MASS-SENSITIVE DETERMINATION OF ß-AGONISTS IN MEAT RAW MATERIALS

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Abstract – This article describes a mass-spectrometry method for determination of muscle growth hormones, ß-agonists. Factors influencing their recovery were determined; methods of ß-agonists extraction and purification using solid-phase extraction are given. The parameters of mass-spectrometric identification of 11 ß-agonists have been developed.

Key Words – LC-MS/MS, β-agonists, solid-phase extraction, meat products.

I. INTRODUCTION

ß-agonists have a strong anti-catabolic effect and contribute to muscle mass gain resulting in their widespread use as growth stimulants in animals. Despite the fact that ß-agonists are not steroid hormones, their pharmacological properties are similar to steroids. Metabolism of pharmacological growth stimulants leads to their excretion from the animal body within 100-200 days, but even later, up to 10% of the initial injected dose may be detected in the organs and tissues of the animal [1-2]. Due to the high biological activity, the residues of such substances represent an exceptional hazard for a person consuming meat produced using hormonal technologies. Therefore, the use of hormones in production of livestock and poultry is legally prohibited in 160 countries, including China, Russia and the countries of the European Union [3-4].

When consumed with food, β-agonists in humans can provoke tachycardia, increased blood pressure and other disorders including lethal outcome [5]. Consumption of such products by people with cardiovascular diseases is of particular hazard [6]. In some countries (USA, Canada, etc.), several natural and synthetic hormonal growth stimulants for farm animals have been officially authorized. Therefore, strict control over the residual content of ß-agonists in products of animal origin is necessary.

II. MATERIALS AND METHODS

Analysis of β-agonists was performed by LC-MS/MS using Agilent 1200 chromatograph with Agilent 6400B triple quadrupole (QQQ) detector. Chromatographic separation was carried out on C18 reversed phase column, Agilent XDB-C18 2.1 x 50 mm, 1.8 μm. Electrospray (ESI) was used as an ionization source. Samples were prepared using BondElut solid-phase extraction sorbents (Agilent, USA).

III. RESULTS AND DISCUSSION

To prepare samples for analysis, a sample of 5 g was placed in a centrifuge tube. 20 cm^3 of a 0.2 M sodium acetate solution with pH of 5.2 was added. 0.250 cm^3 of beta-glucuronidase with activity of 1000 U/cm^3 was added, mixed, and placed in a water bath at 37 °C for 16 hours. The hydrolyzate was centrifuged for 5 minutes at 4000 rpm. 4 cm^3 of the supernatant was transferred into centrifuge tube; 5 cm^3 of 0.1 M perchloric acid solution was added; pH was adjusted to 1 ± 0.3 with perchloric acid; and the sample was centrifuged for 10 minutes. The supernatant was transferred to a test tube; the pH was adjusted to 11.0 with 10 M sodium hydroxide solution. 10 cm^3 of 3M sodium chloride solution and 10 cm^3 of isopropyl alcohol/ethyl acetate mixture (6:4) were added to the tube, mixed, and centrifuged for 5 minutes; extraction was repeated twice. The organic layer of isopropyl alcohol with ethyl acetate was evaporated at 40 °C with the Heidolph Laborota 4003 rotary evaporator. The dry residue was dissolved in 5 cm^3 of a 0.2 M sodium acetate solution with pH 5.2; and solid-phase extraction (SFE) was carried out. To do this, BondElut cartridges (Agilent, USA) were pre-activated with 3 cm^3 of methanol and 3 cm^3 of distilled water. The sample solution was applied to the cartridge at a rate of 1 cm^3/min. The cartridge was washed with 2 cm^3 of distilled water and 2 cm^3 of 2%

formic acid solution. The analytes were eluted with 5 cm^3 of 5% ammonia solution in methanol at a rate of 1 cm^3/min. The eluate was evaporated to dryness with a rotary evaporator at 40 °C. The dry residue was dissolved in 1 cm^3 of 1% formic acid/methanol mixture (9:1). The solution was passed through 0.45 μm membrane filter; and HPLC-MS/MS analysis was performed.

The analysis was carried out on the Agilent 1200 high-performance liquid chromatography system (USA) with the Agilent 6410B triple quadrupole mass spectrometer. The separation was carried out on the Agilent XDB-C18 column (4.6 x 50 mm, 1.8 μm) in a gradient elution mode: for 1 minute, equilibrating the chromatography column with 98% eluent A (1% formic acid solution) and 2% eluent B (acetonitrile/methanol mixture (1:1)) followed by a step gradient to 65% eluent A and 35% eluent B for 3 minutes, and then to 10% eluent A and 90% eluent B for 4 min. The temperature of the column was 40 °C. The volume of the sample injected was 10 μl; the elution rate was 400 μl/min. Ionization was carried out by spraying in an electric field (ESI). The following mass-spectrometric detection parameters were selected for the analysis:

The source temperature was 100 °C;

The temperature of desolvation gas was 350 °C;

The rate of desolvation gas flow was 5 dm^3/min;

The pressure of the spray nozzle was 45 psi. The detection conditions were optimized in manual mode. The voltage in a fragmentor was optimized in increments of 10 V according to the maximum response of the protonated molecular ion. The dissociation energy (CE) was optimized in increments of 1 V according to the maximum response of a characteristic daughter ion. Signal/noise ratio (S/N) of the molecular ion must be at least 1:10. The conditions for recording analytical signals in the mode of multiple-reaction monitoring (MRM) are presented in Table 1.

ß-Agonists were identified by absolute retention time of the chromatographic peaks of the target substances recorded in multiple-reaction monitoring (MRM) mode using Mass Hunter Workstation software (Agilent, USA). Calibration dependence of β-agonist peak areas on the concentration in the sample were plotted. The coefficient of linear correlation for the calibration obtained in ß-agonist peaks was not less than 0.99. The obtained chromatograms for ß-agonist standard samples and calibration curves are given in Figure 1.

Table 1. Parameters of the effect on ions in MRM mode and conditions of electrospray ionization (ESI) with registration of positive ions

Analyte	Molecular ion, m/z	Daughter ions, m/z	Fragment voltage (Frag), V	Dissociation energy (CE), V
Clenbuterol	277.2	203.1 259.1	100	12 5
Salbutamol	240.2	148.2 222.1	100	15 5
Ractopamine	302.2	164.2 284.1	110	12 6
Terbutaline	226.1	152.2 170.2	100	12 6
Salmeterol	416.3	380.3 398.4	130	17 10
Propranolol	260.2	116.2 183.2	120	15 15
Tulobuterol	228.1	154.1 172.2	100	12 5
Cimaterol	220.1	160.2 202.1	90	12 3
Mabuterol	311.2	237.1 293.2	110	13 7
Mapenterol	325.3	237.1 307.2	110	12 5
Zilpaterol	262.2	244.2 202.2	100	7 17

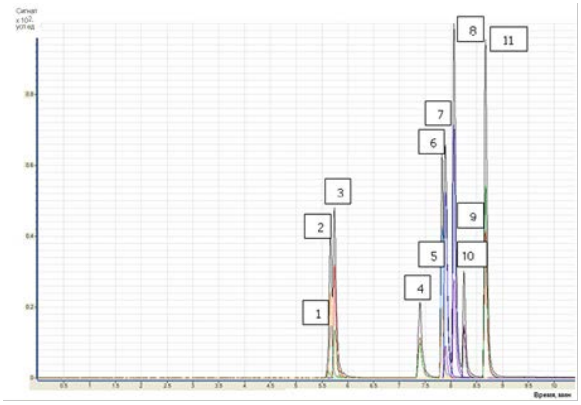

Figure 1. Chromatogram according to the total ion current of ß-agonist calibration solutions; 1 - terbutaline, 2 - cimaterol, 3 - salbutamol, 4 - ractopamine, 5 - tulobuterol, 6 - clenbuterol, 7 - mabuterol, 8 - zilpaterol, 9 - propranolol, 10 - mapenterol, 11 - salmeterol

The main difficulty in developing multicomponent method for determination of ß-agonists of aniline, phenolic and resorcin-type is the differences in polarity and metabolism. Aniline-type β-agonists such as mapenterol,

clenbuterol, and cimbuterol, almost do not generate conjugated forms with glucuronic and sulfuric acids. While phenolic (salbutamol) and resorcin type (terbutapine) β-agonists form polar glucuronides and sulfates in the process of metabolism [14]. Given these peculiar properties, enzymatic preparation of β-glucuronidase from a grape snail (G8885) was used to hydrolyze the conjugated forms; and optimal conditions for enzyme action were selected. Hydrolysis was carried out for 16 hours at 37 °C in acetate buffer, pH 5.2. To precipitate proteins after hydrolysis, a solution of perchloric acid was used.

Studies were conducted to determine the lower limit of quantification (LLOQ) and the recovery during sample preparation. For this purpose, mixture of standard solutions of ß-agonists was added to the samples. The extraction of aniline, phenolic and resorcin type ß-agonists was carried out by liquid-liquid extraction with a mixture of isopropyl alcohol and ethyl acetate. When analyzing the extracts, ionization was suppressed by the matrix due to insufficient purification of the samples. The use of TFE allowed minimizing the "matrix effect" and achieving a stable analytical signal. BondElut cartridges (Agilent, USA) were used for TFE. The composition of the TFE cartridge includes reversed-phase and ion-exchange components, which, due to hydrophobic interactions and ion exchange, simultaneously determine ß-agonists of aniline, phenolic and resorcin type.

One of the main tasks of establishing the method was to minimize the loss of ß-agonists related to the sample preparation procedure. To exclude the presence of organic pollutants in the analyzed sample, the elution conditions for TFE were optimized. After liquid-liquid extraction, the organic solvent was evaporated and the sample was re-dissolved in acetate buffer, pH 5.2, which allowed reducing impurities due to nonpolar interactions. The cartridge was washed with 2 cm^3 of distilled water and 2 cm^3 of 2% formic acid solution, which allowed removing neutral impurities and promoted the selective retention of positively charged β-agonist molecules on the cartridge. Ammonia was added to the eluate to flush the ß-agonists, which resulted in neutralization of amino groups in ß-agonists, as well as the destruction of hydrophobic interactions and ionic bonds. When selecting the elution conditions, it was empirically established that at least 5 cm^3 of eluate is required to flush ß-agonists of aniline, phenolic and resorcin type.

IV. CONCLUSION

The above procedures allow a significant decrease in the total error in the mass-spectrometric analysis technique due to a reduction of the influence and standardization of the sample preparation procedure.

As a result of optimization of the elution conditions, the recovery of target β-agonists was 69 to 87%; the lower limit of quantification for all substances was not less than 1 μg/kg. TFE increases the selectivity of ß-agonists determination and allows minimizing the effect of organic impurities on the result of measurements. By transferring the analytes from a large volume of liquid matrix to solid phase of the sorbent, it is possible to concentrate even trace amounts of ß-agonists. However, usage of TFE does not exclude losses due to flushing from the cartridge; therefore, it is desirable to use isotope-labeled internal standards of ß-agonists to calculate the recovery.

Detectable levels of β-agonists in the organs and tissues of animals range from 1.0 to 100.0 μg/kg. The procedure allows determination of 11 β-agonists.

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