

# Determination of $\beta$ 2-Agonists in Pork Using Agilent SampliQ SCX Solid-Phase Extraction Cartridges and Liquid Chromatography-Tandem Mass Spectrometry

## Application Note

Food Safety

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### Abstract

A method for simultaneous determination of four  $\beta$ 2-agonist residues of terbutaline, salbutamol, clenbuterol and formoterol in pork has been developed and validated. The analytes are purified by liquid-liquid extraction (LLE) and solid-phase extraction (SPE) and quantified by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) operating in positive ion multiple reaction monitoring (MRM) mode. The method provides a sub-ng/g level of limit of detection (LOD) for all four  $\beta$ 2-agonists in pork. The dynamic calibration ranges for these compounds are obtained from 0.25 to 5 ng/g. The overall recoveries range from 78 to 101% with RSD values between 1.8 and 7.2%.

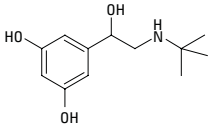
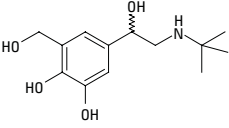
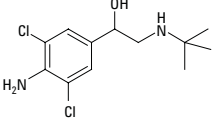
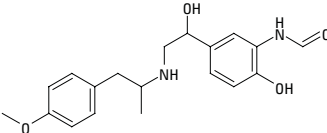


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## Introduction

The  $\beta$ 2-agonists have been used worldwide as illegal growth promoters in pork production. Recent incidences of poisoning have occurred due to high levels of the  $\beta$ -agonist (clenbuterol) in pork. This application note used Agilent's new SPE products to extract and enrich four  $\beta$ -agonists from pork and analysis by LC-MS/MS. Table 1 shows the name and structure of the four  $\beta$ -agonist compounds.

Table 1.  $\beta$ 2-Agonist Compounds Used in this Study

Compound	Log P	Structure
Terbutaline	0.55	
Salbutamol	0.44	
Clenbuterol	2.94	
Formoterol	1.91	

## Experimental

### Reagents and Chemicals

All reagents were MS, HPLC or analytical grade.

Acetonitrile and water were from Scharlau. Ethyl acetate and isopropanol were from Fisher. The standards were purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). Pork was purchased from a local market.

Standard solutions (1.0 mg/mL) were made in methanol individually, and refrigerated at 4 °C. A combined working solution (10  $\mu$ g/mL) was made in methanol-water (10:90) and also stored at 4 °C. The spiked solutions were then made weekly by appropriately diluting the combined working solution in water.

## Equipment and Materials

Agilent 1200 HPLC system

Agilent 6460 Triple Quadrupole LC-MS/MS system

Agilent SamliQ SCX Polymer cartridges, 50  $\times$  3 mL tubes, 60 mg (p/n: 5982-3236)

Agilent ZORBAX Eclipse Plus C18, 50  $\times$  2.1 mm, 1.8  $\mu$ m (p/n: 959741-906)

Agilent Vaccum Manifold processing station (p/n: 5982-9120)

## Sample Preparation

### Liquid-Liquid Extraction

A 2 g amount of pork ( $\pm$ 0.01 g) was weighed into a 15 mL capped polypropylene tube. To the pork, 8 mL of 0.2 M sodium acetate (pH 5.2) solution were added and mixed in a vortex. Next, 100  $\mu$ L  $\beta$ -glucuronidase (1000 U/mL) were added and the tube vortexed thoroughly for 2 minutes. The sample was hydrolyzed at 37 °C for 16 hours.

The hydrolysate was shaken for 15 minutes and centrifuged at 4000 rpm for 10 minutes. A 4 mL amount of supernatant was transferred to another centrifuge tube. A 5 mL amount of 0.1 M perchloric acid solution was added and the pH was adjusted to  $1 \pm 0.3$ . The tubes were then centrifuged at 4000 rpm for 10 minutes. The supernatant was transferred to another tube, and the pH was adjusted to 11 with 10 M sodium hydroxide.

Ten milliliters each of a saturated sodium chloride solution isopropanol-ethyl acetate (60:40) were added to the tubes. The tubes were shaken for 5 minutes. The tubes were centrifuged at 4000 rpm for 5 minutes before the organic layer was carefully transferred to another tube. Isopropanol-ethyl acetate addition, shaking, centrifuging and organic layer transfer were repeated twice, and all supernatants were combined.

Samples were evaporated to dryness with nitrogen at 40 °C. The residue was dissolved in 5 mL of 0.2 M sodium acetate (pH 5.2). The sample was then ready for SPE purification.

### Solid-Phase Extraction

The SPE procedure is shown in Figure 1. Agilent SamliQ SCX cartridges were preconditioned with 3 mL of methanol and then equilibrated with 3 mL water. Five milliliters of the sample solution were then loaded onto a cartridge and passed through the cartridge by gravity (about 1 mL/min). The tubes were rinsed with 2 mL of water and 2 mL 2% formic acid in water. The entire effluent was discarded. Full vacuum was

applied to the cartridge for 3 minutes to completely dry the resin. Finally, the compounds were eluted with 5 mL of 5% ammonia solution in methanol at a rate of 1 mL/min. The eluent was dried with nitrogen flow at 40 °C. The residue was reconstituted in 1 mL of 0.1% formic acid in water/acetonitrile (90:10). The sample was vortexed and ultrasonicated to completely dissolve the residue. The sample was transferred to a 1.5 mL tube and centrifuged at 3000 rpm for 5 minutes. The sample was transferred to a 2 mL chromatography vial for analysis.

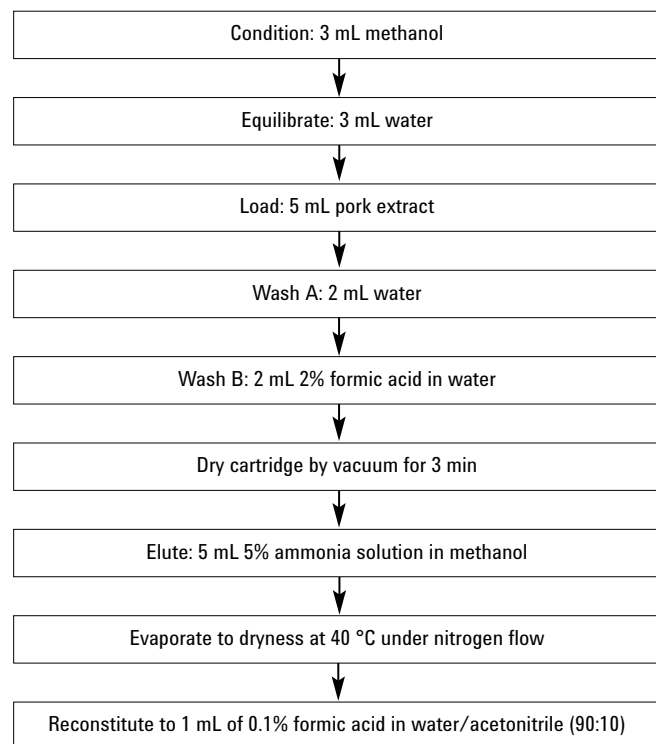


Figure 1. Pork clean up and enrichment – SPE procedure.

## Instrument Conditions

### HPLC Conditions

Column:	Agilent ZORBAX Eclipse Plus C18 2.1 mm × 50 mm 1.8 μm (p/n: 959741-906)		
Flow rate:	0.4 mL/min		
Column temperature:	40 °C		
Injection volume:	5 μL		
Mobile phase:	Water (0.1% FA+2 mM NH <sub>4</sub> Ac, A), Acetonitrile (0.1% FA, B)		
Gradient:	Time (min)	%A	%B
	0	90	10
	0.5	90	10
	1.8	20	80
	2	90	10
	3.5	90	10

### MS Conditions

These four compounds were monitored in the positive mode. The source conditions are shown in Figure 2 and the MRM channels are shown in Table 2.

Figure 2. MS source parameters for these four compounds.

Table 2. Masses Monitored in the MRM

Compound	MRM for quantification	MRM for confirmation
Terbutaline	226.1 → 152.1	226.1 → 125
Salbutamol	240.1 → 148.1	240.1 → 222.1
Clenbuterol	227 → 203	227 → 259.1
Formoterol	345.1 → 149.1	345.1 → 327.1

## Results and Discussion

### Linearity and Limit of Detection

Solutions used to create external calibration curves were prepared by using a combined working solution to spike matrix blanks (0.25, 0.5, 1.0, 2.0 and 5.0 ng/g). Matrix blanks were created by taking pork through the hydrolysis, LLE and SPE procedures. The results for the calibration curves are shown in Table 3. The limits of detection (LOD) were chosen as the concentration of each compound that gave a signal to noise (S/N) ratio greater than 3:1. The LODs are also shown in Table 3.

Table 3. Linearity and LODs of β<sub>2</sub>-Agonists

Compound	Regression equation	R <sup>2</sup>	LOD in pork (ng/g)
Terbutaline	Y = 3470x + 1325.4	0.9972	0.05
Salbutamol	Y = 13099x + 2900.3	0.9921	0.05
Clenbuterol	Y = 27028x + 1143.7	1	0.02
Formoterol	Y = 23251x + 487.44	0.9983	0.02

## Recovery and Reproducibility

The recovery and reproducibility of the method were determined at three levels: pork spiked to a concentration of 0.5, 1.0, and 2.0 ng/g. The analysis was performed with six replicates at each level. The recovery and reproducibility data is shown in Table 4. The chromatograms of spiked pork extracts (1.0 ng/g) are shown in Figure 3.

Table 4. Recoveries and Reproducibility of  $\beta$ 2-agonists in Pork After SPE Employing Agilent's SampliQ SCX: (p/n: 5982-3236), Recovery 90% and RSD 4.4% on Average

Compound	Spiked level (ng/g pork)	Recovery (%)	RSD (n=6)
Terbutaline	0.5	88.7	5.4
	1	98.0	7.2
	2	100.8	5.9
Salbutamol	0.5	100.6	1.8
	1	92.9	2.1
	2	97.4	3.9
Clenbuterol	0.5	82.3	5.0
	1	91.5	6.3
	2	90.6	4.3
Formoterol	0.5	85.1	1.9
	1	83.0	4.0
	2	77.9	2.5

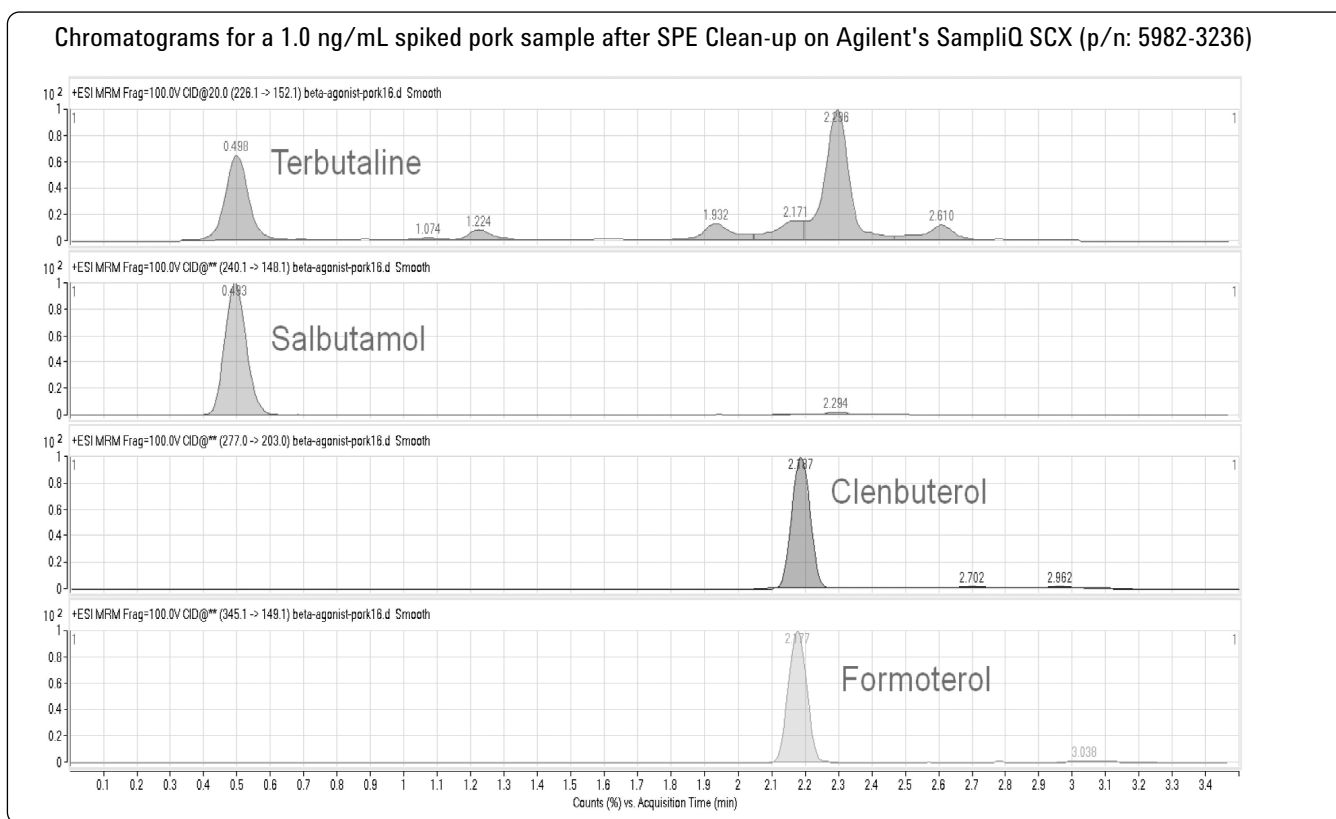


Figure 3. Chromatograms of 1.0 ng/g spiked pork sample extract.

## Conclusions

The result of this study show that Agilent SampliQ SCX can be used as an effective method for purification and enrichment of multiple  $\beta$ 2-agonists in a complex matrix such as pork. The recovery and reproducibility results based on matrix spiked standards are acceptable for  $\beta$ 2-agonists residue determination in pork under Chinese regulations. The impurities and matrix effects are minimal and do not interfere with the quantification of any target compound. The LOQ are significantly lower than the MRLs [1,2].

## References

1. GB/T 21313-2007 "Analysis of  $\beta$ 2-agonists in Foods of Animal Origin by High Performance Liquid Chromatography Tandem Mass Spectrometry"
2. SN/T 1924-2007 "Determination of Clenbuterol, Ractopamine, Salbutamol and Terbutalin Residues in Foodstuffs of Animal Origin for Import and Export -HPLC-MS/MS Method."

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