Rapid Determination of 53 Stimulants Residues in Animal Food by Ultra-high Liquid Chromatography Tandem Mass Spectrometry

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Abstract: A liquid chromatography-tandem mass spectrometry (LC-MS) method for screening 53 stimulants in animal foods was developed. The method uses 2% formic acid-acetonitrile/water (80/20) as the extraction solvent and carries out one-step purification by FaVEx-AP solid phase extraction column. Endeavorsil C18 column was used for separation, and multiple reaction monitoring (MRM) mode was used for data acquisition and analysis. The results showed that the correlation coefficient (R2) was more than 0.99. The detection limit and quantification limit were 0.25-10μg/kg and 1-10μg/kg. The recoveries were 62.25%-123.32% and the relative standard deviation (RSD) was 1.17%-22.03%. The method is rapid, simple and accurate, and is suitable for the determination of 53 kinds of stimulants in animal-derived foods.

1. Introduction

Food stimulants are classified as endogenous and exogenous. Endogenous stimulants: naturally occurring stimulant components in certain foods that are naturally occurring or produced in animals and plants, that is, endogenous stimulant components [1]; Exogenous stimulants: They are not produced naturally in the body and are mainly produced by ingredients artificially added or left in food during production and processing. In China, the main risk of food stimulants comes from exogenous stimulants. Unreasonable eating habits, drug abuse, illegal addition of prohibited drugs to animal-derived foods, etc., may lead to a large number of pesticide residues, antibiotics and hormonal drugs [2]. Today, the most common detection method used for stimulation is solid phase extraction-liquid chromatography mass spectrometry/mass spectrometry. The fastest processing of a batch of samples is a few hours. Purification requires a large amount of solvents. Different drugs use the same type of pretreatment. The pretreatment process of different pretreatment methods, more testing standards [3]. Simultaneous detection of multiple stimulants has become a new development direction. At present, the pretreatment methods commonly used are: solid-phase extraction [4-5], solid-liquid extraction [6-7], matrix solid-phase extraction [8-9], QuEChERS rapid purification [10] and so on. The methods for determination of stimulant residues include gas chromatography-tandem mass spectrometry [11], biochip technology[12], high performance liquid chromatography-tandem mass spectrometry[13] and ultra-high performance liquid chromatography-tandem mass spectrometry [14], etc. UPLC-MS/MS has the advantages of high efficiency, specificity, high sensitivity and simultaneous detection of various compounds, and is the main method for doping detection. A method for simultaneous detection of 53 stimulants in animal-derived foods by ultra-high performance liquid chromatography-mass spectrometry (UPLC-MS/MS) was developed by optimizing the pretreatment methods and instrumental conditions.

2. Materials and Methods

2.1. Materials and reagents

Acetonitrile, methanol(chromatographic pure; Sinopharm Group Chemical Reagent Co. Ltd.); Ammonium formate, formic acid (mass spectrometry grade; Sinopharm Group Chemical Reagent Co.Ltd.); Endeavorsil C18 column (100 mm×2.1 mm×1.8 μm; Beijing Dima Technology); FaVEx-AP Solid Phase Extraction Column (Taiwan Lvjuyan Co.Ltd.) 0.22 μm Needle Membrane Filter Head (Tianjin Jinteng Experimental Equipment Co.Ltd.)

Sample type: Pork, fish, chicken; Sample source: Zhoushan Food and Drug Inspection and Testing Institute sampling project; Sample preparation treatment: shredded by cooking machine for use.

2.2. Instruments and equipment

QTRAP 5500$^+$ Liquid Chromatography-Mass Spectrometer-Distribution Spray Ion (ESI) Source (AB

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SCIEX, USA); ME802E Analytical Balance (Mettler-Toledo Instruments (Shanghai) Co. Ltd.); Milli-Q Ultrapure Water Machine (Sartorius Scientific Instruments Co.Ltd.); Hand-like oscillator (Juyan Technology Co. Ltd.); Constant speed positive pressure column press (Juyan Technology Co.Ltd.)

2.3. Experimental Methods

2.3.1 Sample pretreatment

2.5 g of the sample was weighed, accurately weighed in a 50 mL centrifuge tube, and 10 mL 0.2% formic acid-acetonitrile/water (80/20, v/v) solution was added as the extraction solution, oscillating violently for 15 min. Centrifuge at 5000 r/min for 5 min, take 5 mL of supernatant through the FaVEx-AP purification column, purify at positive pressure, flow out at a flow rate of 1 drop per second, collect all the purified solution, take 2 mL of filter liquid nitrogen and blow, add 1 mL of 10% methanol water (10/90, v/v) for redissolution, pass through the 0.22 μm filter membrane, and then go on the machine for detection.

2.3.2 Preparation of Standard Solution

(1) Preparation of standard stock solution (1mg/mL): Exact and precise removal of 53 standard solutions of stimulants and preparation of 1 mg/mL standard stock solution with methanol, stored at-18 ℃ in the dark.

(2) Preparation of mixed working solution (1.0 μ g/mL): Accurately absorb an appropriate amount of the above-mentioned reserve solutions, dilute with methanol to prepare a mixed standard working solution with a concentration of 1.0 μ g/mL, and store it at-18 °C in the dark.

(3) Preparation of solvent standard curve: Accurately remove the mixed standard working solution, dilute it with 10% methanol water (10: 90, v/v) constant volume solution, and prepare a series of standard solutions of 1, 2, 5, 10, 20, 50, 100ng/mL.

(4) Preparation of matrix standard solution: Accurately remove the blank matrix solution purified by pretreatment 1mL → nitrogen drying → 1mL target compound standard solution redissolve → vortex → oscillation → pass through 0.22 μ m organic filter membrane → go on the machine for detection, and obtain 1, 2, 5, 10, 20, 50, 100ng/mL blank matrix standard solution curves. The standard solution of matrix mixture should be prepared on the spot.

2.3.3 Chromatographic conditions

Liquid chromatography column: Endeavorsil C18 (2.1×100 mm, 1.8 μm); Column temperature 40℃; Injection volume 5 μL; The flow rate is 0.40 mL/min Mobile phase A:2mM ammonium formate (0.01% formate) water, Mobile phase B: acetonitrile; The gradient elution conditions are shown in Table 1.

Table 1. Gradient elution procedure of mobile phase

Time (min)	Velocity of flow (mL/min)	Mobile phaseA(%)	Mobile phaseB(%)
0	80	20	0.4
1	65	35	0.4
3	65	35	0.4
8	50	50	0.4
10	5	95	0.4
16	5	95	0.4

2.3.4 Mass Spectrometry Conditions

Ion source: electrospray ionization (ESI); Atomization voltage (IS) : positive ion mode: 3500V (positive), -3000V (negative), negative ion mode: -1000V (negative); Sheath gas flow: nitrogen, 11L/min; Atomizer temperature (Gas Temp) : 325℃; Atomizing gas (GS1) : 45 psi; Gas Flow: 10L/min; Sheath Gas Flow:11L/min; Scanning mode: positive and negative ion scanning; Monitoring mode: Dynamic Multi-Response Monitoring mode (dMRM)

2.4. Data processing

UPLC-MS is equipped with SCIEX OS data processing software to establish standard curves, suspicious samples are qualitatively confirmed by retention time, qualitative ion, quantitative ion and ion kurtosis ratio matching, and the content of each component is calculated by external standard method.

3. Results and Analysis

3.1. Optimization of Chromatographic and Mass Spectrometric Conditions

3.1.1 Optimization of liquid phase conditions

The chromatographic conditions were optimized to obtain the best separation results and shorter detection time, and the mobile phase favorable for ionization was selected to improve the detection sensitivity of mass spectrometry. General reverse-phase chromatography uses methanol, acetonitrile and a certain concentration of salt. In the positive ion mode of the electrospray ion source, the ionization efficiency of the components to be measured can be improved by adding an appropriate amount of formic acid into the mobile phase, and the detection response intensity can be improved. The peak and response effects of acetonitrile water and methanol water as mobile phases were compared, and the ionization efficiencies of formic acid, ammonia, ammonium formate and ammonium acetate were compared. The elution procedure was adjusted to separate the components. The optimized spectra are shown in Figure 1 below.

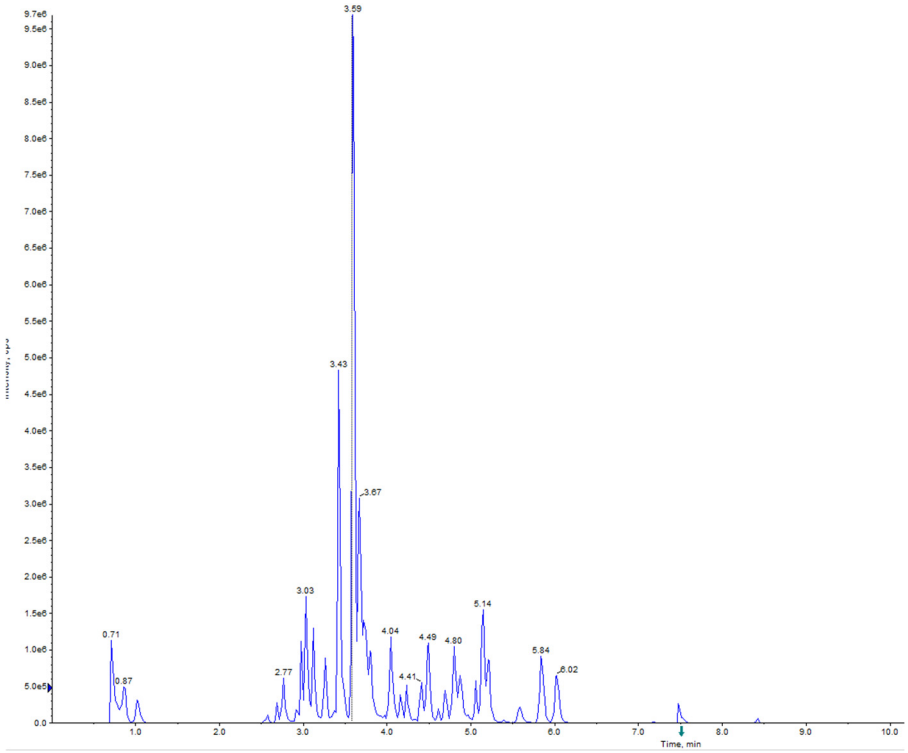

Figure 1. Chromatograms of 53 stimulants

3.1.2 Optimization of mass spectrometry conditions

53 standard solutions were mixed with 1000 ng/L standard solution for mass spectrometry scanning, ESI + mode was selected, and the standard solution was directly implanted into the ion source by pump (needle). First of all, we use first-order mass spectrometry analysis to obtain the existing molecular ion peaks through Q1 to determine the target of the relevant information of the mass spectrometry ion compounds, select an appropriate speed to scan, and after scanning, collect and store the expected data of the collected ions. After the parent ion of the target compound appears, the collision voltage (Collision Energy, CE) is manually adjusted, and a certain Collision Energy is applied to the parent ion to scan, and their secondary fragment ions are obtained, so as to select 2-3 suitable daughter ions and their parent ions to form monitoring ion pairs. On this basis, the MRM ion-pair channel was established, the quantitative ion-pair, conical hole voltage and collision voltage were optimized, and the MRM detection mode was established. Final information for ion pairs and mass spectrometry parameters is shown in Table 2.

Table 2. Compound information and MRM mass spectrometry acquisition parameters

ID	Parent ion (m/z)	Daughter ion (m/z)	Retention time(RT)	Collision voltageCE (eV)
α-zearalanol	321	277	10.2	20
β-zearalanol	321	303	8.86	23
3-O-methyl-Colterol	240.1	166	2.26	10
Arformoterol	345.2	149	4.2	25
Atenolol	267.2	190	1.98	17
Bambuterol	368.1	294.1	4.76	20
Beclometasone	409.2	391.1	8.58	6
Bendroflumethiazide	420	327.9	10.18	29
Betamethasone	393.2	355.2	7.97	5
Boldenone	287.2	135.1	9.59	8
Brombuterol	367	293	4.9	15
Bromchlorbuterol	321.1	303	4.68	8
Bumetanide	365.1	240	11.22	15
Canrenone	341	187	12.04	25
Carteolol	293.2	237.2	2.45	17
Chlorothiazide	293.9	214	2.88	34
Cimbuterol	234.1	216.1	2.51	5
Clenbuterol	277	203	4.88	21
Clencyclohexerol	319.1	203.1	3.22	15
Clenisohexerol	305.1	203	6.15	25
Clenpenterol	291.1	203	5	15
Clenproperol	263.1	245.2	4.03	10

Clorprenaline	214.1	196.1	3.98	8
Colterol	226.1	208.1	1.98	8
Cortisone	361.1	163.1	6.15	20
Dexamethasone	393.2	355.2	8.11	4
Fludrocortisone	381	239	6.24	24
Higenamine Hydrobromide Salt	272.1	255.1	2.17	15
Adrenaline	184.1	166	1.6	8
Hydrocortisone	363.2	121	3.51	24
Hydroxymethylclenbuterol	293.1	275	3.75	8
Isoxsuprine	302.1	284.1	5.05	10
Mabuterol	311.1	237.1	5.32	13
Mapenterol	325.1	237	6.26	23
Metandienone	301.2	149.1	10.47	15
Methylprednisolone	375.2	357.2	7.67	6
Metoprolol	268.1	159	4.24	20
Nandrolone	275.2	239	9.98	15
Olodaterol Hydrochloride	387.2	163	4.59	20
Prednisolone	361.2	343.2	6.15	6
Propranolol	260.2	183.1	6.27	18
Ractopamine	302	284.2	3.59	8
Salbutamol	240.2	222.2	1.83	4
Spironolactone	341.1	186.9	12.04	30
t-Butylnorsynephrine	210.1	192.1	2.15	8
Terbutaline	226.1	152.1	1.76	12
Testosterone propionate	345.2	149	4.32	20
Torasemide	349	290	5.56	15
Trenbolone	271.2	253.2	9.04	20
Tretoquinol	346.2	164.1	3.77	20
Triamterene	254.1	237.2	3.67	30
Tulobuterol	228.1	154	4.42	13
Zilpaterol	262.2	244.1	1.98	8

3.2. Optimization of pretreatment methods

3.2.1 Optimization of extraction solvent

There are many kinds of compounds involved in this experiment, and the polarity difference is large, so the selection of the extraction agent should fully consider the polarity of the target substance and the characteristics of the substrate. In this study, 0.2% formate-acetonitrile/water (70/30 v:v), 0.2% formate-acetonitrile/water (80/20 v:v), 0.2% formate-acetonitrile/water (90/10 v:v), 0.5% formate-acetonitrile/water(80/20 v:v) and 1% formate-acetonitrile/water (80/20 V: V) were investigated v:v), the effect of pure acetonitrile on the extraction rate of 53 stimulants. The spiked concentration was 10ng/g, 3 parallel were detected in each group, and each parallel was measured twice continuously. The average recovery rate of 53 kinds of stimulants was taken as an indicator, and the results were shown in Figure 2. According to the comprehensive comparison of the recovery rate of each compound, 0.2% formic acid-acetonitrile/water (80/20 v:v) acetonitrile water was better for the extraction of most compounds. Therefore, 0.2% formic acid-acetonitrile/water (80/20 v:v) acetonitrile water was selected as the extraction solvent.

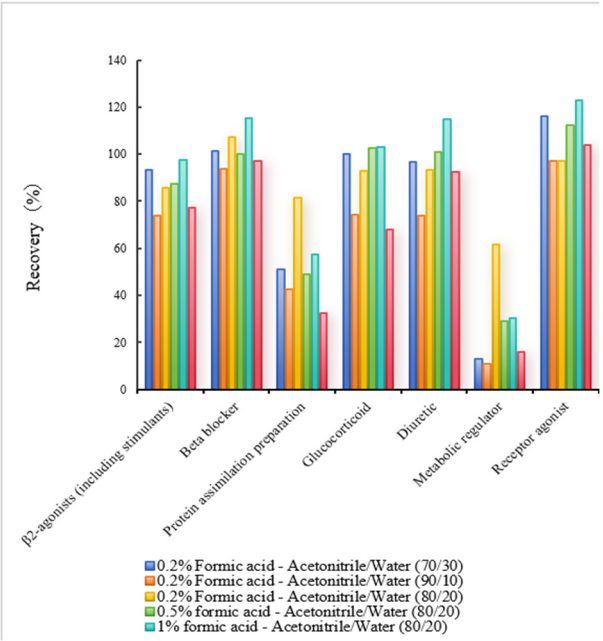

Figure 2. Recovery of stimulants in different extraction solvent

3.2.2 Choice of decontamination methods

During the extraction process, after the sample has been precipitated by protein, there may still be some fatty impurities, which will interfere with the results, cause high baseline noise, and accelerate the contamination of the instrument ion source. In order to improve the purification efficiency and reduce the loss of the measured

object, it is necessary to purify. According to the characteristics of the sample substrate, FaVE-AP purification column, HLB Pro solid phase extraction column and Agilent Captiva EMR-lipid column were selected for detection and analysis. The extraction efficiency of different purification methods was investigated. As shown in Figure 3, compared with HLB Pro solid phase extraction column and Agilent Captiva EMR-lipid column, the FaVE-AP purification column has a higher recovery rate and greater stability. The target compounds range from 90% to 100%, except for metabolic modulators, which are 65%.

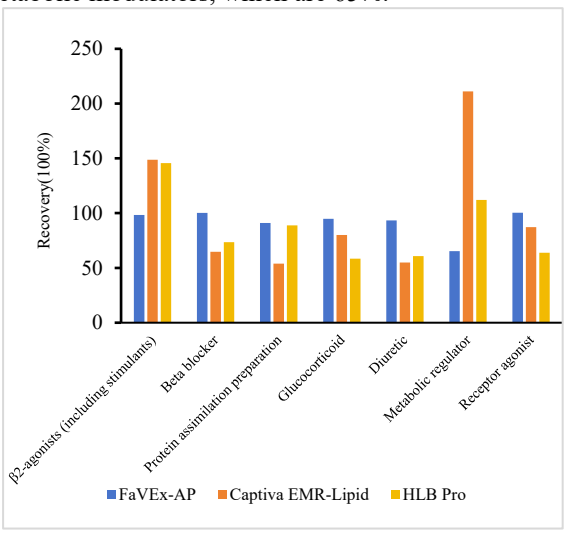

Figure 3. Recovery rate of stimulant under different purification methods

3.3. Methodological Review

3.3.1 Linear range, detection limit and quantification limit of the method

In order to eliminate or weaken the matrix effect, standard curves were established by using matrix matching standard solution. Taking pork samples as an example, the mixed standard solution with different mass concentrations was prepared by diluting blank matrix solution step by step. The concentration gradients were 0, 2, 5, 10, 20, 50, 100, 200 ng/L, and then analyzed and determined on the machine. The matrix standard curves and linear regression equations (R) were drawn with the response area of each compound in the mass spectrum as the ordinate (Y) and the standard working solution concentration of the standard matrix as the abscissa (X). The detection limit (LOD) was calculated by 3 times the signal-to-noise ratio, and the quantitative limit (LOQ) was calculated by 10 times the signal-to-noise ratio. The results are shown in Table 3.

Table 3. Linear equations, correlation coefficients, detection limits and quantitative limits of 53 stimulants

ID	Linear equation	R^2	LOD /(ug/kg)	LOQ /(ug/kg)
α-zearalanol	y = 841.94x + 458.86	0.9989	1.00	2.00
β-zearalanol	y = 492.48x + 93.066	0.9929	1.00	2.00
3-O-methyl-Colterol	y = 547.8x + 528.57	0.9912	1.00	2.00
Arformoterol	y = 1716.2x - 1504.4	0.9947	1.00	2.00
Atenolol	y = 65030x - 18605	0.9974	1.00	2.00
Bambuterol	y = 32650x - 3556.1	0.9937	1.00	2.00
Beclometasone	y = 8551.3x - 257.93	0.9989	1.00	2.00
Bendroflumethiazide	y = 108420x - 54408	0.9961	1.00	2.00
Betamethasone	y = 8779.1x + 1414.3	0.9936	1.00	2.00
Boldenone	y = 30231x - 25020	0.9921	5.00	10.00
Brombuterol	y = 2980.9x + 906.33	0.9949	0.50	1.00
Bromchlorbuterol	y = 2709.3x - 271.93	0.9955	0.30	1.00
Bumetanide	y = 62726x - 34271	0.9962	1.00	2.00
Canrenone	y = 2709.3x - 271.93	0.9955	1.00	2.00
Carteolol	y = 41779x - 21603	0.9904	5.00	10.00
Chlorothiazide	y = 13576x + 577.27	0.9996	0.50	1.00
Cimbuterol	y = 394.31x + 96.958	0.9962	5.00	10.00
Clenbuterol	y = 17172x - 3334.3	0.9916	2.00	10.00
Clencyclohexerol	y = 57077x - 17002	0.9973	1.00	2.00
Clenisohexerol	y = 3278.9x + 190.33	0.9908	1.00	2.00
Clenpenterol	y = 25315x + 52.29	0.9951	1.00	2.00
Clenproperol	y = 5148.1x - 1591	0.992	1.00	2.00
Clorprenaline	y = 5597.5x - 1393.8	0.9959	1.00	2.00
Colterol	y = 12772x + 1647.9	0.9964	1.00	2.00
Cortisone	y = 29782x - 8676.9	0.9971	1.00	2.00
Dexamethasone	y = 2469.9x - 333.65	0.9931	1.00	2.00
Fludrocortisone	y = 3001.1x + 625.63	0.9943	0.40	1.00
Higenamine Hydrobromide Salt	y = 6211.9x + 2458.3	0.9969	0.50	1.00
Adrenaline	y = 3393.8x - 57.57	0.9967	1.00	2.00
Hydrocortisone	y = 97739x - 20894	0.9957	10.00	20.00
Hydroxymethylclenbuterol	y = 50379x - 15584	0.9904	100.00	200.00

Isoxsuprine	y = 49077x - 27692	0.9948	1.00	2.00
Mabuterol	y = 112018x - 40913	0.998	1.00	2.00
Mapenterol	y = 7057.5x - 2800.3	0.995	1.00	2.00
Metandienone	y = 8779.1x + 1414.3	0.9936	1.00	2.00
Methylprednisolone	y = 5404.4x - 583.88	0.9988	0.30	1.00
Metoprolol	y = 4957.4x + 652.48	0.9975	0.50	1.00
Nandrolone	y = 5597.5x - 1393.8	0.9959	0.50	1.00
Olodaterol Hydrochloride	y = 42949x - 24096	0.9929	0.40	1.00
Mabuterol	y = 112018x - 40913	0.998	1.00	2.00
Mapenterol	y = 7057.5x - 2800.3	0.995	1.00	2.00
Metandienone	y = 8779.1x + 1414.3	0.9936	1.00	2.00
Methylprednisolone	y = 5404.4x - 583.88	0.9988	0.30	1.00
Metoprolol	y = 4957.4x + 652.48	0.9975	0.50	1.00
Nandrolone	y = 5597.5x - 1393.8	0.9959	0.50	1.00
Olodaterol Hydrochloride	y = 42949x - 24096	0.9929	0.40	1.00
Prednisolone	y = 17079x - 10031	0.9967	1.00	2.00
Propranolol	y = 4957.4x + 652.48	0.9975	0.50	1.00
Ractopamine	y = 766357x - 181851	0.9971	0.50	1.00
Salbutamol	y = 6926.3x + 1633.3	0.997	0.25	0.50
Spironolactone	y = 0.3313x + 226.74	0.9938	1.00	1.00
T-Butylnorsynephrine	y = 42415x - 6042.1	0.996	5.00	10.00
Terbutaline	y = 49419x - 7362.4	0.9948	1.00	2.00
Testosterone propionate	y = 8577.3x + 386.9	0.9946	0.25	0.50
Torasemide	y = 3372x - 2058.9	0.9914	0.40	1.00
Trenbolone	y = 2309x - 1081.3	0.9967	5.00	10.00
Tretoquinol	y = 5666.5x - 2144.9	0.9908	0.40	1.00
Triamterene	y = 77288x - 16765	0.9954	20.00	50.00
Tulobuterol	y = 6470.7x + 213.72	0.9996	5.00	10.00
Zilpaterol	y = 19094x - 7334	0.9919	0.25	0.50

3.3.2 Accuracy, precision

In order to verify the accuracy of this method in the determination of doping residues, the recovery rate was verified on blank samples. According to the established pretreatment method and instrumental analysis method, according to the three gradient addition ratios of low, medium, and high, 1, 2, and 5 times LQD stimulants were added. Six parallel experiments were carried out for each addition level. The average recovery rate and relative standard deviation (RSD) were calculated to express the precision. The test results are shown in Table 4. The experimental results are shown in Table 4: The average recovery of 1LQD addition level is 56.75% ~ 123.32%, and the RSD is 2.67% ~ 26.8%; The average recovery of 2LQD was 56.75% ~ 125.31%, RSD was 2.67% ~ 27.61%; The average recovery of 5LQD was 50.54% ~ 116.91% and the RSD was 1.17% ~ 11.18%

Table 4. Average recovery and precision of 53 stimulants

ID	Additive amount 1 (μg/kg)		Additive amount 2 (μg/kg)		Additive amount 5 (μg/kg)	
	Recovery%	RSD%	Recovery%	RSD%	Recovery%	RSD%
α-zearzol	73.13	21.49	73.13	21.49	50.54	9.24
β-zearzol	112.73	7.02	112.73	7.02	85.21	5.52
3-methoxycolterol	105.03	13.79	91.94	27.61	87.84	6.32
Formoterol tartrate	82.23	11.28	82.23	11.28	80.34	6.18
Atenolol	81.1	13.26	81.1	13.26	83.52	3.99
Bambuterol hydrochloride	107.42	6.42	107.42	6.42	97.9	2.65
Beclomethasone	97.79	6.4	97.79	6.4	108.02	4.22
Benfluthiazide	123.32	6.87	123.32	6.87	92.1	3.13
Betamethasone	109.09	6.25	109.09	6.25	104.84	4.88
Boldenone	65.84	8.18	65.84	8.18	81.15	7.76
Brobuterol	89.83	11.28	89.83	11.28	79.91	1.49
Cartiolol	91.28	4.45	91.28	4.45	96.23	1.17
Canrenone	115.74	16.47	115.74	15.47	57.89	11.18
Chlorothiazide	86.02	11.93	86.02	11.93	104.6	3.54
Cibuterol	94.56	7.2	94.56	7.2	82.4	4.16
Clenbuterol	101.62	8.64	101.62	8.64	83.44	4.31
Clenceiro	92.94	22.03	92.94	22.03	81.91	10.47
Clensiro	85.27	12.85	85.27	12.85	73.96	6.53

Krumpanter	84.89	10.8	84.89	10.8	83.68	2.46
Clonprolol	91	7.19	91	7.19	91.21	2.57
Clorprenaline	92.21	11.68	92.21	11.68	91.51	7.02
Colterol hydrochloride	76.47	13.1	76.47	13.1	63.43	6.7
cortisone	100.34	8.01	100.34	8.01	102.25	3.88
Dexamethasone	103.45	13.09	103.45	13.09	99.94	3.81
Fluhydrocortisone	106.54	18.03	106.54	18.03	107.41	3.19
Noraconine	84.41	2.67	84.41	2.67	77.7	8.57
Epinephrine	104.59	5.26	95.56	8.76	84.67	7.42
Hydrocortisone	93.49	19.22	93.49	19.22	77.2	2.49
Hydroxymethyl clenbuterol hydrochloride	100.87	5.28	100.87	5.28	96.53	7.4
Isoprim	105.95	14.38	105.95	14.38	83.71	4.11
Mabuteno	95.37		2.99		95.37	
Mapentello	89.27		8.24		89.27	
methandienone	56.75		9.13		56.75	
Methylprednisolone	96.56		5.58		96.56	
Metoprolol	95.56		8.13		95.56	
nandrolone	66.32		13.46		66.32	
Odaterol hydrochloride	81.73		24.06		81.73	
Prednisolone	99.74		9.78		99.74	
Propranolol	72.25		7.36		72.25	
Ractopamine	89.93		16.39		89.93	
Salbutamol	61.59		19.2		61.59	
Spirolactone	116.69		26.8		116.69	
Albuterol EP impurity B	87.08		7.9		87.08	
Terbutaline	72.13		9.85		72.13	
Testosterone propionate	84.99		17.67		84.99	
Tossamide	83.75		3.25		83.75	
Trenbolone	62.76		10.86		62.76	
Trotoquinol	119.73		6.8		121.39	
Aminophenopterine	67.99		7.52		67.99	
Tobuterol	97.35		4.27		97.35	
Zilpaterol	99.56		21.55		125.31	

4. Conclusion

The sample was purified by FaVE-AP column with 0.2% formic acid-acetonitrile/water (80/20 v: v), combined with high performance liquid chromatography-tandem mass spectrometry (HPLC-MS). By optimizing the pretreatment conditions and mass spectrometry parameters and validating the established method, 53 kinds of stimulants in animal food were screened. This method, combined with a new purification method, improved the detection efficiency of sample pretreatment, made the detection more accurate and rapid, and provided a new method for the detection of stimulants in animal food in the future.

Acknowledgments

This paper is one of the phased achievements of the general project of the National Natural Science Foundation of China (32072291).

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