone lined screw top cap were also from Supelco. A VWR 575 Digital Hotplate and stirrers were from VWR International Ltd. (Mississauga, Ont., Canada). Vacuum pump fluid used for the heating bath was from Inland Vacuum Industries, Inc. (Churchville, NY, USA).

2.2. SPME sampling procedure

Five grams of cow milk was weighed into a 15 mL SPME vial using a pipette. A magnetic stirring bar and 2.5 g of sodium chloride were added into the vial afterwards and the vial was then closed with the vial cap. The vial was tightly closed to avoid possible leakage of gas when the vial was heated. Then the vial was placed into a preheated oil bath (90 °C) on a hot plate. The stirring speed was adjusted to make the solution well stirred. After 2 min, the SPME needle then was punched through the cap into the headspace of the vial and the fibre was pushed out from the protection needle to start the sampling in the headspace. The SPME holder should be placed at a height that could result in the tip of the inserted fibre to be suspended about 1.5 cm above the milk sample. After the sampling was finished, the fibre was retracted into the protection needle. The needle was then removed from the sampling vial and inserted into a clean vial to protect the fibre from exposing to laboratory air. The needle was then immediately inserted into the GC injection port for GC/-MS analysis.

2.3. Instrument analysis

A Hewlett Packard gas chromatograph (GC, HP 5890 series) coupled with a mass selective detector (MS, HP 5972 series) (Agilent Technologies, Palo Alto, CA, USA) was used for the analysis. The injection port was fitted with a SPME inlet guide (Supelco, Cat. No. 57356-U) and a pre-drilled Thermogreen LB-2 septa (Supelco, Cat. No. 23168) to secure the SPME fibre holder (Supelco, Cat. No. 57330-U). A special inlet liner for SPME used in this study was also from Supelco (Cat. No. 26375,05). The desorption of SPME fibre in the GC injection port was 10 min at 280 °C with purge gas off. After sample desorption, the fibre was further heated in the injection port for an additional 30 min at 280 °C with purge gas turned on to remove trace residues in the fibre while desorbed phthalate esters were being analyzed, before the next extraction. The fibre was then retracted into the pro-

Table 1
Comparison of relative amount (%) of phthalate esters collected on different SPME fibres from 3.25% Homo-milk spiked with phthalate esters

Phthalate ester vapour pressure (mmHg, 25 °C)	Type	DMP 3.07×10^{-3}[a]	DEP 4.88×10^{-4}[a]	DBP 1.09×10^{-5}[a]	BBP 6.0×10^{-7}[b]	DEHP 2.47×10^{-8}[a]	DOP 7.24×10^{-8}[a]
SPME fibre		Relative amount on fibre[c] (%)					
PDMS-100 μm	Absorbent	4.5	16.2	100	100	100	100
PDMS-30 μm	Absorbent	8.4	13.1	7.8	4.4	7.7	4.2
PDMS-7 μm	Absorbent	9.9	16.7	5.1	0.5	0.7	0.3
PDMS/DVB-65 μm	Absorbent	31.6	18.9	4.0	0.3	1.4	0.9
CW/DVB-65 μm	Absorbent	77.6	54.0	10.8	4.3	1.4	0.5
CAR/PDMS-85 μm	Absorbent	3.7	3.5	2.9	0.5	2.8	0.7
DVB/CAR/PDMS-50/30 μm	Absorbent	48.2	100	36.4	2.5	17.3	3.2
Polyacrylate-85 μm	Absorbent	31.0	46.5	18.3	5.6	11.1	15.7
PDMSDVB-StablFlex-65 μm	Adsorbent	100	59.5	41.6	18.8	25.7	24.9
CW/DVB-StableFlex-70 μm	Adsorbent	37.7	53.2	23.1	5.7	33.5	19.2

[a] Values from [22].
[b] Values from [23].
[c] The relative amount was termed as percentage of the highest amount of the same compound in each column.

tection needle and the needle was inserted into a clean vial, ready for the next sample extraction.

Analytes were separated on a 30 m × 0.25 mm i.d. × 0.25 μm film thickness DB-5 gas chromatographic column (J&W Scientific, Folsom, CA, USA) with the following oven temperature program: the initial temperature was set at 55 °C for 1 min, increased to 280 °C at 15 °C per minute and kept at 280 °C for 15 min.

Selected ion monitoring mode was used for MS operation. Three ions (the first one being the target ion (T) and rest two the qualifying ions (Q1 and Q2)) were selected for each target compound. They were: *m*/*z* 163, 77 and 194 for DMP; *m*/*z* 149, 177 and 104 for DEP; *m*/*z* 149, 223 and 104 for DBP; *m*/*z* 149, 91 and 206 for BBP; *m*/*z* 149, 167 and 279 for DEHP; and *m*/*z* 149, 279 and 104 for DOP. One ion (*m*/*z* 153) was used for the quantification of both DBP-d_4 and DEHP-d_4. All peak area counts of the chromatograms were normalized against the internal standard DBP-d_4 (for DMP, DEP and DBP, respectively) and DEHP-d_4 (for BBP, DEHP and DOP, respectively) before data reduction. Each peak was manually checked by a qualified chemist for the identity and proper integration. However, a peak must have its retention time was within 1% and at least one of its two ion ratios (Q1/T or Q2/T) within ±30% of those of the associated standards to be considered positive.

3. Results and discussion

Phthalate esters are semi-volatile organic chemicals with vapour pressure ranging from 3.07×10^{-3} mmHg for DMP to 2.47×10^{-8} mmHg for DEHP (Table 1) [22,23]. The low vapour pressure means that the concentration of phthalate esters in the headspace of SPME vials would be very low. On the other hand, direct SPME would not be appropriate due to the presence of fat and other biological components in the milk sample matrix. The development of the HS-SPME method was therefore focused on finding a proper extraction fibre and optimizing the extraction conditions such as extraction time and temperature.

3.1. Choice of fibre

Commercially available SPME fibres for SPME can be divided into absorbent-type and adsorbent-type. Absorption type coating is a liquid film, which cross-linked to the silica rod, while adsorbent type fibres are solid particles with pores on the surface. The extraction of absorbent-type fibres is based on partitioning of analytes into a 'liquid-like' phase. In the case of adsorbent type fibres the particle surface interacts physically with the analytes [24]. A total of 10 commercially available fibres with different coating materials and coating thickness (Table 1) were evaluated for their applicability to extract phthalate esters from milk samples. A commercial Homo-milk (3.25% fat) (spiked with 2 μg of each phthalate ester in 5 g milk) was used in the experiment. Table 1 summarizes the relative amount of phthalate esters collected on each fibre (normalized to the highest extracted amount among all phthalate esters) under the sample extraction condition of 90 °C for 60 min (see Section 3.2). Comparison of fibres for their extraction efficiency was also conducted at 50 °C. However, the overall amount of phthalate esters being collected was too low to be used for the purpose of selecting fibres, compared to the results obtained at 90 °C. For DMP and DEP, PDMS/DVB-StablFlex-65 μm and DVB/CAR/PDMS-50/30 μm fibres had the best results. However, for the rest of the phthalate esters listed in Table 1, PDMS-100 μm fibre, a fused silica rod coated with a liquid film of cross-linked polydimethylsiloxane, had the best extraction efficiency. The ability of the coating phase to retain the analyte was also dependent on the coating thickness and the molecular weight of the analytes. It was observed that at the extraction temperature of 90 °C the extraction efficiency of the PDMS coated fibres for phthalate esters, except for DMP and DEP, was drastically reduced when the thickness of the coating was reduced from 100 to 30 μm and 7 μm. Such film thickness

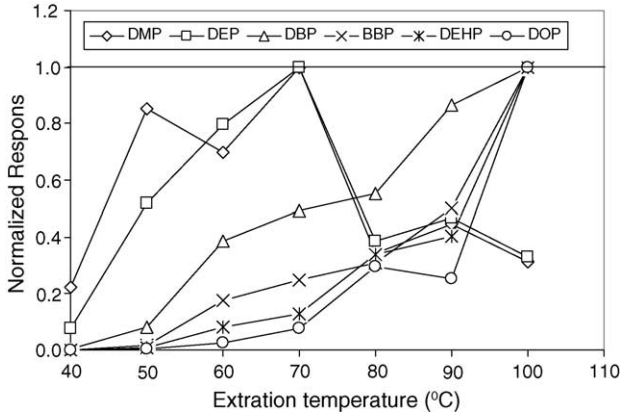

Fig. 1. Effect of extraction temperature on extraction efficiency of PDMS-100 μm fibre. The extraction time was set at 60 min.

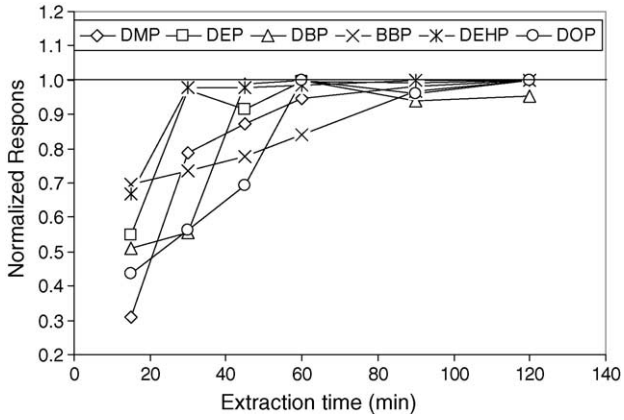

Fig. 2. Effect of extraction time on extraction efficiency of PDMS-100 μm fibre. The extraction temperature was set at 90 °C.

effects on extraction efficiency were however, not evident in the direct SPME of phthalate esters in water which was conducted at 25 °C [19]. Such film thickness dependent extraction efficiency in headspace SPME in the case of higher molecular weight phthalate esters could be attributed to the retaining capacity of the film. The larger the liquid volume is, the more capacity of holding-back the phthalate esters the fibre possesses. For lower molecular weight phthalate esters, the gas–liquid equilibrium dominates the extraction process and therefore the effects of film thickness are not significant.

Although PDMS-100 μm fibre had lower extraction efficiency for DMP and DEP. It was still selected as the extraction fibre for this study since DEHP and DBP are the two major phthalate esters detected in dairy products including milk [9,13]. In addition, DEHP is the most important analyte in this study to achieve the objective of assessing potential migration of DEHP from PVC tubing into milk samples during milking.

3.2. Optimization of HS-SPME extraction and desorption

To compensate for the low vapour pressures of phthalate esters, a high extraction temperature is desirable to accelerate the release of phthalate esters from the milk matrix and increase the concentration of the esters in the headspace. There are two governing equilibriums (gas phase ↔ sample matrix and gas phase ↔ liquid coating of the fibre) in HS-SPME. The high temperature would increase the concentration in the gas phase, however, it also reduces the retaining power of the coating materials, especially for "lighter" esters such as DMP and DEP. Fig. 1 shows the relative extraction efficiency of the PDMS-100 μm fibre in the temperature range of 40 and 100 °C. While the amount of "heavier" phthalate esters being collected on the fibre was proportional to the sample temperature, the extracted amount of DMP and DEP reached a maximum at around 70 °C and then decreased with further increases of sample temperature. The upper bound of the extraction temperature was also limited by the milk matrix as the proteins and other biological components could become clustered if the sample temperature was too high, thereby affecting the effectiveness of the stirring. To balance the extraction efficiency for all targets, a 90 °C sample temperature was selected.

The effects of extraction time on the extraction efficiency of the SPME fibre were illustrated in Fig. 2. The extraction efficiency of PDMS-100 μm fibre reached a plateau within 60 min for most phthalate esters, except for BBP. This time was longer than 40 min required for the direct SPME extraction of phthalate esters in water [19], reflecting the time needed for the analytes to be released from milk matrix into the headspace and diffusion of analytes from headspace into the fibre.

Sodium chloride was added to saturate the milk sample (about 2.5 for 5 g of milk sample) to improve extraction efficiency of the phthalate esters, as addition of salt can result in enhanced ion strength in the sample matrix and in increased vapour pressure in the headspace [21]. Compared to without salt, the extraction efficiency with a salt saturated milk sample was improved for all the phthalate esters as shown Fig. 3. The largest improvement was for DBP (six times).

At the completion of HS-SPME extraction, the fibre was immediately analyzed by GC/MS. The GC desorption tem-

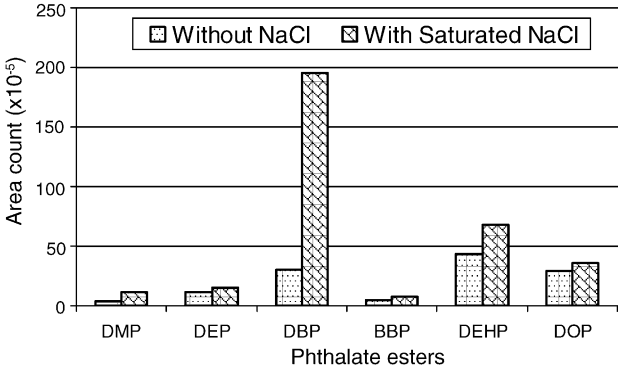

Fig. 3. Effect of salt on the extraction efficiency of PDMS-100 μm fibre. The extraction time and temperature was set at 60 min and 90 °C, respectively.

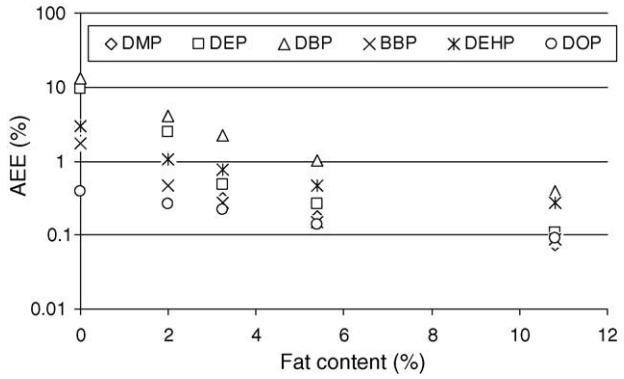

Fig. 4. Effect of fat content on the absolute extraction efficiency (AEE). Conditions same as those in Fig. 3 with saturated NaCl. The power law model was used for best curve fitting. DMP: $y = 2.5478x^{-1.5347}$, $r^2 = 0.9650$; DEP: $y = 5.7975x^{-1.7538}$, $r^2 = 0.9273$; DBP: $y = 11.383x^{-1.4154}$, $r^2 = 0.9988$; BBP: $y = 0.8851x^{-1.0018}$, $r^2 = 0.9926$; DEHP: $y = 1.9393x^{-0.8287}$, $r^2 = 0.9978$; DOP: $y = 0.4306x^{-0.6491}$, $r^2 = 0.9811$.

perature was set at the upper limit (280 °C) of the fibre due to the semi-volatile nature of the phthalate esters. Various desorption times were studied to evaluate the completeness of desorbing phthalate esters from the fibre. The first four "lighter" esters (DMP, DEP, DBP and BBP) could be desorbed with 99% completeness within 5 min. Greater than 99% desorption completeness for all phthalate esters was achieved in 10 min desorption time (99.9% for DMP, 99.8% for DEP, 99.8% for DBP, 99.7% for BBP, 99.1% for DEHP and 99.3% for DOP). The GC purge gas valve was kept closed so that the majority of desorbed analytes could enter the GC column. Once the desorption time of 10 min was reached, the GC purge valve was turned on and the fibre remained in the injection port at 280 °C for an additional 30 min. In this way, the remaining trace residues of the analytes could be cleaned with the large portion (98%) of the desorption stream bypassing the GC/column. The fibre was ready afterwards for the next SPME sample extraction.

3.3. Matrix effects and quantification method

It was observed that the absolute extraction efficiency (AEE) (defined as the percentage of the amount collected on the fibre compared to the amount spiked in the milk sample) was dependent on the fat content in the milk samples. To evaluate the effect of fat concentration, milk samples containing various amount of fat were spiked with 2 μg of each of the phthalate esters. The inverse relationship of AEE and fat content was best described by power law equations when the data from the milk sample containing 0% fat were excluded. The best curve fitting equations for all phthalate esters were listed in the caption of Fig. 4. The AEE of phthalate esters in milk samples was lower than that from direct SPME in water samples [19], especially for samples containing high fat content. For example, the AEE was in the range of 2.9% (0% fat samples) and 0.27% (10.8% fat sample) for DEHP. Due to this low absolute extraction efficiency, it is very important to have the internal standards to correct for the extraction efficiency. In this study, DEHP-d_4 and DBP-d_4 were chosen as internal standards.

Since the AEEs of phthalate esters in the milk samples were dependent on the fat content in the milk, each milk sample therefore has to be considered a unique matrix. To address this matrix effect, the first step was to use the standard addition method to evaluate whether the relative response factor (RRF) was independent of the phthalate concentration in the sample of a given matrix. The RRF of the target analyte to the internal standard can be defined as follows:

$$\mathrm{RRF} = \frac{A_s \times C_{is}}{A_{is} \times C_s} \quad (1)$$

where A_s is the peak area of the analyte, C_{is} the concentration of the internal standard, A_{is} the peak area of the internal standard and C_s is the concentration of the analyte.

The range of standard addition was from 0.01 μg to 0.8 μg g^{-1} of milk sample. It was observed that RRF of an analyte remained the same for a given milk sample and there was little variation among samples with different fat content. An example of such RRFs is given in Table 2, where typical RRFs of DEHP with various fat concentrations in milk samples are summarized. The RRFs of DEHP were constant over the concentration range for each of the sample matrices as evident by the small standard deviation (S.D.) in each sample matrix. Furthermore, the fat content ranged from 2 to 10.8 % has little effects on the RRFs, which was at 1.00 ± 0.05.

Since the RRF remained constant in a given matrix, it is possible to use one-point standard addition (unspiked sample plus one spiking level) to calculate the concentration of the analyte in the sample. Thus for spiked sample, the RRF can be calculated as follows:

$$\mathrm{RRF} = \frac{A_{sp} \times C_{issp}}{A_{issp} \times (C_{sp} + C_s)} \quad (2)$$

where A_{sp} is the peak area of the target analyte in the spiked sample, C_{issp} the concentration of internal standard in the spiked sample, which is equal to C_{is} in the unspiked sample, A_{issp} the peak area of the internal standard in the spiked sample and $(C_{sp} + C_s)$ is the sum of the spiking level (C_{sp}) and the concentration of the target analyte (C_s).

Since the RRF is independent of the concentration in the samples and C_{issp} is the same as C_{is}, Eqs. (1) and (2) can be

Table 2
The response factor of DEHP in different fat content milk

Spiking level (μg g^{-1})	Fat concentration in the milk (%)				
	0	2.0	3.25	5.4	10.8
0.01	1.24	0.99	0.98	1.00	0.97
0.05	1.33	0.99	0.98	0.95	0.99
0.1	1.26	0.97	0.97	0.96	1.04
0.4	1.24	1.03	1.03	1.01	0.96
0.8	1.24	1.02	1.02	1.07	1.08
Mean	1.26	1.01	1.00	1.00	1.01
S.D.	0.04	0.02	0.02	0.04	0.05

Table 3
Method performance

	DMP	DEP	DBP	BBP	DEHP	DOP
Fibreman (pg)	0	0	2.2	0	9	0
S.D. ($n = 27$)	0	0	3.9	0	12	0
$S/N = 3$ (pg)	10	5	–	20	–	20
IDL (pg)	10	5	14	20	45	20
MDL (ng g^{-1})						
0% fat	0.07	0.01	0.02	0.23	0.31	1.0
2.0% fat	0.18	0.04	0.07	0.87	0.84	1.5
3.25% fat	0.63	0.21	0.12	1.5	1.2	1.8
5.4% fat	1.1	0.38	0.27	2.7	1.9	2.9
10.8% fat	2.7	0.91	0.72	4.7	3.3	4.4
Recovery (R.S.D.)						
0.1 μg g^{-1} milk	82 (11)	92 (10)	90 (9)	91 (6)	95 (11)	104 (8)
0.4 μg g^{-1} milk	97 (7)	101 (9)	101 (8)	100 (7)	97 (6)	100 (6)

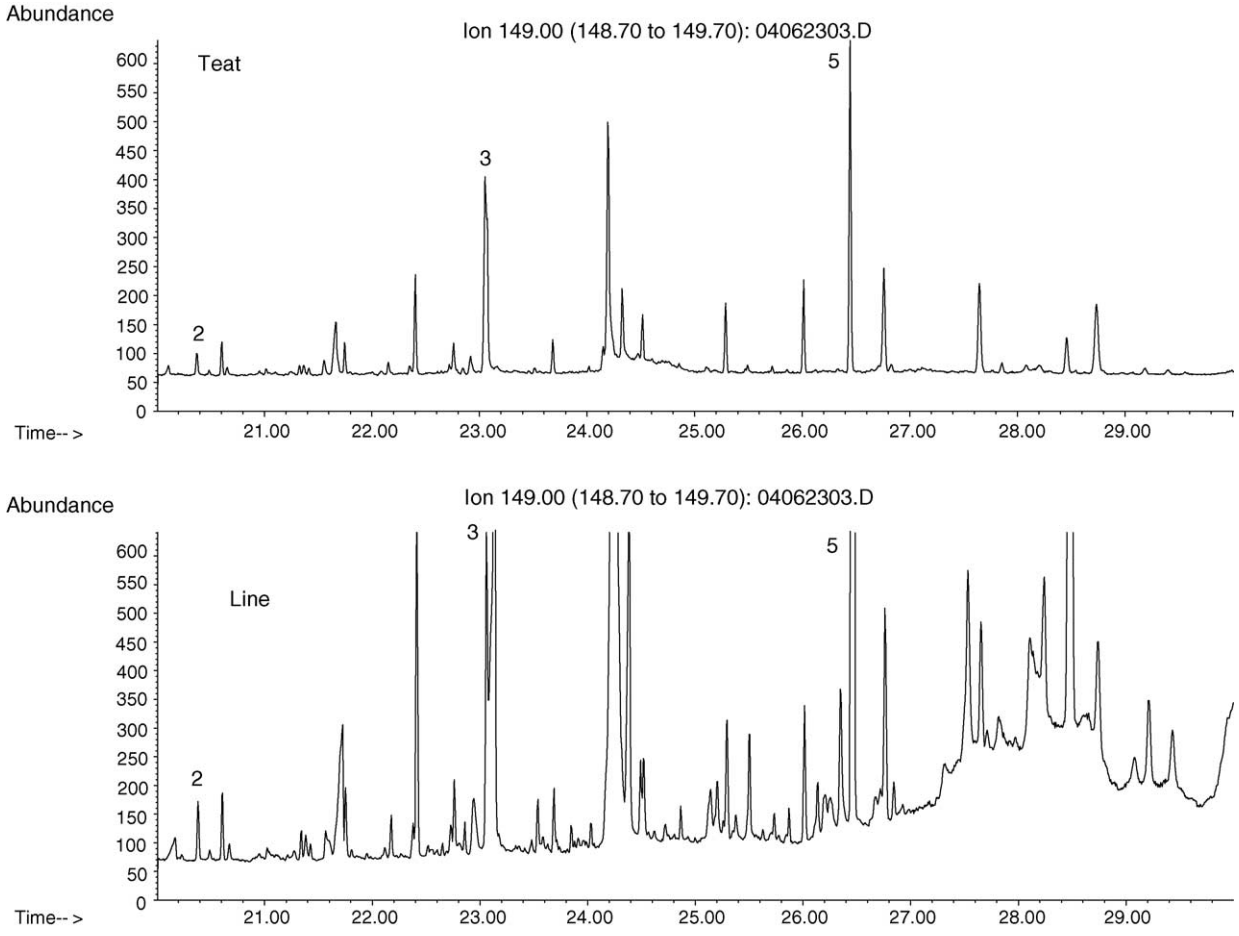

Fig. 5. Selected ion (*m*/*z* 149) chromatograms of cow milk samples (top: teat; bottom: line). Peak identification: (2) DEP (20.38 min), (3) DBP (23.06 min) and (5) DEHP (26.46 min). DMP (19.19 min), BBP (25.50 min) and DOP (27.80 min) were not detected and therefore are not shown in the figure. The same *y*-axis scale of both chromatograms was set for comparison of peak height. Note that the peak height of DEHP (19,000) in the line sample exceeds the scale.

Table 4
Mean concentration (ng g^{-1}) and percentage of difference (in parentheses) of phthalate esters in duplicate cow milk samples

Sample	DMP	DEP	DBP	BBP	DEHP	DOP	% Fat
cow666							
Teat	ND	0.39 (30)	6.12 (18)	ND	13.14 (5.7)	ND	2.93
Line	ND	0.50 (12)	6.23 (13)	ND	208.01 (4.2)	ND	3.05
cow694							
Teat	ND	0.82 (1.1)	9.79 (15)	ND	23.72 (1.5)	ND	4.55
Line	ND	0.65 (16)	9.63 (4.2)	ND	242.39 (2.4)	*ND*	5.38
cow698							
Teat	ND	0.50 (11)	5.06 (18)	ND	14.79 (6.5)	ND	1.36
Line	ND	0.52 (11)	4.35 (0.3)	ND	111.67 (6.9)	ND	1.52
cow557							
Teat	ND	0.71 (15)	7.91 (44)	ND	21.54 (8.7)	ND	5.39
Line	ND	0.86 (4.2)	5.80 (10)	ND	226.25 (8.9)	ND	5.47
cow668							
Teat	ND	0.50 (26)	4.07 (34)	ND	14.64 (12)	ND	3.46
Line	ND	0.58 (28)	4.10 (41)	ND	282.90 (0.4)	ND	4.04
cow616							
Teat	ND	0.66 (5.5)	5.41 (23)	ND	8.40 (30)	ND	3.67
Line	ND	0.66 (16)	4.65 (40)	ND	220.96 (11)	ND	4.63
Mean							
Teat	ND	0.60	6.39	ND	16.04	ND	3.56
Line	ND	0.63	5.79	ND	215.36	ND	4.02

ND = not detected, concentrations below detection limit. Values in parenthesis are in percent.

combined to form Eq. (3) for calculating the concentration of the target analyte (C_s) in the sample.

$$C_s = \frac{A_s \times C_{sp}}{(A_{sp} \times A_{is}/A_{issp} - A_s)} \quad (3)$$

3.4. Detection limit and accuracy

The detection limit (DL) was estimated using two different approaches. For analytes (DMP, DEP, BBP and DOP) that do not have detectable background levels in the SPME fibre, the DL was estimated by the injected amount that resulted in an instrument signal to noise ratio (*S*/*N*) of 3. For analytes (DBP and DEHP) that had detectable background levels, the DL was estimated at three times the standard deviation of the fibre blank plus the mean level in the fibre blanks ($n = 27$) through the study (Table 3). Among all phthalate esters, DEHP had the highest detection limit of 45 pg due to its high background level in the fibre. The relatively high background level of DEHP in the fibre blanks was most likely due to the ubiquitous presence of DEHP in the environment including laboratory air, as DEHP is the most widely used plasticizer present in a variety of soft plastics [25]. The utmost care was taken to minimize the levels in the analytical system including rigorous cleaning of the fibre between samples.

Since the AEE of phthalate esters (Fig. 4) was dependent on the fat content in the milk samples, the method detection limit (MDL) must be calculated by dividing the DL by the AEE. The best curve fitting equations for all the phthalate esters listed in Fig. 4 were used to calculate the AEE at a given fat content, except for 0% fat samples, whose direct measured values were used. For example, the MDL for DEHP varied from 0.31 ng g^{-1} in 0% fat samples to 3.3 ng g^{-1} in 10.8% fat samples. This range of MDL was lower than MDLs from solvent extraction or SPE method, which were reported to be above 10 ng g^{-1} for milk samples [26].

The accuracy for the determination of phthalate esters in milk samples was evaluated at two spiking levels of 500 and 2000 ng in 5 g of the homogenized milk, respectively, with seven replicates for each level. When subtracted by the levels in homogenized milk, the accuracy at both spiking levels was between 90 and 104%, except for DMP at the lower spiking level (82%). The relative standard deviation (R.S.D.) values in this case were around 10% for both spiking levels (Table 3).

3.5. Results from the milk

In the modern dairy farm, milking is done entirely by machine systems employing flexible tubing of various polymers. Concerns of potential migration of DEHP from plasticized PVC milking tubing to raw milk have been raised in the overall strategy of reducing human exposure to DEHP. Using the method described above, milk samples from six cows in a dairy farm were analyzed. Fig. 5 shows the typical selected ion (*m*/*z* 149) chromatograms of the cow milk samples, the top one is a teat sample and the bottom one is the line sample of the same cow. DMP was monitored by the target ion of *m*/*z* 163. Since no DMP was detected in any of the samples, the ion of 163 was not shown in Fig. 5. For each cow, the milk was collected by hand milking (coded as teat sample)

followed by machine milking with plasticized PVC tubing (coded as line sample). All milk samples were collected in duplicate. The duplicate results, expressed in mean value and percentage of difference between the duplicate, indicate the repeatability of the method.

The summary results of phthalate esters in six cow milks are summarized in Table 4. On a per-weight basis, DEHP levels in line samples (111.67–282.90 ng g^{-1}), were 10–20 times higher than the teat samples (8.40–23.72 ng g^{-1}), while the levels of the other two detected phthalate esters (DEP and DBP) were very similar between the two groups of samples. This clearly demonstrated the migration of DEHP from PVC tubing into the raw milk during milk collection. Analysis of the PVC tubing material, which was used for collecting the test milk samples, indicated that the tubing contained 28% by weight of DEHP. DEHP concentrations in commercial milks containing up to 3% fat were reported in the range of 50–130 ng g^{-1}, except for one sample (380 ng g^{-1}) [26]. The levels of DEHP in the teat samples, where the raw milk did not come in contact with PVC tubing, were about 10 times above the detection limit (Table 3).

Besides DEHP, the method was sufficiently sensitive to detect the levels of DBP and DEP in the collected cow milks. DBP was detected in all samples with concentration levels around 4–10 ng g^{-1}, which was at least 10 times above the detection limit listed in Table 3. Levels of DEP in cow milk were found in the range of 0.5–1 ng g^{-1}, which were above the detection limits as well. The other remaining three phthalate esters were not detected in these milk samples.

4. Conclusion

The HS-SPME method described in this paper is sensitive enough to measure the levels of phthalate esters in milk samples directly collected from a dairy farm. The MDLs of this method were in general lower than those previously reported. The method also avoids the solvent-consuming cleaning procedures of the solvent extraction method, which are used to remove fat present in milk samples. A combined standard addition and internal standard approach eliminates the matrix effect primarily attributed to the fat content in milk samples. To the best of our knowledge, this is the first reported analytical method using HS-SPME technique for the measurements of phthalate esters in milk samples.

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