Transferring a Pesticide Screening Solution to the Application of Forensic Screening: Feasibility and Method Transferability Study Including the Application of Enhanced Confirmation Strategies

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Introduction

High-resolution time-of-flight mass spectrometry is known and in use for forensic and toxicological screening since 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 in pesticide screening in food[2] shall be tested on forensic screening samples.

Methods and Experiments

HPLC: Ultimate 3000 Rapid Separation LC ("**RSLC**", Thermo), **Column:** Acclaim RSLC C18 2.1x100 mm, 2.2 µm (Thermo), **Column temp.** 30°C, **Mobile phase:** A = H_2O, B = MeOH (both 5 mM NH_4 formate / 0.01% HCOOH), **Gradient:** multistep gradient 5 – 99.9% in 14 min, **Flow rate:** flow gradient 0.2-0.48 mL/min, **Injection:** 1 µL, **MS:** **impact** (UHR-TOF MS, Bruker Daltonik GmbH). **Ionization:** ESI(+). **Scan range:** *m/z* 30-1000. **Scan mode:** Full scan, bbCID, **Calibration:** external + internal.

All three sites in Helsinki, Freiburg and Bremen were set up with identical hardware configuration and given pesticide screening methods. The following 61 compounds were used in this study 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, retention time, fragmentation energy):

6-Monoacetylmorphine (MAM), 7-Aminoflunitrazepam, 9-Hydroxyrisperidone (Paliperidone), Alprazolam, Amiodarone, , Amisulpride, Amitriptyline, Amphetamine, Benzoylecgonine, Bromazepam, Buprenorphine, Citalopram, Cocaethylen, Cocaine, Codeine, Caffeine, Cotinine, Diazepam, Dihydrocodeine, Diphenhydramin, Doxepin, Ecgonine methyl ester, EDDP, Fentanyl, Flunitrazepam, Lamotrigine, MDA, MDMA (Ecstasy), Methadone, Methamphetamine, Midazolam, Mirtazapine, Morphine, Norbuprenorphine, Norcitalopram, Nordazepam, Nordoxepin, Norfentanyl, Nortilidine, Nortrimipramine, Nortriptyline, O-Desmethyltramadol, O-Desmethylvenlafaxine, Olanzapine, Oxazepam, Oxycodone, Paracetamol, Paroxetine, Pregabalin, Promethazine, Quetiapine, Risperidone, Strychnine, Temazepam, THC-COOH, Tilidine, Tramadol, Trimipramine, Venlafaxine, Zolpidem, Zopiclone

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 (regular and fast switching between high and low collision energy settings resulting in simultaneous fragmentation of all ions present) to build up a screening database containing name, sum formulae and RT. All full scan compound spectra were carefully evaluated for presence of additional ions besides the pseudo-molecular ion. Alternative ions (fragments, adducts) with a relative intensity >10% were defined in the screening database as well. This database was used for for processing of the analyses in full scan mode, whereas a second, extended one was used for the analyses in bbCID mode. Here, additional entries for an M+1 or M+2 isotope of each compound were included, and fragment ions observable in the bbCID data were collected as qualifier ions (QI) for result confirmation (see **fig. 1 – 3** for examples).

Urine and serum samples (after ACN precipitation) were spiked with the compound mixes at four levels (10, 50, 100, 500 ng/ml) and **analyzed on the system in Bremen** 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 on all relevant traces in a 10 ng/ml sample in solvent. The total numbers of findings in all samples were collected and compared to the number of expected findings, thus counting also the events of false positives. **For the runs aqcuired in bbCID mode, additional detection criteria were applied, finally accepting compounds as detected only, when together with the main compound ion at least one diagnostic ion with RT difference <0.05min was detected on full scan or bbCID data level**.

The same processing method and detection criteria were applied on data for **authentic samples from routine screening cases**, which were **run in bbCID mode on the systems in Helsinki** (11 urine samples; autopsy cases) **and Freiburg** (8 urine or serum samples; post mortem & roadside testing cases). Results were compared to findings from established routine screening methods (UPLC-QTOF or GC-MS, LC-MS/MS, Toxtyper).

Results

Database characteristics

The database that was built up for processing of the full scan data contained 73 entries for the 61 compound set. Compared to typical pesticide databases, this is a relatively short one (for pesticides typically the databases contain a number of entries that is about twice the number of compounds). Whereas for a set of pesticides typically about every second compound gives a full scan spectrum with significant intensities of fragments or ammonium/sodium adducts, an almost exclusive ionization as $[M+H]^+$ is observed here. 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. From the bbCID spectra QIs could be assigned for most compounds. Only three compounds (buprenorphine, norbuprenorphine, strychnine) did not show sufficient fragmentation even when using the optimized collision energy settings. For 41 compounds three QIs were assigned, for 6 compounds only one reasonable QI could be assigned.

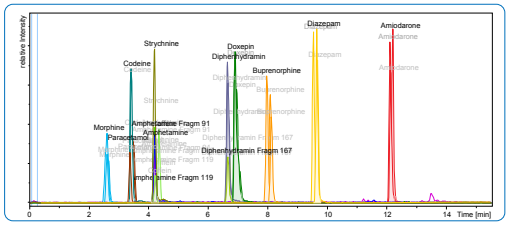

Fig. 1: Overlay of three chromatograms for the same compound mix, acquired at all **three sites: very good system-to-system reproducibility of retention times**.

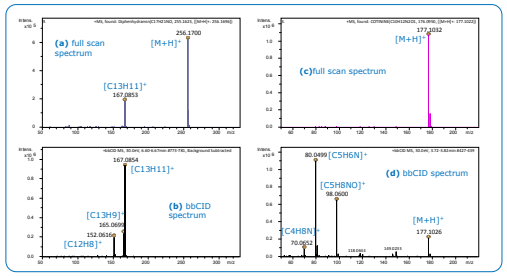

Fig. 2: Full scan and bbCID example spectra for diphenhydramine **(a, b)** and cotinine **(c, d)**.

(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 parts from 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.

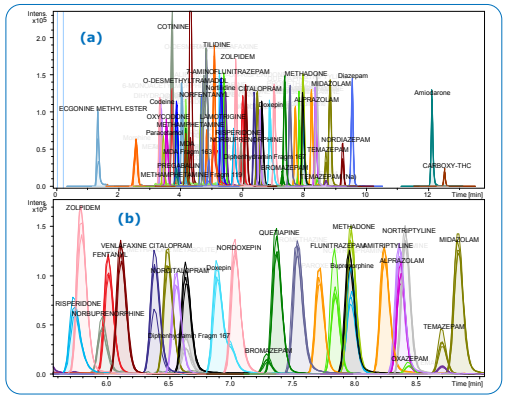

Fig. 4: **Retention time stability over time and in spiked matrix**: **Overlay of four chromatograms** for a mix of all 61 compounds in **solvent at start/end** of complete sequence, in **urine** and in **serum**. Same color for a compound in each chromatogram, **(a)** complete chromatogram, **(b)**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

LC method suitability:

The pesticide LC method including column works well for 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; see **fig. 1** and **4**).

For the spiked urine and serum samples RT values were stable over the complete sequence and independent from matrix (see **fig. 4**).

Screening results for spiked samples:

In the **full scan analyses** all compounds can be detected at all concentration levels, no false negative is observed. **Many compounds would be detectable even at significantly lower levels.** Together with the expected compounds a few additional plausible compounds were detected like caffeine in blank matrix or degradation compounds (e.g. cocaine detection in a mix that contained cocathylene). For the numbers of total, expected and plausible findings see **fig. 5**, which also lists the number of false positives (FP). In sum for all (full scan) analyses 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 relative to expected RT). In total 35 different compounds appeared as FP (see **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).

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

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

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

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. Counted for each analysis: **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 powerpoint slides

level [ng/ml]	Serum: bbCID 500	100	50	10
Mix 1	0	0	0	0
Mix 2	1	1	1	1
Mix 3	0	0	0	0
Mix 4	0	0	0	0
Mix 5	0	0	0	0
Mix 6	0	0	0	0

level [ng/ml]	Urine: bbCID 500	100	50	10
Mix 1	0	0	0	0
Mix 2	1	1	1	1
Mix 3	0	0	0	0
Mix 4	0	0	0	0
Mix 5	0	0	0	0
Mix 6	0	0	0	0

FP statistics for spiked samples after the application of "diagnostic ion concept"

Fig. 6/01

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

In the **bbCID analyses** again 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 (though they would have been detected when using a lower threshold). 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 (see **fig. 6/01**).

Screening results for authentic samples:

The results for the authentic case 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**).

flip-book 2: see separate powerpoint slides

Result summary table for authentic case samples (including "diagnostic ion concept")

Fig. 7/01

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

[1] Rapid Commun Mass Spectrom (2006) 20:1161–1167.

[2] Anal Bioanal Chem (2012) 403: 2891.

Conclusions

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

UHR-TOF MS