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Fully Automated Sample Preparation for the Analysis of Acidic Pesticides and Drug Residues in Potable and Surface Water

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Content

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1. Introduction

Inorganic nutrients becoming pollutants by the concentration of abundance in waterbodies like nitrate have been in public discussion for years. In addition, organic **micropolutants** got relevant since quite a while. The majority of these substances can be found in rather small concentrations, yet constitute extensive threats to men and environment. Almost all substances are artificial or metabolites which, opposite to nitrate, do not appear naturally in the environment.

Todays developments in instrumental analytics offer almost unimagined possibilities to analyse a big number of substances at the same time and reach lowest limits of quantification (LOQ) and detection (LOD). Standardised and by accreditation assured procedures guarantee a maximum of precision and accuracy of the expected measured values. However, the sample preparation is still a labour-intensive, lengthy and instinctive feeling requiring process, but does hardly draw attention. This procedure can be enormously simplified by applying automation. Thus, maximum reproducibility is given and samples can be prepared around the clock, even at weekends and bank holiday.

In the experiment for this application note, a cocktail of agents from the groups of drug residues and antibacterials (Table 1) as well as pesticides (Table 2) was applied the way it is likely to occur in the environment in total or as single substances.

Table 1: Drug residues and antibacterials in the experiment

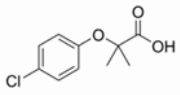
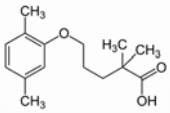
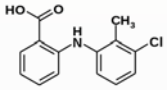
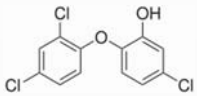
Analyte	Chemical Formula	Molar Mass [g/mol]	Structural Formula	CAS No.
Clofibric acid	$C_{10}H_{11}ClO_3$	214.7		882-09-7
Gemfibrozil	$C_{15}H_{22}O_3$	250.3		25812-30-0
Tolfenamic acid	$C_{14}H_{12}ClNO_2$	261.7		13710-19-5
Triclosan	$C_{12}H_7Cl_3O_2$	289.5		3380-34-5

Table 2: Pesticides used in the experiment

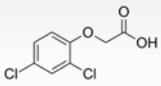
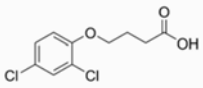
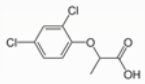
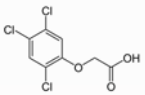
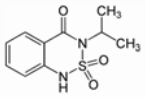
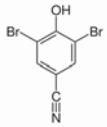
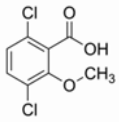
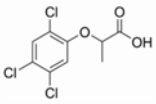
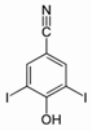
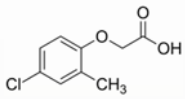
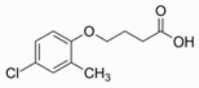
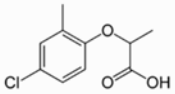
Analyte	Chemical Formula	Molar Mass [g/mol]	Structural Formula	CAS No.
2,4-D	$C_8H_6Cl_2O_3$	221,0		94-75-7
2,4-DB	$C_{10}H_{10}Cl_2O_3$	249,1		94-82-6
2,4-DP (Dichlorprop)	$C_9H_8Cl_2O_3$	235,1		120-36-5
2,4,5-T	$C_8H_5Cl_3O_3$	255,5		93-76-5
Bentazon	$C_{10}H_{12}N_2O_3S$	240,8		25057-89-0
Bromoxynil	$C_7H_3Br_2NO$	276,9		1689-84-5
Dicamba	$C_8H_6Cl_2O_3$	221,0		1918-00-9
Fenoprop	$C_9H_7Cl_3O_3$	269,5		93-72-1
Ioxynil	$C_7H_3I_2NO$	370,9		1689-83-4
MCPA	$C_9H_9ClO_3$	200,6		94-74-6
MCPB	$C_{11}H_{13}ClO_3$	228,7		94-81-5
MCPP (Mecoprop)	$C_{10}H_{11}ClO_3$	214,6		93-65-2

Figure 1: Lake Starnberg

The following application note shows how surface water samples can be prepared fully automated for LC-QTOF-MS analysis by applying SPE and nitrogen supported evaporation with the FREESTYLE XANA robotic system. The results are compared to those of traditionally manually processed samples. By the application of fully automated, sequential but parallel sample preparation, multiple samples can be processed at the same time. Thus, a high sample throughput at low demand of personnel resources is obvious.

2. Background and Regulations

Pesticides enter surface, ground and potable water by diffuse sources, yet mostly deriving from agriculture. The pathways of **pharmaceuticals** are different. They almost completely enter nature coming from municipal waste water and industrial plants. **Drug residues** in the environment can have considerable effects on flora and fauna as well as to human health. From feminisation of fish by **hormone-like substances** to mutation of agent resistant bacteria by continuous uptake of small amounts of **antibiotics**, there are plenty of scenarios.

Because of a huge number of products, agents, tradenames and fields of application, an internationally valid but detailed legal response is hardly feasible. Continuously accelerating advancements in agent development and the substance distribution via the global water cycle make quick and comprehensive response impossible. Nevertheless, subordinate organisations e.g. the American Environmental Protection Agency (US **EPA**) or the European Commission (EC) have been trying to establish minimum standards in their areas of competence.

The **European Water Framework Directive** (EWFD, 2000/60/EG) is listing a couple of substances, which they classify as **priority substances** or even **priority hazardous substances**. These substances need to be especially monitored and analysed. As a legal derivative, the German Surface Waters Ordinance (OGewV) fixes in analogy to the EWFD **environmental quality standards (EQS)** for further distinct substances e.g. Bentazon, Mecoprop and Triclosan.

On the other hand, the German Drinking Water Ordinance (TrinkwV) works with a bulk parameter for insecticides, herbicides, fungicides, etc. Though, it explicitly states that only substances which can be likely found in the watershed have to be analysed.

One example is the pesticide concept of Bavaria. The Bavarian Office for Health and Food Safety as well as the Bavarian Office for the Environment published a joint strategy to analyse pesticides and metabolites in surface and potable water. Depending on the field crops grown, the agents to be subsequently analysed are summarised. This simplifies the work for water providers, water management offices, and public health departments. Furthermore, it allows more transparency for the customer. Comparable approaches are tested in different other states, but depending on the various regional alterations a common way of proceeding is not available yet.

In contrast, the analytical methodology is clearly defined in several countries. Parts of European and non-European countries adapted the EN or at least ISO system whereas the American EPA-methods are well-reputed around the globe. Germany sticks to the German Standard Methods for water, wastewater, and sludge analysis (DEV), European and International (ISO) norms. The DIN EN ISO 11369:1997-11 (F12) describes pesticide analysis after solid liquid extraction whereas the DIN 38407-35:2010-10 (F35) refers to the substances in this application note.

3. Method Development

3.1 Sample Material and Preparation

The filtered water sample (surface or potable water) was equilibrated to pH 2.5 using concentrated formic acid. Thereafter, internal standards (c = 1.2 and 5 µg/mL) were added and the whole sample was homogenised on a magnetic stirrer. To compare the manual against the fully automated preparation, the samples were respectively split into two aliquots.

3.2 Chemicals, Standards, and Consumables

- Ultrapure water, Millipore water purification system
- Acetone, Promochem
- Methanol, Biosolve
- Acetonitrile, Biosolve
- Concentrated formic acid (purity ≥ 98 %), Biosolve
- Standards and isotope marked standards (purity > 95%): 2,4-D, 2,4-D-^{13}C6, 2,4-DB, 2,4-DB-D3, 2,4-DP (Dichlorprop), 2,4-DP(Dichlorprop)-D6, 2,4,5-T, Bentazon, Bentazon-D6, Bromoxynil, Bromoxynil-^{13}C6, Clofibric acid, Clofibric acid-D4, Dicamba, Dicamba-D3, Fenoprop, Gemfibrozil, Gemfibrozil-D6, Ioxynil, MCPA, MCPA-D6, MCPB, MCPB-D6, MCPP (Mecoprop), Mecoprop-D6, Tolfenamic acid, Tolfenamic acid-^{13}C6, Triclosan und Triclosan-D3; e.g. LGC Standards, Sigma-Aldrich, EQ Laboratories GmbH, or HPC Standard GmbH
- SPE cartridges OASIS® HLB, 200 mg, 6 mL, Waters
- Precolumn C18, 3 µm, 2.1 mm, di2chrom
- HPLC separation column Atlantis T3, 3 µm, 150 x 2.1 mm, Waters

3.3 Instrumentation

- 1290 Infinity UHPLC and 6450 Q-TOF-MS, Agilent Technologies with Genius 3010 nitrogen generator, Peak Scientific
- FREESTYLE XANA for water extraction including the modules for SPE and evaporation, LCTech GmbH (configuration details in chapter 3.6)
- AutoTrace SPE workstation, Caliper Life Science or Dionex
- Nitrogen evaporation bench, Barkey
- Magnetic stirrer, IKA®
- pH meter, inoLab

3.4 Solid Phase Extraction and Evaporation with Nitrogen – Fully Automated versus Manual Procedure

The predominantely manually done sample preparation steps were transferred to the fully automated robotic system FREESTYLE XANA. Single SPE steps, i.e. the drying of the SPE cartridges and the second elution had to be adjusted due to device specific technical differences. These deviations are printed in green letters in the following tables 3 and 4.

Two steps were even automated before the introduction of the FREESTYLE XANA: the conditioning and loading of the cartridges via the AutoTrace SPE workstation as well as the evaporation with preheated nitrogen using the evaporation device.

Table 3: Comparison of SPE steps: fully automated versus manual sample preparation

SPE steps	Fully automated	Manual
Conditioning	6 mL methanol 6 mL ultrapure water, pH = 2.5	6 mL methanol 6 mL ultrapure water, pH = 2.5
Loading	500 mL sample, 10 mL/min	500 mL sample, 10 mL/min
Washing	6 mL ultrapure water, pH = 2.5	6 mL ultrapure water, pH = 2.5
Drying	45 min, nitrogen	20 min, preheated nitrogen
1st Elution	4 mL methanol/acetone 3:2, 2 mL/min	7 mL methanol/acetone 3:2
Application time	5 min	5 min
2nd Elution	4 mL methanol/acetone 3:2, 1 mL/min	none
Drying (total removal of solvents)	10 mL nitrogen	some mL of nitrogen

Table 4: Comparison of evaporation parameters: fully automated versus manual sample preparation

Evaporation parameters	Fully automated	Manual
Temperature	Water heater 40 °C	Block temperature 100-110 °C
Blow down with nitrogen	Volume defined to 0.2 mL	Volume defined to 0.1 mL
Rinsing volume	0.2 mL (at the end of the evaporation process)	1-2 mL (manually during the evaporation process)
Backfill to final volume	0.5 mL	0.5 mL

After conditioning the SPE-cartridge in two steps (6 mL of methanol and 6 mL of water), the sample was loaded onto the cartridge and the original vial was rinsed with further 10 mL of water which also were loaded onto the cartridge. Subsequently, the cartridge was washed with water twice and dried for 45 minutes. By the end of the drying period, the sample was eluted twice applying 4 mL of a methanol/acetone mixture at a time. The eluate was directly washed into the EVAporation chamber and the cartridge was dried with 10 mL of nitrogen. In the EVAporation chamber the sample was gently evaporated to 0.2 mL at 40°C by applying nitrogen blow off. Finally, the sample volume was extended to 0.5 mL with a methanol/water mixture and tranferred into a GC vial.
To avoid cross-contamination, the EVAporation chamber was rinsed twice with 5 mL of a methanol/acetone mixture (3:2) as well as once with 5 mL of acetone.

The detailed SPE and evaporation method parameterization on the FREESTYLE XANA is shown in figure 2 and 3.

LCTech FreeStyle - Report on Methods: WAT Date: 05.01.2017 Time: 09:39:05

Name: PALCARZ_6mL.wat

Column:	LCTech_6ml.col		Extension cannula:	yes
Conditioning 1:	ON			
Volume:	6 ml	Dispensing Speed:	10 ml / min	
Suction Speed:	30 ml / min	Waiting time:	0 min	Port : W4 MeOH
Conditioning 2:	ON			
Volume:	6 ml	Dispensing Speed:	10 ml / min	
Suction Speed:	30 ml / min	Waiting time:	0.5 min	Port : W8 H2O 2.5
Conditioning 3:	OFF			
Load 1:	ON			
Volume:	Number of bottles: 1	Transfer Speed	10 ml / min	
rinsing cycle included				
Rinsing volume:	10 ml	Suction Speed:	30 ml / min	
Rinsing volume:	50 ml / min			Port : W8 H2O 2.5
Washing 1:	ON	stay on actual position		
Volume:	6 ml	Dispensing Speed:	10 ml / min	
Suction Speed:	10 ml / min	Waiting time:	0.5 min	Port : W8 H2O 2.5
Washing 2:	OFF			
Drying 1:	ON	stay on actual position		
Time:	45 min			
Washing 3:	OFF			
Elution 1:	ON			
Volume:	4 ml	Dispensing Speed:	2 ml / min	
Suction Speed:	10 ml / min	Waiting time:	5 min	Port : 11 MeOH/Aceton 3:2
Elution in EVA				
Drying 2:	OFF			
Washing 4:	OFF			
Drying 3:	OFF			
Elution 2:	ON			
Volume:	4 ml	Dispensing Speed:	1 ml / min	
Suction Speed:	10 ml / min	Waiting time:	0 min	Port : 11 MeOH/Aceton 3:2
Elution in EVA				
Drying 4:	ON			
defined by volume		Drying volume: 10 ml	Speed: 10 ml / min	
EVA :	ON			
Method:	Palcarz_6ml_2.evp			
Tube filling volume before elution		1 ml		

Figure 2: Parameterization of SPE method on FREESTYLE XANA

LCTech FreeStyle - Report on Methods: EVA Date: 05.01.2017 Time: 09:38:27

Name: Palcarz_6ml_2.evp

	Temperature water heating 40 °C		Temperature bottom cone 40 °C	
	Sample input: Online from GPC or SPE process			
	Batch volume = limit from where concentration starts: 5 ml (fix) + Waiting time: 0.2 min. Vacuum during GPC online sample input: 200 mbar			
	No Vacuum process			
	Nitrogen blow-down: yes	to defined level: 0.2 ml	+ Setup Offset 0 ml	Safetystop after: 90 min.
	Remove Aliquot: no			
	Solvent exchange: no			
	Rinsing, filling up, mixing and transfer into vials:			
	Rinsing volume at the end: 0.2 ml	Rinsing steps: 1 x	Solvent from Port: 10 MeOH/H2O 1:4	
	Fill up to volume: of Port: 10 MeOH/H2O 1:4	0.5 ml	Way of mixing: with gas / air, Volume = 1 ml	
	Concentrate: into vials			
	Nr.: 1 1 [each]	Type: Type1@1 ml	Volume per vial: 0.5 ml	
	Fill Quantitativ: no			
	1. Cleaning cycle Rinsing volume: 5 ml	Rinsing steps: 2 x	Solvent from Port: 11 MeOH/Aceton 3:2	
	2. Cleaning cycle Rinsing volume: 5 ml	Rinsing steps: 1 x	Solvent from Port: 1 Aceton	

Figure 3: Parameterization of evaporation method on FREESTYLE XANA

3.5 Analysis with HPLC-QTOF-MS

The pesticides and drug residues are separated by reversed phase HPLC. The identification and quantification takes place using a time-of-flight mass spectrometer (table 5). An example chromatogramm is shown in figure 8.

Table 5: Chromatographic and mass spectrometric set-up

HPLC	
Parameter	**Set-up**
Master device	1290 Infinity UHPLC, Agilent Technologies
HPLC column	Atlantis T3, 3µm, 150 x 2.1 mm, Waters
Precolumn	C18, 3 µm, 2.1 mm, di2chrom
Injection volume	10 µL (injection and needle rinsing)
Column oven temperature	40 °C
Flow rate	0.3 mL/min
Eluents	A2 formic acid in water (0.1 %), B2 formic acid in methanol (0.1 %)
Gradient	0 min: 10 % B2, 0-14 min: 10 % →100 % B2, 14-18 min 100 % B2
QTOF-MS	
Parameter	**Set-up**
Master device	6550 iFunnel QTOF-LC/MS, Agilent Technologies
Ionisation	ESI negative
Recording mode	MS
Measuring range	m/z 50 -1200
Gas temperature	175 °C
Gas flow	15 L/min
Nebulizer	45 psig
Sheath gas temperature	350 °C
Sheath gas flow	12 L/min
Capillary	3000 V
Nozzle voltage	500 V
Fragmentor	360 V
Scan rate	5 spectra/sec
Reference masses	m/z 112.9856, 966.0007
Collision energy	0 V (Segment 1), 20 V (Segment 2), 40 V (Segment 3)

3.6 Configuration FREESTYLE XANA

1.	FREESTYLE BASIC	P/N 12663-12
2.	FREESTYLE XANA	P/N 14692
3.	FREESTYLE SPE	P/N 12668
4.	FREESTYLE EVA	P/N 13841
5.	Recirculating cooler	P/N 12060
6.	Clamping adapter for 6 mL SPE cartridges	P/N 14893
7.	Caps for 6 mL SPE cartridges	P/N 14923
8.	Reusable needle	P/N 13382
9.	Frame	P/N 11915
10.	Special rack for up to 12 SPE cartridges	P/N 14047
11.	Tray for 60 pcs	P/N 11920

4. Results

4.1 Reproducibility

To determine the reproducibility of the FREESTYLE XANA, the recovery rate was measured using tap water at different days (generating different sample sequences). The recovery rate was determined by an external calibration with both, internal and isotope marked standards. No isotope marked standard was available for Ioxynil, 2,4,5-T and Fenoprop. The standard deviations (s) and the variation coefficients (vc) were usually ≤ 5% (table 6). They were comparable to the ones deriving from the manual handling process, partially they were even significantly better (table 7).

Figure 4: Working station on the FREESTYLE platform

Table 6: Recovery rates of analytes in potable water using fully automated sample preparation

Analyte	Level [µg/l]	Recovery rate in potable water [%]						s	vc [%]
		1	2	3	4	5	Ø		
Bentazon	0,01	79	77	79	81	84	80	2,7	3,3
Bromoxynil	0,1	102	99	97	98	104	100	2,9	2,9
Ioxynil*	0,01	105	105	93	92	95	98	6,5	6,6
2,4-D	0,1	112	104	106	106	110	108	3,3	3,1
MCPA	0,1	114	105	103	104	115	108	5,8	5,4
Clofibric acid	0,1	119	108	114	114	121	115	5,1	4,4
MCPP	0,05	117	111	115	110	117	114	3,3	2,9
2,4-DP	0,1	114	101	105	106	111	107	5,1	4,8
2,4-DB	0,1	109	102	101	110	117	108	6,5	6,1
MCPB	0,1	95	85	86	83	85	87	4,7	5,4
2,4,5-T*	0,1	196	173	167	163	178	175	12,9	7,3
Fenoprop*	0,1	199	173	172	165	183	178	13,2	7,3
Gemfibrozil	0,1	134	126	127	132	122	128	4,8	3,8
Triclosan	0,1	112	107	113	103	107	108	4,1	3,8
Tolfenamic acid	0,1	107	100	101	98	107	103	4,2	4,1

vc = variation coeffizient, s = standard deviation, *no evaluation by isotope marked standard possible

Table 7: Recovery rates of analytes in potable water performing manual sample preparation

Analyte	Level [µg/l]	Recovery rate in potable water [%]						s	vc [%]
		1	2	3	4	5	Ø		
Bentazon	0,01	104	104	87	92	79	93	10,9	11,7
Bromoxynil	0,1	100	110	100	108	96	103	5,9	5,8
Ioxynil*	0,01	129	112	129	113	91	115	15,7	13,6
2,4-D	0,1	102	106	99	108	94	102	5,6	5,5
MCPA	0,1	95	100	100	112	94	100	7,2	7,1
Clofibric acid	0,1	101	100	100	109	92	100	6,0	6,0
MCPP	0,05	106	92	109	115	99	104	8,9	8,6
2,4-DP	0,1	107	117	98	110	103	107	7,2	6,7
2,4-DB	0,1	113	106	100	111	101	106	5,8	5,5
MCPB	0,1	95	105	91	101	96	98	5,5	5,6
2,4,5-T*	0,1	137	224	172	187	142	172	35,5	20,6
Fenoprop*	0,1	172	171	158	172	137	162	15,2	9,4
Gemfibrozil	0,1	120	130	127	126	118	124	5,0	4,0
Triclosan	0,1	110	123	106	95	100	107	10,7	10,0
Tolfenamic acid	0,1	118	118	105	111	100	110	8,0	7,2

vc = variation coeffizient, s = standard deviation, *no evaluation by isotope marked standard possible

4.2 Accuracy of the Mean

Figure 5 shows the average, absolute peak areas (n=3) of the internal standards of endowed surface water samples in comparison of manual and fully automated sample preparation. The peak areas of both procedures are in a comparable range.

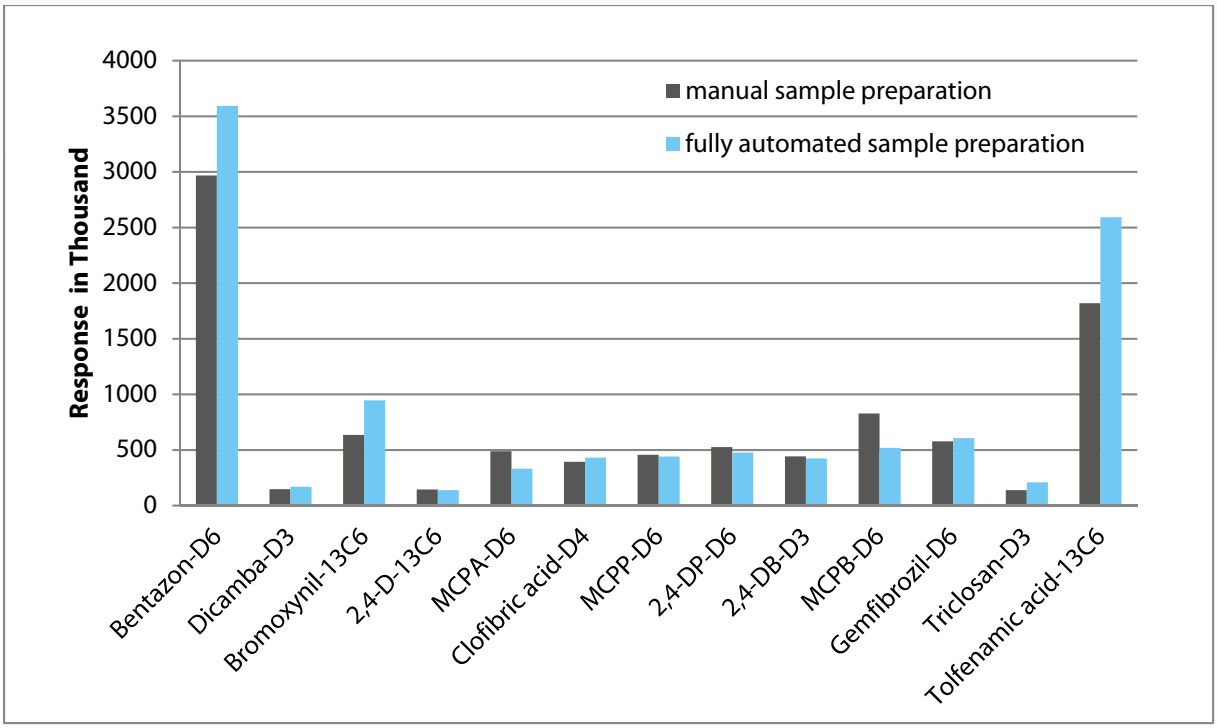


Figure 5: Absolute peak areas of internal standards of endowed surface water samples manual sample preparation and fully automated sample preparation

Figure 6 shows two EI-chromatograms in an overlay mode for each of the 16 analytes of an endowed tap water sample, being processed both ways manually and fully automated. Also here, no significant differences regarding absolute peak areas are observable. Thus, the yield from fully automated sample preparation is partly even significantly better.

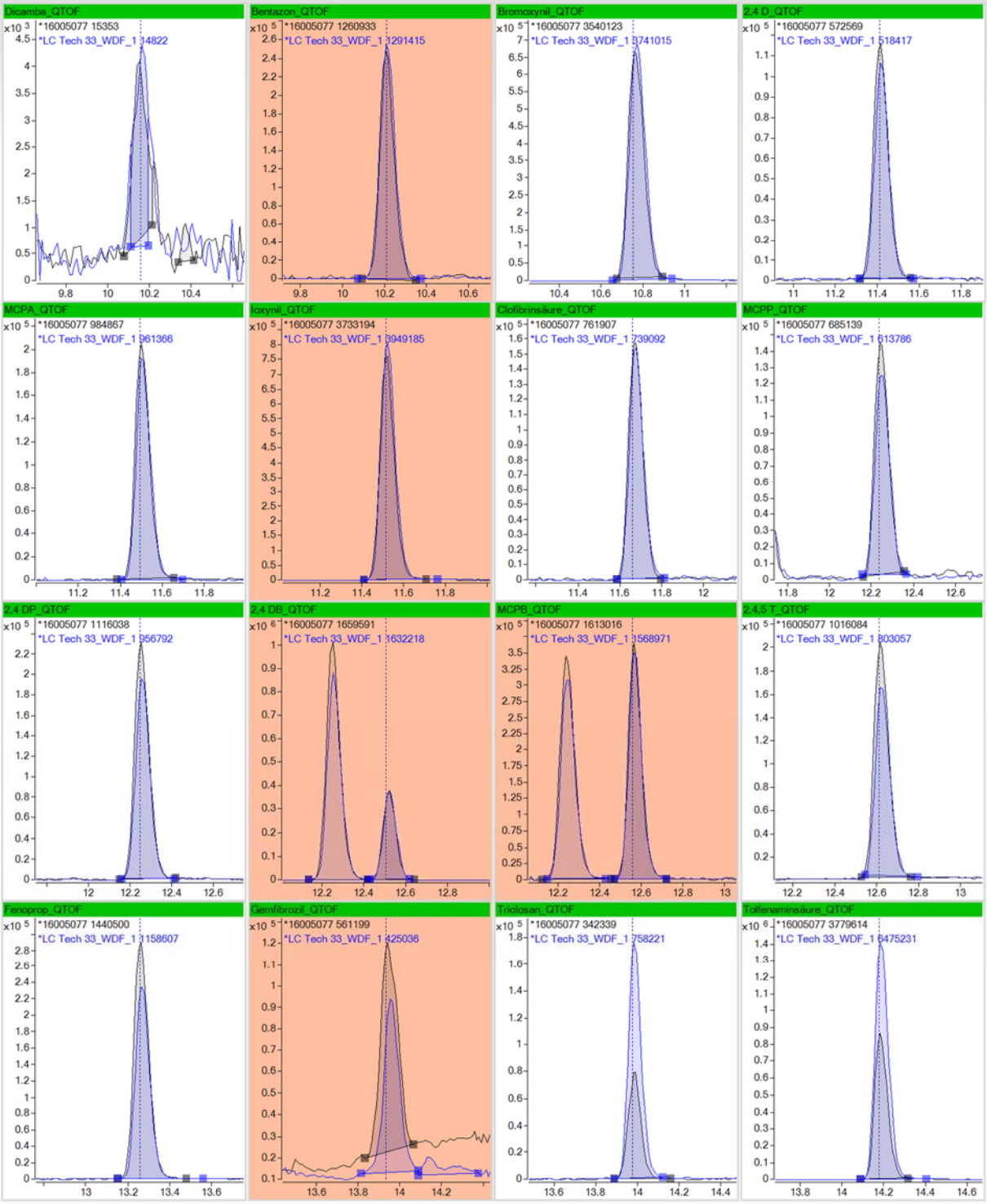


Figure 6: Absolute peak areas of analytes in an endowed tap water sample, prepared manually (grey) and fully automated by FREESTYLE XANA (blue).

4.3 No Cross-Contamination in the Evaporation Chamber

Applying fully automated sample preparation with FREESTYLE XANA, the eluates from SPE are concentrated to a defined volume in the evaporation chamber by using nitrogen. Thereafter, the extracts are directly transferred to a LC vial and can be processed further. After each sample, the evaporation chamber is rinsed. To avoid carryover and prove the rinsing process, blanks were handled directly after higher endowed water samples (c= 2/1/0.5/0.1 µg/L, depending on the sensitivity of the analyte). Noteworthy carryover (>1%) was neither detectable in analytes nor in standards.

4.4 Fully Automated versus Manual Sample Preparation

- FREESTYLE XANA allows to process 24 samples at the time and overnight. Manually max. 6 samples can be handled at the same time.
- With FREESTYLE XANA all preparation steps from conditioning and loading of the SPE cartridge up to the aliquotation of the extracts into the LC-vials were automated. Compared to the earlier process, manual intermediate steps are no longer needed.
- Using FREESTYLE XANA, all sample flasks are rinsed after loading the SPE cartridges. Thus, loss caused by adsorbed materials at glassware could be minimized.
- Using FREESTYLE XANA, the volume of concentration is limited to 0.2 mL whereas manual preparation allows a final volume of 0.1 mL.
- The FREESTYLE XANA software is characterized by simple and intuitive operability.

5. Summary

FREESTYLE XANA allows to process 24 samples at the time at highest reproducibility and overnight. By complete automation personnel resources can be economised and are available for other tasks.

The recovery rates and yield of analytes and internal standards are, matched by measured peak areas, comparable to manual sample preparation.

In the evaporation chamber no cross-contamination could be found.

Figure 7: Automated sample preparation system FREESTYLE XANA

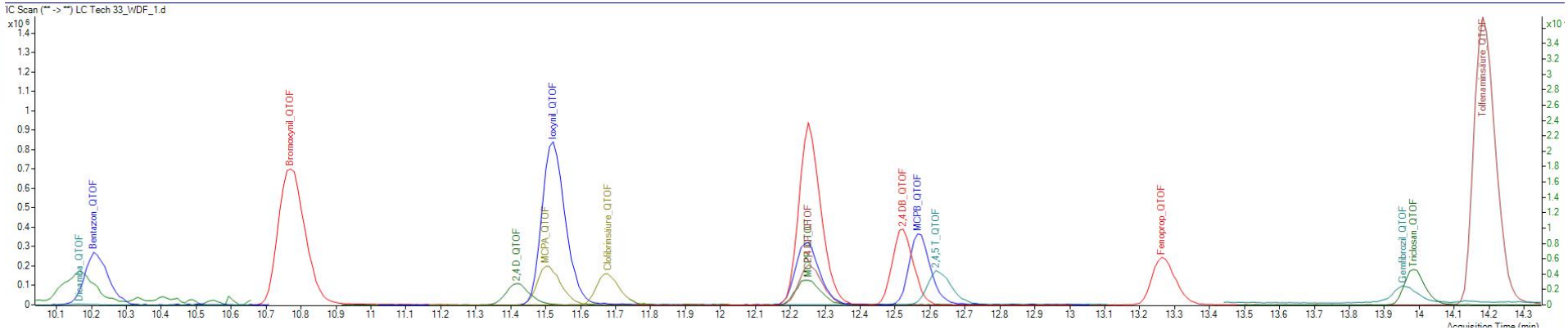


Figure 8: EI-Chromatogramm of an endowed, automatically prepared tap water sample, c = 0.01/0.05/0.1/0.2 µg/l (depending on the sensitivity of the analyte)

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