VETERINARY DRUG RESIDUE

# Simultaneous Determination of Five Hormones in Milk by Automated Online Solid-Phase Extraction Coupled to High-Performance Liquid Chromatography

Kun Liu
Hebei Medical University, School of Public Health, Shijiazhuang 050017, China; Hebei University of Chinese Medicine, College of Chinese Medicine Integrated with Western Medicine, Shijiazhuang 050200, China
Kai Kang
Hebei Medical University, School of Pharmacy, Shijiazhuang 050017, China
Nan Li
Hebei Medical University, School of Public Health, Shijiazhuang 050017, China; Hebei University of Chinese Medicine, College of Chinese Medicine Integrated with Western Medicine, Shijiazhuang 050200, China
Jing An
Hebei Medical University, School of Public Health, Shijiazhuang 050017, China
Kaoqi Lian[1]
Hebei Medical University, School of Public Health, Shijiazhuang 050017, China; Hebei Key Laboratory of Environment and Human Health, Shijiazhuang 050017, China
Weijun Kang
Hebei Medical University, School of Public Health, Shijiazhuang 050017, China

***Background:*** **Many hormones show the effects of protein assimilation and growth promotion, and they are frequently used as veterinary drugs in livestock, which has harmful effects on human health. It is necessary to determine their contamination level in animal-derived food, especially in milk.** ***Objective:*** **In this study, a detailed procedure is described for an automated online solid-phase extraction (SPE)–HPLC method capable of detecting five hormones (i.e., estriol, prednisone acetate, hydrocortisone, diethylstilbestrol, and estrone) in cow milk.** ***Methods:*** **The corresponding milk samples were precipitated by addition of acetonitrile and then purified as well as enriched by a polar-enhanced polymer (PEP) online SPE column. The supernatants were directly injected into the online SPE–HPLC system using methanol–water as the mobile phase mixture.** ***Results:*** **The linearity range of the method was 0.1–25 μg/mL for prednisone acetate, hydrocortisone, and diethylstilbestrol, 0.2–25 μg/mL for estriol, and 0.5–25 μg/mL for estrone, with correlation coefficients (*r*) ranging from 0.9994 to 0.9996. The recovery rates determined at three concentration levels for the five compounds were in the range of 70.82–112.90%. LODs of estriol, prednisone acetate, hydrocortisone, diethylstilbestrol, and estrone were 0.023, 0.005, 0.006, 0.004, and 0.054 μg/mL, respectively.** ***Conclusions:*** **This automated online SPE–HPLC method was both effective and reliable in the simultaneous measurement of five hormones, and the method was successfully applied to the detection of five hormone species in milk.** ***Highlights:*** **An automated online SPE–HPLC method has been developed for the analysis of five hormones in cow milk. Online SPE proved to be a powerful technique for determining five hormones simultaneously. This method ensured simple sample pretreatment and less operation time. The established method was successfully applied to the analysis of five hormone species in milk.**

Estrogens, together with androgens and progestogens, have been demonstrated to promote sexual organ maturation, improve the development of secondary sexuality, and maintain sexual function. They also feature a relationship with the estrous cycle in animals. Moreover, estrogens play an important role in the metabolization of sugar, fat, protein, and minerals. Estrogens can be divided into two subtypes: natural estrogen and synthetic estrogen. Natural estrogen includes estradiol, estriol, and other compounds that may be found in milk. Estrogen in the plasma enters milk through the blood–milk barrier and may also be partially synthesized by mammary glands. The concentration of natural estrogen found in milk is always higher than that in blood, which indicates that hormones in milk may be involved in the regulation of specific functions of the mammary glands and may also contribute to the growth rate of a newborn (1). Endogenous estrogen is difficult to avoid in animal-derived food because these hormones have been shown to regulate animal life activities. The dairy milk that people drink daily is quite different from the milk consumed 100 years ago because of the fact that most of the cows raised

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[1]Corresponding author's e-mail: liankq@hebmu.edu.cn
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in modern industry are genetically modified, and the content of estrogen in milk secreted by different cow breeds also differs significantly (2). Furthermore, in modern dairy production, milk cows are lactating continuously throughout pregnancy, especially during late pregnancy. Therefore, the estrogen levels in serum and milk can be significantly increased. According to previously conducted studies, milk and dairy products constitute 60–70% of the total estrone intake (3), and the levels of estrone cannot be lowered through pasteurization. Nearly $7 \times 10^8$ tons of milk is consumed by humans per year. Several dairy products include yogurt, cheese, butter, and cream. While synthetic estrogen includes diethylstilbestrol, ethylestradiol, and other compounds, synthetic estrogens are estrogen-mimicking compounds with similar geometry and effects as estrogens. These synthetic estrogens are also known as endocrine-disrupting chemicals. Diethylstilbestrol represents a synthetic, nonsteroidal estrogen that may deliver the same pharmacological and therapeutic effects as natural estradiol. From the 1940s to the 1970s, diethylstilbestrol was used to prevent miscarriages and other pregnancy complications in millions of pregnant women; however, it was banned as a human pharmaceutical in 1972 because of serious adverse side effects on the female reproductive system (4). However, as a veterinary drug, diethylstilbestrol can be used to maintain the sexual characteristics of female livestock and has been shown to promote the hyperplasia of endometrium, uterine smooth muscles, and vaginal epithelial cells. In clinical practice, diethylstilbestrol can be used to stimulate the growth of animals and promote the sexual maturity of animals in a given period of time. Glucocorticoids represent a steroidal hormone species secreted by the adrenal cortex and can also be synthesized chemically. Since the late 1940s, synthetic glucocorticoids have been extensively used in the treatment of chronic inflammatory conditions such as rheumatoid arthritis and asthma and for their immunosuppressant action in preventing resistance response after organ transplantation (5). Glucocorticoids are widely administered in livestock production because of their inhibition of inflammatory, allergic, and immunological responses (6). Ingestion of food from animals containing residues of glucocorticoids represents a serious threat to human health.

Above all, hormones like estriol, prednisone acetate, hydrocortisone, diethylstilbestrol, and estrone exhibit the effects of protein assimilation and growth promotion. These compounds are frequently used to improve feed-conversion efficiency and speed up animal growth in livestock, thus providing significant economic benefits in the animal breeding industry (7). These hormones are also used in the treatment of estrogen deficiency disorders in veterinary medicine (3). Therefore, a potential risk for humans exists because of the potential consumption of contaminated food or water. The effects of these hormones include decreasing sperm counts in males, increasing breast cancer occurrences, and reproductive abnormalities as well as adolescent obesity, acne, and prostate abnormalities (2, 8, 9). Therefore, the use of these compounds has been restricted and banned in the European Union and in many other countries (10). Milk is an important source of protein, however, and also a rich source of hormones (11). Unfortunately, the presence of hormones in milk is difficult to quantify. Hence, developing a selective, accurate, and sensitive method for detecting hormones in milk can be described as critical to protect human health.

Because of the small concentration of estrogen present in animal food, suitable extraction methods and cleanup steps for analysis are required. Recently, the determination of hormones in milk has attracted more and more attention. Furthermore, various analytical methods have been reported for the analysis of milk, including immunoassays, HPLC, LC coupled with MS, GC, and GC coupled with MS (GC–MS; 10). Immunoassays have been employed for the determination of a large number of hormonally active substances with great sensitivity, including radioimmunoassays and enzyme-linked immunosorbent assays (ELISA; 12). Nevertheless, these methods generally suffer from cross-reactions due to similar structures of the target analytes (13), limiting their overall applicability. GC–MS offers good separation and precision for analytes; however, GC and GC–MS methods generally require substances to be stable and volatile, and derivatization reactions are often involved, which are complex and may introduce side reactions. HPLC still represents a common method for multitarget determinations of hormones. Because of the low concentrations of these substances in milk, HPLC is commonly combined with offline solid-phase extraction (SPE) or liquid-liquid extraction (14), exhibiting disadvantages of sample loss, time-consuming processes, cross-contamination, poor repeatability, and high consumption of offline SPE columns (15). The disadvantages of offline SPE can be avoided by using an online SPE method, which requires smaller volumes of solvents, reduces overall analysis time, increases automation, and improves reproducibility (16, 17). Online SPE is based on the selective extraction of target analytes by SPE, and target analytes are usually transferred from the extraction column to the analytical column using a column-switching valve. The objective of this study was to develop a rapid and sensitive method for the simultaneous determination of five hormones in milk by automated online SPE coupled with LC with UV detection. An online SPE column was utilized as an automatic device for the extraction, enrichment, and purification of the corresponding analytes. For this purpose, skimmed cow milk was selected as the analysis sample. Several parameters affecting performance were optimized, including the wavelength switching program, methanol percentage during loading phase, loading time, and transfer time. Furthermore, the methodology was validated, resulting in the generation of satisfactory results. The analytical method was used to simultaneously quantify five hormones in cow milk within a relatively short time frame.

## Experimental

### *Samples*

Skimmed dairy milk samples were purchased from the local supermarket, filled in clear brown glass bottles, and stored in the dark at 4°C until processing.

### *Apparatus*

(**a**) *HPLC system*.—Thermo Fisher Dionex UltiMate 3000, equipped with dual-gradient pumps, a column oven with a two-position, six-port switching valve, an automatic sampler, and a UV detector, controlled by Chameleon software (Thermo Fisher Scientific, Waltham, MA).

(**b**) *LC column*.—C18 Diamonsil (250 × 4.6 mm, 5 µm) column (Diamonsil, Beijing, China).

(**c**) *Spectrophotometer*.—TU-1901 double-beam UV-Vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd, Beijing, China).

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(**d**) *Centrifuge*.—D3024R temperature-control centrifuge (Dalong Xingchuang Experimental Instrument Co. Ltd, Beijing, China).

### *Reagents*

(**a**) *Water*.—Ultrapure water was obtained using a purification system (Ulupure, Xi'an, China).

(**b**) *Solvent*.—Methanol and acetonitrile (99.99%, HPLC grade; Oceanpak, Gothenburg, Sweden).

(**c**) *Standard*.—Diethylstilbestrol (99.5%) was purchased from Sigma (St. Louis, MO). Estriol (98.0%), prednisone acetate (99.5%), hydrocortisone (98.7%), and estrone (99.9%) were purchased from National Institutes for Food and Drug Control (Beijing, China). Other chemicals used in the experiment were analytical grade. The chemical structures of the five analytes are shown in Figure 1.

### *Preparation of Standard Solution*

(**a**) *Standard stock solutions (500 μg/mL)*.—Standard stock solutions of standard substances were prepared by dissolving each standard in methanol and were stored at 4°C.

(**b**) *Working solutions*.—Working solutions were prepared by a suitable dilution of the stock solutions in methanol at least once per day.

All glassware was washed thoroughly with detergent and tap water, followed by rinsing with ultrapure water three times and oven-drying before use.

### *Chromatographic Conditions*

(**a**) *Mobile phases*.—Methanol (A) and water (B).

(**b**) *Flow rate and gradient elution*.—The gradient elution program is shown in Table 1.

(**c**) *Column temperature*.—30°C.

(**d**) *Injection volume*.—150 μL.

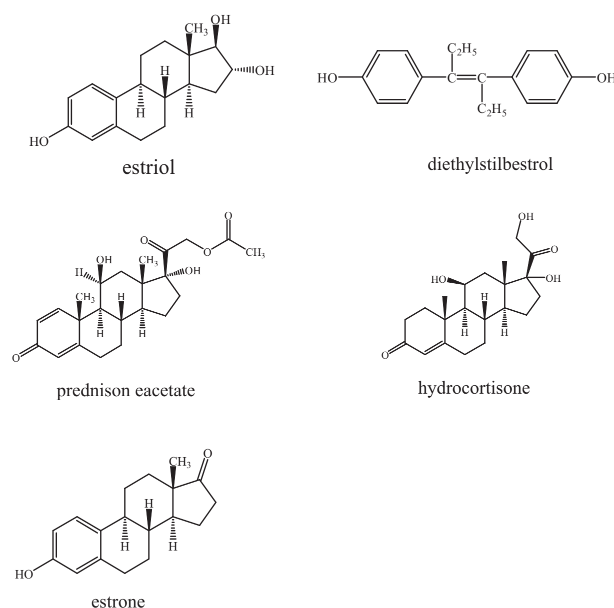


**Figure 1. Chemical structures of the five analytes.**

**Table 1. Time schedule for the sample loading and elution procedure**

| Time, min | Left pump (A), %[a] | Right pump (A), %[a] | Valve location |
|---|---|---|---|
| 0 | 70 | 15 | 1-2 |
| 1.5 | 70 | 15 | 1-6 |
| 1.8 | 70 | 15 | 1-2 |
| 4 | 70 | 15 | 1-2 |
| 4.1 | 70 | 100 | 1-2 |
| 8 | 70 | 100 | 1-2 |
| 8.1 | 70 | 15 | 1-2 |
| 15 | 70 | 15 | 1-2 |

[a] For both pumps, channel A is methanol and channel B is ultrapure water.

(**e**) *Wavelength of detection*.—0–5.5 min, 280 nm; 5.6–9.6 min, 240 nm; and 9.7–15 min, 280 nm.

### *Experimental Process*

The samples were enriched, separated by an online SPE system, and then automatically analyzed using an HPLC system. The left pump of the double-gradient HPLC system was used as the enrichment pump. The function of the latter was to control the loading of analytes and the regeneration of the online SPE column (18, 19). Methanol solution with a concentration of 15% was used as mobile phase, and the sample was enriched onto the polar-enhanced polymer (PEP) SPE column. The impurities flowed out from the liquid pipeline. The right pump was used as the analytical pump, regulating the elution and LC separation processes. Following this procedure, the analytes were transferred from the online SPE column to the analytical column via a column-switching valve to be separated with methanol–water (85 + 15, v/v) as the gradient elution mobile phase. The initial position of the six-port valve was 1-2, and it was switched to position 1-6 after loading, thus connecting the SPE column and the analytical column. The analytes were transferred from the SPE column to the analytical column and UV detector for analysis. Afterward, the valve was switched to position 1-2 again, and the SPE cartridge and analytical column were eluted to remove residual contaminants. Next, the entire system was re-equilibrated. A Hypersep Retain PEP column (10 × 3 mm, 5 μm) was used for SPE. The temperature of the analytical column was set to 30°C. The six-port valve was switched at 1.5 and 1.8 min, respectively. The flow scheme of the online SPE system is shown in Figure 2, and the time schedule for the compartment valve switching is listed in Table 1.

### *Loading of Samples onto the Online SPE Cartridge*

Sample (150 μL) was injected into the sample loop through the automatic sampler and was then delivered to the online SPE column with the mobile phase set to 15% methanol (A) and 85% water (B; 70 + 30, v/v) by the left pump. The loading flow rate was 0.5 mL/min, and the loading time was 1.5 min. The loading phase configuration (with the position of the six-port valve set to 1-2) is illustrated in Figure 2A.

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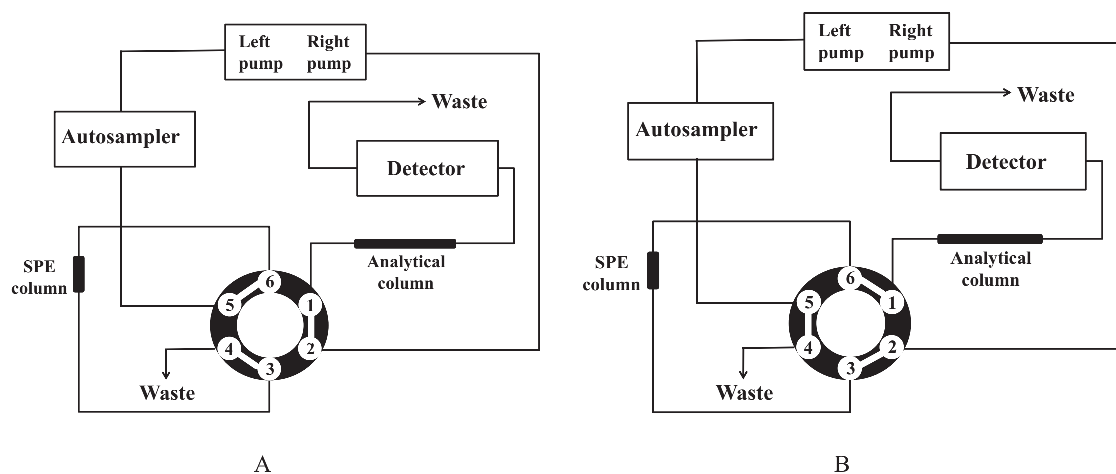


**Figure 2. Flow scheme of online solid-phase extraction system. (A) Loading of samples and re-equilibration; (B) elution and separation.**

### *Elution and Separation*

After loading, the six-port valve was switched to position 1-6 to connect the SPE column and the analytical column. The analytes were transferred from the SPE column to the analytical column and UV detector in a backflush mode for separation and determination at a flow rate of 1 mL/min using a mobile phase composition consisting of 70% (v/v) methanol (A) and 30% ultrapure water (B). The time for this procedure was set to 0.3 min. The elution phase configuration is shown in Figure 2B.

### *Re-Equilibration of the System*

After elution and separation of the samples, the valve was switched to position 1-2, and the SPE cartridge and the analytical column were eluted to remove residual contaminants. The SPE column and analytical column were flushed with 100% methanol at a flow rate of 0.5 mL/min and 1 mL/min, respectively. Then, the entire system was re-equilibrated for the next injection, and a time frame of 15 min was used for analysis. The re-equilibration phase configuration is illustrated in Figure 2A.

### *Sample Preparation*

The blank samples were spiked with a standard working solution of appropriate concentration to obtain standard sample solutions. For analysis, 1.2 mL acetonitrile was added into a centrifuge vial with 0.4 mL homogenized milk sample for protein precipitation and shaken fully, followed by centrifugation at 8000 rpm for 10 min. Then, the supernatant was collected for detection.

To minimize contamination during the experiment process, all solvents were checked for the presence of hormones before use. All glassware was washed with water and detergent, rinsed with tap water and ultrapure water three times, and then heated in an oven for 1 h before use.

## Results and Discussion

### *Method Optimization*

*Optimization of chromatographic conditions*.—To optimize the mobile phase, two organic solvents (methanol and acetonitrile) were evaluated with respect to their effects on signal intensity, resolution factor, and retention times of the analytes. An improved separation effect was observed with methanol, and hence, it was selected for use. For the final optimized procedure, a concentration of 70% methanol was selected as the organic solvent of the mobile phase for the separation of the analytes. Using this condition, sharp and symmetric peaks of estriol, prednisone acetate, hydrocortisone, diethylstilbestrol, and estrone were obtained, and all compounds were baseline-separated.

Five substances were screened in the range of 200–400 nm by UV-Vis spectrophotometry. The results showed that the maximum absorption wavelength was 280 nm for estriol and estrone and 240 nm for prednisone acetate, hydrocortisone, and diethylstilbestrol. In order to achieve a maximum signal response value, a variable-wavelength UV detector was used. The wavelength switching program was set as follows: 0–5.5 min, 280 nm; 5.6–9.6 min, 240 nm; and 9.7–15 min, 280 nm.

*Optimization of online conditions*.—In the sampling stage, samples were enriched on the SPE column with methanol of a certain concentration. Different concentrations of methanol were investigated ranging from 0 to 20%, with increments of 5%. The results prove that the methanol concentration has little effect on the peak area in the scope of investigation. However, the impurities cannot be removed cleanly when the methanol concentration is too low. When the methanol concentration is 15%, better impurity removal can be achieved. Taking into account the effect of impurity removal and organic reagent usage, the loading phase was selected to be 15% methanol.

The loading time for the analytes retained on the SPE column and transfer time from the SPE column to the analytical column were both crucial for recovery. To investigate the effect of valve-switching times, times of 1, 1.3, 1.5, 1.8, 2, and 2.5 min were selected as the first valve-switching times and 1.5, 1.8, 2, 2.3, 2.5, and 3 min were selected as the second valve-switching times. The results showed that, in the case of the loading time being too short, the analytes could not be retained completely; conversely, the analytes were lost. If the transfer time was too short, the analyte recovery was demonstrated to be poor. Conversely, the impurities may have been transferred to the analytical column. Meanwhile, the longer the interval between the two valve switchings was, the worse the obtained peak type. The best result was achieved when the first valve-switching time was 1.5 min and the second was 1.8 min.

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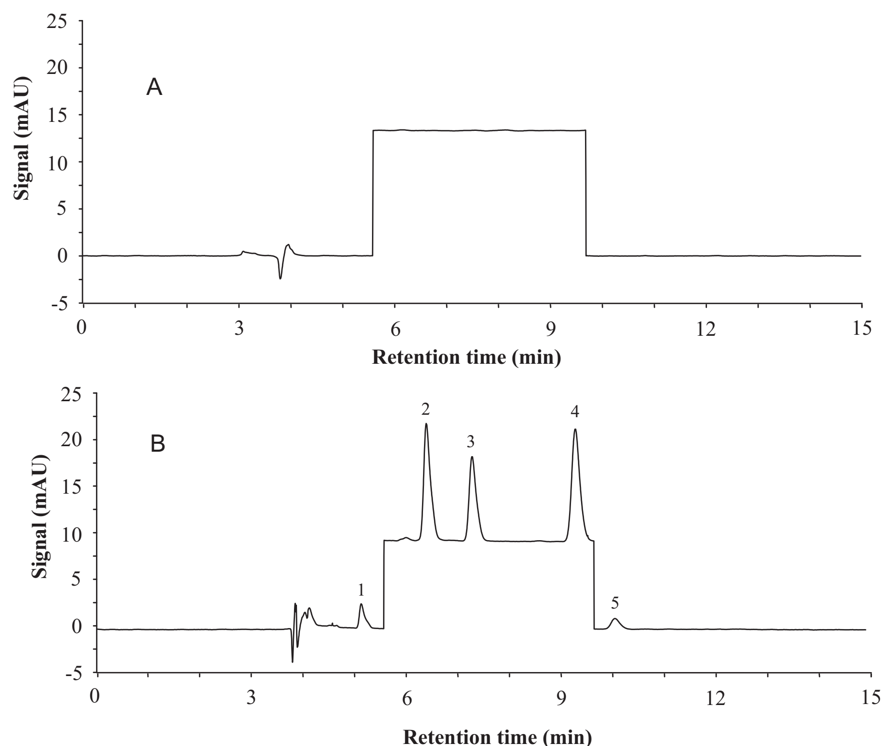


**Figure 3. Chromatograms of (A) blank milk sample and (B) five analytes spiked in milk samples (*C* = 10 μg/mL). Peaks: 1, estriol; 2, prednisone acetate; 3, hydrocortisone; 4, diethylstilbestrol; and 5, estrone.**

### *Method Validation*

A detailed characterization was carried out to validate the suitability of the method for the detection of five hormones in milk. Validation parameters such as selectivity, linearity, sensitivity, LOD, LOQ, accuracy, and precision were evaluated.

*Selectivity*.—Interferences of impurity in milk were assessed by comparing chromatograms of a blank milk sample and spiked milk according to the procedure described above. This online HPLC method was found to be selective for the five substances.

The retention times of estriol, prednisone acetate, hydrocortisone, diethylstilbestrol, and estrone were 5.15, 6.39, 7.26, 9.21, and 10.00 min, respectively. Representative chromatograms of blank milk sample and milk spiked with the five hormones are shown in Figure 3. There were no significant interferences by impurities observed within the retention times for these analytes.

*Linearity and sensitivity*.—Calibration standard solutions were prepared by diluting the corresponding stock solutions to concentrations of 0.2, 0.5, 1, 10, 20, and 25 μg/mL for estriol; 0.1, 0.5, 1, 10, 20, and 25 μg/mL for prednisone acetate, hydrocortisone, and diethylstilbestrol; and 0.5, 1, 5, 10, 20, and 25 μg/mL for estrone. Analyses were carried out under the experimental conditions described above, and standard curves were obtained with the concentration on the abscissa and the corresponding peak area on the ordinate. The LOD was evaluated when the analytes provided a signal three times the background noise. Similarly, the LOQ corresponded to an S/N of 10 (20). The standard curves, correlation coefficients, detection limits, and quantitation limits of the five substances are listed in Table 2.

*Accuracy and precision*.—In order to check the accuracy of the developed method, recovery experiments were carried out on milk samples. Method accuracy was established in terms of relative recovery by adding five standard reference substances to blank milk sample at three levels of concentration. The samples were then injected in triplicates. Standard recovery rates (%) were calculated as follows: (measured value of the standard sample / theoretical value of standard sample) × 100. The accuracy (expressed as recoveries) of the five analytes at three standard levels is shown in Table 3. The obtained values were 77.09–99.18% for estriol, 100.35–105.37% for prednisone acetate, 78.87–92.17% for hydrocortisone, 80.38–95.10% for diethylstilbestrol, and 84.54–104.04% for estrone. The

**Table 2. Linear regression equation, linear range, and correlation coefficient**

| Analytes | Linear equation | Correlation coefficient ($r$) | Linear range, μg/mL | LODs, μg/mL | LOQs, μg/mL |
|---|---|---|---|---|---|
| Estriol | $A = 0.1346c - 0.0093$ | 0.9996 | 0.2–25.0 | 0.023 | 0.078 |
| Prednisone acetate | $A = 0.7815c + 0.0112$ | 0.9995 | 0.1–25.0 | 0.005 | 0.017 |
| Hydrocortisone | $A = 0.7358c - 0.0325$ | 0.9995 | 0.1–25.0 | 0.006 | 0.020 |
| Diethylstilbestrol | $A = 1.1447c - 0.0850$ | 0.9996 | 0.1–25.0 | 0.004 | 0.015 |
| Estrone | $A = 0.1033c - 0.0023$ | 0.9994 | 0.5–25.0 | 0.054 | 0.180 |

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**Table 3. Accuracy and precision of the method**

| Analytes | Blank value | Added, μg/mL | Avg. rec., % | Intraday precision, %[a] | Interday precision, %[a] |
|---|---|---|---|---|---|
| Estriol | ND[b] | 1.0 | 77.09 | | |
| | ND | 10.0 | 99.18 | 2.06 | 4.33 |
| | ND | 20.0 | 97.98 | | |
| Prednisone acetate | ND | 0.5 | 105.37 | | |
| | ND | 10.0 | 101.27 | 1.86 | 3.48 |
| | ND | 20.0 | 100.35 | | |
| Hydrocortisone | ND | 0.5 | 78.87 | | |
| | ND | 10.0 | 92.17 | 1.40 | 2.85 |
| | ND | 20.0 | 84.81 | | |
| Diethylstilbestrol | ND | 0.5 | 80.38 | | |
| | ND | 10.0 | 95.10 | 1.76 | 6.11 |
| | ND | 20.0 | 84.65 | | |
| Estrone | ND | 1.0 | 84.54 | | |
| | ND | 10.0 | 104.04 | | |
| | ND | 20.0 | 95.31 | 1.63 | 5.34 |

[a] Precisions were investigated at a concentration of 10 μg/mL for five analytes.

[b] ND = Not detected.

satisfactory recovery rates indicate that the established method is suitable for the determination of hormones in milk samples. Method precision was determined through intraday relative standard deviations (RSDs) as well as interday RSDs. The intraday precision was determined by assaying five analytes in six spiked samples at a concentration of 10 μg/mL on a single day, whereas the interday precision was determined by analyzing spiked samples on three consecutive days. The results of precision, expressed as RSD (%) of concentration, showed that the intraday precision was in the range of 1.12–3.87%, and the interday precision was 3.48–6.11% for all analytes.

*Stability*.—Method stability was investigated at a concentration of 20 μg/mL for five analytes by repeating analysis after 0, 4, 8, 12, and 24 h under the same analytical conditions. The RSD of five analytes within 24 h were 2.84, 3.14, 4.37, 4.77, and 3.40%, respectively. These results indicate that the stability of the developed method is suitable.

### *Comparison with Previously Established Methods*

Different methods have been established to quantify compounds in food samples (8, 20, 21). For example, Wang et al. (21) have developed an ultra-performance LC (UPLC) coupled to electrospray ionization quadrupole time-of-flight MS (QTOF–MS) method to detect four endocrine-disrupting compound species in milk and powdered milk. This method achieved higher sensitivity, but the recoveries of estrone, estriol, and diethylstilbestrol were 68.3–80.4, 59.4–72.5, and 86.4–135.0%, respectively. Our study showed higher recovery in comparison with the published data. Bárbara et al. (22) reported the detection of a group of estrogenic compounds in dairy products based on hollow-fiber liquid-phase microextraction coupled to LC with recovery rates of 94.0–113, 81.0–106.0, and 93.0–118.0%. Compared to this work, our method required a lower amount of organic solvents, shorter analysis times, and higher recovery rates, and improved intraday and interday precision was achieved. An offline HPLC–MS method was developed by Cui et al. (23) for the simultaneous determination of 12 glucocorticoid species in milk. Milk samples were extracted by sonication, and the fat content was removed with *n*-hexane. The samples were then enriched and purified using a different offline SPE column, separated by C18 column, and detected by MS. In this experiment, average recoveries for 12 glucocorticoids ranging from 69.3 to 94.3%, with RSDs between 3.5 and 16.7%, were obtained. However, these results were not much different from those obtained using our experiment, in which the recovery rates for prednisone acetate and hydrocortisone were 100.35–105.37 and 78.87–92.17%, with RSD values of 0.75–6.75 and 1.61–9.21%, respectively. Zhu et al. (24) analyzed a method based on TurboFlow online SPE combined with LC–tandem MS to confirm 88 wide-ranging veterinary drugs in milk. The linear regression coefficients ($R^2$) were higher than 0.9930. The LODs were in the range of 0.2–2.0 μg/kg, and the LOQs ranged between 0.5 and 10 μg/kg. Average recoveries were between 63.1 and 117.4%, with RSDs (%) in the range of 3.3–17.6%. The parameters of correlation coefficients, recovery rates, and precisions in our experiment were higher than those in the work of Zhu et al. In addition, the method established in this study could reduce amounts of organic solvents and manual labor (25). Therefore, it has proven to be more efficient compared with other offline techniques (25, 26).

## Conclusions

Milk and dairy products are among the most consumed foods in the world (3). Because some studies have revealed that estrogens and glucocorticoids can cause severe illnesses in humans, the detection of hormones in milk is of great importance. Based on some research results that employed LC as the separation technique for the determination of such compounds in milk, this work describes a different sample pretreatment procedure. A simple yet automated and environmentally friendly online SPE method coupled to LC was established for the separation, preconcentration, and determination of five hormones (estriol, prednisone acetate, hydrocortisone, diethylstilbestrol, and estrone) in milk samples using a cycle time of 15 min. This method featured a simplified preparation step, reduced usage of organic reagents, and improved sensitivity as well as reproducibility (27–29). The low detection limit; good linearity, accuracy, and precision; low solvent consumption; and low labor requirements may contribute to applying this method for the quantification of estrogen compounds and glucocorticoids in cow milk. Taken in concert, all of these advantages may render this method highly attractive for future routine analyses (30).

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