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RESEARCH ARTICLE

# Analysis of 13 kinds of steroid hormones in raw milk using modified QuEChERS method combined with UPLC-QTOF-MS

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**Abstract**

Thirteen kinds of steroid hormones in raw milk (cow, goat and buffalo milk) were analyzed with ultra performance liquid chromatography-quadrupole time of flight mass spectrometry (UPLC-QTOF-MS) after extraction and cleanup with the modified QuEChERS method. These steroid hormones included 17β-estradiol, estriol, estrone, diethylstilbestrol, progesterone, melengestrol acetate, megestrol acetate, chlormadinone acetate, 19-nortestosterone, metandienone, boldenone, epitestosterone, and testosterone. The limits of detection for the raw milk basing on 3 times the signal to noise ratios (S/N=3) was in range of 0.07–0.51 μg $kg^{-1}$, and the limits of quantification (basing on S/N=10 method) covered the ranges from 0.23 to 1.7 μg $kg^{-1}$. With matrix external standard method, the substances presented recoveries over the range 74.2–99.7%. Qualitative analysis was also done in the mass/mass spectrum (MS/MS) mode and each debris structure of 13 kinds of steroid hormones was achieved. The methodology was then applied in real raw milk samples which were collected in several areas of China and the progesterone was detected with high level.

**Keywords:** UPLC-QTOF-MS, raw milk, QuEChERS, steroid hormones, acidic alumina

## 1. Introduction

Milk and dairy products are one of the most popular foods in people's life, due to the rich in protein, vitamins, calcium and other essential nutrients. So its quality and safety issues are extremely important. Studies have shown that precocious puberty, breast cancer in women and prostate cancer incidence rising are ascribed the hormone residues in foods (James *et al.* 2011; Key *et al.* 2011; Sandholm *et al.* 2012). Hypertension in men is related to androgen levels (Reckelhoff *et al.* 1998; Wu and Schwartzman 2011). The World Anti-Doping Agency listed the nandrolone, testosterone, methyltestosterone and other steroid hormones as banned steroid doping (Berry 2008; Danaceau *et al.* 2008). However, many dairy farmers abused drugs in violation of the law in order to increase raw milk production, which led to abnormal secretion of hormones in cows and increased the hormone level in raw milk. These would enhance the steroid hormones excessive risks in raw milk. It is necessary to explore an efficient way to detect steroid hormones in raw milk.

Many detection methods have been established for de-

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tecting steroid hormones (Stanczyk and Clarke 2010). Radioimmunoassay and enzyme-linked immunosorbent assay provide higher sensitivity, simple operation and are suitable for large amount measurements (Avila-Poveda *et al.* 2013). However, various hormones are prone to influence each other, resulting in contamination and false-positive in the samples. Recently, prevalent instrumental methods using gas chromatography tandem mass spectrometry (Hansen *et al.* 2011; Pérez *et al.* 2014), liquid chromatography tandem mass spectrometry (Ingrand *et al.* 2003; Farke *et al.* 2011; Gao *et al.* 2013) showed higher sensitivity, good specificity and chromatographic resolution. But their pretreatment processes were tedious and time-consuming. Time-of-flight mass spectrometry (TOF-MS) allows the generation of mass information with higher accuracy and precision, and provides both the parent ions and their fragment information. TOF-MS was a time-saving, high sensitivity and accuracy method. Thus, it was used for determination of 13 kinds of steroid hormones in the study.

QuEChERS is the acronym for a quick, easy, cheap, effective, rugged and safe method, which was developed by U.S. Department of Agriculture, Agricultural Research Service, USA (Anastassiades *et al.* 2011), and often used as a pretreatment method when detecting pesticide residues in fruits and vegetables (Cieślik *et al.* 2011; Furlani *et al.* 2011; Pareja *et al.* 2011). In this experiment, we used the QuEChERS method for handling steroid hormones in raw milk samples. During the purification process, we found that original purification adsorbents would reduce the recoveries and could not achieve the best cleanup results. To obtain a higher recovery and better purification effect, we adjusted the QuEChERS cleanup adsorbents and compared the purification results of different adsorbents.

In this study, we optimized ultra performance liquid chromatography-quadrupole time of flight mass spectrometry (UPLC-QTOF-MS) conditions for analyzing 13 kinds of steroid hormones in raw milk. The modified QuEChERS method was used to pretreat the raw milk samples and provide good analytical results for the target hormones.

## 2. Materials and methods

### 2.1. Materials and standards

Steroid hormones, its purity >99%, were obtained from Germany DR Company. Acetonitrile, formic acid, ammonia water, anhydrous magnesium, Cleanert PSA (primary and secondary amine) and acidic alumina are all analytical grade; and sodium citrate, sodium citrate dibasic sesquihydrate are chromatographic grade. Deionized water was used through the experiment.

The raw milk (cow, goat and buffalo milk) were collected from Shandong and Shanxi provinces and Guangxi Zhuang Autonomous Region, China, at three seasons of 2014. The samples which we received were in frozen state and kept in the refrigerator (–18°C) until analysis.

### 2.2. Instruments

The main instruments were as follows: acquity ultra performance liquid chromatography, Xevo G2-S-flight mass spectrometer (Waters Corporation, U.S.), Heidolph L4000 rotary evaporator (Heidolph Corporation, Germany), 3K30-speed rotating centrifuge (Sigma Corporation, U.S.), IKA-MS3 vortex spin oscillator (IKA Industrial Equipment Corporation, Germany), KQ-500E ultrasonic extractor (Kunshan Ultrasonic Instrument Co. Ltd. China), 0.22 μm organic membrane (Pall Corporation, U.S.), single-channel pipette (100–1000 μL) (Eppendorf Corporation, Germany).

### 2.3. Chromatographic conditions

A Waters BEH C18 (2.1 mm×100 mm, 1.7 μm) was used for separating steroid hormones. Column and sample chamber temperature were set at 35 and 20°C, respectively. The flow rate was 0.3 mL $min^{-1}$. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) in positive ion mode. A gradient elution was applied as follows: Initial 65% A, 1 min 65% A, 10 min 50% A, 10 min 6 s 65% A, 12 min 65% A. While in negative ion mode, the mobile phase consisted of 0.1% ammonia (A) and acetonitrile (B). A gradient elution was applied as follows: Initial 65% A, 1 min 65% A, 5 min 50% A, 9 min 20% A, 10 min 65% A. The injection volume was 2 μL.

### 2.4. Mass spectrum (MS) conditions

Electro spray ionization (ESI) was set in positive electro spray ionization mode ($ESI^+$) and negative electro spray ionization mode ($ESI^-$). MS parameters were as follows: capillary voltage, 3.0 kV in $ESI^+$ and 2.5 kV in $ESI^-$; desolvation gas flow, 800 L $h^{-1}$; ion source temperature, 120°C; desolvation temperature, 420°C; cone voltage, 40 V; scan range, 50–600 m/z.

### 2.5. Final sample pretreatment

10.0 g raw milk was mixed with 10 mL acetonitrile and ultrasonic extracted for 10 min. Then 6.5 g QuEChERS salt (4.0 g anhydrous magnesium, 1.0 g sodium chloride, 1.0 g sodium citrate, 0.5 g sodium citrate dibasic sesquihydrate) was added. QuEChERS salts are commonly used as the drying agent to promote rapidly and satisfactorily separating water and extraction agent to get the analytes in QuEChERS