# Determination of Six Phthalic Acid Esters in Orange Juice Packaged by PVC Bottle Using SPE and HPLC–UV: Application to the Migration Study

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## Abstract

A high-performance liquid chromatographic assay is described for the determination of six phthalic acid esters (PAEs) in orange juice packaged in polyvinyl chloride (PVC) bottle. Samples were extracted by solid-phase extraction (SPE) cartridges and separated by a $C_{18}$ column. The calibration curves were all linear with a correlation coefficient $r > 0.9900$. The limits of detection for the assay ranged from 2.6 to 13.8 ng/mL. Expressed as the within- and between-day coefficient of variation (CV), precision was 1.4–13.4% and 1.9–13.3%, respectively, and relative errors were 7.6–12.8% and –9.0–14.2%, respectively. The recovery ranged from 76.8 to 112.3% with the CV from 0.3 to 11.3%. The proposed methodology was applied for studing the migration of the selected PAEs into orange juice packaged in PVC bottle. Di-ethyl phthalate (DEP) and di-(2-ethylhexyl) phthalate (DEHP) were detected in the orange juice without the other four PAEs. Concentrations would increase with the storage time and reach up to 0.385 μg/mL and 0.662 μg/mL, respectively, when the expiration date arrived. The level of DEHP was about 110 times higher than the limiting one in drink water (6 ppb) regulated by U.S. EPA. Results suggest that PVC plasticized by DEHP should not be used as the packaging material for orange juice.

## Introduction

Phthalic acid esters (PAEs) are a family of plastic additives with a common chemical structure: dialkyl or alkyl/aryl esters of 1,2-benzenedicarboxylic acid. Due to their excellent properties and compatibility with vinyls and other polymers, they are widely used in polymeric materials such as polyvinyl chloride (PVC), polyethylene (PE), polyvinyl acetates (PVA), and so on (1).

Though the relevance of carcinogenicity in humans is still debatable (2,3), a growing number of investigations have proved that some PAEs and their metabolic products are rodent carcinogens (4) and/or reproductive toxicants affecting particularly male reproductive development (5). Exposure to them in adult males may cause the alternation of sperm and semen properties (6,7) and pulmonary function (8).

Being a serious concern in the field of environmental public health, they are classified as toxic substances and endocrine disrupters by most countries including the U.S. and EU (9,10). For example, six PAEs are targeted by the United States Environmental Protection Agency (U.S. EPA) as priority pollutants, including di-methyl phthalate (DMP), di-ethyl phthalate (DEP), di-$n$-butyl phthalate (DBP), butylbenyl phthalate (BBP), di-(2-ethylhexyl) phthalate (DEHP), and di-$n$-octyl phthalate (DOP) (Figure 1). Some PAEs, including BBP, DEHP, DBP, di-isononyl phthalate (DINP), and di-decyl phthalate (DIDP), etc., has been controlled strictly to be additives in plastic materials and articles intended to come into contact with food in EU Directive 2007/19/EC. DMP, DBP, and DOP are also listed as priority pollutants by the State Environmental Protection Administration in China. Therefore, exact determination of PAEs in all kinds of matrices and detailed description of the migration of them in different surroundings are essential.



**Figure 1.** Chemical structures of the six PAEs studied.
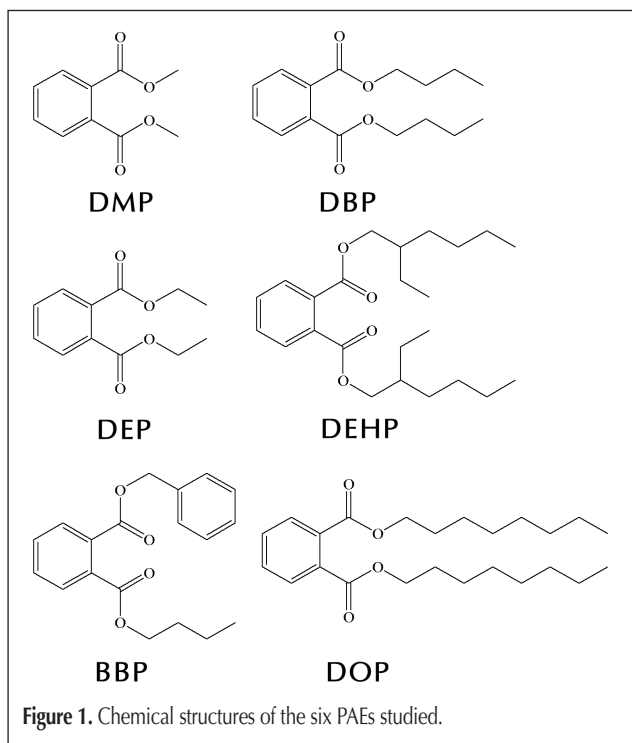
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Due to their inherent separation ability, gas chromatography (GC) (11–29) and high-performance liquid chromatography (HPLC) (30–37) are the most common techniques for the determination of PAEs in plastics (11–13), environmental samples (14–22,26–28,30–35), biological samples (36–37) and some kinds of foods (23–25,29,38–41). Prior to determination, extensive sample treatment involving repetitive liquid–liquid extraction (LLE) (12,17,30) and cleanup steps with large amounts of reagents and solvents was required, which maybe introduced considerable levels of PAEs into the sample matrix.

In recent years, solid-phase extraction (SPE) (13–17,31–34) and solid-phase microextraction (SPME) (18–24) have been extensively investigated to simplify sample pretreatment prior to chromatographic analysis of PAEs to reduce the risk of secondary contamination during sample handling. Compared with SPME, SPE has the advantages of relatively higher absolute extraction yields, shorter extraction times, and better precision. As for materials used as sorbent of SPE, silica-based $C_{18}$ (38) and $C_8$ (27), organic polymers (34), and carbon nanotubes (29,33) have been tried. However, little attention has yet been paid to the development of analytical procedures for simultaneous determination of PAEs in beverages using Waters Oasis MAX cartridge as SPE material. A priority goal in this context is to reduce sample treatment to the minimal steps in order to avoid PAEs contamination and obtain satisfied recovery.

Because they are not chemically but only physically bound to the polymer chains, PAEs normally have high mobility within the polymer matrix and easily diffuse to the surrounding media due to their relatively low molecular weight and large initial concentration. Because foods are the major source of exposure to PAEs, many references have reported the migration of PAEs from flexible PVC films into various foods and food simulants including water, and milk, etc. (23–25,29,38–41) for human exposure assessment in recent decades. However, to our best knowledge, there are no published results on the migration of PAEs from PVC bottles into beverages for storage life as long as 12 months.

This paper reports a SPE-HPLC method with UV detector for analysis of the PAEs targeted as priority pollutants by U.S. EPA (DMP, DEP, DBP, BBP, DEHP, and DOP) in orange juice. The procedure was also applied to study the migration of the six PAEs from PVC bottles into orange juice for a whole storage life.

## Experimental

### Materials and reagents

Standard DMP (99%), DEP (99%), DBP (98%), DEHP (99%), and DOP (99%) were purchased from Sigma Aldrich (St. Louis, MO). BBP (99%) was purchased from TCI (Tokyo, Japan). HPLC-grade acetonitrile was purchased from Fisher Company (Fairlawn, NJ). Ultra-pure water (18 Mohms) was obtained by a PW ultrapure water system (Heal Force Co., Hong Kong, China). All other chemicals were analytical reagents, commercially available and used without further purification. Extraction cartridges (Oasis MAX 1 mL, 30 mg) were purchased from Waters (Milford, MA).

### Instrumentation and operating parameters

HPLC determinations were performed with a Dionex P680C system (Dionex, Sunnyvale, CA) consisting of a P680C pump, a UVD 170 detector, a TCC-100 column oven, and an ASI-100 automatic sample injector. The experimental parameters of the HPLC system, including monitoring wavelength, flow rate, concentration of the mobile phase, and column temperature, were all controlled by the Chromeleon computer software package. The separation was performed on a Phenomenex (Torrance, CA) Luna $C_{18}$ reversed-phase column (250 mm × 4.6 mm i.d., 5-µm particle size) protected by a Phenomenex Luna $C_{18}$ guard column (4 mm × 3 mm i.d., 5-µm particle size). The mobile phase was a mixture of acetonitrile and water with the gradient program: linear gradient from 75% to 85% of acetonitrile in 5 min, then linear gradient from 85% to 100% of acetonitrile in 10 min, and finally isocratic conditions with 100% acetonitrile for 6 min, returned to initial composition at 23 min, and held for 5 min to equilibrate the column. The flow rate was set at 1.0 ml/min. The column effluent was monitored at 226 nm with UV detection. The column temperature was regulated at 35°C. Data were collected and integrated by using Chromeleon software.

### Preparation of standards and controls

Individual stock solutions of six kinds of PAEs at a nominal concentration of 1 mg/mL were prepared separately by adding 50 mg of the analyte into 50 mL of acetonitrile. Mixture stock solution at a nominal concentration of 20 µg/mL was obtained by mixing 200 µL of each kind of the previously mentioned individual stock solution and then diluting to 10 mL with acetonitrile. Individual and mixture working solutions at the concentrations of 2.0, 1.0, and 0.2 µg/mL were obtained by stepwise dilution of the individual and mixture stock solutions. Calibration standard samples at concentrations of 2.0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 µg/mL were obtained by adding working solutions of appropriate concentration and volume into the blank orange juice. Quality control (QC) samples at low (0.05 µg/mL), medium (0.2 µg/mL), and high (1.0 µg/mL) levels were prepared in the same way as calibration standard ones. Calibration standard samples were used for studying the linear relationship and determining limit of detection (LOD) of each kind of PAEs, while QC were used for for determining accuracy and precision of the method. All stock solutions were stored at 4°C for a maximum period of one month. Working solutions, calibration standard samples, and QC samples were prepared just prior to use.

### Sample collection

Several boxes of orange juice packaged by PVC bottles with the same production batch were purchased from local supermarket, and they were stored in the room environment same as shops. Those which had been stored for no more than seven days since production were used as the blank. Samples stored for different periods of time in the room environment were picked out to assay for the presence and migration features of PAEs.

### Sample preparation and extraction procedure

All samples, including blanks, standards, QC, and unknowns,

were extracted using the previously mentioned SPE cartridges. Before the SPE procedures, 1 mL aliquot of the orange juice samples was filtered through 0.45-µm filter (Whatman GF/F, Osmonics, France) to remove suspended solids. Then, the filter was washed with 2 mL 5% acetonitrile aqueous solution. All volumes of resulting solutions were gathered and then uploaded to pass through the cartridges, which were previously activated by 1 mL acetonitrile and balanced by 1 mL water, followed by washing with 1 mL 5% acetonitrile aqueous solution. At last, the analytes were eluted with 1 mL 100% acetonitrile and collected into clean glass tubes. All previously mentioned steps were carried out without a lab vacuum. Occasionally, a 1-mL injector was plugged into the end of the cartridges to force the solutions to pass through it in 5 min. The eluent was evaporated to dryness under a stream of high purity nitrogen gas at 50°C, and the obtained extraction residue was reconstituted in 1 mL 100% acetonitrile. Then, 20 µL of the achieved aliquot was automatically injected into the HPLC system.

## Results and Discussion

### Method development

Considering more background absorption at 205 nm and less sample absorption intensity at 275 nm, the wavelength of 226 nm was chosen as the operating one. To obtain effective and quick separation of the PAEs, a gradient eluting program has been successfully employed for the separation of the homologous series, and the chromatographic peaks of six PAEs were separated independently with the resolution ($R$) being more than 1.5 between any two adjacent peaks.

Due to the widespread applications in consumer products, PAEs are commonly found in the laboratory environment, and this is a major issue when developing methods for determination of PAEs. Thus, for each sequence of analysis, three blanks were measured, and the average blank level of PAEs (if any) were subtracted from the results of samples.

A series of typical chromatograms were obtained (Figure 2). The degree of interference by endogenous orange juice constituents with PAEs was assessed by inspection of chromatograms derived from pretreated blank sample. Figure 2A was the chromatogram of the six PAEs in standard solutions with concentrations being 1 µg/mL. The peaks of the six analytes were resolved thoroughly. The elution time of six PAEs were 3.649 ± 0.013 (DMP), 4.605 ± 0.015 (DEP), 7.968 ± 0.015 (BBP), 8.690 ± 0.017 (DBP), 19.763 ± 0.022 (DEHP), and 20.576 ± 0.021 (DOP) min ($n > 20$), respectively. The coefficients of variation (CV) of the retention times were all less than 0.5%, which showed the stability of the instrumental system. Figure 2B was the chromatogram of the blank orange juice, which illustrated that there were no interfering compounds coming at the elution times for the six PAEs under the selected experimental conditions. Figure 2C showed the chromatogram of QC samples at the concentration of 1.0 µg/mL in which the peaks for the six PAEs could be identified easily and accurately. Figure 2D was the chromatogram of a real orange juice sample, which had been stored for 217 days since production in which DEP and DEHP were found with the concentration of 0.245 µg/mL and 0.248 µg/mL, respectively. Compared with Figure 2C, the interfering peaks were more complicated in the real sample than in the QC, perhaps due to some changes in the matrix of the orange juice or some other substances migrated from the packaging materials into the orange juice.

In the extraction procedure of sample pretreatment, two kinds of SPE cartridges (Waters Oasis HLB cartridge and MAX cartridge) were evaluated in this work. The sorbent of HLB and MAX cartridges consists of macroporous copolymer [poly(divinyl-benzene-co-$N$-vinylpyrrolidone)], and the MAX cartridge is modified by strong anion-exchange quaternary amine groups on the surface. A mixed standard solution was uploaded, and the amount of six PAEs in the effluent was determined. The results showed that the recovery of DEHP and DOP using HLB cartridge was less than 50%, while the good recovery was obtained using MAX cartridge. From the molecular structures of six PAEs, the polarity of DEHP and DOP was relatively lower than other ones because of their much longer carbon chains. Meanwhile, electronegative ester groups in the PAEs and electropositive quaternary amine groups in MAX cartridge generated electrostatic attraction. Therefore, PAEs adsorbed on MAX cartridge more strongly than on HLB, which eventually led to a better recovery. Interestingly, satisfactory recovery could be achieved even when the eluent was not 2% formic acid in acetonitrile, as suggested by the manufacturer, but just only pure acetonitrile, which suggested that the electrostatic attraction is not very strong.
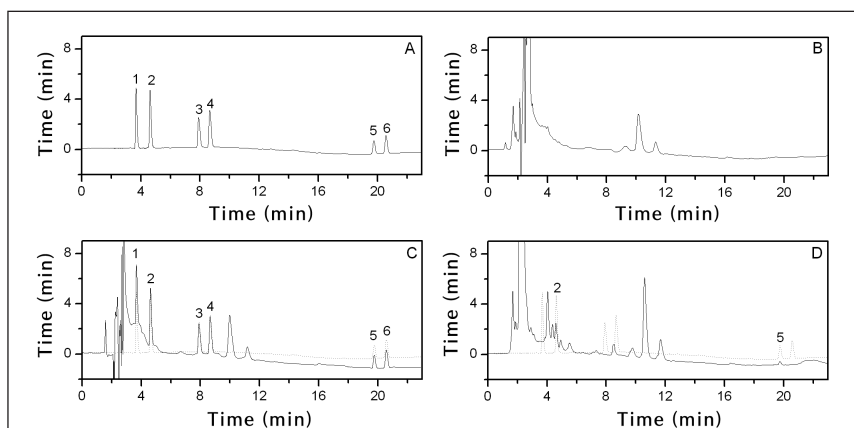
### Method validation

The whole analytical method was validated in terms of linearity, LOD, precision and accuracy, and the recovery. The peak areas of calibration standard samples extracted as described earlier were measured, and



**Figure 2.** A series of typical HPLC chromatograms: mixture working solutions at the concentrations of 1.0 µg/mL injected into the HPLC system directly without SPE (A), the blank orange juice sample (B), QC sample at the concentration of 1.0 µg/mL (C), and a real orange juice sample which had been stored for 217 days since production (D). The dotted line in (C) and (D) is the line shown in (A) for comparison. 1, DMP; 2, DEP; 3, BBP; 4, DBP; 5, DEHP; 6, DOP, respectively;

calibration curves were obtained from the least-squares linear regression of the peak areas $y$ (mAU·min) versus calibration concentration $\rho$ (µg/mL). The linearity obtained for analytes were all good with correlation coefficients ($r$) in the range of > 0.9900. The regression lines were used to calculate concentration of PAEs in the unknown samples. Detailed results of the calibration curves are present in Table I.

**Table I. Regression Equation and LOD of Each Compound (8 points)**

| Compounds | Regression equations | Linear range (µg/mL) | $r$ | LOD (ng/mL) |
|---|---|---|---|---|
| DMP | $y = 0.73534 \times \rho + 0.00175$ | 0.01 ~ 2 | 0.9937 | 2.6 |
| DEP | $y = 0.69124 \times \rho + 0.00078$ | 0.01 ~ 2 | 0.9991 | 4.4 |
| BBP | $y = 0.54453 \times \rho + 0.00015$ | 0.02 ~ 2 | 0.9951 | 7.7 |
| DBP | $y = 0.60444 \times \rho - 0.00129$ | 0.02 ~ 2 | 0.9988 | 7.6 |
| DEHP | $y = 0.28503 \times \rho + 0.00256$ | 0.05 ~ 2 | 0.9919 | 13.8 |
| DOP | $y = 0.35668 \times \rho + 0.00020$ | 0.05 ~ 2 | 0.9946 | 11.1 |

**Table II. Within- and Between-Day Precision and Accuracy for Determination of PAEs in QC Samples**

| Compounds | Conc. added (µg/mL) | Within-day Conc. found* (µg/mL) | CV[†] (%) | Relative error (%) | Between-day Conc. found* (µg/mL) | CV[†] (%) | Relative error (%) |
|---|---|---|---|---|---|---|---|
| DMP | 0.05 | 0.0483 (0.0027) | 5.6 | –3.4 | 0.0475 (0.0029) | 6.1 | –5.0 |
| | 0.2 | 0.204 (0.008) | 3.9 | 2.0 | 0.206 (0.010) | 4.9 | 3.0 |
| | 1 | 1.042 (0.021) | 2.0 | 4.2 | 1.038 (0.023) | 2.2 | 3.8 |
| DEP | 0.05 | 0.0478 (0.0039) | 8.2 | –4.4 | 0.0481 (0.0047) | 9.8 | –3.8 |
| | 0.2 | 0.189 (0.012) | 6.3 | –5.5 | 0.201 (0.009) | 4.5 | 0.5 |
| | 1 | 0.986 (0.046) | 4.7 | –1.4 | 1.021 (0.058) | 5.7 | 2.1 |
| BBP | 0.05 | 0.0521 (0.0031) | 6.0 | 4.2 | 0.0519 (0.0033) | 6.4 | 3.8 |
| | 0.2 | 0.210 (0.011) | 5.2 | 5.0 | 0.214 (0.015) | 7.0 | 7.0 |
| | 1 | 1.031 (0.021) | 2.0 | 3.1 | 1.054 (0.035) | 3.3 | 5.4 |
| DBP | 0.05 | 0.0476 (0.0042) | 8.8 | –4.8 | 0.0509 (0.0048) | 9.4 | 1.8 |
| | 0.2 | 0.205 (0.009) | 4.4 | 2.5 | 0.208 (0.008) | 3.8 | 4.0 |
| | 1 | 1.016 (0.014) | 1.4 | 1.6 | 1.063 (0.049) | 4.6 | 6.3 |
| DEHP | 0.05 | 0.0563 (0.0064) | 11.4 | 12.8 | 0.0571 (0.0076) | 13.3 | 14.2 |
| | 0.2 | 0.214 (0.015) | 7.0 | 7.0 | 0.209 (0.018) | 8.6 | 4.5 |
| | 1 | 1.027 (0.067) | 6.5 | 2.7 | 0.994 (0.072) | 7.2 | –0.6 |
| DOP | 0.05 | 0.0462 (0.0062) | 13.4 | –7.6 | 0.0458 (0.0059) | 12.9 | –8.4 |
| | 0.2 | 0.187 (0.014) | 7.5 | 6.5 | 0.182 (0.014) | 7.7 | –9.0 |
| | 1 | 1.037 (0.015) | 1.4 | 3.7 | 0.983 (0.019) | 1.9 | –1.7 |

* Mean (standard deviation), $n = 6$.
[†] CV = coefficient of variation.

The LOD for the assay of PAEs were calculated, based on 3/1 of the signal-to-noise ratio (42), which were about 2.6 (DMP), 4.4 (DEP), 7.7(BBP), 7.6 (DBP), 13.8 (DEHP), and 11.1 (DOP) ng/mL, respectively (Table I).

The precision and accuracy of within-day and between-day were evaluated by one and three working days in six replicates of QC samples at three different concentrations of PAEs. Precision was presented as CV, and accuracy was expressed as a relative error, [(concentration found – concentration added) / concentration added] $\times$ 100(%)]. Within- and between-day precision was 1.4–13.4% and 1.9–13.3%, and accuracies were –7.6–12.8% and –9.0–14.2%, respectively, as shown in detail in Table II. The results indicate that this method is reliable, reproducible, and accurate.

Recovery was calculated by comparing the peak areas obtained from the QC samples with those obtained by direct injection of the same amount of analytes without SPE. Two replicates were prepared at each concentration level, and each one was injected in triplicate. The mean recoveries for the real sample spiked at three representative concentrations of 0.05, 0.2, and 1.0 µg/mL were in the range of 76.8–112.3% with CV ranging from 0.3 to 11.3% (Table III).

### Migration study

The amounts of PAEs migrated into orange juice samples from packaging bottles were determined by three replicates six times, and the results were given as a function of time. As shown in Figure 3, DEP and DEHP were detected out, and DMP, DBP, BBP, and DOP were not at all. It was accordant with the information supplied by the manufacturer, i.e., DEP and DEHP was added as plasticizers at the concentration of about 3% (g/g) without DMP, DBP, BBP, and DOP. The concentrations of DEP and DEHP in the orange juice were lower than LOD in the first two months and could be detected out in the third month. They would increase with the storage time and reach up to 0.385 µg/mL and 0.662 µg/mL, respectively when the expiration date arrived.

**Table III. Recovery of Each Compounds**

| Compounds | Spiked level (0.05 µg/mL) Recovery* (%) | CV[†] (%) | (0.2 µg/mL) Recovery (%) | CV[†] (%) | (1 µg/mL) Recovery (%) | CV[†] (%) |
|---|---|---|---|---|---|---|
| DMP | 97.5 | 3.5 | 97.5 | 2.4 | 106.7 | 0.5 |
| DEP | 86.4 | 3.4 | 105.1 | 10.6 | 100.3 | 8.2 |
| BBP | 90.8 | 4.1 | 108.2 | 4.3 | 92.0 | 0.6 |
| DBP | 85.6 | 5.5 | 102.4 | 8.6 | 112.3 | 7.8 |
| DEHP | 80.0 | 7.8 | 96.9 | 6.6 | 103.2 | 0.3 |
| DOP | 76.8 | 11.3 | 96.8 | 2.7 | 100.6 | 0.5 |

* Mean recovery, $n = 6$.
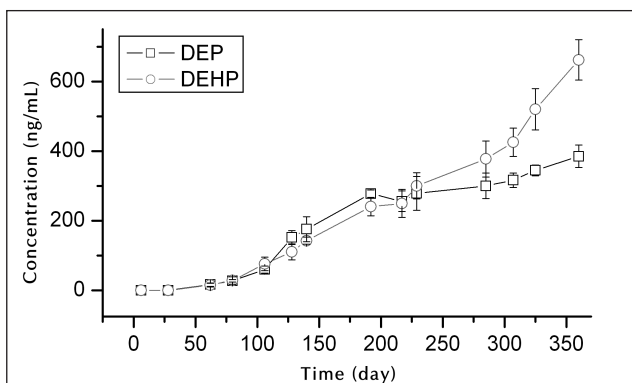[†] CV = coefficient of variation.



**Figure 3.** Concentration of DEP and DEHP in orange juice sample as a function of time.

Considering the weight of packaging bottle was about 25 g and the volume of orange juice was about 500 mL, it could be deduced that the weights of DEP and DEHP in each packaging bottle were about 750 mg, and those of DEP and DEHP in orange juice would be about 190 μg and 330 μg. This result suggested that about 0.025% of DEP and 0.04% of DEHP would migrate from packaging bottle into orange juice when the expiration date arrived (12 months since production date).

The concentration of DEHP was higher than the regulation limit for DEHP in drinking water set by the U.S. EPA (6 ppb) (43) at the end of the third month and about 110 times higher when the expiration date arrived. It suggested that PVC plasticized by DEHP should not be used as the packaging material for orange juice.

The solubility of DEHP in distilled water is controversial up to now. It varied from 1.1 to 1,200 ng/mL determined both experimentally and theoretically (44). A value of 3 ng/mL for the water solubility of DEHP has been recommended by Staples et al. (44), which is "based on available evidence" rather than any one specific experimentally derived value. Unfortunately, the authors do not indicate how they derived their recommended value. In this work, the concentration of DEHP in the orange juice would reach up to 662 ng/mL when expiration date arrived. This value is much higher than the solubility of DEHP in water recommended by U.S. EPA (285 ng/mL) and that one by Staples et al. (3 ng/mL). The facts probably come from the following two reasons: the acidity of the orange juice maybe promotes the solubility of DEHP and a great amount of DEHP is perhaps adsorbed on the natural flesh and cellulose in the orange juice.

## Conclusion

A simple method involving a modified SPE procedure coupled with HPLC–UV analysis was developed and applied to the determination of PAEs contamination in orange juice samples with satisfactory analytical analysis results. It was validated by specificity, linearity, reproducibility, and accuracy for six PAEs. The method was particularly effective for the analysis of DEHP and DOP as exemplified by their high recovery ratios when Waters Oasis MAX cartridges were used instead of HLB ones. The reason is that they were more strongly adsorbed onto the former with the help of the electrostatic attraction.

Analyses of orange juice samples indicated that DEP and DEHP were undoubtedly present in them. In particular, it was found that the levels of DEP and DEHP in orange juice samples would increase with the storage time and reach up to 0.385 μg/mL and 0.662 μg/mL, respectively, at the end of 12 months following production. The results of migration study suggested that the safety of using PVC as packaging material for beverages should be seriously concerned.

## Acknowledgments

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