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RESEARCH PAPER

Determination of 6 Kinds of Sex Hormones in Fish Using Subcritical 1,1,1,2-Tetrafluoroethane Extraction- Gas Chromatography-Tandem Mass Spectrometry

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Abstract: A sample pretreatment method was developed for the analysis of six sex hormones in fish by subcritical 1,1,1,2-tetrafluoroethane (R134a) extraction coupled with gas chromatography-mass spectrometry(GC-MS). After the extraction process was completed, the fatty co-extracted interferent of the sample was removed by freezing filtration. Then the analytes were cleaned up by C_{18} and NH_2 solid phase extraction (SPE) column. At last, the hormones were derivatived by heptafluorobutyric anhydride (HFBA) followed by GC-MS validation. The optima extraction conditions for the six sex hormones were as follows: extraction pressure, 4 MPa; extraction temperature, 30 ºC; extraction cosolvent amount, 6 mL. Under these conditions, the calibration curves showed good linearity with correlation coefficients larger than 0.99 in the hormones concentrations of 5–1000 μg L^{-1}; the detection limits were 0.2–1.0 μg kg^{-1} (S/N = 3). At the spiked level of 1, 5 and 10 μg kg^{-1}, the mean recoveries were between 70.5% and 103.6%, and the relative standard deviations (RSDs) ranged from 2.1% to 12.5%. Finally, diethylstilbestrol was detected in real samples by the proposed method, and the residue content of which was 14.6 μg kg^{-1}.

Key Words: Subcritical fluid extraction; 1,1,1,2-Tetrafluorothane; Sex hormone; Gas chromatography-mass spectrometry; Residue analysis

1 Introduction

Sex hormones are anabolic hormones. They can speed up the growth of animals for the effect of protein assimilation and thus brought significant economic benefits for animal breeding industry. Therefore sex hormones were widely used in animal breeding process in the last century[1]. However, some hormones are chemically stable, not easily to decompose, easily accumulate and remain in animal tissue. Long-term human consumption of animal tissue containing these hormones will lead to metabolic disorders, developmental abnormalities and even induce cancer[2]. Therefore, since the beginning of the 1990s the EU has passed legislation to restrict or prohibit the use of hormones in animal breeding industry[3]. Chinese Ministry of Agriculture has also listed banned drugs in animal feed and drinking water in announcement No. 176[4], explicitly prohibits the use of diethylstilbestrol, estradiol, etc. However, driven by the interests, phenomenon of illegally adding banned hormone drugs in animal feed is still exist in society today. So, continuing to strengthen determination and evaluation of hormone drugs in animal-derived food are still of great significance to the safety of food of animal origin.

Currently, sample pretreatment methods of residue analysis in animal-derived food include liquid-liquid extraction[5,6], ultrasound-assisted extraction[7], microwave-assisted extraction[8], matrix dispersive solid phase extraction (MSPD)[9], accelerated solvent extraction[10], supercritical fluid extraction[11] and so on. The first three methods always require large amounts of organic solvents which are harmful to the operators, such as

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acetonitrile, methanol and tert-butyl methyl ether, etc. Besides, these methods are time-consuming and complicated to operate. MSPD is usually applied as a pre-treatment method for residue analysis in vegetables and fruits, but seldom used for meat products. The last two methods are hard to be widely used as they need specialized equipments which require large investment. The ozone depression potential (ODP) value and global warming potential (GWP) value of 1,1,1,2-tetrafluoroethane (R134a) are 0 and 0.29 respectively, so it is usually used as a kind of new environmental friendly refrigerant. Meanwhile, dielectric constant (ε = 9.5) and the dipole moment (DM = 2.05) of R134a are very close to dichloromethane (ε = 9.08, DM = 1.55), so R134a has similar solvent properties to dichloromethane. Furthermore, R134a is chemically stable and has good safety performance (non-flammable, non-explosive, non-toxic and non-corrosive)[12], thus it has become a new and green extraction medium and attracted more and more researchers' attention[13,14]. In recent years, subcritical R134a extraction has been successfully used as a new sample pre-treatment method for the determination of Persistent Organic Pollutants (Pops) and drug residues, which is deemed to be a potential alternative to supercritical CO_2 for residue analysis[15–17]. In comparison with the traditional liquid-liquid extraction, subcritical R134a extraction has many advantages, such as higher extraction efficiency, easy to be operated, time-saving and environmental friendly, furthermore, it can complete extraction and separation at the same time. Compared with supercritical CO_2 extraction, subcritical R134a extraction can achieve satisfactory extraction efficiency at low pressure and temperature close to room temperature. Besides, it has low requirement on equipment and operating conditions, so it is easier to be spread and applied.

In this study, a new sample pretreatment method was developed for the simultaneous determination of six sex hormones in fish by subcritical R134a extraction coupled with gas chromatography-mass spectrometry (GC-MS). The proposed method has the advantages such as lower solvent consumption, high sensitivity and selectivity, and is simple to be operated.

2 Experimental

2.1 Instruments and reagents

Subcritical R134a extraction device of self-design was shown in Fig.1. 6890-5973 GC-MS (Agilent, USA), Vortex Mixers (Shanghai Kanghua Biochemical instruments company, China) were used in this study.

Methyltestosterone and testosterone propionate were purchased from Dr. Ehrenstorfer GmbH (Germany). Estriol, medroxyprogesterone acetate, β-estradiol acetate and diethylstilbestrol were purchased from Sigma-Aldrich (USA).

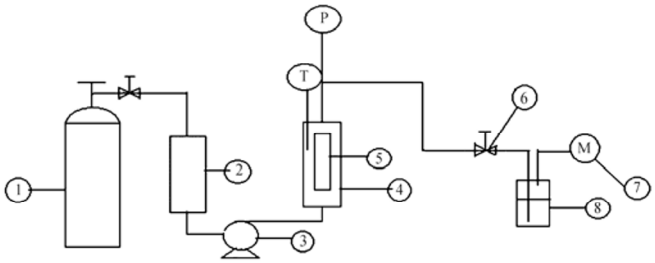

Fig.1 Flow-chart of subcritical R134a extraction

1, R134a tank; 2, condenser; 3, pump; 4, heating jacket; 5, extraction cell; 6, relief valve; 7, flowmeter; 8, Collector

The chemical purity of all the standards were more than 98%. HPLC grade organic solvents, methanol (MeOH), acetonitrile (ACN) and acetone were purchased from Anpel (Shanghai, China). The derivatization reagent HFBA was purchased from Regis (USA); C_{18} (500 mg, 6 mL) and NH_2 (500 mg, 6 mL) cartridges were obtained from Anpel (China); the silica and glass fiber were purchased from Hongyan Chemical Reagent Corporation (Tianjin, China) and Kelong Chemical Reagent Corporation (China); a cylinder of liquefied R134a was obtained from Mexichem (Mexico).

2.2 Preparation of standard solution

A 100 μg mL^{-1} of standard stock solution of six sex hormones mixture in MeOH was prepared and stored at –18 ºC prior to use. Different concentrations of the working standard solution were prepared by diluting the standard stock solution with MeOH at the desired.

2.3 Subcritical R134a extraction

About 4 g ground fish (Tilapia) tissue was placed in a mortar, then 12 g silica and 4 g glass fibre were added to make the sample dispersed homogeneously. Once the mixture was loaded into the extraction cell, moderate amounts of cosolvent were directly spiked onto the mixture. To prevent blocking of the cap vessel holes, the top and the end of the extraction cell were filled with cotton. Then subcritical R134a extraction was conducted under the design conditions. For each experiment, a 20-min static extraction was first carried out, and then followed by a 40-min dynamic extraction. The flow rate was set at 1 mL min^{-1}. The stream of subcritical R134a fluid containing the target compounds was then depressurized through a manual pressure restrictor and the extracts were collected with MeOH. As soon as the experimental process was completed, the extracts were collected and stored at –18 ºC.

2.4 Clean up

The extracts were rapidly passed through a 0.22-μm filter to