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PRELIMINARY RESULTS ON THE ANALYSIS OF STEROIDS IN POULTRY SAMPLES BY DIRECT IMMERSION SOLID-PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT. This paper reports a rapid method for analysis of steroid hormones in poultry serum samples. The target compounds were first extracted by direct immersion on solid-phase microextraction (SPEME) procedure, followed by on fiber derivatization with methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and, final, analysis by gas chromatography with mass spectrometry (GC-MS). The average correlation coefficient of the calibration curve of the steroid hormones was 0.9955. The LOD/(LOQ) values of the steroid hormones in poultry serum samples were in the range of 0.020-0.068/(0.060-0.204) μg L^{-1}. The concentrations of estrone and ß-estradiol in poultry serum were 2.08 μg L^{-1} and 3.61 μg L^{-1}, respectively.

Keywords: *Steroid hormones, SPME, GC-MS*

INTRODUCTION

In recent years, various adverse health effects of endocrine disrupting compounds have been reported [1]. Endocrine disruptor chemicals interfere with chemical from aquatic environment and induce feminization, decrease in fertility or hermaphroditism of aquatic organisms [2, 3]. Estrogens are called the female sex hormones. Steroids are a group of lipophilic, low-molecular weight, biologically active compounds that act as hormones. Various examinations regarding endocrine disrupting chemicals (EDCs) are being conducted across the world. In the environment the hormones are excreted by the humans and animals [4, 5].

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Steroid sex hormones can be found in the livestock wastes such as sheep, cattle, pigs, poultry and other animals, as well as growth regulators in aquaculture [6, 7].

Recently, a number of methods have been reported for the determination of steroid hormones, such as: gas chromatography coupled with mass spectrometry (GC–MS), tandem mass spectrometry (GC–MS–MS), liquid chromatography coupled with mass spectrometry (LC–MS) and tandem mass spectrometry (LC–MS–MS) [8]. GC-MS is the most used techniques for steroid hormones analysis [9, 10]. Over the years, various analytical procedures have been developed for the efficient clean-up of biological matrices, such as liquid–liquid extraction (LLE), solid phase microextraction (SPME), solid–liquid extraction (SLE) and solid phase extraction (SPE). SPME is a simple and fast to use technique, solvent free and single step sample preparation, which is used frequently in the environmental, biological, pharmaceutical and another field analyses [11]. Because SPME uses small volumes of samples, is an ideal method for extraction of steroid hormones from serum samples, because SPE and LLE require large amounts of sample [12, 14].

Prior to GC analysis, due to the poor thermal stability and volatility of steroid hormones, a derivatization step is needed to produce more volatile compounds and to improve the chromatographic separation. The most commonly used derivatisation for steroids before GC–MS analysis is silylation [8, 9]. In general, N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) are used as derivatizing agents [12, 13].

The purpose of this paper is to determine two natural steroid hormones from poultry samples using a sensitive and rapid technique. SPME is the technique employs for the extraction of estrone and ß-estradiol from poultry serum samples. Separation of the target compound from serum samples was realized by direct immersion in serum samples, with on-fiber silylation, followed by derivatizations with MSTFA and analysis by gas chromatographic with mass spectrometry. The proposed method was used for the first time for determination of EDCs from biological poultry samples and is a rapid, sensitive and accurate method.

RESULTS AND DISCUSSION

The aim of this work was to perform preliminary investigations regarding the analysis of steroids hormones in poultry serum samples. The structure of the target compounds analyzed from poultry serum samples are show in Figure 1.

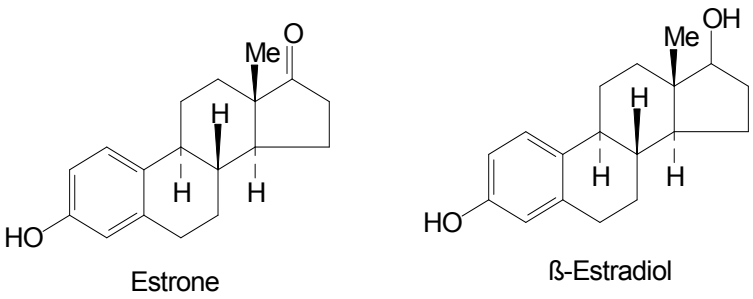


Figure 1. Structures of the steroid hormones

Direct immersion solid-phase microextraction with on fiber derivatization with MSTFA was applied to the poultry serum samples. MSTFA was selected as silylating agent because the products MSTFA silylates are more volatile than BSTFA. The general reaction of derivatization of steroid hormones with MSTFA is presented in Figure 2.

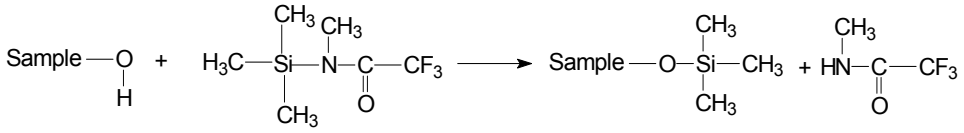


Figure 2. General reaction of steroid hormones derivatization with MSTFA

Estrone and ß-estradiol contained hydroxyl groups can be silylated by replaced labile hydrogens with TMSi groups and to prepare volatile and thermal stable estrogen derivatives for gas chromatography and mass spectrometry. For estrone contained mono-hydroxyl group, the mono-TMSi is formed, ß-estradiol contained bis-hydroxyl groups, the bis-TMSi is formed.

The steroid hormones standards analyzed in this study and their characteristics are given in Table 1.

Table 1. Characteristics of steroid hormones analyzed by GC-MS

Compounds	Molecular mass	Retention time (min.)	Quantitative ions (*m/z*)	Qualitative ions
Estrone	270	20.550	342	257, 218
ß-estradiol	272	21.368	416	129, 285,326

The ions monitored for estrone was *m*/*z* 342 and for ß-estradiol *m*/*z* 416.

The quantification was performed using standard addition method, as presented in the experimental section. The linearity of the calibration curves are presented in the Table 2.

Table 2. Recoveries, limit of detection (LOD) and limit of quantification (LOQ) for steroid hormones from serum poultry samples

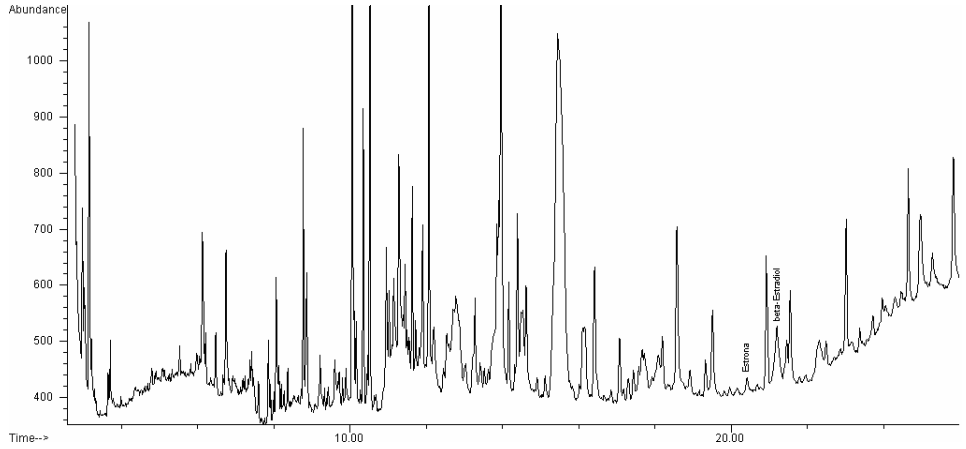
Compounds	Correlation coefficient (R^2)	LOD (μg L^{-1})	LOQ (μg L^{-1})	RSD (%)	Recovery (%)
Estrone	0.996	0.068	0.204	12	95
ß-estradiol	0.995	0.020	0.060	13	90

Limits of detection (LOD) for steroid hormones were calculated as lowest concentration that can be determined with an acceptable level of repeatability and fidelity, by consecutive dilutions of spiked solution of serum and were calculated using the 3s criterion. The limits of quantification (LOQ) for steroid hormones were calculated to the three times of limits of detection.

The relative standard deviation (RSD) of the peaks area of steroid hormones derivatives in the chromatogram (calculated for 6 replicated of a spiked solution containing estrone and ß-estradiol) were 12 % for estrone and 13 % for ß-estradiol.

The recoveries of estrone and ß-estradiol from serum were evaluated by using a serum spiked solution before ether extraction followed by steps presented in experimental section.

The SIM chromatogram of the products ions of estrone in poultry serum sample is presented in Figure 3. The identification of steroid hormones was based on the standard mass spectra of the MS spectral library.



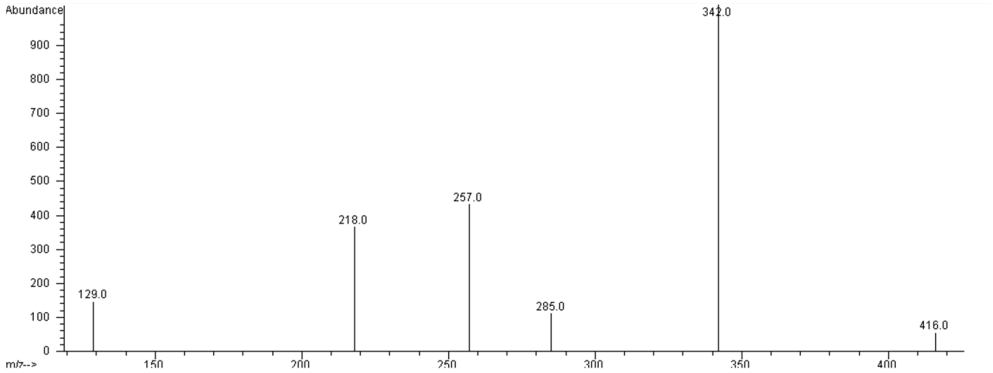


Figure 3. The SIM chromatogram of steroid hormones from poultry serum (and the ions for the quantitative and qualitative analysis of silylation derivatives of estrone)

The extraction time, derivatization time and temperature were selected according to other studies [8, 9].

Temperature is an important factor for SPME process because it controls the diffusion kinetics and the equilibrium between fiber and liquid phase. The obtained average concentrations of estrone and ß-estradiol in poultry serum were: 2.08 μg L^{-1} and 3.61 μg L^{-1}, respectively.

The concentration of estrone and ß-estradiol obtained from poultry serum samples were higher than those obtained by Yang et al. [8] from fish serum.

EXPERIMETAL PART

Chemicals and materials

Steroid hormones including estrone (99+ %) and ß-estradiol (98%) were obtained from Sigma–Aldrich. Methanol, diethyl ether, sodium chloride (NaCl, 99%) and hydrochloric acid (HCl, 37%) was supplied by Merck (Darmstadt, Germany). The derivatization agents MSTFA were also purchased from Sigma–Aldrich. All solvents used were of HPLC grade. Deionized water used in dilutions was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA).

The blood samples were collected from five farmed chickens. The SPME device consists of manual fiber holder supplied from Supelco Inc. (Bellefonte, PA, USA) with 85 μm polyacrylate (PA) fiber supplies by Supelco Inc. (Bellefonte, PA, USA). The fiber was conditioned before use in split mode in the inlet for 2 h at 300 °C according to Supelco's specifications.

For SPME process was used a heater unit and a magnetic stirrer for stirring samples during the process.

Sample preparation

Blood was taken from each poultry sample using heparinized syringe (10 mg mL^{-1} heparin in 0.9 % NaCl). Serum was obtained by separating from plasma by centrifugation for 10 min at 5,000 rpm.

Blood serum is a complex matrix, and contains of proteins, lipids and glucose. In this case, to remove inhibitors extraction, serum was pretreated before of immersing the SPME fiber directly into the serum [12]

An aliquot of 10 mL of the serum was extracted with 50 mL ether three times. The organic phases was combined and evaporated to dryness. The extract was dissolved in methanol and then was transferred to the vials for SPME, MSTFA derivatization and GC-MS analysis.

Stock standards solution of estrone and ß-estradiol (500 µg L^{-1}) were prepared in methanol and stored at -18°C. Working standard mix solutions were performed by dilutions in ultrapure water.

For steroid hormones quantification, the sample was splited into six even aliquots in separate volumetric flasks of the same volume. The first flask was diluted to volume with water. A standard containing the analytes was then added in order to obtain the following concentrations: 1, 10, 50, 75 and 100 µg L^{-1} and then diluted to volume with water. The areas for all calibration levels were measured and linear regression was performed using standard addition method.

Direct immersion SPME analysis

Estrogens were extracted from serum poultry sample by direct immersion of fiber in serum (9 ml serum samples). An amount of 0.9 g NaCl was added to serum to increase of the response of steroids hormones.

In order to control the temperature, sampler vials sealed with a septum were kept in a thermostatic water bath; a magnetic stirring bar was put in for agitation at 250 rpm. Extraction time was 120 min at 45°C. Before SPME, 100 g L^{-1} NaCl was added to sample to enhance the volatilization of the compound.

Derivatization procedure was performed by immersion of the fibre into a sampler vial containing 100 µl MSTFA to a sampler vial sealed with a septum, for 60 min at room temperature (approximately 25 °C).

Finally, the derivatized estrogens were desorbed by introducing the fiber directly into the GC injector.

GC-MS analysis

Analyses were performed using a gas chromatograph (Agilent Technologies, 6890N GC) coupled with mass spectrometer (Agilent Technologies, 5973N MSD) and capillary column of 30 m length×0.25 mm I.D.×0.25 µm HP-3 MS film thickness. The injection port was in splitless

mode. Samples were analyzed in SIM (selected ion monitoring) mode. The temperature program was as following: the initial oven temperature was set at 90 °C, held for 2 min, from 90 to 180 °C via a ramp of 30 °C/min, 180 to 240 °C at a ramp of 10°C/min and 240 to 270 °C at 3 C/min, 270 to 300 °C at 15 °C/min and maintained at 300 °C for 2 min.

CONCLUSIONS

In this paper, a GC-MS method has been successfully utilized for the analysis of the steroid hormones in poultry serum samples. The steroids were extracted from serum by solid phase microextraction and mass spectrometry. The target compounds were first extracted by SPME procedure, followed by derivatization with MSTFA and analysis by GC-MS.

The obtained results confirmed that SPME procedure is very sensitive, and compresses the extraction, concentration and introduction in one step. This method greatly reduces sample preparation time and increases sensitivity comparatively with other extraction methods.

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