

Comparison of SupelMIP™ Beta-agonist and mixed-mode SPE for the extraction of beta-agonists from urine samples

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Beta-2-adrenergic receptor agonists (Beta-agonists) have been clinically and veterinary used in the treatment of cardiovascular and breathing disorders. However, Beta agonists are also used as an illegal muscle growth promoter due to its anabolic effects both in humans and in animals. Although the US Food and Drug Administration, US Department of Agricultural and European Union have banned the use of Beta agonists for humans and livestock, illegal use of the drug still frequently occurs. For example, the Beta agonist clenbuterol is widely used among body builders and athletes due to its anabolic effects. Beta agonists are readily used by farmers to give animals a competitive advantage as well. Due to the potential health risks and competitive advantage associated with Beta agonist use in livestocking and human performance enhancement, residue screening programs for it are conducted worldwide. It is therefore critical to develop a highly selective and sensitive analytical assay to monitor Beta agonist residues in difficult biological matrixes such as urine, retina, tissues, etc.

In this article a summary of the work performed at the Veterinary Institute in Oldenburg, Germany is presented. In order to enhance the methods for analysing Beta agonists the SupelMIP Beta agonist SPE was evaluated. The SupelMIP material was evaluated for an application in urine and the method was compared with a mixed mode material.

Urine is a demanding matrix due to its complexity and high content of contaminants and therefore there is a need for a highly selective extraction method for sample pre-treatment. As an overview minimum required performance levels in different matrices are presented in table1.

Table 1. Minimum required performance levels in different matrices (MRPL).

	Beef/Swine				Poultry			
	Plasma	Urine	Retina	Liver	Feed	Water	Retina	Liver
Brombuterol	< 0.5	< 0.5	< 3.0	< 1.0	< 1.0	< 1.0	< 3.0	< 1.0
Chlorbrombuterol	< 1.0	< 0.5	< 3.0	< 0.2				
Cimaterol	< 2.0	< 3.0	< 10	< 3.0	< 3.0	< 3.0	< 10	< 3.0
Clenbuterol	< 0.2	< 0.2	< 3.0	< 0.2	< 0.2	< 0.2	< 3.0	< 0.2
Clenproperol	< 1.0	< 3.0	< 10	< 1.0	< 1.0	< 1.0	< 10	< 1.0
Mabuterol	< 0.5	< 0.5	< 3.0	< 0.2	< 0.2	< 0.2	< 3.0	< 0.2
Ractopamin	< 3.0	< 3.0	< 10	< 3.0				
Salbutamol	< 0.5	< 0.5	< 3.0	< 0.2	< 0.2	< 0.2	< 3.0	< 0.2
Terbutalin	< 3.0	< 1.0	< 20	< 2.0	< 2.0	< 2.0	< 20	< 2.0
Zilpaterol	< 0.5	< 1.0	< 0.5	< 1.0				

Methods

In this work the SupelMIP SPE Beta agonists (25 mg/10 ml) for monitoring of Beta agonists in calfs urine was evaluated and compared with a mixed mode phase, CS DAU (500 mg/6 mL). Details of the SupelMIP extraction method are presented in table 2.

Results and conclusion

To evaluate the performance of the SupelMIP SPE method it was compared to the mixed mode SPE, CS DAU. LC-MS/MS chromatograms were compared and in figure 1 the chromatograms of Salmeterol extracted from urine using SupelMIP SPE resp. mixed mode SPE are shown. The SupelMIP extract showed to be clean with a very low content of contaminating compounds and having an increased peak height. In figure 2 LC-MS/MS chromatograms for Clenbuterol resp. Clenproperol extracted from urine are shown. The signal strength of Clenbuterol and Clenproperol were increased with a factor of about 10 when using the SupelMIP Beta agonist SPE instead of the mixed mode SPE material. In

Table 3 the Signal to Noise data for a broad range of Beta agonists are presented, both for the target ion and the qualifier. Generally, using SupelMIP Beta-agonist SPE sample pre-treatment for urine samples cleaner extracts are obtained with a low content of interfering contaminants and an increased S/N ratio.

Using mixed mode SPE in the sample pre-treatment of the urine samples, the centrifugation was followed by filtration using a membrane filter. Using the SupelMIP improved performance was obtained excluding the membrane filter step; which resulted in a faster procedure.

In this work it was concluded that the SupelMIP SPE Beta agonists is a highly selective phase for Beta agonists. This high selectivity for the class of compounds allows very clean extracts with low levels of interfering contaminants. Comparing the performance with general mixed-mode phases, a clear enhancement in the signal to noise ratio is obtained using the SupelMIP phase, allowing increased analytical sensitivity and lowered detection levels.

The method as described before fulfills all criteria of the EU Commission Decision 2002/657/EC for confirmatory analysis of substances listed in group A of Annex I of Council Directive 96/23/EC and has meanwhile been adopted for routine analysis in the Veterinary Institute Oldenburg.

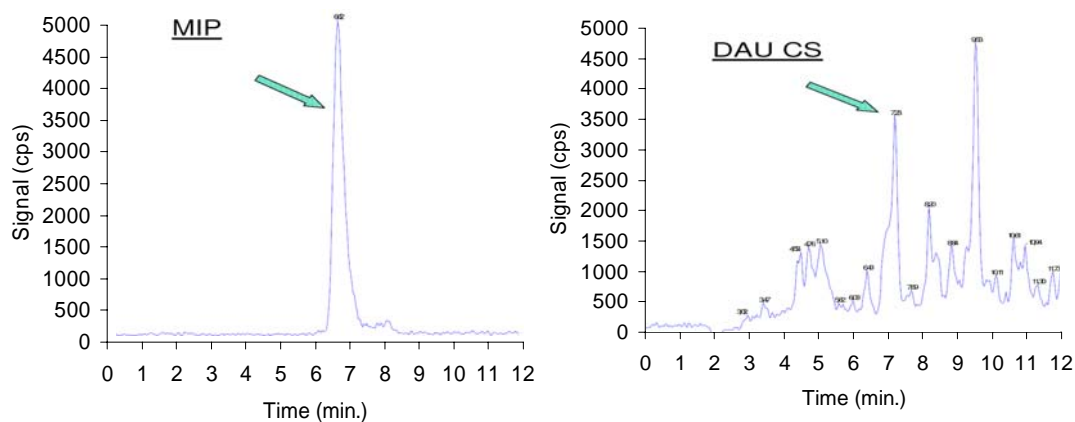


Figure 1. Chromatogram of Salmeterol (0,2 µg/L) at MRM 240 / 148 amu. The Salmeterol peak in respectively chromatogram is shown by the arrows. Compared to the extract using the mixed mode column the SupelMIP extract is cleaner and the level of interfering contaminants are very low.

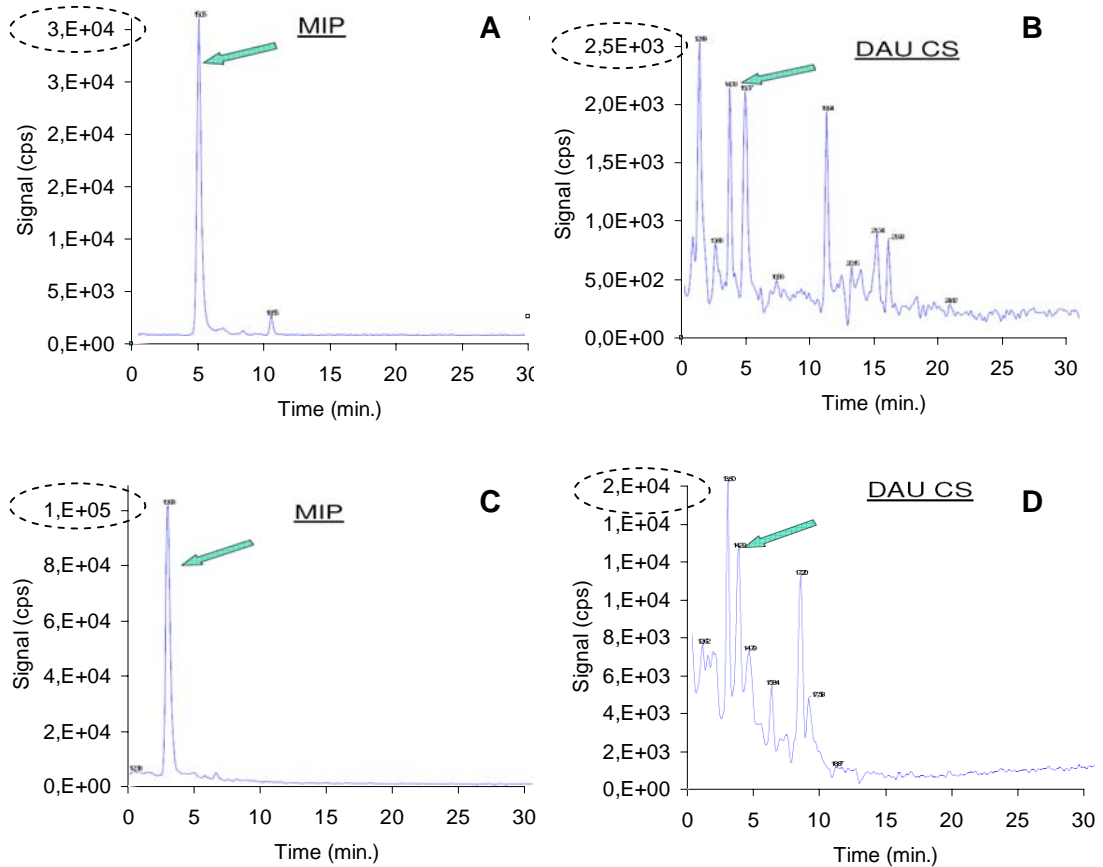


Figure 2. Chromatogram A, B, C and D show urine extracts using SupelMIP resp. CS DAU. Chromatogram A and B show extracts of Clenbuterol (0.05 $\mu\text{g/L}$) at MRM 277 / 203 amu. The signal strength of Clenbuterol is increased with a factor of 10 using the SupelMIP SPE. Chromatogram C and D show extracts of Clenproperol (0.4 $\mu\text{g/L}$) at MRM 263 / 245 amu. In this case the signal strength of Clenproperol is also increased with a factor of 10 using the SupelMIP Beta agonist SPE. Note the differences in scale of the signal intensity between A and B resp. between C and D. The Clenbuterol resp. Clenproperol peak a in respectively chromatograms are shown by the green arrows.

Table 2 Extraction method using SupelMIP Beta-agonist

Extraction method using SupelMIP SPE Beta agonists, 25 mg/10 mL, Cat. No. 53210-U Recommended Flow rates are ~0.5 ml/min during conditioning, sample load and wash and ~0.2 ml/min during elution.	
Sample pre-treatment Hydrolyse of to 5 -10 mL Urine with Glucuronidase/Sulfatase with an activity of 85.000 units/mL (2 h at 37°C, pH 5) (Sigma-Aldrich (Prod. No. G0876)) Adjustment to pH 6-7 followed by centrifugation	
Column conditioning The columns were equilibrated with 1 mL methanol followed by 1 mL DI water and 1 mL 25 mM ammonium or sodium acetate, pH 6.7.	
Sample load 10 ml urine was loaded on the column	
Washing 1 mL DI water followed by full vacuum through cartridge for 2 min 1 mL 1 % acetic acid in acetonitrile 1 mL 50 mM ammonium acetate, pH 6.7 1 mL 60 % acetonitrile/40 % DI water, followed by full vacuum through cartridge for 2 min. to dry the columns.	Analytical method HPLC Agilent 1100 Serie Column: Phenomenex, Synergy Polar-RP, 150 x 2 mm, 4 µm (Phenylphase) Mobile phase: Methanol; Ammoniumacetat 5 mM Gradient: 10 to 80 % Methanol in 15 minutes Mass spectrometer: API 3000, Electrospray-ionization, positiver Modus (ESI+), Multiple Reaction Monitoring (MRM)
Elution 2 x 1 mL MeOH / 10 % Hac The eluate was evaporated u and the sample was reconstituted in mobile phase prior to analysis.	

Table 3 Signal to Noise ratio

	MIP-Phase		DAU CS-Phase	
	Target	Qualifier	Target	Qualifier
Brombuterol	5200	1900	90	30
Chlorbrombuterol	2500	370	120	40
Cimaterol	560	710	100	380
Clenbuterol	1300	140	120	50
Clenproperol	430	1100	100	30
Mabuterol	3000	1100	260	60
Ractopamin	3100	720	100	70
Salbutamol	330	40	40	40
Terbutalin	1300	230	20	70
Zilpaterol	140	220	60	40