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**Untersuchung von Matrixeffekten in der quantitativen Analyse mit
Flüssigkeitschromatographie-Tandem Massenspektrometrie –
Bestimmung, Kompensation und Methodenentwicklung**

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Inhaltsverzeichnis

1	EINLEITUNG	1
1.1	ALLGEMEIN	1
1.1.1	Massenspektrometrie.....	1
1.1.2	Kopplung von Flüssigkeitschromatographie und Tandem-Massenspektrometrie.....	2
1.1.3	Matrixeffekte: Ursachen und Folgen.....	3
1.1.4	Methoden zur Detektion von Matrixeffekten	5
1.1.5	Methoden zur Minimierung von Matrixeffekten.....	5
1.1.6	Methoden zur Kompensation von Matrixeffekten in der quantitativen Analytik	7
1.2	PUBLIKATIONEN DER TEILPROJEKTE	10
1.2.1	Rossmann, J., Schubert, S., Gurke, R., Oertel, R., Kirch, W. Simultaneous determination of most prescribed antibiotics in multiple urban wastewater by SPE-LC–MS/MS. Journal of Chromatography B 969, 2014, 0, 162-170	10
1.2.2	Rossmann, J., Gurke, R., Renner, L.D., Oertel, R., Kirch, W. Evaluation of the matrix effect of different sample matrices for 33 pharmaceuticals by post-column infusion. Journal of Chromatography B, 2015, 1000, 84-94.....	11
1.2.3	Rossmann, J., Renner, L.D., Oertel, R., El-Armouche, A. Post-column infusion of internal standard quantification for liquid chromatography-electrospray ionization-tandem mass spectrometry analysis – Pharmaceuticals in urine as example approach. Journal of Chromatography A, 2018, 1535, 80-87	12
1.3	ZUSAMMENFASSUNG/SUMMARY.....	14
1.4	LITERATURVERZEICHNIS	17
2	ORIGINALPUBLIKATIONEN	19
2.1	ROSSMANN, J., SCHUBERT, S., GURKE, R., OERTEL, R., KIRCH, W. SIMULTANEOUS DETERMINATION OF MOST PRESCRIBED ANTIBIOTICS IN MULTIPLE URBAN WASTEWATER BY SPE-LC–MS/MS. JOURNAL OF CHROMATOGRAPHY B 969, 2014, 0, 162-170.....	19
2.2	ROSSMANN, J., GURKE, R., RENNER, L.D., OERTEL, R., KIRCH, W. EVALUATION OF THE MATRIX EFFECT OF DIFFERENT SAMPLE MATRICES FOR 33 PHARMACEUTICALS BY POST-COLUMN INFUSION. JOURNAL OF CHROMATOGRAPHY B, 2015, 1000, 84-94	29
2.3	ROSSMANN, J., RENNER, L.D., OERTEL, R., EL-ARMOUCHE, A. POST-COLUMN INFUSION OF INTERNAL STANDARD QUANTIFICATION FOR LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION-TANDEM MASS SPECTROMETRY ANALYSIS – PHARMACEUTICALS IN URINE AS EXAMPLE APPROACH. JOURNAL OF CHROMATOGRAPHY A, 2018, 1535, 80-87.....	41
	Erklärungen zur Eröffnung des Promotionsverfahrens	65
	Erklärung zur Einhaltung rechtlicher Vorschriften	68
	Erklärung zur Abgrenzung des Eigenanteils des Verfassers	69
	DANKSAGUNG	70

1 Einleitung

1.1 Allgemein

Quantitative Analysenmethoden spielen eine wichtige Rolle in Medizin, Biologie und Umweltwissenschaften, z. B. bei der klinischen Entwicklung von Arzneimitteln, bei Drogen- und Dopingkontrollen oder dem Umweltmonitoring von anthropogenen Spurenstoffen. Die Quantifizierung von Substanzen mit oft sehr niedriger Konzentration erfolgt häufig in komplexen Probenmatrices wie Urin, Blut, Gewebeextrakten oder Umweltproben. Dafür sind hoch empfindliche, selektive und präzise Methoden erforderlich. Eine sehr leistungsfähige Technik für die quantitative Analyse in komplexen Matrices ist die Flüssigkeitschromatographie (liquid chromatography, LC) gekoppelt mit der Tandem-Massenspektrometrie (MS/MS).

1.1.1 Massenspektrometrie

Die Massenspektrometrie (MS) ist ein Verfahren für die Bestimmung von Molekülmassen und dient der Strukturaufklärung sowie der quantitativen Analytik. Dabei werden die Moleküle zunächst in positiv oder negativ geladene Ionen überführt, wobei zusätzlich ein Zerfall in spezifische Bruchstücke (Fragmentierung) erfolgen kann. Die geladenen Teilchen werden anschließend nach ihrem Masse-Ladungs-(m/z)-Verhältnis aufgetrennt und ihre Signal-Intensität bestimmt. Es lassen sich vier verschiedene Analysatoren unterscheiden: Sektorfeld-, Flugzeit-, Ionenfallen- und Quadrupol-Massenspektrometer. Für die quantitative Analytik spielt das Quadrupol-Massenspektrometer die größte Rolle. Eine höhere Spezifität wird erreicht, indem massenselektierte Ionen einer zweiten Massenanalyse unterzogen werden, der sogenannten Tandem-Massenspektrometrie (MS/MS). Das Triple-Quadrupol-Massenspektrometer ist der am häufigsten verwendete Analysator unter den Tandem-Massenspektrometern und besteht aus drei aufeinanderfolgenden Quadrupolen. Zwei Quadrupole operieren als m/z -Analysatoren und der mittlere Quadrupol funktioniert als eine Kollisionszelle. Im ersten Quadrupol werden ausgewählte Molekülionen selektiert, diese werden im zweiten Quadrupol mittels hoher Energie und Kollisionsgas in spezifische Fragmente dissoziiert, welche dann nachfolgend im dritten Quadrupol wiederum selektiert und in der Intensität analysiert werden.

1.1.2 Kopplung von Flüssigkeitschromatographie und Tandem-Massenspektrometrie

Die Flüssigkeitschromatographie (liquid chromatography, LC) ist ein Trennverfahren, das auf den unterschiedlichen Wechselwirkungen und Verteilungen von Substanzen zwischen einer mobilen und einer stationären Phase beruht. Der Probenextrakt wird in die mobile Phase (Laufmittel) injiziert und mittels diesem durch die stationäre Phase (analytische Trennsäule) transportiert. Dabei kommt es aufgrund von unterschiedlichen Wechselwirkungen und Verteilungen der Substanzen zu einer Auftrennung des Stoffgemisches. Je nach Zielanalyten und Anforderungen können verschiedene Trennmechanismen wie Normalphasen-, Umkehrphasen-, Ionenaustausch- und Größenausschlusschromatographie gewählt werden. Die gebräuchlichste Trenntechnik ist die Umkehrphasen (reversed phase, RP) -Chromatographie, die wässrige polare Lösungen als mobile Phasen und unpolare an Silicagel gebundene C18-Ketten als stationäre Phasen verwendet (auch als C18-modifizierte Kieselgelphasen bezeichnet). Prinzipiell wird die RP-Chromatographie zur Trennung von Verbindungen durch ihre Hydrophobie und Polarität angewendet. Die RP-Chromatographie wird für kleine bis mittelgroße Verbindungen von niedriger bis mittlerer Polarität eingesetzt.

Bei der Kopplung von LC und MS müssen die Zielanalyte aus dem Flüssigkeitsstrom in die Gasphase überführt und gleichzeitig ionisiert werden, was erst durch die Entwicklung von Atmosphärendruck-Ionisierungstechniken (API Quellen) durch Fenn (Fenn et al., 1989) in den 1980er Jahren ermöglicht wurde. Prinzipiell gibt es drei Techniken der Atmosphärendruckionisierung: die Atmosphärendruck-chemische Ionisation (atmospheric pressure chemical ionization, APCI), die Atmosphärendruck-Photoionisation (atmospheric pressure photo ionization, APPI) und die Elektrospray-Ionisation (electrospray ionization, ESI). Die Analyt-Ionen werden bei der APCI durch eine chemische Reaktion mit einem Reaktandgas-Plasma erzeugt und bei der APPI durch eine UV-Lichtquelle.

In der vorliegenden Arbeit wurde die Elektrospray-Ionisierung untersucht, wobei die Ionisierung von der kondensierenden Phase bis zu isolierten Ionen in der Gasphase im Atmosphärendruck erfolgt. Durch diese Technik werden selbst große Moleküle wie Proteine und Peptide leicht ionisiert und dabei nur wenig fragmentiert. Die in über 80 % der LC-MS/MS-Kopplung verwendete Ionisierung mittels Elektrospray-Ionisation ermöglicht darüber hinaus die massenspektrometrische Analyse von kleinen polaren, thermisch labilen oder schlecht verdampfbaren Molekülen der Pharmazeutika und Pestizide.

Seither ist die LC-MS/MS-Kopplung eine weit verbreitete Methode zur quantitativen und qualitativen Analyse in den Bereichen Bio-, Lebensmittel- und Umweltanalytik, da sie für eine Vielzahl von Analyten, Probenmatrices und analytischen Anforderungen geeignet ist.

1.1.3 Matrixeffekte: Ursachen und Folgen

Für eine störungsfreie LC-MS/MS-Analytik müssen Verunreinigungen aus den Proben bestmöglich abgetrennt werden. Jedoch sind die Zielanalyte trotz Probenvorbereitung und nach Flüssigkeitschromatographie oft nicht vollständig von Begleitsubstanzen isoliert. Eine hohe Komplexität der Proben (Probenbestandteile und -beschaffenheit) kann insbesondere bei der quantitativen Analytik mittels LC-MS/MS zu falschen Ergebnissen führen. Je nach Einsatzgebiet der LC-MS/MS-Technik können die Begleitsubstanzen in der Probenmatrix, welche Salze, Fette, Proteine, Zucker und viele weitere Bestandteile ausmachen, die Messung von Zielanalyten beeinflussen und zu Problemen und Fehlern führen. Die durch Probenbestandteile und Verunreinigungen verursachten Folgen werden allgemein als Matrixeffekte bezeichnet. Die „International Union of Pure and Applied Chemistry“ (IUPAC) definiert den Matrixeffekt als Summe aller Einflüsse auf die Messung eines Analyten, die von anderen Komponenten der Probe ausgehen (Guilbault et al., 1989).

Als Ursache für den Matrixeffekt während der LC-MS/MS-Messung werden Wechselwirkungen in der besonders häufig verwendeten ESI vermutet. Der Ionisierungsmechanismus der ESI lässt sich in vier Abläufe unterteilen (Abbildung 1): i) Anlegen einer elektrischen Hochspannung an der Sprühkapillare und Herstellung des Taylorkegels, ii) Erzeugung eines Sprays geladener Tröpfchen, unterstützt durch das Nebulizer Gas, iii) Lösungsmittelverdampfung und Erhöhung der Ladungsdichte innerhalb der Tröpfchen, iv) Rayleighlimit, also coulombsche Abstoßung gleicher Ladungen und Oberflächenspannung befinden sich im Gleichgewicht und es kommt zur „Coulomb Explosion“, ein Auseinanderreißen der Tröpfchen in kleinere Tröpfchen. Dieser Ablauf aus Verdampfung, Erhöhung der Dichte, Abstoßung und „Coulomb Explosion“ wiederholt sich bis die Molekülonen isoliert in der Gasphase vorliegen. Die Ionisierung kann je nach angelegter Spannung im ESI⁺- oder ESI⁻-Modus durchgeführt werden und ermöglicht jeweils die Bildung von positiv oder negativ geladenen Ionen.

Abbildung 1. Schematischer Ablauf der Elektrospray-Ionisierung bei der LC-MS/MS-Kopplung

Innerhalb der Tröpfchen befinden sich neben den Zielanalyten Begleitsubstanzen aus der Probenmatrix. Die Begleitsubstanzen haben aufgrund ihrer Konzentration, Ionisierbarkeit, Oberflächenaktivität oder Moleküloberfläche Einfluss auf die Ionisierungseffektivität von gleichzeitig vorliegenden Zielanalyten. Der Ionisierungsmechanismus ist ein dynamischer Prozess, bei dem es zu Ladungstransferreaktionen kommen kann (Kearle et al., 1993; Enke et al., 1997).

Die Folge davon ist, dass die Ionisierungseffizienz durch Gleichgewichts- oder Verdrängungsmechanismen verändert wird. Damit ist die Signalgröße nicht mehr allein von der Konzentration der Analyten abhängig, sondern auch von der Matrix. Wird der Matrixeffekt nicht beachtet, kann das zu Fehlern bei der Quantifizierung führen.

Matrixeffekte sind ein Hauptgrund für eine verringerte Sensitivität, schlechte Reproduzierbarkeit und Richtigkeit bei der Anwendung von LC-MS/MS vorwiegend, wenn Analyte in geringer Konzentration oder in komplexer Matrix untersucht werden, z. B. Urin-, Plasma-, Abwasser- oder Umweltproben.

1.1.4 Methoden zur Detektion von Matrixeffekten

Der Matrixeffekt kann mit geeigneten Methoden, wie der „post extraction addition“ (Buhrman et al., 1996) oder der „post-column infusion“ (Bonfiglio et al., 1999), quantifiziert und sichtbar gemacht werden. Bei der „post extraction addition“ wird eine bekannte Konzentration einer Substanz mittels LC-ESI-MS/MS in matrixfreier Lösung und in extrahiertem Probenextrakt gemessen und die Messsignale anschließend verglichen. Unterscheiden sich die Signale der beiden Messungen, unterliegt die Substanz während der Messung in dem Probenextrakt einem Matrixeffekt. Die „post extraction addition“ ermöglicht die genaue Quantifizierung des Matrixeffekts eines Probenextrakts für untersuchte Analyte und zu deren Retentionszeiten. Jedoch werden die Matrixeffekte in anderen Bereichen der chromatographischen Trennung als der untersuchten Substanzen nicht erfasst. Die „post-column infusion“ (PCI) ermöglicht die Bestimmung des Matrixeffekt-Profiles für die gesamte chromatographische Trennung. Bei dieser Methode wird eine Analytlösung zwischen chromatographischer Säule und Ionisierungsquelle in den Flüssigkeitsstrom infundiert und liefert kontinuierlich ein gleichbleibendes Signal. Wird ein Matrixextrakt injiziert und chromatographisch getrennt, erreichen dessen Bestandteile aufeinanderfolgend die Ionenquelle und können die Ionisierungseffizienz der infundierten Analytlösung beeinflussen. Die beobachtete Signalunterdrückung oder -verstärkung charakterisiert den Matrixeffekt.

1.1.5 Methoden zur Minimierung von Matrixeffekten

Um den Matrixeffekt zu minimieren, versucht man, die Analyte von der Matrix zu trennen oder die Konzentration störender Probenbestandteile zu verringern. Dafür gibt es zwei prinzipielle Möglichkeiten, die Probenvorbereitung und die chromatographische Trennung. Beide werden meist parallel optimiert. Als Probenvorbereitung dienen beispielsweise Extraktion, Ausfällung oder Verdünnung der Probe.

Der Extraktionsprozess erfolgt zwischen verschiedenen nicht mischbaren Phasen, wobei die durch die physikalisch-chemischen Eigenschaften bedingte Verteilung der Substanzen zwischen den Phasen genutzt wird. Die Phasen können flüssig, fest oder gasförmig sein und verschieden kombiniert verwendet werden. Abhängig von dem Verfahren ist die Extraktion ein Adsorptions-, Lösungs- oder Verteilungsprozess. Für

flüssige Proben sind die Fest- (solid phase extraction, SPE) und Flüssigphasenextraktionsmethoden am meisten etabliert. Die Festphasenextraktion ist aufgrund der Vielfalt an Phasenmaterialien wie Normal-, Umkehr-, Ionenaustausch- und auch kombinierten Phasen für viele Verbindungen und Ionen anwendbar, auch ist sie leichter automatisierbar.

Die Festphasenextraktion aus der flüssigen Probe erfolgt mittels eines geeigneten Extraktionsmaterials, das in eine Kartusche gepackt ist. Je nach gewähltem Festphasenmaterial können Verbindungen von polarem, unpolarem und ionischem Charakter aus der Probe adsorbiert werden. Da alle Substanzen, die ähnliche Eigenschaften aufweisen, an das Extraktionsmaterial gebunden werden, ist das Verfahren unter Umständen unspezifisch. Das Problem verstärkt sich, wenn gleichzeitig verschiedene Analyte mit unterschiedlichen physiko-chemischen Eigenschaften bestimmt werden sollen. Sind die Zielkomponenten aus der flüssigen Probe an die feste Phase gebunden, werden Verunreinigungen wie Salze oder fälschlich gebundene Komponenten mittels Waschlösungen aus der Kartusche ausgewaschen. Anschließend werden die Analyte mittels eines geeigneten Lösungsmittels von der Kartusche eluiert. Das Eluat kann eingeeengt werden, um eine Konzentrationserhöhung der Analyten zu erreichen. Dabei werden jedoch auch nicht abgetrennte, störende Matrixbestandteile angereichert.

Neben der Extraktion stellt die Proteinfällung eine einfache Methode dar, um die Menge an gelösten Proteinverbindungen einer Probe aus Plasma oder Blut zu reduzieren. Auch kann eine einfache Verdünnung der Probe die Gesamtdichte gelöster Moleküle in einer komplexen Probe reduzieren, erfordert aber entsprechend niedrige Nachweisgrenzen. Unter Umständen ist jedoch ein sehr hoher Verdünnungsansatz notwendig, um vernachlässigbare Matrixeffekte zu erreichen.

Die Flüssigkeitschromatographie dient neben der selektiven Trennung von Zielkomponenten auch der Auf- bzw. Abtrennung von Verunreinigungen. Die Elution kann über die zeitliche Änderung der Laufmittelzusammensetzung so optimiert werden, dass alle Bestandteile der Probe aufeinanderfolgend den Detektor erreichen. Damit können Matrixeffekte aufgrund von Co-eluierenden Bestandteilen verringert werden.

Jedoch kann eine intensivierte Trennung lange Retentionszeiten erfordern und insgesamt die Analysenzeiten verlängern. Auch sind die Trennleistung und die Abtrennung von Verunreinigungen jeweils abhängig von der Probenzusammensetzung und müssen dementsprechend für jeden Probenotyp untersucht werden.

1.1.6 Methoden zur Kompensation von Matrixeffekten in der quantitativen Analytik

Da Matrixbestandteile bei der Reinigung komplexer Proben häufig trotz Probenvorbereitung und Chromatographie nicht vollständig abgetrennt werden, wurden verschiedene Methoden entwickelt, um den Matrixeffekt zu berücksichtigen und zu kompensieren (Stuber et al. 2004; Krue et al. 2013).

Die am häufigsten verwendeten Quantifizierungsmethoden sind die Standardaddition (standard addition), die Matrix-Kalibrierung (Matrix-matched external calibration) und der Einsatz von internen Standards oder isotoopenmarkierten internen Standards (isotopically labeled internal standards). Eine alternative, jedoch nicht häufig verwendete Methode ist die Nachsäuleninfusion von isotoopenmarkierten Substanzen.

Bei der Standardaddition wird die Probe mehrfach gemessen: original und mindestens einmal zusätzlich, versetzt mit bekannter Konzentration des Zielanalyten. Über das Verhältnis der Messwerte kann die Konzentration der ursprünglichen Probe berechnet werden. Die Methode ist einfach in der Methodenentwicklung und zuverlässig in der Matrixeffektkompensation der jeweiligen Analyte. Jedoch kommt es durch die Mehrfachbehandlung zu einem erhöhten Arbeits-, Material- und Zeitaufwand und zusätzlich muss der zu erwartende Konzentrationsbereich der Probe für eine richtige Quantifizierung bekannt sein.

Die Matrix-Kalibrierung wird häufig für Proben eines einheitlichen Matrixtyps verwendet, wobei die Kalibrierstandards der Analyte in einer vergleichbaren analytffreien Matrix hergestellt werden. Die Kompensation des Matrixeffekts basiert auf der Annahme von vergleichbarem Verhalten des Analyten in Kalibrierlösungen aus z. B. Serum und Analyt in der jeweiligen Serumprobe. Es wird davon ausgegangen, dass der Matrixeffekt der Kalibrierstandards und jeder einzelnen Serumprobe gleich ist. Das ist jedoch für viele Probentypen nicht der Fall (Abwasserproben, Patientenurin). Hinzu kommt, dass eine analytffreie Vergleichsmatrix nicht immer zur Verfügung steht. Daher ist die Matrix-Kalibrierung u. a. bei Abwasseruntersuchungen nicht möglich.

Die Verwendung von internen Standards (IS), strukturverwandte Substanzen oder isotoopenmarkierte Analoga der Analyte, ist die heutzutage am häufigsten etablierte Quantifizierungsmethode. Der Einsatz von isotoopenmarkierten Analoga ist dabei die bevorzugte Vorgehensweise, da diese das Verhalten der Analyte sehr gut repräsentieren. Die isotoopenmarkierten Analoga unterliegen den gleichen Extraktions- und chromatographischen Trennmechanismen, können aber mittels

Massenspektrometrie, aufgrund anderer Molekülmasse von den Analyten differenziert analysiert werden. Die Standards und Proben werden mit konstanter Konzentration des IS versetzt. Während das Verhältnis von IS und Analyt der Standards eine Funktion für die Analyt-Konzentration liefert, kann das Signalverhältnis von IS und Analyt in der Probe zur Konzentrationsbestimmung herangezogen werden. Gleichzeitig bietet der IS die Möglichkeit, Fehler während der Probenvorbereitung zu korrigieren. Die Quantifizierung über isotopenmarkierte IS ist eine sehr genaue und zuverlässige Methode.

Jedoch ist eine große Anzahl an Standardsubstanzen, für jeden Analyt der isotopenmarkierte Analoga, nötig. Diese sind in ihrer Anschaffung oft sehr teuer und teilweise nicht oder nur temporär erhältlich. Daher wird zur Kompensation häufig eine strukturähnliche Substanz als IS verwendet, die aber nicht exakt den Analyt in Extraktionsverhalten und chromatographischer Trennung repräsentiert. Außerdem wurde bisher beobachtet, dass Analyt und isotopenmarkiertes Analogon sich gegenseitig beeinflussen können. Insgesamt verändert die interne Standardmethode die ursprüngliche Zusammensetzung der Probe und ist daher auch für die Verwendung zur Matrixeffektkompensation limitiert.

Eine alternative Methode zur Matrixkompensation ist die Nachsäuleninfusion von Standardsubstanzen. Dabei wird eine Standardsubstanz verwendet, die die Matrixeffekte resultierend aus der chromatographischen Trennung widerspiegelt und für die Matrixeffektkompensation aller getrennten Analyte herangezogen wird. Die Standardsubstanz wird dem Flüssigkeitsstrom nach der chromatographischen Trennung in gleichbleibender Konzentration infundiert, sodass diese über den gesamten Messzeitraum eine konstante Signalantwort liefert. Wie bei der klassischen IS-Quantifizierung, wird das Signalverhältnis von infundiertem IS und Analyt zur Quantifizierung verwendet und müssen Standards der Analyte vermessen werden, um die Beziehung von infundiertem IS und Analyt zu beschreiben. Dabei können, als zu infundierende Standardsubstanzen, isotopenmarkierte Analoga oder auch in den Proben nicht enthaltene Substanzen verwendet werden. Die Vorteile der Quantifizierung mittels Nachsäuleninfusion eines IS sind ein deutlich reduzierter Verbrauch von Standardsubstanz und eine Quantifizierung unabhängig von isotopenmarkierten IS. Die Nachsäuleninfusion ermöglicht es, verschiedene Analyte unterschiedlicher Fragestellungen und Methoden mit einem infundierten IS zu bestimmen. Die Methode kann um beliebig viele Analyte erweitert werden. Dazu wird der neue Analyt einer Probenreihe eines Matrixtyps in bekannter Konzentration zugesetzt und mit infundiertem

IS über LC-MS/MS gemessen. Der integrierte Analyt und infundierter IS müssen dabei ein konstantes Signal-Verhältnis in den Testmessungen der Probenmatrix zeigen.

Da der nachsäuleninfundierte IS zufällige Fehler während der Probenvorbereitung nicht korrigiert, sollte ein zusätzlicher IS in die Proben integriert werden. Bisher zeigten mehrere Forschergruppen den erfolgreichen Einsatz der Nachsäuleninfusion von IS zur Quantifizierung in verschiedenen Methodenentwicklungen (Choi et al., 1999; Stahnke et al., 2009; Liao et al., 2014; Gonzales et al., 2015).

1.2 Publikationen der Teilprojekte

Übergeordnetes Ziel dieser Arbeit war die Untersuchung und Kompensation des Matrixeffektes für die Analytik von Arzneimitteln in komplexen Probenmatrices mit LC-ESI-MS/MS Technik. Aufgeteilt wurde die Arbeit in drei Teilprojekte:

1.2.1 Rossmann, J., Schubert, S., Gurke, R., Oertel, R., Kirch, W. Simultaneous determination of most prescribed antibiotics in multiple urban wastewater by SPE-LC–MS/MS. *Journal of Chromatography B* 969, 2014, 0, 162-170

Ziel des ersten Teilprojekts war die Entwicklung einer LC-ESI-MS/MS-Analysenmethode für die Bestimmung verschiedener Antibiotika in Abwasserproben in einem Langzeitmonitoring über ein Jahr. Als relevante Antibiotika wurden die am häufigsten verschriebenen Substanzen in Dresden anhand der Verschreibungsdaten der AOK Plus ermittelt. Neben den ausgewählten Mehrklassenantibiotika Amoxicillin, Penicillin V und Piperacillin (Penicilline), Cefotaxim und Cefuroxim (Cephalosporine), Azithromycin, Clarithromycin und Roxithromycin (Makrolide), Ciprofloxacin und Levofloxacin-Ofloxacin (Fluorchinolone), Clindamycin (Lincosamid), Doxycyclin (Tetracyclin), Sulfamethoxazol (Sulfonamid) und Trimethoprim (Dihydrofolat-Reduktase-Hemmer) wurde der bioaktive Metabolit Clindamycin-Sulfoxid, das Reserveantibiotikum Vancomycin (Glycopeptid) und als Tracer der Kläranlage das Antikonvulsivum Carbamazepin und das Antimykotikum Fluconazol ausgewählt. Die chemische Diversität der ausgewählten Antibiotika machte es notwendig, einen RP- und einen HILIC-Trennmechanismus für die Chromatographie von unpolaren und polaren Substanzen anzuwenden. Für die Detektion mit positiver Elektrosprayionisierung (ESI^+) wurde ein Triple-Quadrupol-Massenspektrometer (MS/MS) und Mehrfachreaktionsüberwachung (multiple reaction monitoring, MRM) verwendet. Für die Probenvorbereitung wurde die automatisierte Festphasenextraktion über eine Oasis HLB-Kartusche gewählt. Die Wiederfindung der Analyte, bestimmt in einer Vergleichsmatrix (Urin), variierte von 35 % für Roxithromycin bis zu 103 % für Clindamycin und spiegelt die hohe Diversität der Analyte wider. Die 24-Stunden-Abwassermischproben wurden während unterschiedlicher Reinigungsstufen der Kläranlage Dresden-Kaditz genommen und an ausgewählten Stellen innerhalb des Dresdner Kanalnetzes. Dadurch wiesen die Abwasserproben eine starke Variabilität im Grad der Verunreinigung auf. Neben stark verunreinigten Abwasserproben der Kanalisation wurde häufig auch verdünntes Abwasser an Regentagen oder gereinigtes Abwasser der Kläranlage untersucht. Es gab weder analytfreie Matrixproben noch isotoopenmarkierte Standards für alle Zielanalyte. Um das Problem mit der stark variierenden meist sehr komplexen Matrix zu lösen, wurde mit Standardaddition

quantifiziert und somit der Matrixeffekt der jeweiligen Abwasserprobe berücksichtigt. Als Qualitätskontrolle der Messungen diene eine externe Matrix-Kalibrierung in verdünntem Urin, welche der gleichen Probenvorbereitung unterlag.

Die Analysenmethode wurde mittels der Qualitätskennwerte Linearität, Regressionskoeffizient, Präzision, Genauigkeit, Wiederfindung, Prozesseffektivität und Matrixeffekt innerhalb der Quantifizierungsgrenzen 20 - 20.000 ng/L oder 100 - 100.000 ng/L validiert. Schließlich wurde die Methode erfolgreich zur Quantifizierung der meistverschriebenen Antibiotika im Zu- und Ablauf der städtischen Kläranlage Dresden sowie im Abwasser der städtischen Abwasserkanalisation und im Krankenhausabwasser eingesetzt. Die Ergebnisse zeigten eine breite Anwendbarkeit der analytischen Methode und eine hohe Übertragbarkeit auf andere Abwassersysteme. Der Nachteil der Methode ist der relativ hohe Aufwand, denn für jede Probe sind Mehrfachmessungen erforderlich. Im zweiten Teilprojekt sollte deshalb der Matrixeffekt genauer untersucht werden mit dem Ziel, ihn mit weniger Aufwand kompensieren zu können.

1.2.2 Rossmann, J., Gurke, R., Renner, L.D., Oertel, R., Kirch, W. Evaluation of the matrix effect of different sample matrices for 33 pharmaceuticals by post-column infusion. Journal of Chromatography B, 2015, 1000, 84-94

Ziel dieses Teilprojektes war die Untersuchung des Matrixeffekts von unterschiedlichen Probenmatrices auf die Messung unterschiedlicher Arzneimittel. Die Bestimmung des Matrixeffekts wurde mit der PCI-Technik durchgeführt und sollte Anhaltspunkte zum Mechanismus des Matrixeffektes liefern. Insgesamt wurden 33 verschiedene Arzneimittel der Antibiotika, Antidepressiva, Antiepileptika, Neuroleptika, Betablocker und weitere für die Untersuchung des Matrixeffekts durch Urin, Plasma und verschiedener Abwasser ausgewählt. Die Proben von Urin, Plasma und verschiedener Abwasser wurden jeweils mit SPE wie im ersten Teilprojekt vorbereitet.

Die Ergebnisse der Matrixeffekt-Untersuchung zeigen deutlich einen vom Probentyp abhängigen Matrixeffekt für Urin, Plasma und verschiedene Abwasserproben und zusätzlich einen substanzabhängigen Matrixeffekt. Für 21 Substanzen wurde wiederholt ein sehr ähnliches Matrixeffektprofil aufgrund von Verdrängungsreaktionen mit co-eluierenden Matrixbestandteilen bestimmt, welches auch sehr gut mit dem gemessenen Gesamtionenstrom korreliert werden konnte. Die Matrixeffektprofile konnten so auch die für den jeweiligen Matrixtypen gängige Verteilung der Inhaltsstoffe widerspiegeln. Eine weitere Gruppe von Substanzen zeigte häufig einen ähnlichen Verlauf der Signalunterdrückung, jedoch teilweise mit Abweichungen in der Intensität. Für wenige

Verbindungen wurde wiederholt und überwiegend Signalverstärkung detektiert, auch in Kombination mit Signalunterdrückung. Diese Verbindungen (Sulfamethoxazol, Clindamycin, Gabapentin, Pregabalin, Nadolol, 10,11-dihydro-10-hydroxycarbamazepine (monohydroxy-Derivat des Oxcarbazepine, MHD)) wurden in ihren strukturellen Eigenschaften genau analysiert, es konnte jedoch keine eindeutige Strukturauffälligkeit für die Signalverstärkung identifiziert werden. Die Ergebnisse zeigen, dass der Matrixeffekt nicht hauptsächlich durch Verdrängungsreaktionen erfolgt, sondern auch aufgrund von anderen substanzspezifischen Eigenschaften (z. B. funktioneller Gruppen, Oberflächenaktivität, Molekülgröße, u. a.) und eine Kombination beider Mechanismen umfassen kann.

Diese gewonnenen Erkenntnisse wurden im dritten Teilprojekt eingesetzt, bei dem der Matrixeffekt mittels PCI-Technik kompensiert werden sollte.

1.2.3 Rossmann, J., Renner, L.D., Oertel, R., El-Armouche, A. Post-column infusion of internal standard quantification for liquid chromatography-electrospray ionization-tandem mass spectrometry analysis – Pharmaceuticals in urine as example approach. Journal of Chromatography A, 2018, 1535, 80-87

Ziel dieses Teilprojekts war die Entwicklung einer Nachsäuleninfusion (PCI-Technik) von internen Standards zur Kompensation von Matrixeffekten und damit zur Quantifizierung. Die derzeit meist etablierte Methode der Quantifizierung mittels Einsatz von isotopenmarkierten internen Standards ist limitiert und beinhaltet Nachteile. Die Methodenentwicklung basierte auf den Ergebnissen des vorangegangenen Teilprojekts und fokussierte sich auf die Gruppe der Substanzen mit vergleichbaren Signalsuppressionen.

Wie in der Einleitung bereits beschrieben, müssen Matrixeffekte bei der Probenvorbereitung und der Quantifizierung durch geeignete Methoden berücksichtigt werden. Im vorangegangenen Teilprojekt wurde deutlich, dass für eine große Anzahl Analyte der Matrixeffekt ähnlich ist und auf Verdrängungsmechanismen während der Ionisierung beruht. Dieser Matrixeffekt, aufgrund von Massefluss, ist während der chromatographischen Trennung über die gesamte Laufzeit messbar. Eine PCI-Substanz gibt, als interner Standard, die Matrixeffekte zu den unterschiedlichen Retentionszeitpunkten wieder.

Die Matrixeffektkompensation und -quantifizierung über einen PCI-Standard konnte erfolgreich für die Bestimmung von 16 verschiedenen Arzneimitteln in Urinproben

im ng/L-Bereich entwickelt und validiert werden. Die Methode wurde mit zwei unabhängigen PCI-Standardsubstanzen validiert. Beide Standards, die isotopenmarkierten Analoge von Trimipramin und Venlafaxin, zeigten die Eignung zur Kompensation der Matrixeffekte der 16 Analyte. Bei gleichem Aufwand liefert die PCI-Methode bessere Ergebnisse bezüglich Präzision, Richtigkeit und Nachweisgrenze im Vergleich zur externen Matrix-Kalibrierung. Die Leistung der entwickelten Methode ist ähnlich der Quantifizierung über isotopenmarkierte interne Standards, jedoch ist der Aufwand der PCI geringer, da nur ein isotopenmarkierter Standard erforderlich ist. Allerdings ist die Quantifizierungs- und Kompensationsmethode bisher nicht in kommerziell erhältlichen Quantifizierungssoftwares enthalten. Dadurch ist eine manuelle Auswertung der Rohdaten erforderlich, was kompliziert und zeitaufwendig ist. Wenn die Gerätehersteller und Softwareentwickler das Potential der PCI Methode erkannt haben, steht einer breiten Anwendung dieser Quantifizierungsmethode nichts mehr im Wege.

1.3 Zusammenfassung/Summary

Das übergeordnete Ziel dieser Promotion war die Untersuchung und Kompensation des Matrixeffekts für die Analytik von Arzneimitteln in komplexen Probenmatrices mit LC-ESI-MS/MS-Technik. Zunächst konnte eine einfache analytische Methode für eine breite analytische Anwendbarkeit entwickelt werden. Es zeigte sich jedoch, dass die Matrixeffektkompensation zu einem Mehraufwand bei der Probenvorbereitung führt. Deshalb wurde anschließend der Mechanismus des Matrixeffektes auf die LC-ESI-MS/MS-Technik genauer untersucht. Die gewonnenen Erkenntnisse wurden anschließend eingesetzt, um eine einfache alternative Quantifizierungsmethode mittels der PCI eines internen Standards zu entwickeln.

Im ersten Teilprojekt wurde eine LC-ESI-MS/MS-Methode für die Analytik von häufig verschriebenen Antibiotika in Abwasserproben der Stadt Dresden entwickelt. Da weder Vergleichsmatrix für Abwasser zur Verfügung stand, noch für alle Zielanalyte isotopenmarkierte Standards erhältlich sind, wurde der stark variierende Matrixeffekt der Abwasserproben mittels der Standardaddition kompensiert. Die Ergebnisse der Methodenentwicklung zeigen, dass eine genaue und flexible Methode entwickelt werden konnte, die Matrixkompensation jedoch zu einem erhöhten Zeit- und Materialaufwand führt. Es wurde deutlich, dass neben bisher genutzten Kompensationsmethoden für den Matrixeffekt, wie Standardaddition und interner isotopenmarkierter Standards, neue alternative Strategien getestet werden müssen.

In dem zweiten Teilprojekt wurde daher der Matrixeffektmechanismus von Urin-, Plasma- und verschiedenen Abwasserproben bei der Messung von verschiedenen Arzneimitteln mittels LC-ESI-MS/MS analysiert. Die Ergebnisse der Untersuchungen mittels „post-column infusion“ konnten bisherige Erkenntnisse zu Matrixeffektmechanismen bestätigen und das Verständnis vertiefen. Matrixeffekte sind von der jeweiligen Zusammensetzung der Probenmatrix abhängig, aber auch substanzspezifisch. Dabei kommt es zwischen Analyt und Begleitsubstanzen zu einer Konkurrenz um freie Ladungsträger oder zu einer veränderten Anordnung/Verteilung innerhalb der ESI-Spray-Tröpfchen. Gleichzeitig zeigten die Ergebnisse, dass es auch andere Mechanismen, wie z. B. Ladungstransfers zwischen Analyt und Begleitsubstanzen, geben muss.

Schließlich wurden die Ergebnisse des zweiten Teilprojekts in einer innovativen Methodenentwicklung zur Matrixkompensation und zur Quantifizierung von 16 Arzneimitteln in Urinproben verwendet. Der Matrixeffekt der Substanzen mit

vergleichbarer Signalsuppression konnte über einen einzelnen nachsäuleninfundierten internen Standard kompensiert werden. Die Ergebnisse zeigen einen deutlichen Vorteil der entwickelten Methode gegenüber Matrix-Kalibrierung in Präzision und Richtigkeit oder dem Einsatz von isotoopenmarkierten internen Standards in Aufwand der Methodenentwicklung und Verbrauch von Standardsubstanzen.

Die Ergebnisse der vorliegenden Arbeit zeigen die Bedeutung, Komplexität und den Einfluss der Matrixeffekte in der Anwendung der LC-ESI-MS/MS-Technik. Einerseits sind geeignete Methoden für die Minimierung von Matrixeffekten wie Probenvorbereitung und Chromatographie nötig, andererseits müssen Ionisierungsmechanismen, insbesondere die Wechselwirkungen von Zielanalyten und Begleitsubstanzen, zukünftig Gegenstand weiterer Untersuchungen sein. Die Ergebnisse dieser Arbeit liefern wichtige Beiträge zur Verbesserung der Analytik von komplexen Proben mittels der LC-ESI-MS/MS-Technik.

The overall goal of this Ph.D. thesis was to investigate and compensate the matrix effect of the analysis of drugs in complex sample matrices with LC-ESI-MS/MS technique. First, a simple analytical method for a broad analytical applicability was developed for wastewater analysis. However, the matrix effect compensation embraced the main part effort in sample preparation. Therefore, the mechanism of the matrix effect on the LC-ESI-MS/MS technique was examined in more detail. The findings were used to develop an alternative quantification method using post-column infusion of an internal standard substance.

In the first project, a LC-ESI-MS/MS method was developed to analyze commonly prescribed antibiotics in wastewater samples of Dresden. Since neither comparison matrix for wastewater nor all isotopically-labeled analogs for the target analytes were available, the strongly varying matrix effect of the wastewater samples was compensated by standard addition. The results show that the developed method is precise and flexible, but the matrix effect compensation leads to an increased expenditure of time and materials. Besides previously used matrix effect compensation methods, such as standard addition and internal isotopically-labeled standard, new alternative strategies need to be tested.

Therefore, the matrix effect mechanism of various drugs and sample matrix combinations was examined in the second project using post-column infusion. The results confirmed previous findings on matrix effect mechanisms and deepened our understanding that matrix effects not only depend on the composition of the sample

matrix but are also substance-specific. This results to a competition of free charge carriers between analyte and accompanying substances or to an alternated distribution within the ESI spray droplets. Furthermore, the results indicate that there are other mechanisms, such as charge transfer between analyte and concomitant substances.

The results of the second project were used to invent a method for matrix effect compensation and quantification of 16 drugs in urine samples. The matrix effects of the substances with comparable signal suppression were compensated by a single post-column infused internal standard. The developed method has a significant advantage over the matrix calibration regarding precision and accuracy as well as the use of isotopically-labeled internal standards in effort of method development and consumption of standard substances.

Finally, the results of this work show the importance, complexity and influence of the matrix effects in the application of the LC-ESI-MS/MS-technique. Suitable methods for minimizing matrix effects such as sample preparation and chromatography are needed and ionization mechanisms, in particular the interactions of target analytes with accompanying substances, should be investigated in future studies. The work of this Ph.D. project contributes to the improvement of the analysis of complex samples using the LC-ESI-MS/MS-technique.

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2 Originalpublikationen

2.1 **Rossmann, J., Schubert, S., Gurke, R., Oertel, R., Kirch, W. Simultaneous determination of most prescribed antibiotics in multiple urban wastewater by SPE-LC–MS/MS. Journal of Chromatography B 969, 2014, 0, 162-170**

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Simultaneous determination of most prescribed antibiotics in multiple urban wastewater by SPE-LC–MS/MS

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ABSTRACT

A rapid analytical method was developed for the application of a long-term monitoring (>one year) of the most prescribed and often in hospitals used antibiotics in diverse wastewaters of an urban sewage treatment plant (STP). Additionally to the selected multi-class antibiotics amoxicillin, penicillin V and piperacillin (penicillins), cefotaxime and cefuroxime (cephalosporins), azithromycin, clarithromycin and roxithromycin (macrolids), ciprofloxacin and levofloxacin–ofloxacin (fluoroquinolones), clindamycin (lincosamide), doxycycline (tetracycline), sulfamethoxazole (sulfonamide) and trimethoprim (dihydrofolate reductase inhibitor), the bioactive metabolite clindamycin–sulfoxide, the reserve antibiotic vancomycin (glycopeptide) and as tracer of the STP the anticonvulsant carbamazepine and the anti-fungal fluconazole were involved. The analytical method combines a low-sample-volume solid phase extraction (SPE), followed by a chromatographic separation using a reversed phase (RP) and hydrophilic interaction liquid chromatography (HILIC) technique, respectively, coupled to a triple quadrupole mass spectrometer. Detection was performed with multiple reaction monitoring (MRM) measured with positive electrospray ionization (ESI+). The extraction efficiency of different SPE cartridges and optimized pH-values of the preparation procedure were tested. Finally, the extraction of antibiotics was realized with the Oasis HLB cartridge and a pH adjustment at 3.5. An external calibration curve in diluted blank urine was used for quality control of the sample set of daily composite samples of the STP for the duration of one year monitoring. The squared coefficient of determination (r^2) in the concentration range (20–20,000 ng/L or 100–100,000 ng/L) of the calibration curves for the method was higher than 0.99 for all determined substances. The limit of quantification (LoQ) ranged between 0.8 ng/L (azithromycin) and 245.1 ng/L (vancomycin). Furthermore, a standard addition was used for quantification in wastewater samples. The process efficiencies ranged from 20% (doxycycline) to 134% (cefuroxime) in influent samples and from 31% (doxycycline) to 171% (cefuroxime) in effluent samples of the STP. All selected substances have been found in wastewater samples. Cefuroxime, doxycycline, levofloxacin, piperacillin, sulfamethoxazole and carbamazepine showed highest concentrations up to 6.2 µg/L.

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1. Introduction

In the last few years, pharmaceutical residue analysis has become one of the main research areas in environmental science, since several of these substances are detectable nearly ubiquitous [1,2]. Pharmaceuticals are described as environmental contaminants in low concentrations (ng/L up to µg/L). A high

amount of administered pharmaceuticals passes the human body unchanged by excretion and enters into wastewater. The excreted and unchanged pharmaceuticals pass the sewage treatment plant (STP) and the incomplete removal contribute to environmental presence [3,4].

Antibiotics are a special issue in the analysis of pharmaceutical residuals in the environment due to their potential to cause (multi-)resistance during contact with bacteria [5]. Therefore, the communal canalization and also the STP serves like a hatchery of organisms that have antibiotic resistance [6].

Several analytical methods for different classes of antimicrobial substances were described [7,8], however, for a long-term study of an urban STP, a rapid and simple analytical method is developed to

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quantify the most prescribed antibiotics. The antibiotic substances were selected from the prescriptions in ambulatory medicine and hospital facilities in Dresden, Germany (2012: 525,000 inhabitants), where an antibiotic input of veterinary medicine into the urban STP is negligible.

For the method development the antibiotics amoxicillin, penicillin V and piperacillin (penicillins), cefotaxime and cefuroxime (cephalosporins), azithromycin, clarithromycin and roxithromycin (macrolids), ciprofloxacin and levofloxacin–ofloxacin (fluoroquinolones), clindamycin (lincosamide), doxycycline (tetracycline), sulfamethoxazole (sulfonamide) and trimethoprim (dihydrofolate reductase inhibitor) and the reserve antibiotic vancomycin (glycopeptide) were selected.

The racemic ofloxacin and its (–)-S-enantiomer levofloxacin were determined together without separation. Thus, these substances are mentioned together as levofloxacin since ofloxacin is rarely prescribed. Furthermore, the main metabolite of clindamycin, clindamycin-sulfoxide was involved because of a high amount of possible back transformation in the denitrification process of the STP [9]. Additionally, the anticonvulsant carbamazepine and the antifungal fluconazole were analyzed within the method as tracer of the STP. Carbamazepine fulfills the requirements: less degradation in STP processes, less adhesion to particles, constant concentration levels and so, to be a useful tracer of STPs [10].

The simultaneous determination of those compounds is especially difficult because of big differences in their physicochemical properties [11–16]. To concentrate these substances out of aquatic samples, mostly a solid phase extraction is performed. An optimal sorbent for extraction of many of the selected β -lactam antibiotics, sulfonamides, tetracyclines, trimethoprim, macrolides and carbamazepine from wastewater is the Oasis HLB cartridge and the Strata-X [11,15–18]. Separation of these substances is mostly employed by RP-HPLC, but some of these substances show also high response while using a HILIC column [19]. Especially, fluoroquinolones like levofloxacin and ciprofloxacin [20] and the tetracyclines [21] are substances which showed good retention when separated by the HILIC technique. Other antibiotic substances like aminoglycosides [22], β -lactams, macrolides and quinolones have already been analyzed using HILIC [23].

Chromatographic separation of the substances was performed by a polar endcapped RP column for all selected classes of antibiotics and partially by the zwitterionic HILIC column for the β -lactam amoxicillin, the glycopeptide vancomycin, the macrolide clarithromycin, the lincosamide clindamycin, the fluoroquinolones ciprofloxacin and levofloxacin and the tetracycline doxycycline. This is the first report of a multiclass analysis of antibiotic substances used in human medicine that were separated by the HILIC and RP technique in the analysis of wastewater.

In the present method, antibiotics were quantified by one single standard addition and an external calibration curve measured with the samples of the assay as quality control. Thus, the use of cost-intensive isotopically labeled standards and therefore, the time consuming selection of standards were avoided by the application of this method. Additionally, this method can be modified in the selection of analytes very easily and is usable under other requirements, like investigations of other STPs or hospital facilities. Furthermore, this method will be used for highly contaminated hospital wastewater. In account of this, the concentration range of the calibration solutions (20–200,000 ng/L) for quantification was used as far as possible. A special characteristic of this method development a sample aliquot of 2.5 mL of wastewater was efficient to produce repeatable results. Most of the selected substances were detectable in all wastewater samples.

Finally, the method was successfully applied to the quantification of most prescribed antibiotics in the influent and effluent of the urban STP of Dresden as well as in wastewater of the urban sewage

canalization and in hospital wastewater. Results showed a broad applicability of the analytical method and high transferability to other sewage systems.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (ACN) and methanol, both HPLC grade (HiPerSolv), and ammonium acetate analytical grade were purchased from Merck (Darmstadt, Germany). Formic acid (conc.) LC–MS grade and disodium ethylenediamine tetraacetate (Na_2EDTA) ACS reagent were purchased from Sigma (St. Louis, MO, USA). Water (HPLC grade) was obtained from VWR (Darmstadt, Germany). Acetonitrile, 2 mM ammonium acetate solution and formic acid were used to prepare the mobile phase solvent A (3/97/0.05, v/v/v) and B (95/5/0.05, v/v/v), which were used for the preparation of the antibiotic working solution and for the LC–MS/MS experiments.

Standards of the pharmaceuticals were purchased from Sigma, Fresenius Kabi, Molekula, Fluka and Synopharm-fagron and are listed in Table 1 with corresponding informations.

Stock solutions (1 mg/mL) were prepared in methanol and stored at -20°C for a maximum of three months, except ciprofloxacin which was dissolved in methanol containing 0.5% hydrochloric acid. The working standard solution was weekly prepared in solvent mixture A and B (50/50, v/v) and stored at 4°C .

2.2. Samples

For the method development grab samples of influent and effluent samples were collected at the STP Dresden Kaditz, Germany (740,000 inhabitant equivalent, sewage water of 55 million m^3/a) and stored in amber glass bottles at 4°C until and during the transport into the lab. The stability of the antimicrobial substances and their possible degradation in 24-h composite samples of influent and effluent of the STP during storage was tested for a maximum of seven days in the dark at 4°C and considered during sample preparation. To inhibit bacterial growth and to ligate potential disturbing metal ions (those metal ions could prevent the recovery of doxycycline and other tetracyclines [16] in wastewater) 50 mL of sewage were spiked with the chelating agent Na_2EDTA to a concentration of 0.8 mg/mL, shaken for 5 min, centrifuged at $6000 \times g$ for 5 min and finally filtered through glass fibre filters ($<0.7 \mu\text{m}$, WICOM, Heppenheim, Germany). An aliquot of 2.5 mL of the sewage filtrate was taken for solid phase extraction (SPE). 10% formic acid was used for the pH adjustment of sewage samples and the SPE has been tested at pH 2.5, 3.5 and 6.5 with diluted blank urine (1:40). The tests were done with diluted urine because no blank wastewater was available. The pH adjustment was tested with 100 ng/L, 1000 ng/L and 2000 ng/L spiked samples in a threefold assay.

2.3. Solid phase extraction (SPE) and sorbent testing

The automatic SPE with a Gilson Automatic Sample Processor ASPEC XL (Middleton, WI, USA) was assumed to be the best method for the preparation of a huge number of sewage samples of a STP monitoring. The extraction efficiency of the antibiotics for the SPE was tested with two different commercially available cartridges. The Phenomenex Strata-X (30 mg/1 mL), a polymeric reversed phase sorbent, and the Waters Oasis HLB (30 mg/1 mL), a hydrophilic–lipophilic-balanced reversed phase sorbent, were selected for the sorbent testing. The SPE procedures were performed as from the manufactures proposed [24].

The conditioning of the selected HLB cartridge was made with 1 mL of a mixture of methanol, water and conc. formic acid (90/9/1, v/v/v), secondly, with 1 mL deionized water and thirdly, with 1 mL

Table 1

Selected drugs and corresponding chemical information.

Compound	Class	Molecular weight [g/mol]	Formula	CAS-NO	Producer
Amoxicillin	β -Lactam antibiotic, penicillin	365.40	C ₁₆ H ₁₉ N ₃ O ₅ S	26787-78-0	Sigma
Azithromycin	Makrolide antibiotic	748.98	C ₃₈ H ₇₂ N ₂ O ₁₂	83905-01-5	Sigma
Carbamazepine	Anticonvulsant	236.27	C ₁₅ H ₁₂ N ₂ O	298-46-4	Sigma
Cefotaxime	β -Lactam antibiotic, cephalosporin	455.47	C ₁₆ H ₁₇ N ₅ O ₇ S ₂	63527-52-6	Fresenius Kabi
Cefuroxime	β -Lactam antibiotic, cephalosporin	424.39	C ₁₆ H ₁₆ N ₄ O ₆ S	55268-75-2	Molekula
Ciprofloxacin	Fluorchinolone antibiotic	331.34	C ₁₇ H ₁₈ FN ₃ O ₃	85721-33-1	Fluka
Clarithromycin	Makrolide antibiotic	747.95	C ₃₈ H ₆₉ NO ₁₃	81103-11-9	Sigma
Clindamycin	Lincosamide antibiotic	424.98	C ₁₈ H ₃₃ ClN ₂ O ₅ S	18323-44-9	Sigma
Clindamycin-sulfoxide	Metabolite	440.98	C ₁₈ H ₃₃ ClN ₂ O ₆ S	22431-46-5	Clearsynth
Doxycycline	Tetracycline antibiotic	444.43	C ₂₂ H ₂₄ N ₂ O ₈	564-25-0	Sigma
Fluconazole	Triazole antifungal	306.27	C ₁₃ H ₁₂ F ₂ N ₆ O	86386-73-4	Sigma
Levofloxacin-ofloxacin	Fluorchinolone antibiotic	361.37	C ₁₈ H ₂₀ FN ₃ O ₄	100986-85-4	Sigma
PenicillinV	β -Lactam antibiotic, penicillin	350.39	C ₁₆ H ₁₈ N ₂ O ₅ S	87-08-1	Fluka
Piperacillin	β -Lactam antibiotic, penicillin	517.56	C ₂₃ H ₂₇ N ₅ O ₇ S	66258-76-2	Fresenius Kabi
Roxithromycin	Makrolide antibiotic	837.05	C ₄₁ H ₇₆ N ₂ O ₁₅	80214-83-1	Sigma
Sulfamethoxazole	Sulfonamide bacteriostatic antibiotic	253.28	C ₁₀ H ₁₁ N ₃ O ₃ S	723-46-6	Sigma
Trimethoprim	Dihydrofolate reductase inhibitor	290.32	C ₁₄ H ₁₈ N ₄ O ₃	738-70-5	Synopharm-fagron
Vancomycin	Glycopeptide antibiotic	1449.3	C ₆₆ H ₇₅ Cl ₂ N ₉ O ₂₄	1404-90-6	Sigma

of a 10 mmol/L Na₂EDTA-solution. The prepared sewage sample was loaded onto the SPE-cartridge with a flow rate of 3 mL/min, then washed with 1 mL of deionized water at 1 mL/min and dried with a gentle stream of air. Finally, the elution was performed by 0.5 mL of methanol, water and formic acid (90/9/1, v/v/v).

The extracts were dried under a gentle air stream and reconstituted with 250 μ L of solvent A and B (50/50, v/v) for LC-MS/MS analysis.

2.4. Liquid chromatography systems

The chromatographic separation of the antibiotics was performed by a reversed phase (RP) and a hydrophilic interaction liquid chromatography system (HILIC). Most antibiotics were analyzed using a 150 mm $\times$ 2.0 mm Synergi HydroRP column with 4 μ m particle size (Phenomenex, Aschaffenburg, Germany). A binary gradient at a flow rate of 0.4 mL/min was applied. The following mobile phase gradient was applied with solvent A (3/97/0.05, v/v/v) and solvent B (95/5/0.05, v/v/v) of a mixture of acetonitrile, 2 mM ammonium acetate and formic acid: 0–0.2 min 100% (v/v) solvent A; 0.2–4.2 min a linear gradient was programmed to 0% (v/v) solvent A; 1.4 min 0% (v/v) solvent A; 5.6–5.8 min a steep linear gradient to 100% (v/v) solvent A; 5.8–8.5 min 100% (v/v) solvent A.

Amoxicillin, ciprofloxacin, doxycycline and levofloxacin were determined using a 100 mm $\times$ 3.0 mm Nucleoshell HILIC 2.7 μ m column (Machery-Nagel, Düren, Germany) with the mobile phase gradient: 0–1.0 min 0% (v/v) solvent A; 1.0–3.0 min a linear gradient was programmed to 95% (v/v) solvent A; 1.3 min 95% (v/v) solvent A; 4.3–4.5 min a steep linear gradient to 0% (v/v) solvent A; 4.5–7.5 min 0% (v/v) solvent A. Ciprofloxacin, clarithromycin, clindamycin, levofloxacin and vancomycin were measurable with both systems. The injection volume was 20 μ L and the column temperature was held at 40 °C. The HPLC system consisted of an UltiMate 3000 pump and autosampler from Dionex (Idstein, Germany) and a column oven from Shimadzu (Kyoto, Japan). The system was controlled with the Chromeleon Chromatography Data System from Dionex Softron (Idstein, Germany).

2.5. Mass spectrometry

An API 4000 tandem mass spectrometer from ABSciex, (Framingham MA, USA) with an electrospray interface in the positive mode (ESI+) and ion spray voltage of 5 kV was used. The detection was performed in multiple reaction monitoring (MRM) mode. Two transitions of every compound were taken for quantification and

qualification. The product ion transition with the highest intensity was used for quantification (quantifier) and a second transition was used for confirmation (qualifier). Parameters of the detection were optimized for every compound by infusion of a standard solution (10 ng/mL) in solvents A and B (50/50, v/v). The optimized parameters for every compound are summarized in Table 2. To sustain an adequate number of signal points for every analyte during RP separation the analytes were measured in chosen time windows using scheduled technique. Data acquisition and analysis were performed with Analyst 1.6 (ABSciex, Framingham MA, USA).

2.6. Quantification and method validation

Influent and effluent wastewater samples were prepared in replicates. One pure and one standard spiked surrogate of the sample were prepared by the SPE method. The standard addition was performed by 1000 ng/L for all antibiotics except amoxicillin, cefuroxime, doxycycline and vancomycin. Those were spiked by 5000 ng/L because of higher quantitation limits in the communal and highly contaminated hospital wastewater samples and different linearity ranges. As quality control a 10-point external calibration curve in diluted blank urine in the range from 20 to 20,000 ng/L for all analytes, except 100–100,000 ng/L for amoxicillin, cefuroxime, doxycycline and vancomycin, was prepared and measured with samples of the assay. The quantification was realized in replicates by RP and HILIC chromatographic separation for the antibiotics ciprofloxacin, clarithromycin, clindamycin, levofloxacin and vancomycin.

The squared coefficient of determination for every substance was established by the quadratic regression of the calibration curves ($r^2 > 0.99$).

The method quantification and detection parameters like squared coefficient of determination (r^2), accuracy, precision of the analytical method, lower and upper limit of quantification (LLOQ and ULOQ) were determined in diluted blank urine spiked with known concentrations and prepared like the wastewater samples by SPE and were summarized in Table 6. The limit of detection (LoD) was calculated by the ratio of 3 for signal to noise and the limit of quantification (LoQ) was calculated by the ratio of 10 for signal to noise.

The accuracy of the method was evaluated by the relative deviation of measured concentration and the known added analyte concentration.

For calculating the between-run accuracy, standard solutions (100 ng/L, 1000 ng/L, 2000 ng/L, respectively, 5000 ng/L) in diluted

Table 2
Mass spectrometric parameters for detection^a

Compound	Parent ion	t_R		DP	Quantifier	CE	CXP	Qualifier	CE	CXP
		RP	HILIC							
Amoxicillin	366.0	–	4.27	56	114.0	27	10	208.0	19	12
Azithromycin	749.3	3.44	–	141	591.4	41	14	83.0	77	6
Carbamazepine	237.0	4.36	–	81	194.1	25	16	179.0	49	12
Cefotaxime	456.1	3.33	–	56	396.0	15	10	–	–	–
Cefuroxime	446.9	3.55	–	94	386.0	17	24	–	–	–
Ciprofloxacin	332.2	3.29	4.28	91	288.0	25	18	245.1	33	20
Clarithromycin	749.2	4.17	4.00	101	158.1	39	14	591.4	27	14
Clindamycin	425.0	3.55	4.26	86	126.1	39	8	377.2	27	10
Clindamycin-sulfoxide	441.2	3.32	–	66	377.0	23	10	126.1	45	12
Doxycycline	445.0	–	4.12	86	428.1	25	12	410.1	35	12
Fluconazole	306.9	3.63	–	61	238.0	21	14	220.1	25	18
Levofloxacin	361.9	3.28	4.26	76	318.1	27	20	261.1	37	16
PenicillinV	351.0	4.32	–	76	160.1	15	10	114.0	45	8
Piperacillin	518.2	4.98	–	86	143.1	23	14	160.1	17	28
Roxithromycin	837.3	4.23	–	76	679.4	29	20	158.2	47	14
Sulfamethoxazole	253.9	4.96	–	66	156.1	23	10	92.1	37	6
Trimethoprim	291.0	3.25	–	86	230.1	33	14	261.0	35	14
Vancomycin	725.6	2.88	5.96	71	144.0	23	14	1307.1	21	38

^a DP, EP, CE, CXP in volt, EP constantly 10 V, dwell time constant 25 ms for HILIC measurement

blank urine were prepared with the developed SPE procedure on 5 different days. The relative standard deviation (RSD) was calculated for every concentration of the standard solutions and provides the precision of the developed method.

The within-run accuracy was evaluated by the RSD of 9 different replicate measurements of the same prepared diluted blank urine that was spiked with 1000 ng/L, respectively, 5000 ng/L.

The recovery (RE) of the substances during the extraction procedure was studied in diluted blank urine. Therefore, the analyte peak areas of pre-SPE-spiked (100 ng/L) and post-SPE-spiked (100 ng/L) urine samples were compared.

The process efficiency (PE) of the method was studied in diluted urine and in wastewater samples. Calculations were performed by the comparison of the analyte peak area of pre-SPE-spiked (100 ng/L) sample extracts and standard solutions.

The matrix effect (ME) of the substances for the extraction procedure was studied in diluted urine. Therefore, the analyte peak area of post-SPE-spiked (100 ng/L) urine samples and standard solutions (100 ng/L) were compared.

3. Results and discussion

3.1. Optimization of liquid chromatography and mass spectrometry

The separation of the compounds was performed with a Synergi 4 μ HydroRP 80A, 150 $\times$ 2.0 mm (Phenomenex, Aschaffenburg, Germany), a polar endcapped C18 phase column that provides good retention of polar compounds, and the Nucleoshell HILIC column (Machery-Nagel, Düren, Germany) requiring good retention of hydrophilic and strongly polar organic compounds. 16 of the 18 studied compounds could be separated and analyzed using the Synergi Hydro-RP column. Only amoxicillin and doxycycline were mandatory separated and measured with the HILIC technique. The antibiotics ciprofloxacin, clarithromycin, clindamycin, levofloxacin and vancomycin were analyzable with both analytical columns. These antibiotics have mainly functional groups like carboxylic, carbonyl, hydroxyl or amine, which make them highly polar and they are subject to extremely short retention when using RP. The

Table 3
Recovery and process efficiency of the substances for extraction with Oasis HLB and Strata-X cartridges ($n = 4$) in pure solvent and diluted urine.

pH 3.5	Solvent		Diluted urine			
	RE (%)		RE (%)		PE (%)	
	HLB	Strata-X	HLB	Strata-X	HLB	Strata-X
Amoxicillin	35	35	61	38	75	75
Azithromycin	55	58	59	78	77	69
Carbamazepine	59	63	90	62	80	72
Cefotaxime	39	48	83	94	107	103
Cefuroxime	41	47	91	63	166	165
Ciprofloxacin (hilic)	71	78	80	49	57	49
Clarithromycin	37	35	45	67	32	34
Clindamycin	57	53	103	88	152	149
Clindamycin-sulfoxide	68	62	100	94	151	144
Doxycycline	110	98	88	48	127	105
Fluconazole	55	60	94	95	94	94
Levofloxacin (hilic)	84	73	85	33	44	37
PenicillinV	29	24	45	50	50	65
Piperacillin	43	37	45	50	54	65
Roxithromycin	29	29	35	43	40	36
Sulfamethoxazole	57	47	81	48	106	92
Trimethoprim	81	65	93	92	46	44
Vancomycin	69	64	75	81	60	63

mechanism of the HILIC technique is not exactly the opposite of the RP separation but with this application the separation of strongly polar organic compounds is getting more often used [9,22].

For optimal chromatographic separation and a good peak shape the mobile phase was investigated in acidic additives that promote protonation of basic compounds and in this case increase the sensitivity. Formic acid at adding of 0.05% was established to be a good additive (results not shown).

The API4000 Instrument was tuned for every compound to require optimal mass spectrometric conditions in the ESI+ mode. Optimization was realized by infusion of pure standard solution (10 ng/mL) and a flow rate of 100 μ L/min. Finally, main products of the target analytes were optimized in their declustering potential (DP), collision energy (CE) and cell exit potential (CEP). The most intensive signal was selected for the analyte quantification (quantifier) and a second intensive and stable ion was taken for confirmation (qualifier). For the substances cefotaxime and cefuroxime, only one fragmentation showed acceptable signal intensity for quantification and qualification. The chosen fragment ions, quantifier and qualifier and the optimized MS/MS parameters are summarized in Table 2.

3.2. Solid phase extraction and sorbent testing

For sample preparation, two different commercially available extraction cartridges were tested regarding their analyte recovery (RE) and process efficiency (PE). The cartridges were selected because of previous literature [11,13,17,24,25]. The tests were performed in acidified solvent and diluted blank urine. Table 3 shows the recoveries for the tested cartridges Oasis HLB and Strata-X. The RSD for all measurements were between 1.2% and 18% (Data not shown). When considering the recoveries reached by the extraction out of solvent, there are higher values using the Oasis HLB for 10 of the 18 tested substances, but the differences of the rate of the recoveries are very low up to negligible. The extraction out of diluted urine shows higher recoveries and process efficiencies for the cartridge Oasis HLB for 10 and 12 of the 18 substances, respectively. But for all results of the extraction tests deviations of the extraction efficiencies of the Oasis HLB and Strata-X were highly despite (Table 3).

Table 4

Recovery, matrix effect and process efficiency of the studied compounds in diluted urine ($n=4$), influent and effluent samples of the sewage treatment plant ($n=8$) at pH 3.5.

Compound	Urine			Influent	Effluent
	RE (%)	ME (%)	PE	PE (%)	PE (%)
Amoxicillin	77	−4	74	56	72
Azithromycin	78	39	108	40	73
Carbamazepine	91	−14	78	67	75
Cefotaxime	99	−3	96	87	108
Cefuroxime	94	120	189	134	171
Ciprofloxacin (hilic)	91	−41	53	60	96
Clarithromycin	69	−46	37	37	54
Clindamycin	90	35	120	71	103
Clindamycin-sulfoxide	90	2	91	69	104
Doxycycline	111	−3	107	20	31
Fluconazole	86	−4	83	76	99
Levofloxacin (hilic)	89	−51	43	40	74
Penicillin V	68	7	72	81	79
Piperacillin	77	9	84	77	85
Roxithromycin	51	−13	44	47	52
Sulfamethoxazole	63	61	99	84	98
Trimethoprim	94	−66	31	24	62
Vancomycin	88	−44	49	65	83

When considering the process efficiency, that involves the matrix influence on the extraction efficiency, a number of 12 substances show slightly increased rates while using the Oasis HLB than using the Strata-X. This leads to the assumption that the extraction by the Strata-X cartridge is sometimes more susceptible to matrix influence than the extraction efficiency of the Oasis HLB [26].

Apparent is a low process efficiency for the macrolide antibiotics clarithromycin and roxithromycin, the fluoroquinolones ciprofloxacin and levofloxacin, the penicillin antibiotics penicillin V and piperacillin as well as trimethoprim and vancomycin. It varies from 32% for clarithromycin up to 65% for penicillin V and piperacillin. Similar results were also reported by other authors [17,18]. Comparing the eight substances that have PE less than 65%, a strong matrix effect is suggested for four of them, which show a much higher RE. These are ciprofloxacin, clarithromycin, levofloxacin, trimethoprim and vancomycin where the PE and RE were observably different in the amount. Also for the substances

Fig. 1. Mean recovery of the substances extracted out of diluted urine samples by the Oasis HLB cartridge under different pH-values of 2.5, 3.5 and 6.5.

Table 5Validation data for the 18 studied compounds in diluted urine ($n=5$). Squared coefficient of determination (r^2), LoD (signal/noise = 3), LoQ (signal/noise = 10), LLoQ and ULoQ.

Compound	r^2	LoD _{S/N=3} (ng/L ⁻¹)	LoQ _{S/N=10} (ng/L)	Linearity range (ng/L)	
				LLoQ	ULoQ
Amoxicillin	0.9994	2.8	9.3	100	100,000
Azithromycin	0.9998	0.2	0.8	20	20,000
Carbamazepine	0.9998	0.4	1.2	20	20,000
Cefotaxime	0.9998	10.6	35.2	50	20,000
Cefuroxime	0.9990	29.7	99.0	100	100,000
Ciprofloxacin	0.9980	13.5	45.0	50	20,000
Ciprofloxacin (hilic)	0.9994	2.2	7.3	20	20,000
Clarithromycin	0.9998	0.5	1.6	20	20,000
Clarithromycin (hilic)	0.9992	0.4	1.3	20	20,000
Clindamycin	0.9998	0.4	1.4	20	20,000
Clindamycin (hilic)	0.9996	1.1	3.6	20	20,000
Clindamycin-sulfoxide	0.9998	0.5	1.6	20	20,000
Doxycycline	0.9994	8.8	29.2	100	100,000
Fluconazole	0.9998	1.8	6.1	20	20,000
Levofloxacin	0.9998	0.5	1.6	20	20,000
Levofloxacin (hilic)	0.9996	1.4	4.8	20	20,000
Penicillin V	0.9998	2.3	7.7	20	20,000
Piperacillin	0.9998	3.0	9.9	20	20,000
Roxithromycin	0.9998	1.1	3.8	20	20,000
Sulfamethoxazole	0.9994	5.1	17.1	20	20,000
Trimethoprim	0.9984	3.1	10.2	20	20,000
Vancomycin	0.9998	73.5	245.1	250	100,000
Vancomycin (hilic)	0.9992	59.5	198.4	250	100,000

with a PE higher than 65% the influence of the matrix leads to an enhancing effect while the PE is getting much higher than the RE. These are the substances cefotaxime and cefuroxime, clindamycin, clindamycin-sulfoxide, doxycycline and sulfamethoxazole.

Finally and because of confirming literature [12–14,16] the cartridge Oasis HLB (30 mg/1 mL) was selected for the wastewater monitoring.

To study the dependence of the pH value of the analyte recovery, different pH values of the sample solution were tested. Therefore, diluted urine samples were adjusted to pH value 2.5, 3.5 and 6.5 and tested at three different spiking concentrations of 100 ng/L, 1000 ng/L, 2000 ng/L. The results of the pH test are summarized in Fig. 1 and the ranking of the recovery

of the sorbent testing was confirmed. So, in the pH adjustment test also the penicillin antibiotics amoxicillin, penicillin V, piperacillin, the macrolide clarithromycin and roxithromycin as well as sulfamethoxazole are subject to relatively poor recovery (Fig. 1).

Considering Fig. 1, some of the substances show a high dependency on the pH value of the sample solution. These substances like penicillin V and piperacillin have a zwitterionic nature. Other substances showed much less dependency on the pH value, like carbamazepine, fluconazole and sulfamethoxazole, which are neutral pharmaceuticals. Also, cefotaxime, clarithromycin and clindamycin-sulfoxide showed less dependency on pH adjustment.

Table 6Within-run accuracy and precision for the 18 studied compounds prepared in diluted urine matrix ($n=9$).

Compound	Within-run accuracy			
	Spiked concentration (ng/L)	Mean concentration (ng/L)	RSD (%) ^a	Accuracy (%) ^b
Amoxicillin	5000	5361	11	7.2
Azithromycin	1000	980	4.1	-2.0
Carbamazepine	1000	975	5.1	-2.5
Cefotaxime	1000	1018	7.6	1.8
Cefuroxime	5000	5257	8.4	5.1
Ciprofloxacin	1000	1043	7.4	4.3
Ciprofloxacin (hilic)	1000	1106	8.6	10.6
Clarithromycin	1000	1056	4.6	5.5
Clarithromycin (hilic)	1000	907	6.9	-9.3
Clindamycin	1000	949	5.4	-5.1
Clindamycin (hilic)	1000	973	13.7	-2.7
Clindamycin-sulfoxide	1000	1077	4.4	7.7
Doxycycline	5000	4781	15.3	-4.4
Fluconazole	1000	1055	5.3	5.5
Levofloxacin	1000	1046	8.4	4.6
Levofloxacin (hilic)	1000	1027	11.2	2.7
Penicillin V	1000	976	5.7	-2.4
piperacillin	1000	1076	6.3	7.6
Roxithromycin	1000	1021	4.7	2.1
Sulfamethoxazole	1000	990	5.2	-1.0
Trimethoprim	1000	979	4.0	-2.1
Vancomycin	1000	924	4.6	-7.6
Vancomycin (hilic)	1000	923	5.1	-7.7

^a RSD: relative standard deviation.

^b Accuracy (%) = (mean concentration – theoretical concentration)/(theoretical concentration) × 100.

Substances like azithromycin, ciprofloxacin, clarithromycin, levofloxacin and roxithromycin showed best results in the acidic pH value of 2.5 and 3.5, while penicillin V and piperacillin showed best recovery in the neutral pH range.

Overall, the poorest recovery rates were provided at pH 6.5. The highest recoveries for 14 of the 18 substances were provided with acidified samples for pH 3.5. Other research groups also reported highest recoveries for the studied antibiotic substances at pH 3 for the Oasis HLB cartridge [8,13,15,18]. Therefore, the pH adjustment of 3.5 was used for further sample preparation.

To assure the usability of the Oasis HLB cartridge in analyzing wastewater, grab samples of influent and effluent of the STP were spiked with the antibiotic standards, adjusted to pH 3.5 and were extracted using Oasis HLB cartridges. The results are shown in Table 4. The RSD of all measurements were between 1% and 17% (data not shown). The PE was calculated for influent and effluent of the STP and also for diluted urine. The effluent shows higher results for PE, which is probably a consequence of the reduction of interfering biological compounds during purification steps of the sewage treatment plant.

When comparing the PE of urine and wastewater, the matrix of diluted urine seems to be more influential than the matrix of treated wastewater effluent. In urine matrix, six substances had a PE from 31% to 53%, five substances had a PE from 72% to 84%, six substances had a PE from 91% to 120% and the highest PE of 189% was determined for cefuroxime. For the influent of the STP much lower PE was established. So, six substances had a PE from 20% to 47%, 11 of the 18 substances had a PE from 56% to 87% and only cefuroxime had a PE higher than 100%. In wastewater effluent, only three substances had a PE lower than 54%, 14 substances had PE between 62% and 108% and cefuroxime had a PE of 171%.

The results of the PE for the influent and the effluent of the STP showed that these matrices could not be compared with each other and neither with the matrix of diluted urine. So, there had to be a standard addition for every wastewater sample.

Stability and the possible degradation of the antimicrobial substances were determined by monitoring of the concentration of influent and effluent sewage samples during the storage at 4°C in the dark for the duration of seven days. Results showed that trimethoprim, azithromycin, clarithromycin, roxithromycin, levofloxacin, sulfamethoxazole, vancomycin and also doxycycline were stable in influent and effluent samples within 7 days storage, whereas vancomycin was not analyzed in the effluent of STP (<LoQ).

The substances clindamycin, the metabolite clindamycin-sulfoxide, carbamazepine and fluconazole showed no degradation in the influent samples, whereas these substances were lightly instable in the effluent samples of the STP and showed a degradation of 20 to 40% after a 7-day-storage.

The substances piperacillin, ciprofloxacin, cefuroxime, cefotaxime and amoxicillin were stable in effluent but highly instable in influent samples of the STP. The piperacillin, cefuroxime, cefotaxime and amoxicillin should be measured only in the freshly probed samples, the penicillin V was not analyzed in the influent of the STP (<LoQ) and the ciprofloxacin was reduced in the influent by 33% after seven days storage like mentioned above.

3.3. Quantification and method validation study

Matrix matched calibration does represent the similar matrix effects of the sample extracts but in practice of wastewater analysis it is impossible to find blank wastewater samples. Another method, the use of internal isotopically labeled standards was unpractical for the quantification of that number of samples and for this range of antibiotic substances. Because of that the antibiotics were generally

Table 7
Between-run accuracy and precision (RSD) for the 18 antibiotics prepared in diluted urine matrix (n = 5).

Compound	Between-run accuracy			
	Spiked concentration (ng/L)	Mean concentration (ng/L)	RSD (%) ^a	Accuracy (%) ^b
Amoxicillin	100	106	14.6	6.3
	1000	1003	13.6	0.3
	5000	5142	10.0	2.8
Azithromycin	100	93	7.1	−6.7
	1000	971	7.3	−2.9
	2000	1880	3.9	−6.0
Carbamazepine	100	94	4.5	−6.0
	1000	982	2.4	−1.8
	2000	1966	3.6	−1.7
Cefotaxime	100	101	9.7	0.8
	1000	1029	7.1	2.9
	2000	1996	2.2	−0.2
Cefuroxime	100	93	6.0	−7.1
	1000	967	12.0	−3.3
	5000	5272	6.4	5.4
Ciprofloxacin	100	97	10.6	−3.3
	1000	999	5.4	−0.1
	2000	1994	7.1	−0.3
Ciprofloxacin (hilic)	100	97	8.8	−2.6
	1000	1023	4.6	2.3
	2000	1978	6.7	−1.1
Clarithromycin	100	101	6.6	1.1
	1000	963	9.3	−3.7
	2000	1876	7.0	−6.2
Clarithromycin (hilic)	100	103	8.8	3.2
	1000	976	8.7	−2.4
	2000	1876	11.6	−6.2
Clindamycin	100	102	3.5	1.7
	1000	938	4.2	−6.2
	2000	2018	5.9	0.9
Clindamycin (hilic)	100	100	7.7	0.3
	1000	1032	2.1	3.2
	2000	1988	8.6	−0.6
Clindamycin-sulfoxide	100	102	7.2	2.4
	1000	1007	3.6	0.7
	2000	2054	2.1	2.7
Doxycycline (hilic)	100	91	9.3	−8.8
	1000	1033	7.3	3.3
	5000	5322	5.3	6.4
Fluconazole	100	98	8.1	−2.2
	1000	1013	4.9	1.3
	2000	2012	5.5	0.6
Levofloxacin	100	95	13.5	−4.8
	1000	1024	10.0	2.4
	2000	1934	8.6	−3.3
Levofloxacin (hilic)	100	104	4.4	3.7
	1000	1004	2.4	0.4
	2000	2020	5.9	1.0
Penicillin V	100	102	12.0	1.7
	1000	1038	9.7	3.8
	2000	2004	9.7	0.2
Piperacillin	100	98	6.1	−2.1
	1000	1048	10.5	4.8
	2000	1996	11.5	−0.2
Roxithromycin	100	102	9.8	2.2
	1000	941	8.2	−5.9
	2000	1916	5.1	−4.2
Sulfamethoxazole	100	99	9.6	−0.5
	1000	1052	6.7	5.2
	2000	2155	4.4	7.7
Trimethoprim	100	98	7.3	−2.4
	1000	1024	5.8	2.4
	2000	2048	4.5	2.4
Vancomycin	250	265	14.6	1.1
	1000	913	9.0	0.9
	2000	5258	12.9	1.1
Vancomycin (hilic)	250	255	3.0	1.0
	1000	996	5.1	1.0
	5000	5348	5.4	1.1

^a RSD: relative standard deviation.

^b Accuracy (%) = (mean concentration – theoretical concentration)/(theoretical concentration) × 100.

Table 8
Concentrations of most prescribed antibiotics in the wastewater of the STP in Dresden ($n=400$).

Compound	Influent				Effluent			
	Concentration [ng/L]				Concentration [ng/L]			
	Min	Max	Median	>LLOQ (%)	Min	Max	Median	>LLOQ (%)
Amoxicillin	0	1270	0	40	0	187	0	0
Azithromycin	50	946	285	100	0	956	277	97
Cefotaxime	0	492	85	73	0	217	0	37
Cefuroxime	49	6196	1586	100	0	1957	599	99
Ciprofloxacin (hilic)	78	1570	422	100	19	920	146	100
Clarithromycin	42	1525	425	100	18	1800	366	100
Clindamycin	11	163	41	100	20	882	151	100
Clindamycin sulfoxide	46	857	419	100	34	1302	423	100
Doxycycline	0	2393	259	99	0	1110	0	30
Levofloxacin (hilic)	25	2247	351	100	28	836	150	100
PenicillinV	0	252	0	21	0	0	0	0
Piperacillin	0	2603	355	99	0	1205	274	100
Roxithromycin	0	771	92	99	0	281	84	96
Sulfamethoxazole	12	2204	515	100	18	8263	191	100
Trimethoprim	22	372	186	100	25	554	208	100
Vancomycin	0	664	0	8	0	348	0	2
Carbamazepine	98	5819	860	100	211	3535	1051	100
Fluconazole	5	358	120	100	5	308	121	100

quantified by standard addition. The small changes in the concentration ranges of the substances and matrix conditions between the sample sets (24 h composite samples) allowed the quantitation by one standard addition with every sample.

Method detection and quantitation limits were determined according to Section 2.6 and are presented in Table 5. The calibration curve of the method was determined with concentration levels from 20 to 20,000 ng/L and 100 to 100,000 ng/L, respectively, for doxycycline, amoxicillin and cefuroxime because of high quantitation limits in communal and hospital wastewater samples. The method detection and quantitation limit and the squared coefficient of determination were determined in diluted urine to build up the natural compromise of a multi-analytical method of wastewater analysis. For all substances the squared coefficient of determination (r^2) was higher than 0.99. The limit of detection (LoD) was in the range from 0.2 ng/L for azithromycin to 73.5 ng/L for vancomycin and the limit of quantification (LoQ) was in the range from 0.8 ng/L for azithromycin to 245 ng/L for vancomycin. The LoD and LoQ of the substances trimethoprim, sulfamethoxazole and azithromycin are highly comparable to the limits reached by Petrović et al. [7] in effluent wastewater. Grujić et al. [18] reached much lower limits for trimethoprim, sulfamethoxazole and amoxicillin (LoD and LoQ <10 ng/L).

The within-run accuracy and precision of the method were evaluated by the measurement of 9 replicates that were prepared in diluted urine. Results are shown in Table 6. The accuracies range from −10.8 to 9.1% and the RSD ranges from 1.7 to 13.2%. These deviations were in the range for acceptable limits and provide acceptable instrumental precision.

The between-run accuracy and precision of the method was determined by the measurement of three concentration levels prepared five times. The concentration levels of the solutions were 100, 1000 and 2000 ng/L. The accuracies of the measurements presented in Table 7, ranged from −11.4 to 7.7% and the RSD from 2.1 to 15.6%. These ranges were acceptable for the developed method.

3.4. Occurrence of antibiotics in the wastewater of the sewage treatment plant in Dresden

In Table 8 the results of about 400 samples of 24 h-mixed wastewater of the sewage treatment plant of Dresden are presented.

In the influent samples all measured concentrations were in the range of linearity (>LLOQ). The antibiotics ciprofloxacin, clarithromycin, clindamycin and its metabolite clindamycin-sulfoxide as well as levofloxacin, sulfamethoxazole, and trimethoprim and both tracers carbamazepine and fluconazole were measured in all samples. Cefuroxime, doxycycline, levofloxacin, piperacillin, sulfamethoxazole and carbamazepine showed the highest concentrations. Amoxicillin and penicillin V were found only in the influent. A degradation of antibiotics in the STP was observed for amoxicillin, cefotaxime, cefuroxime, ciprofloxacin, doxycycline, levofloxacin, penicillin V, and sulfamethoxazole. The detailed results of this study are presented in diverse articles. The reported wastewater concentrations were highly comparable with earlier studies in other countries. Thus Lindberg et al. [4] probed five different STPs in Sweden where the determined concentrations of influent and effluent wastewater for sulfamethoxazole and trimethoprim were in the same range of concentration and showed also no or less degradation during sewage treatment. Petrović et al. [7] measured sulfamethoxazole and trimethoprim in the same concentration range in effluent wastewater in Serbia. For ciprofloxacin a highly comparable concentration range in the influent were determined by Lindberg et al. [4] but in the effluent they measured nearly completely degradation for ciprofloxacin. For the β -lactams penicillin V and amoxicillin a completely degradation in the sewage treatment was reported [4] as it was detected in the present monitoring.

4. Conclusions

In this study an analytical method for the fast quantification of the most prescribed antibiotics was developed. The method is addressed to the analysis of composite wastewater samples of an urban sewage treatment plant. The preparation procedure of the low-volume-wastewater samples was adjusted to a pH of 3.5 and the cartridge Oasis HLB was selected for extraction as it resulted in measurements of high process efficiency. The quantification has been realized by one standard addition per sample and the quality control of the preparation procedure was established by a 10-point external calibration curve in diluted blank urine. In wastewater, the process efficiency was highly compound-specific and ranged from 20% for doxycycline to 134% for cefuroxime in the influent and from 31% for doxycycline to 171% for cefuroxime in the effluent samples.

While using this method all selected substances have been found in wastewater samples in concentrations up to 6.2 µg/L. The determined concentrations in wastewater of other countries were highly comparable to our results in amount and degradation efficiency. Thus, an efficient, fast and low-volume method was developed for the quantification of a combination of most prescribed antibiotics including β-lactams and other highly interesting substances of all antibiotic classes. This method can also be applied to a short- or long-term monitoring of other sewage systems.

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Evaluation of the matrix effect of different sample matrices for 33 pharmaceuticals by post-column infusion

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ABSTRACT

Matrix effects that occur during quantitative measurement by liquid chromatography mass spectrometry specifically when using electrospray ionization are a widely recognized phenomenon. Sample matrix compounds affect the ionization process of the target analytes, lead to a low signal response, and flawed analytical results. How these matrix compounds directly influence the ionization process has not yet been completely understood. In the present study, we determined the matrix effect for 33 pharmaceutical substances in sample extracts of urine, plasma and wastewater. Most of the investigated substances were subject to a signal suppression effect. Only for a small subset of the compounds we detected a signal enhancement effect. We investigated the matrix effect profiles in detail to disentangle the influence of different matrices and to correlate the impact of specific components and groups of the analyzed extract in suppressing or enhancing effects in the profile.

Most signal suppression effects were detected in the first half of the chromatographic run-time for the matrix extracts of urine and wastewater. The observed effects are caused by high mass flow of salts and other diverse matrix components that were contained in high concentrations in those biological matrices. We also found signal suppression in the matrix effect profile of plasma samples over a wide time range during the chromatographic separation that were associated with a high content of triglycerides of diverse carbohydrate chain lengths. Here, we provide a broader picture of how 33 substances were influenced during analysis. Our results imply that a high number of the investigated substances had comparable effects of matrix compounds, despite differences in their chemical structure.

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1. Introduction

The analysis of pharmaceutical residues in biological and environmental samples has become a major objective in many biological and water chemical research areas [1]. Mainly, these analyses are performed using the LC-MS/MS technique because of its high accuracy and reproducibility [2,3]. However, during the measurement, the ionization of the target analytes is affected by matrix compounds which are dissolved within the sample extracts. Different ionization techniques (e.g., electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI)) respond

differently in dependence to the matrix components [4–7]. The signal intensity can be suppressed by high mass flows and co-elution of specific compounds, e.g., high concentrations of sugars, proteins, lipids, salts, amines, glycopeptides, phosphocholines or metabolites of the targets. Alternatively, the signal can be enhanced by the accumulation of positively charged ions or by neutralizing charge of the target molecules [8–13]. Moreover, system variables like mobile phase additives, solid phases for extraction or analyte derivatives influence the mechanism of ionization [14–18]. The mechanisms, by which these matrix compounds and variables influence the analytes, are not properly understood, but we understand that they are prone to ionization by ESI [4,7]. First reports based on LC-MS/MS procedures described the quantification of the so-called matrix effect (ME), using techniques such as post-extraction addition or the post-column infusion [19–22]. Those methods enable us to systematically evaluate the matrix effect and to study the influence

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on the instrumental measurement system can. Likewise, the efficiency of the sample preparation procedure can be determined. Many sample species of biological origin such as urine, plasma, oral fluids or food have been probed for their matrix impact on the measurement. Others have evaluated the matrix effect focusing on different plant matrices [23,24]. Stahnke et al. showed that the ME differed less than 10% in suppression effects for 80% of the 129 studied pesticide substances [23].

However, the above mentioned studies provided no insights into the correlation between the dependence between matrix influence and compounds. In those studies [23,24], the ME was investigated with different substances but the authors failed to connect the ME to the chemical structure of the individual target analyte.

To reduce the matrix influence, protein participation, solid phase extraction (SPE), liquid–liquid extraction, sample dilution or flow reduction are used for sample purification [24–29]. The matrix evaluation by Kittlaus et al. [24] demonstrated the beneficial effect of multiple sequential sample preparation steps. However, sample clarification could not completely remove the matrix influence on the measurement [17,21,24,27,30,31]. Due to incomplete removal of matrix compounds, methods for quantification have been implemented to compensate the occurring matrix impact, for example the use of isotopically labeled internal standards.

The aim of this study was to determine the ME that occurred during the measurement of biological and environmental samples by LC–MS/MS using positive ESI. The study was carried out through post-column infusion of 33 pharmaceuticals of different action groups featuring diverse chemical structures. The selected matrices were SPE-extracts of urine, plasma and wastewater samples. The wastewater samples were composite samples of the communal influent and effluent of the sewage treatment plant (STP) Dresden Kaditz, Germany and industrial sewage water of a pharmaceutical producer.

Furthermore, in this study we identified mechanisms of the ME. Thus, we focused our evaluation of the matrix effect on compounds with signal enhancement. The subsets of investigated drugs consisted of β -blocker nadolol, the structurally related atenolol, the metabolite of the anticonvulsant oxcarbazepine, the mono-hydroxy-derivate (MHD) and the structurally related anticonvulsant carbamazepine, the anticonvulsants gabapentin and pregabalin, and the antibiotics clindamycin and sulfamethoxazole.

To our knowledge, the present study is one of the first investigations on the origins of matrix effects that relates the influence of the functional groups of the target analytes using ESI–LC–MS/MS. We show substantial differences in the matrix profiles for the tested compounds in urine, plasma and urban wastewater matrix. For these matrices, the matrix effects were predominantly induced by high mass flows of matrix components. Matrix effects induced by chemical interactions were detected only for several target analytes. The understanding of the underlying causes of the matrix effect will enable novel developments in purification steps and higher sensitivity for the detection in derivatisation applications of the analyzed targets.

2. Materials and methods

2.1. Chemicals, pharmaceutical standards and standard mixtures

Acetonitrile (ACN, HPLC-grade), methanol (MeOH, HPLC-grade) and ammonium acetate (p.A.) were purchased from Merck (Darmstadt, Germany), formic acid (conc., LC–MS grade) and disodium ethylenediamine tetraacetate (Na_2EDTA , ACS reagents) from Sigma (St. Louis, MO, USA) and water (HPLC-grade) from VWR (Darmstadt, Germany). ACN, 2 mM ammonium acetate solution and formic acid were used to prepare solvent A (3/97/0.05, v/v/v) and B

(95/5/0.05, v/v/v), respectively, which were used for the preparation of the pharmaceutical working solution and as mobile phase for LC–MS/MS.

The 33 selected pharmaceuticals, the corresponding chemical information and the producer are listed in Table 1. The selection of the 33 pharmaceutical substances was based on a previous method development for the residue analysis of central nervous active substances out of wastewater (WW) samples. This selection was enlarged by antibiotic, antifungal and beta blocker substances that circumstantially occur in WW.

The stock solution (1 mg/mL) of every substance was prepared in methanol and stored at -20°C for up to three months. For the post-column infusion and sample spiking, a standard mixture of all pharmaceuticals was prepared (1 $\mu\text{g/mL}$) in solvent A/B (50:50; v/v) and stored for four weeks at $4-8^\circ\text{C}$.

2.2. Samples

Sample matrices of plasma, urine and 24 h composite WW were selected for the evaluation. Every matrix type was prepared at least twice (No 1. and No 2.) to ensure a confirmed effect for the selected matrix. Sample extracts were prepared at different days and for different sampling dates.

The WW samples were collected from January to May 2013 from communal and industrial sewage influent at the STP Dresden-Kaditz and its effluent to the river Elbe. The wastewater samples were supplemented with 10 mM Na_2EDTA -solution to a final 0.8 $\mu\text{g/mL}$ concentrated sample solution, centrifuged at $6000 \times g$ for 5 min, filtered with a glass fiber filter ($<0.7 \mu\text{m}$, WICOM, Heppenheim, Germany) and adjusted with formic acid to pH 3.5 prior to analysis. Plasma and urine samples were diluted 10 and 40 times and adjusted with formic acid to pH 3.5. An aliquot of 2.5 mL of the adjusted samples was taken for preparation with SPE. Each sample was run in triplicates.

2.3. Sample matrix preparation

Samples were prepared with a SPE procedure, which was developed for antibiotic substances in sewage water [32]. The sample cleanup was performed with 30 mg Oasis HLB Vac cartridges (Waters, Milford, MA, USA), using the automatic sample processor Abimed ASPEC XL (Gilson, Middleton, WI, USA). The cartridges were conditioned with 1 mL methanol/10% formic acid (9:1), 1 mL water and 1 mL of 10 mmol/L Na_2EDTA -solution. Afterwards, the sample aliquot was loaded and washed with 1 mL water. The substances were eluted with 1 mL of methanol/10% formic acid (9:1) and evaporated until completely dry in a gentle air stream at 60°C . The dry extracts were redissolved in a total volume of 250 μL solvent A/B (50:50; v/v).

2.4. Instrumentation

The chromatographic system UltiMate® 3000 Intelligent LC series, containing binary pump, vacuum solvent degasser unit and sample injector (Thermo Scientific Dionex, Idstein, Germany) was selected for chromatographic separation and controlled with a Chromeleon Chromatography Data System (Dionex Softron, Idstein, Germany). The column oven (Shimadzu, Kyoto, Japan) was maintained at 40°C . The post-column infusion was operated with a P680HPLC Pump of Dionex (Thermo Scientific Dionex, Idstein, Germany). An ABSciex quadrupole mass spectrometer (Sciex API4000, ABSciex, Framingham MA, USA) was used as analytical detector. The system was interfaced by a Z-Spray ESI source that operated in the positive ionization mode. The system was controlled with Analyst 1.6 software.

Table 1
Selected pharmaceuticals and corresponding chemical information.

Pharmaceuticals	Class	Formula	CAS-no	Producer
Amitriptyline	Antidepressant	C ₂₀ H ₂₃ N	50-48-6	Sigma
Atenolol	Beta blocker	C ₁₄ H ₂₂ N ₂ O ₃	29122-68-7	Sigma
Carbamazepine	Anticonvulsant	C ₁₅ H ₁₂ N ₂ O	298-46-4	Sigma
Ciprofloxacin-d8	Isotopically labeled fluorochinolone antibiotic	C ₁₇ H ₁₀ D ₈ FN ₃ O ₃	85721-33-1	Fluka
Citalopram	Antidepressant	C ₂₀ H ₂₁ FN ₂ O	59729-33-8	Cerilliant
Clarithromycin	Makrolide antibiotic	C ₃₈ H ₆₉ NO ₁₃	81103-11-9	Sigma
Clindamycin	Lincosamide antibiotic	C ₁₈ H ₃₃ ClN ₂ O ₅ S	18323-44-9	Sigma
Clozapine	Antipsychotic	C ₁₈ H ₁₉ ClN ₄	5786-21-0	Novartis pharma
Doxepin	Antidepressant	C ₁₉ H ₂₁ NO	1668-19-5	Sigma
Fluconazole	Triazole antifungal	C ₁₃ H ₁₂ F ₂ N ₆ O	86386-73-4	Sigma
Fluoxetine	Antidepressant	C ₁₇ H ₁₈ F ₃ NO	54910-89-3	Sigma
Gabapentin	Anticonvulsant	C ₉ H ₁₇ NO ₂	60142-96-3	Pfizer
Haloperidol	Neuroleptic	C ₂₁ H ₂₃ ClFNO ₂	52-86-8	Sigma
Lamotrigine	Anticonvulsant	C ₉ H ₇ Cl ₂ N ₅	84057-84-1	Sigma
Levetiracetam	Anticonvulsant	C ₈ H ₁₄ N ₂ O ₂	102767-28-2	Sigma
Melperone	Neuroleptic	C ₁₆ H ₂₂ FNO	3575-80-2	TOCRIS bioscience
Mirtazapine	Antidepressant	C ₁₇ H ₁₉ N ₃	61337-67-5	Sigma
Nadolol	Betablocker	C ₁₇ H ₂₇ NO ₄	42200-33-9	Sigma
O-desmethylvenlafaxine	Antidepressant	C ₁₆ H ₂₅ NO ₂	93413-62-8	Wyeth Madison
Olanzapine	Antipsychotic	C ₁₇ H ₂₆ N ₄ S	132539-06-1	Sigma
10,11-dihydro-10-hydroxycarbamazepine/mono-hydroxy-derivate (MHD)	Metabolite of oxcarbazepine	C ₁₅ H ₁₄ N ₂ O ₂	29331-92-8	Cerilliant
Opipramol	Antidepressant	C ₂₃ H ₂₉ N ₃ O	315-72-0	Sigma
Paroxetine	Antidepressant	C ₁₉ H ₂₀ FNO ₃	61869-08-7	Sigma
Pregabalin	Anticonvulsant	C ₈ H ₁₇ NO ₂	148553-50-8	Pfizer
Primidone	Anticonvulsant	C ₁₂ H ₁₄ N ₂ O ₂	125-33-7	Fluka
Quetiapine	Antipsychotic	C ₂₁ H ₂₅ N ₃ O ₂ S	111974-69-7	Sigma
Risperidone	Antipsychotic	C ₂₃ H ₂₇ FN ₄ O ₂	106266-06-2	Sigma
Sulfamethoxazole	Sulfonamide bacteriostatic antibiotic	C ₁₀ H ₁₁ N ₃ O ₃ S	723-46-6	Sigma
Sultiam	Sulfonamide anticonvulsant	C ₁₀ H ₁₄ N ₂ O ₄ S ₂	61-56-3	Sigma
Trimethoprim	Bacteriostatic antibiotic	C ₁₄ H ₁₈ N ₄ O ₃	738-70-5	Synopharm-fagron
Trimipramine	Antidepressant	C ₂₀ H ₂₆ N ₂	739-71-9	Sigma
Topiramate	Anticonvulsant	C ₁₂ H ₂₁ NO ₈ S	97240-79-4	Sigma
Venlafaxine	Antidepressant	C ₁₇ H ₂₇ NO ₂	93413-69-5	Wyeth

2.5. HPLC–MS/MS conditions and continuous post-column infusion

The injection volume of the sample was 10 μ L. The separation was performed using a reversed phase (RP) column (150 $\times$ 2 mm 4 μ m, Synergi HydroRP 80A, Phenomenex, Aschaffenburg, Germany) and Security Guard (4 $\times$ 2 mm, Phenomenex, Aschaffenburg, Germany) with a gradient (%B) as follows: 0 min, 0%; 0.5 min, 30%; 5 min, 50%; 5.5 min, 100%; 6.5 min, 100%; 7 min, 0%; 11 min, 0%. The constant flow of the solvent was set to 0.4 mL/min.

We performed sample analyses using the multiple reaction monitoring (MRM) transition mode and the measurement of the two most intensive precursor ion transitions for quantification (quantifier) and confirmation (qualifier) of the compound. All transitions, retention times (t_R) and optimized conditions like declustering potential (DP), collision energy (CE) and cell exit potential (CEP) of the measurement are summarized in Table 2.

A Q1-full mass scan was applied for every matrix extract in three replicates at selected DPs (50, 80 and 130). For the Q1-full mass scan, the same solvent gradient was used with a runtime of six minutes.

The curtain and collision gas flows (both high purity nitrogen) were 30 L/h and 6 L/h, ion spray voltage was 5 kV, and source temperature was set at 450 °C. The continuous post-column infusion was coupled to the LC–MS/MS system by a T-piece that was introduced between the end of the analytical column and the ionization source. The infusion flow rate was constant at 0.1 mL/min and 30 bar regulated backpressure. Fig. 1 shows a schematic of the experimental set up.

The infusion solution was prepared fresh by a 20-fold dilution of the standard mixture (1 μ g/mL) in solvent A/B (50:50; v/v) for a final concentration of 50 μ g/L.

2.6. Matrix effect profile and signal processing of post-column infusion

The determined ME by post-column infusion provides information of the chromatographic separation for the infused substance which was first described by Bonfiglio et al. in 1999 [21]. The determination was repeated in triplicates for every sample matrix. For visualization of the signal intensity, it was smoothed by building the mean value of five data points. We used eq. 1 to smooth the signal intensity ($SI_{i(M5)}$).

$$SI_{i(M5)} = \frac{SI_{i-2} + SI_{i-1} + SI_i + SI_{i+1} + SI_{i+2}}{N} \quad (1)$$

To calculate the matrix effect ($ME_{\text{Column},i}$ (%)), the signal intensity of the injected sample matrix was normalized to the injection of pure solvent by dividing the data points as presented in Eq. (2).

$$ME_{SI,i}(\%) = \left[\frac{SI_{i(M5)}(\text{sample extract})}{SI_{i(M5)}(\text{sample diluent})} \times 100 \right] - 100 \quad (2)$$

We visualized the matrix effect, the so-called matrix effect profile, by plotting the ME_i of every analyte versus the corresponding chromatographic run time. The matrix effect profile provides an overview of the influence of matrix compounds on the ionization efficiency of the post-column infused analyte.

Fig. 1. Scheme of the continuous post-column infusion instrumentation.

3. Results and discussion

3.1. Post-column infusion for matrix evaluation

We performed the evaluation of the matrix effect by post-column infusion by subjecting a selection of 33 pharmaceuticals (Table 3) from diverse action groups and significant differences in their chemical structure to matrices of urine, plasma and different wastewater samples.

For 21 of the 33 pharmaceuticals, we observed predominant signal suppression. These suppression effects were most likely the result of co-eluting matrix compounds and, as such, did not occur due to the chemical structure of the analytes.

In Fig. 2, we present the matrix effect profile of 31 pharmaceuticals in a urine matrix (Note, with the exception of clindamycin

and sulfamethoxazole that were excluded because of contamination effects during this measurement). We observed that 21 substances were subject to signal suppression with a comparable impact (Fig. 2a). In Fig. 2b, we plotted the ten other substances, which did not have comparable ME because the amount of suppression was different or even signal enhancement was detected.

Comparison of the retention times (t_R) (Table 2) and the corresponding profiles (Fig. 2) demonstrates that the suppression effects occurring between 3 and 5 min were significantly perturbing the signal response of most analytes. This observation and the permanently changing matrix effects of different sample types, confirmed that the evaluation was not feasible for single sample extract and that it had to be constantly corrected during measurements [10].

Table 2
Instrumental parameters of 33 pharmaceuticals for the measurement.

Pharmaceutical	Molecular weight (g/mol)	t_R (min)	DP (V)	Quantifier	CE (V)	CXP (V)	Qualifier	CE (V)	CXP (V)
Amitriptyline	277.93	5.9	66	233.1	25	12	105	31	8
Atenolol	266.98	2.6	71	145.3	35	12	190.3	29	14
Carbamazepine	236.98	5.5	81	194.1	25	16	179	49	12
Ciprofloxacin-d8	340.23	3.0	81	296.2	27	26	249.2	35	14
Citalopram	325.03	4.6	91	108.9	37	20	262	27	0
Clarithromycin	747.95	6.0	101	158	39	14	591	27	14
Clindamycin	424.98	3.3	86	126.1	39	8	377.2	27	10
Clozapine	326.99	4.1	86	269.9	31	16	192	59	12
Doxepine	280.33	4.8	76	107	29	6	115.2	55	10
Fluoxetine	309.98	6.3	46	44.1	41	8	148.1	13	14
Fluconazole	306.94	3.4	61	238	21	14	220.1	25	18
Gabapentin	172.05	3.0	31	154	19	10	95.3	52	8
Haloperidol	375.98	5.1	106	165	33	16	123	59	10
Lamotrigine	255.92	3.2	81	211	37	18	145	53	10
Levetiracetam	171.1	2.7	41	126.1	19	22	154.2	11	14
Melperon	264.19	3.8	66	165.2	27	14	123.2	43	10
MHD	255.05	3.6	61	194.1	29	16	194.1	29	16
Mirtazapine	266.03	3.3	91	195	31	16	72	31	6
Nadolol	310.17	2.8	86	254.2	23	14	201.2	33	16
O-desmethyl-venlafaxine	264.02	3.0	56	107.1	45	8	246.1	17	14
Olanzapine	312.91	2.7	86	256	31	16	83.9	33	14
Opipramol	364.04	4.3	71	171	31	14	143	41	12
Paroxetine	330.32	5.2	101	70	47	6	191.9	29	12
Pregabalin	159.93	2.7	56	97.1	21	8	55.1	33	4
Primidone	218.88	3.3	66	162.1	17	14	91.2	35	6
Quetiapine	383.97	4.3	86	253	31	16	221	49	18
Risperidone	411.29	3.7	86	191.1	39	18	110.2	77	6
Sultiam	291.06	3.6	76	185.1	27	12	105.2	53	10
Sulfamethoxazole	253.92	4.4	66	156.1	23	10	92.1	37	6
Topiramate	356.91	5.0	46	264	21	16	282	17	16
Trimethoprim	290.98	2.9	86	230.1	33	14	261	35	14
Trimipramine	295.03	6.2	71	100.1	23	8	208	33	12
Venlafaxine	278.51	3.7	46	58	47	10	260.1	17	16

EP (V) constant at 10 volt; dwell time: quantifier 15 ms, qualifier 10 ms.

Table 3

Chemical structures of the studied pharmaceuticals arranged by the detected matrix effects (high comparability of suppression amount and trend, significant signal enhancement or no signal induction).

	<i>amitriptyline</i>	<i>doxepine</i>	<i>Fluoxetine</i>	<i>quetiapine</i>	<i>o-desmethylvenlafaxine</i>

	<i>citalopram</i>	<i>trimipramine</i>	<i>olanzapine</i>	<i>risperidone</i>

<i>predominant signal suppression</i>	<i>clarithromycin</i>	<i>ciprofloxacin</i>	<i>opipramol</i>	<i>haloperidol</i>

	<i>clozapine</i>	<i>venlafaxine</i>	<i>paroxetine</i>	<i>trimethoprim</i>

	<i>primidone</i>	<i>mirtazapine</i>	<i>lamotrigine</i>	<i>melperone</i>

	<i>sultiam</i>	<i>topiramate</i>	<i>clindamycin</i>	<i>fluconazole</i>

<i>partly signal enhancement / no induction</i>	<i>levetiracetam</i>	<i>carbamazepine</i>	<i>gabapentin</i>	<i>atenolol</i>

	<i>sulfamethoxazole</i>	<i>MHD</i>	<i>pregabalin</i>	<i>nadolol</i>

3.2. Signal suppression study

The matrix effect evaluation for every sample extract was prepared at least two times to ensure a reproducibility of the occurring ME. The sample extract preparations were done at different days and for different dates of sampling. Each sample was measured

three times. The displayed matrix effect profile is always a sum of the signal of at least 16 pharmaceuticals (Table 3, predominant signal suppression), because of contamination samples or partly higher differences in the suppression trend. In Fig. 3, we exemplify two analyzed profiles (No 1. and No 2.) for urine, plasma and different wastewater extracts, respectively, for the retention

Fig. 2. Matrix effect profiles for 31 pharmaceuticals by post-column infusion and injection of sample extract of urine, (a) Matrix effect profile for 21 substances with highly comparable signal suppression; (b) ten substances that differ in amount of signal suppression or a signal enhancement was detected.

time interval from 2 to 8 min of the chromatographic separation. Considering the chemical structure (Table 3, predominant signal suppression) as well as the retention time (Table 2) of the studied substances, we did not observe a preference for a specific functional group or a comparable retention behavior to underlie the same matrix effect.

Apparently, we found a highly comparable trend regarding the ME of different analytes in the same sample matrix (Fig. 3, dark

grey area). The two detected profiles per matrix suggested a good reproducibility in signal suppression in the first half segment of the chromatographic separation. However, the height and shape of the suppression trend varied significantly. The sample extracts were separated with a polar end-capped reversed phase analytical column. The interval of the signal suppression revealed information about the polarity of the eluting matrix compounds. With the selected analytical RP column, a high amount of polar compounds

Fig. 3. Matrix effect profiles (No 1. and No 2.) for different sample extracts of (a) urine, (b) plasma, (c) industrial WW, (d) WW of the influent of STP, and (e) WW of the effluent of STP (grey area: signal suppression effects, dark grey area: fluctuation range of the signal suppressions of the summarized substances (comparable trend of ME)).

were briefly retained and left the chromatographic column in the first half till the mean time segments.

Analyzing every ME profile, we identified substantial differences in the trends of the signal suppression. For the urine matrix (Fig. 3a), intensive signal suppression occurred for both measured urine samples in the time segment from 2.5 to 5.5 min. The amount of signal suppression for the plasma extracts (Fig. 3b) was much lower but almost constant between 2.5 to 7.0 min, indicative of a high content of nonpolar compounds. For urine and plasma the ME appeared to be smeared peaks. In contrast, the ME showed multiple single peaks in the industrial WW (Fig. 3c), caused by high mass flow suppression of matrix compounds at exact time sections. WW samples taken from the influent and effluent of the STP showed comparable profile trends to the urine extract, but with somewhat

different intensities of suppression (Fig. 3d). As expected, the suppressing effect was stronger in the extract of the influent sample (Fig. 3d) than in the effluent sample (Fig. 3e). This reduction is an effect of the WW cleanup and complements the fact that some matrix components have already been removed during purification steps of the STP. The ME profiles in Fig. 3(a), (d) and (e) indicate the effect of suppression in early time segments. These effects in biological matrices are often associated with the presence of glycopeptides and amines that are polar and elute early [7]. In contrast to this fact, plasma does contain triglycerides/phospholipids (fatty acids) in high concentration along with monosaccharides and proteins. Fatty acids contain nonpolar carbohydrate chains with varying length profiles. The influence of length of the carbohydrate chains in separation experiments varies in such a way that

Fig. 4. Q1-full mass scan of urine, plasma, effluent wastewater (WW) and influent wastewater (WW) extract and mass spectra at 3.5 and 5.5 min. (TIC of 50–1000 m/z , DP80).

they are retained consecutively and cause an apparent stable signal suppression effect over long- and late-term time intervals as demonstrated by others [4,7,33,34].

The Q1-full mass scans (Fig. 4) represent the total ion-flow during the chromatographic separation. In general, using a DP of 130 resulted in lower absolute total ion current (TIC) intensities for the urine and wastewater matrices except for plasma, which increased the TIC intensity (data not shown). We analyzed the mass spectra of the Q1-full mass scan, using DP 80, at retention times 3.5 and 5.5 min in more detail.

We detect a higher mass flow during the initial part of the separation for the matrices urine, influent and effluent wastewater. The maximum mass flow occurred at approximately 3.5 min. We

detected a second substantial increase of the TIC for the wastewater matrices at approximately 5.5 min although less significant for the effluent wastewater. For the plasma matrix we found a smaller peak at 3.5 min, followed by a more broadened but higher peak at 5.5 min.

From the presented data, we can conclude that the influent wastewater showed the highest mass load while plasma contained the lowest mass load. The mass load in the WW samples consisted of a high number of molecules with diverse polarity, which was less concentrated in the effluent WW. The urine, influent and effluent WW matrices showed higher levels of polar substances, while the plasma samples contained higher concentrations of nonpolar substances.

Fig. 5. Matrix effect profiles of sample extracts of urine, plasma and influent WW for (a) sulfamethoxazole (b) clindamycin (c) gabapentin and (d) pregabalin and a comparison of (e) nadolol and (g) MHD with their structural analogues (f) atenolol and (h) carbamazepine.

If we directly compare the TIC and the ME (Figs. 3 and 4), we find a strong overlap of the data. The spectra of high mass flows correlate well with higher suppression effects in the ME profiles.

On the basis of the mass spectra we detected the highest amount of molecules with the highest molar masses at both time points in the influent WW matrix. For the effluent WW, we detected lower amounts of molecules as a result of the WW treatment. For the later time section at 5.5 min, we measured higher concentrations of larger molecules (higher molecular weight) in all mass spectra. We detected the lowest concentrations of larger molecules in the urine sample and the lowest amount of small molecules in plasma samples. In conclusion, the mass spectra show the strong correlation in

the analysis of the ME (Fig. 3) and the TIC (Fig. 4). We observed the elution of smaller polar substances at earlier time points and the elution of larger nonpolar substances at later time points.

3.3. Signal enhancement study

The following analyses focuses on potential reasons for the higher and lower signal enhancement, respectively, regarding the chemical structure of the analyzed pharmaceuticals (see Table 3).

We plotted the ME profiles for two different sample extracts of the mentioned substances to visualize the recurrence of the enhancement effect (Fig. 5).

The analytes sulfamethoxazole, clindamycin, gabapentin, pregabalin, nadolol and MHD, showed a predominant signal enhancement pattern in those regions where the other substances were affected by high signal suppression (Fig. 5).

We made an in-depth analysis of the profiles and the chemical structure of MHD and nadolol comparing the profiles and chemical structures (Table 3) of carbamazepine and atenolol, which are structural analogues. All other studied substances (Fig. 5) were compared in the profiles and structures among themselves and the structural differences to the substances that show signal suppression effect. Signal enhancement was predominant for all analyzed substances in any exemplified sample matrix. Despite the areas of signal enhancement, several substances showed signal suppression. However, these suppression effects were consistently found in the first time segment of separation and appeared to be caused by high-punctuated mass flow of organic matrix compounds that were not well retained. The comparison of the matrix effect profiles of the structural analogues nadolol (Fig. 5e) and atenolol (Fig. 5f) as well as MHD (Fig. 5g) and carbamazepine (Fig. 5h) illustrate that a minor change in the chemical structure (Table 3) can cause a strong difference in the trend of the profile.

Clindamycin (Fig. 5b) featured the highest signal enhancement effect up to 120% in urine matrix and also for a long retention time-interval. For the other substances, enhancement effects of up to 60% were detected.

All substances displayed in Fig. 5 that contain a hydroxyl-group showed very high enhancement effects (clindamycin, pregabalin, gabapentin, nadolol, MHD). Atenolol and carbamazepine (Fig. 5f and h) showed little enhancement from 4.0 to 6.0 min of up to 20%. Both substances have an amide as functional group. Regarding the tested plasma matrices, the enhancement effect was higher and reproducible observed in the later time segments compared to urine. This effect can be explained by the higher content of plasma proteins (albumine, lipoproteins, immunoglobulins) and fatty acids in plasma and their diverse number of carbohydrates chains which were retained late [35]. The urine matrix showed suppression effects in the first half time segment for sulfamethoxazole, nadolol, atenolol, MHD and carbamazepine.

The ME profiles of gabapentin and pregabalin (Fig. 5c and d) were highly similar with the exception that gabapentin was measured in the WW sample up to 2 µg/L. Both substances have the gamma-aminobutyric acid as a common basic structural component. Each of the pharmaceutical substances contains alkyl rests at the β-position, gabapentin contains a cyclohexane and pregabalin contains an isobutan. Although the structures differ in their alkyl substituents, it becomes clear that the alkyl substituent had no relevance for the ME.

The comparison of nadolol and atenolol (Fig. 5e and f) showed that these two pharmaceuticals have structural differences in their functional groups. Whereas nadolol has two hydroxyl groups and one methyl group, atenolol contains one amino group (primary). For nadolol a high signal enhancement of up to 60% was detected, whereas atenolol showed much lower signal enhancement of up to 20%. In contrast, we found strong signal suppression for atenolol compared to nadolol with much lower suppression effects.

Carbamazepine and MHD (Fig. 5g and h) are structurally highly comparable. MHD contains one additional hydroxyl group at the azepine and, consequently, has no conjugated double bonds which are found in dibenzazepine. Analyzing the ME profiles of those two structurally similar substances we observed significant differences in the matrix effect profiles. While carbamazepine showed strong signal suppression in the time segment from 3.0 to 4.0 min, MHD had shows lower suppression effects. In the time interval between 4.2 and 6.0 min, MHD experienced signal enhancement of up to 45%, compared to carbamazepine with an enhancement of approximately 20%.

We observed an extremely high signal enhancement in the ME profiles of clindamycin for plasma and urine. The chemical structure of clindamycin includes three hydroxyl groups. Nadolol, that contains two additional terminal hydroxyl groups, showed a stronger signal enhancement than atenolol with only one carbonyl group. Moreover, carbamazepine and MHD have the same basic structural elements of dibenzazepine and one N-standing carbonyl group, but MHD has one additionally hydroxyl group at the azepine and, thus, no conjugated double bound system. Hence, MHD also showed a significantly stronger signal enhancement in the time section of 2.0–3.8 min than its structural analog. In the same time section we detected signal suppression for carbamazepine that contains no hydroxyl group. Together, the results strongly suggest that the hydroxyl-group does play an essential role in enhancing matrix effects of those substances.

The strongest enhancement effect was detected for clindamycin with up to 120%. For the other substances, we observed comparable signal enhancement effects between 40% and up to 60%.

4. Conclusions

In this study, we evaluated the matrix effect for the sample extracts of urine, plasma and urban wastewater for 33 chemical and therapeutically different pharmaceuticals. The evaluation was performed by the post-column infusion method that provided a ME profile for the entire chromatographic run time.

We found a comparable matrix effect profile for the sample extracts for the majority of the studied compounds. The studied matrices (urine, plasma, and wastewater) were predominantly characterized by a signal suppression effect. Only six of the investigated 33 substances showed a constant signal enhancement effect independent of the analyzed matrix sample. The structural analysis of all substances revealed that strong signal enhancement was associated with at least one hydroxyl-group, with the exception of sulfamethoxazole. Altogether, the obtained results clearly indicate that a correction for the ME must be synchronized simultaneously and constantly during measurements. Moreover, this evaluation study presents a starting point for continuing efforts to understand the fundamental ME occurring in mass spectrometry and bioanalysis.

In a following investigation we are studying the influence of substituents and possible mechanisms of adduct formation and their impact on ME. We anticipate that due to the strong differences of the ME in the analyzed samples, further extract components like hormones and vitamins should be considered for signal enhancement. Finally, this study not only confirms that matrix effects are the result of the measurement system, but also demonstrates that it is also a result of varying matrix compounds and of the chemical structure of the target substances.

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Post-column infusion of internal standard quantification for liquid chromatography-electrospray ionization-tandem mass spectrometry analysis – Pharmaceuticals in urine as example approach

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ABSTRACT

Liquid chromatography with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) technique is gaining more and more attraction as the method of choice for multi-sample analysis. However, it is strongly susceptible to the influence of matrix components. Matrix effects are the main source of substantial losses in detection sensitivity and have to be compensated via complex quantification methods. In this work, we introduce a sophisticated quantification method for the LC-ESI-MS/MS analysis of 16 substances in urine samples using a single continuously post-column infused internal standard (PCI-IS) for matrix effect correction. The performance of the introduced technique was proven by the simultaneous quantification using internal standards. Our results demonstrate that a single post-column infused internal standard suffices to analyze multiple target analytes. The introduced method is a new approach to analyze complex matrices and represents a powerful alternative to the classic internal standard methodology. The proposed technique significantly reduces the required steps for sample preparation, costs of additional stable isotopically-labeled internal standards, and self-induced matrix effects.

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1. Introduction

Liquid chromatography coupled with tandem mass spectrometric detection and electrospray ionization (LC-ESI-MS/MS) is the method of choice for rapid drug analysis in biological and environmental samples [1,2]. However, the sensitivity of the method is strongly affected by variations in sample composition [3], sample preparation [4–6] and the type of ionization [7,8]. Common indicators for occurring matrix effects (ME) during MS measurement are reductions in signal intensity, accuracy and reproducibility. The presence of matrix compounds can result in either lower or higher ionization rates of the target analytes, which implies that the actual analyte concentration is inaccurately determined. The influence of matrix compounds on the molecular detection in MS is not completely understood; however, it is clear that those effects are prone to electrospray ionization [2,9,10]. In the last decades, other contributing factors during ESI have been examined, such as enhancing and suppressing effects by the sample matrix [11–15], solvent additives [16–18] electrospray source design [19],

ionization mode [20], mobile phase composition [21,22] and chromatographic techniques [23–25]. However, also internal standards (IS) can be common sources of interferences, for example isotopically labeled analog internal standards (SIL-IS) of the target analytes [26–28].

In order to compensate for MEs, alternative methods for quantification have been developed, e.g. the external matrix matched calibration (external calibration), standard addition method, or the use of IS or SIL-IS. While external matrix matched calibration does not compensate for the individual sample matrix and standard addition method is induced with higher sample numbers and volume, the use of analog SIL-IS is currently the method of choice to compensate for ME and quantitative analysis [29]. The method is based on the assumption that analyte and analog SIL-IS have the highest probability of similar physical and chemical properties, and thus share comparable ME.

However, limited availability and high costs of analog SIL-IS led to quantify several analytes by one SIL-IS or IS substances with comparable behavior in response and retention time that are not present in the actual sample [30,31]. However, even minimal differences in retention time, even of the SIL-IS analog of the analyte, can in some cases result in different ME and cause substantial problems during quantification [32–37]. It has been conclusively shown

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that increased concentrations and variety of target molecules and IS during calibration and in sample solutions can strongly affect the ionization efficiency due to self-induced ME which can ultimately result in the loss of the linear relationship [26–28,37].

To avoid the above-mentioned disadvantages, a new technique was introduced: post-column infusion of IS (PCI-IS). The PCI-IS method was first described by Choi et al. for the quantification of the insecticide tebufenozide and the corresponding metabolite hydroxyl-tebufenozide by the post column infused isotopically labeled analog tebufenozide [^{13}C] in extracted wheat hay samples. They succinctly showed that PCI-IS provides the opportunity to quantify analytes independent of elution times [38]. Another study—exemplifying the high similarity of the ME for more than 100 pesticides—showed that ME were predominantly depending on retention time [39]. Therefore, the applicability of the PCI-IS correction by a single monitor substance was shown, however it did not result in the development of a defined methodology. Gonzales et al. developed a matrix compensation method by using eight isotopically labeled PCI-ISs targeting 18 different drugs (i.e. β -blockers, hypertension drugs, analgesics, lipid-lowering drugs, opioid, antidiabetic and antidepressant) at a concentration range 3–1000 mg/L in urine sample matrices [28]. The effectivity of this approach was rather low as eight PCI-ISs were required to quantify 18 drugs and they did not clearly demonstrate the suitability of the selected PCI-IS. Liao et al. adapted the PCI-IS technique to successfully analyze six benzodiazepines (10–500 $\mu\text{g/L}$) in urine samples and to analyze eleven amino acids (3–200 mg/L) in plasma matrices [40,41], showing an improvement using a single PCI-IS at low infusion concentrations (10–100 ng/min) for quantification. In our method development, we aimed to further reduce the selected SIL-IS in a multi-target analytical method in the lower ng/L range [31]. Previously, we found high comparability of signal suppression for more than 20 pharmaceuticals in different wastewater, urine and plasma matrices [42].

In this work, we decided to use a urine matrix to establish a PCI-IS approach using two isotopically labeled analogs for 16 analyte substances (antidepressants, anticonvulsants, neuroleptics, two metabolites and one antibiotic) with comparable ME profiles that can be successfully separated and detected at low concentration (ng/L) within three minutes. Our PCI-IS method showed vast improvements in accuracy compared to the quantification by IS and SIL-IS. The successful development strongly suggests that the PCI-IS method is useful to investigate large numbers of analytes using few PCI-IS in the presence of complex ME and that it is suitable for detecting trace levels during high-throughput analyses.

2. Experimental section

2.1. Targets, standards and chemicals

To develop the PCI-IS method, we have chosen 14 pharmaceuticals that are active compounds of anticonvulsants, antidepressants, neuroleptics (antipsychotic drugs), antibiotic and two related metabolites of Fluoxetine and Venlafaxine.

The selected substances and 11 SIL-ISs, the corresponding chemical information and their manufacturer are listed in Table S1. Stock solutions (1 mg/mL) of each target substance were prepared in methanol or purchased directly from the producer and stored at -20°C for up to twelve months, until a decrease in the response was detected. The SIL-ISs were purchased at 1 mg/mL or 0.1 mg/mL stock solution and stored at -20°C for up to twelve months. Acetonitrile (Merck, Darmstadt, Germany), 2 mM ammonium acetate solution (Merck, Darmstadt, Germany) and formic acid (Sigma, St. Louis, MO, USA) (conc.) were used to prepare solvent A (3/97/0.05, v/v/v) and B (95/5/0.05, v/v/v), respectively, which were used for

the preparation of the working solution and as mobile phase for LC-ESI-MS/MS. For ME profiles, sample spiking and calibration solutions, a standard mixture containing all target analytes was prepared (1 $\mu\text{g/mL}$) in solvent A and B (A/B; 50:50; v/v) and stored for up to four weeks at $4-8^\circ\text{C}$. We used the same procedure for the SIL-ISs. For the PCI-IS method, Venlafaxine d6 and Trimipramine d3 (Cerilliant, Round Rock, TX, USA) were combined in one infusion solution while the concentrations were adjusted at 1.25 ng/mL for Venlafaxine d6 and at 2.5 ng/mL for Trimipramine d3 in solvent A/B (50:50). Information on used additives and chemicals are given in the supporting information.

2.2. Sample preparation and solid phase extraction

Urine samples were received from healthy, non-medicated volunteers. The urine samples were diluted 5-, 10- and 20-fold and were adjusted to pH 3.5 with formic acid prior to solid phase extraction (SPE) procedure or standard spiking. For the study of recovery (RE) and process efficiency (PE) the water samples were also adjusted to pH 3.5 with formic acid prior to standard spiking and SPE procedure.

The applied SPE procedure was modified from a prior sample preparation method for antibiotic substances detection in sewage water [43]. The sample cleanup was performed with 30 mg Oasis HLB Vac cartridges (Waters, Milford, MA, USA) using the automatic sample processor Abimed ASPEC XL (Gilson, Middleton, WI, USA). More detailed information on the SPE procedure is described in the supporting information. The SPE extracts were dried in an air stream at 60°C and dissolved in a final volume of 250 μL solvent A/B (50:50; v/v) for LC-MS/MS analysis.

2.3. Continuous post-column infusion methodology and data processing

The post-column infusion was operated with a P680 HPLC pump (Thermo Scientific Dionex, Idstein, Germany). An ABSciex quadrupole mass spectrometer (Sciex API4000, ABSciex, Framingham MA, USA) was used as detector. The system was interfaced by an orthogonal ESI source that operates in the positive ionization mode. The system was controlled with Analyst 1.6 software. Quantification by SIL-IS, external calibration methodology, and calculated target analyte area for PCI-IS method were prepared with the MultiQuant software version 3.0. Detailed information on the chromatographic system, the reversed phase gradient, the MS system and detection parameters (Table S2 and Table S3) can be found in the supporting information.

For PCI-IS correction and ME visualization, the listed signal intensity data information of the infused PCI-ISs was exported of the Analyst software. The data points of the signal intensity of the infused substances were smoothed by building the mean value of five consecutive data points. For the so-called ME profile the signal intensity of the injected sample matrix was normalized to the signal intensity of the injection of pure solvent and plotted versus the corresponding chromatographic run time.

For the PCI-IS method the intensities of the PCI-IS were summarized for a time window that was adjusted to the peak width of the corresponding target analyte. The area of the analyte was divided by the sum of the PCI-IS intensity.

A standard calibration in 10-fold diluted urine was used for the quantification by linear regression of target analyte concentration and the individual ratio of the target analyte area to PCI-IS intensity sum. Finally, the derived function of the linear regression was applied to quantify unknown samples by their ratio of target analyte area to PCI-IS intensity sums that were similarly affected by ME.

2.4. Matrix effects, recovery and process efficiency

The ME, RE and PE were determined by measuring four replicates of water, 10- and 5-fold diluted urine samples at concentrations of 250, 500 and 850 ng/L target analyte and SIL-IS, and comparing the signal areas. For ME determination, blank samples were extracted and spiked with standards solution of target analytes and SIL-IS (post-SPE-spiked matrix). For RE determination, samples were spiked with standard mixtures of targets and SIL-ISs before extraction (pre-SPE-spiked matrix). As a reference, we used the target standard and SIL-ISs in pure solvent (standard), respectively. The PE was determined by comparison of pre-SPE-spiked matrix and the respective standard. ME, RE and PE were calculated by using the following equations:

$$\text{ME (\%)} = \left(\frac{\text{signal area}_{\text{post-SPE-spiked matrix}}}{\text{signal area}_{\text{standard}}} \times 100 \right) - 100 \quad (1)$$

$$\text{RE (\%)} = \frac{\text{signal area}_{\text{pre-SPE-spiked matrix}}}{\text{signal area}_{\text{post-SPE-spiked matrix}}} \times 100 \quad (2)$$

$$\text{PE (\%)} = \frac{\text{signal area}_{\text{pre-SPE-spiked matrix}}}{\text{signal area}_{\text{standard}}} \times 100 \quad (3)$$

2.5. Calibration solutions and method test samples

The validation data of the developed method, lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), within-run and between-run accuracy and precision were determined according to the International conference on harmonisation (ICH, Harmonised tripartite guideline) validation of analytical procedures: text and methodology [44] and was improved by a random test of accuracy and precision in different urine samples. The calibration curves for the validation procedure were prepared using concentrations of 31.25, 62.5, 125, 250, 500, 1000, 2000 and 4000 ng/L twice in 10- and 20-fold diluted urine ($n=4$). LLOQ was set the lowest calibration solution within a 20% relative standard deviation (RSD) while ULOQ was defined as the highest concentration of the calibration line with a coefficient of regression (r^2) of 0.99. Within-run and between-run accuracy and precision were tested with spiked concentrations of 125, 500 and 1250 ng/L in urine samples (one urine specimen, 10-fold diluted) that were prepared three times a day for within-run accuracy and precision ($n=3$) and that were prepared on three consecutive days for between-run accuracy and precision ($n=9$).

To assess within-run accuracy and precision ($n=12$) of the PCI-IS method regarding various MEs in urine samples, six urine specimens in 10- and 20-fold dilution were tested at four spiked concentrations (125, 500, 1250 and 2000 ng/L) and compared to quantification by external matrix matched quantification (without correction) and by the SIL-IS quantification method according to

[31]. Thus, samples were additionally spiked with 1000 ng/L of SIL-IS standard mixture. We selected SIL-IS with similar retention times and correlated PE for seven substances without available SIL-IS analogs.

The calibration standards for PCI-IS quantification of various urine samples were prepared in one blank 10-fold diluted urine sample by serial dilution to concentrations of 31.25, 62.5, 125, 250, 500, 1000, 2000 and 4000 ng/L and were spiked with 1000 ng/L of SIL-ISs standard mixture. The quantification by external calibration, PCI-IS correction and SIL-IS method were applied by a non-weighted linear regression.

3. Results and discussion

This study describes the analytical method development for LC-ESI-MS/MS using the quantification by post column standard substance infusion for the detection of 16 pharmaceutical substances in urine samples. The pharmaceuticals and metabolites were originally selected because they were discovered at various concentrations in the sewage system [31,45]. Here, we developed an effective method for trace level analysis and for reducing the number of used SIL-ISs. Our PCI-IS method is based on our previous studies on the ME during LC-ESI-MS/MS measurements that indicated mass-load dependent suppression effects and comparable ME for several analytes [42]. As the applicability of the PCI-IS methodology was shown successfully in previous work [38,40], we selected similar behaving compounds for the PCI-IS method development using two related SIL-IS for urine analysis to evaluate the ME. A detailed schematic of the workflow of the PCI-IS technique is shown in Fig. 1.

The selected compounds (see Table 1 for chemical structures) and available SIL-IS were post-column infused and showed comparable MEs and consistent behavior among themselves for all tested urine samples. Thus, we concluded that a post-column infused standard substance can monitor and correct for MEs for similarly behaving substances. The ME profile of the examined substances during injection of a urine sample is shown on the left and the subsequent ME compensation and quantification by one post-column infused standard is shown on the right of Fig. 1. During method development, we identified five substances (quetiapine, olanzapine, opipramol, clozapin and mirtazapine) that had to be excluded because they were underestimated during subsequent quantification method testing (in urine samples b, c and d).

A structural analysis indicates that some substances have similarities that might be responsible for the respective therapeutic applications, such as the tricyclic antidepressants Doxepin, Amitriptyline and Trimipramine or the selective serotonin reuptake inhibitors (SSRI) Fluoxetine and Norfluoxetine (metabolite), Paroxetine, Citalopram and Sertraline that contain halogenated phenols. Other similarities can be observed for Haloperidol

Fig. 1. Workflow of the PCI-IS technique: matrix effect examination (left) and the resulting correction applicability of PCI-ISs for 16 analytes due to identical behavior in urine matrices.

Table 1Chemical structures of investigated compounds of the PCI-IS method ranked by their retention time, t_R (min).

Compound	t_R	Structure	Compound	t_R	Structure
Trimethoprim	4.09		Haloperidol	6.07	
ODV	4.14		Paroxetine	6.11	
Lamotrigine	4.33		Carbamazepine	6.54	
Primidone	4.49		Norfluoxetine	6.62	
Venlafaxine	4.86		Amitriptyline	6.70	
Melperone	5.03		Trimipramine	6.87	
Citalopram	5.68		Fluoxetine	6.91	
Doxepin	5.79		Sertraline	7.07	

and Melperone—both neuroleptics—that contain a butyrophenone structure. Other pharmaceuticals such as Carbamazepine, Lamotrigine, Primidone, Venlafaxine, ODV and Trimethoprim contain amines such as pyrimidine, triazine-phenyl and methyl-amines or have partially comparable structures such as tricyclic or phenyl substituents. Overall, the examined structures consist of amines, substituted phenyls, ketones and halogenated phenols (Table 1). However, we are unable to make assumptions for ME mechanisms solely based on structural similarities, since the ME is more complex and difficult to prescribe.

3.1. Selection of the PCI-IS

While the potential PCI-IS has to represent the ME of 16 substances in the sample matrix, we only selected available SIL-IS analogs of the target substances to analyze their ME, stability, their associated costs and availability. The 16 substances and 11 available SIL-IS analogs were combined in one infusion solution and compared for their resulting ME profiles in different urine specimens. We found consistent trends of the analyte and potential PCI-ISs which is represented by constant ratios of analyte to SIL-IS intensities in different urine samples. The maximum deviation of the

analyte and potential PCI-IS ratio was set at $\pm 15\%$ relative standard deviation (RSD) in a range of $\pm 20\%$ of the expected retention time (t_R) for the analyte. For Trimipramine d3 and Venlafaxine d6 (and other SIL-IS), we found a constant ratio for all 16 substances. Hence, Trimipramine d3 and Venlafaxine d6 were selected as test PCI-ISs due to their high signal intensity. The infusion solution concentrations were 1.25 ng/mL of Venlafaxine d6 with a maximum SI of 39000 cps and 2.5 ng/mL of Trimipramine d3 with a maximum signal intensity of 150000 cps. This graded procedure provides a more dynamic intensity range and allows specific quantification.

3.2. Recovery, process efficiency and matrix effect determination

The recovery (RE), process efficiency (PE) and ME of the examined substances were determined in 10- and 5-fold diluted urine samples ($n = 4$), for concentrations of 250, 500 and 850 ng/L (Table S5 and S6). The RE was also determined in acidified water (Table S4) ($n = 4$).

Most substances showed a matrix dependent RE (Tables S4, S5 and S6). Lamotrigine $^{13}\text{C}_6$ could not be extracted from water samples with a RSD lower than 20%. Overall, 34% of all RE values from water were lower than 50%, while no substance in urine

matrix had such a low RE. Deviations in the RE higher than 10% for water and urine matrix were determined for all substances, except for Carbamazepine, Carbamazepine d10 and Haloperidol (compare Table S4, S5 and S6). The RE of substances in different diluted urine samples was highly similar within a $\pm 10\%$ variation, except for Primidone, ODV and Lamotrigine (Table S5 and S6). A concentration dependence of RE was observed for most substances and for every matrix, but in general was more pronounced for water (Tables S4, S5 and S6).

For the majority of the tested SIL-IS and their target analogs, we found a strong correlation of RE that varied within 15%, except for Lamotrigine, Norfluoxetine and Paroxetine and those SIL-IS analogs with a variance of up to 58% RE. A closer inspection of the RE in different matrices indicates that the calibration in a comparable matrix includes the influence of the matrix type to RE rates. Also, we found the RE is concentration dependent, as a certain amount is lost during sample preparation. Thus, the external calibration for a comparable matrix includes a certain concentration and sample matrix dynamic.

The ME was determined by the post extraction addition method for one exemplarily urine sample in 10- and 5-fold dilution. For all substances and analog SIL-IS, the ME correlated well within a 15% variation, except for Paroxetine and Paroxetine d6 in 10-fold diluted urine with an observed variation of 21% (Tables S5 and S6).

In Table S5 and S6, we present the ME of the examined substances and SIL-IS by their retention times. The substances with high ME of -50% to -68% are substances in the range of 4–4.3 min retention time, potentially due to the co-elution of a high amount of organic and inorganic compounds. At higher retention times—from 4.5 to 6.5 min—the ME drops while the concentrations of substances with lower polarity and higher molecular structure are decreasing in urine matrices. Further, in later separation sections, the eluting substances are subject to lower but still detectable ME, depending on the dilution factor. The ME of the different diluted urine were consistent and showed that a 10-fold dilution is not sufficient for ME elimination. Small differences between target analytes and SIL-IS are most likely the result of experimental noise.

The ME profiles of all examined urine samples, for the post extraction determination g)–h), for urine specimens that were taken as method test samples a)–f) and for calibration solutions i) demonstrate the high variability of urine specimens depending on volunteers' nutrient uptake, daytime of sampling, medication intake, etc. (Fig. S1). The high suppression at earlier time points could be related to an overall high concentration of creatinine and its metabolites in urine samples. Later eluting substances are varying for individual volunteers. However, small deviations in the retention time and the content of the urine matrix can cause significant variations in the ME and the subsequent quantification.

The PE is determined to compare extraction efficiencies of substances within a single sample matrix. The PE is dynamic for different matrices and can be used for differentiation when different matrices are studied. Thus, the PE is used to correlate the IS for substances in the absence of an analog SIL-IS. The results for the selection of the related IS by comparable PE and t_R are listed in Table S9. For the subsequent comparison of the PCI-IS method and IS quantification, combinations of compound and IS were selected that showed results within a range of $\pm 20\%$ accuracy.

3.3. Validation of PCI-IS method in urine samples

The coefficient of regression (r^2), the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) of the PCI-IS method were determined by the measurement of calibration solutions in a range from 31.25 to 4000 ng/L, prepared in 10- and 20-fold diluted urine, twice ($n=4$). The quantification by the regenerated calibration function does provide linearity of

Fig. 2. Comparison of the different quantification methods by results (% AC, $n=48$) within $\pm 20\%$ accuracy (AC).

every target analyte and calibration standards with a maximum of 15% RSD. We determined a high coefficient of regression of the PCI-IS method (Trimipramine d3 and Venlafaxine d6) for all substances at 0.99 (Table S7). The ULOQ was 4000 ng/L for all substances. The LLOQ was 62.5 ng/L for nine substances, 125 ng/L for seven substances—following a correction with Trimipramine d3—and 31.25 ng/L for two, 62.5 ng/L for three substances and 125 ng/L for eleven substances—following a correction with the Venlafaxine d6 (for more details compare Table S7).

The within- and between-run accuracy and precision were determined with spiked urine samples at three different concentration levels for the quantification by the developed PCI-IS method. The within-run ($n=3$) precision was within 19% and the accuracy within $\pm 17.3\%$ for all spiked concentrations. The between-run ($n=9$) precision was within 15% and the accuracy for three samples and three consecutive days within $\pm 18.3\%$ (Table S8).

3.4. PCI-IS method test

The quantification potential of the PCI-IS method was assessed by spiking urine samples with selected pharmaceuticals from six different volunteers in 10- and 20-fold dilution with concentrations of 125, 500, 1250 and 2000 ng/L (a total of 48 samples). The quantification potential was determined for the PCI-ISs Trimipramine d3 and Venlafaxine d6 and were compared to the quantification by external calibration (without correction) and IS quantification. The performance of the respective quantification method is shown in Fig. 2 (results $\pm 20\%$ accuracy (Table S9)). For the substances Haloperidol, Lamotrigine, Primidone and Sertraline the lowest spiked concentration of 125 ng/L was determined with RSD above 30%, which we then proceeded to exclude from analysis for both PCI-ISs.

The results of our introduced PCI-IS methodology showed significant improvements in accuracy and precision within $\pm 20\%$ for both tested PCI-ISs. For 13 substances, more than 90% of the results were quantified within 93–104% accuracy and less than 15% RSD with the PCI-IS approach. Comparing the results with external calibration and after PCI-IS correction in dependence of the respective urine specimen a)–f), the successful ME correction performance of the PCI-IS approach is clearly observed (Fig. 3).

Venlafaxine, Melperone, Lamotrigine and ODV are shown by external calibration quantification (left) and after correction by the PCI-ISs (right), respectively. While ODV could be quantified in the first urine sample a) with high accuracy, the substances Venlafaxine, Melperone and Lamotrigine were subject to higher deviations related to their retention times. This observation was also made for the two last urine samples e) and f). On the right graphs (Fig. 3), the successful ME compensation is shown by the PCI-IS methodology with a higher accuracy for all urine examined specimen (for Trimipramine d3 and Venlafaxine d6). Thus, the quantification by a single external calibration is not sufficient for determining differ-

Fig. 3. Accuracy (%) of determination of Venlafaxine, Melperone, Lamotrigine and o-desmethyl-Venlafaxine (ODV) in spiked samples of six different urine specimens in 10- and 20-fold dilution for the concentrations 125, 500, 1250 and 2000 ng/L, in dependence of the urine specimen a)–f) (colored and shaped), quantified by external calibration (left) and after PCI-IS trimipramine d3 or venlafaxine d6 correction (right).

ent urine specimens due to high RSD and insufficient accuracy. The ME compensation by the PCI-IS approach was improved for Doxepin, Lamotrigine, Melperone, ODV, Primidone, Trimethoprim and Venlafaxine due to a RSD of less than 15% compared to a previous RSD of 17–34%. All results of the PCI-IS method test are listed in Table S10.

Further, the SIL-IS method was subject to false results and lower amounts of correctly quantified samples compared to the PCI-IS approach. In Fig. 4, the ME compensation performance of the SIL-IS methodology and the PCI-IS correction is shown for selected substances by the RSD of the measurement in different urine samples.

Fig. 4 shows that the RSD of all substances was above 15% in the different urine samples as determined by external calibration (except for Citalopram); and the quantification by ISs reduces the RSD of all substances, even if no substance analogs SIL-IS are available as in the case of Melperone, Primidone, ODV and Venlafaxine. Using PCI-IS correction–Trimipramine d3 and Venlafaxine d6–the RSD was significantly reduced to less than 10% (Fig. 4), except for Lamotrigine and Primidone.

Our results show the potential to use PCI-ISs for correcting several target analytes. Nevertheless, this method development was exclusively based on urine analysis and for the specifically selected target analytes. For the inclusion of other substances into the method, the parameters have to be adjusted for reliable quan-

tification according to their behavior in the sample environment. Therefore, new substances and PCI-IS must produce a consistent correlation for an extensive assay of the discovered sample species. Thus, the PCI-IS technique is applicable for a wide range of matrices such as plasma, blood and other sample types for the quantitative analysis with highly consistent matrix compositions, which has been previously shown for the analysis in urine and plasma matrix [40,46]. The introduced PCI-IS method has the potential to directly extract the ME profile, and identify ME or differences in the native behavior of the sample matrix type during the entire chromatographic run.

With the described method, we successfully tackle the limited quantification range and disadvantages of the traditional IS method quantification, which are additional experimental errors caused by sample spiking, an additionally induced ME due to high mass load, varying behavior in the sample preparation procedure or during ionization processes for the analyte and standard. The PCI-IS methodology is a further improvement to the IS method due to I) the reduction of sample preparation steps, II) the substantially reduced consumption of IS, III) the minimized sample manipulation and IV) a simplified sample preparation effort. In summary, we describe a simple yet precise analysis for 16 selected compounds by the PCI-IS method.

Fig. 4. Comparison of quantification by external calibration, by IS method and by PCI-IS correction Trimipramine d3 and Venlafaxine d6 represented by the relative standard deviation (RSD, %) for the analysis of Citalopram, Doxepin, Lamotrigine, Melperone, o-desmethyl-Venlafaxine (ODV), Primidone, Trimethoprim and Venlafaxine for spiked concentrations of 125, 500, 1250 and 2000 ng/L in six urine specimens in 10- and 20-fold dilution ($n = 12$).

4. Conclusions

We developed a PCI-IS approach to quantify 16 pharmaceuticals in various urine samples with a strongly reduced IS consumption. In comparison to SIL-IS quantification, we show that both methods were highly comparable. However, the PCI-IS application showed great potential in improving the traditional IS method. With the presented method, we can reduce the sample preparation steps and the consumption of the expensive SIL-IS. The PCI-IS method was successfully applied for two isotopically labeled analogs of the target analytes Trimipramine and Venlafaxine for the analysis of 16 pharmaceutical substances. Both infused standards show acceptable quantification results and confirm that the ME is predominantly retention time dependent. To our knowledge, this is the first report of a method development using the PCI-IS approach in an extensive study for 16 different target substances at ng/L concentrations and a drastically reduced consumption of internal standard substances. Additionally, we make the circumstantial comparison to the classic methodology of SIL-IS for nine of the 16 tested substances. The described method is especially suitable for screening approaches and will improve the analyses for various matrix types with reduced material and standard substance consumption. We believe that the PCI-IS methodology can be extended to other target analytes with varying retention times but the technique must be tested extensively for their ME consistent behavior. Sample preparation and the analysis by LC-ESI-MS/MS will be considered separately in future approaches. Smart and accurate sample purification is of utmost importance to avoid false results during the analysis but the preparation effort has to be adjusted to costs and benefit. While the traditional IS method is error prone and can distort results, our PCI-IS approach does not affect sample preparation, extraction processes, and chromatographic separation during the ionization process. With more extensive examination of this technique, we are confident to improve it to a widely-established ME compensation and quantification method for future mass spectrometric and potential high-throughput applications. The method has the potential to be combined with direct sampling [47], dilute-and-shoot or the quantification by specific matrix contents [48]. Further, we think for future method development and a stronger

appeal to a wider scientific community, it is necessary to design software tools that enable raw data extraction for a more basic and simpler data handling to quantify mass spectrometric analyses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.chroma.2018.01.001>.

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Supporting information:

Post-column infusion of internal standard quantification for liquid chromatography-electrospray ionization-tandem mass spectrometry analysis – Pharmaceuticals in urine as example approach

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Contents

In this supporting document detailed information about the used pharmaceuticals, the stable isotopically labeled internal standards (SIL-IS) as purchasing information and parameters of the measurement by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) in *Table S1*, *S2* and *S3* are presented. The results of the method validation of the 16 selected pharmaceutical substances are listed in *Table S4*, *S5*, *S6*, *S7*, *S8* and *S9*. In *Figure S1* matrix effect profiles of the investigated urine specimens are shown. The results of the comparison of the developed post-column infusion of internal standard (PCI-IS) method to quantification by IS and external matrix matched calibration are presented in *Table S10*.

Chemicals and solvents

Acetonitrile (ACN, HPLC-grade), methanol (MeOH, HPLC-grade) and ammonium acetate (p.A.) were purchased from Merck (Darmstadt, Germany), formic acid (conc., LC-MS grade) and disodium ethylenediaminetetraacetic acid disodium salt dihydrate (Na₂EDTA, ACS reagents) from Sigma (St. Louis, MO, USA) and water (HPLC-grade) from VWR (Darmstadt, Germany). ACN, 2 mM ammonium acetate solution and formic acid were used to prepare solvent A (3/97/0.05, v/v/v) and B (95/5/0.05, v/v/v), respectively, which were used for the preparation of the pharmaceutical working solution and as mobile phase for LC-MS/MS.

Table S1: Selected drugs, isotopically labeled standards and corresponding chemical information.

substance	Class	Formula	CAS-No	Producer
Amitriptyline	antidepressant	C ₂₀ H ₂₃ N	50-48-6	Sigma
Carbamazepine	anticonvulsant	C ₁₅ H ₁₂ N ₂ O	298-46-4	Sigma
Citalopram	antidepressant	C ₂₀ H ₂₁ FN ₂ O	59729-33-8	Cerilliant
Doxepin	antidepressant	C ₁₉ H ₂₁ NO	1668-19-5	Sigma
Fluoxetine	antidepressant	C ₁₇ H ₁₈ F ₃ NO	54910-89-3	Sigma
Haloperidol	neuroleptic	C ₂₁ H ₂₃ ClFNO ₂	52-86-8	Sigma
Lamotrigine	anticonvulsant	C ₉ H ₇ Cl ₂ N ₃	84057-84-1	Sigma
Melperone	neuroleptic	C ₁₆ H ₂₂ FNO	3575-80-2	TOCRIS bioscience
Norfluoxetine	metabolite	C ₁₆ H ₁₆ F ₃ NO	107674-50-0	Cerilliant
<i>O</i> -desmethyl-Venlafaxine (ODV)	metabolite	C ₁₆ H ₂₅ NO ₂	93413-62-8	Wyeth
Paroxetine	antidepressant	C ₁₉ H ₂₀ FNO ₃	61869-08-7	Sigma
Primidone	anticonvulsant	C ₁₂ H ₁₄ N ₂ O ₂	125-33-7	Fluka
Sertraline	antidepressant	C ₁₇ H ₁₇ NCl ₂	79559-97-0	Sigma
Trimethoprim	bacteriostatic	C ₁₄ H ₁₈ N ₄ O ₃	738-70-5	Synopharm-fagron
Trimipramine	antidepressant	C ₂₀ H ₂₆ N ₂	739-71-9	Sigma
Venlafaxine	antidepressant	C ₁₇ H ₂₇ NO ₂	93413-69-5	Wyeth
Amitriptyline d3	isotopically labeled standards	C ₂₀ H ₂₀ D ₃ N	342611-00-1	Cerilliant
Carbamazepine d10		C ₁₅ H ₂ D ₁₀ N ₂ O	132183-78-9	Cerilliant
Citalopram d6		C ₂₀ D ₆ H ₁₅ FN ₂ O	1190003-26-9	Cerilliant
Doxepin d3		C ₁₉ H ₁₈ D ₃ NO	347840-07-7	Cerilliant
Fluoxetine d6		C ₁₇ H ₁₂ D ₆ F ₃ NO		Cerilliant
Lamotrigine ¹³ C, ¹⁵ N ₄		¹³ C ₉ H ₇ Cl ₂ N ₃ ¹⁵ N ₄		Cerilliant
Norfluoxetine d6		C ₁₆ H ₁₀ D ₆ F ₃ NO		Cerilliant
Paroxetine d6		C ₁₉ H ₁₄ D ₆ FNO ₃		Cerilliant
Trimethoprim d9		C ₁₄ H ₉ D ₉ N ₄ O ₃	1189460-62-5	Witega
Trimipramine d3		C ₂₀ H ₂₃ D ₃ N ₂		Cerilliant
Venlafaxine d6		C ₁₇ H ₂₁ D ₆ NO ₂	1062606-12-5	Cerilliant

Solid phase extraction procedure

The cartridges were pre-conditioned with 1 mL methanol/10% formic acid (9:1), 1 mL water and 1 mL of 10 mmol/L Na₂EDTA-solution. Afterwards, the sample aliquot of 1 mL was loaded and washed with 1 mL water. The substances were eluted with 1 mL of methanol/10% formic acid (9:1) and evaporated until completely dry in a gentle air stream at 60°C. After every sample treatment the needle was washed with 1 mL of ethanol/water (1:9) to avoid carry over.

HPLC-MS/MS instrumentation and continuous post-column infusion

The chromatographic system UltiMate® 3000 Intelligent LC series, containing binary pump, vacuum solvent degasser unit and sample injector (Thermo Scientific Dionex, Idstein, Germany) was selected for chromatographic separation and controlled with a Chromeleon Chromatography Data System (Dionex Softron, Idstein, Germany). The column oven (Shimadzu, Kyoto, Japan) was kept at 40°C.

The injection volume of the sample was 10 µL. The separation was performed using a reversed phase (RP) column (150 × 2mm 4µm, Synergi HydroRP 80A, Phenomenex, Aschaffenburg, Germany) and Security Guard (4 × 2mm, Phenomenex, Aschaffenburg, Germany) with a gradient (%B) as follows: 0 min, 0%; 0.3 min, 30%; 3.8 min, 50%; 4.5 min, 100%; 6 min, 100%; 7 min, 0%; 10 min, 0%. The constant flow of the solvent was set to 0.4 mL/min.

We performed sample analyses using the multiple reaction monitoring (MRM) transition mode and the measurement of the two most intensive precursor ion transitions for quantification (quantifier) and confirmation (qualifier) of the compound. All substance and SIL-IS information like transitions, retention times (*t_R*) and optimized conditions like declustering potential (DP), collision energy (CE) and cell exit potential (CXP), of the measurement are summarized in Table S2 and Table S3.

The curtain and collision gas flows (both high purity nitrogen) were 30 L/h and 6 L/h, ion spray voltage was 5 kV, and source temperature was set at 450°C. The continuous post-column infusion was coupled to the LC-ESI-MS/MS system by a T-piece that was introduced between the end of the analytical column and the ionization source. The infusion flow rate was constant at 0.1 mL/min and 30 bar regulated backpressure.

Table S2: Instrumental parameters of 16 substances for the measurement. parent ion ($[M+H]^+$) $[m/z]$, declustering potential (DP), quantifier and qualifier in $[m/z]$, collision energy (CE), cell exit potential (CXP)

Pharmaceutical	parent ion	DP	quantifier qualifier	CE	CXP
Amitriptyline	277.93	66	233.1 105	25 31	12 8
Carbamazepine	236.98	81	194.1 179	25 49	16 12
Citalopram	325.03	91	108.9 262	37 27	20 0
Doxepin	280.33	76	107 115.2	29 55	6 10
Fluoxetine	309.98	46	44.1 148.1	41 13	8 14
Haloperidol	375.98	106	165 123	33 59	16 10
Lamotrigine	255.92	81	211 145	37 53	18 10
Melperone	264.19	66	165.2 123.2	27 43	14 10
Norfluoxetine	296.2	36	134.1 -	11 -	12 -
o-desmethyl-Venlafaxine (ODV)	264.02	56	107.1 246.1	45 17	8 14
Paroxetine	330.32	101	70 191.9	47 29	6 12
Primidone	218.88	66	162.1 91.2	17 35	14 6
Sertraline	307.1	46	276.1 158.9	17 33	16 12
Trimethoprim	290.98	86	230.1 261	33 35	14 14
Trimipramine	295.03	71	100.1 208	23 33	8 12
Venlafaxine	278.51	46	58 260.1	47 17	10 16

DP, CE, CXP in volt, EP constant at 10 volt; dwell time: quantifier 15ms, qualifier 10ms

Table S3: Instrumental parameters of 11 isotopically labeled internal standards (SIL-IS) for the measurement. parent ion ($[M+H]^+$) $[m/z]$, declustering potential (DP), quantifier and qualifier in $[m/z]$, collision energy (CE), cell exit potential (CXP)

SIL-IS	parent ion	DP	quantifier qualifier	CE	CXP
Amitriptyline d3	281.1	66	233.1	23	20
			117.2	31	10
Carbamazepine d10	247.2	81	204.2	29	18
			187.2	51	16
Citalopram d6	331.1	76	109.2	35	10
			262.1	29	16
Doxepin d3	283.2	71	107.2	31	18
			77.2	75	6
Fluoxetine d6	316.2	56	44.2	33	8
			154.2	13	14
Lamotrigine $^{13}C,^{15}N_4$	262.8	91	216.0	37	18
			46.1	63	6
Norfluoxetine d6	302.2	46	140.2	11	12
			-	-	-
Paroxetine d6	336.1	76	198.3	31	18
			76.2	49	14
Trimethoprim d9	300.3	96	234.2	33	20
			263.9	35	24
Trimipramine d3	298.2	56	130.2	25	8
			61.2	47	4
Venlafaxine d6	284.2	46	64.2	37	4
			121.2	33	10

DP, CE, CXP in volt, EP constant at 10 volt; dwell time: quantifier 15ms, qualifier 10ms; dwell time of Trimipramine d3 and Venlafaxine d6 20ms

Table S4: Recovery, RE (%) and relative standard deviation, RSD (%) (n=4) of target analytes and isotopically analogs (SIL-IS) for sample preparation by SPE in acidified water (H₂O) for spiked concentrations of 250, 500 and 850 ng/L, retention time (t_R min)

H ₂ O	t _R	250 ng/L		500 ng/L		850 ng/L	
		RE	RSD	RE	RSD	RE	RSD
Trimethoprim d9	4.04	66	12.0	39	12.5	20	12.7
Trimethoprim	4.09	73	7.7	44	6.4	20	9.3
ODV	4.14	29	3.8	15	8.9	8	9.6
Lamotrigine	4.33	-	-	31	11.5	33	16.9
Lamotrigine ¹³ C, ¹⁵ N ₄	4.34	-	-	-	-	-	-
Primidone	4.49	65	11.0	87	12.0	67	9.9
Venlafaxine	4.86	81	4.1	67	8.5	59	5.3
Melperone	5.03	67	7.5	66	4.2	63	6.6
Citalopram d6	5.67	74	8.3	65	15.0	80	9.5
Citalopram	5.68	72	6.7	73	7.6	71	5.9
Doxepin d3	5.79	67	7.9	61	8.0	77	7.1
Doxepin	5.79	74	7.6	65	9.5	71	4.4
Haloperidol	6.07	79	7.0	65	6.7	78	6.4
Paroxetine d6	6.10	47	12.4	46	12.5	67	7.1
Paroxetine	6.11	42	7.1	50	5.2	59	7.9
Carbamazepine d10	6.46	65	5.2	71	7.2	75	5.9
Carbamazepine	6.54	67	6.7	69	6.1	73	6.7
Norfluoxetine d6	6.60	42	9.9	24	10.4	54	8.4
Norfluoxetine	6.62	44	11.5	25	7.9	49	7.0
Amitriptyline d3	6.69	73	8.2	59	8.0	72	5.5
Amitriptyline	6.70	60	5.4	61	8.8	73	8.2
Trimipramine	6.87	61	8.2	66	4.2	72	6.3
Fluoxetine d6	6.88	46	7.3	30	8.3	52	6.7
Fluoxetine	6.91	41	5.8	28	14.2	52	13.4
Sertraline	7.07	46	8.7	37	10.8	50	7.7

Table S5: Recovery, RE (%), matrix effect, ME (%), and process efficiency, PE (%) with related relative standard deviation, RSD (%) (n=4) of target analytes and isotopically analogs (SIL-IS) for sample preparation by SPE in 10-fold diluted urine for spiked concentrations of 250, 500 and 850 ng/L. t_R (min)

Urine diluted 10-fold	t_R	250 ng/L			500 ng/L			850 ng/L		
		RE	RSD	ME	RSD	ME	PE	RSD	RE	RSD
Trimehoprim d9	4.04	72	15.0	-46	14.9	40	14.9	40	83	11.7
Trimehoprim	4.09	72	11.3	-43	13.9	38	5.9	92	6.6	-60
ODV	4.14	97	4.9	-38	5.3	62	5.4	91	10.5	-35
Lamotrigine	4.33	51	6.1	-25	11.4	54	15.0	80	9.9	-36
Lamotrigine $^{13}C, ^{15}N_4$	4.34	109	16.3	-36	13.3	87	13.7	103	4.9	-43
Primidone	4.49	70	12.4	-14	4.0	93	13.0	84	12.7	-9
Venlafaxine	4.86	88	5.5	-19	6.2	71	4.8	85	7.2	-23
Melperone	5.03	89	5.5	-22	4.7	70	6.6	85	10.0	-16
Citalopram d6	5.67	82	13.8	-2	12.1	79	7.2	77	1.6	-10
Citalopram	5.68	69	9.2	1	9.2	69	7.5	80	8.0	-5
Doxepin d3	5.79	76	7.4	8	7.6	82	3.3	83	1.4	-11
Doxepin	5.79	77	9.8	5	12.9	79	8.3	82	4.9	-9
Haloperidol	6.07	80	7.5	-4	8.8	77	5.7	82	3.7	-11
Paroxetine d6	6.10	63	11.0	-7	8.7	58	8.8	69	5.7	-17
Paroxetine	6.11	77	14.0	-28	2.7	55	13.8	60	9.9	-14
Carbamazepine d10	6.46	72	12.9	-1	8.8	69	13.8	70	5.8	-1
Carbamazepine	6.54	62	13.6	-1	5.9	62	13.1	68	3.0	-2
Norfluoxetine d6	6.60	68	0.7	-21	7.2	54	7.2	67	2.2	-6
Norfluoxetine	6.62	61	0.1	-12	7.3	53	7.3	73	5.7	-1
Amiripryline d3	6.69	91	2.4	-4	4.2	87	4.8	78	4.8	-12
Amiripryline	6.70	85	7.0	-7	8.0	79	5.6	82	3.7	-2
Trinipranine	6.87	76	1.2	-13	5.5	66	5.5	85	1.7	-11
Fluoxetine d6	6.88	69	10.7	-10	4.5	62	9.9	74	6.0	-10
Fluoxetine	6.91	62	3.1	-3	10.9	60	10.9	75	8.3	-12
Sertraline	7.07	57	15.4	-11	15.8	45	16.9	62	4.3	-19

Table S6: Recovery, RE (%), matrix effect, ME (%), and process efficiency, PE (%) with related relative standard deviation, RSD (%) (n=4) of target analytes and isotopically analogs (SIL-IS) for sample preparation by SPE in 5-fold diluted urine for spiked concentrations of 250, 500 and 850 ng/L, t_R (min)

Urine diluted 5-fold	t _R	250 ng/L			500 ng/L			850 ng/L											
		RE	RSD	ME	RSD	ME	RSD	PE	RSD	ME	RSD	PE	RSD						
Trimethoprim d9	4.04	103	14.8	-72	11.3	28	13.4	55	20.9	-55	25.8	21	11.0	70	7.7	-65	5.3	25	9.2
	4.09	82	13.4	-65	2.6	28	13.1	59	22.2	-70	6.3	22	19.3	78	18.3	-69	8.4	23	16.2
Trimethoprim																			
ODV	4.14	94	6.9	-51	5.7	48	8.4	100	1.0	-50	1.1	50	0.5	97	2.8	-55	3.1	44	4.1
Lamotrigine	4.33	-	-	-50	14.3	82	11.4	69	11.6	-45	11.9	37	2.6	84	15.0	-46	11.4	48	13.2
Lamotrigine ¹³ C, ¹⁵ N ₄	4.34	78	5.5	-46	14.4	94	13.3	109	11.9	-54	2.0	44	2.0	83	9.2	-48	4.6	43	7.9
Primidone	4.49	110	13.5	-39	5.0	63	16.7	99	2.3	-23	3.1	76	2.0	101	13.5	-26	4.5	70	16.5
Venlafaxine	4.86	87	14.4	-35	4.2	56	14.8	83	1.1	-34	6.8	55	6.7	82	4.3	-25	5.7	61	7.1
Melperone	5.03	83	6.6	-29	4.5	59	7.7	81	0.8	-29	1.2	58	0.9	86	2.6	-30	5.6	60	6.2
Citalopram d6	5.67	82	17.4	-24	1.9	66	14.8	78	3.1	-18	8.0	64	7.4	86	4.0	-16	9.9	72	10.6
Citalopram	5.68	78	14.6	-27	5.4	61	11.9	81	0.2	-25	2.7	61	2.7	90	4.4	-30	5.9	62	4.3
Doxepin d3	5.79	86	11.4	-23	11.7	66	2.6	74	5.8	-18	9.3	61	7.2	85	4.4	-16	5.7	72	3.5
Doxepin	5.79	81	9.7	-18	10.5	66	11.0	85	2.1	-21	2.2	68	0.8	82	5.4	-19	5.5	66	4.1
Haloperidol	6.07	77	12.7	-25	6.1	58	13.3	77	2.3	-29	5.7	54	5.3	75	7.8	-19	5.8	61	6.7
Paroxetine d6	6.10	65	14.6	-30	7.9	45	14.8	64	0.0	-21	8.3	50	8.3	78	7.1	-23	7.7	60	6.1
Paroxetine	6.11	70	6.8	-33	3.5	49	8.3	59	0.7	-26	3.2	43	3.1	71	9.5	-27	2.1	52	9.5
Carbamazepine d10	6.46	75	9.4	-1	0.4	74	9.4	65	6.1	-3	9.1	63	6.7	72	0.9	0	5.5	72	5.5
Carbamazepine	6.54	70	14.7	-3	6.3	68	15.4	64	4.4	-5	7.0	61	5.5	74	4.1	-10	1.6	66	3.8
Norfluoxetine d6	6.60	88	14.0	-18	7.3	76	11.5	68	1.8	-8	5.9	63	5.6	80	3.1	-7	3.9	74	3.9
Norfluoxetine	6.62	68	7.8	-11	8.9	61	9.4	63	1.2	-1	3.0	63	2.8	79	1.8	-9	2.1	71	2.8
Amitriptyline d3	6.69	76	17.2	-15	4.8	65	16.4	70	2.4	-12	4.5	62	3.8	90	7.7	-21	3.5	71	7.6
Amitriptyline	6.70	77	13.1	-12	4.8	68	13.9	71	0.1	-9	6.2	64	6.2	87	8.4	-18	9.4	72	10.4
Trinipramine	6.87	81	8.9	-26	5.5	59	10.4	76	3.6	-17	3.8	63	1.0	81	2.1	-11	6.2	72	6.6
Fluoxetine d6	6.88	78	15.9	-19	1.9	66	13.7	77	4.1	-15	6.1	65	4.5	81	4.6	-16	3.6	68	5.3
Fluoxetine	6.91	71	14.9	-10	10.7	69	13.0	75	7.0	-19	7.0	60	0.6	73	2.6	-15	4.9	62	5.5
Sertraline	7.07	64	7.6	-20	13.8	53	19.8	67	13.6	-22	14.2	52	4.1	70	0.7	-23	3.0	54	3.0

Figure S1: ME profiles of the PCI-IS Trimipramine d3 during measurement of 10-fold diluted urine samples of six different volunteers a)-f) (n=4), 5- and 10-fold diluted urine g)-h) of the ME determination by post extraction addition (n=8) and 10-fold diluted urine i) of the external calibration (n=8)

Table S7: Method validation data of 16 pharmaceuticals for the quantification by PCI-IS
 Trimipramine d3 and Venlafaxine d6 in 10- and 20-fold diluted urine, linearity (r^2), LLoQ_{RSD<20%}
 and ULoQ (ng/L), n=4

	Trimipramine d3			Venlafaxine d6		
	r^2	LLoQ	ULoQ	r^2	LLoQ	ULoQ
Amitriptyline	0.9983	62.5	4000	0.9990	125	4000
Carbamazepine	0.9995	62.5	4000	0.9992	125	4000
Citalopram	0.9970	125	4000	0.9988	125	4000
Doxepin	0.9957	62.5	4000	0.9965	62.5	4000
Fluoxetine	0.9944	62.5	4000	0.9988	125	4000
Haloperidol	0.9981	125	4000	0.9961	125	4000
Lamotrigine	0.9928	125	4000	0.9986	125	4000
Melperone	0.9981	62.5	4000	0.9992	31.25	4000
Norfluoxetine	0.9950	62.5	4000	0.9964	31.25	4000
ODV	0.9987	125	4000	0.9990	125	4000
Paroxetine	0.9984	125	4000	0.9968	125	4000
Primidone	0.9961	125	4000	0.9811	125	4000
Sertraline	0.9988	125	4000	0.9993	125	4000
Trimethoprim	0.9975	62.5	4000	0.9978	62.5	4000
Trimipramine	0.9975	62.5	4000	0.9988	62.5	4000
Venlafaxine	0.9996	62.5	4000	0.9978	125	4000

Table S8: Method validation data of 16 pharmaceuticals for the quantification by PCI-IS trimipramine d3 and venlafaxine d6, mean conc, ng/L, accuracy (%) and relative standard deviation, RSD (%) for spiked concentrations of 125, 500 and 1250 ng/L in 10-fold diluted urine

Trimipramine d3
within-run (n=3)

	ng/L	accuracy	RSD	ng/L	accuracy	RSD	ng/L	accuracy	RSD
Amitriptyline	119	95,4	8,9	468	93,5	7,2	1265	101,2	0,3
Carbamazepine	123	98,3	3,2	459	91,7	7,0	1283	102,7	3,5
Citalopram	122	97,2	8,9	448	89,7	5,2	1433	114,6	3,8
Doxepin	121	96,4	10,7	538	107,6	2,1	1255	100,4	2,0
Fluoxetine	140	112,0	2,8	464	92,8	1,5	1194	95,5	0,1
Haloperidol	130	103,9	13,1	503	100,6	4,4	1416	113,2	1,8
Lamotrigine	114	91,3	6,4	503	100,5	0,5	1270	101,6	5,1
Melperone	120	95,7	1,9	497	99,4	5,0	1287	102,9	0,7
Norfluoxetine	116	93,0	11,2	481	96,2	0,8	1250	100,0	2,1
ODV	113	90,6	5,4	443	88,7	8,7	1290	103,2	6,4
Paroxetine	133	106,2	14,4	482	96,3	5,0	1366	109,2	3,9
Primidone	129	103,4	0,7	414	82,7	5,1	1274	102,0	8,3
Sertraline	93	74,2	14,5	415	83,0	1,5	1328	106,2	1,4
Trimethoprim	101	81,1	1,5	418	83,6	6,8	1206	96,5	9,2
Trimipramine	107	85,3	0,3	457	91,5	5,3	1227	98,1	7,2
Venlafaxine	114	91,3	5,2	550	109,9	12,8	1289	103,1	7,9

between-run (n=9)

	ng/L	accuracy	RSD	ng/L	accuracy	RSD	ng/L	accuracy	RSD
Amitriptyline	118	94,5	8,7	465	92,9	7,0	1277	102,1	5,3
Carbamazepine	125	100,3	5,3	451	90,3	9,0	1290	103,2	9,2
Citalopram	119	94,9	11,0	458	91,5	10,3	1453	116,2	2,2
Doxepin	117	93,6	10,6	506	101,3	6,3	1227	98,1	3,5
Fluoxetine	134	106,9	4,7	475	95,0	7,7	1237	99,0	5,7
Haloperidol	134	106,9	7,9	504	100,8	6,4	1475	118,0	4,5
Lamotrigine	111	88,7	9,5	464	92,7	10,7	1240	99,2	10,5
Melperone	116	92,9	5,9	482	96,5	5,4	1285	102,8	3,6
Norfluoxetine	121	96,9	12,2	483	96,7	3,2	1240	99,2	5,8
ODV	115	92,0	5,9	459	91,8	6,3	1348	107,9	7,3
Paroxetine	133	106,0	10,7	505	101,0	7,5	1337	107,0	6,7
Primidone	123	98,2	8,9	441	88,1	5,2	1287	103,0	8,2
Sertraline	112	89,9	16,4	433	86,7	6,2	1383	110,7	6,3
Trimethoprim	101	81,1	2,6	439	87,8	5,7	1230	98,4	8,1
Trimipramine	111	89,0	4,8	486	97,3	5,1	1279	102,4	6,0
Venlafaxine	107	85,3	11,0	503	100,7	11,2	1211	96,9	7,7

Table S8: continued

Venlafaxine d6
within-run (n=3)

	ng/L	accuracy	RSD	ng/L	accuracy	RSD	ng/L	accuracy	RSD
Amitriptyline	115	92,2	6,1	416	83,2	4,8	1391	111,3	2,5
Carbamazepine	107	86,0	1,3	456	91,1	4,7	1371	109,7	1,3
Citalopram	131	105,2	8,3	504	100,9	4,0	1215	97,2	0,5
Doxepin	119	95,2	11,2	488	97,6	0,8	1290	103,2	3,6
Fluoxetine	108	86,5	6,0	471	94,2	1,0	1256	100,5	1,0
Haloperidol	118	94,0	13,3	458	91,5	0,7	1188	95,1	4,1
Lamotrigine	141	113,0	5,4	511	102,3	0,3	1179	94,3	7,0
Melperone	115	92,1	8,8	451	90,2	3,0	1327	106,1	1,0
Norfluoxetine	120	96,1	13,9	467	93,5	4,3	1375	110,0	1,6
ODV	117	93,4	11,5	439	87,9	5,3	1277	102,1	5,6
Paroxetine	129	103,3	14,4	483	96,6	11,3	1403	112,2	3,4
Primidone	123	98,5	0,5	430	86,1	6,3	1357	108,5	9,9
Sertraline	110	88,0	9,1	414	82,8	1,9	1306	104,5	5,1
Trimethoprim	101	81,2	3,3	435	87,1	0,6	1195	95,6	3,6
Trimipramine	105	84,2	3,4	417	83,4	3,5	1346	107,7	6,3
Venlafaxine	110	88,2	7,1	517	103,5	19,0	1224	97,9	14,0

between-run (n=9)

	ng/L	accuracy	RSD	ng/L	accuracy	RSD	ng/L	accuracy	RSD
Amitriptyline	112	89,5	7,7	439	87,8	7,3	1406	112,5	4,3
Carbamazepine	110	87,7	8,2	474	94,8	7,6	1388	111,0	9,0
Citalopram	132	105,3	7,9	502	100,4	5,7	1231	98,5	3,2
Doxepin	113	90,7	8,8	450	89,9	7,4	1299	104,0	7,1
Fluoxetine	108	86,1	11,6	474	94,9	8,9	1303	104,2	5,1
Haloperidol	114	91,2	11,2	481	96,1	9,1	1218	97,4	6,3
Lamotrigine	133	106,6	11,1	463	92,7	10,8	1144	91,5	11,0
Melperone	113	90,8	8,9	446	89,1	3,6	1340	107,2	3,2
Norfluoxetine	122	97,2	15,4	459	91,7	3,6	1338	107,1	3,1
ODV	115	91,9	8,8	449	89,8	4,7	1277	102,2	5,0
Paroxetine	119	95,0	12,0	509	101,8	9,8	1377	110,2	6,1
Primidone	118	94,7	6,7	447	89,4	7,0	1356	108,5	8,3
Sertraline	111	88,6	8,7	414	82,8	5,0	1317	105,3	6,8
Trimethoprim	102	81,3	3,2	439	87,8	4,7	1219	97,6	5,9
Trimipramine	106	84,9	4,6	424	84,8	6,3	1345	107,6	6,3
Venlafaxine	110	88,0	6,4	469	93,9	14,4	1128	90,2	11,2

Table S9: Results by external calibration, IS quantification and correction by Trimipramine d3 and Venlafaxine d6 of 16 substances for spiked concentrations 125, 500, 1250 and 2000 ng/L in six urine specimens diluted 10- and 20-fold; (n=48)*, accuracy, AC (%), relative standard deviation, RSD (%), results within 20% accuracy, n (%) *Haloperidol, Lamotrigine, Primidone and Sertraline with RSD_{125 ng/L} >20%, n=36

	external calibration			IS quantification			PCI-IS method					
				IS			Trimipramine d3			Venlafaxine d6		
	AC	RSD	n _(20%AC)		AC	RSD	n _(20%AC)	AC	RSD	n _(20%AC)	AC	RSD
Amitriptyline	96.2	11.4	88	Amitriptyline d3	100.9	14.0	85	96.6	9.4	94	97.7	9.4
Carbamazepine	92.1	9.2	90	Carbamazepine d10	94.3	11.4	85	96.2	9.8	94	93.7	9.7
Citalopram	107.6	15.9	71	Citalopram d6	104.7	14.5	77	94.9	10.3	92	101.5	9.7
Doxepin	101.6	18.2	71	Doxepin d3	96.1	11.0	88	95.6	10.6	92	94.7	8.5
Fluoxetine	98.6	10.5	92	Fluoxetine d6	92.7	15.0	77	101.3	14.4	85	95.2	11.5
Haloperidol	108.2	11.7	80	Paroxetine d6	88.2	12.8	74	102.5	13.6	94	103.7	11.5
				Doxepin d3	96.9	16.9	77					
Lamotrigine	110.7	34.0	26	Lamotrigine ¹³ C, ¹⁵ N ₄	86.0	16.2	51	98.0	16.4	77	96.1	19.6
Melperone	100.8	17.4	65	Citalopram d6	112.6	14.8	71	96.0	11.2	88	103.6	11.4
				Fluoxetine d6	77.1	19.5	31					
Norfluoxetine	100.0	12.7	85	Norfluoxetine d6	102.8	13.9	79	97.8	12.4	92	97.5	13.5
ODV	115.2	18.5	71	Trimethoprim d9	112.2	15.9	65	101.8	10.0	94	96.9	9.6
				Lamotrigine ¹³ C, ¹⁵ N ₄	89.2	27.3	46					
Paroxetine	104.2	13.6	88	Paroxetine d6	110.1	20.1	71	100.0	15.0	88	101.3	12.3
Primidone	110.8	20.6	49	Lamotrigine ¹³ C, ¹⁵ N ₄	113.8	26.4	51	94.8	15.3	77	102.7	14.6
				Citalopram d6	89.1	15.8	74					
Sertraline	95.4	9.2	94	Norfluoxetine d6	89.8	11.7	77	100.9	8.4	97	97.7	8.1
				Fluoxetine d6	88.3	13.8	74					
Trimethoprim	113.8	20.9	50	Trimethoprim d9	98.4	14.5	79	94.8	13.9	83	98.5	11.9
Trimipramine	94.4	10.9	92	Amitriptyline d3	91.9	17.6	77	97.3	9.0	96	94.3	10.4
				Fluoxetine d6	79.2	16.4	44					
Venlafaxine	104.5	21.9	58	Citalopram d6	92.2	17.6	71	92.7	10.6	92	94.8	11.5
				Paroxetine d6	62.4	55.6	31					

Table S10: Results by external calibration, IS and PCI-ISs quantification of 16 substances for spiked concentrations 125*, 500, 1250 and 2000 ng/L in six urine specimens diluted 10- and 20-fold; (n=12), mean concentration, conc. (ng/L), relative standard deviation, RSD (%), accuracy, AC (%) *Haloperidol, Lamotrigine, Primidone and Sertraline with RSD_{125 ng/L} >20%

	Amitriptyline			Carbamazepine			Citalopram			Doxepin		
	ng/L	AC	RSD	ng/L	AC	RSD	ng/L	AC	RSD	ng/L	AC	RSD
external calibration		96.3	10.4		92.2	7.0		107.5	13.5		101.6	16.7
	115	92.1	14.5	109	87.1	8.8	148	118.0	14.3	136	109.0	20.0
	503	100.7	8.1	493	98.6	6.2	570	114.0	11.8	520	104.0	14.9
	1244	99.5	11.4	1204	96.3	8.3	1280	102.4	13.2	1,273	101.9	17.4
	1857	92.8	7.4	1738	86.9	4.6	1915	95.8	14.7	1,831	91.6	14.5
IS method		100.8	11.7		94.2	7.6		104.5	9.5		96.0	8.5
	136	108.8	10.7	128	102.3	7.8	148	118.3	7.5	128	102.4	9.2
	535	107.1	13.2	495	99.1	10.4	562	112.3	11.4	504	100.8	10.0
	1215	97.2	10.5	1167	93.3	6.3	1216	97.3	9.3	1,185	94.8	6.5
	1801	90.0	12.3	1647	82.3	5.6	1802	90.1	9.8	1,721	86.1	8.4
Trimipramine d3		96.6	8.6		96.2	7.7		94.8	6.8		95.6	7.7
	118	94.6	10.0	124	99.3	7.8	118	94.3	10.3	131	104.5	11.8
	503	100.7	7.1	506	101.2	8.2	531	106.1	4.5	481	96.2	4.7
	1237	98.9	10.5	1217	97.4	9.1	1085	86.8	6.3	1198	95.9	5.9
	1844	92.2	6.6	1738	86.9	5.8	1834	91.7	6.1	1719	86.0	6.1
Venlafaxine d6		97.7	8.0		93.7	7.7		101.5	7.7		94.6	6.5
	126	100.4	11.2	120	95.6	8.7	123	98.2	12.4	118	94.3	10.5
	505	101.1	7.2	494	98.8	6.8	549	109.8	5.2	512	102.3	4.7
	1231	98.5	8.0	1196	95.7	8.5	1279	102.3	5.8	1122	89.7	4.7
	1816	90.8	5.7	1696	84.8	6.7	1916	95.8	7.5	1845	92.3	6.0
external calibration	Fluoxetine			Haloperidol			Lamotrigine			Melperone		
	ng/L	AC	RSD	ng/L	AC	RSD	ng/L	AC	RSD	ng/L	AC	RSD
		98.5	7.3		108.3	10.6		110.8	33.9		100.9	16.7
	134	106.8	7.9	-	-	-	-	-	-	125	100.2	18.4
	520	104.0	5.8	568	113.5	10.7	552	110.4	32.5	538	107.5	15.5
	1191	95.3	6.9	1369	109.6	12.2	1426	114.0	35.7	1278	102.2	16.4
	1762	88.1	8.4	2034	101.7	8.8	2159	108.0	33.5	1870	93.5	16.3
IS method		92.6	10.9		96.8	13.4		86.0	11.8		112.6	12.5
	130	103.8	10.4	-	-	-	-	-	-	144	114.9	12.9
	488	97.6	14.0	544	108.7	16.3	481	96.2	17.2	613	122.6	16.3
	1118	89.5	10.3	1183	94.6	10.8	1058	84.6	10.2	1390	111.2	11.5
	1591	79.6	9.0	1741	87.0	13.1	1541	77.1	8.1	2035	101.7	9.4
Trimipramine d3		101.1	6.9		102.2	9.9		98.1	16.2		96.0	9.7
	150	119.8	8.4	-	-	-	-	-	-	123	98.4	13.4
	523	104.6	5.5	565	113.0	14.4	504	100.7	15.2	500	100.0	10.3
	1163	93.0	6.4	1167	93.4	9.7	1239	99.1	17.3	1218	97.4	7.0
	1741	87.0	7.6	2005	100.2	5.7	1888	94.4	16.0	1766	88.3	8.0
Venlafaxine d6		95.0	8.1		103.7	9.6		96.2	19.3		103.7	9.8
	122	97.2	8.4	-	-	-	-	-	-	128	102.7	14.4
	532	106.5	7.3	547	109.4	14.3	493	98.5	16.9	552	110.3	9.7
	1073	85.9	6.9	1297	103.7	8.1	1232	98.5	22.1	1316	105.3	6.9
	1806	90.3	9.8	1961	98.1	6.4	1830	91.5	18.7	1926	96.3	8.2

Table S10: continued

	Norfluoxetine			ODV			Paroxetine			Primidone		
	ng/L	AC	RSD	ng/L	AC	RSD	ng/L	AC	RSD	ng/L	AC	RSD
external calibration		100.0	9.0		115.4	17.7		104.2	10.5		110.9	19.9
	136	108.4	9.5	143	114.2	20.4	134	107.4	20.7	-	-	-
	537	107.5	7.9	582	116.4	16.1	548	109.7	6.9	578	115.7	19.6
	1221	97.7	9.8	1547	123.8	17.2	1315	105.2	8.0	1430	114.4	19.5
	1728	86.4	8.6	2145	107.3	17.1	1893	94.7	6.3	2055	102.8	20.5
IS method		102.3	9.6		112.9	17.5		109.9	10.8		89.1	14.4
	142	113.9	9.4	100	80.3	24.3	172	137.9	14.9	-	-	-
	539	107.9	9.7	616	123.2	18.0	552	110.3	9.3	476	95.2	14.6
	1227	98.1	9.0	1645	131.6	14.7	1259	100.7	10.3	1130	90.4	14.5
	1785	89.3	10.3	2328	116.4	13.0	1811	90.6	8.5	1634	81.7	14.2
Trimipramine d3		97.8	10.0		101.9	7.8		99.9	10.4		94.0	13.6
	123	98.1	13.0	119	95.4	7.7	142	113.5	14.6	-	-	-
	535	107.1	7.9	536	107.2	8.4	517	103.5	8.1	502	100.4	14.3
	1231	98.4	9.1	1363	109.1	8.3	1194	95.6	10.9	1224	97.9	14.8
	1753	87.7	9.8	1920	96.0	6.8	1738	86.9	7.9	1730	86.5	11.8
Venlafaxine d6		97.3	9.5		97.0	7.0		101.2	9.0		102.6	13.0
	122	97.4	13.0	114	91.4	6.6	126	100.5	14.1	-	-	-
	561	112.2	7.1	507	101.4	5.5	571	114.2	6.3	561	112.2	13.1
	1113	89.0	7.5	1302	104.2	10.7	1165	93.2	8.0	1222	97.8	13.7
	1815	90.8	10.4	1822	91.1	5.4	1933	96.7	7.8	1956	97.8	12.1
	Sertraline			Trimethoprim			Trimipramine			Venlafaxine		
	ng/L	AC	RSD	ng/L	AC	RSD	ng/L	AC	RSD	ng/L	AC	RSD
external calibration		95.4	9.2		113.9	20.3		94.5	8.3		104.5	20.8
	-	-	-	139	111.1	20.6	108	86.3	10.3	138	110.2	24.4
	500	99.9	7.0	591	118.2	21.0	508	101.6	9.2	539	107.8	20.7
	1221	97.6	7.2	1489	119.1	21.9	1244	99.5	8.5	1305	104.4	19.6
	1774	88.7	8.9	2143	107.1	17.5	1806	90.3	5.3	1910	95.5	18.5
IS method		88.8	13.9		98.4	10.0		87.9	12.4		91.9	16.6
	-	-	-	139	111.0	14.9	93	74.1	16.4	111	88.8	22.8
	454	90.7	11.3	505	101.1	9.6	526	105.1	14.1	497	99.5	15.3
	1152	92.2	9.5	1199	95.9	8.2	1232	98.6	7.1	1156	92.5	13.9
	1733	86.6	13.2	1711	85.5	7.2	1805	90.3	9.5	1737	86.8	13.6
Trimipramine d3		100.9	8.4		95.0	11.5		97.4	7.3		93.1	9.5
	-	-	-	107	85.2	17.9	117	93.7	9.2	113	90.7	11.8
	531	106.2	5.0	496	99.2	6.0	516	103.2	6.8	484	96.8	8.2
	1283	102.7	6.2	1283	102.7	13.9	1264	101.1	8.3	1232	98.5	9.8
	1877	93.8	8.5	1856	92.8	8.2	1833	91.6	4.9	1731	86.6	8.4
Venlafaxine d6		97.7	8.1		98.6	10.2		94.4	6.9		95.2	9.3
	-	-	-	117	93.4	12.5	106	84.8	7.5	106	85.1	13.2
	506	101.3	5.7	518	103.6	7.6	512	102.5	7.8	505	101.1	8.7
	1247	99.7	6.9	1305	104.4	13.5	1244	99.5	8.4	1266	101.3	9.6
	1842	92.1	8.2	1859	93.0	7.0	1816	90.8	3.7	1871	93.5	5.8

Erklärungen zur Eröffnung des Promotionsverfahrens

1. Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.
2. Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts habe ich Unterstützungsleistungen von folgenden Personen erhalten:
Bei der Erarbeitung der dieser Dissertation zugrundeliegenden Veröffentlichungen haben folgende Personen mitgewirkt: Lars David Renner, Reinhard Oertel, Robert Gurke, Sara Schubert, Wilhelm Kirch und Ali El-Armouche.
3. Weitere Personen waren an der geistigen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die Hilfe eines kommerziellen Promotionsberaters in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.
4. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.
5. Die Inhalte dieser Dissertation wurden in folgender Form veröffentlicht:

Publikationen als Erstautor:

Rossmann, J., Schubert, S., Gurke, R., Oertel, R., Kirch, W. „Simultaneous determination of most prescribed antibiotics in multiple urban wastewater by SPE-LC–MS/MS.”

Journal of Chromatography B 969, 2014, 0, 162-170,

DOI: 10.1016/j.jchromb.2014.08.008.

Rossmann, J., Gurke, R., Renner, L.D., Oertel, R., Kirch, W. „Evaluation of the matrix effect of different sample matrices for 33 pharmaceuticals by post-column infusion.”

Journal of Chromatography B, 2015, 1000, 84-94,

DOI: 10.1016/j.jchromb.2015.06.019.

Rossmann, J., Renner, L.D., Oertel, R., El-Armouche, Ali. „Post-column infusion of internal standard quantification for liquid chromatography-electrospray

ionization-tandem mass spectrometry analysis – Pharmaceuticals in urine as example approach.”

Journal of Chromatography A, 2018, 1535, 80-87,

DOI: 10.1016/j.chroma.2018.01.001.

Publikationen als Co-Autor:

Gurke, R., Rossmann, J., Schubert, S., Sandmann, T., Rößler, M., Oertel, R., Fauler, J. „Development of a SPE-HPLC–MS/MS method for the determination of most prescribed pharmaceuticals and related metabolites in urban sewage samples”

Journal of Chromatography B, 2015, 990, 23-30,

DOI: 10.1016/j.jchromb.2015.03.008.

Oertel, R., Baldauf, J., Rossmann, J. „Development and validation of a hydrophilic interaction liquid chromatography-tandem mass spectrometry method for the quantification of the antidiabetic drug metformin and six others pharmaceuticals in wastewater”

Journal of Chromatography A, 2018, 1556, 73–80,

DOI: 10.1016/j.chroma.2018.04.068.

Vorträge:

Hohenroda 2014, 24. Doktorandenseminar der Arbeitsgruppe Separation Science der Gesellschaft deutscher Chemiker GDCh-Fachgruppe Analytische Chemie, *“Post-column infusion of monitor substances for the quantification of pharmaceuticals in wastewater”*

Dresden 2013, ANTI-Resist Symposium, *“Investigating the problems of mass spectrometric detection during pharmaceutical residue analysis of biological and aquatic samples- development of a new approach for wastewater analysis”*

Posterpräsentationen:

Amsterdam 2013, HPLC, 45th and 39th International Symposium on High Performance Liquid Phase Separations and Related Techniques, *“Permanent post-column infusion as a challenge of matrix effect compensation in quantitative LC-MS/MS analysis of pharmaceuticals in sewage of Dresden”*

Liège 2014, Drug Analysis 2014, *10th International Symposium on Drug Analysis*
25th International Symposium on Pharmaceutical and Biomedical Analysis,
 “Postcolumn infusion of isotopically labelled standards for quantification as a new
 approach for high-throughput analysis of complex samples by LC-MS/MS”

Leipzig 2014, LC-MS in der Umweltanalytik, HELMHOLTZ CENTER FOR
 ENVIRONMENTAL RESEARCH –UFZ, “Matrix effect Evaluation of 33 Pharmaceuticals
 for different biological Sample Matrix by Postcolumn Infusion”

Saarbrücken 2018, European Mass Spectrometry Conference EMSC 2018,
35ème Journées Française de Spectrométrie de Masse, *51. Jahrestagung der*
Deutschen Gesellschaft für Massenspektrometrie, “Continuous post-column
 standard substance infusion for LC-ESI-MS/MS analysis – An effective
 quantification approach”

6. Ich bestätige, dass es keine zurückliegenden erfolglosen Promotionsverfahren gab.
7. Ich bestätige, dass ich die Promotionsordnung der Medizinischen Fakultät Carl Gustav Carus der Technischen Universität Dresden anerkenne.
8. Ich habe die Zitierrichtlinien für Dissertationen an der Medizinischen Fakultät der Technischen Universität Dresden zur Kenntnis genommen und befolgt.
9. Ich bin mit den “Richtlinien zur Sicherung guter wissenschaftlicher Praxis, zur Vermeidung wissenschaftlichen Fehlverhaltens und für den Umgang mit Verstößen” der Technischen Universität Dresden einverstanden.

Dresden, 24.08.2018

Julia Rossmann

Erklärung zur Einhaltung rechtlicher Vorschriften

Hiermit bestätige ich die Einhaltung der folgenden aktuellen gesetzlichen Vorgaben im Rahmen meiner Dissertation

- ☐ das zustimmende Votum der Ethikkommission bei Klinischen Studien, epidemiologischen Untersuchungen mit Personenbezug oder Sachverhalten, *Aktenzeichen der zuständigen Ethikkommission*.....
- ☐ die Einhaltung der Bestimmungen des Tierschutzgesetzes
Aktenzeichen der Genehmigungsbehörde zum Vorhaben/zur Mitwirkung
.....
- ☐ die Einhaltung des Gentechnikgesetzes
Projektnummer.....
- ☐ die Einhaltung von Datenschutzbestimmungen der Medizinischen Fakultät und des Universitätsklinikums Carl Gustav Carus.

Obige Punkte sind für die vorliegende Arbeit nicht zutreffend.

Dresden, 24.08.2018

Julia Rossmann

Erklärung zur Abgrenzung des Eigenanteils des Verfassers

Bei den dieser Dissertation zugrunde liegenden Fachartikeln bin ich alleiniger Erstautor, wurde aber in Teilen bei der Planung der Experimente sowie beim Verfassen der Manuskripte durch die in den Artikeln gekennzeichneten Koautoren unterstützt. Die Liste der Antibiotika und die Planung der Methodenentwicklung zur Abwasseranalytik wurde in Zusammenarbeit mit Sara Schubert erstellt. Die Probenvorbereitung im Monitoringprogram der Antibiotikauntersuchung wurde durch Ute Mann, Kerstin Weber und Sara Schubert durchgeführt. Die anschließende Analytik und Auswertung der Antibiotikakonzentrationen in den mehr als 300 Abwasserproben wurde von Dr. Reinhard Oertel durchgeführt. Die Auswahl weiterer zentral aktiver Arzneimittel habe ich in eigenständiger Arbeit erweitert, wofür ich die ambulanten Verschreibungsdaten der AOK Plus analysierte. Die Methodenentwicklung der Nachsäuleninfundierten Internen Standard Quantifizierung, die Messungen zu Matrixeffektuntersuchungen, Validierung und Methodentests sowie alle Auswertungen wurden selbstständig von mir durchgeführt. Die Erarbeitung der Manuskripte sowie die Einarbeitung der Gutachten der Reviewer war hauptsächlich meine Aufgabe. Unterstützung durch meine Koautoren habe ich vor allem in der Strukturierung der Texte und Korrektur der englischen Sprache erhalten.

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