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Determination of Phenolic Environmental Estrogens in Eggs by High Performance Liquid Chromatography and Sample Preparation with Matrix Solid Phase Dispersion

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Simultaneous determination of the phenolic environmental estrogens in egg by high performance liquid chromatography (HPLC) and sample preparation with matrix solid phase dispersion (MSPD) was developed. A 0.2 g of sample was placed into an agate mortar and gently blended with 0.4 g florisil to obtain a homogeneous mixture. This mixture was introduced into a Teflon cartridge. The estrogens fraction was eluted from the cartridge with dichloromethane. The residue was dissolved with methanol after evaporation of the solvent (dichloromethane). The estrogens (bisphenol A, diethylstilbestrol and nonylphenol) were separated on a ZORBAX stable bound (4.6 mm × 50 mm, 1.8 μm) C_{18} column with acetonitrile and water (80:20) as the mobile phase and detected with photodiode array detector. This method provides good reproducibility and sensitivity for the quantification of bisphenol A, diethylstilbestrol and nonylphenol. The relative standard derivations of overall intra-day variations were less than 2.6 % and the relative standard derivations of inter-day variations were less than 2.9 %. The standard recoveries (three different concentrations of markers: 0.5, 1.0 and 5.0 μg) were ranged from 93-104 %.

Key Words: Phenolic environmental estrogens, High performance liquid chromatography, Matrix solid phase dispersion, Egg.

INTRODUCTION

Bisphenol A, diethylstibestrol and nonylphenol belong to the phenolic environmental estrogens, have estrogen activity and antagonizing androgen effect. These compounds can enter human body through food chain and cumulated in human body. The toxicity of estrogens had received more and more attentions[1-4]. Therefore, the determination of their residues in food is very important.

HPLC is an analytical method with the advantage of direct detection and simple operation for phenolic environmental estrogens. Several works on the HPLC methods for the quantitation of estrogens were reported[5-8]. However, these methods usually need a tedious samples preparation or long time for chromatographic separation. Matrix solid-phase dispersion (MSPD) has also been successfully applied for the

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isolation of target molecules from biological matrices[8-12]. This procedure can considerably reduce the sample size and the solvent consumption. Therefore, in this paper, a simple and sensitive HPLC analytical method for the simultaneous determination of bisphenol A, diethylstibestrol and nonylphenol using ZORBAX stable bound rapid analysis column and sample preparation MSPD was developed and this method had been applied to the analysis of bisphenol A,diethylstibestrol and nonylphenol in egg samples with good results.

EXPERIMENTAL

The HPLC analysis was performed on a Waters 2695 Alliance separation system with a 996 photodiode array detector (Waters Corporation, Milford., MA 01757, USA). A ZORBAX Stable Bound column (4.6 mm × 50 mm, 1.8 μm) (Agilent Technologies Inc, Santa Clara., CA 95051, USA) was utilized. The florisil used in analytical experiment was 400 mesh purchased from Merck company (Germany).

The bisphenol A, diethylstilbestrol and nonylphenol reference standards were obtained from Shanghai Usea Biotech Company (Shanghai P.R. China). The purities were ≥ 98 %.

HPLC grade acetonitrile (mobile phase) and dichloromethane (for sample preparation) were provided by Fisher Scientific Inc (Madison, WI 53711, USA). The ultrapure water used was obtained from a Milli-Q50 SP Water system (Millipore Inc, MA 01730 Bedford). The mobile phase used is acetonitrile and water (80:20) at a flow-rate of 2.5 mL min^{-1}. The optimal detected wavelength was 270 nm. The sample injection volume is 20 μL. The chromatogram of estrogens standards and egg sample at 270 nm is shown in Fig. 1.

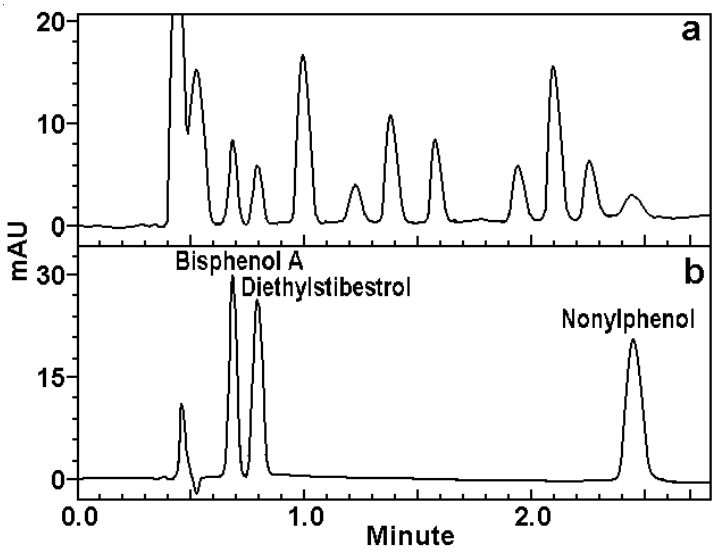

Fig. 1. Chromatogram of estrogens standard (b) and egg sample (a)

Preparation of sample: The egg samples were dried by vacuum at room temperature and then pulverized to 80 mesh. A 0.2 g of sample was placed into an agate mortar containing 0.4 g of florisil (400 mesh), the mixture was gently blended with a pestle. Once the mixture was homogeneous, it was then transferred into the top of an 8 mm × 20 mm Teflon cartridge (Fig. 2) containing 0.2 g florisil (400 mesh). The cartridge was eluted with 10 mL dichloromethane to obtain the fraction containing estrogens. The estrogens fraction eluent was evaporated to dryness by nitrogen stream and the residue was dissolved precisely in 1.0 mL methanol. This methanol solution was filtered through a 0.45 μm syringe filter and ready for HPLC analysis.

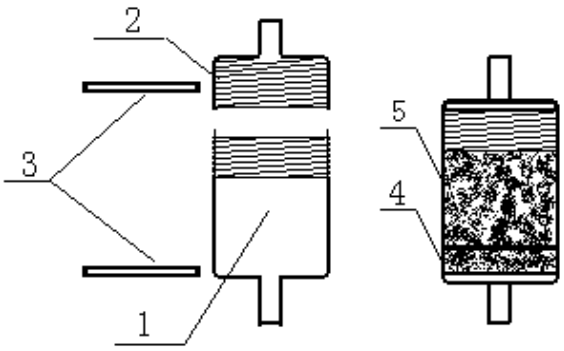

Fig. 2. The cartridge (1) tube for fill in samples, (2) screw cap for sealing the tube), (3) sieve plate, (4) florisil, (5) the mixture of sample and florisil

Preparation of standard solution: To prepare standard solutions, an accurately weighed amount of bisphenol A, diethylstilbestrol and nonylphenol which were dissolved in methanol for HPLC. Five concentrations were chosen, with the range 0.1-25 μg mL^{-1}, respectively.

RESULTS AND DISCUSSION

Matrix solid-phase dispersion (MSPD) has been successfully applied for the isolation of target molecules from biological matrices. The mechanism of MSPD includes sample homogenization, cellular disruption, exhaustive extraction, fractionation and purification into a simple process. Matrix solid-phase dispersion technology involves blending of a small amount of matrix with an appropriate sorbent followed by washing and elution of compounds with a small volume of solvent. The procedure can considerably reduce the sample size and the solvent consumption. Therefore, MSPD was selected as sample preparation method in this work.

Different parameters that affect MSPD extraction such as dispersant agent and eluent solvent were studied. The polar solid phase (silica gel, alumina and florisil) and non-polar solid phase (C_{18}, graphite carbon black) were tested for matrix dispersion. High recoveries (> 95 %) were obtained when use C_{18} (methanol-water system as eluent) and florisil (dichloromethane as eluent) as dispersant agent. The florisil is

cheaper than the C_{18}. Therefore, the florisil (400 mesh) was selected as dispersant agent and the dichloromethane was selected as eluent in this experiment.

To evaluate the elution volume, 4, 6, 8, 10, 12, 14 and 16 mL dichloromethane to perform elution were studied. The results show that the estrogens can be eluted from cartridge completely with 10 mL chloromethane used. However, further increase of eluent volume can cause more interfering compounds eluted from the cartridge. Therefore, 10 mL of dichloromethane was selected as eluent in this experiment.

Optimal chromatographic condition was obtained after testing different mobile phase systems with two reversed-phase columns (C_8 and C_{18}). In the case of the C_8 column, the bisphenol A and diethylstibestrol could not be resolved as a baseline separation. However, bisphenol A and diethylstibestrol were resolved well with a baseline separation when using the C_{18} column. Furthermore, among various mobile phases examined, the mobile phase of acetonitrile and water (80:20) was found to be the best separation. Therefore, acetonitrile-water (80:20) was selected as mobile phase in this experiment. To shorten the chromatographic separation time, a ZORBAX stable bound rapid analysis column (4.6 mm × 50 mm, 1.8 μm) was used in this experiment. With this rapid analysis column, the estrogens were separated completely within 2.5 min (Fig. 1). In comparison to the previous literature[5-8], this is one of the most rapid methods to separation estrogens. The absorption spectrum of bisphenol A, diethylstilbestrol and nonylphenol were obtained with a 996 photodiode array detector. Results show that the analyts have the strong absorption at 270 nm. Therefore, the 270 nm was selected as detecting wavelength.

Under the optimum conditions, the regression equations of estrogens were established based on the standard samples injected and their peaks area. The limits of detection are calculated by the ratio of signal to noise (S/N = 3). The results were shown in Table-1. The reproducibility of this method was also examined for 1.0 μg mL^{-1} of the estrogens. The relative standard deviations (n = 9) were shown in Table-1.

TABLE-1
REGRESSION EQUATION, COEFFICIENT AND DETECTION LIMIT

Components	Regression equation C (μg mL^{-1})	Linearity range (μg mL^{-1})	Coefficient	Detection limits (ng mL^{-1})	RSD % (n = 9)
Bisphenol A	$A = 4.16\times10^5$ C - 202	0.08~20	r = 0.9994	10	2.0
Diethylstilbestrol	$A = 4.74\times10^5$ C + 116	0.05~18	r = 0.9995	8	1.9
Nonylphenol	$A = 3.87\times10^5$ C + 162	0.1~25	r = 0.9994	12	2.2

Method recovery and precision: The recovery tests were carried out by adding bisphenol A, diethylstilbestrol and nonylphenol to the samples (three different concentrations of markers: 0.5, 1.0 and 5.0 μg). The sample was prepared as above and injected for HPLC analysis to calculate the amount of the estrogens. The results shown that the recoveries (n = 5) were ranged from 93-104 %.

The measurements of intra-day and inter-day variability (determination of the same samples for 7 times) were utilized to determine the precision of the developed method. The results show that the relative standard derivation of overall intra-day variations were less than 2.6 % and of inter-day variations were less than 2.9 %.

Conclusion

The proposed HPLC method enables simultaneous determination of bisphenol A, diethylstilbestrol and nonylphenol because of good separation and resolution of the chromatographic peaks within 2.5 min. In comparison to the routine chromatographic method, more than 80 % of separation time was saved. It is one of the most rapid methods for chromatographic analysis of the estrogens. The matrix solid-phase dispersion was used as sample preparation method. Matrix solid-phase dispersion combines both sample homogenization and extraction of the analyzed compounds in one step. It considerably reduced the sample size and the solvent consumption. The method precision and recovery are higher than that of traditional solvent extraction and solid phase extraction method. Thus, this method is rapid, high sensitive and selective and provides good reproducibility and accurateness for the quantification of the estrogens.

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