# Targeted lipidomics of low abundant lipid signaling molecules: Clinical analysis of steroids and endocannabinoids

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## TABLE OF CONTENTS

**SUMMARY**

Lipidomics is the study of all or a fraction of the lipids in a biological sample as well as their metabolism, biological processes, and features, such as diseases. Although lipidomics is one of the most recent -omics study areas, its significance is expanding as more people realize that other factors besides genes and proteins affect the condition of biological systems. Over the past ten years, lipidomics has grown quickly since it has introduced new areas of research into the role of lipids in cell biology, health, and disease and it is a continuously growing research field. By identifying changes in lipid states and searching for pathogenic mechanisms that result in lipid-associated disorders, lipidomics can benefit in the discovery and development of new drugs.

A well-defined specific group of lipids is accurately quantified using targeted lipidomics. Targeted lipidomics focuses on certain compounds of interest rather than trying to cover the whole lipid classes. Compared to non-targeted lipidomics, less qualitative data is acquired, but targeted lipidomics is more sensitive and enables accurate analysis of the targeted analytes. In fact, while high resolution mass spectrometers are employed in non-targeted lipidomics, very sensitive mass spectrometers, such as triple quadrupoles (QqQ), are used in targeted lipidomics. The utilization of quadrupole-based instruments for focused applications has increased over the past couple of decades as advances in methodology enhanced the technology's availability. To enable comparisons of a certain group of lipids (such as biomarkers, low abundant compounds, or pathways), such as those usually seen in clinical, biochemical, or industrial research applications, efforts have been undertaken to improve both the selectivity and throughput. Over the past ten years, innovations in technology have significantly increased the scanning speed of triple quadrupole instruments, enabling the measurement of more than 600 transitions per second and more than 200 lipid targets on a contemporary triple quadrupole instrument.

In this cumulative doctoral dissertation, LC-MS based lipidomics assays have been developed to establish the best conditions to study low abundant clinically relevant lipids such as steroids and endocannabinoid species.

Within the first study, a targeted LC-MS/MS method of salivary cortisol and cortisone was developed and then validated. Since saliva and other non-invasive sampling matrices

have been preferred, cortisol and cortisone are commonly used as indicators of stress. Due to the fact that cross-reactivities make immunoassays less specific, they are nonetheless often employed for measuring steroid hormone levels. Currently LC-MS/MS-based sensitive methods are mostly used for quantification of steroid hormones. For that reason, the goal of this study was to develop a novel microflow UHPLC-ESI-MS/MS technique with MRM data acquisition for a large-scale long-term neuroimaging stress study for measuring salivary cortisol and cortisone that, thanks to its microflow regime, enables improved sensitivity and is more environmentally friendly. Positive pressure elution mode and offline SPE with Oasis PRIME HLB in 96-well plate format enabled excellent sample preparation with high throughput and the ability to effectively remove matrix effects. With the acquired SPE enrichment factor of 14, the capillary chromatography scale's microflow regime (20 µL $min^{-1}$) favored effective electrospray ionization and produced a sensitive cortisol/cortisone steroid quantification technique (LLOQ of cortisol/cortisone, 72/62 pg $mL^{-1}$, respectively). The evaluation of cortisol and cortisone concentrations in various batches of samples from normal clinical stress study samples (4056 total injections with 1983 study samples) was effective in the end. Furthermore, the instrument performance of the five capillary columns under investigation varied throughout time, including the retention time variations within each batch, across batches, and from lot to lot during the study took 2 years. The research shows that, if appropriate internal standards can be utilized, micro-UHPLC-ESI-MS/MS is sufficient and reliable enough to conduct a comprehensive clinical investigation with more than 1000 samples over a long period of time.

The second study involved the development and validation of a new UHPLC–ESI-MS/MS method for endocannabinoid separation and determination in low-quantity CSF (cerebrospinal fluid) samples. Endocannabinoids are fatty acid derivatives produced from within the body that activate cannabinoid receptors. In the real samples, six analytes of endocannabinoids (1/2-AG, 2-AGE, AEA, LEA, PEA, and OEA) could be measured in a single chromatographic run with good sensitivity, high accuracy, and in a short analysis time (5.5 min). The approach described here is straightforward, robust, and capable of high throughput due to its quick analysis time and single protein precipitation/extraction phase. As far as we are aware, this is the first validated technique that used two

calibration procedures in CSF samples: surrogate calibrant and surrogate matrix method especially for quantification of 2-AG, 2-AGE, AEA, LEA, PEA, and OEA in CSF.

The third project intended to develop quick, sensitive, reliable, and reproducible LC-MS/MS technique for melatonin and cortisol measurement in saliva to investigate the sleep-awake rhythm in healthy volunteers and compare the sleep-awake cycles with Parkinson patients. The technique must be quick and simple, requiring the least amount of sample preparation and it was evaluated using a range of saliva samples from healthy participants to monitor changes in cortisol and melatonin concentrations over the course of a 24-hour period in a circadian rhythm. The run time of 6 minutes per sample in clinical research allowed for a high throughput. Different parameters were compared to improve the sensitivity. A RPLC-ESI-MS/MS method was developed with LOQs of 15 pg/mL for melatonin and 104 pg/mL for cortisol in saliva. Despite several optimizations being made to increase the LC-MS/MS sensitivity and the detection limits, the melatonin amount that could be measured in saliva samples was already above the baseline values, thereby further research is needed.

## ZUSAMMENFASSUNG

Unter Lipidomik versteht man die Untersuchung aller oder eines Teils der Lipide in einer biologischen Probe sowie ihres Stoffwechsels, biologischer Prozesse und Funktion, wie z. B. Krankheiten. Obwohl Lipidomik zu den neueren Forschungsgebieten der Omics gehört, nimmt ihre Bedeutung zu, da immer mehr Menschen erkennen, dass neben Genen und Proteinen auch andere Faktoren den Zustand biologischer Systeme beeinflussen. In den letzten zehn Jahren ist die Lipidomik schnell gewachsen, da sie neue Forschungsbereiche zur Rolle von Lipiden in der Zellbiologie, Gesundheit und Krankheit eröffnet hat, und es handelt sich um ein kontinuierlich wachsendes Forschungsgebiet. Durch die Identifizierung von Veränderungen im Lipidzustand und die Suche nach pathogenen Mechanismen, die zu Lipid-assoziierten Störungen führen, kann Lipidomik bei der Entdeckung und Entwicklung neuer Medikamente von Nutzen sein.

Mithilfe der gezielten Lipidomik wird eine genau definierte spezifische Gruppe von Lipiden genau quantifiziert. Die gezielte Lipidomik konzentriert sich auf bestimmte interessierende Verbindungen, anstatt zu versuchen, die gesamten Lipidklassen abzudecken. Im Vergleich zur nicht gezielten Lipidomik werden weniger qualitative Daten erfasst, die gezielte Lipidomik ist jedoch empfindlicher und ermöglicht die Analyse der gezielten Analyten. Während in der nicht gezielten Lipidomik hochauflösende Massenspektrometer eingesetzt werden, werden in der fokussierten Lipidomik sehr empfindliche Massenspektrometer wie Triple Quadrupole (QqQ) verwendet. Der Einsatz quadrupolbasierter Instrumente für gezielte Anwendungen hat in den letzten Jahrzehnten zugenommen, da Fortschritte in der Methodik die Verfügbarkeit der Technologie verbesserten. Um Vergleiche einer bestimmten Gruppe von Lipiden (z. B. Biomarkern, Verbindungen mit geringem Vorkommen oder Signalwegen) zu ermöglichen, wie sie üblicherweise in klinischen, biochemischen oder industriellen Forschungsanwendungen vorkommen, wurden Anstrengungen unternommen, um sowohl die Selektivität als auch den Durchsatz zu verbessern. In den letzten zehn Jahren haben technologische Innovationen die Scangeschwindigkeit von Dreifach-Quadrupol-Instrumenten deutlich erhöht und die Messung von mehr als 600 Übergängen pro Sekunde und mehr als 200 Lipidzielen auf einem modernen Triple-Quadrupol-Instrument ermöglicht.

In dieser kumulativen Doktorarbeit wurden LC-MS-basierte Lipidomik-Assays entwickelt, um die besten Bedingungen für die Untersuchung von niedrig abundanten Lipiden wie Steroiden und Endocannabinoidarten zu schaffen.

Im Rahmen der ersten Studie wurde eine gezielte LC-MS/MS-Methode für Speichelcortisol und Kortison entwickelt und anschließend validiert. Da Speichel und andere nicht-invasive Probenahmematrizen bevorzugt werden, werden Kortisol und Kortison häufig als Indikatoren für Stress verwendet. Da Immunoassays aufgrund von Kreuzreaktivitäten weniger spezifisch sind, werden sie dennoch häufig zur Messung des Steroidhormonspiegels eingesetzt. Zur Quantifizierung von Steroidhormonen werden derzeit überwiegend LC-MS/MS-basierte empfindliche Methoden eingesetzt. Aus diesem Grund war das Ziel dieser Studie die Entwicklung einer neuartigen Mikrofluss-UHPLC-ESI-MS/MS-Technik mit MRM-Datenerfassung für eine groß angelegte langfristige Neuroimaging-Stressstudie zur Messung von Kortisol und Kortison im Speichel zu entwickeln. Dank ihres Mikroflusses Regime ermöglicht die Methode eine verbesserte Empfindlichkeit und ist umweltfreundlicher. Der Überdruck-Elutionsmodus und die Offline-SPE mit Oasis PRIME HLB im 96-Well-Plattenformat ermöglichten eine hervorragende Probenvorbereitung mit hohem Durchsatz und der Fähigkeit, Matrixeffekte effektiv zu entfernen. Mit dem erworbenen SPE-Anreicherungsfaktor von 14 begünstigte das Mikroflussregime der Kapillarchromatographie-Skala (20 µL $min^{-1}$) eine effektive Elektrospray-Ionisierung und erzeugte eine empfindliche Quantifizierungstechnik für Kortisol/Kortison-Steroide (LLOQ von Kortisol/Kortison, 72/62 pg $mL^{-1}$). Die Bewertung der Kortisol- und Kortisonkonzentrationen in verschiedenen Probenchargen aus Studienproben mit normalem klinischem Stress (insgesamt 4056 Injektionen mit 1983 Studienproben) erwies sich letztendlich als wirksam. Darüber hinaus schwankte die Geräteleistung der fünf untersuchten Kapillarsäulen im Laufe der Zeit, einschließlich der Schwankungen der Retentionszeit innerhalb jeder Charge, zwischen den Chargen und von Charge zu Charge während der Studie, die zwei Jahre dauerte. Die Forschung zeigt, dass Mikro-UHPLC-ESI-MS/MS bei Verwendung geeigneter interner Standards ausreichend und zuverlässig genug ist, um eine umfassende klinische Untersuchung mit mehr als 1000 Proben über einen langen Zeitraum durchzuführen.

Meine zweite Studie umfasste die Entwicklung und Validierung einer neuen UHPLC-ESI-MS/MS-Methode zur Trennung und Bestimmung von Endocannabinoiden in Zerebrospinalflüssigkeitsproben geringer Menge. Endocannabinoide sind körpereigene Fettsäurederivate, die Cannabinoidrezeptoren aktivieren. In den realen Proben konnten sechs Endocannabinoid-Analyten (1/2-AG, 2-AGE, AEA, LEA, PEA und OEA) in einem einzigen chromatographischen Lauf mit guter Empfindlichkeit, hoher Genauigkeit und in kurzer Analysezeit gemessen werden (5.5 Minuten). Die hier beschriebene Methode ist unkompliziert, robust und aufgrund ihrer kurzen Analysezeit und der Fällungs-/Extraktionsphase einzelner Proteine für einen hohen Durchsatz geeignet. Soweit uns bekannt ist, ist dies die erste validierte Technik, die zwei Kalibrierungsverfahren in Zerebrospinalflüssigkeitsproben verwendet: Surrogatkalibrantmethode und Surrogatmatrixmethode, insbesondere zur Quantifizierung von 2-AG, 2-AGE, AEA, LEA, PEA und OEA in Zerebrospinalflüssigkeitsproben.

Ziel des dritten Projekts war die Entwicklung einer schnellen, empfindlichen, zuverlässigen und reproduzierbaren LC-MS/MS-Technik zur Messung von Melatonin und Cortisol im Speichel, um den Schlaf-Wach-Rhythmus bei gesunden Probanden zu untersuchen und die Schlaf-Wach-Zyklen mit denen von Parkinson-Patienten zu vergleichen. Die Technik muss schnell und einfach sein und den geringsten Aufwand an Probenvorbereitung erfordern. Sie wurde anhand einer Reihe von Speichelproben gesunder Teilnehmer evaluiert, um Veränderungen der Cortisol- und Melatoninkonzentrationen über einen Zeitraum von 24 Stunden in einem zirkadianen Rhythmus zu überwachen. Die Laufzeit von 6 Minuten pro Probe in der klinischen Forschung ermöglichte einen hohen Durchsatz. Um die Empfindlichkeit zu verbessern, wurden verschiedene Parameter verglichen. Es wurde eine RPLC-ESI-MS/MS-Methode mit LOQs von 15 pg/ml für Melatonin und 104 pg/ml für Kortisol im Speichel entwickelt. Der nachweisbare Melatoninspiegel lag jedoch bereits über den Grundwerten in Speichelproben, obwohl verschiedene Optimierungen durchgeführt wurden, um die Empfindlichkeit zu verbessern, sind weitere Untersuchungen erforderlich.

## LIST OF PUBLICATIONS INCLUDED IN THIS THESIS

### Publication I

Aydin, E.; Drotleff, B; Noack, H; Derntl, B; Lämmerhofer M. Fast accurate quantification of salivary cortisol and cortisone in a large-scale clinical stress study by micro-UHPLC-ESI-MS/MS using a surrogate calibrant approach. *J. Chromatogr. B 2021,* 1182 (2021): 122939, DOI: 10.1016/j.jchromb.2021.122939.

### Publication II

Aydin, E.; Cebo, M.; Mielnik, J.; Richter, H.; Schüle, R.; Sievers-Engler, A.;Mlynarz, P.; Lämmerhofer M. UHPLC-ESI-MS/MS assay for quantification of endocannabinoids in cerebrospinal fluid using surrogate calibrant and surrogate matrix approaches. *J. Pharm. Biomed. Anal*., 222, (2023) 115090, DOI: 10.1016/j.jpba.2022.115090.

## AUTHOR CONTRIBUTIONS

### Publication I

Fast accurate quantification of salivary cortisol and cortisone in a large-scale clinical stress study by micro-UHPLC-ESI-MS/MS using a surrogate calibrant approach.

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General concept

Method development and Validation

Sample preparation and analysis

Data processing and interpretation

LC-MS instruments maintenance

Main writing of the manuscript

**Bernhard Drotleff**

Supervision

Method development

Discussion of results and interpretation

LC-MS instruments maintenance

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Collection of saliva samples

Proofreading of the manuscript

**Prof. Dr. Birgit Derntl**

Coordination and financing of the biological part of the project

Proofreading of the manuscript

**Prof. Dr. Michael Lämmerhofer**

Generation, initiation, coordination, and financing of the project

Discussion of results and interpretation

Partial writing and editing of the manuscript

Proofreading and final approval of the manuscript

Corresponding author

**Publication II**

UHPLC-ESI-MS/MS assay for quantification of endocannabinoids in cerebrospinal fluid using surrogate calibrant and surrogate matrix approaches.

**Ece Aydin**

General concept

Method development and Validation

Sample preparation and analysis

Data processing and interpretation

LC-MS instruments maintenance

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Discussion of results and interpretation

Partial writing and editing of the manuscript

Proofreading and final approval of the manuscript

Corresponding author

## LIST OF OTHER PUBLICATIONS PREPARED DURING THE RESEARCH PERIOD

### Publication I

Kuhn, L.; Noack, H.; Wagels, L.; Prothmann, A.; Schulik, A.; Aydin, E.; Nieratschker, V.; Derntl, B.; Habel, U. Sex-dependent multimodal response profiles to psychosocial stress. *Cerebral Cortex* 2022, DOI: 10.1093/cercor/bhac086.

## LIST OF UNFINISHED PROJECTS DURING THE RESEARCH PERIOD

### Manuscript I

Aydin[a], E., Alfadel[a], T., Michael Lämmerhofer[a,*] Development and validation of LC–MS/MS assay for the quantification of salivary cortisol, melatonin and its active metabolites using synthesized isotopically-labelled surrogate calibrant

## LIST OF POSTER PRESENTATIONS

***48TH International Symposium on High-Performance Liquid Phase Separations and Related Techniques*** (HPLC 2019), University Milano-Bicocca. Milano Italy 16 - 20 June 2019.

**Poster Title**: Quantification of Salivary Cortisone and Cortisol without Derivatization by micro-LC QTRAP-MS/MS Using a Surrogate Calibrant Approach

Ece Aydin, Bernhard Drotleff, Hannes Noack,Birgit Derntl, Michael Lämmerhofer

***DPhG Jahrestagung 2021***, 28 September - 1 October 2021.

**Poster Title**: Fast accurate quantification of salivary cortisol and cortisone in a large-scale clinical stress study by micro-UHPLC-ESI-MS/MS using a surrogate calibrant approach

Ece Aydin, Bernhard Drotleff, Hannes Noack,Birgit Derntl, Michael Lämmerhofer

***26th International Symposium on Separation Sciences (ISSS 2022)***, Ljubljana, Slovenia 28 June - 1 July 2022.

**Poster Title**: Sensitive UHPLC-ESI-MS/MS method for quantification of endocannabinoids in cerebrospinal fluid using surrogate calibrant and surrogate matrix approaches

*Awarded with a Poster Prize*

Ece Aydin, Malgorzata Cebo, Justyna Mielnik, Hardy Richter, Piotr Młynarz, Michael Lämmerhofer

***38th International Symposium on Microscale Separations and Bioanalysis (MSB 2022)***, Liege, Belgium 3-6 July 2022.

**Poster Title**: Quantification of endocannabinoids in cerebrospinal fluid using surrogate calibrant and surrogate matrix approaches by UHPLC-ESI-MS/MS

Ece Aydin, Malgorzata Cebo, Justyna Mielnik, Hardy Richter, Piotr Młynarz, Michael Lämmerhofer

**ABBREVIATON**

AA Arachidonic acid

AEA Anandamide

1-AG 1-Arachidonoylglycerol

2-AG 2-Arachidonoylglycerol

cAMP Cyclic adenosine monophosphate

CB1 Cannabinoid receptor type 1

CB2 Cannabinoid receptor type 2

CEM Continuous electron multipliers

CSF Cerebrospinal fluid

DHEA Dihydroepiandrosterone

EC Endocannabinoid

FT-ICR Fourier transform-ion cyclotron resonance

FWHM Full width half maximum

HILIC Hydrophilic interaction liquid chromatography

HPA Hypothalamus-pituitary-adrenal

HSD11B2 11β-hydroxysteroid dehydrogenase type 2 enzyme

IUPAC International Union of Pure and Applied Chemistry

Q Quadrupole

QTOF Quadrupole time-of-flight

LEA Linoleoylethanolamide

LIT Linear ion trap

MAG Monoalcylglycerol

MCP Microchannel plate

MRM Multiple reaction monitoring

MTBE Methyl tert-butyl ether

NAE N-acylethanolamine

NMR Nuclear magnetic resonance

OEA Oleoylethanolamide

PEA Palmitoylethanolamide

RF Resonance frequency

SFC supercritical fluid chromatography

SRM Selected reaction monitoring

THF Tetrahydrofuran

TOF Time-of-flight

QqQ Triple quadrupole

# 1. INTRODUCTION

## 1.1. STEROIDS

### 1.1.1. DEFINITION OF STEROIDS

Steroids, derived from cholesterol, are lipophilic molecules that have common 17-carbon skeleton “gonane” (i.e., cyclopentanoperhydrophenanthrene) (Figure 1) [1-3]. This structure is a combination of three hexagonal rings (A, B, C) forming the phenanthrene core and a five-carbon cyclopentane ring (D). The prefix perhydro- indicates saturation with hydrogen atoms [1]. The numbering of carbon atoms in cholesterol is essential in naming steroids (Figure 2).

They differ from each other if various functional groups are attached to the rings (A, B, C or D).

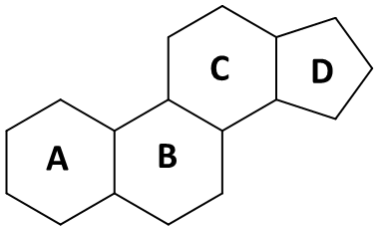


*Figure 1: Cyclopentanoperhydrophenanthrene (i.e., gonane) nucleus.*

Steroids in vivo are present at low levels, with the biologically active form of steroids being present in much lower levels. Although steroid levels are low, they are crucial and are the pathogenesis of many illnesses. Their biological capacities are influenced by both genetic and nongenomic factors. Based on these two impacts, studies reveal that steroids have a role in the emergence and progression of several disorders, such as polycystic ovarian syndrome, infertility, hypogonadism, hirsutism, etc. Therefore, it is crucial to accurately quantify steroid levels [3-5].

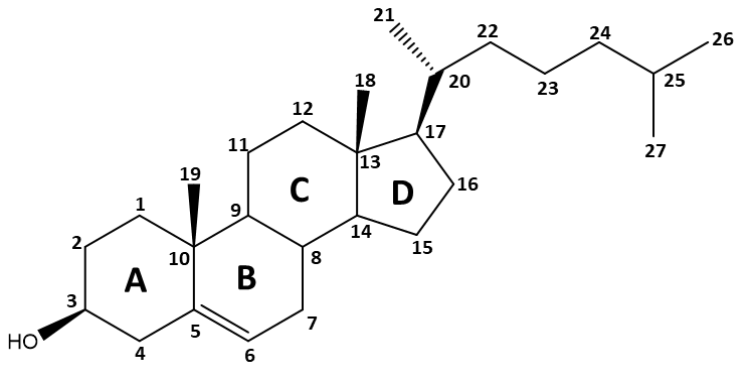


*Figure 2: Structure of an example of the numbering of the carbon atoms in steroids (structure represents cholesterol).*

### 1.1.2. CLASSIFICATION OF STEROIDS

Endocrine glands regulate the functioning of certain cells by secreting endogenous active substances called hormones. Hormones are also used as drugs due to their pharmacological effects depending on the dose as well as their physiological effects. Hormones play an important role in coordinating and maintaining physiological and behavioral responses for specific biological purposes such as water and electrolyte balance, acid-base balance, for blood pressure, muscle, for adipose tissue, energy production, metabolism, stress, reproduction, growth, and development. Physiological functions usually occur under the control of more than one hormone. According to their chemical and physicochemical properties, they are divided into three groups as peptide hormones, steroid hormones and amino acid hormones [6].

In the endocrine glands (adrenal cortex, testis, and ovary) that secrete steroid hormones, all steroids are synthesized from cholesterol. The source of the first substance, cholesterol, is the low-density lipoprotein in the plasma. Steroids are lipophilic and clinically important compounds. Neurosteroids are also produced in the central nervous system. Since all steroid hormones are derived from cholesterol, they are insoluble in plasma and other body fluids. Adrenal glands located on each of human kidneys, are secreting some essential hormones of human body (adrenaline, noradrenaline, aldosterone, cortisol). They are endocrine glands that resemble triangles. They consist of adrenal glands, adrenal cortex, and adrenal medulla [6].

The adrenal cortex is divided into three main anatomical regions: aldosterone-producing zona glomerulosa, the zona fasciculata and reticularis, which together produce cortisol and adrenal androgens. The cortex of the adrenal gland is responsible for the biosynthesis of mineralocorticoids and glucocorticoids, as well as the production of adrenal androgen precursors and androgens. Zona glomerulosa (*latin for "lump zone"*) secretes mostly aldosterone. Zona fasciculata (*latin for "bundle zone"*) secretes mostly cortisol. Cortisol and aldosterone are 21 carbon steroids. Zona reticularis (*latin for "network zone"*) secretes mostly sex hormones (dihydroepiandrosterone (DHEA) and androstenedione). All these hormones are called androgens. Unlike the shell region, the medulla consists of a single region and the medulla synthesizes catecholamines. More than 30 steroids are produced in the adrenal cortex, and steroids can be divided into three classes based on their function: mineralocorticoids, glucocorticoids, and androgens [6]

The receptors for lipid-soluble steroids are not on the cytoplasmic membrane of target cells but are usually in the nucleus. There is a separate type of receptor for each type of steroid hormone. There are 6 types of steroid hormone receptors in total: glucocorticoids (e.g., cortisol), mineralocorticoids (e.g., aldosterone), estrogens (e.g., estrone), progestins (Gestagens) (e.g., progesterone), androgens (e.g., testosterone), and vitamin D receptors. All corticosteroids containing steroid hormones such as cortisol and aldosterone secreted from the adrenal cortex contain a pregnane skeleton with 21 carbon atoms. They are mainly metabolized in the liver, and they are inactivated.

In addition to the traditional nomenclature of steroids, systematic IUPAC (International Union of Pure and Applied Chemistry) nomenclatures are used also for classification of steroids [7]. In systematic nomenclature, estrane for 18-carbons, androstane for 19-carbons, pregnane for 21-carbons, cholestane for 27-carbons, gonane for 17-carbons and cholane for 24-carbons are used [1, 8]. Most of the steroid compounds are of these six skeletal types (besides Vitamin D, in which ring B is opened) (Figure 3) [7].

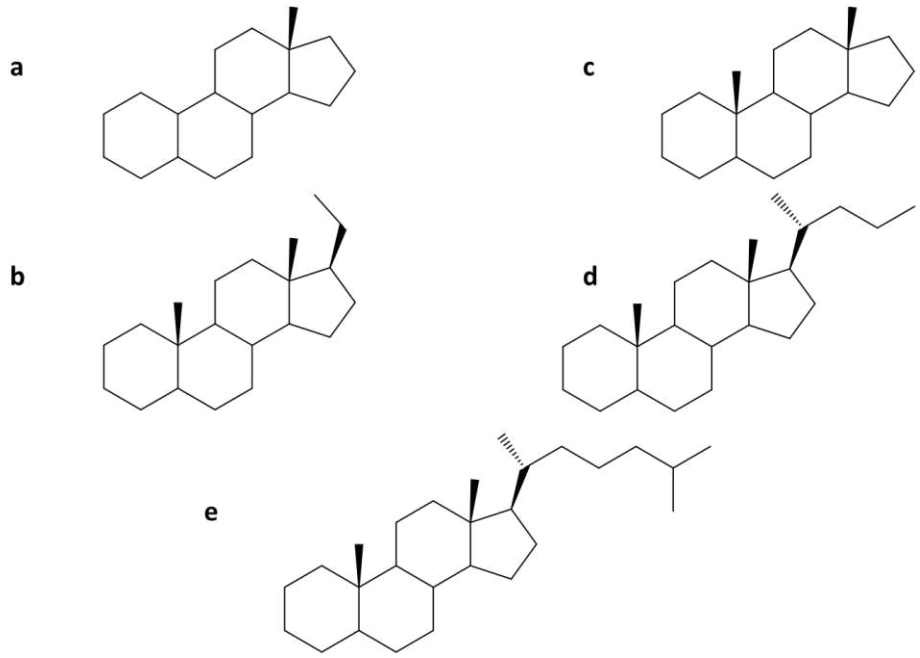


*Figure 3: (a) Estrane; (b) pregnane; (c) androstane; (d) cholane; (e) cholestane.*

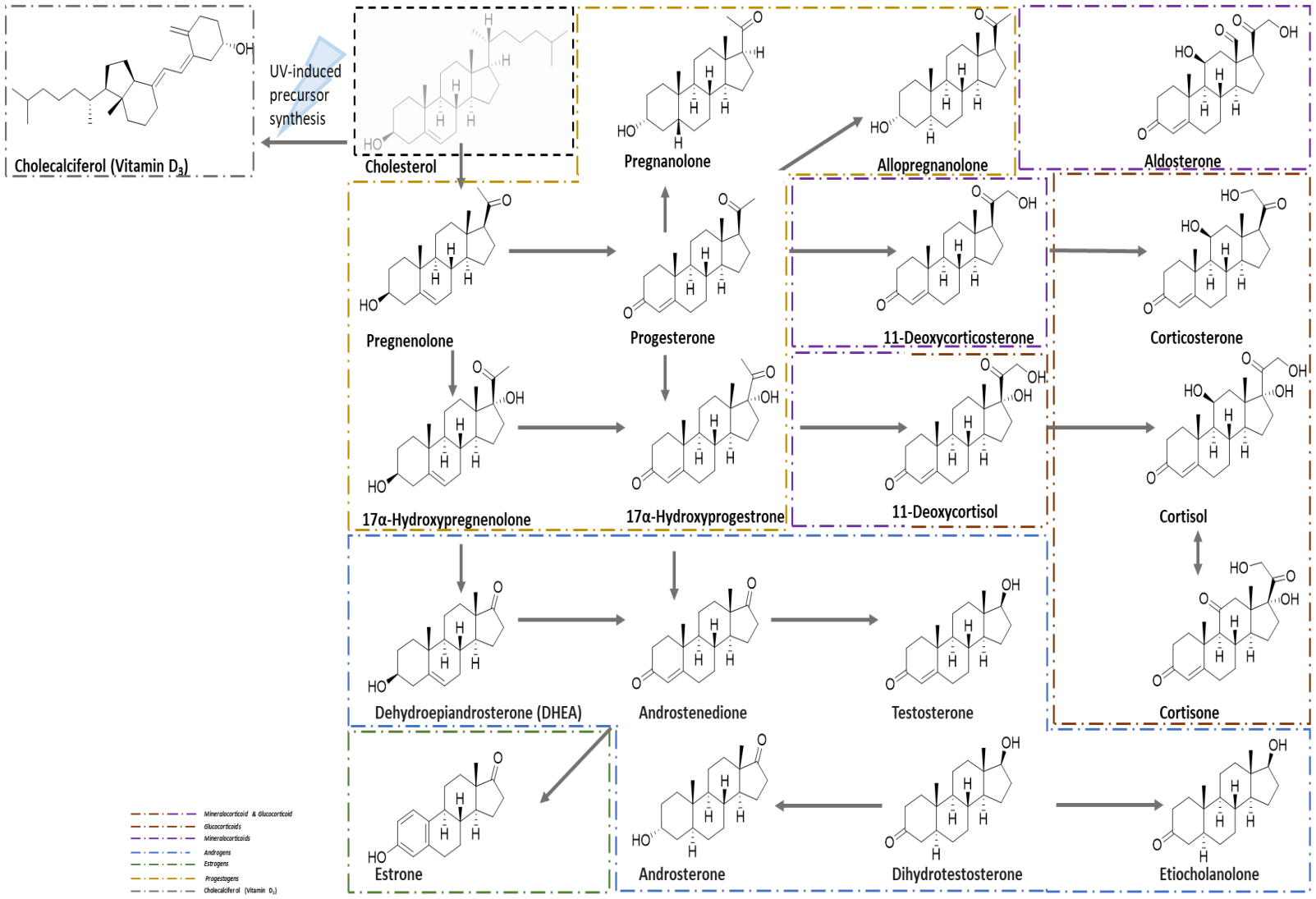
Cholecalciferol (Vitamin D3)
UV-induced precursor synthesis
Cholesterol
Pregnanolone
Allopregnanolone
Aldosterone
Pregnenolone
Progesterone
11-Deoxycorticosterone
Corticosterone
17α-Hydroxypregnenolone
17α-Hydroxyprogestrone
11-Deoxycortisol
Cortisol
Dehydroepiandrosterone (DHEA)
Androstenedione
Testosterone
Cortisone
Estrone
Androsterone
Dihydrotestosterone
Etiocholanolone
Mineralocorticoid & Glucocorticoid
Glucocorticoids
Mineralocorticoids
Androgens
Estrogens
Progestogens
Cholecalciferol (Vitamin D3)

*Figure 4: Steroid hormone biosynthesis pathway for 6 major chemical classes of steroid hormones: [9, 10]*

### 1.1.3. STUDIED STEROID CLASSES

#### 1.1.3.1. GLUCOCORTICOIDS

Glucocorticoids are 21-carbon steroid hormones that are naturally secreted from the adrenal glands to maintain homeostasis in daily rhythm and when the organism is under stress. It is both secreted in the body and produced synthetically. Glucocorticoids are mainly produced in the zona fasciculata, and the main steroid hormone is cortisol [11, 12].

They are also used as drugs due to their anti-inflammatory, immunosuppressive, antiallergic and various effects and in the absence of adrenal function disorders (e.g. Addison's disease, Cushing's disease) where they are used as replacement therapy and secondly in the absence of adrenal or endocrine disorders (e.g., allergic rhinitis, bronchial asthma, atopic dermatitis, gouty arthritis, rheumatoid arthritis, and others). Glucocorticoids affect metabolism in various ways, stimulate gluconeogenesis and reduce glucose utilization by cells [12].

Cortisol, the most effective glucocorticoid in humans, is synthesized from pregnenolone. In its basic structure, there is a cyclopentanoperhydrophenanthrene ring system consisting of three 6-carbon cyclohexane rings and a 5-carbon cyclopentane ring. The human body releases the steroid hormone cortisol in relation to mental and physical stress. Stress responses are triggered by stressful stimuli that activate the hypothalamus-pituitary-adrenal (HPA) axis [13]. Nearly every organ system, including the neurological, immunological, cardiovascular, respiratory, reproductive, musculoskeletal, and covering systems, can be impacted by cortisol. Additionally, cortisol regulates several physiological processes, including blood pressure, glucose levels, and carbohydrate metabolism. It also serves as a biomarker for many disorders. It is crucial for maintaining the homeostasis of the endocrine, immunological, renal, skeletal, and cardiovascular systems. Depending on how frequently the hormone circulates in the body, cortisol has different impacts on health. The body might suffer from several disorders as a result of excessive or insufficient cortisol production.

Cortisol is metabolized to inactive cortisone particularly in kidneys by the 11β-hydroxysteroid dehydrogenase type 2 enzyme (HSD11B2) and exerts a secondary glucocorticoid effect (Figure 5). Cortisol concentration in tissues and body fluids is kept under control by 11β-hydroxysteroid dehydrogenase type 1 and type 2 enzymes. Therefore, an analytical method for quantification of both cortisol and cortisone would potentially be useful in the diagnosis of many disorders.

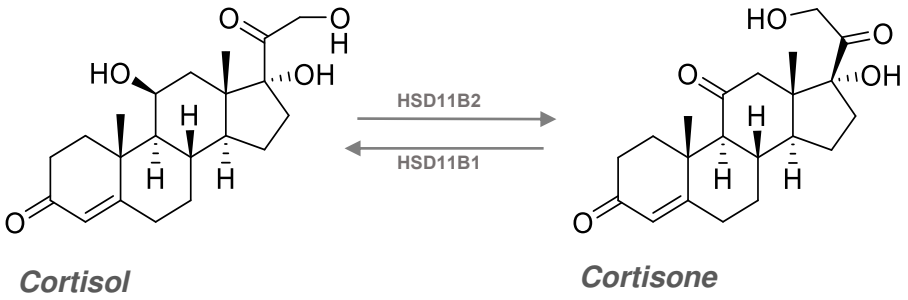


*Figure 5: Interconversion of cortisone and cortisol [14]*

## 1.2. ENDOCANNABINOIDS AND ENDOCANNABINOID-LIKE COMPOUNDS

Endocannabinoids (ECs) are endogenous lipids that can bind to Cannabinoid receptor type 1 (CB1) and Cannabinoid receptor type 2 (CB2) receptors. CB1 is more common in the central nervous system and produces the psychotropic effects of exogenous cannabinoids. CB1 is involved in the release of ion channels-dependent neurotransmitters in the brain, analgesia, and body temperature control is found in neurons in the hypothalamic nucleus that controls energy balance and body weight. CB2 is more commonly found in inflammatory and immune tissues and mediates the anti-inflammatory effects of endocannabinoids. CB1 and CB2 are coupled to the G protein and act by lowering the intracellular cAMP (cyclic adenosine monophosphate) level. The decrease in cAMP level reduces the amount of synaptic transmitter, resulting in essentially inhibitory effects on the target cell [15-18].

ECs are synthesized from arachidonic acid (AA), a phospholipid precursor [19, 20].

### 1.2.1. STUDIED CLASSES OF ENDOCANNABINOIDS AND ENDOCANNABINOID-LIKE COMPOUNDS

#### 1.2.1.1. ACYLETHANOLAMINES

N-acylethanolamines (NAEs) are generated from a fatty acid linked to ethanolamine (Figure 6) and has a broad range of biological functions, including regulating metabolism and appetite [21, 22]. Anandamide (AEA), is one of this class of NAEs, and was the first endocannabinoid to be discovered [23]. It is an endogenous fatty acid derivative that is synthesized and bound from the phospholipids of the cell membrane by biosynthetic means. It is formed by the catalysis of N-arachidonic phosphatidylethanolamine by phospholipase D [24]. The single nitrogen atom in ethanolamine that holds the compound together is represented by the suffixes "amine" and "amide" in these names. In ethanolamine, the nitrogen atom is referred to as an "amine" because it is thought of as a free terminal nitrogen, whereas it is assumed of as an "amide" when it is considered of in association with the adjacent carbonyl group of the acyl subunit. Depending on the author, these compounds may go by names that start with "amide" or "amine." General structure of NAEs can be seen in Figure 6. OEA (oleoyl ethanolamide), PEA (palmitoyl ethanolamide), LEA (Linoleoyl ethanolamide) are the most important N-acylethanolamines.

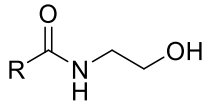


*Figure 6: General chemical structure of N-acylethanolamines*

OEA appears to be the most significant endocannabinoid-like molecule in the control of appetite, fat metabolism, and energy balance, indicating its therapeutic promise in the treatment of the obesity epidemic [25, 26]. PEA is produced when needed from membrane endocannabinoid-like phospholipids and has a variety of pharmacological effects. Through modulating endocannabinoid signaling and indirectly activating cannabinoid receptors, PEA supports the endocannabinoids. This is known as the entourage effect [25]. LEA is a homologue of anandamide, is produced by the enzymatic

hydrolysis of the corresponding acyl phosphatidyl ethanolamides by phospolipase D9 [27, 28].

#### 1.2.1.2. MONOALCYLGLYCEROLS

In monoacylglycerols (also known as "monoglycerides"), just one of the hydroxyl groups of the alcohol glycerol is esterified with a long-chain fatty acid. As shown in Figure 7, they can exist in three isomeric forms: *sn*-1, *sn*-2 and *sn*-3 [29].

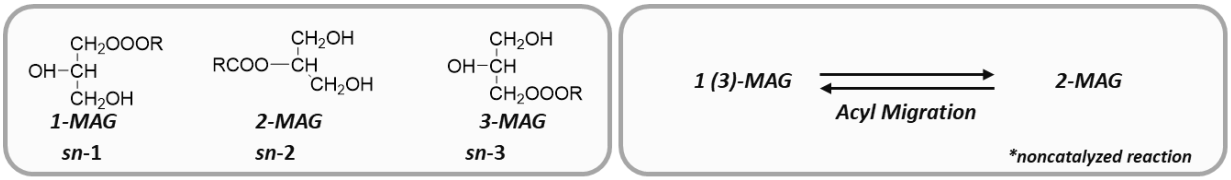


*Figure 7: Stereoisomers of monoacyl-sn-glycerols*

The second discovered endocannabinoid, is 2-AG (2-Arachidonoylglycerol) is a distinctive species of monoacylglycerol. As with anandamide, 2-AG target both CB1 and CB2 receptors. 2-AG is prone to molecular rearrangement in water-based media, where the arachidonoyl moiety changes from the 2-position to the 1-position of glycerol, unlike anandamide, as anandamide is chemically stable in organic solvents and aqueous solutions. This non-enzymatic isomerization, also known as acyl migration, results in the formation of 1-arachidonoyl-sn-glycerol (1-AG), which is thermodynamically more stable than 2-AG. The process continues until it achieves an equilibrium at a 1:9 ratio of 2-AG and 1-AG [30]. ECs that are available for purchase, are typically supplied as solutions in organic solvents. Furthermore, ECs are frequently extracted from biological samples with organic solvents, such as methanol and chloroform mixtures. The evaporation of the organic solvents and later recovery of the residues in organic solvents for further analysis are essential steps in EC analysis. The nearly full conversion of ethanolic 2AG solutions to 1AG is brought about by evaporation. In contrast, there is no notable 2AG/1AG isomerization when 2AG solutions in toluene evaporate. Therefore, it is essential to pay attention to the extraction solvent, pH conditions, and even the evaporation phase during analytical techniques [14].

2-AGE (2-Arachidonyl glyceryl ether or noladin ether) is a third discovered endocannabinoid by Hanus et al. (2001) [31] that is a stable analog of the endogenous cannabinoid 2-AG. It has a strong affinity for cannabinoid receptors [31, 32].

Because of their lability and lipophilicity, endocannabinoid system components are difficult to extract from biological samples. Further, the analytical technique that are able to directly measure these compounds, are restricted by their low abundance (picomol/g sample) and absence of any electrochemical or fluorescent functional groups [33].

## 1.3. LIPIDOMICS

The "comprehensive characterisation of lipids in biological systems" is referred to as lipidomics [34]. It is the comprehensive study of cellular lipid networks and processes in biological systems. The entire lipid profile found within a cell, tissue, organism, or ecosystem is called as the "lipidome" and is a subset of the "metabolome," which also includes other significant groups of biological molecules (such as amino acids, sugars, glycolysis & TCA intermediates, and nucleic acids). With the rapid development of technologies like mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, fluorescence spectroscopy, dual polarization interferometry, and computational methods, as well as the growing understanding of the role of lipids in a variety of metabolic diseases like obesity, atherosclerosis, stroke, hypertension, and diabetes, the field of lipidomics has emerged relatively recently. This rapidly developing field completes to the enormous advancements in genomics and proteomics, which together form the biological system family [35].

### 1.3.1. SEPARATION TECHNIQUES IN LIPIDOMICS

Due to innovations in mass spectrometry, lipidomics has become one of the most promising study areas since its introduction in 2003 [36]. In general, lipid analysis can be carried out by either chromatographic separation before MS detection or by directly injecting the lipid extract of a sample into the MS (shotgun lipidomics) [37]. Direct infusion (shotgun) methods were widely used in the early stages of lipidomics research, as their speed in analysis, relative ease of use, and ability to identify many lipid classes in a single run have increased in importance over other methods. These techniques often employed tandem MS in a class-specific or targeted manner, making it straightforward to detect and

subsequently identify unknowns. Following this, advances in computational approaches and liquid-chromatography (LC) separation were made quickly. Several benefits of LC-MS-based approaches over direct infusion procedures include more accurate identification of particular lipid species, even at trace levels. Additionally, modern LC devices enable more efficient separation, shorter analytical times, and using less solvent [38, 39].

Liquid chromatography (LC), that allows to separate steroid classes or endocannabinoid classes based on their physicochemical characteristics, is a highly helpful technique for the study of lipids due to their broad range of polarity [14]. Figure 8 shows the number of studies published about targeted lipidomics since it is released in 2003.

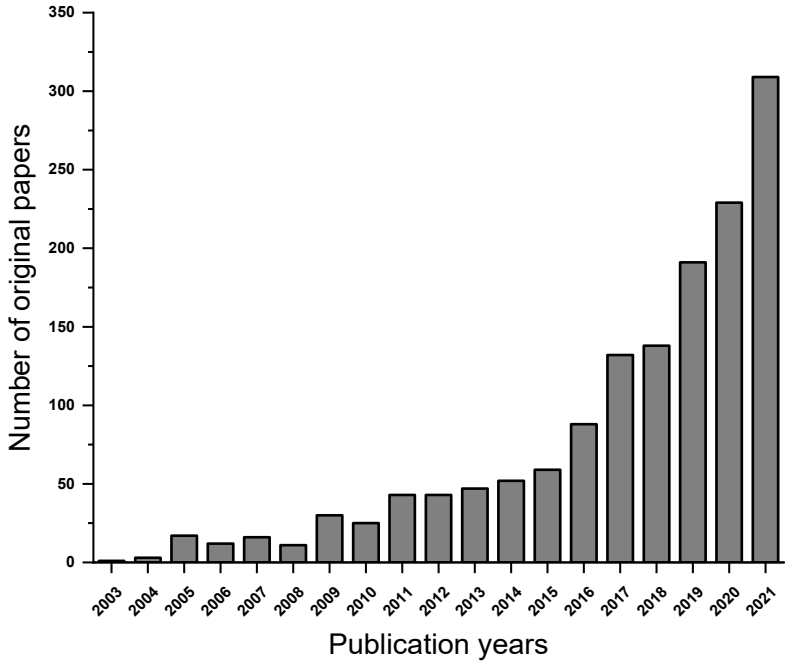


*Figure 8: Number of original papers published over 18 years dedicated to targeted lipidomics and different instrumental platforms. Web of Knowledge (www.webofknowledge.com) databases used for citation analysis.*

The reversed phase LC (RPLC), normal phase LC (NPLC), and hydrophilic interaction LC (HILIC) are the three most essential LC configurations for steroid and endocannabinoid studies.

#### 1.3.1.1. REVERSED PHASE LIQUID CHROMATOGRAPHY

Reversed-phase liquid chromatography (RP-LC) is the most widely used type of liquid chromatography for the analysis of endocannabinoids and steroids. The term reversed phase derives from this separation mode, which consists of a polar mobile phase containing binary mixtures of water and organic solvents and a non-polar stationary phase. Polar solutes interact with the mobile phase, which is polar like itself, prefer to drift on the mobile phase and retain little in the column. Apolar solutes, on the other hand, interact with apolar stationary phase and are hold more in the column. Sample components are attached by stationary phase non-specific hydrophobic interaction. In this separation mode, the retention is low if the solute is very polar. Increasing the polarity of the mobile phase increases the solute retention [40]. In RP-LC, solvent power is defined as a function of polarity, and the elution power of the RP-LC solvent varies depending on the polarity of the solvent. Polar solvents (such as water) provide weak elution in RP-LC, while non-polar solvents (such as tetrahydrofuran) provide strong elution.

Polymeric hydrocarbon structures and at the surface chemically functionalized silica particles are generally used in RP-LC as the stationary phase. The stationary phase type commonly used in RP-LC is C18 modified silica columns with octadecyl derivatives. Apart from C18, silica-based stationary phase types that carry C8, C4, -CN, and polar embedded C18 groups are also widely used. The porosity, pore size, surface area and particle structure and size of the stationary phase types are very important [41].

Since all unconjugated steroids are hydrophobic, reversed phase chromatography is more efficient for steroid separations. In RP-LC, the steroids are injected in a mobile phase with a high polarity, such 5% methanol in water (e.g.), and they all quickly adhere to the stationary phase at the front of the column, where they interact strongly with the hydrophobic resin coating. The gradient is set up to elute the more polar steroids first, followed by the less polar steroids, while increasing the amount of methanol (e.g.) in the mobile phase. Only a small portion of the sequence of steroid elution can be predicted

from the structure of the compounds, and changing solvent combinations, such as aqueous acetonitrile vs methanol, might change elution properties.

Mobile phase additives enhance the separation in RP-LC and the detection of endocannabinoids and lipid, particularly when they are used in MS systems. The most used additives are volatile buffers such as ammonium formate and ammonium acetate (2-20 mM); acetic or formic acid (0.1-2%) to support protonation or ammonia (0.1-1%) to support deprotonation. The use of various additives in mixtures is somewhat common, often at low concentrations of up to 10 mM for salts and up to 0.2% for acids. These additions improve LC separation and enhance ionization in the MS source even further [42].

#### 1.3.1.2. NP-LC, HILIC and SFC

The hydrophilic interaction liquid chromatography (HILIC) method is a variant of normal phase liquid chromatography used for the separation of hydrophilic substances. In HILIC, the stationary phase is silica or a polar substance in which silica has been modified with groups such as cyano, amino, and diol. The mobile phase, on the other hand, is highly organic with polar-water mixtures and is less polar than the stationary phase [43]. Organic solvent such as acetonitrile (60-97 %) with an amount of water (3-40 %) or acetone is a standard mobile phase used for HILIC. Water-miscible solvents such as tetrahydrofuran (THF) and dioxane can also be used. Since HILIC uses a mobile phase containing a high amount of organic solvents, the column back pressure is low, which simplifies the column usage at high flow rates and shortens the analysis time [44].

In normal-phase liquid chromatography, the stationary phase is highly polar (e.g., silica or polar functionalized silica), while the mobile phase is non-polar, such as hexane or THF. Here, the retention times of polar analytes interacting with the polar column filling material are longer than the less polar analytes. Therefore, the more polar sample components leave the column later and separation takes place.

Elution of analytes in supercritical fluid chromatography (SFC) is performed by using a supercritical fluid or subcritical fluids as the mobile phase. $CO_2$ is essentially mainly employed mobile phase, since it is inert, has relatively low critical temperatures and pressures (31 °C and 74 bar), is nonflammable, is inexpensive, and is environmentally

safe [8]. SFC is positioned as an alternative method for NP-LC due to fluidized $CO_2$'s comparable polarity to hexane. However, the eluotropic strength of $CO_2$ may be altered using modifiers, making the usage for RP-LC possible [45]. Since the 1980s, supercritical $CO_2$ has been utilized as an eluent, but its usage has been restricted by insufficient reproducibility and poor chromatographic performance [46].

#### 1.3.1.3. COMPARISON OF LC SYSTEMS

In NP-LC, the eluents—typical alkanes or chlorinated solvents—are incompatible with ESI-MS, which makes this method unsuitable for current lipidomics techniques and restricts comprehensive steroid and endocannabinoid identification [47].

Single lipid species from the same class are separated using RP-LC [48]. Conversely, HILIC separates lipids based on their polar head groups, which means that while lipid classes are differentiated from one another, all of the lipid species within a class elute extremely near to one another. For the investigation of complex lipids, RP-LC and HILIC can be used as an alternative or supplementary approach [49, 50].

## 1.4. MASS SPECTROMETRY FOR STEROIDS AND ENDOCANNABINOIDS

Mass spectrometry is an analytical technique to separate and analyze ions based on their mass to charge ratio (m/z). An ion source, a mass analyzer, a detector, and a data processing system are main components of a mass spectrometer.

### 1.4.1. IONIZATION

#### 1.4.1.1. ELECTROSPRAY IONIZATION

Electrospray ionization (ESI) is a soft ionization technique that has become the most widely used ionization mode for LC-MS systems regarding its high ionization efficiency and the applicability to a large variety of chemical substances. The first development of ESI was made by Malcolm Dole who contended to find out molecular mass of oligomers of synthetic polymers in 1968 [51]. John Fenn who was Nobel Prize laurate in 2002 coupled mass spectrometer with electrospray to analyze proteins and other large biological molecules by ESI by 1980s [52].

In a typical ESI, analyte and the organic solvent is pumped through a small stainless steel capillary needle where a high voltage is applied to create fine charged droplets. Then, the solvent is evaporated with the help of nitrogen gas while formed ions moving from the needle tip toward the instrument orifice. At the tip, positive ions start to accumulate as droplets which form a Taylor cone. The opposite occurs in the negative ion mode. In the meantime, the surface tension forces keeping the spherical structure of microdroplets become imbalanced and the inside of microdroplets are charged by the repulsive forces.

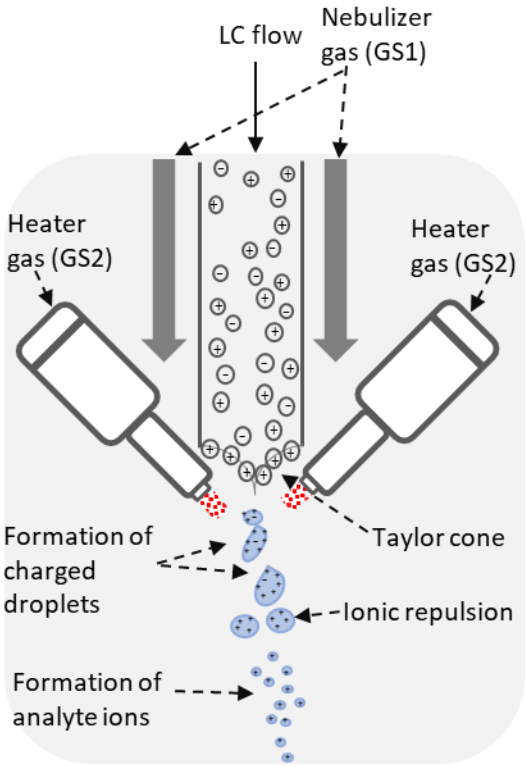


*Figure 9: Schematic representation of the ESI mechanism (Based on AB Sciex instrumentation)*

Finally, the generated ions at the droplet surface are emitted into the gaseous phase once the critical point is reached.

The emitted ions are transferred to the mass analyzer by a skimmer cone to be analyzed in the mass spectrometer [53]. The schematic representation of the ESI mechanism is presented in Figure 9.

### 1.4.2. MASS ANALYZERS

The main function of the mass analyzer is to separate the ions produced in the ion source according to their different mass–charge ratios (m/z) in electromagnetic fields. The quality of analytical performance is characterized by various parameters such as resolution, mass resolving power, m/z range and acquisition rate [42, 54]. Mass resolving power (and mass resolution, respectively) is defined as m/dm, where m indicates the mass and dm indicates the peak width necessary for separation at mass m (difference between two resolved ions in case of mass resolution). Mass resolving power is usually mentioned as full width at half maximum (FWHM), and it is the ratio of *m/z* of an ion to its peak width (measured at half of the peak height). The smaller the number of FWHM is, the better is resolving power therefore separation of ions with different *m/z* is better achieved by the mass analyzer. Resolving power is used as analytical performance parameter and usually a large number (up to 2,000,000). Mass accuracy is referred to as measurement error, and it is a metric describing the difference the measured m/z of an ion and theoretical m/z of that ion which is usually specified in terms of parts per million (ppm). Nevertheless, mass accuracy is not a constant value, but instable in a short time. Therefore, recommended frequently instrument calibration will ensure mass accuracy. Spectrum acquisition rate is the time needed to acquire scan of an m/z spectrum and its unit is Hz. High number of scans per peak is needed to deconvolute full scan mass spectra and to integrate correct peaks. The duration required to produce ions in ion source is orders of magnitude lower than the time they travel through the mass analyzer. Hence, spectrum acquisition rate is quite dependent on the specific instrument and the data system as well as the actual chromatographic peak width.

The mass analyzers can be also categorized according to their resolution as low- and high-resolution mass spectrometers. Later on, another significant parameter has been also added that refers to mass accuracy which provides further information by determining the elemental formula of a certain analyte. There are many types of mass analyzers such as single mass spectrometers including quadrupole (Q) and time-of-flight (TOF), linear ion-trap (LIT), and Fourier transform-ion cyclotron resonance (FT-ICR), and tandem mass spectrometers such as triple quadrupole (QqQ), and quadrupole time of-flight (QTOF). The choice of proper mass analyzer depends on the performance,

availability and cost effectiveness of the instrument and the purpose of analysis. Currently, quadrupole, LIT, QTOF, orbitrap and FT-ICR are mainly used in steroid and endocannabinoid assays. They are all available commercially from different manufactures and their exact parameters will differ depending on specifications. Table 1 compares typical analytical parameters of different mass analyzers.

*Table 1: Comparison of typical analytical characteristics of mass analyzers [18, 19]*

| Mass Analyzers | Mass Resolution* ($x10^3$) | Mass Accuracy (ppm) | Acquisition Rate (Hz) |
|---|---|---|---|
| **Quadrupole** | 1 | 100-1500 | 10-20 |
| **Orbitrap** | 100-800 | <5 | 10-40 |
| **TOF** | 40 | <5 | 10-100 |
| **QTOF** | 10-60 | <5 | 10-100 |
| **LIT** | 2 | 100-500 | 10-30 |
| **Ion Trap** | 4-20 | 100 | 10-30 |
| **FT-ICR** | 1000 | <1-2 | 0.5-2 |

*Mass resolution at full width half maximum (FWHM)

Recently, several combinations of two or more mass analyzers in one instrument (i.e., hybrid MS) are also frequently used. Especially triple quadrupoles (QqQs) or QTraps (combination of quadrupoles and linear ion traps) were used commonly for quantitative research in steroid and endocannabinoid assays. For this thesis two different MS instruments were used: QqQ API 4000 and QTrap Sciex 4500 LC/MS (AB SCIEX, Ontario, Canada). Thus, the following chapters will explain the technique and working properties of this mass analyzer.

#### 1.4.2.1. QUADRUPOLE

Quadrupole is a set of four conducting rods arranged in parallel with a gap in the middle (Figure 10). Its principles were first described by Paul and Steinwegen at the University of Bonn in 1953 [55]. By continuously changing the voltages applied to the rods, the m/z value of more than one molecule can be scanned. The effect of applying the two

electrostatic fields at right angles to each other, one of which is oscillating (resonating), is to create a resonance frequency (RF) for each m/z value, ions which resonate at the frequency of the quadrupole are to pass through it and be detected. In this way, ions across the mass range of the mass spectrum are chosen as the RF of the quadrupole is varied [56].

One quadrupole or more quadrupoles can be used in MS (i.e., QTRAP). When using a single quadrupole, only the m/z of the precursor ion is measured, so the specificity of the method is low. When more than one quadrupole is used, the ionized molecules are not separated according to their precursor mass alone. In the first quadrupole, the precursor ions of the analytes are filtered out. Then, in the q2 area, also called the collision cell, the process known as collision induced dissociation takes place and the precursor ion is fragmented using Argon or nitrogen gas and the product ions are released. The resulting product ion is passed through a second quadrupole (Q3). Thus, the molecule to be measured is measured more specifically by allowing only the determined precursor and product ion to pass through the quadrupoles to the detector. The data reaching the MS detector is for ion passage of a single analyte. The QTRAP 4500 is a quadrupole-linear

ion trap mass spectrometer and is operated with a DuoSpray ion source (Figure 11) consisting of an ESI probe.

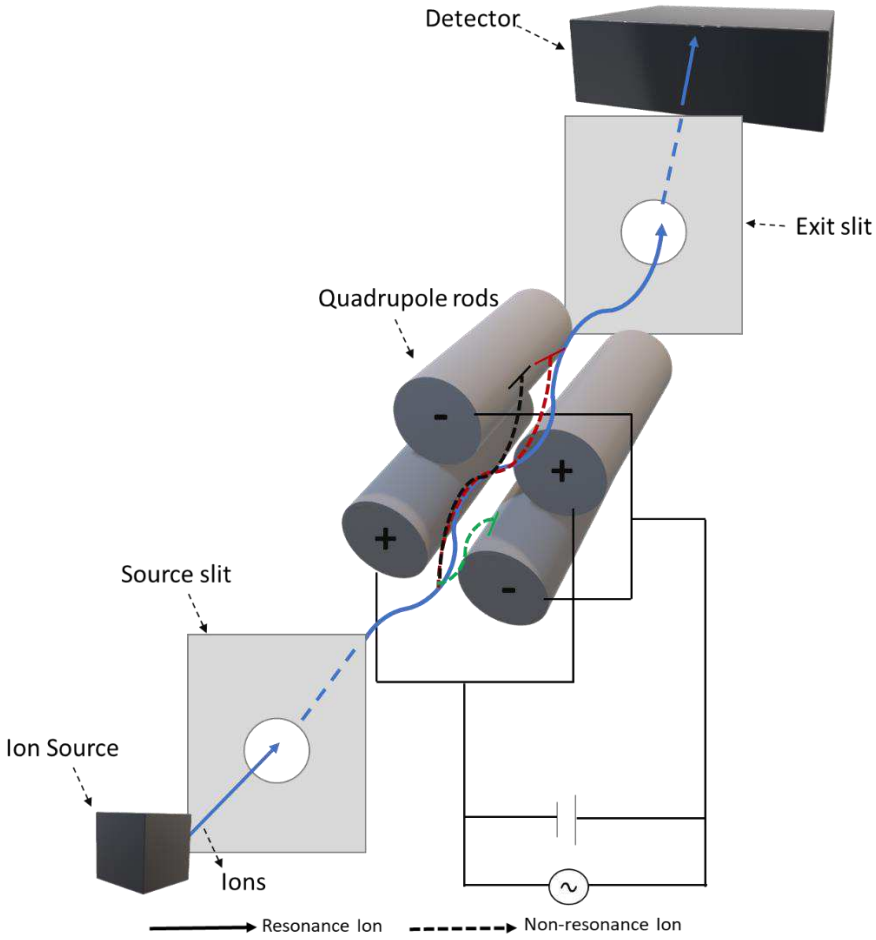


*Figure 10: Schematic illustration of a quadrupole mass analyzer (Adapted from [13, 16])*

The equations of travel of the detected ions following the stable trajectory are defined by the “Mathieu differential equations”. In Figure 12, Mathieu Stability diagrams for the stable regions for ions in quadrupole mass analyzer is shown. The amplitudes of the said oscillations are limited for any time interval.

They have “stable” trajectories if they are characterized by non-increasing amplitude, and they have “unstable” trajectories if they are characterized by ever-increasing amplitude. While ions with unstable trajectory are neutralized by impacting a quadrupole rod, those with stable trajectory pass through the mass analyzer. The stable region is characterized by two parameters:

$a = 4eU/mr_0^2\omega^2$ and

$q = 2eV/mr_0^2\omega^2$

and defined as operational parameters. Here $\omega=2\pi f$ is defined as the angular frequency (rad/s). When V, U, ω and r0 parameters are given, only ions with a certain mass number or a certain mass number range reach the detector by passing through the field and their oscillation amplitudes remain smaller than r0 at a certain value. All other ions hit the electrodes and are then taken out as gas [57-59].

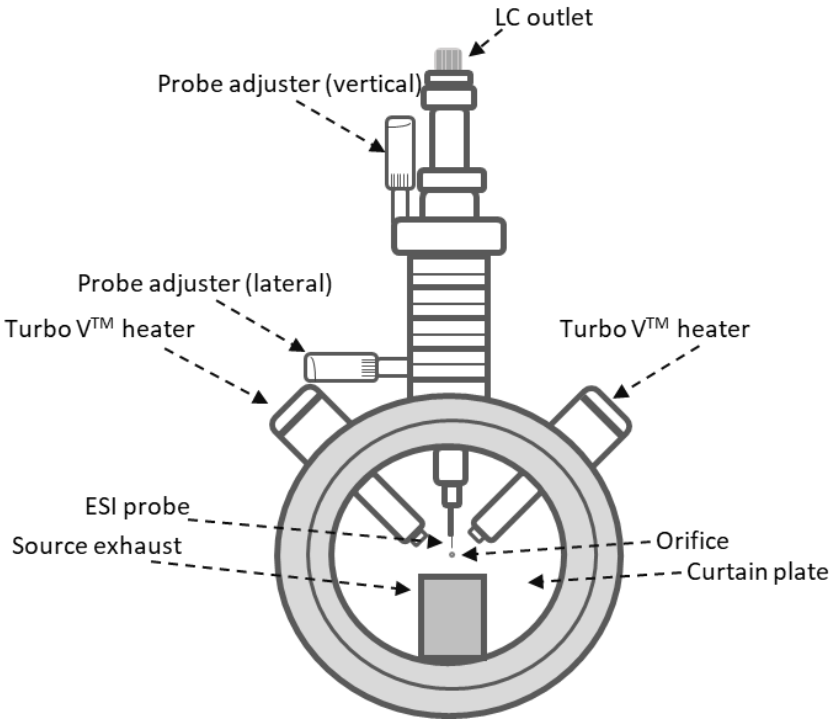


*Figure 11: Schematic build-up of the DuoSpray ion source available for QTRAP 4500 (Sciex, Washington DC, USA) (Based on AB Sciex instrumentation)*

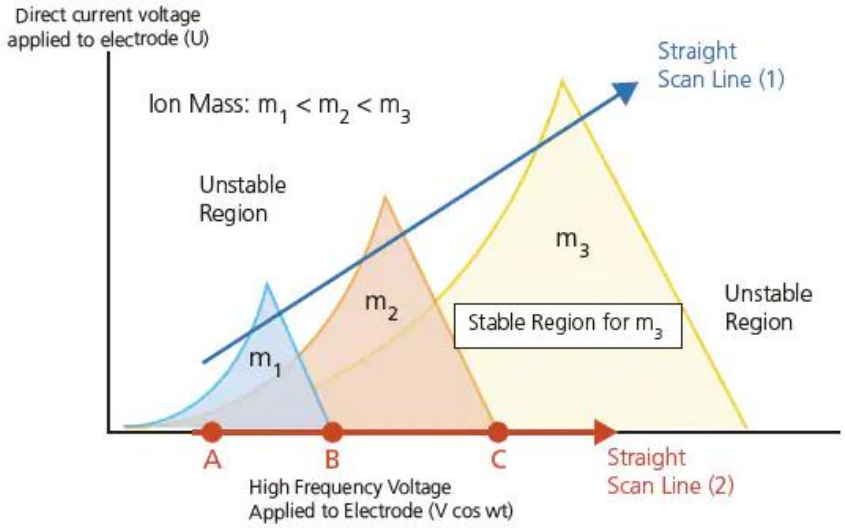


*Figure 12: Mathieu Stability diagrams for the stable regions for ions in quadrupole mass analyzer [13, 17]*

Selected reaction monitoring (SRM), also known as multiple reaction monitoring (MRM) if more than one analyte is measured at the same, is a technique used in tandem mass spectrometry in which a precursor ion's fragmentation reaction product is selected for detection in the second stage of the mass spectrometer and an ion of a particular mass is selected in the first stage. The use of SRM mode increases sensitivity and signal repeatability because the device focuses on only one transition at all transition times.

### 1.4.2.2. TIME-OF-FLIGHT (TOF)

The first linear TOF mass spectrometer, first described by Stephens [60] in 1946 and designed by Wiley and McLaren [61] in 1955, was the first device to be commercialized. In a time-of-flight analyzer, ions are accelerated in an electric field and move in a fieldless (free) drift region, finally reaching the detector. These ions are possessed of same kinetic energy that results in different velocities of ions of different m/z values, thus a correlation between the time required to cross the tube and their m/z can be established in an equation for conservation of energy, from potential energy to kinetic energy:

$E_k = zeV = mv^2/2$

where z is the number of charges of the ion, e is the charge of electron, V is the potential applied to the ions, m is ion mass and v is its velocity. v can be expressed as the ratio between the distance of the drift tube (d) and the time required to cross it (t), which leads to the equation:

$m/z=2eV(t/d)^2$

Light ions reach greater velocities than heavy ions and require less time to reach the detector. The measurement of time differences in the detector allows the dependence of the masses of the ions to be determined. TOF analyzers working principle can be seen in Figure 13.

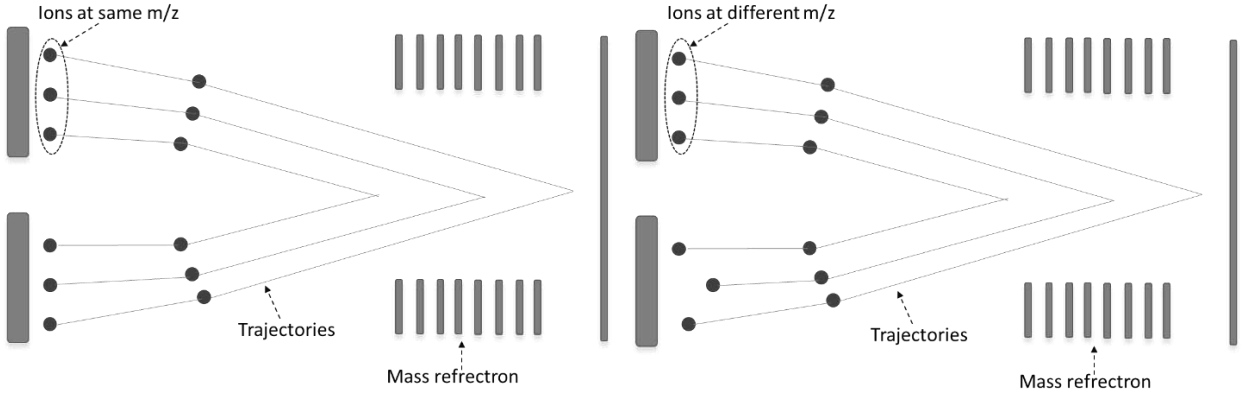


*Figure 13: Schematic working principle of an ion mirror of TOF analyzer [18]*

### 1.4.3. DETECTOR SYSTEM

The last component of the mass spectrometer is the detector, and it records an induced charge or current created when an ion passes by or collides with a surface [47]. Detector systems transform ions into a signal that computers can monitor and store. Electron multipliers are the most commonly used detectors in MS systems. Sciex's QqQ and QTrap devices are equipped with continuous electron multipliers (CEM). TOF analysers require detectors that can calculate ion arrival time very precisely and this analyzer is the microchannel plate (MCP).

Because single ions emit a very little current when they reach the detector, this signal must be amplified to obtain high instrument sensitivity. A strong counter potential accelerates ions entering from the mass analyser in the first phase of detection. They

collide with the detector's surface, releasing a slew of secondary particles. The secondary particles in negative mode analysis are positive ions, whereas the generated particles in positive mode analysis are electrons. As secondary particles (both positive ions and electrons) contact with the surface, the cascade of impacts begins. Multipliers generally enhance the signal $10^6$ times as the cascade progresses. The electron current may then be monitored with ease.

### 1.4.4. SCAN MODES

The quality of the results will mostly depend on the type of mass analyzer utilized and the specific type of measurement or scan mode used, since there are several different types of mass spectrometers now available.

In general, there are two types of techniques: targeted and untargeted techniques for MS data acquisition.

Triple quadrupole (QqQ) instruments have been considered for a long time as the gold standard for targeted quantitative measurements with its common mode multiple reaction monitoring (MRM) due to its utmost speed, sensitivity, dynamic range, and multiplexing capability. However, they are unable to achieve the best performance in qualitative evaluations because to their shorter duty cycle in full scan mode. For qualitative tests, QqQ mass analyzers are less sensitive and have a slower scan speed than QTOF or ion trap-based mass analyzers. However, the disadvantage is that these full scan-based devices sometimes are unable to offer the same specifications in sensitivity and dynamic range as a QqQ for specialized quantitative analysis.

The standard ion path of a QqQ mass spectrometer provides the basis for the mass analyzer part of a QTRAP System. But unlike traditional QqQ systems, a QTRAP System's third quadrupole (Q3) can also be used as a LIT. The QTRAP System offers full capabilities as a QqQ mass spectrometer owing to the dual functionality of Q3, but it also has more efficacious qualitative scan functions.

High quality data of preselected analytes are acquired by targeted acquisition, which is most frequently carried out using QqQ equipment.

### 1.4.4.1. SELECTED REACTION MONITORING (SRM)

A quantitative analytical assay known as "selected reaction monitoring" (SRM) is performed using a triple-quadrupole (Figure 14), quadrupole-ion trap. SRM exclusively

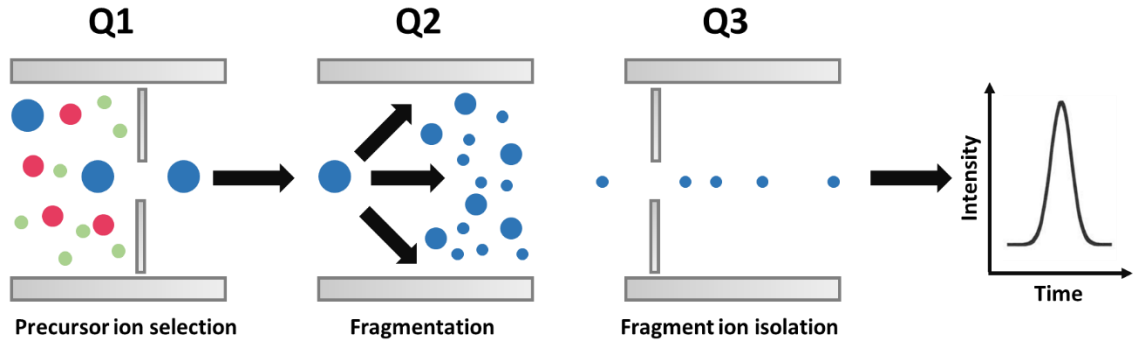


*Figure 14: Schematic depiction of a triple-quadrupole system in MRM/SRM mode and one fragment´s recorded MRM signal*

monitors a single fixed mass window (typically with unit mass resolution 0.7 Da), whereas MRM (Multiple Reaction Monitoring) rapidly scans over several (extremely narrow) mass windows, capturing evidence of several fragment ion masses concurrently: MRM, then, is the process of applying SRM to numerous product ions derived from one or more precursor ions.

### 1.4.4.2. MULTIPLE REACTION MONITORING (MRM)

The most common technique of using a triple quadrupole MS/MS for quantitative analysis, which enables increased sensitivity and selectivity, is MRM. One particular precursor ion of interest is filtered by the first quadrupole. Ions produced in an ion source with a different m/z can't travel through Q1. By colliding the precursor ion with a neutral collision gas, such as nitrogen, the collision cell is created to generate a distinctive product ion. Collision Induced Dissociation is the term of this procedure (CID). The third quadrupole is where the produced product ions are moved, and only ions with a certain m/z are permitted to pass through. In Q3, all further product ions are filtered out.

As a result, MRM mode functions as a double mass filter, are greatly reducing noise and enhancing selectivity.

#### 1.4.4.3. MRM$^3$

Despite being widely acknowledged as the most quantitatively accurate and sensitive MS-based approach, SRM has limitations due to a comparatively low resolution in both precursor and fragment m/z filtering. Due to interferences that might result from the decreased resolution, it is possible for quantitative readouts to be made by integrating peak groups that are unrelated to the target analyte. By obtaining high resolution MS/MS, PRM and HR-MRM can minimize these impacts. Using a method known as MRM cubed (MRM$^3$), implemented on QTRAP instruments, is another way to overcome this problem. Here, a linear ion trap (LIT) utilizes as the third quadrupole (Q3), allowing the capture of MRM product ions and enabling the performance of a second fragmentation step. This improvement makes it possible to filter and capture an ion that was previously produced in the collision cell. The specificity and sensitivity can then be improved by further fragmenting this ion and accumulating it in the LIT prior to detection. Although a full MS3 spectrum can also be obtained, if specific experiments have been performed, the MRM3 mode can also be used in a targeted approach [62, 63].

### 1.4.5. DATA PROCESSING IN TARGETED LIPIDOMICS

With the help of hyphenated mass spectrometry (MS) techniques, targeted lipidomics analysis measures the concentrations of pre-selected lipids in samples. Higher sensitivity and specificity are made possible by targeted lipidomics when identifying and quantifying lipids. Low abundant lipids may be accurately identified and quantified when triple quadrupole mass spectrometry is used in MRM mode. The data analytics, however, is still a critical factor because of internal standards and quantitation calculations.

These factors that need to be taken into consideration to achieve the goal of accurate analysis:

1) Ability to choose one or more internal standards for the same or different lipid classes when assigning internal standards.
2) Ion abundance adjustments that are normalized based on internal standards are normalized depending on the internal standards chosen.
3) Using the chosen internal standards, normalization of the ion abundance adjustments.

4) Isotopic levels of lipids may overlap, in which case a correction is necessary to separate one or more overlapped lipids.
5) For lipid quantification calculations, it is necessary to monitor the spiking IS concentrations.
6) The lipid concentrations are calculated using the correlation between the ion abundances of IS and lipids.
7) The replicated data must be aligned across the replicates/samples in order to remove redundant and missing peaks.

Quantitative mass spectrometry approaches are used for absolute, relative and semi-quantitative quantification in lipidomic studies. Relative quantification, which refers to determining the ratio of lipid components between two samples without needing to know the exact amount, is frequently utilized in biomarker discovery and other studies. In LC-MS-based research, relative quantification techniques are frequently used [64]. Stoichiometric variations between lipids are not required, although relative quantification is frequently adequate when relative changes are of importance, such as between diseased and control populations [65].

When accurate quantitative levels within 10–20% cannot be achieved yet stoichiometric differences between lipid species are of importance, semi-quantification is employed. For semi-quantification, both an internal calibrant and an external calibration are frequently utilized [66, 67].

Any efforts to measure lipid molecular species at the concentration level are better referred to as "accurate" (or absolute) quantification. Accurate quantification is the approach to quantify lipid molecular species at the concentration level within quantitative bias since absolute quantitation is impossible due to insufficient standards. The methodology must be used in combination with the appropriate standards, just like with any quantitative strategy, to be beneficial. Accurate quantification of lipids at the total composition level is attainable with a single internal standard when utilizing an infusion or normal phase methodology that allows all lipids of a particular class to be assessed nearly simultaneously, as long as the method is developed to target common precursor ions and neutral losses for a given lipid class [64]. For each lipid being measured, accurate

quantification often uses internal standards that have been isotopically labelled and/or external calibration curves that are matrix-matched. Because of the huge diversity of the lipidome, the lack of relevant standards to account for this diversity, and the expense of purchasing hundreds of standards, this quantitative technique has limited applicability to untargeted lipidomic research.

## 1.5. SAMPLE PREPARATION TECHNIQUES AND EXTRACTION FOR STEROIDS AND ENDOCANNABINOIDS

### 1.5.1. PROTEIN PRECIPITATION

With the advent of tandem mass spectrometry, protein precipitation has become a popular sample preparation protocol in bioanalysis. The analysis of low molecular analytes like lipids requires the removal of proteins from the sample. Proteins contaminate column and ion source and lead quickly to performance decline in LC separations and MS sensitivity. The precipitation process is used to remove proteins from sample matrix. In accordance with the purpose of the research, the precipitation of proteins can be done in the fraction obtained by centrifugation and filtration or directly in the sample matrix. Many proteins can be precipitated by the addition of water-miscible organic solvents such as acetone, ethanol, methanol, acetonitrile, mixtures of those organic solvents or salting-out reagents such as $ZnSO_4$ [3]. Many lipids like steroids are bound to plasma proteins; for the determination of the total concentration, it is mandatory to release protein-bound analyte. This is achieved when organic solvents are used for protein precipitation. There are currently just a few LC-MS/MS steroid and endocannabinoid profiling techniques that only rely on protein precipitation, other techniques utilize protein precipitation in combination with other sample preparation techniques (e.g., salting out and SPE). Protein precipitation methods have been the widely used sample preparation procedures for steroids [3, 68, 69] and endocannabinoids [23, 70, 71].

### 1.5.2. EXTRACTION

When developing an extraction protocol for lipids, such as steroids or endocannabinoids, many factors must be taken into account. As previously stated, lipids can have a wide range of polarity, and extraction protocols only cover a subset of all classes of interested analytes. As a result, the type of lipids or endocannabinoids recovered from a sample is

determined by the solvent used for extraction. Because certain lipids and endocannabinoids have some interaction with proteins and polysaccharides in particular regions of the cell, the kind of sample and the location of the lipids in the sample might be important. Van der Waals forces, hydrogen bonds, and ionic bonds can all be used to interact between lipids and those components [72].

#### 1.5.2.1. LIQUID-LIQUID EXTRACTION

Liquid-liquid extraction has been the most frequently used sample preparation method for years and is based on the principle that the target analyte is dispersed between two immiscible liquids, one usually water and the other organic solvent. In this way, the analytes are separated from the sample medium according to their polarity, enriched in the organic solvent phase. Methyl tert-butyl ether (MTBE), ethyl ether, dichloro-methane, and n-hexane are the most often utilized steroid extraction agents in laboratories [73, 74].

Liquid-liquid extraction has some drawbacks such as excessive solvent consumption, formation of emulsion phase during extraction, obtaining extracts that do not have the required purity, insufficient removal of solvents and inability to obtain sensitive quantitative results [75].

For steroid analyses [74, 76, 77] and endocannabinoids studies [78, 79], liquid-liquid extraction technique is commonly used.

#### 1.5.2.2. SOLID PHASE EXTRACTION

Solid phase extraction (SPE) is a separation method in which dissolved or suspended compounds are separated from other compounds in a liquid mixture based on their physical and chemical characteristics [75]. SPE method needs a particular SPE column (SPE cartridge), which eventually raises the study cost.

Conditioning, sample loading, washing, and elution are the four essential processes in a solid phase extraction (Figure 15). In the first stage, a non-polar or slightly polar solvent is equilibrated with the cartridge to moisten the surface and enter the bound phase. After that, the sample is introduced into the cartridge. The analytes in the sample will remain on the sorbent as the sample passes through the stationary phase, while the other components flow through the cartridge. The cartridge is rinsed with buffer or solvent in

the third stage to eliminate any remaining contaminants. The analyte is then eluted using a non-polar solvent or a pH-controlled buffer.

There are a variety forms of SPE stationary phases: reversed phase (non-polar), normal phase (polar), ion exchange (anion/cation), as well as mixed-mode phases, which include the characteristics of more than one type of SPE material. Non-polar SPE stationary phases contain C18, C8, C6, C4, C2, phenyl, cyclohexyl, and cyanopropyl nonpolar functional groups. Van der Waals forces are responsible for the interaction between the analyte and sorbent surface.

SPE, in contrast to LLE, is suited for both medium-polar and non-polar steroids, and it may more efficiently separate the analyte from the matrix and increase analyte recovery rates [3].

For sample preparation method of steroids [68, 80, 81] and endocannabinoids [82, 83], SPE has been a high demand.

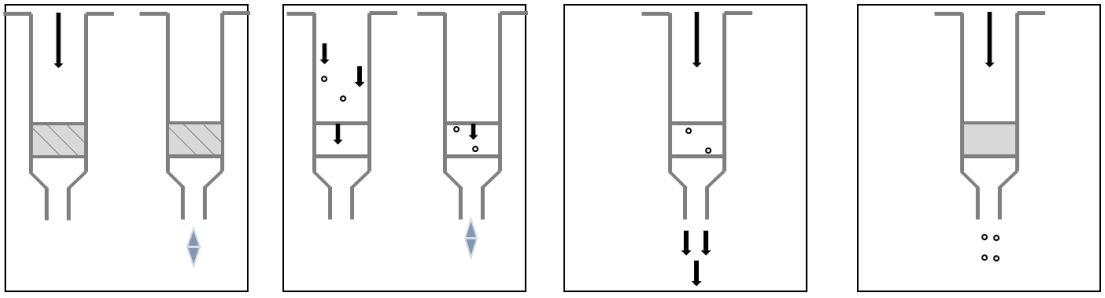


*Figure 15: Schematic working principle of SPE where ▼is the contaminants and ○ is the analyte.*

#### 1.5.2.2.1. ON-LINE SOLID PHASE EXTRACTION

In recent years there has been considerable interest in on-column extraction as a high-throughput on-line extraction technique [84]. SPE, in general, is a manual off-line method that expands the procedure considerably and introduces errors in the laboratory workflow (analyte loss, degradation and/or adsorption during solvent evaporation, errors while tube handling). Online SPE addresses all the aforementioned problems by automating the sample preparation and analyte enrichment steps [85].

Off-line SPE methods have the disadvantage that they can be laborious and time-consuming to complete, sometimes needing several stages to produce a concentrated extract appropriate for instrumental analysis, of which just a small amount is actually injected onto the chromatographic column [24]. On-line SPE has a number of benefits over off-line SPE. Through increased sample throughput and shorter sample preparation times, the use of on-line SPE techniques has enabled the creation of faster approaches. It is possible to automate the stages of conditioning, washing, and elution, and some systems allow you to extract one sample while another is being analyzed by LC. Minimal risk of contamination of the sample or sample extract, elimination of analyte losses by evaporation or by degradation during sample preconcentration, and enhanced precision and accuracy are other significant advantages of on-line coupling. In contrast to off-line SPE methods, where just a small amount of the extract is injected into the column, higher sensitivity can be achieved in on-line setups due to the transfer and analysis of the whole of the extracted species to the analytical system [86, 87].

#### 1.5.2.3. AUTOMATED SOLID PHASE EXTRACTION

Although they may be operated manually, paralleled sample preparation solutions for LC-MS/MS based on 96-position arrays are frequently carried out on generic liquid handling robotic systems (see Fig. 16). These devices are set up to create extracts into a secondary sample carrier, which are then manually transferred into the LC-system's autosampler, providing an "off-line" solution. Although this method is discontinuous, it has the advantage that a single sample preparation module may be used for a number of LC-MS/MS systems [88].

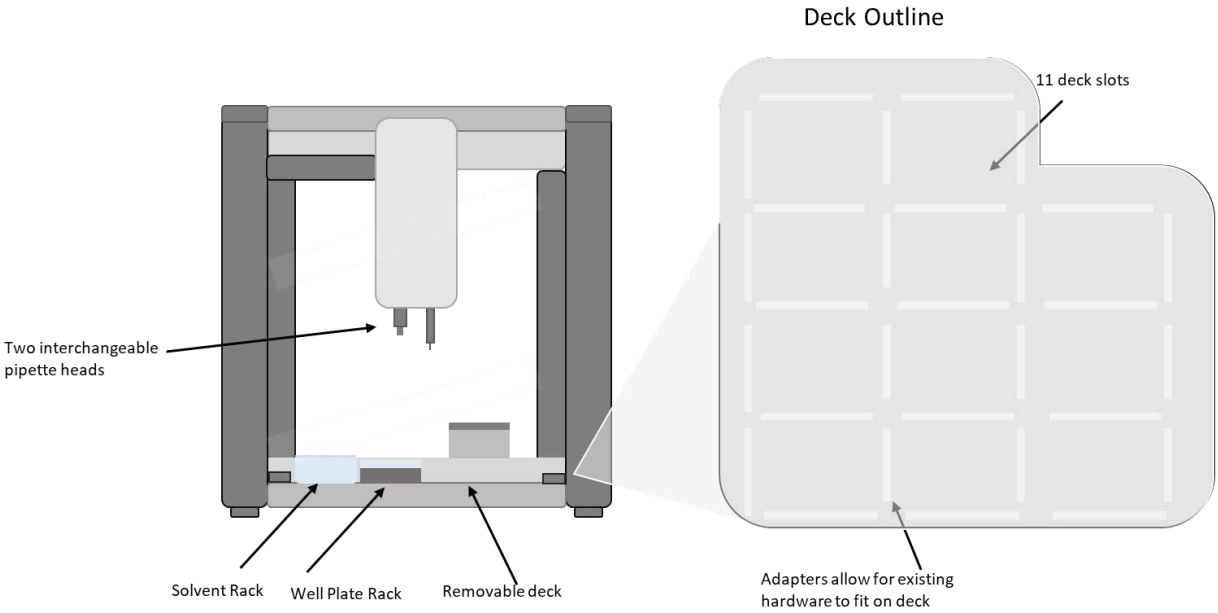


*Figure 16: Schematic build-up of Liquid Handling station (left) and deck outline (right) (OT-2, Opentron, New York, USA) (Based on Opentron instrumentation)*

They stand for "open" systems that enable variable configurations for a wide range of equipment (such as shakers, pipette arms, vacuum stations, and grippers for micro titer plates). Starting over a decade back, complete liquid handling systems have been employed to handle LC-MS/MS batch analyses.

In addition to spiking the internal standard solution to a sample aliquot, robotic liquid handling systems for sample preparation may also read the bar codes on main tubes and create a sample list [88, 89].

A typical work-flow protocol involves scanning the bar code on sample tubes, re-suspending whole samples (if necessary), transferring a sample aliquot into a well, adding the internal standard solution to the well, mixing, conditioning the extraction material (single cartridge or cell in a 96-position array), applying the sample spiked with the internal standard to the SPE material, parallel washing, and parallel elution of the extract from the sample. Most systems operate 4 to 8 pipetting channels. It is technically challenging to produce a quick enough travel of fluids through the SPE materials without using vacuum or positive pressure [88-90].

For batch studies of large series without the need for rapid turnaround times for individual samples, automated SPE can be practical. While the daily handling may be simplified to a few basic interventions like re-filling liquids and consumables, discarding trash, and handling the plates, the setup and programming of a sample preparation module unquestionably demands professionals.

For large-volume aqueous solution extractions, automated SPE devices automate the three processes of SPE (cartridge conditioning, sample loading, and elution).

Automated SPE only needs an operator for 15 to 30 minutes before running unsupervised for two to three hours. Due to the time savings, one can do other duties while the SPE is being carried out. For a normal vacuum manifold extraction, labor accounts for more than half of the sample preparation cost. Automated SPE allows for unattended operation, which lowers the cost of analysis while greatly improving output. An analyte recovery of >90% may regularly be achieved with a robust, customized sample preparation method that is tailored to your analytical aims and carried out by knowledgeable personnel [89, 91, 92].

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## 1.7. LIST OF FIGURES

## 1.8. LIST OF TABLES

## 2. AIM OF THE WORK

The general aim of my research at University of Tübingen was to develop new methods and optimize workflows for the analysis of lipids, in particular steroids and endocannabinoids (ECs) for routine clinical assays. The developed methods were then applied for analyzing steroids and ECs of human saliva and cerebrospinal fluid (CSF). My work was divided into 3 main analytical projects, and for 3 of them, the goals were to develop new LC-MS/MS methods. My research also included analysis of several sets of real samples for routine clinical research.

The first study was aiming to establish a new sensitive and accurate analytical method developed to quantify cortisol and cortisone in saliva in a large-scale stress study via micro-UHPLC-ESI-MS/MS with surrogate calibrant method. In this method, samples were collected in Salivette® Cortisol. The research also described the instrument performance (retention time variability of targeted analytes) within each batch, between different batches and lot to lot of investigated capillary columns over time for a full clinical study with large number of clinical samples (close to 2000) over an extended period.

In a second study, the goal was to develop a new sensitive and simple UHPLC-ESI-MS/MS method for analyzing ECs by a simple monophasic extraction procedure in human CSF using different quantification approaches such as surrogate calibrant and surrogate matrix. A challenge in this study was to develop suitable analytical method for the retention and separation of all target analytes due to structural similarity and low abundance.

In a third study, it was aimed to develop a new method for salivary melatonin and cortisol in UHPLC system. In this method, samples were collected in passive drool. For this reason, a range of saliva samples from healthy volunteers were taken to monitor the change in cortisol and melatonin concentration levels over the course of 24 hours in a circadian rhythm. The optimization of several parameters was made and in conclusion, with LOQs of 15 pg/mL for melatonin and 104 pg/mL for cortisol in saliva the method was established. But as the baseline of the melatonin in a circadian rhythm was below 15 pg/mL, the method could not be published, thus more research needs to be done.

# 3. RESULTS AND DISCUSSION

## 3.1. Publication I Fast accurate quantification of salivary cortisol and cortisone

# Fast accurate quantification of salivary cortisol and cortisone in a large-scale clinical stress study by micro-UHPLC-ESI-MS/MS using a surrogate calibrant approach

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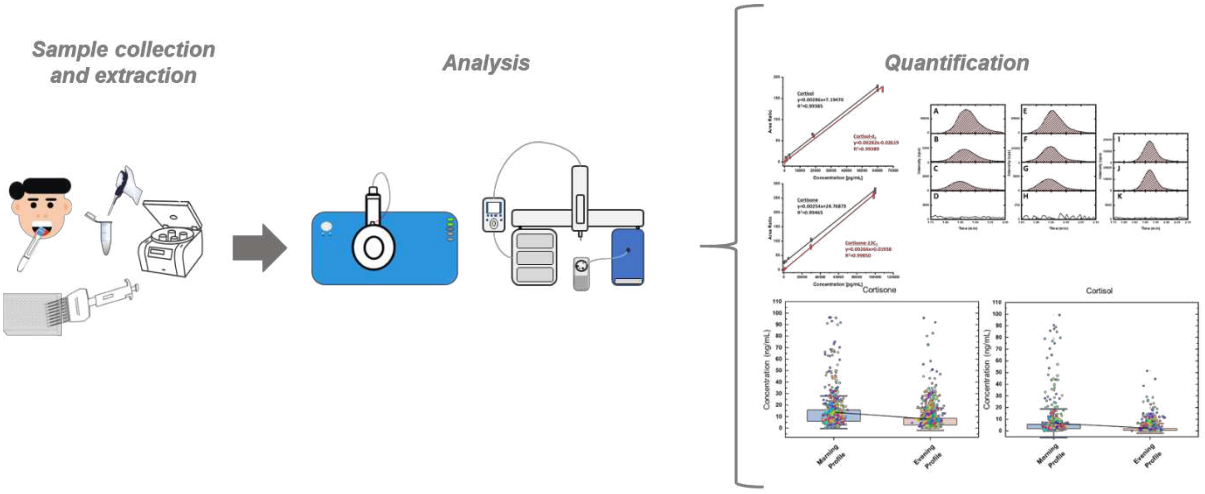


**Graphical Abstract**

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# Fast accurate quantification of salivary cortisol and cortisone in a large-scale clinical stress study by micro-UHPLC-ESI-MS/MS using a surrogate calibrant approach

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ABSTRACT

Cortisol and cortisone are common markers for stress and thus preferentially analyzed in matrices that allow non-invasive sampling such as saliva. Though the major drawback of immunoassays is lack of specificity due to cross reactivities, they are still most commonly used for quantification of steroid hormones. To overcome such problems, sensitive methods based on liquid chromatography-mass spectrometry are becoming more and more accepted as the golden standard for steroid bioanalysis as they achieve accurate quantification at trace levels for multiple analytes in the same run. Along this line, the aim of this study was the development of a new microflow UHPLC-ESI-MS/MS method for the measurement of salivary cortisol and cortisone, which due to its microflow regime provides enhanced sensitivity and is more ecofriendly. The developed method implemented sample preparation by Solid-Phase Extraction (SPE) in a 96-well plate format. Data acquisitions were carried out in MRM (multiple reaction monitoring) mode. The quantitative determination of endogenous compounds in saliva remains a challenge since analyte-free matrix is lacking. Hence, a surrogate calibrant approach with cortisol-$d_4$ and cortisone-$^{13}C_3$ was applied for the target compounds in the presented method. A number of factors were optimized and the method validated. The lower limit of quantitation (LLOQ) was 72 and 62 pg $mL^{-1}$ for cortisol and cortisone, respectively. Linear calibration was achieved in the range from 0.062 to 75.5 ng $mL^{-1}$ for cortisol-$d_4$ and 0.072 to 44 ng $mL^{-1}$ for cortisone-$^{13}C_3$. The performance of the method was also evaluated via proficiency test for salivary cortisol. Finally, it was applied successfully to evaluate cortisol and cortisone concentrations in multiple batches in routine clinical stress study samples (4056 total injections with 1983 study samples). Moreover, the instrument performance (in particular retention time variability) within each batch, between different batches and lot-to-lot of 5 investigated capillary columns over time is described. The work documents that micro-UHPLC-ESI-MS/MS is suitable and robust enough to carry out a full clinical study with greater than 1000s of samples over an extended period if adequate internal standards can be used.

## 1. Introduction

According to the World Health Organization (WHO), stress has been classified as the health epidemic of the 21st century. Hence, detection of reliable and easily accessible physiological markers for stress is of great scientific interest, potentially enabling explanation and modelling of individual differences in stress vulnerability [1,2]. Glucocorticoids, and in particular cortisol and cortisone, are therefore widely investigated in (psychosocial) stress research [3,4], where the need arises to take several samples in order to investigate individual reactivity and recovery of the hypothalamus–pituitary-adrenal axis (HPA). In this project, we were interested in exploring the physiological and neural substrates

*Abbreviations:* **$NH_4F$, ammonium fluoride**; **ELISA, Enzyme-linked immunosorbent assay**; **ER, extraction recovery**; **ESI, electrospray ionization**; **FDA, United States Food and Drug Administration**; **IS, Internal standard**; **QC, Quality Control**; **LLOQ, Lower limit of quantitation**; **LOD, Limit of detection**; **LUM, Luminescence Immunoassay**; **ME, Matrix effect**; **MeCN, Acetonitrile**; **MeOH, Methanol**; **MM, Master mix**; **MRM, multiple reaction monitoring**; **PE, process efficiency**; **RF, response factor**; **RSD, relative standard deviation**; **SPE, Solid-Phase Extraction**; **ULOQ, Upper limit of quantitation**; **$ZnSO_4$, Zinc sulfate**.

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of stress induction in healthy women and men using a validated stress paradigm developed for the magnetic resonance imaging (MRI) environment [5].

There are a variety of pre-analytical and analytical factors to be considered in the course of steroid hormone analysis such as cortisol. In plasma, cortisol is to about 90 % bound to cortisol-binding globulin and to minor extent to albumin [6]. Hence, a low percentage is available in free form. Due to its invasive sampling, it was not of first choice in this work. Hair has been suggested for the measurement of chronic stress and has been established as preferred method for long-term and retrospective determination of endogenous stress markers [7–12]. For the current project with focus on short term stress response and multiple sampling time points in an MRI tube, salivary cortisol measurement seemed more convenient and suitable for non-invasive sampling in this context. In saliva, cortisol is present in free form and can be directly measured along with its 11β-hydroxysteroid dehydrogenase (11β-HSD) metabolite cortisone, which has been shown to be a better marker than salivary cortisol due to its stronger correlation with serum total and free cortisol and higher salivary concentration [13]. For these reasons, determination of cortisol and cortisone in saliva has become a viable and preferred option in the diagnosis of endocrine diseases [14–19] as compared to other types of biological samples such as plasma and serum which require invasive sampling [20,21]. Sweat is yet another matrix but still less common in cortisol analysis [22]. Volumetric absorptive micro sampling, which enables minimally invasive collection of fixed-volume capillary blood, could become a promising alternative to saliva in the future as recently proposed [23].

Cortisol and cortisone concentrations in plasma, saliva and urine are underlying diurnal variations following a circadian rhythm with usually highest concentrations in the morning [24,25] and higher levels normally in males than females [1]. Hence, sampling in this study has been undertaken by consideration of the diurnal rhythm. It has been reported that cortisol in saliva is stable for a week at 4 °C and for a month when stored frozen [6]. Its relatively high stability facilitates the implementation of the study plan and allows collection of samples until analyzed in larger analytical batches.

Salivary cortisol and cortisone are therefore widely analyzed as stress biomarkers and specific sampling devices have been developed for saliva matrix [26,27]. For many years, immunoassays were preferentially utilized to analyze steroid hormones including the glucocorticoid cortisol in body fluids [28]. Despite their potential drawback of cross-reactivities and the resulting lack of assay specificity, they are still most commonly used for quantification of steroid hormones including cortisol and cortisone [4,28–30]. In fact, reactivity with structurally related components or metabolites is a problem in particular at low concentrations. Furthermore, poor agreement between distinct commercially available immunoassays has contributed to the lack of reliability of these assays. Owing to the structural similarities between distinct steroids, it is, however, mandatory to validate the immunoassays to ensure the absence of cross-reactivities with other interfering molecules [31]. In clinical routine testing, it may still be the dominating assay format due to its excellent high throughput capacity [6], but seems to become increasingly replaced by liquid-chromatography tandem mass spectrometry assays [32]. For research purposes, endocrinology societies and leading journals in the field of clinical chemistry recommend the use of more reliable approaches in clinical assays such as the preferential use of LC-MS/MS which can differentiate between structurally very similar steroids and allow the selective analysis of a panel of target analytes [4,28,32–35]. Numerous LC-MS/MS assays have already been proposed for glucocorticoids, especially for cortisol and cortisone in biological samples [36–39] and the state-of-the-art for these steroid hormones has been reviewed recently [38]. Derivatization can bring more sensitivity, which is crucial for low abundant steroids in biofluids, such as estradiol in male saliva, being capable of reaching accurate quantification at trace levels [40–42]; it is, however, usually not required with modern LC-MS/MS instruments for salivary cortisol and cortisone. In a few studies, it has been shown that microflow LC-MS/MS has the potential to further increase the sensitivity of direct steroid analysis methods through increased ionization efficiency in the lower flow regimes [40,43]. While these methods benefit from less solvent consumption and align them with future green technologies, it is still to be elucidated whether they are robust enough to carry out large scale clinical studies that run over an extended period of time.

In this work, we developed and validated a sensitive accurate analytical method for the measurement of salivary cortisol and cortisone using microflow UHPLC-MS/MS without compound derivatization. To eliminate problems with matrix effects and enrich the target analytes, solid phase extraction by a 96-well plate format was used for sample preparation. In the literature, there are several examples of quantitative analytical methods that have been reported so far for the determination of cortisol [23,37,44–46] and cortisone [46,47] using standard HPLC coupled to a triple-quadrupole tandem MS instrument. Since there is no analyte-free matrix available for matrix-matched calibration, a surrogate calibrant approach has been adopted [48,49]. Validation of the assay was carried out according to regulatory guidelines [50]. The final validated micro-UHPLC-ESI-MS/MS assay was employed for the analysis of saliva samples from the neuroimaging stress study to elucidate the practical utility and robustness of the micro-UHPLC format. Inter-day batch performance of the micro-UHPLC-MS/MS system spanning a period of almost two years is discussed to address the question whether microflow LC-MS/MS assays can fulfil the robustness requirements of large clinical bioanalysis studies.

## 2. Experimental

### 2.1. Materials

Cortisol was purchased from Cayman Chemical (Ann Arbor, MI, USA). Cortisone was supplied by Sigma Aldrich (Munich, Germany). Cortisone-2,3,4-$^{13}C_3$ was purchased from IsoSciences (King of Prussia, PA, USA). Cortisol-9,11,12,12-$d_4$ and cortisone-2,2,4,6,6,9,21,21-$d_8$ were acquired from Toronto Research Chemicals (Toronto, Ontario, Canada) (for structures of analytes see Fig. 1). Methanol (MeOH) and acetonitrile (MeCN) were ultra LC MS-grade and obtained from Carl Roth (Karlsruhe, Germany). As mobile phase additive, ammonium fluoride ($NH_4F$, 98 %, ACS grade) was purchased from Merck (Sigma Aldrich) (Munich, Germany). Zinc sulfate ($ZnSO_4$, as $ZnSO_4{\cdot}7H_2O$) was acquired from Sigma Aldrich (Munich, Germany). Distilled water for LC-MS was deionized by Elga Purelab Ultra purification system (Celle, Germany).

### 2.2. Preparation of stock solutions, calibrants and quality control samples

To prepare the standard stock solutions of cortisone, cortisol, cortisone-$^{13}C_3$, cortisone-$d_8$ and cortisol-$d_4$, each was dissolved in MeOH at 1 mg $mL^{-1}$ and stored at −20 °C until needed.

An internal standard stock mix solution was prepared from 1 mg $mL^{-1}$ by diluting to a final concentration of 15,000 ng $mL^{-1}$ in MeOH. Twenty (20) μL of internal standard stock mix solution was then further diluted with 200 mL of 50 mg $mL^{-1}$ $ZnSO_4$ (50:50 MeOH:Water, v:v; prepared from 89 mg $mL^{-1}$ $ZnSO_4{\cdot}7H_2O$) to obtain the precipitation solution containing 1.5 ng $mL^{-1}$ of IS.

Two master mixes ($MM_{Low}$ as low-concentrated standard solution and $MM_{High}$ as high-concentrated standard solution) prepared from above adjusted stock solutions, were employed to prepare four levels of quality control samples. $MM_{High}$ was used to prepare $QC_{Intermediate}$ ($QC_{Inter}$), $QC_{High}$, calibrants 5, 6, 7 and 8, and $MM_{Low}$ for $QC_{3*LLOQ}$, $QC_{Mid}$, and calibrants 1, 2, 3, 4. Calibrant 0 (Cal0), which is pooled matrix spiked with only internal standard (IS), was also prepared. QCs and calibrants were prepared by pooling saliva in 5 mL volumetric flasks, spiked with corresponding amounts of the respective MM. Five aliquots (each containing 1000 μL pooled saliva) were pipetted into the

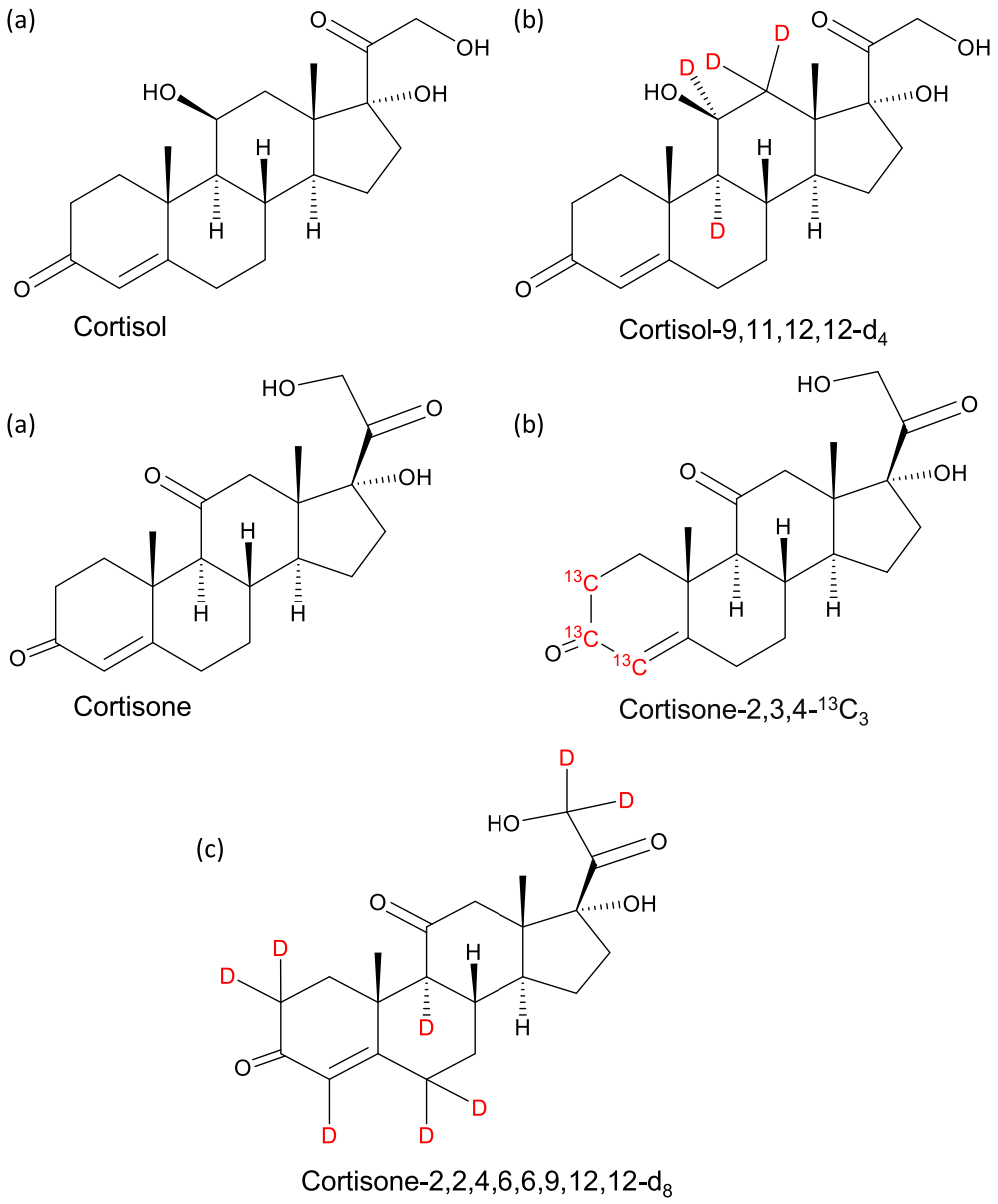


**Fig. 1.** Chemical structures of analytes: (a) targeted analytes, cortisone and cortisol; (b) surrogate calibrants, cortisone-$^{13}C_3$ and cortisol-$d_4$, (c) deuterated internal standard, cortisone-$d_8$.

Salivette® Cortisol tubes and kept at −20 °C until needed. MM concentrations and the final concentrations of surrogate calibrants in calibration and QC samples can be found in supplementary information in Table S1 and Table S2, respectively.

### 2.3. Collection of saliva samples

The study was performed in agreement with the Declaration of Helsinki as revised in 2008 and was approved by the Ethics Committee of the Medical Faculty of Tübingen University. All participants gave their written informed consent.

One hundred and seventy-two healthy donors (78 female, 18–35 years) took part in the neuroimaging stress study. The participants were instructed not to drink, eat or smoke prior or during the saliva collection. Non-stimulated saliva samples (around 1 mL) were collected in Salivette® Cortisol tubes (synthetic fiber neutral collection device) (Sarstedt, Nümbrecht, Germany) by rolling the contained synthetic fiber swab in the mouth for two minutes. The participants were instructed to donate twelve saliva samples during a single day (five times in the morning and seven times during the afternoon between 1 p.m. and 7 p.m. when participants performed on a psychosocial stress induction paradigm in the MR-scanner). The samples (in the Salivette® Cortisol tube) were stored at −20 °C until assayed. Saliva samples for method development and validation were, after informed consent, kindly donated by members of our working group (5 healthy donors, 2 females and 3 males) and pooled.

### 2.4. Sample preparation

The scheme of the entire experimental procedure is shown in supplementary information (Fig. S1).

Prior to analysis, the samples in the Salivette® Cortisol tubes were slowly thawed at 4 °C for approximately 2 h, centrifuged at 2000 × g for 15 min at 4 °C to obtain a clear fluid (removing residual cells and particles in the saliva) and 700 µL of each supernatant was transferred into an Eppendorf tube. For protein precipitation in the saliva samples, 350 µL MeOH was added to saliva samples and vortexed. After vortexing, 700 µL of a precipitation solution containing 50 mg $mL^{-1}$ $ZnSO_4$ in

MeOH:Water (50:50; v:v) spiked with cortisone-$d_8$ as IS (at the concentration of 1.5 ng $mL^{-1}$) was added to the mixture and vortexed. The Eppendorf tubes were then centrifuged at 15,026 × g for 15 min, and 1500 µL of supernatant were slowly loaded onto an Oasis PRIME hydrophilic-lipophilic balance (HLB) Solid-Phase Extraction (SPE) 96-well plate (1 cc / 30 mg, Waters, Milford, MA, USA), which does not require pre-conditioning and equilibration steps prior to loading, without disturbing the pellet. SPE was carried out in 96-well plate format with nitrogen flow under positive pressure using an Agilent Positive Pressure Manifold 96 Processor (PPM-96) (Agilent Technologies, Santa Clara, CA, USA). The preparation layout for the 96-well plate as used in this assay is shown in supplementary information (Fig. S2). Each well was then washed with 1000 µL MeOH:$H_2O$ (50:50; v/v). After the washing step, 700 µL conical round well collection plates (Waters, Milford, MA, USA), that were found suitable due to the low reconstitution volume, were placed in the PPM-96 SPE unit. With these collection plates, the following steps of drying and reconstitution were processed without any need of transferring the samples. Then, the analytes were eluted with 2 × 300 µL MeOH. Positive pressure of 3–5 psi was applied for loading the samples, for the washing step and for elution; positive pressure of 25 psi was applied after the end of each washing and elution step to dry the sorbent bed. Finally, the samples on the well plate were evaporated to dryness under nitrogen protection with an EZ2 evaporator (GeneVac EZ 2 Evaporator, GeneVac Ltd, Ipswich, UK) and each well was reconstituted by 50 µL MeOH-$H_2O$ (30:70; v/v). Prior to UHPLC-MS/MS analysis, the collection plates were sealed with collection plate mats (Captiva pierceable 96-well collection plate cover, Agilent Technologies), vortexed for 30 s and centrifuged at 2000 × g for 2 min at 4 °C to prevent clogging of the LC flow path.

### 2.5. Micro-UHPLC-ESI-MS/MS instrumentation and conditions

An Eksigent MicroLC 200 Plus UHPLC System with CTC Analytics HTC-xt PAL autosampler and a QTRAP 4500 quadrupole-linear ion trap mass spectrometer (Sciex, Framingham, MA, USA) equipped with a Turbo V source and 50 µm ID Hybrid PEEKSIL electrode with a stainless-steel tip was employed for LC-MS/MS analysis. Quantification was achieved using the mass spectrometer in MRM (multiple reaction monitoring) mode with dwell time, declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) as summarized in Table 1. MS conditions for MRM including compound-dependent and ion source dependent parameters were first optimized by direct infusion of each analyte at the concentration of 1000 ng $mL^{-1}$ in 30 % MeOH containing 0.2 mM $NH_4F$ into the MS to obtain the optimal signal intensity. All of the experiments were carried out in negative electrospray ionization (ESI) mode. The main source parameters were set as follows: nebulizer gas, GS1 (zero grade air) 20 psi, drying gas, GS2 (zero grade air) 0 psi and CAD, collision gas ($N_2$) medium; ion spray voltage −4000 V; probe temperature 200 °C. For the chromatographic separation, a HALO C18 capillary column (50 × 0.5 mm, 2.7 µm) equipped with a guard column YMC-Triart C18 (0.5 mm × 5 mm, 3 µm) and a pre-filter (placed after the column) (mini-microfilter assembly with a filter capsule, porosity 2 µm) (Sigma-Aldrich) was used. Final chromatographic parameters were optimized at 20 µL $min^{-1}$ mobile phase flow rate, 30 °C column temperature, 4 °C autosampler temperature and 5 µL injection volume. After each analytical batch (about 169 injections), the LC flow path was cleaned with MeCN:$H_2O$ (50:50; v/v) after the sequence ended. The mobile phases consisted of HPLC grade water with 0.2 mM $NH_4F$ as eluent A and MeCN with 0.2 mM $NH_4F$ as eluent B. MeCN was used as needle wash. Gradient elution was carried out with the following gradient profile: 0 min, 25 % B; 2.5 min 37 % B; 2.7 min 100 % B; 3.2 min, 100 % B; followed by 0.8 min re-equilibration with 25 % eluent B. Run time was set to 4 min with a retention time of 1.9 min for cortisol and 2.0 min for cortisone. In order to obtain peak areas of analytes and IS, Analyst software 1.6.3. Version was employed (Sciex).

### 2.6. Sample injection order and design of the analytical batch

Each analytical batch consisted of blanks (i.e. solvent solution; no analyte and no IS spiked), zero-calibrators (Cal0; pooled saliva without spiked calibrants but spiked with IS working solution only; sometimes also termed extraction blank), 8 non-zero-calibrators (pooled saliva spiked with calibrants; Cal1-8), quality controls ($QC_{3xLLOQ}$, $QC_{Mid}$, $QC_{Inter}$ and $QC_{High}$), and study samples. Study samples were interspersed in-between QCs and injected in random order. The first injections were blanks, and they were followed by (multiple) injection(s) of zero-calibrator Cal0. Quality controls and Cal0 were utilized for column/system equilibration and also for control of the quantitative performance of the instrument during the analytical sequence. Each analytical batch consisted of around 169 injections including 20 QCs and 32 non-zero calibrants and multiple injections of zero calibrator. The QC samples were repeatedly analyzed every approx. 25 study samples.

Every analytical batch took around 17 h to quantify 83 study samples. During analysis, the samples were kept at 4 °C in the autosampler tray. The analytical order is summarized in supplementary information in Table S3.

Before each analytical batch, a system suitability test was run by injecting $MM_{high}$ and $MM_{low}$ in six replicates to assess system back pressure, retention time compliance of analytes, surrogate calibrants and IS, their peak symmetries as well as peak areas (signal intensities) to monitor response factors (balanced/matched signal intensities of target analyte and corresponding surrogate calibrant).

### 2.7. Parallelism assessment

Surrogate calibration requires identical detector responses for analytes and corresponding surrogate calibrants in order to avoid bias. Therefore, it is essential to evaluate parallelism between the target analyte standard addition curve and the surrogate calibration curve in pooled saliva (matrix-matched). Prior to preparation of calibrants and QCs, detector responses of each analyte/surrogate calibrant pair in the

**Table 1**
MRM compound dependent parameters set up for targeted analysis of cortisol and cortisone in saliva samples.

| Analyte | Q1 [m/z] | Q3 [m/z] | Transition | Dwell Time (ms) | DP (V) | EP (V) | CE (V) | CXP (V) |
|---|---|---|---|---|---|---|---|---|
| Cortisone | 359.2 | 136.9 | Quantifier | 45 | -70 | -10 | -39 | -9 |
| | | 301.1 | Qualifier | 45 | -70 | -10 | -20 | -11 |
| Cortisone-$^{13}C_3$ | 362.2 | 136.9 | Quantifier | 45 | -71 | -10 | -39 | -9 |
| | | 304.1 | Qualifier | 45 | -71 | -10 | -20 | -11 |
| Cortisol | 361.2 | 297.1 | Quantifier | 45 | -75 | -10 | -32 | -11 |
| | | 282.0 | Qualifier | 45 | -75 | -10 | -40 | -10 |
| Cortisol-$d_4$ | 365.2 | 301.1 | Quantifier | 45 | -80 | -10 | -34 | -11 |
| | | 286.0 | Qualifier | 45 | -80 | -10 | -40 | -10 |
| Cortisone-$d_8$ | 367.2 | 138 | Quantifier | 30 | -75 | -10 | -40 | -9 |
| | | 307.2 | Qualifier | 30 | -75 | -10 | -20 | -11 |

Q1: first quadrupole, Q3: third quadrupole, DP: declustering potential, EP: entrance potential, CE: collision energy, CXP: collision cell exit potential

MMs were matched via adjusting surrogate calibrant concentrations [51]. Area response ratios (surrogate calibrant/target analyte response factors, RFs) of 1.00 ± 0.10 were found acceptable (as shown in Table 2). In order to control for parallelism and accurate calibration during study sample measurements, RFs were determined prior to each batch by individually injecting 3 replicates of a dilution of each MM ($MM_{Low}$ and $MM_{High}$ were diluted by factors of 1:10 and 1:50, respectively). Nominal concentrations of surrogate calibrant calibration levels were adjusted by multiplying them by the corresponding RF. Parallelism was monitored during validation and study batches.

### 2.8. Calibration and method validation

Calibration curves were established by spiking different amounts of MMs and adding the same amount of precipitation mix to pooled saliva. To check for parallelism, linear functions derived from standard addition of target analytes in pooled saliva were determined. The calibrants were processed using the same sample preparation procedure as the saliva study samples, as described in Section 2.4. Calibrants were freshly prepared each analysis day. After optimization, the newly developed method was successfully validated according to guidelines of United States Food and Drug Administration (FDA) [50], including parameters of precision and accuracy, limits of detection and lower limit of quantitation, linearity, carryover, assay specificity, dilution effect, matrix effect, extraction recovery, and process efficiency for the two targeted compounds. The limit of detection (LOD) and the limit of quantification (LOQ) of the method for each compound were set at concentrations for which signals of surrogate calibrant spiked to pooled saliva were 3 and 10 times the signal in chromatograms of blank (non-spiked) pooled saliva (spiked with IS) from 6 replicates, respectively. The calibration functions were obtained by means of plotting the surrogate calibrant peak area to IS peak area ratio versus concentration. Linearity was determined over three different days using weighted least-square linear regression (weighting factor was set to 1/x for all compounds) with eight different calibration levels.

Matrix effects, extraction recoveries and process efficiencies were determined via surrogate calibrants according to protocols suggested by Matuszewski et al. [52]. For the determination of the matrix effect (ME), peak area ratios of post-extraction spiked matrix to corresponding MeOH standard solutions were calculated. Extraction recovery (ER) was determined by calculations of the peak area ratio of pre-extraction spiked matrix and post-extraction spiked matrix. To establish process efficiency, peak area ratios of pre-extraction spiked matrix to corresponding MeOH standard solutions were calculated.

Samples for inter-laboratory proficiency testing were obtained from T-IBL (IBL International GmbH).

The stability of the target compounds in saliva was assessed under different storage conditions by reanalyzing two levels of QCs: 6 h on ice, storage for 6 months and 1 year at −20 °C, three freeze–thaw cycles between −20 °C and on ice during 24 h, and for 10 h, 24 h and 48 h in autosampler. Furthermore, the stability of the target compounds in MMs was also assessed at 4 °C for 1 month.

### 2.9. Data analysis and quantification

Data was processed using MultiQuant 3.0 with automated integration (Sciex). The Gaussian smooth (width 2 data points), noise percentage of 90 % and peak splitting factor of 2, baseline subtraction window of 0.10 min was set additionally. Microsoft Excel 2016 (Microsoft, Redmond, WA, USA) was used for additional data evaluation. Graphs and figures were generated by using Origin 2019 (Originlab, Northampton, MA, USA), chemical structures were generated by ChemDraw (PerkinElmer Informatics).

## 3. Results and discussion

### 3.1. Method development and assay characteristics

#### 3.1.1. Sampling and sample preparation

This method was developed for the analysis of cortisol and cortisone in samples from a neuroimaging stress study in female and male participants, where hypothalamic–pituitary-adrenal (HPA) activity was assessed using repeated saliva samples taken from young healthy participants. As we needed several samples (in total, n = 12; cortisol awakening profile n = 5, psychosocial stress induction in the afternoon, n = 7) from each donor within 24 h and sampling was also performed while participants were lying in the MR-scanner, non-invasive sampling was preferred and hence saliva selected as matrix of first choice. Two saliva sampling approaches were initially tested: passive drool and Salivette®. Passive drool typically includes methods that describe the direct collection of saliva from the patient́s mouth by spitting or drooling the sample matrix into an appropriate container. For increased adherence, straws or more sophisticated sampling devices like the Saliva Collection Aid (Salimetrics, State College, PA, USA) can be utilized. In contrast, the Salivette® system consists of a cotton or synthetic fiber roll that is inserted into the mouth. After absorption of a sufficient sample volume (up to 1.4 mL), the roll is returned into the corresponding Salivette® tube, which is then centrifuged to extract the sample matrix from the roll (see section 2.3 and 2.4). Whereas passive drool showed advantages in terms of recoveries, the Salivette® system was more convenient in the context of the present neuroimaging study. For MRI measurement, participants were positioned supine on the patient table of the MR Scanner with their head tightly bedded within a head coil. As excess head movements would require repositioning and localization procedures passive drool saliva selection was impossible and salivette sampling was performed throughout this study in the course of method development, validation and application.

Evidently, saliva is less complex in its composition than plasma but contains a variety of constituents comprising electrolytes, mucins, enzymes and other proteins, and cells. To remove these matrix constituents and enrich the target analytes, an SPE protocol previously developed for estradiol and testosterone analysis in plasma was adapted [48]. To cope with the large number of samples, SPE in well plate format with Oasis PRiME HLB was utilized. After protein precipitation with $ZnSO_4$ solution in MeOH-water (containing IS), the supernatant could be loaded onto

**Table 2**
Control table for parallelism during validation.

| Analyte | Day | Slope surrogate calibrant | Slope target analyte | Slope ratio[1,2] | Slope ratio precision Intra-Day (%) | Slope ratio Standard Error Intra-Day | Slope ratio precision Between-Days (n = 3) (%) | Slope ratio Standard Error Between-Days (n = 3) |
|---|---|---|---|---|---|---|---|---|
| **Cortisone** | 1 | 0.00266 | 0.00254 | 1.047 | 1.065 | 0.753 | 1.442 | 0.832 |
| | 2 | 0.00274 | 0.00269 | 1.019 | 5.465 | 3.864 | | |
| | 3 | 0.00261 | 0.00252 | 1.036 | 0.010 | 0.007 | | |
| **Cortisol** | 1 | 0.00282 | 0.00286 | 0.986 | 0.209 | 1.511 | 1.430 | 0.825 |
| | 2 | 0.00309 | 0.00308 | 1.003 | 2.137 | 1.349 | | |
| | 3 | 0.00282 | 0.00278 | 1.014 | 1.907 | 0.148 | | |

[1] Slope ratio of slope surrogate calibrant to slope target analyte
[2] Slope ratio of 1.00 ± 0.05 was deemed acceptable

dry Oasis PRiME HLB wells. SPE was performed using a positive pressure manifold for 96-well plate format. Various factors of the SPE procedure were optimized with single cartridges. An enrichment factor of 14 could be finally realized.

The MeOH content of the MeOH-water mixture in washing, elution and reconstitution step was investigated and optimized. In this study, pooled saliva samples were extracted in the same way as described in Section 2.4. Aliquots of these extracts were spiked with standards at $QC_{Mid}$ level. To determine optimum MeOH content at washing step, 8 pooled saliva samples were loaded to cartridges and washed with 1 mL of one of the following concentrations of MeOH: 0 %, 5 %, 10 %, 20 %, 30 %, 40 %, 50 % and 60 % (v/v in water) and each was eluted with 2 × 300 µL of 100 % MeOH (v/v), and then reconstituted with 50 µL of 20 % MeOH (v/v in water). 50 % MeOH was chosen, as it showed the highest recovery among all concentrations (see Suppl. Fig. S3). Furthermore, to evaluate the effect of elution solvent content, 5 freshly prepared pooled saliva samples were loaded to cartridges and each washed with 1 mL of 50 % MeOH (v/v in water) and eluted with 2 × 300 µL of one of the following concentrations of MeOH: 60 %, 70 %, 80 %, 90 %, 100 % (v/v in water) and reconstituted with 50 µL of 20 % MeOH (v/v in water). For elution, the highest recovery was obtained with 100 % MeOH (see Suppl. Fig S4), which was consequently selected for the elution step. For optimization of the reconstitution solvent, 7 freshly prepared pooled saliva samples were loaded to cartridges and each washed with 1 mL of 50 % MeOH (v/v in water) and eluted with 2 × 300 µL of 100 % (v/v) and reconstituted with 50 µL of one of the following concentrations of MeOH: 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 % MeOH (v/v in water). When 30 % MeOH was used, the recovery was highest among all concentrations (see Suppl. Fig. S5) and peaks were well refocussed on the capillary column. Therefore, 30 % MeOH was selected for reconstitution.

A critical factor turned out to be the type of sample collection cap mats. As mentioned in section 2.4, the collection plates were sealed with pierceable collection plate mat (Agilent Captiva pierceable 96-well collection plate cover). Alternatively, round collection plate mats from Waters were tested. However, it was found that these latter mats were not compatible with the Eksigent Micro LC 200 autosampler, as particles from the mat were observed in the LC system although the mats were dimpled, which resulted in enormously increased back-pressure and clogging. Therefore, it is recommended to use pierceable and pre-slit mats with microflow LC systems, as they have less tendency to lead to clogging of the sensitive microflow path by “stamped-out” particles.

#### 3.1.2. Micro-UHPLC-ESI-MS/MS method

Microflow LC is attracting increasing scientific interest, since it provides higher ESI sensitivity [53] with reduced reagent consumption [54] while being a good compromise between standard- and nano-flow LC systems in terms of sensitivity and robustness. However, miniaturization may be accompanied by problems like a clogged flow path especially when real clinical samples with proteinaceous matrix are analyzed. For this reason, special care must be taken particularly when working with long sequences of around 169 samples in an analytical batch such as in the present case. A careful sample preparation, such as by SPE, is certainly advantageous. However, also other factors should be considered. Therefore, initially a mini-microfilter assembly (with a filter capsule, porosity 2 µm) (obtained from Sigma Aldrich) was placed before the column for column protection and prevention of clogging of the micro ESI electrode. Since this assembly was not robust enough for a long sequence, a guard column was installed to the micro-LC system. The utilization of a guard column YMC-Triart C18 (0.5 mm × 5 mm, 3 µm) together with the mini-microfilter assembly placed after the column resulted in satisfactory robustness enabling over 1000 injections.

The utilized micro-LC system can cope with maximum backpressures up to 10,000 psi (69 MPa) only. Thus, the chromatographic separation was performed with a C18 core–shell capillary column, 2.7 µm HALO fused C18 column (0.5 mm × 50 mm). It has UHPLC performance at moderate backpressures. The effect of flow rate on sensitivity was tested in the range of 15 to 40 µL $min^{-1}$. Sensitivity increased with a decrease of the flow rate from 40 to 20 µL $min^{-1}$ at little expense of analysis time while 15 µL $min^{-1}$ gave significantly lower signal intensity. Hence, a flow rate of 20 µL $min^{-1}$ was finally selected and allowed fast analysis cycles in 4 min (including re-equilibration) (see Fig. 2).

For the above-mentioned flow rate range, a 50 µm ID ESI electrode is recommended for the ion source and was used herein. For reasons of assay specificity (see below), negative ESI mode was selected. Initially, we used nitrogen as ion source gas (nebulizer and turbo gas; Gas 1 and Gas 2). However, we observed adverse effects of arcing (electric discharge) on the tip of the ESI probe (between spray needle and spray cone) during routine operation preferentially at higher temperatures in negative ESI mode. It had a deleterious effect on sensitivity which therefore tended to decrease dramatically (see supplementary Fig. S6 for examples showing a comparison of sensitivity with and without arcing). Other factors that may promote arcing are corrosion and contamination of the ESI needle (e.g. with salt), high voltage and low flow rate as typical for the micro-LC system. The gas supply was therefore changed to zero grade air. Zero grade air was found to be less susceptible to arcing, as this phenomenon related to Paschens Law [55] (effect in gases like $N_2$, zero air described elsewhere [56]) was not observed anymore. The source parameters and collision energies were optimized for the target analytes and calibrants as well as IS (see Table 1).

### 3.2. Method validation

#### 3.2.1. Selectivity and assay specificity

For each analyte, two SRM transitions (i.e. precursor-product ion pairs) were selected (see Table 1 and in suppl. Fig. S7 for mass spectra of each target analyte and their product ions). They were evaluated for interferences to assure assay specificity. For this purpose, six individual lots of saliva were analyzed measuring SRM transitions of surrogate calibrants and IS in both positive and negative ion electrospray mode for ensuring that they are free of interferences. No interference peaks for surrogate calibrants and IS were observed in negative ion mode. However, poor selectivity was found in positive ion mode. Therefore, further MS analyses were performed in negative ion mode. Fig. 2 shows the typical chromatograms of targeted analytes in real sample as well as surrogate calibrants and IS acquired from pooled saliva matrix.

Assay specificity was verified in saliva matrix by injecting analyte at highest calibration level and measuring all other MRM transitions checking for absence of interferences. Besides, steroids with similar or same precursor ion *m/z* may interfere when saliva matrix is quantified in real samples. For this reason, two transitions were measured, one as quantifier and one as qualifier ion for each target analyte and assay specificity was confirmed by constant SRM ratios throughout the study of real sample analysis according to Decision 2002/657/EC [57].

#### 3.2.2. Matrix effect, extraction recovery and process efficiency

All evaluations of ME, ER, and PE were done at three different concentration levels of surrogate calibrants equal to $QC_{3*LLOQ}$, $QC_{Mid}$, $QC_{High}$ in quadruplicate (Table 3). ME was between 88.2 and 114.6 % for cortisol-$d_4$ and between 94.0 and 114.2 % for cortisone-$^{13}C_3$, respectively. ER was found to be between 89.9 and 114.2 % for cortisol-$d_4$ and between 83.6 and 101.2 % for cortisone-$^{13}C_3$, respectively. PE was determined to be between 100.1 and 104.8 % and between 95.4 and 105.4 % for cortisol-$d_4$ and cortisone-$^{13}C_3$, respectively. The results indicate that sample preparation by SPE was successful to remove matrix components efficiently so that matrix effects are of minor extent and compensated by isotope labelled internal standards.

#### 3.2.3. Assay sensitivity as well as intra-assay, inter-day and inter-laboratory accuracy and precision

The limit of detection (LOD) and lower limit of quantitation (LLOQ) were 20 and 71.7 pg $mL^{-1}$ for cortisol-$d_4$, 10 and 61.9 pg $mL^{-1}$ for

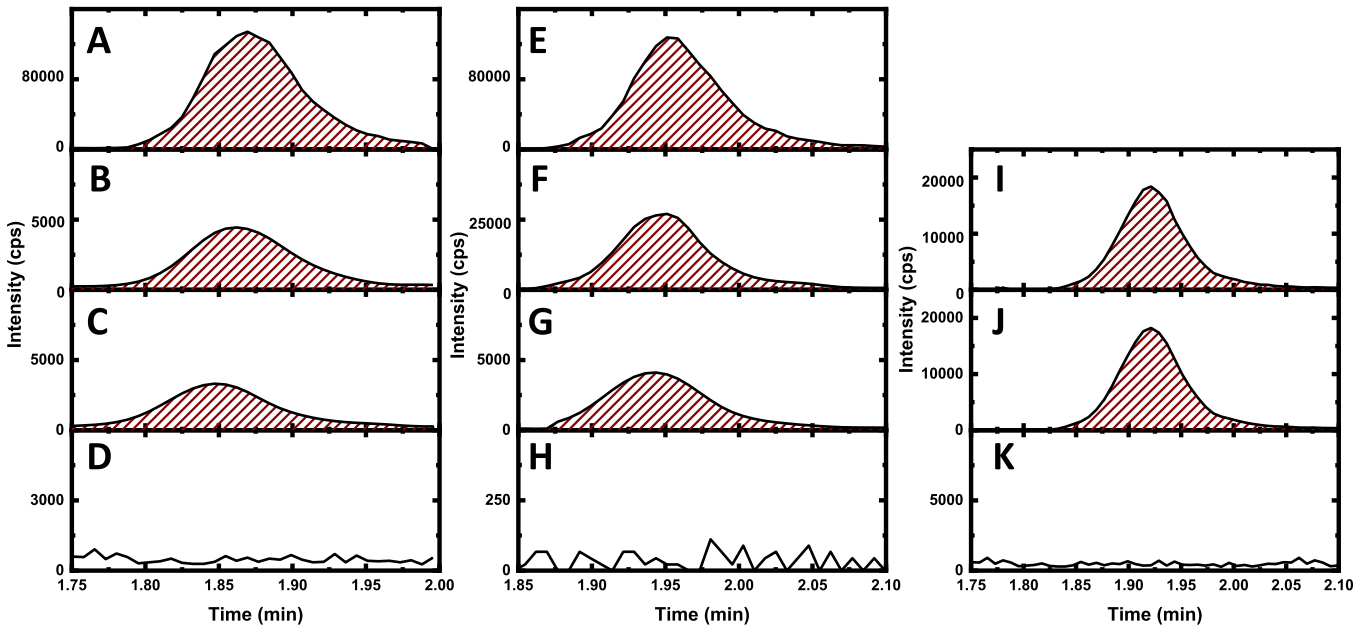


**Fig. 2.** Typical MRM chromatograms. A: Cortisol in real sample (42,000 pg $mL^{-1}$; B: Cortisol in real sample at 3 × LLOQ (180 pg $mL^{-1}$); C: Cortisol-$d_4$ spiked at LLOQ (215.04 pg $mL^{-1}$); D: Cortisol-$d_4$ in pooled saliva ; E: cortisone in real sample (70,000 pg $mL^{-1}$; F: Cortisone in real sample at 3 × LLOQ (150 pg $mL^{-1}$); G: Cortisone-$^{13}C_3$ spiked at 3x LLOQ (185.74 pg $mL^{-1}$); H: Cortisone-$^{13}C_3$ in pooled saliva ; I: Cortisone-$d_8$ spiked in real sample-1 (900 pg $mL^{-1}$); J: Cortisone-$d_8$ spiked in real sample-2 (900 pg $mL^{-1}$); K: Cortisone-$d_8$ in pooled saliva.

**Table 3**
Summary of the validation for Matrix Effect, Process Efficiency, Extraction Recovery*

| | | Analyte | | | |
|---|---|---|---|---|---|
| | | Cortisol-$d_4$ | | Cortisone-$^{13}C_3$ | |
| | | Accuracy [%] | Precision [%] | Accuracy [%] | Precision [%] |
| **Matrix Effect** | $QC_{3xLLOQ}$ | 91.78 | 3.46 | 94.04 | 1.96 |
| | $QC_{Mid}$ | 114.64 | 3.33 | 114.23 | 1.54 |
| | $QC_{High}$ | 88.15 | 2.54 | 95.71 | 3.74 |
| **Extraction Recovery** | $QC_{3xLLOQ}$ | 114.15 | 0.48 | 112.1 | 1.45 |
| | $QC_{Mid}$ | 89.9 | 1.44 | 83.55 | 2.4 |
| | $QC_{High}$ | 113.54 | 0.69 | 101.23 | 1.11 |
| **Process Efficiency** | $QC_{3xLLOQ}$ | 104.77 | 1.67 | 105.42 | 2.85 |
| | $QC_{Mid}$ | 103.06 | 4.78 | 95.43 | 3.71 |
| | $QC_{High}$ | 100.09 | 1.74 | 96.9 | 4.15 |

*The measurements were done in quadruplicate.

cortisone-$^{13}C_3$, respectively (Table 4). Linear calibration was achieved in the range from 0.072 to 44 ng $mL^{-1}$ for cortisone-$^{13}C_3$ and 0.062 to 75.5 ng $mL^{-1}$ for cortisol-$d_4$.

Parallelism of calibration functions between analyte standard addition curves in pooled saliva and matrix-matched surrogate calibration curves was verified during three inter-day measurements to ensure the

**Table 4**
Validation results for precision and accuracy.

| Analyte | | | Cortisol-$d_4$ | | | Cortisone-$^{13}C_3$ | | |
|---|---|---|---|---|---|---|---|---|
| | | | Day 1 | Day 2 | Day 3 | Day 1 | Day 2 | Day 3 |
| $QC_{LLOQ}$ | Within-run (n = 5) | Accuracy [%] | 110.0 | 107.9 | 97.1 | 103.3 | 108.0 | 100.5 |
| | | Precision [%] | 11.4 | 6.2 | 8.6 | 13.5 | 6.1 | 7.0 |
| | Between-runs (n = 15) | Accuracy [%] | 105.0 | | | 103.9 | | |
| | | Precision [%] | 6.6 | | | 3.2 | | |
| $QC_{3xLLOQ}$ | Within-run (n = 5) | Accuracy [%] | 92.3 | 100.3 | 88.2 | 89.6 | 100.1 | 93.7 |
| | | Precision [%] | 3.7 | 4.4 | 2.1 | 5.6 | 4.8 | 4.1 |
| | Between-runs (n = 15) | Accuracy [%] | 93.6 | | | 94.5 | | |
| | | Precision [%] | 6.6 | | | 7.8 | | |
| $QC_{Mid}$ | Within-run (n = 5) | Accuracy [%] | 95.2 | 100.5 | 96.7 | 110.0 | 109.3 | 109.4 |
| | | Precision [%] | 4.9 | 3.8 | 9.3 | 4.9 | 4.8 | 4.2 |
| | Between-runs (n = 15) | Accuracy [%] | 97.5 | | | 109.6 | | |
| | | Precision [%] | 2.8 | | | 4.8 | | |
| $QC_{High}$ | Within-run (n = 5) | Accuracy [%] | 104.5 | 98.5 | 114.8 | 103.6 | 102.3 | 103.2 |
| | | Precision [%] | 2.1 | 3.8 | 1.8 | 4.4 | 7.2 | 5.9 |
| | Between-runs (n = 15) | Accuracy [%] | 105.9 | | | 103.0 | | |
| | | Precision [%] | 7.8 | | | 7.5 | | |

accuracy of quantification via surrogate calibrant method (see Suppl. Fig. S8 for exemplary standard addition and surrogate calibration functions). The maximum difference of the slopes of cortisol/cortisol-$d_4$ and cortisone/cortisone-$^{13}C_3$ (slope of surrogate calibrant divided by slope of target analyte) were found to be 1.442 and 1.430 %, respectively. The difference of slope ratio between non-zero calibrants from the first and second calibration range measured at the beginning and end of the analytical batch was also calculated. The slope ratio difference of cortisol/cortisol-$d_4$ was found to be 0.209, 2.137, 1.907 % for three inter-day measurements. For cortisone/cortisone-$^{13}C_3$, the slope ratio difference was 1.065, 5.465, 0.010 %. Thus, all values fell within the acceptable range of 10 % deviation of parallelism. The detailed results are summarized in Table 2.

The intra-assay and inter-day precision and accuracy were assessed in saliva by using four quality controls prepared by spiking pooled saliva with different concentrations using surrogate calibrants at 4 distinct levels: $QC_{LLOQ}$ (61.9 pg $mL^{-1}$ for cortisone-$^{13}C_3$, 71.7 pg $mL^{-1}$ for cortisol-$d_4$), $QC_{3xLLOQ}$ (185.7 pg $mL^{-1}$ for cortisone-$^{13}C_3$, 215.0 pg $mL^{-1}$ for cortisol-$d_4$), $QC_{Mid}$ (1,298.4 pg $mL^{-1}$ for cortisone-$^{13}C_3$, 1,033.6 pg $mL^{-1}$ for cortisol-$d_4$), $QC_{High}$ (2,163.9 pg $mL^{-1}$ for cortisone-$^{13}C_3$, 1,722.6 pg $mL^{-1}$ for cortisol-$d_4$). These four QCs were measured in quintuplicate (n = 5) on three different days to determine the within-day and between-day precision and accuracy, respectively. The results are shown in Table 5. Precisions (determined as RSD%) were always <15 % and accuracies (assessed as % recovery) between 88.2 and 115.0 % in the entire range, both within-day and between-day. Consequently, validation has fulfilled the FDA acceptance criteria for bioanalytical assays.

Besides, the inter-laboratory accuracy was assessed by proficiency testing. Saliva samples obtained from T-IBL with cortisol reference levels at three distinct concentrations (high-concentrated sample specified as Sample 1, low-concentrated sample as Sample 2, and mid-concentrated sample as Sample 3; see supplementary information in Table S4 and S5) were analyzed by the present validated micro-UHPLC-ESI-MS/MS method (termed 'our method' in supplementary information in Table S4 and S5). The proficiency test comprised different methods consisting of Luminescence Immunoassay (LUM), enzyme-linked immunosorbent assay (ELISA) and MS. The number of participant laboratories was sixty-six and twenty laboratories determined cortisol by MS methods. $|Z\text{-score}| \leq 2.0$ is deemed as satisfactory; $2.0 < |Z\text{-score}| < 3.0$ is considered questionable; $|Z\text{-score}| \geq 3.0$ is categorized as unsatisfactory. Based on the Z-score results, our method was regarded as satisfactory for all concentration levels (calculated Z-score was 0.032, 0.155, −0.724 for Sample 1, Sample 2 and Sample 3, respectively, when all methods were included and 0.857, 1.66, −1.521 when only MS methods were considered). More than 90 % of the laboratories showed satisfactory results. The detailed results can be found in supplementary information in Figure S9 and Table S4 and Table S5. No proficiency test was available for salivary cortisone.

**Table 5**
Summary of detection and quantification limits, linearity and linear range for salivary cortisol and cortisone analysis.

| | Analyte | | | | | |
|---|---|---|---|---|---|---|
| | Cortisol-$d_4$ | | | Cortisone-$^{13}C_3$ | | |
| Linear Range (in matrix) (pg/mL) | 71.7–44,017.8 | | | 61.9–75,555.2 | | |
| Slope | Day 1 | Day 2 | Day 3 | Day 1 | Day 2 | Day 3 |
| | 0.00282 | 0.00309 | 0.00282 | 0.00266 | 0.00274 | 0.00261 |
| Intercept | 0.02619 | -0.1088 | -0.0079 | 0.01938 | 0.00552 | 0.04836 |
| LOD (pg/mL) (in matrix) | | 20.0 | | | 10.0 | |
| LOQ (pg/mL) (in matrix) | | 71.7 | | | 61.9 | |

### 3.2.4. Dilution integrity

In some cases, not enough saliva sample volume was obtained (< 700 μL). In order to keep the sample preparation procedure identical, those samples were filled up to 700 μL by adding water to have a uniform sample processing procedure for all samples throughout the study and ultimately adjust the results mathematically. Since this resulted in a different sample dilution, it must be demonstrated during validation that the dilution does not have a negative impact on the integrity of the quantitative results, as explained in FDA guidelines [50]. This was assessed by diluting QC samples by adding water for expected dilutions, herein within the range of the assay using a respective dilution factor of 2.8. As can be seen from Supp. Table S6, the dilution effects were within the acceptable range (precision and accuracy <±15 % and passed the acceptance criteria of the FDA guideline).

### 3.2.5. Carry over

Carryover should be monitored and if any, during assay development, it should be minimized. To ensure absence of interference from any carryover, blanks were injected subsequently to a QC or a calibrant at highest concentration according to the FDA guideline [50]. The carryover was ≤20 % of LLOQ and thus the acceptance criteria requested by the FDA guideline for carryover was met.

### 3.2.6. Stability

The stability of target analytes in saliva was investigated by simulating the handling and transport conditions of the samples under different time periods and storage conditions including five types of stability experiments: freeze/thaw stability, bench-top stability, long-term stability, stock solution stability and extract stability. For this matter, fresh QC samples at two levels ($QC_{3xLLOQ}$ and $QC_{High}$) were prepared and measured in quintuplicate for all stability experiments. For freeze/thaw stability, QCs were stored at −20 °C for 24 h and they were frozen and unfrozen in three cycles. To assess bench-top stability, QCs were kept for 6 h on ice and for long-term stability tests, they were stored for 6 months and 1 year at −20 °C. To conduct extract stability, QCs were reanalyzed that were kept in an autosampler tray (4 °C) for 10 h, 24 h and 48 h. The stability of MMs ($MM_{Low}$ and $MM_{High}$) was also investigated under a storage condition of 4 °C for 1 month. All prepared QC samples for stability assays were compared with freshly prepared QCs.

The results of the stability tests are summarized in Supp. Table S7. It becomes evident that the analytes can be considered stable in the stock solutions. Changes in analyte concentration for long-term and freeze/thaw stability were all within common acceptance limits for accuracy (±15 % for $QC_{High}$ and ±20 % at $QC_{3xLLOQ}$) and hence can be considered sufficiently stable. Bench-top and extract stability were slightly outside common acceptance limits but still <20 % which is the acceptable accuracy limit at 3xLLOQ. Since extracts were always freshly analyzed, no significant bias from instability was expected.

## 3.3. Analyses of clinical study samples

The validated micro-UHPLC-ESI-MS/MS method was finally utilized to measure saliva samples as part of a neuroimaging stress study. An overview of the study size and number of injections is given in Suppl. Table S8. In total, 1983 samples were analyzed in 24 analytical batches over about 2 years. Comprising calibrants, quality controls, and blanks 4056 injections were made on 5 columns. This corresponds to an average of about 811 injections per column and is certainly less than is possible with a common standard microbore (2.1 mm ID) UHPLC column. Column longevity can be probably increased if the batch size is reduced (from currently 169 injections per batch; 83 samples per batch) to about half of the injections per batch. It would assure more frequent

column cleaning with less pressure build up and less column failure.

To assess the long-term, batch and column lot-to-lot performance of this micro-HPLC-ESI-MS/MS method, the retention data for analytes, calibrants and ISs were monitored over all analytical batches. Typical intra-batch retention times were <5 % RSD (e.g. average RSD of 0.45 % for all 5 compounds for batch 2 with 169 injections). The column lot-to-lot variability (e.g. for columns 1 to 3 which were equipped with the same hardware) was on average for the 5 substances around 2.8 % RSDs for the comparison of the mean retention times (i.e. mean of the means on the 3 columns). Absolute peak areas were showing a typical variability of 26 % RSD for cortisone-$d_8$ (IS) in the QCs over all batches. Here, it must be emphasized that this covers a period of almost two years with several other studies having taken place on the same instrument between the sample batches. Calibration and IS adequately compensated for the inter-day variance in instrument sensitivity. The majority of the batches were running without problems complying with the quality criteria of the FDA guideline. Four analytical batches, however, had to be repeated due to either leakage, arcing or not fulfilling compliance to the QC acceptance criteria. Finally, analyzed and utilized sample batches were all fulfilling the acceptance criteria set forth by the FDA guideline. An overview of the calibration and validation results over all 24 batches is given in Suppl. Table S9 for cortisol and in Suppl. Table S10 for cortisone. As can be seen, method sensitivity (as assessed by the slope of the calibration curves) varied slightly between different days and analytical batches, respectively, which emphasizes the importance of adequate daily calibration and isotope labelled internal standards. Linearity of weighted calibration functions (1/x) was adequate over all batches with an average coefficient of determination $r^2$ of 0.9954 (±0.0032) for cortisol and $r^2$ of 0.9958 (±0.0029) for cortisone. Mean precisions (as RSD) of the QCs over all batches were <9 % for cortisol and <7 % for cortisone at 3 levels and mean accuracy (as RSD) of the QCs over all batches was on average within ±9 % for cortisol and ±8 % for cortisone.

The results of the clinical study samples with distributions of cortisol and cortisone concentrations in dependence on the diurnal sampling time are depicted as Box plots in Fig. 3. Except for 3 (out of 1983) samples, the concentrations were all above the LLOQ and within the range of the established micro-UHPLC-ESI-MS/MS assay, documenting the applicability of the method. Median as well as lower and upper quartiles for cortisol were 3.25 [1.93 to 5.48] ng $mL^{-1}$ for the morning samples and 0.99 [0.53 to 2.1] ng $mL^{-1}$ for the afternoon samples, and for cortisone 9.33 [5.88 to 15.33] ng $mL^{-1}$ for the morning samples and 4.93 [2.78 to 8.34] ng $mL^{-1}$ for the afternoon samples. A detailed discussion and interpretation of the results will be published elsewhere along with other psychological, physiological and neuroimaging data.

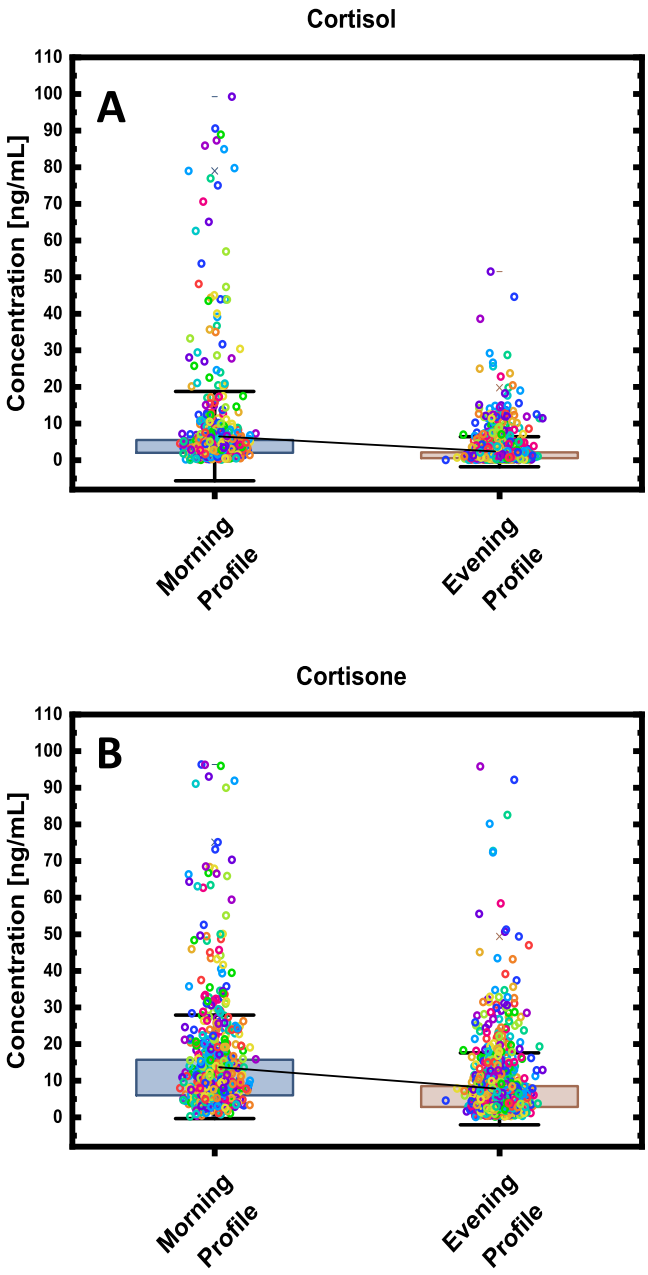


**Fig. 3.** Distribution of cortisone (a) and cortisol (b) concentrations illustrated by Box plots for two sample collection groups: Morning profile (5 samples per each donor) upon waking up and afternoon profile (7 samples per each donor) before going to bed with 5 to 13 min intervals between individual samples.

## 4. Conclusion

In this work we presented a new micro-UHPLC-ESI-MS/MS method with MRM data acquisition for a large-scale long-term neuroimaging stress study. Offline SPE with Oasis PRIME HLB in 96-well plate format and positive pressure elution mode enabled effective sample preparation with high throughput and capability to efficiently eliminate matrix effects. The microflow regime (20 µL $min^{-1}$) of the capillary chromatography scale favorably supported efficient electrospray ionization and, together with the achieved SPE enrichment factor of 14, it resulted in a sensitive cortisol/cortisone steroid analysis method (LLOQ of cortisol/cortisone, 72/62 pg $mL^{-1}$, respectively). It performed fairly well in comparison to other published LC/MS-MS methods which showed LLOQs in the range from 5 to 280 pg $mL^{-1}$ for salivary cortisol and from 3 to 500 pg $mL^{-1}$ salivary cortisone [4,58–60]. The chromatographic separation on a core–shell C18 packed capillary column was achieved in <3 min, with a total run time of 4 min. In addition, the instrument performance over time was monitored and some adjustments for column setup and gas supply were carried out to optimize the performance and robustness of the micro-LC setup. Surrogate calibration with isotope labelled calibrants and complementary isotope labelled internal standard were selected as an effective strategy to cope with the problem of unavailability of blank matrix for these endogenous analytes. Validation documented the applicability for the intended purpose of accurate cortisol and cortisone analysis in saliva. With the participation in a proficiency testing, it was shown that the method was accurate and reliable.

Last but not least, this validated method was successfully implemented in routine clinical analysis of a large number of samples (1983 study samples and 4056 injections in total analyzed in 24 batches of 169 injections per batch). This study clearly confirms the real-life applicability of micro-LC-MS, which was shown to be robust enough to perform a large-scale clinical study. At present we can make this claim only for analytes for which proper isotope-labelled internal standards are available and implemented as in the present case. Overall, this method may

represent a green alternative to established standard flow HPLC-ESI-MS/MS methods for steroids including cortisol.

## CRediT authorship contribution statement

**Ece Aydin:** Investigation, Methodology, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Bernhard Drotleff:** Investigation, Conceptualization, Methodology, Supervision, Writing – review & editing. **Hannes Noack:** Investigation, Writing – review & editing. **Birgit Derntl:** Conceptualization, Supervision, Writing – review & editing, Resources. **Michael Lämmerhofer:** Conceptualization, Methodology, Supervision, Writing – review & editing, Resources.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122939.

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**Supporting Information for**

# Fast accurate quantification of salivary cortisol and cortisone in a large-scale clinical stress study by micro-UHPLC-ESI-MS/MS using a surrogate calibrant approach

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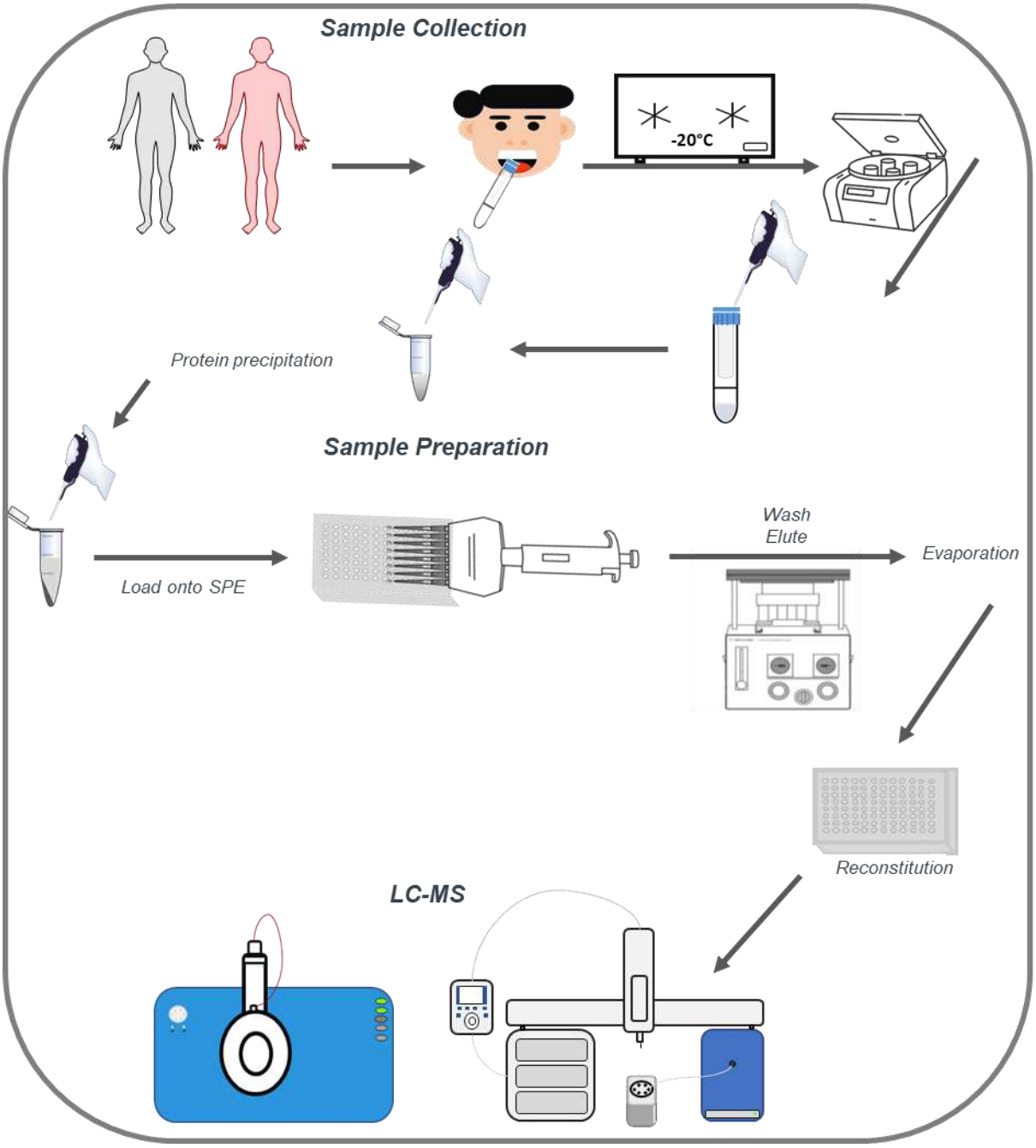


**Fig. S1** Scheme of the experimental procedure.

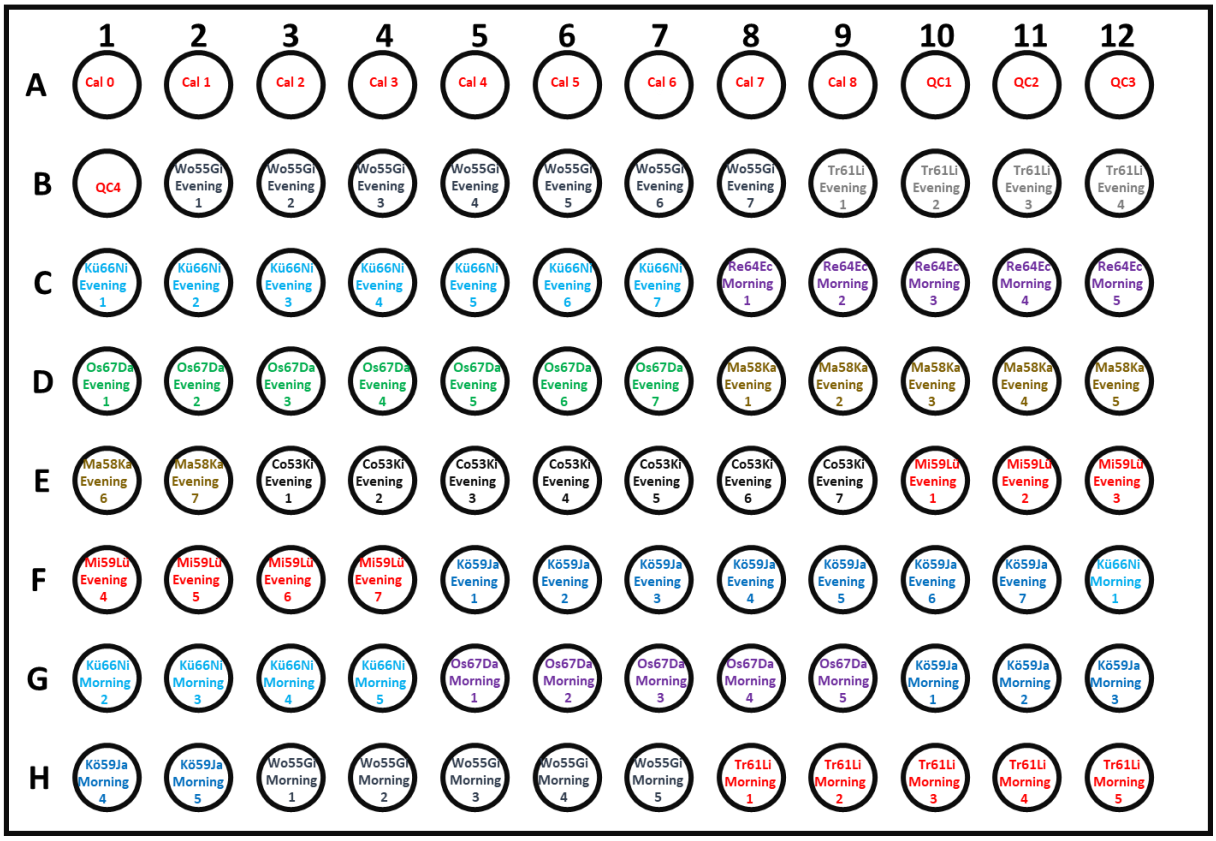


**Fig. S2** Scheme of a 96 Well Plate template example for sample preparation. Cal and QC represent here calibrants and quality control samples, respectively and the rest are patient samples that are encoded.

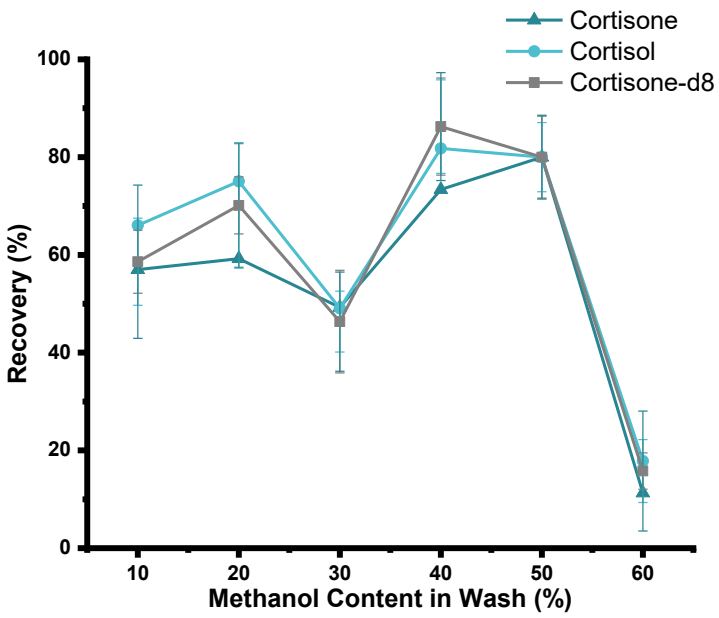


**Fig. S3** Effect of methanol content in (MeOH/$H_2O$) of wash step of solid phase extraction.

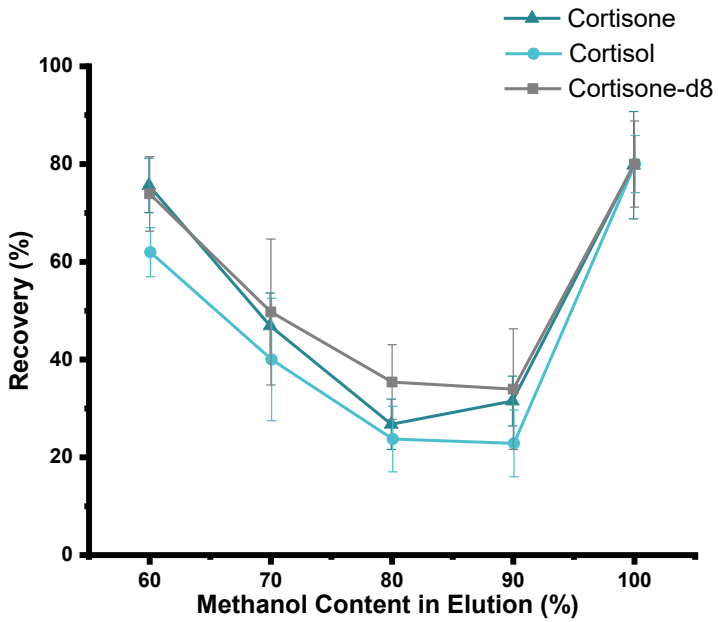


**Fig. S4** Effect of methanol content in (MeOH/$H_2O$) of elution step of solid phase extraction.

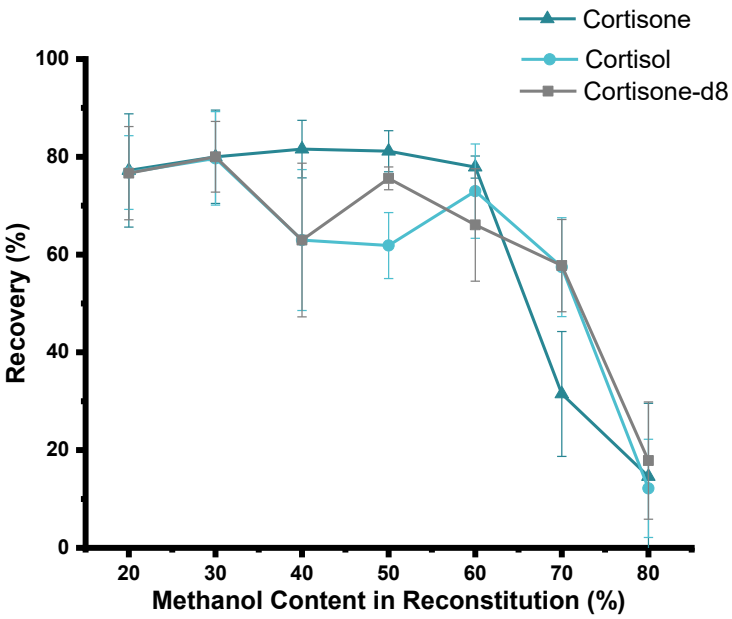


**Fig. S5** Effect of methanol content in ($MeOH/H_2O$) of reconstitution step of solid phase extraction.

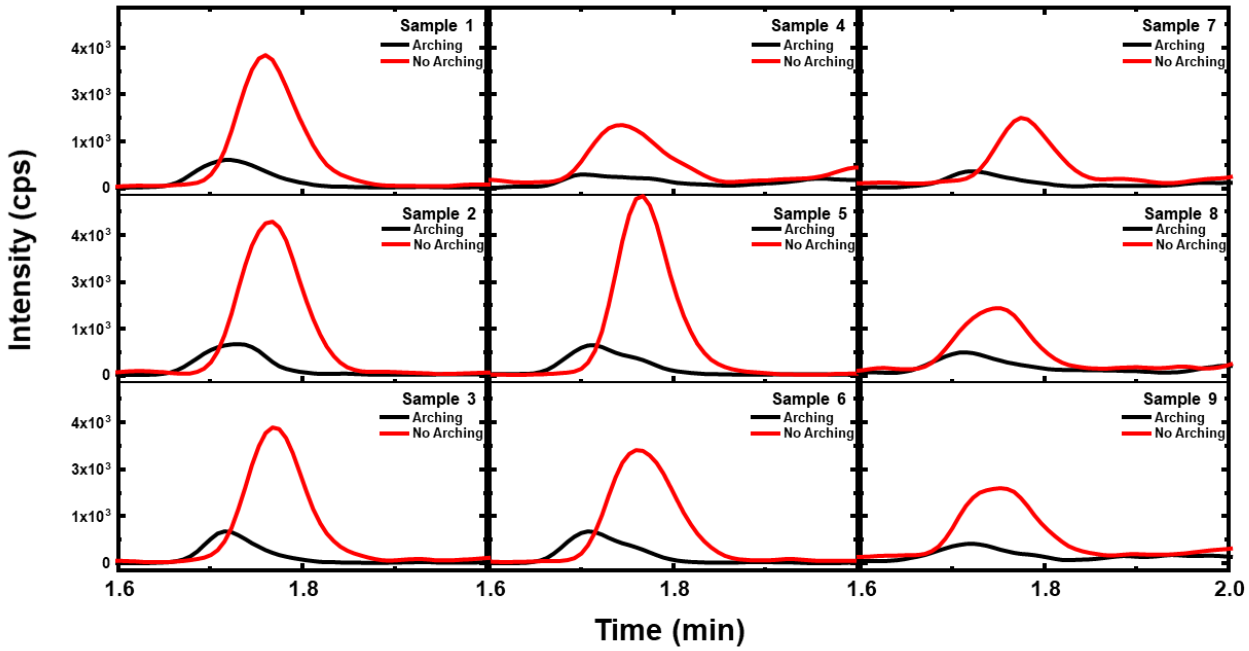


**Fig. S6** Chromatograms of internal standard in different experimental samples showing the effect of arching in the ESI probe on the ionization efficiency and sensitivity, respectively. The samples mentioned as “arching” were re-measured again without arching (indicated as “no arching”).

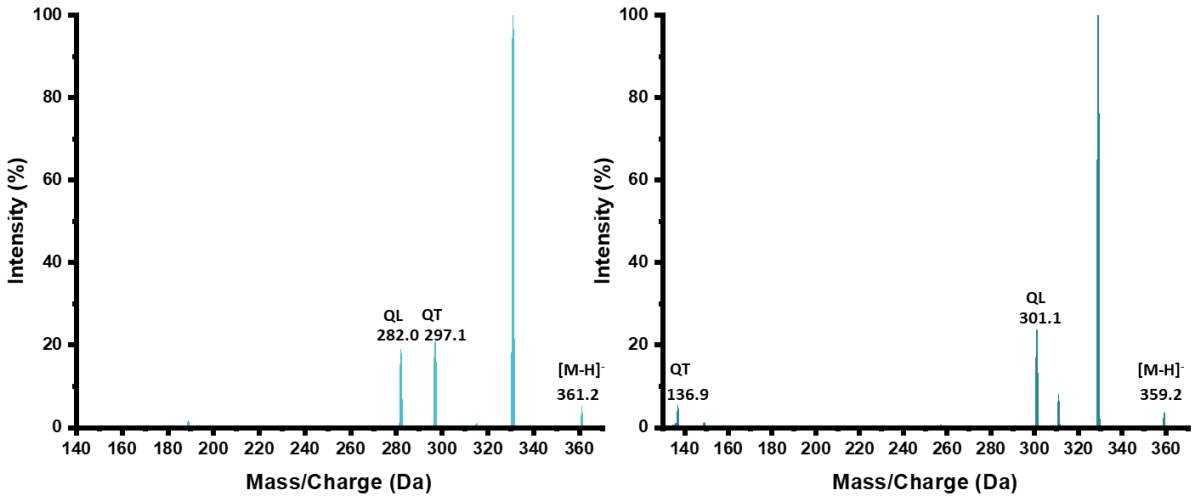


**Fig. S7** Mass spectra of cortisol (a) and cortisone (b), respectively, and their product ions in solvent solution with 0.2 mM $NH_4F$, injected with syringe by direct infusion. Most abundant product ions of cortisol and cortisone with m/z value of 331.2 and 329.1 were not selected as qualifier and quantifier transitions, as two huge interfering peaks were observed in matrix samples. Spectra were acquired from product ion scan. Collision energy ramped between 20-40 V. QL and QT represents here qualifier ion and quantifier ion, respectively.

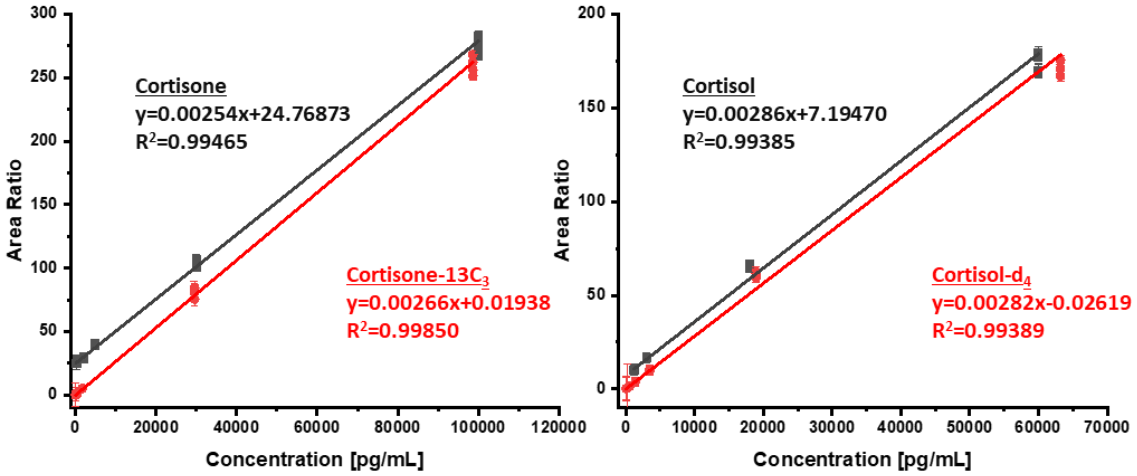


**Fig. S8** Parallelism of standard addition curve and corresponding surrogate calibrant curve for cortisol and cortisol-$d_4$, cortisone and cortisone-$^{13}C_3$ during validation exemplarily shown for one day.

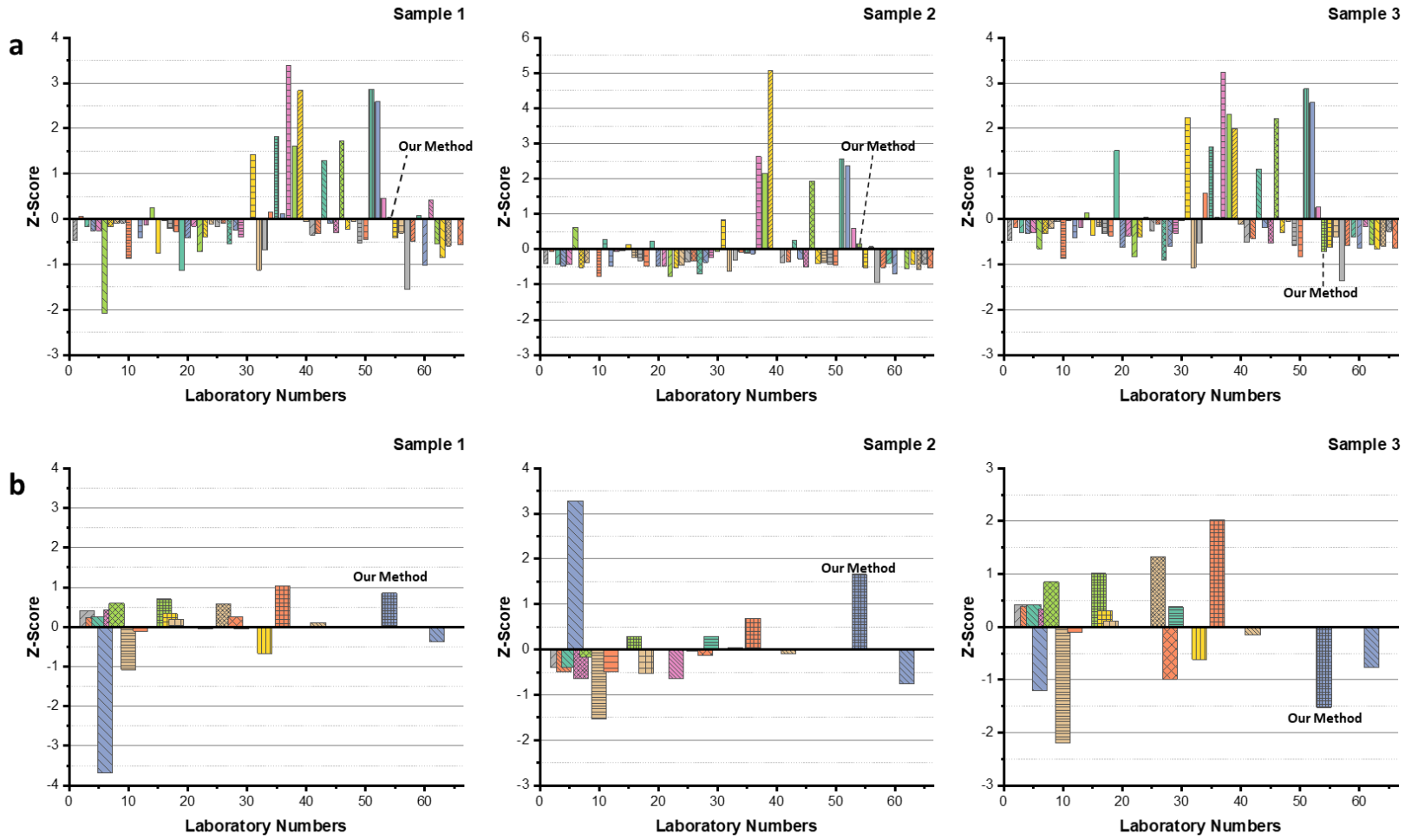


**Fig. S9** Z-Scores of proficiency test reported for cortisol concentrations in saliva. Our method was used in this proficiency test. Calculated Z-score for Sample 1 was 0.032 for among all methods (LUM, ELISA, MS, RIA and Roche) (a) and 0.857 for MS methods (b), for Sample 2 was 0.155 for among all methods (LUM, ELISA, MS, RIA and Roche) (a) and 1.66 for MS methods (b), for Sample 3 was -0.724 for among all methods (LUM, ELISA, MS, RIA and Roche) (a) and -1.521 for MS methods (b) (satisfactory $|z| \leq 2$; questionable $2 < |z| \leq 3$; unsatisfactory $|z| > 3$).

**Table S1** Master Mix Concentrations

| | Type of Master Mix, used | Master Mix concentration (nM) | Volume (µL) | Absolute amount (nmol) |
|---|---|---|---|---|
| | | Cortisone-$^{13}C_3$ / Cortisol-$d_4$ | | Cortisone-$^{13}C_3$ / Cortisol-$d_4$ |
| Cal 1 / $QC_{LLOQ}$ | $MM_{Low}$ | 3.61/3.90 | 6.25 | 0.023/0.024 |
| Cal 2 | $MM_{Low}$ | 3.61/3.90 | 9.38 | 0.034/0.037 |
| Cal 3 | $MM_{Low}$ | 3.61/3.90 | 25.00 | 0.090/0.098 |
| Cal 4 | $MM_{Low}$ | 3.61/3.90 | 50.00 | 0.181/0.195 |
| Cal 5 | 1:10 dilution of $MM_{High}$* | 25.83/15.52 | 10.00 | 0.258/0.155 |
| Cal 6 | 1:10 dilution of $MM_{High}$* | 25.83/15.52 | 25.00 | 0.646/0.388 |
| Cal 7 | $MM_{High}$* | 258.31/155.18 | 15.00 | 3.875/2.328 |
| Cal 8 | $MM_{High}$* | 258.31/155.18 | 50.00 | 12.916/7.759 |
| $QC_{3xLLOQ}$ | $MM_{Low}$ | 258.31/155.18 | 18.75 | 0.069/0.072 |
| $QC_{Mid}$ | 1:10 dilution of $MM_{High}$* | 25.83/15.52 | 7.50 | 0.194/0.116 |
| $QC_{Inter}$ | 1:10 dilution of $MM_{High}$* | 25.83/15.52 | 12.50 | 0.323/0.194 |
| $QC_{High}$ | $MM_{High}$* | 258.31/155.18 | 35.00 | 9.041/5.431 |

* MM represents here Master Mix. $MM_{High}$ was used to extend the calibration to higher concentrations. Final surrogate analyte concentrations in $MM_{Low}$ and $MM_{High}$: Cortisone-$^{13}C_3$: 1.24 ng/mL and 88.71 ng/mL; Cortisol-$d_4$ 1.43 ng/mL and 56.87 ng/mL, respectively.

**Table S2** Concentration of surrogate calibrants in calibration and quality control samples*

| | Dilution Factor to Cal 8 | Final Concentration of Cortisone-$^{13}C_3$ | Final Concentration of Cortisol-$d_4$ |
|---|---|---|---|
| Cal 1 / $QC_{LLOQ}$ | 2000 | 61.9 | 71.7 |
| Cal 2 | 1333.3 | 92.9 | 107.5 |
| Cal 3 | 500 | 247.7 | 286.7 |
| Cal 4 | 250 | 495.3 | 573.4 |
| Cal 5 | 50 | 1731.2 | 1378.1 |
| Cal 6 | 20 | 4327.9 | 3445.2 |
| Cal 7 | 3.3 | 29571.6 | 18956.5 |
| Cal 8 | | 98572.1 | 63188.4 |
| $QC_{3xLLOQ}$ | 666.7 | 185.7 | 215.0 |
| $QC_{Mid}$ | 66.7 | 1298.4 | 1033.6 |
| $QC_{Inter}$ | 40.0 | 2163.9 | 1722.6 |
| $QC_{High}$ | 1.4 | 69000.5 | 42917.5 |

*Concentrations are given in pg $mL^{-1}$.

**Table S3** Typical sequence of the measurement of a batch*

| Analytical Order | Sample Name | Type of Samples | Analytical Order | Sample Name | Type of Samples | Analytical Order | Sample Name | Type of Samples |
|---|---|---|---|---|---|---|---|---|
| 1 | Blank | Solvent Solution | 27 | $QC_{High}$ | QCs | 53 | Re-E-3 | Sample |
| 2 | Blank | Solvent Solution | 28 | Blank | Solvent Solution | 54 | Re-E-4 | Sample |
| 3 | Blank | Solvent Solution | 29 | Cal 0 | Zero-calibrator | 55 | Re-E-5 | Sample |
| 4 | Cal 0** | Zero-calibrator | 30 | Wo-E-1 | Sample | 56 | Re-E-6 | Sample |
| 5 | Cal 0** | Zero-calibrator | 31 | Wo-E-2 | Sample | 57 | Re-E-7 | Sample |
| 6 | Cal 1 | Non-zero Calibrators | 32 | Wo-E-3 | Sample | 58 | Blank | Solvent Solution |
| 7 | Cal 1 | Non-zero Calibrators | 33 | Wo-E-4 | Sample | 59 | Cal 0 | Zero-calibrator |
| 8 | Cal 2 | Non-zero Calibrators | 34 | Wo-E-5 | Sample | 60 | $QC_{3xLLOQ}$ | QCs |
| 9 | Cal 2 | Non-zero Calibrators | 35 | Wo-E-6 | Sample | 61 | $QC_{Mid}$ | QCs |
| 10 | Cal 3 | Non-zero Calibrators | 36 | Wo-E-7 | Sample | 62 | $QC_{Inter}$ | QCs |
| 11 | Cal 3 | Non-zero Calibrators | 37 | Tr-E-1 | Sample | 63 | $QC_{High}$ | QCs |
| 12 | Cal 4 | Non-zero Calibrators | 38 | Tr-E-2 | Sample | 64 | Blank | Solvent Solution |
| 13 | Cal 4 | Non-zero Calibrators | 39 | Tr-E-3 | Sample | 65 | Cal 0 | Zero-calibrator |
| 14 | Cal 5 | Non-zero Calibrators | 40 | Tr-E-4 | Sample | 66 | Os-E-1 | Sample |
| 15 | Cal 5 | Non-zero Calibrators | 41 | Tr-E-5 | Sample | 67 | Os-E-2 | Sample |
| 16 | Cal 6 | Non-zero Calibrators | 42 | Tr-E-6 | Sample | 68 | Os-E-3 | Sample |
| 17 | Cal 6 | Non-zero Calibrators | 43 | Tr-E-7 | Sample | 69 | Os-E-4 | Sample |
| 18 | Cal 7 | Non-zero Calibrators | 44 | Kü-E-1 | Sample | 70 | Os-E-5 | Sample |
| 19 | Cal 7 | Non-zero Calibrators | 45 | Kü-E-2 | Sample | 71 | Os-E-6 | Sample |
| 20 | Cal 8 | Non-zero Calibrators | 46 | Kü-E-3 | Sample | 72 | Os-E-7 | Sample |
| 21 | Cal 8 | Non-zero Calibrators | 47 | Kü-E-4 | Sample | 73 | Ma-E-1 | Sample |
| 22 | Blank | Solvent Solution | 48 | Kü-E-5 | Sample | 74 | Ma-E-2 | Sample |
| 23 | Cal 0 | Zero-calibrator | 49 | Kü-E-6 | Sample | 75 | Ma-E-3 | Sample |
| 24 | $QC_{3xLLOQ}$ | QCs | 50 | Kü-E-7 | Sample | 76 | Ma-E-4 | Sample |
| 25 | $QC_{Mid}$ | QCs | 51 | Re-E-1 | Sample | 77 | Ma-E-5 | Sample |
| 26 | $QC_{Inter}$ | QCs | 52 | Re-E-2 | Sample | 78 | Ma-E-6 | Sample |

| Analytical Order | Sample Name | Type of Samples | Analytical Order | Sample Name | Type of Samples | Analytical Order | Sample Name | Type of Samples |
|---|---|---|---|---|---|---|---|---|
| 79 | Ma-E-7 | Sample | 105 | Kö-E-4 | Sample | 131 | Tr-M-1 | Sample |
| 80 | Co-E-1 | Sample | 106 | Kö-E-5 | Sample | 132 | Tr-M-2 | Sample |
| 81 | Co-E-2 | Sample | 107 | Kö-E-6 | Sample | 133 | Tr-M-3 | Sample |
| 82 | Co-E-3 | Sample | 108 | Kö-E-7 | Sample | 134 | Tr-M-4 | Sample |
| 83 | Co-E-4 | Sample | 109 | Kü-M-1 | Sample | 135 | Tr-M-5 | Sample |
| 84 | Co-E-5 | Sample | 110 | Kü-M-2 | Sample | 136 | Blank | Solvent Solution |
| 85 | Co-E-6 | Sample | 111 | Kü-M-3 | Sample | 137 | Cal 0 | Zero-calibrator |
| 86 | Co-E-7 | Sample | 112 | Kü-M-4 | Sample | 138 | $QC_{3xLLOQ}$ | QCs |
| 87 | Mi-E-1 | Sample | 113 | Kü-M-5 | Sample | 139 | $QC_{Mid}$ | QCs |
| 88 | Mi-E-2 | Sample | 114 | Kü-M-6 | Sample | 140 | $QC_{Inter}$ | QCs |
| 89 | Mi-E-3 | Sample | 115 | Kü-M-7 | Sample | 141 | $QC_{High}$ | QCs |
| 90 | Mi-E-4 | Sample | 116 | Os-M-1 | Sample | 142 | Blank | Solvent Solution |
| 91 | Mi-E-5 | Sample | 117 | Os-M-2 | Sample | 143 | Cal 0 | Zero-calibrator |
| 92 | Mi-E-6 | Sample | 118 | Os-M-3 | Sample | 144 | Cal 1 | Non-zero Calibrators |
| 93 | Mi-E-7 | Sample | 119 | Os-M-4 | Sample | 145 | Cal 1 | Non-zero Calibrators |
| 94 | Blank | Solvent Solution | 120 | Os-M-5 | Sample | 146 | Cal 2 | Non-zero Calibrators |
| 95 | Cal 0 | Zero-calibrator | 121 | Kö-M-1 | Sample | 147 | Cal 2 | Non-zero Calibrators |
| 96 | $QC_{3xLLOQ}$ | QCs | 122 | Kö-M-2 | Sample | 148 | Cal 3 | Non-zero Calibrators |
| 97 | $QC_{Mid}$ | QCs | 123 | Kö-M-3 | Sample | 149 | Cal 3 | Non-zero Calibrators |
| 98 | $QC_{Inter}$ | QCs | 124 | Kö-M-4 | Sample | 150 | Cal 4 | Non-zero Calibrators |
| 99 | $QC_{High}$ | QCs | 125 | Kö-M-5 | Sample | 151 | Cal 4 | Non-zero Calibrators |
| 100 | Blank | Solvent Solution | 126 | Wo-M-1 | Sample | 152 | Cal 5 | Non-zero Calibrators |
| 101 | Cal 0 | Zero-calibrator | 127 | Wo-M-2 | Sample | 153 | Cal 5 | Non-zero Calibrators |
| 102 | Kö-E-1 | Sample | 128 | Wo-M-3 | Sample | 154 | Cal 6 | Non-zero Calibrators |
| 103 | Kö-E-2 | Sample | 129 | Wo-M-4 | Sample | 155 | Cal 6 | Non-zero Calibrators |
| 104 | Kö-E-3 | Sample | 130 | Wo-M-5 | Sample | 156 | Cal 7 | Non-zero Calibrators |

| Analytical Order | Sample Name | Type of Samples | Analytical Order | Sample Name | Type of Samples | Analytical Order | Sample Name | Type of Samples |
|---|---|---|---|---|---|---|---|---|
| 157 | Cal 7 | Non-zero Calibrators | 162 | $QC_{3xLLOQ}$ | QCs | 167 | Cal 0 | Zero-calibrator |
| 158 | Cal 8 | Non-zero Calibrators | 163 | $QC_{Mid}$ | QCs | 168 | Blank | Solvent Solution |
| 159 | Cal 8 | Non-zero Calibrators | 164 | $QC_{Inter}$ | QCs | 169 | Blank | Solvent Solution |
| 160 | Blank | Solvent Solution | 165 | $QC_{High}$ | QCs | | | |
| 161 | Cal 0 | Zero-calibrator | 166 | Blank | Solvent Solution | | | |

*QC and Cal represent here Quality control sample and calibrant, respectively, and the rest are patient samples that are coded.

**Cal 0 represents here non-zero calibrant which was not spiked with any Master Mix solution.

**Table S4**. Proficiency test results from all laboratories for salivary cortisol*

| | Methods | Lab. Code | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample 1** | All Methods | (µg/dL) | 1.266 | 1.640 | 1.475 | 1.417 | 1.420 | 0.123 | 1.480 | 1.533 | 1.540 | 0.982 | 1.601 | 1.304 | 1.504 | 1.780 | 1.062 | 1.569 | 1.449 | 1.404 | 0.797 |
| | LUM | | 1.266 | 1.640 | | | | | | | | | | | | | | | | | |
| | ELISA | | | | | | | | | | | | | | 1.5 | 1.78 | | | | | |
| | MS | | | | 1.475 | 1.417 | 1.420 | 0.123 | 1.480 | 1.533 | | 0.982 | | 1.304 | | | | 1.569 | 1.449 | 1.404 | |
| | Others (Roche, RIA) | | | | | | | | | | 1.540 | | 1.601 | | | | 1.062 | | | | 0.797 |
| **Sample 2** | All Methods | | 0.076 | 0.11 | 0.072 | 0.069 | 0.072 | 0.174 | 0.065 | 0.078 | 0.114 | 0.040 | 0.141 | 0.069 | 0.108 | 0.110 | 0.127 | 0.091 | 0.083 | 0.068 | 0.137 |
| | LUM | | 0.076 | 0.11 | | | | | | | | | | | | | | | | | |
| | ELISA | | | | | | | | | | | | | | 0.108 | 0.110 | | | | | |
| | MS | | | | 0.072 | 0.069 | 0.072 | 0.174 | 0.065 | 0.078 | | 0.040 | | 0.069 | | | | 0.091 | 0.083 | 0.068 | |
| | Others (Roche, RIA) | | | | | | | | | | 0.114 | | 0.141 | | | | 0.127 | | | | 0.137 |
| **Sample 3** | All Methods | | 0.667 | 0.79 | 0.736 | 0.732 | 0.736 | 0.587 | 0.728 | 0.775 | 0.837 | 0.496 | 0.848 | 0.688 | 0.783 | 0.920 | 0.714 | 0.790 | 0.725 | 0.708 | 1.497 |
| | LUM | | 0.667 | 0.79 | | | | | | | | | | | | | | | | | |
| | ELISA | | | | | | | | | | | | | | 0.783 | 0.920 | | | | | |
| | MS | | | | 0.736 | 0.732 | 0.736 | 0.587 | 0.728 | 0.775 | | 0.496 | | 0.688 | | | | 0.790 | 0.725 | 0.708 | |
| | Others (Roche, RIA) | | | | | | | | | | 0.837 | | 0.848 | | | | 0.714 | | | | 1.497 |

| | Methods | Lab. Code | **20** | **21** | **22** | **23** | **24** | **25** | **26** | **27** | **28** | **29** | **30** | **31** | **32** | **33** | **34** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample 1** | All Methods | (μg/dL) | 1.306 | 1.486 | 1.094 | 1.324 | 1.520 | 1.480 | 1.529 | 1.216 | 1.423 | 1.319 | 1.615 | 2.605 | 0.805 | 1.117 | 1.710 |
| | LUM | | | | | | | 1.48 | | | | | | 2.605 | | | |
| | ELISA | | 1.31 | | 1.09 | | 1.52 | | | 1.22 | | | 1.62 | | 0.81 | | |
| | MS | | | | | 1.324 | | | 1.529 | | 1.423 | 1.319 | | | | 1.117 | |
| | Others (Roche, RIA) | | | 1.486 | | | | | | | | | | | | | 1.710 |
| **Sample 2** | All Methods | | 0.068 | 0.069 | 0.040 | 0.065 | 0.070 | 0.080 | 0.082 | 0.047 | 0.079 | 0.091 | 0.109 | 0.193 | 0.054 | 0.084 | 0.106 |
| | LUM | | | | | | | 0.080 | | | | | | 0.193 | | | |
| | ELISA | | 0.068 | | 0.040 | | 0.070 | | | 0.047 | | | 0.109 | | 0.054 | | |
| | MS | | | | | 0.065 | | | 0.082 | | 0.079 | 0.091 | | | | 0.084 | |
| | Others (Roche, RIA) | | | 0.069 | | | | | | | | | | | | | 0.106 |
| **Sample 3** | All Methods | | 0.598 | 0.707 | 0.514 | 0.697 | 0.880 | 0.750 | 0.819 | 0.479 | 0.607 | 0.732 | 0.846 | 1.799 | 0.413 | 0.641 | 1.100 |
| | LUM | | | | | | | 0.750 | | | | | | 1.799 | | | |
| | ELISA | | 0.598 | | 0.514 | | 0.880 | | | 0.479 | | | 0.846 | | 0.413 | | |
| | MS | | | | | 0.697 | | | 0.819 | | 0.607 | 0.732 | | | | 0.641 | |
| | Others (Roche, RIA) | | | 0.707 | | | | | | | | | | | | | 1.100 |

| | Methods | Lab. Code | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample 1** | All Methods | (µg/dL) | 2.890 | 1.682 | 4.000 | 2.730 | 3.600 | 1.561 | 1.350 | 1.375 | 2.513 | 1.530 | 1.387 | 2.820 | 1.440 | 1.562 | 1.226 |
| | LUM | | | | | | | | | | | 1.53 | | | | | |
| | ELISA | | 2.89 | | 4 | 2.73 | 3.6 | | 1.35 | | | | 1.39 | 2.82 | | | 1.23 |
| | MS | | | 1.682 | | | | | | 1.375 | | | | | | | |
| | Others (Roche, RIA) | | | | | | | 1.561 | | | 2.513 | | | | 1.440 | 1.562 | |
| **Sample 2** | All Methods | | 0.103 | 0.102 | 0.366 | 0.320 | 0.600 | 0.113 | 0.078 | 0.080 | 0.138 | 0.088 | 0.066 | 0.300 | 0.076 | 0.078 | 0.072 |
| | LUM | | | | | | | | | | | 0.088 | | | | | |
| | ELISA | | 0.103 | | 0.366 | 0.320 | 0.600 | | 0.078 | | | | 0.066 | 0.300 | | | 0.072 |
| | MS | | | 0.102 | | | | | | 0.080 | | | | | | | |
| | Others (Roche, RIA) | | | | | | | 0.113 | | | 0.138 | | | | 0.076 | 0.078 | |
| **Sample 3** | All Methods | | 1.530 | 0.882 | 2.220 | 1.830 | 1.700 | 0.816 | 0.647 | 0.683 | 1.326 | 0.780 | 0.641 | 1.790 | 0.738 | 0.841 | 0.619 |
| | LUM | | | | | | | | | | | 0.780 | | | | | |
| | ELISA | | 1.530 | | 2.220 | 1.830 | 1.700 | | 0.647 | | | | 0.641 | 1.790 | | | 0.619 |
| | MS | | | 0.882 | | | | | | 0.683 | | | | | | 0.841 | |
| | Others (Roche, RIA) | | | | | | | 0.816 | | | 1.326 | | | | 0.738 | 0.841 | |

| | Methods | Lab. Code | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 | 66 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample 1** | All Methods | (µg/dL) | 1.280 | 3.626 | 3.430 | 1.922 | 1.621 | 1.303 | 1.379 | 0.504 | 1.257 | 1.649 | 0.880 | 1.901 | 1.217 | 0.995 | 1.180 | 1.584 | 1.202 |
| | LUM | | | | | | | | | | | | 0.88 | | | | | 1.58 | |
| | ELISA | | 1.28 | 3.63 | | | | 1.3 | 1.38 | 0.5 | 1.26 | 1.65 | | 1.901 | | 1 | | | 1.2 |
| | MS | | | | | | **1.621** | | | | | | | | 1.217 | | | | |
| | Others (Roche, RIA) | | | | 3.430 | 1.922 | | | | | | | | | | | 1.180 | | |
| **Sample 2** | All Methods | | 0.070 | 0.360 | 0.340 | 0.170 | 0.129 | 0.065 | 0.121 | 0.024 | 0.065 | 0.076 | 0.047 | 0.114 | 0.062 | 0.074 | 0.060 | 0.074 | 0.064 |
| | LUM | | | | | | | | | | | | 0.047 | | | | | 0.074 | |
| | ELISA | | 0.070 | 0.360 | | | | 0.065 | 0.121 | 0.024 | 0.065 | 0.076 | 0.114 | | | 0.074 | | | 0.064 |
| | MS | | | | | | **0.129** | | | | | | | | 0.062 | | | | |
| | Others (Roche, RIA) | | | | 0.340 | 0.170 | | | | | | | | | | | 0.060 | | |
| **Sample 3** | All Methods | | 0.510 | 2.069 | 1.940 | 0.972 | 0.558 | 0.600 | 0.694 | 0.290 | 0.618 | 0.699 | 0.591 | 0.795 | 0.627 | 0.585 | 0.610 | 0.748 | 0.594 |
| | LUM | | | | | | | | | | | | 0.591 | | | | | 0.748 | |
| | ELISA | | 0.510 | 2.069 | | | | 0.600 | 0.694 | 0.290 | 0.618 | 0.699 | 0.795 | | | 0.585 | | | 0.594 |
| | MS | | | | | | **0.558** | | | | | | | | 0.627 | | | | |
| | Others (Roche, RIA) | | | | 1.940 | 0.972 | | | | | | | | | | | 0.610 | | |

***** Lab-codes are anonymized. Light grey shaded background indicates In-House method results.

**Table S5**. Statistical Results of Proficiency Test

| Sample 1 | | | | Sample 2 | | | | Sample 3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| All Methods (μg/dL) | | MS Methods (μg/dL) | | All Methods (μg/dL) | | MS Methods (μg/dL) | | All Methods (μg/dL) | | MS Methods (μg/dL) | |
| Our Method | 1.621 | Our Method | 1.621 | Our Method | 0.129 | Our Method | 0.129 | Our Method | 0.558 | Our Method | 0.558 |
| **Mean** | **1.598** | **Mean** | **1.338** | **Mean** | **0.114** | **Mean** | **0.083** | **Mean** | **0.862** | **Mean** | **0.697** |
| sd | 0.707 | sd | 0.330 | sd | 0.096 | sd | 0.028 | sd | 0.420 | sd | 0.092 |
| Mean-sd | 0.892 | Mean-sd | 1.008 | Mean-sd | 0.018 | Mean-sd | 0.055 | Mean-sd | 0.442 | Mean-sd | 0.606 |
| Mean-2sd | 0.185 | Mean-2sd | 0.678 | Mean-2sd | -0.077 | Mean-2sd | 0.027 | Mean-2sd | 0.023 | Mean-2sd | 0.514 |
| Mean-3sd | -0.521 | Mean-3sd | 0.348 | Mean-3sd | -0.173 | Mean-3sd | -0.001 | Mean-3sd | -0.397 | Mean-3sd | 0.423 |
| Mean+sd | 2.305 | Mean+sd | 1.668 | Mean+sd | 0.210 | Mean+sd | 0.111 | Mean+sd | 1.281 | Mean+sd | 0.789 |
| Mean+2sd | 3.011 | Mean+2sd | 1.999 | Mean+2sd | 0.305 | Mean+2sd | 0.138 | Mean+2sd | 1.701 | Mean+2sd | 0.881 |
| Mean+3sd | 3.718 | Mean+3sd | 2.329 | Mean+3sd | 0.401 | Mean+3sd | 0.166 | Mean+3sd | 2.121 | Mean+3sd | 0.972 |
| Z-Score | 0.032 | Z-Score | 0.857 | Z-Score | 0.155 | Z-Score | 1.660 | Z-Score | -0.560 | Z-Score | -0.768 |

**Table S6**. Dilution effects of cortisol and cortisone in saliva during validation

| Analyte | Concentration Levels | Dilution Factor | Accuracy (RE, %) | Precision (CV, %) |
|---|---|---|---|---|
| **Cortisone** | $QC_{3xLLOQ}$ | **2.8** | 85.6 | 7.9 |
| | $QC_{Mid}$ | | 100.7 | 5.4 |
| | $QC_{ULOQ}$ | | 97.6 | 7.8 |
| **Cortisol** | $QC_{3xLLOQ}$ | **2.8** | 90.9 | 4.7 |
| | $QC_{Mid}$ | | 110.2 | 9.5 |
| | $QC_{ULOQ}$ | | 106.8 | 2.9 |

**Table S7** Summary of the validation for stability test*

| | | Analyte | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Cortisol-$d_4$** | | | **Cortisone-$^{13}C_3$** | | |
| **Freeze/ Thaw Stability [%]** | $QC_{3xLLOQ}$ | 101.51 ± 0.08 | | | 88.49 ± 3.87 | | |
| | $QC_{High}$ | 83.39 ± 2.74 | | | 92.33 ± 1.31 | | |
| **Long Term Stability [%]** | $QC_{3xLLOQ}$ | 6 months<br>91.19 ± 1.12 | 1 year<br>80.45 ± 3.8 | | 6 months<br>80.78 ± 0.58 | 1 year<br>78.9 ± 5.90 | |
| | $QC_{High}$ | 6 months<br>86.2 ± 2.53 | 1 year<br>79.2 ± 5.27 | | 6 months<br>101.11 ± 1.48 | 1 year<br>83.11 ± 4.67 | |
| **Bench-top Stability [%]** | $QC_{3xLLOQ}$ | 90.00 ± 8.89 | | | 82.26 ± 0.44 | | |
| | $QC_{High}$ | 94.22 ± 0.68 | | | 82.88 ± 4.61 | | |
| **Extract Stability [%]** | $QC_{3xLLOQ}$ | 10h<br>92.19 ± 1.23 | 24h<br>90.89 ± 2.97 | 48h<br>85.19 ± 1.05 | 10h<br>83.32 ± 8.75 | 24h<br>85.20 ± 4.23 | 48h<br>80.09 ± 0.59 |
| | $QC_{High}$ | 10h<br>87.1 ± 0.83 | 24h<br>80.9 ± 5.41 | 48h<br>79.9 ± 6.23 | 10h<br>91.23 ± 9.37 | 24h<br>88.83 ± 7.29 | 48h<br>87.5 ± 3.01 |
| **Stock Solution Stability [%]** | $MM_{Low}$** | 109.59 ± 3.28 | | | 105.04 ± 2.91 | | |
| | $MM_{High}$** | 99.97 ± 14.45 | | | 107.51 ± 6.74 | | |

*The samples are measured in quintuplicate.

**MM represents here Master Mix, $MM_{Low}$ and $MM_{High}$ were diluted with dilution rate of 1:10 and 1:50, respectively.

**Table S8:** Overview of clinical study size

| | n |
|---|---|
| Total number of samples | 1983 |
| Total number of injections (incl calibrants, QCs, blanks) | 4056 |
| Number of batches | 24 |
| Injections per batch | 169 |
| Study samples per batch | 83 |
| Average injections per column | 811 |
| Total number of columns required | 5 |

**Table S9**: Inter-batch assay performance for cortisol

| | Calibration Cortisol-$d_4$ | | | Precision [%] | | | Accuracy [%] | | |
|---|---|---|---|---|---|---|---|---|---|
| **Batch** | **slope** | **intercept** | **$r^2$** | **$QC_{Low}$** | **$QC_{Mid}$** | **$QC_{High}$** | **$QC_{Low}$** | **$QC_{Mid}$** | **$QC_{High}$** |
| Batch 1 | 0.0028 | -0.0262 | 0.9939 | 11.4 | 3.7 | 3.9 | 110 | 92 | 111 |
| Batch 2&3[a] | 0.0022 | -0.1283 | 0.9990 | 5.5 | 5.4 | 7.0 | 108 | 99 | 103 |
| Batch 4 | 0.0024 | -0.0753 | 0.9962 | 7.3 | 5.3 | 5.3 | 104 | 105 | 102 |
| Batch 5 | 0.0019 | 0.4050 | 0.9905 | 4.0 | 14.6 | 4.0 | 92 | 91 | 113 |
| Batch 6 | 0.0027 | 0.5640 | 0.9901 | 3.7 | 14.5 | 4.5 | 88 | 91 | 114 |
| Batch 7 | 0.0031 | 0.5998 | 0.9953 | 5.4 | 5.1 | 2.0 | 107 | 99 | 113 |
| Batch 8 | 0.0017 | 0.0256 | 0.9916 | 7.2 | 7.6 | 8.8 | 107 | 115 | 95 |
| Batch 9 | 0.0011 | 0.5374 | 0.9989 | 11.7 | 7.9 | 4.8 | 104 | 86 | 102 |
| Batch 10&11[a] | 0.0011 | 0.5920 | 0.9998 | 9.3 | 7.9 | 4.9 | 101 | 92 | 103 |
| Batch 12 | 0.0017 | -0.0778 | 0.9931 | 15.6 | 10.5 | 9.5 | 93 | 106 | 103 |
| Batch 13&14[a] | 0.0011 | 0.5374 | 0.9998 | 13.8 | 7.9 | 4.9 | 101 | 92 | 103 |
| Batch 15 | 0.0014 | -0.0256 | 0.9967 | 6.5 | 9.7 | 6.7 | 109 | 108 | 109 |
| Batch 16 | 0.0016 | -0.0191 | 0.9990 | 9.2 | 8.8 | 5.2 | 98 | 93 | 91 |
| Batch 17 | 0.0017 | 0.0311 | 0.9922 | 5.3 | 7.3 | 6.5 | 106 | 115 | 87 |
| Batch 18 | 0.0013 | 0.0208 | 0.9989 | 5.4 | 9.3 | 2.8 | 95 | 102 | 105 |
| Batch 19 | 0.0014 | 0.0221 | 0.9926 | 4.0 | 6.2 | 7.4 | 93 | 91 | 95 |
| Batch 20 | 0.0010 | 0.0163 | 0.9974 | 16.1 | 3.4 | 3.0 | 98 | 112 | 106 |
| Batch 21 | 0.0017 | 0.0087 | 0.9923 | 13.8 | 4.8 | 6.2 | 96 | 91 | 86 |
| Batch 22 | 0.0010 | 0.0081 | 0.9935 | 2.4 | 0.3 | 6.0 | 94 | 108 | 114 |
| Batch 23 | 0.0015 | 0.0002 | 0.9971 | 9.9 | 7.6 | 7.8 | 87 | 108 | 112 |
| Batch 24 | 0.0018 | -0.0990 | 0.9957 | 9.2 | 5.1 | 5.1 | 108 | 103 | 104 |
| **Mean** | 0.0017 | 0.1389 | 0.9954 | 8.4 | 7.3 | 5.5 | 100 | 100 | 104 |
| **sd** | 0.0006 | 0.2653 | 0.0032 | 4.1 | 3.4 | 1.9 | 7.2 | 9.0 | 8.6 |

[a] Due to the arcing on ESI probe, contamination at the LC flow path (by cause of particles from different types of 96 well plate collection mats) and leakage, these batches needed to be re-measured.

**Table S10**: Inter-batch assay performance for cortisone

| | Calibration Cortisone-$^{13}C_3$ | | | Precision [%] | | | Accuracy [%] | | |
|---|---|---|---|---|---|---|---|---|---|
| **Batch** | **slope** | **intercept** | **$r^2$** | **$QC_{Low}$** | **$QC_{Mid}$** | **$QC_{High}$** | **$QC_{Low}$** | **$QC_{Mid}$** | **$QC_{High}$** |
| Batch 1 | 0.0027 | 0.0194 | 0.9985 | 13.5 | 4.9 | 3.9 | 103 | 110 | 111 |
| Batch 2&3[a] | 0.0024 | -0.0238 | 0.9898 | 5.3 | 7.6 | 3.0 | 100 | 102 | 89 |
| Batch 4 | 0.0021 | -0.0057 | 0.9961 | 6.5 | 5.2 | 5.6 | 104 | 102 | 93 |
| Batch 5 | 0.0018 | 0.0394 | 0.9963 | 5.0 | 5.3 | 1.7 | 114 | 109 | 114 |
| Batch 6 | 0.0013 | -0.3453 | 0.9961 | 6.6 | 5.0 | 5.5 | 109 | 101 | 100 |
| Batch 7 | 0.0024 | -0.0238 | 0.9896 | 6.2 | 5.1 | 5.5 | 108 | 101 | 100 |
| Batch 8 | 0.0017 | 0.1214 | 0.9937 | 4.7 | 6.7 | 9.2 | 92 | 90 | 102 |
| Batch 9 | 0.0012 | 0.2429 | 0.9999 | 10.3 | 5.6 | 7.5 | 97 | 106 | 106 |
| Batch 10&11[a] | 0.0014 | 0.0200 | 0.9900 | 9.7 | 4.9 | 6.9 | 96 | 95 | 102 |
| Batch 12 | 0.0011 | 0.2144 | 0.9975 | 2.2 | 11.2 | 4.8 | 96 | 99 | 109 |
| Batch 13&14[a] | 0.0012 | 0.1302 | 0.9957 | 11.5 | 5.2 | 7.2 | 104 | 106 | 97 |
| Batch 15 | 0.0030 | 0.1189 | 0.9975 | 2.6 | 5.7 | 5.2 | 101 | 89 | 102 |
| Batch 16 | 0.0028 | 0.0368 | 0.9979 | 2.3 | 4.8 | 3.9 | 115 | 103 | 109 |
| Batch 17 | 0.0032 | 0.0298 | 0.9954 | 6.5 | 4.5 | 9.1 | 103 | 86 | 89 |
| Batch 18 | 0.0027 | 0.0843 | 0.9957 | 4.3 | 5.7 | 6.2 | 91 | 105 | 108 |
| Batch 19 | 0.0032 | 0.0095 | 0.9982 | 7.8 | 5.2 | 5.0 | 106 | 99 | 95 |
| Batch 20 | 0.0024 | 0.0188 | 0.9975 | 1.9 | 0.2 | 5.6 | 105 | 103 | 110 |
| Batch 21 | 0.0029 | 0.0443 | 0.9973 | 3.3 | 5.2 | 8.9 | 110 | 105 | 104 |
| Batch 22 | 0.0032 | 0.0234 | 0.9975 | 3.4 | 2.2 | 4.2 | 92 | 93 | 111 |
| Batch 23 | 0.0032 | 0.0343 | 0.9964 | 6.2 | 6.1 | 4.8 | 92 | 91 | 102 |
| Batch 24 | 0.0013 | -0.0147 | 0.9944 | 8.2 | 4.8 | 3.9 | 101 | 85 | 93 |
| **Mean** | 0.0022 | 0.0369 | 0.9958 | 6.1 | 5.3 | 5.6 | 102 | 99 | 102 |
| **sd** | 0.0008 | 0.1137 | 0.0029 | 3.2 | 2.0 | 2.0 | 7.2 | 7.5 | 7.5 |

[a] Due to the arcing on ESI probe, contamination at the LC flow path (by cause of particles from different types of 96 well plate collection mats) and leakage, these batches needed to be re-measured.

## 3.2. Publication II Quantification of endocannabinoids in cerebrospinal fluid

# UHPLC-ESI-MS/MS assay for quantification of endocannabinoids in cerebrospinal fluid using surrogate calibrant and surrogate matrix approaches

Ece Aydin[a], Malgorzata Cebo[a], Justyna Mielnik[a,b], Hardy Richter[c,d], Rebecca Schüle[e,f], Adrian Sievers-Engler[a], Piotr Młynarz[b], Michael Lämmerhofer[a,*]

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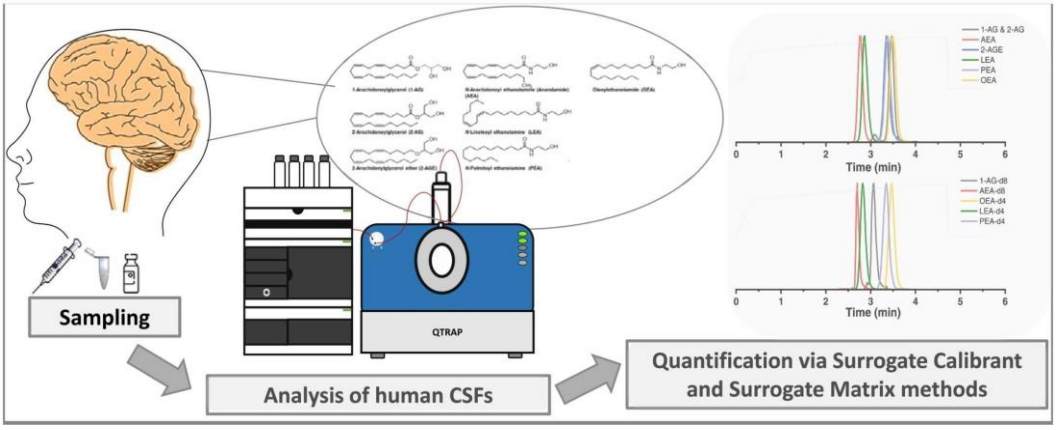


**Graphical Abstract**

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# UHPLC-ESI-MS/MS assay for quantification of endocannabinoids in cerebrospinal fluid using surrogate calibrant and surrogate matrix approaches

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ABSTRACT

Endocannabinoids are endogenous lipids with the main function recognized to act as neuromodulators through their cannabinoid receptors. Dysregulation of the endocannabinoid system is implicated in various pathologies, such as inflammatory and neurodegenerative diseases. In this study we describe a sensitive UHPLC-MS/MS method for the analysis of trace levels of 7 endocannabinoids in cerebrospinal fluid samples. The analytes covered comprised 1- and 2-arachidonoylglycerol 1- and 2-AG (which were analysed as sum due to their interconversion), 2-arachidonylglycerol ether 2-AGE, anandamide AEA, N-linoleoyl ethanolamide LEA, N-palmitoyl ethanolamide PEA and N-oleoyl ethanolamide OEA. Analytes were extracted from the biofluid by a simple monophasic procedure involving protein precipitation with acetonitrile (MeCN). The analytical method is based on chromatographic separation of the analytes with solid-core (core-shell, superficially porous) particle column Cortecs C18+ . Gradient elution with changing proportion of water and acetonitrile and constant concentration of formic acid provided reasonable separation of analytes, close elution of analytes and their internal standards and minimized matrix effects in biological samples. For specific detection of the endocannabinoids a triple-quadrupole tandem mass spectrometer with electrospray ionisation (ESI) and selected reaction monitoring (SRM) mode was used, and it provided good assay selectivity. The developed method required a minute volume of the biological samples (50 μL) and achieved excellent sensitivity (the lower limit of detection was between 4.15 and 30.18 pM of the biological sample). Linear calibration was achieved in the range from 25 to 10,545 pM for AEA, 90–3802 pM for 1-AG, 90–724 pM for 2-AG, 12–5226 pM for LEA, 33–13,942 for OEA, 34–23,850 pM for 2-AGE, 72–30,190 for PEA and 10–4218 for AEA-$d_4$ in CSF. The method was validated and revealed relative errors in the range of − 14.7 to + 12.3% at LLOQ and − 14.1 to + 14.2% for the remaining validation range. Precisions were in the acceptable range (< 20% RSD at LLOQ, and <15% for the remaining levels) as well. It was finally used to quantify endocannabinoids in human cerebrospinal fluid obtained from 118 donors. Accurate quantification of endogenous compounds in biological samples was achieved by using two different principal

*Abbreviations:* aCSF, artificial CSF; 1- and 2-AG, 1- and 2-arachidonoylglycerol; AEA, *N*-arachidonoyl ethanolamide (Anandamide); 2-AGE, 2-arachidonylglycerol ether; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; CSF, cerebrospinal fluid; ESI, electrospray ionisation; IS, internal standard; LEA, *N*-linoleoyl ethanolamide; LOD, limit of detection; LOQ, limit of quantification; MeCN, acetonitrile; MeOH, methanol; MM, master mix; OEA, *N*-oleoyl ethanolamide; PEA, *N*-palmitoyl ethanolamide; QC, quality control; SLE, supported liquid extraction; SPE, solid phase extraction; SPME, solid phase micro-extraction; SRM, selected reaction monitoring.

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approaches (surrogate matrix for AEA, 2-AG, OEA, 2-AGE, LEA and PEA, and surrogate calibrant for AEA only) and they were evaluated by use of the Passing-Bablok regression. Concentrations (median) of CSF samples of patients suffering from CNS infection and controls were found to be around 160 pM for 1- and 2-AG, 86 pM for AEA, 62 for 2-AGE, 58 for LEA, 93 pM for PEA, and 83 pM for OEA.

## 1. Introduction

The endocannabinoid system is involved in many physiological processes, and it is part of fundamental regulatory mechanisms. Its dysregulation might result in various psychological and/or neurological disorders, like brain trauma, neuroinflammation, anxiety, depression, and stroke [1].

Endocannabinoids are the ligands of 2 main receptors: cannabinoid receptor 1 and 2 (CB1 and CB2), and these receptors are abundant in the central nervous system. 2-Arachidonoyl glycerol (2-AG) and anandamide (AEA) are the most important and best described endogenous ligands to these receptors [2]. However, the other endocannabinoids, 2-arachidonylglycerol ether (2-AGE), *N*-linoleoyl ethanolamide (LEA), *N*-palmitoyl ethanolamide (PEA) and *N*-oleoyl ethanolamide (OEA) have a significantly lower affinity to the CB1 and CB2 receptors than AEA, but they indirectly modulate the system by the so-called "entourage effect" [2]. All mentioned endocannabinoids are lipid mediators, originating from membrane phospholipids and triglycerides.

Monitoring levels of endocannabinoids in the brain is crucial in understanding the mechanism of diseases and to develop cannabinoid-based therapies. Cerebrospinal fluid (CSF) is a body fluid which is relatively easy to obtain; its chemical composition reflects the brain status [3].

Most of the analytical methods published so far for quantification of endocannabinoids in biological matrices focused on human plasma [4–7], human serum [8,9], mouse serum [10], saliva [4,9,11], hair [12], mouse brain tissue [10,13], human brain tissue [14], rat brain tissue [15], urine [9], breast milk [9], amniotic fluid [9], peritoneal fluid [9], and human cells [16].

Endocannabinoids in biofluids are typically analysed with LC-MS/MS methods after protein precipitation and lipid extraction [17]. A critical issue with this approach is rapid non-enzymatic isomerisation of 2-AG to 1-arachidonoylglycerol (1-AG) [18]. This phenomenon is observed in protic solvents like water and alcohols, but to lower extent in acetonitrile (MeCN). The isomerisation might already appear in biological samples as they are water based and is thus hard to suppress [19]. Isomerization of 2-AG to 1-AG occurs both inside and outside the body; since 1-AG is not active, isomerization of 2-AG reduces its ability in activating cannabinoid receptors in the brain and central nervous system. Because 2-AG concentrations are typically given as the cumulative values of 1- and 2-isomers, interpretations in different studies is vague due to somewhat unclear 2-AG quantities under physiological conditions. Isomerization was also discovered to be a post-isolation artefact [20]. Analytical methods used in endocannabinoid research have been recently reviewed by Marchioni et al. [21].

At the moment, there has been reported one work on analysis of endocannabinoids in human CSF with utilization of nano-LC-MS/MS [22]. Endocannabinoid analysis in human CSF poses some challenges. On the one hand, as they are endogenous compounds there is no blank matrix for uncompromised calibration and validation. Standard addition has the disadvantage that the calibration is done in a higher concentration range but not in the relevant range of endogenous concentrations levels. Surrogate calibrant methodology is often suggested as elegant strategy for the accurate quantification of endogenous substances such as steroids [23–25]. It is based on two variants of stable isotope-labelled standards one of which, preferably the $^{13}$C-labelled one, is used as internal standard (IS) and the other one as surrogate calibrant, which shows the same detector response and detection sensitivity (i.e., the same slope of the calibration function as the target analyte). Due to limitations of availability of stable isotope-labelled standards of endocannabinoids for surrogate calibrant method, for which there is a need of two labelled analogues, another quantification method, surrogate matrix is also commonly used. Furthermore, the sample volume of CSF is limited (total CSF in brain is between 125 and 150 mL, of which approximately 20% is contained within the ventricles) [26], which results in limitations regarding sample volume that can be taken and thus the ability of analyte enrichment in the course of sample preparation, but also matrix availability for method development and validation. Hence, a sensitive analytical method was proposed to analyse endocannabinoids in CSF using surrogate matrix and surrogate calibrant approaches in this validated method. Our developed method requires very low amount of the biological sample - only 50 μL and it is based on an easy and fast one-step monophasic liquid extraction incorporating protein precipitation. The method utilizes UHPLC with commercially available solid-core Cortecs C18+ column (2.7 μm). The solid-core (core-shell, superficially porous) particle column enables fast separations at low backpressure with total run time of 5.5 min. Selective tandem MS/MS detection by SRM provides high sensitivity and low limits of detection. To the best of our knowledge, no study has been reported for the quantification of endocannabinoids in CSF comparing a combined surrogate calibrant and surrogate matrix-based approach.

## 2. Experimental

### 2.1. Materials

1-Arachidonoylglycerol (1-AG), 2-arachidonoylglycerol (2-AG), 2-arachidonylglycerol ether (2-AGE), *N*-arachidonoyl ethanolamide (anandamide, AEA), *N*-linoleoyl ethanolamide (LEA), *N*-palmitoyl ethanolamide (PEA), *N*-oleoyl ethanolamide (OEA), 5Z,8Z,11Z,14Z-eicosatetraenoic acid 2-glycerol-1,1,2,3,3-$d_5$ ester (2-AG-$d_5$), *N*-(2-hydroxyethyl-1,1,2,2-$d_4$)-5Z,8Z,11Z,14Z-eicosatetraenamide (AEA-$d_4$), *N*-(2-hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide-5,6,8,9,11,12,14,15-$d_8$ (AEA-$d_8$), *N*-(2-hydroxyethyl-1,1,2,2-$d_4$)-9Z,12Z-octadecadienamide (LEA-$d_4$), *N*-(2-hydroxyethyl-1′,1,2,2′-$d_4$)-9Z-octadecenamide (OEA-$d_4$) and *N*-(2-hydroxyethyl)-hexadecanamide-7,7,8,8-$d_4$ (PEA-$d_4$) were purchased from Cayman Chemical (Ann Arbor, MI, USA). The chemical structures of analytes are shown in Fig. 1.

MeCN (LC-MS grade) was obtained from Carl Roth (Karlsruhe, Germany). Ethanol (gradient grade), formic acid, sucrose, potassium chloride (KCl), magnesium chloride hexahydrate ($MgCl_2.6H_20$ ), calcium chloride ($CaCl_2$), monosodium phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$), glucose and sodium bicarbonate ($NaHCO_3$) were from Sigma Aldrich (Merck, Munich, Germany). Ultrapure water was produced in-house by Elga Purelab Ultra (Celle, Germany).

### 2.2. Cerebrospinal fluid samples

One hundred eighteen (118) CSF samples obtained in the clinical routine underwent further analysis. The indications for CSF acquisition were the differential diagnostic assessment in suspected CNS infection, headache disorders, polyneuropathy, brain tumors, neurodegenerative and neuroinflammatory disorders. The study was approved by the institutional ethics committee (document no. 552/2020BO) and complied with the declaration of Helsinki and good clinical practice guidelines.

### 2.3. Sample preparation

#### 2.3.1. Sample extraction

CSF samples were stored after collection at − 80 °C and slowly thawed on ice just before extraction. Once unfrozen, 50 μL of each sample were taken and placed in Eppendorf tubes. Then, IS Stock Solution 3 (for details see supplementary material) was added to each sample to obtain a final volume of 1 mL. Samples were vortexed (5 s) and left at − 20 °C for 20 min for protein precipitation. They were then centrifuged (4000 x g, 4 °C, 5 min). The supernatant was transferred to fresh Eppendorf tubes and the solvent was evaporated in GeneVac EZ-2 high performance evaporator (SP Scientific, Ipswich, UK) under nitrogen protection. The dry samples were re-constituted in 50 μL of MeCN: $H_2O$ (50:50; v/v), vortexed (5 s), sonicated (2 min) and then transferred to autosampler vials. The samples were analysed in a random order with calibrants and quality controls.

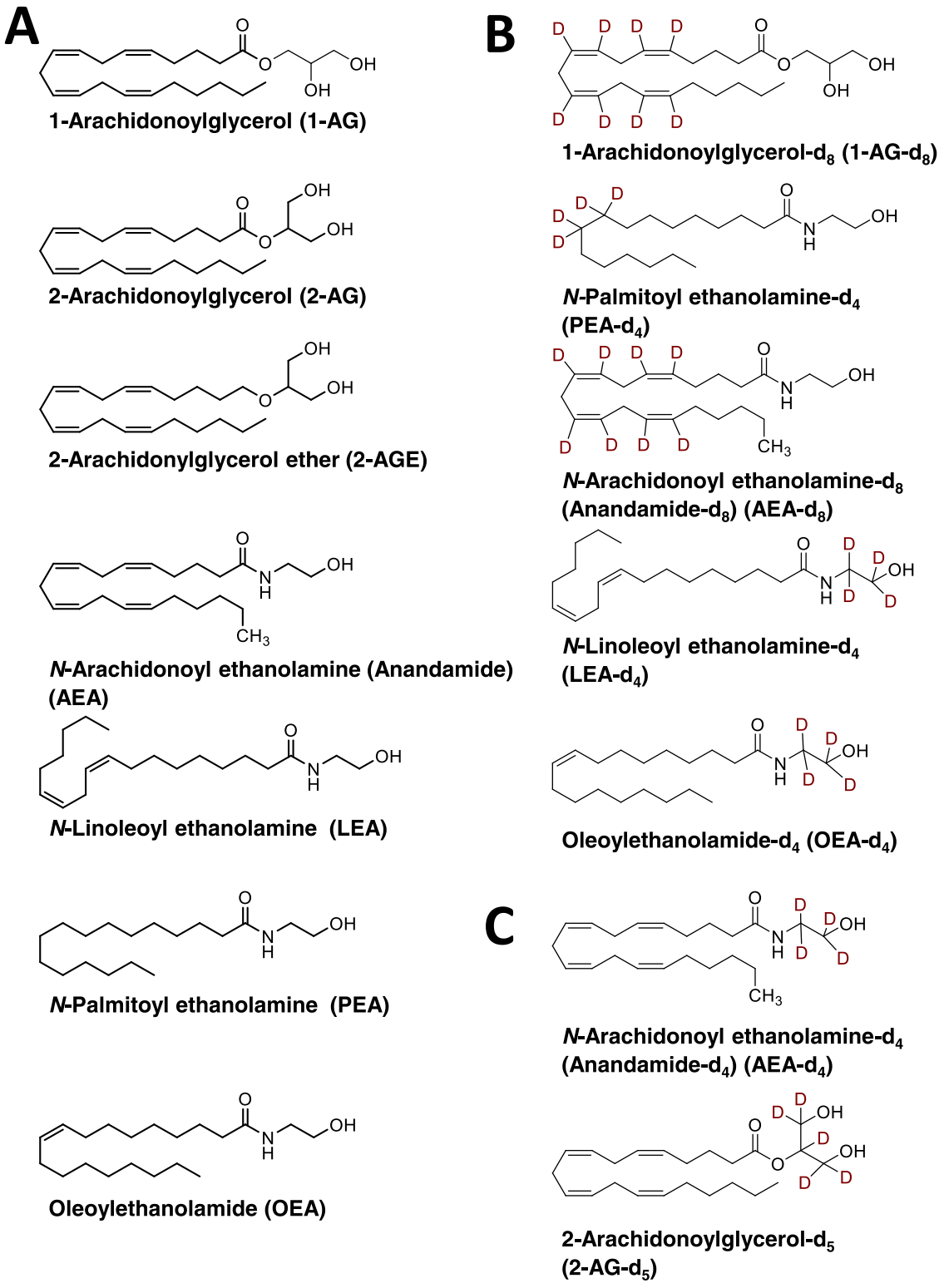


**Fig. 1.** Chemical structures of (A) targeted analytes, (B) internal standards, (C) surrogate calibrants.

The artificial CSF (aCSF) was prepared according to protocols of Shirin et al. [26] for surrogate matrix approach. aCSFs were extracted the same way as human (authentic) CSF.

#### 2.3.2. Preparation of calibrants, quality control samples and stock solutions

Calibrants and QCs were prepared from pooled matrix - aCSF or CSF -, different pools were used for calibrants and for QCs. A surrogate matrix pool (aCSF) was prepared at the final concentration of 234 mM sucrose, 3.6 mM KCl, 1.2 mM $MgCl_2$ (prepared from $MgCl_2.6H_2O$), 2.5 mM $CaCl_2$, 1.2 mM $NaH_2PO_4$ (prepared from $NaH_2PO_4{\cdot}H_2O$), 12 mM glucose and 25 mM $NaHCO_3$ by dissolving in water according to Shirin et al. [26] and stored at − 80 °C until assayed. An authentic matrix pool (CSF) for preparation of calibrants and QC samples was prepared from minimally 5 individual lots for method development, validation and QCs embedded in study sample batches and stored at − 80 °C until assayed. The preparation of standard stock solutions (Master mixes, MM) of analytes is described in detail in the supplementary material. Final concentrations of MM, surrogate calibrants, calibrants in surrogate matrix approach, and QC samples can be found in supplementary Tables S1, S2 and S3, respectively.

Twenty-four aliquots of 1200 µL pooled CSF and aCSF were placed in falcon tubes to prepare duplicates of each calibrants and QCs. They were spiked with different volumes of analyte stock solutions (MMs). The concentrations of the spiked analytes in the final samples ranged from 20.08 to 35031.02 pM; two of the twenty-four aliquots were not spiked (used as Cal0, zero calibrant). Each non-zero calibrant and QCs were spiked with corresponding amounts of the respective MMs.

Samples were treated in the same way as calibrants and QCs without spiking MM solutions (as explained in 2.3.1). They were re-constituted with the same amount of MeCN as the initial volume of the pooled CSF and aCSF (i.e., 50 µL). Before the sample batch, 1:100 diluted MMs were injected six times separately for system suitability test to evaluate the performance of the analytical system. At the end of the sample batch, the consistent response from the analytes throughout the batch was confirmed by another six separate injections.

In each analytical batch, blanks (solvent solutions, MeCN:$H_2O$ (50:50; v/v)) were first injected (after confirmed system suitability), followed by multiple Cal0s and non-zero calibrants (for column and system equilibration as well as instrument performance control during analytical sequence), then QCs and then the sample batch. In each analytical batch approximately 30 study samples were measured, and after their acquisitions, QCs, non-zero calibrants and zero calibrants followed again. Each analytical batch consisted in total of 12 QCs and 28 non-zero calibrants and 4 Cal0s.

### 2.4. UHPLC method

The chromatographic separation was performed on an Agilent 1290 Infinity II LC (Agilent, Waldbronn, Germany) which was composed of a binary pump, a degasser, a multisampler system, and a column compartment.

Samples were kept at 4 °C in the autosampler and the analytical batch was immediately analysed after sample preparation. The injection volume was 20 µL. A Cortecs C18+ (Waters, Milford, MA, USA) column (50 ×2.1 mm, 2.7 µm) and matching Cortecs C18 guard column (2.7 µm) was used for the separation of the sample components prior to MS detection. The column oven was set to 45 °C. Mobile phase component A was water with 0.1% formic acid (v/v) and the component B was MeCN with 0.1% formic acid (v/v). The flow rate was 0.2 mL $min^{-1}$ and the following gradient profile used for elution: From 50% B at 0 min to 75% B in 0.35 min, then the percentage of B was increased from 75% B at 0.35 min to 98% at 4 min, held constant at 98% for the next 0.7 min, then dropped quickly (in 0.01 min) to the starting conditions of 50% B to re-equilibrate the column for 0.79 min. After each analytical batch (about 30 samples), the system was cleaned with MeCN:$H_2O$ (50:50; v/v). Needle wash solvent was Ethanol:$H_2O$ (50:50; v/v). The chromatographic separation of the analytes is shown in Fig. 2.

### 2.5. MS-method

Mass spectrometric detection was performed on a QTRAP 4500 mass spectrometer with a Turbo V electrospray ionization (ESI) source (Sciex, Concord, ON, Canada). A 100 µm ID Hybrid PEEKSIL electrode with a stainless-steel tip was employed for LC-MS/MS analysis. The samples were analysed in positive ESI-MS polarity mode using data acquisition by an advanced scheduled multiple reaction monitoring (sMRM) algorithm which provided a sufficient measurement sensitivity and specificity. The ESI source parameters were optimized by injecting analyte solution with syringe by direct infusion. The source voltage was set to 5500 V, source temperature was 600 °C, nebulizer gas, GS1 (zero grade air) was 10 psi, drying gas, GS2 (zero grade air) was 20 psi and CAD, collision gas ($N_2$) was set to Medium. The target cycle time was set to 0.54 s and the detection window to 60 s. For each ion transition dwell time was fixed. The peak areas of analytes and ISs were processed using MultiQuant 3.0 (Sciex). MS settings are summarized in Table 1.

### 2.6. Suitability of surrogate calibrant and matrix approaches, and parallelism

Before starting the validation, identical detector responses of each equimolar mixtures of surrogate calibrants and corresponding targeted analytes were verified and adjusted (if different), respectively. The detector responses of each analyte/surrogate calibrant pair in the MMs were matched prior to the preparation of calibrants and QCs by adjusting the concentrations of surrogate calibrants.

Parallelism was evaluated in the pre-validation period between the target analyte standard addition curve and the surrogate calibration curve in pooled CSF (matrix-matched) (surrogate calibrant approach) and between the target analyte standard addition curve in pooled CSF and the surrogate matrix curve (surrogate matrix approach) to ensure reliable quantification [23,24,27]. For parallelism control, area response ratios (response factors, RFs between surrogate calibrant and authentic analyte; between surrogate matrix and authentic analyte for each method) of 1.00 ± 0.10 were found acceptable (see Table 2). Prior to measurement, RFs were determined individually injecting 6 replicates of a dilution of each stock solution (MMs, $MM_{High}$, $MM_{Mid}$ and $MM_{Low}$ were diluted by factor of 1:100). Validation only started after parallelism was successfully shown. Parallelism was monitored during validation and each study batch (as shown in Table 2 and Figure S1). The

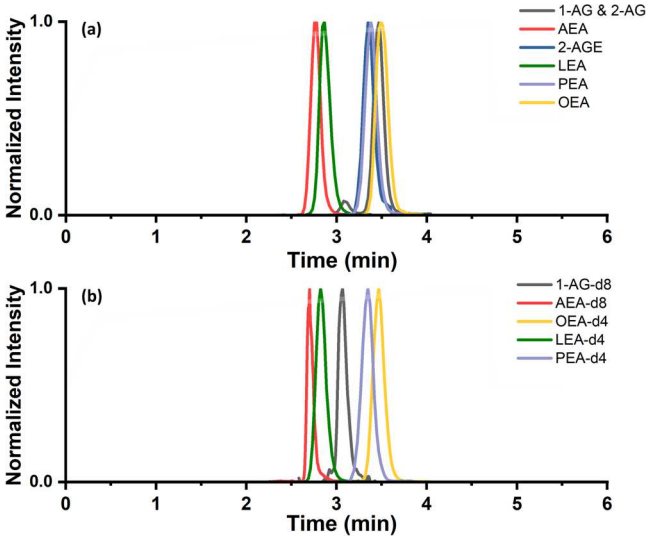


**Fig. 2.** Chromatographic separation of analytes (A) and internal standards (B) on Cortecs C18+ with optimized conditions.

**Table 1**
SRM transitions and target specific MS parameters of analytes used for the present UHPLC-MS/MS analysis.

| Analyte | Retention time (min) | Q1 [*m/z*] | Q3 [*m/z*] | Transition | Dwell Time (ms) | DP (V) | CE (V) | CXP (V) |
|---|---|---|---|---|---|---|---|---|
| 1-AG & 2-AG | 3.51 & 3.12 | 379.3 | 77.0 | Quantifier | 35 | 40 | 90 | 5 |
| | | | 105.1 | Qualifier | | | 65 | 10 |
| AEA | 2.71 | 348.3 | 62.0 | Quantifier | 35 | 51 | 29 | 4 |
| | | | 91.2 | Qualifier | | | 67 | 14 |
| 2-AGE | 3.41 | 365.2 | 273.2 | Quantifier | 35 | 39 | 66 | 8 |
| | | | 90.9 | Qualifier | | | 11 | 10 |
| LEA | 2.82 | 324.2 | 62.1 | Quantifier | 35 | 41 | 37 | 16 |
| | | | 81.0 | Qualifier | | | 43 | 12 |
| PEA | 3.46 | 300.3 | 62.1 | Quantifier | 35 | 16 | 29 | 8 |
| | | | 71.2 | Qualifier | | | 41 | 20 |
| OEA | 3.55 | 326.3 | 62.1 | Quantifier | 35 | 61 | 35 | 10 |
| | | | 69.1 | Qualifier | | | 49 | 10 |
| 1-AG-$d_8$ | 3.14 | 387.1 | 294.2 | Quantifier | 35 | 37 | 21 | 28 |
| | | | 275.0 | Qualifier | | | 17 | 10 |
| AEA-$d_8$ | 2.74 | 357.4 | 62.0 | Quantifier | 35 | 49 | 47 | 12 |
| | | | 95.0 | Qualifier | | | 47 | 6 |
| AEA-$d_4$ | 2.76 | 352.0 | 91.1 | Quantifier | 35 | 51 | 61 | 14 |
| | | | 104.9 | Qualifier | | | 45 | 8 |
| LEA-$d_4$ | 2.85 | 328.3 | 66.1 | Quantifier | 35 | 16 | 36 | 6 |
| | | | 95.1 | Qualifier | | | 41 | 14 |
| PEA-$d_4$ | 3.49 | 304.2 | 66.0 | Quantifier | 35 | 37 | 21 | 18 |
| | | | 287.4 | Qualifier | | | 19 | 10 |
| OEA-$d_4$ | 3.59 | 330.3 | 66.1 | Quantifier | 35 | 71 | 37 | 10 |
| | | | 313.3 | Qualifier | | | 21 | 12 |

Q1: first quadrupole, Q3: third quadrupole, DP: declustering potential, EP: entrance potential, CE: collision energy, CXP: collision cell exit potential

**Table 2**
Demonstration of parallelism for surrogate matrix and surrogate calibrant method during validation.

| Method | Analyte | Day | Slope surrogate calibrant or matrix | Slope target analyte | Slope ratio[1,2] | Slope ratio precision[3] Intra-Day (%) | Slope ratio Standard Error[4] Intra-Day (%) | Slope ratio precision Between-Days (n = 3) (%) | Slope ratio Standard Error Between-Days (n = 3) (%) |
|---|---|---|---|---|---|---|---|---|---|
| Surrogate Matrix | AEA | 1 | 0.420 | 0.418 | 1.006 | 1.240 | 0.877 | 1.134 | 2.421 |
| | | 2 | 0.474 | 0.482 | 0.984 | 1.914 | 1.353 | | |
| | | 3 | 0.337 | 0.337 | 1.000 | 2.200 | 1.556 | | |
| | 2-AGE | 1 | 0.010 | 0.010 | 1.020 | 0.197 | 0.139 | 4.657 | 2.689 |
| | | 2 | 0.012 | 0.012 | 0.951 | 0.005 | 0.004 | | |
| | | 3 | 0.011 | 0.010 | 1.040 | 0.110 | 0.078 | | |
| | PEA | 1 | 0.376 | 0.377 | 0.998 | 1.013 | 0.716 | 2.945 | 0.399 |
| | | 2 | 0.336 | 0.354 | 0.949 | 6.615 | 4.678 | | |
| | | 3 | 0.288 | 0.288 | 1.002 | 0.409 | 0.289 | | |
| | 2-AG | 1 | 0.001 | 0.001 | 1.000 | 0.009 | 0.006 | 3.326 | 1.920 |
| | | 2 | 0.004 | 0.004 | 0.972 | 0.355 | 0.251 | | |
| | | 3 | 0.003 | 0.003 | 1.038 | 0.324 | 0.229 | | |
| | 1-AG | 1 | 0.029 | 0.030 | 0.970 | 0.198 | 0.140 | 4.523 | 2.611 |
| | | 2 | 0.038 | 0.040 | 0.965 | 0.025 | 0.018 | | |
| | | 3 | 0.030 | 0.029 | 1.046 | 0.342 | 0.242 | | |
| | OEA | 1 | 0.011 | 0.011 | 0.965 | 0.085 | 0.060 | 2.394 | 1.471 |
| | | 2 | 0.010 | 0.010 | 1.010 | 0.058 | 0.041 | | |
| | | 3 | 0.009 | 0.009 | 1.001 | 0.036 | 0.025 | | |
| | LEA | 1 | 0.010 | 0.010 | 1.032 | 0.194 | 0.137 | 0.946 | 0.344 |
| | | 2 | 0.009 | 0.009 | 1.022 | 0.017 | 0.012 | | |
| | | 3 | 0.008 | 0.008 | 1.013 | 0.063 | 0.045 | | |
| Surrogate Calibrant | AEA-$d_4$ | 1 | 0.621 | 0.592 | 1.050 | 0.550 | 0.389 | 2.980 | 1.720 |
| | | 2 | 0.591 | 0.597 | 0.990 | 0.645 | 0.456 | | |
| | | 3 | 0.615 | 0.599 | 1.026 | 0.311 | 0.220 | | |

[1] Slope ratio of surrogate calibrant or matrix to target analyte
[2] Slope ratio of 1.00 ± 0.05 was deemed acceptable
[3] Slope ratio precision was determined from standard deviation of slope ratios
[4] Standard error was calculated by dividing standard deviation of slope ratio by square root of amount of slopes

response factor (RF) using the parallelism approach was calculated using the slopes of a normalized response function of the instrument, i.e., by plotting the relative response over the concentrations. This is a way to compute the RF, which considers the concentration of the analyte and the internal standard, however, other options are also possible [28].

### 2.7. Method validation

The validation of the method followed largely the guideline of U.S. Food and Drug Administration [29]. The concentration-response relationship, accuracy, precision, LLOQ, carry over, selectivity, matrix effect, extraction recovery, stability was evaluated for validation. The linear response function estimations (in MeCN), the goodness of fit of the calibration function by linear regression in both matrices (surrogate matrix and CSF) and their limits of detection (LODs) as well as limits of quantitation (LOQs) were determined together with within and between batch accuracy and precision.

Within batch precision and between batch precision were

determined by calculating the R.S.D. (%) at each concentration of QCs in pooled CSF and aCSF prepared at four different levels, six measurements at each level (n = 6). Within batch and between batch accuracy was calculated as the relative error (RE) of each concentration of QCs in pooled CSF and aCSF prepared at four different levels, six measurements at each level (n = 6), i.e. (calculated concentration − nominal concentration) / (nominal concentration) × 100%. While the between accuracy and precision were evaluated by analyzing three batches on various validation days, the within batch accuracy and precision were evaluated by selecting QC samples using six replicates on the same day.

For surrogate calibrant method, LODs and LOQs of each method for each compound were established at concentrations at which signals of corresponding surrogate calibrant to pooled human CSF were three and ten times the signal in chromatograms of blank (not spiked) pooled human CSF for six separate measurements and they were spiked with IS. For surrogate matrix method LODs and LOQs were set as the lowest concentration on the calibration curve for which S/N was three and ten, respectively.

Matrix effects, extraction recoveries and process efficiencies were investigated according to protocols suggested by Matuszewski et al. [30]. Three sets of samples were prepared for these investigations: pooled matrix (aCSF and CSF) spiked with standards and ISs before extraction (pre-spiked); pooled matrix (aCSF and CSF) spiked with standards and ISs after extraction (post-spiked); standard and ISs in reconstitution solvent. For the matrix effect (ME) evaluation, peak area ratios (in %) of post-extraction spiked matrix to corresponding MeCN standard solutions were calculated. Extraction recovery (ER) was evaluated by calculations of the peak area ratio (in %) of pre-extraction spiked matrix and post-extraction spiked matrix. To determine process efficiency, peak area ratios (in %) of pre-extraction spiked matrix to corresponding MeCN standard solutions were calculated.

Stability of the targeted compounds in aCSF and CSF was determined by analyzing fresh QCs at two levels under five different storage conditions: 6 h on ice (at 4 °C); storage for one month and three months at − 80 °C; three freeze-thaw cycles between − 80 °C and on ice (at 4 °C); 10, 24 h and 48 h in autosampler. Moreover, the stability of the target compounds in MMs was also determined at 4 °C for one month.

Carry over was evaluated by injecting blank samples after a QC or a calibrant at highest concentration according to FDA guidelines [29].

Calibrants were prepared by standard addition to pooled CSF and by a concentration series in aCSF, respectively. The endogenous levels were obtained from the -x-intercepts of standard addition curves. Corrected matrix-matched calibration functions were then obtained by considering both the endogenous and spiked levels.

### 2.8. Data processing and quantification

The data were pre-processed with Analyst (Sciex) and the quantification was done with MultiQuant 3.0 (Sciex) with automated integration. The Gaussian smooth width was 1.0 points, retention time half window was set to 30.0 s, minimal peak width was equal to 3 points and minimum peak area was 500 cps. Noise percentage was set to 40%, peak splitting was 1 point, and the baseline subtraction window was equal to 0.20 min. The peak area ratio (analyte to IS) was used as the response for the calibration functions. 1-AG and 2-AG were integrated together (two peaks calculated as sum). The concentration-response relationship was evaluated by preparation of calibration curves in three different batches. The ratio of analyte peak area to internal standard peak area was plotted against endocannabinoid concentration (nmol $L^{-1}$). Calibration functions were established in MultiQuant as weighted linear regressions 1/x. Further calculations were made with Excel 2007 (Microsoft, Redmond, WA, USA). Graphs and figures were generated by using Origin 2019 (Originlab, Northampton, MA, USA) and Excel 2007, chemical structures were generated by ChemDraw (PerkinElmer Informatics). Passing-Bablok regression was performed to compare the performance between surrogate calibrant and surrogate matrix methodologies for AEA (CRAN R Repository Package "mcr", Version 1.2.2, function mcreg(), method used: PaBa).

## 3. Results and discussion

### 3.1. Optimization of UHPLC-MS/MS method

Efficient extraction of targeted endocannabinoids and removal of interferences is highly important for a sensitive and specific assay even when tandem MS and SRM are used for detection. As for the complexity of biological matrices and the low concentrations of target analytes, proper sample preparation techniques are required to address the challenges given by these two restrictions. There is no single standard procedure for endocannabinoid extraction at the time. Different groups use different protocols such as two phase liquid-liquid extraction with a single organic solvent or a mixture of polar and non-polar solvents (e.g., ethyl acetate:cyclohexane (50:50; v/v) [4], toluene [7], ethyl acetate [16], hexane:isopropanol (9:1; v/v) [13]), ethyl acetate:hexane (9:1; v/v) [31], different adaptions of the traditional lipid extraction methods of Folch and Bligh and Dyer (such as with chloroform [32], with chloroform:MeOH (2:1; v/v) or with methanol:chloroform:water (1:2:1; v/v/v) [14]), supported liquid extraction (SLE) [11], protein precipitation with MeCN [5], with MeOH [12], with acetone [33], with methanol:MeCN:acetic acid (50:50:0.1; v/v) [10], isopropanol or solid phase extraction (SPE) [9]. Without any doubt, sample pre-concentration by SPE will need less prior sample preparation steps, i.e., the biofluid (plasma, CSF) can be directly applied to the SPE cartridge, and thus less time, yet it is primarily beneficial for analyte enrichment if the sample volume is large enough such as for plasma. However, this might typically not be the case for CSF, since the largest quantities are used up for clinical routine assessment. Indeed, most of the CSF samples of the current study were less than 100 μL. Therefore, another sample preparation strategy needed to be used. Whilst 2-AG quickly isomerizes into 1-AG particularly in protic solvents such as water and alcohols, it does so to a lesser extent in acetonitrile (MeCN) [18,34]. Following a comparison of multiple extraction techniques and different compositions of solvent mixtures in a preliminary study, protein precipitation with MeCN was finally selected for endocannabinoid extraction herein. The main reason for selecting MeCN was the finding that acetonitrile-based protein precipitation showed less isomerisation of 2-AG to 1-AG. Initially, a number of distinct columns and mobile phase conditions were screened (see Suppl. Fig. S2) with the goal to reduce analyte overlaps and minimize the risk of interferences, while assuring coelution of IS and as far as possible also of surrogate calibrants. For instance, the M+2 isotopologue LEA of the precursor ion could impart an interference on the quantifier ion of OEA as both have the same group specific fragment ion with *m/z* 62.1 (ethanolammonium ion). The columns for screening studies were chosen to cover a broad variety of stationary phase surface properties, for instance, carbon load (it was between 5.7%−20%) and also distinct morphologies (fully and superficially porous particles). Either impurity peaks, high background noise or poor resolution between some targeted analytes were observed on some columns. Adjustments in mobile phase compositions, column temperature and gradient profile could not assist to eliminate the critical coelutions. Therefore, Cortecs C18 + column was finally chosen for the analysis, even though the analyte peaks were broader on the Cortecs C18 + column as compared to other screened columns (see Suppl. Fig. S2). Furthermore, with the Cortecs C18 + column the analysis time could be significantly shortened. In spite of the lowest carbon loading amongst the tested columns (5.7%) it yielded good selectivity and was found to be the most suitable choice.

Endocannabinoid concentrations were expected to be low, hence the main criterion for selection of the phase system was the signal response, evidently favouring more efficient UHPLC separation systems.

In order to find the most sensitive and selective chromatographic method, different mobile phase compositions were tested. Mobile phase

A was always water with or without additive and mobile phase B was MeCN or MeOH with or without additive. There were two different additives with different compositions tested: ammonium acetate (2–20 mM) and formic acid (0.1–1%). Finally, 0.1% formic acid was selected which showed better MS response.

Fig. 2 shows the separation of the target analytes (Fig. 2A) and their internal standards (Fig. 2B) with the optimized UHPLC method using the core-shell column Cortecs C18+. The different analytes are partly separated, which reduces their mutual matrix effect on each other, and they eluted as symmetrical peaks. The two critical analytes, LEA and OEA (as well as their $d_4$-labelled ISs), could be chromatographically baseline separated avoiding isotopologue interferences. At the same time, good co-elution of analytes with their internal standards was achieved, which adequately compensated for matrix effects, and which hence provided high confidence in the quantitative results. 2-AGE and PEA as well as OEA and 1-AG coeluted which, however, could be readily distinguished by selective SRM transitions in MS/MS detection (see Suppl. Fig.S3 for product ion spectra). The two isomers (2-AG and 1-AG) were separated at baseline. The sum of 1-AG and 2-AG was employed for further data analysis in this study (2-AG concentrations could be obtained from the respective peak are ratio of the sum, yet represents not only endogenous levels but also the fraction resulting from interconversion during sampling and sample preparation).

Owing to high chemical structural similarities, endocannabinoids show very similar product ion spectra. Most of the targeted analytes have the product ion at $m/z$ 62 with the highest intensity, which is formed due to loss of the ethanolamine moiety. The MS/MS method used in the sMRM mode provides high selectivity and sensitivity. The co-eluted compounds are separately detected with different SRMs transitions. The transitions (quantifier and/or qualifier ion) that were used were in accordance with previous studies [5,8,12,14]. To this end, for each analyte the two most sensitive SRM transitions (i.e., precursor-product ion pairs) were selected (See Figure S3 for Product ion spectra of each target analyte and their product ions). For monitoring assay specificity during the analytical batches, the peak area ratio of quantifier to qualifier ions of targeted analytes was evaluated throughout the study to check for potential interferences. The average ratios of quantifier and qualifier ions (based on peak area) and their maximum deviations found during the validation are shown in Table S4. Throughout the study, a constant quantifier/qualifier ratio was found and indicated an appropriate assay specificity (as can be seen in Table S4).

The dwell time was fixed individually for each analyte peak based on chosen retention time window width (Table 1).

### 3.2. Calibration and method validation

#### 3.2.1. Assay specificity and sensitivity

For assay specificity, special attention had to be given for interferences, and thereupon sMRM transitions of surrogate calibrants and ISs of six different lots of CSF sample were acquired to prove that they do not comprise any interferences. In the CSF matrix, assay specificity was

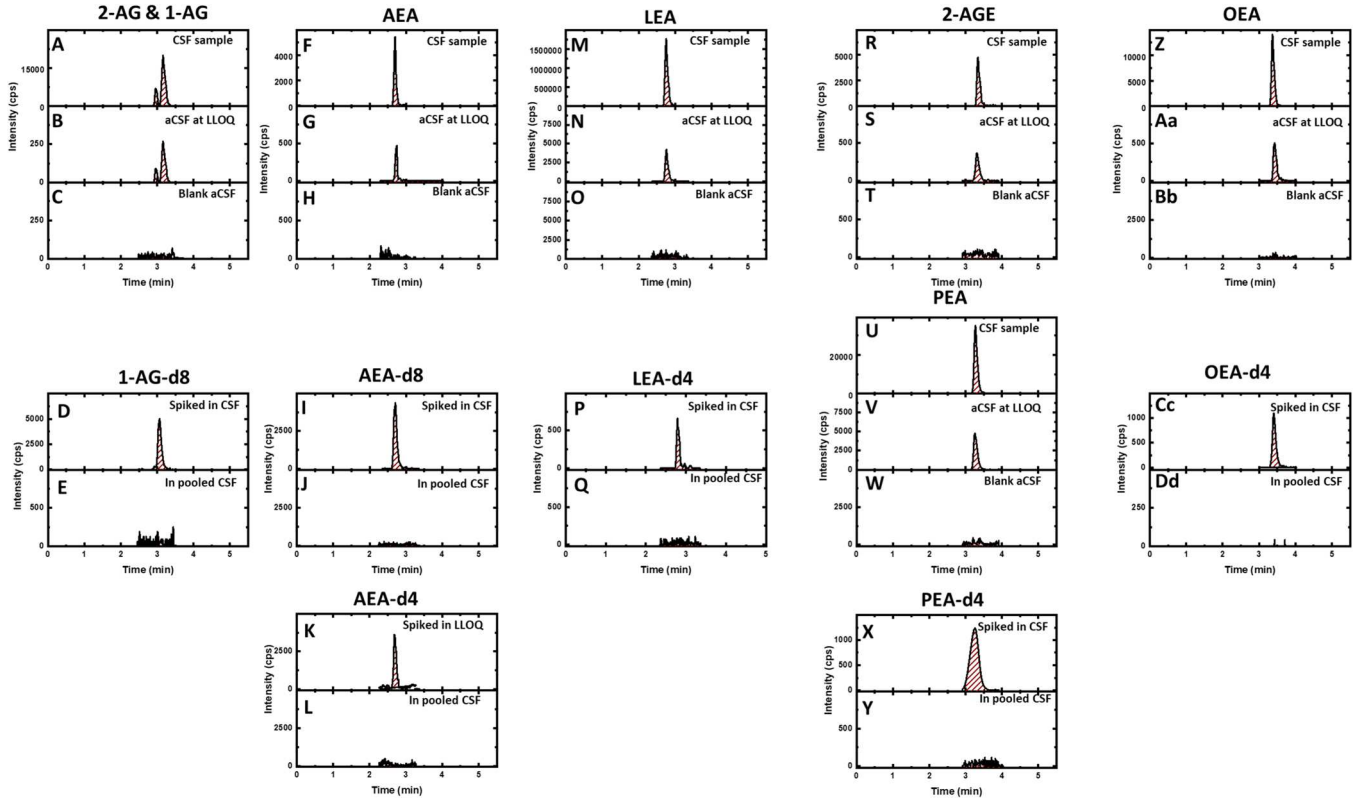


**Fig. 3.** Typical MRM chromatograms. A: 2-AG & 1-AG in authentic CSF sample (17463.7 pM); B: 2-AG & 1-AG spiked in surrogate CSF sample at LLOQ (83.2 pM); C: 2-AG & 1-AG in pooled surrogate CSF sample (first eluting 2-AG); D: 1-AG-$d_8$ spiked in authentic CSF sample (9560pM); E: 1-AG-$d_8$ in pooled authentic CSF sample; F: AEA in authentic CSF sample (175.2 pM); G: AEA spiked in surrogate CSF sample at LLOQ (25.1 pM); H: AEA in pooled surrogate CSF sample; I: AEA-$d_8$ spiked in authentic CSF sample (3560 pM); J: AEA-$d_8$ in pooled authentic CSF sample; K: AEA-$d_4$ spiked in authentic CSF sample at LLOQ (20.1 pM); L: AEA-$d_4$ in pooled authentic CSF sample; M: LEA in authentic CSF sample (8878.2 pM); N: LEA spiked in surrogate CSF sample at LLOQ (13.4 pM); O: LEA in pooled surrogate CSF sample; P: LEA-$d_4$ spiked in authentic CSF sample (8190 pM); Q: LEA-$d_4$ in pooled authentic CSF sample; R: 2-AGE in authentic CSF sample (228.0 pM); S: 2-AGE spiked in surrogate CSF sample at LLOQ (32.6 pM); T: 2-AGE in pooled surrogate CSF sample; U: PEA in authentic CSF sample (583.9 pM); V: PEA spiked in surrogate CSF sample at LLOQ (83.4 pM); W: PEA in pooled surrogate CSF sample; X: PEA-$d_4$ spiked in authentic CSF sample (3040 pM); Y: PEA-$d_4$ in pooled authentic CSF sample; Z: OEA in authentic CSF sample (1488.4 pM); Aa: OEA spiked in surrogate CSF sample at LLOQ (35.4 pM); Bb: OEA in pooled surrogate CSF sample; Cc: OEA-$d_4$ spiked in authentic CSF sample (8240 pM); Dd: OEA-$d_4$ in pooled authentic CSF sample.

confirmed by injecting each analyte at the highest calibration level and monitoring all other MRM transitions for interferences. None of the compounds in CSF samples were found to interfere with analytes to be quantified in this assay. In Fig. 3, the typical chromatograms of targeted analytes in real sample as well as surrogate calibrants and IS acquired from pooled matrix are shown.

Our described method had LOQs in CSF at 25.1, 90.5, 33.2, 34.2, 12.4 and 71.9 pM for AEA, 2-AG, OEA, 2-AGE, LEA and PEA, respectively, and in aCSF at 30.2, 92.5, 35.2, 30.4, 20.5 for surrogate matrix approach and 10 pM for $AEA_{-d4}$ for surrogate calibrant approach. It is evident that the method is very sensitive with similar LOQs in CSF and aCSF. In the latter matrix, the LOQs appeared to be, however, slightly higher. The surrogate calibrant approach confirmed the low LOQ for AEA in CSF, i.e., in presence of matrix.

### 3.2.2. Extraction recovery, matrix effect and process efficiency

Extraction recovery (as assessed by comparison of pre- and post-extraction spike), matrix effect (post-extraction-spike vs standard solution) and process efficiency (pre-extraction-spike vs standard solution) were evaluated at three QC concentration levels of each matrix ($QC_{Low}$, $QC_{Mid}$, $QC_{High}$) in quadruplicate (Table 3). The average extraction recoveries were 110.9% and 114.7%, for 1-AG and 2-AG; 84.1% for AEA, 111.9% for 2-AGE, 83.9% for LEA and 80.4% for PEA, 94.1% for OEA in surrogate matrix approach and 94.6% for AEA-$d_4$ for surrogate calibrant approach. The recoveries over 100% might be due to experimental errors originating from pipetting and evaporation but are within acceptable margins in accordance with validation guidelines. There are a very few outliers with recoveries of analytes just below 85% (especially PEA and LEA) which could be explained by random errors and/or adsorption of commonly used glass ware and plastic ware [18]. Note also that these extraction recoveries were calculated with uncorrected peak areas, not normalized by IS, which is more prone to errors. Such errors should be largely corrected by ISs in the quantification workflow of real samples.

The results of matrix effect showed slight ion enhancement in the matrix. The average matrix effect for 1-AG and 2-AG is equal to 102.4 and 110.4, for AEA it is 114.8%, for 2-AGE it is equal to 100.2%, for LEA it equals to 110.2% and for PEA 111.4% and for OEA 88.4% in surrogate matrix approach and average matrix effects equals to 104.7% for AEA-$d_4$ for surrogate calibrant approach (see Table 3).

Process efficiency ranged between 83.5% and 119.4% among all targeted analytes and all concentration levels.

These results prove that monophasic extraction by acetonitrile-mediated protein precipitation successful in efficiently removing matrix components, resulting in minor matrix effects that are corrected using stable isotope-labelled internal standards.

### 3.2.3. Evaluation of the surrogate calibrant and surrogate matrix strategies

Due to lack of blank matrix for the calibration of the endogenous target analytes of this study, two distinct approaches of calibration were adopted: a surrogate calibrant approach [34] and surrogate matrix approach, not yet evaluated before. The surrogate calibrant approach relies on two distinct isotope labelled standards, one used as IS and the other as calibrant. It requires parallelism of the surrogate calibration curve and analyte standard addition curve in real matrix (i.e., same response for analyte and surrogate calibrant in presence of matrix). Two different isotopically labelled analogues were commercially available for AEA and 2-AG. During method development, parallelism was not achieved for 2-AG. Hence, only AEA was quantified by a surrogate calibrant approach. AEA-$d_8$ gave a significantly different detector response compared to AEA, while it was more similar for AEA-$d_4$. It was not possible to achieve parallelism for calibration functions between AEA and AEA-$d_8$, yet parallelism between AEA and AEA-$d_4$ was adequate. For this reason, AEA-$d_4$ was selected as surrogate calibrant and AEA-$d_8$ as IS.

For other targeted endocannabinoids appropriate dual isotope-labelled standards were not available. Hence, for these compounds (2-AGE, LEA, OEA, PEA) a different approach, surrogate matrix, was applied for calibration. Its validity also relies on parallelism of the calibration lines between the standard addition series of the analyte in authentic matrix and a standard series in the surrogate matrix. For this reason, parallelism was thoroughly evaluated (see Suppl. Fig. S1).

AEA was evaluated with both calibration approaches, i.e., in human CSF (surrogate calibrant method) and aCSF (surrogate matrix) and analysed in the same analytical batch to compare the performance of the two different approaches. 2-AGE,1/2-AG, LEA, OEA and PEA were quantified in human CSF using surrogate matrix method only. Response factors for unlabelled analytes and surrogate calibrants were determined by injecting six replicates of each MMs.

For the surrogate matrix approach, two artificial CSFs (aCSF) were prepared according to protocols of Shirin et al. [26] and Jiang et al. [35] before validation. aCSF prepared from the protocol of Shirin et al. generated MS responses that were comparable to CSF. It was therefore chosen as surrogate matrix for validation and study measurements. It is noted however that other approaches for surrogate matrix preparation are also possible [28].

Isotopically labelled analogues of targeted compounds such as AEA-$d_8$ (IS of AEA), 1-AG-$d_8$ (IS of 2-AG and 1-AG), PEA-$d_4$ (IS of PEA and 2-

**Table 3**
Extraction Recovery, Matrix Effect, Process Efficiency for analysis of endocanna-binoids (surrogate matrix and surrogate calibration method).

| Method | | Surrogate Matrix | | | | | | | | Surrogate Calibrant |
|---|---|---|---|---|---|---|---|---|---|---|
| | Analyte | 1-AG | 2-AG | AEA | 2-AGE | LEA | PEA | OEA | AEA-$d_4$ | AEA-$d_4$ |
| Extraction recovery | $QC_{Low}$ | mean | 120.0 | 109.5 | 81.9 | 118.8 | 85.6 | 75.1 | 86.5 | 95.5 |
| | | sd | 2.1 | 6.1 | 10.3 | 3.5 | 8.3 | 6.4 | 6.8 | 3.6 |
| | $QC_{Mid}$ | mean | 112.1 | 115.1 | 85.6 | 112.5 | 77.6 | 86.3 | 95.4 | 88.7 |
| | | sd | 6.1 | 3.9 | 5.8 | 4.6 | 8.8 | 4.1 | 5.7 | 0.4 |
| | $QC_{High}$ | mean | 100.5 | 119.4 | 84.9 | 104.4 | 88.5 | 79.7 | 100.5 | 99.6 |
| | | sd | 11.2 | 15.4 | 9.0 | 9.3 | 8.5 | 9.4 | 6.9 | 12.5 |
| Matrix effect | $QC_{Low}$ | mean | 105.6 | 109.5 | 115.3 | 87.6 | 111.2 | 107.4 | 88.6 | 90.4 |
| | | sd | 6.1 | 12.3 | 9.2 | 5.2 | 8.2 | 4.6 | 12.3 | 9.9 |
| | $QC_{Mid}$ | mean | 99.3 | 110.5 | 119.9 | 102.2 | 111.7 | 112.0 | 90.8 | 107.5 |
| | | sd | 11.7 | 15.2 | 12.6 | 6.3 | 2.4 | 4.9 | 6.7 | 8.7 |
| | $QC_{High}$ | mean | 102.4 | 111.1 | 109.3 | 110.7 | 107.6 | 114.7 | 84.9 | 116.1 |
| | | sd | 7.5 | 6.9 | 11.4 | 4.5 | 12.4 | 9.2 | 6.5 | 9.4 |
| Process efficiency | $QC_{Low}$ | mean | 110.5 | 108.6 | 94.7 | 100.8 | 106.5 | 84.6 | 89.1 | 93.6 |
| | | sd | 6.5 | 1.2 | 0.8 | 2.1 | 0.9 | 2.5 | 1.5 | 3.6 |
| | $QC_{Mid}$ | mean | 105.4 | 119.4 | 110.7 | 105.6 | 93.8 | 97.5 | 92.8 | 86.5 |
| | | sd | 9.9 | 5.5 | 6.7 | 7.0 | 0.6 | 2.1 | 6.9 | 7.5 |
| | $QC_{High}$ | mean | 101.3 | 116.4 | 91.0 | 115.6 | 100.1 | 83.5 | 99.7 | 114.5 |
| | | sd | 0.7 | 2.1 | 6.9 | 6.7 | 3.6 | 6.4 | 10.9 | 6.9 |

*The measurements are done in quadruplicate.

AGE), LEA-$d_4$ (IS of LEA), OEA-$d_4$ (IS of OEA), were used as internal standards for signal correction.

The restricted availability and high cost of isotopically labelled analogues are one of the obstacles of the surrogate calibrant approach. For surrogate matrix method development, much more attention needs to be paid. The choice of surrogate matrix, sample extract purity, mobile-phase composition can all have a significant impact on parallelism [27]. Furthermore, the challenge of establishing parallelism with a surrogate matrix assay rises with the number of analytes tested. While the number of analytes in a surrogate calibrant assay may become more difficult to manage as the number of analytes increases, parallelism is primarily a matter of following the response balance method, i.e., the response needs to be adjusted for each calibrant.

Parallelism was achieved and verified during validation. Parallelism of calibration functions between analyte standard addition curves in pooled CSF and matrix-matched surrogate calibration curves or surrogate matrix curves was confirmed to ensure the accuracy of quantification (see Figure S1). The maximum deviations of the slope ratios of surrogate calibrant or surrogate matrix and targeted analyte (i.e., slope of surrogate calibrant or surrogate matrix divided by slope of targeted analyte) from 1.00 (indicating parallelism) were found to range from 0.95% to 4.66% for AEA, 2-AGE, PEA, 2-AG, 1-AG, OEA, LEA and AEA-$d_4$ (see Table 2). The slope ratio value of each analyte for each validation day was comparable, obtained values varied only slightly between batches (Table 2) and hence it can be concluded that the results were reliable. The slope ratio difference between non-zero calibrants from the first and second calibration ranges was screened at the start and end of the analytical batch. For three inter-day measurements, the slope ratio precision was found to range from 0.01% to 6.62% for AEA, 2-AGE, PEA, 2-AG, 1-AG, OEA, LEA and AEA-$d_4$ (see Table 2 for detailed results). Consequently, all results were within the acceptable limits of $\pm$ 10% deviation of parallelism (i.e., from 1.00). Precisions matched those of above validation study and bias remained within acceptable limits. Table 2 summarizes the detailed results.

### 3.2.4. Concentration-response relationship, precision, and accuracy

The method was validated following the FDA guideline and the validation was performed using pooled human CSF quality controls (QCs) and artificial CSF QCs. The method was fairly sensitive, lowest LOD and LLOQ in CSF ranged from 3.4 to 30.2 pM and 12.4–90.5 pM, respectively, for all analytes. In aCSF, lowest LOD and LLOQ ranged 6.8–30.8 pM and 20.5–92.5 pM, respectively, for all analytes (see Table 4 for detailed results). The quality of the calibration function was assessed by the % relative error (% RE) of the back-calculated concentrations of the calibrators applying the acceptance requirement of $< \pm$ 20% at LLOQ and $< \pm$ 15% for all other concentration levels. The mean of the RE for calibrator levels on 3 different days was calculated to be 11.6% for concentrations at LLOQ and 8.6% for all calibration levels, and complied with the above acceptance criteria (for detailed results see suppl. Table S5). All back-calculated calibrators (i.e. 100%) met the above criteria (acceptance limit: 75% and a minimum of six non-zero calibrator levels should meet the above criteria in each validation run). Linear calibration was achieved in the range from 90.5 to 3802 pM for 1-AG, 90.5–724.2 pM for 2-AG, 25.1–10,544.9 pM for AEA, 34.2–23,850.8 pM for 2-AGE, 12.4–5225.6 pM for LEA, 71.9–30,189.6 for PEA, 33.2–13,941.9 for OEA, 10.0–4217.9 for AEA-$d_4$ in CSF.

Within batch and between-batch accuracies and precisions showed very good results easily fulfilling the guideline's acceptance criteria for a validated method. Within batch accuracy and precision in both methods ranged from 85.2% to 112.3% and 0.36–14.4%, respectively, for all analytes. Between-batch accuracy and precision in both methods varied from 86.2% to 108.6% and 0.7–10.0%, respectively, for all analytes. All the results are summarized in Table 4, Table 5, and Table 6. These results demonstrate that calibration with the surrogate calibrant approach (for AEA) and surrogate matrix approach (2-AGE, PEA, 1/2-AG, OEA, and LEA), respectively, provide precise and accurate quantification of endocannabinoids in CSF.

### 3.2.5. Carryover

Cross-contamination between samples (carryover) was investigated in all validation and study batches by injecting blanks after injection of each QC at highest concentration or each calibrant at highest concentration as it was specified in FDA guideline [29]. The carryover was $\leq$ 20% of the average LLOQ response in each batch and thereby the acceptance criteria requested by the FDA guideline for carryover was met.

### 3.2.6. Stability

Stability experiments were performed under five different time periods and handling conditions for the stability of the target analytes in artificial and authentic CSF. For that purpose, fresh QCs at two concentrations ($QC_{3xLLOQ}$ and $QC_{High}$) were prepared. QC samples were stored at 6 h on ice (at 4 °C) for bench-top stability test. Extracted QCs were kept for 10 h, 24 h and 48 h in the autosampler (at 4 °C) to assess extract stability. Freeze/thaw stability was evaluated by analyzing QCs stored at − 80 °C for 24 h and frozen and unfrozen in three cycles. For long-term stability, QCs were kept at − 80 °C for one month and three months. Stock solution stability test was performed by analyzing all stock solutions ($MM_{Low}$ and $MM_{High}$ and internal standard stock solution) kept at 4 °C for one month.

**Table 4**
Linear response function estimation and concentration range for endocannabinoids in aCSF and CSF (surrogate matrix and surrogate calibration method).

| Analyte | | | 1-AG | 2-AG | AEA | 2-AGE | LEA | PEA | OEA |
|---|---|---|---|---|---|---|---|---|---|
| IS | | | 1-AG-$d_8$ | | AEA-$d_8$ | PEA-$d_4$ | LEA-$d_4$ | PEA-$d_4$ | OEA-$d_4$ |
| Surrogate Calibrant | | | | | AEA-$d_4$ | | | | |
| **LOD (S/N = 3)** | **[pM]** | | 0.126 | 0.315 | 0.290 | 3.038 | 0.270 | 0.249 | 2.713 |
| **LOQ (S/N = 10)** | | | 0.378 | 0.945 | 0.870 | 9.114 | 0.809 | 0.748 | 8.138 |
| **Concentration-response relationship (in MeCN)** | **Range** | **[pM]** | 0.38–946 | 0.95–946 | 0.87–8688 | 9.11–9114 | 0.81–80878 | 0.75–7487 | 8.14–8138 |
| | **$R^2$** | | 0.997 | 0.997 | 0.995 | 0.996 | 0.995 | 0.998 | 0.995 |
| **Concentration-response relationship (in CSF)** | **Range** | **[pM]** | 91–3802 | 91–724 | 25–10545 | 34–23851 | 1215133.000 | 72–30190 | 33–13942 |
| | **$R^2$** | **Day 1** | 0.993 | 0.995 | 0.998 | 0.996 | 0.993 | 0.991 | 0.997 |
| | | **Day 2** | 0.996 | 0.995 | 0.995 | 0.996 | 0.997 | 0.996 | 0.997 |
| | | **Day 3** | 0.994 | 0.991 | 0.994 | 0.998 | 0.999 | 0.994 | 0.997 |
| **Concentration-response relationship (in aCSF) (surrogate matrix method)** | **Range** | **[pM]** | 90–700 | 92–689 | 30–5501 | 30–24540 | 20–5505 | 56–20216 | 35–7206 |
| | **$R^2$** | **Day 1** | 0.999 | 0.993 | 0.991 | 0.993 | 0.990 | 0.990 | 0.993 |
| | | **Day 2** | 0.994 | 0.996 | 0.995 | 0.994 | 0.990 | 0.994 | 0.997 |
| | | **Day 3** | 0.991 | 0.994 | 0.999 | 0.997 | 0.990 | 0.996 | 0.996 |
| **Concentration-response relationship (surrogate calibration method)** | **Range** | **[pM]** | | | 10–4218 | | | | |
| | **$R^2$** | **Day 1** | | | 0.999 | | | | |
| | | **Day 2** | | | 0.992 | | | | |
| | | **Day 3** | | | 0.998 | | | | |

**Table 5**
Validation results for precision and accuracy (surrogate matrix method).

| | | $QC_{LLOQ}$ | | | | $QC_{3xLLOQ}$ | | | | $QC_{Mid}$ | | | | $QC_{High}$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Within-run (n = 5) | | Between-runs (n = 15) | | Within-run (n = 5) | | Between-runs (n = 15) | | Within-run (n = 5) | | Between-runs (n = 15) | | Within-run (n = 5) | | Between-runs (n = 15) | |
| **Analyte** | | **Accuracy [%]** | **Precision [%]** | **Accuracy [%]** | **Precision [%]** | **Accuracy [%]** | **Precision [%]** | **Accuracy [%]** | **Precision [%]** | **Accuracy [%]** | **Precision [%]** | **Accuracy [%]** | **Precision [%]** | **Accuracy [%]** | **Precision [%]** | **Accuracy [%]** | **Precision [%]** |
| **1-AG** | **Day 1** | 89.55 | 2.50 | 88.90 | 3.11 | 97.45 | 3.54 | 90.04 | 5.39 | 95.45 | 9.50 | 93.29 | 5.90 | 86.56 | 6.59 | 93.09 | 4.01 |
| | **Day 2** | 90.50 | 0.54 | | | 85.02 | 2.12 | | | 87.98 | 5.78 | | | 93.14 | 4.78 | | |
| | **Day 3** | 86.55 | 6.30 | | | 87.65 | 10.50 | | | 96.45 | 2.36 | | | 99.56 | 0.66 | | |
| **2-AG** | **Day 1** | 90.24 | 6.45 | 97.10 | 7.29 | 108.05 | 5.14 | 98.50 | 3.17 | 89.79 | 3.69 | 96.01 | 6.46 | 98.79 | 1.35 | 94.79 | 0.73 |
| | **Day 2** | 91.50 | 5.78 | | | 90.15 | 0.90 | | | 92.68 | 6.91 | | | 86.47 | 0.47 | | |
| | **Day 3** | 109.50 | 9.65 | | | 97.40 | 3.46 | | | 105.56 | 8.78 | | | 99.10 | 0.36 | | |
| **AEA** | **Day 1** | 95.39 | 3.62 | 90.63 | 7.14 | 109.45 | 5.87 | 100.59 | 7.50 | 88.33 | 3.62 | 101.12 | 5.06 | 98.06 | 9.31 | 91.57 | 5.13 |
| | **Day 2** | 88.46 | 3.39 | | | 99.24 | 14.41 | | | 110.22 | 2.41 | | | 90.49 | 1.87 | | |
| | **Day 3** | 88.04 | 14.40 | | | 93.07 | 2.20 | | | 104.81 | 9.16 | | | 86.17 | 4.21 | | |
| **2-AGE** | **Day 1** | 85.34 | 7.11 | 94.49 | 5.77 | 85.16 | 2.53 | 92.82 | 7.97 | 90.91 | 7.15 | 101.57 | 6.65 | 104.10 | 7.81 | 103.83 | 10.04 |
| | **Day 2** | 112.29 | 5.00 | | | 101.25 | 13.10 | | | 111.31 | 3.95 | | | 108.36 | 9.02 | | |
| | **Day 3** | 85.85 | 5.19 | | | 92.05 | 8.29 | | | 102.49 | 8.85 | | | 99.03 | 13.30 | | |
| **LEA** | **Day 1** | 85.24 | 7.34 | 86.19 | 5.25 | 89.50 | 4.00 | 98.37 | 4.45 | 101.11 | 8.89 | 108.60 | 5.28 | 97.34 | 3.06 | 92.84 | 4.24 |
| | **Day 2** | 87.29 | 5.44 | | | 107.36 | 3.95 | | | 111.79 | 4.45 | | | 86.82 | 3.99 | | |
| | **Day 3** | 86.01 | 2.98 | | | 98.26 | 5.41 | | | 113.79 | 2.92 | | | 94.36 | 5.65 | | |
| **PEA** | **Day 1** | 86.50 | 1.38 | 90.15 | 1.39 | 108.07 | 4.61 | 106.72 | 9.44 | 88.41 | 6.40 | 101.02 | 4.78 | 86.27 | 5.37 | 90.52 | 9.07 |
| | **Day 2** | 94.98 | 10.74 | | | 114.19 | 6.24 | | | 106.60 | 6.88 | | | 86.19 | 6.02 | | |
| | **Day 3** | 88.97 | 1.40 | | | 97.90 | 14.27 | | | 108.05 | 3.15 | | | 99.09 | 12.76 | | |
| **OEA** | **Day 1** | 86.52 | 4.20 | 85.76 | 3.36 | 85.20 | 3.47 | 97.23 | 5.77 | 87.42 | 2.17 | 91.20 | 4.62 | 104.11 | 7.81 | 93.58 | 4.34 |
| | **Day 2** | 85.57 | 1.35 | | | 109.00 | 4.33 | | | 90.46 | 6.02 | | | 88.31 | 3.91 | | |
| | **Day 3** | 85.19 | 4.55 | | | 97.48 | 9.51 | | | 95.73 | 5.66 | | | 88.33 | 1.29 | | |

**Table 6**
Validation results for precision and accuracy (surrogate calibrant method).

| Surrogate calibrant | | | AEA-$d_4$ | | |
|---|---|---|---|---|---|
| | | | Day 1 | Day 2 | Day 3 |
| $QC_{LLOQ}$ | Within-run (n = 5) | Accuracy [%] | 95.45 | 105.32 | 109.54 |
| | | Precision [%] | 3.26 | 4.05 | 9.89 |
| | Between-runs (n = 15) | Accuracy [%] | | 103.44 | |
| | | Precision [%] | | 5.73 | |
| $QC_{3xLLOQ}$ | Within-run (n = 5) | Accuracy [%] | 89.50 | 105.48 | 96.66 |
| | | Precision [%] | 5.47 | 9.65 | 3.26 |
| | Between-runs (n = 15) | Accuracy [%] | | 97.21 | |
| | | Precision [%] | | 6.13 | |
| $QC_{Mid}$ | Within-run (n = 5) | Accuracy [%] | 99.87 | 101.54 | 100.01 |
| | | Precision [%] | 0.54 | 1.06 | 3.65 |
| | Between-runs (n = 15) | Accuracy [%] | | 100.47 | |
| | | Precision [%] | | 1.75 | |
| $QC_{High}$ | Within-run (n = 5) | Accuracy [%] | 89.70 | 97.54 | 106.78 |
| | | Precision [%] | 8.97 | 5.78 | 6.98 |
| | Between-runs (n = 15) | Accuracy [%] | | 98.01 | |
| | | Precision [%] | | 7.24 | |

All samples for stability test were measured in quintuplicate. The stability results are summarized in Table S6. Briefly, the freeze/thaw stability was proven for all compounds with a maximum overall bias of 14.45% at both levels of QC samples. The accuracy of freeze/thaw stability for $QC_{High}$ of 2-AG was less than 15%, therefore 2-AG should not go through more than one freeze-thaw cycle. For all analytes except OEA at $QC_{3xLLOQ}$, the effects of long-term stability for both QC levels were all within normal variation of the method, for OEA, the $QC_{3xLLOQ}$ was + 15.01% from the nominal concentration. The accuracy and precision of bench top stability monitored for $QC_{3xLLOQ}$ and $QC_{High}$ respectively, were in acceptable range, < 15%, for all compounds. The results for extract stability were slightly outside the common acceptance limits of 15% for precision (% CV) and 85–115% for accuracy, but still less than 20% for accuracy which is the acceptable limit at 3xLLOQ. Stock solutions found to be stable at 4 °C for one month for both QC levels within ± 15% of the nominal concentrations.

### 3.3. Performance comparison to previous works

The LOQ of this presented method for 2-AG (90.5 pM) was lower (i.e. the method more sensitive) than the previously reported approaches using LC-MS/MS in various matrices (756.6 pM in human cells) [16], (158.9 pM in saliva, 1055.5 pM in plasma) [4], (98.4 pM in plasma) [5], (2704.9 pM in human brain tissue) [14]. The LOQ at 7.6 pM for 2-AG in human CSF was reported by Kantae and his colleagues [22] using nano LC system. Another working group [6] reported the LOQ of 2-AG at 18.9 pM in human plasma with the sample volume of 400 µL. On the other side, measurement times in those described assays were frequently between 6.5 and 43 min. The LOQ achieved by the method presented herein was 25.1 pM for AEA and lower than in other recently reported LC-MS/MS assays in different matrices such as in plasma at 34.8 pM [6], at 37.8 pM [7], in brain tissues at 121.6 pM [14], in brainstem at 173.8 pM [15]. There are also some assays that reported slightly lower LOQ (between 0.1 and 17.4 pM) for AEA [4,5,16,22]. Yet, in the above-mentioned methods that determined AEA, the run times were typically between 7.9 and 25 min. Larger sample volumes (2 mL and 0.5–2 mL) were typically needed for those assays in which run times were shorter (4 and 3.5 min, respectively) than in our method.

### 3.4. Concentrations of analytes in CSF samples

One hundred eighteen (118) human CSF samples of patients, mainly with CNS infections, were analysed by the validated method to determine the concentrations of endocannabinoids. All targeted analytes could be detected above LOQ in the large majority of samples. Except for 20 (out of 118) samples, the concentrations of OEA in CSF samples were all above the LLOQ (88 samples for 1-AG and 2-AG, 64 samples for AEA, 69 samples for LEA, 91 samples for PEA and 71 samples for 2-AGE were above the LLOQ) and within the range of the established UHPLC-ESI-MS/MS assay, documenting the general applicability of the method (note, with newest QTRAP instruments significantly lower LOQs can be achieved by the newly developed method yielding even less samples below LOQ). The concentrations measured for AG which was determined as the sum of 1-AG and 2-AG resulted a median of 160.4 pM, for PEA this was 92.7 pM, for LEA 57.9 pM, for OEA 60.0 pM, for 2-AGE 62.1 pM and for AEA 86.0 pM, respectively. Both AEA and LEA were much lower abundant, and their amounts were close to the LOQ levels. The results are summarized in Table 7. Details of the results in view of the pathophysiological background will be reported and discussed elsewhere. The presented data document that the developed method has acceptable applicability. However, some further sensitivity improvements are recommended for future studies which could be easily achieved by transferring the method to the newest generation of tandem mass spectrometers.

Finally, Passing-Bablok regression analysis of the results of the study samples without those below LOQ (n = 103) between the two approaches, i.e., surrogate calibrant and surrogate matrix calibration method, for AEA was utilized to verify the suitability of the surrogate matrix approach and confirm that it provides comparable results. As shown in Fig. 4, a high correlation is found between the two methods with a Pearson's correlation coefficient of > 0.999 ($p < 0.05$), a slope of 1.03 ( ± 0.06) and an intercept of 0.22 ( ± 0.11) (values in parenthesis representing the 95% confidence intervals). It can be concluded that the surrogate matrix approach yields comparable results as the surrogate calibrant approach, but slightly overestimates the concentrations. The minor deviations though were considered acceptable for our purpose. To advance the method, the surrogate matrix could be further fine-tuned to get values closer to 1.00 for the slope and 0.00 for the intercept.

## 4. Conclusion

In this study, a UHPLC–ESI-MS/MS method has been developed and successfully validated for the determination of endocannabinoids in low quantity CSF samples. Six analytes (1/2-AG, 2-AGE, AEA, LEA, PEA and

**Table 7**
Summarized results of endocannabinoid concentrations in 120 CSF samples of patients suffering CNS infection and controls.

| Analyte | CSF samples above LLOQ (%) | Concentrations (pM) | | |
|---|---|---|---|---|
| | | Median | Lower quartiles | Upper quartiles |
| 1-AG & 2-AG | 74.6 | 160.4 | 110.4 | 334.9 |
| AEA | 53.8 | 86.0 | 32.1 | 147.9 |
| 2-AGE | 59.7 | 62.1 | 45.6 | 94.6 |
| LEA | 58.5 | 57.9 | 18.6 | 292.4 |
| PEA | 76.5 | 92.7 | 55.6 | 132.1 |
| OEA | 83.2 | 60.0 | 49.6 | 71.2 |

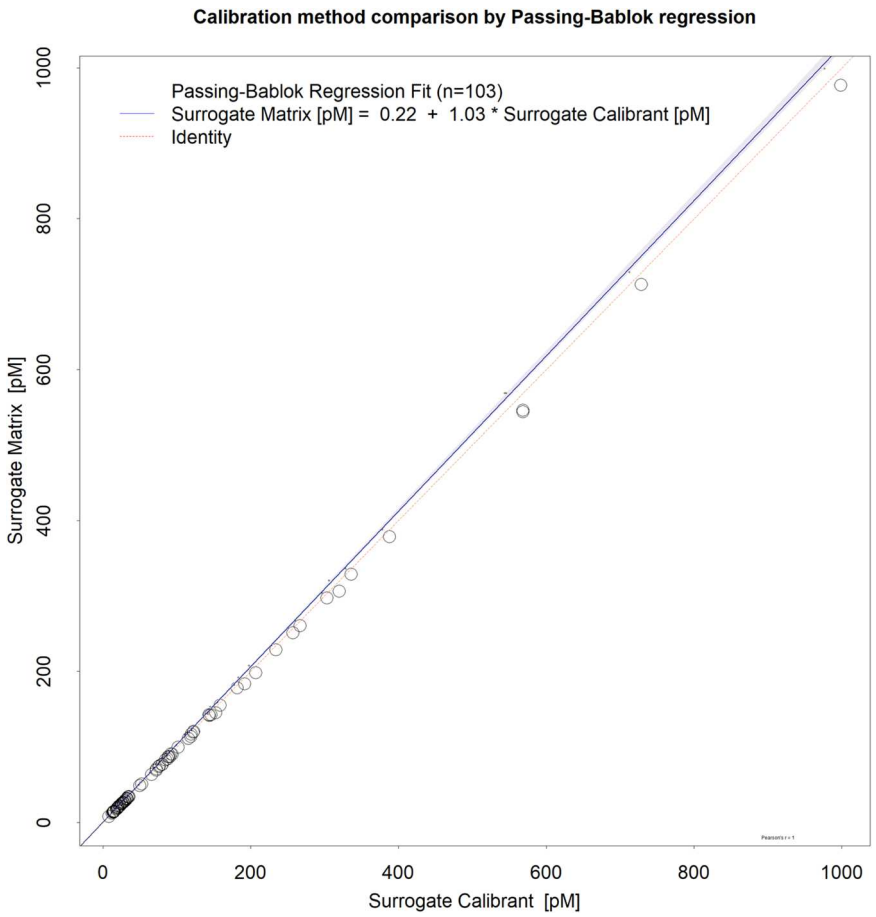


**Fig. 4.** Method comparison between Surrogate Calibrant method and Surrogate Matrix method by Passing-Bablok regression. The solid line represents here the regression line, the red dashed line corresponds to the 95% confidence interval for the regression line.

OEA) could be quantified in a single chromatographic run with good sensitivity, high accuracy, and with a short analysis time in the real samples. The method presented here is simple, robust and has, due to its short analysis time (5.5 min) and single protein precipitation/extraction step, high throughput capability. To the best of our knowledge, this is the first validated method which utilized two calibration approaches in CSF samples: surrogate calibrant and surrogate matrix method specifically for quantitation of 2-AG, 2-AGE, AEA, LEA, PEA and OEA in CSF. Only limited information is currently available on endocannabinoid concentrations in CSF and hence this report sheds some light on this dark spot of clinical lipidomics.

## CRediT authorship contribution statement

**Ece Aydin:** Investigation, Methodology, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review and editing. **Malgorzata Cebo:** Investigation, Conceptualization, Methodology, Formal analysis, Writing – review and editing. **Justyna Mielnik:** Formal analysis **Hardy Richter:** Conceptualization, Writing – review and editing. **Rebecca Schüle:** Conceptualization, Neurology Biobank supervisor. **Adrian Sievers-Engler:** Formal analysis. **Piotr Młynarz:** Investigation, Writing – review and editing. **Michael Lämmerhofer:** Conceptualization, Methodology, Supervision, Writing – review and editing, Resources.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2022.115090.

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# Supplementary Material

## UHPLC-ESI-MS/MS assay for quantification of endocannabinoids in cerebrospinal fluid using surrogate calibrant and surrogate matrix approaches

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# 1. Preparation of calibrants, quality control samples and stock solutions

Standard stock solutions (Master mixes) of analytes were dissolved in MeCN and prepared in required concentrations for each analyte (1-AG, 2-AG, AEA, 2-AGE, AEA, OEA, PEA and LEA). Master Mixes (MM, $MM_{High}$ as high concentrated stock solution, $MM_{Mid}$ as medium concentrated stock solution, $MM_{Low}$ as low concentrated stock solution) were prepared by serial dilution ($MM_{Mid}$ by 1:10 dilution of $MM_{High}$, $MM_{Low}$ by 1:10 dilution of $MM_{Mid}$). They were used to prepare four levels of quality control samples and seven different concentration levels of calibrants for each matrix (aCSF and CSF). $MM_{High}$ was used to prepare $QC_{High}$, calibrants 6 and 7, and $MM_{Mid}$ for $QC_{Intermediate}$ ($QC_{Inter}$), calibrant 5 and $MM_{Low}$ for $QC_{3*LLOQ}$, $QC_{LLOQ}$ and calibrants 1, 2, 3 and 4. Calibrant 0 (Cal0 or zero calibrant, pooled matrix) was also prepared but only spiked with ISs. Three IS stock solutions were prepared. 1000 µL of IS Stock Solution 2 was prepared by 1:10 dilution of IS Stock Solution 1 in MeCN (IS concentrations at IS Stock Solution 1: AEA-$d_8$: 35.6 nM; PEA-$d_4$: 30.4 nM; LEA-$d_4$: 81.9 nM; OEA-$d_4$: 82.4 nM; 1-AG-d8: 95.6 nM in MeCN). Then, 120 mL of IS Stock Solution 3 was prepared by 1:500 dilution of IS Stock Solution 2 in MeCN for all batches. IS Stock Solution 3 was added at the same concentrations as in the samples.

**Table S1** Master Mix Concentrations

| | Type of Master Mix, used | Master Mix concentration (nM) | | | | | | Volume (µL) | Absolute amount (nmol) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AEA/AEA-$d_4$ | OEA | 2-AGE | PEA | LEA | 1-AG/2-AG | | AEA/AEA-$d_4$ | OEA | 2-AGE | PEA | LEA | 1-AG/2-AG |
| Cal 1/ $QC_{LLOQ}$ | $MM^*_{Low}$ | 14.6/11.5 | 21.9 | 18.0 | 56.1 | 8.3 | 44.3 | 0.7 | 10.3/8.2 | 15.6 | 12.8 | 39.8 | 5.9 | 31.4 |
| Cal 2 | $MM_{Low}$ | 14.6/11.5 | 21.9 | 18.0 | 56.1 | 8.3 | 44.3 | 1.5 | 21.8/17.3 | 32.9 | 27.0 | 84.1 | 12.5 | 66.4 |
| Cal 3 | $MM_{Low}$ | 14.6/11.5 | 21.9 | 18.0 | 56.1 | 8.3 | 44.3 | 3.0 | 43.7/34.5 | 65.8 | 54.0 | 168.3 | 25.0 | 132.8 |
| Cal 4 | $MM_{Low}$ | 14.6/11.5 | 21.9 | 18.0 | 56.1 | 8.3 | 44.3 | 5.0 | 72.8/57.5 | 109.6 | 90.0 | 280.5 | 41.6 | 221.4 |
| Cal 5 | $MM_{Mid}$ | 145.5/115.1 | 219.3 | 180.0 | 560.9 | 83.2 | 442.8 | 3.0 | 436.6/345.2 | 657.8 | 540.0 | 1682.8 | 249.7 | 1328.3 |
| Cal 6 | $MM_{High}$ | 1455.3/1150.7 | 2192.7 | 1799.9 | 5609.4 | 832.2 | 4427.8 | 1.5 | 2182.9/1726.1 | 3289.0 | 2699.9 | 8414.1 | 1248.3 | 6641.7 |
| Cal 7 | $MM_{High}$ | 1455.3/1150.7 | 2192.7 | 1799.9 | 5609.4 | 832.2 | 4427.8 | 3.0 | 4365.8/3452.2 | 6578.0 | 5399.8 | 16828.1 | 2496.6 | 13283.4 |
| $QC_{3xLLOQ}$ | $MM_{Low}$ | 1455.3/1150.7 | 21.9 | 18.0 | 56.1 | 8.3 | 44.3 | 2.1 | 31.1/24.6 | 46.9 | 38.5 | 120.0 | 17.8 | 94.8 |
| $QC_{Mid}$ | $MM_{Mid}$ | 145.5/115.1 | 219.3 | 180.0 | 560.9 | 83.2 | 442.8 | 1.4 | 208.1/164.6 | 313.6 | 257.4 | 802.1 | 119.0 | 633.2 |
| $QC_{High}$ | $MM_{High}$ | 1455.3/1150.7 | 2192.7 | 1799.9 | 5609.4 | 832.2 | 4427.8 | 2.5 | 3638.2/2876.9 | 5481.6 | 4499.8 | 14023.4 | 2080.5 | 11069.5 |

*MM represents here Master Mix. $MM_{High}$ was used to extend the calibration to higher concentrations.

**Table S2** Concentrations of surrogate calibrants in calibration and quality control samples*

| | **Dilution Factor to Cal 7** | **Final Concentration of AEA-$d_4$** |
|---|---|---|
| Cal 1/ $QC_{LLOQ}$ | 420 | 20.1 |
| Cal 2 | 200 | 42.2 |
| Cal 3 | 100 | 84.4 |
| Cal 4 | 60 | 140.6 |
| Cal 5 | 10 | 843.7 |
| Cal 6 | 2 | 4218.3 |
| Cal 7 | | 8436.6 |
| $QC_{3xLLOQ}$ | 140.0 | 60.3 |
| $QC_{Mid}$ | 21.0 | 401.7 |
| $QC_{High}$ | 1.2 | 7030.5 |

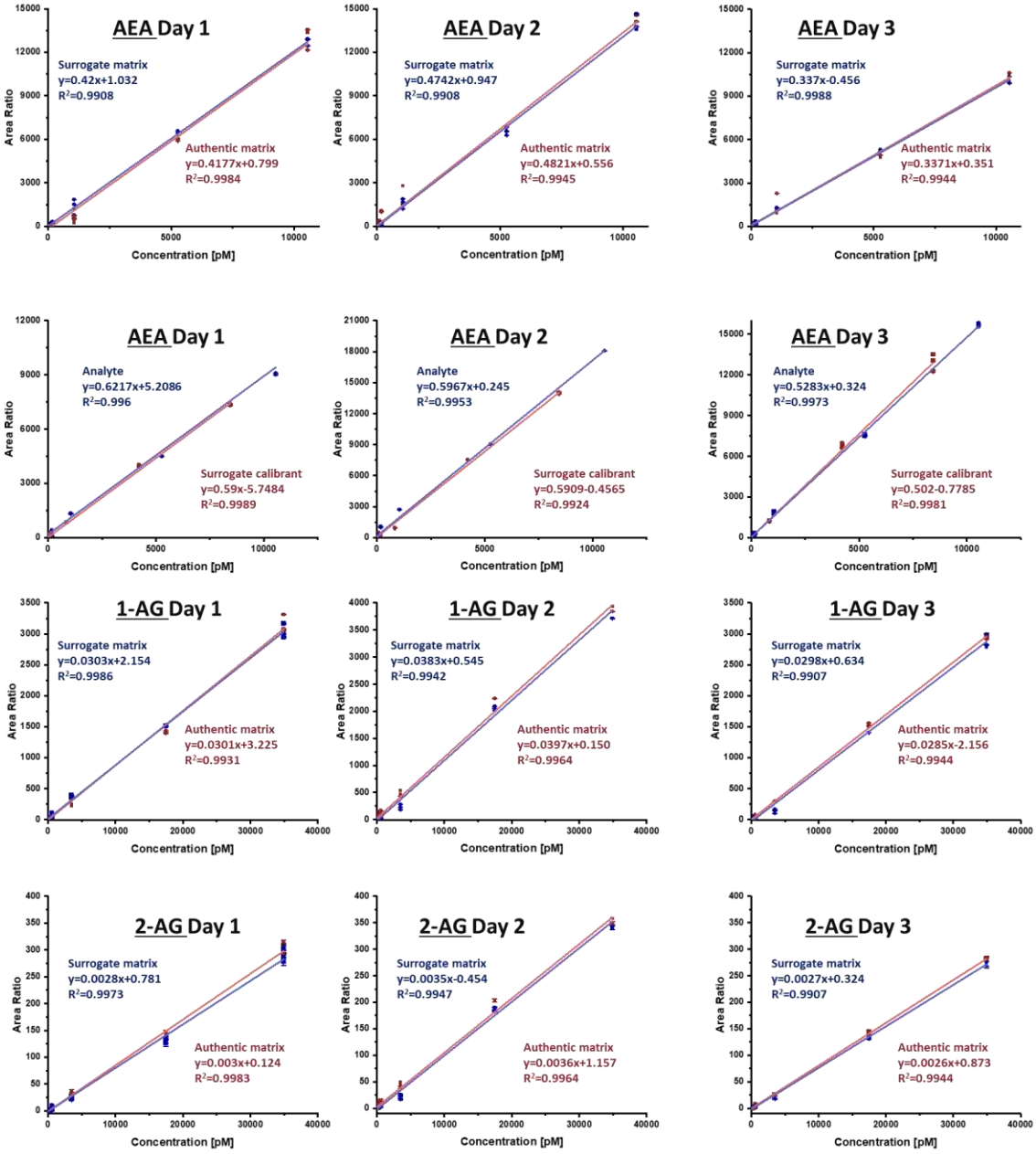
*Concentrations are given in pM.

**Table S3** Concentration of calibrants and quality control samples in surrogate matrix method*

| | Dilution Factor to Cal 7 | Final Concentration of AEA | Final Concentration of 2-AGE | Final Concentration of LEA | Final Concentration of OEA | Final Concentration of PEA | Final Concentration of 1-AG&2-AG |
|---|---|---|---|---|---|---|---|
| Cal 1/ $QC_{LLOQ}$ | 420 | 25.1 | 32.6 | 13.4 | 35.4 | 83.4 | 83.2 |
| Cal 2 | 200 | 52.7 | 68.4 | 28.1 | 74.4 | 175.2 | 124.7 |
| Cal 3 | 100 | 105.5 | 136.8 | 56.1 | 148.8 | 350.3 | 332.6 |
| Cal 4 | 60 | 175.8 | 228.0 | 93.6 | 248.1 | 583.9 | 665.3 |
| Cal 5 | 10 | 1054.6 | 1368.3 | 561.4 | 1488.4 | 3503.1 | 3492.7 |
| Cal 6 | 2 | 5272.9 | 6841.3 | 2806.9 | 7441.9 | 17515.5 | 33264.2 |
| Cal 7 | | 10545.8 | 13682.5 | 5613.7 | 14883.8 | 35031.0 | 34927.4 |
| $QC_{3xLLOQ}$ | 140.0 | 75.3 | 97.7 | 40.1 | 106.3 | 250.2 | 249.5 |
| $QC_{Mid}$ | 21.0 | 502.2 | 651.6 | 267.3 | 708.8 | 1668.1 | 1663.2 |
| $QC_{High}$ | 1.2 | 8788.2 | 11402.1 | 4678.1 | 12403.2 | 29192.5 | 29106.2 |

*Concentrations are given in pM.

# 2.Suitability of surrogate calibrant and matrix approaches and parallelism / dilution linearity



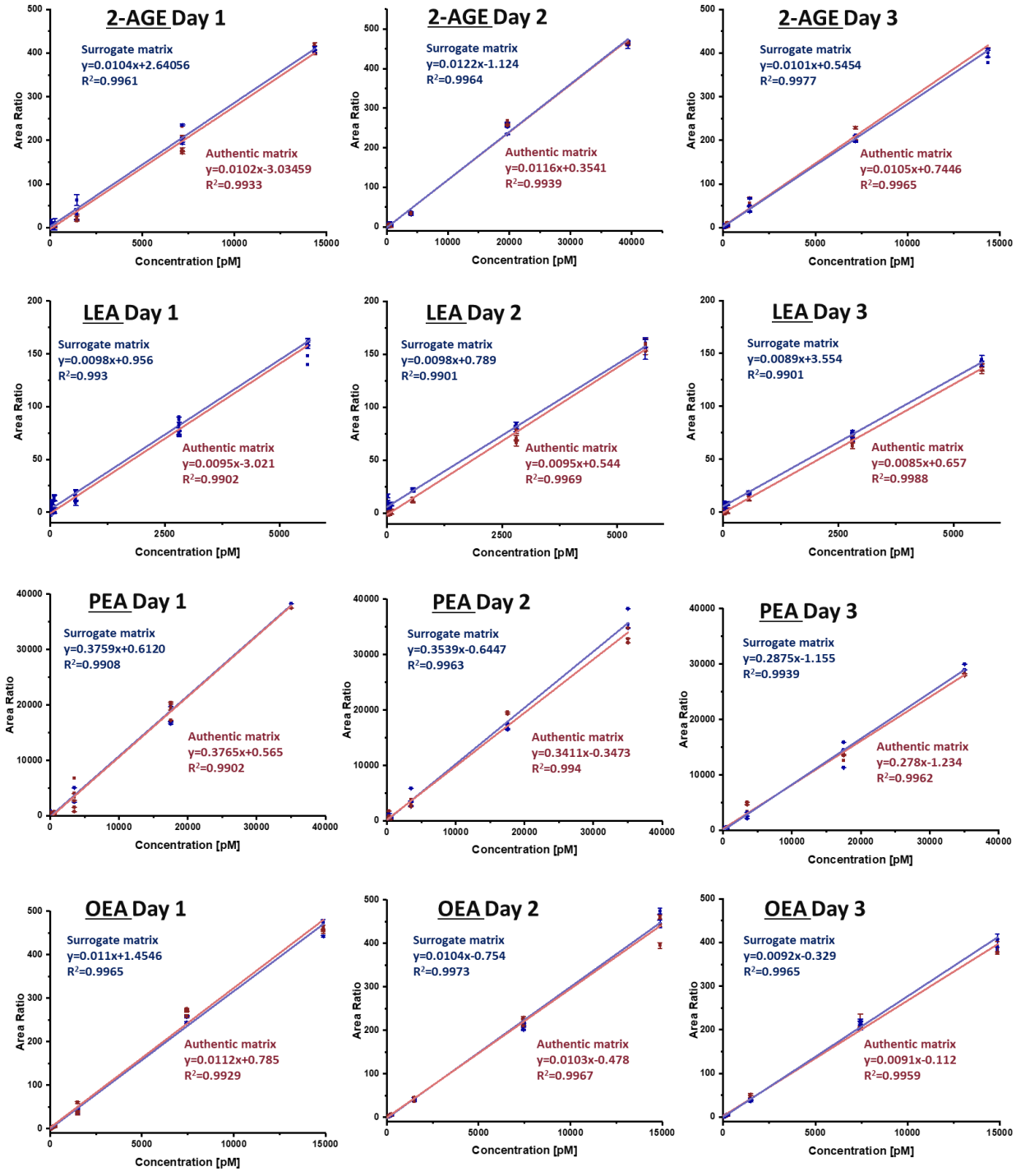



**Fig. S1** Results for parallelism of standard addition curve and corresponding surrogate matrix and surrogate calibrant curve for targeted analytes during validation.

## 3. Optimization of UHPLC-MS/MS method

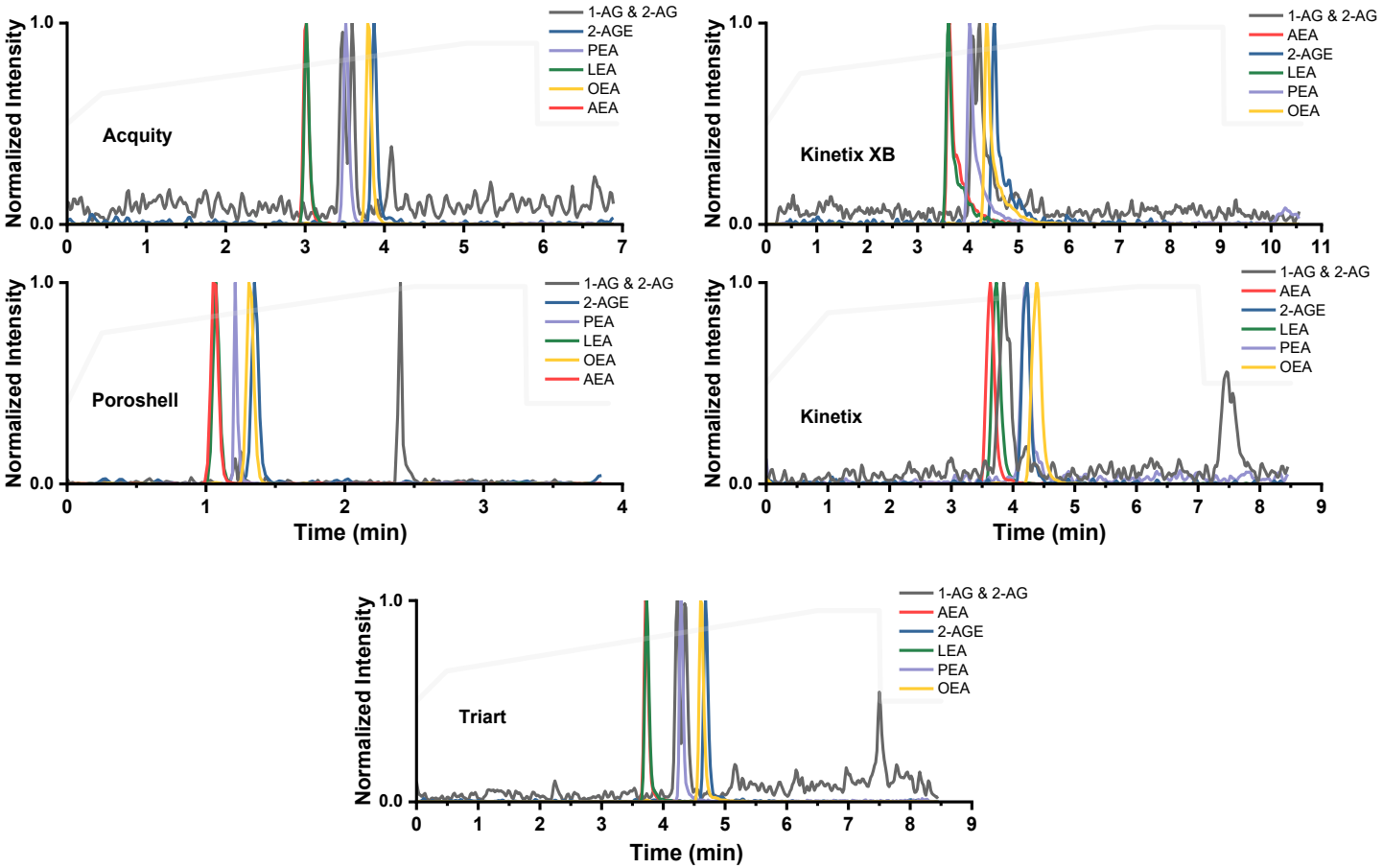



**Fig. S2** Results for column screening (Poroshell HPH (C18 50*3 mm, 1.9 um) with flow rate 0.87 ml/min; Acquity CSH C18 (100*2.1 mm, 1.7um) with flow rate 0.5 ml/min; Kinetix XB C18 (100*1mm, 2.6um) with flow rate 0.87 ml/min; Triart C18 (100*2 mm, 1.9um) with flow rate 0.4 ml/min; Kinetix C18 (100*3mm, 2.6um) with flow rate 0.3 ml/min).

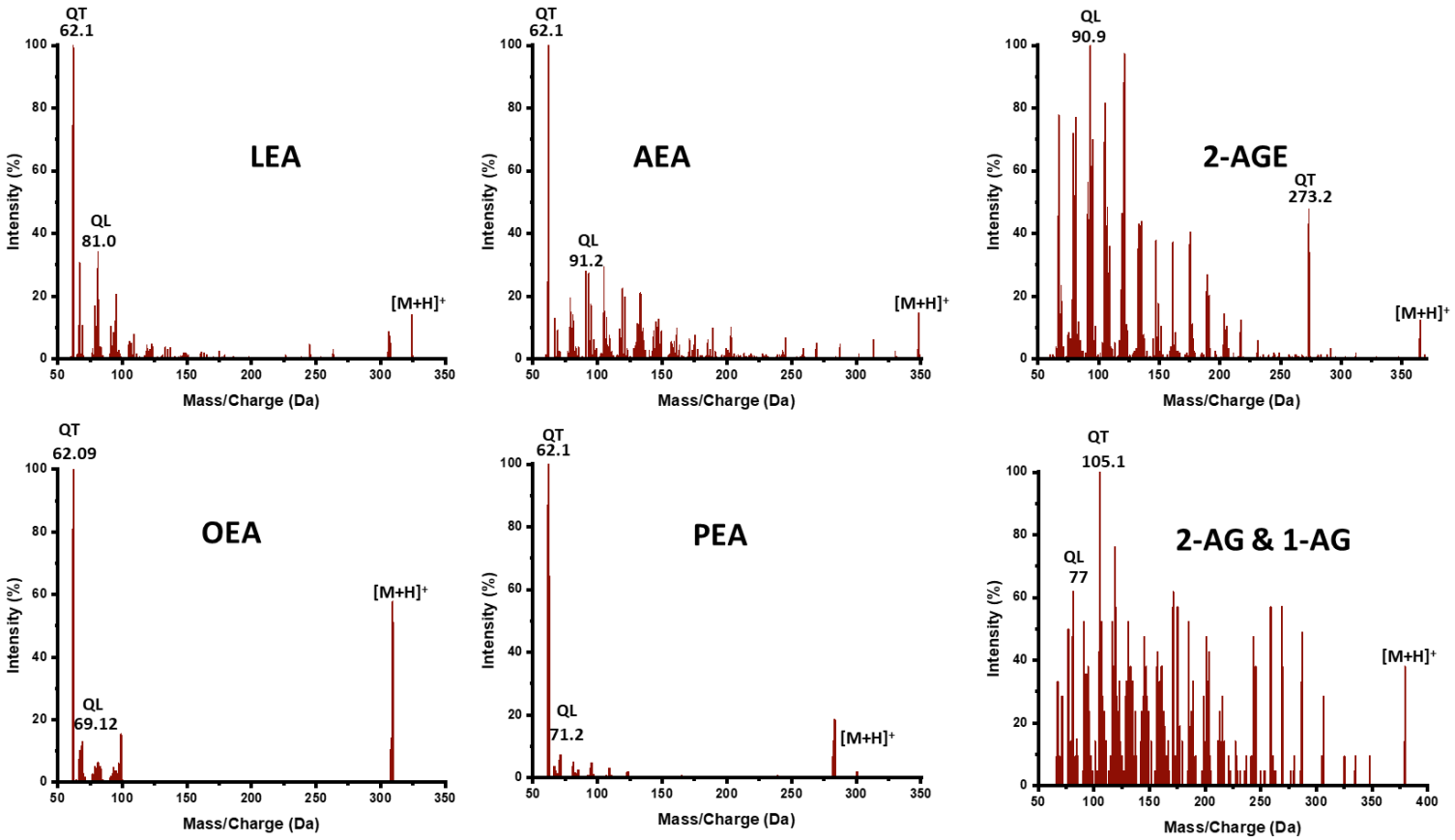



**Fig. S3** Product ion spectra of targeted analytes in MeCN with 0.1% formic acid, injected with syringe by direct infusion. Spectra were acquired from product ion scan. Collision energy ramped between 21-90 V. QL and QT represents here qualifier ion and quantifier ion, respectively.

**Table S4** Details of retention times of target analytes and mean ion ratios during validation

| Analyte | Ion ratio* | | | Retention time (min) |
|---|---|---|---|---|
| | In CSFs | In aCSFs | In Standards** | |
| PEA | 5.05 ± 0.75 | 5.14 ± 0.77 | 5.15 ± 0.10 | 3.46 |
| AEA | 1.22 ± 0.18 | 1.22 ± 0.06 | 1.30 ± 0.05 | 2.71 |
| 2-AGE | 4.12 ± 0.36 | 4.10 ± 0.33 | 4.12 ± 0.28 | 3.41 |
| LEA | 3.20 ± 0.44 | 3.14 ± 0.45 | 3.07 ± 0.36 | 2.82 |
| OEA | 5.35 ± 0.55 | 5.41 ± 0.78 | 5.44 ± 0.19 | 3.55 |
| 1-AG | 2.14 ± 0.32 | 2.14 ± 0.30 | 2.11 ± 0.47 | 3.51 |
| 2-AG | 1.51 ± 0.07 | 1.51 ± 0.08 | 1.55 ± 0.20 | 3.12 |

*Average Ion ratios of quantifier to qualifier ions. Average ion ratios are represented here within their standard deviations.

**Master Mix that was measured before and after measurement of the batch as system suitability test.

**Table S5** Statistical data of back-calculation from calibrators run in quadruplicate during validation (n=12)**

| | | Cal 1 | | | Cal 2 | | | Cal 3 | | | Cal 4 | | | Cal 5 | | | Cal 6 | | | Cal 7 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 3 | Day 1 | Day 2 | Day 3 | Day 1 | Day 2 | Day 3 | Day 1 | Day 2 | Day 3 | Day 1 | Day 2 | Day 3 | Day 1 | Day 2 | Day 3 | Day 1 | Day 2 | Day 3 |
| **AEA-$d_4$** | Nominal Concentration | | 20.1 | | | 42.2 | | | 84.4 | | | 140.6 | | | 843.7 | | | 4218.3 | | | 8436.6 | |
| | %Relative Error* | 11.9 | 12.9 | 11.4 | 9.1 | 9.3 | 9.7 | -5.1 | 1.9 | 6.9 | 8.1 | 7.9 | 8.9 | -0.4 | 4.3 | 5.2 | -0.2 | 11.5 | 14.2 | 12.9 | 10.2 | 14.5 |
| **AEA** | Nominal Concentration | | 25.1 | | | 52.7 | | | 105.4 | | | 175.7 | | | 1054.5 | | | 5272.4 | | | 10544.9 | |
| | %Relative Error* | 10.8 | 11.5 | 12.9 | 8.5 | 7.8 | 8.9 | 6.9 | 11.5 | 14.5 | 9.2 | 8.7 | 9.9 | 11.1 | 12.4 | 14.5 | 0.9 | 3.5 | 2.7 | 11.9 | 14.6 | 13.6 |
| **2-AGE** | Nominal Concentration | | 34.2 | | | 71.8 | | | 143.5 | | | 239.2 | | | 1435.3 | | | 7176.5 | | | 14353.0 | |
| | %Relative Error* | -5.5 | -9.8 | -11.2 | -7.2 | -8.9 | -10.4 | 7.3 | 11.2 | 0.5 | 10.1 | 10.9 | 11.7 | -0.7 | 4.5 | 10.7 | -1.0 | -3.1 | -2.1 | -8.1 | -8.3 | -8.9 |
| **LEA** | Nominal Concentration | | 12.4 | | | 26.1 | | | 52.3 | | | 87.1 | | | 522.6 | | | 2612.8 | | | 5225.6 | |
| | %Relative Error* | 11.1 | 19.2 | 18.4 | -10.5 | -9.5 | 4.3 | -8.9 | 7.3 | 5.4 | -7.3 | 9.2 | -6.1 | -0.3 | 4.5 | 3.9 | -9.1 | -8.3 | -7.9 | 13.9 | 13.1 | 6.3 |
| **OEA** | Nominal Concentration | | 33.2 | | | 69.7 | | | 139.4 | | | 232.4 | | | 1394.2 | | | 6971.0 | | | 13941.9 | |
| | %Relative Error* | -10.3 | 5.1 | 2.4 | 14.1 | 14.9 | 12.3 | -7.2 | 3.6 | 10.6 | 9.1 | 11.5 | -13.6 | 2.1 | 11.7 | 14.7 | -1.0 | -6.8 | -5.4 | -12.7 | -14.4 | -14.9 |
| **PEA** | Nominal Concentration | | 71.9 | | | 150.9 | | | 301.9 | | | 503.2 | | | 3019.0 | | | 15094.8 | | | 30189.6 | |
| | %Relative Error* | 19.2 | 19.7 | 11.5 | 13.1 | 0.9 | 11.4 | 9.1 | 6.9 | 6.7 | -10.6 | -3.6 | 9.7 | 1.0 | -2.1 | 12.6 | 0.3 | 10.2 | 10.6 | -0.3 | -3.6 | -14.6 |
| **1-AG&2-AG** | Nominal Concentration | | 90.5 | | | 190.1 | | | 380.2 | | | 633.7 | | | 3802.0 | | | 19009.9 | | | 38019.8 | |
| | %Relative Error* | 19.1 | 5.7 | 4.7 | 13.6 | 14.7 | 5.4 | 8.1 | 10.1 | 11.9 | -11.5 | 9.4 | 6.9 | -0.3 | 11.5 | -2.4 | -0.7 | -3.9 | 9.4 | -0.6 | -8.9 | -11.2 |

*%RE=(mean measured conc. (n=12)-nominal conc.)/nominal conc. x100%

**Concentrations and SD are given in pM.

**Table S6** Summary of the validation for stability test*

| Analytes/ Surrogate Calibrant | | Freeze/Thaw Stability [%] | Long Term Stability [%] | | Bench-top Stability [%] | Extract Stability [%] | | | Stock Solution Stability [%]** |
|---|---|---|---|---|---|---|---|---|---|
| **2-AG** | $QC_{3xLLOQ}$ | 111.51 ± 0.08 | 1 month | 3 months | 100.19 ± 8.89 | 10h | 24h | 48h | 101.51 ± 3.13 |
| | | | 80.78 ± 0.58 | 78.9 ± 5.90 | | 92.19 ± 1.23 | 90.89 ± 2.97 | 85.19 ± 1.05 | |
| | $QC_{High}$ | 83.39 ± 2.74 | 1 month | 3 months | 92.19 ± 1.24 | 10h | 24h | 48h | 88.09 ± 6.77 |
| | | | 101.11 ± 1.48 | 83.11 ± 4.67 | | 87.1 ± 0.83 | 80.9 ± 5.41 | 79.9 ± 6.23 | |
| **1-AG** | $QC_{3xLLOQ}$ | 96.11 ± 6.30 | 1 month | 3 months | 100.59 ± 4.28 | 10h | 24h | 48h | 100.01 ± 9.78 |
| | | | 88.11 ± 3.55 | 80.1 ± 12.90 | | 90.23 ± 10.04 | 88.65 ± 0.88 | 80.99 ± 12.5 | |
| | $QC_{High}$ | 88.77 ± 9.67 | 1 month | 3 months | 84.69 ± 9.34 | 10h | 24h | 48h | 96.0 ± 5.52 |
| | | | 110.44 ± 6.87 | 85.06 ± 7.65 | | 85.11 ± 6.27 | 84.1 ± 0.58 | 81.6 ± 3.87 | |
| **AEA** | $QC_{3xLLOQ}$ | 90.00 ± 8.89 | 1 month | 3 months | 83.71 ± 2.74 | 10h | 24h | 48h | 90.00 ± 8.89 |
| | | | 90.01 ± 12.99 | 84.92 ± 6.53 | | 90.99 ± 4.87 | 90.03 ± 6.85 | 88.99 ± 6.57 | |
| | $QC_{High}$ | 94.22 ± 0.68 | 1 month | 3 months | 87.50 ± 6.49 | 10h | 24h | 48h | 94.22 ± 0.68 |
| | | | 86.18 ± 3.66 | 78.9 ± 9.94 | | 87.19 ± 6.85 | 85.87 ± 4.32 | 79.93 ± 0.17 | |
| **2-AGE** | $QC_{3xLLOQ}$ | 85.01 ± 13.24 | 1 month | 3 months | 79.9 ± 6.23 | 10h | 24h | 48h | 105.04 ± 2.90 |
| | | | 115.01 ± 0.47 | 83.1 ± 4.63 | | 112.55 ± 7.40 | 95.33 ± 6.49 | 87.80 ± 3.61 | |
| | $QC_{High}$ | 93.11 ± 0.97 | 1 month | 3 months | 92.59 ± 1.21 | 10h | 24h | 48h | 100.78 ± 13.2 |
| | | | 80.78 ± 0.58 | 78.9 ± 5.90 | | 90.45 ± 9.04 | 86.03 ± 3.00 | 83.98 ± 1.12 | |
| **LEA** | $QC_{3xLLOQ}$ | 109.59 ± 3.28 | 1 month | 3 months | 101.11 ± 1.49 | 10h | 24h | 48h | 109.59 ± 3.28 |
| | | | 108.84 ± 0.73 | 105.36 ± 6.36 | | 86.33 ± 3.87 | 86.07 ± 6.97 | 85.00 ± 4.21 | |
| | $QC_{High}$ | 99.97 ± 14.45 | 1 month | 3 months | 90.00 ± 8.88 | 10h | 24h | 48h | 99.97 ± 14.45 |
| | | | 91.71 ± 9.97 | 88.11 ± 7.67 | | 89.11 ± 0.07 | 85.1 ± 11.24 | 78.48 ± 9.64 | |
| **PEA** | $QC_{3xLLOQ}$ | 89.11 ± 6.51 | 1 month | 3 months | 89.78 ± 0.58 | 10h | 24h | 48h | 100.22 ± 4.68 |
| | | | 85.1 ± 3.25 | 79.15 ± 0.78 | | 92.12 ± 3.88 | 85.20 ± 1.12 | 85.09 ± 6.88 | |
| | $QC_{High}$ | 92.14 ± 6.11 | 1 month | 3 months | 85.19 ± 1.03 | 10h | 24h | 48h | 109.58 ± 14.45 |
| | | | 90.69 ± 1.12 | 86.15 ± 3.92 | | 88.55 ± 0.91 | 80.12 ± 10.00 | 77.56 ± 3.15 | |
| **OEA** | $QC_{3xLLOQ}$ | 86.15 ± 6.8 | 1 month | 3 months | 83.39 ± 9.66 | 10h | 24h | 48h | 99.67 ± 6.01 |
| | | | 91.65 ± 2.13 | 82.05 ± 15.01 | | 92.09 ± 5.67 | 90.08 ± 1.31 | 85.09 ± 6.88 | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $QC_{High}$ | 100.5 ± 0.19 | 1 month | 3 months | 99.91 ± 14.4 | 10h | 24h | 48h | 95.03 ± 14.91 |
| | | | 96.51 ± 1.28 | 88.67 ± 6.91 | | 100.03 ± 5.27 | 87.00 ± 2.36 | 85.55 ± 4.80 | |
| **AEA-$d_4$** | $QC_{3xLLOQ}$ | 114.48 ± 9.73 | 1 month | 3 months | 101.94 ± 0.77 | 10h | 24h | 48h | 109.59 ± 3.28 |
| | | | 112.63 ± 0.78 | 103.11 ± 10.39 | | 99.33 ± 6.21 | 95.11 ± 3.30 | 96.07 ± 3.69 | |
| | $QC_{High}$ | 92.60 ± 7.59 | 1 month | 3 months | 88.51 ± 13.18 | 10h | 24h | 48h | 99.97 ± 14.45 |
| | | | 86.71 ± 7.69 | 80.37 ± 5.78 | | 87.08 ± 11.24 | 86.32 ± 5.01 | 85.11 ± 6.75 | |

*The samples are measured in quintuplicate.

**For stock solution stability, MM samples are utilized instead of QC samples: MM represents here Master Mix, $MM_{Low}$ and $MM_{High}$ were diluted with dilution rate of 1:10 and 1:50, respectively.

### 3.3. Manuscript I

# Development and validation of an LC–MS/MS assay for the quantification of salivary cortisol and melatonin

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**Keywords**

Steroidomics, QTRAP, melatonin, uHPLC, Mass Spectrometry, Surrogate Calibration

**Abbreviations**

$NH_4Ac$, Ammonium acetate

$NH_4F$, Ammonium fluoride

$NH_4FA$, Ammonium formate

DLMO, Dim light melatonin offset

ER, Extraction recovery

ESI, Electrospray ionization

EtAc. Ethyl acetate

FA, Formic acid

HPA, Hypothalamic-pituitary-adrenal

IPA, isopropanol

QC, Quality Control

LLE, Liquid-liquid extraction

LLOQ, the lower limit of quantitation

LOD, the limit of detection

ME, Matrix effect

MeCN, Acetonitrile

MeOH, Methanol

MM, Master mix

PE, Process efficiency

PPT, Protein precipitation

RF, Response factor

SPE, Solid-Phase Extraction

SPP, Superficially porous particles

SRM, Selected reaction monitoring

ULOQ, the upper limit of quantitation

**Abstract**

To investigate the sleep-awake rhythm in healthy volunteers and compare the sleep-awake cycles with Parkinson patients, this work aimed to develop a rapid, sensitive, robust, reliable, and reproducible LC-MS/MS technique for melatonin and cortisol quantification in saliva. It was aimed to achieve this by utilizing deuterated internal standards and surrogate analytes. The technique needed to be fast as well, with the least amount of sample preparation necessary.

Firstly, the unique MS settings for each targeted analyte were optimized. The specific MS parameters for each targeted analyte were determined and, the S/N of the targeted analytes in neat solution were compared among different mobile phases. After comparing several extraction techniques (SPE, LLE, SLE and PPT etc.) for extracting analytes from pooled salivary samples, the objective was to choose the technique that required the fewest sample preparation steps and was most affordable for routine analysis. Following that, the chromatographic column based on the results of screening several reversed-phase columns was chosen. Different mobile phases and their compositions in neat solution and pooled saliva were compared after the column and extraction technique were chosen.

After optimization of above-mentioned parameters, the concentrations of the various mobile phase additives (Formic acid (FA), ammonium acetate ($NH_4Ac$), ammonium formate ($NH_4FA$)) were compared and commercially available internal standards for each targeted analyte were determined. After that, several gradient and separation techniques were evaluated for decision of the fastest and most effective method. Following that the temperature of the column compartment is optimized and the limit of detection (LOD), the limit of quantification (LOQ), calibration curve was determined. Then, using a variety of saliva samples from healthy volunteers, the optimized method was tested to track the change of cortisol and melatonin concentration levels over the period of 24 hours in a circadian rhythm. To observe the matrix effect, process efficiency, process recovery, and change in melatonin and cortisol peak intensity, the impact of sample quantities and extraction solvent volume was examined. To conclude, a RPLC-ESI-MS/MS technique with LOQs of 15 pg/mL for melatonin and 104 pg/mL for

cortisol in saliva was established. The high throughput in a clinical study is made possible by the run duration of 6 minutes per sample. Most of the real samples taken throughout the day were close to the LOQ and could not be reliably quantified, hence further adjustment of the LOQs for melatonin would be advised. Moreover, as there are not many commercially available isotopically labelled internal standard for melatonin, for surrogate calibration approach an isotopically labelled internal standard could be synthesized using its active metabolites. This will be the subject of more research.

### 3.3.1 Introduction

Parkinson’s disease is the second most frequent neurodegenerative disorder, which affects nearly 315 per 100.000 human beings along with the characteristics of motor symptoms including rigidity, bradykinesia, tremor, and impaired balance [1, 2]. It is common for patients to have sleep disturbance, which according to the Baark staging theory may contribute to the effect of Parkinson’s disease on other brain regions besides substantia nigra resulting in the development of some non-motor-symptoms [3, 4].

Human behavioral, physiological, and biochemical changes show 24-hour rhythms called circadian rhythms as physiological and behavioral processes are controlled in 24-hour cycle [5, 6]. Among these, for instance, endocrine rhythms of melatonin and corticosteroids, also the sleep-awake cycle follows a rhythm [7]. This rhythm is regulated by the circadian pacemaker, which is in the suprachiasmatic nuclei. The pacemaker runs in a non-periodic environment closely however not equal to 24h. Other environmental alterations entrain the circadian pacemaker mainly light as an activator and darkness throughout different ways namely a direct retinal projection “the retinohypothalamic tract” and the geniculohypothalamic tract [8].

Melatonin is an endogenous hormone and is secreted from pineal gland with a circadian rhythm [9, 10]. This circadian rhythm differs according to living species. This difference is related to the hours and durations of the hormone's nocturnal peak [11]. Melatonin has antioxidant, oncostatic, anti-inflammatory and anticonvulsant effects and important physiological functions such as regulation of circadian rhythms and regulation of the reproductive axis. Cortisol is the major corticosteroid in the human body, is released at the endpoint of the hypothalamic-pituitary-adrenal (HPA) axis, helps the adaption of the body to stress situations and conditions through providing the body with energy and suppressing non-emergency biological processes including sleep [12-14]. Melatonin and cortisol are typical neuroendocrine biomarkers in circadian rhythms [15, 16]. Normally, endogenous concentrations of cortisol go up after awakening, and largely diminish during the evening [17] which is normally almost on the contrary for melatonin, highest at night and moderate after awakening [18, 19].

Melatonin and cortisol were studied in different matrices in previous studies: cortisol was analyzed in saliva [15, 16, 20-22], in plasma [15, 23], in serum [24] in human CSF [25], in urine [26] in human eccrine sweat [27] and in hair [28, 29]; melatonin was analyzed in plasma [30, 31], in serum [32] in human CSF [32], in urine [26].

Previously several traditional methods were reported for quantification of salivary melatonin and cortisol [33-36]. These immunoassay methods tend to have a potential risk of cross-reactivity among similar chemical structures, therefore, specificity of these method of analysis is poor comparing to chromatographic methods [37]. Chromatographic methods became a popular technique for the quantifications of melatonin and cortisol using GC [38, 39], HPLC coupled with DAD [40] UHPLC coupled with FD and DAD or MS/MS.

Studies on melatonin should be able to detect low melatonin levels in order to determine the dim light melatonin offset (DLMO) in studies on the circadian phase studies [20, 41]. Additionally, it is predicted that salivary melatonin levels will be around 30% of the overall plasma melatonin concentration. The sensitivity of any assay used to quantify melatonin in saliva is obviously burdened [41].

### 3.3.2. Experimental

#### 3.3.2.1. Chemicals and reagents

Cortisol was purchased from Cayman Chemical (Ann Arbor, MI, USA). Melatonin (N-acetyl-3-(2-aminoethyl)-5-methoxyindole) was supplied by Sigma-Aldrich (Munich, Germany). Cortisol-9,11,12,12-$d_4$ and cortisone-2,2,4,6,6,9,21,21-$d_8$ were acquired from Toronto Research Chemicals (Toronto, Ontario, Canada). Melatonin-$d_4$ was purchased from Cayman Chemical (Ann Arbor, MI, USA) (for structures of analytes see Figure 1). Methanol (MeOH) and Acetonitrile (MeCN) were Ultra LC MS-grade and Ethyl acetate was obtained from Carl Roth (Karlsruhe, Germany). As mobile phase additive, ammonium acetate ($NH_4Ac$) was purchased from Merck (Sigma Aldrich) (Munich, Germany). Distilled water for LC-MS was deionized by Elga Purelab Ultra purification system (Celle, Germany).

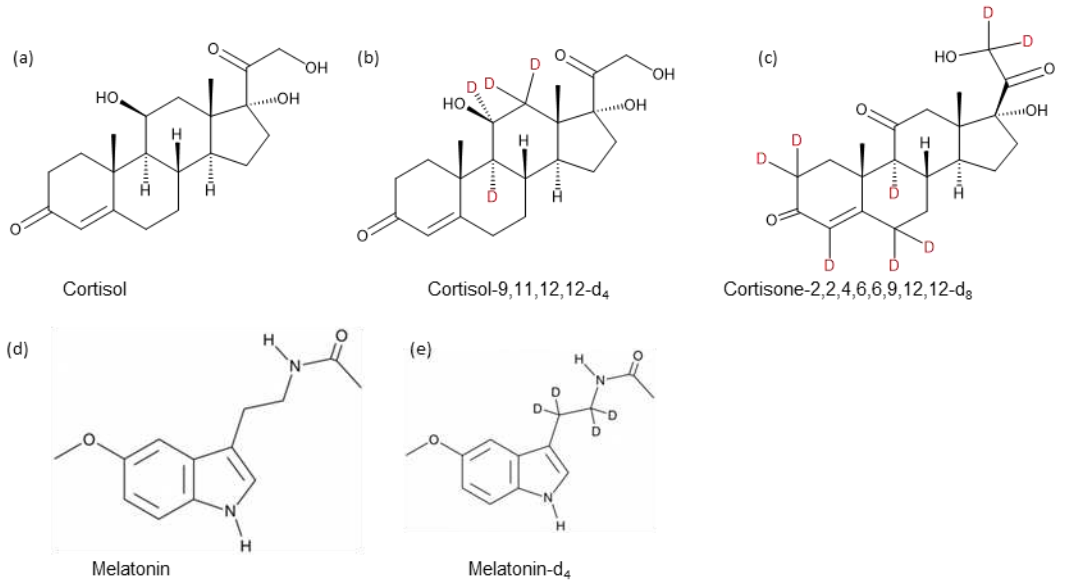


Fig. 1. Chemical structures of analytes: (a) cortisol, (b) cortisol-$d_4$, (c) cortisone-$d_8$, (d) melatonin, (e) melatonin-$d_4$

### 3.3.3. Circadian cycle prototype

At one day, throughout the course of 24 hours, saliva samples were taken from 4 healthy volunteers (2 male, 2 female) for the circadian cycle prototype experiment. The samples were collected as soon as the volunteers woke up, in the morning, midday, afternoon, early evening, night, and just before bed.

The sample was kept frozen at -20°C until the day of sample processing, at which point it was brought to room temperature to defrost. Each sample was then centrifuged, and a 250 µL quantity of saliva was taken from the supernatant. The samples were prepared in accordance with the LLE protocol.

### 3.3.4. Sample pre-treatment

Samples in the Eppendorf tubes were slowly thawed at 4 °C for approximately 2 hours. They were then centrifuged at 13200 rpm for 10 minutes at 4 °C to obtain a clear fluid by removing residual cells and particles in the saliva. 500 µL of each saliva supernatant was transferred into another Eppendorf tube. LLE was carried out by adding 1500 µL

ethyl acetate containing ISs (at 200, 4000 pg/mL concentration of melatonin-$d_4$, cortisone-$d_8$, respectively) and shaken for 1.5 hours at 1500 rpm in ice box. After that, the supernatant was transferred to freezer and stored there allowing the organic and aqueous phases to separate at -20°C for 30 min. The supernatant was then evaporated to dryness under nitrogen protection with an EZ2 evaporator (GeneVac EZ 2 Evaporator, GeneVac Ltd, Ipswich, UK) and each was reconstituted by 50 µL MeOH-$H_2O$ (10:90; v/v) prior to UHPLC-MS/MS analysis.

### 3.3.5. UHPLC–MS/MS instrumentation and conditions

The chromatographic instrumentation consisted of an Agilent 1290 Infinity II UHPLC system equipped with an autosampler system (G7167B, and G7120A, Agilent Technologies). UHPLC system was coupled to an AB Sciex QTRAP 4500 mass spectrometer with a Turbo V electrospray ionization (ESI) source (Sciex, Ontario, Canada). The measurements were performed in positive and negative polarity mode using selected reaction monitoring (SRM). By directly injecting each analyte into the MS to obtain the optimal signal intensity at a concentration of 1,000 ng $mL^{-1}$ in 10% MeOH containing 10 mM $NH_4Ac$, the MS settings for SRM, including compound-dependent and ion source dependent parameters, were initially tuned.

The samples were kept at 4°C in the autosampler and immediately analysed after their preparation. All of the experiments were carried out in negative electrospray ionization (ESI) mode. The main source parameters were set as follows: in positive mode: nebulizer gas, GS1 (zero grade air) 40 psi, drying gas, GS2 (zero grade air) 0 psi and CAD, collision gas ($N_2$) Low; ion spray voltage +4500 V; ion source temperature 550 °C; in negative mode: nebulizer gas, GS1 (zero grade air) 20 psi, drying gas, GS2 (zero grade air) 0 psi and CAD, collision gas (N2) Medium; ion spray voltage -4000 V; ion source temperature 400 °C. Q1 and Q3 resolution was set to unit. The chromatographic separation was carried out using Kinetex (50 x 2.1mm, 2.6 µm) C18 column. The column was kept at 20°C during the analysis. The injection volume was 20 µL. Mobile phase A was $H_2O$ with 10 mM $NH_4Ac$ (v/v) and mobile phase B was ACN with 10 mM $NH_4Ac$ (v/v). The flow rate was 0.5 mL $min^{-1}$ and the gradient elution was carried out with following gradient profile without isocratic delay: From 5% B at 0 min to 70% B in

3.76 min, then the percentage of B was increased from 70% B at 3.76 min to 100% at 3.77 min, held constant at 100% for the next 1.25 min, then dropped quickly (in 0.1 min) to the starting conditions of 5% B to re-equilibrate the column for 1 min. Run time was set to 6 min with a retention time of 2.2 min for cortisol and 1.9 min for melatonin.

SRMs were optimized individually for each analyte and are summarized in 3.3.8.2.

### 3.3.6. Determination of matrix effects, extraction recoveries and process efficiencies

According to Matuszewski's technique [42], matrix effects, extraction recoveries and process efficiencies were determined. Three sets of samples were prepared for this purpose: Pooled saliva matrix spiked with standards and ISs before extraction (pre-extraction spiked); pooled saliva matrix spiked with standards and ISs after extraction (standards and ISs added with the reconstitution solvent to dried extracts) (post-extraction spiked); and solvent ($H_2O$/MeOH (1:10; v/v)) spiked with standards and ISs. In each set, the theoretical concentration was the same. Peak areas in post-extraction spiked samples and the associated standard solution were used to calculate the matrix effect (spiked solvent). The ratio between peak areas in pre-extraction spiked samples and corresponding post-extraction spiked samples was used to calculate extraction recovery. Process efficiency was calculated from the peak areas of pre-spiked saliva matrix and spiked solvent.

### 3.3.7. Data analysis and quantification

Data was processed using MultiQuant 3.0 with automated integration (Sciex). The Gaussian smooth (width 2 data points), noise percentage of 90% and peak splitting factor of 2, baseline subtraction window of 0.10 min was set additionally. Graphs and figures were generated by using Origin 2019 (Originlab, Northampton, MA, USA) and Excel 2007, chemical structures were generated using MarvinSketch 20.19., 2019 (ChemAxon).

### 3.3.8. Results and Discussion

3.3.8.1. Sample preparation

Especially for the current UHPLC approach efficient extraction with high recoveries, proper enrichment, and efficient removal of interfering matrix components are crucial. High sensitivity is supported by the huge injection volume, but it also increases the possibility of matrix interferences and matrix effects. Therefore, certain sample preparation techniques are required. However, there is no standard procedure together for melatonin and saliva extraction reported in the literature. For that reason, different extraction methods were tested during the method development as follows: solid phase extraction (SPE) (used SPE cartridge: Oasis HLB Prime (3 mL / 60 mg, Waters, Milford, MA, USA)), different supported liquid extraction (SLE) procedures (used SLE cartridges: Chem Elut S (400 mg) and Isolute SLE+ (400 µL)), and five protein precipitation (PPT) solvent or solvent mixtures (Acetone, isopropanol (IPA), ACN, MeOH, ACN:MeOH, ACN:MeOH:Acetone and Ethyl acetate (EtAC):MeOH). Different proportions (Solvent volume:saliva volume) were compared for PPT methods can be found in Table 1

Table 1: Comparison of ratios (Solvent volume:saliva volume, v:v) tested for protein precipitation method

| **Solvent** | Aceton | IPA | MeCN | MeOH | MeCN:MeOH * | MeCN:MeOH:Acetone | EtAC:MeOH** |
|---|---|---|---|---|---|---|---|
| **Solvent Ratio** | 2.5, 3, 4 | 2.5, 3, 4 | 2.5, 3, 4 | 2.5, 3, 4 | 1,2,3 | 1,2,3 | 1,2,3 |
| **Saliva Ratio** | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

*with increasing percentage of MeOH from 10% up to 50%

**with increasing percentage of MeOH from 10% up to 70 %

For all extraction/PPT methods sample volume was kept constant to compare the methods. For LLE and for PPT, 3 different extraction volumes (750, 1000, and 1250 µL and 1750, 2000 and 2250 µL, respectively) were compared. For primary experiments, different sample volumes of pooled saliva were used for sample preparation method optimization for each extraction method, which was required. For SLE, to promote the flow, 250 µL aliquots of pooled saliva was diluted (1:1) (v: v) with aq. buffer prior to loading. Then, the mix was taken to the Eppendorf tube. 250 µl of aqueous 4% $H_3PO_4$

including standard mix was added to the mix and then vortexed. Samples were loaded to each SLE cartridge. Vacuum (-0.2 bar) or pressure (3 psi) was applied for 2–5 seconds to initiate loading. It was waited 5 min for sample to completely absorb and form extraction layer. Water immiscible extraction solvent (2x500 µl ethyl acetate) was applied and allowed to flow for 5 mins under gravity. The sample was evaporated under GeneVac® until dryness. The residue was reconstituted in 50 µl 10% MeOH. For SPE, to the pooled saliva (700 µL) in Eppendorf tube, 350 µl MeOH was added and then vortexed. To the mixture 700 µL $ZnSO_4$ (50 mg/mL in 50% MeOH) including standard mix was added, vortexed and centrifuged at maximum speed for 15 min. The supernatant was loaded onto Oasis SPE Prime HLB 3cc cartridge. Each well was washed with 50% MeOH, dried. Each well was then eluted with 2x 300 µl 100% MeOH. Samples were evaporated under GeneVac® until dryness. The residue then was reconstituted in 50 µL 10% MeOH. For PPT, to the pooled saliva (250 µL) in Eppendorf tube 1750 or 2000 or 2250 µl precipitating solution including standard mix was added and vortexed, allowed to rest in a refrigerator at 4 ◦C for 30 min to further precipitate the proteins. Then it was centrifuged at 20,000 RCF again for ten minutes to create a protein pellet. A circa 1875 µl aliquot of supernatant was evaporated under GeneVac® until dryness. The residue was reconstituted in 50 µl 10% MeOH. For LLE, the sample preparation protocol was same as explained in 5.6. Extraction recoveries and matrix effects were determined for all analytes for all methods by spiking internal standard before or after extraction.

PPT using acetone was insufficient, therefore it was excluded from further experiments. PPT using IPA was sufficient, however the detection of melatonin and cortisol was not observed. PPT with ACN (2.5:1, 3:1, 4:1, v:v ) was partly sufficient with ACN: Saliva (4:1), as no detection was occurred for cortisol. PPT with MeOH (2.5:1, 3:1, 4:1, v:v) was partly sufficient with MeOH: saliva (4:1, v:v), as no detection was occurred for cortisol. Extra peaks were seen in SPE and SLE methods even with water extraction, indicating that the substance of the cartridges interferes with the technique being utilized. As a result, these methods were excluded from further testing. Due to the strong matrix effect and ion suppression for PPT, the interpretation was too challenging, as the process efficiency was insufficient. The possible explanation would be that

interferences may emerge because the matrix effect and recovery are excessively high. Therefore, final extraction method was chosen as LLE with ethyl acetate. The volumes of extraction solvent (EtAc) were also compared to gain more sensitivity. The extraction volumes were 2250, 3000 and 3750 µL EtAC. Outcomes are essentially the same, therefore the technique was not changed.

Methanol solutions of different concentrations (1 pg $mL^{-1}$ to 10 ng $mL^{-1}$ for all targeted analytes) were prepared to check for detector linearity range and instrument detection limits. Linearity range was found to be from 7.5 pg $mL^{-1}$ to 1750 pg $mL^{-1}$ for Melatonin-$d_4$, 100 pg $mL^{-1}$ to 10000 pg $mL^{-1}$ for Cortisone-$d_8$, 250 pg $mL^{-1}$ to 10000 pg $mL^{-1}$ for Cortisol-$d_4$, 1 pg $mL^{-1}$ to 500 pg $mL^{-1}$ for Melatonin and 75 pg $mL^{-1}$ to 10000 pg $mL^{-1}$ for Cortisol. Instrument LOD were found to be 3 pg $mL^{-1}$ for Melatonin-$d_4$, 30 pg $mL^{-1}$ for Cortisone-$d_8$, 85 pg $mL^{-1}$ for Cortisol-$d_4$, 0.5 pg $mL^{-1}$ for Melatonin and 20 pg $mL^{-1}$ for Cortisol.

#### 3.3.8.2. Final method development

Baseline melatonin concentrations in saliva may be relatively low (in pg/mL range), which provides an issue for assay sensitivity. Therefore, a proper method development was necessary.

Testing several mobile phase compositions could help to find the sensitive and selective chromatographic technique. To determine which mobile phase will provide the optimum selectivity and sensitivity (as measured by the S/N ratio for chosen analyte), many mobile phases were screened (i.e., resolution between melatonin and cortisol). Mobile phase A was always water with additive and mobile phase B was 100% of either ACN or MeOH (v/v); an additive was always added also to the mobile phase B to increase elution strength. These additives tested were: Formic acid (FA), ammonium acetate ($NH_4Ac$), ammonium formate ($NH_4FA$). ACN and MeOH were evaluated in the initial experiment as mobile phase B. 5 mM $NH_4FA$, 10 mM $NH_4FA$, 5 mM $NH_4Ac$, 10 mM $NH_4Ac$, 0.1% FA (v/v), 2 mM $NH_4Ac$ with 0.1% FA (v/v) were the six different types of additives applied. For each type of mobile phase B, a different preparation of mobile phase A was made. MeOH generally had good sensitivity (notably when buffer salts were added), although the peaks were considerably broader than with ACN. As a result,

MeOH had poor resolution of melatonin and cortisol. ACN provided narrower peaks, and the addition of NH4Ac improved the sensitivity of detection.

The following reversed-phase columns were screened to determine which reversed-phase column would be best for analytical separation: Eclipse bonus RP C18 50x2.1 mm, 1.8 µm (Agilent, Waldbronn, Germany); Eclipse plus C18 50x3 mm, 1.8 µm (Agilent, Waldbronn, Germany); Kinetex C18 20x2.1 mm, 2.6 µm (Phenomenex, Torrance, CA, USA); Kinetex C18 50x2.1 mm, 2.6 µm (Phenomenex, Torrance, CA, USA); Poroshell 120 C18 50x3mm, 1.8 µm (Agilent, Waldbronn, Germany); Cortecs C18+ 50x2.1 mm, 2.7 µm (Waters, Milford, MA, USA), and Acquity CSH C18 100x2.1mm, 1.7 µm (Waters, Milford, MA, USA). Eclipse Bonus RP column, which contains an embedded amide linkage in the C14-alkyl chain, did provide a noisy baseline. Eclipse plus column provided less noisy baseline than Eclipse Bonus RP column, along with other columns that their column LOQ was not low enough. Poroshell 120 C18 column packed with superficially porous particles (SPP) gave broader peaks under the same gradient conditions. Solid-core Cortecs C18+ column and Acquity CSH C18 gave peak broadening and less sensitivity (assessed by the S/N ratio for targeted compounds). Shorter Kinetex C18 column (20x2.1 mm, 2.6 µm) resulted bad chromatographic resolution compared to longer Kinetex C18 column (50x2.1 mm, 2.6 µm), despite good gradient optimization and method transfer. In contrast, Kinetex C18 column (50x2.1 mm, 2.6 µm) showed good peak shape, highest column LOQ. Due to its performance, it was decided to use a Kinetex C18 (Phenomenex, Torrance, USA) column (50 x 2.1 mm, 2.6 µm) for further measurements.

To assess sensitivity (S/N), five different flow rates between 0.3 and 1 mL/min were examined. Low flow rates (0.3 to 0.5 mL/min) produced the best sensitivity for targeted compounds. The final method's flow rate was determined upon at 0.5 mL/min as a compromise between runtime and efficiency.

Ion source parameters were tuned by automatic optimization using flow injection to maximum sensitivity after screening chromatographic column and choosing mobile phase for quantitative analysis of the targeted compounds. The collision energy (CE) was systematically optimized, among other compound specific parameters (such as

declustering potential, and cell exit potential) and the instrument specific parameters (including GS1, GS2, ion source temperature, Cur, and CAD, and ion source voltage). The collision energies were 31 and 39 V for Melatonin (233.1→174.1 and 233.1→159.1, respectively), 19 and 35 V for Melatonin-$d_4$ (237.1→178.2 and 237.1→162.2, respectively), -32 and -40 V for cortisol (361.1→297.1 and 361.1→282.0, respectively), -12 and -42 V for cortisol-$d_4$ (365.1→334.9 and 365.1→285.6, respectively) and -20 and -40 V for cortisone-$d_8$ (367.1→306.9 and 367.1→137.9 respectively). Declustering potential values were 17 V for Melatonin, 61 V for Melatonin-$d_4$, -75 V for cortisol, -85 V for cortisol-$d_4$ and -85 V for cortisone-$d_8$. For the measurement in positive mode, the ion source parameters were as follows: CAD low, CUR, 20, GS1 40, GS2 0, IS voltage 4500 volts and ion source temperature 550 °C. For the measurement in negative mode, the ion source parameters were as follows CAD medium, CUR 35, GS1 20, GS2 0, IS voltage 4000 volts and ion source temperature 400 °C. The dwell time was set to 55 ms for all compounds. Cell exit potential values 19 V for Melatonin, 8 V for Melatonin-$d_4$, -11 V for Cortisol and Cortisol-$d_4$ and -9 V for Cortisone-$d_8$.

The column temperature was adjusted in the following step. Five temperatures ranging from 20 to 50 °C were evaluated. Higher S/N was once again considered as the deciding factor. For most of the compounds (including internal standards), 20 °C produced the best results. The temperature of 20°C was used in the final method.

Isotopically labelled analog of targeted compounds such as Cortisone-$d_8$ (Internal Standard of Cortisol), Melatonin-$d_4$ (Internal Standard of Melatonin) were used as internal standards for signal correction. Cortisol-$d_4$ was intended to use as a surrogate calibrant at the beginning of the method. At that time minimum amount of 10 mg was available for Melatonin-$d_7$ at the market, and which the study could not afford. Therefore, Melatonin's active metabolites such as 5-methoxytryptamine and N-acetyl hydroxytryptamine were thought to be used by either acetylation (of 5-methoxytryptamine with Acetic-1-$^{13}$C anhydride, or Acetic-2-$^{13}$C anhydride or of 5-methoxytryptamine with Acetyl-1-$^{13}$C chloride, or Acetyl-2-$^{13}$C chloride) or methylation ((of N-acetyl hydroxytryptamine using Di(methyl-$^{13}$C) sulfate)), if the LC method would have been decided for the validation.

The pre-validation experiments established several criteria to demonstrate that the method can yield accurate results when analyzing real samples. The circadian cycle prototype experiment was done to stimulate the circadian melatonin and cortisol rhythms. From that outcome it was observed that the method was insufficient for the detection of melatonin of volunteers after awaking despite all series of method optimization experiments (see Figure 2 for the exemplary chromatograms of the non-spiked sample collected from one of volunteers). Disappointingly, it fails at this stage after series of optimization, therefore the method was not validated, and it could not be implemented to measure clinical samples.

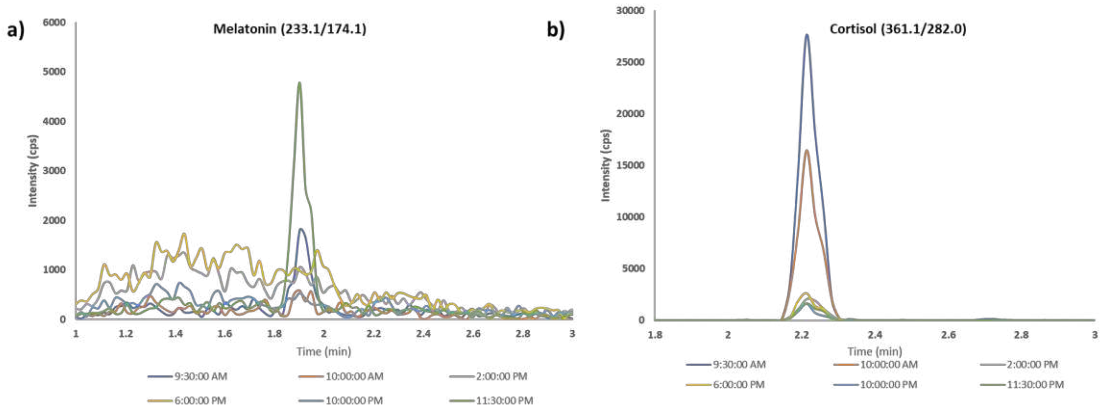


Fig. 2. Exemplary chromatograms from non-spiked volunteer sample for melatonin (a) and cortisol (b). Samples were taken at different time during circadian rhythm. Samples were treated with final sample preparation method.

3.3.8.2.1. Matrix effect, extraction efficiency and process efficiency

Extraction recovery (measured by comparing pre- and post-extraction spike), matrix effect (post-extraction spike vs standard solution), and process efficiency (pre-extraction spike vs standard solution) were assessed in quadruplicate at three QC concentration levels for each matrix ($QC_{Low}$, $QC_{Mid}$, and $QC_{High}$) ($QC_{Low}$ was prepared at concentration level of 3 times of LLOQ to cover the concentration level from LLOQ to 3 times of LLOQ; $QC_{Mid}$, was prepared at concentration level of 5 times of LLOQ to cover the concentration level from 3 times of LLOQ to 5 times of LLOQ and $QC_{High}$ was prepared at concentration level of 10 times of LLOQ to cover the concentration level from 5 times

of LLOQ to ULOQ). Melatonin extraction recoveries were often greater than 90% (90.3% ± 10.3%) and cortisol extraction recoveries were often greater than 115% (115.6% ± 5.6%). The recoveries above 100% may be the result of pipetting and evaporation-related experimental errors. Matrix effect was around 69-91.6% (mean 75.6 ± 8.2). This proposes that the current LLE method is adequate for current quantification method for salivary melatonin and cortisol. To compensate for high matrix effect, isotopically labelled IS was included. Average process efficiency was equal to 80.6 (± 15.2) over all compounds.

### 3.3.9. Conclusion

In this project, we aimed to develop a short, simple, cheap, and fast LC-MS method for the determination of melatonin and cortisol in salivary samples to monitor the circadian cycle. For this purpose, several parameters were tested. From these results, it was deduced that, despite several method optimization assays, this approach was inadequate for the detection of volunteers' melatonin level after awakening. Unfortunately, after a number of optimization attempts, it fails at this point; as a result, the method could not be validated and could not be used to assess clinical samples. We suggest for future to utilize either an expensive but better and simple sample preparation technique (i.e., SPE), and/or derivatization (if possible), and low-flow LC system. If then this fast research method could quantify low levels of endogenous melatonin from saliva extract with sensitive system and sample preparation method.

## Figure captions

**Figure 1.** Chemical structures of analytes: (a) cortisol, (b) cortisol-$d_4$, (c) cortisone-$d_8$, (d) melatonin, (e) melatonin-$d_4$

**Figure 2.** Exemplary chromatograms from non-spiked volunteer sample for melatonin (a) and cortisol (b). Samples were taken at different time during circadian rhythm. Samples were treated with final sample preparation method.

## Table captions

**Table 1:** Comparison of ratios (Solvent volume:saliva volume, v:v) tested for protein precipitation method

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