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An unified approach to identify bioactive substances in complex matrices

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Background

The detection of unknown or unexpected bioactive compounds is of importance for various fields of research, e.g. finding new drugs. For food and feed safety the presence of unknown or unexpected bioactive compounds may lead to severe risks for consumers. In addition, after a human or animal intoxication incident inspection services will try to elicit the source or the compound causing the intoxication.

Objective

In this study a general approach for bioactive driven identification of unknowns was explored, a fictive case was created with an unknown beta-agonist in urine. A simple artificial case description together with a prepared sample, was send to various research groups, i.e. a biosensor & bioassay group (BB) and an analytical group specialized in chemical analysis of veterinary drugs. After the screening in the BB group, this group collaborated with the analytical group, using a bioassay guided LC fractionation approach for identification and confirmation of the active fraction, using high resolution mass spectrometry in combination with powerful statistical and structure elucidation tools.

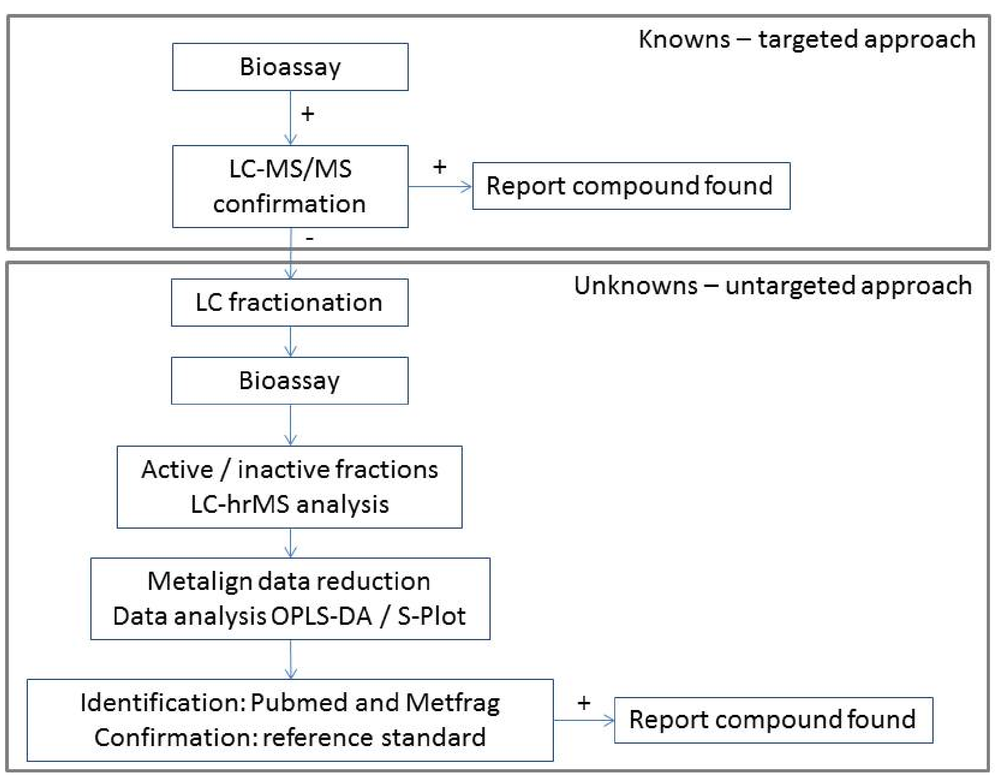

Figure 1. Unified approach to detect unknowns in complex matrices.

Experimental

Bio-assay analysis

For testing the suspect urine sample on the presence of a steroid hormone a yeast estrogen and yeast androgen bioassay were performed.
For testing the suspect sample on the presence of a β-agonist a competitive radioligand binding assay was performed.

LC-Fractionation

The fractionation system consisted of a Waters Acquity UPLC coupled to two Gilson 234 autosamplers which were used as fraction collectors. The column effluent was split in a 50:50 ratio and diverted towards the two fraction collectors. During the UPLC high resolution fractionation each well was sampled during 3 minutes which corresponds to 600 µL. After fractionation, bioactivity screening was performed in order to identify the bioactive fractions of interest.

LC-MS analysis

The LC fractions collected were diluted 1:1 with water and 10 µl was injected on the Thermo Fisher Q-Exactive Orbitrap MS.
Data were acquired by continuously alternating scan events: first a full-scan (*m/z* 135-1000) followed by five all-ion fragmentation events (*m/z* 150±110 Da, *m/z* 250±110 Da, *m/z* 350±110 Da, *m/z* 450±110 Da and *m/z* 750±510 Da). The fragmentation normalised collision energy was stepped from 14, 40, 80 %.

Full Scan	m/z 135 - 1000 (Res. 70000)	Scan Range (res. 35000)
AIF MS	m/z 150 ± 110 Da	m/z 50-225
AIF MS	m/z 250 ± 110 Da	m/z 50-330
AIF MS	m/z 350 ± 110 Da	m/z 50-430
AIF MS	m/z 450 ± 110 Da	m/z 50-535
AIF MS	m/z 750 ± 510 Da	m/z 70-1045

Figure 2. Various scan events applied with normalised collision energies of 14, 40 and 80 %.

Results

In this study the urine sample was found negative in the estrogen assay, but both the androgen and beta-agonist assay showed activity.
After analysing the urine sample in our routine analysis the androgen activity could fully explained by the finding of the presence of **testosteron**, but for the beta-agonist activity no known beta-agonist was found.

The suspected fractions were analysed with the Q-Exactive Orbitrap and by applying statistical data-analysis the following masses were found most abundant: ***m/z* 321.036** and ***m/z* 323.034**.
Based on the isotopic pattern and the exact mass the following elemental composition was determined: **$C_{12}H_{18}ON_2BrCl$**.
Using the available fragmentation data a correlated structure was determined which could be related to the compound: **bromochlorobuterol**.

Conclusions

The present work describes an approach to identify unknown bioactive compounds in complex matrices. The strengths of both biological effect or bindingassays and analytical chemistry in combination with powerful statistical tools and in-silico fragmentation prediction prove that identification of unknown actives at low concentration levels in complex matrices is possible.

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