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Comprehensive Automation of the SPE-GC/MS Analysis of Opioids, Cocaine and Metabolites from Serum and Other Matrices

Oliver Lerch
Gerstel GmbH & Co. KG, Eberhard-Gerstel-Platz 1, D-45473 Mülheim an der Ruhr, Germany

Oliver Temme, Thomas Daldrup
University Hospital Düsseldorf, Institute of Legal Medicine, Department of Forensic Toxicology, Moorenstrasse 5, D-40225 Düsseldorf, Germany

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Abstract
Analyzing blood serum for opioids, cocaine and metabolites is a routine task in forensic laboratories. The most commonly used methods involve several manual or partly-automated sample preparation steps such as protein precipitation, solid phase extraction, evaporation and derivatization followed by GC/MS or LC/MS determination.

In this study a comprehensively automated method is compared with a validated, partly-automated routine method. Following manual protein precipitation, the automated method relies on a MultiPurpose Sampler (MPS) to perform all remaining sample preparation steps. These include solid phase extraction (SPE), evaporation of the eluate, derivatization and introduction to the GC/MS. Quantitative analysis of close to 170 serum samples, as well as more than 50 samples of other matrices like urine, different tissues and heart blood, was performed using both methods. Cocaine, benzoylecgonine, methadone, morphine, codeine, 6-monoacetylmorphine, dihydrocodeine and

7-aminoflunitrazepam were determined quantitatively and the methods were found to produce equivalent analytical results even near the limits of quantification [1].

Introduction

Toxicological chromatographic analysis of biological fluids or tissues usually requires sample preparation for cleanup and enrichment. Recently in conjunction with very sensitive and selective mass spectrometers protein precipitation alone [2] or "dilute and shoot" methods [3,4] were also used though these methods may suffer from sample dependent matrix effects that can compromise the accuracy of the results. Although the number of LC-MS/MS methods is rapidly increasing [5,6,7,8] GC-MS/(MS) is still the standard routine analysis technique in many forensic laboratories [5,9,10,11].

Solid Phase Extraction (SPE) is the most widely used extraction technique for toxicological analysis of biological fluids and tissues [10,11,12]. Typically polypropylene cartridges with a fixed sorbent bed (typically mixed mode cation exchange cartridges) are used.

A range of methods for the analysis of opioids, cocaine and metabolites in different matrices was published. The compounds were analyzed in matrices like urine [10,13,14], whole blood, serum, plasma [6,8,10], saliva [7], hair [9] or post-mortem samples [15].

In this study a validated, partly-automated SPE-GC/MS analysis method for opioids, cocaine and metabolites was completely automated. Analysis results of both methods were compared. Automation was performed by different modules attached to a GERSTEL MultiPurpose Sampler MPS. This allowed mimicking the manual workflow of sample dilution, SPE, evaporation, derivatization and sample injection.

Experimental

Instrumentation. The system employed for automation of the sample preparation is based on a MultiPurpose Sampler (MPS, GERSTEL). It was configured with two syringes, a 2.5 mL syringe with gas supply for the sample preparation steps and a 10 µL syringe used for sample injection into a Cooled Injection System CIS 4 (GERSTEL) coupled to a 7890 GC/5975 MSD (Agilent Technologies). The MPS was equipped with modules for solid phase extraction (SPE), for evaporation of solvents under controlled vacuum and temperature (MultiPosition eVAPoration station, mVAP), for shaking under controlled temperature conditions (Agitator) and for supplying large volumes of solvents (Solvent Filling Station 2, SFS 2, all GERSTEL). The complete system is shown in figure 1.

Figure 1. Setup used for the automated analysis of blood serum for THC and metabolites. Dual Head MultiPurpose Sampler (MPS) equipped with solvent bottle holder, agitator, standard wash station, trays for eluate vials, SPE cartridges and samples, SPE module, two solvent filling stations (SFS), MultiPosition eVAPoration station (mVAP) and solvent bottle holder (from left to right).

Measurements with the partly-automated, validated analysis method were conducted on a 7890 GC/5975 MSD (Agilent Technologies). A 7683B autosampler was used for injection into a hot Split/Splitless inlet (both Agilent Technologies). A bench-top instrument was used for automation of the solid phase extraction. All other sample preparation steps were done manually.

Materials. All analytes and deuterated analogues were certified standards purchased from Lipomed AG or LGC Promochem GmbH. All solvents and salts were of analytical grade and purchased from VWR. N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) for silylation was purchased from MachereyNagel or Sigma-Aldrich. Bond Elut Certify 130 mg, 3 mL format SPE cartridges from Agilent Technologies were used. For automated SPE these cartridges were cut at the top, equipped with a transport adapter and a disposable syringe needle (canula) (figure 2).

Blood, urine and tissue samples were taken from authentic forensic cases of the Institute of Legal Medicine in Düsseldorf.

Figure 2. Top: Solid phase extraction cartridge configured for automated GERSTEL SPE.
Bottom: Standard solid phase extraction cartridge.

Preparation of standards and solutions. For calibration multi-compound calibration solutions and one multi-compound internal standard solution containing deuterated analogues of every analyte were prepared in methanol. The calibration ranged from 25 to 1500 ng/mL (methadone), from 50 to 1500 ng/mL (benzoylecgonine), from 5 to 150 ng/mL (codeine), from 5 to 300 ng/mL (cocaine, dihydrocodeine, morphine), and from 2.5 to 150 ng/mL (7-aminoflunitrazepam, 6-monoacetylmorphine) respectively and was calculated for 0.6 mL serum sample (nine levels). Each 20 µL of the internal standard solution was added to samples, calibration samples or quality control samples.

Manual sample pretreatment. All liquids (urine, blood, serum) were handled in the same way:

1. Protein precipitation by drop-wise addition of a mixture of 0.6 mL sample, 0.1 mL water and 20 µL internal standard solution to a mixture of 1 mL acetonitrile and 0.1 mL isopropanol.
2. Mixing and centrifugation.
3. Transfer of an aliquot (0.75 mL) of the supernatant to individual vials (093640-046-00 with cap 093640-075-00) for both analysis methods.

Tissues (brain and kidney, native and lyophilized) were homogenized with an Ultra Turrax. An aliquot of approximately 0.6 g was weighed and handled like the liquid samples as described above, except the acetonitrile/isopropanol solution was added to the sample/standard mixture.

These protein precipitation steps could be automated by employing a centrifuge combined with the MPS, but this was not within the scope of this study.

Automated sample preparation

The following list describes the completely automated method. Differences to the partly-automated method are given in [brackets].

1. Condition the SPE cartridge with 2 mL methanol and 2 mL phosphate buffer (pH 7.9).
2. Dilute the supernatant of the protein precipitation in the SPE syringe [in a vial] and add the diluted sample to the SPE cartridge.
3. Wash the cartridge with 2 mL water, 2 mL acetic acid, and 2 mL methanol.
4. Dry cartridge briefly using a flow of nitrogen.
5. Elute with 1.9 mL [2 mL] of dichloromethane/isopropanol/ammonia. The first 0.6 mL are discarded and the following 1.3 mL are collected [2 mL are collected] in a vial (093640-046-00, with cap 093640-102-00).
6. Evaporate the eluate to dryness at 70°C, 8 kPa and 300 rpm in the mVAP station [manually at 60°C under nitrogen].
7. Reconstitute in 200 µL isooctane/pyridine/MSTFA 14/5/1 v/v/v [isooctane/MSTFA 19/1 v/v].
8. Shake for 5 min at 90°C [30 min at 90°C] for derivatization.
9. Inject 2 µL into the CIS [2 µL into the split/splitless injector].

Calibration solutions were treated analogous to the eluates.

Analysis Conditions. In the following, parameters for the completely automated method are listed. Whenever these differ from the parameters used in the partly-automated method, the original parameters are listed in [brackets].

MPS: 2 µL injection volume
CIS: 50°C; 12°C/s; 280°C (5 min) [270°C isothermal]
Inlet Liner: Quartz wool deactivated [glass wool]
Injection Mode: Splitless, 3 min [2 min]
Pneumatics: He, constant flow, 1 mL/min
Oven: 140°C (1 min); 120°C/min; 225°C (5.29 min) 120°C/min; 275°C (5.2 min)
Post Run: 300°C (2.5 min)
Column: Rxi-5Sil MS, Restek [HP-5ms, Agilent Technologies] 30 m, d_i = 0.25 mm, d_f = 0.25 µm
MSD Mode: Selected ion monitoring (SIM)
SIM Masses: see Table 1

Table 1. Quantifier and qualifier ions for analytes and internal standards.

Compound	Quantifier [m/z]	Qualifier [m/z]
Cocaine	182	303, 198
Cocaine-d_3	185	306, 201
Benzoylecgonine	361	256, 346
Benzoylecgonine-d_3	364	259, 349
Methadone	223	294, 236
Methadone-d_9	226[a], 303[b]	303[a], 318[b], 242
Morphine	429	220, 401
Morphine-d_3	432	223, 404
Codeine	371	234, 343
Codeine-d_3	374	237, 346
6-Monoacetylmorphine	399	340, 400
6-Monoacetylmorphine-d_3	402	343, 403
Dihydrocodeine	373	315, 358
Dihydrocodeine-d_6	379	318, 364
7-Aminoflunitrazepam	326[b], 355[a]	326[a], 356[a], 327[b], 354[b]
7-Aminoflunitrazepam-d_7	362	333, 363

[a] Qualifier ion used in partly-automated analysis method.
[b] Qualifier ion used in fully automated analysis method.

According to guidelines of the Society of Toxicological and Forensic Chemistry (GTFCh, Germany) a blank injection of pure derivatization solution was done after every sample, quality control or calibration sample.

Results and Discussion

The validated, partly-automated routine analysis method (table 2) could be successfully automated using the MPS starting with the dilution of the sample after protein precipitation and ending with the injection into the GC/MS. Some modifications were necessary to establish the automated method:

Table 2. Limit of detection (LOD), limit of quantification (LOQ) and upper limit of calibration (ULOC) for each compound using the validated, partly-automated reference method.

Analyte	LOD [ng/mL]	LOQ [ng/mL]	ULOC [ng/mL]
Cocaine	1.1	3.5	300
Benzoylecgonine	9	47	1500
Methadone	4.2	16.7	1500
Morphine	1.2	4.9	300
Codeine	0.4	2.6	150
6-Monoacetylmorphine	0.3	0.8	150
Dihydrocodeine	0.8	4.2	300
7-Aminoflunitrazepam	0.6	2.5	150

The dilution of the supernatant after protein precipitation was partly done in the autosampler syringe. Therefore a 0.75 mL aliquot of the supernatant was diluted with 0.75 mL phosphate buffer and 0.75 mL of this mixture was aspirated. After that another 1.75 mL phosphate buffer was aspirated resulting in the final dilution (same as in the reference method). This solution was added to the SPE cartridge and the process was repeated once to transfer the entire sample.

The elution volume was reduced from 2 mL to 1.9 mL. The first 0.6 mL were discarded and the last 1.3 mL were collected based on the established elution profile (figure 3).

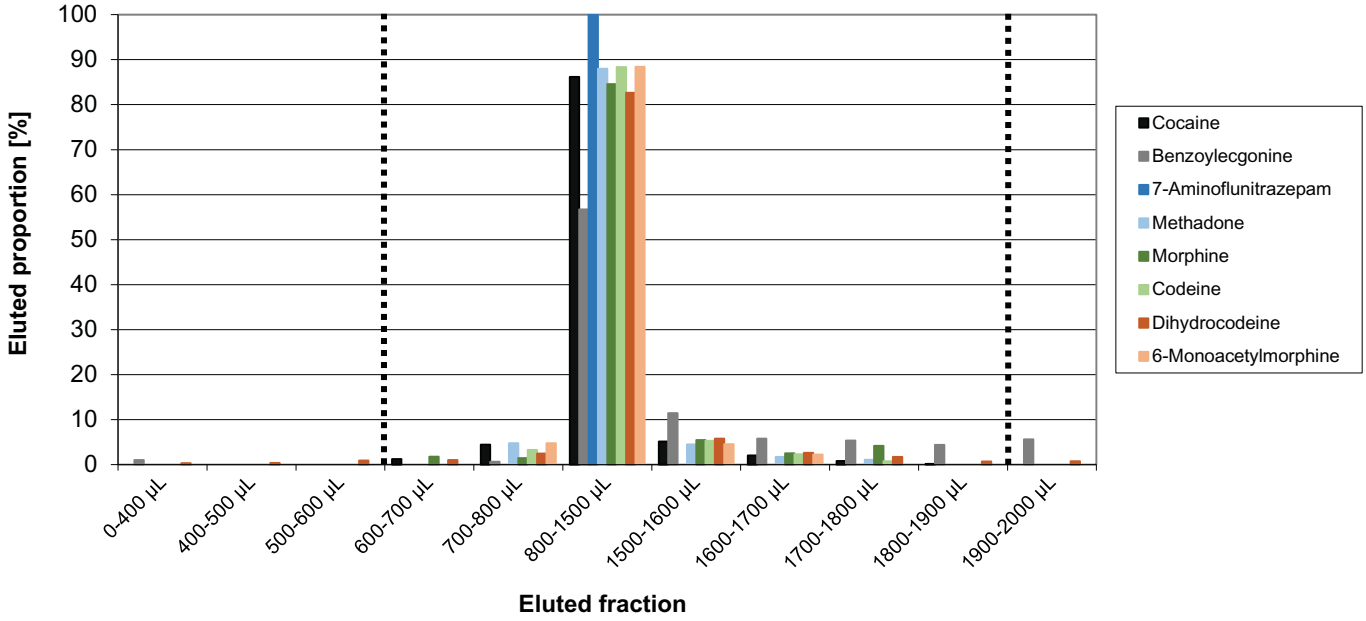

Figure 3. Profile of analyte elution from Bond Elut Certify SPE cartridge. Eventually collected fractions between dashed lines.

The derivatization time could be shortened from 30 min to 5 min with shaking at 90°C (see figure 4) by employing a mixture of isooctane/pyridine/MSTFA 14/5/1 (v/v/v) instead of isooctane/MSTFA 19/1 (v/v) which was used originally.

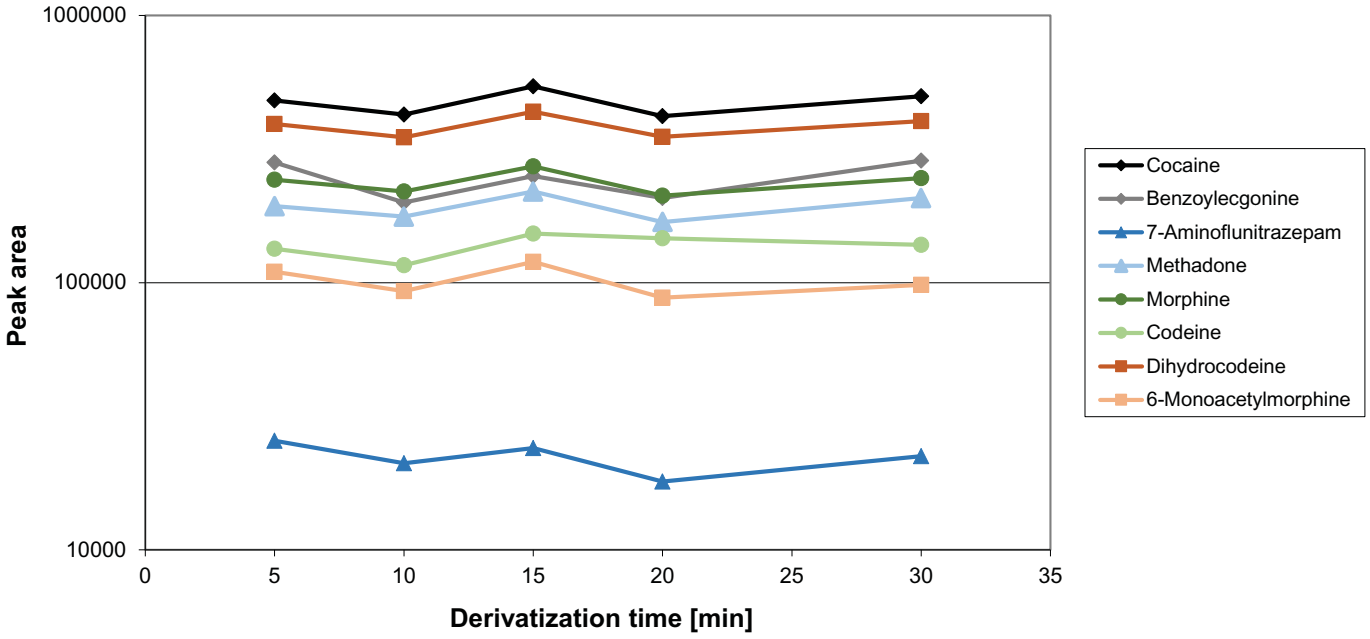

Figure 4. Optimization of derivatization time (with shaking) at 90°C with a mixture of isooctane/pyridine/MSTFA 14/5/1 v/v/v. 5 min with shaking is sufficient for complete derivatization.

Almost 170 serum samples and more than 50 samples of other matrices like urine, different tissues and heart blood were analyzed by both methods. Results are equivalent as can be seen in the double logarithmic line- and Bland-Altman-plots (figures 6 and 7). This is true for serum samples and also for alternative matrix samples. Although results between the limit of quantification and the limit of detection may not be reported routinely, they are included in the line plots (dashed red lines in figures 6). Even in this concentration range the method equivalence is obvious. Since only a couple of samples were positive for dihydrocodeine and 7-aminoflunitrazepam these results are not plotted. Samples and quality control samples were also in good concordance for these compounds.

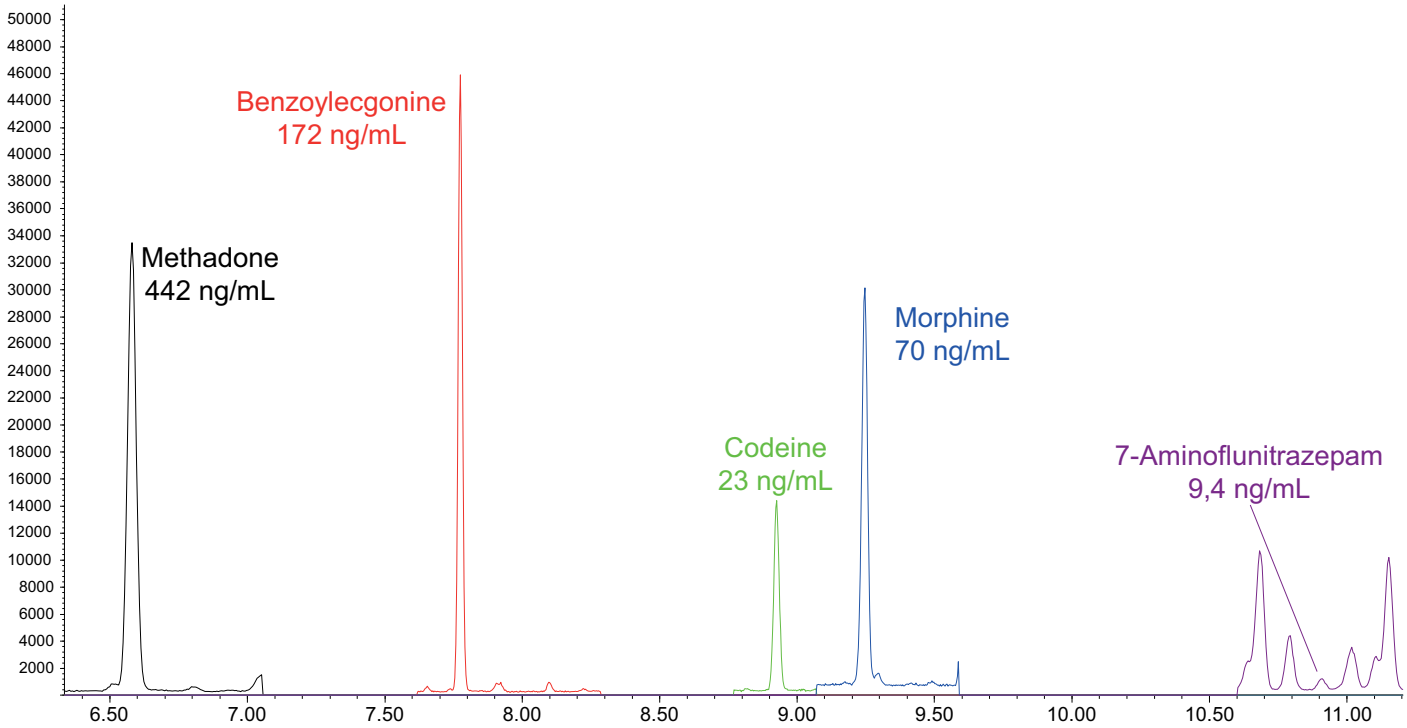

Figure 5. Extracted-ion chromatogram resulting from a real serum sample. Quantified compounds are named.

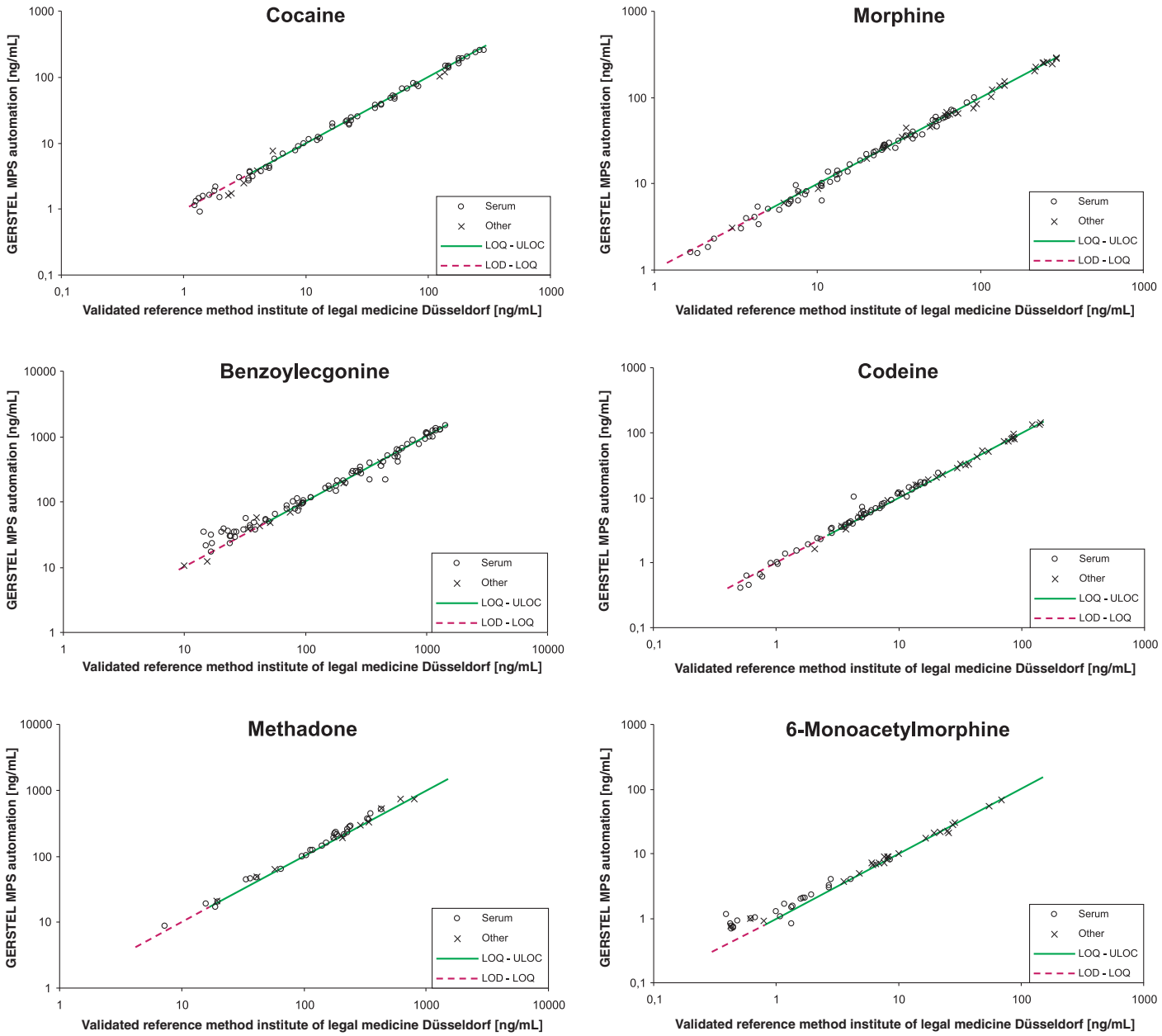

Figure 6. Correlation of determined analyte concentrations in double logarithmic scale. Line with a slope of one – representing complete equivalence of results – is shown.
ng/mL: Nanogram per milliliter or nanogram per gram for tissue respectively; Other: Other matrices than serum - urine, blood, lyophilized kidney tissue, heart blood, lyophilized and native brain tissue; LOD: Limit of detection; LOQ: Limit of quantification; ULOC: Upper limit of calibration.

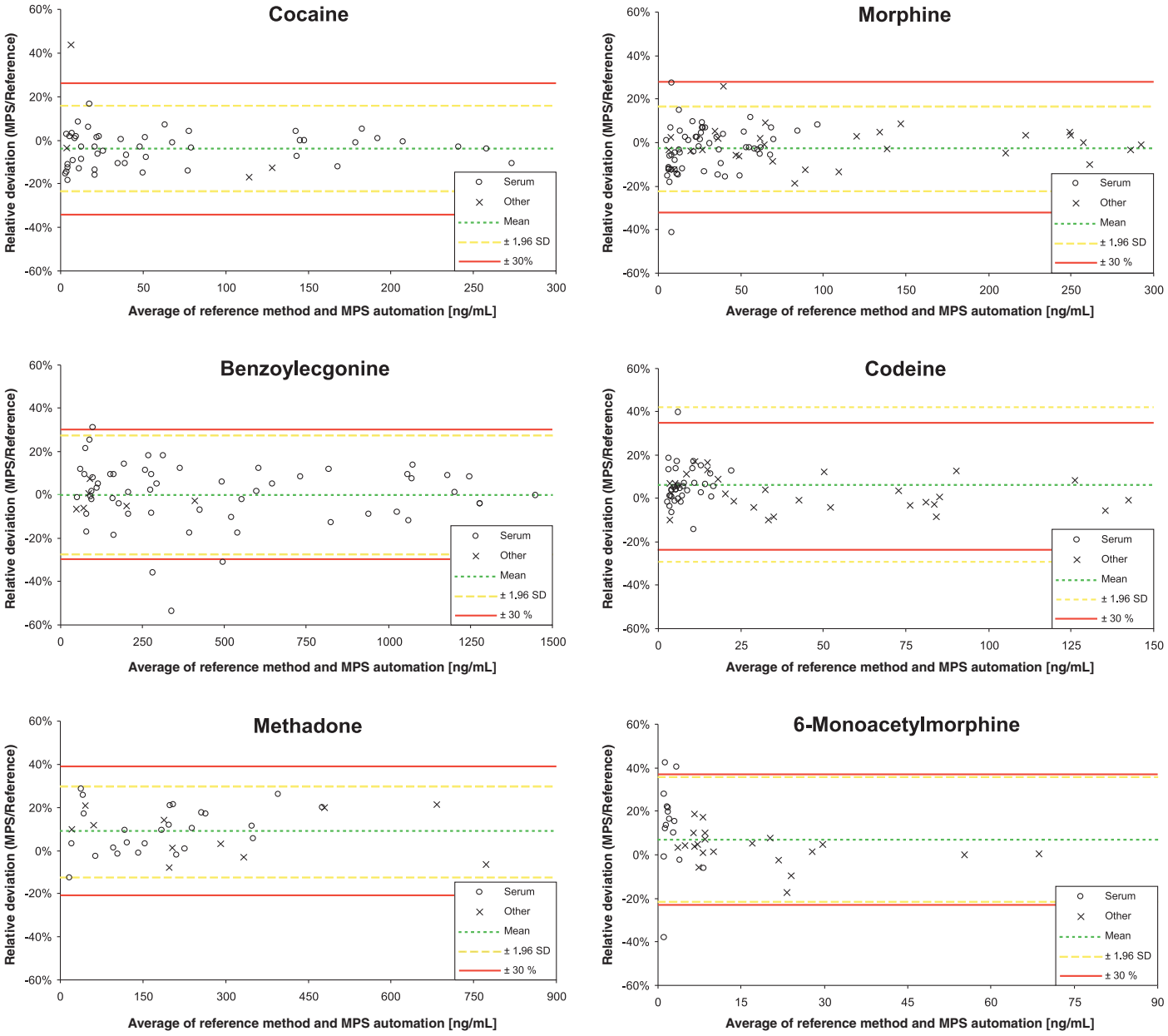

Figure 7. Relative deviations of measured concentrations displayed in Bland-Altman-plots.
ng/mL: Nanogram per milliliter or nanogram per gram for tissue respectively; Other: Other matrices than serum - urine, blood, lyophilized kidney tissue, heart blood, lyophilized and native brain tissue; LOD: Limit of detection; LOQ: Limit of quantification; ULOC: Upper limit of calibration; SD: Standard deviation of relative deviations.

No carryover for any of the compounds could be detected when extracting blank serum after real samples. By overlapping sample preparation steps with the GC/MS run a throughput of around 26 samples per day could be achieved which is comparable with the partly-automated reference method.

The analyses were performed in different laboratories by different personnel at different times revealing the ruggedness of the instrumentation and methods and the suitability for routine forensic analysis tasks.

Conclusions

The following achievements were made:

- Comprehensive automation of a validated, partly-automated analysis method for opioids, cocaine and metabolites from blood serum and other matrices.
- Analysis results of both methods are equivalent on the basis of GTFCh recommendations.
- The automated method proved to be rugged and suitable for routine analysis in forensic laboratories.
- The automated method saves manual work and reduces the risk of human error. It generates a throughput of 29 samples per day, which is similar to the reference method and corresponds to the GC/MS analysis time.
- The analysis system is highly flexible and can mimic manual sample preparation workflows. Therefore it can be employed for easy automation of other validated GC or LC analysis methods or for standalone automation of sample preparation.

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G L O B A L A N A L Y T I C A L S O L U T I O N S

GERSTEL GmbH & Co. KG
Eberhard-Gerstel-Platz 1
45473 Mülheim an der Ruhr
Germany
☎ +49 (0) 208 - 7 65 03-0
🖷 +49 (0) 208 - 7 65 03 33
@ gerstel@gerstel.com
🌐 www.gerstel.com

GERSTEL Worldwide

GERSTEL, Inc.
701 Digital Drive, Suite J
Linthicum, MD 21090
USA
☎ +1 (410) 247 5885
🖷 +1 (410) 247 5887
@ sales@gerstelus.com
🌐 www.gerstelus.com

GERSTEL AG
Wassergrabe 27
CH-6210 Sursee
Switzerland
☎ +41 (41) 9 21 97 23
🖷 +41 (41) 9 21 97 25
@ swiss@ch.gerstel.com
🌐 www.gerstel.ch

GERSTEL K.K.
1-3-1 Nakane, Meguro-ku
Tokyo 152-0031
SMBC Toritsudai Ekimae Bldg 4F
Japan
☎ +81 3 5731 5321
🖷 +81 3 5731 5322
@ info@gerstel.co.jp
🌐 www.gerstel.co.jp

GERSTEL LLP
Level 25, North Tower
One Raffles Quay
Singapore 048583
☎ +65 6622 5486
🖷 +65 6622 5999
@ SEA@gerstel.com
🌐 www.gerstel.com

GERSTEL Brasil
Av. Pascoal da Rocha Falcão, 367
04785-000 São Paulo - SP Brasil
☎ +55 (11)5665-8931
🖷 +55 (11)5666-9084
@ gerstel-brasil@gerstel.com
🌐 www.gerstel.com.br

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