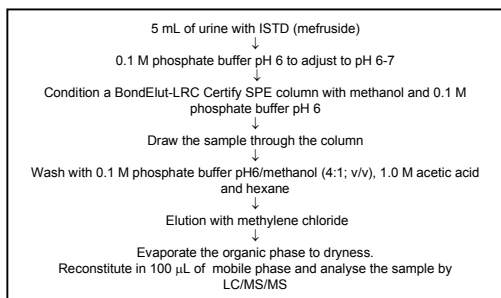


## Introduction

Furosemide is, as a doping agent, the most frequently detected diuretic in sport drug testing programs. In this study a qualitative confirmation method was developed to detect furosemide in an urinary matrix based on a LC/MS/MS procedure using the negative Electrospray Ionization mode. The method was validated according to the EUROCHEM Guide for method validation, which follows ISO 17025 quality requirements. Parameters, which were considered to be essential, were extraction recovery, limit of detection (LOD), limit of identification (LOI) and selectivity.

## Experimental

### Extraction procedure



### Instrumental conditions

Instrument: HPLC TSP P4000 with a LCQ Advantage	
Column: Hypersil ODS (125mm × 2.1 mm, 5 µm)	
Mobile phase: solvent A: 100% CH <sub>3</sub> CN; B: 2 mM HCOONH <sub>4</sub>	
Flow rate: 0.3 mL/min	
Gradient program:	
initial composition	10% A
gradient	10% A for 2 min 75% A in 20 min 75% A for 2 min
Mass spectrometric parameters	
ionisation	ESI in negative mode
sheath gas flow rate	60 units
aux gas flow rate	20 units
capillary temperature	300 °C
tune method	specific
acquisition mode	MS/MS
number of segments	2
Furosemide conditions	
precursor ion	m/z 329; m/z 331
collision energy	28%
isolation width	1 unit
ISTD conditions	
precursor ion	m/z 381
collision energy	35%
isolation width	1 unit

## Results and Discussion

### Extraction recovery

The extraction recovery obtained using a special developed BondElut-LRC Certify Solid Phase Extraction (SPE) method was **93%**.

### Limit of detection (LOD)

The LOD, defined as the lowest amount in a sample, that can be distinguished from background noise, was calculated based on a calibration curve in the range of a pre-estimated value of the LOD and linear regression analysis. The following formula was applied:

$$LOD = \frac{3.3 \times S_{y/x}}{b}$$

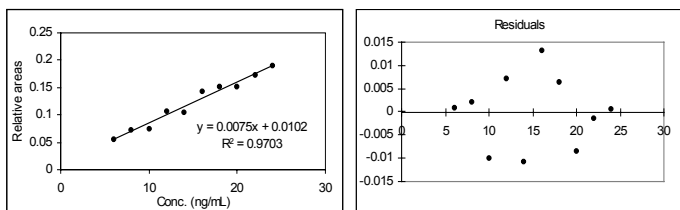


Figure 1. Calibration curve and residuals for the limit of detection (LOD)

The LOD proved to be **4 ng/mL**. The limit of identification (LOI) was defined as the lowest amount of the compound of interest in a sample that fulfils *in house* validated identification criteria using equipment specific library search algorithm routines. These routines were applied by comparing the spectrum of interest with that of a reference spectrum obtained from a blank sample fortified with an amount for furosemide and analysed under identical experimental conditions as the spectrum of interest. The LOI for furosemide was identical to the LOD.

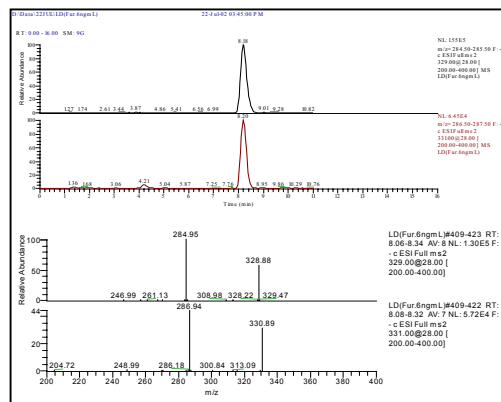


Figure 2. Chromatograms and spectra of a sample containing furosemide (6 ng/mL).

### Selectivity

Selectivity was determined by checking for interferences caused by known and unknown compounds that could be present in authentic samples. An interference was defined as a signal of a diagnostic ion with a signal-to-noise ratio > 3 and a resolution < 1.0. Resolution ( $R_s$ ) was determined by fortifying the sample, which was associated with the interference, with a certain amount of furosemide. The following formula was applied:

$$R_s = 2 \times \frac{(t_2 - t_1)}{(w_{b1} - w_{b2})}$$

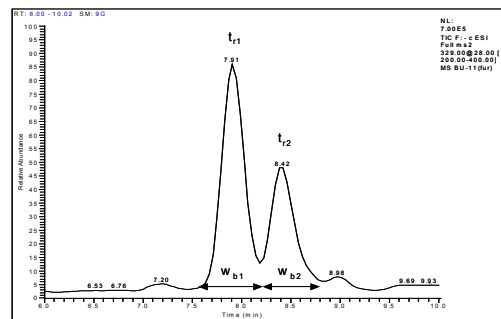


Figure 3. An example of the presence of an interference ( $t_2$ ) in a furosemide sample ( $t_1$ )

No known compounds were found to interfere. In relation to unknown compounds, some interferences were noted, but these did not lead to false suspected samples.

## Conclusion

The LC/MS/MS combined with a SPE method proved to be a suitable qualitative confirmation method of the furosemide presence in human urine samples.

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