

Detection of Clenbuterol at Sub-ppb Levels Using Molecularly Imprinted Polymer SPE Methods for Sample Preparation



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Introduction

- Clenbuterol belong to the class of a beta-agonists
 - known for its growth promoting properties, increases the ratio of muscle-to-fat in farm animals.
 - athletes can abuse clenbuterol because of the anabolic effects it provides.
- Clenbuterol can adversely affect heart and lung functions.
 - It was banned from use in humans and in animal feeds to minimize the population exposure in both USA and EU.
 - Maximum residue limits at trace levels (0.1-0.3 ppb)
- It is necessary to develop a sensitive essay for analysis of clenbuterol in biological matrices such as urine, retina, tissues, etc.

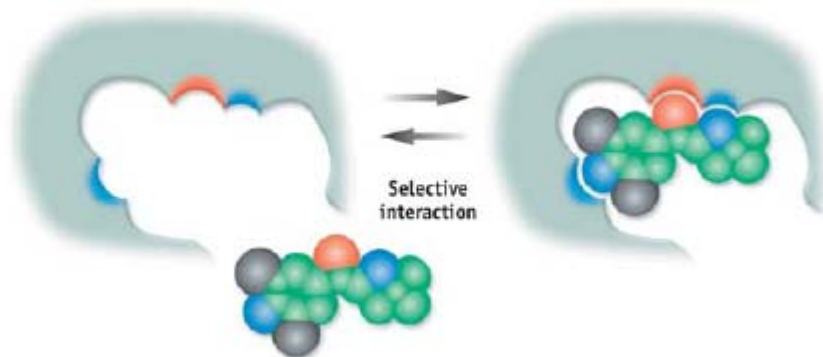


Introduction

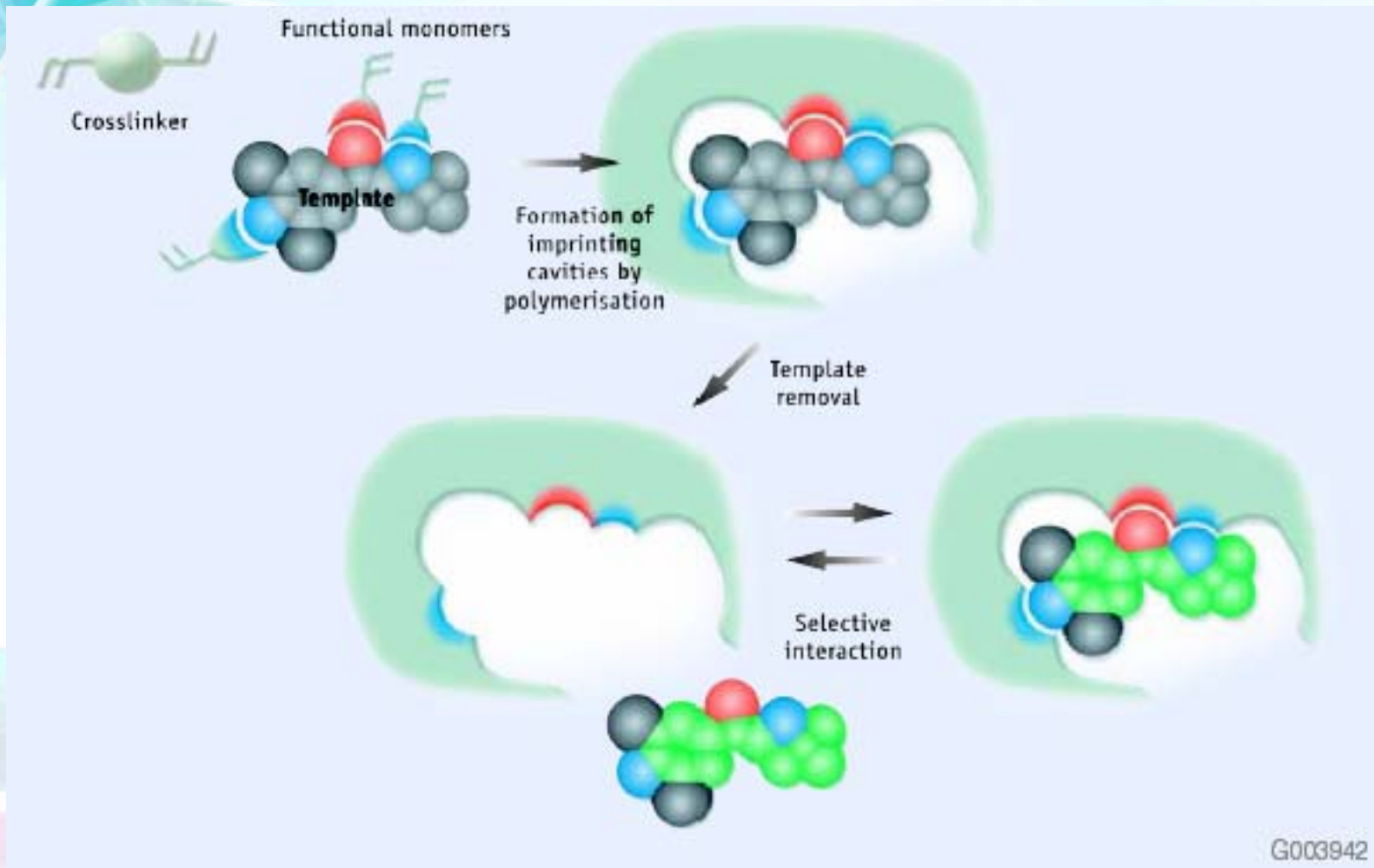
- Sample for testing: 0.1 ng/ml in urine
- Different retention mechanisms were evaluated for sample clean-up by Solid Phase Extraction
 - mixed-mode SPE (reversed phase and cation exchange)
 - polymeric SPE (reversed phase)
 - molecular-imprinted polymer SPE (reversed phase, cation exchange, hydrogen bonding)

What are Molecularly Imprinted Polymers?

- Highly cross-linked polymers
- Engineered to extract a single analyte or a class of structurally related analytes of interest with an extremely high degree of selectivity
- Selectivity is introduced during MIP synthesis
 - a template molecule, designed to mimic the analyte, guides the formation of specific cavities or imprints that are sterically and chemically complementary to the analyte(s) of interest



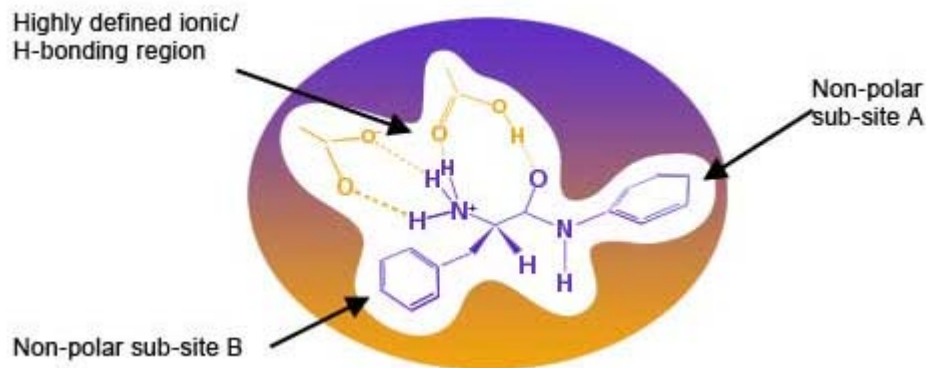
How are MIPs Made?



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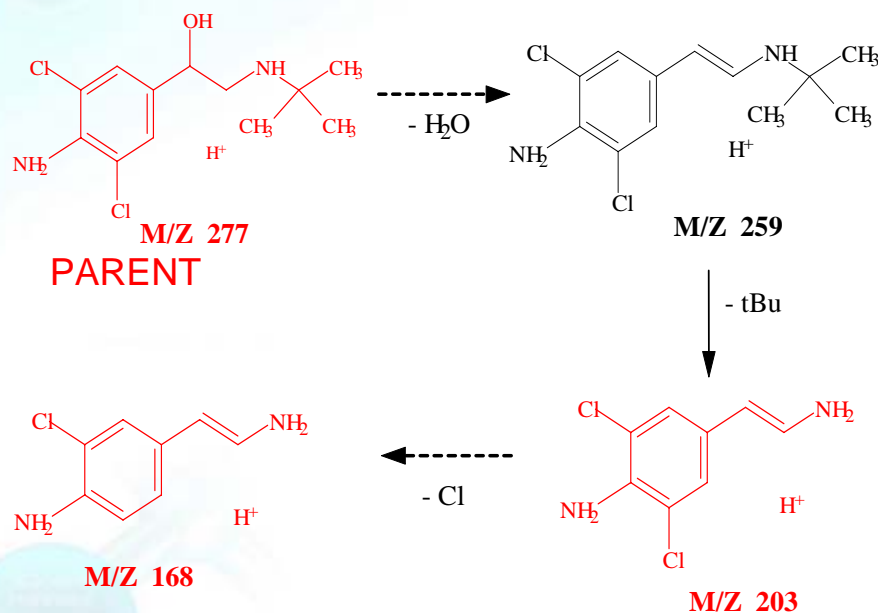
How is Selectivity Improved Using SupelMIP SPE?

- By careful design of the imprinting site, either by molecular modeling, experimental design, or screening methods, the binding cavities can be engineered to offer multiple interactions with the analyte(s) of interest.
- Multiple non-covalent interaction points (ion-exchange, reversed-phase with polymer backbone, and hydrogen bonding) between the MIP phase and analyte functional groups allow for stronger and more specific analyte retention.
- Improved selectivity is then introduced through the use of stronger wash conditions during sample prep methodology.
- Because extraction selectivity is improved, lower background is observed allowing analysts to achieve lower detection limits.

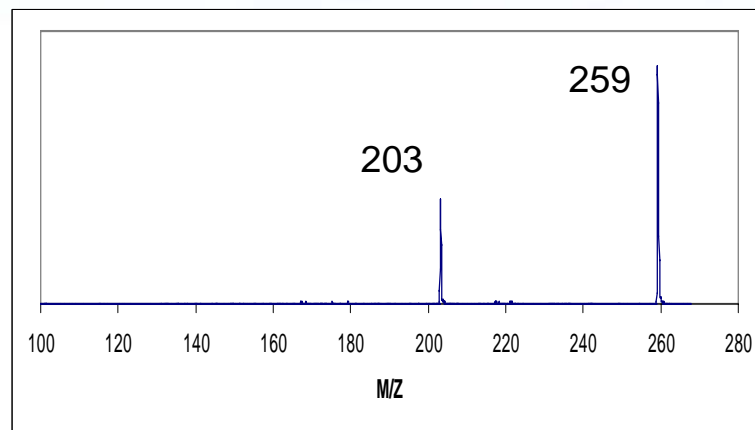


LC-MS Method

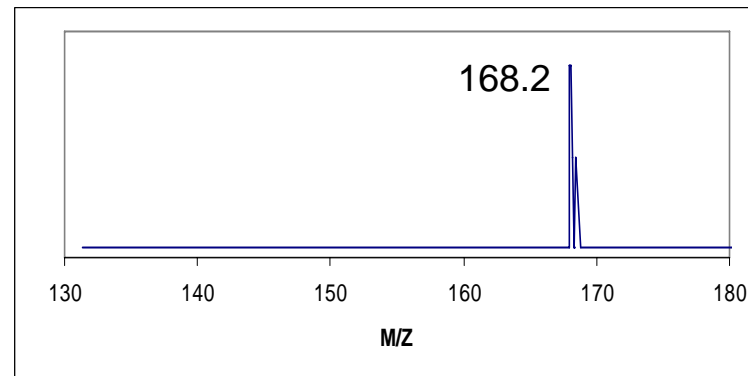
Daughter-ion Formation for Clenbuterol



Product Ion scan



MS³ 259/203 scan



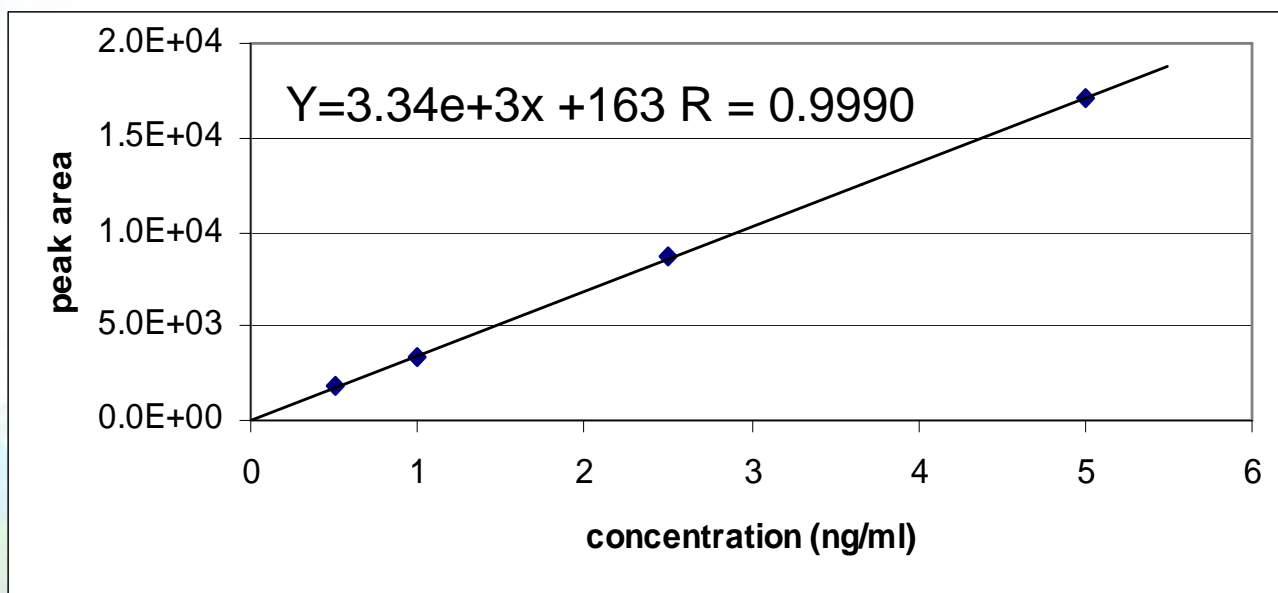
Experimental

Analytical LC-MS-MS Method

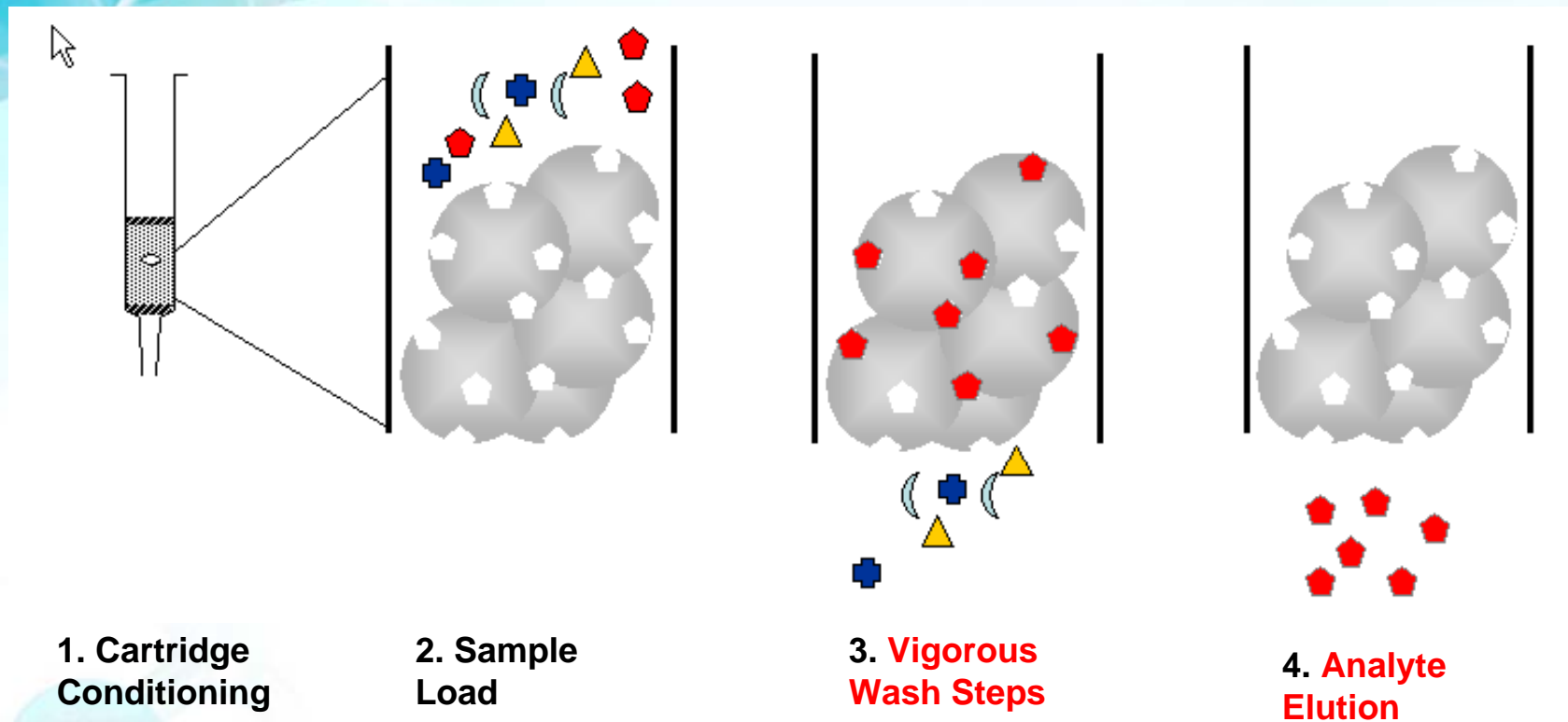
- Instrument: Agilent 1100 Stack with Applied Biosystems 3200 Q-trap
- Column: Ascentis Express-C18, 5 cm x 2.2 mm, 3 um (Supelco)
- Mobile phase: ammonium formate in 80:20 water:acetonitrile at 200ul/min
- Injection: 10 uL
- Ion source conditions: Turbo-Ion spray ESI+, CUR 35, IS 3200, TEM 425, GS1 40, GS2 45, DP 70, EP 10
- MRM transitions monitored in **Experiment 1**: 277.2/203.1, 277.2/168.2
- **Experiment 2**: Q3 full ion scan from 150-500 M/Z

Quantification of Clenbuterol

- External calibration curve was constructed with 4 standard solutions.
- Sum of both MRM transitions was used for calibration



General SPE Procedure (illustrated for MIPs)



1. Cartridge
Conditioning

2. Sample
Load

3. Vigorous
Wash Steps

4. Analyte
Elution

Molecularly Imprinted Polymer SPE

Molecularly Imprinted Polymer

SupelMIP-Clenbuterol 25 mg/10ml Large Reservoir Cartridge

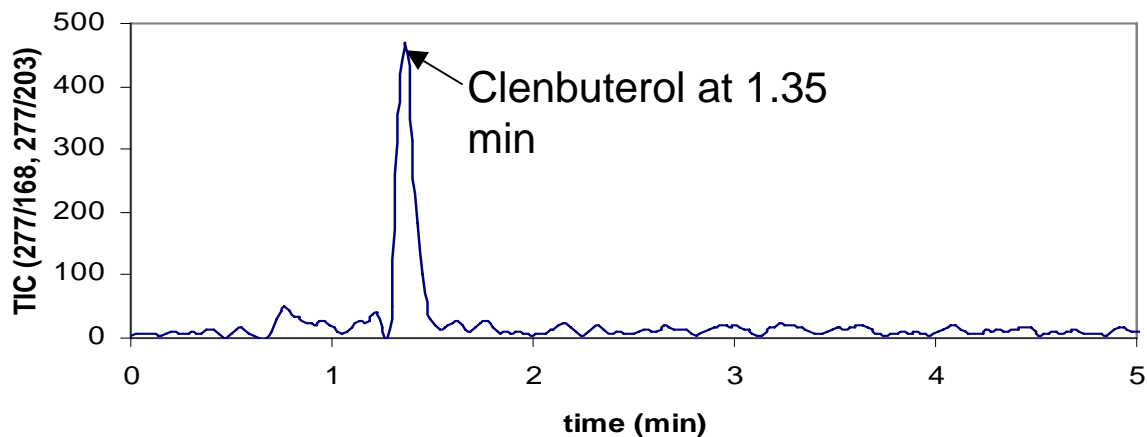
Condition: 1ml methanol, 1ml water, 1 ml ammonium acetate

Load: urine sample diluted 1:1 with ammonium acetate buffer

Wash: 1 ml of water, Vacuum pulled, 1ml 2% acetic acid in acetonitrile, 1ml 0.5M ammonium acetate buffer pH 5, 1ml 70% acetonitrile in water, Vacuum pulled

Elution: 2 x 1ml 10% acetic acid in methanol, Vacuum pulled between fractions

Evaporate and reconstitute in mobile phase (0.1 ml-1ml)



S/N = 30

0.1 ng/ml clenbuterol in urine after SupelMIP SPE cleanup

Generic Polymer SPE

Hydrophilic polymer (from Josefson et al. J. Chromat. A 1120 (2006) 1-12)

Cartridge: generic hydrophilic polymer, 30mg/1ml

Condition: 1 ml methanol, 1 ml water

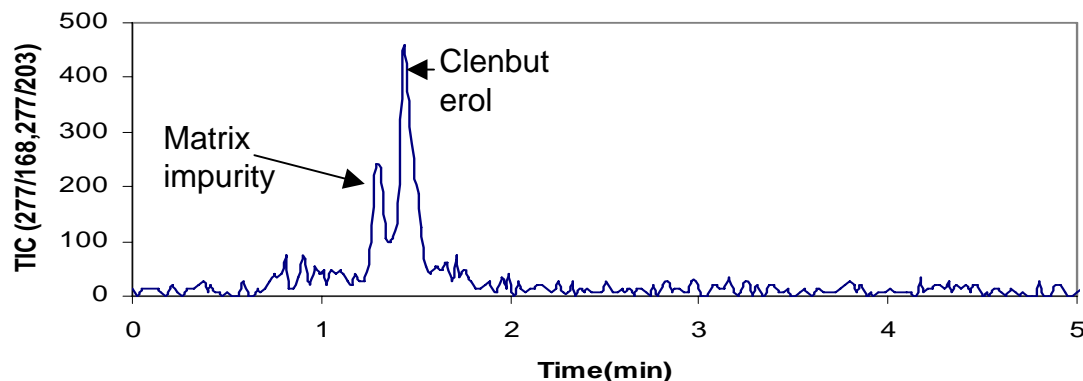
Load: 1ml human urine, diluted 1:1 with 2% NH₄OH/H₂O

Wash: 1 ml water, 0.5 mL 30:68:2 MeOH:H₂O:NH₄OH

Elute: 2 x 1ml methanol

Evaporate and reconstitute in 0.1-1 ml of the mobile phase

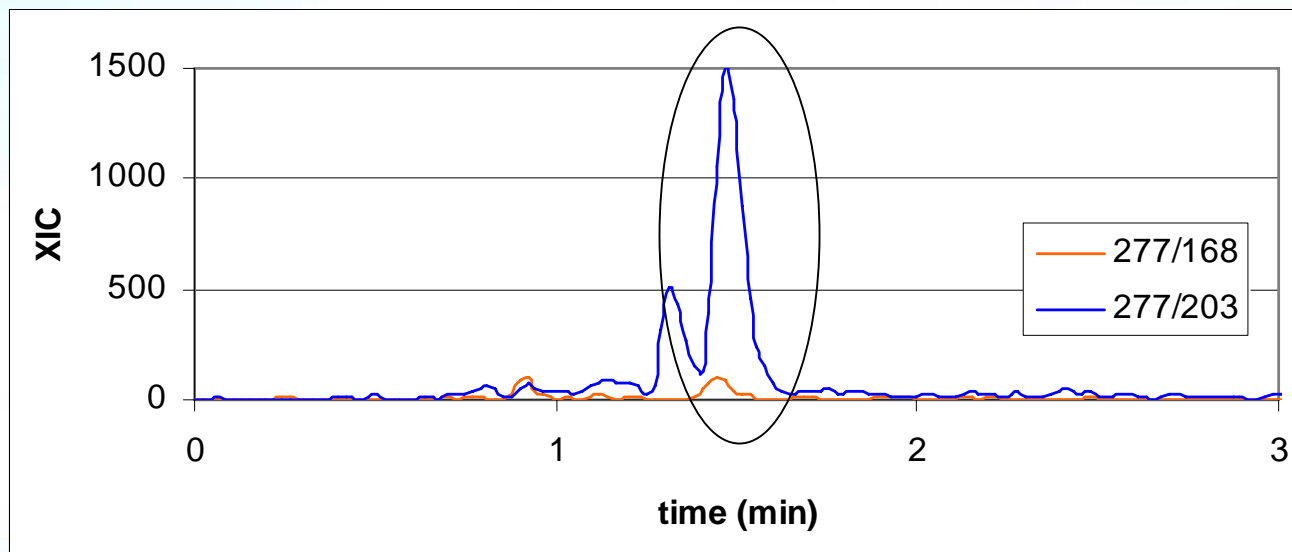
Note: samples were yellow in color



S/N = 33

0.1 ng/ml clenbuterol in urine cleaned with generic polymer SPE

2 monitored MRM transitions for clenbuterol in the sample after polymeric SPE



Mixed-Mode SPE

Mixed-Mode

Cartridge: Supelco Discovery MCAX 100mg/1ml

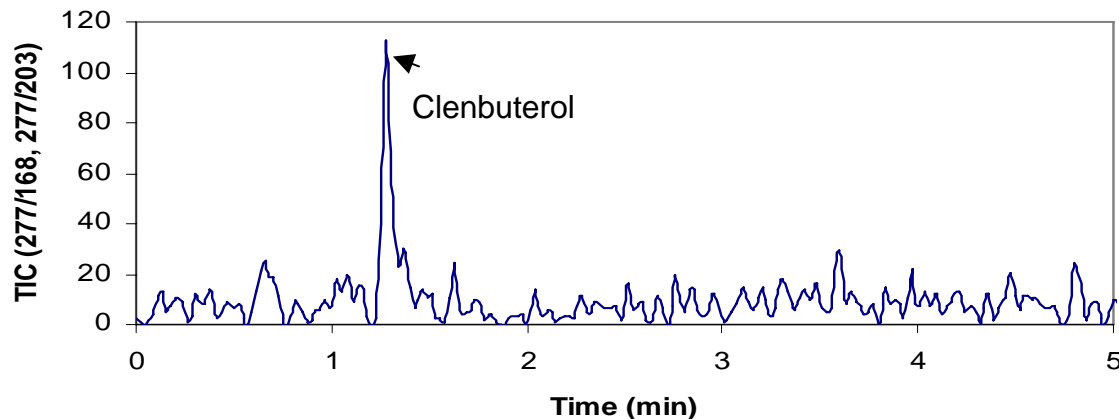
Condition: 2 ml methanol, 2 ml ammonium acetate (10mM)

Load: urine diluted 1:1 with 10mM ammonium acetate

Wash: 2 ml 10mM ammonium acetate, 2ml 10% acetic acid, 2 ml methanol

Elute: 2x 1 ml 2% ammonium hydroxide in methanol

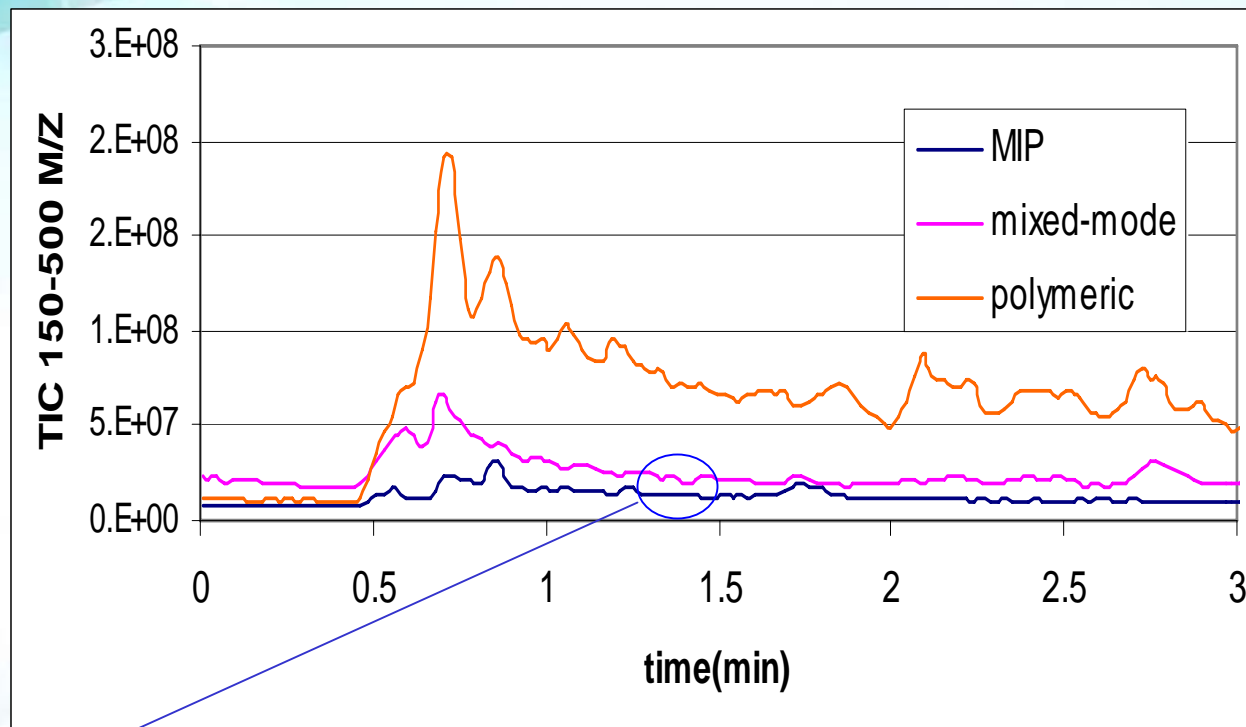
Evaporate and reconstitute into 0.1-1 ml mobile phase



S/N = 8

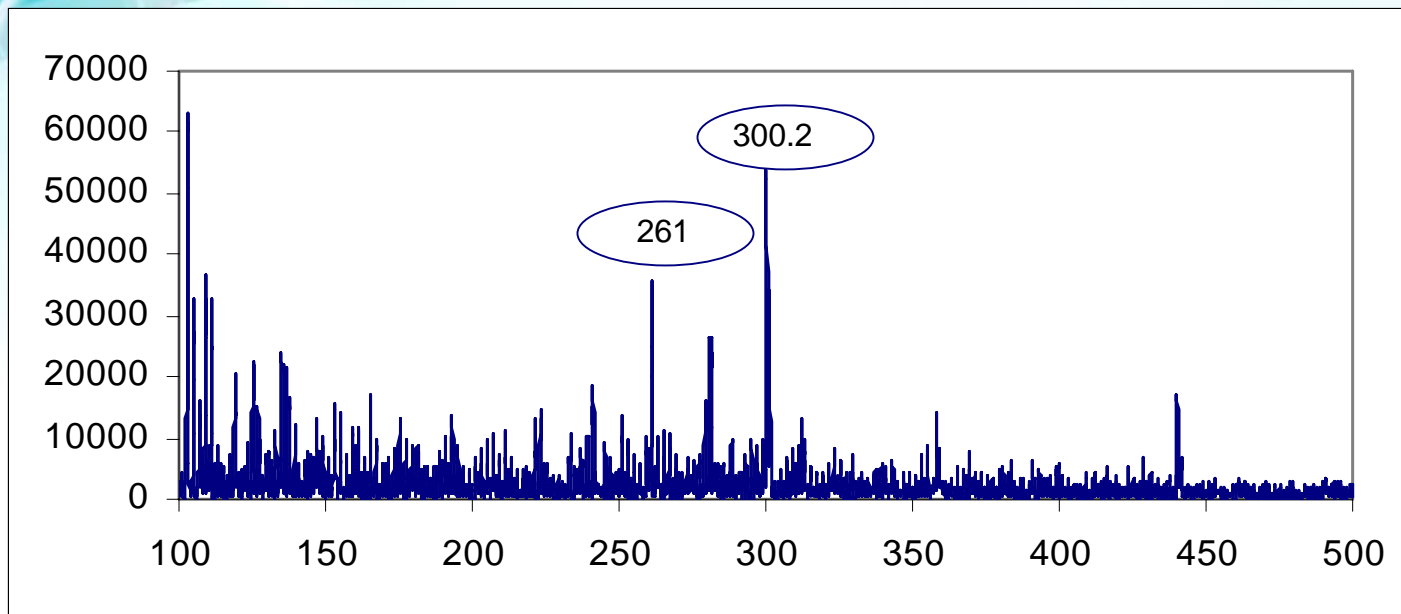
0.1 ng/ml clenbuterol in urine cleaned with Mixed-mode SPE

Investigation of Matrix Noise

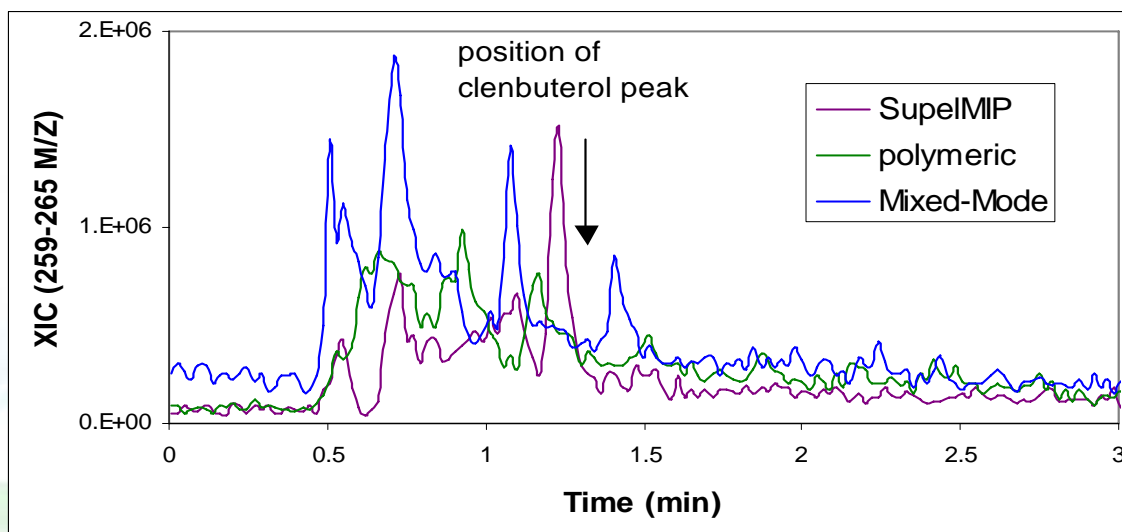
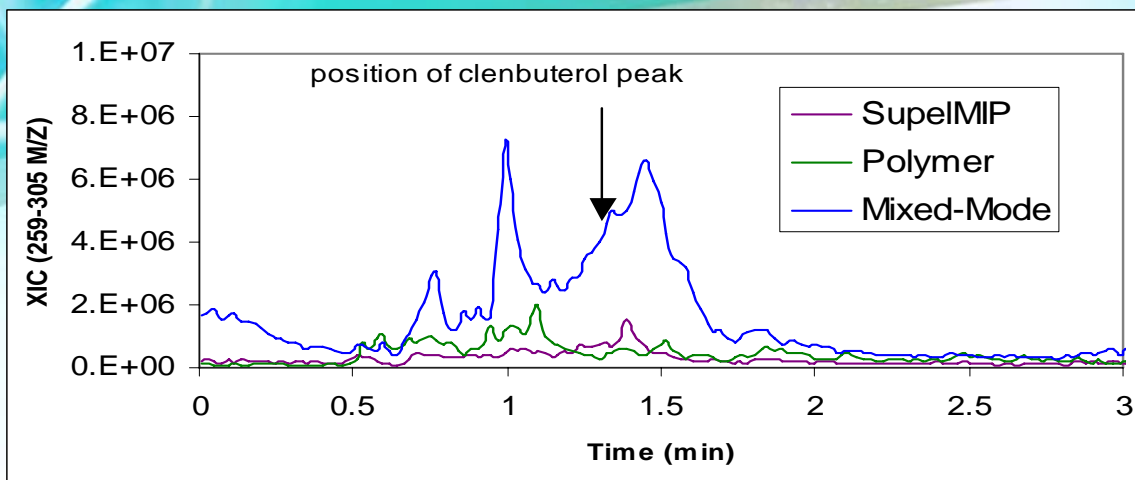


- The advantage of the Q-trap LCMS instrument is the possibility of the full range ion scan concurrently with the analyte detection by MRM. Q3 scan was done as experiment 2 in the same period. Most abundant interference peaks were identified in the samples cleaned with SupelMIP SPE.

Investigation of Matrix Noise



Interferences from Q3 scan over the time range of clenbuterol peak (1.2-1.5 min) in the samples cleaned with SupelMIP SPE



XIC for 2 major **matrix peaks** in samples cleaned with different SPE methods

Recovery of Clenbuterol from Urine at 0.1 ng/ml

	SupelMIP SPE	Polymeric SPE	Mixed Mode SPE
SPE sample	104%	137%*	62%

* Integration error due to the overlapping impurity peak

Recovery of Clenbuterol from Urine at 1.0 ng/ml

	SupelMIP SPE	Polymeric SPE	Mixed Mode SPE
SPE sample	75%	69%	121%

Investigation of Matrix Effects

- Blank urine samples were cleaned with one of the SPE methods and spiked with clenbuterol directly before analysis
- The “matrix-matched” samples were compared to the standards prepared in buffer to determine if any ion suppression effects are present

Recovery of clenbuterol from Post-SPE Spiked Samples at 1 ng/ml

	SupelMIP SPE	Polymeric SPE	Mixed Mode SPE
SPE sample	91%	109%	145%*

Summary

- Good recovery values and good clean-up was achieved with SupelMIP SPE. Multiple interactions allow for stronger binding during SPE procedure. The use of stronger washing conditions result in a better sample clean-up
- Interference peaks were observed at MRM 277/203 in samples cleaned with polymeric SPE, the samples looked the dirtiest with yellow color. The background total ion count was the highest for these samples.
- Clean up with mixed-mode SPE resulted in a low recovery (60%) at the 0.1 ng/ml spiking concentration. After evaluation and the absence of the ion-suppression effects, the low recovery should be attributed to the breakthrough losses.

Summary

Evaluation of SPE methods

	SupelMIP	Polymeric	Mixed-mode
S/N for 0.1 ppb	30	33	8
Total background	low	high	medium
Recovery at sub-ppb level	good	Good	low
Matrix ionizaion effects	None	None	none

Acknowledgments

- Supelco Scientists Carmen Santasania and Daniel Shollenberger for their help with LC-MS work
- MIP Technologies AB, Sweden for development MIPs and the SPE methods for using MIPs