Determination of estrogens and bisphenol A in bovine milk by automated on-line C_{30} solid-phase extraction coupled with high-performance liquid chromatography–mass spectrometry

Wei Yan[a], Ying Li[a], Lixia Zhao[a], Jin-Ming Lin[a,b,*]

[a] State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, P.O. Box 2871, Beijing 100085, China
[b] Department of Chemistry, Tsinghua University, Beijing 100084, China

ARTICLE INFO

ABSTRACT

Using triacontyl bonded silica (C_{30}) as on-line solid-phase extraction (SPE) material and a specially designed on-line analytical system which allowed large sample volume injection, a high speed and robust on-line SPE-HPLC–MS method was established for the analysis of five estrogens and bisphenol A (BPA) in milk samples. The milk sample is pretreated with acetonitrile for protein precipitation and then treated with primary secondary amine for the removal of polar impurities in the matrix. Then the pretreated sample can be automatically loaded by a LC pump. For effective extraction, an offshoot with NH_4Ac solution of high-flow rate was employed to dilute the loaded sample by a mixing tee before sample was loaded onto the C_{30} extraction column. In this way, large volume injection (1 mL in this experiment) could be achieved. Some important parameters such as sample loading flow rate, sample dilution ratio and injection volume were optimized. Under the optimized conditions, the recoveries for all analytes range from 71.4 to 97.1% and reproducibility represented as RSDs are less than 15.0% ($n = 5$) with milk samples spiked at 0.6 and 15 ng/mL of each analyte. To the authors' knowledge, it constitutes the first work describing a C_{30} on-line SPE-LC–MS analytical method for the screen and monitoring of these estrogens and BPA in milk.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Steroid hormones, a large group of lipophilic, low-molecular weight, estrogen active compounds which act as hormones, have been drawn extensively scientific, societal and political attentions. Prominent among these compounds are naturally occurring 17β-estradiol (E_2), estrone (E_1), estriol (E_3) and synthetic diethylstilbestrol (DES), ethinylestradiol (EE_2). Widespread evidences showed that these estrogens with highest estrogenic activity can exhibit adverse effect on reproductive processes in wildlife and humans at low-ng/L concentration [1–5]. Furthermore, new findings indicated that some of these estrogens or their metabolites play a role in human carcinogenesis, such as breast, prostate and ovarian cancer [6–9], which has become another hotspot these years about the specific biological effect and potential threat for human bodies.

In this context, various sensitive and high-resolution analytical methods, such as GC–MS, LC–MS or LC–MS–MS were established for the investigation of those biologically active estrogens. Up to now,

however, most research interests of this issue seem to focus on the estrogens in environmental waters and surface sediment matrices because they are considered to be the final sink of steroid estrogens excreted by animals and humans, whereas the study on milk matrix has only a few reports [10–14]. Actually, steroid hormones can be transported from blood flow by an active transport within the mammary gland and synthesized by the mammary gland and finally excreted to milk. What's more, for the purpose of animal fattening or growth promotion, large amount of steroid hormones and synthetic hormones were illegally used. So it can be believed that milk is an important source of estrogens which has been neglected for long time. It has been reported that approximately 60–80% of estrogens come from milk and dairy products in Western diets [9].

Besides, bisphenol A (BPA), an industrial chemical which imitates the hormone estrogens, has been widely researched about its occurrence and fate in different environments in past years. It has been most commonly believed as been "weakly" estrogenic since the tolerable daily intake (TDI) of BPA in Europe is $0.050\,mg\,(kg\,body\,weight)^{-1}\,day^{-1}$, according to European Food Safety Authority (EFSA) in its risk assessment on BPA published in January 2007 [15]. Very recently, however, the adverse effect of BPA leached from baby bottles and drinks packaging to children has caught high-scientific attention. According to the report

from National Toxicology Program (NTP), the low-level exposure to BPA during development can cause changes in behavior and the brain, prostate gland, mammary gland, and the age at which females attain puberty. For this reason, increasing attention has been paid on BPA in milk in academic field and a lot of extraction methods has been developed, including solid-phase extraction (SPE) [16], molecularly imprinted polymers (MIPs) [17] and matrix solid-phase dispersion (MSPD) [18].

Though these methods have been reported for the quantitative analysis of estrogens in biological fluids, most approaches require time-consuming and cumbersome extraction procedures prior to the LC analysis, which seriously limit their application, especially when a large amount of samples should be detected. On-line SPE technique method, compared to other offline SPE methods, has becoming particularly attractive these years because of numerous advantages, such as high throughput, no loss of analytes and small consumption of organic solvent, etc. To date, although on-line SPE coupled to LC/MS or LC/MS/MS has been widely applied to the trace analysis of organic compounds in environmental waters and biofluids [19–21], little information could be obtained about the determination method of steroid hormones in milk [22].

Triacontyl bonded silica (C_{30}), a kind of extraction sorbent with the properties of long alky chains, relatively large particle sizes but with acceptable active surfaces areas for high recoveries, has been initially applied as reversed-phase liquid chromatography (RPLC) stationary phase to improve the separation of the geometric isomers. Our previous studies show the good performance and versatile applications in the extractions of polycyclic aromatic hydrocarbons (PAHs) in air and estrogens in environmental waters [23,24]. The main purpose of this work is to develop a high speed and robust on-line SPE-HPLC–MS method for high-throughput analysis of five estrogens and BPA in milk samples. C_{30} was firstly employed and subjected to on-line SPE for the sample cleanup and extraction of interested estrogens. The results testified that C_{30} column could utilize high-flow rates without significant increases in the backpressure or deteriorated chromatographic performance. The pretreated sample was loaded directly by LC pump to achieve the large volume injection (1 mL) for increased sensitivities and improved limits of detection (LODs). Finally, the proposed method was validated by evaluating sensitivity, linearity, repeatability, and accuracy.

2. Experimental

2.1. Chemicals and reagents

Ultra-pure water (18.3 MΩ) was obtained by purification of deionized water through a Milli-Q system (Millipore, Bedford, MA, USA). Pure standards of bisphenol A (BPA, purity > 99%) and estrogens, both natural and synthetic, including estrone (E_1, purity ≥ 99%), 17β-estradiol (E_2, purity ≥ 99%), estriol (E_3, purity ≥ 97%), diethylstilbestrol (DES, purity ≥ 99%), 17α-ethynylestradiol (EE_2, purity ≥ 98%), were purchased as powders from Sigma–Aldrich (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were purchased from Fisher (Fair Lawn, NJ, USA). Ammonium acetate was purchased from Wako (Osaka, Japan). Primary secondary amine (PSA), a weak anion exchanger sorbent which is used in dispersive SPE for reducing matrix effect, was obtained from Macherey-Nagel (Düren, Germany).

2.2. Preparation of standard solution

Standard solutions of individual analyte (1 mg/mL) were prepared by dissolving 10 mg of each compound in 10 mL of methanol. Working solution of the individual and mixed standards was prepared at various concentrations by appropriate dilution of the stock solutions in methanol. All these solutions were stored at −20 °C and could be stable for at least 6 months.

2.3. Sample preparation

Different brands of pasteurized, homogenized whole milk samples (3.5% fat) were purchased from retail markets in Beijing, China. These samples were frozen at −20 °C until analysis. When were used, milk samples were thawed, sonicated, vortex mixed, and divided into aliquots.

Milk matrix was complex, composed of proteins and lipids. Therefore, some cleanup pretreatment before on-line extraction and LC–MS analysis seems essential. First, most protein in milk sample was precipitated by adding acetonitrile into the milk sample (sample:acetonitrile, 1:2, v/v). The mixture was oscillated for about 3 min and then centrifuged at 10,000 rpm for 15 min. The supernatant was collected and transferred to another 15 mL Teflon centrifuge tube.

For further clean up, add 150 mg PSA into 10 mL supernatant, shake the sample vigorously for about 3 min and centrifuge at 10,000 rpm for 10 min. The supernatant was collected again for sample on-line loading.

For several previous studies, *Helix pomatia juice* has often been used for enzymatic hydrolysis of steroid glucuronides or sulfates during the sample preparation procedure. However, the usefulness of this technique is controversial. Some paper [25,26] indicated that the conjugated proportion could be omitted, since the proportion of cleavable conjugated testosterone was lower than 20% and that of conjugated 17β-estradiol was lower than 5%. For this reason, hydrolysis procedure is not used in proposed method.

2.4. Instrumentation

The on-line extraction and analysis was performed using a Shimadzu LC–MS 2010A system which consisted of four LC-ADvp pumps, a DGU-14A degasser, a six-port switching valves, a SIL-HTc autosampler and a single quadrupole MS.

The extraction column was tailor-made that C_{30} material was packed into a guard cartridge (10.0 mm × 2.1 mm, ID). The C_{30} material was obtained from ChromaNik Technologies (Osaka, Japan). The physical characteristic of C_{30} is as follow: the particle size is about 180–250 μm; the pore size is 10 nm; the surface area is around 300 m^2/g. Analytical separations were performed using Symmetry-C_{18} column (150 mm × 2.0 mm, 5 μm) purchased from Waters (Milford, MA, USA).

MS was using electrospray ionization (ESI) interface in negative ionization mode and the general MS conditions were set as follows: sheath and auxiliary gas, nitrogen; flow rate of nebulizer gas, 1.5 L/min; drying gas, 0.04 MPa; curved desolvation line (CDL) temperature, 250 °C; block heater, 240 °C; detector voltage, 1.75 kV; probe voltage, 4.5 kV.

2.5. On-line procedure

The whole on-line procedure, including sample loading, cleanup and elution, was programmed by the data system of LC–MS solution Ver 3.0 Workstation. The schematic view of the on-line procedure was shown in Fig. 1.

First, in the I position, pretreated milk sample was loaded by pump 'c' at 0.4 mL/min. In order to overcome the problem that the analytes in high-content organic solution cannot be effectively retained by extraction column, a dilution line with 15 mM ammonium acetate solution was employed and delivered at 3.6 mL/min by pump 'd'. The sample loading line and the dilution line were

Table 1
The time procedure of on-line SPE-HPLC–MS method.

| t (min)	Pump A (%)	Pump B (%)	Pump C (mL/min)	Pump D (mL/min)	Position
0.00	10	90	0.4	3.6	I
2.50	–	–	0.0	–	–
3.50	–	–	–	–	–
3.51	–	–	–	0.0	II
5.50	50	50	–	–	–
9.50	80	20	–	–	–
14.00	–	–	–	–	–
18.00	40	60	–	–	–
20.00	10	90	–	–	–
45.00	10	90	–	–	I

mixed through mixing tee and then the diluted sample was loaded onto the extraction column (sample loading step).

The dilution line continually delivered ammonium acetate solution at 3.6 mL/min for 1 min after 2.5 min loading process (washing step) and then the valve was switched from position I to position II. The retained analytes were then backflushed onto the analytical column by pumps 'a' and 'b' and separated under a binary gradient at 0.2 mL/min (elution step). The gradient and the process were shown in Table 1.

3. Results and discussion

3.1. Optimization of the on-line SPE process

The determination performance is strongly dependent on the efficiency of SPE process. In order to achieve the best recoveries and maximum sensitivity for the determination of estrogens and BPA using the proposed method, specific parameters were optimized, including the loading flow rate, the dilution line and the injection volume.
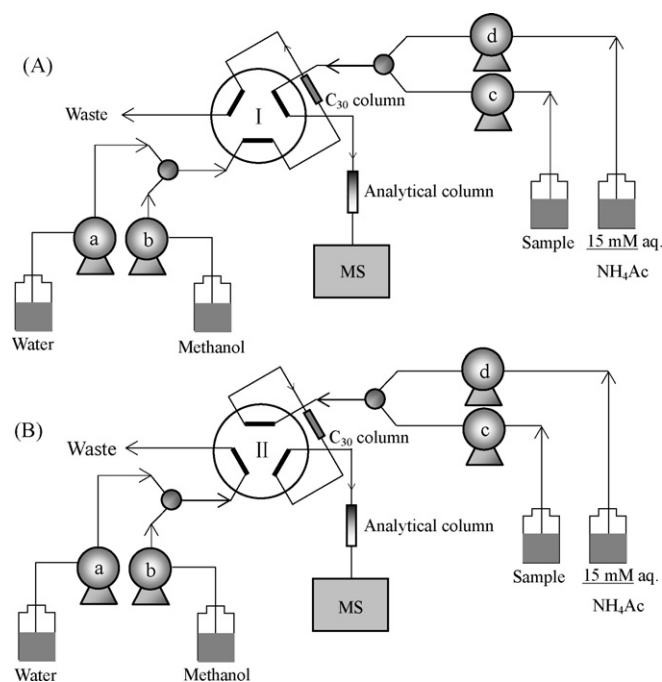
Fig. 1. Schematic diagrams of the proposed on-line SPE-HPLC–MS method for the determination. (A) In the position I of six-port valve, pretreated milk sample was loaded onto the C_{30} extraction column by pumps 'c' and 'd' and the analytical column was equilibrated by pumps 'a' and 'b'. (B) In the position II of six-port valve, the retained analytes on the C_{30} extraction column was eluted by pumps 'a' and 'b', and then was separated and detected by LC–MS.

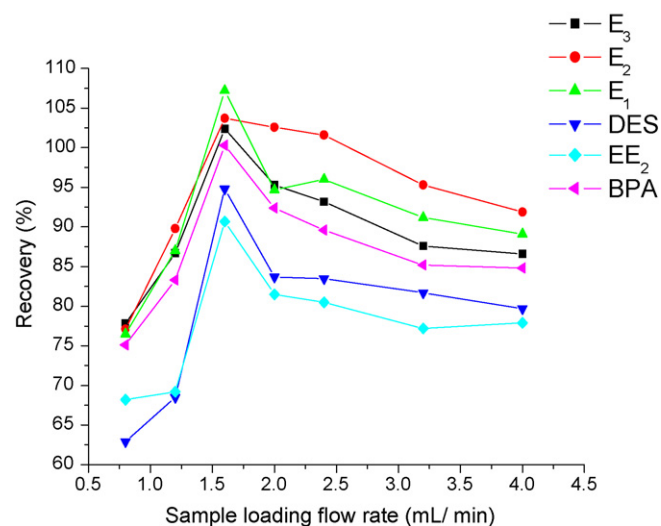### 3.1.1. Optimization of sample loading flow rate

In order to obtain the best sample loading flow rate, 0.8–4 mL/min flow rates were investigated. The ratio of sample and dilution line (pure water) was 1:3. The recoveries were obtained by the comparison of the signal responses between the on-line SPE with 1 mL 1 ng/mL standard samples and 10 μL 100 ng/mL injected by autosampler.

Because of the special structure of C_{30} SPE material, especially the big particle size and long carbon chain, the study as shown in Fig. 2, found that the use of high-sample loading flow rate was feasible without the introduction of excessive column backpressure, which problem was often encountered when traditional C_{18} column was used for biological fluids analysis. When the flow rate was increased to 4 mL/min, the maximum backpressure of C_{30} SPE column was only about 4.0 MPa. In the latter real sample analysis, the maximum backpressure increased a little (lower than 5.0 MPa) after several times injection, which mean that the matrix impurities could be effectively flushed out of the SPE column without clogging.

Besides, despite the surface area of C_{30} material is only about 300 m^2/g, far lower than that of some commercial SPE columns (Oasis HLB, 810 m^2/g; Supelclean ENVI-18, 475 m^2/g), the recoveries of all analytes were effective and the influence of flow rate was little. Since the influence of flow rate from 2.0 to 4.0 mL/min was neglectable, 4.0 mL/min was selected for the effective sample loading.

3.1.2. The dilution line

Because milk sample is a complex matrix which contained lipids, protein, salts, etc., an additional step of lipids and proteins removal

Fig. 2. Effect of sample loading flow rate on the recovery of 1 mL 1 ng/mL standard samples.

is essential to be proceeded before SPE procedure. In this study, acetonitrile as organic solvent was used for protein precipitation. After this treatment, analytes were transferred into acetonitrile solution with high concentration (about 67%). Then another problem arose that the analytes in acetonitrile solution could not be effectively retained on the SPE column since the retention mechanism is based on the hydrophobic interaction.

To cover this problem, a dilution line with 15 mmol/L NH$_4$Ac solution was designed in this study. There are two reasons for this design. One is apparent that the diluted line increased the polarity of the loaded sample which helped the analytes retain on the C$_{30}$ column. Besides, as reported previously [27], NH$_4$Ac solution has been shown to effectively suppress the interaction of the analytes with the macromolecules in milk sample (such as protein) and increase the solubility of protein. The other reason is that the sample dilution itself is a way to decrease the concentration of sample matrix which facilitates washing these impurities away from the SPE column to the waste [28].

As shown in Fig. 3, the response signals of all analytes were increased with the increasing ratios of dilution, especially when the ratios of dilution were from 1:2 to 1:6. Since the effect of dilution is not very significant between 1:8 and 1:10, we selected 1:9 as final ratio of dilution.

3.1.3. Optimization of injection volume

Large volume injection (LVI) is another advantage of the propose method. It is obvious that LVI can improve the sensitivity and LOD of the on-line analytical method since it provide bigger concentration factor. In this study, 1.0, 2.0, 3.0, 4.0, 5.0 mL sample volumes were investigated (loading sample:diluted solution = 1:9). The injection times were from 2.5 to 12.5 min. In Fig. 4, it can be shown that the injection volume is increased up to 5.0 mL with proportional increase of signal response which proved the feasibility of LVI in this on-line SPE-LC–MS method. When higher sample volumes were applied, the recoveries of most analytes became to decline. The possible reason was that the injection amount exceeded the limit of SPE column binding capability.

Though as much as 5.0 mL standard sample could be loaded without evident chromatographic deterioration, in the following real sample study, we found that they are still a lot of unknown peaks which suppress the target signals when LVI is carried out. Considering the intolerable matrix interference, we chose 1.0 mL as final injection volume. In this way, the concentration factor of
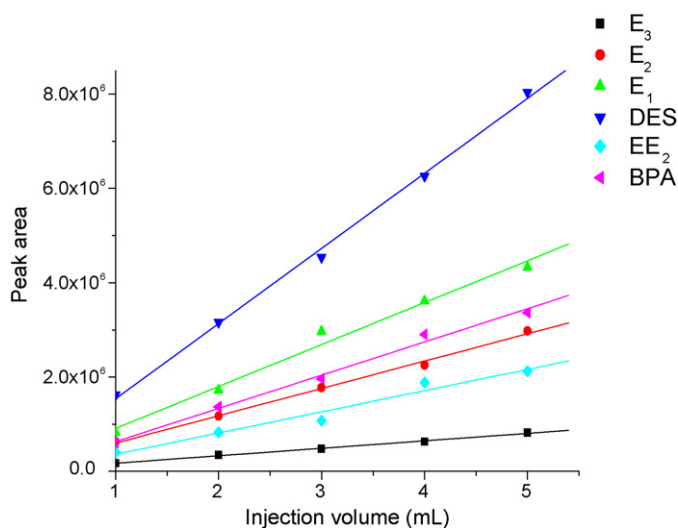

Fig. 4. Corresponding peak areas to different sample injected volumes.

100 could be obtained, compared to the 10 μL sample volume of autosampler loading mode.

3.2. Matrix removal

For any on-line SPE-LC–MS method, it is a challenge to deal with the matrix effect, especially when biological fluids are detected.

It is well known that milk is a kind of complex biological fluids containing different macromolecule substances, such as proteins, lipids. The existence of these compounds can cause the serious SPE column damage and ionizing suppression. There are several protein precipitation methods, including heat, acid, salt and organic precipitation. In this study, acetonitrile is selected as precipitant at a 2:1 ratio to milk sample. By this treatment, above 90% protein could be effectively removed [29].

Though the proteins were effectively removed by acetonitrile, there are still large amount of polar substances in milk sample such as amino acids, carbohydrates, which also could interfere with the identification and quantification of target compounds. In order to improve the performance of on-line SPE, dispersive SPE with primary secondary amine (PSA) was employed for the further cleanup. This method was introduce by Anastassiades et al. [30] and has gained wide acceptance for the determination of various analytes in recent years [31–33]. In the proposed method, PSA was used to adsorb and eliminate the impurities which were polar with –OH and –COOH in samples. By these treatments, a relative clean chromatogram could be obtained. In Fig. 5, it could be clearly shown that though there still some peaks of co-elutes existed in the chromatogram, the matrix effect is greatly reduced, compared to the result of the same method without dispersive SPE procedure. Furthermore, in the experiment of repeatability evaluation, we found that with appropriate rinse of C$_{30}$ SPE column (5 min, 3.6 mL/min NH$_4$Ac solution) after each analysis, there has no obvious drift of the MS response after consecutive injections (more than eight times). The result demonstrates this method has strong resistance towards the matrix effect.

Besides, the 1 min washing step with 3.6 mL NH$_4$Ac solution (from 2.5 to 3.5 min) also plays very important role in the whole on-line SPE process. In our experiment, we found that it is very easy to cause the high backpressure of SPE without the washing step. The reason is that there are a lot of soluble residues in the SPE column after samples have been loaded. Then if the valve switch and elution step was carried out without washing step, the soluble residues turn to be precipitates with high-content organic solution and hard to be flush out from the SPE column.
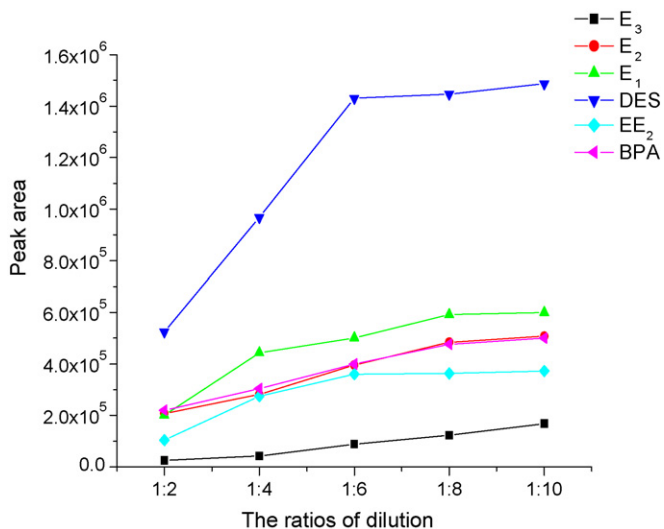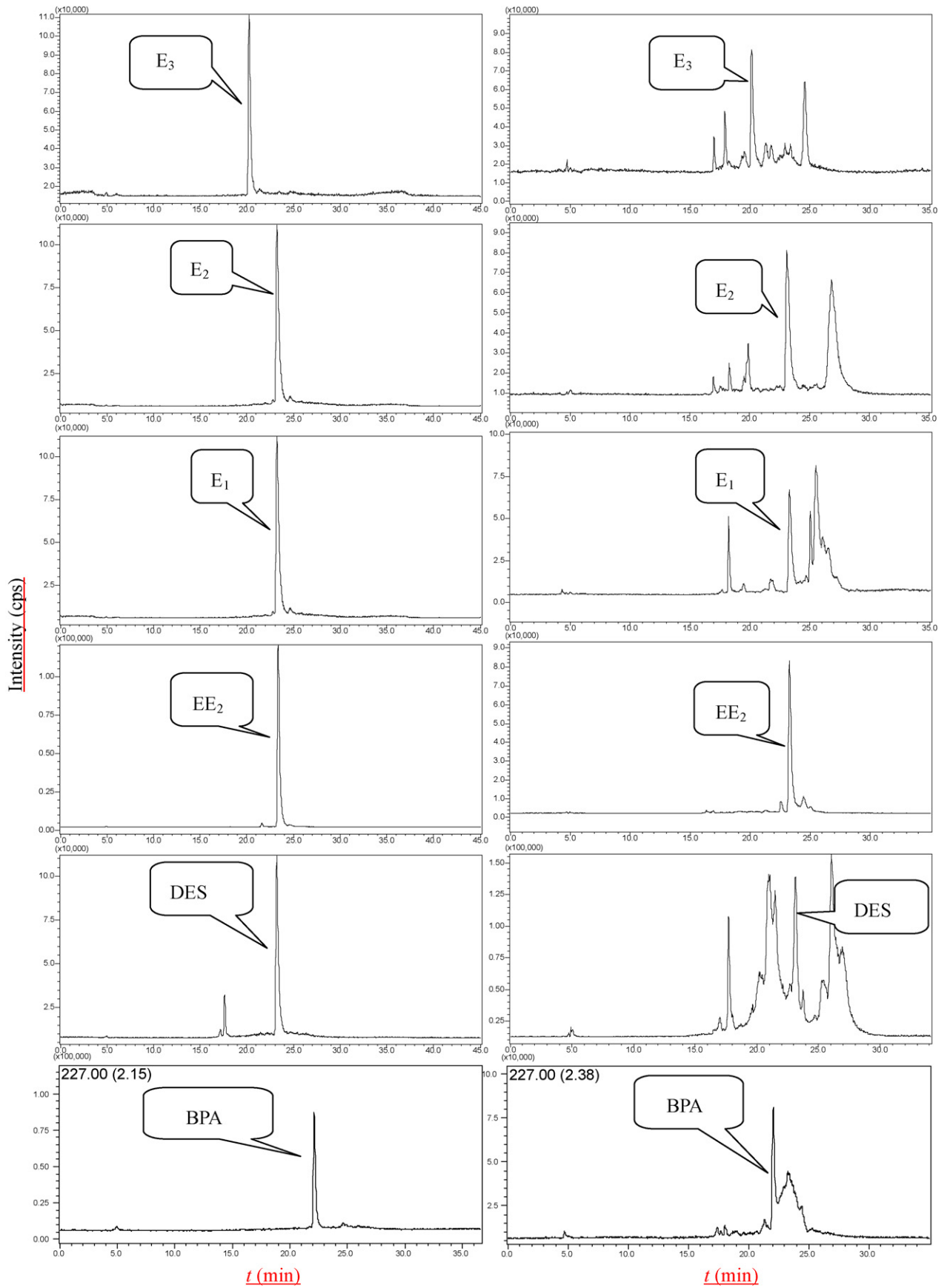

Fig. 3. Effect of the ratios of dilution on the response signals of 1 mL 1 ng/mL standard samples.

Fig. 5. LC–MS Chromatograms obtained from the direct on-line analysis of 10 mL 1.0 ng/mL standard solution (A) and 1 mL milk sample spiked at 3.0 ng/mL of each analyte (B).

Table 2
Limit of detection (ng/mL), calibration curve correlation, recovery mad reputability at two concentrations, and matrix effect evaluation for each analyte.

Analyte	LOD (ng/mL)	R^2	Recovery (RSD)% ($n = 5$)		ME%
			0.6 ng/mL	15 ng/mL	
E_3	0.30	0.995	79.4 (12.8)	71.4 (13.4)	83.6
E_2	0.10	0.994	84.7 (8.6)	82.9 (10.1)	87.9
E_1	0.08	0.997	77.6 (12.9)	72.6 (14.7)	85.3
DES	0.05	0.997	84.9 (10.6)	83.8 (9.9)	94.9
EE_2	0.20	0.998	96.7 (13.7)	94.5 (11.6)	107.8
BPA	0.20	0.996	97.1 (15.0)	92.4 (13.2)	101.3

3.3. Matrix effect

It is well known that matrix effects due to co-extracted and co-eluting matrix substances can seriously affect the analyte signals, such as enhance or suppress the analyte responses. This phenomenon is particularly prevalent for soft ion technologies, such as ESI.

To evaluate the matrix effect, we compared the MS area responses of standards in spiked milk samples (a) with those spiked in purified water (b). The ratio ($a/b \times 100$) is defined as the matrix effect (ME%) [4]. A value of 100% means that there is no matrix effect. There is enhancement if the value is >100% and signal suppression if the value is <100%.

The result in Table 2 indicated that the matrix effects for all analytes were between 83.6 and 107.8%. It can be concluded that the sample pretreatment is successful and no serious matrix effect is observed.

3.4. Method performance

Under the optimized condition, the method performance was evaluated by the determination of the sensitivity, linearity, repeatability, and accuracy of the method. The result was shown in Table 2.

Calibration curves were established using least-square linear regression from injection of 1 mL spiked mike sample at concentrations from 0.3 to 15.0 ng/mL for each analyte. All the R^2 values were higher than 0.994, which demonstrated exact linear signal response.

The LOD of each analyte was investigated from the spiked milk sample at the signal-to-noise ratio (S/N) ratio of 3:1. The results in Table 2 show that the all the LODs of analytes were at low-ng/mL level.

The accuracy and repeatability of this method were also evaluated by recoveries of spiked samples at 0.6 and 15 ng/mL. Most recovery percentages were between 75 and 100%, except E_1 and E_3 at the spiked concentration of 15 ng/mL. The RSD values seem relative high which were at range of 8.6–15.0%. It is most probably due to the matrix effect which not only causes ion suppression but also introduces the unstable ionization.

Compared to traditional off-line SPE coupled with HPLC–single quadruple MS method [16], lower LODs could be obtained in this method. It most probably owes to the LVI mode which enhances the method sensitivity. Besides, the proposed method introduces less matrix effect than the traditional one, because no significant signal suppression was observed while only 52% of recovery without internal standard was obtained by traditional method [16].

4. Conclusion

A high speed and robust on-line SPE-HPLC–MS method was established for high-throughput analysis of five estrogens and BPA in milk samples. For an 8 h working day, 8–10 analysis could be accomplished and no noticeable deterioration of sensitivity and C_{30} column backpressure were observed after totally 100 injections.

There are mainly two advantages in our experiment. The one is that triacontyl bonded silica (C_{30}) is firstly introduced as on-line cleanup and SPE material. The results demonstrated that the properties of long alky chains and relatively large particle sizes enable the C_{30} SPE column to treat samples at high-flow rate (ca. 4 mL/min) with satisfactory recovery and low backpressure.

The other one is large volume injection. It is obvious that LVI can improve the sensitivity and LOD of the on-line analytical method since it provide bigger concentration factor. In our study, 1 mL milk sample could be loaded which breaks the injection volume limit of autosampler and a concentration factor as high as 100 times could be obtained. As for the sample treatment procedure, dispersive SPE, which is described as QuEChERS (quick, easy, cheap, effective, rugged, and safe) is combined with protein precipitation by organic solvent. This combination is very effective to remove most of polar and nonpolar matrix substances. It is especially important for the single quadruple MS.

In the optimized conditions, the proposed method was also validated by milk sample spiked with analytes mixture. Though the LODs were not low enough for actual milk detection, the main purpose was to describe a simple and fast on-line LC–MS method for determination of estrogens in milk samples with a new on-line SPE material (C_{30}). With satisfactory recovery, repeatability and efficiency, We believe that this analytical method is promising to be applied for the analysis of estrogens and BPA in biological matrices with other more sensitive detectors, such as triple quadrupole MS.

Acknowledgment

References

[1] E.P.M. Grist, N.C. Wells, P. Whitehouse, G. Brighty, M. Crane, Environ. Sci. Technol. 37 (2003) 1609.
[2] M. Kuster, M.J.L. de Alda, D. Barceló, Trends Anal. Chem. 23 (2004) 790.
[3] Y.K.K. Koh, T.Y. Chiu, A. Boobis, E. Cartmell, J.N. Lester, M.D. Scrimshaw, J. Chromatogr. A 1173 (2007) 81.
[4] A. Salvador, C. Moretton, A. Piram, R. Faure, J. Chromatogr. A 1145 (2007) 102.
[5] H. Noppe, K. Verheyden, W. Gillis, D. Courtheyn, P. Vanthemsche, H.F. De Brabander, Anal. Chim. Acta 586 (2007) 22.
[6] X. Xu, T.D. Veenstra, S.D. Fox, J.M. Roman, H.J. Issaq, R. Falk, J.E. Saavedra, L.K. Keefer, R.G. Ziegler, Anal. Chem. 77 (2005) 6646.
[7] X. Xu, J.M. Roman, H.J. Issaq, L.K. Keefer, T.D. Veenstra, R.G. Ziegler, Anal. Chem. 79 (2007) 7813.
[8] J. Russo, I.H. Russo, J. Steroid Biochem. Mol. Biol. 102 (2006) 89.
[9] L.-Q. Qin, P.-Y. Wang, T. Kaneko, K. Hoshi, A. Sato, Med. Hypotheses 62 (2004) 133.
[10] H. Mallekinejad, P. Scherpenisse, A.A. Bergwerff, J. Agric. Food Chem. 54 (2006) 9785.
[11] H. Noppe, B. Le Bizec, K. Verheyden, H.F. De Brabander, Anal. Chim. Acta 611 (2008) 1.
[12] F. Courant, J.P. Antignac, J. Laille, F. Monteau, F. André, B. Le Bizec, J. Agric. Food Chem. 56 (2008) 3176.
[13] F. Toldra, M. Reig, Trends Food. Sci. Technol. 17 (2006) 482.
[14] B. Shao, R. Zhao, J. Meng, Y. Xue, G. Wu, J. Hu, X. Tu, Anal. Chim. Acta 548 (2005) 41.
[15] Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a Request from the Commission Related to 2,2-Bis(4-Hydroxyphenyl) Propane (Bisphenol A), European Food Safety Authority (EFSA), Parma, 2007. http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178710289744.htm.
[16] N.C. Maragou, E.N. Lampi, N.S. Thomaidis, M.A. Koupparis, J. Chromatogr. A 1129 (2006) 165.
[17] D.K. Alexiadou, N.C. Maragou, N.S. Thomaidis, G.A. Theodoridis, M.A. Koupparis, J. Sep. Sci. 31 (2008) 2272.
[18] B. Shao, H. Han, X. Tu, L. Huang, J. Chromatogr. B 850 (2007) 412.
[19] X. Ye, L.J. Tao, L.L. needham, A.M. Calafat, Talanta 76 (2008) 865.
[20] K.E. Pickl, C. Magnes, M. Bodenlenz, T.R. Pieber, F.M. Sinner, J. Chromatogr. B 850 (2007) 432.
[21] M. Li, Y. Alnouti, R. Leverence, H. Bi, A.I. Gusev, J. Chromatogr. B 825 (2005) 152.

[22] H. Noppe, B. Le Bizec, K. Verheyden, H.F. De Brabamder, Anal. Chim. Acta 611 (2008) 1.
[23] K. Li, H. Li, L. Liu, Y. Hashi, T. Maeda, J.-M. Lin, J. Chromatogr. A 1154 (2007) 74.
[24] W. Yan, L. Zhao, Q. Feng, J.-M. Lin, J. Sep. Sci. 31 (2008) 3581.
[25] P. Marchand, B. Le Bizec, C. Gade, F. Monteau, F. André, J. Chromatogr. A 867 (2000) 219.
[26] S. Hartmann, H. Steinhart, J. Chromatogr. B 704 (1997) 105.
[27] M. Liu, W. Yan, J.-M. Lin, Y. Hashi, L.-B. Liu, Y. Wei, J. Chromatogr. A 1198 (2008) 87.

[28] A.-C. Liu, T.-Y. Lin, L.-W. Su, M.-R. Fuh, Talanta 75 (2008) 198.
[29] C. Polson, P. Sarkar, B. Incledon, V. Raguvaran, R. Grant, J. Chromatogr. B 785 (2003) 263.
[30] M. Anastassiades, S.J. Lehotay, D. Štajnbaher, F.J. Schenck, J. AOAC Int. 86 (2003) 412.
[31] M. Liu, Y. Hashi, Y.Y. Song, J.-M. Lin, J. Chromatogr. A 1097 (2005) 183.
[32] L.-B. Liu, Y. Hashi, Y.P. Qin, H.X. Zhou, J.-M. Lin, J. Chromatogr. B 845 (2007) 61.
[33] F. Ji, L. Zhao, W. Yan, Q. Feng, J.-M. Lin, J. Sep. Sci. 31 (2008) 961.