A method transferability study:
Adapting a QTOF-MS pesticide screening method to the application of forensic screening using enhanced confirmation strategies

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Introduction

High-resolution time-of-flight mass spectrometry (HR-MS) is used for forensic and toxicological screening for several years[1]. The accurate mass based inherent characteristics like sensitive wide scope screening together with retrospective and general unknown analysis capabilities make it an ideal tool for this work. A recently developed solution for pesticide screening in food shall therefore be tested for suitability and expandability into this application in general. Transferability of the screening setup and methods between three installation sites is evaluated. Concurrently, a variety of options for enhanced result confirmation (concept of "diagnostic ions"), which has proven to be a useful tool for efficient reduction of false-positive findings[2] shall be tested on forensic screening samples.

Methods and Experiments

HPLC: Ultimate 3000 Rapid Separation LC (Thermo)
Column: Acclaim RSLC C18 2.1x100 mm, 2.2 µm (Thermo)
Mobile phase: A: H2O, B: MeOH (5 mM NH_4formate/0.01 % HCOOH),
Gradient: 14 min multistep gradient 5 - 99.9 % eluent B with a flow gradient 0.2 - 0.48 mL/min.
MS: impact (UHR-TOF MS, Bruker Daltonik GmbH).
Scan mode: ESI(+) Full scan (m/z 30 - 1000), bbCID.

For this study, 61 compounds based on practical relevance in post-mortem and routine drug screening, covering a variety of compound classes plus the full range of relevant properties (exact mass, RT, fragmentation energy), were choosen.

Databases: Six solvent based mixes were prepared containing up to 11 compounds each at a concentration of 1 µg/mL and analyzed at all three sites in broad-band CID (bbCID) data acquisition mode to build up a database containing name, sum formulae and RT. Full scan compound spectra were evaluated for presence of additional ions besides the pseudo-molecular ion like fragments or adducts. If their relative intensity was higher 10 %, these ions were also defined in the database. This screening database was used for for processing of the analyses in full scan mode.
For bbCID mode a second database was used containing additional entries for an M+1 or M+2 isotope of each compound and fragment ions observable in the bbCID data as qualifier ions (QI) for result confirmation (fig. 1 - 3).

Urine and serum samples were spiked after ACN precipitation with compound mixes at four levels (10, 50, 100, 500 ng/mL) and analyzed in full scan and bbCID mode. For automated processing using DataAnalysis 4.1 and TargetAnalysis 1.3 the intensity threshold for compound detection was set to allow detection in a 10 ng/ml sample in solvent on all relevant traces. The total numbers of findings were compared to the number of expected findings, including false positives. For the runs aqcuired in bbCID mode, additional detection criteria were applied, finally accepting compounds as detected only, when the main compound ion and at least one diagnostic ion with RT difference < 0.05 min was detected on full scan or bbCID data level.

Authentic samples from routine screening cases, which were run in bbCID mode on the systems in Helsinki (11 post mortem urine samples) and Freiburg (8 urine/serum samples; post mortem & roadside testing) were analysed and processed using the same processing method and detection criteria. Results were compared to findings from routine analysis.

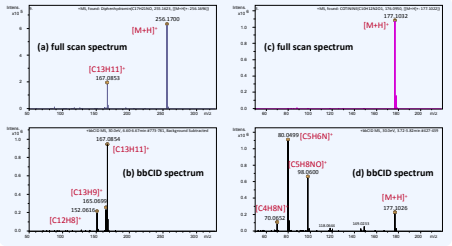

Fig. 2: Full scan and bbCID example spectra for diphenhydramine **(a, b)** and cotinine **(c, d)**.

(a)

m/z	RT	sum formula	name
300.1594	3.40	C18H21NO3	Codeine
195.0877	4.35	C8H10N4O2	Caffeine
177.1022	3.75	C10H12N2O	Cotinine
285.0789	9.53	C16H13ClN2O	Diazepam
302.1751	3.36	C18H23NO3	Dihydrocodeine
256.1696	6.63	C17H21NO	Diphenhydramin
167.0855	6.63	C13H11^1+	Diphenhydramin Fragm 167
280.1696	6.88	C19H21NO	Doxepin

(b)

m/z	RT	sum formula	name	QI 1	QI 2	QI 3
316.0080	7.28	C14N3H10OBr	Bromazepam	182.0839	209.0947	288.0131
316.0080	7.28	C14N3H10O^81Br	Bromazepam (^81Br)			
300.1594	3.40	C18H21NO3	Codeine	58.0651	215.1067	243.1016
300.1594	3.40	C17^13CH21NO3	Codeine (^13C)			
177.1022	3.75	C10H12N2O	Cotinine	70.0651	80.0495	98.0600
177.1022	3.75	C9^13CH12N2O	Cotinine (^13C)			
285.0789	9.53	C16H13ClN2O	Diazepam	154.0418	228.0575	257.0840
285.0789	9.53	C16H13^37ClN2O	Diazepam (^37Cl)			
256.1696	6.63	C17H21NO	Diphenhydramin	152.0621	165.0699	167.0855
256.1696	6.63	C16^13CH21NO	Diphenhydramin (^13C)			
167.0855	6.63	C13H11^1+	Diphenhydr. Fragm 167	152.0621	165.0699	167.0855

Fig. 3: Example for the TargetAnalysis databases:
(a) database used for full scan data, **(b)** database used for bbCID data with additional definitions for M+1/M+2 traces and qualifier ions.

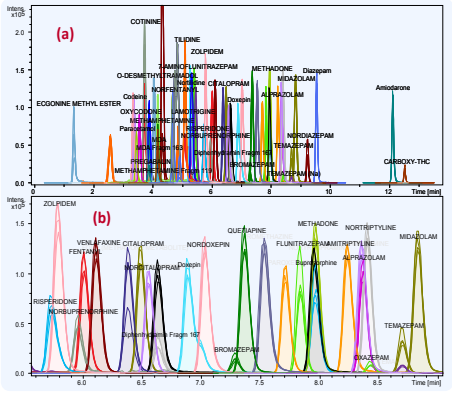

Fig. 4: Retention time stability in spiked matrix over time: Overlay of four chromatograms for a mix of all 61 compounds in solvent at start/end of complete sequence, in urine and in serum. (a) complete chromatogram, (b) expanded chromatogram range (5.5 - 9.0 min).

level [ng/ml]	Serum: full scan 500	100	50	10	Serum: bbCID 500	100	50	10
Mix 1	✓	✓	✓	✓	✓	✓	✓	✓
Mix 2	✓	✓	✓	✓	✓	✓	✓	✓
Mix 3	✓	✓	✓	✓	✓	✓	✓	✓
Mix 4	✓	✓	✓	✓	✓	✓	✓	✓
Mix 5	✓	✓	✓	✓	✓	✓	✓	✓
Mix 6	✓	✓	✓	✓	✓	✓	✓	✓
missed:					Mix 1: ZOPICLONE			

level [ng/ml]	Urine: full scan 500	100	50	10	Urine: bbCID 500	100	50	10
Mix 1	✓	✓	✓	✓	✓	✓	✓	✓
Mix 2	✓	✓	✓	✓	✓	✓	✓	✓
Mix 3	✓	✓	✓	✓	✓	✓	✓	✓
Mix 4	✓	✓	✓	✓	✓	✓	✓	✓
Mix 5	✓	✓	✓	✓	✓	✓	✓	✓
Mix 6	✓	✓	✓	✓	✓	✓	✓	✓
missed:					Mix 6: Paracetamol			

level [ng/ml]	Serum: full scan 500	100	50	10	Serum: bbCID 500	100	50	10
Mix 1	29	27	29	24	41	36	29	36
	12	12	12	11	22	22	21	18
	5	4	4	3	8	6	5	2
	12	11	13	10	11	8	3	16
Mix 2	29	25	25	23	32	27	33	31
	10	10	10	10	20	20	20	19
	1	1	1	1	2	2	2	2
	18	14	14	12	10	5	11	10
Mix 3	30	31	33	27	44	50	41	36
	15	15	16	13	26	28	26	21
	4	4	3	2	7	5	4	3
	11	12	14	12	11	17	11	12
Mix 4	28	30	20	28	30	31	29	28
	9	9	9	9	18	18	18	17
	2	2	2	2	4	4	4	3
	17	19	9	17	8	9	7	8
Mix 5	31	22	29	25	27	29	28	26
	9	9	9	9	18	18	18	17
	1	1	1	1	2	2	2	2
	21	12	19	15	7	9	8	7
Mix 6	27	29	26	30	38	39	36	36
	14	14	14	14	26	26	26	24
	2	1	1	1	2	2	2	2
	11	14	11	15	10	11	8	10
in sum:	657	274	50	333	813	507	79	227

Serum:	full scan				bbCID			
blank	10	0	1	9	9	0	1	8

level [ng/ml]	Urine: full scan 500	100	50	10	Urine: bbCID 500	100	50	10
Mix 1	40	43	35	46	52	44	50	43
	12	12	12	13	22	22	22	22
	6	2	1	1	4	2	2	2
	22	29	22	32	26	20	26	19
Mix 2	38	36	36	39	42	38	44	40
	10	10	10	10	20	20	20	19
	1	1	1	1	2	2	2	2
	27	25	25	28	20	16	22	19
Mix 3	36	32	34	34	44	46	49	39
	15	15	15	14	26	27	27	19
	3	3	2	2	5	4	4	2
	18	14	17	18	13	15	18	18
Mix 4	27	34	36	35	45	37	46	36
	9	9	9	9	18	18	19	17
	2	2	2	1	4	3	3	2
	16	23	25	25	23	16	24	17
Mix 5	35	32	32	38	41	39	42	43
	9	9	9	9	18	18	18	15
	1	1	1	1	2	2	2	2
	25	22	22	28	21	19	22	26
Mix 6	32	35	39	38	50	45	46	41
	14	14	14	14	26	26	26	23
	1	1	1	1	2	2	2	2
	17	20	24	23	22	17	18	16
	862	276	39	547	1042	508	61	473

Urine:	full scan				bbCID			
blank	24	0	1	23	28	0	1	27

Fig. 5: Detection statistics for spiked samples ✓: all compounds found, ✓: one or more compounds missed. **Black**: total # of findings, **blue:** # expected findings, **green:** # plausible positive findings, **red:** # remaining false positives (for bbCID analyses: **before applying the "diagnostic ion concept"**).

flip-book 1: see separate slides
Fig. 6/01

Fig. 6: Result examples for spiked samples and the application of the diagnostic ion concept.

Results

The **database built up for processing of the full scan data** contained a total of 73 entries. Compared to typical pesticides, where half of the compounds show significant intensities of fragments or adducts, an almost exclusive ionization as $[M+H]^+$ is observed. Diphenhydramin (fig. 2a), amphetamine and related compounds present significant fragmentation, and for temazepam and pregabalin some sodium adduct formation is observed. The higher compound stability is also reflected in the collision energy settings of the acquisition method for bbCID. With the settings optimized for pesticides only insufficient fragmentation could be achieved for the forensic compound set. Adequate fragmentation could be achieved by increasing the high energy setting from 25 eV to 30 eV.

The **extended database for bbCID data processing** contained 136 entries including the M+1 or M+2 isotope trace definitions. QIs could be assigned for most compounds, only three analytes (buprenorphine, norbuprenorphine, strychnine) did not show sufficient fragmentation even when using optimized collision energy settings. For 41 compounds three QIs were assigned, for 6 compounds only one reasonable QI could be assigned.

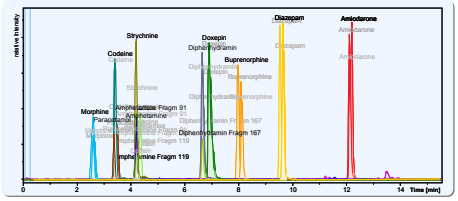

Fig. 1: Overlay of three chromatograms for the same compound mix acquired at all three sites

LC method suitability:
The LC conditions of the pesticide method sufficiently separates the selected set of forensic compounds. The compounds are evenly spread across the chromatogram, with good peak shapes also for early eluting compounds (ecgonine methyl ester, morphine) and compounds known for chromatographic issues (e.g. bromazepam, olanzapine). RT reproducibility between the three sites was better than 0.2 min (0.35 min for olanzapine; fig. 1 and 4). RT values were stable over the complete sequence and independent from matrix (see fig. 4) for spiked urine and serum samples

Screening results for spiked samples:
In the full scan analyses all compounds can be detected at all concentration levels. Accordung to the observed signal-to-noise ratios, many of them would be detectable at lower levels. No false negative is observed. Few additional compounds like caffeine in blank matrix or degradation compounds (e.g. cocaine detection in a mix that contained cocathylene) were detected. The numbers of total expected and plausible findings and false positives (FP) are listed in (fig. 5). For all full scan analyses a total of 333 FP were detected in serum. This is more than the number of 274 expected findings. The higher matrix load of the urine samples explaines the even higher number of 547 FP versus 276 expected. These FP typically arise due to the low detection threshold (750 cts) on traces with high noise levels (e.g. MDMA, norbuprenorphine) or from low intensity peaks within the RT detection window (± 0.5 min). In total, 35 different compounds appeared as FP (fig. 6/02). About one half of them was detected only few times (< 10x), whereas only seven compounds were causing ~75 % of all FP (MDMA, Norbuprenorphine, MDMA (13C), Paracetamol, Mirtazepine, Methamphetamine Fragm 91, Amphetamine Fragm 91).

In the bbCID analyses all compounds can be detected at least on one of their traces, but after applying the detection criteria, zopiclone and paracetamol were missed on 10 ng/µl level due to missing confirmatory finding. Thus, applying the "diagnostic ion concept" does not significantly compromise the detection of true positive compounds, but does have an impressive impact on the FP rate: the FP were completely removed, only tramadol can not be removed as finding if O-desmethyl venlafaxine is present in the sample (fig. 6/01).

Screening results for authentic samples:
The results for the authentic samples were in good agreement with findings from routine analysis. Applying the diagnostic ion concept again completely removes the false positive findings, with the only exception of tramadol in presence of O-desmethylvenlafaxine (fig.7).

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Fig. 7/01

Fig. 7: Result summary and examples for authentic case samples.

Conclusion

- A pesticide screening solution is successfully transferred to the field of forensic screening and is working reproducibly at 3 different sites.
- The presented workflow offers wide scope screening capabilities with high sensitivity.
- Application of the „diagnostic ion concept" is a powerful tool to remove false positive findings, thus allowing for a robust screening method with low detection threshold and wide retention time window to avoid false negative results.
- The method provides correct results in authentic forensic samples.

[1]Rapid Commun Mass Spectrom (2006) 20:1161-1167.
[2]Anal Bioanal Chem (2012) 403: 2891.