Rapid supercritical fluid chromatography analysis for 18 phthalate esters and bisphenol A in dairy products

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Abstract A rapid and environmental method based on supercritical fluid chromatography (SFC) was established for the simultaneous analysis of 18 phthalate esters (PAEs) and bisphenol A (BPA) in dairy products. The stationary phases, organic modifiers, back pressures and column temperatures were investigated for the optimal separation. The BEH chromatographic column (100 mm x 3.0 mm, 1.7 mm) with sub-2-micron particles was performed for the detection of target compounds. 18 PAEs and BPA can be effectively separated within 5.5 min with acetonitrile as solvent modifier at the optimal conditions. The correlation coefficients ($R^2$) of 18 PAEs and BPA were more than 0.997 in the linear range of 0.3-10.0 mg/L, except for BMPP, DNOP and BPA in the range of 0.6-10.0 mg/L. The limits of quantification (LOQs) for PAEs and BPA were 0.15-0.60 mg/kg. The spiked recoveries and relative standard deviation (RSDs) were in the range of 88.2-120.5% and 0.69-10.06% for powdered milk infant formula samples, 89.6-114.2% and 0.69-10.06% for milk drinks samples, respectively. The SFC method can be used as a simple and efficient alternative route for the detection of 18 PAEs and BPA in dairy products.

1. Introduction

In recent years, human health has seriously suffered from food contaminant, which has become the focus of the government and customers in the whole world. Endocrine disrupting chemicals (EDCs) mainly deriving from food processing or storage have been observed in human and other organisms, which probably affect the subsequent generations [1-3]. Aware of this, specific migration limit (SML) of EDCs in food has been set by European Union and other countries [4,5].

Phthalate esters (PAEs) have been widely used as plasticizers in food contact materials, which are the most common type of EDCs with carcinogenic and reproductive toxicity [6-8]. Bisphenol A (BPA), another representative kind of EDCs which used in the synthesis of polycarbonate plastics, can-coating material and epoxy adhesives, can damage the genital system and immune system through migrating from packaging materials into food chain [9-11]. At present, various analysis techniques have been developed for assessing the levels of these EDCs in food. The most common methods are based on gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS), on account of their good responsibility and stability [12-15]. However, the development of chromatography methods has been limited by long detection time (often no less than 10 min) and high analytical costing, as well as poor environmental conservation [16].

Supercritical fluid chromatography (SFC) is an attractive analytical technique for its remarkable advantages such as faster separation, higher resolution, greater peak capacities, along with less solvent...
consumption compared with frequently-used GC and LC [17,18]. The previous work has been demonstrated that SFC technology could be applied to analyze a wide range of compounds, especially suitable for the high-throughput analysis and routine detection, including vitamins [19], fatty acids [20,21], polycyclic aromatic hydrocarbons [22], bioamines [23], and PAEs [24]. However, little work has been focused on the simultaneous detection of PAEs and BPA using SFC technology so far.

Dairy products are indispensable in daily life, particularly for children, also for most adults. In recent years, several health incidents have been caused by dairy products [25], which were mainly rooted in the migration of contamination from plastics packaging and food containers [26-28]. In the present work, we developed a rapid SFC method in combination with sub-2-micron column to provide a high resolution separation for simultaneously analysis of 18 PAEs and BPA in dairy products. The new analytical method may save analysis time and organic solvent consumption immensely while improving efficiency and reliability.

2. Experimental and Methods

2.1. Materials and Reagents

The standards were bis(4-methyl-2-pentyl) phthalate (BMPP); diisobutyl phthalate (DIBP); diallyl phthalate (DAP); diiso-amyl phthalate (DIPP); dipropyl phthalate (DPRP); di-n-butyl phthalate (DBP); diethyl phthalate (DEP); dimethyl phthalate (DMP); bis(2-ethylhexyl) phthalate (DEHP); di-n-hexyl phthalate (DHXP); di-n-hexyl phthalate (DHP); di-n-octyl phthalate (DNOP); butyl benzyl phthalate (BBP); dinonyl phthalate (DNP); diphenyl phthalate (DPhP); dicyclohexyl phthalate (DCHP); bis(2-n-butoxyethyl) phthalate (DBEP); bis(2-ethoxyethyl) phthalate (DEEP); bis(2-methoxyethyl) phthalate (DMEP); and bisphenol A (BPA). They were all supplied from Dr. Ehrenstorfer (Augsburg, Germany). HPLC grade methanol, isopropanol, acetonitrile, n-hexane, dichloromethane, and water were purchased from Merck (Darmstadt, Germany). Food grade CO₂ (99.99%) was obtained from Tonghui Gas (Chongqing, China). All dairy products samples were obtained from the local supermarkets in Chongqing. The cartridges of Oasis HLB (6 cc/200 mg, glass) were purchased from Waters (Milford, USA).

2.2. Preparation of standards

Different concentrations of standard mixture of 18 PAEs and BPA were prepared for constructed the calibration curves. 25.00 mg of each standard was introduced into a volumetric flask, dissolved and diluted to 25 mL with methanol to obtain a standard mixture of 1000 mg/L. Then the standard mixture was stepwise diluted to 10.0, 5.0, 2.0, 1.0, 0.6 and 0.3 mg/L with methanol for linearity measurements.

2.3. Instruments and chromatographic conditions

The qualitative and quantitative analysis of 18 PAEs and BPA were performed on UPC² system (Waters, Milford, USA) equipped with sampler manager, binary solvent manager, convergence manager, column manager and PDA detector. The separation was carried out with four stationary phase of Acquity UPC² HSS C18 SB, Acquity UPC² CSH F-P, Acquity UPC² BEH 2-EP and Acquity UPC² BEH, all of them at 100 × 3.0 mm with 1.7 µm particles. The mobile phase used in gradient elution was a co-solvent of CO₂ with methanol, isopropanol or acetonitrile. The elution gradient was (eluent A, CO₂; eluent B, acetonitrile): 0-1 min, 3-4.5% B, 1.5-1.2 mL/min; 1-2.5 min, 4.5% B, 1.2-1.0 mL/min; 2.5-3.5 min, 4.5-15% B, 1.0-1.5 mL/min; 3.5-4.5 min, 15-20% B, 1.5 mL/min; 4.5-5.0 min, 20-25% B, 1.5 mL/min; 5.0-5.5 min, 25-3% B, 1.5 mL/min. The column temperature was observed in the range of 50-65°C, and the back pressure was optimized from 1700 to 2000 psi. The injection volume was 5.0 µL, and the PDA detection was performed at 220 nm.

2.4. Method validation

Method linearity was evaluated at different concentration of standards mixture in the range of 0.3-10.0 mg/L (except for BMPP, DNOP and BPA were 0.6-10.0 mg/L), and each point injected in triplicates.
According to the requirements of ICH guidelines [29], the precision was assessed at three concentration levels (0.6, 5.0 and 10.0 mg/kg for powdered milk infant formula samples and milk drinks samples) in seven replicates. The LODs and LOQs were calculated based on 3 and 10 times of signal-to-noise (S/N) ratio, and the resolution was obtained by the peak purity from Empower software.

2.5. Sample preparation
Powdered milk infant formula: Firstly, powdered milk (1.0 g) was reconstituted with HPLC (5 mL) water in a pre-rinsed glass tube. Secondly, NaCl (2.0 g) and acetonitrile (5 mL) were introduced in the tube, and proceeded a ultrasonic treatment for 10 min, centrifuged for 5 min at 5000 r/min, then collected the supernatant (5 mL). The Oasis HLB cartridge was activated with methanol (5 mL) and HPLC water (5 mL) in order to avoid any impurity. After that, the supernatant (5 mL) was placed on the top of cartridge and eluted with dichloromethane/methanol (4:1, v/v) and methanol orderly. Lastly, the extract was dried with N₂ flow at 45℃ and diluted to the final volume of 1.0 mL with methanol for SFC analysis.

Milk drinks: Milk drinks sample (1.0 g) was introduced into a clean glass tube, the subsequent operation was conducted according to the same method as described for powdered milk infant formula.

3. Results and discussion

3.1. Optimization of chromatographic conditions
The stationary phases were optimized primarily to obtain high resolution and reasonability for separating 18 PAEs and BPA in dairy product. Four different chromatographic columns including Acquity UPC² HSS C18 SB with non-polarity, CSH fluoro-phenyl with moderate polarity, BEH 2-EP and BEH with strong polarity were investigated [30]. Since the distinction of the nature of stationary phases, the selection of column had the greatest influence on separation efficiency. The strongest retention capacity of 18 PAEs and BPA was observed on BEH phase (Figure 1a), probably due to its strong polarity [31].

The organic modifiers included methanol, isopropanol and acetonitrile were participated to regulate the adequate solvent strength for compounds. Compared to methanol and isopropanol, acetonitrile was the most befitting modifier on account of better retention, peak symmetries and resolution (Figure 1b). The column temperature was further evaluated from 50-65℃, which had significant effect on selectivity and retention time by changing the density of supercritical mobile phase. The appropriate temperature was chosen as 65℃ (Figure 1c). The back pressure in the present work was varied from 1700-2000 psi to achieve a baseline resolution for PAEs and BPA. The elution strength was increased with the enhancement of pressure due to the density of supercritical fluid increased. As a result, the satisfactory separation was appeared at 1800 psi (Figure 1d).
Figure 1. The influence of (a) stationary phases; (b) organic modifiers; (c) column temperatures; (d) back pressures on separation of UV absorbents

As analysis under the above optimized chromatographic conditions, the standard mixtures of 18 PAEs and BPA could achieve an effective separation within 5.5 min (Figure 2). Symmetrical peak shapes were obtained, and the resolution was exceeded 1.5 for all eluting peaks.

Figure 2. Chromatogram of standard mixture solution of the 18 PAEs and BPA. Peaks numbering: 1. BMPP; 2. DIBP; 3. DAP; 4. DIPP; 5. DPRP; 6. DBP; 7. DMP; 8. DEHP; 9. DHXP; 10. DHP; 11. DNOP; 12. BBP; 13. DNP; 14. DPhP; 15. DCHP; 16. DBEP; 17. DEEP; 18. DMEP; 19. BPA

3.2. Linearity, LODs, and LOQs

The reliability and accuracy of the method performance was investigated via using standards mixture, method blanks and spiked dairy products. The calibration curves for 18 PAEs and BPA were evaluated in the concentration range of 0.3-10.0 mg/L (0.3, 1.0, 2.0, 5.0 and 10.0 mg/L), except for BMPP, DNOP and BPA in the range of 0.6-10.0 mg/L (0.6, 1.0, 2.0, 5.0 and 10.0 mg/L). The method sensitivity was tested according to the S/N ratio and assured by calculation of peak areas via Empower software. The LODs and LOQs were obtained from the concentration corresponding to S/N=3 and S/N=10, respectively. The retention time, correlation coefficients ($R^2$), LODs and LOQs for 18 PAEs and BPA were listed in Table 1. For DAP, DBP, DHP and DNOP, the $R^2$ values were no less than 0.997, and the $R^2$ for the other 14 PAEs and BPA were all more than 0.999. For all the target compounds, the LODs and LOQs were 0.05-0.20 mg/kg and 0.15-0.60 mg/kg in dairy products samples, indicating a good linearity and reliability of the SFC method.

| No. | Compound | Retention time / min | $R^2$ | LODs$^a$ / (mg/kg) | LOQs$^a$ / (mg/kg) | LODs$^b$ / (mg/kg) | LOQs$^b$ / (mg/kg) |
|-----|----------|----------------------|-------|-------------------|-------------------|-------------------|-------------------|
| 1   | BMPP     | 2.36                 | 0.9996| 0.20              | 0.60              | 0.10              | 0.30              |
| 2   | DIBP     | 2.48                 | 0.9995| 0.10              | 0.30              | 0.05              | 0.15              |
The results suggested that this method could achieve by injecting three different levels (0.6, 3.3, and 10.0 mg/kg) of PAEs and BPA in seven replicates. As shown in Table 2, the spiked recoveries and RSDs were in the range of 88.2-120.5% and 1.55-13.51% for powdered milk infant formula samples, 89.6-114.2% and 0.69-10.06% for milk drinks samples, respectively. The results suggested that this method could achieve the requirements completely for daily detection of 18 PAEs and BPA in dairy products.

### 3.3. Recoveries and RSDs

To evaluate the feasibility of this SFC method, the recoveries and RSDs were subsequently detected for powdered milk infant formula and milk drinks samples.

#### Table 2. Recoveries and RSDs for PAEs and BPA in powdered milk infant formula and milk drinks samples

| Sample Matrix | 0.6 mg/kg | 5.0 mg/kg | 10.0 mg/kg |
|---------------|-----------|-----------|------------|
|               | Rec<sup>a</sup> (%) | RSD<sup>a</sup> (%) | Rec<sup>b</sup> (%) | RSD<sup>b</sup> (%) | Rec<sup>c</sup> (%) | RSD<sup>c</sup> (%) | Rec<sup>d</sup> (%) | RSD<sup>d</sup> (%) |
| BMPP          | 104.5     | 10.82     | 106.5      | 5.75        | 101.5     | 7.11       | 96.1       | 6.99        | 98.9     | 5.03       | 103.0      | 3.81       |
| DIBP          | 110.9     | 3.70      | 107.4      | 3.70        | 107.7     | 2.04       | 94.3       | 2.25        | 103.2     | 3.65       | 98.7       | 1.74       |
| DAP           | 103.5     | 5.86      | 109.2      | 2.24        | 98.4      | 4.81       | 113.0      | 1.14        | 100.5     | 3.95       | 96.4       | 0.88       |
| DIPP          | 106.3     | 4.86      | 98.7       | 4.44        | 103.3     | 3.41       | 105.4      | 7.40        | 97.9      | 2.34       | 97.0       | 2.15       |
| DPRP          | 96.0      | 2.81      | 104.1      | 3.23        | 99.9      | 2.40       | 97.8       | 5.13        | 100.6     | 2.22       | 103.8      | 1.54       |
| DBP           | 107.2     | 4.39      | 102.7      | 7.08        | 109.3     | 2.08       | 96.4       | 5.07        | 101.2     | 3.66       | 99.3       | 0.69       |
| DMP           | 94.1      | 6.91      | 109.5      | 3.73        | 109.5     | 6.22       | 108.2      | 2.73        | 97.8      | 1.89       | 98.2       | 1.42       |
| DEHP          | 97.3      | 2.90      | 97.4       | 4.30        | 101.1     | 3.61       | 106.3      | 1.88        | 99.0      | 2.69       | 103.5      | 1.09       |
| DHXP          | 93.4      | 3.63      | 105.1      | 5.95        | 111.1     | 3.75       | 97.0       | 2.68        | 104.6     | 2.80       | 101.4      | 2.01       |
| DHP           | 106.3     | 13.51     | 114.0      | 10.06       | 101.7     | 8.13       | 90.5       | 7.45        | 102.5     | 4.78       | 97.6       | 1.67       |
| DNOP          | 120.5     | 9.81      | 89.6       | 6.61        | 98.9      | 7.50       | 107.9      | 5.64        | 103.7     | 3.20       | 95.5       | 3.10       |
| BBP           | 101.0     | 5.19      | 109.2      | 3.46        | 99.2      | 2.30       | 92.7       | 2.59        | 100.7     | 2.15       | 105.2      | 2.23       |

<sup>a</sup> Sample matrix of infant milk powder

<sup>b</sup> Sample matrix of milk drinks
| Compound | DNP | DPDP | DCHP | DBEPP | DEEP | DMEP | BPA |
|----------|-----|------|------|--------|------|------|-----|
|          | 100.6 | 101.1 | 105.5 | 113.7 | 107.4 | 116.3 | 88.2 |
|          | 3.71  | 5.90  | 2.42  | 3.12   | 4.82  | 2.65  | 8.64 |
|          | 93.3  | 104.6 | 97.6  | 112.8  | 114.2 | 94.9  | 90.2 |
|          | 4.78  | 4.62  | 6.47  | 3.69   | 2.37  | 2.60  | 8.27 |
|          | 99.0  | 99.2  | 99.0  | 99.8   | 100.1 | 104.4 | 92.6 |
|          | 1.74  | 2.17  | 3.29  | 2.02   | 4.80  | 2.23  | 6.07 |
|          | 106.6 | 97.7  | 107.1 | 93.3   | 109.5 | 97.8  | 105.3|
|          | 3.04  | 3.25  | 2.17  | 3.36   | 3.57  | 1.70  | 5.06 |
|          | 99.8  | 103.2 | 103.2 | 101.8  | 103.7 | 107.1 | 97.2 |
|          | 1.66  | 1.98  | 1.98  | 2.81   | 2.04  | 2.17  | 3.88 |
|          | 98.7  | 99.2  | 99.2  | 99.0   | 103.2 | 103.2 | 104.4|
|          | 3.64  | 1.88  | 1.88  | 1.29   | 1.98  | 1.98  | 2.99 |

Table 1: Sample matrix of infant milk powder

**3.4. Application to real samples**

PAEs and BPA were analyzed by the SFC method in twenty dairy products (ten powdered milk infant formula samples and ten milk drinks samples) with different brands and manufacturers, which were bought from the local supermarkets in Chongqing. Among these selected samples, the target compounds were observed in three of them, including two powdered milk infant formula samples and one milk drinks samples. DBP was appeared in two samples, DMP, BBP and DBEP were tested in the range of 0.061-0.313 mg/kg, and BPA was detected only in one sample at the concentration of 0.141 mg/kg. The levels of PAEs tested in this work were basically consistent with that published by Ren et al. in dairy products [32]. With regard to BPA, the content detected in our work was slightly higher than the concentration in infant formula (45-113 μg/kg) in the previous report [33]. The difference between the levels of target compounds was likely to result from the distinction of milk source, manufacturing technique, packing materials, transportation and storage conditions.

**4. Conclusions**

In the present work, a rapid and environmental method based on SFC was developed for the simultaneous analysis of 18 PAEs and BPA in dairy products. Under the optimized chromatographic conditions, all the target compounds were efficiently separated within 5.5 min with good resolution and selectivity. The LODs and LOQs for all the compounds were 0.05-0.20 mg/kg and 0.15-0.60 mg/kg in dairy products samples, respectively. This SFC method exhibited significant advantages such as convenient, reliable, high-efficiency and low-cost, which may further provide an alternative route for the simultaneous detection of PAEs and BPA in dairy products.

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