Aus dem Institut für Biochemie

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Geschäftsführender Leiter: Univ.-Prof. Dr. Mario Thevis

Entwicklung massenspektrometrischer Nachweismethoden für dopingrelevante Analyten in Atemluft und getrockneten Blutstropfen als komplementäre Matrices im Anti-Doping Kontext

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1 Zusammenfassender Überblick und Diskussion

1.1 Einleitung und Zielsetzung

Das Doping-Kontroll-System

In den 1960er Jahren begann ein Umdenken im professionellen Humansport, nachdem ein Todesfall bei einem international ausgestrahlten Sportevent mit leistungssteigernden Substanzen in einen Zusammenhang gebracht wurde.[1] Im Fokus standen dabei nicht nur die Idealvorstellung eines ehrlichen Sportgeists, sondern auch die Gesundheit der Athlet*innen. Die ersten Dopingkontrollen folgten bereits in den 70er Jahren und wurden hauptsächlich von den Sportfachverbänden organisiert, bevor 1999 die Welt Anti-Doping Agentur (WADA) gegründet wurde.[2,3] Mit diesem Schritt wurden Anti-Doping Bestimmungen erstmalig auf internationaler Ebene harmonisiert, organisiert und kontrolliert. Darüber hinaus engagiert sich die WADA zusätzlich für Forschung, Entwicklung und Aufklärung im Anti-Doping Kontext. Die Umsetzung der von der WADA gestellten Richtlinien wird auf nationaler Ebene von nationalen Anti-Doping Agenturen sichergestellt.[4,5] Der sogenannte *World Anti-Doping Code* (WADC) bildet das Fundament des Doping-Kontroll-Systems und gilt zusammen mit insgesamt acht internationalen Standards.[6-13] Mit dem Ziel einer Harmonisierung logistischer und analytischer Prozesse, werden spezifische Anforderungen für Laboratorien in sogenannten *Technical Documents* definiert.[9] Bestandteil der internationalen Standards ist eine jährlich aktualisierte Verbotsliste (*Prohibited List*), die eine Zusammenstellung der Substanzen und Methoden darstellt, die im professionellen Sport verboten sind. Dabei kann das Verbot auf eine bestimmte Applikationsart oder den Zeitpunkt der Einnahme beschränkt sein. Während die Substanzklassen S0-S5 zu jeder Zeit verboten sind (Substanzen ohne Zulassung; Anabolika; Peptidhormone, Wachstumsfaktoren, verwandte Substanzen und Mimetika; Beta-2 Agonisten; Hormon- und Stoffwechselmodulatoren; Diuretika und maskierende Substanzen), beschränkt sich das Verbot der Gruppen S6-S9 auf den Tag eines Wettkampfes (Stimulanzien; Narkotika; Cannabinoide; Glukokortikoide). Beta-Blocker, die als Gruppe P1 in der Verbotsliste aufgeführt werden, sind nur in ausgewählten Sportarten verboten.[14] Die Kontrollen lassen sich hauptsächlich in Trainingskontrollen

(*out-of-competition*, OOC) und Wettkampfkontrollen (*in-competition*, IC) kategorisieren. Trainingskontrollen können dabei unangekündigt zu einem beliebigen Zeitpunkt sowie an einem beliebigen Ort erfolgen und sind mit einem hohen organisatorischen Aufwand verbunden, gelten aufgrund der Unvorhersehbarkeit jedoch als sehr effektiv. Dahingegen werden Wettkampfkontrollen in der Regel im Anschluss an einen Wettkampf durchgeführt.[11,15]

Herkömmliche Matrices – Urin und Blut

Mit der Einführung von Dopingkontrollen wurden leistungssteigernde Substanzen hauptsächlich in Urinproben nachgewiesen, die bis heute den Großteil der analysierten Probenmatrices darstellen.[1,16] Da die renale Exkretion den primären Eliminationsweg niedermolekularer Substanzen darstellt, kann ein Großteil der dopingrelevanten Substanzen in Urin als Probenmatrix identifiziert werden.[17] Jährlich veröffentlicht die WADA einen zusammenfassenden Bericht über alle Doping-Kontroll-Proben, die in den WADA-akkreditierten Laboratorien untersucht wurden. Im Jahr 2021 wurden demzufolge insgesamt 270 803 Proben analysiert, davon waren ca. 80% Urinproben.[18] Verbindungen, die aufgrund ihrer physikochemischen Eigenschaften kaum intakt über die Niere ausgeschieden werden, werden durch Biotransformationsprozesse chemisch verändert und anschließend als Metaboliten mit dem Urin eliminiert.[19] Sowohl die pharmakokinetischen als auch die pharmakodynamischen Eigenschaften von Biotransformationsprodukten können im Vergleich zur Muttersubstanz verändert sein, sodass sie über einen längeren Zeitraum im Urin nachweisbar sind. Mit Einbezug dieser Metaboliten in die Dopinganalytik wird dementsprechend sowohl das Spektrum der nachweisbaren Substanzen als auch der Zeitraum der Nachweisbarkeit erweitert.[20,21] Aufgrund der langjährigen Erfahrung zeichnet sich Urin als Probenmatrix insbesondere durch einen hohen Forschungs- und Kenntnisstand aus. Der Ablauf einer Urin-Probenahme wird durch einen Leitfaden der WADA reglementiert und dementsprechend harmonisiert. Dieser gibt ein Mindestvolumen von 90 mL vor.[15,22] Dadurch wird dem analytischen Labor ein Probenvolumen zur Verfügung gestellt, das die Durchführung von Mehrfach- sowie Wiederholungsanalysen ermöglicht und darüber hinaus Spielraum für Methodenoptimierungen hinsichtlich der Sensitivität zum Beispiel durch die Erhöhung

des Ausgangsvolumen bietet. Unter gewissen Umständen kann diese Volumenvorgabe von den Athlet*innen durch eine einzelne Urinabgabe nicht erfüllt werden. Dies kann mit einer physischen Belastung, die aufgrund einer Dehydratation auftritt, verbunden sein und wird dementsprechend häufig bei Dopingkontrollen nach Wettkämpfen oder während Trainingseinheiten beobachtet.[23] In solchen Fällen werden mehrere Urinabgaben durchgeführt und die gesammelten Proben im Anschluss vereint, sodass die gesamte Dopingkontrolle einen unvorhersehbaren Zeitraum in Anspruch nimmt.[24]

Darüber hinaus beschreibt der Leitfaden zur Probenahme Maßnahmen zur Vorbeugung von Täuschungs- und Manipulationsversuchen wie den Austausch des Urins mit körperfremden Flüssigkeiten oder die Zugabe von proteolytisch aktiven Substanzen.[25] Aus diesem Grund befinden sich die Athlet*innen gemäß den Vorgaben ab dem Zeitpunkt des Eintreffens des Doping-Kontrollpersonals unter dessen stetiger Aufsicht einschließlich der tatsächlichen Urinabgabe. Dieser Ablauf wird als erheblicher Eingriff in die Privatsphäre der Athlet*innen kritisiert.[23] Insgesamt betrachtet stellt die Prozedur der Urin-Probenahme einen erheblichen Nachteil dieser Matrix dar. Um die Integrität der Proben zu gewährleisten, wird von der WADA eine ständige Kühlung während des Transports in das analytische Labor empfohlen, doch aufgrund der Größe des Sammelcontainers wird dies in der Praxis aus logistischen und finanziellen Gründen häufig nicht umgesetzt. In der Konsequenz können sich Zersetzungsprozesse nachteilig auf die Stabilität der dopingrelevanten Substanzen auswirken.[26] Die anschließende gekühlte Lagerung der Proben stellt analytische Labore ebenfalls vor logistische Herausforderungen und führt zu einem hohen Anspruch an entsprechende Räumlichkeiten.

Mit der Offenlegung von Blutdoping, das die Verabreichung von roten Blutkörperchen zum Beispiel in Form von Erythrozyten-Konzentraten oder Vollblut beschreibt, stieg das Interesse an alternativen Matrices zu Urin, sodass Ende der 80er Jahre erstmalig auch Blutproben im Anti-Doping Kontext entnommen und analysiert wurden.[1,27,28] In Folge dessen etablierten sich Blutproben als fester Bestandteil des Doping-Kontroll-Systems.[11] Dies basierte im Wesentlichen auf den Vorteilen für den Nachweis von Makromolekülen oder Wirkstoffen, deren Struktur von Peptiden abgeleitet ist und die aufgrund einer geringen renalen Elimination eine deutlich höhere Konzentration im Blut aufweisen. Darunter fallen zum Beispiel rekombinante Analoga der Hormone Somatotropin oder

Insulin.[29,30] Im Jahr 2009 wurde zudem der Biologische Athletenpass (*Athlete Biological Passport*) eingeführt. Dabei werden festgelegte Biomarker aus regelmäßig entnommenen Vollblutproben der Athlet*innen gemessen und zu einem individuellen hämatologischen Profil zusammengetragen.[31] Darauf basierend wird ein persönlicher Referenzbereich definiert, so dass anhand auffälliger Abweichungen die Applikation von Erythropoese-stimulierenden Substanzen (Gruppe S2 der WADA Verbotsliste) oder die Anwendung von Blutdoping detektiert werden kann.[32] Dieses Prinzip wurde wenige Jahre später auf das individuelle Steroidmuster registrierter Athlet*innen übertragen, das routinemäßig aus Urinproben erfasst wird und den Missbrauch von anabolen Steroiden (Gruppe S1 der WADA Verbotsliste) anzeigen soll.[33] Die Gewinnung von Blutproben im Rahmen einer Dopingkontrolle ist in einem Leitfaden der WADA hinsichtlich der Planung, Durchführung der Blutentnahme sowie den Transport- und Lagerungsbedingungen definiert.[15,34] Die Entnahme erfolgt mittels Venenpunktion in der Armbeuge und muss von dafür qualifiziertem Kontrollpersonal, den sogenannten *Blood Collection Officers*, durchgeführt werden.[34] Neben den personellen Anforderungen stellt auch die invasive Natur des Entnahmeprozesses eine Hürde für die Probenahme dar. Um die Integrität der Proben zu gewährleisten, ist der Transport an strenge Vorgaben gebunden. Die stetige Kühlung hat dabei eine hohe Priorität und muss mittels Temperatur-Logger dokumentiert werden.[11,34] Dementsprechend ist die Entnahme von Blutproben logistisch gesehen mit vergleichsweise viel Aufwand und Kosten verbunden und wirkt sich insgesamt limitierend auf die Zugänglichkeit dieser Probenmatrix aus.

Komplementäre Matrices

Die Frage nach der am besten geeigneten Probenmatrix ist für die Bioanalytik, insbesondere im medizinischen und forensischen Bereich, von großer Bedeutung und bildet dementsprechend die Grundlage für ein eigenes Forschungsgebiet. Im Fokus steht dabei die Charakterisierung verschiedener Matrices im Hinblick auf die Entnahmeprozedur, die Verfügbarkeit und Zugänglichkeit, den Einfluss auf die Analytik und Erkenntnisse und Rückschlüsse, die daraus gewonnen werden können. Dadurch konnte die Anwendbarkeit von zum Beispiel Speichel, Haaren, getrockneten Blutstropfen oder Atemluft als Alternative oder Ergänzung zu bereits etablierten Matrices wie Vollblut,

Serum, Plasma oder Urin demonstriert werden.[21,35-38] Die präventive Dopingforschung kann als vergleichsweise junges Forschungsfeld von diesen Erkenntnissen profitieren.[39-42] Als vielversprechend kristallisierten sich Atemluft und getrocknete Blutstropfen heraus, die im Folgenden näher beschrieben werden.[43,44]

Atemluft

Für den Nachweis von flüchtigen Substanzen, insbesondere im Kontext von Alkoholkonsum im Straßenverkehr, hat sich Atemluft als Matrix bereits Mitte des 20. Jahrhunderts etabliert.[45,46] Mit der Veröffentlichung von Forschungsarbeiten über den erfolgreichen Nachweis von nicht-flüchtigen Substanzen aus Atemluftkondensat, gewann die Matrix in den 90er Jahren erneut an Interesse.[47] Das Kondensat wird mithilfe einer Kühlzelle gesammelt und enthält neben kondensiertem Wasser aus der Gasphase auch Aerosolpartikel und wasserlösliche flüchtige Substanzen.[48] Die Aerosolpartikel bestehen dabei aus einer grenzflächenaktiven Substanz, die im Allgemeinen als *Surfactant* (*surface active agent)* bezeichnet wird und neben Neutrallipiden und Proteinen zu einem Großteil aus Phospholipiden besteht. Es wird von spezialisierten Lungenzellen, den Pneumozyten Typ II, in die Lungenbläschen sezerniert und kleidet die Atemwege aus, um dort die Oberflächenspannung zu reduzieren.[49] Im Jahr 2009 wurde von Johnson und Morawska eine Theorie zum Entstehungsprozess des Atemaerosols veröffentlicht, das sogenannte *Bronchiole Film Burst Model*. Demzufolge verengen sich tiefer gelegene Atemwege während der Exspiration, dem Vorgang des Ausatmens, sodass es zur Blockade durch das *Surfactant* kommt. Im Zuge der Inspiration, dem Vorgang des Einatmens, werden die Atemwege belüftet und weiten sich. Dabei bleiben kleinste Partikel des *Surfactants* in den Atemwegen zurück, die mit dem Luftstrom der darauffolgenden Ausatmung nach außen getragen werden.[50] Dieser Prozess ist in **Abbildung 1.1** schematisch dargestellt.

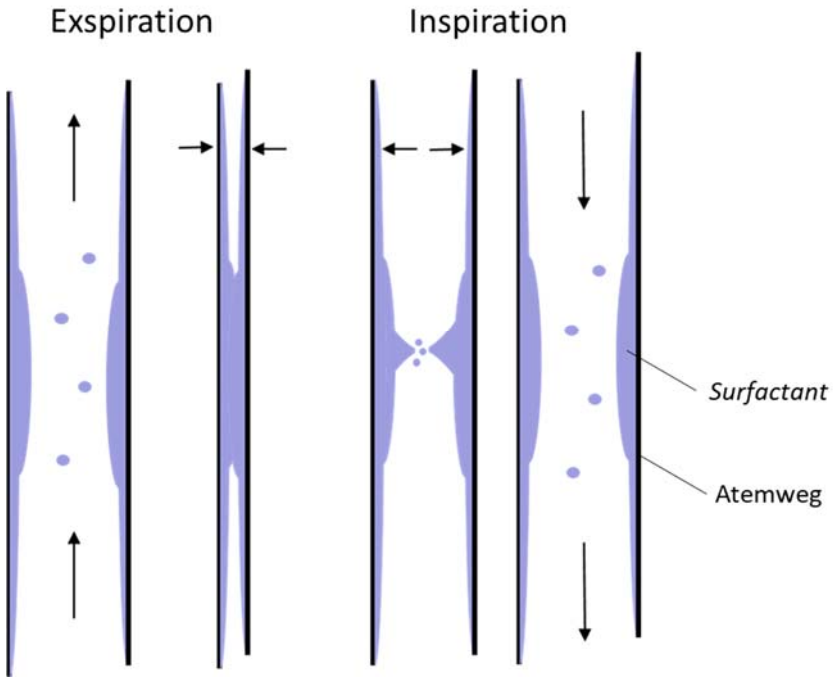

Abbildung 1.1 Aerosolbildung in den unteren Atemwegen nach dem *Bronchiole Film Burst Model* von Johnson und Morawska (2009)[50]

Für die Bewegung von nicht-flüchtigen Substanzen vom Blutkreislauf in das *Surfactant*, muss das Lungenepithel überwunden werden. Die Transportwege umfassen aktive Mechanismen zum Beispiel durch Transmembran-Proteine oder eine passive Diffusion und hängen von den physikochemischen Eigenschaften des entsprechenden Moleküls ab. Neben der Molekülgröße und Lipophilie kann auch die Bindung an Plasmaproteine einen Einfluss auf den Transportprozess haben.[51-53] Die Nutzbarkeit von Atemluftkondensat in der Dopinganalytik wird allerdings aufgrund der Verdünnung durch das kondensierte Wasser limitiert.[48] Zudem sind die zur Verfügung stehenden Gerätschaften aufgrund der notwendigen Kühlzelle schlecht portabel, sodass sie für eine Dopingkontrolle nicht praktikabel wären.[54] Die Entwicklung von Sammeltechniken, die auf Adsorptions- und Filtrationsmechanismen basieren, ermöglichte die Verwendung eines trockenen Mediums und initiierte schließlich Pilotprojekte im Anti-Doping Kontext.[44] Als Probensammelgefäß wurde dabei eine Kartusche des schwedischen Unternehmens SensAbues® mit Sitz in Stockholm verwendet. Der Aufbau der Kartusche ist in **Abbildung 1.2** dargestellt.

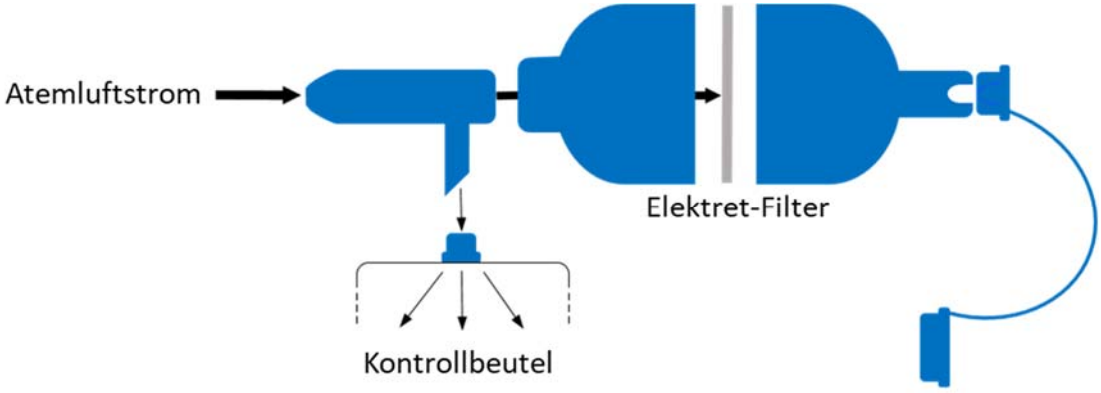

Abbildung 1.2 ExaBreath Probensammelgefäß für Atemaerosol der Firma SensAbues®

Das Kernelement der Kartusche bildet ein elektrostatisch geladener Elektret-Filter aus Polymerfasern, der Partikel des abgeatmeten Aerosols abfängt und in ein Plastikgehäuse integriert ist. Während der Probenahme wird die Kartusche mit einem Mundstück verbunden, das verschiedene Funktionen erfüllt.[55] Im Vorfeld dieser Arbeit konnte in einer Studie gezeigt werden, dass größere Partikel und Tröpfchen, die nicht aus den tieferen Atemwegen stammen, effektiv mittels eingebauter Barrieren aus Plastik abgefangen werden, um somit einer Kontamination des Filters mit zum Beispiel oralem Sekret entgegenzuwirken.[56] Ein angeschlossener Plastikbeutel dient als Indikator für die korrekte Durchführung der Beatmung und für die Vollständigkeit der Probenahme. Nach Angaben des Herstellers haben nach einer durchschnittlichen Sammelzeit von drei Minuten ca. 30 L Atemluft den Filter durchströmt. Nach Abschluss der Probenahme wird das Mundstück verworfen und die Kartusche kann verschlossen per Briefversand an ein entsprechendes Labor geschickt werden.[55] Ursprünglich wurden diese Probensammelgefäße für den Nachweis von Missbrauchssubstanzen im Kontext des illegalen Drogenkonsums entwickelt. In einer im Jahr 2012 veröffentlichten Publikation wird die Anwendung eines ersten Prototyps beschrieben, der für die erfolgreiche Detektion verschiedener Stimulanzien und Narkotika eingesetzt wurde.[57] Im Zusammenhang mit diesem Anwendungsbereich folgten weitere Veröffentlichungen, die mithilfe von Ausscheidungsstudien die Anwendbarkeit der entwickelten Kartusche demonstrierten.[58-63] Die Motivation für Forschungsarbeiten im Bereich der

Dopinganalytik basierte vor allem auf der Einfachheit der Probenahme, die für die Athlet*innen weder einen invasiven noch einen intrusiven Eingriff darstellt. Dieser Vorteil wurde in einer veröffentlichten Feldstudie mit insgesamt 521 Teilnehmer*innen bestätigt, bei der Atemluft-, Speichel- und Urinproben gesammelt und miteinander verglichen wurden. Sowohl Athlet*innen als auch das Kontrollpersonal gaben bezüglich der Probenahme eine Präferenz für Atemluft als Matrix an. Darüber hinaus erforderte die Probenahme von Atemluft im Durchschnitt ein kürzeres Zeitfenster, was in der Praxis zu einer höheren Probenahmerate beitragen könnte.[64] Verschiedene publizierte Ausscheidungsstudien mit dopingrelevanten Substanzen lassen die Annahme eines vergleichsweise kurzen Nachweisfensters zu.[59,63,65] Dies könnte sich einerseits nachteilig auf die Identifizierung von Substanzen auswirken, die zu jeder Zeit verboten sind, andererseits bietet es Vorteile für Substanzen, die nur während eines Wettkampfs verboten sind (Gruppe S6-S9 der WADA-Verbotsliste). In diesem Zusammenhang stellt die Interpretation der aus Urinproben gewonnenen Daten hinsichtlich der tatsächlichen Aufnahme und Wirkung einer Substanz in vielen Fällen aufgrund des langen Nachweisfensters eine Herausforderung dar. Für Substanzen, die eine vergleichsweise kurze Nachweisbarkeit in Atemluft aufweisen, können Daten aus Atemluftproben wertvolle ergänzende Informationen liefern, um das Applikationsfenster einzugrenzen.[39,44,65]

Getrocknete Blutstropfen

Über die erste praktische Anwendung von getrocknetem Kapillarblut wurde bereits 1963 berichtet. In dem von Robert Guthrie beschriebenen Test für Phenylketonurie, eine der häufigsten angeborenen Stoffwechselerkrankungen, wird bei Neugeborenen Kapillarblut aus der Ferse entnommen und anschließend auf Filterpapier getrocknet.[66,67] Das Potential von getrockneten Blutstropfen (*dried blood spots*, DBS) weckte daraufhin nicht nur in der klinischen Diagnostik Interesse, sondern auch im Bereich der forensischen Analytik hinsichtlich der missbräuchlichen Verwendung von Rauschmitteln.[68-71] Um die Jahrtausendwende folgten erste Pilotstudien in der Dopinganalytik zum Nachweis diverser Substanzklassen aus DBS sowie Machbarkeitsstudien und Stabilitätsuntersuchungen.[72-77] Im Jahr 2021 veröffentlichte die WADA schließlich ein

technisches Dokument, das die Prozedur der Probenahme, Transportprozesse, analytische Untersuchungen und Lagerungsvorgänge im Zusammenhang mit DBS harmonisiert.[78] Dadurch wurden DBS-Proben ein offizieller Teil der Anti-Doping Teststrategie und die daraus gewonnenen Erkenntnisse justiziabel.[43,74] Es folgte die erstmalige Einführung in die Routineanalytik bei den olympischen und paralympischen Winterspielen 2022 in Peking.[79,80] Die Vorteile wurden unter anderem in der minimal-invasiven Probenahme gesehen. Da für einen *spot* nur 15-50 µL Kapillarblut benötigt werden, ist eine Venenpunktion für die Blutgewinnung nicht mehr notwendig. Das Kapillarblut wird in der Regel durch einen Stich mit einer Lanzette aus der Fingerbeere oder dem Oberarm gewonnen. Im Vergleich zu einer Blutentnahme mittels Venenpunktion wird dadurch das Infektionsrisiko gesenkt und die Notwendigkeit für einen qualifizierten *Blood Collection Officer* entfällt.[78,81] In einer Studie von Solheim *et al.* aus dem Jahr 2021 mit 108 Teilnehmer*innen wurden die Verfahren zur Probenahme von Urin, venösem Blut und DBS miteinander verglichen. Dabei gaben sowohl die Athlet*innen als auch das beteiligte Kontrollpersonal eine Präferenz für DBS an.[81] Die entnommenen Blutstropfen werden in der Regel auf einem Filtermaterial getrocknet und zusammen mit einem Trocknungsmittel versiegelt. Die finale DBS Probe kann im Vergleich zu Urinproben deutlich platzsparender transportiert und gelagert werden. Basierend auf Stabilitätsstudien ist anzunehmen, dass der Transport in ein entsprechendes analytisches Labor bei Raumtemperatur akzeptabel ist.[75,82-84] Im Vergleich zu herkömmlichen Urin- und venösen Blutproben können logistische Strukturen hinsichtlich des Transports und der Lagerung dadurch vereinfacht und kostengünstiger gestaltet werden.[41,74] Durch die Simplizität der gesamten Doping-Kontrolle wird eine autonome Probenahme der Athlet*innen im Kontext einer Fern-Dopingkontrolle mit Videoüberwachung ermöglicht. Mit dem Aufkommen der COVID-19-Pandemie gewann diese Art der Doping-Kontrolle erstmalig an Aufmerksamkeit, da in den Jahren 2020-2022 die Reduzierung des persönlichen Kontakts zwischen den Athlet*innen und dem Kontrollpersonal zur Eindämmung der Viruserkrankung immer wieder im Vordergrund stand.[85,86] Doch auch langfristig gesehen könnten Fernkontrollen zu einer verbesserten Flächendeckung der durchgeführten Dopingkontrollen beitragen. Des Weiteren könnte die Organisation von Trainingskontrollen deutlich vereinfacht werden, sodass die Probenahmerate erhöht

werden kann und somit eine größere Daten- und Informationsmenge für die Interpretation von auffälligen Analyseergebnissen zur Verfügung steht. Mit Blick auf Substanzen, die ausschließlich während des Wettkampfes verboten sind, können Blutkonzentrationen gewinnbringende Kenntnisse über den Zeitpunkt der Applikation und die Wirksamkeit der Substanz während des relevanten Zeitraums liefern.[87,88]

Während das geringe Probenvolumen seitens der Probenahme Vorteile mit sich bringt, ist es aus analytischer Sicht mit Limitationen verbunden. Da die zur Verfügung stehende Gesamtmenge der relevanten Analyten im gleichen Maße reduziert wird, ist der Anspruch an die Sensitivität entsprechender Nachweisverfahren vergleichsweise hoch. Um diesen Anforderungen gerecht zu werden, ist eine geeignete Probenvorbereitung der komplexen Matrix sowie eine hochsensitive instrumentelle Analytik notwendig.[68] Gemäß dem von der WADA veröffentlichten technischen Dokument muss eine DBS Probe aus mindestens zwei A-Proben und einer B-Probe bestehen.[78] Dies begrenzt die Anzahl der möglichen Probenaufarbeitungen, für die jeweils mindestens ein *Spot* notwendig ist, und damit die Anzahl realisierbarer Analysen. Dadurch wird die Durchführung von Wiederholungs- und Mehrfachanalysen auf ein Minimum begrenzt.

Ziel dieser Doktorarbeit

Das initiale Ziel dieser Doktorarbeit bestand in der Untersuchung der Nutzbarkeit verschiedener komplementärer Matrices für die Dopinganalytik. Im Zuge der Promotion rückten in diesem Zusammenhang Atemluft und DBS in den Fokus, die entsprechend ihres jeweiligen Forschungsstandes charakterisiert werden sollten. Im Vordergrund stand dabei stets die Entwicklung von massenspektrometrischen Nachweismethoden für dopingrelevante Substanzen, die sowohl in der Routineanalytik als auch für die präventive Dopingforschung Anwendung finden können.

Zu Beginn der Promotion konnten vorangegangene Forschungsarbeiten die Vorteile von Atemluft als Probenmatrix demonstrieren, sodass es sich als vielversprechende Matrix im Anti-Doping Kontext herauskristallisierte.[41,44] Insbesondere die nicht-invasive sowie nicht-intrusive Probenahme und allgemeine logistische Rahmenbedingungen weckten das Interesse.[64] Zahlreiche grundlegende Fragestellungen hinsichtlich der Anwendbarkeit von Atemluft im Anti-Doping Kontext waren allerdings noch ungeklärt. Diese bezogen sich

unter anderem auf die Art und Weise der Probenahme und der daraus resultierende Einfluss auf analytische Prozesse, zum Beispiel mit Blick auf die Probenvorbereitung und Matrixeffekte. Das erste Projekt dieser Arbeit beschäftigte sich deshalb mit der Charakterisierung des Geräts *ExaBreath* der Firma SensAbues® (Stockholm, Schweden) mit Bezug auf die Effizienz der Sammeltechnik und die Robustheit des Elektret-Filters. Darüber hinaus sollte der Kenntnisstand über die Stabilität der Matrix erweitert werden, um Anforderungen an Transport- und Lagerungsbedingungen definieren zu können. Zu diesem Zweck sollte ein Nachweisverfahren für eine Vielzahl von dopingrelevanten Substanzen mittels Flüssigkeitschromatographie und Tandem-Massenspektrometrie (*liquid chromatography with tandem mass spectrometry*, LC-MS/MS) entwickelt werden, welches darüber hinaus als Screening-Verfahren für authentische Atemluftproben dienen sollte.

Dabei sollte ein System für die Simulation des Atemaerosols genutzt werden, um Atemluftproben authentisch mit dopingrelevanten Analyten anzureichern. Vor Beginn der Promotion wurde die allgemeine Nachweisbarkeit von nicht-flüchtigen Substanzen in Atemluft bereits im Rahmen verschiedener Ausscheidungsstudien demonstriert.[44,57,60,61,63,89-93] Sowohl die pulmonale Eliminierung als auch der Transportprozess der Analyten vom Blutkreislauf in das Atemaerosol ist jedoch bis heute nicht vollständig aufgeklärt. Darüber hinaus lassen sich ermittelte Daten und Erkenntnisse aufgrund der Vielfalt an chemischen Strukturen von dopingrelevanten Substanzen nicht grundsätzlich von einer Substanzklasse auf eine andere übertragen. Die Durchführung von Applikationsstudien hat eine entsprechend hohe Relevanz, um die Nachweisbarkeit verschiedener Analyten in Atemluft zu charakterisieren und sollte ein wichtiger Bestandteil der Doktorarbeit sein. Im Rahmen eines Machbarkeitsnachweises der entwickelten LC-MS/MS Methode wurden im Zuge des ersten Projekts Ausscheidungsversuche mit Glukokortikoiden und Stimulanzien durchgeführt, die darüber hinaus Informationen über das pulmonale Ausscheidungsprofil der applizierten Substanzen liefern sollten. Wie bereits in der Literatur beschrieben, konnten dabei inter- und intraindividuelle Fluktuationen von ermittelten Atemluftkonzentrationen beobachtet werden.[62,94] Daraus resultierte die Fragestellung, ob das Geschlecht oder der Konsum von Zigaretten einer Testperson als Einflussfaktoren auf die Anatomie und Physiologie der

menschlichen Lunge in Korrelation zu den beobachteten Fluktuationen stehen.[95,96] Dies wurde im Zuge des zweiten Projekts dieser Arbeit mithilfe von Ausscheidungsstudien mit Propranolol (Beta-Blocker, Gruppe P1 der WADA Verbotsliste) und Pseudoephedrin (Stimulans, Gruppe S6 der WADA Verbotsliste) untersucht. Im Kontext der Robustheit sollte dadurch die Nutzbarkeit der Matrix Atemluft in der Dopinganalytik weiter charakterisiert werden.

Im Vergleich zu Atemluft war der Forschungsstand für DBS als komplementäre Matrix für den Nachweis dopingrelevanter Substanzen bereits wesentlich fortgeschrittener. Zahlreiche Publikationen beschrieben die damit verbundenen Vorteile gegenüber venösem Blut und Urin und demonstrierten die prinzipielle Anwendbarkeit im Anti-Doping Kontext.[41,68,73,74,81,87,88] Doch erst im Laufe der hier dargelegten Promotion wurde ein technisches Dokument der WADA veröffentlicht, durch das die Matrix DBS zum festen Bestandteil des Doping-Kontroll-Systems und damit juristisch relevant wurde.[78] Um den daraus folgenden Konsequenzen für die Routineanalytik gerecht werden zu können, war die Entwicklung eines umfassenden Nachweisverfahrens für DBS notwendig. Die Erlaubnis für die Freigabe von Analyseergebnissen ist dabei verbindlich an die Akkreditierung durch die WADA geknüpft. Die vollständige Implementierung eines neuen Nachweisverfahrens war dementsprechend an ein erfolgreiches Audit gebunden und stellte das Ziel für das dritte Projekt dieser Arbeit dar.[9] Dafür sollte ein bereits bestehendes Nachweisverfahren zu einer umfassenden *Screening* Methode (*Initial Testing Procedure*, ITP) für einen Großteil der niedermolekularen Analyten der WADA Verbotsliste erweitert und gemäß der relevanten Richtlinien validiert und charakterisiert werden. Es wurde angestrebt, ein breit gefächertes Nachweisverfahren anbieten zu können, das zudem flexibel und adaptiv auf weitere Entwicklungen und Erkenntnisse in der DBS Analytik angepasst werden kann. Dies bezieht sich beispielsweise auf die Einführung von Grenzwerten für positive Befunde oder neue verbotene Substanzen durch die WADA. Da zukünftig eine erhöhte Nachfrage für Analysen von DBS erwartet wird, hatte zudem die Effizienz des Nachweisverfahrens eine hohe Relevanz und sollte mithilfe einer automatisierten Probenvorbereitung optimiert werden.

1.2 Übersicht über den ersten Artikel

A. Garzinsky, A. Thomas, O. Krug & M. Thevis. Probing for the presence of doping agents in exhaled breath using chromatographic/mass spectrometric approaches. *Rapid Communications in Mass Spectrometry*. 2021

Im Rahmen dieser Studie wurde eine massenspektrometrische Nachweismethode für ein breites Spektrum an Substanzen der WADA Verbotsliste aus Atemluftproben entwickelt. Ausscheidungsstudien mit Vertretern von Glukokortikoiden und Stimulanzien zeigten die prinzipielle Anwendbarkeit des Verfahrens. Die Publikation beschreibt zudem ein System für die Simulation des Atemaerosols, mithilfe dessen die Effizienz und die Robustheit des Probensammelgefäßes *ExaBreath* sowie die Stabilität von ausgewählten Analyten untersucht wurden.

Bisher wurden im Rahmen der Entwicklung und Validierung von massenspektrometrischen Nachweisverfahren sowie für die Abschätzung von Konzentrationen in positiven Atemluftproben *ExaBreath* Kartuschen in der Regel künstlich mit relevanten Analyten angereichert. In diesem Zusammenhang wurden Lösungen von Referenzsubstanzen auf den Elektret-Filter pipettiert und dadurch punktuell aufgetragen.[57,63,65,89,90] Dabei stellte sich die Frage nach der Übertragbarkeit der ermittelten Daten auf eine durch Beatmung angereicherte *ExaBreath* Kartusche, in der sich die im *Surfactant* gelösten Substanzen in Form kleinster Aerosolpartikel verteilen.[50] Darüber hinaus blieb bis zu diesem Zeitpunkt die Frage offen, ob die Effizienz des elektrostatisch geladenen Filters im Laufe des Beatmungsprozesses durch die zunehmende Feuchtigkeitsbeladung beeinflusst wird. Zum Zweck der Untersuchung dieser Fragestellungen wurde ein Aerosolgenerator für die Simulation des Atemaerosols genutzt, mithilfe dessen die Verteilung der Aerosolpartikel innerhalb der Kartusche sowie die Effizienz und Robustheit des Elektret-Filters charakterisiert und bewertet wurden. In diesem Zusammenhang wurde der Einfluss einer Feuchtigkeitsbeladung von bis zu ca. 500 µL getestet. Aus den daraus gewonnenen Daten lässt sich schließen, dass das Atemaerosol effizient durch den Elektret-Filter der *ExaBreath* Kartusche gesammelt und dabei nicht durch die bei einer regulären Beatmung entstehende Feuchtigkeitsbeladung

beeinträchtigt wird. Die Ergebnisse zur Untersuchung der Verteilung der Aerosolpartikel bestätigen diese Beobachtung. Sie zeigen zudem, dass die Partikel nicht nur an den Elektret-Filter, sondern auch an die Wand des Kartuschen-Gehäuses adsorbieren. Im Zuge der Optimierung einer Probenextraktion kann demzufolge eine erhöhte Wiederfindung erzielt werden, indem die gesamte Kartusche anstelle des separaten Elektret-Filters mit Lösemittel ausgespült wird.

Des Weiteren wurde in diesem Projekt die Stabilität dopingrelevanter Substanzen in dem Sammelgefäß untersucht, um Transport- und Lagerungsbedingungen im Zusammenhang mit einer Doping-Kontrolle definieren zu können.[11] Dafür wurden mithilfe des Aerosolgenerators künstlich angereicherte *ExaBreath* Atemluftproben bei -20 °C, 4 °C oder Raumtemperatur für zwei, sieben oder 28 Tage gelagert. Unabhängig von der Lagerungstemperatur konnten alle Modellsubstanzen nach einer Lagerungszeit von 28 Tagen detektiert werden. Entsprechend lässt sich annehmen, dass der Versand von Atemproben bei Raumtemperatur über einen Zeitraum von sieben Tagen, der üblicherweise den routinemäßigen Dopingkontrollprozess abdeckt, akzeptabel ist.

Als Basis für die Detektion der verwendeten Modellsubstanzen diente ein Nachweisverfahren, das bereits für ein Pilotprojekt entwickelt wurde und im Rahmen dieses Projekts auf insgesamt 49 repräsentative Analyten aus allen Substanzgruppen der WADA-Verbotsliste erweitert wurde.[44] Sowohl die Probenvorbereitung als auch die instrumentelle Analytik wurden außerdem hinsichtlich der Anzahl und der variierenden physikochemischen Natur der Substanzen optimiert. Abschließend wurde das qualitative Nachweisverfahren in Anlehnung an Richtlinien der WADA zur Methodenvalidierung hinsichtlich Selektivität, Nachweisgrenzen, Identifizierungsgrenzen, Robustheit, Verschleppung, Matrixeffekte und Wiederfindung charakterisiert.[9] Aufgrund der zu erwartenden niedrigen Konzentrationen in Atemluft, wurde ein besonderes Augenmerk auf eine hohe Sensitivität der Methode gelegt, die durch die ermittelten Nachweisgrenzen zwischen 1-500 pg/Kartusche demonstriert wird. Auffällig war die Beobachtung von signifikanten Matrixeffekten, die sich sowohl als Ionensuppression als auch als Ionenverstärkung äußerten. Zur Aufklärung dieser Effekte wurden hochauflösende Massenspektren von extrahierten unbehandelten *ExaBreath* Kartuschen aufgenommen. Dabei wurden Polyethylenglykole (PEGs) unterschiedlicher Länge identifiziert, die

vermutlich aus dem Elektret-Filter extrahiert werden und bereits in Studien einer anderen Arbeitsgruppe beobachtet wurden.[97]

Als Machbarkeitsnachweis der entwickelten LC-MS/MS Methode, aber auch zur Aufklärung pulmonaler Eliminationsprofile, dienten Applikationsstudien in Form einer oralen Einmalgabe von Vertretern der Glukokortikoide (Prednisolon) und Stimulanzien (Octodrin, Isopropylnorsynephrin und Methylhexanamin) als Modellsubstanzen. Die Ergebnisse zeigen, dass die Detektion der verabreichten Substanzen in Atemluftproben grundsätzlich möglich ist. Abhängig vom Analyten gibt die Studie Hinweise auf ein vergleichsweise kurzes Nachweisfenster in Atemluft, was sich limitierend auf die Nutzbarkeit im Anti-Doping Kontext auswirken kann.

Insgesamt konnte in diesem Teilprojekt gezeigt werden, dass die *ExaBreath* Kartuschen grundsätzlich für die Anwendung bei Dopingkontrollen geeignet sind. Die Simulation des Atemaerosols wurde erfolgreich umgesetzt, sodass eine authentische qualitative Anreicherung von *ExaBreath* Kartuschen ermöglicht wurde. Darüber hinaus wurden im Rahmen von Applikationsstudien einige dopingrelevante Substanzen erfolgreich in Atemluftproben nachgewiesen, doch weitere Forschungsarbeiten sind erforderlich, um den Zusammenhang zwischen pharmakokinetischen Eigenschaften von dopingrelevanten Substanzen und ihrer Nachweisbarkeit in Atemluft aufzuklären.

1.3 Übersicht über den zweiten Artikel

A. Garzinsky, A. Thomas & M. Thevis. Probing for factors influencing exhaled breath drug testing in sports — Pilot studies focusing on the tested individual's tobacco smoking habit and sex. *Rapid Communications in Mass Spectrometry*. 2022

In dieser Publikation werden Ausscheidungsstudien mit den dopingrelevanten Substanzen Propranolol und Pseudoephedrin beschrieben, bei denen Atemluft- sowie DBS-Proben gesammelt und im Anschluss mittels LC-MS/MS analysiert wurden. Die dabei generierten Daten wurden im Hinblick auf das Geschlecht und den Konsum von Zigaretten verglichen. Dabei konnte kein Einfluss der untersuchten Faktoren auf die Nachweisbarkeit der Substanzen in Atemluft beobachtet werden.

Zum Zeitpunkt der Projektdurchführung konnten frühere Studien bereits zeigen, dass der Nachweis dopingrelevanter Substanzen nach oraler Verabreichung in Atemluft grundsätzlich möglich ist, jedoch wurden bei verschiedenen Ausscheidungsstudien inter- sowie intraindividuelle Fluktuationen hinsichtlich der ermittelten Konzentrationen beobachtet.[62,94] Die Frage nach der Ursache dieser Fluktuationen und ob sie sich mit gewissen Eigenschaften der beprobten Individuen in einen Zusammenhang bringen lassen, war die Motivation für die Durchführung dieser Studie. Aufgrund des Einflusses auf die Anatomie und Physiologie der Lunge lag der Fokus dabei auf dem biologischen Geschlecht und dem Konsum von Zigaretten.[95,96] Zu diesem Zweck wurden insgesamt 19 Proband*innen, davon fünf Nichtraucher, fünf Nichtraucherinnen, vier Raucher und fünf Raucherinnen, jeweils zwei dopingrelevante Modellsubstanzen als orale Einmalgabe verabreicht. Dabei handelte es sich um den Beta-Blocker Propranolol (Gruppe P1 der Verbotsliste) und das Stimulans Pseudoephedrin (Gruppe S6 der Verbotsliste). Im Anschluss wurden über einen Zeitraum von 24 Stunden *ExaBreath* Atemluftproben sowie DBS Proben gesammelt.

Um eine semiquantitative Abschätzung der Konzentrationen von Propranolol und Pseudoephedrin in Atemluftproben und DBS zu ermöglichen, wurde zunächst eine Nachweismethode mittels hochauflösender Massenspektrometrie entwickelt und hinsichtlich Selektivität, Nachweis- und Identifizierungsgrenze, Präzision, Linearität und Verschleppung charakterisiert. Die mit dieser Methode gewonnenen Daten der

Ausscheidungsstudie zeigten signifikante interindividuelle Variationen in jeder der untersuchten Bevölkerungsgruppe. Dabei war kein Zusammenhang zum biologischen Geschlecht oder dem Konsum von Zigaretten erkennbar. Auch eine Korrelation zwischen Atemluftkonzentrationen und DBS-Konzentrationen konnte nicht beobachtet werden. Bei beiden Applikationsstudien wurden zudem anhand von unregelmäßigen Ausscheidungsprofilen intraindividuelle Variationen detektiert, die zum Beispiel mit verschiedenen Atemmanövern (Atemzugvolumen, Atemfrequenz) des Individuums erklärt werden können.[98] Der Vergleich mit zusätzlich ermittelten Daten aus DBS-Proben zeigte, dass unterschiedliche Konzentrationen in Atemluft nicht auf eine vom Analyten abhängige unterschiedliche Resorption in den Blutkreislauf zurückzuführen sind.

Insgesamt wurde durch dieses Projekt gezeigt, dass sich die Matrix Atemluft robust gegenüber dem Geschlecht und dem Zigarettenkonsum eines Individuums verhält. Die Anwendbarkeit von Atemluft in der Dopinganalytik konnte mit diesen Ergebnissen bestätigt werden, doch für eine Evaluierung der allgemeinen Robustheit ist die Untersuchung weiterer Einflussfaktoren notwendig.

1.4 Übersicht über den dritten Artikel

A. Garzinsky, A. Thomas, S. Guddat, C. Görgens, J. Dib & M. Thevis. Dried blood spots for doping controls — Development of a comprehensive initial testing procedure with fully automated sample preparation. *Biomedical Chromatography*. 2023

In diesem Projekt wurde ein umfangreiches Nachweisverfahren als ITP für insgesamt 233 niedermolekulare Verbindungen aus DBS entwickelt, die als Teil der WADA Verbotsliste dopingrelevant sind. Das Verfahren zeichnet sich dabei durch eine vollautomatisierte Probenvorbereitung mit anschließender adaptiver hochauflösender massenspektrometrischer (HRMS) Analyse aus. Die Methode wurde konform mit den Richtlinien der WADA validiert und erfolgreich in die Routineanalytik implementiert.[9]

Als Folge der Einführung von DBS als Probenmatrix in das Doping-Kontroll-System wurde ein wachsender Beitrag von DBS-Proben zur Routineanalytik erwartet. Um dem nachzukommen wurde angestrebt, ein geeignetes Nachweisverfahren für einen großen Teil der niedermolekularen Verbindungen der Verbotsliste zu entwickeln. Dieses Verfahren sollte für die vorläufige Identifizierung auffälliger Proben dienen, bevor entsprechende Befunde in einem Bestätigungsverfahren überprüft werden. Besondere Herausforderungen stellten dabei die hohen Anforderungen an die Sensitivität aufgrund des geringen Probenvolumens sowie die Komplexität der Matrix dar.

Die Methodenentwicklung und -validierung wurden mit FTA™ DMPK-C Karten durchgeführt und erfolgten teilweise mit aus der Fingerbeere gewonnenem Kapillarblut oder venösem Vollblut, das mit dopingrelevanten Substanzen künstlich angereichert wurde. Für die vollautomatisierte Probenvorbereitung wurde ein *MultiPurpose Sampler*, verbunden mit einem *dried blood spot autosampler*, der Firma Gerstel (Mülheim an der Ruhr, Deutschland) eingesetzt. Die instrumentelle Analytik wurde mittels eines Ultra-Hochleistungs-Flüssigkeitschromatographen (UHPLC) gekoppelt an ein hochauflösendes Massenspektrometer durchgeführt. Da der Ansatz ein breites Spektrum an unterschiedlichen chemischen Strukturen umfasst, war das Ziel des Projekts, möglichst vielseitige, flexible und anpassungsfähige chromatographische und massenspektrometrische Parameter zu konzipieren. Dementsprechend wurde das Massenspektrometer sowohl im *Full-Scan* als auch im *Data Independent Acquisition* (DIA)

Modus betrieben. Der DIA Messmodus ermöglicht neben einer retrospektiven Auswertung der Daten auch eine einfache und schnelle Adaption an Entwicklungen der WADA Verbotsliste. Die Identifizierung eines Analyten erfolgte jeweils anhand des Vorläufer-Ions aus *Full-Scan* Daten sowie eines entsprechenden Produkt-Ions aus den DIA Daten. Um die Anzahl der Datenpunkte pro Signal zusätzlich zu erhöhen, wurden zwei separate Injektionen im positiven und im negativen Ionisierungsmodus durchgeführt.

Der Ansatz wurde in Übereinstimmung mit den Richtlinien der WADA für qualitative Nachweisverfahren, den allgemeinen Anforderungen an die Kompetenz von Prüf- und Kalibrierlaboratorien (ISO/IEC 17025:2017) und dem technischen Dokument für DBS für insgesamt 233 dopingrelevante Substanzen aus allen Gruppen der Verbotsliste validiert.[9,78] Entsprechend wurden folgende Parameter berücksichtigt: Selektivität, Nachweisgrenze, Reproduzierbarkeit und Verschleppung. Des Weiteren wurde die Methode auf ihre Robustheit überprüft mit Hinblick auf eine alternative manuelle Probenvorbereitung, ein alternatives System zur Kapillarblutentnahme (Tasso-M20) sowie die Stabilität des Probenextraktes im *autosampler* der UHPLC. Anhand der untersuchten Parameter wurde die Erfüllung von vorgegeben Richtwerten bestätigt und die Leistungsfähigkeit des Nachweisverfahrens demonstriert.

Um die Anwendbarkeit des Testverfahrens zu demonstrieren, wurden vier exemplarische dopingrelevante Substanzen von gesunden Freiwilligen als orale Einmaldosis eingenommen und anschließend gesammelte DBS für die Analyse mittels des entwickelten Ansatzes zur Verfügung gestellt. Bei den Substanzen handelte es sich um die Glukokortikoide Dexamethason und Prednison sowie den Beta-Blocker Propranolol und das Stimulans Pseudoephedrin, deren Applikation nach WADA-Regularien nur während des Wettkampfes verboten ist. In allen DBS Proben, die binnen eines für einen Wettkampftag realistischen Zeitraums nach der oralen Einnahme gesammelt wurden, konnte die entsprechende Substanz mittels der entwickelten Methode identifiziert werden.

Das entwickelte Nachweisverfahren wurde abschließend erfolgreich in die Routineanalytik implementiert, um die zunehmende Bedeutung von DBS in der Dopinganalytik zu berücksichtigen und bietet eine Grundlage für optimierte Ansätze für die Analytik spezifischer Substanzklassen.

1.5 Zusammenfassende Diskussion mit Ausblick

Mit den in der Dopinganalytik etablierten Matrices Urin und Blut kann zwar der Großteil des Spektrums der im professionellen Sport verbotenen Substanzen nachgewiesen werden, doch insbesondere mit Blick auf die Prozedur der Probenahme, logistische Anforderungen und finanzielle Aspekte werden nachteilige Aspekte deutlich.[11,15,17,20-24,26,34] Demzufolge konnte ein stetig anwachsendes Interesse an komplementären Matrices in den letzten Jahrzehnten beobachtet werden, das sich in der Anzahl entsprechender Forschungsarbeiten wiederspiegelt.[40-42,44,76] Die hier vorgelegte Dissertation knüpft an diese Studien an und fokussiert sich dabei auf Atemluft und DBS. Im Zuge der drei Projekte, deren Publikationen dieser Arbeit zugrunde liegen, wurde die Nutzbarkeit dieser Matrices im Anti-Doping Kontext untersucht und entsprechende Nachweismethoden für dopingrelevante Substanzen entwickelt.

Sowohl der erste als auch der zweite Artikel befassen sich mit der Matrix Atemluft. Zum Zeitpunkt der Projektdurchführung hatte sich das Probensammelgefäß *ExaBreath* der Firma SensAbues® im Bereich der Toxikologie etabliert und wurde demzufolge auch für die Dopinganalytik in Betracht gezogen. Im Rahmen des ersten Projekts wurde ein System für die Simulation von Atemaerosol eingeführt, mithilfe dessen erstmalig eine authentische künstliche Anreicherung von *ExaBreath* Atemluftproben mit dopingrelevanten Substanzen erreicht wurde. Dadurch wurden Untersuchungen zur Verteilung des Aerosols innerhalb der Kartusche ermöglicht, die die Praktikabilität der Sammelgefäße demonstrierten. Der derzeitige Aufbau des Aerosolgenerators ist jedoch ausschließlich für eine qualitative künstliche Anreicherung von Atemluftproben geeignet. Ausblickend ist die Entwicklung eines Systems erstrebenswert, das eine quantitative Anreicherung erlaubt. Dadurch kann eine authentische Methodenentwicklung und -validierung ermöglicht und die Vergleichbarkeit von künstlich angereicherten und beatmeten Atemluftproben verbessert werden.

Im Zuge des ersten Projekts wurden signifikante Matrixeffekte beobachtet, die sich sowohl als Ionensuppression als auch als Ionenverstärkung äußerten. Diese könnten mit koeluierenden PEGs in einem Zusammenhang stehen, die in Extrakten der *ExaBreath* Kartuschen identifiziert wurden und vermutlich aus dem Elektret-Filter stammen.[97] Für eine quantitative Bestimmung wird dadurch eine korrespondierende isotopenmarkierte

Referenzsubstanz notwendig, was bei einem umfassenden Nachweisverfahren neben erhöhten Kosten auch einen erhöhten laborseitigen Arbeitsaufwand verursacht und sich somit limitierend auf die Nutzbarkeit der *ExaBreath* Kartuschen auswirkt. Eine zusätzliche Herausforderung für quantitative Analysen von Atemluftproben ergibt sich aus dem Fehlen eines Normalisierungsfaktors. Da mit den bislang kommerziell erhältlichen Probensammelgefäßen keine Aussage über das tatsächlich gesammelte Volumen an Aerosol getroffen werden kann, werden Konzentrationsangaben in bislang publizierten Studien auf die Anzahl der extrahierten Einheiten des genutzten Sammelgefäßes bezogen. Dadurch wird die Vergleichbarkeit von Studien, die verschiedene Probensammelgefäße verwenden, eingeschränkt. Da die Partikelbeladung eines definierten Volumens Atemluft in Abhängigkeit zum durchgeführten Atemmanöver steht, ist eine Normalisierung von quantitativen Bestimmungen mithilfe des Atemluftvolumens nicht möglich.[98,99] Eine endogene Substanz, die beispielsweise als Bestandteil des *Surfactants* sezerniert wird, könnte genutzt werden, um die tatsächliche Aerosolmenge einer Atemluftprobe abzuschätzen und quantitative Analyseergebnisse zu normieren. Für den Nachweis dopingrelevanter Substanzen, deren Verbot an einen Grenzwert gebunden ist, wäre diese Vereinheitlichung zwingend erforderlich und somit eine Voraussetzung für eine zukünftige Nutzbarkeit der Matrix im Anti-Doping Kontext. In einer bereits veröffentlichten Studie stand das Dipalmitoylphosphatidylcholine als Hauptkomponente des *Surfactants* als potentieller Normalisierungsfaktor im Fokus. Da die dabei erhobenen Daten jedoch keine erfolgreiche Normalisierung der ermittelten Konzentrationen in Atemluftproben zeigten, sind weitere Forschungsarbeiten auf diesem Gebiet notwendig.[100,101] Zum aktuellen Zeitpunkt ist Atemluft als Probenmatrix ausschließlich für qualitative Bestimmungen geeignet.[53]

Bei der Durchführung von Applikationsstudien zeigte sich, dass die prinzipielle Nachweisbarkeit von dopingrelevanten Substanzen in Atemluft nicht von einer Substanzklasse auf eine andere übertragbar ist. Während einige der getesteten Modellsubstanzen der Stimulanzien für einen Zeitraum von bis zu 48 Stunden identifizierbar waren, konnte Prednisolon als Vertreter der Glukokortikoide nur in Atemluftproben einiger Proband*innen detektiert werden. Dies könnte auf pharmakodynamische und pharmakokinetische Parameter der Substanzen

zurückzuführen sein, wie beispielsweise die Bioverfügbarkeit, Eliminierungsprozesse und Bindungen an Plasmaproteine. Darüber hinaus wird die Diffusion der Analyten vom zirkulierenden Blut durch das Lungenepithel in das *Surfactant* durch die physikochemischen Eigenschaften wie die Lipophilie, Polarität und Größe des Moleküls beeinflusst.[102] Für die Anwendung der Matrix im Anti-Doping Kontext bedeutet dies, dass weitere umfassende Ausscheidungsversuche durchgeführt werden müssen, um eine Einschätzung der Nutzbarkeit von Atemluft zu ermöglichen, insbesondere hinsichtlich der Substanzklassen, die in einem realistischen Konzentrationsbereich nachweisbar sind.

Neben den Eigenschaften der Substanz kann auch die Physiologie des Individuums einen Einfluss auf die Nachweisbarkeit in Atemluft haben. Im Rahmen des zweiten Projekts wurden deshalb Vergleichsstudien durchgeführt, bei denen das biologische Geschlecht sowie der Konsum von Zigaretten mit Daten aus Ausscheidungsversuchen mit zwei dopingrelevanten Substanzen in Relation gebracht wurden. Dabei konnte kein Zusammenhang aufgezeigt werden, doch es wurden signifikante inter- und intra-individuelle Schwankungen bezüglich der detektierten Atemluftkonzentration beobachtet. Diese könnten auf individuelle Atemmanöver während der Probenahme zurückzuführen sein. Um weitere Faktoren wie zum Beispiel den Einfluss von physischer Aktivität vor der Probenahme bewerten zu können, ist die Durchführung von weiteren Forschungsarbeiten notwendig.

Insgesamt konnten die Untersuchungen zur Nutzbarkeit von Atemluft als Matrix in der Dopinganalytik demonstrieren, dass dopingrelevante Substanzen grundsätzlich unter Verwendung der *ExaBreath* Kartuschen nachgewiesen werden können. Das getestete Probensammelgefäß erwies sich als effizient und geeignet für die Probenahme bei Dopingkontrollen. Darüber hinaus konnte die Robustheit der Matrix gegenüber dem Geschlecht und dem Konsum von Zigaretten gezeigt werden. Für den Nachweis einiger Substanzklassen ist eine vergleichsweise hohe Sensitivität erforderlich, die zum jetzigen Zeitpunkt eine Limitation darstellt. Dennoch konnte bislang eine stetige Weiterentwicklung der für die instrumentelle Analytik verwendeten Gerätschaften beobachtet werden und ist dementsprechend auch zukünftig zu erwarten. Dadurch bieten sich für die Dopinganalytik stetig neue Möglichkeiten an und könnte auch die Nachweisbarkeit einiger Substanzklassen in Atemluft langfristig verbessern. Für Vertreter

der Substanzklasse der Stimulanzien, die in den hier beschriebenen Applikationsstudien verwendet wurden, konnte bereits ein ausreichendes Nachweisfenster in Atemluft erreicht werden. Das bedeutet, dass Daten aus Atemluftproben, die ergänzend zu etablierten Matrices bei Dopingkontrollen gesammelt werden, bereits jetzt unterstützende Informationen für die Interpretation von auffälligen Analyseergebnissen für gewisse Substanzen liefern könnten. Dies ist besonders wertvoll für Substanzklassen, die ausschließlich während des Wettkampfs verboten sind. Die herausragende Simplizität der Probengewinnung von Atemluft könnte Interesse bei Sportverbänden wecken, die bislang nicht den WADC unterzeichneten und nicht Teil eines international organisierten und harmonisierten Doping-Kontroll-Systems sind. Mit der Anwendung von Atemluft könnte dadurch die Einführung von Dopingkontrollen bei entsprechenden Sportarten initiiert werden.

Im Rahmen des dritten Projekts wurde erfolgreich ein Nachweisverfahren mit automatisierter Probenvorbereitung für insgesamt 233 dopingrelevante Substanzen validiert und in die Routineanalytik implementiert. Im Jahr 2022 wurde der Ansatz bereits für die Analyse von über 200 DBS-Proben angewendet und ausblickend kann für die kommenden Jahre ein stetig wachsender Beitrag von DBS-Proben zur Routineanalytik erwartet werden. Dabei muss derzeit noch beachtet werden, dass bei der praktischen Anwendung des Verfahrens ein Teil der validierten Substanzen nicht in die Auswertung von analytischen Daten einbezogen wird. Darunter fallen alle Substanzen, für die offizielle Grenzwerte für die Berichterstattung von auffälligen Befunden in Urin- oder Blutproben definiert sind. Diese werden durch die WADA festgelegt und sollen die Freigabe von positiven Fällen harmonisieren.[103,104] In einer Veröffentlichung von Thevis *et al.* aus dem Jahr 2022 werden auf der Grundlage von Daten aus Applikationsstudien entsprechende Grenzwerte für DBS in der Dopinganalytik vorgeschlagen.[74] Bis zur offiziellen Einführung solcher Grenzwerte durch die WADA, werden bei der Auswertung von Analyseergebnissen von DBS-Proben ausschließlich Substanzen berücksichtigt, für die es keinen Grenzwert (*Decision Limit* oder *Minimum Reporting Level*) für Urin- oder Blutproben gibt. Die Veröffentlichung eines entsprechenden Dokuments kann für die nahe Zukunft erwartet werden. Dies hat zur Folge, dass quantitative Bestimmungen oder Abschätzungen in DBS ermöglicht werden müssen und die Leistung der entsprechenden

Nachweismethoden angepasst werden muss. Um eine einfache und schnelle Reaktion auf Entscheidungen der WADA hinsichtlich neuer verbotener Substanzen oder Grenzwerte zu ermöglichen, wurde die Entwicklung einer möglichst anpassungsfähigen massenspektrometrischen Methode für das dritte Projekt angestrebt. Dieses Ziel wurde mithilfe des *full scan* sowie des *Data Independent Acquisition* (DIA) Messmodus umgesetzt. Beim DIA Messmodus wird die Generierung von Produkt-Ionen-Spektren anhand eines festgelegten Massenbereichs induziert. Dadurch wird die simultane Detektion von Produkt-Ionen verschiedener Vorläufer-Ionen erreicht, was im Vergleich zu einer datenabhängigen und zielgerichteten kollisionsinduzierten Dissoziation Zeit spart und somit die Anzahl der generierten Datenpunkte pro Experiment erhöht.[105] Die Auswertung der erzeugten Daten kann demzufolge flexibel an Fragestellungen angepasst werden. Somit ist zudem eine retrospektive Auswertung der Daten möglich. Um der zu erwartenden erhöhten Anzahl an Proben gerecht werden zu können, wurde eine automatisierte Probenvorbereitung angestrebt. Dadurch sollen neben personellen Ressourcen auch Kosten eingespart werden. Das im Rahmen des Projekts verwendete System kann für die vollautomatisierte Vorbereitung von bis zu 48 Proben gleichzeitig verwendet werden. Hervorzuheben ist dabei die effiziente Extraktionstechnik, bei der das Lösungsmittel mit erhöhter Temperatur (80 °C) und unter Druck durch den *Spot* strömt, sodass eine hohe Wiederfindung mit wenig Zeitaufwand erzielt wird. Nachteilig wirkt sich dabei die limitierte Flexibilität bezüglich des DBS-Sammelsystems aus. Das Extraktionssystem ist ausschließlich für ein Kartensammelsystem konstruiert. Die dazugehörigen Bauteile sind auf das Greifen und Halten von Karten einer definierten Größe ausgelegt, sodass DBS, die mit einem davon abweichenden System (wie z.B. Tasso-M20) gesammelt wurden, nicht mit dem hier verwendeten Extraktionssystem aufgearbeitet werden können. Für diesen Fall wurde im Kontext der Robustheit eine manuelle Probenvorbereitung validiert, die von einer Laborkraft durchgeführt werden muss. Nach Implementierung der Nachweismethode in die Routineanalytik konnte eine Tendenz für die Nutzung von Tasso-M20 Sammelsystemen beobachtet werden. Dieses Sammelsystem wird als Verbund vertrieben. Es wird am Oberarm angebracht und mittels Knopfdrucks ausgelöst. Das Kapillarblut wird automatisch auf vier Probenzylinder aufgebracht und in dieser Form getrocknet.[106] Hinsichtlich der Simplizität der

Probenahme bietet dieses System im Vergleich zu DBS-Sammelkarten entsprechend Vorteile. Die Reliabilität von quantitativen Bestimmungen wird bei Tasso-M20 Geräten durch die Reproduzierbarkeit der volumetrischen Absorption des Kapillarbluts bedingt. Der Hersteller gibt dafür eine Standardabweichung von weniger als 5% an.[106] Im Vergleich dazu beruht die Reproduzierbarkeit von ermittelten DBS-Konzentrationen ausgehend von Sammelkarten auf der Theorie, dass das Filterpapier pro Kubikzentimeter ein definiertes Volumen Kapillarblut aufnimmt. Dieses Volumen wird durch den Anteil der Blutzellen am Gesamtblutvolumen, dem individuellen Hämatokrit-Wert, beeinflusst, was sich auf die Ausdehnung des Blutstropfens aus der Sammelkarte auswirkt. Da das automatisierte Extraktionssystem einen festgelegten Durchmesser des *Spots* extrahiert, kann es daher bei quantitativen Bestimmungen zu Verzerrungen aufgrund von schwankenden Hämatokrit-Werten kommen, die als *hematocrit bias* bekannt sind.[74,107] Um diesem systematischen Fehler entgegenzuwirken, soll zukünftig eine nicht-destruktive Hämatokrit-Bestimmung mittels Nahinfrarot in die Routineanalytik von DBS-Proben eingeführt werden. Quantitative Analyseergebnisse sollen anschließend mithilfe des Hämatokrit-Werts normalisiert werden.[108-110] In diesem Zusammenhang bietet das Tasso-Sammelsystem den Vorteil, dass die Probenzylinder regulär vollständig extrahiert und der systematische Fehler somit grundsätzlich vermieden werden soll.[111] Doch erst im Laufe der Zeit wird sich herausstellen, welches DBS-Sammelsystem sich langfristig etabliert und ob es international zu einer Harmonisierung kommen wird. Ausblickend bedeutet dies, dass eine Anpassung des automatisierten Extraktionssystems an weitere Sammelsysteme notwendig sein könnte, um das Ziel einer effizienten Probendurchlaufquote weiterhin erfüllen zu können.

Im Rahmen dieser Doktorarbeit konnten wichtige Kenntnisse zur Nutzbarkeit von Atemluft und DBS in der Dopinganalytik gewonnen werden. Es wurde gezeigt, dass der Nachweis verschiedener Modellsubstanzen in Atemluft grundsätzlich möglich ist und wertvolle ergänzende Informationen liefern kann. Das untersuchte Probensammelgefäß erwies sich als effizient und robust, doch insbesondere hinsichtlich der Reliabilität von quantitativen Bestimmungen wurden Limitationen beobachtet. Zukünftig ist die Durchführung umfangreicher Applikationsstudien erforderlich, um pulmonale Ausscheidungsprofile von weiteren dopingrelevanten Substanzen zu charakterisieren und

somit die tatsächliche Nutzbarkeit der Matrix in der Dopinganalytik bewerten zu können. Für DBS wurde ein umfangreiches Nachweisverfahren entwickelt, das bereits erfolgreich in der Routineanalytik angewendet wird. Ausblickend ist die Veröffentlichung weiterer Vorgaben und Richtlinien der WADA für Prozesse im Zusammenhang mit DBS zu erwarten, die in das Verfahren eingearbeitet werden müssen. Diesem kann durch die Adaptivität der massenspektrometrischen Parameter des entwickelten Verfahrens mit wenig Aufwand nachgekommen werden. Dadurch trägt diese Forschungsarbeit maßgeblich dazu bei, die Dopinganalytik an neueste Entwicklungen anzupassen.

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2 Probing for the presence of doping agents in exhaled breath using chromatographic/mass spectrometric approaches

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2.1 Abstract

Rationale: Exhaled breath (EB) has been demonstrated to be a promising alternative matrix in sports drug testing due to its non-invasive and non-intrusive nature compared with urine and blood collection protocols. In this study, a pilot-test system was employed to create drug-containing aerosols simulating EB in support of the analytical characterization of EB sampling procedures, and the used analytical method was extended to include a broad spectrum of prohibited substances.

Methods: Artificial and authentic EB samples were collected using sampling devices containing an electret filter, and doping agents were detected by means of liquid chromatography and tandem mass spectrometry with unispray ionization. The analytical approach was characterized with regard to specificity, limits of detection, carry-over, recovery and matrix effects, and the potential applicability to routine doping controls was shown using authentic EB samples collected after single oral dose applications of glucocorticoids and stimulants.

Results: The analytical method was found to be specific for a total of 49 model substances relevant in sports drug testing, with detection limits ranging from 1 to 500 pg per cartridge. Both ion suppression (−62%) and ion enhancement (+301%) effects were observed, and all model compounds applied to EB sampling devices were still detected after 28 days of storage at room temperature. Authentic EB samples collected after the

oral administration of 10 mg of prednisolone resulted in prednisolone findings in specimens obtained from 3 out of 6 participants up to 2 h. In octodrine, dimethylamylamine (DMAA) and isopropylnorsynephrine post-administration EB samples, the drugs were detected over a period of 50, 48, and 8 h, respectively.

Conclusions: With the analytical approach developed within this study, the identification of a broad spectrum of prohibited doping agents in EB samples was accomplished. Application studies and stability tests provided information to characterize EB as a potential matrix in sports drug testing.

2.2 Introduction

The fight against doping has evolved into an internationally organized and omnipresent objective, governed by the World Anti-Doping Agency (WADA).[1,2] Alongside fair competitions, the health of the athletes is a major concern.[3] Currently used matrices for doping control purposes are mostly urine, serum and whole blood samples to address the full spectrum of prohibited substances and methods of doping. The renal pathway is a major route for the elimination of drugs from the blood circulation and the detection of metabolites can significantly broaden the detection window,[4] but urine sampling must be performed under supervision to ensure the integrity of the sample, which is a substantial intrusion into privacy.[5] In addition, doping control samples are often collected after physical activity causing dehydration of the athletes, which can complicate result interpretation on the one hand and lead to a prolonged duration of the urine sampling procedure on the other.[6] The actual effect of a substance at the time of sampling can be estimated more precisely by determining the concentration in whole blood or plasma samples.[7] Furthermore, due to a limited excretion into urine, blood is the preferred matrix for molecules of comparably high molecular mass such as protein-derived therapeutics.[8] Apart from the invasive nature of the blood collection procedure, it necessitates strictly harmonized collection, transportation and storage conditions.[9]

In this context, the interest in alternative matrices that are less intrusive or invasive has increased remarkably in recent years.[10] In addition to e.g. oral fluid, hair and dried blood spots, exhaled breath (EB) was found to be a promising matrix, especially for sports drug testing purposes.[11-14] The collection and analysis of exhaled breath condensate (EBC) initially revealed the potential to detect non-volatile substances in EB, but target analyte concentrations have been diluted by resulting volumes of condensed water vapor, and differing collection devices have complicated the comparison of test results.[15] With new collection methods using adsorption and filtration mechanisms, the collection of exhaled particles on a dry medium was achieved.[16,17] One collection device utilizing a thin electret filter to capture particles by electrostatic forces has been established in drug abuse testing,[18-20] and pilot studies concerning its applicability in doping controls followed.[14] From the clients'/tested individuals' point of view, EB sampling was well received,[21,22] and also in terms of sampling costs, stability of the analytes, and a minimized risk of sample

manipulation, EB samples are expected to offer adequate characteristics. The maximum detection time for target analytes in EB is expected to be shorter than for urine and blood, which was confirmed for several substances in earlier studies.[14,20,23] While this limits the utility of EB testing in the context of retrospectivity, it might offer an advantage for the analysis of substances that are banned in-competition only.

Various studies provide proof that drug testing in EB is feasible, and artificial EB samples used for method developments and validations are commonly prepared by spiking the analytes (diluted in appropriate solvent) directly onto the electrostatic filter of the sampling device.[16,18,20,23-33] However, in authentic EB samples, the EB aerosol is distributed through small droplets[34,35] and further research into the comparability of authentic and artificial EB samples is warranted, especially regarding the interactions between the small aerosol droplets and the electrostatic filter. In this study, a system was developed that enables the simulation of EB aerosols containing selected doping agents in order to examine the retention efficiency of the electret filter and the stability of the analytes after the sampling procedure. As an additional objective of this study, a method was developed to indicate the presence of a broad range of prohibited substances in EB samples by means of liquid chromatography and tandem mass spectrometry (LC/MS/MS). Proof-of-concept was accomplished by the analysis of collected EB samples after single application of representatives of glucocorticoids and stimulants.

2.3 Experimental

2.3.1 Chemicals and materials

EB sampling devices were obtained from SensAbues AB (Stockholm, Sweden). Methanol and acetonitrile for sample preparation and LC/MS/MS analysis were of analytical grade and purchased from VWR International GmbH (Darmstadt, Germany). Ethyl acetate, ammonium acetate and acetic acid were from Merck (Darmstadt, Germany). Detailed information about the vendors/providers of the reference standards is given in **Table 2.3** (supporting information).

2.3.2 Stock solutions and working solutions

Stock solutions of all model compounds (**Table 2.1**) were prepared at a concentration of either 1 mg/mL or 100 µg/mL in methanol. Mixed working solutions at concentrations of 10 µg/mL, 0.1 µg/mL and 0.01 µg/mL in a mixture of water and acetonitrile (50:50, *v/v*) were generated by dilution of the stock solutions. A mixture used as internal standard solution contained deuterated compounds, listed in **Table 2.2**, in varying concentrations (5 to 500 ng/mL) depending on the detection limit. All stock and working solutions were stored at −20 °C. The solutions used for the aerosol generation were freshly prepared by diluting the working solutions to a concentration of 20 ng/mL in a mixture of acetonitrile and water (95:5, *v/v*).

2.3.3 EB sampling

Authentic EB sampling was conducted according to the manufacturer's instructions.[19] During breathing through a mouthpiece into the cartridge, a plastic bag mounted on the mouthpiece inflates and indicates the completeness of the sampling procedure, implying that approximately 30 L of exhaled breath passed through the filter. After completion, the mouthpiece including the plastic bag is removed and the cartridge is sealed and stored at room temperature until analysis. Blank specimens for assay characterization were provided by a total of 7 female and 9 male healthy volunteers aged 23–47 years.

Table 2.1 Key parameter of the analytical test method and main results of the assay characterization. US: Unispray, CV: cone voltage, CE: collision energy, ISTD: internal standard, LOD: limit of detection, LOI: limit of identification

Substance Class (Class in Prohibited List)		Compound	Ion Mode (US+ / US-)	Precursor Ion (m/z) // CV [V]	Quantifier Ion (m/z) // CE [eV]	Qualifier Ion (m/z) // CE [eV]	ISTD	LOD/LOI [pg/cartridge]	Matrix Effects [%]	Recovery [%]
Prohibited at all times	**S1:** Anabolic Agents	Metandienone	+	301.1 // 28	121.0 // 24	149.1 // 14	Tes-D_3	10 / 10	124	105
		Dehydrochlormethyl-testosterone (DHCMT)	+	335.2 // 2	155.0 // 34	149.1 // 16	Sta-D_3	100 / 500	192	98
		Testosterone	+	289.2 // 20	97.1 // 25	109.1 // 25	Tes-D_3	50 / 50	146	107
		Stanozolol	+	329.2 // 28	95.1 // 45	81.1 // 45	Sta-D_3	500 / 500	224	94
		Ostarine	-	388.1 // 50	118.1 // 20	269.2 // 15	Dex-D_4	10 / 10	304	64
		Andarine	-	440.1 // 50	150.0 // 25	261.0 // 20	Dex-D_4	10 / 10	278	81
		Trenbolone	+	271.1 // 16	107.0 // 34	83.0 // 26	Dex-D_4	500 / 500	130	100
	S2: Agents affecting erythropoiesis	Desidustat	+	333.3 // 20	261.1 // 9	279.2 // 11	Dex-D_4	50 / 100	116	98
		Vadadustat	-	305.0 // 25	204.0 // 25	261.0 // 12	Tes-D_3	100 / 500	162	102
		Molidustat	+	315.3 // 20	207.2 // 25	137.2 // 38	Amph-D_6	100 / 100	54	76
	S3: Beta-2-Agonists	Salbutamol	+	240.3 // 20	148.2 // 15	166.2 // 10	Sal-D_6	1 / 1	97	93
		Terbutaline	+	226.2 // 34	152.1 // 14	125.1 // 22	Sal-D_6	1 / 10	107	90
		Formoterol	+	345.41 // 20	149.1 // 18	121.0 // 31	Met-D_9	1 / 10	65	92
	S4: Hormone and metabolic modulators	Meldonium	+	147.1 // 4	58.2 // 12	59.2 // 12	Mel-D_3	10 / 10	83	83
		Anastrozole	+	294.1 // 32	225.1 // 20	142.0 // 40	Dex-D_4	1 / 10	105	96
		Exemestane	+	297.1 // 2	121.1 // 16	149.0 // 14	Sta-D_3	500 / 500	189	106
	S5: Diuretics	Hydrochlorothiazide	-	296.0 // 70	269.0 // 18	205.1 // 23	Hct-$^{13}C_1$,D_2	50 / 50	58	94
		Triamterene	+	254.0 // 2	237.1 // 24	168.0 // 32	Amph-D_6	10 / 10	47	64
		Torasemide	+	349.3 // 20	264.2 // 17	168.2 // 45	Pre-D_6	1 / 10	73	94
		Indapamide	+	366.8 // 20	132.1 // 17	117.2 // 42	Dex-D_4	500 / 1000	107	91

Table 2.1 (Continued)

Substance Class (Class in Prohibited List)		Compound	Ion Mode (US+ / US-)	Precursor Ion (m/z) // CV [V]	Quantifier Ion (m/z) // CE [eV]	Qualifier Ion (m/z) // CE [eV]	ISTD	LOD/LOI [pg/cartridge]	Matrix Effects [%]	Recovery [%]
Prohibited in-competition	**S6:** Stimulants	Amphetamine	+	136.2 // 20	119.2 // 6	91.1 // 13	Amph-D_6	10 / 10	48	79
		Pseudoephedrine	+	166.1 // 24	133.0 // 20	117.0 // 20	Eph-D_3	10 / 10	48	78
		Methylphenidate	+	234.0 // 22	84.1 // 18	91.0 // 36	Met-D_9	10 / 100	54	79
		25-I-NBOMe	+	428.0 // 25	121.0 // 24	91.0 // 45	Tes-D_3	100 / 500	278	25
		Pentylone	+	236.2 // 6	188.1 // 16	175.1 // 18	Met-D_9	10 / 10	53	71
		X-Ethylmethcathinone	+	192.2 // 25	145.0 // 21	174.0 // 11	Met-D_9	10 / 10	66	68
		Cocaine	+	304.2 // 20	105.0 // 30	150.0 // 25	Coc-D_3	10 / 10	66	62
		Methylhexanamine	+	116.1 // 12	57.0 // 10	99.1 // 6	Amph-D_6	100 / 500	36	85
		Octodrine	+	130.1 // 18	71.0 // 10	57.0 // 12	Met-D_9	100 / 100	71	80
		Heptaminol	+	146.1 //2	128.0 // 8	146.1 // 14	Sal-D_6	50 / 50	106	92
		Isopropylnorsynephrine	+	196.0 //34	91.0 // 24	136.0 // 14	Mor-D_3	10 / 10	84	93
	S7: Narcotics	Pethidine	+	248.1 // 26	70.0 // 26	174.1 // 26	Coc-D_3	1 / 10	76	74
		Morphine	+	286.2 // 30	165.0 // 35	201.0 // 27	Mor-D_3	50 / 50	116	92
		Methadone	+	310.4 // 20	265.3 // 13	105.1 // 28	Tes-D_3	1 / 1	192	68
		Oxycodone	+	316.0 // 2	241.0 // 26	298.1 // 16	Amph-D_6	1 / 1	37	83
		Codeine	+	300.2 // 4	165.0 // 36	215.1 // 22	Cod-D_3	1 / 1	71	89
		Hydrocodone	+	300.2 // 60	128.0 // 48	171.0 // 20	Hyd-D_3	50 / 500	44	84
	S8: Cannabinoids	JWH-073	+	328.3 // 20	155.1 // 23	127.2 // 42	Sta-D_3	10 / 10	258	69
		AM-1220	+	383.4 // 20	98.2 // 25	112.2 // 20	Dex-D_4	100 / 100	401	23
		JWH-019	+	356.1 // 10	155.0 // 24	127.0 // 46	Sta-D_3	10 / 10	1228	76
	S9: Glucocorticoids	Dexamethasone	+	393.3 // 40	373.3 // 7	355.3 // 10	Dex-D_4	50 / 50	117	92
			-	450.8 // 32	361.1 // 18	307.0 // 30		50 / 50	149	103
		Prednisolone	+	361.4 // 20	343.4 // 10	147.2 // 26	Pre-D_6	100 / 100	98	96
			-	419.0 // 42	329.0 // 10	187.0 // 32		100 / 100	160	101

Table 2.1 (Continued)

Substance Class (Class in Prohibited List)		Compound	Ion Mode (US+ / US-)	Precursor Ion (m/z) // CV [V]	Quantifier Ion (m/z) // CE [eV]	Qualifier Ion (m/z) // CE [eV]	ISTD	LOD/LOI [pg/cartridge]	Matrix Effects [%]	Recovery [%]
Prohibited in-competition		Methylprednisolone	+	375.2 // 30	161.0 // 25	321.2 // 10	Mep-D_2	100 / 100	118	88
		Prednisone	+	359.2 // 20	147.0 // 28	313.2 // 12	Pre-D_6	500 / 500	94	92
			-	416.8 // 24	327.0 // 12	299.1 // 18		100 / 100	156	89
Prohibited in particular sports	**P1:** Beta-Blockers	Carvedilol	+	407.4 // 20	100.2 // 27	224.2 // 22	Dex-D_4	100 / 500	318	14
		Propranolol	+	260.2 // 20	116.2 // 17	183.2 // 18	Pro-D_7	10 / 10	103	58
		Metoprolol	+	268.2 // 2	116.1 // 18	74.2 // 20	Met-D_9	10 / 10	55	93
		Bisoprolol	+	326.3 // 80	116.1 // 16	74.1 // 22	Pro-D_7	1 / 10	58	77
		Acebutolol	+	337.4 // 20	116.2 // 25	98.2 // 19	Amph-D_6	10 / 10	37	89

Table 2.2 Test method characteristics for internal standards. US: Unispray, CV: cone voltage, CE: collision energy

Internal Standards	Ion Mode (US+ / US-)	Precursor Ion (*m/z*) // CV [V]	Product Ion (*m/z*) // CE [eV]	Matrix Effects [%]
Amphetamine-D_6 (Amph-D_6)	+	142.0 // 4	93.0 // 14	42
Meldonium-D_3 (Mel-D_3)	+	150.1 // 4	61.2 // 12	70
Ephedrine-D_3 (Eph-D_3)	+	169.1 // 24	117.0 // 12	53
Methylphenidate-D_9 (Met-D_9)	+	243.0 // 54	93.0 // 25	60
Salbutamol-D_6 (Sal-D_6)	+	246.3 // 4	148.2 // 18	104
Propranolol-D_7 (Pro-D_7)	+	267.2 // 18	72.0 // 20	99
Morphine-D_3 (Mor-D_3)	+	289.2 // 20	201.3 // 25	107
Testosterone-D_3 (Tes-D_3)	+	292.2 // 25	109.1 // 21	136
Codeine-D_3 (Cod-D_3)	+	303.1 // 76	215.1 // 24	54
Hydrocodone-D_3 (Hyd-D_3)	+	303.2 // 60	199.1 // 30	47
Cocaine-D_3 (Coc-D_3)	+	307.2 // 40	185.0 // 20	73
Stanozolol-D_3 (Sta-D_3)	+	332.2 // 16	81.0 // 42	238
Prednisolone-D_6 (Pre-D_6)	+	367.2 // 20	312.4 // 11	94
	-	424.8 // 26	333.1 // 16	151
Methylprednisolone-D_2 (Mep-D_2)	+	377.1 // 12	161.1 // 18	107
Dexamethasone-D_4 (Dex-D_4)	+	397.4 // 20	377.4 // 7	116
	-	455.4 // 20	363.3 // 15	151
Hydrochlorothiazide-$^{13}C_1$,D_2 (Hct-$^{13}C_1$,D_2)	-	298.7 // 54	270.0 // 18	57

2.3.4 Sample preparation

Ventilated or aerosolized specimens were fortified with 10 µL of the mixture of internal standards via pipetting onto the electret filter, and the sampling device was subsequently extracted with 4 mL of methanol. For the elution of the solvent, the outlet of the sampling device was placed on a glass test tube and centrifuged at 1800 *g* for 1 min. The resulting eluate was evaporated to dryness in a stream of compressed air at 50 °C, reconstituted with 100 µL of a mixture of acetonitrile and water (5:95, *v/v*), and subjected to LC/MS/MS analysis with an injection volume of 10 µL.

2.3.5 LC/MS/MS

LC/MS/MS analyses were performed on an Aquity I-Class ultra-performance liquid chromatography (UPLC) system interfaced via a unispray (US) ionization source to a Xevo TQ-XS triple quadrupole mass spectrometer (Waters, Eschborn, Germany). The liquid

chromatograph was equipped with an Poroshell C-8 analytical column (50 × 3.0 mm, 2.7 µm particle size; Agilent). The LC system was operated at a flow of 0.4 mL/min with 5 mM aqueous ammonium acetate buffer (pH 4.5, solvent A) and acetonitrile with 5% solvent A and 0.1% acetic acid (solvent B). The gradient started at 5% B increasing to 15% B from 1 to 2 min followed by an increase to 60% from 2 to 9 min and ended with a final increase to 100% B from 9 to 12 min and re-equilibration at 1% B for 3 min. The US source was operated in positive mode using an impactor voltage of 3.1 kV and in negative mode using an impactor voltage of 1 kV. The desolvation temperature was set to 500 °C. The mass spectrometer recorded two diagnostic precursor/product ion pairs per analyte in multiple reaction monitoring (MRM) mode. The collision gas was argon (purity grade 5.0) and nitrogen (provided by a nitrogen generator; CMC Eschborn, Germany) was used to support the ionization process in the ion source. Collision energies and declustering potentials were optimized for each ion transition. A summary of the main parameters is shown in **Table 2.1**.

2.3.6 Assay characterization

The characterization of the assay was derived from the requirements for validation of an initial testing procedure approved by WADA and described in the International Standards for Laboratories and the Laboratory Technical Note for the Validation of Methods of Analysis for Doping Control Testing with adaptations for EB.[36] In this context, selectivity, limits of detection (LODs), limits of identification (LOIs), robustness and carryover were determined. In addition, matrix effects and recovery were investigated. Selectivity was assessed by examining 10 blank samples (5 female, 5 male) for interfering signals at the expected retention time using diagnostic ion transitions. The average noise for each analyte was calculated using the same set of blank samples. In order to estimate the LODs, obtained signals from respectively six EB samples prepared at 1, 10, 50, 100, 500 or 1000 pg had to exceed the average noise plus a threefold standard deviation by at least a factor 3 at the expected retention time at one precursor/product ion pair. Accordingly, for the estimation of the LOIs, the signal-to-noise ratio had to exceed a factor of 3 at two precursor/product ion pairs. LOD samples prepared at 500 pg were re-analyzed after storage for 1 day in the autosampler to assess the stability of the sample extract. The

carryover effect was investigated in three blank samples each after analysis of EB samples prepared at 1, 2 and 5 ng. Matrix effects were examined by comparing peak areas of blank EB samples prepared by adding 500 pg of the model substances either into the blank EB sample extract or into 100 µL of the solvent with six replicates each. Analyte recoveries were measured by fortifying six blank EB samples with 500 pg of the model substances or by adding 500 pg of the model substances into the methanolic extract of the filter, and ratios were calculated by using the ISTD-normalized peak areas.

2.3.7 Proof-of-concept

Authentic EB samples were prepared and analyzed after oral administration of a single dose of 10 mg prednisolone (produced by GALENpharma GmbH, Kiel, Germany) to six healthy male volunteers aged 30–47 years. Blank EB samples were provided shortly before intake, and post-administration EB samples were collected for a further 3 days at 2, 4, 6, 8, 24, 28, 32, 48 and 72 h. In the case of one volunteer, EB samples were collected at 0.5, 1, 1.5, 2, 3, 6.5 and 8.5 h after intake only. The EB samples were stored at −20 °C until analysis via LC/MS/MS as described above. In addition, calibration standards were prepared using the standard stock solution of prednisolone and ventilated blank EB samples. Written consent was obtained from all participants as well as ethical approval from the local ethics committee of the German Sport University Cologne (#107/2018).

Additional excretion studies were performed with representatives of the class of stimulants. After oral administration of 20 mg of octodrine (Sigma-Aldrich, Schnelldorf, Germany) dissolved in a mixture of water and ethanol, EB samples were collected after 1, 7, 18, 25, 44 and 50 h from a 62-year-old healthy male volunteer. Further EB samples were collected from two healthy male volunteers aged 46 and 47 years after the oral administration of a dietary supplement declared to contain, amongst other ingredients, octodrine and isopropylnorsynephrine (IPNS) at 20 mg and 200 mg per 11 g, respectively. The identity and quantity were examined using the standard addition method. For this purpose, 55 mg were dissolved in 10 mL of purified water and a dilution of 1:10000 was fortified with 10 ng/mL of the respective internal standard and either 0, 1, 2, 5, 10 or 20 ng/mL of octodrine, methylhexanamine (DMAA, which was also observed in the supplement) and IPNS. The prepared solutions were measured via LC/MS/MS and the

unknown concentrations were calculated using the x-intercept after linear regression. Following the intake of 5.5 g of a powdered supplement dissolved in water (half of a single dose recommended by the manufacturer), EB samples were provided shortly before and after 1, 2, 4, 6, 8, 12 or 13, 24, 30 or 36 and 48 h. Together with calibration standards for octodrine, DMAA and IPNS, all EB samples were prepared and analyzed using LC/MS/MS. Written informed consent was obtained from all proof-of-concept study participants.

2.3.8 Aerosol test generator

In order to simulate authentic drug-containing exhaled breath aerosols, the portable test aerosol generator (model 3073; TSI, Shoreview, MN, USA) was assessed (**Figure 2.1**). The aerosol generator is designed to produce a steady stream of aerosol by using a submerged two-stream nozzle. An air flow is pumped through the nozzle at an adjustable pressure, resulting in a vacuum inside the nozzle so that the surrounding solution is drawn in. A solution with 20 ng/mL in acetonitrile/water (95:5, *v/v*) of selected substances that are prohibited in-competition and in particular sports is used as simulating solvent. Aerosol droplets are formed as a result of the shear forces within the nozzle and larger droplets are retained by the solution. Before connecting to the EB sampling device, the aerosol generator was switched on for 30 min to equilibrate the apparatus and the solution in the nozzle. The EB cartridge was placed onto the aerosol outlet and connected with a plastic tubing connector that was further sealed off with a sleeve tube, and the aerosol was collected for 10 min at an adjusted nozzle pressure of 200 hPa.

Figure 2.1 Schematic illustration of the aerosol test generator (model 3073 by TSI)

2.3.9 Retention efficacy and analyte collection area

In order to estimate potential saturation or analyte “break-through” during the sample application procedure, the drug retention efficacy of the collection device was tested. A total of six EB collection devices were ventilated by means of the aerosol generator for 10, 20, 30, 40, 50 or 60 min. In addition, a second EB collection device was attached to the exit of each first device without the mouthpiece. Subsequently, both EB collection devices were prepared and analyzed. To estimate the moisture load during the aerosolizing process, aerosol produced from purified water was transferred to a glass separation funnel that was placed in iced water and filled with ethyl acetate. Water originating from the aerosol sedimented in the glass separation funnel and was weighed after an operating period of 60 min.

To determine whether the exhaled aerosol is captured solely by the filter or also adsorbs on the cartridge housing wall, a total of 12 artificial drug-containing EB samples were prepared by using the aerosol generator with blank EB samples. While six samples were extracted as described above by rinsing the entire cartridge with 4 mL of methanol, only the analytes retained by the electret filter were measured from the remaining samples. For that purpose, the membrane was separated from the plastic collection device, placed in a glass funnel that was attached to a glass tube, and extracted with 4 mL of methanol. For further information about the location of the aerosol inside the cartridge, five additional blank EB samples were fortified with the model substances using the aerosol generator. In addition to the electret filter, both hemispheres of the cartridge housing were disconnected, placed in a glass funnel and rinsed separately four times with 1 mL of methanol. Following centrifugation (1 min at 1800 *g*) of the composite consisting of the glass tube and the glass funnel, the solvent was evaporated and analyzed as described above.

2.3.10 Stability testing

In order to determine the stability of the model compounds, ten EB sampling devices were ventilated by five healthy volunteers (50 samples in total) and the aerosol generator was employed to apply different model compounds to the electret filters. Subsequently, nine cartridges per subject (45 samples in total) were stored for 2, 7 or 28 days at −20 °C, 4 °C

or room temperature (RT). The remaining five samples were directly stored at −80 °C and used as reference to evaluate the analyte stability. A total of 20 additional EB samples were collected and 400 pg of the model compounds were directly applied to the electret filter through pipetting. Again, two samples were directly frozen at −80 °C and the remaining 18 cartridges were stored for 2, 7 or 28 days at −20 °C, 4 °C or RT (2 samples per storage condition). After storage at the respective temperature, all samples were frozen at −80 °C until analysis. Prior to sample extraction, 10 µL of the mixture of the internal standards was added.

2.4 Results and Discussion

2.4.1 Assay characterization

Based on an approach developed in a pilot study investigating EB as an alternative matrix for doping control purposes,[14] the set of detectable compounds has been expanded to include more representative analytes of the Prohibited List. The method was further optimized considering the large number and the diverse physicochemical nature of the substances. For the detection and identification of the analytes, respectively two characteristic precursor/product ion pairs with the most intense signal abundance were selected. Ion transitions consisted of either the protonated or the deprotonated molecule ($[M + H]^+$, $[M - H]^-$) and the corresponding product ions. Several glucocorticoids such as dexamethasone, prednisolone and prednisone were monitored in both positive and negative mode with the acetate adduct being the precursor ion ($[M + OAc]^-$).

The characterization of the method was conducted with authentic EB blank samples that were fortified with a mixture of all model substances at the concentrations expected in authentic doping control samples.[23,29] Representative MRM chromatograms of all quantifier ion transitions after extraction of an EB sampling device fortified with 500 pg of the model compounds are shown in **Figure 2.4** (supporting information). The analysis of ten blank samples, provided by five male and five female volunteers, yielded ion chromatograms without interfering signals at relevant retention times, and all ion transitions were found to be specific. The estimated LODs ranged from 1 to 500 pg per cartridge (LOIs 1–1000 pg/cartridge). The investigation of potential carry-over effects demonstrated that EB samples containing ostarine, vadadustat, molidustat and synthetic cannabinoids such as JWH-073 and JWH-019 at concentrations higher than 5 ng necessitate a solvent injection prior to the next sample injection. The sample extract was found to be stable in the autosampler for 24 h, enabling respective batch run-times. For the purposes of this study, recovery refers to the extent to which the substances are retrieved from the fortified cartridges, and was determined to span from 14% to 107% with an average value of 83%. The overall matrix (including contributions/components from the exhaled breath as well as the collection device) exhibited a substantial impact on the ionization of the analytes causing both ion suppression (up to −62%) and ion

enhancement (up to +301%) with one exception (JWH-019) that repeatedly yielded a signal abundance enhancement of a factor of 12.28 (**Table 2.1**). While ion suppression appears to predominantly occur for analytes eluting between 2.5 and 5.5 min, ion enhancement is observed for late-eluting analytes, e.g. synthetic cannabinoids. Using full MS data of the extracted EB collection devices, polymeric leachables (polyethylene glycol) were found to co-elute from the cartridges. The retention times of the species yielding the most abundant signals in the total ion chromatogram correlate with the occurrence of ion suppression, implying polymeric leachables as a possible reason for the observed matrix effects as reported earlier.[32] Since the impact of the recovery and the matrix effect on the measured peak area varies significantly from substance to substance, labelled internal standards are required for quantification purposes. However, the assay characterization (as summarized in **Table 2.1**) demonstrates the capability to indicate the presence of a prohibited substance in an EB doping control sample in the context of an initial testing procedure.

2.4.2 Proof-of-concept

Several administration studies have corroborated the utility of EB in detecting the use of substances such as drugs of abuse,[14,16,18,21,25-30,33,37-40] with the Bronchiole Fluid Film Burst Model representing one theory as to how exhaled aerosols are generated.[41] However, various questions have yet remained unaddressed, particularly concerning drug elimination processes of non-volatile substances via the lung and their transfer from the bloodstream into the breath aerosol. Consequently, administration studies of different substance classes are still of utmost relevance.

Due to their stimulating, immunological and metabolic effects, glucocorticoids are potentially misused for performance enhancement and are therefore banned in sport competition. For a proof-of-concept study, EB samples were analyzed after a single-dose systemic application of 10 mg of prednisolone with six male study participants. No signals indicating the presence of prednisolone were detected in the chromatograms of the corresponding MRM transitions of any of the EB samples collected before drug intake. In the case of two volunteers, prednisolone and prednisone were identified in EB specimens sampled 2 h post-administration by unequivocal peaks obtained for two

precursor/product ion transitions each in both positive and negative ion mode. Conversely, EB samples collected from those participants at later time points, as well as all EB samples provided by three additional study participants, tested negative for prednisolone and prednisone using the established analytical approach. Of note, EB sampled by one participant closer to the time of drug intake (30 min) yielded abundant signals exclusively for prednisolone whilst no prednisone was observed. Test results for prednisolone analyzed from EB samples collected before and after drug administration are shown in **Figure 2.2**. Overall, the findings demonstrate that prednisolone can be detected in EB using LC/MS/MS. In addition, prednisone was identified on a few occasions, due to the conversion of prednisolone by the liver.[42,43] Nevertheless, neither prednisolone nor prednisone was detected in any EB sample provided by three out of six participants, which could be attributed to the selection of collection points. The maximum detection time was possibly exceeded with the chosen time interval of 2 h and/or the known high affinity of prednisolone and prednisone for plasma protein binding potentially affected the concentration of the drug in the bronchiolar lining fluid and thus in the exhaled breath aerosol.[41,44] Accordingly, prednisolone and prednisone amounts in EB samples might remain below the LOD of the test method.

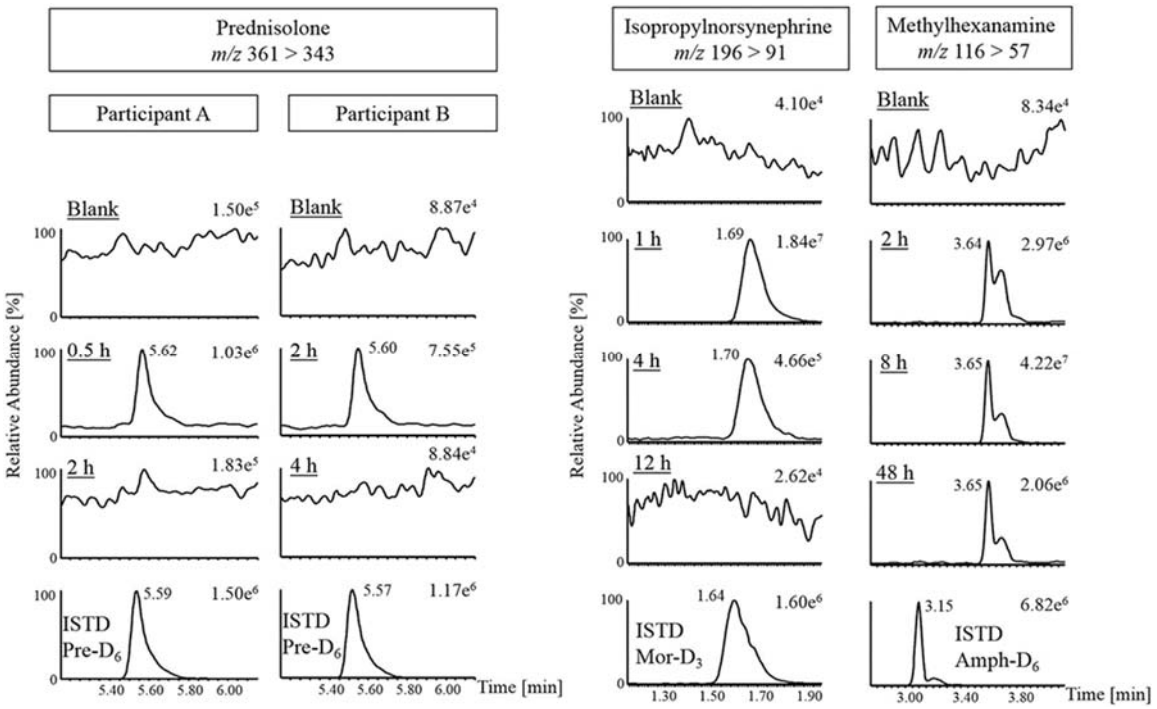

Figure 2.2 MRM chromatograms (quantifier ion transition, positive ionization mode, Table 1) of EB samples collected before and at several time points after a single dose application of prednisolone, isopropylnorsynephrine or methylhexanamine

Further excretion studies were performed with representatives of selected stimulants, representing the class of prohibited substances that is most frequently detected in in-competition doping control samples.[45] A variety of stimulants have been observed as (illicit) additions in dietary supplements, with e.g. DMAA as well as octodrine being marketed as fat-burners and pre-workout supplements.[1,46,47] All EB samples collected after the oral administration of 20 mg octodrine resulted in abundant signals in extracted MRM chromatograms. Up to 7 h after intake, octodrine was identified with two precursor/product ion transitions with a maximum value observed 1 h post-administration. Interestingly, heptaminol (that was found to be an abundant urinary metabolite of octodrine)[48] was not detected in any EB sample, thus offering a means to differentiate the misuse of octodrine from a (likewise prohibited) use of heptaminol. An additional proof-of-concept study was conducted with a dietary supplement declared to contain a variety of stimulants including octodrine and IPNS. The identity and quantity of the active agents were verified using LC/MS/MS and the product was found to contain no detectable amounts of octodrine and about 28 mg of IPNS per 11 g (recommended

serving), corresponding approximately to the labelled declaration (20 mg per serving). Moreover, DMAA was identified with an estimated amount of 32 mg per serving, which is not declared by the manufacturer. Accordingly, the dose administered in the excretion study is equivalent to about 16 mg of DMAA and 14 mg of IPNS. No abundant signals were obtained in MRM chromatograms of DMAA and IPNS after extraction of blank EB samples provided by the participants. All EB samples collected after the intake of the dietary supplement resulted in positive findings for DMAA within a time frame of 48 h. After a rapid increase in peak abundance, maximum drug levels (equivalent to approximately 10 and 12 ng per cartridge) were obtained in EB samples after 2 h (volunteer 1) and after 6 h (volunteer 2). IPNS was detectable for 4 and 8 h after supplement ingestion, with the most abundant signals recorded when analyzing EB samples collected after 1 or 2 h. Maximum levels were estimated to be equivalent to 590 pg and 18 pg IPNS per cartridge, respectively. **Figure 2.2** shows chromatograms of DMAA and IPNS after analysis of EB samples collected at several time points. In the case of one participant's EB samples, detected peak areas for DMAA and IPNS fluctuate, which can be due to variable airflow breathed into the EB collection devices. In addition, according to literature data, varying particle concentrations per exhaled volume depending on the breathing process would also lead to inconsistent results.[26,49] The question of whether physical activity affects the breathing process with respect to the exhaled particle concentration is of particular relevance in the field of anti-doping testing. First studies demonstrated that endogenous substances originating from the bronchiolar lining fluid may be useful to normalize the measured drug concentration in relation to the amount of exhaled particles in EB samples, but further research needs to be conducted.[31]

2.4.3 Aerosol test generator

The aerosol generator tested within this study was found to effectively generate drug-containing aerosols as all model substances dissolved in the generator working solution were successfully detected in the sampling devices using LC/MS/MS. To generate an aerosol as authentic as possible, particle size and concentration were adapted to reported literature values by adjusting the nozzle pressure to 200 hPa, which was found to be the lowest stably produced pressure of the instrument.[34] Peak areas of several compounds

obtained after aerosolization of EB samples using the specified experimental parameters were similar to those measured after applying 400 pg of the model substances to EB sampling devices by pipetting. However, deviations between the model substances do not allow for a concrete quantitative assessment. These variations may be caused by the variable solubility of the model substances in the aerosol droplets. The absolute amount of the substances in the generated aerosol can only be estimated from the experimental conditions. When larger series of collection devices were aerosolized, the reproducibility of the applied drug amount was observed to be limited, which could be because the concentration of the substances inside the nozzle increases over time during operation. Although the tested instrument in its current setup is not accommodated to accurate quantitative analyte application, the generation of a drug-containing aerosol and its application to the collection devices were realized. Hence, an opportunity is provided to address questions regarding the distribution and the stability of substances in EB samples and to further characterize features of the EB collection devices.

2.4.4 Retention efficacy and analyte collection area

To date, it has not yet been determined to what extent the target analytes are actually captured by the electrostatic filter during the ventilation process. By simulating an aerosol flow, the retention efficacy of the filter can be estimated. The substance break-through was assessed by connecting a second EB collection device to the first EB collection device attached to the aerosol generator. Both EB collection devices were prepared and analyzed, and no drug residues were detected at the method's LOD in the second collection cartridge (data not shown). These results suggest that negligible amounts (if any) pass through the device at the conditions used for ventilation. Assuming that the functional principle of the filter is based on electrostatic forces, it was expected that the moisture load during ventilation would impact the retention performance. To evaluate this impact, the experiment was carried out at prolonged aerosolization times to increase the moisture load onto the filter. When the instrument is operated for 60 min, the estimated volume of aerosol produced is 500 µL, which should be equivalent to, if not exceed, the expected moisture load prevailing during breath collection.[15] No substance break-through was detected in the second cartridge after an aerosolization time of

60 min, implying that the moisture load during breath collection does not impact the retention efficacy of the electret filter.

The aerosol generator simulates the distribution of small droplets inside the EB sampling device and thus allows us to investigate whether the aerosol is only captured by the electret filter or also adsorbs on the cartridge housing wall. With six replicates each, either the whole cartridge or only the filter was extracted and the ISTD-normalized peak areas obtained for the different model compounds were used to calculate the ratios "$Extraction_{Membrane}/Extraction_{Cartridge}$". Depending on the analyte, the ratios varied from 57 to 98%, indicating that the overall analyte recovery can be reduced by up to 43% if only the electrostatic filter is extracted. When both hemispheres of the cartridge housing were rinsed and analyzed separately, all model compounds were detected in the extract of the part proximal to the mouthpiece. In contrast, no detectable amounts of the model substances were found in the distal part positioned behind the electret filter, supporting the observation of an efficient retention of the breath aerosol droplets by the membrane.

2.4.5 Stability testing

For the application of EB in doping control, the substances of interest must be stable on the electrostatic filter during the transportation process from the location of the sample collection to the laboratory conducting the analysis. To investigate the stability of the substances, EB sampling devices were fortified with the model substances either using the aerosol generator or by pipetting, and the containers were stored for different time periods at different temperatures. Independent from the storage temperature, all model compounds were detectable after a storage period of 28 days. This applies to both the drug-containing samples generated by using the aerosol generator and the samples that were manually fortified with the substances by pipetting. Overlaid chromatograms obtained after extraction of EB samples stored either at −80 °C (reference sample) and at RT for 7 days from all model substances dissolved in the generator working solution are shown in **Figure 2.3**. Consequently, shipping and storing of EB samples at RT for a period of time that commonly covers the routine doping control process appears acceptable.

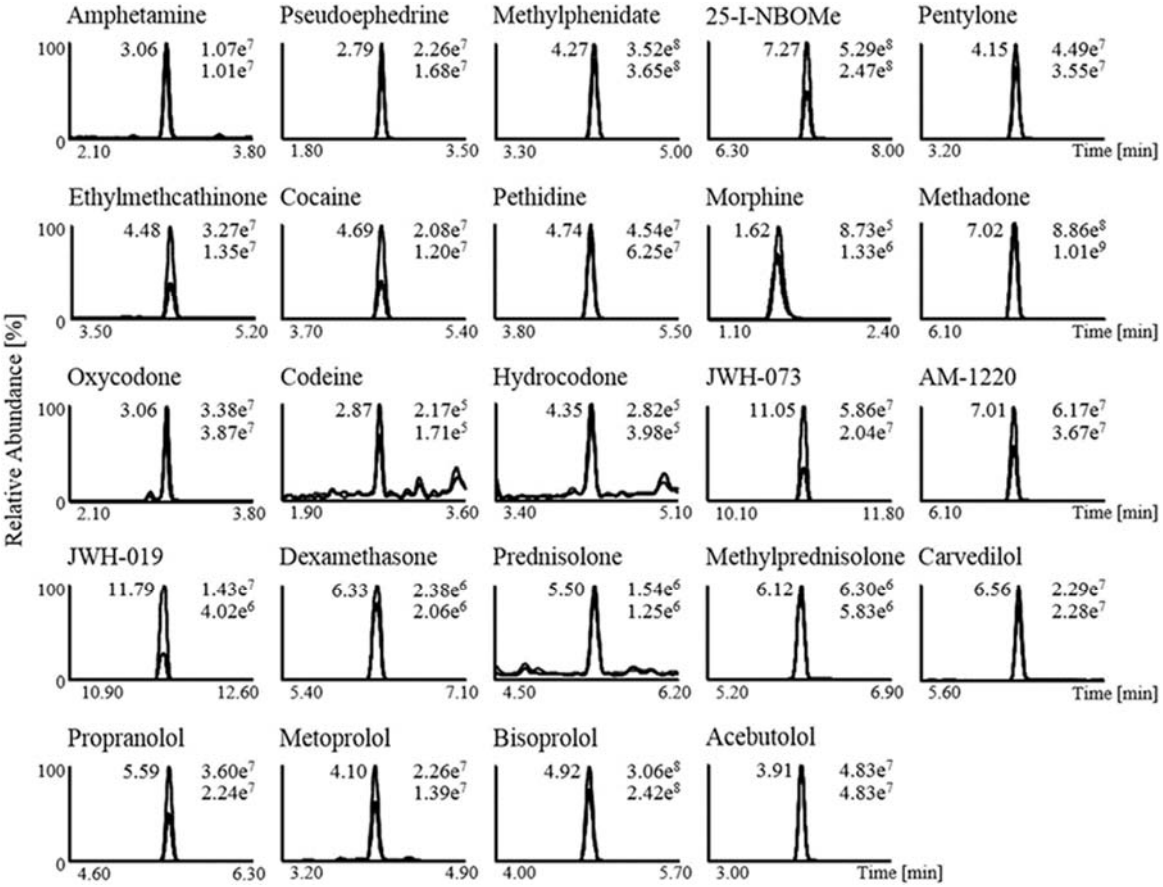

Figure 2.3 Overlaid MRM chromatograms (quantifier ion transition, **Table 2.1**) of a stability reference EB sample (stored at −80 °C, upper intensity value) and an EB sample stored at room temperature for 7 days (lower intensity value). Model substances were applied to the collection devices by means of the aerosol test generator (200 hPa, 10 min)

2.5 Conclusions

The increasing attention towards EB as a matrix for drug testing was largely motivated by its simplicity of sampling especially with regard to the non-invasiveness and non-intrusiveness of the procedure. As part of this study, the development of an analytical approach indicating the presence of an expanded number of prohibited doping substances in EB samples was achieved. Excretion studies with representatives of glucocorticoids and stimulants demonstrated its applicability in principle. Further research is required to understand the correlation between pharmacokinetic properties such as the distribution of a substance and its detectability in EB. Although the generation of a drug-containing aerosol has been accomplished, further optimizations are needed to achieve quantitative application of the substances to EB samples for authentic method developments.

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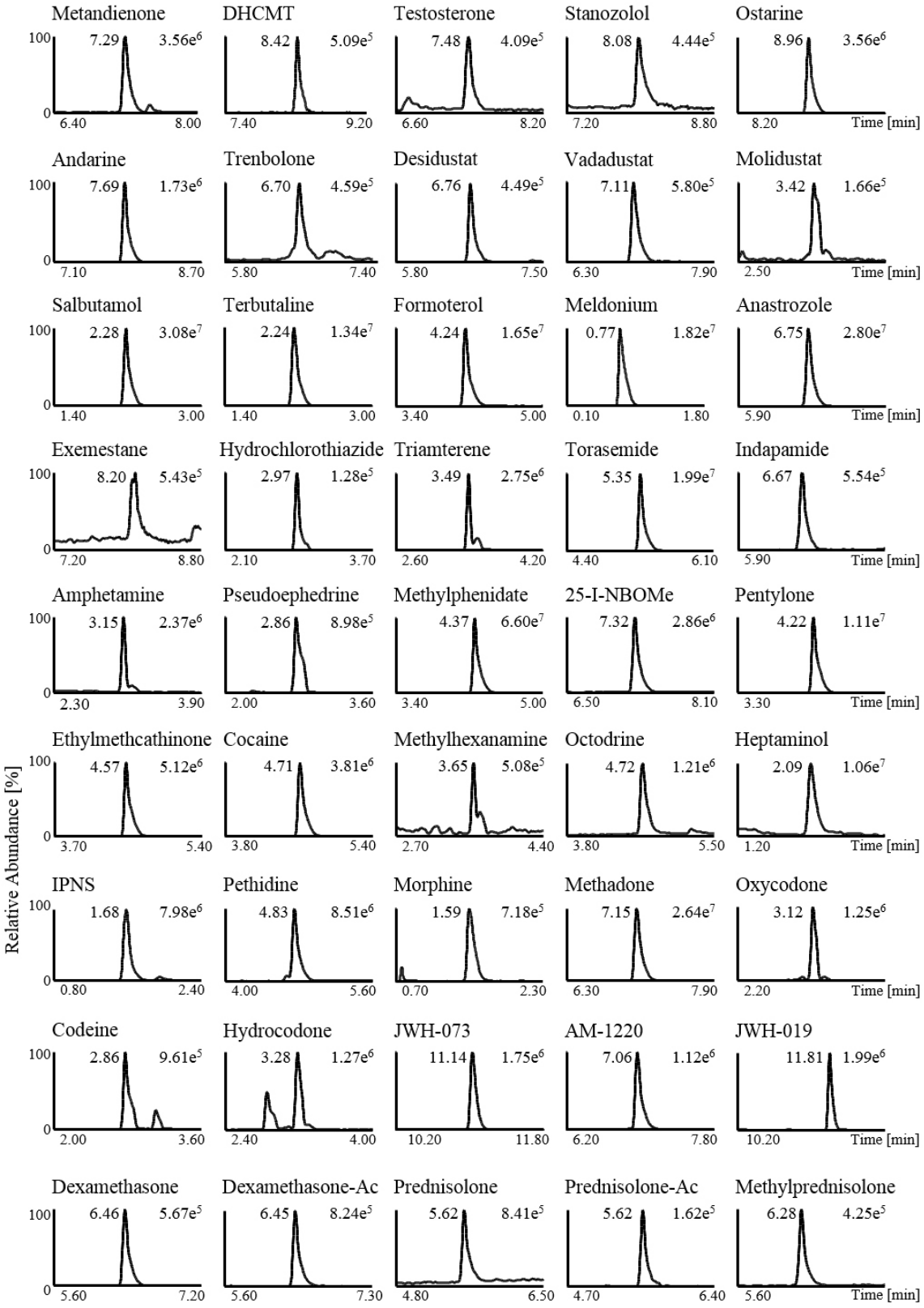
pulmonary function variables. J Aerosol Med Pulm Drug Deliv. 2010;23:371-379. doi:10.1089/jamp.2009.0809.

2.8 Supporting Information

2.8.1 Figure 2.S1



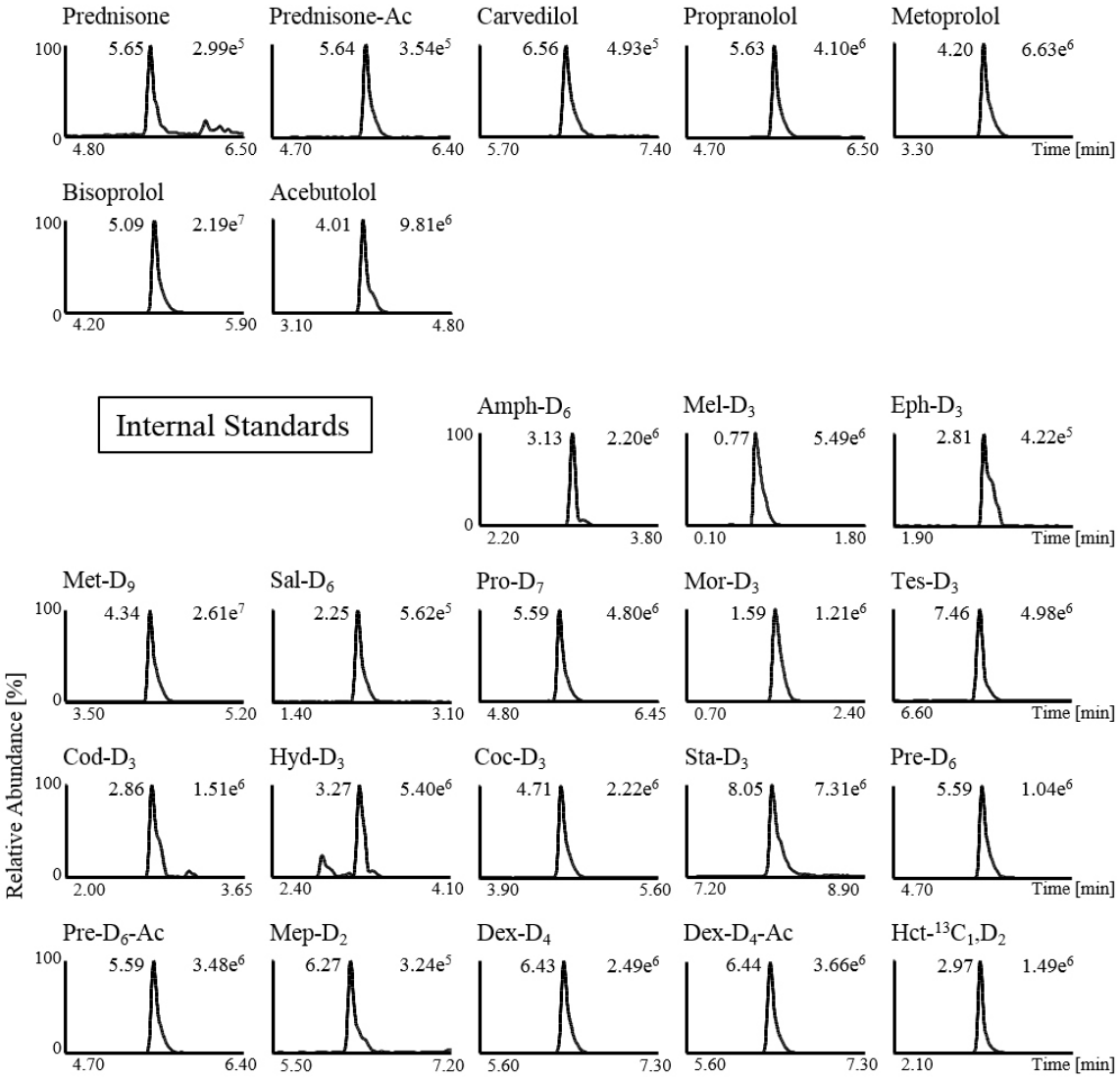


Figure 2.S1 MRM chromatograms of the quantifier ion transitions of the model compounds (**Table 2.1**) applied to an EB sampling device by pipetting (500 pg) and of the internal standards (**Table 2.2**)

2.8.2 Table 2.S1

Table 2.S1 CAS Registry Number of the reference standards purchased from following vendors/providers: Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany), LGC Standards GmbH (Wesel, Germany), Toronto Research Chemicals (Toronto, Canada), CDN Isotopes (Quebec, Canada), Hycultec GmbH (Beutelsbach, Germany), Merck (Darmstadt, Germany)

Substance	CAS Registry Number	Vendor / Provider
Metandienone	72-63-9	Sigma-Aldrich Chemie GmbH
Dehydrochlormethyltestosterone (DHCMT)	2446-23-3	LGC Standards GmbH
Testosterone	58-22-0	Sigma-Aldrich Chemie GmbH
Stanozolol	10418-03-8	Sigma-Aldrich Chemie GmbH
Ostarine	841205-47-8	In-house synthesis
Andarine	401900-40-1	In-house synthesis
Trenbolone	10161-33-8	Sigma-Aldrich Chemie GmbH
Desidustat	1616690-16-4	Hycultec GmbH
Vadadustat	1000025-07-9	Hycultec GmbH
Molidustat	1154028-82-6	Sigma-Aldrich Chemie GmbH
Salbutamol	18559-94-9	LGC Standards GmbH
Terbutaline	23031-25-6	LGC Standards GmbH
Formoterol	67346-49-0	LGC Standards GmbH
Meldonium	76144-81-5	Sigma-Aldrich Chemie GmbH
Anastrozole	120511-73-1	Sigma-Aldrich Chemie GmbH
Exemestane	107868-30-4	Sigma-Aldrich Chemie GmbH
Hydrochlorothiazide	58-93-5	Sigma-Aldrich Chemie GmbH
Triamterene	396-01-0	LGC Standards GmbH
Torasemide	56211-40-6	Tablet extraction
Indapamide	26807-65-8	LGC Standards GmbH
Amphetamine	300-62-9	LGC Standards GmbH
Pseudoephedrine	90-82-4	LGC Standards GmbH
Methylphenidate	113-45-1	LGC Standards GmbH
25-I-NBOMe	919797-19-6	LGC Standards GmbH
Pentylone	698963-77-8	LGC Standards GmbH
4-Ethylmethcathinone	1225622-14-9	LGC Standards GmbH
Cocaine	50-36-2	Merck
Methylhexanamine	105-41-9	LGC Standards GmbH
Octodrine	543-82-8	LGC Standards GmbH
Heptaminol	372-66-7	LGC Standards GmbH
Isopropylnorsynephrine	7104-41-8	Sigma-Aldrich Chemie GmbH
Pethidine	57-42-1	LGC Standards GmbH
Morphine	57-27-2	LGC Standards GmbH
Methadone	76-99-3	LGC Standards GmbH
Oxycodone	76-42-6	LGC Standards GmbH
Codeine	76-57-3	LGC Standards GmbH

Table 2.S1 (Continued)

Substance	CAS Registry Number	Vendor / Provider
Hydrocodone	125-29-1	Merck
JWH-073	208987-48-8	LGC Standards GmbH
AM-1220	137642-54-7	LGC Standards GmbH
JWH-019	209414-08-4	LGC Standards GmbH
Dexamethasone	50-02-2	Sigma-Aldrich Chemie GmbH
Prednisolone	50-24-8	Sigma-Aldrich Chemie GmbH
Methylprednisolone	83-43-2	Sigma-Aldrich Chemie GmbH
Prednisone	53-03-2	Sigma-Aldrich Chemie GmbH
Carvedilol	72956-09-3	Sigma-Aldrich Chemie GmbH
Propranolol	525-66-6	Sigma-Aldrich Chemie GmbH
Metoprolol	51384-51-1	LGC Standards GmbH
Bisoprolol	66722-44-9	Sigma-Aldrich Chemie GmbH
Acebutolol	37517-30-9	LGC Standards GmbH
Amphetamine-D_6 (Amph-D_6)	73758-26-6	LGC Standards GmbH
Meldonium-D_3 (Mel-D_3)	76144-81-5	Toronto Reseach Chemicals
Ephedrine-D_3 (Eph-D_3)	285979-73-9	Sigma-Aldrich Chemie GmbH
Methylphenidate-D_9 (Met-D_9)	1219804-02-0	Sigma-Aldrich Chemie GmbH
Salbutamol-D_6 (Sal-D_6)	not available	RIVM, European Union Reference Laboratory
Propranolol-D_7 (Pro-D_7)	344298-99-3	LGC Standards GmbH
Morphine-D_3 (Mor-D_3)	67293-88-3	LGC Standards GmbH
Testosterone-D_3 (Tes-D_3)	77546-39-5	Sigma-Aldrich Chemie GmbH
Codeine-D_3 (Cod-D_3)	70420-71-2	LGC Standards GmbH
Hydrocodone-D_3 (Hyd-D_3)	136765-36-1	LGC Standards GmbH
Cocaine-D_3 (Coc-D_3)	65266-73-1	Sigma-Aldrich Chemie GmbH
Stanozolol-D_3 (Sta-D_3)	88247-87-4	LGC Standards GmbH
Prednisolone-D_6 (Pre-D_6)	not available	Sigma-Aldrich Chemie GmbH
Methylprednisolone-D_2 (Mep-D_2)	not available	CDN Isotopes
Dexamethasone-D_4 (Dex-D_4)	not available	CDN Isotopes
Hydrochlorothiazide-$^{13}C_1$,D_2 (Hct-$^{13}C_1$,D_2)	1190006-03-1	Toronto Research Chemicals

3 Probing for factors influencing exhaled breath drug testing in sports— Pilot studies focusing on the tested individual's tobacco smoking habit and sex

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3.1 Abstract

Rationale: Exhaled breath (EB) was found to be a promising matrix in the field of sports drug testing due to the non-invasive and non-intrusive sampling procedure, but significant inter-individual variations regarding detected drug concentrations have been observed in previous studies. To investigate whether the detectability of doping agents in EB is affected by sex or tobacco smoking, two administration studies were conducted with male and female smokers and nonsmokers concerning the elimination of the beta blocker propranolol and the stimulant pseudoephedrine into EB.

Methods: Following the administration of 40 mg propranolol or 30 mg pseudoephedrine, a total of 19 participants, including female and male nonsmokers as well as female and male smokers, collected EB and dried blood spot (DBS) samples over a period of 24 h. Respective analyte concentrations were determined using liquid chromatography and high-resolution tandem mass spectrometry, and semi-quantitative assays were characterized with regard to selectivity, limit of detection and identification, precision, linearity, and carryover.

Results: Both propranolol and pseudoephedrine were identified in post-administration EB samples from female and male nonsmokers as well as female and male smokers, and the

maximum detected drug levels ranged from 9 to 2847 pg/cartridge for propranolol and from 26 to 4805 pg/cartridge for pseudoephedrine. The corresponding DBS levels were in a range of 4–30 ng/mL for propranolol and 55–186 ng/mL for pseudoephedrine.

Conclusions: Neither the consumption of cigarettes nor the sex appears to represent a decisive criterion as to the detectability of propranolol or pseudoephedrine in EB, but inter-individual variations regarding the detected drug levels were observed among all studied population groups.

3.2 Introduction

To ensure fair conditions in professional sports competitions, doping controls were established under the transnational coordination of the World-Anti-Doping-Agency (WADA), which has established rules and regulations, including an annual list of prohibited substances and methods of doping.[1-3] Currently, mainly urine samples, which provide a broad analyte spectrum and long detection windows due to the detection of metabolites, and, to a lesser extent, blood samples are used for analytical purposes.[4-6] Recently, dried blood spots (DBS) have been introduced as a useful additional matrix for doping controls.[7-16] Urine samples are collected under visual control, invading the privacy of athletes,[17] whereas blood collection is an invasive procedure, or in the case of DBS, a minimally invasive procedure.[18] Besides analytical aspects, the sampling procedure is a major concern when alternative matrices are examined and established. In comparison to urine and blood, the sampling of exhaled breath (EB) is simple, fast, and neither invasive nor intrusive.[19,20]

Among other options, EB is collected using a small sampling device composed of a mouthpiece with trapping barriers for oral fluid, a volume control device, and a cartridge equipped with an electrostatic membrane that captures exhaled particles.[21-23] Previous studies have demonstrated that the detection window in EB is comparatively short, indicating, predominantly, benefits for the detection of substances that are prohibited exclusively in competition, such as stimulants, narcotics, cannabinoids, and glucocorticoids.[24-26] In addition, doping substances in EB were observed to be stable at room temperature over a period of time, which covers transport from the sampling site to the analytical laboratory, saving cost and ensuring analytical robustness.[27] Overall, EB was found to be a promising matrix complementing blood and urine.[28]

According to the bronchiole fluid film burst model, nonvolatile substances diffuse from the bloodstream into the bronchiolar lining fluid, which is exhaled in the form of an aerosol.[29] Previous studies have already demonstrated that the detection of doping-relevant substances in EB after oral administration is generally feasible, but inter-individual variations were observed with respect to the concentration determined.[25,28,30-40] The objective of this study was to investigate whether these variations correlate to the

sex of the individual providing the EB sample. For this purpose, five of both female and male volunteers collected EB and additional DBS samples after oral administration of two model substances, the beta-blocker propranolol and the stimulant pseudoephedrine, for 24 h. Both compounds represent prohibited substances according to the WADA regulations, with propranolol banned in specific disciplines and pseudoephedrine prohibited in competition only. Respective concentrations were determined using liquid chromatography and high-resolution tandem mass spectrometry (LC–MS/MS).

According to the literature, tobacco smoke may influence the bronchiolar lining fluid regarding its composition and secretion.[41] Consequently, the question arose whether the consumption of cigarettes also affects the detectability of non-volatile substances in EB, because research on the robustness of the matrix towards external agents is still scarce. For further characterization of the matrix and thus its potential scope of application, propranolol and pseudoephedrine were additionally administered to nine smokers (five female, four male).

3.3 Experimental

3.3.1 Chemicals and materials

EB sampling devices were obtained from SensAbues AB (Stockholm, Sweden). DBS samples were collected using QIAcards FTA DMPK-C distributed by Qiagen (Venlo, Netherlands), and 20 µL capillaries coated with ethylenediaminetetraacetic acid (EDTA) were purchased from Sarstedt (Hildesheim, Germany). Administration studies were conducted with commercially available preparations of propranolol (Propranolol AL 40, Aliud Pharma, Laichingen, Germany) and pseudoephedrine in combination with ibuprofen (BoxaGrippal, Sanofi, Paris, France). Standard solutions for pseudoephedrine as well as $^{2}H_{7}$-propranolol were purchased from LGC (Teddington, UK) and for propranolol and $^{2}H_{3}$-ephedrine from Sigma-Aldrich (St. Louis, Missouri, USA). Methanol, acetonitrile, and formic acid for sample preparation and LC–MS/MS analysis were of analytical grade and purchased from VWR International GmbH (Darmstadt, Germany).

3.3.2 Stock solutions and working solutions

Stock solutions of propranolol and pseudoephedrine as well as labeled internal standards (IS) were prepared at a concentration of 1 mg/mL in methanol. Combined working solutions at concentrations of 10, 0.1, 0.01, and 0.001 µg/mL in methanol were generated by dilution of the stock solutions. Likewise, the mixed IS solution was prepared with a concentration of 100 ng/mL for $^{2}H_{7}$-propranolol and 200 ng/mL for $^{2}H_{3}$-ephedrine.

3.3.3 EB and DBS sampling

Authentic EB samples were collected according to the manufacturer's instructions.[42] The sampling procedure involved breathing through a mouthpiece connected to a plastic bag that inflates and thereby indicates the completeness of the sampling. Afterward, the mouthpiece and the volume control were discarded, and the stopper-plugged cartridge containing the adsorbing membrane was stored at −20 °C until analysis.

For DBS sampling, blood was obtained from the fingertip using a disposable lancet, and 20 µL per spot was applied to the DBS card using an EDTA-coated capillary. The assay

characterizations were conducted using blank specimens provided by six female and six male healthy volunteers, aged 24–48 years.

3.3.4 Sample preparation

Prior to EB sample preparation, 5 µL of the IS solution containing 500 pg of $^{2}H_{7}$-propranolol and 1 ng of $^{2}H_{3}$-ephedrine was pipetted onto the electrostatic filter. For elution of the retained substances, the cartridge was rinsed with 4 mL of methanol and centrifuged at 1800 × *g* for 5 min after placing it on a glass test tube. The obtained sample extract was evaporated to dryness at 50 °C and reconstituted with a 100 µL mixture of 95% H_2O and 5% acetonitrile for LC–MS/MS analysis.

DBS samples were excised from the sampling card and transferred into a plastic test tube in quarters. The substances of interest were extracted by incubation with 200 µL of methanol/purified water 95/5 (*v/v*) containing 0.1% formic acid and 5 µL of the IS solution for 30 min at 37 °C and 500 rpm. After brief centrifugation, 200 µL of the sample extracts was transferred to a new plastic test tube and evaporated under reduced pressure. The dry extract was reconstituted with 100 µL of H_2O/acetonitrile 95/5 (*v/v*) and centrifuged for 5 min at 17 000 × *g* before transferring to a glass vial for LC–MS/MS analysis.

3.3.5 Liquid chromatography–tandem mass spectrometry

LC–MS/MS analyses were performed on a Thermo Vanquish UHPLC coupled to a Thermo Orbitrap Exploris 480 (Thermofisher, Dreieich, Germany). The LC system was equipped with an Agilent (Waldbronn, Germany) Poroshell 120 EC-C_{18} analytical column (50 × 3.0 mm, 2.7 µm particle size) connected to an EC 4/3 Nucleoshell RP 18 Plus precolumn (5 µm particle size) provided by Macherey-Nagel (Düren, Germany). The analytes were separated using a gradient with water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid at a flow rate of 0.3 mL/min. The gradient started at 5% of solvent B with a steep increase to 20% B within 0.5 min, followed by an increase to 75% B in 3.5 min. After a final increase to 100% B within 0.5 min, which was maintained for an additional 0.5 min, the column was re-equilibrated for 3 min at starting conditions, resulting in an overall run time of 8 min.

High-resolution mass spectrometric analysis was performed using positive electrospray ionization (ESI) with an ionization voltage of 2.6 kV. Precursor/product ion pairs were

detected using parallel reaction monitoring experiments with an orbitrap resolution set to 30 000 FWHM and optimized collision energies for each transition (**Table 3.1**).

Table 3.1 Overview of the monitored precursor/product ion pairs for propranolol and pseudoephedrine

Analyte	Ion Mode	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*) // NCE [%]	Qualifier Ion (*m/z*) // NCE [%]	ISTD
Propranolol	ESI+	260.1645	116.1069 // 40	98.0966 // 40	D_7-propranolol
Pseudoephedrine	ESI+	166.1226	148.1120 // 30	117.0699 // 60	D_3-ephedrine

Abbreviation: ESI, electrospray ionization

3.3.6 Assay characterization

The assay was characterized enabling a semi-quantitative estimation of propranolol and pseudoephedrine in EB and DBS samples, including specificity, limit of detection (LOD) and identification (LOI), precision, linearity, and carryover as adapted from WADA guidelines.[4] Both EB and DBS blank samples were provided by three female and three male volunteers. For the characterization of DBS analysis, venous blood was collected in EDTA-coated tubes and fortified with different concentrations of the analytes, except for the determination of the specificity. About 20 µL per spot was pipetted onto a sampling card and dried for a minimum of 2 h. EB samples were enriched with the analytes by pipetting standard solutions directly onto the electrostatic filter of blank samples.

For specificity, six blank samples of EB and DBS were prepared and examined for interfering signals. In the case of DBS, spots from both fingertip and venous blood were included. For the determination of the LOD and LOI, six repetitions each were measured with different concentrations: 1, 2, 5, 10, and 50 pg/cartridge for propranolol and 10, 20, 50, 100, and 500 pg/cartridge for pseudoephedrine in exhaled breath and 0.5, 1, 2, and 5 ng/mL for propranolol and pseudoephedrine in DBS. The precision was calculated for 50 and 500 pg/cartridge for exhaled breath and 2 and 50 ng/mL for DBS for both propranolol and pseudoephedrine with six replicates each. The linearity for EB samples was determined up to 1000 pg/cartridge for propranolol with six replicates each for the concentrations 5, 10, 50, 100, 500, and 1000 pg/cartridge and up to 5000 pg/cartridge for pseudoephedrine with six replicates each for the concentrations 20, 50, 100, 500, 1000, 2000, and 5000 pg/cartridge. For DBS samples, linearity was determined up to 100 ng/mL

with six replicates each for the concentrations 0.5, 1, 2, 5, 10, 50, and 100 ng/mL for both propranolol and pseudoephedrine. The carryover effect was estimated by measuring three blank samples subsequent to the highest concentration of the linearity determination.

3.3.7 Administration study

To evaluate the detectability of propranolol and pseudoephedrine as model substances for doping agents in exhaled breath with regard to sex-specific or smoking-induced variations, two excretion studies were performed. A total of 19 volunteers ranging in age from 23 to 48 years participated, including 10 women and 9 men, of whom 5 and 4, respectively, reported to smoke cigarettes daily. The smoking activity varied inter-individually from 1 to 60 cigarettes per day with 5 to 32 years of active use. After a single oral administration of a commercially available medication containing 40 mg propranolol or 30 mg pseudoephedrine (combined preparation with 200 mg ibuprofen), a total of seven EB samples were collected after 1, 2, 4, 6, 8, 12, and 24 h, as well as four DBS samples after 2, 6, 12, and 24 h. Shortly before the intake, the participants provided a blank sample for each matrix. All samples were stored at −20 °C until analysis. Written consent was obtained from all participants as well as the ethical approval from the local ethics committee of the German Sport University Cologne (#107/2018).

3.4 Results and Discussion

3.4.1 Assay characterization

To estimate the concentration of propranolol and pseudoephedrine in EB and DBS samples for comparative purposes, a semi-quantitative LC–MS/MS method was developed. Mass spectrometric analysis was conducted in targeted MS^2 mode with two characteristic precursor/product ion pairs per analyte optimized regarding their collision energies. The main parameters are summarized in **Table 3.2**.

Table 3.2 Main results of the assay characterization

Analyte	Matrix	LOD	LOI	Precision [%]		Linearity R^2	Selectivity
Propranolol	Exhaled Breath	5 pg/c	10 pg/c	4 (50 pg/c)	6 (500 pg/c)	0.999 (5-1000 pg/c)	yes
	DBS	500 pg/mL	1 ng/mL	10 (5 ng/mL)	9 (50 ng/mL)	0.999 (0.5-100 ng/mL)	yes
Pseudoephedrine	Exhaled Breath	20 pg/c	500 pg/c	11 (20 pg/c)	7 (500 pg/c)	0.999 (20-5000 pg/c)	yes
	DBS	0.5 ng/mL	1 ng/mL	7 (5 ng/mL)	3 (50 ng/mL)	0.999 (0.5-100 ng/mL)	yes

Abbreviations: LOD, limit of detection; LOI, limit of identification; pg/c, picogram/cartridge

The analytical assay was found to be specific for all monitored ion traces as no interfering signals were detected at relevant retention times in blank EB and DBS samples for both propranolol and pseudoephedrine. For the determination of the LOD and the LOI, six blank EB and blood samples each were fortified with different concentrations of the analytes. The LOD was defined as the lowest concentration that yields signals at the correct retention time for two ion transitions in all replicates. The LOI was specified as the lowest concentration with a relative abundance (quantifier/qualifier ratio) within a 20% deviation range compared to a value determined with a highly concentrated sample.

For propranolol, an LOD of 5 pg/cartridge in EB and 0.5 ng/mL in DBS and an LOI of 10 pg/cartridge in EB and 1 ng/mL in DBS were determined. In the case of pseudoephedrine, the LOD in EB was defined solely using the quantifier ion transition owing to the distinct difference in sensitivity between the two precursor/product ion pairs. In DBS and for the determination of the LOI, both ion transitions were considered. Accordingly, an LOD of 20 pg/cartridge in EB and 0.5 ng/mL in DBS and an LOI of

500 pg/cartridge in EB and 1 ng/mL in DBS were obtained for pseudoephedrine. The precision was calculated for 50 and 500 pg/cartridge in EB and 2 and 50 ng/mL in DBS using the quantifier ion transition for each analyte. All coefficients of variation were found to be in an acceptable range of less than 15% (**Table 3.2**). The detection of propranolol was determined to be linear from 5 to 1000 pg/cartridge in EB and from 0.5 to 100 ng/mL in DBS. In the case of pseudoephedrine, linearity was established for a concentration range of 20–5000 pg/cartridge in EB and 0.5–100 ng/mL in DBS. No carryover effect was observed for any of the analytes. Overall, the analytical assay was successfully characterized with respect to the detection of propranolol and pseudoephedrine in EB and DBS as well as the quantitative estimation in realistic concentration ranges.

3.4.2 Administration study

In this study, variations in the detectability of doping substances in EB were investigated with regard to a potential influence of the sex or tobacco smoking. For this purpose, elimination studies with two representative doping substances were conducted including a single oral administration of 40 mg of the beta-blocker propranolol and 30 mg of the stimulant pseudoephedrine. Subsequently, EB and DBS samples were collected for a total period of 24 h after administration. The concentration of the substances was estimated using LC–MS/MS, and the results were compared with respect to the sex and cigarette smoking.

Propranolol was detected in post-administration EB samples collected by 18 of the 19 study participants. EB samples collected by one female nonsmoker yielded concentrations for propranolol that were below the established LOD. For 13 participants, the analyte was found in the first sampled post-administration specimen, and in the case of 3 participants, propranolol was still present in EB samples 24 h after drug intake. The maximum levels were measured between 1 and 12 h post-administration and ranged from 9 to 2847 pg/cartridge. Regarding DBS, all samples provided 2 h after drug intake were found to contain propranolol, and the 24 h-DBS still tested positive in the case of 12 participants. Except for three cases, the maximum concentrations, ranging from 1.7 to 29.8 ng/mL, were determined in the first sample collected 2 h after administration.

After the intake of pseudoephedrine, EB samples from 18 of 19 participants tested positive for the stimulant with a detection window of up to 12 h. As observed in the propranolol study, but for a different participant, all signals detected in EB samples provided by one female nonsmoker were below the assessed LOD. Maximum concentrations were detected between 1 and 8 h after the administration, with values ranging from 26 to 2328 pg/cartridge. For DBS, all provided samples yielded signals for pseudoephedrine within the monitored time frame of 24 h. In each case except for one, the highest concentration was measured in the first sample collected after 2 h and ranged from 57.2 to 186.0 ng/mL.

Both of the investigated substances were detectable in EB samples without an observable relation to sex and smoking activity. **Figure 3.1** shows exemplarily extracted ion chromatograms of EB and DBS samples collected after administration of propranolol and pseudoephedrine by one participant from each of the population groups investigated, respectively. However, it was observed that the measured levels vary substantially between individuals. In the data obtained solely from nonsmoking female volunteers, maximum concentrations of propranolol in post-administration EB samples ranged from 0 to 51 pg/cartridge and from 0 to 2215 pg/cartridge for pseudoephedrine. In comparison, EB samples provided by nonsmoking male participants yielded maximum concentrations from 12 to 2847 pg/cartridge for propranolol and from 26 to 2328 pg/cartridge for pseudoephedrine. Accordingly, measured values differ by up to a factor of 1000, irrespective of the sex or any other known potential influence. In the case of EB samples collected after the administration of propranolol by female nonsmokers, the variance is comparably low. However, considering the elimination study with pseudoephedrine, the data indicate that lower concentrations found for propranolol are not attributable to the female sex exclusively but rather to the inter-individual variations, in general.

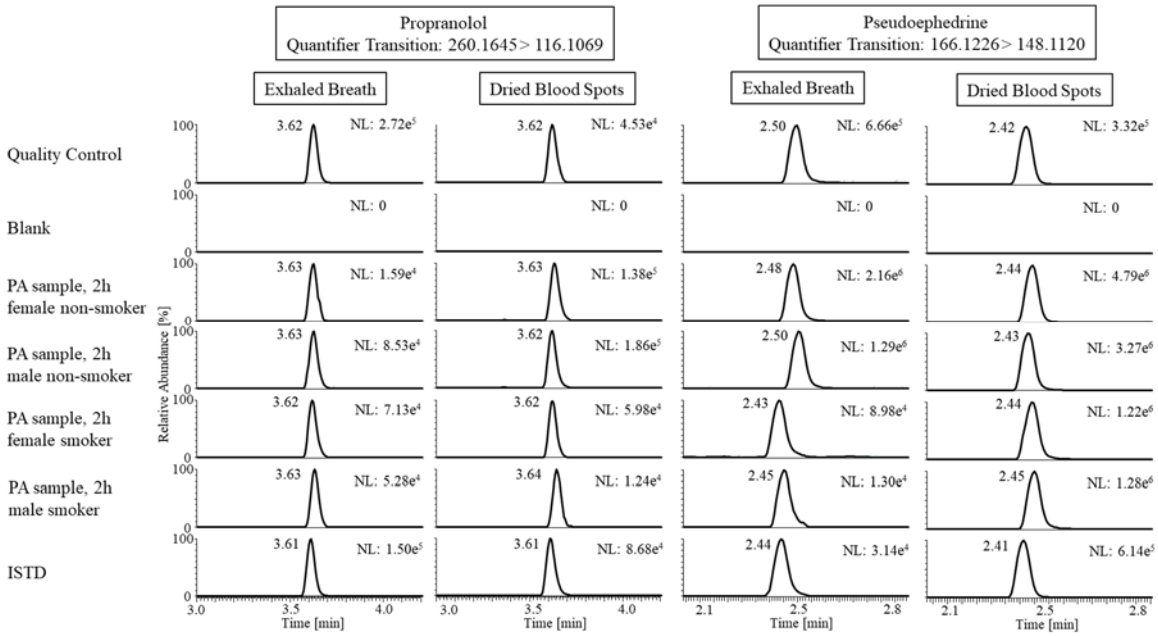

Figure 3.1 Extracted ion chromatograms of the quantifier transition for propranolol and pseudoephedrine from exhaled breath (EB) and dried blood spots (DBS) samples. For quality control, blank samples were fortified with either 500 pg per cartridge (EB) or 0.5 ng/ mL (DBS) of the respective analyte. Chromatograms from post-administration (PA) samples were obtained from a female nonsmoker, a male nonsmoker, a female smoker and a male smoker 2 h after administration of the substance. The corresponding peak of the internal standard is displayed as a dashed line

Taking into account all maximum levels for propranolol with the exception of one outlier with a comparably high concentration, a standard deviation of 202 pg/cartridge is calculated. When the standard deviations are considered separately for nonsmokers and smokers, values of 150 and 230 pg/cartridge, respectively, are obtained. This shows the present inter-individual variance regarding the detectability of propranolol in EB, but also demonstrates that the variance is not affected by smoking tobacco. Comparable data were obtained from EB samples collected during the excretion study with pseudoephedrine. Whereas one participant's EB samples were exclusively tested negative for pseudoephedrine, five other cases comprising all populations investigated yielded maximum values in the nanogram range, manifesting the observation of significant inter-individual differences regarding the detectability in EB. **Figure 3.2** provides an overview of the maximum levels of propranolol and pseudoephedrine found in post-administration

EB samples outlining the occurrence of significant inter-individual variations in each population group investigated.

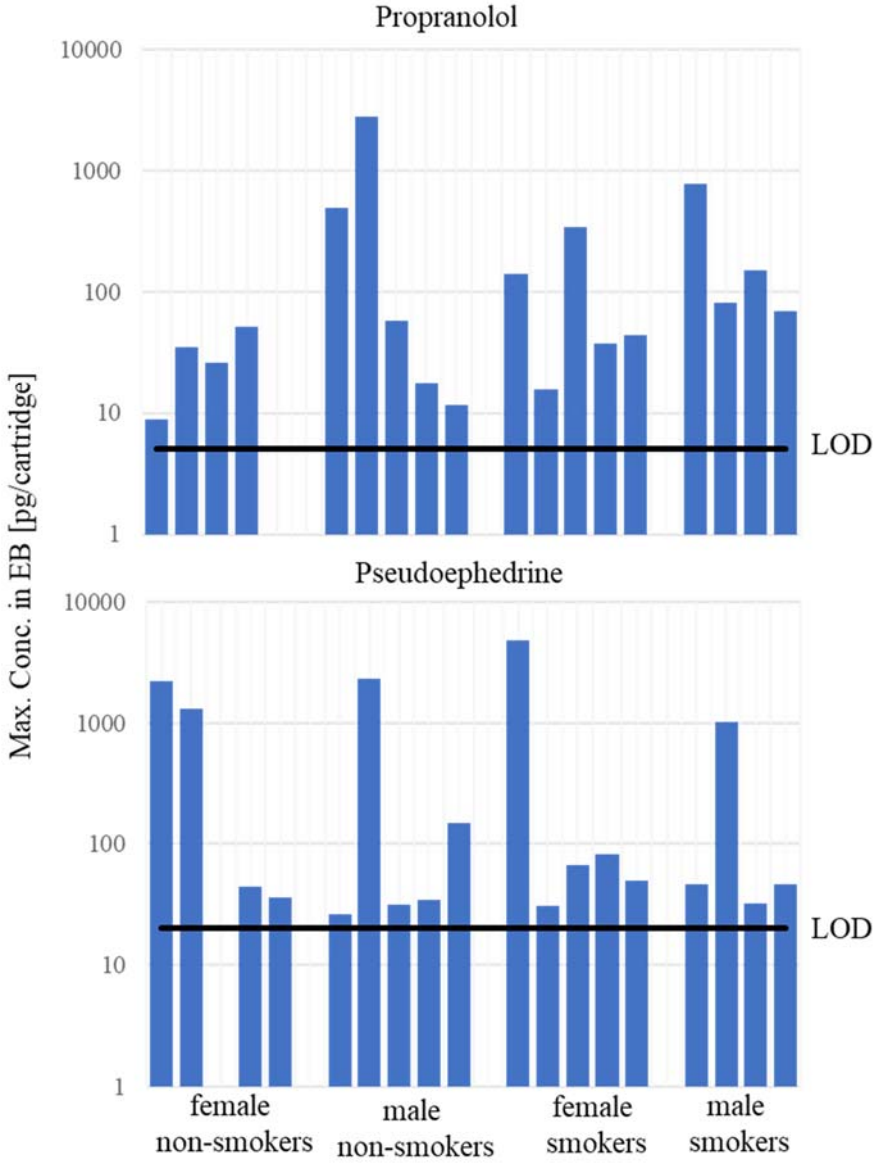

Figure 3.2 Overview of the maximum levels for propranolol (upper chart) and pseudoephedrine (lower chart) detected in post-administration exhaled breath (EB) samples obtained from female and male nonsmokers as well as female and male smokers. Due to the inter-individual differences, a logarithmic representation was chosen allowing high and low concentrations to be displayed in the same chart

The measured maximum levels of propranolol in DBS obtained from nonsmokers ranged from 7.9 to 29.8 ng/mL, which equates to a variance of 40%. However, no correlation was observed between the variations in measured concentrations in EB and DBS samples. This

finding was confirmed by the pseudoephedrine study. When EB samples were tested positive with pseudoephedrine levels in a nanogram range, the stimulant was detected with low or average concentrations in the corresponding DBS samples, indicating that the determined inter-individual differences are not due to a varying absorption of the substance into the blood (**Figure 3.3**).

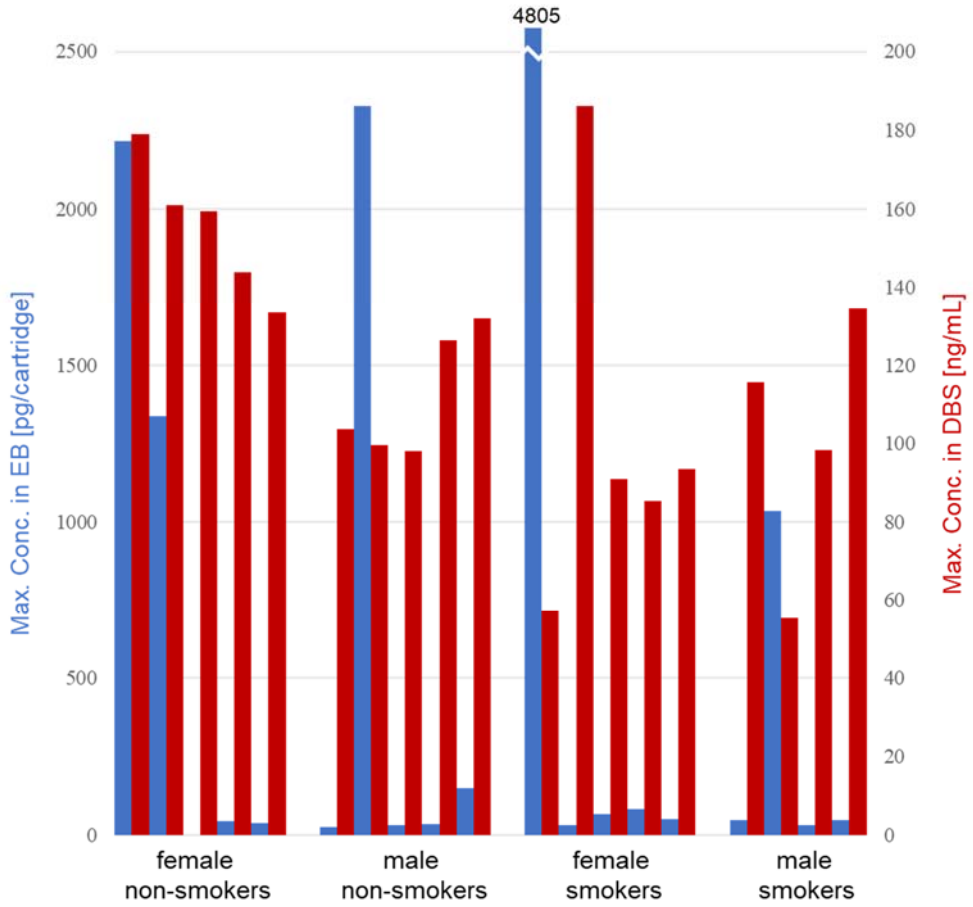

Figure 3.3 This chart compares the respective maximum levels for pseudoephedrine detected in post administration exhaled breath (EB) and dried blood spots (DBS) samples obtained from female and male nonsmokers as well as female and male smokers. The EB-concentration given in pg/ cartridge is displayed in blue and refers to the left y-axis, and the DBS-concentration given in ng/ mL is displayed in red and refers to the right y-axis

In the case of five participants, including male and female nonsmokers, as well as male and female smokers, irregular propranolol profiles were detected in EB samples. After an increase of concentrations in the first samples, a decrease was observed, which was followed by another increase to higher concentrations again in samples collected at later

time points. In contrast, the corresponding DBS concentrations of the beta blocker followed an expected pattern with a rapid rise and subsequent decline of the drug concentration. For one participant (male, nonsmoker), this observation was also found to a significant extent in the pseudoephedrine study, which is shown in **Figure 3.4**. Here, 898 pg/cartridge was detected in the EB sample collected after 2 h, the values decreased

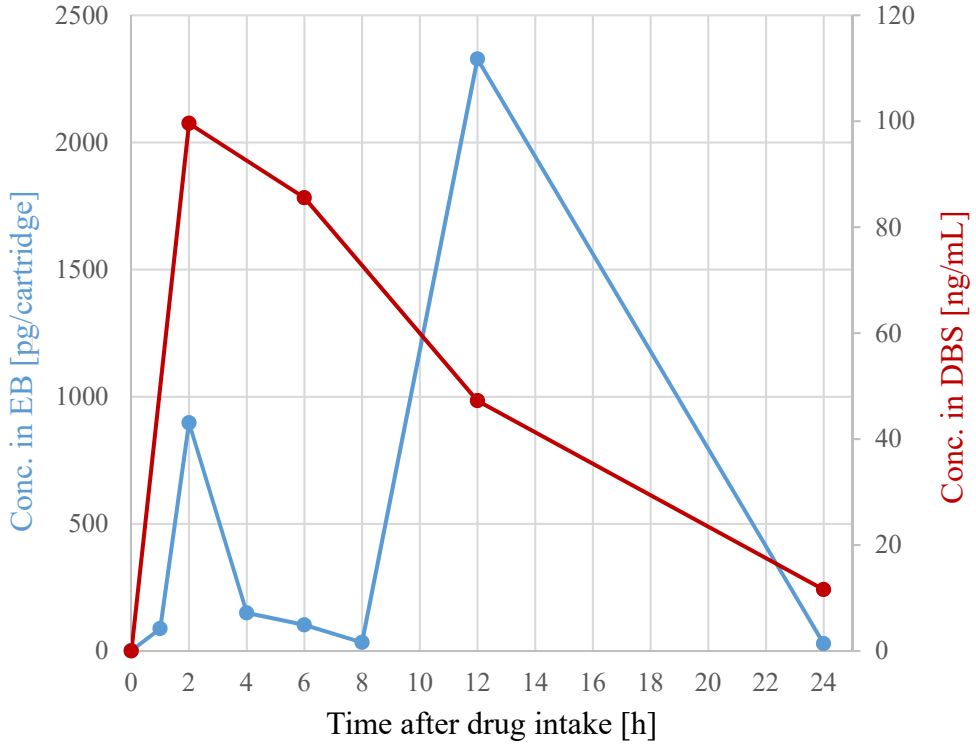

Figure 3.4 Comparison of pseudoephedrine levels in EB (blue, left y-axis) and DBS (red, right y-axis) samples collected by a male nonsmoker after the intake of 30 mg, outlining an irregular drug profile in exhaled breath as observed in several cases

to around 100 pg/cartridge in the following samples and a new increase was detected after 12 h with 2328 pg/cartridge.

This observation indicates that the concentration of nonvolatile substances in EB is also affected by intra-individual variances, which could be due, for example, to the breathing maneuver. According to the theory published by Johnson and Morawska, the number of exhaled aerosol droplets would increase during deep inhalation and exhalation compared to shallow breathing.[29,43] A normalization factor, for example an endogenous substance originating from the bronchiole lining fluid, would therefore be of great interest to investigate the source of these variances and to allow smoothing of the measured

concentrations of doping substances in EB. This would be of utmost interest for interpretation of EB data from drug users or athletes. First studies have evaluated phosphatidylcholines with respect to their suitability, but further research and studies are required to optimize these or new substances as markers.[44,45] Prospectively, further investigation of the robustness of the matrix is needed, for example with regard to intense physical activity or certain lung diseases. It is of additional interest, whether the results obtained from the study with pseudoephedrine and propranolol are congruent with other substances. As a representative of stimulants, the most frequently abused substance class banned in competition, pseudoephedrine is characterized by its accessibility and low toxicity.[46] Given the structural similarities to other phenylethylamine derivatives, which include a variety of banned stimulants, the findings of this study would be expected to be confirmed with analogues of this substance. The results of the administration study with propranolol demonstrate the detectability of beta-blockers in exhaled breath, which has scarcely been investigated to date, but also indicate that the observed findings do not necessarily depend on the analyte, but rather on the individual lung constitution and breathing pattern.

3.5 Conclusions

Previous excretion studies have already demonstrated that drug testing from exhaled breath is generally feasible, but also indicated that detected concentrations vary inter-individually. In this study, EB samples collected after the administration of the doping agents propranolol and pseudoephedrine were compared with regard to the sex and the use of cigarettes of the participants to elucidate whether the detectability in EB is altered by these factors. The observation of significant inter-individual variations was confirmed for all population groups studied, but no correlation was found with the sex or the consumption of cigarettes. A cross-comparison with additionally collected DBS samples indicated that the varying concentrations detected in EB are not due to the varying resorption of the substances into the blood. Overall, EB is a promising matrix in the context of sports drug testing, but further research is required to characterize its benefits and limitations, as well as its application as a complementary matrix.

3.6 Acknowledgements

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4 Dried blood spots for doping controls—Development of a comprehensive initial testing procedure with fully automated sample preparation

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4.1 Abstract

Currently, primarily urine, whole blood and serum samples are analyzed for doping-relevant substances in professional sports, but recently dried blood spots (DBS) have been introduced as complementary matrix, offering advantageous features, e.g. a minimally invasive sampling procedure. In order to cope with the increased application of DBS, a comprehensive initial testing procedure (ITP) was developed, optimized and validated, comprising a total of 233 substances representing all groups on the World Anti-Doping Agency's (WADA's) Prohibited List. The sample preparation was conducted by employing a fully automated system using an efficient flow-through extraction of a 4 mm diameter spot followed by LC–HRMS/MS analysis. The procedure was successfully validated in terms of selectivity, limit of detection, reproducibility, carryover and robustness with respect to an alternative manual sample preparation, an alternative dried blood collection device and the sample extract stability, and was thus found to meet the required criteria of the relevant guidelines published by WADA for routine application. As a proof-of-concept, DBS samples were analyzed after the administration of the glucocorticoids prednisone and dexamethasone, as well as the stimulant pseudoephedrine and the beta-

blocker propranolol. All substances were detected in post-administration samples for at least 4 h and up to 24 h after intake, depending on the collection time period, using the developed testing procedure. In particular, for substances that are only banned in-competition, data obtained from DBS samples can be useful for the interpretation of adverse analytical findings. In conclusion, the developed ITP accounts for the anticipated increasing relevance of DBS in anti-doping analysis in the future and provides a foundation for optimized approaches for specific substance classes.

4.2 Introduction

The first use of dried blood microsamples was reported in 1961, when a neonatal screening for metabolic diseases was developed (Guthrie, 1961). The interest increased rapidly, and besides clinical diagnostics, applications in the field of drug-of-abuse testing followed (Henion et al., 2013; Lehmann et al., 2013; Lim, 2018; Niemiec, 2021). The potential of dried blood spots (DBS) as a complementary matrix for anti-doping controls was discovered about two decades ago (Peng et al., 2000), leading to further studies and investigations targeting various groups of prohibited substances and methods (Thevis, 2021; Thevis et al., 2022; Thomas et al., 2011, 2012; Yuan et al., 2021). In particular, in terms of the organization and conduction of the sampling procedure, DBS offer certain benefits compared with whole blood, serum and urine samples, which are predominantly used for sports drug testing (Saugy et al., 2009; Vandevenne et al., 2000; World Anti-Doping Agency, 2021b). In contrast to a venous blood sample, DBS require only small volumes of capillary blood, approximately 10–50 µL per spot, collected from the fingertip or upper arm in a minimally invasive procedure, eliminating the need for venipuncture and thus a qualified phlebotomist (World Anti-Doping Agency, 2023). Owing to the simplicity of the sampling procedure, DBS can be collected by a trained doping control officer or even by the athletes themselves, enabling remote testing, which has gained interest during the COVID-19 pandemic (Fedoruk, 2020). Collecting urine specimens may be time-consuming in cases where athletes are incapable of providing urine, for example owing to dehydration after physical activity (World Anti-Doping Agency, 2014). When the sampling procedures of whole blood, urine and DBS were compared regarding the convenience in a study with 108 participants, both athletes and doping control officers stated a preference for DBS as a matrix (Solheim et al., 2021). Moreover, stability studies with numerous substances from the Prohibited List demonstrated the detectability of these substances after storage for at least 1 week and up to 1 year at room temperature (Mazzarino et al., 2022; Moretti et al., 2018; Thevis et al., 2022; Thomas et al., 2011). Together with the convenient size of DBS cards, both transport from the sampling site to the analytical laboratory and long-term storage are considerably simplified and of lower cost. Overall, these benefits allow for the acquisition of a larger number of blood samples

and thus the generation of blood concentration data, providing valuable information in addition to data obtained from urine samples for result interpretation, in particular with regard to substances prohibited only in-competition (Thevis et al., 2020, 2021). However, from an analytical point of view, the small sample volume implies certain challenges, as it requires highly sensitive mass spectrometric instruments along with specific sample preparation procedures owing to the complex sample matrix (Henion et al., 2013).

In September 2021, a technical document was released by the World Anti-Doping Agency (WADA), harmonizing DBS testing with respect to sample collection and transport, as well as analytical testing and sample storage, giving juridical relevance to adverse analytical findings obtained from dried blood (World Anti-Doping Agency, 2023). This was followed by the implementation of DBS in routine testing at the Olympic and Paralympic Winter Games in Beijing in 2022 for the first time (Wang et al., 2022). Accordingly, a further growth of the contribution of DBS to routine doping controls is expected. In this context, the objective of this study was the development of an initial testing procedure (ITP) comprising a wide range of substances that are banned both in-competition and out-of-competition according to the Prohibited List published by WADA, following up on previous projects addressing the extraction and analysis of various model substances from DBS (Thomas et al., 2011, 2012). The method developed in this study comprises an extended list of analytes and is based on a multi-target approach that has already been established for urine samples and is applied in routine analysis (Görgens et al., 2016). Additionally, in order to cope with the increased sample throughput associated with routine application, fully automated sample preparation was required for the approach. A particular challenge regarding the instrumental setup included the demanding sensitivity requirements owing to the small sample volume and the broad spectrum of chemical structures. In addition, the consistency between the detection limits of the approach and realistic concentrations of prohibited substances in DBS had to be considered and was evaluated using authentic post-administration samples and literature data.

4.3 Experimental

4.3.1 Chemicals and materials

Methanol and acetonitrile that were used for the automated sample preparation and instrumental analysis as well as formic acid and hydrochloric acid were of analytical grade and purchased from VWR International (Darmstadt, Germany). Ultrapure water was generated using a Barnstead GenPure Purification System distributed by Fisher Scientific (Schwerte, Germany). An overview of the suppliers of all standard solutions and reference substances is given in **Table 4.2**.

4.3.2 Stock solutions and working solutions

Stock solutions of reference substances that were used to produce mixed standard solutions were either prepared in methanol or purchased as a solution with concentrations ranging from 10 to 1000 µg/ml and stored at −20 °C. Mixed stock solutions were prepared with adjusted concentrations of each compound in methanol and stored at −20 °C for up to 1 year. Before usage, mixed working solutions were freshly generated by combining the total of 10 mixed stock solutions in equal parts. For the preparation of a quality control sample, venous blood was enriched with the working solution and 20 µl were pipetted onto the collection cards per spot as described under **Section 4.3.3**, resulting in analyte concentrations given in **Table 4.1**, which were considered as target performance level. The internal standard (ISTD) working solution, containing $^{2}H_{6}$-amphetamine (0.1 µg/ml), $^{2}H_{9}$-clenbuterol (0.1 µg/ml), $^{2}H_{3}$-stanozolol (0.1 µg/ml), $^{2}H_{4}$-dexamethasone (0.1 µg/ml), $^{2}H_{5}$-furosemide (0.02 µg/ml) and $^{13}C_{1}$, $^{2}H_{2}$-hydrochlorothiazide (0.02 µg/ml), was prepared by the appropriate dilution of stock solutions (100 µg/ml, stored at −20 °C) with methanol. The ISTD working solution is permanently connected to the automated DBS autosampler and accordingly stored at room temperature and freshly prepared on a monthly basis.

4.3.3 DBS and blood sampling

Both DBS and venous blood provided by six male and six female healthy volunteers aged 24–48 years were used for method development and validation and for all sample

collections, approval of the local ethics committee of the German Sport University Cologne, #139/2021, and written informed consent was obtained. For blank DBS, 20 µl of blood obtained from a fingertip prick was pipetted onto a Whatman FTA DMPK-C card purchased from Merck (Darmstadt, Germany) and dried for at least 2 h at room temperature. Fortified DBS on collection cards were generated from venous whole blood samples containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant and stored at 4 °C for no longer than 7 days. Aliquots of venous blood were fortified with the substances of interest at various concentrations and 20 µl per spot was pipetted onto the card. Thereby, the added amount of the organic standard solution did not exceed 10% of the venous blood volume in order to avoid agglutination. After a drying time of a minimum of 2 h, all DBS samples were stored in a plastic bag together with a desiccant sachet at 4 °C and protected from daylight until analysis.

Table 4.1 Overview of included substances as well as key parameters of the LC–HRMS/MS analysis together with defined target performance levels (TPL) and determined limit of detections

Group on Prohibited List (by WADA)	Compound	Precursor Ion (Full MS)		Product Ion (DIA)	Ret. Time	TPL	LOD	
		Molecular Ion	Exact Mass [*u*]	Exact Mass [*u*]	[min]	Conc. [ng/mL]	[ng/mL]	[ng/20 µL spot]
S1	Clenbuterol	$[M+H]^+$	277.0869	203.0137	4.70	50	2.5	0.05
	Dehydrochlormethyl-testosterone	$[M+H]^+$	335.1772	289.1353	8.56	50	25	0.5
	Metandienone	$[M+H]^+$	301.2162	283.2056	7.43	50	5	0.1
	Methyltestosterone	$[M+H]^+$	303.2319	285.2213	8.05	50	10	0.2
	S-22 (Ostarine)	$[M-H]^-$	388.0904	118.0299	8.80	25	0.25	0.005
	S-23	$[M-H]^-$	415.0467	144.9863	10.24	50	0.5	0.01
	S-24	$[M-H]^-$	381.0857	241.0598	9.45	50	0.5	0.01
	S-4 (Andarine)	$[M-H]^-$	440.1064	261.0487	7.79	50	2.5	0.05
	Stanozolol	$[M+H]^+$	329.2587	329.2587	7.06	25	25	0.5
	Testosterone	$[M+H]^+$	289.2162	271.2056	7.66	50	5	0.1
	Trenbolone	$[M+H]^+$	271.1693	253.1586	6.92	50	2.5	0.05
	Nandrolone propionate	$[M+H]^+$	331.2267	257.1899	11.25	500	25	0.5
	Testosterone propionate	$[M+H]^+$	345.2424	271.2056	11.47	500	50	1
	Trenbolone enanthate	$[M+H]^+$	383.2580	253.1586	12.29	500	500	10
	Testosterone acetate	$[M+H]^+$	331.2268	97.0638	10.66	500	25	0.5
	Zilpaterol	$[M+H]^+$	262.1550	244.1439	3.19	50	2.5	0.05
S2	Daprodustat	$[M-H]^-$	392.1816	291.1713	11.48	100	20	0.4
	Desidustat	$[M-H]^-$	331.0936	331.0936	5.95	100	100	2
	FG-2216	$[M-H]^-$	279.0167	178.0066	7.84	25	12.5	0.25
	Roxadustat	$[M+H]^+$	353.1139	296.0919	9.50	50	5	0.1
	IOX-2	$[M+H]^+$	353.1139	296.0918	8.85	100	20	0.4
	Vadadustat	$[M+H]^+$	307.0480	250.0263	8.47	25	12.5	0.25

Table 4.1 (Continued)

Group on Prohibited List (by WADA)	Compound	Precursor Ion (Full MS)		Product Ion (DIA)	Ret. Time	TPL	LOD	
		Molecular Ion	Exact Mass [*u*]	Exact Mass [*u*]	[min]	Conc. [ng/mL]	[ng/mL]	[ng/20 µL spot]
S3	Bambuterol	$[M+H]^+$	368.2180	294.1448	5.03	50	2.5	0.05
	Bamethane	$[M+H]^+$	210.1489	192.1382	3.63	250	12.5	0.25
	Brombuterol	$[M+H]^+$	364.9859	290.9129	4.97	50	2.5	0.05
	Cimaterol	$[M+H]^+$	220.1443	143.0607	3.22	50	10	0.2
	Cimbuterol	$[M+H]^+$	234.1601	160.0868	3.61	250	12.5	0.25
	Clenpenterol	$[M+H]^+$	291.1025	132.0682	5.11	50	2.5	0.05
	Clenproperol	$[M+H]^+$	263.0712	168.0444	4.32	250	12.5	0.25
	Fenoterol	$[M+H]^+$	304.1543	135.0807	3.70	250	2.5	0.05
	Formoterol	$[M+H]^+$	345.1809	149.0962	4.73	500	5	0.1
	Higenamine	$[M+H]^+$	272.1281	255.1008	3.58	250	125	2.5
	Indacaterol	$[M+H]^+$	393.2173	375.2063	6.04	250	2.5	0.05
	Isoxsuprine	$[M+H]^+$	302.1751	150.0915	5.10	50	2.5	0.05
	Mabuterol	$[M+H]^+$	311.1133	237.0398	5.17	250	12.5	0.25
	Mapenterol	$[M+H]^+$	325.1289	237.0398	5.72	50	0.5	0.01
	Olodaterol	$[M+H]^+$	387.1914	207.0762	4.99	50	2.5	0.05
	Pirbuterol	$[M+H]^+$	241.1546	167.0819	2.98	250	12.5	0.25
	Procaterol	$[M+H]^+$	273.1602	231.1124	3.41	250	12.5	0.25
	Ractopamine	$[M+H]^+$	302.1751	107.0493	4.34	250	2.5	0.05
	Reproterol	$[M+H]^+$	390.1772	221.1038	3.54	250	12.5	0.25
	Ritodrine	$[M+H]^+$	288.1594	288.1594	3.84	500	5	0.1
	Salbutamol	$[M+H]^+$	240.1594	148.0758	3.19	250	2.5	0.05
	Salmeterol	$[M+H]^+$	416.2795	380.2573	6.77	50	2.5	0.05
	Terbutaline	$[M+H]^+$	226.1438	152.0705	3.17	250	12.5	0.25
	Tulobuterol	$[M+H]^+$	228.1150	154.0417	4.66	250	2.5	0.05

Table 4.1 (Continued)

Group on Prohibited List (by WADA)	Compound	Precursor Ion (Full MS)		Product Ion (DIA)	Ret. Time	TPL	LOD	
		Molecular Ion	Exact Mass [*u*]	Exact Mass [*u*]	[min]	Conc. [ng/mL]	[ng/mL]	[ng/20 µL spot]
S3	Tretoquinol	$[M+H]^+$	346.1649	164.0706	4.35	250	125	2.5
	Vilanterol	$[M+H]^+$	486.1809	158.9762	6.50	1000	50	1
S4	Anastrozole	$[M+H]^+$	294.1713	225.1386	6.75	25	1.25	0.025
	Clomifene	$[M+H]^+$	406.1932	100.1126	8.33	25	2.5	0.05
	Exemestane	$[M+H]^+$	297.1849	279.1743	8.32	50	25	0.5
	Meldonium	$[M+H]^+$	147.1128	58.0651	0.71	250	25	0.5
	Trimetazidine	$[M+H]^+$	267.1703	181.0858	3.26	50	2.5	0.05
S5	5-oxo-Mefruside	$[M-H]^-$	395.0133	395.0133	6.17	500	5	0.1
	Aminochlorobenzen-disulphonamide	$[M-H]^-$	283.9561	283.9561	3.38	500	5	0.1
	Acetazolamide	$[M-H]^-$	220.9798	220.9798	3.57	500	25	0.5
	Althiazide	$[M-H]^-$	381.9751	340.9362	6.55	500	25	0.5
	Amiloride	$[M+H]^+$	230.0552	189.0173	3.34	50	25	0.5
	4-Aminotrifluoro-methylbenzen-disulfonamide	$[M-H]^-$	317.9825	317.9825	4.42	500	25	0.5
	Azosemide	$[M-H]^-$	368.9990	325.9824	7.30	500	50	1
	Bemetizide	$[M-H]^-$	400.0187	293.9412	7.39	500	25	0.5
	Bendroflumethiazide	$[M-H]^-$	420.0294	289.0445	7.51	250	2.5	0.05
	Benzthiazide	$[M-H]^-$	429.9751	307.9566	7.00	500	5	0.1
	Benzylhydrochlorothiazide	$[M-H]^-$	386.0031	293.9415	6.86	500	5	0.1
	Brinzolamide	$[M+H]^+$	384.0716	217.0115	4.42	250	2.5	0.05
	Bumetanide	$[M-H]^-$	363.1009	319.1110	8.01	500	25	0.5
	Butizide	$[M-H]^-$	352.0187	352.0187	6.68	250	12.5	0.25
	Chlorazanil	$[M+H]^+$	222.0541	153.0213	5.34	250	2.5	0.05
	Chlorothiazide	$[M-H]^-$	293.9406	293.9406	3.73	250	2.5	0.05

Table 4.1 (Continued)

Group on Prohibited List (by WADA)	Compound	Precursor Ion (Full MS)		Product Ion (DIA)	Ret. Time	TPL	LOD	
		Molecular Ion	Exact Mass [*u*]	Exact Mass [*u*]	[min]	Conc. [ng/mL]	[ng/mL]	[ng/20 µL spot]
S5	Chlortalidone	$[M-H]^-$	337.0044	146.0245	5.24	500	5	0.1
	Clopamide	$[M+H]^+$	346.0987	169.0162	5.39	250	2.5	0.05
	Conivaptane	$[M+H]^+$	499.2129	181.0644	6.43	250	2.5	0.05
	Cyclopenthiazide	$[M-H]^-$	378.0344	314.0735	7.56	250	2.5	0.05
	Cyclothiazide	$[M-H]^-$	388.0187	321.9716	7.22	500	50	1
	Diclofenamide	$[M-H]^-$	302.9062	302.9062	5.22	500	25	0.5
	Dorzolamide	$[M+H]^+$	325.0345	135.0266	3.33	250	2.5	0.05
	Epithiazide	$[M-H]^-$	423.9469	309.9718	6.81	500	5	0.1
	Eplerenone	$[M+H]^+$	415.2115	415.2115	6.56	500	25	0.5
	Furosemide	$[M-H]^-$	328.9993	285.0109	6.51	500	25	0.5
	Hydrochlorothiazide	$[M-H]^-$	295.9561	268.9460	3.93	500	5	0.1
	Hydroflumethiazide	$[M-H]^-$	329.9825	302.9718	4.75	250	12.5	0.25
	Hydroxytriamterene	$[M+H]^+$	270.1098	253.0827	3.67	250	25	0.5
	Mebutizide	$[M-H]^-$	380.0500	380.0500	7.81	500	25	0.5
	Methazolamide	$[M-H]^-$	234.9954	57.9756	4.23	500	5	0.1
	Methyclothiazide	$[M-H]^-$	357.9484	321.9719	6.15	500	25	0.5
	Metolazone	$[M-H]^-$	364.0517	256.9798	6.41	500	5	0.1
	Mozavaptane	$[M+H]^+$	428.2333	238.0861	5.70	250	2.5	0.05
	Piretanide	$[M+H]^+$	363.1009	236.1065	7.52	500	25	0.5
	Polythiazide	$[M-H]^-$	437.9625	259.9476	7.47	250	12.5	0.25
	Probenecide	$[M-H]^-$	284.0951	198.0594	8.18	250	2.5	0.05
	Quinethazone	$[M+H]^+$	290.0361	273.0101	4.44	500	5	0.1
	Relcovaptane	$[M-H]^-$	618.0863	/	7.89	500	50	1

Table 4.1 (Continued)

Group on Prohibited List (by WADA)	Compound	Precursor Ion (Full MS)		Product Ion (DIA)	Ret. Time	TPL	LOD	
		Molecular Ion	Exact Mass [*u*]	Exact Mass [*u*]	[min]	Conc. [ng/mL]	[ng/mL]	[ng/20 µL spot]
S5	Tolvaptane	$[M+H]^+$	449.1626	252.1016	8.61	250	2.5	0.05
	Trichlomethiazide	$[M-H]^-$	377.8938	241.9787	5.90	500	5	0.1
	Xipamide	$[M-H]^-$	353.0357	274.0633	7.72	250	2.5	0.05
S6	1,3-Dimethylbutylamine	$[M+H]^+$	102.1277	85.1014	3.33	5000	250	5
	2,5-Dimethoxyphen-ethylamine	$[M+H]^+$	182.1176	182.1176	4.09	250	12.5	0.25
	2-amino-6-methylheptane (Octodrine)	$[M+H]^+$	130.1590	57.0698	4.92	250	25	0.5
	3,4-Methylenedioxyethyl-amfetamine (MDEA)	$[M+H]^+$	208.1338	163.0750	4.28	125	6.25	0.125
	4-methylhexan-2-amine	$[M+H]^+$	116.1434	58.0733	4.05	2500	125	2.5
	5-(2-aminopropyl)-2,3-dihydrobenzofuran (5-APDB)	$[M+H]^+$	178.1226	133.0650	4.01	1250	12.5	0.25
	6-(2-aminopropyl)-benzofuran	$[M+H]^+$	176.1070	131.0494	4.42	250	2.5	0.05
	Bromantane	$[M+H]^+$	322.0801	133.1010	9.28	250	250	5
	Amfepramone (Diethylpropione)	$[M+H]^+$	206.1539	100.1123	4.04	250	12.5	0.25
	Amiphenazol	$[M+H]^+$	192.0590	192.0590	3.41	1250	625	12.5
	Amphetamine	$[M+H]^+$	136.1121	119.0855	3.75	2500	125	2.5
	Benfluorex	$[M+H]^+$	352.1519	159.0414	6.79	125	1.25	0.025
	Benzoylecgonine	$[M+H]^+$	290.1387	168.1018	4.40	125	6.25	0.125
	Benzphetamine	$[M+H]^+$	240.1747	122.0965	5.64	125	1.25	0.025
	Cafedrine	$[M+H]^+$	358.1874	207.0874	4.49	250	2.5	0.05
	Carphedone	$[M+H]^+$	219.1128	174.0914	4.87	250	25	0.5
	Cathinone	$[M+H]^+$	132.0807	117.0576	3.31	1250	12.5	0.25
	Clobenzorex	$[M+H]^+$	260.1201	125.0154	5.89	125	1.25	0.025

Table 4.1 (Continued)

Group on Prohibited List (by WADA)	Compound	Precursor Ion (Full MS)		Product Ion (DIA)	Ret. Time	TPL	LOD	
		Molecular Ion	Exact Mass [*u*]	Exact Mass [*u*]	[min]	Conc. [ng/mL]	[ng/mL]	[ng/20 µL spot]
S6	Cocaine	$[M+H]^+$	304.1543	182.1175	5.01	25	0.25	0.005
	Cropropamide	$[M+H]^+$	196.1332	168.1380	6.07	2500	250	5
	Crothetamide	$[M+H]^+$	182.1176	154.1227	5.36	2500	1250	25
	Cyclazodone	$[M+H]^+$	217.0972	217.0972	5.50	250	12.5	0.25
	Desmethylselegiline	$[M+H]^+$	174.1277	174.1277	4.33	250	50	1
	Diethylnorephedrine, N,N-	$[M+H]^+$	208.1696	117.0701	4.11	125	1.25	0.025
	Dimethoxy-4-ethylamphetamine, -2,5- (DOEt)	$[M+H]^+$	224.1645	192.1141	5.55	250	2.5	0.05
	Dimethoxy-4-methylamphetamine, -2,5- (DOM)	$[M+H]^+$	210.1489	193.1219	5.00	250	12.5	0.25
	Dimethoxyamphetamine, -2,5- (DMA)	$[M+H]^+$	196.1332	151.0755	4.37	250	12.5	0.25
	Dimethoxyamphetamine, -3,4	$[M+H]^+$	196.1332	179.1067	3.77	1250	12.5	0.25
	Dobutamine	$[M+H]^+$	302.1751	107.0493	4.33	250	2.5	0.05
	Ephedrine	$[M+H]^+$	166.1226	148.1121	3.50	250	2.5	0.05
	Ethamivane	$[M+H]^+$	224.1281	151.0389	5.45	250	2.5	0.05
	Etilamfetamine	$[M+H]^+$	164.1433	119.0853	4.22	250	125	2.5
	Etilefrine	$[M+H]^+$	182.1176	164.1070	2.77	250	25	0.5
	Famprofazone	$[M+H]^+$	378.2540	217.1332	6.16	125	1.25	0.025
	Fenbutrazate	$[M+H]^+$	368.2220	191.1063	6.96	125	1.25	0.025
	Fencamfamine	$[M+H]^+$	216.1747	129.0696	5.32	125	1.25	0.025
	Fencamine	$[M+H]^+$	385.2346	236.1144	4.47	250	125	2.5
	Fenethylline	$[M+H]^+$	342.1925	224.1142	4.75	25	1.25	0.025

Table 4.1 (Continued)

Group on Prohibited List (by WADA)	Compound	Precursor Ion (Full MS)		Product Ion (DIA)	Ret. Time	TPL	LOD	
		Molecular Ion	Exact Mass [*u*]	Exact Mass [*u*]	[min]	Conc. [ng/mL]	[ng/mL]	[ng/20 µL spot]
S6	Fenfluramine	$[M+H]^+$	232.1308	159.0414	5.48	125	1.25	0.025
	Fenproporex	$[M+H]^+$	189.1386	119.0855	4.09	250	12.5	0.25
	Fluoroamphetamine, -4	$[M+H]^+$	154.1027	109.0449	3.99	250	2.5	0.05
	Furfenorex	$[M+H]^+$	230.1539	81.0339	5.13	250	2.5	0.05
	Heptaminol	$[M+H]^+$	146.1539	128.1433	3.05	625	62.5	1.25
	Isometheptene	$[M+H]^+$	142.1590	142.1590	4.57	1250	62.5	1.25
	Lisdexamfetamine	$[M+H]^+$	264.2070	84.0809	3.33	125	6.25	0.125
	Methcathinone (Ephedrone)	$[M+H]^+$	164.1070	146.0961	3.50	250	12.5	0.25
	Mebeverine	$[M+H]^+$	430.2588	430.1070	6.46	125	1.25	0.025
	Mefenorex (Chlorpropylamphetamine)	$[M+H]^+$	212.1201	91.0546	5.04	125	1.25	0.025
	Mephentermine	$[M+H]^+$	164.1433	133.1013	4.26	250	12.5	0.25
	Mesocarb	$[M+H]^+$	323.1503	177.0656	7.78	250	2.5	0.05
	Metamfepramone	$[M+H]^+$	178.1226	133.0650	3.60	250	12.5	0.25
	Methamphetamine	$[M+H]^+$	150.1277	119.0855	3.94	250	12.5	0.25
	Methedrone	$[M+H]^+$	194.1176	176.1075	3.94	250	2.5	0.05
	Methoxyamphetamine, p-(PMA)	$[M+H]^+$	166.1226	121.0650	3.99	1250	12.5	0.25
	Methoxyphenamine	$[M+H]^+$	180.1383	121.0647	4.38	250	2.5	0.05
	Methylamphetamine, 4-	$[M+H]^+$	150.1277	133.1012	4.47	250	12.5	0.25
	Methylendioxyamphetamine (MDA)	$[M+H]^+$	180.1019	163.0749	3.90	250	12.5	0.25
	Methylendioxymethamphetamine (MDMA)	$[M+H]^+$	194.1176	163.0749	4.05	250	2.5	0.05
	Methylenedioxypyrovalerone (MDPV)	$[M+H]^+$	276.1594	126.1276	4.97	125	1.25	0.025

Table 4.1 (Continued)

Group on Prohibited List (by WADA)	Compound	Precursor Ion (Full MS)		Product Ion (DIA)	Ret. Time	TPL	LOD	
		Molecular Ion	Exact Mass [*u*]	Exact Mass [*u*]	[min]	Conc. [ng/mL]	[ng/mL]	[ng/20 µL spot]
S6	Methylephedrine	$[M+H]^+$	180.1383	162.1275	3.61	250	2.5	0.05
	Methylmethcathinone (Mephedrone)	$[M+H]^+$	178.1226	145.0887	4.22	1250	12.5	0.25
	Methylnaltroxen	$[M+H]^+$	356.1856	338.1768	3.73	250	2.5	0.05
	Methylphenethylamine	$[M+H]^+$	136.1121	91.0544	3.80	250	12.5	0.25
	Methylphenidate	$[M+H]^+$	234.1489	84.0812	4.73	125	1.25	0.025
	Modafinilic Acid	$[M-H]^-$	273.0579	167.0871	6.35	1250	62.5	1.25
	N-ethyl-1-phenyl-2-butanamine	$[M+H]^+$	178.1590	178.1590	4.66	125	6.25	0.125
	Nikethamide	$[M+H]^+$	179.1179	108.0447	3.84	250	12.5	0.25
	Norfenfluramine	$[M+H]^+$	204.0995	159.0416	5.11	125	1.25	0.025
	Norpseudoephedrine (Cathine)	$[M+H]^+$	152.1070	134.0964	3.28	2500	25	0.5
	Pemoline	$[M+H]^+$	177.0659	106.0655	4.20	1250	62.5	1.25
	Pentetrazole	$[M+H]^+$	139.0978	96.0806	4.03	625	31.25	0.625
	Phendimetrazine	$[M+H]^+$	192.1383	148.1124	3.90	250	12.5	0.25
	Phenylethylamine, 2-	$[M+H]^+$	122.0964	122.0964	3.24	2500	1250	25
	Pholedrine	$[M+H]^+$	166.1226	135.0802	3.07	250	12.5	0.25
	p-Hydroxyprenylamine	$[M+H]^+$	346.2165	212.1428	6.63	125	6.25	0.125
	Pipradrol	$[M+H]^+$	268.1696	250.1603	5.20	125	1.25	0.025
	p-OH Amphetamine	$[M+H]^+$	135.0804	107.0494	2.98	250	25	0.5
	Prenylamine	$[M+H]^+$	330.2216	212.1431	7.43	125	1.25	0.025
	Prolintane	$[M+H]^+$	218.1903	72.0810	5.41	25	0.25	0.005
	Propylamphetamine	$[M+H]^+$	166.1226	148.1121	3.50	125	6.25	0.125
	Propylhexedrine	$[M+H]^+$	178.1590	91.0548	4.66	1250	62.5	1.25

Table 4.1 (Continued)

Group on Prohibited List (by WADA)	Compound	Precursor Ion (Full MS)		Product Ion (DIA)	Ret. Time	TPL	LOD	
		Molecular Ion	Exact Mass [*u*]	Exact Mass [*u*]	[min]	Conc. [ng/mL]	[ng/mL]	[ng/20 µL spot]
S6	Pseudoephedrine	$[M+H]^+$	156.1747	156.1747	5.03	1250	12.5	0.25
	Pyrrolidinovalerophenone, alpha- (a-PVP)	$[M+H]^+$	232.1696	126.1278	4.85	25	1.25	0.025
	Ritalinic Acid	$[M+H]^+$	220.1332	84.0811	4.28	250	2.5	0.05
	Selegiline	$[M+H]^+$	188.1434	188.1434	4.49	250	12.5	0.25
	Sibutramine	$[M+H]^+$	280.1826	125.0154	6.88	250	12.5	0.25
	Strychnine	$[M+H]^+$	335.1754	264.1017	4.18	125	1.25	0.025
	Tuaminoheptane	$[M+H]^+$	116.1434	57.0700	4.25	1250	62.5	1.25
S7	3-Methylfentanyl	$[M+H]^+$	351.2431	202.1587	6.07	5	0.25	0.005
	Acetylmorphine, 6-	$[M+H]^+$	328.1543	211.0739	3.90	125	1.25	0.025
	Alfentanil	$[M+H]^+$	417.2609	165.1018	5.62	25	1.25	0.025
	Buprenorphine	$[M+H]^+$	468.3108	468.3108	5.98	125	1.25	0.025
	Codeine	$[M+H]^+$	300.1594	243.1020	3.61	125	1.25	0.025
	Diacetylmorphine	$[M+H]^+$	370.1649	211.0750	4.83	1250	62.5	1.25
	EDDP Perchlorate	$[M+H]^+$	278.1903	234.1288	6.21	62.5	0.625	0.0125
	Fentanyl	$[M+H]^+$	337.2274	188.1432	5.74	25	1.25	0.025
	Hydrocodone	$[M+H]^+$	300.1594	199.0765	3.97	125	1.25	0.025
	Hydromorphone	$[M+H]^+$	286.1438	286.1438	3.22	125	6.25	0.125
	Methadone	$[M+H]^+$	310.2165	265.1582	6.63	62.5	0.625	0.0125
	Morphine	$[M+H]^+$	286.1438	185.0595	2.89	500	5	0.1
	Norcodeine	$[M+H]^+$	286.1438	286.1438	3.53	125	1.25	0.025
	Norfentanyl	$[M+H]^+$	233.1648	233.1648	4.38	50	2.5	0.05
	Norhydrocodone	$[M+H]^+$	286.1438	199.0750	3.92	62.5	3.125	0.0625
	Norhydromorphone	$[M+H]^+$	272.1281	272.1281	3.12	125	6.25	0.125
	Norpethidine	$[M+H]^+$	234.1489	160.1130	5.01	125	12.5	0.25

Table 4.1 (Continued)

Group on Prohibited List (by WADA)	Compound	Precursor Ion (Full MS)		Product Ion (DIA)	Ret. Time	TPL	LOD	
		Molecular Ion	Exact Mass [*u*]	Exact Mass [*u*]	[min]	Conc. [ng/mL]	[ng/mL]	[ng/20 µL spot]
S7	Oxycodone	$[M+H]^+$	316.1543	298.1438	3.84	125	1.25	0.025
	Oxymorphone	$[M+H]^+$	302.1387	284.1280	3.05	125	6.25	0.125
	Pentazocine	$[M+H]^+$	286.2165	218.1539	5.36	62.5	0.625	0.0125
	Pethidine	$[M+H]^+$	248.1645	174.1277	5.04	62.5	0.625	0.0125
	Racemoramide	$[M+H]^+$	393.2537	236.1428	6.51	62.5	0.625	0.0125
	Remifentanil	$[M+H]^+$	377.2071	228.1225	5.01	25	1.25	0.025
	Sufentanyl	$[M+H]^+$	387.2101	111.0264	6.36	25	1.25	0.025
	Tramadol	$[M+H]^+$	264.1958	58.0658	4.68	125	1.25	0.025
S8	AM-1220	$[M+H]^+$	383.2118	112.1120	6.64	25	1.25	0.025
	JWH-018	$[M+H]^+$	342.1852	342.1852	11.70	25	5	0.1
	JWH-019	$[M+H]^+$	356.2009	356.2009	11.86	25	25	0.5
	JWH-073	$[M+H]^+$	328.1696	328.1696	11.48	25	2.5	0.05
	JWH-122	$[M+H]^+$	356.2009	356.2009	11.85	25	25	0.5
S9	Betamethasone	$[M+HCOO^-]^-$	437.1981	361.1837	6.53	50	25	0.5
	Budesonide	$[M+H]^+$	431.2428	413.2322	7.88	50	25	0.5
	Dexamethasone	$[M+HCOO^-]^-$	437.1981	361.1838	6.88	50	25	0.5
	Fluticasone propionate	$[M+HCOO^-]^-$	545.1826	/	9.88	50	10	0.2
	Methylprednisolone	$[M+HCOO^-]^-$	419.2075	343.1931	6.4	50	10	0.2
	Prednisolone	$[M+HCOO^-]^-$	405.1919	329.1773	5.91	50	10	0.2
	Prednisone	$[M+HCOO^-]^-$	403.1762	327.1617	5.96	50	10	0.2
	Triamcinolone	$[M+HCOO^-]^-$	439.1773	345.1523	5.33	50	10	0.2
	Triamcinolone acetonide	$[M+HCOO^-]^-$	479.2087	386.0059	6.84	50	10	0.2

Table 4.1 (Continued)

Group on Prohibited List (by WADA)	Compound	Precursor Ion (Full MS)		Product Ion (DIA)	Ret. Time	TPL	LOD	
		Molecular Ion	Exact Mass [*u*]	Exact Mass [*u*]	[min]	Conc. [ng/mL]	[ng/mL]	[ng/20 µL spot]
P1	Acebutolol	$[M+H]^+$	337.2122	72.0808	4.55	25	1.25	0.025
	Bisoprolol	$[M+H]^+$	326.2326	116.1070	5.29	25	1.25	0.025
	Carvedilol	$[M+H]^+$	407.1965	283.1441	6.30	25	0.25	0.005
	Metoprolol	$[M+H]^+$	268.1907	116.1070	4.67	25	1.25	0.025
	Opipramol	$[M+H]^+$	364.2383	171.1492	5.52	25	2.5	0.05
	Propranolol	$[M+H]^+$	260.1645	116.1070	5.61	25	1.25	0.025
Others	Bemethyl	$[M+H]^+$	179.0637	151.0325	3.89	250	12.5	0.25
	Fluconazole	$[M+H]^+$	307.1113	220.0684	4.76	250	2.5	0.05
	Ketoconazole	$[M+H]^+$	531.1560	489.1446	6.31	500	50	1
	Lomerizine	$[M+H]^+$	469.2297	181.0859	7.80	50	0.5	0.01
	Miconazole	$[M+H]^+$	414.9933	414.9933	8.19	250	2.5	0.05

Abbreviations: DIA, Data independent acquisition; LOD, limit of detection; TPL, target performance level; WADA, World Anti-Doping Agency.

4.3.4 Sample preparation

The sample preparation was performed in a fully automated procedure using a Multi-Purpose Sampler coupled to a dried blood spot autosampler manufactured by Gerstel (Mülheim an der Ruhr, Germany). First, the substances were extracted from the spot via flow-through desorption at 80 °C using 1500 µl of a mixture of methanol and water at a ratio of 80:20 (*v/v*) combined with 30 µl of the mixed ISTD working solution with a dispensation flow of 5,000 µl/min. During this process, a fixed diameter of 4 mm was extracted and the duration of a single extraction was estimated to require 3.8 min. Subsequently, the sample extract was acidified by adding 5 µl of 0.3 M hydrochloric acid and evaporated to dryness under reduced pressure at 50 °C for 35 min. Following reconstitution with 120 µl of a mixture of purified water and acetonitrile 80:20 (*v/v*), the samples were prepared for LC–HRMS/MS analysis. The overall preparation of a batch of six samples had an estimated duration of 90 min, allowing up to 48 samples to be extracted in succession. However, the evaporator of the device used for this project was limited to six samples, so an additional 70 min had to be scheduled for the complete preparation of each additional six samples.

4.3.5 Liquid chromatography–tandem mass spectrometry

For liquid chromatography, a Thermo Vanquish UHPLC system was employed, equipped with an Agilent Poroshell 120 EC-C_{18} analytical column (50 × 3.0 mm, 2.7 µm particle size) combined with an EC 4/3 Nucleoshell RP 18 Plus precolumn provided by Macherey & Nagel (5 µm particle size). After sample injection of 10 µl, chromatographic separation was achieved using gradient elution with ultrapure water mixed with 0.1% formic acid as aqueous solvent (A) and acetonitrile mixed with 0.1% formic acid as organic solvent (B). The gradient started at 0% B, which was maintained for 0.5 min followed by an increase to 40% B within the next 4.5 min. The proportion of solvent B was increased to 70% at 10 min and then held at 100% B for a further minute. Subsequently, the column was re-equilibrated at starting conditions for 5 min resulting in a total run time of 16 min.

High-resolution mass spectrometry was performed using a Thermo Orbitrap Exploris 480 operated in full scan mode and data independent acquisition (DIA) mode. Full MS data were monitored within a mass range of 100–800 *m/z* with an Orbitrap resolution set to

45,000 full width at half maximum (FWHM). The corresponding product ions were generated using four timed DIA experiments adjusted to different mass ranges with stepped normalized collision energies of 30 and 55%: 100–200 *m/z* from 0 to 6.5 min, 200–400 *m/z* throughout the whole run time; 400–500 *m/z* from 5 to 16 min; and 500–600 *m/z* from 6 to 16 min. All DIA experiments were conducted at an Orbitrap resolution of 30,000 (FWHM). For ionization, the mass spectrometer was interfaced with a heated electrospray ion source that was operated in positive or negative mode in two separate runs with ionization voltages of 3.5 and −3.5 kV, respectively. Raw data were analyzed using the TraceFinder software (version 4.1) by Thermo Fisher (Waltham, MA, USA).

4.3.6 Assay validation

In compliance with the guidelines of the WADA, the General Requirements for the Competence of Testing and Calibration Laboratories (ISO/IEC 17025:2017), the International Standard for Laboratories (World Anti-Doping Agency, 2021a) and the Technical Document for Dried Blood Spots were considered for the validation process (World Anti-Doping Agency, 2023). Accordingly, the following parameters were included: selectivity, limit of detection (LOD), reproducibility of the detection, carryover, robustness in terms of an alternative manual sample preparation and an alternative dried blood collection device (Tasso-M20), and the sample extract stability.

The selectivity was assessed by testing 12 blank DBS provided by six male and six female volunteers aged 24–49 years for interfering signals at the respective retention times. Additionally, 10 DBS from whole blood collected in EDTA-coated tubes (five males, five females) were tested for selectivity as they were used to generate enriched DBS for positive controls and the assessment of other validation parameters. Both batches were divided and six or five replicates respectively were each prepared and analyzed on two different days. Since DBS have only recently been introduced as a matrix in sports drug testing, there are no minimum required performance levels established for prohibited substances to date, which is why the validation plan was based on individually determined detectable concentrations as summarized in **Table 4.1** and referred to as the target performance level (TPL) in the following. For LOD estimation, 10 DBS each were generated from blood samples enriched at 50% TPL, 20% TPL, 10% TPL, 5% TPL and 1% TPL. After

sample preparation and analysis via LC–HRMS/MS on two different days of five replicates each, the LODs were set to the lowest concentration with a detection rate of 100%. In order to evaluate the reproducibility of the detection, 10 DBS were generated using whole blood samples enriched at the TPL, divided into two batches each consisting of five replicates and analyzed on two different days. The carryover was determined for both automated spot extraction and liquid chromatography, as both components of the approach potentially lead to a carryover effect. For this purpose, DBS were generated at two different concentrations, at the TPL and at 4 × TPL. The enriched DBS were processed and followed by three blank DBS samples each at the respective preparation or analytical step to be investigated. In order to verify the robustness of the sample preparation, a manual procedure was evaluated as an alternative to the fully automated approach. In this protocol, the DBS was first punched out using a device with a comparable diameter extracted during the automated sample preparation and transferred to a plastic tube. Subsequently, 800 µl of a mixture of methanol and purified water at a ratio of 80:20 (*v/v*) and 20 µl of the mixed internal standard solution were added. A lower extraction volume was used for manual sample preparation considering that the extraction is equally efficient compared with the automated flow-through procedure despite a lower volume, attributable to the extended extraction time in the ultrasonic bath. Larger volumes were not required for competitive performance. After the incubation in an ultrasonic bath for 20 min and centrifugation at 13,000 *g* for 5 min, the supernatant was transferred to a fresh plastic tube and 5 µl of aqueous hydrochloric acid (0.3 M) was added. The solvent was evaporated to dryness under reduced pressure at 45 °C and the residue was reconstituted using 120 µl of a mixture of purified water and acetonitrile 80/20 (*v/v*). Owing to the extraction of a larger amount of matrix components compared with the automated sample preparation, a final centrifugation step at 13,000 *g* for 10 min was added before transferring 100 µl to a vial and subsequent analysis via LC–HRMS/MS. This alternative sample preparation was evaluated in terms of selectivity using six blank DBS samples (three males, three females) and detectability at the TPL and 50% TPL using six DBS generated with fortified whole blood samples (three males, three females). In addition to DBS collection cards, collection devices for the upper arm are approved for doping control purposes. Therefore, Tasso-M20 (Tasso, Seattle, WA, USA) collection

devices were tested for their selectivity (five females, five males) and detectability at the TPL (three females, three males) in the context of robustness. For each sample, one Tasso volumetric pellet was used, corresponding to 17.5 µl (with a coefficient of variation [CV] of <5%) of blood according to the manufacturer's instructions. For the generation of positive quality control samples, Tasso-M20 devices were opened and 5 µl of the mixed standard working solution was added to the volumetric pellets. After drying for 30 min, 20 µl of whole blood was pipetted onto the fortified pellets in order to obtain comparable results to DMPK-C cards regarding analyte concentrations and matrix effects. After an additional drying period of at least 2 h, Tasso devices were sealed according to the manufacturer's instructions and stored at 4 °C until analysis. The sample preparation was conducted manually as described above. In order to test the stability of the sample extracts, six DBS were generated from enriched whole blood samples (three males, three females) at the TPL and prepared using the fully automated approach. The samples were analyzed via LC–HRMS/MS on the day of preparation (d0), after 2 days of storage in the autosampler of the instrument at 4 °C (d2) and after 5 days of storage in the autosampler of the instrument at 4 °C (d5).

4.3.7 Proof-of-concept

In order to demonstrate the applicability of the testing procedure, several substances that are prohibited only in-competition were ingested by healthy volunteers and post-administration DBS samples were analyzed using the validated ITP. For this purpose, DBS samples were collected as authentically as possible according to the corresponding Technical Document provided by WADA (World Anti-Doping Agency, 2021c). Following the oral application of 3 mg of the glucocorticoid dexamethasone (two tablets containing 1.5 mg, GALENpharma, Kiel, Germany) to one male healthy volunteer at the age of 61 years, a total of seven DBS samples were collected after 2, 6, 12, 19, 24, 31 and 48 h. An additional administration study was conducted with 5 mg of prednisone (acis Arzneimittel, Grünwald, Germany), which also belongs to the group of glucocorticoids. Two healthy volunteers (female and male, 28 and 49 years, respectively) collected post-administration DBS samples after 1, 2, 3 and 4 h or after 2, 4 and 6 h. As a representative of beta-blockers, 40 mg propranolol (Aliud Pharma, Laichingen, Germany) was

administered to two healthy volunteers (female and male, 27 and 47 years, respectively) and a total of four post-administration DBS samples were collected for a period of 24 h after 2, 6, 12 and 24 h. Similarly, in a further study, two healthy volunteers (female and male, 27 and 47 years, respectively) collected DBS samples after the ingestion of 30 mg of the stimulant pseudoephedrine (combined preparation with 200 mg ibuprofen, BoxaGrippal, Sanofi, Paris, France). In addition to post-administration DBS samples, blank DBS samples were collected in all cases prior to drug intake. Signed written informed consent was received from all participants, as well as ethical approval from the local ethics committee of the German Sport University Cologne (no. 107/2018).

4.4 Results and Discussion

4.4.1 Assay development

For method development, sample preparation steps from previous projects on DBS were applied, and the instrumental analysis was adapted from a comprehensive ITP for urine. The automated sample preparation system performed the extraction using a flow-through desorption technology. In this process, the spot was rinsed with a defined volume of the extraction solvent under pressure and at an elevated temperature, giving an efficient and fast extraction. During this procedure, the internal standard was introduced, allowing for compensating potential extraction- and matrix-related analyte losses. Subsequently, the automated processing involved acidification in order to retain alkaline and readily volatile molecules, for example certain stimulants, dissolved in solution during evaporation. Afterwards, the sample extract was evaporated under reduced pressure and reconstituted to the final working solution.

Considering that the approach was intended to comprise a broad spectrum of diverse chemical structures, both chromatographic and mass spectrometric parameters were devised to be flexible and versatile. The application of high-resolution mass spectrometry in a full scan and DIA mode allowed the detection of a broad range of analytes with a time-saving monitoring technique, which increased the number of data points, and provided the ability to adapt the approach for additional compounds. The identification of each analyte was ascertained by the presence of the precursor ion in the full scan data and a confirming product ion in the DIA data at the corresponding retention time. In order to further increase the number of data points per signal, two separate injections were performed in positive and negative ionization mode, respectively. A summary of the mass spectrometric parameters for each analyte is provided in **Table 4.1**.

4.4.2 Assay validation

For the validation of the approach, a total of 233 compounds related to sports drug testing were evaluated. According to WADA's Prohibited List, these included 16 substances from the group of anabolic agents (S1), six substances that increase the production of erythropoietin (S2), 26 beta-2-agonists (S3), five substances from the group of hormones

and metabolic modulators (S4), 42 diuretic and masking agents (S5), 88 stimulants (S6), 25 narcotics (S7), five synthetic cannabinoids (S8), nine substances from the group of glucocorticoids (S9), six beta-blockers (P1) and five substances that are not assigned to a group but are or were relevant for monitoring programs or constitute confounding factors (**Table 4.1**). The analytes mainly represent the intact drug, and partly active metabolites circulating in blood.

For selectivity evaluation, both full scan and DIA data of 12 blank DBS samples were tested for interfering signals at the corresponding retention time of each compound, which showed the approach to be selective for all 233 compounds. In addition, the selectivity of 10 venous whole blood samples was verified as they were used to generate enriched DBS samples required for several validation parameters. In order to establish the limit of detection, various dilutions of the TPL were prepared and 10 DBS were analyzed for each concentration. The LOD values listed in **Table 4.1** correspond to the lowest concentration with a detection rate of 100%. In order to demonstrate the reproducibility of the detection, 10 DBS samples were enriched and analyzed at the TPL and all compounds were reproducibly identified in all samples by two ions, the precursor ion extracted from full scan data and one product ion extracted from DIA data. An overview of chromatograms for each compound is presented in **Figure 4.S1**. A highly concentrated sample often implies the risk of a carryover effect. For the developed approach, two sources may be involved, the automated extraction step during sample preparation and the carryover on the analytical column during instrumental analysis. Hence, the carryover effect was determined for each of the two processing steps, once at the TPL and once at the fourfold TPL. For the majority of the substances, no carryover was detected at the TPL during the automated sample preparation. In cases where carryover was observed, it was estimated to be about 1% or less, with a maximum of 2%, excluding amiloride and lisdexamfetamine, for which carryover values of ca. 3% and 6%, respectively, were found in the full MS data. For the fourfold TPL concentration, the carryover for most of the substances was not detected or was <1%. However, there was a notable carryover for amiloride, lisdexamfetamine, norcodeine and norhydromorphone of approximately 7, 10, 3 and 4%, respectively.

For certain substances, the fourfold TPL was comparably high and not to be expected in authentic doping control samples, i.e. the determined carryover effect was not anticipated to be of practical significance. The carryover effect associated with liquid chromatography was found to be insignificant for most analytes. A carryover was detected in the DIA scan for daprodustat, at approximately 2% at the TPL and approximately 3% at the fourfold TPL. Furthermore, carryovers of about 3 and 2% at the fourfold TPL were determined for trimetazidine and ketoconazole, respectively.

Under certain circumstances, the automatic sample preparation may not be applicable, e.g. if the blood spot is not properly applied and forms an inadequately widespread dried sample. Here, the automated spot detection might fail. Furthermore, the automatic sample preparation is not applicable for collection devices intended for the upper arm, e.g. Tasso-M20 devices. Consequently, a manual sample preparation was evaluated in the context of robustness with respect to selectivity and detectability at the TPL and 0.5 × TPL. Selectivity samples were collected from the fingertip, and detectability samples were generated using venous whole blood that was fortified with the substances at different concentration levels and subsequently spotted onto the collection cards with six replicates for each parameter. All negative samples were found to be selective for the analytes of interest. Furthermore, all analytes were identified with a detection rate of 100% in the fortified samples, with the exceptions of higenamine with detection rates of 67% (TPL) and 17% (0.5 × TPL) and tretoquinol with detection rates of 67% (TPL) and 50% (0.5 × TPL).

With the increasing attention to DBS, a number of collection devices have been developed and introduced to the market. The blood collection material could affect the extraction and, owing to various matrix effects, also the instrumental analysis. In order to address this concern, additional collection devices distributed by Tasso were examined regarding selectivity (n = 10) and detectability (n = 6) at the TPL. Compared with DMPK-C cards, which were used within this project and are based on untreated cellulose, Tasso M-20 devices employ a hydrophilic porous blood collection material. While Tasso blank samples tested negative, all analytes were identified in fortified samples with signal intensities comparable with those found for DMPK-C cards enriched at the same concentration.

In the case of extended run times, for example owing to a large number of samples, the samples are stored in the autosampler for a certain period of time before analysis. For this reason, the stability of the sample extracts was examined using six samples, each enriched at the TPL and prepared to be measured on day 0, 2 and 5 after storage in the autosampler at 4 °C. For evaluation of the results, peak areas were compared with the corresponding peak area of the analysis on day 0. For all analytes, no degradation was observed after 2 days of storage in the autosampler. However, a decrease in peak area was determined after 5 days for several substances, especially for steroid esters and synthetic cannabinoids. The following substances were found to be affected by storage at 4 °C for 5 days: dehydrochloromethyltestosterone (−40%), nandrolone propionate (−70%), testosterone propionate (−80%), trenbolone enanthate (−95%), testosterone acetate (−50%), IOX-2 (−30%), vadadustat (−40%), eplerenone (−40%), bromantane (−90%), JWH-018 (−60%), JWH-019 (−80%), JWH-073 (−30%) and JWH-122 (−80%).

However, all validation experiments were conducted exclusively with freshly prepared DBS. Thus, the stability and the extractability of the analytes after a certain storage time were not investigated, but previous studies demonstrated that various analytes were still detectable after a storage time of up to 1 year (Mazzarino et al., 2022; Moretti et al., 2018; Thevis et al., 2022; Thomas et al., 2011). Follow-up research is required to verify the extractability and stability in DBS for all substance groups included in the developed approach, in order to address realistic scenarios such as prolonged transport from the sample collection site to the analytical laboratory.

4.4.3 Proof-of-concept and evaluation of limits of detection

An overview of established limits of detection is shown in **Table 4.1**. It was defined by a sample number of 10 and a detection rate of 100%. Minimum required performance levels have not yet been specified for DBS and the expected plasma concentrations that should be reliably detected are still undetermined for many substances. Accordingly, the concentration of the mixed standard working solution (TPL), which was used for the development and optimization of the method as well as for the determination of validation parameters, had to be estimated on the basis of preliminary experiments and

was notably high for several substances, as subsequent optimization processes resulted in a significantly higher sensitivity compared with pre-measurements.

Among the 233 evaluated substances, 14 were found to allow for a LOD <1 ng/ml, 103 were found to have a LOD ≤5 ng/ml, 95 were found to have a LOD ≤50 ng/ml, eight were found to have a LOD ≤100 ng/ml, and 13 were found to have a LOD >100 ng/ml. The variations found for LODs can be attributed to various sources. For example, differing recovery efficiencies as well as ionization efficiencies or abundant background signals on the corresponding mass traces as observed for certain molecules of comparably low molecular mass may be involved. Regarding the suitability of the LOD for a substance in the context of a real doping control sample, the pharmacodynamic and pharmacokinetic processes need to be considered. In particular, therapeutic doses and associated plasma concentrations, as well as metabolism and excretion patterns, are relevant parameters when it comes to establishing a required detection limit. For this purpose, administration studies are inevitable and literature data are especially valuable in order to reassess the applicability of the testing procedure.

For several substances that are frequently misused, post-administration plasma level data are already available (World Anti-Doping Agency, 2020). Accordingly, studies have demonstrated that the determined LOD is compliant, among others, in the cases of the anabolic agent ostarine (Coss et al., 2016), the metabolic modulators meldonium (Cai et al., 2011), clomiphene (Mikkelson et al., 1986) and anastrozole (Yu et al., 2011), the diuretic hydrochlorothiazide (Barbhaiya et al., 1982) and the stimulants methylphenidate (Srinivas et al., 1993) and cocaine (Wilkinson et al., 1980).

After oral administration of 5 mg of the glucocorticoid prednisone to two healthy volunteers, post-administration DBS samples were tested for suspicious signals using the developed ITP. DBS samples that were collected before the administration were found to be negative for all included substances. In addition to signals for prednisone, signals for prednisolone were found with greater intensity in all post-administration samples obtained, in both the full scan and DIA measurements, owing to the conversion by the liver (Jenkins & Sampson, 1967). An overview of extracted chromatograms is shown in **Figure 4.1**. Accordingly, all post-administration samples meet the criteria, and would be

analyzed using an individually developed Confirmation Procedure validated following WADA guidelines in the next step, thus fulfilling the requirements for an ITP.

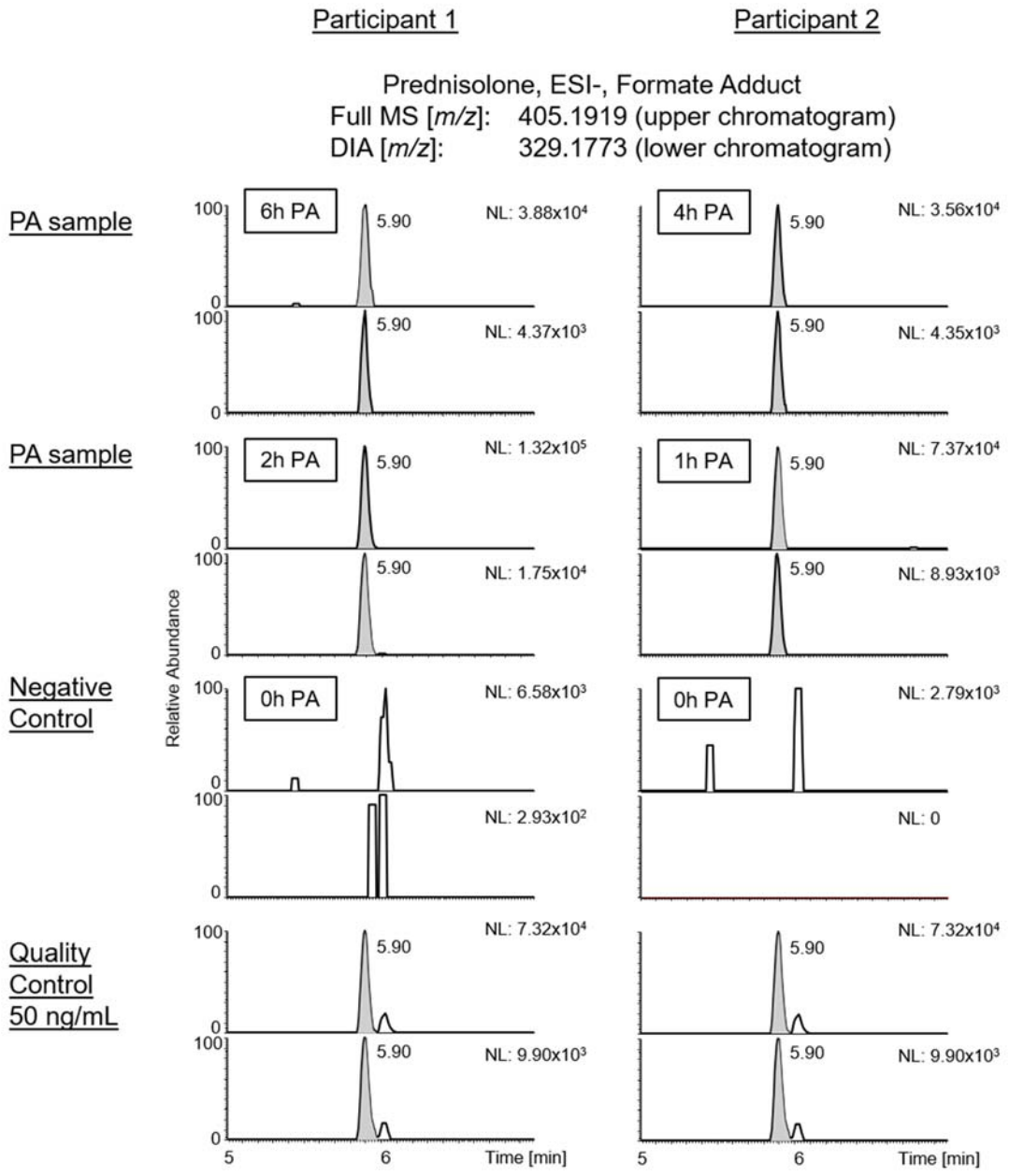

Figure 4.1 This figure shows extracted ion chromatograms from dried blood spot (DBS) samples obtained from two participants after the administration of 5 mg of prednisone. In addition to the negative and positive control, post-administration (PA) samples are shown after 2 and 6 h (participant 1) and after 1 and 4 h (participant 2), respectively. Depicted in each case are chromatograms of the formate adduct of prednisolone obtained from both full mass spectrum data [extracted mass (*m/z*): 405.1919] in the respective upper chromatogram and data independent acquisition (DIA) data [extracted mass (*m/z*): 329.1773] in the respective lower chromatogram. ESI, electrospray ionization; NL, normalization level.

After the administration of 3 mg of the glucocorticoid dexamethasone to one healthy participant, DBS samples were collected over a total period of 48 h. Similar to all of the conducted administration studies, a pre-application sample was obtained and tested negative for all substances investigated. However, DBS samples collected after 2 and 6 h yielded a suspicious signal in the DIA scan together with a less prominent signal in the full scan at the correct retention time, while all samples collected at later time points, more specifically after 12, 19, 24, 31 and 48 h, were found to be negative for dexamethasone. This case demonstrates the benefit of DBS regarding the interpretation of positive findings of substances that are only prohibited in-competition (S6–S9 on the WADA Prohibited List), which applies for example to the group of glucocorticoids, by allowing for a more substantiated estimation of the timepoint of the intake as well as the activity at the time of sampling of the respective substance.

Further proof-of-concept studies involved the intake of 40 mg of the beta-blocker propranolol and 30 mg of the stimulant pseudoephedrine to two participants in each case and four DBS samples were collected within a period of 24 h after ingestion. Prior to ingestion, an additional DBS sample was provided, which was found to be negative for all investigated substances after analysis by means of the developed ITP. In contrast, unequivocal signals for propranolol or pseudoephedrine were detected in all post-administration samples in both full scan and DIA data. The intensity of the signals decreased significantly over time post ingestion. Extracted ion chromatograms for both the negative and positive controls as well as post-administration samples collected after 2 and 24 h are shown for both substances for one participant each in **Figure 4.2**. In the case of pseudoephedrine, a threshold value is defined for urine.

Accordingly, an adverse analytical finding is reported when the detected urinary concentration is >150 µg/ml. In addition to such thresholds, analytical performance characteristics (minimum required performance level) as well as minimum reporting levels are expected to be established by WADA in the future, and analyzing threshold substances will then require the quantification of compounds in DBS. A well-known limitation in this context is the impact of the hematocrit value on the interpretation of determined concentrations in DBS (Capiau et al., 2018). For this reason, the implementation of an automated non-destructive hematocrit determination by means of

near-infrared spectroscopy is prospectively intended (Oostendorp et al., 2016). Previous projects yielded promising results in this regard but indicated a need for further research (Lange et al., 2020), while more recent studies demonstrated robust and reliable hematocrit measurements using near infrared (Delahaye et al., 2021).

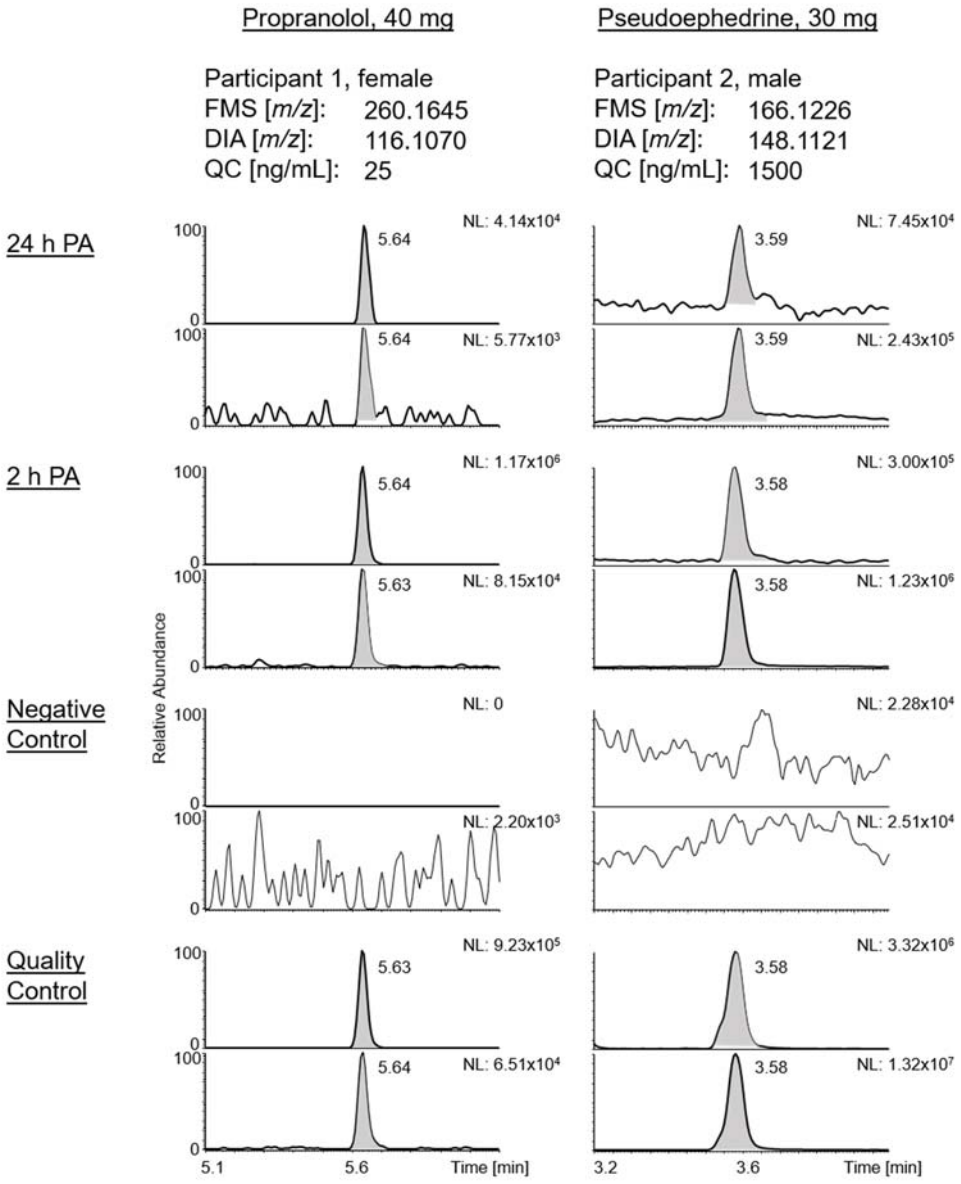

Figure 4.2 This figure presents extracted ion chromatograms of propranolol and pseudoephedrine from both full mass spectra (FMS, respective upper chromatogram) and data independent acquisition (DIA, respective lower chromatogram) data from DBS samples collected 2 and 24 h after the administration of 40 or 30 mg, respectively. In addition, chromatograms of the corresponding negative and positive control are shown. QC, quality control; PA: Post-administration; NL, normalization level.

4.5 Conclusions

Over recent years, DBS have been found to be a beneficial complementary matrix and thus were successfully introduced by WADA into the routine sports drug testing program in 2021. The ITP developed during this project aims at addressing the increasing use of DBS that is anticipated for future anti-doping programs. Accordingly, a comprehensive screening procedure for substances from all groups of the Prohibited List was successfully validated and implemented into the routine program. The automated sample preparation ensures the accomplishment of a high sample throughput, additionally saving laboratory staff time and costs. When considering a batch of six samples, the automated sample preparation requires 90 min, compared with the manual sample preparation involving ~180 min, depending on the experience of the laboratory staff. Several limits of detection were found to be appropriate with regard to authentic excretion samples collected after the intake of prohibited substances, but further studies are required to evaluate the applicability of the method in the context of anti-doping testing. In addition, the ITP will be adapted and optimized in the future with regard to introduced thresholds for both the required analytical performance and the reporting of adverse analytical findings.

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4.8 Supporting Information

4.8.1 Table 4.S1

Table 4.S1 Overview of the manufacturers and distributors of all standard solutions and reference substances

Substance	Manufacturer / Distributor	Headquarters
1,3-Dimethylbutylamine	LGC Standards	Teddington, United Kingdom
2,5-Dimethoxyphenethylamine	NMI Natural and Medical Sciences Institute	Reutlingen, Germany
2-amino-6-methylheptane (Octodrine)	Sigma-Aldrich	St. Louis, Missouri, United States
3,4-Methylenedioxyethyl-amfetamine (MDEA)	LGC Standards	Teddington, United Kingdom
3-Methylfentanyl	Lipomed AG	Arlesheim, Switzerland
4-Aminotrifluoromethylbenzen-disulfonamide	Sigma-Aldrich	St. Louis, Missouri, United States
4-methylhexan-2-amine	Cerilliant	Round Rock, Texas, United States
5-(2-aminopropyl)-2,3-dihydrobenzofuran (5-APDB)	LGC Standards	Teddington, United Kingdom
5-oxo-Mefruside	Toronto Research Chemicals	Toronto, Canada
6-(2-aminopropyl)benzofuran	LGC Standards	Teddington, United Kingdom
Acebutolol	Bayer	Leverkusen, Germany
Acetazolamide	European Directorate for the Quality of Medicines & HealthCare	Straßburg, France
Acetylmorphine, 6-	LGC Standards	Teddington, United Kingdom
Alfentanil	Cerilliant	Round Rock, Texas, United States
Althiazide	LGC Standards	Teddington, United Kingdom
AM-1220	LGC Standards	Teddington, United Kingdom
Amfepramone (Diethylpropione)	Toronto Research Chemicals	Toronto, Canada
Amiloride	European Directorate for the Quality of Medicines & HealthCare	Straßburg, France
Aminochlorobenzendisulphonamide	Sigma-Aldrich	St. Louis, Missouri, United States
Amiphenazol	LGC Standards	Teddington, United Kingdom
Amphetamine	LGC Standards	Teddington, United Kingdom
Anastrozole	Sigma-Aldrich	St. Louis, Missouri, United States
Azosemide	LGC Standards	Teddington, United Kingdom
Bambuterol	LGC Standards	Teddington, United Kingdom
Bamethane	Toronto Research Chemicals	Toronto, Canada
Bemethyl	In-house synthesis	German Sport University, Cologne, Germany
Bemetizide	Schwarz Pharma	Monheim am Rhein, Germany
Bendroflumethiazide	European Directorate for the Quality of Medicines & HealthCare	Straßburg, France
Benfluorex	Toronto Research Chemicals	Toronto, Canada
Benzoylecgonine	Sigma-Aldrich	St. Louis, Missouri, United States
Benzphetamine	Cayman Chemical Company	Ann Arbor, Michigan, United States
Benzthiazide	Sigma-Aldrich	St. Louis, Missouri, United States
Benzylhydrochlorothiazide	Toronto Research Chemicals	Toronto, Canada
Betamethasone	Sigma-Aldrich	St. Louis, Missouri, United States
Bisoprolol	Sigma-Aldrich	St. Louis, Missouri, United States
Brinzolamide	Cayman Chemical Company	Ann Arbor, Michigan, United States
Bromantane	Cayman Chemical Company	Ann Arbor, Michigan, United States
Brombuterol	LGC Standards	Teddington, United Kingdom
Budesonide	Sigma-Aldrich	St. Louis, Missouri, United States
Bumetanide	European Directorate for the Quality of Medicines & HealthCare	Straßburg, France

Table 4.S1 (Continued)

Substance	Manufacturer / Distributor	Headquarters
Buprenorphine	LGC Standards	Teddington, United Kingdom
Butizide	Toronto Research Chemicals	Toronto, Canada
Cafedrine	LGC Standards	Teddington, United Kingdom
Carphedone	NMI Natural and Medical Sciences Institute	Reutlingen, Germany
Carvedilol	LGC Standards	Teddington, United Kingdom
Cathinone	Cerilliant	Round Rock, Texas, United States
Chlorazanil	Heumann Pharma	Nürnberg, Germany
Chlorothiazide	Sigma-Aldrich	St. Louis, Missouri, United States
Chlortalidone	European Directorate for the Quality of Medicines & HealthCare	Straßburg, France
Cimaterol	LGC Standards	Teddington, United Kingdom
Cimbuterol	LGC Standards	Teddington, United Kingdom
Clenbuterol	LGC Standards	Teddington, United Kingdom
Clenpenterol	LGC Standards	Teddington, United Kingdom
Clenproperol	LGC Standards	Teddington, United Kingdom
Clobenzorex	LGC Standards	Teddington, United Kingdom
Clomifene	Toronto Research Chemicals	Toronto, Canada
Clopamide	LGC Standards	Teddington, United Kingdom
Cocaine	LGC Standards	Teddington, United Kingdom
Codeine	Cerilliant	Round Rock, Texas, United States
Conivaptane	LGC Standards	Teddington, United Kingdom
Cropropamide	LGC Standards	Teddington, United Kingdom
Crothetamide	Toronto Research Chemicals	Toronto, Canada
Cyclazodone	Santa Cruz Biotechnology	Dallas, Texas, United States
Cyclopenthiazide	LGC Standards	Teddington, United Kingdom
Cyclothiazide	Sigma-Aldrich	St. Louis, Missouri, United States
Daprodustat	LGC Standards	Teddington, United Kingdom
Dehydrochlormethyltestosterone	NMI Natural and Medical Sciences Institute	Reutlingen, Germany
Desidustat	Hycultec	Hycultec, Germany
Desmethylselegiline	LGC Standards	Teddington, United Kingdom
Dexamethasone	Sigma-Aldrich	St. Louis, Missouri, United States
Diacetylmorphine	LGC Standards	Teddington, United Kingdom
Diclofenamide	LGC Standards	Teddington, United Kingdom
Diethylnorephedrine, N,N-	LGC Standards	Teddington, United Kingdom
Dimethoxy-4-ethylamphetamine, -2,5- (DOEt)	Cayman Chemical Company	Ann Arbor, Michigan, United States
Dimethoxy-4-methylamphetamine, -2,5- (DOM)	Cayman Chemical Company	Ann Arbor, Michigan, United States
Dimethoxyamphetamine, -2,5- (DMA)	LGC Standards	Teddington, United Kingdom
Dimethoxyamphetamine, -3,4	Lipomed AG	Arlesheim, Switzerland
Dobutamine	LGC Standards	Teddington, United Kingdom
Dorzolamide	LGC Standards	Teddington, United Kingdom
EDDP Perchlorate	Cerilliant	Round Rock, Texas, United States
Ephedrine	LGC Standards	Teddington, United Kingdom
Epithiazide	LGC Standards	Teddington, United Kingdom
Eplerenone	Sigma-Aldrich	St. Louis, Missouri, United States
Ethamivane	LGC Standards	Teddington, United Kingdom
Etilamfetamine	LGC Standards	Teddington, United Kingdom
Etilefrine	LGC Standards	Teddington, United Kingdom
Exemestane	Sigma-Aldrich	St. Louis, Missouri, United States
Famprofazone	LGC Standards	Teddington, United Kingdom
Fenbutrazate	LGC Standards	Teddington, United Kingdom
Fencamfamine	Toronto Research Chemicals	Toronto, Canada

Table 4.S1 (Continued)

Substance	Manufacturer / Distributor	Headquarters
Fencamine	NMI Natural and Medical Sciences Institute	Reutlingen, Germany
Fenethylline	NMI Natural and Medical Sciences Institute	Reutlingen, Germany
Fenfluramine	LGC Standards	Teddington, United Kingdom
Fenoterol	LGC Standards	Teddington, United Kingdom
Fenproporex	NMI Natural and Medical Sciences Institute	Reutlingen, Germany
Fentanyl	Cerilliant	Round Rock, Texas, United States
FG-2216	Cayman Chemical Company	Ann Arbor, Michigan, United States
Fluconazole	LGC Standards	Teddington, United Kingdom
Fluoroamphetamine, -4	Lipomed AG	Arlesheim, Switzerland
Fluticasone propionate	LGC Standards	Teddington, United Kingdom
Formoterol	TLC Pharmaceutical Standards	Newmarket, Canada
Furfenorex	Toronto Research Chemicals	Toronto, Canada
Furosemide	Sigma-Aldrich	St. Louis, Missouri, United States
Heptaminol	LGC Standards	Teddington, United Kingdom
Higenamine	ChromaDex	Los Angeles, California, United States
Hydrochlorothiazide	Sigma-Aldrich	St. Louis, Missouri, United States
Hydrocodone	LGC Standards	Teddington, United Kingdom
Hydroflumethiazide	LGC Standards	Teddington, United Kingdom
Hydromorphone	LGC Standards	Teddington, United Kingdom
Hydroxytriamterene	LGC Standards	Teddington, United Kingdom
Indacaterol	LGC Standards	Teddington, United Kingdom
IOX-2	Tocris Bioscience	Bristol, United Kingdom
Isometheptene	NMI Natural and Medical Sciences Institute	Reutlingen, Germany
Isoxsuprine	LGC Standards	Teddington, United Kingdom
JWH-018	LGC Standards	Teddington, United Kingdom
JWH-019	Cayman Chemical Company	Ann Arbor, Michigan, United States
JWH-073	Cayman Chemical Company	Ann Arbor, Michigan, United States
JWH-122	Cayman Chemical Company	Ann Arbor, Michigan, United States
Ketoconazole	Sigma-Aldrich	St. Louis, Missouri, United States
Lisdexamfetamine	LGC Standards	Teddington, United Kingdom
Lomerizine	LGC Standards	Teddington, United Kingdom
Mabuterol	LGC Standards	Teddington, United Kingdom
Mapenterol	LGC Standards	Teddington, United Kingdom
Mebeverine	LGC Standards	Teddington, United Kingdom
Mebutizide	DoCoLab	Gent, Belgium
Mefenorex (Chlorpropylamphetamine)	NMI Natural and Medical Sciences Institute	Reutlingen, Germany
Meldonium	Toronto Research Chemicals	Toronto, Canada
Mephentermine	LGC Standards	Teddington, United Kingdom
Mesocarb	Toronto Research Chemicals	Toronto, Canada
Metamfepramone	LGC Standards	Teddington, United Kingdom
Metandienone	NMI Natural and Medical Sciences Institute	Reutlingen, Germany
Methadone	LGC Standards	Teddington, United Kingdom
Methamphetamine	Cerilliant	Round Rock, Texas, United States
Methazolamide	Sigma-Aldrich	St. Louis, Missouri, United States
Methcathinone (Ephedrone)	Cerilliant	Round Rock, Texas, United States
Methedrone	LGC Standards	Teddington, United Kingdom
Methoxyamphetamine, p-(PMA)	LGC Standards	Teddington, United Kingdom
Methoxyphenamine	Sigma-Aldrich	St. Louis, Missouri, United States
Methyclothiazide	LGC Standards	Teddington, United Kingdom
Methylamphetamine, 4-	LGC Standards	Teddington, United Kingdom
Methylendioxyamphetamine (MDA)	LGC Standards	Teddington, United Kingdom

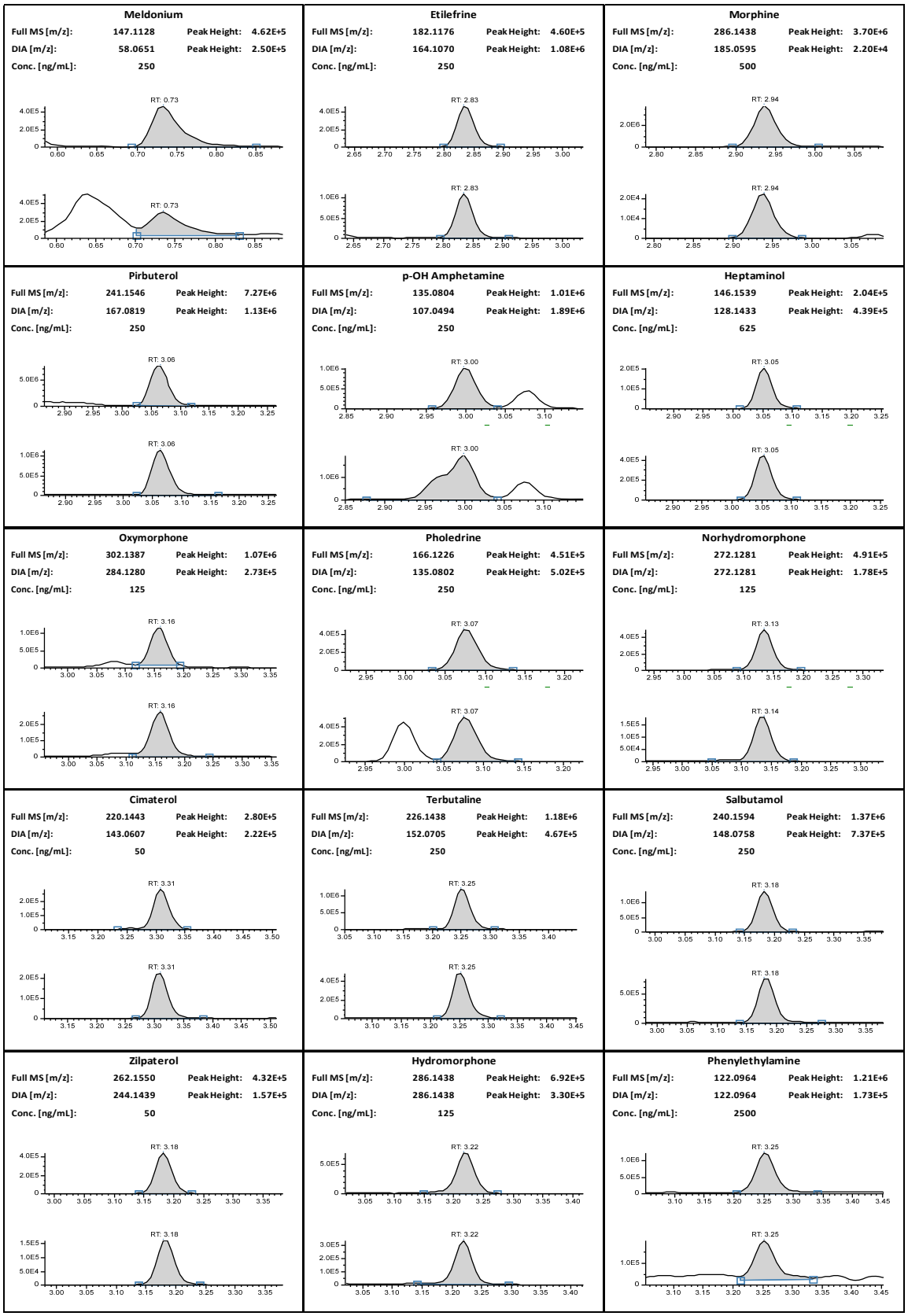
Table 4.S1 (Continued)

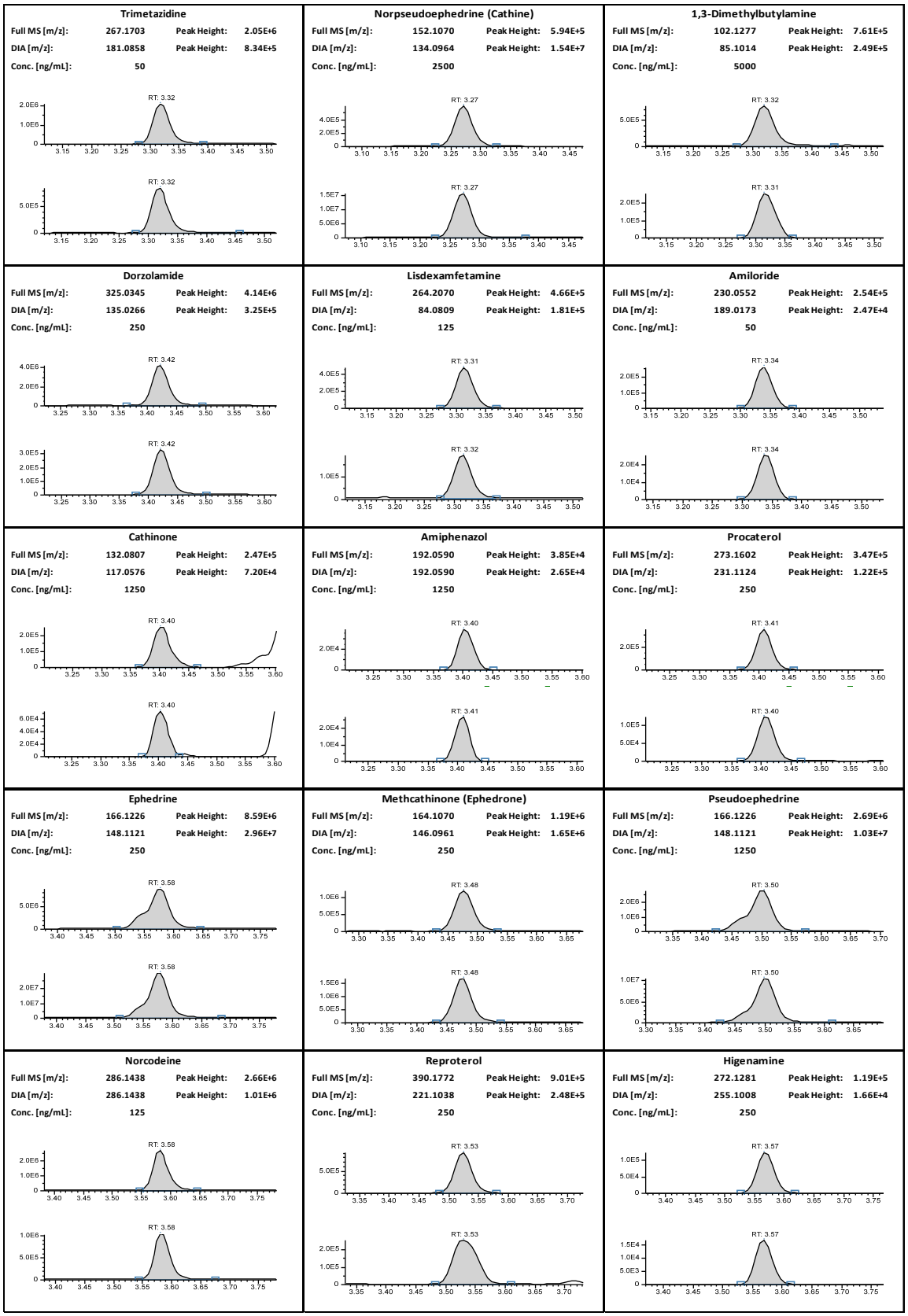
Substance	Manufacturer / Distributor	Headquarters
Methylendioxymethamphetamine (MDMA)	LGC Standards	Teddington, United Kingdom
Methylenedioxypyrovalerone (MDPV)	Cerilliant	Round Rock, Texas, United States
Methylephedrine	Clearsynth	Mumbai, India
Methylmethcathinone (Mephedron)	LGC Standards	Teddington, United Kingdom
Methylnaltroxen	LGC Standards	Teddington, United Kingdom
Methylphenethylamine	Sigma-Aldrich	St. Louis, Missouri, United States
Methylphenidate	LGC Standards	Teddington, United Kingdom
Methylprednisolone	Sigma-Aldrich	St. Louis, Missouri, United States
Methyltestosterone	Sigma-Aldrich	St. Louis, Missouri, United States
Metolazone	European Directorate for the Quality of Medicines & HealthCare	Straßburg, France
Metoprolol	LGC Standards	Teddington, United Kingdom
Miconazole	LGC Standards	Teddington, United Kingdom
Modafinilic Acid	LGC Standards	Teddington, United Kingdom
Morphine	Cerilliant	Round Rock, Texas, United States
Mozavaptane	Sigma-Aldrich	St. Louis, Missouri, United States
Nandrolone propionate	Cayman Chemical Company	Ann Arbor, Michigan, United States
N-ethyl-1-phenyl-2-butanamine	NMI Natural and Medical Sciences Institute	Reutlingen, Germany
Nikethamide	Toronto Research Chemicals	Toronto, Canada
Norcodeine	LGC Standards	Teddington, United Kingdom
Norfenfluramine	LGC Standards	Teddington, United Kingdom
Norfentanyl	LGC Standards	Teddington, United Kingdom
Norhydrocodone	Cerilliant	Round Rock, Texas, United States
Norhydromorphone	Cerilliant	Round Rock, Texas, United States
Norpethidine	LGC Standards	Teddington, United Kingdom
Norpseudoephedrine (Cathine)	Lipomed AG	Arlesheim, Switzerland
Olodaterol	LGC Standards	Teddington, United Kingdom
Opipramol	Sigma-Aldrich	St. Louis, Missouri, United States
Oxycodone	LGC Standards	Teddington, United Kingdom
Oxymorphone	LGC Standards	Teddington, United Kingdom
Pemoline	Toronto Research Chemicals	Toronto, Canada
Pentazocine	Cerilliant	Round Rock, Texas, United States
Pentetrazole	LGC Standards	Teddington, United Kingdom
Pethidine	LGC Standards	Teddington, United Kingdom
Phendimetrazine	LGC Standards	Teddington, United Kingdom
Phenylethylamine, 2-	Sigma-Aldrich	St. Louis, Missouri, United States
Pholedrine	LGC Standards	Teddington, United Kingdom
p-Hydroxyprenylamine	In-house synthesis	German Sport University, Cologne, Germany
Pipradrol	LGC Standards	Teddington, United Kingdom
Pirbuterol	LGC Standards	Teddington, United Kingdom
Piretanide	LGC Standards	Teddington, United Kingdom
p-OH Amphetamine	Sigma-Aldrich	St. Louis, Missouri, United States
Polythiazide	Pfizer	New York City, New York, United States
Prednisolone	Sigma-Aldrich	St. Louis, Missouri, United States
Prednisone	Sigma-Aldrich	St. Louis, Missouri, United States
Prenylamine	Toronto Research Chemicals	Toronto, Canada
Probenecide	LGC Standards	Teddington, United Kingdom
Procaterol	Sigma-Aldrich	St. Louis, Missouri, United States
Prolintane	LGC Standards	Teddington, United Kingdom
Propranolol	Sigma-Aldrich	St. Louis, Missouri, United States
Propylamphetamine	Cayman Chemical Company	Ann Arbor, Michigan, United States
Propylhexedrine	LGC Standards	Teddington, United Kingdom
Pseudoephedrine	LGC Standards	Teddington, United Kingdom

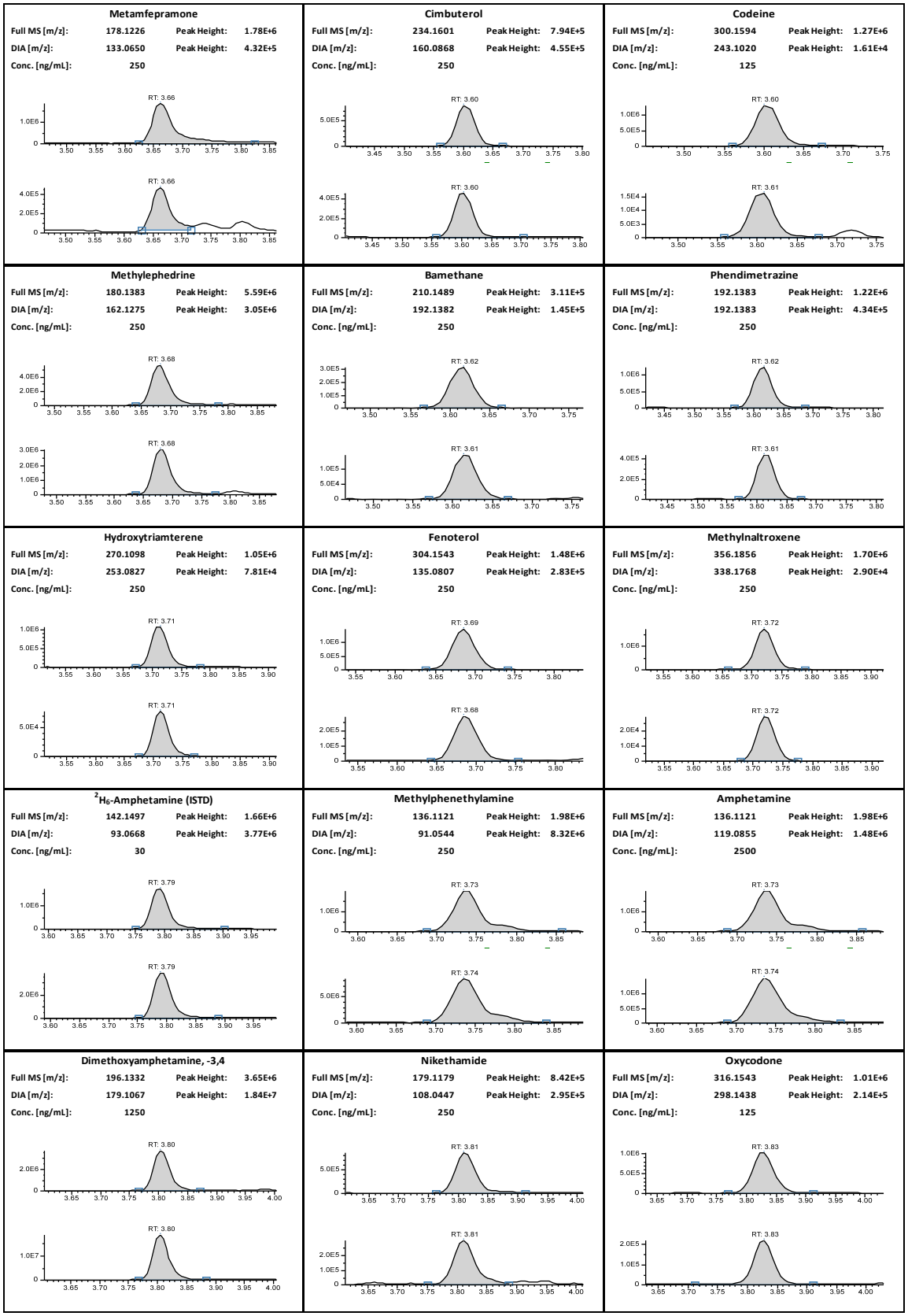
Table 4.S1 (Continued)

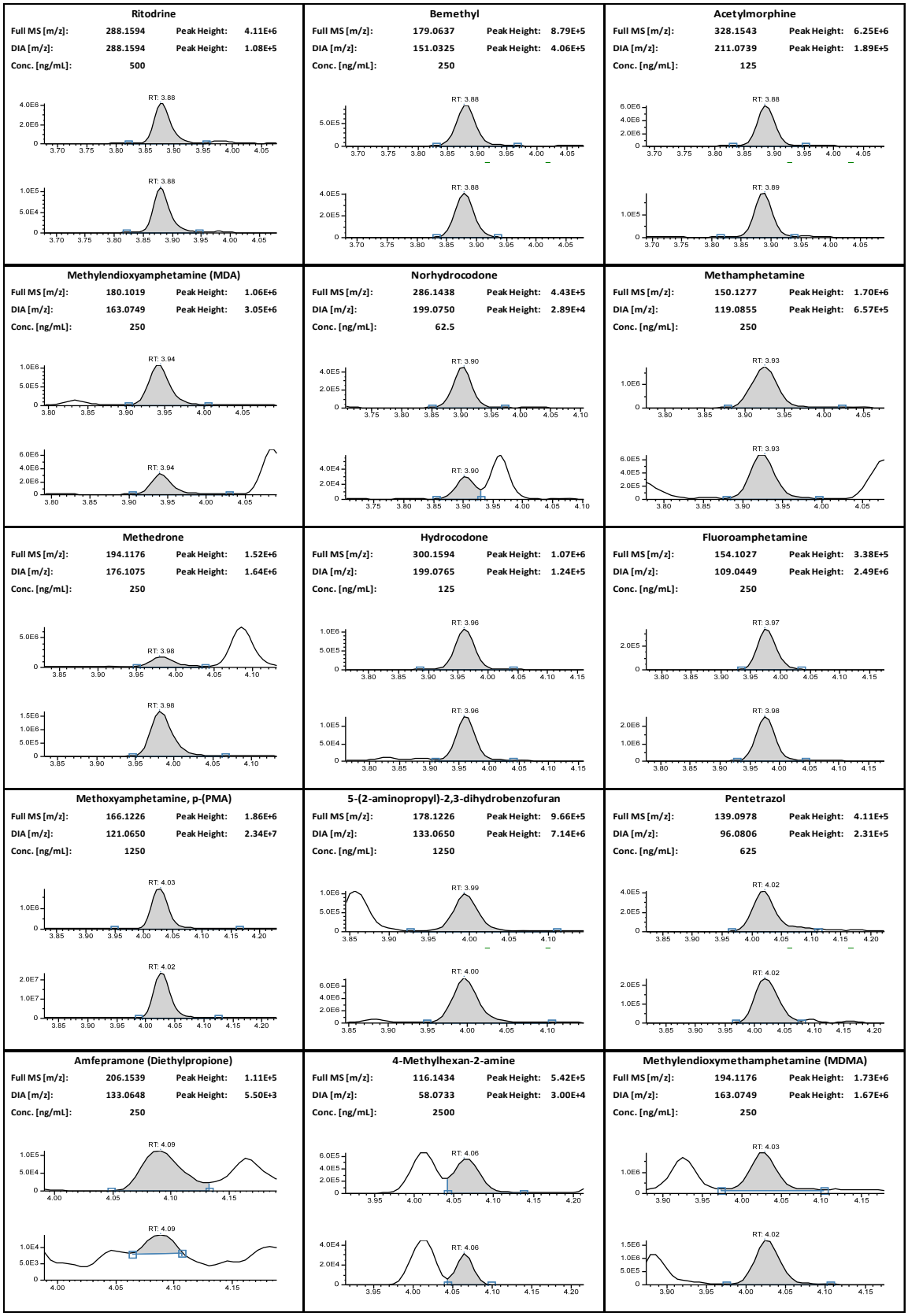
Substance	Manufacturer / Distributor	Headquarters
Pyrrolidinovalerophenone, alpha-(α-PVP)	Cerilliant	Round Rock, Texas, United States
Quinethazone	LGC Standards	Teddington, United Kingdom
Racemoramide	NMI Natural and Medical Sciences Institute	Reutlingen, Germany
Ractopamine	LGC Standards	Teddington, United Kingdom
Relcovaptane	Sigma-Aldrich	St. Louis, Missouri, United States
Remifentanil	Cayman Chemical Company	Ann Arbor, Michigan, United States
Reproterol	Toronto Research Chemicals	Toronto, Canada
Ritalinic Acid	Sigma-Aldrich	St. Louis, Missouri, United States
Ritodrine	MERCK	Darmstadt, Germany
Roxadustat	LGC Standards	Teddington, United Kingdom
S-22 (Ostarine)	In-house synthesis	German Sport University, Cologne, Germany
S-23	In-house synthesis	German Sport University, Cologne, Germany
S-24	In-house synthesis	German Sport University, Cologne, Germany
S-4 (Andarine)	In-house synthesis	German Sport University, Cologne, Germany
Salbutamol	NMI Natural and Medical Sciences Institute	Reutlingen, Germany
Salmeterol	LGC Standards	Teddington, United Kingdom
Selegiline	LGC Standards	Teddington, United Kingdom
Sibutramine	LGC Standards	Teddington, United Kingdom
Stanozolol	LGC Standards	Teddington, United Kingdom
Strychnine	LGC Standards	Teddington, United Kingdom
Sufentanyl	Cerilliant	Round Rock, Texas, United States
Terbutaline	LGC Standards	Teddington, United Kingdom
Testosterone	NMI Natural and Medical Sciences Institute	Reutlingen, Germany
Testosterone acetate	LGC Standards	Teddington, United Kingdom
Testosterone propionate	LGC Standards	Teddington, United Kingdom
Tolvaptane	Toronto Research Chemicals	Toronto, Canada
Tramadol	LGC Standards	Teddington, United Kingdom
Trenbolone	LGC Standards	Teddington, United Kingdom
Trenbolone enanthate	LGC Standards	Teddington, United Kingdom
Tretoquinol	Toronto Research Chemicals	Toronto, Canada
Triamcinolone	Sigma-Aldrich	St. Louis, Missouri, United States
Triamcinolone acetonide	Sigma-Aldrich	St. Louis, Missouri, United States
Trichlomethiazide	MERCK	Darmstadt, Germany
Trimetazidine	LGC Standards	Teddington, United Kingdom
Tuaminoheptane	LGC Standards	Teddington, United Kingdom
Tulobuterol	LGC Standards	Teddington, United Kingdom
Vadadustat	LGC Standards	Teddington, United Kingdom
Vilanterol	Toronto Research Chemicals	Toronto, Canada
Xipamide	Beiersdorf	Hamburg, Germany
Zilpaterol	Toronto Research Chemicals	Toronto, Canada

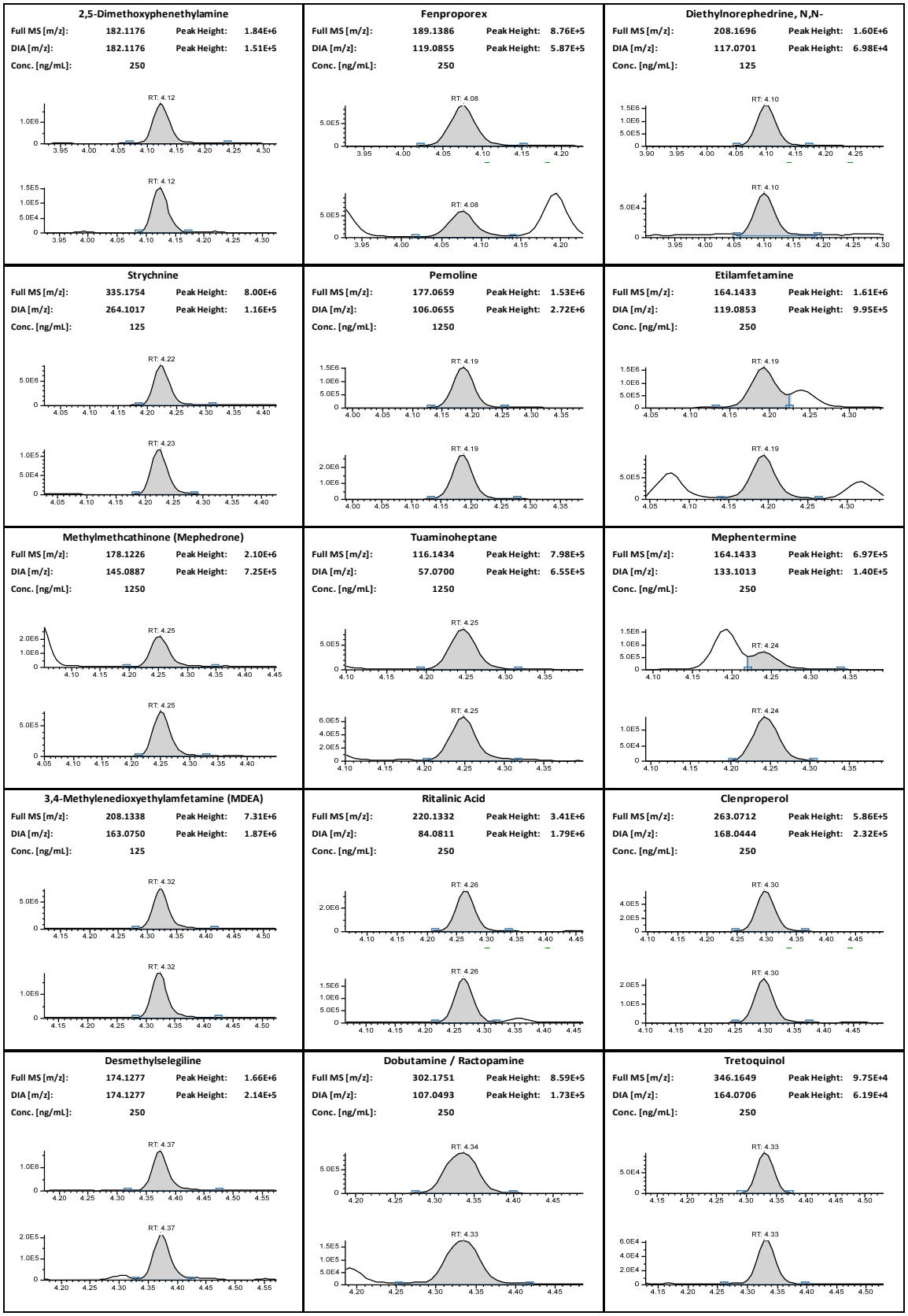
4.8.2 Figure 4.S1

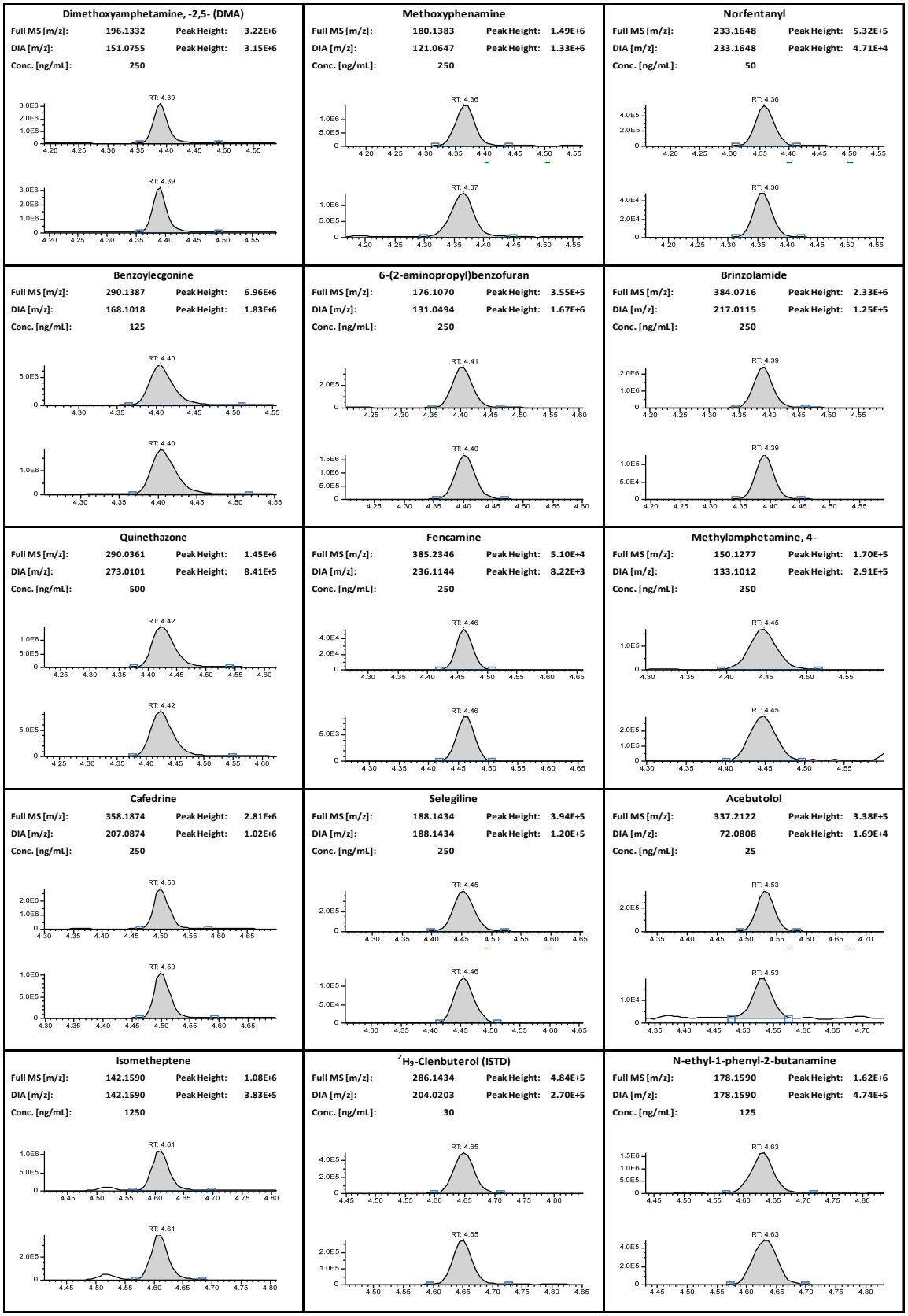


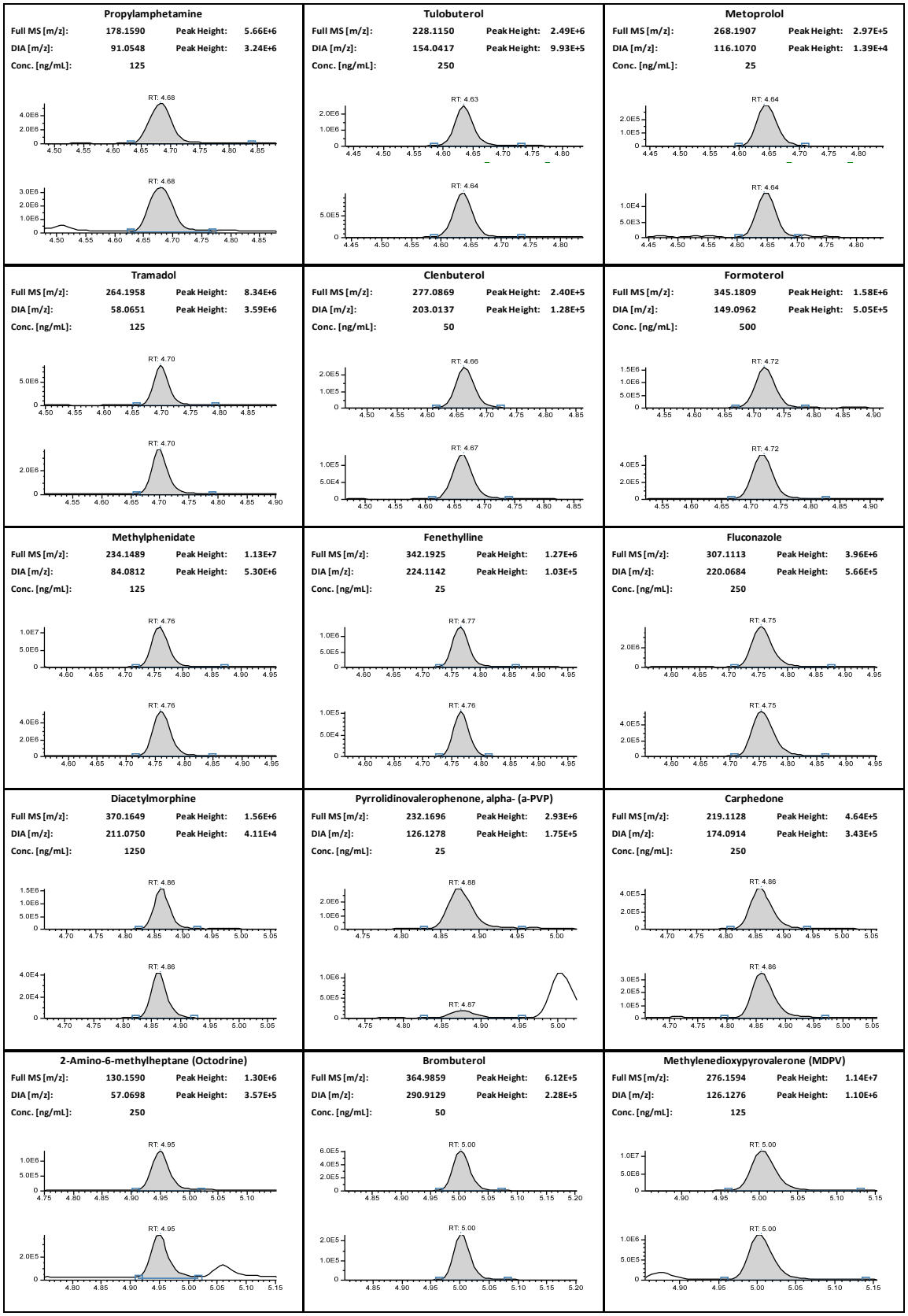

Trimetazidine
Full MS [m/z]: 267.1703 Peak Height: 2.05E+6
DIA [m/z]: 181.0858 Peak Height: 8.34E+5
Conc. [ng/mL]: 50
Norpseudoephedrine (Cathine)
Full MS [m/z]: 152.1070 Peak Height: 5.94E+5
DIA [m/z]: 134.0964 Peak Height: 1.54E+7
Conc. [ng/mL]: 2500
1,3-Dimethylbutylamine
Full MS [m/z]: 102.1277 Peak Height: 7.61E+5
DIA [m/z]: 85.1014 Peak Height: 2.49E+5
Conc. [ng/mL]: 5000
Dorzolamide
Full MS [m/z]: 325.0345 Peak Height: 4.14E+6
DIA [m/z]: 135.0266 Peak Height: 3.25E+5
Conc. [ng/mL]: 250
Lisdexamfetamine
Full MS [m/z]: 264.2070 Peak Height: 4.66E+5
DIA [m/z]: 84.0809 Peak Height: 1.81E+5
Conc. [ng/mL]: 125
Amiloride
Full MS [m/z]: 230.0552 Peak Height: 2.54E+5
DIA [m/z]: 189.0173 Peak Height: 2.47E+4
Conc. [ng/mL]: 50
Cathinone
Full MS [m/z]: 132.0807 Peak Height: 2.47E+5
DIA [m/z]: 117.0576 Peak Height: 7.20E+4
Conc. [ng/mL]: 1250
Amiphenazol
Full MS [m/z]: 192.0590 Peak Height: 3.85E+4
DIA [m/z]: 192.0590 Peak Height: 2.65E+4
Conc. [ng/mL]: 1250
Procaterol
Full MS [m/z]: 273.1602 Peak Height: 3.47E+5
DIA [m/z]: 231.1124 Peak Height: 1.22E+5
Conc. [ng/mL]: 250
Ephedrine
Full MS [m/z]: 166.1226 Peak Height: 8.59E+6
DIA [m/z]: 148.1121 Peak Height: 2.96E+7
Conc. [ng/mL]: 250
Methcathinone (Ephedrone)
Full MS [m/z]: 164.1070 Peak Height: 1.19E+6
DIA [m/z]: 146.0961 Peak Height: 1.65E+6
Conc. [ng/mL]: 250
Pseudoephedrine
Full MS [m/z]: 166.1226 Peak Height: 2.69E+6
DIA [m/z]: 148.1121 Peak Height: 1.03E+7
Conc. [ng/mL]: 1250
Norcodeine
Full MS [m/z]: 286.1438 Peak Height: 2.66E+6
DIA [m/z]: 286.1438 Peak Height: 1.01E+6
Conc. [ng/mL]: 125
Reproterol
Full MS [m/z]: 390.1772 Peak Height: 9.01E+5
DIA [m/z]: 221.1038 Peak Height: 2.48E+5
Conc. [ng/mL]: 250
Higenamine
Full MS [m/z]: 272.1281 Peak Height: 1.19E+5
DIA [m/z]: 255.1008 Peak Height: 1.66E+4
Conc. [ng/mL]: 250

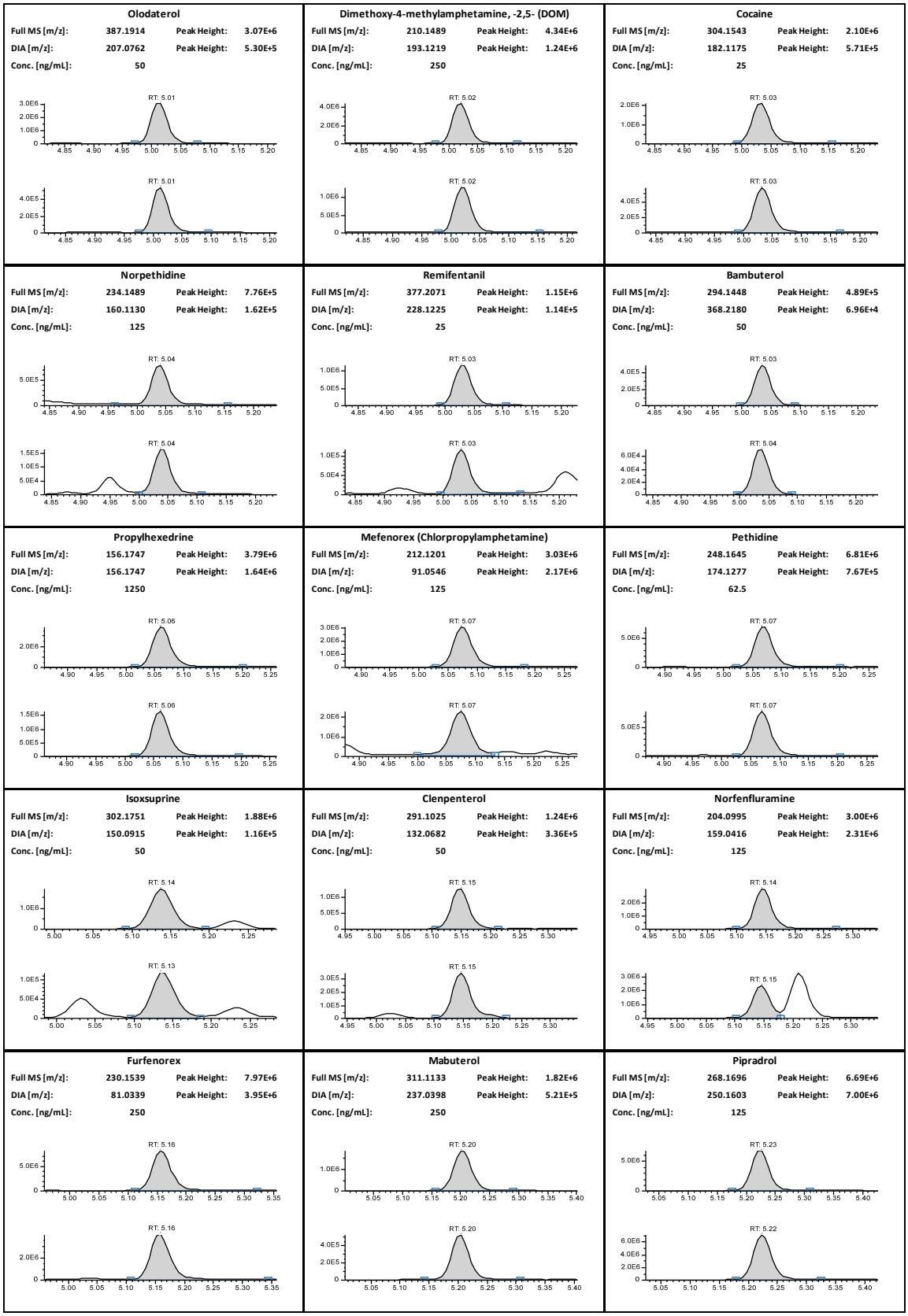

Metamfepramone
Full MS [m/z]: 178.1226 Peak Height: 1.78E+6
DIA [m/z]: 133.0650 Peak Height: 4.32E+5
Conc. [ng/mL]: 250
Cimbuterol
Full MS [m/z]: 234.1601 Peak Height: 7.94E+5
DIA [m/z]: 160.0868 Peak Height: 4.55E+5
Conc. [ng/mL]: 250
Codeine
Full MS [m/z]: 300.1594 Peak Height: 1.27E+6
DIA [m/z]: 243.1020 Peak Height: 1.61E+4
Conc. [ng/mL]: 125
Methylephedrine
Full MS [m/z]: 180.1383 Peak Height: 5.59E+6
DIA [m/z]: 162.1275 Peak Height: 3.05E+6
Conc. [ng/mL]: 250
Bamethane
Full MS [m/z]: 210.1489 Peak Height: 3.11E+5
DIA [m/z]: 192.1382 Peak Height: 1.45E+5
Conc. [ng/mL]: 250
Phendimetrazine
Full MS [m/z]: 192.1383 Peak Height: 1.22E+6
DIA [m/z]: 192.1383 Peak Height: 4.34E+5
Conc. [ng/mL]: 250
Hydroxytriamterene
Full MS [m/z]: 270.1098 Peak Height: 1.05E+6
DIA [m/z]: 253.0827 Peak Height: 7.81E+4
Conc. [ng/mL]: 250
Fenoterol
Full MS [m/z]: 304.1543 Peak Height: 1.48E+6
DIA [m/z]: 135.0807 Peak Height: 2.83E+5
Conc. [ng/mL]: 250
Methylnaltroxene
Full MS [m/z]: 356.1856 Peak Height: 1.70E+6
DIA [m/z]: 338.1768 Peak Height: 2.90E+4
Conc. [ng/mL]: 250
$^{2}H_{6}$-Amphetamine (ISTD)
Full MS [m/z]: 142.1497 Peak Height: 1.66E+6
DIA [m/z]: 93.0668 Peak Height: 3.77E+6
Conc. [ng/mL]: 30
Methylphenethylamine
Full MS [m/z]: 136.1121 Peak Height: 1.98E+6
DIA [m/z]: 91.0544 Peak Height: 8.32E+6
Conc. [ng/mL]: 250
Amphetamine
Full MS [m/z]: 136.1121 Peak Height: 1.98E+6
DIA [m/z]: 119.0855 Peak Height: 1.48E+6
Conc. [ng/mL]: 2500
Dimethoxyamphetamine, -3,4
Full MS [m/z]: 196.1332 Peak Height: 3.65E+6
DIA [m/z]: 179.1067 Peak Height: 1.84E+7
Conc. [ng/mL]: 1250
Nikethamide
Full MS [m/z]: 179.1179 Peak Height: 8.42E+5
DIA [m/z]: 108.0447 Peak Height: 2.95E+5
Conc. [ng/mL]: 250
Oxycodone
Full MS [m/z]: 316.1543 Peak Height: 1.01E+6
DIA [m/z]: 298.1438 Peak Height: 2.14E+5
Conc. [ng/mL]: 125

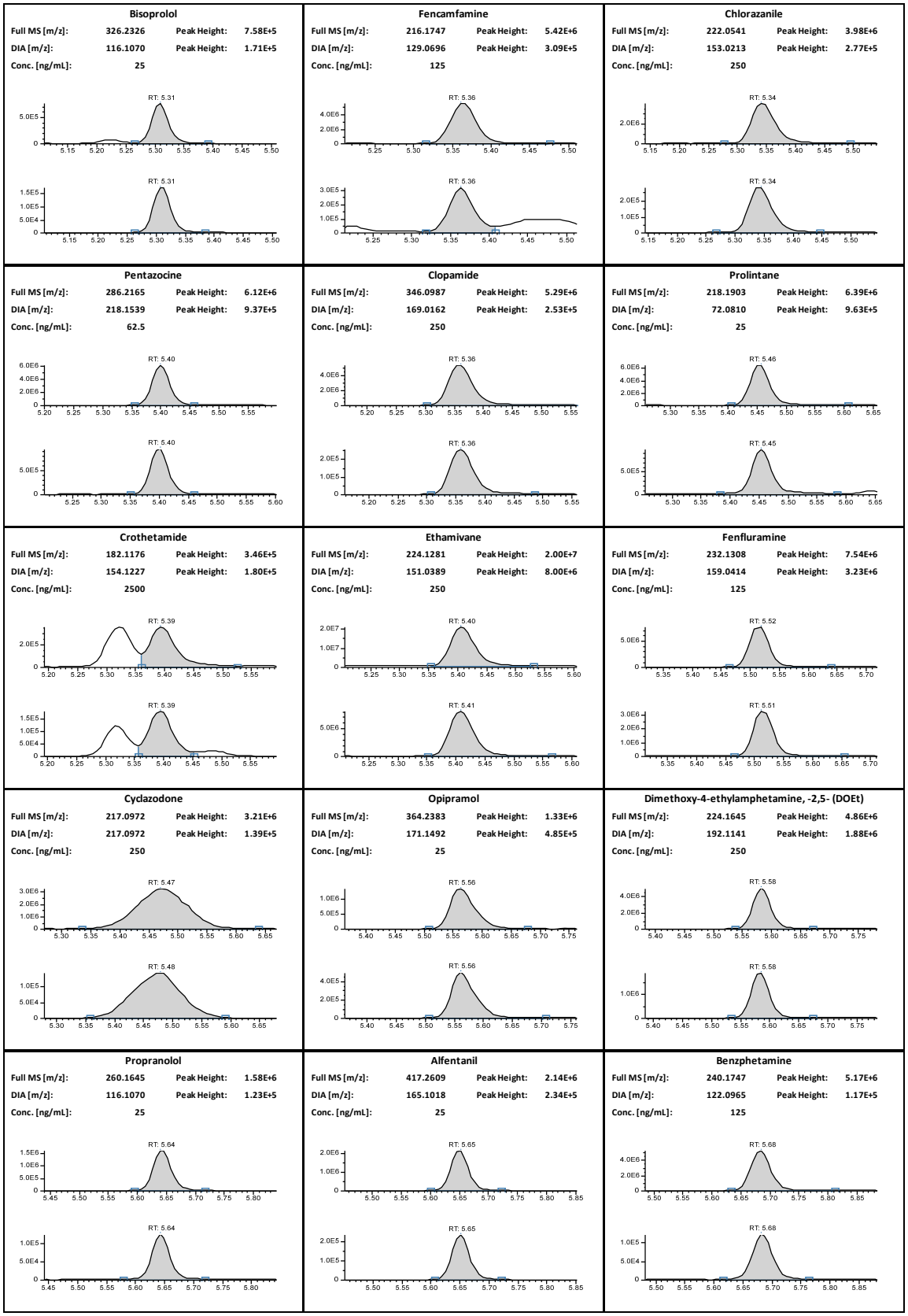

Ritodrine
Full MS [m/z]: 288.1594 Peak Height: 4.11E+6
DIA [m/z]: 288.1594 Peak Height: 1.08E+5
Conc. [ng/mL]: 500
Bemethyl
Full MS [m/z]: 179.0637 Peak Height: 8.79E+5
DIA [m/z]: 151.0325 Peak Height: 4.06E+5
Conc. [ng/mL]: 250
Acetylmorphine
Full MS [m/z]: 328.1543 Peak Height: 6.25E+6
DIA [m/z]: 211.0739 Peak Height: 1.89E+5
Conc. [ng/mL]: 125
Methylendioxyamphetamine (MDA)
Full MS [m/z]: 180.1019 Peak Height: 1.06E+6
DIA [m/z]: 163.0749 Peak Height: 3.05E+6
Conc. [ng/mL]: 250
Norhydrocodone
Full MS [m/z]: 286.1438 Peak Height: 4.43E+5
DIA [m/z]: 199.0750 Peak Height: 2.89E+4
Conc. [ng/mL]: 62.5
Methamphetamine
Full MS [m/z]: 150.1277 Peak Height: 1.70E+6
DIA [m/z]: 119.0855 Peak Height: 6.57E+5
Conc. [ng/mL]: 250
Methedrone
Full MS [m/z]: 194.1176 Peak Height: 1.52E+6
DIA [m/z]: 176.1075 Peak Height: 1.64E+6
Conc. [ng/mL]: 250
Hydrocodone
Full MS [m/z]: 300.1594 Peak Height: 1.07E+6
DIA [m/z]: 199.0765 Peak Height: 1.24E+5
Conc. [ng/mL]: 125
Fluoroamphetamine
Full MS [m/z]: 154.1027 Peak Height: 3.38E+5
DIA [m/z]: 109.0449 Peak Height: 2.49E+6
Conc. [ng/mL]: 250
Methoxyamphetamine, p-(PMA)
Full MS [m/z]: 166.1226 Peak Height: 1.86E+6
DIA [m/z]: 121.0650 Peak Height: 2.34E+7
Conc. [ng/mL]: 1250
5-(2-aminopropyl)-2,3-dihydrobenzofuran
Full MS [m/z]: 178.1226 Peak Height: 9.66E+5
DIA [m/z]: 133.0650 Peak Height: 7.14E+6
Conc. [ng/mL]: 1250
Pentetrazol
Full MS [m/z]: 139.0978 Peak Height: 4.11E+5
DIA [m/z]: 96.0806 Peak Height: 2.31E+5
Conc. [ng/mL]: 625
Amfepramone (Diethylpropione)
Full MS [m/z]: 206.1539 Peak Height: 1.11E+5
DIA [m/z]: 133.0648 Peak Height: 5.50E+3
Conc. [ng/mL]: 250
4-Methylhexan-2-amine
Full MS [m/z]: 116.1434 Peak Height: 5.42E+5
DIA [m/z]: 58.0733 Peak Height: 3.00E+4
Conc. [ng/mL]: 2500
Methylendioxymethamphetamine (MDMA)
Full MS [m/z]: 194.1176 Peak Height: 1.73E+6
DIA [m/z]: 163.0749 Peak Height: 1.67E+6
Conc. [ng/mL]: 250

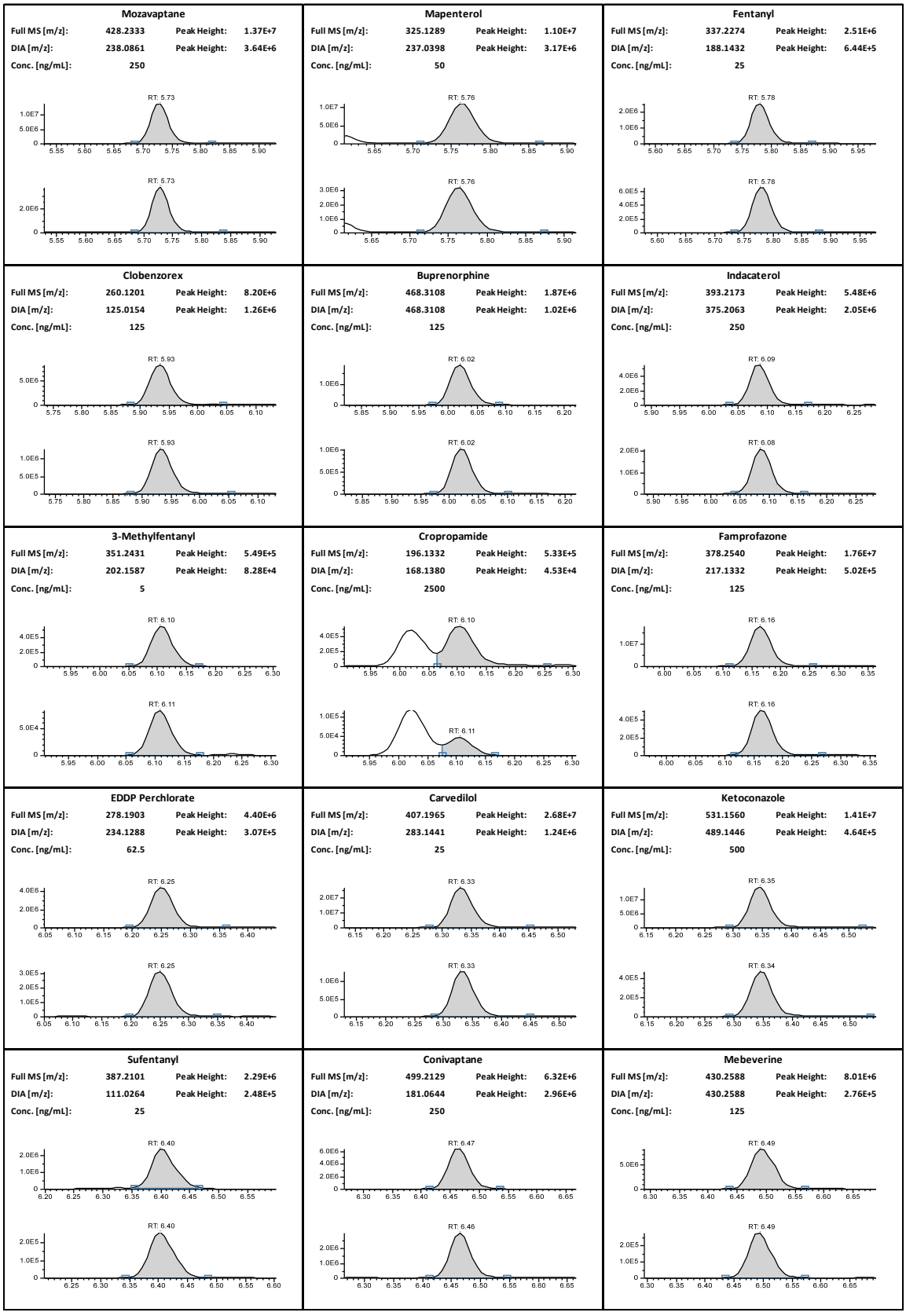

2,5-Dimethoxyphenethylamine
Full MS [m/z]: 182.1176 Peak Height: 1.84E+6
DIA [m/z]: 182.1176 Peak Height: 1.51E+5
Conc. [ng/mL]: 250
Fenproporex
Full MS [m/z]: 189.1386 Peak Height: 8.76E+5
DIA [m/z]: 119.0855 Peak Height: 5.87E+5
Conc. [ng/mL]: 250
Diethylnorephedrine, N,N-
Full MS [m/z]: 208.1696 Peak Height: 1.60E+6
DIA [m/z]: 117.0701 Peak Height: 6.98E+4
Conc. [ng/mL]: 125
Strychnine
Full MS [m/z]: 335.1754 Peak Height: 8.00E+6
DIA [m/z]: 264.1017 Peak Height: 1.16E+5
Conc. [ng/mL]: 125
Pemoline
Full MS [m/z]: 177.0659 Peak Height: 1.53E+6
DIA [m/z]: 106.0655 Peak Height: 2.72E+6
Conc. [ng/mL]: 1250
Etilamfetamine
Full MS [m/z]: 164.1433 Peak Height: 1.61E+6
DIA [m/z]: 119.0853 Peak Height: 9.95E+5
Conc. [ng/mL]: 250
Methylmethcathinone (Mephedrone)
Full MS [m/z]: 178.1226 Peak Height: 2.10E+6
DIA [m/z]: 145.0887 Peak Height: 7.25E+5
Conc. [ng/mL]: 1250
Tuaminoheptane
Full MS [m/z]: 116.1434 Peak Height: 7.98E+5
DIA [m/z]: 57.0700 Peak Height: 6.55E+5
Conc. [ng/mL]: 1250
Mephentermine
Full MS [m/z]: 164.1433 Peak Height: 6.97E+5
DIA [m/z]: 133.1013 Peak Height: 1.40E+5
Conc. [ng/mL]: 250
3,4-Methylenedioxyethylamfetamine (MDEA)
Full MS [m/z]: 208.1338 Peak Height: 7.31E+6
DIA [m/z]: 163.0750 Peak Height: 1.87E+6
Conc. [ng/mL]: 125
Ritalinic Acid
Full MS [m/z]: 220.1332 Peak Height: 3.41E+6
DIA [m/z]: 84.0811 Peak Height: 1.79E+6
Conc. [ng/mL]: 250
Clenproperol
Full MS [m/z]: 263.0712 Peak Height: 5.86E+5
DIA [m/z]: 168.0444 Peak Height: 2.32E+5
Conc. [ng/mL]: 250
Desmethylselegiline
Full MS [m/z]: 174.1277 Peak Height: 1.66E+6
DIA [m/z]: 174.1277 Peak Height: 2.14E+5
Conc. [ng/mL]: 250
Dobutamine / Ractopamine
Full MS [m/z]: 302.1751 Peak Height: 8.59E+5
DIA [m/z]: 107.0493 Peak Height: 1.73E+5
Conc. [ng/mL]: 250
Tretoquinol
Full MS [m/z]: 346.1649 Peak Height: 9.75E+4
DIA [m/z]: 164.0706 Peak Height: 6.19E+4
Conc. [ng/mL]: 250

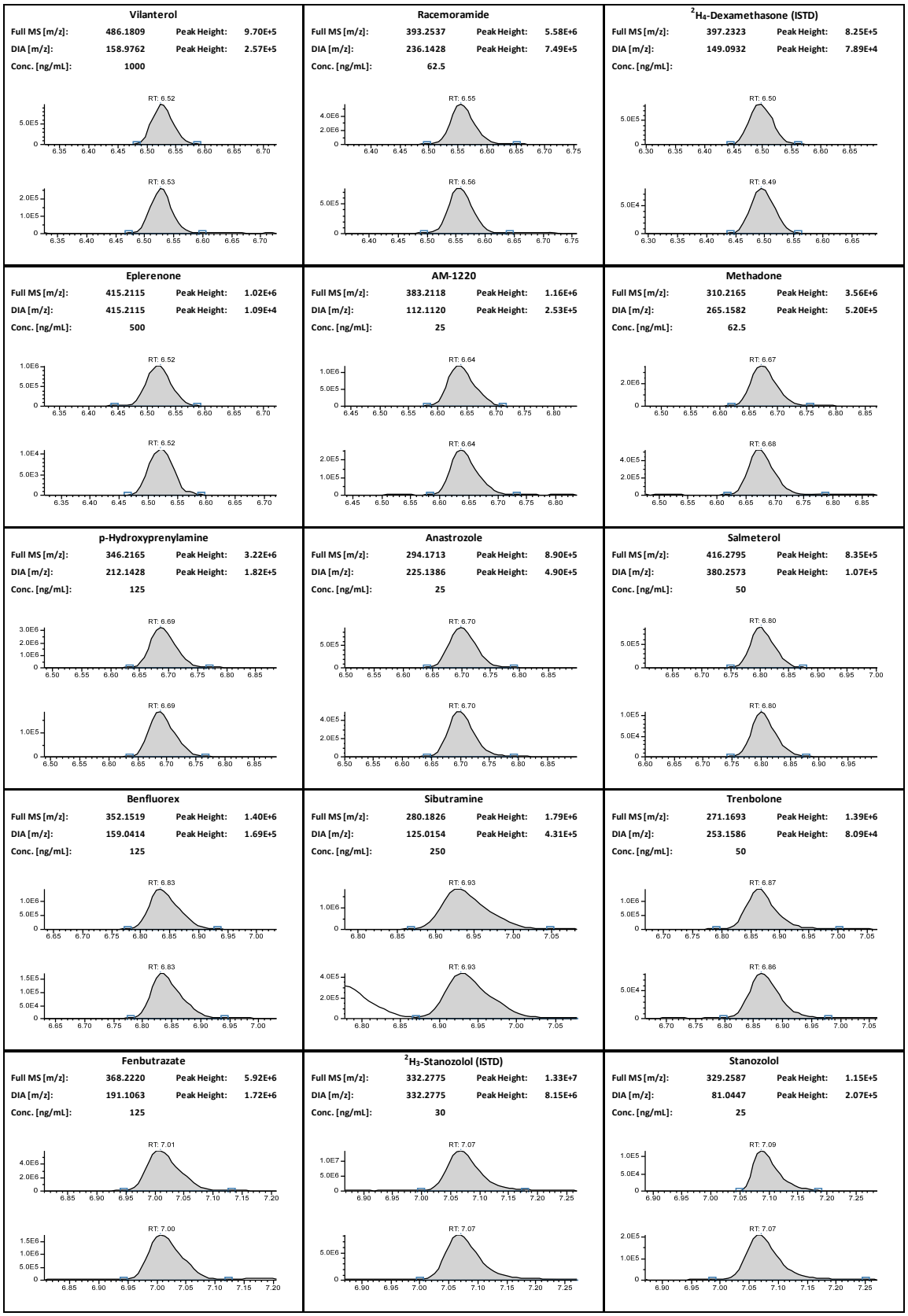

Dimethoxyamphetamine, -2,5- (DMA)
Full MS [m/z]: 196.1332 Peak Height: 3.22E+6
DIA [m/z]: 151.0755 Peak Height: 3.15E+6
Conc. [ng/mL]: 250
Methoxyphenamine
Full MS [m/z]: 180.1383 Peak Height: 1.49E+6
DIA [m/z]: 121.0647 Peak Height: 1.33E+6
Conc. [ng/mL]: 250
Norfentanyl
Full MS [m/z]: 233.1648 Peak Height: 5.32E+5
DIA [m/z]: 233.1648 Peak Height: 4.71E+4
Conc. [ng/mL]: 50
Benzoylecgonine
Full MS [m/z]: 290.1387 Peak Height: 6.96E+6
DIA [m/z]: 168.1018 Peak Height: 1.83E+6
Conc. [ng/mL]: 125
6-(2-aminopropyl)benzofuran
Full MS [m/z]: 176.1070 Peak Height: 3.55E+5
DIA [m/z]: 131.0494 Peak Height: 1.67E+6
Conc. [ng/mL]: 250
Brinzolamide
Full MS [m/z]: 384.0716 Peak Height: 2.33E+6
DIA [m/z]: 217.0115 Peak Height: 1.25E+5
Conc. [ng/mL]: 250
Quinethazone
Full MS [m/z]: 290.0361 Peak Height: 1.45E+6
DIA [m/z]: 273.0101 Peak Height: 8.41E+5
Conc. [ng/mL]: 500
Fencamine
Full MS [m/z]: 385.2346 Peak Height: 5.10E+4
DIA [m/z]: 236.1144 Peak Height: 8.22E+3
Conc. [ng/mL]: 250
Methylamphetamine, 4-
Full MS [m/z]: 150.1277 Peak Height: 1.70E+5
DIA [m/z]: 133.1012 Peak Height: 2.91E+5
Conc. [ng/mL]: 250
Cafedrine
Full MS [m/z]: 358.1874 Peak Height: 2.81E+6
DIA [m/z]: 207.0874 Peak Height: 1.02E+6
Conc. [ng/mL]: 250
Selegiline
Full MS [m/z]: 188.1434 Peak Height: 3.94E+5
DIA [m/z]: 188.1434 Peak Height: 1.20E+5
Conc. [ng/mL]: 250
Acebutolol
Full MS [m/z]: 337.2122 Peak Height: 3.38E+5
DIA [m/z]: 72.0808 Peak Height: 1.69E+4
Conc. [ng/mL]: 25
Isometheptene
Full MS [m/z]: 142.1590 Peak Height: 1.08E+6
DIA [m/z]: 142.1590 Peak Height: 3.83E+5
Conc. [ng/mL]: 1250
$^{2}H_{9}$-Clenbuterol (ISTD)
Full MS [m/z]: 286.1434 Peak Height: 4.84E+5
DIA [m/z]: 204.0203 Peak Height: 2.70E+5
Conc. [ng/mL]: 30
N-ethyl-1-phenyl-2-butanamine
Full MS [m/z]: 178.1590 Peak Height: 1.62E+6
DIA [m/z]: 178.1590 Peak Height: 4.74E+5
Conc. [ng/mL]: 125

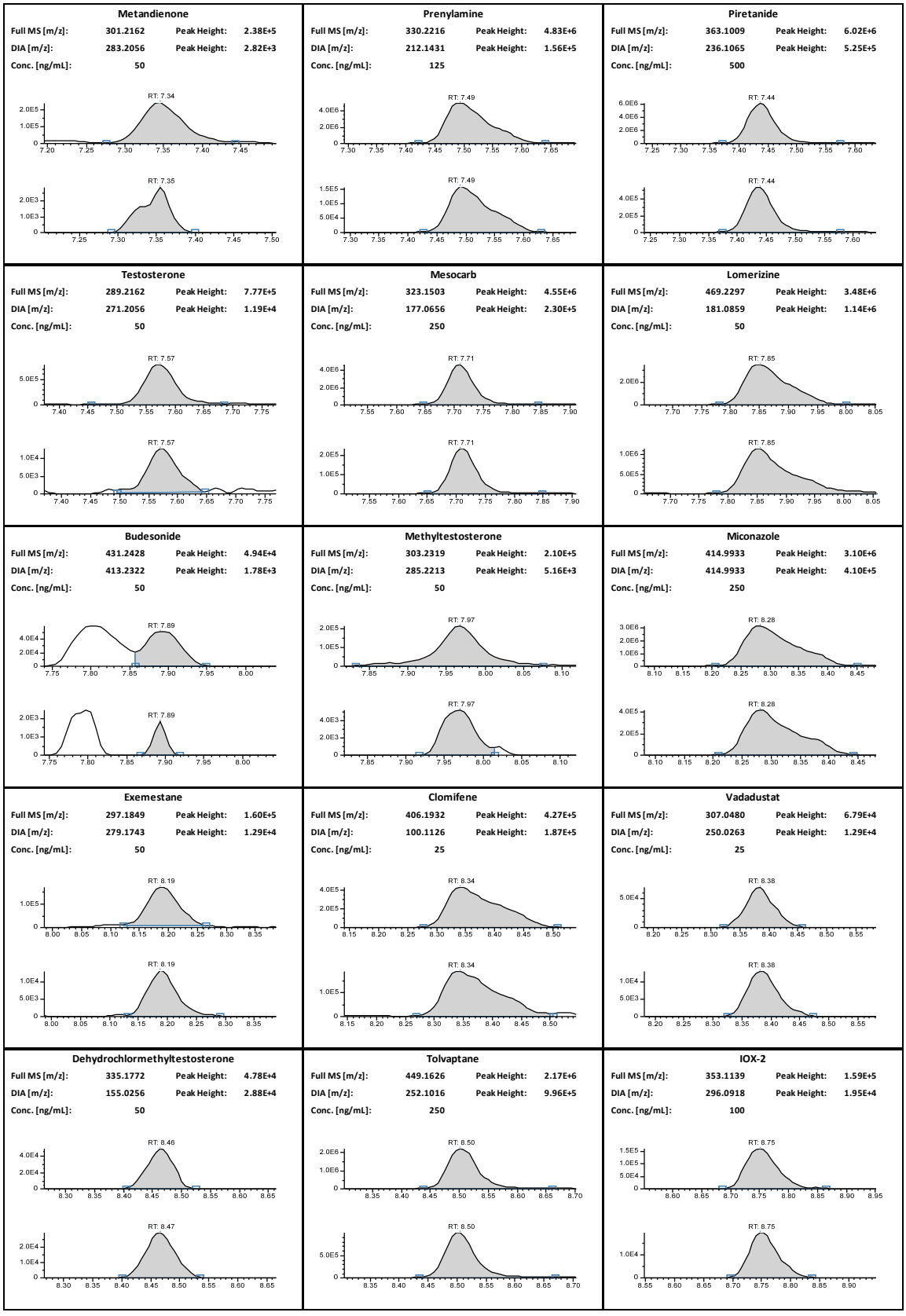

Propylamphetamine
Full MS [m/z]: 178.1590 Peak Height: 5.66E+6
DIA [m/z]: 91.0548 Peak Height: 3.24E+6
Conc. [ng/mL]: 125
Tulobuterol
Full MS [m/z]: 228.1150 Peak Height: 2.49E+6
DIA [m/z]: 154.0417 Peak Height: 9.93E+5
Conc. [ng/mL]: 250
Metoprolol
Full MS [m/z]: 268.1907 Peak Height: 2.97E+5
DIA [m/z]: 116.1070 Peak Height: 1.39E+4
Conc. [ng/mL]: 25
Tramadol
Full MS [m/z]: 264.1958 Peak Height: 8.34E+6
DIA [m/z]: 58.0651 Peak Height: 3.59E+6
Conc. [ng/mL]: 125
Clenbuterol
Full MS [m/z]: 277.0869 Peak Height: 2.40E+5
DIA [m/z]: 203.0137 Peak Height: 1.28E+5
Conc. [ng/mL]: 50
Formoterol
Full MS [m/z]: 345.1809 Peak Height: 1.58E+6
DIA [m/z]: 149.0962 Peak Height: 5.05E+5
Conc. [ng/mL]: 500
Methylphenidate
Full MS [m/z]: 234.1489 Peak Height: 1.13E+7
DIA [m/z]: 84.0812 Peak Height: 5.30E+6
Conc. [ng/mL]: 125
Fenethylline
Full MS [m/z]: 342.1925 Peak Height: 1.27E+6
DIA [m/z]: 224.1142 Peak Height: 1.03E+5
Conc. [ng/mL]: 25
Fluconazole
Full MS [m/z]: 307.1113 Peak Height: 3.96E+6
DIA [m/z]: 220.0684 Peak Height: 5.66E+5
Conc. [ng/mL]: 250
Diacetylmorphine
Full MS [m/z]: 370.1649 Peak Height: 1.56E+6
DIA [m/z]: 211.0750 Peak Height: 4.11E+4
Conc. [ng/mL]: 1250
Pyrrolidinovalerophenone, alpha- (a-PVP)
Full MS [m/z]: 232.1696 Peak Height: 2.93E+6
DIA [m/z]: 126.1278 Peak Height: 1.75E+5
Conc. [ng/mL]: 25
Carphedone
Full MS [m/z]: 219.1128 Peak Height: 4.64E+5
DIA [m/z]: 174.0914 Peak Height: 3.43E+5
Conc. [ng/mL]: 250
2-Amino-6-methylheptane (Octodrine)
Full MS [m/z]: 130.1590 Peak Height: 1.30E+6
DIA [m/z]: 57.0698 Peak Height: 3.57E+5
Conc. [ng/mL]: 250
Brombuterol
Full MS [m/z]: 364.9859 Peak Height: 6.12E+5
DIA [m/z]: 290.9129 Peak Height: 2.28E+5
Conc. [ng/mL]: 50
Methylenedioxypyrovalerone (MDPV)
Full MS [m/z]: 276.1594 Peak Height: 1.14E+7
DIA [m/z]: 126.1276 Peak Height: 1.10E+6
Conc. [ng/mL]: 125

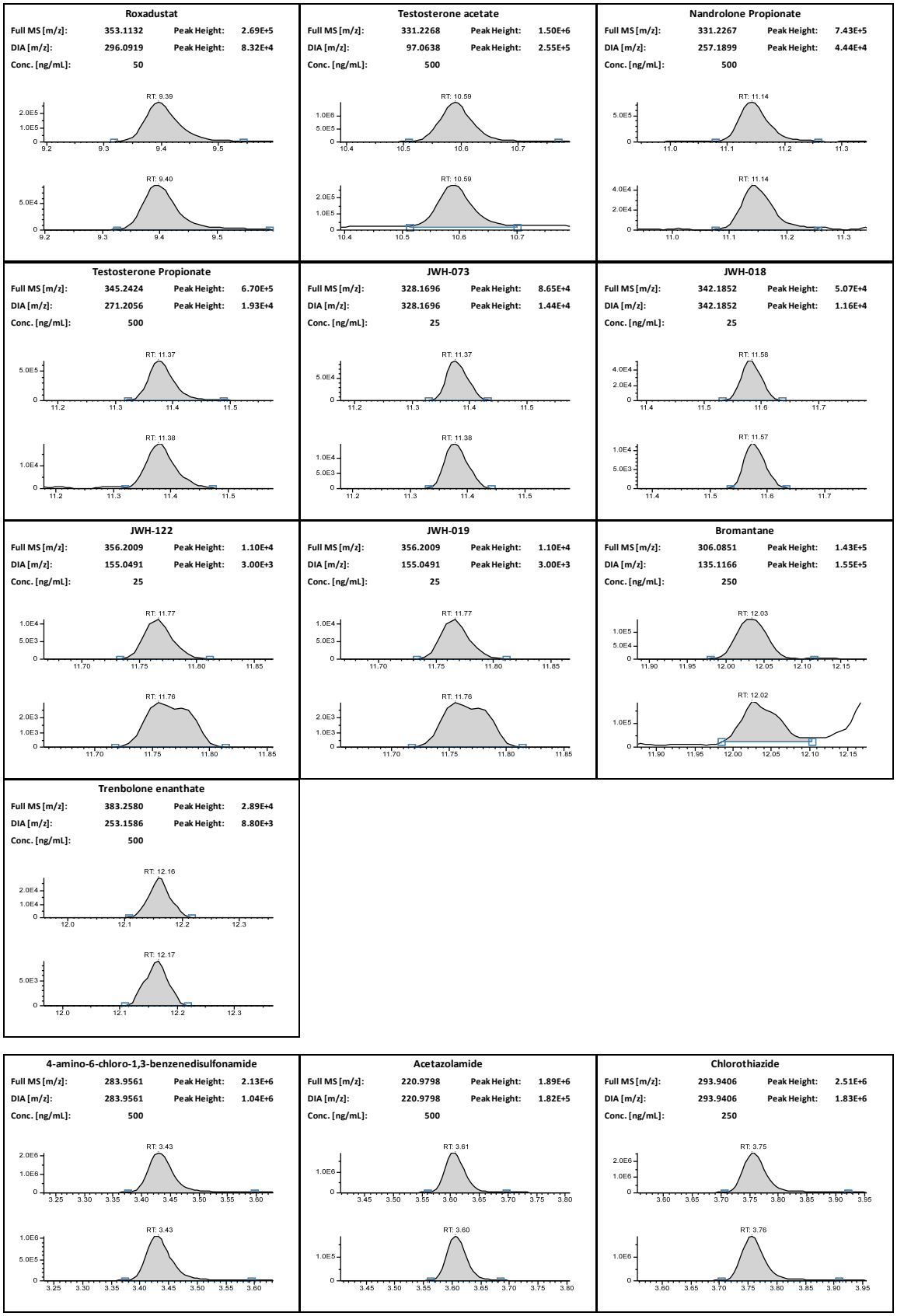

Olodaterol
Full MS [m/z]: 387.1914 Peak Height: 3.07E+6
DIA [m/z]: 207.0762 Peak Height: 5.30E+5
Conc. [ng/mL]: 50
Dimethoxy-4-methylamphetamine, -2,5- (DOM)
Full MS [m/z]: 210.1489 Peak Height: 4.34E+6
DIA [m/z]: 193.1219 Peak Height: 1.24E+6
Conc. [ng/mL]: 250
Cocaine
Full MS [m/z]: 304.1543 Peak Height: 2.10E+6
DIA [m/z]: 182.1175 Peak Height: 5.71E+5
Conc. [ng/mL]: 25
Norpethidine
Full MS [m/z]: 234.1489 Peak Height: 7.76E+5
DIA [m/z]: 160.1130 Peak Height: 1.62E+5
Conc. [ng/mL]: 125
Remifentanil
Full MS [m/z]: 377.2071 Peak Height: 1.15E+6
DIA [m/z]: 228.1225 Peak Height: 1.14E+5
Conc. [ng/mL]: 25
Bambuterol
Full MS [m/z]: 294.1448 Peak Height: 4.89E+5
DIA [m/z]: 368.2180 Peak Height: 6.96E+4
Conc. [ng/mL]: 50
Propylhexedrine
Full MS [m/z]: 156.1747 Peak Height: 3.79E+6
DIA [m/z]: 156.1747 Peak Height: 1.64E+6
Conc. [ng/mL]: 1250
Mefenorex (Chlorpropylamphetamine)
Full MS [m/z]: 212.1201 Peak Height: 3.03E+6
DIA [m/z]: 91.0546 Peak Height: 2.17E+6
Conc. [ng/mL]: 125
Pethidine
Full MS [m/z]: 248.1645 Peak Height: 6.81E+6
DIA [m/z]: 174.1277 Peak Height: 7.67E+5
Conc. [ng/mL]: 62.5
Isoxsuprine
Full MS [m/z]: 302.1751 Peak Height: 1.88E+6
DIA [m/z]: 150.0915 Peak Height: 1.16E+5
Conc. [ng/mL]: 50
Clenpenterol
Full MS [m/z]: 291.1025 Peak Height: 1.24E+6
DIA [m/z]: 132.0682 Peak Height: 3.36E+5
Conc. [ng/mL]: 50
Norfenfluramine
Full MS [m/z]: 204.0995 Peak Height: 3.00E+6
DIA [m/z]: 159.0416 Peak Height: 2.31E+6
Conc. [ng/mL]: 125
Furfenorex
Full MS [m/z]: 230.1539 Peak Height: 7.97E+6
DIA [m/z]: 81.0339 Peak Height: 3.95E+6
Conc. [ng/mL]: 250
Mabuterol
Full MS [m/z]: 311.1133 Peak Height: 1.82E+6
DIA [m/z]: 237.0398 Peak Height: 5.21E+5
Conc. [ng/mL]: 250
Pipradrol
Full MS [m/z]: 268.1696 Peak Height: 6.69E+6
DIA [m/z]: 250.1603 Peak Height: 7.00E+6
Conc. [ng/mL]: 125

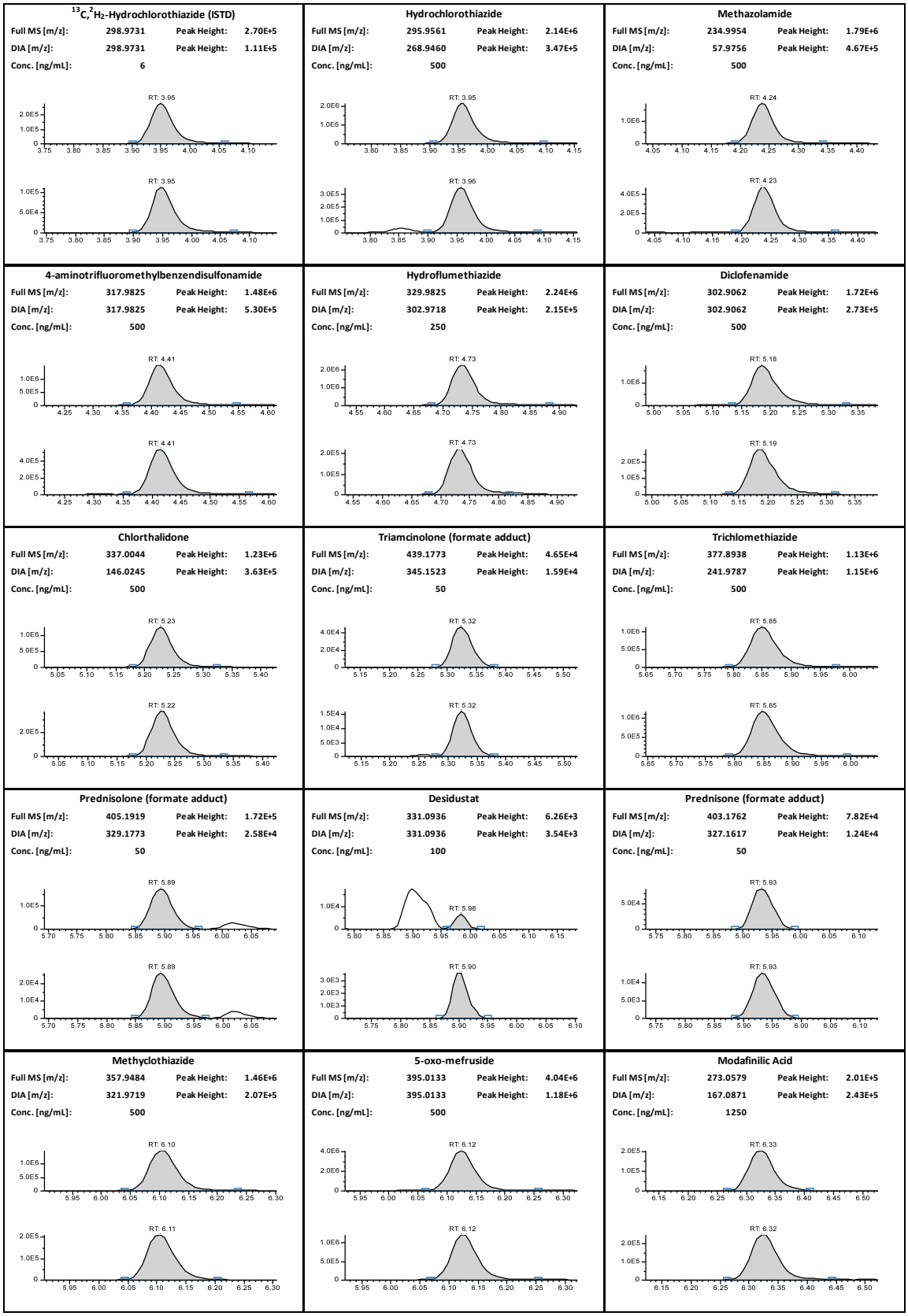

Bisoprolol
Full MS [m/z]: 326.2326 Peak Height: 7.58E+5
DIA [m/z]: 116.1070 Peak Height: 1.71E+5
Conc. [ng/mL]: 25
Fencamfamine
Full MS [m/z]: 216.1747 Peak Height: 5.42E+6
DIA [m/z]: 129.0696 Peak Height: 3.09E+5
Conc. [ng/mL]: 125
Chlorazanile
Full MS [m/z]: 222.0541 Peak Height: 3.98E+6
DIA [m/z]: 153.0213 Peak Height: 2.77E+5
Conc. [ng/mL]: 250
Pentazocine
Full MS [m/z]: 286.2165 Peak Height: 6.12E+6
DIA [m/z]: 218.1539 Peak Height: 9.37E+5
Conc. [ng/mL]: 62.5
Clopamide
Full MS [m/z]: 346.0987 Peak Height: 5.29E+6
DIA [m/z]: 169.0162 Peak Height: 2.53E+5
Conc. [ng/mL]: 250
Prolintane
Full MS [m/z]: 218.1903 Peak Height: 6.39E+6
DIA [m/z]: 72.0810 Peak Height: 9.63E+5
Conc. [ng/mL]: 25
Crothetamide
Full MS [m/z]: 182.1176 Peak Height: 3.46E+5
DIA [m/z]: 154.1227 Peak Height: 1.80E+5
Conc. [ng/mL]: 2500
Ethamivane
Full MS [m/z]: 224.1281 Peak Height: 2.00E+7
DIA [m/z]: 151.0389 Peak Height: 8.00E+6
Conc. [ng/mL]: 250
Fenfluramine
Full MS [m/z]: 232.1308 Peak Height: 7.54E+6
DIA [m/z]: 159.0414 Peak Height: 3.23E+6
Conc. [ng/mL]: 125
Cyclazodone
Full MS [m/z]: 217.0972 Peak Height: 3.21E+6
DIA [m/z]: 217.0972 Peak Height: 1.39E+5
Conc. [ng/mL]: 250
Opipramol
Full MS [m/z]: 364.2383 Peak Height: 1.33E+6
DIA [m/z]: 171.1492 Peak Height: 4.85E+5
Conc. [ng/mL]: 25
Dimethoxy-4-ethylamphetamine, -2,5- (DOEt)
Full MS [m/z]: 224.1645 Peak Height: 4.86E+6
DIA [m/z]: 192.1141 Peak Height: 1.88E+6
Conc. [ng/mL]: 250
Propranolol
Full MS [m/z]: 260.1645 Peak Height: 1.58E+6
DIA [m/z]: 116.1070 Peak Height: 1.23E+5
Conc. [ng/mL]: 25
Alfentanil
Full MS [m/z]: 417.2609 Peak Height: 2.14E+6
DIA [m/z]: 165.1018 Peak Height: 2.34E+5
Conc. [ng/mL]: 25
Benzphetamine
Full MS [m/z]: 240.1747 Peak Height: 5.17E+6
DIA [m/z]: 122.0965 Peak Height: 1.17E+5
Conc. [ng/mL]: 125

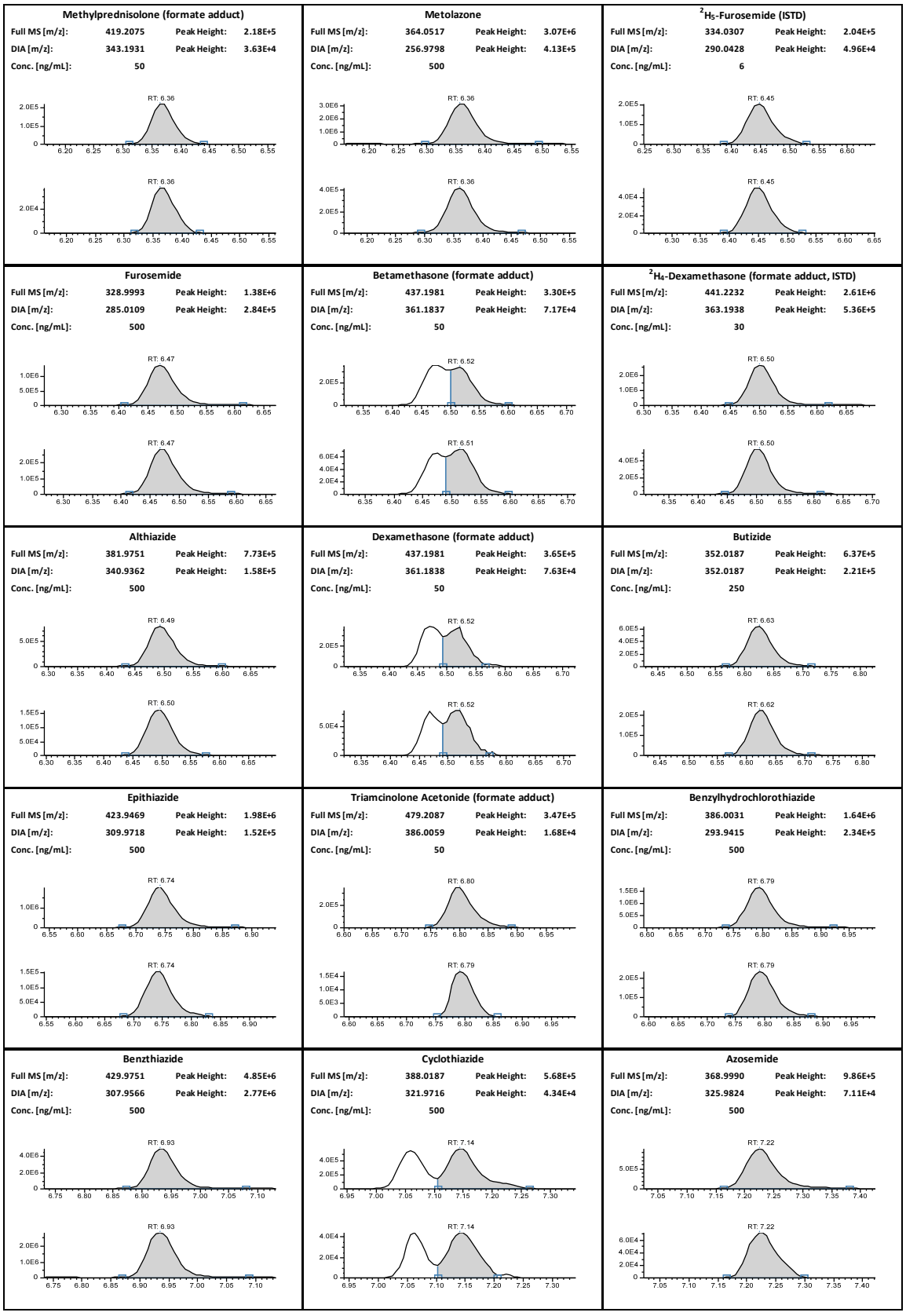

Mozavaptane
Full MS [m/z]: 428.2333 Peak Height: 1.37E+7
DIA [m/z]: 238.0861 Peak Height: 3.64E+6
Conc. [ng/mL]: 250
Mapenterol
Full MS [m/z]: 325.1289 Peak Height: 1.10E+7
DIA [m/z]: 237.0398 Peak Height: 3.17E+6
Conc. [ng/mL]: 50
Fentanyl
Full MS [m/z]: 337.2274 Peak Height: 2.51E+6
DIA [m/z]: 188.1432 Peak Height: 6.44E+5
Conc. [ng/mL]: 25
Clobenzorex
Full MS [m/z]: 260.1201 Peak Height: 8.20E+6
DIA [m/z]: 125.0154 Peak Height: 1.26E+6
Conc. [ng/mL]: 125
Buprenorphine
Full MS [m/z]: 468.3108 Peak Height: 1.87E+6
DIA [m/z]: 468.3108 Peak Height: 1.02E+6
Conc. [ng/mL]: 125
Indacaterol
Full MS [m/z]: 393.2173 Peak Height: 5.48E+6
DIA [m/z]: 375.2063 Peak Height: 2.05E+6
Conc. [ng/mL]: 250
3-Methylfentanyl
Full MS [m/z]: 351.2431 Peak Height: 5.49E+5
DIA [m/z]: 202.1587 Peak Height: 8.28E+4
Conc. [ng/mL]: 5
Cropropamide
Full MS [m/z]: 196.1332 Peak Height: 5.33E+5
DIA [m/z]: 168.1380 Peak Height: 4.53E+4
Conc. [ng/mL]: 2500
Famprofazone
Full MS [m/z]: 378.2540 Peak Height: 1.76E+7
DIA [m/z]: 217.1332 Peak Height: 5.02E+5
Conc. [ng/mL]: 125
EDDP Perchlorate
Full MS [m/z]: 278.1903 Peak Height: 4.40E+6
DIA [m/z]: 234.1288 Peak Height: 3.07E+5
Conc. [ng/mL]: 62.5
Carvedilol
Full MS [m/z]: 407.1965 Peak Height: 2.68E+7
DIA [m/z]: 283.1441 Peak Height: 1.24E+6
Conc. [ng/mL]: 25
Ketoconazole
Full MS [m/z]: 531.1560 Peak Height: 1.41E+7
DIA [m/z]: 489.1446 Peak Height: 4.64E+5
Conc. [ng/mL]: 500
Sufentanyl
Full MS [m/z]: 387.2101 Peak Height: 2.29E+6
DIA [m/z]: 111.0264 Peak Height: 2.48E+5
Conc. [ng/mL]: 25
Conivaptane
Full MS [m/z]: 499.2129 Peak Height: 6.32E+6
DIA [m/z]: 181.0644 Peak Height: 2.96E+6
Conc. [ng/mL]: 250
Mebeverine
Full MS [m/z]: 430.2588 Peak Height: 8.01E+6
DIA [m/z]: 430.2588 Peak Height: 2.76E+5
Conc. [ng/mL]: 125

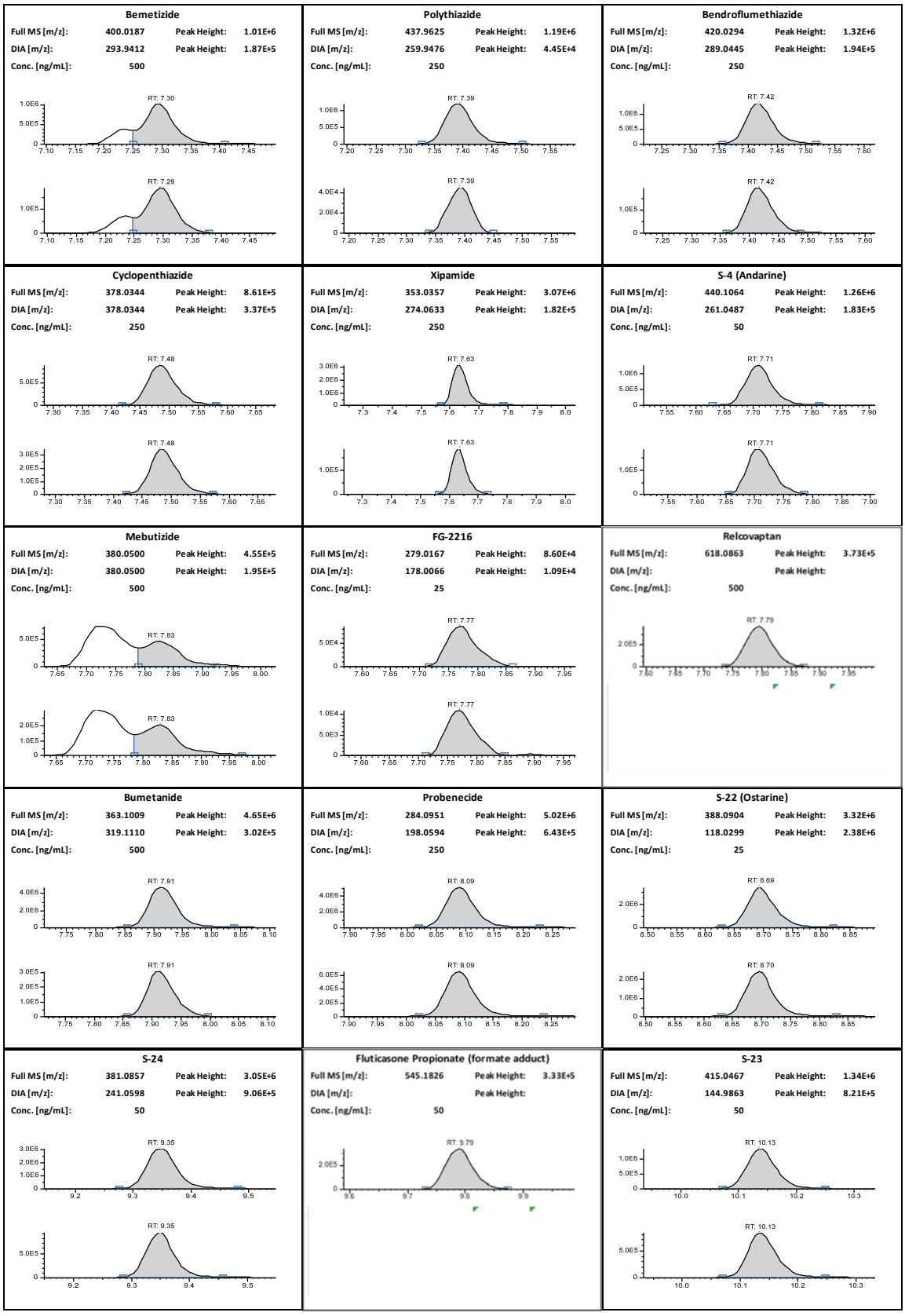

Vilanterol
Full MS [m/z]: 486.1809 Peak Height: 9.70E+5
DIA [m/z]: 158.9762 Peak Height: 2.57E+5
Conc. [ng/mL]: 1000
Racemoramide
Full MS [m/z]: 393.2537 Peak Height: 5.58E+6
DIA [m/z]: 236.1428 Peak Height: 7.49E+5
Conc. [ng/mL]: 62.5
2H4-Dexamethasone (ISTD)
Full MS [m/z]: 397.2323 Peak Height: 8.25E+5
DIA [m/z]: 149.0932 Peak Height: 7.89E+4
Conc. [ng/mL]:
Eplerenone
Full MS [m/z]: 415.2115 Peak Height: 1.02E+6
DIA [m/z]: 415.2115 Peak Height: 1.09E+4
Conc. [ng/mL]: 500
AM-1220
Full MS [m/z]: 383.2118 Peak Height: 1.16E+6
DIA [m/z]: 112.1120 Peak Height: 2.53E+5
Conc. [ng/mL]: 25
Methadone
Full MS [m/z]: 310.2165 Peak Height: 3.56E+6
DIA [m/z]: 265.1582 Peak Height: 5.20E+5
Conc. [ng/mL]: 62.5
p-Hydroxyprenylamine
Full MS [m/z]: 346.2165 Peak Height: 3.22E+6
DIA [m/z]: 212.1428 Peak Height: 1.82E+5
Conc. [ng/mL]: 125
Anastrozole
Full MS [m/z]: 294.1713 Peak Height: 8.90E+5
DIA [m/z]: 225.1386 Peak Height: 4.90E+5
Conc. [ng/mL]: 25
Salmeterol
Full MS [m/z]: 416.2795 Peak Height: 8.35E+5
DIA [m/z]: 380.2573 Peak Height: 1.07E+5
Conc. [ng/mL]: 50
Benfluorex
Full MS [m/z]: 352.1519 Peak Height: 1.40E+6
DIA [m/z]: 159.0414 Peak Height: 1.69E+5
Conc. [ng/mL]: 125
Sibutramine
Full MS [m/z]: 280.1826 Peak Height: 1.79E+6
DIA [m/z]: 125.0154 Peak Height: 4.31E+5
Conc. [ng/mL]: 250
Trenbolone
Full MS [m/z]: 271.1693 Peak Height: 1.39E+6
DIA [m/z]: 253.1586 Peak Height: 8.09E+4
Conc. [ng/mL]: 50
Fenbutrazate
Full MS [m/z]: 368.2220 Peak Height: 5.92E+6
DIA [m/z]: 191.1063 Peak Height: 1.72E+6
Conc. [ng/mL]: 125
2H3-Stanozolol (ISTD)
Full MS [m/z]: 332.2775 Peak Height: 1.33E+7
DIA [m/z]: 332.2775 Peak Height: 8.15E+6
Conc. [ng/mL]: 30
Stanozolol
Full MS [m/z]: 329.2587 Peak Height: 1.15E+5
DIA [m/z]: 81.0447 Peak Height: 2.07E+5
Conc. [ng/mL]: 25


Metandienone
Full MS [m/z]: 301.2162 Peak Height: 2.38E+5
DIA [m/z]: 283.2056 Peak Height: 2.82E+3
Conc. [ng/mL]: 50
Prenylamine
Full MS [m/z]: 330.2216 Peak Height: 4.83E+6
DIA [m/z]: 212.1431 Peak Height: 1.56E+5
Conc. [ng/mL]: 125
Piretanide
Full MS [m/z]: 363.1009 Peak Height: 6.02E+6
DIA [m/z]: 236.1065 Peak Height: 5.25E+5
Conc. [ng/mL]: 500
Testosterone
Full MS [m/z]: 289.2162 Peak Height: 7.77E+5
DIA [m/z]: 271.2056 Peak Height: 1.19E+4
Conc. [ng/mL]: 50
Mesocarb
Full MS [m/z]: 323.1503 Peak Height: 4.55E+6
DIA [m/z]: 177.0656 Peak Height: 2.30E+5
Conc. [ng/mL]: 250
Lomerizine
Full MS [m/z]: 469.2297 Peak Height: 3.48E+6
DIA [m/z]: 181.0859 Peak Height: 1.14E+6
Conc. [ng/mL]: 50
Budesonide
Full MS [m/z]: 431.2428 Peak Height: 4.94E+4
DIA [m/z]: 413.2322 Peak Height: 1.78E+3
Conc. [ng/mL]: 50
Methyltestosterone
Full MS [m/z]: 303.2319 Peak Height: 2.10E+5
DIA [m/z]: 285.2213 Peak Height: 5.16E+3
Conc. [ng/mL]: 50
Miconazole
Full MS [m/z]: 414.9933 Peak Height: 3.10E+6
DIA [m/z]: 414.9933 Peak Height: 4.10E+5
Conc. [ng/mL]: 250
Exemestane
Full MS [m/z]: 297.1849 Peak Height: 1.60E+5
DIA [m/z]: 279.1743 Peak Height: 1.29E+4
Conc. [ng/mL]: 50
Clomifene
Full MS [m/z]: 406.1932 Peak Height: 4.27E+5
DIA [m/z]: 100.1126 Peak Height: 1.87E+5
Conc. [ng/mL]: 25
Vadadustat
Full MS [m/z]: 307.0480 Peak Height: 6.79E+4
DIA [m/z]: 250.0263 Peak Height: 1.29E+4
Conc. [ng/mL]: 25
Dehydrochlormethyltestosterone
Full MS [m/z]: 335.1772 Peak Height: 4.78E+4
DIA [m/z]: 155.0256 Peak Height: 2.88E+4
Conc. [ng/mL]: 50
Tolvaptane
Full MS [m/z]: 449.1626 Peak Height: 2.17E+6
DIA [m/z]: 252.1016 Peak Height: 9.96E+5
Conc. [ng/mL]: 250
IOX-2
Full MS [m/z]: 353.1139 Peak Height: 1.59E+5
DIA [m/z]: 296.0918 Peak Height: 1.95E+4
Conc. [ng/mL]: 100


Roxadustat
Full MS [m/z]: 353.1132 Peak Height: 2.69E+5
DIA [m/z]: 296.0919 Peak Height: 8.32E+4
Conc. [ng/mL]: 50
RT: 9.39
RT: 9.40
Testosterone acetate
Full MS [m/z]: 331.2268 Peak Height: 1.50E+6
DIA [m/z]: 97.0638 Peak Height: 2.55E+5
Conc. [ng/mL]: 500
RT: 10.59
RT: 10.59
Nandrolone Propionate
Full MS [m/z]: 331.2267 Peak Height: 7.43E+5
DIA [m/z]: 257.1899 Peak Height: 4.44E+4
Conc. [ng/mL]: 500
RT: 11.14
RT: 11.14
Testosterone Propionate
Full MS [m/z]: 345.2424 Peak Height: 6.70E+5
DIA [m/z]: 271.2056 Peak Height: 1.93E+4
Conc. [ng/mL]: 500
RT: 11.37
RT: 11.38
JWH-073
Full MS [m/z]: 328.1696 Peak Height: 8.65E+4
DIA [m/z]: 328.1696 Peak Height: 1.44E+4
Conc. [ng/mL]: 25
RT: 11.37
RT: 11.38
JWH-018
Full MS [m/z]: 342.1852 Peak Height: 5.07E+4
DIA [m/z]: 342.1852 Peak Height: 1.16E+4
Conc. [ng/mL]: 25
RT: 11.58
RT: 11.57
JWH-122
Full MS [m/z]: 356.2009 Peak Height: 1.10E+4
DIA [m/z]: 155.0491 Peak Height: 3.00E+3
Conc. [ng/mL]: 25
RT: 11.77
RT: 11.76
JWH-019
Full MS [m/z]: 356.2009 Peak Height: 1.10E+4
DIA [m/z]: 155.0491 Peak Height: 3.00E+3
Conc. [ng/mL]: 25
RT: 11.77
RT: 11.76
Bromantane
Full MS [m/z]: 306.0851 Peak Height: 1.43E+5
DIA [m/z]: 135.1166 Peak Height: 1.55E+5
Conc. [ng/mL]: 250
RT: 12.03
RT: 12.02
Trenbolone enanthate
Full MS [m/z]: 383.2580 Peak Height: 2.89E+4
DIA [m/z]: 253.1586 Peak Height: 8.80E+3
Conc. [ng/mL]: 500
RT: 12.16
RT: 12.17
4-amino-6-chloro-1,3-benzenedisulfonamide
Full MS [m/z]: 283.9561 Peak Height: 2.13E+6
DIA [m/z]: 283.9561 Peak Height: 1.04E+6
Conc. [ng/mL]: 500
RT: 3.43
RT: 3.43
Acetazolamide
Full MS [m/z]: 220.9798 Peak Height: 1.89E+6
DIA [m/z]: 220.9798 Peak Height: 1.82E+5
Conc. [ng/mL]: 500
RT: 3.61
RT: 3.60
Chlorothiazide
Full MS [m/z]: 293.9406 Peak Height: 2.51E+6
DIA [m/z]: 293.9406 Peak Height: 1.83E+6
Conc. [ng/mL]: 250
RT: 3.75
RT: 3.76


$^{13}C,^{2}H_2$-Hydrochlorothiazide (ISTD)
Full MS [m/z]: 298.9731 Peak Height: 2.70E+5
DIA [m/z]: 298.9731 Peak Height: 1.11E+5
Conc. [ng/mL]: 6
Hydrochlorothiazide
Full MS [m/z]: 295.9561 Peak Height: 2.14E+6
DIA [m/z]: 268.9460 Peak Height: 3.47E+5
Conc. [ng/mL]: 500
Methazolamide
Full MS [m/z]: 234.9954 Peak Height: 1.79E+6
DIA [m/z]: 57.9756 Peak Height: 4.67E+5
Conc. [ng/mL]: 500
4-aminotrifluoromethylbenzendisulfonamide
Full MS [m/z]: 317.9825 Peak Height: 1.48E+6
DIA [m/z]: 317.9825 Peak Height: 5.30E+5
Conc. [ng/mL]: 500
Hydroflumethiazide
Full MS [m/z]: 329.9825 Peak Height: 2.24E+6
DIA [m/z]: 302.9718 Peak Height: 2.15E+5
Conc. [ng/mL]: 250
Diclofenamide
Full MS [m/z]: 302.9062 Peak Height: 1.72E+6
DIA [m/z]: 302.9062 Peak Height: 2.73E+5
Conc. [ng/mL]: 500
Chlorthalidone
Full MS [m/z]: 337.0044 Peak Height: 1.23E+6
DIA [m/z]: 146.0245 Peak Height: 3.63E+5
Conc. [ng/mL]: 500
Triamcinolone (formate adduct)
Full MS [m/z]: 439.1773 Peak Height: 4.65E+4
DIA [m/z]: 345.1523 Peak Height: 1.59E+4
Conc. [ng/mL]: 50
Trichlomethiazide
Full MS [m/z]: 377.8938 Peak Height: 1.13E+6
DIA [m/z]: 241.9787 Peak Height: 1.15E+6
Conc. [ng/mL]: 500
Prednisolone (formate adduct)
Full MS [m/z]: 405.1919 Peak Height: 1.72E+5
DIA [m/z]: 329.1773 Peak Height: 2.58E+4
Conc. [ng/mL]: 50
Desidustat
Full MS [m/z]: 331.0936 Peak Height: 6.26E+3
DIA [m/z]: 331.0936 Peak Height: 3.54E+3
Conc. [ng/mL]: 100
Prednisone (formate adduct)
Full MS [m/z]: 403.1762 Peak Height: 7.82E+4
DIA [m/z]: 327.1617 Peak Height: 1.24E+4
Conc. [ng/mL]: 50
Methyclothiazide
Full MS [m/z]: 357.9484 Peak Height: 1.46E+6
DIA [m/z]: 321.9719 Peak Height: 2.07E+5
Conc. [ng/mL]: 500
5-oxo-mefruside
Full MS [m/z]: 395.0133 Peak Height: 4.04E+6
DIA [m/z]: 395.0133 Peak Height: 1.18E+6
Conc. [ng/mL]: 500
Modafinilic Acid
Full MS [m/z]: 273.0579 Peak Height: 2.01E+5
DIA [m/z]: 167.0871 Peak Height: 2.43E+5
Conc. [ng/mL]: 1250


Methylprednisolone (formate adduct)
Full MS [m/z]: 419.2075 Peak Height: 2.18E+5
DIA [m/z]: 343.1931 Peak Height: 3.63E+4
Conc. [ng/mL]: 50
Metolazone
Full MS [m/z]: 364.0517 Peak Height: 3.07E+6
DIA [m/z]: 256.9798 Peak Height: 4.13E+5
Conc. [ng/mL]: 500
$^{2}H_5$-Furosemide (ISTD)
Full MS [m/z]: 334.0307 Peak Height: 2.04E+5
DIA [m/z]: 290.0428 Peak Height: 4.96E+4
Conc. [ng/mL]: 6
Furosemide
Full MS [m/z]: 328.9993 Peak Height: 1.38E+6
DIA [m/z]: 285.0109 Peak Height: 2.84E+5
Conc. [ng/mL]: 500
Betamethasone (formate adduct)
Full MS [m/z]: 437.1981 Peak Height: 3.30E+5
DIA [m/z]: 361.1837 Peak Height: 7.17E+4
Conc. [ng/mL]: 50
$^{2}H_4$-Dexamethasone (formate adduct, ISTD)
Full MS [m/z]: 441.2232 Peak Height: 2.61E+6
DIA [m/z]: 363.1938 Peak Height: 5.36E+5
Conc. [ng/mL]: 30
Althiazide
Full MS [m/z]: 381.9751 Peak Height: 7.73E+5
DIA [m/z]: 340.9362 Peak Height: 1.58E+5
Conc. [ng/mL]: 500
Dexamethasone (formate adduct)
Full MS [m/z]: 437.1981 Peak Height: 3.65E+5
DIA [m/z]: 361.1838 Peak Height: 7.63E+4
Conc. [ng/mL]: 50
Butizide
Full MS [m/z]: 352.0187 Peak Height: 6.37E+5
DIA [m/z]: 352.0187 Peak Height: 2.21E+5
Conc. [ng/mL]: 250
Epithiazide
Full MS [m/z]: 423.9469 Peak Height: 1.98E+6
DIA [m/z]: 309.9718 Peak Height: 1.52E+5
Conc. [ng/mL]: 500
Triamcinolone Acetonide (formate adduct)
Full MS [m/z]: 479.2087 Peak Height: 3.47E+5
DIA [m/z]: 386.0059 Peak Height: 1.68E+4
Conc. [ng/mL]: 50
Benzylhydrochlorothiazide
Full MS [m/z]: 386.0031 Peak Height: 1.64E+6
DIA [m/z]: 293.9415 Peak Height: 2.34E+5
Conc. [ng/mL]: 500
Benzthiazide
Full MS [m/z]: 429.9751 Peak Height: 4.85E+6
DIA [m/z]: 307.9566 Peak Height: 2.77E+6
Conc. [ng/mL]: 500
Cyclothiazide
Full MS [m/z]: 388.0187 Peak Height: 5.68E+5
DIA [m/z]: 321.9716 Peak Height: 4.34E+4
Conc. [ng/mL]: 500
Azosemide
Full MS [m/z]: 368.9990 Peak Height: 9.86E+5
DIA [m/z]: 325.9824 Peak Height: 7.11E+4
Conc. [ng/mL]: 500


Bemetizide
Full MS [m/z]: 400.0187 Peak Height: 1.01E+6
DIA [m/z]: 293.9412 Peak Height: 1.87E+5
Conc. [ng/mL]: 500
Polythiazide
Full MS [m/z]: 437.9625 Peak Height: 1.19E+6
DIA [m/z]: 259.9476 Peak Height: 4.45E+4
Conc. [ng/mL]: 250
Bendroflumethiazide
Full MS [m/z]: 420.0294 Peak Height: 1.32E+6
DIA [m/z]: 289.0445 Peak Height: 1.94E+5
Conc. [ng/mL]: 250
Cyclopenthiazide
Full MS [m/z]: 378.0344 Peak Height: 8.61E+5
DIA [m/z]: 378.0344 Peak Height: 3.37E+5
Conc. [ng/mL]: 250
Xipamide
Full MS [m/z]: 353.0357 Peak Height: 3.07E+6
DIA [m/z]: 274.0633 Peak Height: 1.82E+5
Conc. [ng/mL]: 250
S-4 (Andarine)
Full MS [m/z]: 440.1064 Peak Height: 1.26E+6
DIA [m/z]: 261.0487 Peak Height: 1.83E+5
Conc. [ng/mL]: 50
Mebutizide
Full MS [m/z]: 380.0500 Peak Height: 4.55E+5
DIA [m/z]: 380.0500 Peak Height: 1.95E+5
Conc. [ng/mL]: 500
FG-2216
Full MS [m/z]: 279.0167 Peak Height: 8.60E+4
DIA [m/z]: 178.0066 Peak Height: 1.09E+4
Conc. [ng/mL]: 25
Relcovaptan
Full MS [m/z]: 618.0863 Peak Height: 3.73E+5
DIA [m/z]: Peak Height:
Conc. [ng/mL]: 500
Bumetanide
Full MS [m/z]: 363.1009 Peak Height: 4.65E+6
DIA [m/z]: 319.1110 Peak Height: 3.02E+5
Conc. [ng/mL]: 500
Probenecide
Full MS [m/z]: 284.0951 Peak Height: 5.02E+6
DIA [m/z]: 198.0594 Peak Height: 6.43E+5
Conc. [ng/mL]: 250
S-22 (Ostarine)
Full MS [m/z]: 388.0904 Peak Height: 3.32E+6
DIA [m/z]: 118.0299 Peak Height: 2.38E+6
Conc. [ng/mL]: 25
S-24
Full MS [m/z]: 381.0857 Peak Height: 3.05E+6
DIA [m/z]: 241.0598 Peak Height: 9.06E+5
Conc. [ng/mL]: 50
Fluticasone Propionate (formate adduct)
Full MS [m/z]: 545.1826 Peak Height: 3.33E+5
DIA [m/z]: Peak Height:
Conc. [ng/mL]: 50
S-23
Full MS [m/z]: 415.0467 Peak Height: 1.34E+6
DIA [m/z]: 144.9863 Peak Height: 8.21E+5
Conc. [ng/mL]: 50

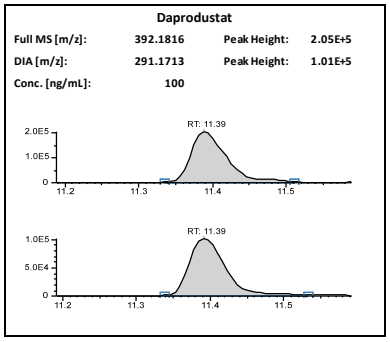


Figure 4.S1 Overview of extracted chromatograms from both full MS data (upper chromatogram) and DIA data (lower chromatogram) of all included compounds.

5 Zusammenfassung

Im Kontext von Dopingkontrollen konnten sich Urin- und Blutproben für den Nachweis von leistungssteigernden Substanzen etablieren. Dennoch sind diese Matrices insbesondere hinsichtlich ihrer Probengewinnung sowie logistischer Prozesse mit Nachteilen verbunden. Aus diesem Grund stieg das Interesse für komplementäre Matrices in den letzten Jahrzehnten stetig an. Die hier dargelegte Doktorarbeit beschreibt in Form von drei publizierten wissenschaftlichen Artikeln Untersuchungen zur Nutzbarkeit von Atemluft und getrockneten Blutstropfen (DBS) in der Dopinganalytik sowie die Entwicklung von Nachweismethoden mittels Flüssigkeitschromatographie gekoppelt mit Tandem-Massenspektrometrie (LC-MS/MS).

Im Fokus des ersten Projekts stand die Entwicklung einer umfassenden LC-MS/MS Methode für ein breites Spektrum an niedermolekularen dopingrelevanten Substanzen für Atemluft. Der Ansatz wurde hinsichtlich der Selektivität, Nachweisgrenzen, Identifizierungsgrenzen, Robustheit, Verschleppung, Matrixeffekte und Wiederfindung charakterisiert und Ausscheidungsstudien mit verschiedenen dopingrelevanten Modellsubstanzen demonstrierten die prinzipielle Anwendbarkeit. Die Publikation beschreibt darüber hinaus die erstmalige Anwendung eines Systems für die Simulation des Atemaerosols, mithilfe dessen das Probensammelgefäß *ExaBreath* der Firma SensAbues® hinsichtlich ausreichender Effizienz und Robustheit für die Dopinganalytik charakterisiert wurde.

Im Rahmen des zweiten Projekts wurden exemplarische Ausscheidungsstudien mit den dopingrelevanten Substanzen Propranolol und Pseudoephedrin durchgeführt, bei denen Atemluft- und DBS-Proben gesammelt und im Anschluss mittels einer hochauflösenden LC-MS/MS Methode analysiert wurden. Die Datenauswertung erfolgte unter Einbeziehung des Geschlechts der Studienteilnehmer*innen sowie des Zigarettenkonsums als mögliche Einflussfaktoren auf die Nachweisbarkeit in Atemluft. Die Matrix erwies sich als robust gegenüber den untersuchten Parametern, doch es wurden inter- und intraindividuelle Fluktuationen hinsichtlich der ermittelten Konzentrationen in Atemluft detektiert.

Das dritte Projekt ging aus der Veröffentlichung eines technischen Dokuments der Welt Anti-Doping Agentur (WADA) hervor, das die Anwendung von DBS offiziell in das Doping-Kontroll-System etablierte. Um dem daraus resultierenden Beitrag von DBS-Proben zur Routineanalytik nachzukommen, wurde ein umfangreiches Nachweisverfahren für insgesamt 233 niedermolekulare dopingrelevante Verbindungen entwickelt. Das Verfahren zeichnet sich dabei durch eine vollautomatisierte Probenvorbereitung mit anschließender adaptiver LC-HRMS/MS Analyse aus. Die Methode wurde konform mit den Richtlinien der WADA validiert und erfolgreich in die Routineanalytik implementiert.
Im Rahmen dieser Doktorarbeit konnten wichtige Kenntnisse zur Nutzbarkeit von Atemluft und DBS in der Dopinganalytik gewonnen werden. Es wurde gezeigt, dass der Nachweis verschiedener Modellsubstanzen in Atemluft grundsätzlich möglich ist und wertvolle ergänzende Informationen liefern kann, doch zukünftig ist die Durchführung weiterer Applikationsstudien erforderlich. Das entwickelte Nachweisverfahren für DBS findet erfolgreich Anwendung in der Routineanalytik und zeichnet sich durch die Adaptivität für neueste Entwicklungen in diesem Bereich aus.

6 Abstract

In the context of doping controls, urine and blood samples are well established matrices for the detection of performance-enhancing substances. However, these matrices are associated with disadvantages, particularly with regard to their collection procedures, logistical processes and financial aspects. For this reason, the interest in complementary matrices has been growing constantly. The thesis presented herein describes investigations regarding the applicability of exhaled breath and dried blood spots (DBS) for doping control purposes as well as the development of detection methods using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

The first project focused on the development of a comprehensive LC-MS/MS method for a broad range of doping agents of low molecular weight for exhaled breath. The approach was characterized in terms of selectivity, detection limits, identification limits, robustness, carryover, matrix effects, and recovery. The applicability was demonstrated based on several administration studies using doping-relevant substances. In addition, the article describes a system for simulating the exhaled breath aerosol, enabling the sample collection device ExaBreath manufactured by SensAbues® to be characterized in terms of sufficient efficiency and robustness.

The second project involved the conduction of exemplary excretion studies with the doping agents propranolol and pseudoephedrine. After the oral application, exhaled breath and DBS samples were collected and subsequently analyzed using LC-MS/MS. For data analysis, the sex of the study participants as well as cigarette consumption were considered as possible impacting factors on the detectability of doping substances in exhaled breath. The matrix was shown to be robust to the investigated parameters, but significant inter- and intraindividual variations were detected with respect to the determined concentrations in exhaled breath.

The third project emerged from the publication of a technical document by the World Anti-Doping Agency (WADA) establishing the application of DBS in the context of doping controls. In order to address the resulting contribution of DBS samples to routine analysis, a comprehensive detection method was developed comprising a total of 233 substances representing all groups on the WADA's Prohibited List. The approach is characterized by

a fully automated sample preparation with subsequent adaptive high-resolution LC-MS/MS analysis. After validation in compliance with WADA guidelines, the method was successfully implemented into routine analysis.

In the framework of this thesis important knowledge on the applicability of exhaled breath and DBS for doping analysis was gained. It was shown that the detection of different model substances in exhaled breath is generally feasible and can provide valuable complementary information. However, the conduction of comprehensive application studies is required in the future. The developed detection method for DBS is being successfully applied in routine analysis and is characterized by its adaptability to new developments in this field.

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Abkürzung	Erläuterung
°C	Grad Celsius
µg	Mikrogramm
µL	Mikroliter
Amph-D_6	Amphetamin-D_6
c	Kartusche (cartridge)
Coc-D_3	Cocain-D_3
Cod-D_3	Codein-D_3
COVID-19	Coronavirus-Krankheit-2019
DBS	Getrockneter Blutrstropfen (*dried blood spot*)
Dex-D_4	Dexamethason-D_4
DIA	Datenunabhängige Erfassung (*data independent acquisition*)
DMAA	Dimethylamylamin
DMPK	Arzneimittelmetabolismus-Pharmakokinetik (*drug metabolism-pharmacokinetics*)
e.g.	zum Beispiel (*exempli gratia*)
EB	Atemluft (*exhaled breath*)
EBC	Atemluftkondensat (*exhaled breath condensate*)
EDTA	Ethylendiamintetraessigsäure (*ethylenediaminetetraacetic acid*)
Eph-D_3	Ephedrin-D_3
ESI	Elektrospray-Ionisation
et al.	und andere (*et alii*)
eV	Elektronenvolt
FMS	Gesamtmassenspektrum (*full mass spectrum*)
FWHM	Halbwertsbreite (*full width at half maximum*)
g	g-Kraft
h	Stunde
Hct-$^{13}C_1$,D_2	Hydrochlorothiazid-$^{13}C_1$,D_2
hPa	Hektopascal
HRMS	Hochauflösende Massenspektrometrie (*high resolution mass spectrometry*)
Hyd-D_3	Hydrocodon-D_3
IC	während eines Wettkampfes (in-competition)
IEC	Internationale Elektrotechnische Kommission
IPNS	Isopropylnorsynephrin
ISO	Internationale Organisation für Normung
ISTD / IS	Interner Standard
ITP	Vorläufiges Testverfahren (*initial testing procedure*)
kV	Kilovolt
L	Liter
LC	Flüssigkeitschromatographie (*liquid chromatography*)
LC-MS/MS	Flüssigkeitschromatographie-Tandem-Massenspektrometrie (*liquid chromatographic - tandem mass spectrometry*)
LOD	Nachweisgrenze (*limit of detection*)

Abkürzung	Erläuterung
LOI	Identifizierungsgrenze (*limit of identification*)
M	Mol
m/z	Masse-zu-Ladungs-Verhältnis
Mel-D_3	Meldonium-D_3
Mep-D_2	Methylprednisolon-D_2
Met-D_9	Methylphenidat-D_9
mg	Milligramm
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimol
Mor-D_3	Morphin-D_3
MRM	*multiple reaction monitoring* Messmodus
MS / MS^2	(Tandem-) Massenspektrometrie ((*tandem*) *mass spectrometry*)
n	Anzahl
NC	Negativkontrolle (*negative control*)
NCE / CE	(Normalisierte) Kollisionsenergie ((*normalized) collision energy*)
ng	Nanogramm
NL	Normalisierungslevel
OAc^-	Acetat-Ion
OOC	außerhalb eines Wettkampfes (out-of-competition)
PA	Nach Verabreichung (*post-administration*)
PEGs	Polyethylenglykole
pg	Pikogramm
pH	negativer dekadischer Logarithmus der Wasserstoff-Ionen-Aktivität (*pondus hydrogenii*)
Pre-D_6	Prednisolon-D_6
PRM	*parallel reaction monitoring* Messmodus
Pro-D_7	Propranolol-D_7
QC	Qualitätskontrolle (*quality control*)
R^2	Bestimmtheitsmaß
rpm	Umdrehungen pro Minute (*revolutions per minute*)
RT	Raumtemperatur
Sal-D_6	Salbutamol-D_6
Sta-D_3	Stanozolol-D_3
Tes-D_3	Testosteron-D_3
TPL	*target performance level*
UHPLC / UPLC	Ultra-Hochleistungsflüssigkeitschromatographie (*ultra (high) performance liquid chromatography*)
US	Unispray-Ionisierung
V	Volt
v/v	Bezug auf das Volumen eines Stoffes
WADA	Welt Anti-Doping Agentur (*World Anti-Doping Agency*)
WADC	Welt Anti-Doping Code (*World Anti-Doping Code*)
z.B.	zum Beispiel