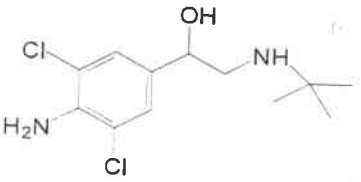
ANALYSIS OF CLENBUTEROL IN FEEDSTUFFS AND MEA PRODUCTS BY LC/MS

NGUYEN THI THU THUY, LAM VAN XU, PHAM THI ANH
CHU PHAM NGOC SON, TRAN KIM TINH

1. INTRODUCTION

Illegal use of the β-agonist clenbuterol in meat-producing livestock is linked to its abil induce weight gain and a greater proportion of muscle to fat tissues.



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1-(4-amino-3,5-dichlorophenyl)-2-(*tert*-butylamino) ethanol ($C_{12}H_{18}Cl_2N_2O$).

Though a few countries have approved it for therapeutic uses in food producing anim the doses needed for this purpose are much lower than those required as growth promoter. M proposed by Codex Alimentarius are 0.2 µg/kg for muscle and 0.6 µg/kg for liver [1]. Sev outbreaks of illness in Europe and Hongkong involving consumption of pork with clenbut residues around 160-291 ppb were reported. In Viet Nam, use of clenbuterol and seventeen o chemicals as feedstuff additives for growth promotion of livestock has been banned since 20 However, clenbuterol may be still available through the so-called "black market".

To enforce the ban, different analytical methods have been developed to determ clenbuterol in feedstuff and in pork meat including ELISA, GC, GC-MS, HPLC, HPLC-M Due to its high selectivity and sensitivity, HPLC-MS analysis is often the method of choice the quantification of trace levels of polar contaminants if preceded by a well conceived sam preparation procedure. In fact several methods of clenbuterol analysis with complex sam preparation procedures were reported [2, 3].

This paper presents a simple, low cost method of analysis of clenbuterol in feedstuff and meat which consists in extraction, clean-up with Strata SCX SPE column and Clenbuter determination by LC/MS complying with the requirement of the 0.2 µg/kg MRL in meat.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

- Standard: Clenbuterol hydrochloride (95% chemical purity) from Sigma Aldrich Pty. L (New South Wales, Australia);

- Methanol, HPLC grade;
- Sodium hydroxide, AR grade;
- Ammonia solution 30%;
- Acetonitrile, HPLC grade;
- Deionised water;
- Phosphate buffer 0.1 M dipotassium hydrogenphosphate, pH = 6 was prepared by dissolving 17.4 g K_2HPO_4 in 100 ml of deionised water and adjusting the pH = 6 with H_3PO_4.
- Strata SCX SPE column (500 mg/3 ml).

2.2 Calibration Standards and Sample Preparation

2.2.1. Calibration Standards

- Primary stock solution was prepared by dissolving 1.17 mg of clenbuterol hydrochloride (95% pure) in 10 ml methanol, the solution kept in refrigerator was stable for six month.

- Working standards solution of clenbuterol hydrochloride were prepared by dilution of stock solution with mobile phase (methanol:deionised water, 0.1% HCOOH 15:85) to give concentrations of 0.585, 1.17; 2.34; 5.85; 11.7; 23.4 µg/l.

2.2.2. Sample Preparation

The animal feed and pork meat were purchased at local market (Can Tho City - Viet Nam). An adequate portion (approprimately 0.3 kg) of feed or meat cut into small slices was well homogenized in a laboratory mixer. A 5 gram portion of the test sample was weighted into a 50 ml test tube and mechanically extracted using 20 ml of buffer (K_2HPO_4 pH = 6). Next, the extract was centrifuged for 15 minutes at 5000 rpm, the supernatant was filtered over a plug of glass wool and pH adjusted to 6. The operation was twice repeated.

2.2.3. Solid Phase Extraction

- Sample was loaded into a Strata SCX SPE column preconditioned by adding sequentially 4 ml methanol, 4 ml deionised water, 4 ml buffer (pH = 6).

- The column was washed with 4 ml buffer, 4 ml deionised water and 2 ml acetonitrile.

- Elution was performed with 4 ml methanol:ammonia (95:5). After evaporation of the solvent, the residue was redissolved in 1 ml mobile phase and transferred into a microvial for analysis.

2.3. Analysis by LC/MS

- Column: Purospher Star C_{18} 125 mm × 4.6 mm × 5 µm with guard column
- Mobile phase: Gradient with A: Methanol (0.1% HCOOH) and B: Water (0.1% HCOOH).

Minutes	Mobile phase
0- 5	A:B (15:85)
5-8	A:B (50:50)
8-15	A:B (100: 0)
15-23	A:B (15:85)

- Flow rate: 0.5 ml/min;
- Injected volume: 20 µl;
- Interface: ESI (+);
- Nebulizing gas flow: 1.5 ml/min;
- Detector Volt. (eV): 1.5;
- CDL: 250°C;
- Heating block: 200°C;
- Interface Voltage: 4.5 kV;
- CDL Volt. Mode: tuning file (CDL Voltage 25 V);
- Q-Array Bias Voltage: 20 V; Q- Array RF Voltage: 150 V;
- SIM *m/z*: 277 for Clenbuterol.

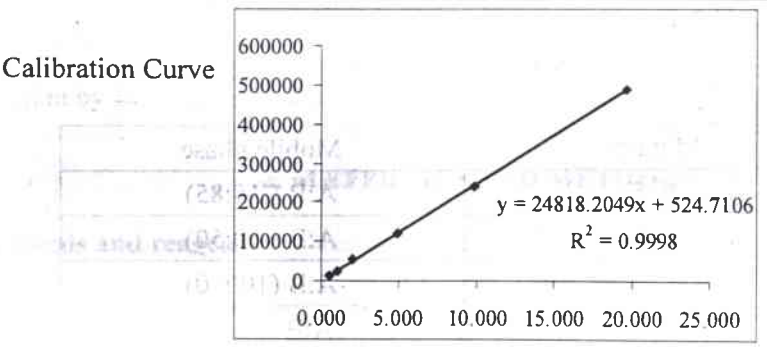
3. RESULT AND DISCUSSION

3.1. Calibration curve

Typical LC/MS chromatograms of Clenbuterol were presented in Fig.1. Clenbuterol was separated over 9 minutes. The linearity of the proposed method was assessed with various concentrations of the standard (0.585 – 23.400 µg/l calculated as Clenbuterol, HCl with 95% purity or 0.491 – 19.642 µg/l calculated as Clenbuterol free base).The correlation coefficient was better than 0.999 (Table 1).

Table 1. Peak intensities vs Clenbuterol conc.

$C_{o\ Clenb.HCl\ 95\%}$, *ppb*	$C_{Clenbuterol}$, *ppb*	*Area*
0.585	0.491	12103
1.170	0.982	24230
2.340	1.964	53136
5.850	4.910	121120
11.700	9.821	241674
23.400	19.642	489274



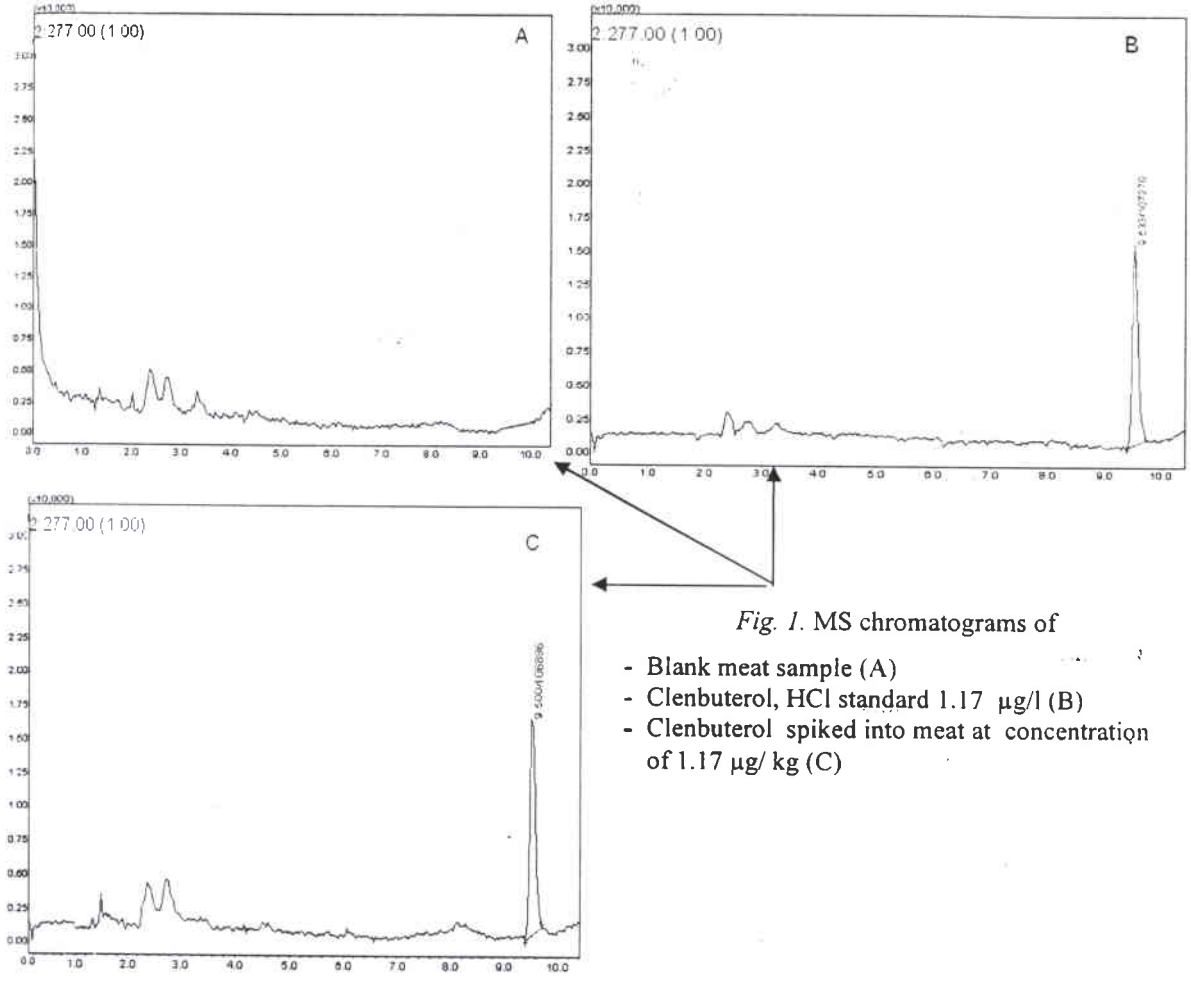


Fig. 1. MS chromatograms of

- Blank meat sample (A)
- Clenbuterol, HCl standard 1.17 µg/l (B)
- Clenbuterol spiked into meat at concentration of 1.17 µg/ kg (C)

3.2. Recovery (%) was determined for both matrices meat and feedstuff (Tables 2, 3).

Table 2. Recoveries and RSD For Clenbuterol analyzed by LC/MS (in meat)

Clenbuterol, HCl Std (ng) spiked into 5 g meat	$C_{clenbuterol, HCl}$ in meat(µg /kg)	$C_{Recovery}$ (µg/ kg) (n=6)	Recovery % (RSD %)
1.571	0.314	0.270	86.1 (7.21)
3.437	0.687	0.601	87.5 (5.22)
5.893	1.179	1.039	88.2 (3.25)
15.713	3.143	2.550	81.1 (3.37)

Table 3. Recoveries and RSD for Clenbuterol analyzed by LC/MS (in Feedstuff)

Clenbuterol, HCl Std (ng) spiked in 5 g feedstuff	$C_{clenbuterol, HCl}$ in feedstuff (µg /kg)	$C_{Recovery}$ (µg/ kg) (n = 6)	Recovery % (SD%)
3.437	0.687	0.552	80.35 (7.50)
5.893	1.179	0.945	80.15 (6.25)
15.713	3.143	2.577	82.28 (5.39)

3.3. LOD values were determined for Clenbuterol, HCl spiked respectively into meat and feedstuff.

Table 4. LOD values

	LOD (meat) (µg /kg)	**LOD (Feedstuff) (µg /kg)**
$LOD_{average}$	0.0379	0.0497
RSD %	6.7020	7.4970

3.4. Some analytical results are reported in Table 5

From October /2006 to May /2007, we analyzed 30 meat samples and 50 feedstuffs (collected in Cantho City and some provinces in Mekong Delta - Viet Nam), among these, 4 of meat samples and 7 of feedstuffs samples were contaminated.

Table 5. Analytical results of Clenbuterol in meat and feedstuff*

Samples	**Meat** *(µg/kg)*	**Samples**	**Feedstuff** *(µg/kg)*
01	21.94	01	197.07
02	8.71	02	147.99
03	45.14	03	181.88
04	39.46	04	216.75
		05	89.50
		06	39.86
		07	45.97

*** Results given in Clenbuterol. HCl (95% pure), values in Clenbuterol free base are obtained by multiplying with conversion factor 0.839.**

4. CONCLUSION

In conclusion, our analytical method comprising a much simpler sample preparation procedure followed by single quad LC/MS analysis permits a low LOD of about 0.04 µg/kg for

meat and 0.05 µg/kg for feedstuff and a good recovery around 80% for both matrices. The method was successfully applied to the analysis of contaminated meat and feedstuff.

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TÓM TẮT

PHÂN TÍCH CLENBUTEROL TRONG THỨC ĂN CHĂN NUÔI VÀ SẢN PHẨM THỊT BẰNG LC/MS

Hợp chất Clenbuterol thuộc họ β-agonist vẫn còn được sử dụng không hợp pháp làm chất phụ gia trong thức ăn gia súc, nhằm mục tiêu tăng trọng heo đồng thời làm giảm lớp mỡ dưới da trong thịt heo thương phẩm. MRL theo *Codex Alimentarius* là 0.2 µg/kg cho thịt và 0.6 µg/kg cho gan. Ở Việt Nam, Clenbuterol được phát hiện chủ yếu bằng phương pháp sàng lọc ELISA. Việc sử dụng kĩ thuật GC/MS hay LC/MS bị giới hạn do chưa có phương pháp chuẩn bị mẫu thích hợp. Phối hợp với Đại học Cần Thơ, phòng thí nghiệm của Trung tâm Đào tạo và Phát triển Sắc kí EDC-HCM đã xây dựng thành công phương pháp kiểm nghiệm Clenbuterol trong thịt và thức ăn gia súc đạt độ nhạy thấp và tỉ lệ thu hồi cao. Phương pháp nầy đã được áp dụng để kiểm tra clenbuterol trong thức ăn và thịt gia súc.Chi tiết của phương pháp và một số kết quả phân tích trên thức ăn và thịt gia súc được trình bày trong báo cáo này.

Từ khóa. clenbuterol, feedstuff, meat, LC/MS.

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Address:

Nguyen Thi Thu Thuy,

Department of Chemistry, Can Tho University, Can Tho, Vietnam.

Lam Van Xu, Pham thi Anh, Chu Pham Ngoc Son,

Center of Education and Development of Chromatography, Ho Chi Minh City, Vietnam.

Tran Kim Tinh, Laboratory of Instrumental Analysis, Can Tho University, Can Tho, Vietnam.