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Quick and high-throughput quantification of β-agonist residues in bovine liver, meat, milk, kidney, poultry, and egg using dispersive solid phase extraction

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ABSTRACT

A reliable liquid chromatography coupled to quadrupole-Orbitrap high-resolution mass spectrometry (LC-Q-Orbitrap HRMS) method was developed for the simultaneous identification and quantification of 13 β-agonist residues in bovine liver, meat, milk, kidney, poultry, and egg. Dispersive-solid phase extraction (d-SPE) using acetonitrile (ACN) was used to prepare the samples. The analyte in the extracts was separated on a reversed-phase Accucore aQ (50 mm × 2.1 mm, 2.6 μm) using a mobile phase of an aqueous solution containing 2 mM ammonium acetate and acetonitrile (ACN) 0.1 % formic acid. The method was validated in accordance with Commission Implementing Regulation (CIR) EU 2021/808 at six different concentrations ranging from 0.1 to 5 μg/kg. The mean recoveries ranged from 65 to 94 %, while repeatability and reproducibility values were all below 13 %. The linearity, as correlation coefficients (R^2) ranged from 0.9955 to 0.9999. The decision limit (CCα) and detection capability (CCβ) ranges were 0.11–0.13 μg/kg and 0.12–0.15 μg/kg, respectively. The limits of detection (LOD) and limits of quantification (LOQ) were in the range of 0.004–0.048 μg/kg and 0.010–0.075 μg/kg, respectively. Of the 180 samples that were collected from local markets in Egypt, 21.11 % had β-agonist residues. The mean concentration (μg/kg) and detection frequency (%) of the most frequently found β-agonist in the samples were as follows: terbutaline (2.63 μg/kg and 90 %), ractopamine (5.14 μg/kg and 23.3 %). The method's applicability was verified by successfully completing two rounds of proficiency testing (PT).

1. Introduction

The presence of veterinary medicine residues in the environment is now a major concern because of their potential to promote the growth of drug-resistant bacteria and increase the incidence of antibiotic resistant infections. β-adrenergic agonists (β-Agonists) are a class of synthetic phenylethanolamine derivatives characterized by their aromatic group, aliphatic nitrogen-containing group, and β-hydroxyl group [1,2]. β-agonists are highly regarded for their ability to boost protein synthesis, promote muscle development, and reduce fat deposition, establishing their role as pivotal in altering nutrient partitioning and advancing growth in animal husbandry [3–5]. Traditionally used in both human and veterinary drugs as tocolytics, bronchodilators, and cardiac tonics, these compounds are widely applied in treating respiratory disorders [6,7]. Recently, β-agonists have also been used improperly to enhance protein synthesis and improve the efficiency of feed conversion in livestock management [8,9]. Nevertheless, residual traces of β-agonists in food products can potentially lead to harmful impacts on human health, including foodborne illnesses, neurological disorders, cardiovascular diseases, muscle spasms, or even death [10,11]. Therefore, to maintain the superior quality of animal-derived food products and safeguard consumer health, The detection of residues in food from animals is a major concern for human public. As a result, since 1996, the use of β-agonists in livestock raised for food has been banned by the European Union. [12], other countries, such as the USA, Mexico, and Brazil, allow their use within established maximum residue limits (MRLs) set by national regulations or Codex Alimentarius. Codex Alimentarius, a joint FAO/WHO food standards program, plays a crucial role in setting international standards for food safety, including MRLs for β-agonists. These MRLs are designed to ensure that residues in food products remain below levels considered harmful to human health [13–15]. Despite these regulations, concerns remain regarding the potential for illegal or uncontrolled use of β-agonists, leading to residues exceeding MRLs and posing potential risks to human health. However,

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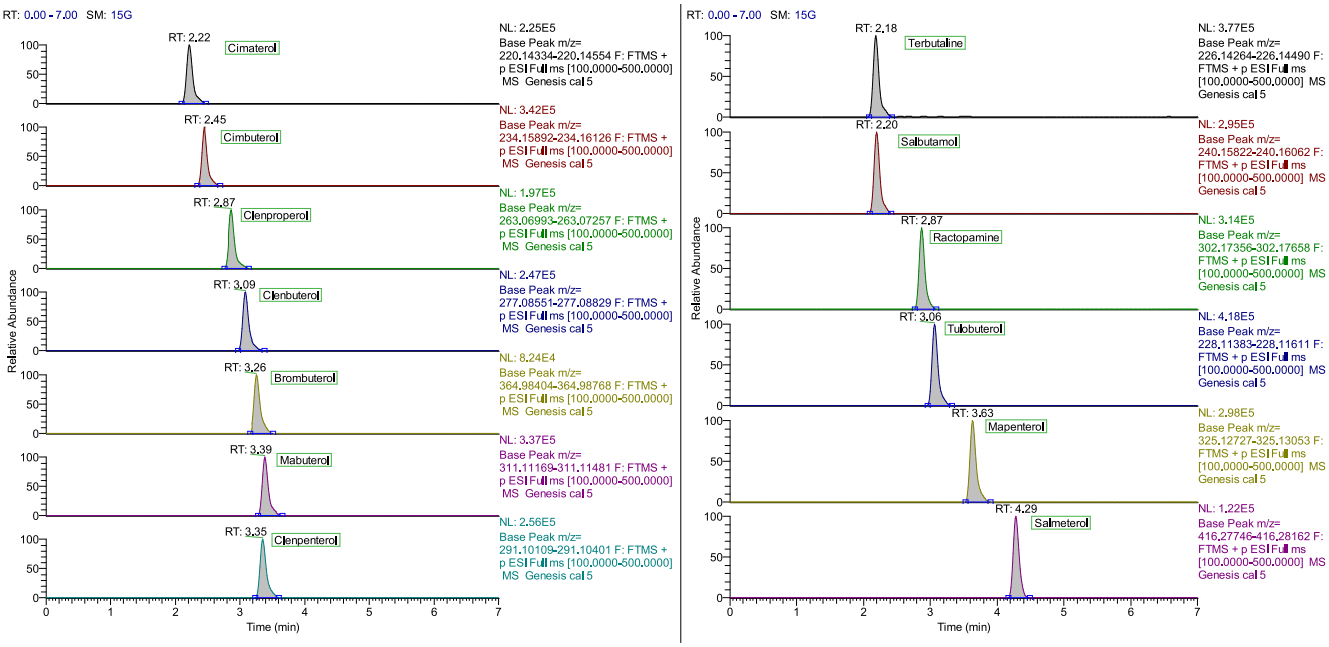

Fig. 1. Typical chromatograms of transitions for β-Agonist analytes at a concentration of 1 μg/mL.

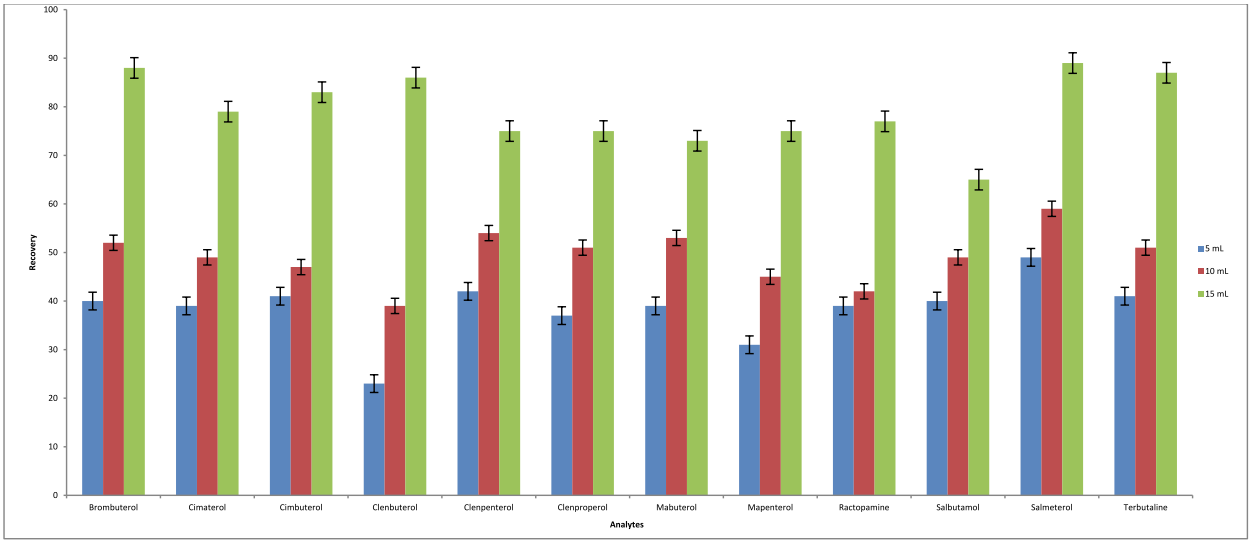

Fig. 2. Effect of different volumes of acetonitrile (5, 10, and 15 mL) on the recovery rates of the β-Agonist analytes.

these compounds are being used illicitly in some countries as growth enhancers due to the ability to improve growth rates and feed efficiency, while other countries allow their use in a restricted manner, always respecting the MRL. In the past decade, there have been numerous reports in the literature about the prevalent fraudulent use of β-agonists in livestock [16,17]. Hence, it is critically important to develop a quick and accurate technique for the determination of β-agonists, a point that underscores the necessity for regular monitoring and the implementation of specialized analytical techniques. During the last two decades, a range of analytical techniques has been developed to detect β-agonist residues across different animal-derived food [18]. Currently, the primary methods employed for identifying β-agonist residues in food of animal origin samples encompass the enzyme-linked immunosorbent assay (ELISA) [19,20]. Antibody-based immunoassays are straightforward, cost-effective, and particularly well-suited for rapid screening purposes. However, their limited sensitivity continues to hinder their widespread application. Gas chromatography-mass spectrometry (GC–MS) [21,22], most GC–MS techniques require derivatization, complicating the operational process. High performance liquid chromatography (HPLC) [23], HPLC-UV [24], liquid chromatography tandem mass spectrometry (LC-MS/MS) [25,26]. Conversely, LC-MS/MS techniques offer several advantages for extensive screening of a variety of prohibited veterinary drugs and other contaminant residues across diverse food matrices [27,28]. However, when considering the broader uses of mass spectrometry techniques in food safety, Orbitrap mass spectrometry has a substantial advantage over LC-MS/MS due to its remarkable high-resolution mass spectra capabilities when combined with a variety of chromatography techniques. Accurate mass measurements, structural information, and the determination of previously identified chemicals in complicated combinations are its strong suits [29]. Additionally, quadrupole Orbitrap (Q-Orbitrap) and high-resolution mass spectrometry (HRMS) have enhanced the data acquisition speed and resolution significantly [30–32]. Many of these studies require a complex and lengthy sample preparation process [33], and the

Table 1
Molecular formula, retention time, and mass spectrometry parameters for the β-agonist compounds.

Analyte	Retention time	Molecular formula	Precursor ion	Product ion *m/z*
Brombuterol	3.26	$C_{12}H_{18}N_2OBr_2$	364.98586	292.9107, 290.9127, 213.9927, 211.9944, 183.9756, 132.0682,
Cimaterol	2.22	$C_{12}H_{17}N_3O$	220.14444	202.13307, 160.08630, 145.07547, 143.05986
Cimbuterol	2.45	$C_{13}H_{19}N_3O$	234.16009	216.14871, 160.08632, 143.05987, 57.070440
Clenbuterol	3.09	$C_{12}H_{18}C_{12}N_2O$	277.08690	259.07546, 203.01312, 168.04422, 132.06795, 57.070500
Clenpenterol	3.35	$C_{13}H_{20}C_{12}N_2O$	291.10255	273.09080, 203.01292, 167.03642, 132.06777, 71.085940
Clenproperol	2.87	$C_{11}H_{16}C_{12}N_2O$	263.07125	245.05962, 203.01292, 168.04418, 132.06776
Mabuterol	3.39	$C_{13}H_{18}ClF_3N_2O$	311.11325	293.10138, 237.03906, 217.03299, 57.070430
Mapenterol	3.63	$C_{14}H_{20}ClF_3N_2O$	325.12890	307.11712, 237.03915, 217.03311, 71.085960
Ractopamine	2.87	$C_{18}H_{23}NO_3$	302.17507	284.16438, 164.10701, 136.07570, 121.06475, 107.04904, 91.05415
Salbutamol	2.20	$C_{13}H_{21}NO_3$	240.15942	222.14890, 166.08637, 148.07577, 57.070710
Salmeterol	4.29	$C_{25}H_{37}NO_4$	416.27954	398.26736, 380.25687, 232.16865, 230.15308, 91.054390
Terbutaline	2.18	$C_{12}H_{19}NO_3$	226.14377	170.08075, 152.07025, 125.05957, 57.070530
Tulobuterol	3.06	$C_{12}H_{18}ClNO$	228.11497	154.04187. 118.06511, 119.07281, 172.05247

efficiency is suboptimal since the target samples must undergo enzymatic hydrolysis at 37 °C for 16 h to release β-agonists [1,16,34]. Many of these studies have required extensive sample pretreatment steps, preventing the entire test from being completed within a single day. In this study we introduce a simple and novel pre-treatment method based on the dispersive solid phase extraction (d-SPE) procedure. This method is complemented by the development and validation of a quantitative technique for the detection and determination of thirteen β-agonist residues in bovine liver, meat, milk, kidney, poultry, and egg. Our approach utilizes liquid chromatography coupled with quadrupole-

Table 2
Linearity, range, limits of detection (LOD) and limits of quantification (LOQ) of β-agonists in liver, meat, milk, kidney, poultry, and egg.

Analyte	Liver			Meat			Milk			Kidney			Poultry			Egg		
	Range (μg/kg)	R^2	LOD (μg/kg)	LOQ (μg/kg)	R^2	LOD (μg/kg)	LOQ (μg/kg)	R^2	LOD (μg/kg)	LOQ (μg/kg)	R^2	LOD (μg/kg)	LOQ (μg/kg)	R^2	LOD (μg/kg)	LOQ (μg/kg)	R^2	LOD (μg/kg)	LOQ (μg/kg)
Brombuterol	0.1–5	0.9997	0.009	0.020	0.9996	0.004	0.010	0.9996	0.008	0.015	0.9998	0.018	0.030	0.9990	0.021	0.035	0.9994	0.009	0.018
Cimaterol	0.1–5	0.9995	0.015	0.030	0.9999	0.009	0.015	0.9991	0.010	0.019	0.9975	0.030	0.067	0.9997	0.018	0.028	0.9987	0.012	0.018
Cimbuterol	0.1–5	0.9997	0.019	0.029	0.9984	0.008	0.012	0.9994	0.019	0.038	0.9985	0.008	0.019	0.9996	0.025	0.037	0.9977	0.024	0.036
Clenbuterol	0.1–5	0.9995	0.011	0.029	0.9991	0.007	0.013	0.9989	0.020	0.040	0.9992	0.024	0.048	0.9987	0.009	0.013	0.9994	0.009	0.013
Clenpenterol	0.1–5	0.9991	0.019	0.028	0.9998	0.006	0.015	0.9998	0.009	0.019	0.9998	0.039	0.057	0.9999	0.007	0.010	0.9995	0.017	0.032
Clenproperol	0.1–5	0.9998	0.007	0.019	0.9997	0.009	0.020	0.9995	0.017	0.025	0.9975	0.017	0.029	0.9955	0.019	0.028	0.9997	0.039	0.059
Mabuterol	0.1–5	0.9990	0.012	0.028	0.9966	0.008	0.019	0.9990	0.039	0.058	0.9992	0.009	0.017	0.9998	0.035	0.049	0.9996	0.006	0.010
Mapenterol	0.1–5	0.9990	0.020	0.035	0.9992	0.008	0.021	0.9994	0.006	0.010	0.9993	0.010	0.019	0.9974	0.008	0.013	0.9991	0.010	0.015
Ractopamine	0.1–5	0.9987	0.019	0.031	0.9980	0.010	0.030	0.9998	0.010	0.031	0.9981	0.029	0.043	0.9975	0.007	0.012	0.9990	0.048	0.072
Salbutamol	0.1–5	0.9976	0.025	0.048	0.9999	0.009	0.025	0.9981	0.031	0.059	0.9991	0.010	0.019	0.9992	0.018	0.030	0.9997	0.021	0.031
Salmeterol	0.1–5	0.9987	0.027	0.059	0.9955	0.007	0.020	0.9979	0.008	0.015	0.9987	0.039	0.050	0.9993	0.008	0.021	0.9978	0.038	0.057
Terbutaline	0.1–5	0.9993	0.008	0.012	0.9998	0.019	0.030	0.9995	0.007	0.012	0.9999	0.039	0.068	0.9981	0.024	0.036	0.9978	0.027	0.040
Tulobuterol	0.1–5	0.9999	0.009	0.018	0.9974	0.010	0.028	0.9997	0.018	0.038	0.9990	0.018	0.053	0.9998	0.039	0.060	0.9987	0.048	0.075

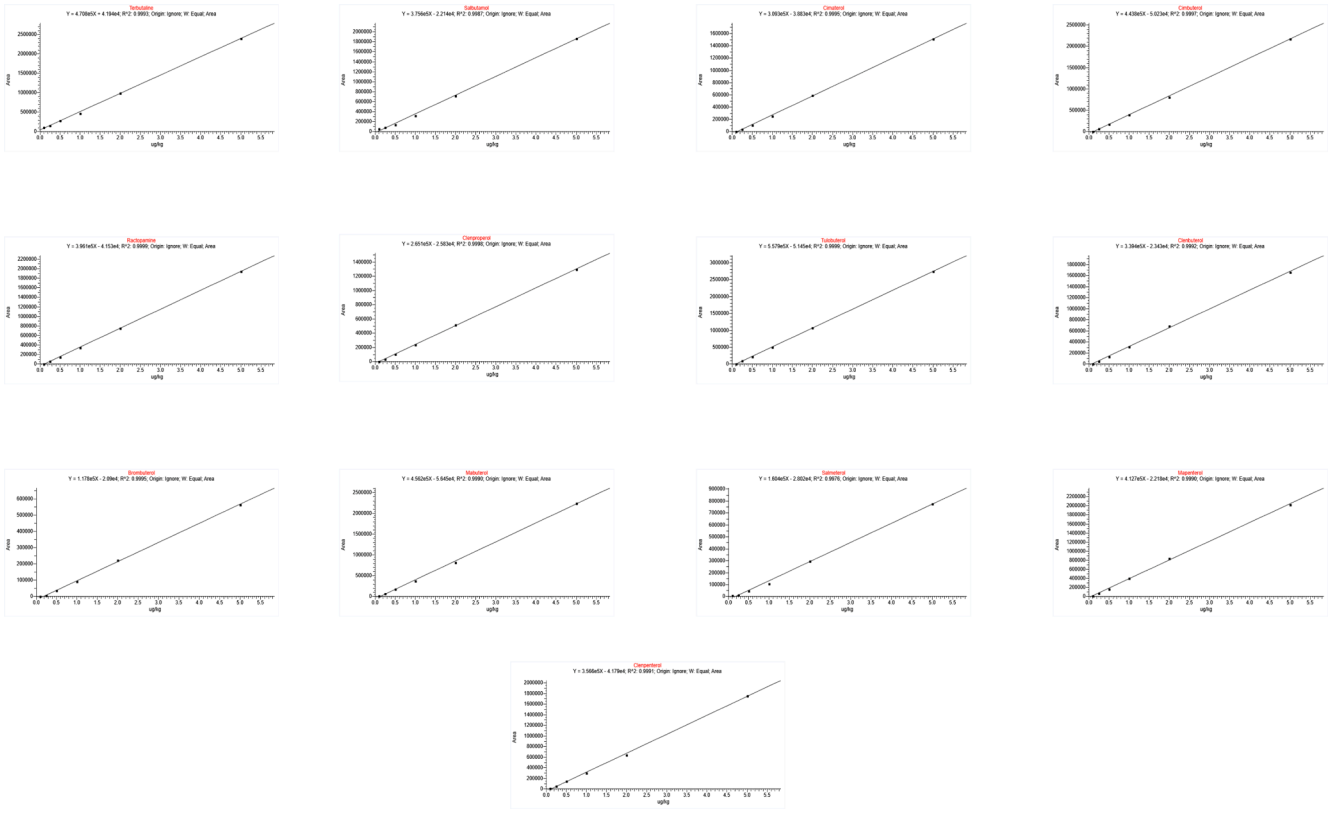

Fig. 3. Matrix matched calibration curve at six different points of β-Agonist analytes.

Orbitrap high resolution mass spectrometry (LC-Q-Orbitrap HRMS). Furthermore, our research contributes to food safety assurance by gathering a significant quantity of samples from the Egyptian market. This observation contributes to evaluating the health implications of antibiotic residues in widely consumed food of animal origin products. The presence of these residues would raise concerns regarding consumer health if the MRLs are exceeded in a given products, which is particularly important given the scarcity of studies conducted in this specific area within the Egyptian context.

2. Materials and methods

2.1. Chemicals and reagents

All the analytical standards of β-Agonists utilized in this work were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and were of high purity (95 %). The LC-MS grade acetonitrile (ACN), n-hexane (99 %), and methanol (MeOH) were purchased from CARLO ERBA (Milan, Italy). Ammonia solution (37 %) was obtained from Fisher Scientific (Leicestershire, United Kingdom). The chemicals used in this study were ammonium acetate, per chloric acid, sodium chloride (NaCl), and pure formic acid (99 %) all obtained from Sigma-Aldrich (Darmstadt, Germany). The d-SPE tubes containing primary secondary amine (PSA), were sourced from Supleco (Pennsylvania, USA). The acquisition of ultra-pure water was accomplished by the utilization of a MilliQ UF-Plus system, manufactured by Millipore in Germany. Individual compound stock solutions were prepared in MeOH at a concentration of (1000 μg/mL), which were then stored in glass bottles at a temperature of −18 °C, within their designated validity period. The mixed standard of working solution used in the experiment was diluted and then stored at a temperature of −4 °C.

2.2. Apparatus

The Geno/Grinder-Shaker, manufactured in the United States. The centrifuge was obtained from Hermle (Gosheim, Germany). Similarly, the rotary evaporator was supplied by Heidolph (Schwabach, Germany). The pH-meter was obtained from Mettler Toledo (Greifensee, Switzerland) and calibrated before being used calibration standards, including pH 4, pH 7, and pH 10.

2.3. Sample collection

The study involved collecting 180 samples, including bovine liver, meat, milk, kidney, poultry, and eggs from various local markets across Egypt, ensuring a comprehensive representation of consumer products. To minimize selection bias, the samples were randomly selected. Sterile tools and containers were used for collection; subsequently, the samples were clearly labeled and immediately cooled in an icebox for transportation to the laboratory on the same day. Upon arrival, they were stored at −20 °C to preserve their condition until analysis.

2.4. Sample preparation

In the standard assay protocol, 2.00 ± 0.02 g of homogenized samples were weighed into 50 mL polypropylene centrifuge tubes. To each tube, 2.0 mL of water was added followed by the addition of 5 mL of acetonitrile (ACN). Next, 5 mL of n-hexane was added to the mixture. The tubes were then mechanically shaken for 3 min at 700 rpm. After shaking, the resulting mixture is left at room temperature for 30 min (hydrolysis). The samples were centrifuged at 4500 rpm and 4 °C for 10 min to facilitate the separation of the supernatant. The supernatant was transferred to a new tube containing (PSA). The extraction process was repeated twice more by adding an additional 5 mL of ACN and 5 mL of n-hexane each time, followed by shaking and centrifugation under the same conditions. The supernatants from these steps were combined in

Table 3
Recovery and coefficient of variation (CV) repeatability; reproducibility in liver, meat, milk, kidney, poultry, and egg.

Analyte	Liver			Meat			Milk			Kidney			Poultry			Egg		
	Recovery(%)	CV_r (%)[a]	CV_{rw}(%)[b]	Recovery(%)	CV_r(%)[a]	CV_{rw}(%)[b]	Recovery(%)	CV_r(%)[a]	CV_{rw}(%)[b]	Recovery(%)	CV_r(%)[a]	CV_{rw}(%)[b]	Recovery(%)	CV_r(%)[a]	CV_{rw}(%)[b]	Recovery(%)	CV_r(%)[a]	CV_{rw}(%)[b]
Brombuterol	88	6	9	81	4	6	82	5	8	88	6	9	81	5	8	87	4	7
Cimaterol	79	10	13	76	6	8	79	6	8	75	6	8	91	6	9	85	3	5
Cimbuterol	83	5	7	84	5	7	88	5	7	77	5	8	89	5	8	73	4	6
Clenbuterol	86	5	8	88	6	8	76	7	9	80	7	10	84	7	8	86	6	8
Clenpenterol	75	5	7	73	5	7	87	4	5	85	8	11	88	9	12	88	7	8
Clenproperol	75	6	8	80	6	8	81	8	10	74	5	8	87	4	7	91	5	7
Mabuterol	73	5	6	79	5	7	86	5	8	78	7	10	84	5	8	87	4	6
Mapenterol	75	5	7	82	4	7	82	7	9	86	9	12	90	8	11	81	6	7
Ractopamine	77	8	10	84	4	6	80	9	11	71	8	10	79	7	9	86	5	7
Salbutamol	65	9	12	69	7	8	79	4	6	81	8	11	86	5	8	90	5	6
Salmeterol	89	7	10	92	5	8	91	5	8	89	5	8	92	6	9	79	4	6
Terbutaline	87	10	12	81	7	8	77	8	10	79	4	7	75	4	7	71	4	7
Tulobuterol	87	5	7	87	10	12	80	4	7	86	8	11	94	5	6	84	8	10

[a] coefficient of variation (repeatability), [b]coefficient of variation (reproducibility).

the same tube to collect a total of 15 mL of ACN. This combined supernatant was then shaken for 1 min at 700 rpm and centrifuged for 5 min at 4500 rpm and 4 °C. The final clear supernatant was transferred into a 50 mL flask. The solvent was evaporated at 40 °C using a rotary evaporator. The dry residue was reconstituted in 2.0 mL of 0.08 % perchloric acid in ACN. The reconstituted solution was then filtered through a 0.45 μm PTFE membrane filter into an amber glass vial, rendering it suitable for analysis. The sample was then ready for injection into the HPLC Orbitrap HRMS for further analysis.

2.5. Chromatographic conditions

Chromatographic separation was carried out on a Thermo Scientific Vanquish High Performance Liquid Chromatography system (Thermo Scientific, Bremen, Germany). Separation was achieved using three distinct columns: a reversed-phase ZORBAX Eclipse Plus C18 (150 mm × 4.6 mm, 5 μm), Thermo Accucore VDX (100 mm × 2.6 mm, 1.9 μm), and Thermo Accucore aQ (50 mm × 2.1 mm, 2.6 μm). The mobile phase consisted of eluent A (2 mM ammonium acetate) and eluent B (ACN with 0.1 % formic acid). The gradient program commenced with 5 % eluent B for the first 0.5 min, increased linearly to 55 % over 3 min, then escalated to 95 % over 2 min then returned to 5 % eluent B within 1.5 min re-equilibration period, making the total run time 7 min. The flow rate was set at 0.4 mL/min. The column temperature was maintained at 40 °C, the sample tray was kept at 25 °C, and the injection volume was 2 μL.

2.6. Mass spectrometry conditions

Mass spectrometric analysis was performed with a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany), equipped with a heated electrospray ionization (HESI) source operating in positive ionization mode. The HESI source temperature was set at 350 °C, and the capillary temperature was 325 °C. An electrospray voltage of 3.70 kV was applied. The S-lens RF level was adjusted to 50 V. Sheath and auxiliary gas flows were regulated to 50 and 12 units, respectively. The automatic gain control (AGC) target was set to 3.10^6, and the maximum injection time (IT) was 100 ms. Full scan data acquisition was conducted in positive ion mode at a mass resolution of 70,000 FWHM, scanning from m/z 100 to 500. The instrument was set to Full MS/vDIA scan type, with a resolution of 70,000 for Full MS and 17,500 for vDIA. Data acquisition and processing were executed using TraceFinder software (version 4.1, Thermo Fisher Scientific, Bremen, Germany).

2.7. Method validation

The method validation was conducted in accordance with the EU Commission Implementing Regulation (CIR) 2021/808. [35]. The validation process involved a thorough examination of key parameters, including linearity, repeatability, reproducibility, recovery, decision limit (CCα), and detection capability (CCβ). Linearity was comprehensively assessed in both the solvent and matrix. Calibration curves were designed at six different point levels. Clear linearity was tested across concentration ranging from 0.1 to 5 μg/kg. Precision encompass both repeatability and within laboratory reproducibility. Validation was performed using bovine liver, meat, milk, kidney, poultry, and egg samples that had been confirmed to be free from the target analytes (blank samples). The testing levels included six concentrations (0.1, 0.25, 0.5, 1, 2, and 5 μg/kg). For each concentration, six replicates were analyzed on the same day using matrix-matched calibration curves. This process was repeated over three different days, introducing variations in time, operator, and the calibration status of the LC-HRMS/MS equipment. The decision limit (CCα) is the threshold value above which a sample can be deemed non-compliant, with an associated error probability of α. Conversely, the value of 1 – α represents the statistical confidence, expressed as a percentage, that the established threshold has

Table 4
Decision limits (CCα), detection capability (CCβ), and matrix effect (ME) for β-agonists in liver, meat, milk, kidney, poultry, and egg.

Analyte	Liver			Meat			Milk			Kidney			Poultry			Egg		
	CCα (μg/kg)	CCβ (μg/kg)	ME (%)	CCα (μg/kg)	CCβ (μg/kg)	ME (%)	CCα (μg/kg)	CCβ (μg/kg)	ME (%)	CCα (μg/kg)	CCβ (μg/kg)	ME (%)	CCα (μg/kg)	CCβ (μg/kg)	ME (%)	CCα (μg/kg)	CCβ (μg/kg)	ME (%)
Brombuterol	0.12	0.14	−16	0.11	0.13	−21	0.12	0.14	−25	0.12	0.14	−17	0.11	0.12	−24	0.12	0.15	−10
Cimaterol	0.11	0.14	−14	0.12	0.14	−19	0.12	0.14	−24	0.11	0.13	−20	0.11	0.13	−22	0.11	0.14	−18
Cimbuterol	0.11	0.13	−19	0.12	0.13	−25	0.11	0.13	−20	0.11	0.12	−18	0.11	0.12	−25	0.11	0.14	−19
Clenbuterol	0.13	0.14	−22	0.11	0.14	−14	0.11	0.13	−18	0.11	0.14	−22	0.12	0.12	−23	0.12	0.14	−17
Clenpenterol	0.12	0.14	−15	0.11	0.14	−16	0.11	0.12	−26	0.12	0.14	−14	0.11	0.14	−21	0.11	0.13	−20
Clenproperol	0.11	0.12	−14	0.12	0.14	−19	0.12	0.13	−25	0.13	0.15	−22	0.13	0.15	−20	0.11	0.14	−15
Mabuterol	0.12	0.13	−24	0.13	0.13	−24	0.12	0.13	−24	0.11	0.14	−23	0.11	0.13	−11	0.11	0.14	−11
Mapenterol	0.13	0.14	−23	0.11	0.12	−29	0.11	0.12	−27	0.11	0.13	−27	0.12	0.14	−18	0.11	0.15	−10
Ractopamine	0.11	0.15	−21	0.13	0.14	−11	0.12	0.13	−25	0.12	0.14	−21	0.11	0.12	−14	0.12	0.14	−15
Salbutamol	0.11	0.14	−25	0.11	0.14	−12	0.11	0.12	−23	0.11	0.13	−22	0.13	0.15	−16	0.11	0.13	−21
Salmeterol	0.12	0.13	−11	0.11	0.13	−17	0.11	0.14	−27	0.11	0.12	−13	0.11	0.14	−15	0.12	0.14	−14
Terbutaline	0.11	0.14	−8	0.12	0.14	−18	0.12	0.13	−29	0.12	0.14	−28	0.12	0.13	−20	0.12	0.14	−22
Tulobuterol	0.12	0.14	−17	0.11	0.12	−20	0.12	0.13	−21	0.12	0.14	−23	0.11	0.15	−28	0.12	0.15	−24

Table 5
β-agonist residues in liver, meat, milk, kidney, poultry, and egg. samples (n 180; positive samples = 38) collected from Egyptian retail markets.

Species	Sample number	Detected number	Analyte	Range (μg/kg)		Mean	SD	Frequency	
				Minimum	Maximum			No.	(%)
Liver	30	27	Ractopamine	3	11.22	5.14	2.93	7	23.3
			Terbutaline	0.09	14.6	2.63	3.29	27	90
Meat	30	0	–	–	–	–	–	–	–
Milk	30	8	Terbutaline	0.51	1.99	0.98	0.60	8	26.6
Kidney	30	0	–	–	–	–	–	–	–
Poultry	30	3	Ractopamine	0.05	0.17	0.10	0.06	3	10
Egg	30	0	–	–	–	–	–	–	–

Table 6
Outcomes of the Food Analysis Performance Assessment Scheme (FAPAS), proficiency testing (PT) utilizing the validated method for two rounds analysis.

FAPAS round	Species	Analyte	Assigned value (μg/kg)	Found (μg/kg)	Z-score	Observations
02,432	Liver	Clenbuterol	0.3	0.26	−0.3	Satisfactory
02,468	Liver	Mabuterol	0.2	0.19	−0.1	Satisfactory
		Ractopamine	1	0.95	−0.2	Satisfactory
		Tulobuterol	0.3	0.24	−0.4	Satisfactory
		Other analytes	0	0	0	Satisfactory

been exceeded. The detection capability (CCβ) is defined as the smallest quantity of the analyte that can be detected or quantified with a likelihood of error represented by β. According to the European Commission [35], for analytes with no specified MRLs, CCα should be calculated by analysing at least 20 representative blank samples at the lowest concentration of 0.1 μg/kg, which allows for the calculation of the signal-to-noise ratio within the expected time window for the analyte. Twenty fortified blank samples at each concentration level were analysed to provide a solid foundation for CCβ. The concentration at which 5 % or fewer false compliant results remain is considered the method's CCβ. Likewise, the limit of detection (LOD) which is the samllest amount of an analyte in a sample that can be detected, although not necessarily quantified. Moreover, limit of quantitation (LOQ) is the concentration level at which the analyte can not only be reliably detected but also measured with a specified degree of accuracy and precision. LOD is calculated as three times the standard deviation of the lowest concentration divided by the slope of the calibration curve, and LOQ is five times the same standard deviation over the slope.

Matrix effect (ME) experiments were carried out to quantify the degree of ion suppression or enhancement. The ME was determined by dividing the slope of matrix-matched calibration curves (Slope M) with that of the calibration curves in ACN solvent (Slope S), using the following equation: $ME\% = 100 \times ((Slope\ M)/(Slope\ S)) - 1$.

This measures the impact of the matrix on the analysis of the analyte. A 100 % result signifies no matrix effects. Results exceeding 100 % suggest ion enhancement, and those under 100 % denote ion suppression. Positive results increase the ion signal, whereas negative results decrease it. It is crucial to manage matrix effects (ME) for precise measurement of β-agonist compounds in different matrices. Therefore, matched matrix calibration was employed in this research.

2.8. Proficiency testing (PT)

Method validation accuracy was ensured by analysis of two PT samples from Food Analysis Performance Assessment Scheme (FAPAS). The sample rounds examined (02432 and 02468) targeted specific analytes. This Rounds aimed to detect β-Agonist compounds. These proficiency testing samples were crucial in affirming the precision and reliability of the analytical methods used in this study.

3. Results and discussion

3.1. Optimization of the liquid chromatography conditions

In the stationary phase: the chromatographic analysis was conducted using three different columns: ZORBAX Eclipse Plus C18 (150 mm × 4.6

mm, 5 μm), Thermo Accucore VDX (100 mm × 2.6 mm, 1.9 μm), and Thermo Accucore aQ (50 mm × 2.1 mm, 2.6 μm). The Zorbax column and Thermo VDX exhibited bad peak shapes for the target compounds, and their separation times were notably extended. In contrast, the Thermo Accucore aQ column demonstrated significantly enhanced peak shapes and considerably shorter retention times for the analytes. These findings are clearly shown in Fig. 1. These findings are in alignment with the outcomes observed in previous research [36]. In the mobile phase: in our optimization process, we compared the effects of ACN and MeOH as organic solvents by infusing each standard solution and assessing their impact on sensitivity. The infusion results indicated that ACN, when utilized as the organic solvent, significantly bolstered the signal intensity for each β-agonist analytes relative to the outcomes obtained with MeOH. The superior performance of ACN in separating analytes has been well-documented in previous research [37]. To enhance and stabilize the ionization process, the addition of acids and salts to the mobile phase was explored [38,39]. We integrated 0.1 % formic acid with ACN to enhance the ionization efficiency and sensitivity of analytes accepted for positive ionization. The methodical inclusion of formic acid is aligned with our goal of optimizing the detection capabilities of our HPLC-Orbitrap HRMS system. In the aqueous phase, both ammonium acetate and ammonium formate were evaluated, with ammonium acetate showing a more pronounced increase in mass spectrometry sensitivity compared to ammonium formate. Therefore, ammonium acetate was chosen for subsequent experiments. We then examined the effects of varying concentrations of ammonium acetate, ranging from 0.5 to 2 mM. Our observations revealed that higher concentrations significantly improved peak shapes for all compounds under study. As a result, a concentration of 2 mM ammonium acetate was selected for our study.

3.2. Optimization of the preparation procedure

Sample preparation is a critical component in analytical procedures. Various pre-treatment techniques have been developed to date for detecting the unauthorized use of β-Agonists. Shen et al. [40] utilized techniques such as immunoaffinity chromatography, liquid–liquid extraction (LLE) as reported by Keskin, Özer, & Temizer [41], and SPE as described by Sanghvi et al. [42]. Given the complicated nature of biological matrices and the minimal quantities found in samples, these techniques may not completely eliminate salts and endogenous substances like fat, phospholipids, and aliphatic acids, potentially leading to matrix effects. In our experimental approach, we tested various volumes of ACN and n-Hexane specifically 5 mL, 10 mL, and 15 mL to optimize the recovery rates of all analytes. The outcomes of these tests are shown in Fig. 2, which illustrates the impact of solvent volume on recovery efficiency. The optimal protocol was found to involve using 15 mL of ACN and 15 mL of hexane, applied across three extraction cycles, to attain maximum extraction efficacy. The larger volume of ACN likely disrupts the analyte-matrix interactions more effectively, facilitating better solubilization and extraction of the compounds. While increasing the volume of ACN beyond 15 mL did not result in significantly better recoveries. Given the balance between extraction efficiency and the economic and time costs associated with larger solvent volumes, 15 mL of ACN was selected as the optimal volume. This choice reflects a compromise that maximizes recovery rates while maintaining reasonable processing times and costs. Food matrices, particularly those of animal origin, contain high levels of fats, proteins, and phospholipids. These components can significantly impact matrix effects in analytical methods. PSA is effective in removing these substances, thereby reducing matrix effects and enhancing analytical performance. Additionally, by eliminating interfering substances, PSA improves the recovery rates of β-agonists from complex matrices, ensuring accurate and reliable detection.

3.3. Optimization of q-orbitrap HRMS parameters

The Q-Exactive Orbitrap/MS instrument was operated in Full MS/vDIA scanning mode, utilizing positive ion modes. The initial full mass scan was employed for screening and quantifying target compounds, as well as for retrospective analysis of unknown substances. To confirm the identity of the target compound, the generation of fragment ions was crucial. When a target compound was identified and its signal intensity surpassed the predefined threshold, it was selected using the quadrupole and directed to the higher-energy collision dissociation collision cell through the C-trap for fragmentation [43]. All resulting fragments from the collision cell were gathered within the C-trap and subsequently introduced into the Orbitrap mass analyzer [44]. This workflow allows for a comprehensive analysis of the compounds. It initiates with a full MS scan, followed by a series of data-independent scans focusing on fragment ions with applied fragmentation energy. Detailed information for the 13 analytes, including compound names, exact precursor masses, characteristic fragment ions, mass accuracy, and molecular formula, is presented in Table 1.

3.4. Method validation

According to the CIR 2021/808 [35] all validation parameters complied with the set requirements. Throughout the development, validation, and analysis of real samples, the analyte was positively identified and confirmed when the retention time (RT), precursor ion, and product ion met the established criteria. The calibration curves exhibited excellent linearity over various concentration ranges for the analytes within the matrices, constructed at six concentration levels, the range was 0.1–5 μg/mL, as detailed in Table 2. The correlation coefficients (R^2) for both matrix-matched standard calibration curves exceeded 0.9955 as shown in Fig. 3. The LOD and LOQ were in the range of 0.004–0.048 μg/kg and 0.010–0.075 μg/kg, respectively. In the liver samples, clenbuterol demonstrated the lowest LODs at 0.007 μg/kg, while terbutaline showed the lowest LOQs at 0.012 μg/kg. Conversely, salmeterol exhibited higher LOD and LOQ values, with 0.027 μg/kg and 0.059 μg/kg, respectively. For meat samples, the lowest LOD and LOQ observed for brombuterol were 0.004 μg/kg and 0.010 μg/kg, respectively. In contrast, Terbutaline showed the highest LOD and LOQ at 0.019 μg/kg and 0.030 μg/kg, respectively. It also appears in the other matrices, as detailed in Table 2. Mastrianni et al. [45] reported LOD and LOQ in meat samples at 0.2–0.7 and 0.6–2.2 μg/kg, respectively. Our findings demonstrate enhanced sensitivity, yielding LOD and LOQ values that surpass those obtained in their study. To assess selectivity, twenty samples from all matrices were analysed for potential interfering substances. The results indicated that the retention times of the target compounds were free from interference peaks. To assess the precision of this method, blank samples spiked with six concentrations (0.1, 0.25, 0.5, 1, 2, and 5 μg/kg) were utilized to determine repeatability and within-laboratory reproducibility, for a total of 18 samples spiked per level. They were analysed across three different days using standard solutions freshly prepared daily. Recovery rates for the liver matrix ranged from 65 % to 89 %, and the coefficients of variation (CVs) did not exceed 13 %. In the meat matrix, recovery rates varied between 69 % and 92 %, with CVs remaining below 12 %. For the milk matrix, recoveries were within the 76 % to 91 % range, and CVs did not surpass 11 %. For kidney matrix recovery rates ranged from 71 % to 89 %, and CVs below 12 %. For the poultry matrix, recovery rates ranged from 75 % to 94 %, and CVs did not exceed 12 %. For the egg matrix, recovery rates varied between 71 % and 91 %, with CVs remaining below 10 %. The data compiled in Table 3 highlights the method's considerable accuracy.

This study evaluated the values of CCα and CCβ for every target compound, and Table 4 summarises the findings. The CCα values for samples varied from 0.11 to −0.13 μg/kg, and the CCβ values fell within the range of 0.12 to 0.15 μg/kg. These results indicate that the developed method is appropriate for detecting β-agonists. analytes in liver,

meat, milk, kidney, poultry, and egg matrices. The matrix effects ranged from −29 % to −8 %. Milk, with a −29 % signal suppression, showed a higher degree of ionization interference. Given milk's relatively homogeneous matrix, this suppression level was anticipated and is consistent with the presence of proteins and fats that can impact the ionization process. as shown in Table 4.

3.5. Real samples analysis

The developed method was used to analyze β-agonist residues in 180 samples, which included 30 samples each of bovine liver, meat, milk, kidney, poultry, and eggs. All these samples were sourced from various local markets across Egypt. Out of the 180 samples, a total of 38 showed the presence of ractopamine and terbutaline, resulting in an overall detection rate of 21.11 %, as shown in Table 5. The detection frequencies showed considerable variation among the different food matrices, ranging from a minimum of 23.3 % for ractopamine to a maximum of 90 % for terbutaline. These findings are consistent with the outcomes reported in previous studies [11,18,37,45].

3.6. Proficiency testing (PT)

As part of the Food Analysis Performance Assessment Scheme (FAPAS), proficiency testing (PT) samples round 02,432 and 02,468 were analysed to validate assay performance and calculation methodology. In round 02432, the optimized assay confirmed clenbuterol, concentrations of 0.26 μg/kg. For the 02,468PT round found mabuterol concentrations of 0.19 μg/kg, ractopamine concentrations of 0.95 μg/kg and Tulobuterol concentrations of 0.24 μg/kg. There were no findings for the other analytes, as shown in Table 6. The Z-score was computed for the obtained results and was found to be within the acceptable range of $|z| < (2, -2)$, as specified by the FAPAS reports.

4. Conclusions

The study presents LC-Q-Orbitrap HRMS technology to quantify 13 β-agonist in bovine liver, meat, milk, kidney, poultry, and egg. A simplified cleanup method based on d-SPE. The analytical separation and detection method greatly shortened the analysis time and enhanced the throughput of samples. Ensuring high method sensitivity and the ability to determine residues in the range from 0.1 to 5 μg/kg. The validation procedure included selectivity, linearity, LOD, LOQ, trueness, repeatability, reproducibility, CCα, and CCβ. All validation parameter values met the intended use and established criteria. The developed method was successfully applied for the analysis of two proficiency testing (PT), and real samples, including 38 positive samples out of 180 samples, were detected. The method provides an integrated strategy for the screening and quantification of β-agonist residues in an effective manner. Continuous monitoring studies should be conducted regularly to determine the presence of β-Agonist residues in food of animal origin, poultry meat, and egg, identify their sources, and implement secure preventive and remedial strategies.

CRediT authorship contribution statement

Omar Khaled: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Lamia Ryad:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Investigation, Data curation. **Nermine Gad:** Writing – review & editing, Validation, Resources, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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