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Review

Recent advances of liquid chromatography–(tandem) mass spectrometry in clinical and forensic toxicology

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ABSTRACT

Liquid chromatography (LC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) has become increasingly important in clinical and forensic toxicology as well as doping control and is now a robust and reliable technique for routine analysis in these fields. In recent years, methods for LC–MS(/MS)-based systematic toxicological analysis using triple quadrupole or ion trap instruments have been considerably improved and a new screening approach based on high-resolution MS analysis using benchtop time-of-flight MS instruments has been developed. Moreover, many applications for so-called multi-target screening and/or quantification of drugs, poisons, and or their metabolites in various biomatrices have been published. The present paper will provide an overview and discuss these recent developments focusing on the literature published after 2006.

Contents

Abbreviations: LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; STA, systematic toxicological analysis; HPLC, high-performance liquid chromatography; DAD, diode array detection; GC–MS, gas chromatography–mass spectrometry; TOF, time-of-flight; MRM, multiple reaction monitoring; IDA, information dependent acquisition; EPI, enhanced product ion; LIT, linear ion trap; CES, collision energy spread; LLE, liquid–liquid extraction; SPE, solid-phase extraction; AT, Agilent Technologies; CID, collision induced dissociation; UPLC, ultra-performance liquid chromatography; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photo ionization; WADA, World Anti-Doping Agency; EU, European Union; IP, identification point; SIM, selected-ion monitoring; QC, quality control; SMC, succinylmonocholine; IS, internal standard.

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Introduction

In recent years, liquid chromatography (LC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) has become increasingly important in the field of clinical and forensic toxicology, workplace drug testing, and doping control. Evolving from an experimental technique in the early 1990s it reached the state of maturity in the early 2000s and has meanwhile become a reliable and robust analytical technique for routine analysis. Most importantly for the above-mentioned fields, LC–MS(/MS) has helped closing the gap with respect to hydrophilic, thermolabile, and non-volatile analytes that were not sufficiently covered by the established gold standard technique gas chromatography–mass spectrometry (GC–MS).

The position of LC–MS(/MS) in clinical and forensic toxicology has been reviewed several times in the past [1–14]. The present paper will provide an overview on very recent developments of LC–MS(/MS)-based analysis in clinical and forensic toxicology focusing on methods for systematic toxicological analysis (STA) and multi-analyte procedures published after 2006. Recently published papers from the field doping control that deal with similar developments will also be included.

Systematic toxicological screening analysis by LC–MS(/MS)

In clinical and forensic toxicology, the compounds involved in a clinical or forensic case are often unknown. In these fields, so-called STA or general unknown screenings for identification of toxicologically relevant compounds in biological matrices is an important part of daily routine work. Analytical methods for STA should ideally cover hundreds of relevant drugs, poisons, and/or their metabolites and allow unambiguous compound identification [5,15,16]. In most forensic and clinical toxicology laboratories, GC–MS and high-performance liquid chromatography (HPLC) with diode array detection (DAD) are still primarily used for STA. However, LC–MS and LC–MS/MS have become increasingly important in recent years, because they combine the high selectivity of mass spectrometric detection with the possibility to directly analyze aqueous samples and hydrophilic, thermolabile and non-volatile analytes. A major drawback of LC–MS(/MS) in STA is the lack of reference libraries that can be used on different apparatus types due to insufficient reproducibility of LC–MS(–MS) mass spectra obtained with different instrument types. A review of LC–MS-based methods for (STA) published before 2007 is available in the literature [17]. Here, only updates or improvements of previously published methods, new approaches, and methods published after 2006 are considered.

Recently published or updated LC–MS-based screening procedures covering at least hundreds of toxicologically relevant compounds can be divided into two general approaches: In the first approach, triple quadrupole, ion trap, or hybrid mass spectrometers are used to generate information-rich product ion spectra which can be searched against libraries of reference mass spectra previously recorded on the same or similar type of apparatus. The second approach is based on high-resolution mass spectrometry with benchtop time-of-flight (TOF) mass spectrometers, where compounds are identified by comparison of accurate masses as measured in the sample with accurate mass databases of toxicologically relevant compounds. Key information about LC–MS(/MS)-based STA procedures is summarized in Table 1.

STA based on product ion spectra

Weinmann and co-workers have recently updated their previous multi-target screening method for 301 drugs which was based on an a so-called survey scan using multiple reaction monitoring (MRM), information dependent acquisition (IDA), and enhanced product ion (EPI) scanning at three collision energies (20, 35 and 50 eV) [18]. They described the generation of a new ESI-MS/MS library containing 1253 compounds including drugs, pharmaceuticals, and toxic organic compounds of forensic and clinical importance [19]. This new library was generated on a hybrid triple quadrupole instrument in which the third quadrupole can be used as a linear ion trap (LIT). In contrast to the previous version, the new library does not only contain EPI spectra recorded at three distinct collision energies, but also spectra recorded with the so-called collision energy spread (CES) feature of the LIT function. Using the latter, information-rich EPI spectra representing three collision energies (here 35 ± 15 V) can be recorded within the same time frame as single collision energy spectra, thus dramatically reducing IDA cycle time. The new library established on a hybrid triple quadrupole/LIT instrument was then also used with an older

Table 1
Methods for systematic toxicological screening analysis.

Analytes	Sample	Work-up	Stationary phase	Mobile phase	Detection mode	Apparatus type	Validation data	Ref.
700 drugs, pharmaceuticals, and toxic compounds	U	Dilution	Restek Allure PFP Propyl (50×2.1 mm, 5 μm)	Gradient water and ACN with 2 mM AF and 0.2% FA each	ESI+MRM (survey), IDA, EPI	QQQ, QQQ/LIT	Comparison to routine GC–MS results	[20]
	U, S	LLE						
780 toxicologically relevant compounds	B, U	SPE (HCX)	Zorbax SB-Aq (150×2.1 mm, 3.5 μm)	Gradient 0.1% FA and MeOH	ESI+, PIS with collision energy ramping	Ion trap	LODs of 24 model drugs	[25]
320 pesticides and metabolites	B	PP, SPE (WAX)	Intersil ODS3 C18 (100×1 mm, 3 μm)	Gradient 10 mM AF and ACN	ESI+ and ESI−, PIS in MS^2 and MS^3	Ion trap	LOD of 320 pesticides, recovery and matrix effect of 14 model compounds	[26]
over 300 common drugs and metabolites	U	LLE (acidic and basic)	Acquity UPLC HSS T_3 (100×2.1 mm, 1.8 μm)	Gradient 0.05% FA and MeOH	ESI+, scan aperture voltages: 10 and 45 V	TOF	Transferability between 6 labs, specificity, recovery, matrix effects, method comparison	[35]
Approx. 50500 compounds	H	incubation with 0.1 HCl, LLE	Zorbax Eclipse C18 (150×2.1 mm, 3.5 μm)	gradient 0.1% FA and ACN	ESI+, full-scan, metabolomics	TOF	Tested on 108 compounds of various drug classes	[32,33]
	B	PP, LLE						
	U	Dilution						

Abbreviations: U, urine; S, serum; B, blood; H, hair; LLE, liquid–liquid extraction; SPE, solid-phase extraction; HCX, mixed-mode sorbent with hydrophobic and cation exchange properties; PP, protein precipitation; WAX, mixed-mode sorbent with hydrophobic and weak anion exchange properties ACN, acetonitrile; AF, ammonium formate; FA, formic acid; MeOH, methanol; ESI, electrospray ionization; MRM, multiple reaction monitoring; IDA, information dependent acquisition; PIS, product ion scan; QQQ, triple quadrupole; QQQ/LIT, triple quadrupole/linear ion trap; TOF, time-of-flight; GC–MS, gas chromatography–mass spectrometry; LOD, limit of detection.

generation triple quadrupole instrument of the same manufacturer and the single collision energy spectra matched very well. Good agreement of single collision energy as well as CES spectra was also observed when the library was run on two newer generation hybrid triple quadrupole/LIT instruments of the same manufacturer. While this clearly is a step into the direction of an LC–MS/MS library than can be used on different instruments, it must be considered that all instruments in the described study were from the same manufacturer and hence work with similar ion paths and geometry. In a second publication, the group of Weinmann reported an update of their target screening method increasing the number of targets from 301 to 700 compounds [20]. This increase became possible because of a new software feature, the so-called scheduled MRM allowing the setting of time windows for transitions monitored in the survey scan. Hence, the overall number of transitions in the method could be considerably increased while even reducing the cycle time as compared to the previous method [18]. Sample preparation consisted either of a simple dilution of urine samples or liquid–liquid extraction (LLE) of serum or urine samples with 1-chlorobutane. The method was evaluated using spiked and authentic samples and the results were generally satisfactory. However, there were situations were compounds could not be identified because the respective peaks did not trigger the IDA mode or because the EPI quality was not sufficient for identification. Other drawbacks are the currently limited number of metabolite spectra included in the library and that only positive ionization is possible due to polarity switching being too slow for repeated switching between polarities within the same run.

An approach similar to the one described above was published by Sauvage et al. in 2006 [21]. These authors also used a hybrid triple quadrupole/LIT instrument with IDA and EPI with CES (40 ± 25 V) to generate product ion spectra for library search after preparation of samples by non-selective polymer-based solid-phase extraction (SPE). However, the survey scan was performed in the so-called enhanced MS mode, i.e. a single stage full-scan mode with ion accumulation in the LIT for increased sensitivity. The three most intense ions of each background-subtracted scan were selected for fragmentation in the EPI mode and excluded from IDA after four occurrences. With this procedure, EPI spectra are recorded of any precursor ion being the most intense in the survey scans potentially including (unknown) metabolites. In a recent update paper, Sauvage et al. [22] systematically searched for EPI spectra with fragmentation patterns similar to those of parent compounds. Through interpretation of the fragments, they were able to tentatively identify a considerable number of metabolites and the respective EPI spectra could be added to the database after confirmation by literature data, incubation experiments with rat liver microsomes or drug ingestion by healthy volunteers. An interesting example of this approach was published by Picard et al. [23]. Elliott and Smith used EPI spectra obtained at three distinct collision energies (20, 30 and 50 V) and with CES (35 ± 15 V) to identify the designer drugs benzylpiperazine and 3-trifluoromethylpiperazine in postmortem samples [24].

Liu et al. [25] established an LC–MS/MS database for the analysis of drugs in postmortem specimens using an ion trap mass spectrometer. Here, information-rich product spectra were generated using collision energy ramping, meaning that precursor ions were fragmented by collision induced dissociation (CID) at collision energies between 0.3 and 2.0 V leading to information-rich product ion spectra containing fragments at the low as well as high mass range. The NIST MS Search algorithm was used for library searching. The spectra of 15 tested model compounds were found to be highly stable over a period of 6 months. Application of the described method to 12 postmortem cases showed that generally more compounds were found by the new LC–MS/MS procedure as compared to an in-house GC–MS method. However, in all cases opiates were found indicating that the selection was most probably not representative for the whole spectrum of cases generally seen in postmortem toxicology.

Dulaurent et al. [26] reported a screening procedure for 320 pesticides and metabolites in blood. They first established two libraries of MS^2 and MS^3 spectra of 320 pesticides and metabolites. The spectra were generated on a linear ion trap instrument using the base peak ion of the parent scan as the precursor ion for the MS^2 spectrum and the base peak of the latter as precursor ion for the MS^3 spectrum. The resulting MS^2 and MS^3 libraries contained 450 and 430 spectra, respectively, partly including negative and positive polarity spectra of the same compound as well as some spectra of isotope peaks or adduct ions. The spectra of six model pesticides were found to be fairly stable over the studied period of five months. For the screening of pesticides in blood, samples were first worked up by protein precipitation and SPE with a mixed-mode polymeric weak anion exchange sorbent. Subsequently, they were analyzed by LC–MS/MS using data-dependent scanning and polarity switching after each cycle. Library searching was performed using the NIST MS Search algorithm and automated spectra comparison of MS^2 spectra based on signal intensity, retention time and match quality. If an MS^2 spectrum was identified by the automated method, the MS^3 spectrum was manually checked for confirmation. As already pointed out by the authors, the major drawback of the described screening method is the long cycle time of 2.45 s bearing the risk of missing smaller peaks. This can only be avoided by using separate injections for each polarity doubling analysis time or by dumping the time consuming third MS stage increasing the risk of false positive results. Other problems were that some pesticides were not even detectable after direct infusion or injection of pure standards on column. Nevertheless, the described approach could become a useful tool for screening of pesticides and many other compounds in complex matrices due to the additional selectivity resulting from the MS^3 spectra.

STA based on high-resolution mass spectrometry

The group of Ojanpera was the first to apply the concept of high-resolution mass spectrometry to STA in forensic toxicology. They first generated a database of theoretical accurate masses of toxicologically relevant compounds and metabolites, which could be established without reference substances. This database was then used for forward searching of the respective compounds in the full-scan LC–time-of-flight mass spectrometry (TOF-MS) data obtained from analysis of urine samples after enzymatic hydrolysis and mixed-mode SPE [27–30]. Experience with this approach showed that accurate mass and isotopic patterns alone were not sufficient for unambiguous compound identification. However, with the reference mass database having been set up using theoretical mass data, retention data which could have been used for corroboration of tentative compound identifications via accurate mass were only available for a subset of compounds. An alternative way of supporting compound identification is additional identification of metabolites and/or the use of CID to generate fragments providing additional structural information of the compound in question. Since this requires knowledge about metabolic data, which are often not available for new drugs of abuse, or reference standards for recording CID mass spectra, which are not available for many analytes, the authors recently reported an *in silico* approach to generate the necessary data [31]. In this approach, expected metabolites for certain compounds were predicted by a metabolite prediction software, so that the respective accurate mass data could be added to the database. Moreover, fragmentation patterns of compounds, for which no reference standards were available, were predicted using another software tool. An application study showed that the metabolite prediction software correctly predicted major metabolites of quetiapine, but also several others that were not found in authentic samples. On the other hand, the program failed to predict any hydroxyl metabolites of which several isomers were detected in authentic samples. However, the prediction of fragmentation patterns by the fragmentation software was found to be very helpful in differentiation of isobaric metabolites. In summary, the described *in silico* approach certainly looks like a promising way to generate useful data to support accurate mass-based compound

identification. However, more application data will be necessary to demonstrate whether or not this approach will ultimately be sufficiently reliable for compound identification without reference standards.

The group of Polettini also used TOF-MS for compound identification but the approach to generating an accurate mass database was quite different. By setting certain search limits they established a subset of 55,000 compounds of toxicological relevance from the Pubchem Compound database, including drugs, poisons, chemicals as well as a considerable number of phase I and phase II metabolites [32]. Due to the large number of compounds in the database, it is not surprising that the number of compounds with identical chemical formulae ranged from 1 to 39 with an average of 1.82 precluding unambiguous compound identification via accurate molecular mass alone. This was confirmed in an application study in which the database was tested by analyzing authentic samples from postmortem cases using CE-TOF-MS after simple dilution (urine), protein precipitation and SPE (blood) or LLE (hair). In none of the samples, a compound could be unambiguously identified. However, the best hits with respect to mass error and isotopic pattern were generally supported by presence of peaks with m/z values indicating the presence of metabolites of the compounds in question. In a second study, it was therefore evaluated, whether the presence of metabolites could effectively shorten the hitlist [33]. For this purpose a "metabolomic" approach was used. In a first step the number of a predefined set of functional groups was calculated for each candidate compound using a software tool. Subsequently, the mass shifts for 23 major biotransformation reactions were calculated and the peaks with the respective masses were searched in the data files obtained from analysis of authentic samples by LC–TOF-MS or capillary electrophoresis coupled to TOF-MS. Finally, detected potential metabolites were checked for congruence with the functional groups of the candidates. Application to 108 compounds from various drug classes showed that with this approach the average number of hits could be reduced by half. However, in many cases it was still insufficient to obtain unambiguous results. The authors therefore concluded that it will be necessary to ultimately include retention data into the search strategy and suggested generating such data by theoretical calculations based on physicochemical properties of the molecules. It will be interesting to see, if this will effectively shorten the hitlists any further. However, even if it would ultimately lead to a single hit for each peak, it seems doubtful that compound identification partly based on theoretical considerations and calculations on metabolism and retention behavior would be considered "unambiguous" by the scientific community or in court. In a widely accepted guidance document issued by the European Union [34], a single ion is not considered sufficient for compound identification, even if it has been measured by high-resolution mass spectrometry and the retention time is correct (also see Section 3.4). Hence it seems necessary that the presence of candidate compounds be confirmed by another method. Nevertheless, the described approach could become a powerful tool for simultaneous screening for a very large number of compounds, if used in combination with an unselective sample preparation.

Lee et al. [35] published a toxicological screening method based on LLE of urine samples under acidic and basic conditions followed by ultra-performance liquid chromatography (UPLC)–TOF-MS analysis of the combined extracts. The procedure was set up in a collaborative work by six laboratories using the same instruments. Over 300 mass spectra were included in the library, 102 of which were metabolite spectra taken from real sample analysis. The problem of identification by accurate mass only was addressed by including chromatographic retention data into the search strategy and by recording spectra at two aperture voltages, one leaving the intact pseudomolecular ion, one leading to CID and more fragment-rich spectra increasing the confidence in identification considerably. However, using two aperture voltages increased the MS cycle time of the method necessitating a slower chromatography and hence partly compromising the major advantage of UPLC over HPLC even though the resulting run-time of 17 min is certainly acceptable even from an emergency toxicology point of view. In an initial application study with 30 authentic urine samples, the results obtained with the new UPLC–TOF-MS method were in very good agreement with those obtained with a combination of GC–MS, UPLC–MS/MS, and HPLC-DAD. Matrix effect experiments with 29 model compounds showed only moderate effects (signal in spiked extracts 70–130% of neat standards) for the majority of the compounds. The strongest ion suppression effect was observed for ecgonine methyl ester (40 reduction of signal).

Methods for screening and/or quantification of a limited number of analytes in biological matrices

Multi-analyte procedures allowing simultaneous analysis of various or multiple drugs from one specific drug class or a number of closely related drug classes are often used in clinical and forensic toxicology as well as doping control. They help saving time and resources during method development and validation and limit the number of methods to be established in the laboratory despite a broad spectrum of analytes to be covered. In the following, multi-analyte LC–MS(/MS) methods published after 2006 will be discussed. Key method parameters are summarized in Tables 2–6.

Target analytes

The majority of the methods reviewed here targeted classic drugs of abuse such as amphetamines [36–58], opiates [37–42,45,46,48,49,52–69], cocaine [37–42,45,46,48,49,52–58,60,62,64,65,68,70], cannabinoids [39–42,57,58], phencyclidine; [41,42,53–55] and therapeutic drugs with abuse potential or relevant in the context of driving under the influence of drugs [38–43,46,52,57,58,61,71–73]. Others covered diuretics [74–76], beta-blockers [74,76–78], stimulants [74,76], steroids [74,76,77,79], and beta-adrenergic drugs [76,77,79], which are primarily relevant to doping analysis. Therapeutic drugs covered by the listed procedures belonged to the following drug classes: beta-blockers [74,76–78] calcium-channel antagonists [78], angiotensin-II-receptor antagonists [78,80], antiarrhythmic drugs [78], antidepressants (tricyclics and selective serotonin reuptake inhibitors) [81], and low-dosed antipsychotics [82]. Inoue et al. [83] described a method determination of organophosphorus insecticides in human serum. A method for simultaneous determination of five toxic plant alkaloids was published by Qiu et al. [84].

More exotic analytes were targeted by Pichini et al. [47]. These authors described a screening method for "hallucinogenic designer drugs" in urine which covered compounds from major classes of emerging designer drugs: phenethylamines (2Cs and 2C-B-fly), tryptamines, and piperazines. Very recently, Wohlfahrt et al. [85] published a method for screening of altogether 36 designer amphetamines, phenethylamines, tryptamines and piperazines in serum. Because most of these drugs are not or only poorly detected by immunoassay procedures for drugs of abuse analysis and not included in the confirmation strategies of most laboratories, the above methods could help gathering more information on the prevalence of these compounds among drug users.

In another interesting publication, natural hallucinogens such as psilocin and bufotenine were included in a screening procedure for classic hallucinogens such as lysergide [72]. Considering that these "natural hallucinogens" are not covered by most conventional screening methods and that there is an increasing popularity of herbal drugs, this is certainly a reasonable approach.

Biosamples and work-up

Urine is a well established matrix for screening analysis, because it can be obtained non-invasively in comparatively large volumes and

Table 2
Multi-analyte procedures for screening and/or quantification in urine.

Analytes	Work-up	Stationary phase	Mobile phase	Detection mode	Apparatus type	Validation data	Ref.
22 drugs relevant to doping	Enzymatic hydrolysis, LLE	Acquity BEH 18 (50×2.1 mm, 1.7 μm)	Gradient, water and ACN with 0.1% FA each	ESI+, scan, 3 aperture voltages	TOF	LOD, LOQ, linearity	[79]
25 opioid drugs	Enzymatic hydrolysis, LLE	Gemini C18 (100×2 mm, 3 μm)	Gradient, aq. AA and ACN with 0.1% FA	ESI+, MRM (2)	QQQ/LIT	Selectivity, linearity, accuracy, precision, LOD, LOQ, matrix effect	[66]
72 xenobiotics relevant to doping	Enzymatic hydrolysis, LLE	Discovery C18 (150×2.1 mm, 5 μm)	Gradient, water and ACN with 0.1% FA	ESI + and ESI−, MRM (1 or 2)	QQQ	Selectivity, repeatability, LOD, recovery, ion suppression, robustness	[76]
42 drugs of abuse and metabolites	96-well, enzymatic hydrolysis, dilution	Zorbax Eclipse XDB-C18 (50×4.6 mm, 1.8 μm)	Gradient, water and ACN with 0.1% FA each	ESI+, MRM (1)	QQQ	Recovery, precision, carryover	[41]
64 compounds or metabolites relevant to doping	enzymatic hydroylsis, LLE	Zorbax Eclipse Plus C18 (100×2.1 mm, 1.8 μm)	Gradient, 5 mM AF in 0.1% FA and ACN with water	ESI+, scan, optimized for M + H	TOF	selectivity, carryover	[77]
7 amphetamines	LLE	Atlantis dC18 (20×2.1 mm, 3 μm)	Gradient, AF buffer and ACN	ESI +,MRM (2)	QQQ	Selectivity, linearity, accuracy, precision, LLOQ, ULOQ, recovery, matrix effect, carryover	[50]
24 diuretics	Dilution	Chrompak Intersil ODS-3 (100×3 mm, 3 μm)	Gradient, 0.1% FA and ACN	ESI + and ESI−, SRM survey, IDA, EPI	QQQ/LIT	Selectivity, LOD, matrix effect	[75]
Morphine, codeine, ethylmorphine and glucuronides, 6-acetylmorphine	Dilution	Luna C18 (100×2 mm, 3 μm)	Gradient, water and ACN with 25 mM FA	ESI +,SRM (2)	QQQ	Selectivity, linearity, accuracy, precision, LOD, LLOQ, matrix effect, stability	[67]
Multiple drugs of abuse and metabolites	Automated SPE (HLB)	XTerra MS C 18 (50×3.0 mm, 2.5 μm) two runs	Gradient, 5 mM AA with 0.05% acetic acid and ACN (+ mode) 5 mM AA with 0.05% conc. NH3 and ACN (− mode)	ESI + ESI−, MRM (2)	QQQ	Specificity, linearity, accuracy, precision, LOD, recovery, matrix effect, carryover	[54]
12 illicit drugs of abuse	(enzymatic hydrolysis), SPE (HLB)	Atlantis dC18 (100×2.1 mm, 3 μm)	Gradient, ACN and AF buffer	ESI +,MRM (2)	QQQ	Specificity, linearity, accuracy, precision, LOD, LLOQ, ULOQ, recovery, matrix effect	[49]
Amphetamine, methamphetamine, MDA, MDMA	Dilution	Luna C18 (200×2.0 mm, 3 μm)	Gradient, 25 mM FA in water and ACN	ESI +,SRM (2)	QQQ	Specificity, linearity, precision, LOD, LLOQ	[51]
Opiates and cocaine	SPE (HCX)	UPLC BEH C18 (50×2.1 mm, 1.7 μm)	Gradient, 2 mM ammonium bicarbonate and MeOH	ESI +,MRM (1)	QQQ	Specificity, linearity, accuracy, precision, LOD, LOQ, matrix effect, carryover, stability	[65]
Multiple hallucinogens, chlorpheniramine, ketamine, ritalinic acid, and metabolites	SPE (HCX)	Sunfire C8 (200×2.1 mm, 3.5 μm)	Gradient, 10 mM AF and ACN/MeOH	ESI +,MRM (2)	QQQ	Selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, matrix effect, proficiency test	[72]
6-acetylmorphine glucuronides of morphine, codeine, ethylmorphine	SPE (HLB)	Luna C18 (100×2.0 mm, 3 μm)	Gradient, 25 mM FA in water and ACN	ESI +,SIM (2)	Q	Specificity, linearity, precision recovery,	[63]
5 toxic alkaloids	SPE (HCX)	XBridge Shield RP18 (250×3.0 mm, 5 μm)	Gradient, 10 mM ammonium bicarbonate and ACN	ESI +,MRM (3)	Ion trap	linearity, precision, LOQ, recovery, matrix effect	[84]
10 hallucinogenic designer drugs	(enzymatic hydrolysis) SPE (HCX)	Hipersil Gold ultra pure (150×4.6 mm, 5 μm)	Gradient, 10 mM ammonium bicarbonate (pH 7.3) and ACN	APCI +,SIM (3) (three fragmentor voltages)	Q	Selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, matrix effect, stability, carryover	[47]
21 Diuretics, 19 beta-blockers, 8 stimulants, 2 steroids	SPE (polymer)	Atlantis T3 (100×2.1 mm, 3 μm)	Gradient, 10 mM AF and ACN	ESI+, ESI−, MRM (?)	QQQ	Selectivity, Precision, LOD, recovery, carryover, stability	[74]
		BEH Shield RP18 (100×2.1 mm, 1.7 μm)	Gradient, 0.1% formic acid in water and methanol	ESI+, ESI−, MRM	QQQ		

Abbreviations: LLE, liquid–liquid extraction; SPE, solid-phase extraction; HCX, mixed-mode sorbent with hydrophobic and cation exchange properties; HLB, polymer sorbent with hydrophilic and lipophilic properties; ACN, acetonitrile; AF, ammonium formate; FA, formic acid; AA, ammonium acetate; MeOH, methanol; ESI, electrospray ionization; MRM, multiple reaction monitoring; IDA, information dependent acquisition; PIS, product ion scan; QQQ/LIT, hybrid triple quadrupole/linear ion trap; QQQ, triple quadrupole; TOF, time-of-flight; GC–MS, gas chromatography–mass spectrometry; LOD, limit of detection, LLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

because drugs and/or their metabolites are concentrated in urine [5]. Concentrations of drugs or poisons in blood, plasma or serum generally show the best correlation with pharmacologic effects. Therefore, these matrices are preferred for quantitative analysis when interpretation of concentrations and effects are required. Oral fluid can be easily taken non-invasively and detection times of most drugs in this matrix are more or less similar to those in blood. Therefore, oral fluid has been extensively used in road-side and workplace drug testing as well as in certain therapeutic drug monitoring applications. Hair is a biomatrix ideally suited for monitoring long-term exposure to drugs, e.g. in compliance monitoring of detoxification treatment. The perinatal biomatrices meconium, placenta, and umbilical cord can be used to monitor in-utero exposure to drugs (of abuse).

Table 3
Multi-analyte procedures for screening and/or quantification in whole blood, plasma, or serum.

Analytes	Sample	Work-up	Stationary phase	Mobile phase	Detection mode	Apparatus type	Validation data	Ref.
Designer amphetamines, tryptamines and piperazines	S	SPE (HCX)	Synergi Polar-RP (150×2 mm, 4 μm)	Gradient, 1 mM AF with 0.1% FA and MeOH with 0.1% FA	ESI+,MRM (2–3)	QQQ	Selectivity, LOD, recovery, matrix effect	[85]
19 drugs of abuse and metabolites	P	PP	C18 Alltima (250×4.6 mm, 5 μm)	Gradient, water and ACN with 5 mM FA each	ESI+,MRM (2)	QQQ	Selectivity, linearity, precision, accuracy, LLOQ, recovery, matrix effect, stability, carryover	[42]
25 opioid drugs	B	LLE	Gemini C18 (100×2 mm, 3 μm)	Gradient, AA and ACN with 0.1% FA	ESI+,MRM (2)	QQQ/LIT	Selectivity, linearity, accuracy, precision, LOD, LLOQ, matrix effect	[66]
Morphine, 6-acetylmorphine, codeine, dihydrocodeine, oxycodone, hydrocodone, hydromorphone, noscapine, papaverine, and metabolites	B	SPE	Synergi Polar-RP (150×2 mm, 4 μm)	Gradient, 1 mM AF with 0.1% FA and ACN	ESI+,MRM (2–3)	QQQ	For morphine and its glucuronides only: selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, matrix effect, stability	[59]
19 drugs of abuse and metabolites	B	Automated SPE (HCX)	Varian Pursuit C18 (100×3 mm, 3 μm)	Gradient, F/8% ACN and MeOH	ESI+,MRM (2)	QQQ	Selectivity, linearity, precision, accuracy, recovery, matrix effect, LOD, LLOQ, ULOQ, carryover, proficiency testing	[38]
Midazolam, morphine, and metabolites	P	SPE (HLB)	Aquity UPLC BEH C18 (2.1×100 mm, 1.7 μm)	Gradient, water and MeOH with 0.1% FA	ESI+, MRM, 1 transition per analyte	QQQ	Linearity, accuracy, precision, LLOQ, stability, recovery, matrix effect	[61]
18 basic drugs and metabolites	S	Online extraction Allure PFP (10×2.1 mm, 3 μm)	Allure PFP (30×2.1 mm, 3 μm)	Gradient, 1 mM AF and ACN with 0.1% FA each	ESI+,MRM (2)	QQQ/LIT	Selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, matrix effect, carryover	[37]
6 AT II receptor antagonists + 1 metabolite	P	PP	Luna phenyl hexyl (50×2 mm, 3.5 μm)	Gradient, water and ACN with 0.1% FA and 1 mM AF	ESI+, MRM (2)	QQQ	Selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, matrix effect, stability	[80]
10 organophosphates	S	PP	XTerra MS C18 (100×2.1 mm, 3.5 μm)	Gradient, 10 mM AF and MeOH	APCI+, APCI− SIM (1)	Q	Selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, stability	[83]
43 benzodiazepines and metabolites, zolpidem, zopiclone	P	SPE (polymer)	UPLC BEH C18 100×2.1 mm, 1.7 μm)	Gradient, 0.05% FA in water and ACN	ESI+,SIM (1) and full-scan	Q	Linearity, precision, LOD, matrix effect	[73]
14 antidepressants and metabolites	P	Dilution, online-SPE (HCX), disposable cartridges	Gemini C18 (150×2 mm, 5 μm)	Gradient, 10 mM ammonium hydrogen carbonate, ACN	ESI+,MRM (2)	QQQ	Selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, matrix effect	[81]
7 low-dosage antipsychotics	B	LLE	Zorbax Stable Bond Cyano (50×2.1 mm, 3.5 μm)	Gradient, MeOH, ACN, 20 mM AF	ESI+,MRM (2)	QQQ	Selectivity, linearity, precision, accuracy, recovery, matrix effect	[82]
5 toxic alkaloids	B	SPE (HCX)	XBridge Shield RP18 (250×3.0 mm, 5 μm)	Gradient, 10 mM ammonium bicarbonate and ACN	ESI+,MRM (3)	Ion trap	Linearity, precision, LOQ, recovery, matrix effect	[84]
Alprazolam, flunitrazepam, metabolites	B	96 well SPE (HLB)	XBridge Shield (100×2.1 mm, 3.5 μm)	Gradient, 20 mM acetate buffer and ACN	APPI, SIM (1)	Q	Selectivity, linearity, accuracy, precision, LLOQ, matrix effect	[71]
14 cardiovascular drugs	B	Automated SPE (HCX)	Atlantis dC18 (150×2.1 mm, 3.0 μm)	Gradient, 10 mM AF and ACN	ESI+, SIM (2)	Q	Selectivity, linearity, accuracy precision, LOD, LLOQ, recovery, matrix effect,	[78]

Abbreviations: LLE, liquid–liquid extraction; SPE, solid-phase extraction; HCX, mixed-mode sorbent with hydrophobic and cation exchange properties; HLB, polymer sorbent with hydrophilic and lipophilic properties; ACN, acetonitrile; AF, ammonium formate; FA, formic acid; AA, ammonium acetate; MeOH, methanol; ESI, electrospray ionization; MRM, multiple reaction monitoring; IDA, information dependent acquisition; PIS, product ion scan; QQQ/LIT, hybrid triple quadrupole/linear ion trap; QQQ, triple quadrupole; TOF, time-of-flight; GC–MS, gas chromatography–mass spectrometry; LOD, limit of detection, LLOQ. lower limit of quantification; ULOQ, upper limit of quantification.

Sample preparation for the above-mentioned described matrices may consist of simple dilution, e.g. of urine samples, or involve several sample pretreatment and extraction steps.

Urine sample are often submitted to enzymatic cleavage of glucuonic or sulfuric acid conjugates of drugs and/or their phase I metabolites. This kind of treatment is a prerequisite for sensitive GC–MS analysis of drugs excreted into urine in conjugated form. However, it is not strictly required in LC–MS(/MS) analysis, because this technique allows direct analysis of such conjugates, e.g. glucuronides of midazolam [61] or opiates [59,61,63,67]. Nevertheless, many of the recently published methods for LC–MS(/MS)-based urine analysis still include enzymatic conjugate cleavage.

Complex matrices like meconium, placenta, or umbilical cord generally require a homogenization, “protein precipitation” and/or

Table 4
Multi-analyte procedures for screening and/or quantification in oral fluid.

Analytes	Work-up	Stationary phase	Mobile phase	Detection mode	Apparatus type	Validation data	Ref.
19 drugs of abuse and metabolites	PP	C18 Alltima (250×4.6 mm, 5 μm)	Gradient, water and ACN with 5 mM FA each	ESI+, MRM (2)	QQQ	Selectivity, linearity, precision, accuracy, LLOQ, recovery, matrix effect, stability, carryover	[42]
23 illicit and medicinal drugs, metabolites	SPE (HLB)	Atlantis dC18 (50×2.1 mm, 3 μm)	Gradient, ACN and 0.1% aq. FA	ESI+, MRM (2)	QQQ	Selectivity, linearity, accuracy, precision, LLOQ, ULOQ, recovery, matrix effect	[39]
29 drugs of abuse	Automated SPE (HCX)	Acquity UPLC HSS T3 C18 (100×2.1 mm, 1.8 μm)	Gradient, 2 mM AA and MeOH	ESI+,MRM (2)	QQQ	Selectivity, linearity, precision, accuracy, LLOQ, ULOQ, recovery, matrix effect, carryover	[40]
24 illicit drugs and medicines	LLE	Atlantis T3 (100×2.1 mm, 3 μm)	Gradient, water and ACN with 2 mM AF	ESI, + MRM (2)	QQQ	Selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, carryover	[58]
32 drugs	LLE	Atlantis dC18 (50×2.1 mm, 3.5 μm)	Gradient, 5 mM AA and ACN	ESI, + MRM (1)	QQQ	Selectivity, linearity, accuracy, precision, LOQ, recovery, matrix effect, device recovery	[57]
13 drugs of abuse	LLE (amphetamines, phencyclidine) SPE (opiates, cocaine BZE)	Pinnacle II C18 (50×4.6, 5 μm) for amphetamines Allure PFP propyl (50×2.1, 5 μm) for PCP, opiates cocaine, BZE	Gradient, 5 mM AA and MeOH with 0.1% FA for amphetamines 0.1% FA and MeOH/ACN for PCP, opiates, cocaine, BZE	ESI+, MRM (2)	QQQ/LIT	Linearity, accuracy, precision, LOD, LLOQ matrix effect	[55]
21 drugs of abuse	Automated SPE (HCX)	Allure PFP Propyl (50×2.1 mm, 5 μm)	Gradient, 0.1% FA and 2 mM AF in water and ACN	ESI+, MRM (2)	QQQ	Selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, matrix effect, carryover	[53]

Abbreviations: LLE, liquid–liquid extraction; SPE, solid-phase extraction; HCX, mixed-mode sorbent with hydrophobic and cation exchange properties; HLB, polymer sorbent with hydrophilic and lipophilic properties; ACN, acetonitrile; AF, ammonium formate; FA, formic acid; AA, ammonium acetate; MeOH, methanol; ESI, electrospray ionization; MRM, multiple reaction monitoring; IDA, information dependent acquisition; PIS, product ion scan; QQQ, triple quadrupole; QQQ/LIT, hybrid triple quadrupole/linear ion trap; TOF, time-of-flight; GC–MS, gas chromatography–mass spectrometry; LOD, limit of detection, LLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

dilution step prior to analyte extraction. In hair analysis, a washing step is always necessary to remove potential external contamination.

A look at the "work-up" columns in Tables 1–6 shows that conventional LLE and SPE are employed in the vast majority of LC–MS(/MS) methods independent of the sample matrix. These will not be further discussed here.

Only few authors have used simple dilution for preparation of urine samples [51,63,67,75]. The dilution factors were five-fold [51,63,67] and 50-fold [75], respectively. Eichhorst et al. [41] also used (ten-fold) dilution in their procedure, but only after urine samples had been submitted to enzymatic conjugate cleavage.

A simple work-up method for blood, plasma or serum samples is protein precipitation. Ferreiros et al. [80] used a zinc sulphate/methanol mixture for precipitation of serum samples. The precipitation reagent was slowly dropped to the samples to avoid precipitation of protein cluster potentially trapping the analytes. Sergi et al. [42] used precipitation with pure methanol for work-up of plasma and oral fluid samples. After precipitation and centrifugation, the supernatants were filtered prior to injection. Inoue et al. [83] also used filtration prior to injection for supernatants obtained after protein precipitation with acetonitrile. These authors chose this simple procedure to avoid heating of the sample during solvent evaporation to avoid a known thermal instability of the analyte dichlorvos.

In order to streamline and speed up the extraction process, several authors used sample work-up in the 96-well format allowing parallel processing of almost 100 samples at a time. Eichhorst et al. [41] used this format for enzymatic hydrolysis as well as for centrifugation and dilution of urine samples. Speed of analysis was an important aspect for their method which was developed to replace fully automated immunoassay-based drug screening. Another application of SPE in 96-well format was the extraction of benzodiazepine (metabolites) from haemolyzed blood [71].

Another alternative to conventional SPE is online-SPE in which one LC column is used for extraction and another one for separation. Transfer of the analyte onto the extraction column and of the extracted analyte onto the separation column is achieved by column switching techniques as exemplified by Ferreiros Bouzas et al. [37]. Instead of LC extraction columns, disposable cartridges have also been used for online-SPE [81].

LC separation

As for sample preparation, the chromatographic conditions of the vast majority of the methods reviewed here are not particularly novel (see columns "stationary phase" and "mobile phase" in Tables 1–6) and will not be further discussed.

However, some of the procedures employed fast LC or UPLC, i.e. LC on stationary phases with sub-2 μm particle sizes resulting in a much higher separation power as compared to conventional LC columns. Due to the higher separation power of such columns, multiple analytes can be separated in comparatively short run-times. Eichhorst et al. [41] achieved separation of 42 compounds within 5 min. However, due to the short run-time only one transition per compound could be monitored, because otherwise the peak widths would have been too narrow for monitoring a sufficient number of data points per peak in the given cycle time. A similar problem was described by Lee et al. [35]. They recorded TOF-MS spectra at two aperture voltages extending the MS cycle time so much, that chromatography had to be "slowed down" to guarantee a sufficient number of points per peak. Touber et al. [79] reported separation of 22 analytes in 6 min, Berg et al. [65] separation of 8 compounds in 4 min. The latter used a mobile phase with ammonium hydrogen carbonate buffer (pH 10.2) and reported improved chromatography with increased retention of more polar analytes. In

Table 5
Multi-analyte procedures for screening and/or quantification in hair.

Analytes	Extraction	Stationary phase	Mobile phase	Detection mode	Apparatus type	Validation data	Ref.
6 amphetamines and ketamine	0.01% FA	Synergi Polar (150×2 mm, 4 μm)	Isocratic, 0.1% acetic acid and MeOH	ESI,+SRM (2)	Ion trap	Selectivity, linearity, precision, accuracy, LOD, LLOQ, recovery	[43]
15 opioid analgesics	MeOH	Synergi Max-RP (150×2 mmd, 4 μm)	Gradient, water and ACNwith 5 mM AF	ESI+,MRM (2)	QQQ	Selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, matrix effect	[69]
Morphine, codeine, 6-acetylmorphine, cocaine, benzoylecgonine	MeOH	XBridge phenyl (150×4.6 mm, 3.5 μm)	Gradient, 10 mM AA and MeOH	ESI+,MRM (1)	QQQ	Selectivity, linearity, accuracy, precision, LOD, LLOQ, matrix effect	[68]
22 illicit and medicinal drugs	ACN/25 mM FA	Zorbax SB-Phenyl (100×2.1, 3.5 μm)	Gradient, 25 mM FA and ACN	ESI+,MRM (1)	QQQ	Selectivity, linearity, accuracy, precision, LLOQ, recovery, matrix effect	[52]
Drugs of abuse and stimulants	Buffer and SPE (HCX)	Polaris C18 (100×2.0 mm, 3 μm)	Gradient, water and ACN with 0.1% FA	ESI+,MS^2 and MS^3 confirmation	Ion trap	Selectivity, linearity, accuracy, precision, LOD, LLOQ, process efficiency	[48]
Amphetamines, diazepam and its metabolites, cocaine and metabolites and opiates	SPE (HCX)	Synergi Hydro RP (150×2.1 mm, 4 μm)	Gradient, 3 mM AF with 0.001% FA and ACN	ESI+,MRM (1)	Ion trap	Linearity, accuracy, precision, LOD, LLOQ, recovery, matrix effect, stability	[46]
Opiates, amphetamines, cocaine	MeOH	Purospher RP18 (125×3 mm, 5 μm)	Gradient, 0.1% FA and ACN	APCI+,full-scan MS/MS	Ion trap	Selectivity, linearity, accuracy, precision, LOD, LOQ	[45]

Abbreviations: LLE, liquid–liquid extraction; SPE, solid-phase extraction; HCX, mixed-mode sorbent with hydrophobic and cation exchange properties; HLB, polymer sorbent with hydrophilic and lipophilic properties; ACN, acetonitrile; AF, ammonium formate; FA, formic acid; AA, ammonium acetate; MeOH, methanol; ESI, electrospray ionization; MRM, multiple reaction monitoring; IDA, information dependent acquisition; PIS, product ion scan; QQQ, triple quadrupole; TOF, time-of-flight; GC–MS, gas chromatography–mass spectrometry; LOD, limit of detection, LLOQ. lower limit of quantification; ULOQ, upper limit of quantification.

contrast to what one may expect when using a basic mobile phase pH with basic analytes, ionization still worked well in the ESI mode.

A direct comparison of conventional LC–MS/MS and UPLC–MS/MS was published by Murray and Danaceau [74]. With the latter, 49 compounds partly analyzed in positive partly in negative polarity could be separated within 6 min, while 12.5 min were needed with conventional LC. However, the very short run-time with UPLC–MS/MS could only be achieved because of a very short polarity switching time of 20 ms of the used MS/MS instrument.

Ionization, detection mode, and compound identification

Electrospray ionization (ESI) is by far the most often used ionization technique. Of the multi-analyte procedures considered here only three used atmospheric pressure chemical ionization (APCI) [44,45,47] and only one atmospheric pressure photo ionization (APPI) [71]. Because most toxicologically relevant compounds have basic properties, positive ionization mode was generally applied. The negative mode was only used for diuretics [74–76], barbiturates [54], some organophosphates [83]. and pesticides [26].

The reliability of compound identification very much relies on the detection mode of the method. The World Anti-Doping Agency (WADA) has released a document specifying criteria for compound identification in doping analysis [86], but currently there are no widely accepted criteria as to how much mass spectrometric and chromatographic information is required for a compound's identification in clinical or forensic toxicology. Some authors have referred to a document issued by the European Union (EU) Commission that contains detailed information about mass spectrometric identification of drug residues in foodstuffs [34]. In this system, so-called identification points (IP) are earned per ion the selected-ion monitoring (SIM) mode (1 IP) or per transition monitored in MS^n (1.5 IP). In high-resolution mass spectrometry, 2 IP are earned for a single ion. However, it must be considered that a prerequisite for earning IPs is that the retention time and ratios of the monitored ions are within narrow margins of those of a quality control (QC) standard or calibrator. A minimum 3 IPs is required for compound identification. Hence, monitoring of two transitions or three

Table 6
Multi-analyte procedures for screening and/or quantification in perinatal drug analysis.

Analytes	Sample	Work-up	Stationary phase	Mobile phase	Detection mode	Apparatus type	Validation data	Ref.
Nicotine, opioids, cocaine and metabolites	Fetal postmortem brain	Homogenized, SPE (HCX)	Synergi Hydro RP (75×2.0, 4 μm)	Gradient, water and ACN with 0.1% FA each	ESI+MRM (2)	QQQ/LIT	Selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, matrix effect, stability, carryover	[60]
20 drugs of abuse a nd metabolites	Meconium	"PP" SPE (HCX)	Synergi Hydro RP (150×2.0, 4 μm)	1 mM AF and ACN	ESI+MRM (2)	QQQ	Selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, matrix effect, carryover, dilution integrity, stability	[56]
Methadone, opiates, cocaine, and metabolites	Umbilical cord	PP SPE (HCX)	Synergi Polar-RP (75×2 mm, 4 μm)	Gradient, 0.1% FA and ACN	ESI+SRM (3 MS^2 or 2 MS^3)	Ion trap	Selectivity, linearity, accuracy, precision, recovery, matrix effect, carryover, hydrolysis, dilution integrity, stability	[64]
10 amphetamine-related drugs	Meconium	SPE (HCX)	Synergi Polar-RP (150×2.0 mm, 4 μm)	Gradient, 10 mM AA with 0.01% FA and ACN	APCI+MRM (2)	QQQ	Selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, matrix effect, stability	[44]
Methadone, cocaine, opiates and metabolites	Placenta	SPE (HCX)	Synergi Polar-RP (75×2 mm, 4 μm)	Gradient, 0.1% FA and ACN	ESI+SRM (3 MS^2 or 2 MS^3)	Ion trap	Selectivity, linearity, accuracy, precision, LOD, LLOQ, recovery, matrix effect, process efficiency, carryover, hydrolysis, dilution integrity, stability	[62]

Abbreviations: LLE, liquid–liquid extraction; SPE, solid-phase extraction; HCX, mixed-mode sorbent with hydrophobic and cation exchange properties; HLB, polymer sorbent with hydrophilic and lipophilic properties; ACN, acetonitrile; AF, ammonium formate; FA, formic acid; AA, ammonium acetate; MeOH, methanol; ESI, electrospray ionization; MRM, multiple reaction monitoring; IDA, information dependent acquisition; PIS, product ion scan; QQQ/LIT, hybrid triple quadrupole/linear ion trap; QQQ, triple quadrupole; TOF, time-of-flight; GC–MS, gas chromatography–mass spectrometry; LOD, limit of detection, LLOQ. lower limit of quantification; ULOQ, upper limit of quantification.

ions in single stage MS as used in most methods listed in Tables 2–6 fulfill these requirements, provided retention and ion ratios are in compliance with the acceptance intervals.

Several authors evaluated compliance with the above-mentioned EU recommendations and generally found them fulfilled [39,44,56,70]. Pistos et al. [70] performed a systematic study on compliance of cocaine, benzoylecgonine and ecgonine methyl ester identification with the IP system. The analytes were extracted from 1 mL of blood using LLE, separated on a XTerra MS C8 column (250 mm × 2.1 mm, 5 μm) using an ammonium formate/acetonitrile gradient, and detected by single quadrupole MS with an ESI+ interface and SIM. Analyzing certified reference materials, samples from proficiency tests and from real cases, they found that in real cases benzoylecgonine met the criteria in 23 out 27 samples, while ecgonine methyl ester and cocaine only met the criteria in 16 and 10 samples, respectively. The authors attributed their findings to poor in source fragmentation and low concentrations. However, it is somewhat difficult to follow their thoughts, because ion ratio findings are only reported for one ratio per compound while at least three ions (and hence two ratios) have to be monitored to acquire a sufficient number of IPs. Also, the authors speak about transitions throughout the paper while obviously referring to ions resulting from single stage MS in-source fragmentation.

Concheiro et al. [49] reported a comparatively small within-day variability of ion ratios, but considerably larger variability between days. These findings were confirmed by Roman et al. [82] who also reported considerable variability of ion ratios recorded over 26 days with CVs ranging from 9.9 to 22.3%. These findings highlight, that at least for certain compounds it can be important to adjust the reference ion ratios according to quality control or calibration samples analyzed on the same day.

Apart from the mentioned guidance documents, the need to monitor more than one ion or transition has been underlined by the recent findings of Sauvage et al. [87]. They reported case examples where monitoring of a single transition could have lead to false compound identifications and how this was avoided by the EPI-based compound identification described in reference [21]. The need to monitor more than one ion or transition is also acknowledged by most other authors as can be seen from the fact that the majority used at least two transitions in tandem MS or three ions in single stage MS (Tables 1–6). Moreover, those who have not done so are generally aware of the fact that monitoring fewer ions is not sufficient for reliable compound identification. Oiestad et al. [57] commented that a false positive diazepam result caused by promethazine might have been avoided by looking at two transitions per compound. Mazzarino et al. [76] stated that one or two transitions are sufficient for screening in doping analysis, because "suspicious" results will be further studied by specific confirmation methods.

Method validation issues

Selectivity

Multi-analyte methods are primarily used in situations where more than on drug and its metabolites must be expected to be present in the sample. A number of authors have taken this into account and performed additional selectivity experiments [38,44,47,52,53,56,57,60,64,78,81–83]. For this, they spiked (high concentrations) of numerous potentially interfering drugs or metabolites into blank matrix or QC samples with low analyte concentrations. After analysis of these samples, spiked blank samples were checked for interfering peaks while for the spiked QC samples it was checked whether the analyte concentrations could be determined with sufficient accuracy despite the presence of potentially interfering compounds.

Oiestad et al. [57] checked for interference from other drugs by spiking high concentration of 47 drugs from various drug classes in to blank oral fluid. Promethazine was found to cause a false positive result for diazepam, but this was not considered important, because nordazepam was also included in the method and, according to the authors, would also be expected in the samples in case of true positive diazepam finding. However, this seems questionable if the oral fluid sample is collected shortly after diazepam ingestion or administration.

When testing the selectivity of their method for determination of drugs of abuse in oral fluid, Fritch et al. [53] not only checked for interference from other drugs, but also included various foods and beverages into their selectivity experiments. They found high phenmetrazine concentrations interfered with detection of methamphetamine, while the tested foods and beverages did not interfere. Nevertheless, checking for his kind of interference study can be generally recommended in oral fluid analysis.

Ferreiros et al. [80] analyzed authentic plasma samples from cardiovascular patients receiving various other drugs besides the targeted angiotensin-II-receptor antagonists and found no interference from these drugs or their metabolites. The group of Beck used authentic urine samples tested positive for potentially interfering drugs to check for interferences with their analytical method [63,67].

Kuepper et al. [88] reported on a matrix peak present in plasma and urine samples that interfered with most transitions of succinylmonocholine (SMC), the main metabolite of the muscle relaxant succinylcholine. The peak could neither be chromatographically separated nor could it be removed from the sample without removing the analyte as well. The only possibility to differentiate SMC from the unknown interfering compound was monitoring of two minor transitions of SMC which were not present in the unknown interference. These findings clearly highlight the need for extensive selectivity experiments and for monitoring at least two selective transitions per compound.

Matrix effect

Matrix effects refer to "the direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample" [89]. Matrix effects can either reduce the analyte response (ion suppression) or enhance it (ion enhancement). Both can considerably compromise the accuracy of quantification and ion suppression may in the worst case even lead to false negative results. Meanwhile, it has been widely accepted that experiments on matrix effects are an essential part in the validation of any LC–MS(MS)-based methods. Nevertheless, there are still publications in which potential matrix effects have not been adequately addressed [43,45,58,74,77,83].

Saar et al. [90] systematically compared different methods for extraction of 19 antipsychotics in human blood with respect to extraction efficiency and matrix effects. The authors evaluated nine different LLE procedures (combination of three buffers and three extraction solvents) and one SPE method. They also used spiked antemortem and postmortem blood samples, the latter further including non-decomposed and severely decomposed samples. For antemortem and non-decomposed postmortem blood, they found that LLE with Trizma buffer and 1-chlorobutane yielded acceptable extraction efficiencies (generally well over 60%) and matrix effects (−26 to +59%). These results were further comparable to those obtained with SPE. For postmortem samples, the mentioned procedures also yielded the best of the tested results. However, the majority of analytes showed considerable matrix effects (suppression or enhancement) in some of the tested decomposed samples. These results clearly show that methods intended for postmortem toxicology should consider a much higher risk of matrix effects. This was also confirmed by Roman et al. [82] who reported particularly strong matrix effects in one of five tested postmortem blood samples. In contrast, Taylor et al. [59] observed only moderate matrix effects in five different postmortem blood samples tested during method validation. This could either be attributable to the sample preparation and

chromatographic separation in this study or simply be a coincidental finding, because five samples are of course not representative for all postmortem blood samples.

Another paper including systematic experiments on matrix effects was recently published by Marchi et al. [91]. The aim of this study was to propose a simple and low-cost strategy for developing sample preparation methods for multi-analytes. Using a mixture of 34 model compounds representing compounds with different physicochemical properties, they evaluated different mixed-mode cation and anion exchange sorbents SPE as well as a so-called HLB sorbent. A mixed-mode cation exchange sorbent was found to be the best compromise in extracting all analytes and therefore used in further optimization experiments. The optimized method was then evaluated with respect to matrix effects for the full set of compounds. The authors found that most compounds were affected by more or less intense ion suppression and that suppression effects were reproducible between matrix batches in most cases. This shows that matrix-based calibration is particularly important in LC–MS(/MS) analysis, especially when no deuterated internal standards (IS) are available.

Another systematic study on ion suppression/enhancement was very recently published by Remane et al. [92]. They studied effects of non-deuterated analytes on their respective deuterated analogues with respect to concentration of the non-deuterated compounds and ionization mode. Using APCI, a statistically significant suppression effect was observed for only one out of 14 tested standards, whereas in ESI mode 12 showed statistically significant suppression effects and another showed significant enhancement. While this may not be a problem when using the deuterated compound as internal standard for the respective non-deuterated analyte, it may cause serious quantification bias when the deuterated compound is also used as IS of compounds other than the respective non-deuterated compound. Therefore, the authors concluded that such ion suppression/enhancement studies are essential for such situations and recommended to use APCI unless other reasons speak against this. However, the data presented in this reference partly show a large variability of ionization in the APCI mode, which could become a problem of its own when using this ionization mode. Also, matrix effects may well be present despite using APCI as found by Kelly et al. [44] who observed matrix effects ranging from −14% to up to almost 50%.

Many authors reported considerable matrix effects for at least part of the monitored analytes [37–39,54,57,64,67,82]. Particularly pronounced matrix effects were observed in analysis of oral fluid, diluted urine, and of highly complex matrices such as brain. When analyzing preserved oral fluid samples, Concheiro et al. [39] observed almost 100% ion enhancement for tetrahydrocannabinol whereas extensive ion suppression was observed for cocaine (−74%), zolpidem (−68%) and zopiclone (−75%). The latter was attributed to effects caused by the sampling buffer. Besides these findings, matrix effects were in part found to be highly variable between matrix batches as indicated by a CV of over 37% in the case of methamphetamine. Badawi et al. [40] found moderate matrix effects from neat oral fluid and an oral fluid buffer mixture from the Statsure sampling device, whereas synthetic oral fluid suppressed the signal of 9 analytes (7 benzodiazepines, zolpidem, and methadone) by almost 100%. Oiestad et al. [57] also reported in part large matrix effects that for some drugs further showed a large variability between blank matrix batches. Only part of these could be compensated by deuterated internal standards since such were only available for part of the analytes. However, the authors considered these findings acceptable for a screening only method. Massive ion suppression was also reported in analysis of brain samples despite previous clean-up by SPE. Signals were reduced by up to 90%, but this reduction was effectively compensated by stable-isotope-labeled IS [60]. Considerable ion suppression was also reported for morphine (−30%) and its glucuronides (−50%) in analysis of diluted urine, but this was again compensated for by stable-isotope-labeled IS [37,67].

The presented examples clearly show, that testing for and dealing with matrix effects is one of the key issues in LC–MS(/MS) analysis. In this context it is important to state that even extensive matrix effects may be acceptable as long as they are reproducible between matrix batches and as long as the sensitivity of the method is still acceptable. For these reasons, Viswanathan et al. [93] proposed an acceptance limit of <15% CV for the variability of matrix effects between matrix batches.

Conclusions and perspectives

LC–MS(/MS) has become increasingly important in clinical and forensic toxicology especially for multi-target screening and/or quantification of drugs, poisons and their metabolites in conventional and alternative biomatrices. In recent years, there has been considerable progress in LC–MS(/MS) based STA thanks to information-rich product ion spectra. However, the problem of reference libraries not being transferable between different instrument types still remains to be solved. With the increasing availability of benchtop TOF-MS instruments an alternative screening approach based on high-resolution MS has been developed, which at least theoretically could encompass compounds for which no reference standards are available. Currently the versatility of this approach in routine analysis is hampered by the problem of differentiating between isomeric compounds. It will be interesting to see whether the currently proposed *in silico* or metabolomic approaches will ultimately help to overcome this problem. The very recent development of benchtop orbitrap mass spectrometers has further opened the possibility to combine information-rich mass spectra and high-resolution MS in a single approach [94].

With respect to analyte separation, UPLC has the potential to dramatically reduce chromatographic run-times. This potential can currently not be fully exploited, because MS cycle times in multi-analyte LC–MS(/MS) are the limiting factor for analysis time. However, given the speed of development in MS hard- and software development this problem may be overcome in the future.

Besides these new developments, there are well-known points of debate which hopefully will come to conclusion in the near future. The first is the need for widely accepted criteria for mass spectrometry-based compound identification in clinical and forensic toxicology. The second relates to the type and extent of experiments to assess matrix effects and again to establishing widely accepted acceptance criteria for this parameter.

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