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Effect of protein on the detection of stilbene estrogens in milk

Xingmei Zhang, Qiaoqiao Deng, Peipei Liu, Guizhi Li and Yongming Liu*

Fluorescence spectrometry was used to investigate the binding interactions of bovine serum albumin (BSA) with three stilbene estrogens (hexestrol, diethylstilbestrol and dienoestrol). And further research into the binding ratios between stilbene estrogens and actual milk sample was carried out by equilibrium dialysis method. Meanwhile, the effect of protein on extraction efficiency of stilbene estrogens in milk sample was investigated in detail. The results show that stilbene estrogens strongly bound with milk sample. In 70% (V/V) ethanol-water extracting solution, the protein matrix of sample takes place a slow but full denaturation, which causes the drugs bonded with protein to be released. Then appropriate amount of K$_2$HPO$_4$ was added to above extracting solution to form a stable aqueous two phase system. Following, the fat-soluble stilbene estrogen residues were extracted into upper phase with high extraction efficiency. Purification steps were omitted in this work because the fat-soluble impurities were extracted less in 70% (V/V) ethanol-water solution than those in hydrophobic solvents as liquid-liquid extraction procedure.

The proposed approach was satisfactorily applied to the quick determination of stilbene estrogen residues in milk by High Performance Liquid Chromatography (HPLC). Overall recoveries were 83.2~93.8% with RSD values less than 4.52%, and the detection limits were in the range of 11.7~20.7 ng/g. The sample preparation method was straightforward, efficient, economically advantageous and environment-friendly.

Introduction

Stilbene estrogens are synthetic estrogenic substances, including hexestrol (HEX), diethylstilbestrol (DES) and dienoestrol (DEN). Due to their anabolic effects, stilbene estrogens have been widely used for animal fattening in animal husbandry, especially in dairy farming. This means that their residues may be found in milk samples. In spite of their great usefulness, stilbene estrogens are responsible for several detrimental effects, such as endocrine disorders and even cancer in hormone-dependent organs (e.g., breast, ovaries, prostate and testicles) for the excess of stilbene estrogens in the human diet produces. Therefore, rapid and accurate determination of stilbene estrogens in milk samples becomes an important task that must be addressed for the food safety and human health.

However, the key problem for accurate and effective determination of drug residues in milk samples is sample preparation. Because the trace drug residues in milk samples coexist with large amount of protein and fat which
could affect the extraction efficiency of the drugs. Several studies have been reported on stilbene estrogen residues extraction from milk samples using liquid-liquid extraction followed by solid-phase extraction purification method\(^4\). The disadvantages of these methods are complicated for steps of extraction and purification, time-consuming, a large amount of harmful solvent-consuming, and analyte-lossing for some ones. E.M. Malone and C.T. Elliottbetal simplified the purification steps by using matrix solid-phase dispersion method\(^5\). But in their method, poisonous acetonitrile was used as extraction solvent. Therefore, to develop a rapid, efficient and environment-friendly sample pretreatment method for laboratories becomes an urgent need.

Studying the existing state of drug residues in matrix is the premise for developing the sample pretreatment techniques. Recently, the effect of matrixes on the extraction efficiency of drug residues has already attracted the analyst’s attention\(^6\). Protein is the main matrix material in the determination of stilbene estrogen residues in milk samples. In the previous reports, some small drug molecules are highly bound to proteins\(^7\)\(^-\)\(^11\). Logically, the coexistence of the protein matrix and stilbene estrogens will inevitably decrease the extraction efficiency of the drugs in milk samples. Therefore, the interactions between stilbene estrogens and protein (or actual milk sample) should be studied deeply. For this reason, fluorescence spectrometry and equilibrium dialysis method were jointly applied to study the binding interactions in this work.

Recent research activities are oriented towards the development of simple, quick, efficient, and economical sample pretreatment methods. Aqueous two phase extraction (ATPE) technology is a green and efficient extraction technology which developed in recent years. Phase separation from a homogeneous solution by creating a second immiscible phase, ATPE technology has been investigated as an alternative to the traditional liquid-liquid extraction. Previous researches indicated that water-soluble organic solvent (such as ethanol, isopropanol and acetonitrile etc.), inorganic salt and water could form simple aqueous two phase system, which has been used in the determination of drug residues\(^12\)\(^-\)\(^14\).

In the survey by Kai Griebenow et al.\(^15\) and our previous research\(^8\)\(^,\)\(^16\), proteins are more denatured in aqueous-organic mixtures than in the corresponding pure organic solvents. The protein denaturation may give rise to the bonded drugs being released. This paper attempt to select the ATPE of water and ethanol as extraction system for extract the drugs in protein matrix sample effectively. Since the high polarity of the ethanol-water solution, the fat-soluble impurities would be extracted less in extraction solution. Subsequent purification steps could be simplified probably. This research aims to establish a simple, rapid and high efficient novel method for milk sample pretreatment.

**Experimental**
Reagents and standards

Methanol, acetonitrile, ethanol, isopropanol and tetrahydrofuran of HPLC grade were purchased from Kernel Chemical Reagent Company (Tianjin, China); Bovine serum albumin (BSA, purity>98%) with molecular weight of 68000, was purchased from Sigma Chemical Company, stock solution of 1.0×10⁻³ mol/L was prepared in Tris-HCl buffer (pH=7.4). K₂HPO₄ (AR) and NaH₂PO₄ (AR) were obtained from Guoyao Chemical Ltd (Shanghai, China). Dialysis bags MD34 (3500, Viskase) are made in USA. Stilbene estrogens (purity 92.0–97.0%) were obtained from National Institute for the Control of Pharmaceutical and Biological Products. Stock solutions of 1000 µg/mL were prepared in methanol medium. Working standard solutions were prepared by diluting stock standard solutions with methanol and were stored in a freezer at 4 °C.

Apparatus

A Qilinbeier QL-861 vortex apparatus (Haimen, China) was used for initial extraction procedure. THZ-312 thermostatic oscillator (Shanghai, China) was used in equilibrium dialysis procedure. Fluorescence measurement was carried on a Varian Cary Eclipse fluorescence spectrophotometer equipped with a Varian Cary single cell peltier accessory (Victoria, Australian) to maintain the temperature at 293 K, 298 K and 310 K, respectively. The analyses were performed by Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA), which equipped with a diode array detector (DAD) and reversed-phase Zorbax Eclipse C₁₈ column (Agilent, 250 mm×4.6 mm, 5.0 µm particle size). The optimized mobile phase was composed of 61% (v/v) methanol and 39% (v/v) 0.01 mol/L NaH₂PO₄ aqueous solutions. Before use, the mobile phase was filtered and degassed. The flow rate was kept at 1.0 mL/min. The DAD wavelength was set at 230 nm for three analytes. The column oven temperature was maintained at 40 °C. The injection volume was 20 µL.

Procedures

Fluorescence quenching study. 3.0 mL BSA solution (2.0×10⁻⁴ mol/L) prepared in Tris–HCl buffer (pH=7.4) was transferred to 1.0 cm quartz cell. Then a portion of single stilbene estrogen solution was gradually injected into the cell using microsyringe. The accumulated volume was smaller than 100 µL to avoid unnecessary volume increment. Under the fluorescence spectrophotometer with an entrance and exit slit wide of both 5 nm, fluorescence quenching spectra were obtained in the range of 290–450 nm with λex=280 nm.

Equilibrium dialysis study. 40 mL high purity water was added to 13 beakers (200 mL) respectively, 1.0 mL milk was accurately pipetted in dialysis bags, a series of different volumes of stock standard solutions were gradually injected into the milk using microsyringe. The volumes were all smaller than 100 µL to avoid unnecessary volume increment. Then 9.0 mL high purity water was added to every dialysis bag. Sealed with clips
at both ends of the dialysis bags, the solutions in the dialysis bags were mixed evenly and then immersed into the water of beakers. Then all the beakers were put into the thermostatic oscillator, equilibrium dialysis conducted for 24 hours under a constant temperature of 25 °C, the solutions outside of the dialysis bags were transferred for HPLC analysis after filtered with 0.45 μm micro-pore filter membrane.

**Sample preparation.** Previously homogenized milk samples (1.0 mL) were accurately pipetted into 20 mL teflon centrifuge tubes, and 2.0 mL high purity water and 7.0 mL ethanol were added. The tubes were capped tightly and vortexed for approximately 1 min. Then K$_2$HPO$_4$ (1.5 g) was added to the sample tubes. The tubes were capped tightly and vortexed for another 30 s immediately followed by standing for 30 min. Then the supernatant was determined by HPLC system after filtered with 0.45 μm micro-pore filter membrane.

**Results and discussion**

**Binding interactions between stilbene estrogens and protein**

Protein is the main matrix material in the determination of stilbene estrogens in milk sample. Because BSA is the protein which molecular structure and molecular weight has been confirmed, usually, it was used as model to study the interactions of drugs and protein in theory$^{17,18}$. In this section we investigated the interactions between stilbene estrogens and BSA by fluorescence quenching method.

![Fig.1](image.png)

**Fig.1** The quenching fluorescence spectra of BSA by DES at 298 K

$\lambda_{ex}/\lambda_{em} = 280/345$ nm; $C_{BSA}=2.0 \times 10^4$ mol/L

$10^6 C_{DES}$(mol/L), 1→11: 0.00, 1.24, 2.48, 3.72, 4.96, 6.20, 7.44, 8.68, 9.92, 11.16, 12.40

The quenching fluorescence spectra were shown in Fig.1 (take DES as an example). The intrinsic fluorescence
of BSA at 345 nm could be quenched regularly with increasing the concentration of the stilbene estrogens. According to the literature\textsuperscript{10-11,19}, the binding sites $n$, binding constants $K_B$ and thermodynamic parameters ($\Delta H$, $\Delta G$, $\Delta S$) between BSA and three drugs were calculated. The results were shown in Table 1. That the binding sites $n$ were about 1 and the binding constants $K_B$ > $10^4$ L/mol, manifested that three drugs existed with a high binding affinity to BSA. The $\Delta G$ values of all the three drugs were negative, showing that the binding processes were spontaneous. $\Delta H < 0$ and $\Delta S < 0$ indicated that the acting forces of BSA-HEX and BSA-DES were mainly hydrogen bond and van der Waals forces. While $\Delta H > 0$ and $\Delta S > 0$ in the BSA-DEN procedure indicated the hydrophobic effect involved predominantly.

As protein is the dominating matrix in milk samples, the strong binding interactions between three stilbene estrogens and protein would influence the extraction efficiency of drugs. Therefore, the effect of protein must be eliminated to get good recoveries for the extraction of stilbene estrogen residues in milk samples.

\textbf{Table 1} The binding sites $n$, binding constants $K_B$ and thermodynamic parameters of stilbene estrogens-BSA

<table>
<thead>
<tr>
<th>Analytes</th>
<th>$T$ (K)</th>
<th>$n$</th>
<th>$K_B$ (L/mol)</th>
<th>$\Delta H$ (kJ/mol)</th>
<th>$\Delta G$ (kJ/mol)</th>
<th>$\Delta S$ (J/mol/K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEX</td>
<td>293</td>
<td>0.97</td>
<td>$3.29 \times 10^4$</td>
<td>-32.61</td>
<td>-20.36</td>
<td>-41.11</td>
</tr>
<tr>
<td>DES</td>
<td>298</td>
<td>0.96</td>
<td>$5.15 \times 10^4$</td>
<td>-346.94</td>
<td>-29.50</td>
<td>-1047.66</td>
</tr>
<tr>
<td>DEN</td>
<td>298</td>
<td>1.01</td>
<td>$3.53 \times 10^4$</td>
<td>54.17</td>
<td>26.25</td>
<td>266.53</td>
</tr>
<tr>
<td></td>
<td>303</td>
<td>1.03</td>
<td>$3.92 \times 10^4$</td>
<td>54.17</td>
<td>27.86</td>
<td>264.61</td>
</tr>
</tbody>
</table>

\textbf{Binding ratios between stilbene estrogens and actual milk sample}

In the above study, BSA was used as protein model to get the binding constants $K_B$ and other thermodynamic parameters of interactions between protein and stilbene estrogens in theory. In order to verify the real existing state of the drugs in milk sample, in this section, actual milk sample was selected as research object. And equilibrium dialysis method\textsuperscript{20-22} was used.

Binding ratios between three stilbene estrogens and milk sample were shown in Table 2. The calculation formula
of binding ratio: Binding ratio = \( \frac{m_{\text{absorbed}}}{m_0} \times 100\% \), where, \( m_0 \) the mass of drug initially added in dialysis bag; \( m_{\text{absorbed}} \) the mass of drug milk sample adsorbed.

**Table 2** Binding ratios between three stilbene estrogens and milk sample

<table>
<thead>
<tr>
<th>( m_0 (\mu g) )</th>
<th>1.00</th>
<th>2.00</th>
<th>5.00</th>
<th>10.0</th>
<th>15.0</th>
<th>20.0</th>
<th>30.0</th>
<th>40.0</th>
<th>50.0</th>
<th>60.0</th>
<th>70.0</th>
<th>80.0</th>
<th>90.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>( m_{\text{absorbed}} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DES (\mu g)</td>
<td>1.00</td>
<td>2.00</td>
<td>4.48</td>
<td>9.04</td>
<td>14.4</td>
<td>19.5</td>
<td>29.3</td>
<td>39.3</td>
<td>49.3</td>
<td>59.0</td>
<td>69.3</td>
<td>74.0</td>
<td>88.2</td>
</tr>
<tr>
<td>DEN (\mu g)</td>
<td>1.00</td>
<td>2.00</td>
<td>4.66</td>
<td>8.72</td>
<td>14.7</td>
<td>19.5</td>
<td>29.1</td>
<td>39.0</td>
<td>49.5</td>
<td>58.9</td>
<td>69.4</td>
<td>78.8</td>
<td>88.1</td>
</tr>
<tr>
<td>HEX (\mu g)</td>
<td>1.00</td>
<td>2.00</td>
<td>4.49</td>
<td>8.76</td>
<td>14.3</td>
<td>19.5</td>
<td>29.1</td>
<td>38.8</td>
<td>49.6</td>
<td>59.0</td>
<td>69.3</td>
<td>78.3</td>
<td>88.0</td>
</tr>
<tr>
<td>Binding ratio DEN (%)</td>
<td>100</td>
<td>100</td>
<td>93.1</td>
<td>98.1</td>
<td>97.3</td>
<td>97.1</td>
<td>97.5</td>
<td>98.9</td>
<td>98.2</td>
<td>99.1</td>
<td>98.5</td>
<td>97.8</td>
<td></td>
</tr>
<tr>
<td>Binding ratio HEX (%)</td>
<td>100</td>
<td>100</td>
<td>89.7</td>
<td>96.0</td>
<td>97.5</td>
<td>97.6</td>
<td>98.3</td>
<td>98.6</td>
<td>98.3</td>
<td>99.0</td>
<td>98.0</td>
<td>98.0</td>
<td>98.0</td>
</tr>
</tbody>
</table>

As shown in Table 2, the adsorption capacities of milk for three drugs were quite similar. The calculated results revealed that the binding ratios between stilbene estrogens and actual milk sample were all in the range of 87.2% ~100%, indicating the strong binding interactions between them. Referencing the results of fluorescence quenching study, we could infer that drugs mainly combined with the protein in milk sample. Thus, how to destroy the strong binding interactions and to extract the drugs fully becomes a top priority.

**Effects of different matrices on extraction efficiency**

The effects of protein and butter on extraction efficiency of stilbene estrogens were investigated. 0.5 mL water or BSA solution (1.0×10^{-3} mol/L) and 20.0 mg butter in duplicate were transferred to six tubes, respectively. Then 5.0 \( \mu L \) stock standard solutions were added to each tube and all stand for 30 min. The sample solutions were prepared by using the extraction method in reference 6.

**Table 3** The effects of different matrices on extraction efficiency of stilbene estrogens (n=3)

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Water as matrix</th>
<th>BSA as matrix</th>
<th>Butter as matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E (%)</td>
<td>RSD (%)</td>
<td>E (%)</td>
</tr>
<tr>
<td>1-DES</td>
<td>88.2</td>
<td>2.18</td>
<td>78.7</td>
</tr>
<tr>
<td>2-DEN</td>
<td>92.1</td>
<td>1.97</td>
<td>82.5</td>
</tr>
<tr>
<td>3-HEX</td>
<td>99.1</td>
<td>1.23</td>
<td>80.6</td>
</tr>
</tbody>
</table>

Note: E- extraction efficiency (%).
The results in Table 3 showed that based on BSA as matrix, the extraction efficiency (78.7–82.5%) was lower than those based on water as matrix (88.2–99.1%) and butter as matrix (89.9–96.6%). It suggested that the interactions between stilbene estrogens and BSA decreasing the extraction efficiency of stilbene estrogens in milk sample. Meanwhile, the extraction efficiency based on butter as matrix was close to those for water as matrix. It indicated that butter had little influence on extraction efficiency of stilbene estrogens.

Selection of extraction solvent

As previous reported, protein is more denatured in aqueous-organic mixtures than in the corresponding pure organic solvents. While pretreating the milk sample, as long as suitable water-soluble organic solvent with appropriate concentration was chosen as extracting agent, the protein in milk may be denaturation completely and the drugs combined with the protein could be dissociated. Four common organic extraction solvents (acetonitrile, ethanol, isopropanol and tetrahydrofuran) were chosen to conduct this work. K$_2$HPO$_4$ was selected for phase separation because of its high solubility in water and referencing some existing researches. Further experimental explores found that stable aqueous two phase systems (ATPS) could form when appropriate amount of K$_2$HPO$_4$ added to the above four organic extraction solvents, respectively.

This paper discussed the extraction efficiency of stilbene estrogens using the four ATPS as extracting methods. The influence of different extraction solvents was studied as follows: 1.0 mL milk was pipetted into four 20 mL teflon centrifuge tubes, respectively, and 5.0 µL three stock standard solutions were added to every tube. The samples were left to stand for 30 min. Then the same volume fraction of four organic solvents was added, respectively. The following procedures were performed according to the “Sample preparation” section.

Experimental results showed that when isopropanol and tetrahydrofuran were used as extraction solvents, too many impurities were extracted in solutions because of the strong dissolving capacity of these two organic solvents. Certainly it would affect the determination of three drugs. But when using acetonitrile and ethanol as extraction reagents, higher extraction efficiency for drugs achieved and fewer impurities left in extraction solutions. Focusing on the concept of environmental protection and non-toxic harmless for analyst in modern sample pretreatment technology, ethanol was the best to be selected as the organic extraction solvent for pretreatment of the milk sample.

Effect of ethanol concentration on extraction efficiency

In order to further clarify the effect of protein denaturation on the extraction of stilbene estrogens and to find
suitable concentration of ethanol to extract drugs, different volume fraction ($\phi$) of ethanol from 50% to 90% was used. Operated as follows: In five 20 mL teflon centrifuge tubes, 1.0 mL milk was pipetted and 5.0 $\mu$L three stock standard solutions were added, respectively. The samples were left to stand for 30 min. Then 4.0 mL, 3.0 mL, 2.0 mL, 1.0 mL, 0.0 mL high purity water and 5.0 mL, 6.0 mL, 7.0 mL, 8.0 mL, 9.0 mL ethanol were added to every tube in turn, forming 50%, 60%, 70%, 80% and 90% volume fractions ($\phi$) of ethanol solution. The tubes were capped tightly and vortexed for approximately 1 min. Then same amount of K$_2$HPO$_4$ were added to five sample tubes, respectively. The following procedures were performed according to the “Sample preparation” section. The extraction efficiency was calculated as follows:

$$E = \frac{V_o C_o}{m_o}$$

Where: $E$- extraction efficiency (%); $V_o$- the volume of upper phase obtained after aqueous two phase formed; $C_o$- the concentration of stilbene estrogens detected in upper phase; $m_o$- the mass of stilbene estrogens standard added in milk.

The results in Fig.2 showed that the extraction efficiency is highest when the volume fraction of ethanol is about 70%. This demonstrated that in $\phi$ (ethanol) 70% aqueous solution, protein denaturation reactions occurred slowly and the stilbene estrogens bound with the protein would be released wholly. That would result in higher recoveries of drugs. However, in $\phi$ (ethanol) < 60% aqueous solution, the protein couldn’t denature completely, some drugs would be bound with the protein still. Moreover, in $\phi$ (ethanol) 80%~90% aqueous solutions, protein denatured so rapidly that drugs being packaged into the protein. Therefore, $\phi$ (ethanol) 70% aqueous solution was selected as extraction solvent.

Due to high polar for the $\phi$ (ethanol) 70% aqueous solution, less fat-soluble impurities were extracted to the extraction solution. Meanwhile, liquid chromatogram of drugs-free sample showed that the retention times of impurities were all within 10 min, so the impurities had no influences on the determination of three drugs. Therefore, purification steps could be omitted. This is highly effective to minimize the loss of drug residues and to reduce the workload for this experiment.
Conditions of forming ethanol-K$_2$HPO$_4$-water ATPS

Experimental results showed that the mixture of ethanol-water in any ratio could form ATPS after adding K$_2$HPO$_4$. Because of the different distribution coefficients of drugs in organic phase and aqueous phase, drugs were entering into the upper phase of ATPS, and a large number of protein matrixes were leaving in lower phase. Thus, not only the enrichment of drugs but also the elimination of matrix interferences could be realized at one step.

Referencing the phase diagram of ethanol-K$_2$HPO$_4$-water system under 25 °C and normal pressures conditions$^{24}$, appropriate amount of K$_2$HPO$_4$ was used to form ATPS in different φ (ethanol).

Experiment results showed that when 1.5 g K$_2$HPO$_4$ was added to 10.0 mL φ (ethanol) 70% solution, ATPS formed. The volume of upper phase solution was 8.75 mL and the solution could be analyzed directly by HPLC after filtered.

Method validation

External calibration curves were obtained by linear least-squares regression, plotting peak area (y) versus concentration (x). Three stilbene estrogens all had good linear response within the range of 0.05–10.0 μg/mL. The mean values of correlation coefficients (R$^2$) were higher than 0.999 for all drugs. The limits of detection (LODs) were obtained by dilution method step by step. The retention times, linear equations and LODs for three drugs were shown in Table 4.

A brand of milk sample was determined by HPLC after pretreatment. Liquid chromatogram showed that three stilbene estrogens were not detected. It was taken as drugs-free sample in follow-on work.

The intra-day and inter-day precisions of the method were evaluated by the analysis of samples spiked with
three stilbene estrogens at three different fortification levels, 0.1, 0.2, and 0.5 μg/mL, carried out within one day and for three consecutive days, respectively. Data were included in Table 5. Under optimized conditions, the extraction recoveries were 83.2–93.8%, the intra-day precision for three drugs proved to be 1.50–3.98%, and the inter-day precision were acceptable with values of 3.21–4.52%. The results showed that the method has good precision. The chromatogram of the stilbene estrogens in spiked milk sample are displayed in Fig.3, which indicate a good separation.

![Liquid chromatogram of milk sample spiked with 0.5 μg/mL stilbene estrogens](image)

**Fig.3** Liquid chromatogram of milk sample spiked with 0.5 μg/mL stilbene estrogens

1-DES  2-DEN  3-HEX

### Table 4 Linear equations, correlation coefficients (r) and detection limits (LODs) of stilbene estrogens

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Linear equation</th>
<th>Correlation coefficients</th>
<th>Retention time(min)</th>
<th>LODs (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-DES</td>
<td>y = 69.09x +3.4243</td>
<td>0.9996</td>
<td>17.3</td>
<td>19.3</td>
</tr>
<tr>
<td>2-DEN</td>
<td>y = 124.6x + 6.4935</td>
<td>0.9997</td>
<td>19.5</td>
<td>11.7</td>
</tr>
<tr>
<td>3-HEX</td>
<td>y = 76.12x +4.6236</td>
<td>0.9995</td>
<td>21.3</td>
<td>20.7</td>
</tr>
</tbody>
</table>

### Table 5 The recoveries, intra-day and inter-day precisions of spiked milk sample

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Spiked (μg/ mL)</th>
<th>Recoveries and intra-day precision (n=5)</th>
<th>Recoveries and inter-day precision (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Recovery (%)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>1-DES</td>
<td>0.1</td>
<td>87.2</td>
<td>3.98</td>
</tr>
<tr>
<td>1-DES</td>
<td>0.2</td>
<td>84.9</td>
<td>2.33</td>
</tr>
<tr>
<td>1-DES</td>
<td>0.5</td>
<td>89.4</td>
<td>2.13</td>
</tr>
<tr>
<td>1-DES</td>
<td>0.1</td>
<td>88.5</td>
<td>3.31</td>
</tr>
<tr>
<td>2- DEN</td>
<td>0.2</td>
<td>91.3</td>
<td>1.84</td>
</tr>
</tbody>
</table>
Conclusions

This study researched the effect of protein on the extraction of stilbene estrogen residues in milk samples. In 70% (v/v) ethanol-water solution, the strong binding interactions between protein and stilbene estrogens were destroyed completely. As matrix protein was precipitated, the stilbene estrogens, bound with protein originally, could be released to the highest extent. On the basis of the results, a new sample pretreatment method was developed by using 70% (v/v) ethanol-water solution as the extraction solution, followed by adding K₂HPO₄ to form a stable aqueous two phase. Meanwhile, needing no efforts for further tedious purification steps becomes a highlight of this study. This developed method has good analytical performance and was suitable for the fast and effective determination of the stilbene estrogen residues in milk samples. Obviously, the superiority of the method was environment friendly.

Notes and references

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An environmentally friendly method to extract and detect stilbene estrogens in milk using ethanol-K$_2$HPO$_4$-water system coupled with HPLC.