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DETERMINATION OF PROTEINOGENIC AMINO ACIDS BY HIGH-PERFORMANCE SEPARATION TECHNIQUES

STANOVENÍ PROTEINOGENNÍCH AMINOKYSELIN VYSOKOÚČINNÝMI SEPARAČNÍMI TECHNIKAMI

Doctoral thesis

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ABSTRACT (EN)

Proteinogenic amino acids are key components of living organisms. Thus, the latest metabolomics research has focused on developing fast and sensitive methods for the determination of amino acids. In this context, this thesis contains two studies describing development of high-performance separation techniques for the quantification of amino acids.

In the first study, a capillary electrophoresis method was developed for the determination of free amino acids in tobacco plants, particularly focusing on optimizing the extraction of amino acids from solid plant materials. The extraction procedure was optimized using design of experiments (DoE) to obtain the highest possible extraction yield of amino acids. Factors such as volume and concentration of the extraction solvent (hydrochloric acid) were assessed as the most significant. Subsequently, the optimal values of these factors were determined using response surface methodology (RSM). Lastly, proteinogenic amino acids were quantified using capillary electrophoresis with contactless conductivity detection and calibration with internal standard, which improved the precision of the method.

The second study aimed at developing a supercritical fluid chromatography method for the determination of free proteinogenic amino acids in human plasma. The most important part of this study was to improve the solubility of proteinogenic amino acids in a CO_2-rich mobile phase. Firstly, the polarity of mobile phase was increased by adding water and ammonium formate to the CO_2/methanol mixture. Secondly, the polarity of amino acids was decreased by derivatization with 1-chlorobutane. The derivatization step greatly enhanced the solubility of amino acids in the mobile phase, thus substantially improving the shapes of the amino acid peaks. Proteinogenic amino acids were quantified using tandem mass spectrometry detection and a calibration curve with deuterated internal standards, which provided an acceptable method precision.

In this thesis, both methods were compared, focusing on separation efficiency, limits of quantification, and acquisition and operating costs. This comparison showed that the separation efficiency was better in capillary electrophoresis method than in supercritical fluid chromatography. On one hand, the latter was faster than the former and enabled the quantification of concentrations up to five orders of magnitude lower.

On the other hand, operation of a supercritical fluid chromatography with tandem mass spectrometry not only requires more qualified operators but also more stable laboratory conditions and higher acquisition and operating costs.

ABSTRAKT (CZ)

Proteinogenní aminokyseliny patří mezi základní stavební jednotky živých organismů. Proto byl v poslední době výzkum v metabolomice zaměřen na vývoj rychlých a citlivých analytických metod pro stanovení aminokyselin. Tato práce obsahuje dvě studie popisující vývoj vysoce účinných separačních metod pro kvantifikaci aminokyselin.

V první studii byla vyvinuta metoda kapilární elektroforézy pro stanovení aminokyselin v rostlinách tabáku. Nedílnou součástí této studie byla optimalizace extrakce aminokyselin z rostlinného materiálu. Extrakce byla optimalizována postupem "design of experiments" (DoE), pomocí kterého byly vyhodnoceny faktory ovlivňující výtěžek extrakce aminokyselin. Nejdůležitějšími faktory byly objem a koncentrace extrakčního činidla (kyselina chlorovodíková), jejichž optimální hodnoty byly určeny pomocí "response surface methodology" (RSM). Poté byly aminokyseliny separovány pomocí kapilární elektroforézy s bezkontaktní vodivostní detekcí a stanoveny za použití kalibrace s vnitřním standardem, což přispělo ke zlepšení přesnosti metody.

Druhá studie byla zaměřena na vývoj metody pro stanovení aminokyselin v lidské plasmě pomocí superkritické fluidní chromatografie. Jedním z cílů této studie bylo zvýšit rozpustnost aminokyselin v mobilní fázi bohaté na CO_2. Nejdříve byla zvýšena polarita mobilní faze (CO_2/methanol) přídavkem vody a mravenčanu amonného. Polarita aminokyselin byla zároveň snížena derivatizací 1-chlorbutanem. Derivatizace zvýšila rozpustnost aminokyselin v mobilní fázi, proto se zlepšily tvary píků aminokyselin. Aminokyseliny byly separovány pomocí superkritické fluidní chromatografie s tandemovou hmotnostní detekcí. Stanovení aminokyselin v lidské plasmě bylo provedeno pomocí kalibrace s deuterovanými standardy aminokyselin.

V této práci byly obě metody porovnány z hlediska jejich separační účinnosti, mezí stanovitelnosti a provozních nákladů. Použití kapilární elektroforézy pro stanovení aminokyselin zajistilo lepší separační účinnost. Avšak použití superkritické fluidní chromatografie umožnilo stanovení až o pět řádů nižších koncentrací aminokyselin, navíc byla tato metoda podstatně rychlejší. Využití superkritické fluidní chromatografie s tandemovou hmotnostní detekcí ovšem klade vyšší nároky nejen na zkušenosti

operátora, ale i na stabilní laboratorní prostředí. Superkritická fluidní chromatografie také vyžaduje vyšší pořizovací a provozní náklady než kapilární elektroforéza.

Keywords: amino acids, capillary electrophoresis, conductivity detection, mass spectrometry, supercritical fluid chromatography

Klíčová slova: aminokyseliny, hmotnostní spektrometrie, kapilární elektroforéza, superkritická fluidní chromatografie, vodivostní detekce

CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS

Ala	alanine
AOT	bis(2-ethylhexyl)sulfosuccinate
APCI	atmospheric-pressure chemical ionization
API	atmospheric-pressure ionization
APPI	atmospheric-pressure photoionization
Arg	arginine
Asn	asparagine
Asp	aspartic acid
BBB	brain-blood barrier
BEH	ethylene bridged hybrid
BGE	background electrolyte
C^4D	capacitively coupled contactless conductivity detection
CCD	contactless conductivity detection
CE	capillary electrophoresis
CEM	chain ejection model
CID	collision-induced dissociation
CITP	capillary isotachophoresis
ClogP	decadic logarithm of calculated partition coefficient
CNS	central nervous system
CRM	charged residue model
cSFC	capillary supercritical fluid chromatography
Cys	cysteine
dansyl	dimethylaminonaphthalenesulfonyl chloride
DNFB	2,4-dinitrofluorobenzene
DoE	Design of Experiments
EAAs	essential amino acids
EI	electron ionization
EOF	electroosmotic flow
ESI	electrospray ionization
FID	flame ionization detector
FMOC	9-fluorenylmethyl chloroformate
FS	full scan
GABA	γ-aminobutyric acid
GC	gas chromatography
Gln	glutamine
Glu	glutamic acid

Gly	glycine
HEC	hydroxyethyl cellulose
His	histidine
IEC	ion-exchange chromatography
IEM	ion evaporation model
Ile	isoleucine
LC	liquid chromatography
Leu	leucine
LIF	laser-induced fluorescence
LOD	limit of detection
LOQ	limit of quantification
Lys	lysine
Met	methionine
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NPLC	normal-phase liquid chromatography
OPA	O-phthalaldehyde
PEG	polyethylene glycol
Phe	phenylalanine
PITC	phenyl isothiocyanate
Pro	proline
PTH	phenylthiohydantion
PVA	polyvinyl alcohol
QQQ	triple quadrupole mass analyzer
RSD	relative standard deviation
RPLC	reversed-phase liquid chromatography
Ser	serine
SFC	supercritical fluid chromatography
SIM	selected ion monitoring
SPE	solid-phase extraction
Thr	threonine
Trp	tryptophan
TSP	thermospray ionization
Tyr	tyrosine
UPLC	ultra-performance liquid chromatography
UPSFC	ultra-performance supercritical fluid chromatography
UV-VIS	ultraviolet-visible electromagnetic radiation
Val	valine

e	elementary charge
γ	surface tension
ε	dielectric constant
ε_0	permitivity of vacuum
H	height equivalent to a theoretical plate
L	column length
m/z	mass-to-charge ratio
N	number of theoretical plates
η	dynamic viscosity
η_0	viscosity at capillary surface
μ_{EOF}	mobility of electroosmotic flow
ψ	electrokinetic potential
R	droplet radius
R^2	coefficient of determination
t_{mig}	migration time
t_R	retention time
w	peak width
ζ	zeta potential
z_R	charge number

1 INTRODUCTION

Proteinogenic amino acids are essential components of living organisms biosynthetically incorporated into peptides and proteins. In addition, these amino acids participate in the synthesis of key endogenous compounds, including hormones and nucleotides. Accordingly, changes in the concentrations of free amino acids in human plasma may indicate the presence of inborn aminoacidopathy. Similarly, liver disease can be predicted using the Fischer ratio, i.e., the ratio of branched chain amino acids to aromatic amino acids [1,2]. In plants, amino acids are involved in total nitrogen supply and in the biosynthesis of secondary plant metabolites, such as phenolic compounds and glucosinolates [3]. Therefore, analytical methods for the determination of amino acids have been extensively used in plant and human metabolomics.

Capillary electrophoresis (CE) is a suitable separation technique not only for proteins and peptides but also for small molecules thanks to its high separation efficiency in the range of hundreds of thousands of theoretical plates per meter. Thus, CE can be applied in metabolomics even when using conventional detectors with low selectivity, e.g., spectrophotometric (UV-VIS) or capacitively coupled contactless conductivity (C^4D) detectors. These detectors have low concentration sensitivity due to the low sample volume that can be introduced into a capillary and/or to their short absorption path (UV-VIS detectors, for example) [4-6]. Despite their low sensitivity, C^4D and UV/VIS detectors suffice for separations of abundant analytes such as amino acids. However, only few proteinogenic amino acids (tryptophan, phenylalanine, tyrosine) absorb UV/VIS radiation above 190 nm. Thus, C^4D is the detection technique of choice for amino acids, especially those that do not absorb in UV-VIS range above 190 nm [7,8].

Even before the widespread use of capillary electrophoresis in amino acid separation, chromatography-based methods had long been applied for the separation of proteinogenic amino acids. Actually, one of the first chromatography methods used for the separation of amino acids was gas chromatography (GC). However, GC separations require the derivatization of amino acids to increase their volatility. Considering this drawback, soon after its introduction, liquid chromatography (LC) quickly became a popular method for the separation of amino acids because LC is faster than GC and

requires no derivatization before separation on column. Subsequently, a new separation technique emerged combining GC with LC – supercritical fluid chromatography (SFC). Although the SFC technique initially used GC-like instrumentation and separation principles, later improvements in the former, particularly in the last two decades, caused a gradual shift from GC- to LC-like instrumental setups. SFC replaced normal-phase mode of liquid chromatography (NPLC) because of similar polarity of mobile and stationary phase of NPLC to the polarity of mobile and stationary phase used in SFC. Moreover, SFC is more environmentally friendly than NPLC because it uses CO_2 and an organic co-solvent or even water as a mobile phase and its polarity can be changed using co-solvents, thereby enabling the separation of a wide spectrum of compounds ranging from vitamin D and its metabolites [9] to nucleotides and lipids [10].

The analysis of complex biological samples requires a high degree of detection selectivity. Unsurprisingly, mass spectrometry (MS) in connection with separation techniques has recently become one of the most commonly used detection methods for such purpose. Currently, MS is the prevailing detection technique in GC separation. After separation by GC, analyte molecules are mostly ionized by electron ionization (EI), which promotes in-source ion fragmentation; thus, successive fragmentation in a collision cell is seldom required in GC-MS. Conversely, the combination of "weak" ionization techniques, e.g., electrospray ionization (ESI) with a liquid-phase separation technique, such as CE and LC, demands further fragmentation of molecular ions in a collision cell and scanning for product ions to improve detection selectivity. This combination method is referred to as tandem mass spectrometry (MS/MS).

Based on the above, this thesis was divided into two parts. The first study described the determination of proteinogenic amino acids by CE-C^4D focusing on amino acid extraction. The extraction process was optimized using Design of Experiments (DoE) to achieve the highest amino acid yield possible. The second study aimed to determine amino acids in human plasma by SFC-MS/MS, which provided shorter run times than the CE-C^4D method. However, the total analysis time of SFC-MS/MS was prolonged due to the necessary derivatization of amino acids. Overall, this thesis describes the determination of proteinogenic amino acids by both aforementioned techniques, using parameters such as total analysis time, separation efficiency and detection selectivity to compare the suitability of both techniques for various

applications. This thesis also compares acquisition and operating costs of both techniques.

2 AIMS OF THE THESIS

The overall aim of the thesis was to compare two separation techniques – capillary electrophoresis and supercritical fluid chromatography – in the determination of proteinogenic amino acids. The studies included in this thesis focused on the following goals:

Study I (i) To optimize the extraction process of proteinogenic amino acids from tobacco plants using design of experiments, including fractional factorial design and the response surface method, and (ii) to validate a method for the determination of amino acids in plant extracts using capillary electrophoresis with contactless conductivity detection.

Study II (iii) To develop an ultra-performance supercritical fluid chromatography-tandem mass spectrometry method for the determination of proteinogenic amino acids in human plasma; (iv) to find a suitable derivatization process to decrease amino acid polarity and (v) to optimize mobile phase composition and gradient to reach the shortest analysis time possible and to improve peak shape.

3 PROTEINOGENIC AMINO ACIDS

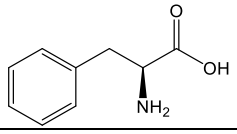
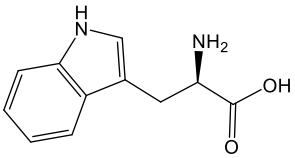
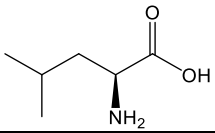
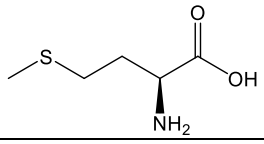
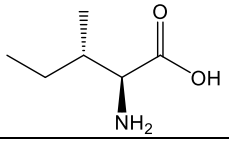
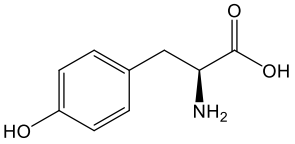
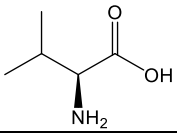
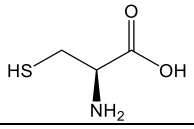
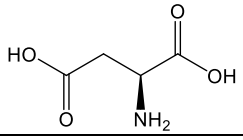
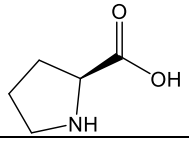
3.1 Amino acid structure and properties

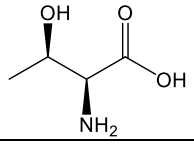
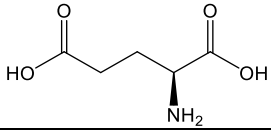
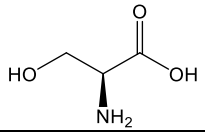
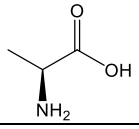
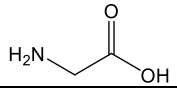
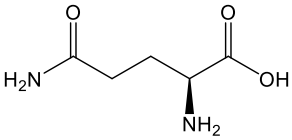
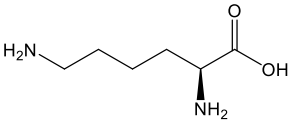
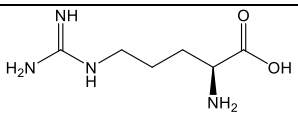
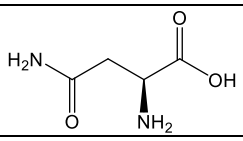
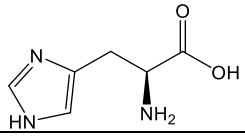
Table 1 lists the proteinogenic amino acids, including their structures, and one-letter codes as well as three-letter codes used throughout this thesis. All amino acids contain a basic amino group and an acidic carboxyl functional group (**Table 1**), which accounts for their zwitterionic character, and all proteinogenic amino acids are *L* enantiomers with an amino group bonded to an α-carbon. However, some proteinogenic amino acids, e.g., Lys and Arg, contain multiple amino groups, thus increasing their basicity, whereas others, such as Asp and Glu, contain more than one carboxyl group. Therefore, when separating amino acids by capillary electrophoresis, one must consider the overall charge of an amino acid at a specific pH.

The overall charge is characterized by the isoelectric point (p*I*), which is the mean pK_a of all acid-base functional groups. Thus, separation selectivity can be optimized by slightly changing the pH of the background electrolyte [11]. In supercritical fluid chromatography, one must consider not only the ionizable functional groups but also the amino acid side chains (**Table 1**), which control amino acid polarity.

Amino acid polarity can be calculated, using the ChemDraw software (PerkinElmer, USA), as the decadic logarithm of the calculated partition coefficient (ClogP) by comparing the solubility of an amino acid in 1-octanol with its solubility in water. Then, the feasibility of an SFC analysis can be predicted, to some extent, based on the ClogP values, which range from –3.73 for *L*-His, the most polar amino acid, to –1.56 for *L*-Phe, the least polar amino acid (**Table 1**) [10].

Table 1 List of proteinogenic *L*-amino acids and respective abbreviations, structures, ClogP and p*I* values, sorting the amino acids in increasing order of polarity

***L*-amino acid**	**Three-letter code**	**One-letter code**	**Structure**	**ClogP**	**p*I*** [12]
phenylalanine	Phe	F		–1.56	5.76
tryptophan	Trp	W		–1.57	5.88
leucine	Leu	L		–1.67	6.04
methionine	Met	M		–1.73	5.71
isoleucine	Ile	I		–1.76	6.04
tyrosine	Tyr	Y		–2.23	5.63
valine	Val	V		–2.29	6.02
cysteine	Cys	C		–2.35	5.15
aspartic acid	Asp	D		–2.41	2.98
proline	Pro	P		–2.41	6.30

threonine	Thr	T		–2.50	5.60
glutamic acid	Glu	Z		–2.69	3.08
serine	Ser	S		–2.81	5.70
alanine	Ala	A		–3.12	6.11
glycine	Gly	G		–3.21	6.06
glutamine	Gln	Q		–3.38	5.65
lysine	Lys	K		–3.42	9.47
arginine	Arg	R		–3.52	10.76
asparagine	Asn	N		–3.54	5.43
histidine	His	H		–3.73	7.64

3.2 Amino acids in plants and humans

In addition to their structural functions as basic building blocks of peptides and proteins, some proteinogenic amino acids play key roles in stabilizing the structure of large protein units through hydrogen bonds between individual amino acid residues and through disulfide bridges between two Cys molecules [13]. Moreover, abiotically formed amino acids have been implicated in the RNA-based life hypothesis of evolution, according to which early RNA structures might have benefited from the presence of free amino acids, especially positively charged Lys and Arg. These amino acids help to stabilize the secondary structure of nucleic acids because they alter hairpin folding dynamics through electrostatic interactions [14].

In plants, amino acids are synthesized from inorganic compounds. The nitrogen used for biosynthesis derives from various sources, such as nitrate and ammonium salts, which are absorbed through the plant root system. Following this nitrogen uptake, nitrates are reduced to ammonium, which, together with ammonium absorbed from the soil, serves as a building block for the synthesis of organic compounds containing nitrogen. Our first study included experiments with *Nicotiana tabacum* L. cv. Petit Havana SR1, which belongs to a group of C3 plants. In C3 plants, the main metabolic pathway of nitrogen assimilation is ammonium incorporation into Gln, resulting in the presence of amide group. Then, Gln is transformed to Glu via an enzyme-catalyzed (Gln synthetase and Glu synthase) reaction with 2-oxoglutarate. Eventually, other proteinogenic amino acids are formed by transamination, deamination or transmethylation reactions [15]. Once the amino acids are synthesized, they participate in the plant metabolism as chelating agents or phytohormone precursors. Amino acids can also chelate micronutrient metals such as Fe, Zn, Mn and Cu, and they help plants to assimilate metal ions from the soil and to transport them throughout the organism [16,17]. In addition, Trp and Met serve as phytohormone precursors for indole-3-acetic acid and ethylene, respectively [18,19], and other amino acids, such as Met and Phe, are precursors of glucosinolates involved in the plant defense system. When the plant is exposed to biotic stress, glucosinolates are enzymatically degraded into isothiocyanates, which act as natural deterrents against grazing by ruminants [20].

In contrast to plants, the human organism is unable to synthesize some proteinogenic amino acids, termed essential amino acids (EAAs), namely Leu, Ile, Lys, Met, Phe, Thr, Trp and Val. Therefore, the human diet must contain EAAs from meat and/or plant sources [21]. Although all proteinogenic amino acids are equally important when it comes to protein synthesis, some of them also participate in other major biochemical pathways, including hormone production from aromatic amino acids. For instance, Trp acts as a precursor of serotonin, which is subsequently transformed into melatonin in an enzymatic reaction (**Fig. 1**).

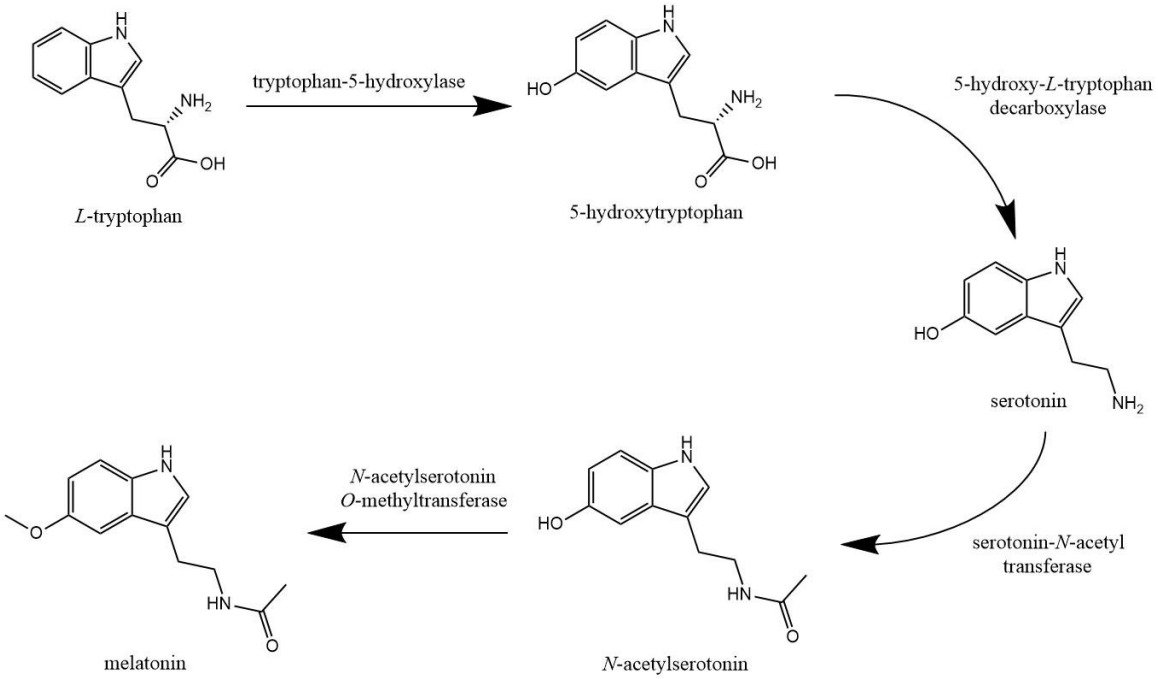


Fig. 1 Enzymatic transformation of tryptophan into serotonin and melatonin

Moreover, serotonin is synthesized in serotonergic neurons located in the central nervous system (CNS), inside the blood-brain barrier (BBB). In turn, melatonin is produced in the pineal gland located outside the BBB; thus, the blood tryptophan concentration easily decreases during short photoperiods. Accordingly, under those conditions, the amount of tryptophan transported through the BBB to serotonergic neurons declines, which may cause seasonal affective disorders, for example, winter depression [22]. Similarly, Phe serves as precursor of Tyr, which is subsequently converted into thyroid hormones – triiodothyronine and thyroxine – which in turn stimulate mitochondrial activity and are thus regulators of human energy [23]. Another amino acid associated with the nervous system is Gln, which provides the basic

structure for the biosynthesis of Glu – an excitatory neurotransmitter. In addition, Glu decarboxylation results in the non-proteinogenic amino acid γ-aminobutyric acid (GABA), which acts as an inhibitory neurotransmitter in human cerebral cortex [24,25].

Considering the above, free amino acids have been increasingly screened in plant and human tissues to assess the overall status of organisms and their development. For example, some human diseases can be predicted based on the concentrations of specific amino acids in the blood stream, which are therefore used as biomarkers. Using the Fischer ratio, which is correlated with some liver diseases such as hepatic fibrosis or hepatocellular carcinoma, the quantification of proteinogenic amino acids can be a powerful tool in clinical practice [26]. In *Study I* of this thesis, we used the CE-C^4D method to determine the concentration of proteinogenic amino acids in leaves of *Nicotiana tabacum* plants grown under various conditions. The findings helped us assess the effects of abiotic stress and of different nitrogen sources on the distribution of free amino acids. In *Study II*, we developed an ultra-performance supercritical fluid chromatography-tandem mass spectrometry (UPSFC-MS/MS) method for the separation of 19 proteinogenic amino acids in a 6-minute run. The sensitivity of this method enables us to quantify amino acids at low concentrations, thereby allowing the use of very low volumes of human plasma or the analysis of samples with low amino acid concentrations. The UPSFC-MS/MS method could become widely used in clinical practice considering its speed and high sample throughput.

3.3 Analysis of amino acids-containing samples

Historically, amino acids were first detected in 1910, when Siegfried Ruhemann developed a selective reaction for amino acids. This reaction was based on the combination of primary amines with ninhydrin, forming a blue dye termed Ruhemann's purple [27]. Since then, the ninhydrin reaction has been extensively used in the identification and quantification of amino acids using the ninhydrin reaction as a post-column derivatization step and thus improving the sensitivity of photometric detection [28,29]. Subsequently, other techniques emerged and were applied to enhance the detection sensitivity, particularly the separation of amino acids as their copper complexes by ion-exchange chromatography (IEC), which not only enabled their

photometric detection but also changed the selectivity of the separation process [30]. Gradually, IEC was replaced by reversed-phase high-performance liquid chromatography (RP-HPLC) in the separation of amino acids because RP-HPLC has shorter analysis times and higher sensitivity. Various derivatization reagents for pre-column derivatization of amino acids in HPLC were studied, e. g., *O*-phthalaldehyde (OPA), dimethylaminonaphthalenesulfonyl chloride (dansyl) or phenyl isothiocyanate (PITC) [31]. From commonly used derivatization reagents for amino acids, the PITC reaction (**Fig. 2**) provides more stable phenylthiohydantoin (PTH) derivatives at faster reaction rates than other derivatization techniques [32].

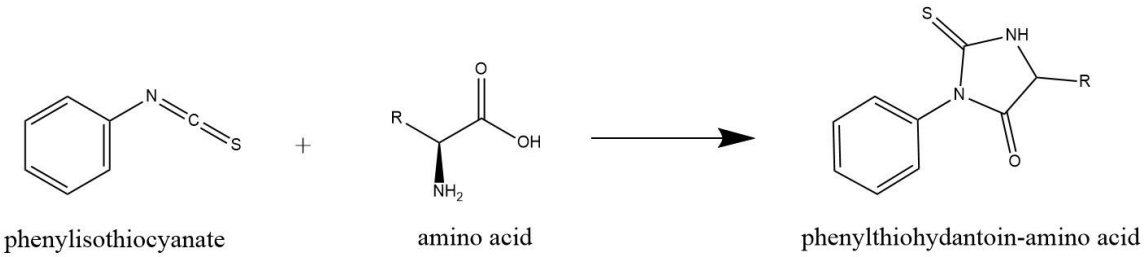


Fig. 2 Derivatization of an amino acid with phenyl isothiocyanate at basic pH yielding the phenylthiohydantoin of the corresponding amino acid (*R* – amino acid side chain)

Before the rise of mass spectrometric detectors in separation science, GC methods had also been used for the separation of amino acids presumably because the separation efficiency of GC is higher than that of HPLC, even efficiently separating *D* and *L* enantiomers when using a chiral stationary phase [33]. However, similarly to HPLC-UV, the separation of amino acids by GC requires pre-column derivatization to increase the volatility of amino acids. Hence, various derivatization reactions have been established for GC analysis predominantly using esterification mechanisms. More specifically, amino acids have been analyzed as *N*-heptafluorobutyryl isobutyl esters using either flame ionization (FID) [34,35] or nitrogen-selective detectors [36].

Meanwhile, capillary electrophoresis was introduced as a new separation technique based on different separation principles. Unsurprisingly, CE rapidly became a widely used method for the separation of ionogenic analytes, i.e., amino acids, thanks to its simple setup, undemanding instrumentation and low operating costs. However, amino acid detection in CE is not an easy task. Similarly to other separation techniques,

direct UV-VIS detection is only sensitive to amino acids containing a suitable chromophore in their structure.

Some amino acids can be identified even by direct UV-VIS detection but only after complexation and preconcentration with Cu^{2+} ions [37] or derivatization with chromophore-containing reagent, e.g., 9-fluorenylmethyl chloroformate (FMOC) binding to the amino functional group [38]. This laborious derivatization step can be avoided by indirect UV-VIS detection of amino acids using an indirect probe added to a background electrolyte (BGE). An indirect probe is an amino acid co-ion that significantly absorbs UV-VIS radiation above 190 nm. Generally, the selection of a specific indirect probe depends on the pH of the background electrolyte. BGEs with basic pH values are frequently used for amino acid separation; accordingly, amino acids carry the negative charge of the dissociated carboxyl group. Under these conditions, aromatic carboxylic acids such as salicylic acid, benzoic acid or trimellitic acid act as indirect probes for amino acids at pH above 10 [39,40].

More recently, laser-induced fluorescence (LIF) detection has been applied to CE in the separation of amino acids, thus substantially increasing the detection sensitivity. Similarly to UV-VIS detection, LIF detection has been used in both direct and indirect modes of operation. Direct LIF detection requires derivatization of amino acids with a fluorophore-containing reagent, such as fluorescein isothiocyanate [41] or 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde [42]. Indirect LIF detection requires no derivatization of amino acids, but a fluorophore of the same charge as that of the amino acid must be added to BGE. Subsequently, the fluorescent co-ion is displaced by a nonfluorescent amino acid ion, thereby decreasing the signal. Indirect LIF detection has been applied in the separation of amino acids using methylene blue at acidic pH [43] and fluorescein at basic pH [44] as probes.

Amino acids are electrochemically active compounds and, therefore, can be detected by electrooxidation using copper electrodes in a wall-jet configuration. Under such conditions, referred to as amperometric detection, amino acids were oxidized at a constant electrode potential, measuring the induced electric current [45]. In contrast to amperometric detection methods, capacitively coupled contactless conductivity detection (C^4D) uses an electrochemical-based detector that requires no direct contact

with the BGE. Hence, C^4D is an easy-to-use detection technique that measures electric conductivity inside the separation capillary (see **section 4.3** for details) [46].

The emergence of mass spectrometry as a detection technique has considerably changed the field of separation science. MS detection, especially its multiple reaction-monitoring (MRM) mode, has significantly improved the selectivity and sensitivity of detection. However, in GC-MS, amino acids require derivatization due to separation by GC. In GC-MS, derivatization by silylation reagents has become popular because they bind to both amino and carboxyl functional groups. Accordingly, silylation not only increases the volatility needed for GC separation but also provides EI-MS spectra rich in characteristic fragments [47]. Moreover, in contrast to GC, no derivatization is necessary in liquid phase separation techniques such as HPLC or CE. In HPLC-MS, amino acids have been mainly analyzed in reversed phase separation mode using methanol/water or acetonitrile/water mixtures and a volatile salt (ammonium formate) with a volatile acid (formic acid) as the mobile phase [48,49]. Alternatively, because they are highly polar/ionogenic compounds, amino acids can also be separated in HILIC mode using a high percentage of acetonitrile in the mobile phase and silica particles with cross-linked diol groups on their surface as the stationary phase [50].

When using MS detection with CE, one must consider the composition of the BGE. Most buffers commonly used in CE, such as phosphate or TRIS buffers, cannot be used for amino acid detection by MS because the high content of nonvolatile salts in the BGE subsequently leads to their precipitation in the CE-MS interface. For the same reason, BGE additives often used to tune separation processes, such as surfactants (CTAB, SDS) and chiral selectors (cyclodextrins), cannot be used in such experimental setups. Therefore, in CE-MS, BGE usually consists of a volatile buffer, i.e., ammonium formate [51]. Nevertheless, no baseline separation of amino acids in CE-MS is necessary when using the MRM mode, except for Ile and Leu because these isomeric amino acids provide identical MRM transitions. Thus, the buffer concentration must be optimized for their baseline separation [52].

4 CAPILLARY ELECTROPHORESIS IN THE DETERMINATION OF PROTEINOGENIC AMINO ACIDS

4.1 Extraction of amino acids from solid materials

Generally, every step of the sampling procedure and sample preparation should be thoroughly considered in the amino acid analysis of any solid material. Inappropriate sample handling or sample preparation might distort the final concentration distribution of individual free amino acids. Particularly when analyzing samples from living organisms, the sampling procedure should ensure that all metabolic transformations are halted after collecting the sample. For such purposes, deep-freezing in liquid nitrogen is the most commonly used technique, and deep-freezing with subsequent lyophilization (vacuum drying) has become a well-established method for the preparation of samples from plant materials in which all water content sublimates from solid material during vacuum drying, thereby avoiding an undesired freeze-thaw cycle [53].

According to the well-known rule of thumb "*similia similibus solvuntur*", amino acid extraction requires using an appropriate extraction reagent to disintegrate the material and to transfer amino acids to a liquid phase. Historically, a Soxhlet extractor has been applied for large-scale extractions of plant materials using aqueous ethanol as an extraction solvent [54]. However, in such extraction procedures, the evaporation of the extraction solvent required to preconcentrate the amino acids usually takes several hours, thus significantly prolonging the analysis. Hence, extractions with different solutions, or their mixtures, have also been studied over time to enhance the extraction efficiency.

In amino acid extraction, an extraction mixture consisting of methanol, chloroform and water (2:1:0.8) has been shown to be more efficient than traditionally used 80% ethanol [55]. Amino acid extraction using organic solvents has also been tested in water-in-oil microemulsions of bis(2-ethylhexyl) sulfosuccinate (AOT) in *n*-heptane. Based on the results, the authors of this study suggested that hydrophilic amino acids dissolve only in the polar cavities of AOT globules, whereas less polar amino

acids, i.e., Trp and Phe also dissolve in the organic phase, albeit less than in water, and that Trp incorporates in the interfacial layer of a globule by hydrophobic interactions [56].

The pH of the medium determines the ionic nature of amino acids, which in turn determines their solubility in acidified aqueous solutions. Thus, amino acid extraction using mixtures of organic solvents with water and hydrochloric acid (HCl) has been tested, and the results showed that acidified acetonitrile had the lowest extraction efficiency for amino acids, followed by aqueous, acidic ethanol. As expected, deionized water with HCl had the highest yield of amino acid extraction from lyophilized yeast [57].

When adding an extraction solvent to a dried sample, it is necessary to ensure total sample wetting. The wetting process and the transfer of amino acids from the material to the bulk solution can be enhanced by ultrasound- [58] or microwave-assisted extraction [59]. However, the drawback of using aqueous solutions lies in the complicated preconcentration of amino acids by evaporation, requiring multiple extraction steps, which makes this approach impractical. When the use of an aqueous extraction solution is inevitable and the concentration of the amino acids analyzed is low, solid-phase extraction (SPE) becomes a powerful tool for amino acid preconcentration. In SPE, amino acid extracts are introduced in a conditioned SPE column containing silica-based particles with a C18 stationary phase. Eventually, amino acids are eluted from the SPE cartridge with 10% ethanol [60,61].

In *Study I*, we used an aqueous solution of HCl for the extraction of proteinogenic amino acids from *Nicotiana tabacum* L. cv. Petit Havana SR1 leaves. The leaf samples were deep frozen immediately after the collection, and then the samples were lyophilized. To increase the extraction yield, several factors affecting the extraction process were optimized using Design of Experiments (DoE). In total, 4 factors were assessed: HCl volume, HCl concentration, shaking and sonication time. The most important factors were the extraction solvent volume and concentration. The optimal extraction solution volume-to-mass ratio was 14, i.e., 14 µl of HCl solution per 1 mg of dried sample. When possible, the mass of all tobacco samples was kept at 50 mg, and the samples were extracted with 700 µL of 8.2 mM hydrochloric acid. Subsequently, shaking and sonication, the two factors with the

weakest effect on amino acid extraction yield, enhanced the extraction. Eventually, an extract was filtered through a 0.45-μm filter and immediately analyzed because protein precipitation in the extract could alter the CE analysis or even clog the separation capillary.

4.2 Capillary electrophoresis with suppressed electroosmotic flow

Electroosmotic flow (EOF) is a key factor of CE. In the most frequently used fused silica capillaries, siloxane groups on the inner surface of the capillary are hydrolyzed to silanol groups. Silanol groups covering the capillary surface dissociate when they come into contact with an aqueous solution. The silanol group dissociation increases when the pH of the solution is sufficiently high. The silica surface contains two types of silanol groups of variable ratio, (I) isolated silanol groups with pK_a ~ 4.9 and (II) vicinal silanol groups of pK_a ~ 8.5, with a total silanol group surface area density of approx. 4.9×10^{14} cm^{-2} [62]. Therefore, the overall pI of all silanol groups should be experimentally determined when needed. Helmholtz derived the **Equation 1** that describes EOF mobility:

$$\mu_{EOF} = \frac{\varepsilon \zeta}{4 \pi \eta} \quad (1),$$

where μ_{EOF} is the electroosmotic flow mobility, ε stands for dielectric constant, ζ is the zeta potential at the slipping plane and η is the bulk dynamic viscosity. However, the ratio between the electroosmotic flow mobility and the electrophoretic mobility of a solute is independent of the bulk viscosity. Therefore, electroosmotic and electrophoretic mobility decrease at the same rate when increasing bulk viscosity. However, an increase in viscosity within the double layer of the capillary wall can slow down EOF according to the more general **Equation 2** for electroosmotic mobility:

$$\mu_{EOF} = \frac{\varepsilon}{4 \pi} \int_0^{\zeta} \frac{1}{\eta_0} d\psi (x) \quad (2),$$

where $\psi(x)$ is electrokinetic potential at a distance x from the capillary wall [63]. Consequently, EOF mobility is inversely proportional to capillary surface viscosity (η_0).

Some CE applications require eliminating the electroosmotic flow to increase the separation efficiency, especially in protein analysis. There are 3 main procedures for EOF elimination: pH-assisted EOF suppression, permanent coating of the capillary surface with a polymer, and dynamic coating of the capillary surface with hydrophilic polymers. Because EOF mobility depends on the pH value of BGE, the electroosmotic flow can be substantially slowed down using acidic buffers. However, pH-assisted EOF suppression does not entirely eliminate solute adsorption to the capillary surface with a residual negative charge. Hence, the pH-suppressed EOF method found its place in the separation of anions that do not interact with the capillary surface [64]. In contrast to pH-suppressed EOF, polymer coatings not only suppress EOF but also eliminate unwanted interactions between positively charged solutes and the negatively charged capillary surface. Permanently coated capillaries have been previously prepared using two types of procedures (I) covalent bonding to silanol groups and (II) *in situ* polymerization of monomer units. In procedure (I), at first, a bifunctional silane, e.g., 3-(trimethoxysilyl)propyl methacrylate, reacts with silanol groups, forming siloxane bonds. Then, monomers (e.g., acrylamide) are introduced in the capillary and polymerize with the second functional group of the sublayer (**Fig. 3**).

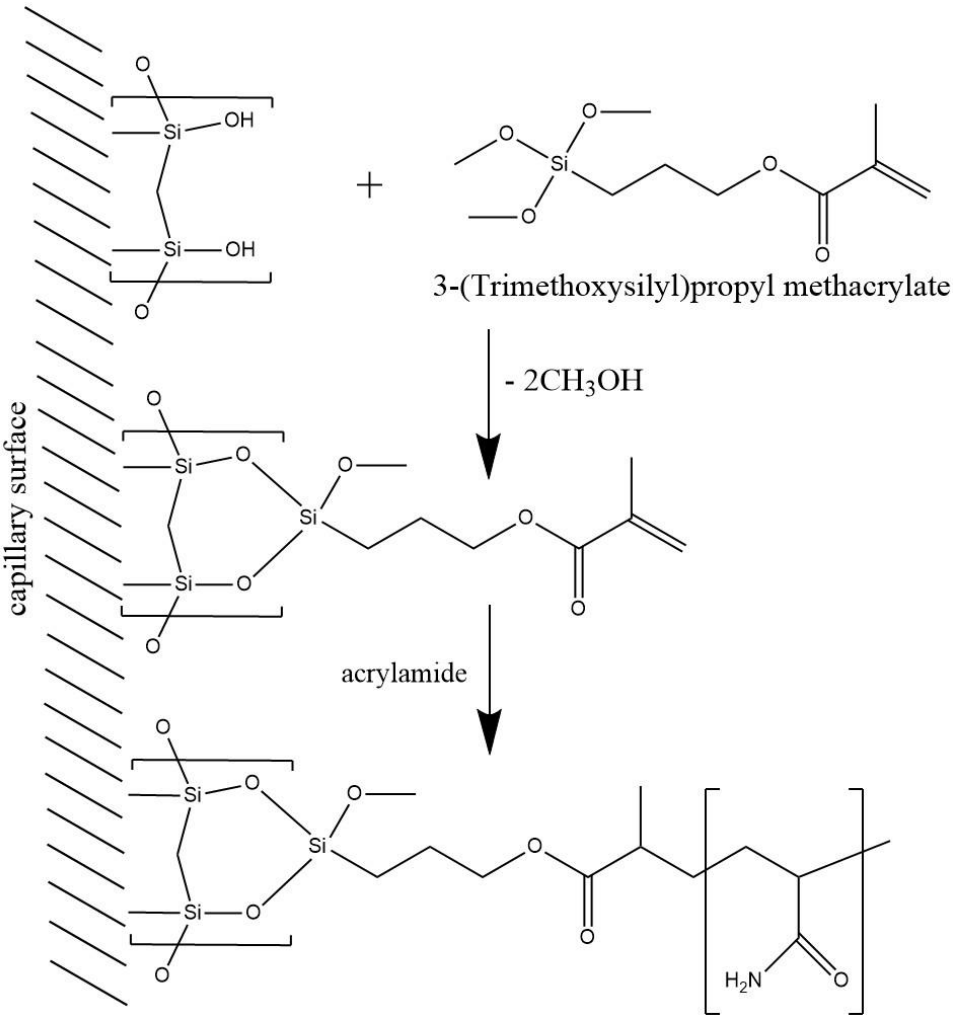


Fig. 3 Permanent polymer coating using 3-(trimethoxysilyl)propyl methacrylate as a sublayer for polymerization with acrylamide monomer units

In procedure (II), polymers such as poly(acrylamide) or polyvinyl alcohol (PVA) can be directly polymerized *in situ* from monomer units with subsequent thermal immobilization, without the sublayer. However, the permanent polymer coating of a capillary wall tends to be a time-consuming technique with low reproducibility [65]. Thus, to achieve coatings with higher reproducibility and stability, dynamic coating procedures have been applied in various CE methods. In those methods, hydrophilic polymers such as PVA [66], polyethylene glycol (PEG), hydroxyethyl cellulose (HEC) or their mixture have been used [67]. In particular, PEG [68] and HEC [8,69] coatings have been shown to effectively improve the separation efficiency by EOF suppression and by elimination of solute adsorption on the capillary wall in amino acid analyses.

Based on the above, we directly added 0.1% HEC (*w*/*v*) to a BGE (1.8 M acetic acid) of low pH (~ 2.2) in *Study I*. The HEC dynamic coating substantially improved the peak resolution (**Fig. 4**) because EOF was significantly slowed down.

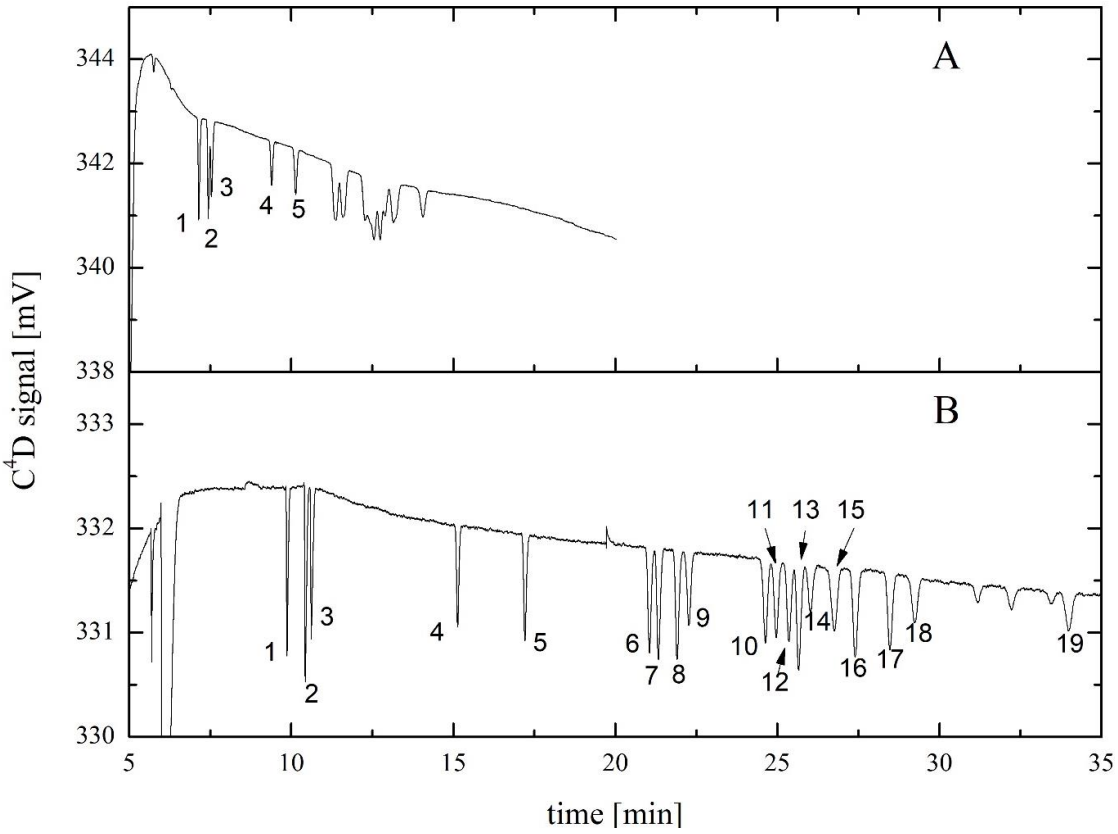


Fig. 4 Electropherograms of 240 μM standard mixture of amino acids. Separation conducted in (A) 1.8 M acetic acid and (B) 1.8 M acetic acid with 0.1% HEC (*w*/*v*). Peak identification: 1 – Lys, 2 – Arg, 3 – His, 4 – Gly, 5 – Ala, 6 – Val, 7 – Ile, 8 – Leu, 9 – Ser, 10 – Thr, 11 – Asn, 12 – Met, 13 – Trp, 14 – Gln, 15 – Glu, 16 – Phe, 17 – Tyr, 18 – Pro, 19 – Asp. Capillary dimensions: l_{tot} 80 cm; l_{det} 66.5 cm; ID 50 μm; OD 363 μm. Voltage 30 kV, electric current 12 μA

4.3 Contactless conductivity detection in capillary electrophoresis

Most inorganic ions and many organic compounds, e.g., some saccharides, carboxylic acids and amino acids insufficiently absorb UV radiation above 190 nm. Thus, considerable research efforts have focused on the development of new detection

techniques for UV non-absorbing analytes in recent decades. Conductivity detection has become the most significant detection technique supplementary to conventional UV/VIS detection. Initially, conductivity detection was used in capillary isotachophoresis (CITP) to measure the resistance of an electrolyte between two electrodes [70,71]. However, the electrodes were in direct contact with the electrolyte inside a capillary, which caused changes in the electrode surface through redox reactions, thereby decreasing the reproducibility of detection. This obstacle was overcome with the development of the contactless conductivity detector (CCD), which eliminates the direct contact between electrodes and electrolyte [72]. This detector consists of a generator that transmits a high-frequency signal through emitting electrodes and through a capacitive cell (separation capillary). Subsequently, the signal is collected by receiving electrodes and amplified, and the final distribution of the electromagnetic field depends on the permittivity, permeability and conductivity of the electrolyte inside the capillary. Because permittivity and permeability change insignificantly in diluted aqueous solutions, in practice, the final signal only depends on the molar ionic conductivity of ions passing through the detector. In CE, separated zones are then detected based on differences in the molar ionic conductivity of analyte ions and of their co-ions in the BGE.

The CCD arrangement was later improved by capacitive coupling of AC voltage. This new arrangement was referred to as capacitively coupled contactless conductivity detector (C^4D). In contrast to the CCD arrangement used in CITP, in CE, the C^4D consisted of two tubular electrodes – two capacitors placed around the capillary with a gap between each other, where the changes in conductivity were measured [73]. Since then, the C^4D technique has been extensively used in CE of amino acids [7,8,68,69,74-76].

In our first study (*Study I*), amino acids were separated by CE and amino acid zones were detected using C^4D. In this study, 1.8 M acetic acid was used as a BGE; hence, we measured differences in the molar ionic conductivity of amino acid cations and hydronium ions. Because the molar ionic conductivity of amino acids is significantly lower than that of hydronium ions, amino acid zones were displayed as negative peaks (**Fig. 4, section 4.2**).

4.4 Method validation in capillary electrophoresis

In every analytical method, validation is an essential step before applying any method to the analysis of real samples. Several basic validation parameters are considered: selectivity, sensitivity, linear dynamic range, accuracy and precision.

Selectivity assessment includes the reliable assignment of an analyte to a peak in an electropherogram, i.e., identification. Identification is easier when UV/VIS detection is used in CE and spectra acquired from the separation of standard mixtures are compared with spectra from the separation of real samples. Moreover, inspecting peak purity in the real sample helps to detect any possible interference of an unknown compound with an analyte of interest [77]. However, in CE-C^4D, no peak purity assessment or spectra comparison is possible. Therefore, in *Study I*, we assessed selectivity based on the standard addition to the extract of a tobacco leaf. To identify highly abundant amino acids, such as *L*-Arg, *L*-His and *L*-Gln, we diluted the sample and added the corresponding standards to reliably identify the amino acids.

Sensitivity, in general, is assessed as the slope of calibration curve. Sensitivity is often described by the limit of detection (LOD) and by the limit of quantification (LOQ) of the method. Generally, LOD and LOQ values represent the concentrations that correspond to signal-to-noise ratios of 3 and 10, respectively [78]. In *Study I*, we used the standard deviation of noise to calculate the LOD and LOQ values from calibration curves of the variation of concentration as a function of peak height. Then, we determined the LOD and LOQ using the calibration curve, extrapolating the concentrations corresponding to 3-fold and 10-fold standard deviations of noise values, respectively. Eventually, we assessed sensitivity as the slope of a calibration curve, which helped us compare the sensitivity of the method for particular amino acids more efficiently than the LOD and LOQ values that were very close to each other for individual amino acids.

The **linear dynamic range** of a method is determined using a calibration curve. The coefficient of determination (R^2) of a calibration curve is often the first indicator of acceptable linearity. In addition, the linear dynamic range corresponds to concentrations that can be determined with acceptable accuracy and precision. In *Study I*, the calibration curves of amino acids were linear across more than two orders of magnitude.

Accuracy determines how close the concentration calculated from calibration is to the true concentration. In our first study, no reference material or blank matrix was available. Therefore, accuracy could not be evaluated, either through analysis of a reference material or through addition of a known amino acid amount to a blank matrix. Hence, the accuracy of the method was expressed as the percentage difference of the slopes of two calibration curves: the calibration curve constructed using standard solutions and the calibration curve measured in a real extract. In real-extract calibration, known amounts of standards were added to a real extract, and a calibration curve was constructed based on the standard addition method.

Precision was determined as the relative standard deviation (RSD) of peak areas within 6 repeated measurements. We found that precision substantially improved when amino acid peak areas were corrected to the peak area of an internal standard (aniline).

STUDY I - Design of experiments for amino acid extraction from tobacco leaves and their subsequent determination by capillary electrophoresis

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RESEARCH PAPER

Design of experiments for amino acid extraction from tobacco leaves and their subsequent determination by capillary zone electrophoresis

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Abstract In this study, we optimized a method for the determination of free amino acids in *Nicotiana tabacum* leaves. Capillary electrophoresis with contactless conductivity detector was used for the separation of 20 proteinogenic amino acids in acidic background electrolyte. Subsequently, the conditions of extraction with HCl were optimized for the highest extraction yield of the amino acids because sample treatment of plant materials brings some specific challenges. Central composite face-centered design with fractional factorial design was used in order to evaluate the significance of selected factors (HCl volume, HCl concentration, sonication, shaking) on the extraction process. In addition, the composite design helped us to find the optimal values for each factor using the response surface method. The limits of detection and limits of quantification for the 20 proteinogenic amino acids were found to be in the order of 10^{-5} and 10^{-4} mol l^{-1}, respectively. Addition of acetonitrile to the sample was tested as a method commonly used to decrease limits of detection. Ambiguous results of this experiment pointed out some features of plant extract samples, which often required specific approaches. Suitability of the method for metabolomic studies was tested by analysis of a real sample, in which all amino acids, except for *L*-methionine and *L*-cysteine, were successfully detected. The optimized extraction process together with the capillary electrophoresis method can be used for the determination of proteinogenic amino acids in plant materials. The resulting inexpensive, simple, and robust method is well suited for various metabolomic studies in plants. As such, the method represents a valuable tool for research and practical application in the fields of biology, biochemistry, and agriculture.

Keywords Amino acids · Capillary electrophoresis · Central composite design · Factorial design · Response surface method · Tobacco

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Introduction

The proper nitrogen supply is very important for sustainable agriculture. It was well documented that low nitrogen supply leads to reduced growth, reduced biomass, and nitrate concentration in plants [1]; however, it was found that in *Arabidopsis thaliana*, under these conditions, protein content was unaltered and total free amino acids and many individual amino acids were increased [2]. From this and other results [3], it follows that the determination of amino acid concentration is very important for evaluation of physiological situation of plants under various nutrition conditions or stress treatment.

Efficient separation methods are needed for fast screening and determination of proteinogenic amino acids in order to clarify the abovementioned processes. The suitability of capillary zone electrophoresis (CZE) [4–9] and high-performance liquid chromatography (HPLC) [10–13] has already been proven by several studies.

Although CZE with mass spectrometry detection provides high selectivity [14], it is convenient to utilize CZE in connection with UV/VIS detection in order to reduce initial and operating costs of the instrumentation. However, non-

aromatic amino acids such as alanine, arginine, aspartic acid, and threonine are characterized by low molar absorptivities at wavelengths above 210 nm. Derivatization of amino acids contributes to more sensitive UV/VIS detection [15]; nevertheless, time consumption and reproducibility of the derivatization procedure are the factors that should be taken into consideration. On the other hand, CZE with capacitively coupled contactless conductivity detector (C^4D) offers sensitive detection of all amino acids without any need for derivatization and has been applied to separation of selected amino acids [6, 9]. CZE-C^4D method providing baseline separation of all 20 proteinogenic amino acids was reported in the literature [7]. Amino acids were separated as cations within 35 min in an acidic background electrolyte with 0.1% hydroxyethyl cellulose (HEC) as an EOF suppressing agent. It is important to note that the study was focused on qualitative analysis; no quantitation of the analytes was performed.

Extraction of analytes belongs among crucial steps of biological sample analysis. An optimization of this step should be conducted to maximize the extraction yield. The full factorial design, so-called OFAT (one-factor-at-a-time), followed by the response surface method (RSM) has commonly been used for various applications [16–18]. Nevertheless, the fractional factorial design requires fewer experiments for an evaluation of statistically significant factors, thus reduces time and material consumption [19]. In addition, there are different options how to conduct RSM such as central composite design [20–22] and Box-Behnken design [23]. In contrast with Box-Behnken design, central composite design includes factorial design, hence is used more often in analytical chemistry [24].

Solvent extractions of free amino acids from a wide range of biological samples have been reported [5, 25–29]. Aqueous mixtures with ethanol [5, 25–27] or with pure water [28, 29] have been used among others, but acidic solutions such as diluted hydrochloric acid of approximate pH 2 [10] are more suitable for amino acid extraction since they are positively charged; thus, they are readily dissolved in aqueous solution.

In this work, we propose an optimized procedure for extraction of free amino acids from *Nicotiana tabacum* leaves. The most suitable extraction conditions were investigated using the fractional factorial design followed by the central composite face-centered design (CCFD) and RSM. Diluted hydrochloric acid was applied as an extraction agent [10] and the determination of the amino acids was conducted by CZE-C^4D [7] with aniline as an internal standard.

Materials and methods

Chemicals and reagents

L-amino acids, namely, glycine (99%), alanine (98%), arginine (98%), asparagine (98%), aspartic acid (98%), cysteine (98%), glutamine (99%), glutamic acid (99%), histidine (98%), isoleucine (98%), leucine (98%), lysine (98%), methionine (98%), phenylalanine (98%), proline (99%), serine (99%), threonine (98%), tryptophan (98%), tyrosine (98%), and valine (98%), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aniline, used as an internal standard, was purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid p.a. (35%) and acetic acid p.a. (99%) were purchased from Lach-Ner (Neratovice, Czech Republic). Hydroxyethyl cellulose was purchased from Ashland (Convington, KY, USA). Sodium hydroxide p.a. was purchased from Penta (Prague, Czech Republic). Solutions were prepared using deionized water supplied by a Milli-Q water purification system from Millipore (Bedford, MA, USA). All amino acid standard solutions (5 mmol l^{-1}) were prepared in 10 mmol l^{-1} hydrochloric acid.

Instrumentation

All electrophoretic experiments were conducted in a fused-silica capillary (Polymicro Technologies, Phoenix, USA) using a G7100A Capillary Electrophoresis Instrument (Agilent Technologies, Waldbronn, Germany) with a contactless conductivity detector. The detector consisted of two cylindrical electrodes, 4 mm long with 1-mm insulation gap. Inner diameter of the electrodes was 400 μm. The detector was operated at a frequency of 1.84 MHz with an amplitude of 44 V. Background noise in the 1.8 mol l^{-1} acetic acid with 0.1% *w*/*w* hydroxyethyl cellulose background electrolyte was 25 μV. A freeze-dryer (Finn-Aqua Santasalo-Sohlberg, Hürth, Germany) was used for lyophilization of the samples. An Elmasonic S 15 ultrasonic cleaner (Elma, Singen, Germany), a Vibramax 100 vibration platform shaker (Heidolph Instruments, Schwabach, Germany), and a Force 7 microcentrifuge (Denver Instrument, Bohemia, NY, USA) were used for sample pretreatment.

Sample preparation

The leaves of *N. tabacum* L. cv. Petit Havana SR1 were collected and frozen within 5 s in liquid nitrogen to stop metabolic transformations. Afterwards, the samples were stored in a freezer at −80 °C. Prior to an analysis, the samples were dried by lyophilization (20 h) and ground with a mortar and pestle.

Sample extraction

A solution of hydrochloric acid was used as an extraction solvent for free amino acids. Hydrochloric acid was added to 50 mg of a ground sample, after that, the sample was sonicated and shaken. The sample was centrifuged for 5 min at 7000 rpm. Following this, the supernatant was removed and filtered through a 0.45-μm PVDF syringeless filter. Eventually, 10 μl of aniline (5 mmol l^{-1}) and 90 μl of the

filtered extract were mixed, so that the final concentration of aniline was 0.5 mmol l^{-1}.

Electrophoretic conditions

The dimensions of the fused-silica capillary were as follows: 50 μm ID, 363 μm OD, 80.0 cm of total length, and 66.5 cm to detector. The separation capillary was washed with 1 mol l^{-1} NaOH (10 min) followed by deionized water (10 min) before every set of measurements. The capillary was washed with the background electrolyte (BGE) between individual measurements for 2 min. Afterwards, a voltage of 30 kV was applied (2 min) and then the samples were introduced hydrodynamically by a pressure of 5 kPa (5 s). All electrophoretic experiments were conducted at 30 kV inducing an electric current of 12 μA in 1.8 mol l^{-1} acetic acid with 0.1% *w/w* hydroxyethyl cellulose as a BGE.

Design of experiments

MiniTab 16 software (State College, PA, USA) was used for the fractional factorial design as well as for the central composite face-centered design with response surface method [19]. At first, four factors were selected for factorial design: HCl concentration, HCl volume, sonication time, and shaking time. Afterwards, two levels of each factor were established. Concentration of HCl varied from 1 mmol l^{-1} (pH 3.0) to 15 mmol l^{-1} (pH 1.9); the higher level was limited by the risk of acidic protein hydrolysis. The lower level of HCl volume was 0.64 ml considering sufficient hydration of the sample, while the higher level was 1.50 ml limited by dilution of the amino acids. Sonication time was set to 0 and 10 min as the lower and higher level, respectively. This enabled an evaluation of sonication effect. Finally, shaking time varied between 5 and 20 min. The set of experiments for the fractional factorial design is presented in Table S1 (see Electronic Supplementary Material, ESM). Moreover, the CCFD with RSM was conducted; thus, axial points and the central point were added to the experimental matrix. The response as a function of individual factors and their interactions was estimated based on CCFD results. General form of the model can be seen in Eq. 1.

$$Y = b_0 + b_1A + b_2B + b_3C + b_4D + b_{21}BA + b_{23}BC + b_{24}BD + b_{11}A^2 + b_{22}B^2 + b_{44}D^2 \quad (1)$$

Where, Y is the response (the area of an amino acid peak divided by the area of aniline peak), b_0 refers to model coefficient, b_1, b_2, b_3, and b_4 are the coeffients of the factors. A is HCl volume, B is HCl concentration, C is shaking time, and D is sonication time.

Results and discussion

Capillary electrophoresis method

As the 2.3 mol l^{-1} concentration of acetic acid in BGE reported in [7] did not provide baseline separation of all 20 amino acids under our experimental conditions, a suitable concentration of BGE was investigated within the range from 1.7 to 2.4 mol l^{-1} with optimum at 1.8 mol l^{-1} providing the best resolution of the peaks. Figure 1a shows an electropherogram obtained under standard separation conditions. There is a very large system peak, after which the detector signal only slowly returns back to the baseline, thus evaluation of the peaks of fast migrating analytes was problematic. However, we found out that the system can be equilibrated and the system peak can dramatically be reduced by application of voltage (30 kV) for 2 min before every analysis (Fig. 1b). Separation and quantification of all 20 amino acids could be performed under the adjusted conditions.

Method parameters

Calibration curves for all 20 amino acids were measured within a concentration range dependent on particular amino acid. The calibration curves for *L*-Cys and *L*-Gln had to be measured immediately after standard solution preparation. During several days, *L*-Cys started oxidizing to cystine as well as *L*-Gln was successively transformed to *L*-Glu. All calibration data are shown in Table 1.

The sensitivity of the method varied between 1829 for *L*-Gly and 3799 mV s mol^{-1} l for *L*-Trp with the coefficients of determination above 0.99 with the exceptions of *L*-Met and *L*-Asp. Limits of detection (LOD) and quantification (LOQ) were calculated as 3- and 10-fold the standard deviation of the noise. The detection of all amino acids is feasible in order of 10^{-5} mol l^{-1} excluding *L*-Asp as the amino acid with the highest LOD. LOQs of most amino acids were shown to be at the lower values of 10^{-4} mol l^{-1}. It is apparent from Table 1 that, in the extract sample, concentrations of 17 amino acids were found to be above their LOQs with the highest abundance of *L*-Pro, *L*-Glu, and *L*-Asp.

In several studies [9, 30–32], LOD and separation efficiency were significantly improved by addition of organic solvent, such as acetonitrile, to sample, which has led to decreased sample conductivity and thus field amplification and sample stacking. Compared with these studies, amino acid LODs of our method are about one order of magnitude higher. In our case, samples were dissolved in 0.01 M HCl and their conductivity was relatively high. Addition of acetonitrile should thus improve LOD and separation efficiency. Presence of 50 or 75% *v/v* acetonitrile in standard mixture of amino acids in 0.01 M HCl significantly increased amino acid responses when electrokinetic injection was applied. However,

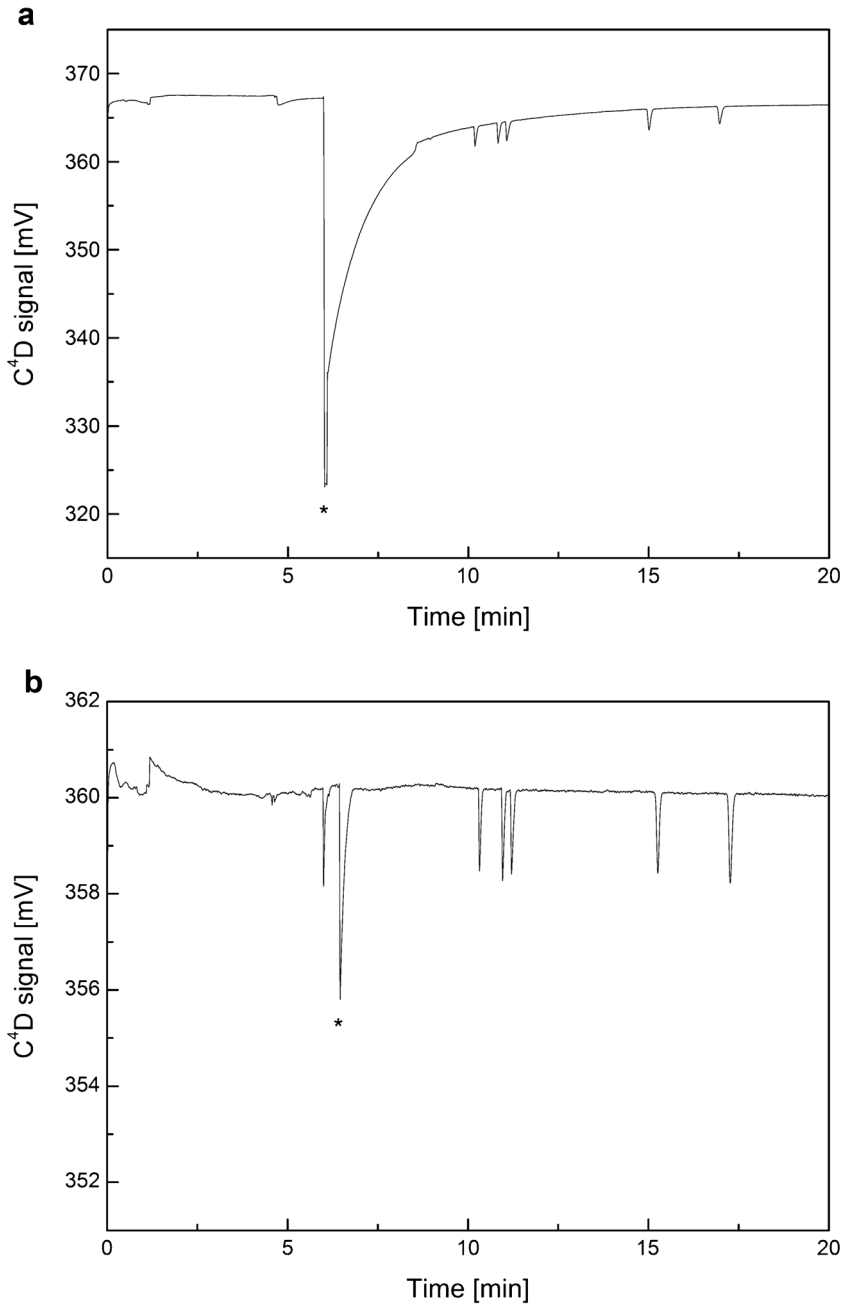

Fig. 1 System peak (marked with *asteriks*) in the electropherogram—original (**a**) and decreased by the application of voltage (30 kV, 2 min) before the analysis (**b**). Background electrolyte 1.8 M acetic acid (pH 2.25) with 0.1% *w*/*w* HEC. Voltage 30 kV, electric current 12 μA

separation efficiency did not increase enough to maintain baseline resolution of Arg/His, Val/Ile, and Trp/Gln peak pairs. Comparison of separation efficiency is rather delicate matter as it depends on peak size. To assess the effect of acetonitrile addition on separation efficiency, mixtures of 0.25 mM amino acid standards in 0.01 M HCl with water or acetonitrile 1:1 *v*/*v* were injected hydrodynamically using a pressure of 5 kPa for 5 s. Resulting separation efficiencies for Lys as the fastest migrating amino acid were 455,000 and 524,000 plates m^{-1} for sample diluted with water and acetonitrile, respectively. For Asp, as the slowest migrating amino acid, the efficiencies were 132,000 and 266,000 plates m^{-1} for sample diluted with water and acetonitrile, respectively. Therefore, increase of efficiency can be seen in samples diluted with acetonitrile; however, it should be noted that peak areas were roughly 30% lower, which apparently increased their calculated efficiency values. When acetonitrile was added to tobacco extract sample and electrokinetic injection was used, no signal increase was observed and signals of slowly migrating amino acids decreased or even were not detected. The behavior described above may be ascribed to two factors. First, pH of samples and BGE are not equal and lie in the area of pK_A values of amino acid carboxylic groups. Therefore, effective charge of amino acids is

Table 1 Calibration parameters with limits of detection (LOD) and quantification (LOQ) and concentrations found in an extract from *Nicotiana tabacum*. Amino acids are ordered according to increasing sensitivity of detection

Amino acid	Slope [mV s mol^{-1} l]	Intercept [mV s]	R^2	LOD [.10^{-4} M]	LOQ [.10^{-4} M]	Extract concentration[a] [.10^{-4} M]
Gly	1829	0.043	0.9993	0.3	1.0	8.3 ± 0.4
Cys	2182	0.008	0.9910	0.5	2.2	<LOD
Pro	2468	−0.058	0.9956	0.6	1.8	4.0 ± 0.3
Ala	2525	0.006	0.9978	0.3	1.0	3.6 ± 0.2
Ser	2604	−0.037	0.9990	0.4	1.3	11.7 ± 0.7
Gln	2772	0.001	0.9998	0.5	1.5	36.3 ± 2.0
Met	2817	−0.013	0.9839	0.5	1.5	<LOD
Asp	2889	−0.062	0.9864	1.1	2.3	6.4 ± 0.6
His	2897	0.005	0.9966	0.2	0.6	2.3 ± 0.3
Asn	2904	−0.010	0.9995	0.4	1.2	14.8 ± 1.0
Lys	2984	0.013	0.9999	0.2	0.6	1.1 ± 0.1
Thr	3063	−0.047	0.9980	0.5	1.5	4.0 ± 0.4
Val	3098	−0.036	0.9951	0.4	1.2	1.8 ± 0.1
Ile	3234	−0.003	0.9992	0.3	0.8	1.5 ± 0.1
Arg	3288	−0.032	0.9965	0.2	0.7	1.7 ± 0.2
Phe	3383	0.008	0.9968	0.4	1.1	2.2 ± 0.1
Leu	3414	−0.038	0.9976	0.2	0.9	1.7 ± 0.2
Glu	3445	−0.027	0.9994	0.3	1.1	6.6 ± 0.4
Tyr	3658	−0.019	0.9986	0.3	1.0	1.1 ± 0.1
Trp	3799	−0.013	0.9978	0.3	0.9	<LOQ

[a] Median of three repeated measurements with confidence interval $\alpha = 0.95$, under optimized conditions

higher in sample zone than in BGE and sample stacking already occurs to certain extent. Second, addition of acetonitrile influences pK_A values and complicates the overall situation. This combined with specific composition of tobacco extract samples probably limits benefits usually arising from addition of acetonitrile into sample. Here, addition of acetonitrile can be beneficial to some extent while working with standard samples, however, does not bring any improvement for analysis of tobacco extract samples.

CCFD and RSM models

In order to optimize the extraction procedure specifically for plant samples and metabolomic studies, the fractional factorial design was used to assess the effect of each factor on the response (peak area of an individual amino acid) resulting in a Pareto chart for each amino acid. The examples of Pareto charts for *L*-His, *L*-Thr, and *L*-Leu as representative amino acids are presented in Fig. 2. If the value of standardized effect exceeds the determined border, the factor is found to be statistically significant based on *t* statistics characterized by *P* values. The *P* values were calculated by dividing each linear coefficient by its standard error. The volume of hydrochloric acid was evaluated as a statistically significant factor for all amino acids. The CCFD was conducted, whereby the significance of HCl volume was confirmed by analysis of variance (ANOVA) comprised in CCFD. The ANOVA divided the total variance among the individual factors and the residual error. Accordingly, the highest portion of variance was assigned to HCl volume and CCFD allowed realization of RSM and determination of optimal values for each factor (Fig. 2).

The CCFD estimated the coefficients of quadratic regression model (for general form, see Eq. 1) for each amino acid. The examples of such models for *L*-His, *L*-Thr, and *L*-Leu are given below.

$$\frac{A_{His}}{A_{IS}} = 0.105 - 0.023A + 0.005B - 0.003C - 0.001D + 0.001BA + 0.003BD - 0.012A^2 - 0.024B^2 - 0.020D^2 \quad (2)$$

$$\frac{A_{Thr}}{A_{IS}} = 0.880 - 0.207A + 0.027B - 0.013C + 0.012D + 0.003BA - 0.025BC + 0.025BD - 0.095A^2 - 0.241B^2 + 0.012D^2 \quad (3)$$

$$\frac{A_{Leu}}{A_{IS}} = 0.466 - 0.126A - 0.010B - 0.004C + 0.019D - 0.001BA - 0.027BC + 0.021BD - 0.066A^2 - 0.143B^2 - 0.085D^2 \quad (4)$$

Equation 2 corresponds to *L*-His, Eq. 3 to *L*-Thr, and Eq. 4 to *L*-Leu. The model coefficient describes the response at the maximum in RSM plots seen in Fig. 2. The variables *A*, *B*, *C*, and *D* are the factors as described in Eq. 1. Each coefficient estimates the change in the response of the variable if the other predictors are held constant. As an illustration, if HCl volume (*A*) in Eq. 3 increases by 1 ml and the other factors remain the same, the response decreases approximately by 0.207. In order

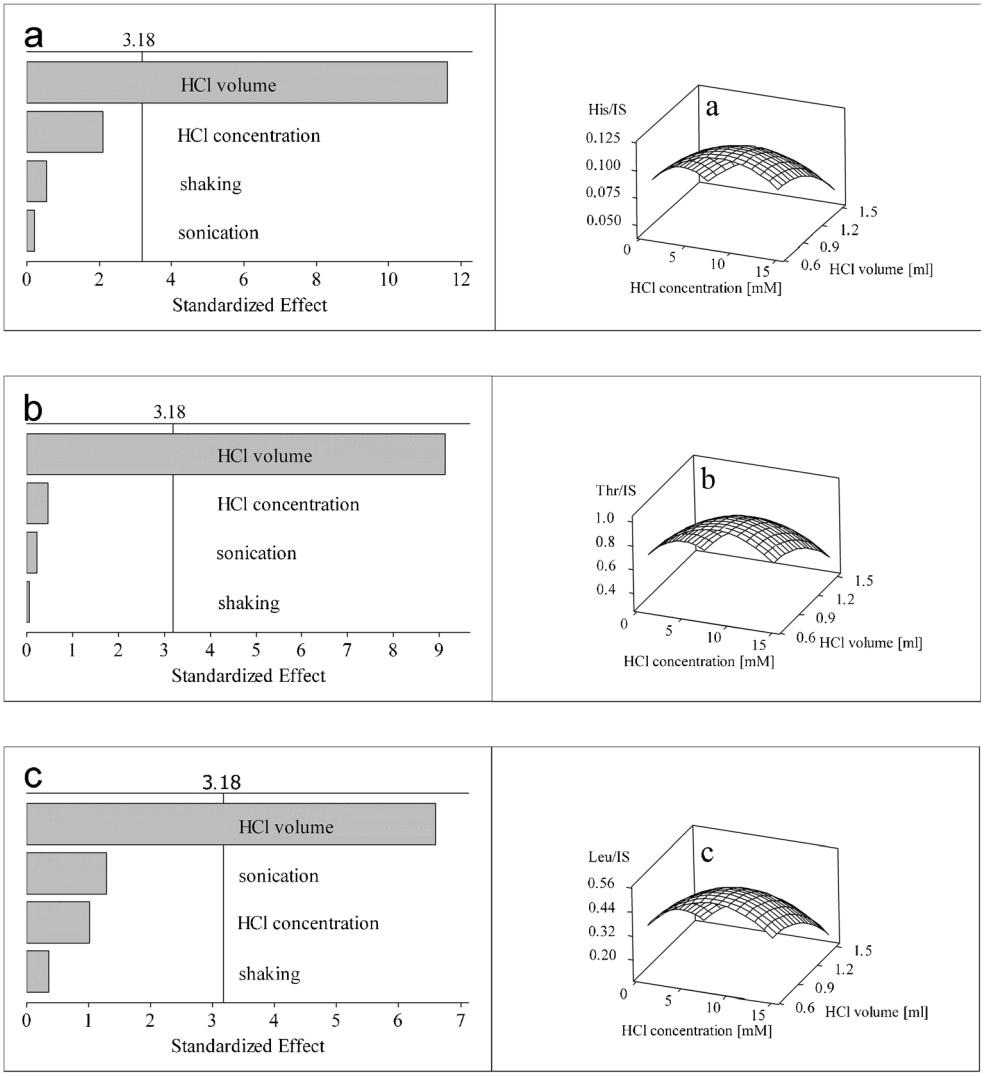

Fig. 2 Pareto charts for the tested factors and RSM plots for selected amino acids. **a** His. **b** Thr. **c** Leu

to obtain the equations describing the plots in Fig. 2, the factors *C* and *D* had to be held at their optimal values. Therefore, the quadratic members comprising *C* and *D* were excluded from the equations. The CCFD determined the optimal factor values for each amino acid; therefore, an average value of each factor was considered as the optimal one. These optimized values are summarized in Table 2.

After that, a real sample of tobacco leave extract was processed under the optimized conditions (average values in Table 2) and measured by CZE-C^4D. The result of such analysis can be seen in Fig. 3. Two amino acids, namely, *L*-Cys and *L*-Met, were not found in the electropherogram because their concentration levels were below the LODs. The amount of the other amino acids corresponds to the data given in Table 1. As seen in Fig. 3b, the excessive abundance of *L*-Arg and *L*-His caused overlapping of their peaks with other peaks in electropherogram. The sample had to be 10-fold diluted for better quantification.

Table 2 Optimized values for the investigated factors

Amino acid	HCl volume [ml]	HCl concentration [mmol l^{-1}]	Shaking [min]	Sonication [min]
Lys	0.64	5.4	20	6
Arg	0.64	5.7	20	7
His	0.64	8.6	5	5
Gly	0.90	7.1	5	5
Ala	0.64	7.1	20	6
Val	0.70	8.8	5	6
Ile	0.64	10.5	5	6
Leu	0.64	8.5	5	6
Ser	0.66	9.2	5	6
Thr	0.64	8.8	5	5
Asn	0.80	8.8	5	5
Trp	0.80	8.9	5	5
Gln	0.64	1.0	5	0
Glu	1.0	13.0	5	5
Phe	0.64	8.9	5	6
Tyr	0.74	9.0	5	5
Pro	0.64	8.9	5	5
Asp	0.64	9.6	5	6
Average values	0.70	8.2	8	5

Method validation

Relative standard deviations (RSD) of six repeated measurements of the amino acid standards varied from 5.7% (*L*-Ile) to 9.8% (*L*-Gln), for peak area, except *L*-Met which had RSD 30.9%. Therefore, aniline was used as an internal standard and the areas of all amino acid peaks were corrected to the peak area of aniline. Using the internal standard, RSDs decreased significantly as seen in Fig. 4. Aniline was a suitable internal standard since its peak did not interfere with the other peaks in the electropherogram (Fig. 3).

The results, as shown in Fig. 4, indicate that correction to aniline contributed to decrease of RSDs of all amino acids excluding *L*-Asn in the standard sample. The RSDs of most amino acids fluctuated between 2 and 5% after correction to aniline. Furthermore, accuracy of the method was tested by standard addition of amino acid standards to real matrix (Table 3). It is apparent from Table 3 that the differences between the slopes of the calibration curves measured with standards in 0.01 M HCl and the slopes obtained with sample matrix are for most amino acids below 10%. The results

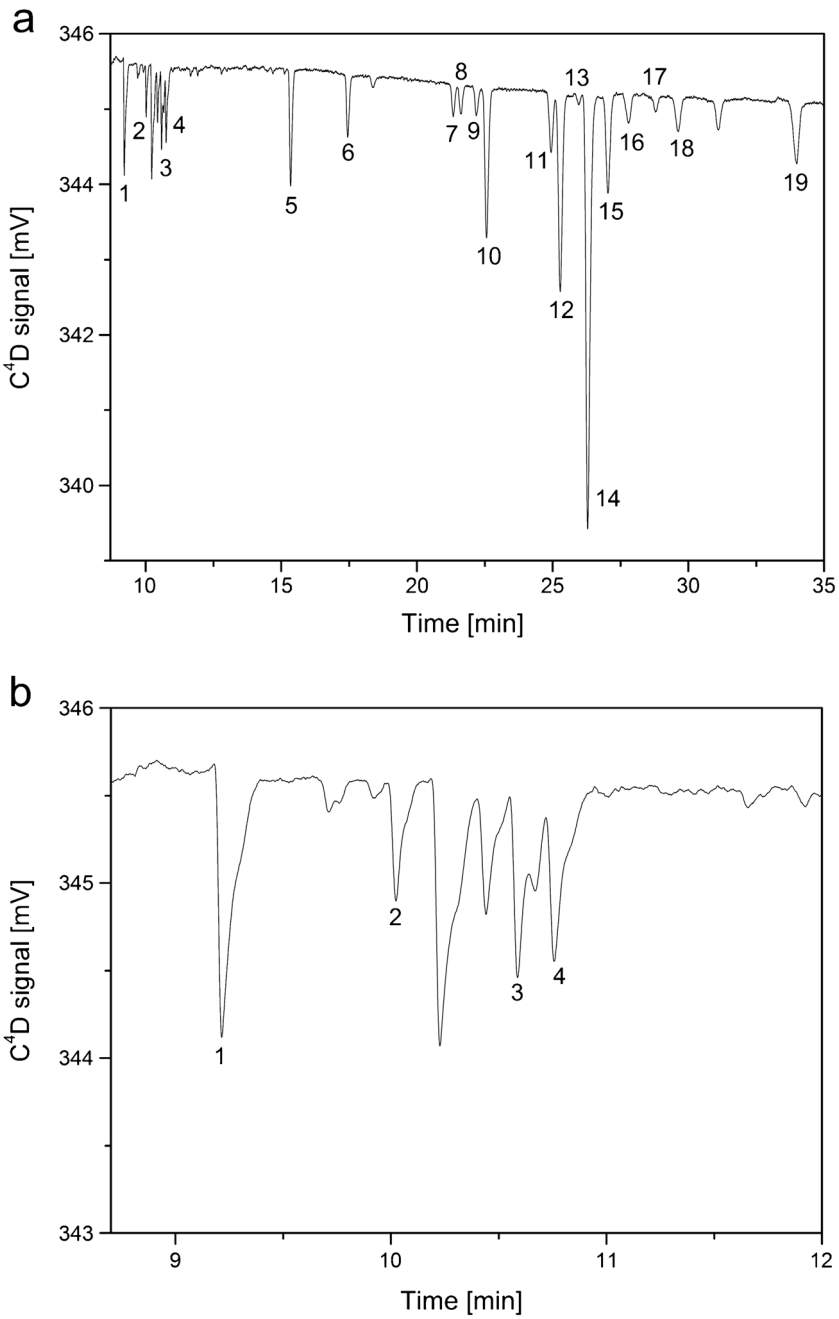

Fig. 3 Electropherogram of a tobacco leaf extract measured under optimized conditions. All detected peaks (**a**) and detail of the 8.5 to 12.0 min time range (**b**). Peak identification: *1* aniline, *2* Lys, *3* Arg, *4* His, *5* Gly, *6* Ala, *7* Val, *8* Ile, *9* Leu, *10* Ser, *11* Thr, *12* Asn, *13* Trp, *14* Gln, *15* Glu, *16* Phe, *17* Tyr, *18* Pro, *19* Asp. Separation was conducted in 1.8 M acetic acid (pH 2.25) with 0.1% *w*/*w* HEC. Voltage 30 kV, electric current 12 μA

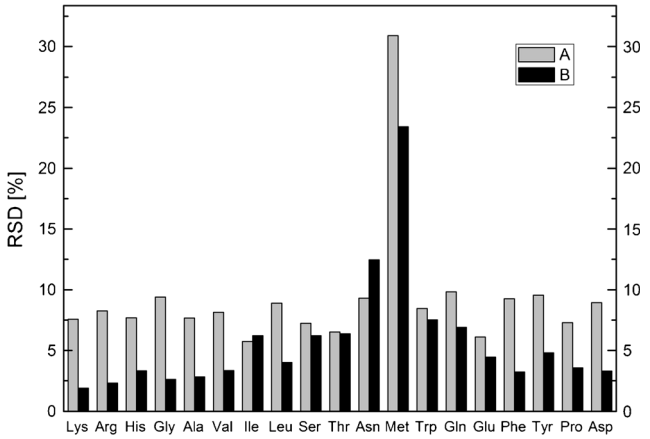

Fig. 4 Comparison of repeatability of amino acid peak areas without (*A*) and with (*B*) aniline as an internal standard, $n = 6$. Separation conditions: background electrolyte 1.8 M acetic acid (pH 2.25) with 0.1% *w*/*w* HEC. Voltage 30 kV, electric current 12 μA

show that when aniline is used as an internal standard precision and accuracy of the optimized method are sufficient and the method is suitable for determination of amino acid profiles in plant materials.

Conclusion

The optimization of extraction process of free amino acids from tobacco leave extracts employing central composite face-centered design coupled with response surface method has been conducted. This design of experiments helped us to evaluate the most significant factors affecting the extraction yield as well as it helped us to find the most convenient values for each factor.

Capillary electrophoresis with contactless conductivity detection has been utilized for separation and detection of 20 proteinogenic amino acids in 1.8 mol l^{-1} acetic acid as a background electrolyte within 34 min. We found out that application of 30 kV voltage for 2 min before every analysis can successfully reduce a system peak occuring in the electropherograms. We also managed to increase repeatability of quantification by using aniline as an internal standard. Following this, the calibration curves of the amino acids were measured and their limits of detection and quantification were determined. An attempt to decrease limits of detection by addition of acetonitrile to sample brought only partially positive results in case of standard mixture and failed in case of tobacco extract sample. This underlines specific features of plant samples and necessity of careful sample treatment method development and validation. Viability of the method have been tested on an extract from tobacco leaves. Using the optimized method, 17 amino acids were successfully quantified, *L*-Trp was detected but could not be reliably quantified, and concentrations of *L*-Met and *L*-Cys were below the limit of detection. Eventually, possible matrix effect was investigated by comparison of calibration slopes measured with standard samples and those measured in the extract. The results show that the complete optimized method provides good precision and accuracy for determination of amino acids in plant materials.

Table 3 Comparison of calibration curve slopes measured with samples in 0.01 M HCl and in real extract matrix

Amino acid	0.01 M HCl slope [mV s mol^{-1} l]	Extract slope [mV s mol^{-1} l]	Difference [%]
Gly	1829	2016	10
Ser	2604	2697	3
Pro	2570	2666	4
Ala	2525	2659	5
Asp	2889	3184	10
Asn	2904	2723	6
His	2897	2417	17
Gln	2772	2691	3
Lys	2984	3317	11
Val	3098	3177	3
Ile	3234	3307	2
Arg	3288	2962	10
Thr	3063	3106	1
Phe	3383	3574	6
Leu	3414	3342	6
Glu	3445	3872	12
Tyr	3658	3831	5
Trp	3799	3568	6

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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5 AMINO ACID SEPARATION BY SUPERCRITICAL FLUID CHROMATOGRAPHY WITH MASS SPECTROMETRY DETECTION

5.1 Supercritical fluid chromatography

The first experiments with supercritical fluids were conducted in the 1960s, specifically on the solubility of porphyrins. Porphyrins decompose at high temperature, which precludes their separation by GC. To solve this problem, porphyrin solubility in gases such as trifluoromethane and chlorodifluoromethane was tested. For this purpose, the gases were heated and pressurized beyond their critical points, resulting in supercritical fluids of density and solvating power similar to those of liquids whilst their diffusivity and viscosity remained similar to those of gases. In contrast to conventional GC separation, porphyrins dissolved in supercritical fluids at significantly lower temperatures without being degraded and were eluted through the capillary column [79]. In addition to chlorofluorocarbons, light hydrocarbons and N_2O have also been used as mobile phases in SFC.

All aforementioned gases have been considered hazardous to environment and unsuitable for separation of thermolabile compounds. As a result, CO_2 has become a widely used mobile phase in SFC, as a safer and more environmentally friendly alternative to previously used gases. Moreover, reaching the critical point of CO_2 requires mild conditions (31°C and 7.4 MPa) achievable using conventional chromatographic instrumentation. Initially, SFC separations were conducted in capillary columns using GC instrumentation, resulting in capillary supercritical fluid chromatography (cSFC) [80]. This cSFC method used long GC capillaries and a typical GC detection technique – FID. Moreover, placing a splitter at the end of the column enabled dual detection simultaneously using FID and UV/VIS [81].

The development of HPLC instrumentation brought up an idea to use packed columns not only for HPLC but also for SFC separations, which led to the establishment of modern SFC [82]. Subsequently, sub-2 μm stationary phase particles were applied for

separations in LC and in SFC, which resulted in more efficient techniques – ultra-performance liquid chromatography (UPLC) and ultra-performance supercritical fluid chromatography (UPSFC), respectively. UPSFC provides a shorter analysis time and higher separation efficiency than UPLC thanks to the high diffusivity and low viscosity of its mobile phase. Therefore, SFC gradually replaced NPLC in the separation of low-polarity analytes because the use of toxic solvents (*n*-heptane) is avoided [83]. During the last two decades, SFC has also been applied to separations of more polar compounds, thus replacing reversed-phase liquid chromatography (RPLC), to some extent. In order to separate polar compounds by SFC, the compounds must be soluble in mobile phase, which requires modifying the mobile phase by adding organic co-solvents to CO_2. On one hand, a co-solvent increases the mobile phase elution strength and density. On the other hand, after co-solvent addition, the SFC measurement is shifted to subcritical conditions. Notwithstanding, the high diffusivity and low viscosity of the mobile phase remains unchanged after co-solvent addition [84].

Various organic co-solvents have been tested over time. Alcohol co-solvents, especially methanol, ethanol and isopropanol, up to 40% in the mobile phase, became popular in SFC because they improve peak shape of hydrogen bond donor species [85]. Similarly to LC separations, additives to mobile phase in SFC help to improve peak shape by modifying the stationary phase surface, by increasing the polarity of the mobile phase and by changing the pH of the mobile phase. Again, similarly to LC, common additives are used in SFC, e.g., formic acid, acetic acid, ammonium formate, ammonium acetate, water. Organic acids (formic and acetic acid) are usually added in amounts ranging from 0.1% to 1%, and they decrease the pH and help to improve peak shape and ionization efficiency when using MS detection. Salts (ammonium formate, ammonium acetate) increase the mobile phase polarity and improve peak shape and are typically added in concentrations up to 20 mM. Water (up to 5%) increases the solvating power of the mobile phase and brings a HILIC-like separation mode to SFC [86].

In *Study II* of this thesis, we developed and optimized a UPSFC-MS/MS method for the determination of proteinogenic amino acids in human plasma. In our method, we used a Viridis BEH column and CO_2 with a methanol/water mixture and formic acid and ammonium formate as mobile phase. Peak shape and separation efficiency substantially improved when water and ammonium formate were added to the mobile phase. For

example, **Figure 5** compares the *L*-proline butyl ester peak obtained in the mobile phase composed of (**A**) CO_2/methanol with 0.1% formic acid vs (**B**) CO_2/methanol/water with 0.1% formic acid and 20 mM ammonium formate.

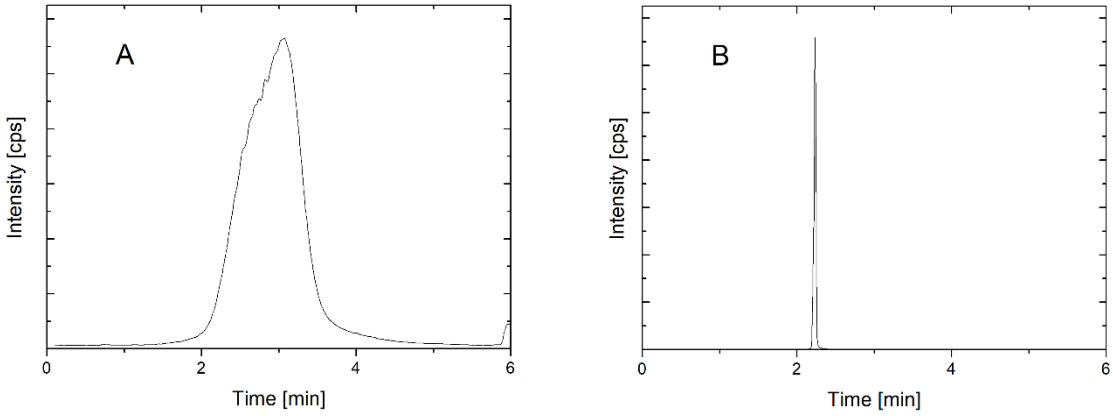

Fig 5 Peak shape comparison of *L*-Pro butyl ester eluted in (A) CO_2/methanol with 0.1% formic acid and in (B) CO_2/methanol/water with 0.1% formic acid and 20 mM ammonium formate. BEH column, slower gradient than in the final method.

5.2 Amino acid derivatization

As described above (**section 3.3**), most amino acids require incorporating a suitable chromophore into their structures for UV/VIS detection. Derivatization can be conducted in either pre- or post-column arrangements. Post-column derivatization has been previously performed in online setups using a copper solid-state reactor, which improved the sensitivity of UV/VIS detection of amino acids complexed with Cu^{2+} ions [87]. In post-column derivatization using conventional derivatization reagents, matrix effects are suppressed, thus improving the detection selectivity [88]. Accordingly, in pre-column derivatization in LC with UV/VIS detection, amino acids have been mostly derivatized by FMOC [89], by OPA [90], by dansyl chloride [91], and by 2,4-dinitrofluorobenzene (DNFB) [92]. All these derivatization reagents selectively react with amines (**Fig. 6**).

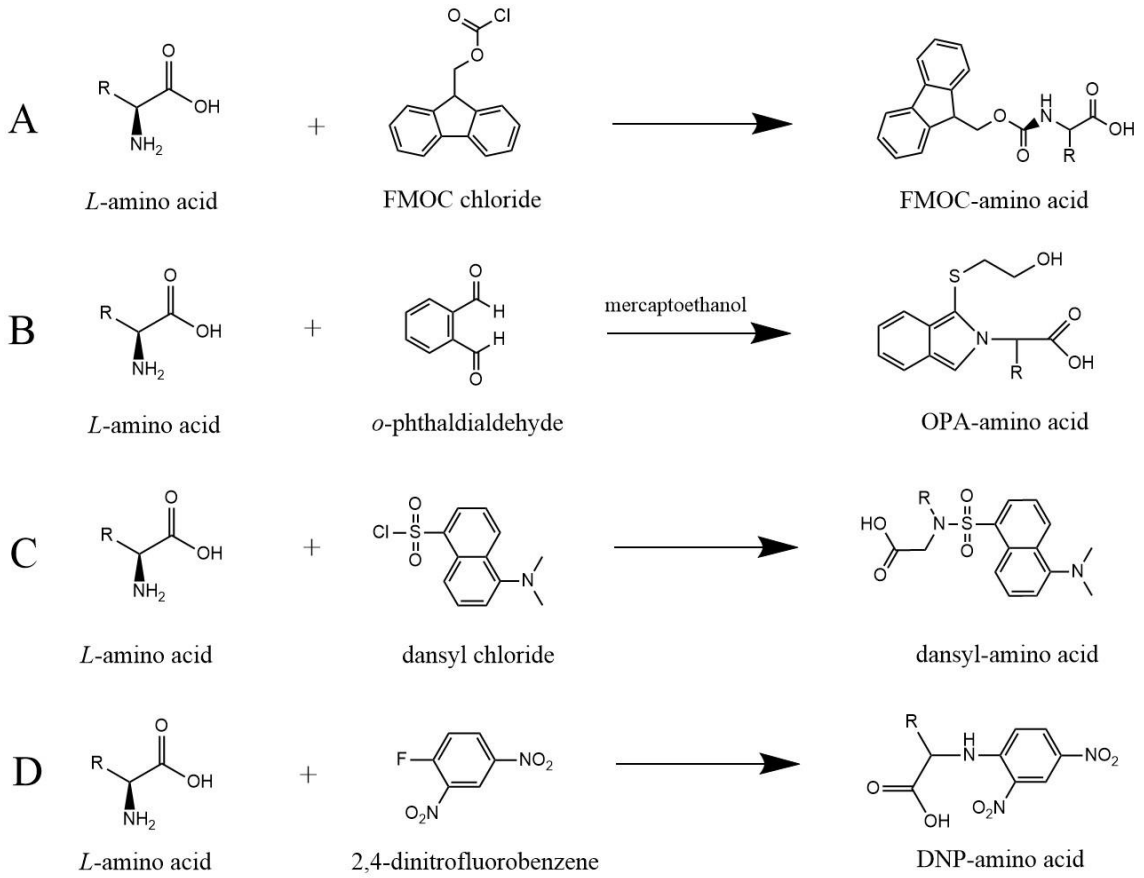

Fig. 6 Derivatization of the amino group of amino acids with conventional reagents yielding (A) FMOC-amino acid, (B) OPA-amino acid, (C) dansyl-amino acid and (D) DNP-amino acid, performing all reactions under basic pH conditions

Such reagents bind to amino groups, thereby preventing the amino acids from carrying a positive charge, whereas the carboxyl groups remain underivatized. In addition, low-polarity groups are incorporated into the amino acid structure, which helps to improve the retention of amino acids on the RPLC column. In LC-UV/VIS, such derivatizations enable the detection of proteinogenic amino acids.

In LC-MS, underivatized amino acids have been successfully separated and detected in IEC [93], in RPLC [48] and in HILIC [94] modes followed by ESI in positive mode. Under these conditions, the loss of formic acid, water, ammonia or CO_2 is monitored in MRM mode. However, the selectivity and sensitivity of these methods are often low because the molecular masses of most amino acids lie in the range of unspecific signals derived from a sample matrix or from mobile phase constituents. Moreover, the small size of amino acids limits the selection of specific and suitable MRM transitions.

Therefore, derivatization of amino acids with bulky derivatization reagents improves the selectivity and the signal-to-noise ratio.

Commonly used derivatization reagents such as FMOC have also been applied to the separation of amino acids by LC-MS/MS. Because FMOC binds to the amine group of an amino acid and the carboxylic group dissociates, the amino acid carries a negative charge and must therefore be ionized in negative ESI. After collision-induced dissociation (CID) of derivatized species in a collision cell, the most intense ion is predominantly created by cleavage of the bond between an amino acid and a derivatized group, i.e., FMOC (**Fig. 7**) [95].

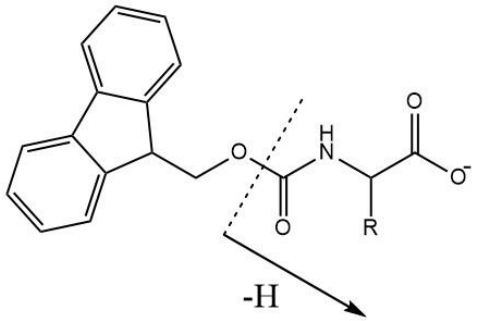

Fig. 7 Main fragmentation pathway of FMOC-amino acid in collision-induced dissociation

Sometimes, the amino group-modified amino acids are analyzed by LC-MS/MS. Consequently, the amino groups are not available for derivatization. When increasing the sensitivity and selectivity through derivatization of such modified amino acids, the carboxyl groups of amino acids remain the only site for potential derivatization. Thus, new derivatization approaches have been developed using 1-bromobutane [96] and 1-chlorobutane [97], which derivatize the amino and carboxyl groups of amino acids. However, when using 1-chlorobutane, monobutylated amino acids are the most abundant species (**Fig. 8**). In contrast to the aforementioned derivatizations, esterification with 1-chlorobutane requires only the derivatization reagent and no other additive, such as salts or buffers; moreover, the derivatization process is faster (7.5 min at 60°C) than other conventional derivatizations.

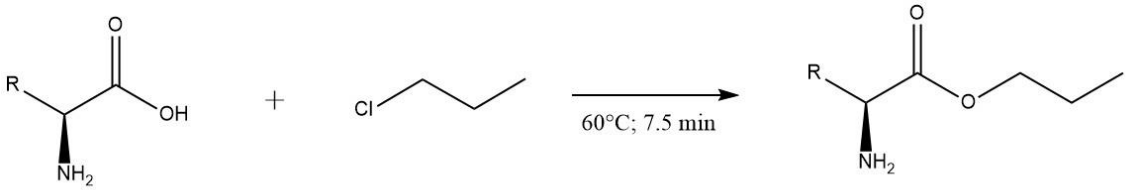

Fig. 8 An amino acid combines with 1-chlorobutane to form the butyl ester of the corresponding amino acid

In SFC-MS, similarly to LC-MS, amino acids have been separated in both underivatized and derivatized forms. However, in SFC-MS, amino acid polarity is a limiting factor not only in the separation process but also in ionization by ESI. Surprisingly, SFC-MS/MS has already been used to separate underivatized amino acids using methanol as a co-solvent and adding water and ammonium formate to increase the solubility of amino acids in a CO_2-rich mobile phase [98]. However, such a method had low sensitivity, especially to Gly and *L*-Ser; moreover, amino acids showed asymmetric peaks. Therefore, they were derivatized for SFC-MS using conventional derivatization reagents such as FMOC [86,99] and PITC [100] but only in connection with UV/VIS detectors.

In *Study II*, we developed an UPSFC-MS/MS method for the quantification of proteinogenic amino acids in human plasma. To decrease amino acid polarity, amino acids were derivatized with 1-chlorobutane, as shown in **Fig 8**, predominantly producing monobutyl esters of amino acids. Accordingly, the ClogP value of *L*-His, the most polar amino acid, increased from –3.73 to 0.16, whilst the ClogP value of *L*-Phe increased from -1.56 to 2.57. Derivatization significantly improved the separation efficiency and sensitivity for all 19 proteinogenic amino acids. Notwithstanding, the experiments with both underivatized and derivatized cysteine were highly unrepeatable, most likely due to sample preparation and derivatization, during which two cysteine molecules easily oxidize, forming cystine.

5.3 Mass spectrometry

J. J. Thomson laid the foundations of mass spectrometry in the early 20th century while studying cathode rays. Later, together with F. Aston, he constructed the first mass spectrometer to study ionic charge in gas phase. Ions were generated by gas discharge tubes and passed through electric and magnetic fields, which deflected their trajectories into parabolic shapes, detecting these ions on a photographic plate. From the 1940s, after improving the resolving power, MS was extensively used in industry to control production processes once the instrumentation became commercially available [101]. During the 1950s, MS was first coupled with gas chromatography using an EI source [102]. Since the MS source and analyzer had to be kept under very low pressures, an interface between GC and MS could be easily constructed for such setups.

In GC, the effluent is already in gas phase; thus, no evaporation is required, and the vacuum remains unchanged in MS. However, in LC-MS, the high volumes of liquid entering the MS source would cause loss of vacuum. Unsurprisingly, MS was coupled with liquid chromatography using the ionization technique applied in GC, i.e., EI, only two decades later.

Initially, the LC-MS interface using EI was constructed using two approaches: either splitting the column effluent and introducing low volumes of mobile phase (<1 μL/min) directly into the MS source or using an offline technique termed moving belt in which the solvent was evaporated from the collected LC effluent fractions outside the MS and then transported to an MS ion source from which the dry sample was desorbed. Subsequently, in the presence of volatile buffers in mobile phase, ions were generated without additional ionization. Unsurprisingly, the use of EI in LC-MS was discontinued once higher flowrates (1-2 mL/min) were achieved in MS, which led to the development of a thermospray (TSP) ionization source.

The TSP source consists of a heated capillary that helps the LC effluent to evaporate at very fast rates, thereby creating aerosols in the desolvation chamber. As a result, ions are formed through droplet fragmentation, ion evaporation, and ion desolvation and then enter the MS analyzer through a sampling cone. In addition, advances in TSP ionization instrumentation have led to the introduction of atmospheric pressure ionization (API) techniques such as chemical ionization at atmospheric pressure

(APCI), photoionization at atmospheric pressure (APPI) and electrospray ionization (ESI) in connection with liquid phase separation methods [103,104].

5.3.1 Electrospray ionization

The earliest electrospray experiments date back to 1914 when J. Zeleny studied discharges between the end of a capillary and a grounded electrode placed against the capillary tip. After applying a potential difference of several thousand volts between the capillary tip and the electrode, the liquid "sprayed" from the capillary tip [105]. Although the electrospray process had long been known, the first advanced electrospray experiments were performed by M. Dole in the 1960s to characterize high-molecular-mass synthetic polymers. For such purpose, he applied electrospray to generate gas-phase polystyrene ions, which were then collected with a Faraday cage detector [106]. After nearly two decades, electrospray was finally coupled with MS, which resulted in a soft ionization technique – ESI. Since then, such technique has been used for ionization of intact biopolymers, i.e., proteins, because it enables multiple charging and avoids in-source fragmentation [107].

Over the years, the robustness of ESI-MS in connection with liquid phase separation techniques prompted its wide spread use in the determination of small organic molecules [108]. In LC-ESI-MS, for instance, analyte molecules are carried in the mobile phase through a heated capillary with a metal tip, which is held at an electrical potential of several kV. Then, the mobile phase at the end of capillary is shaped into a Taylor cone, which emits a mist of solution droplets (**Fig. 9**). Moreover, the stability of the spray and the evaporation of the solution are assisted by a nebulizer gas (nitrogen) flowing coaxially around the capillary.

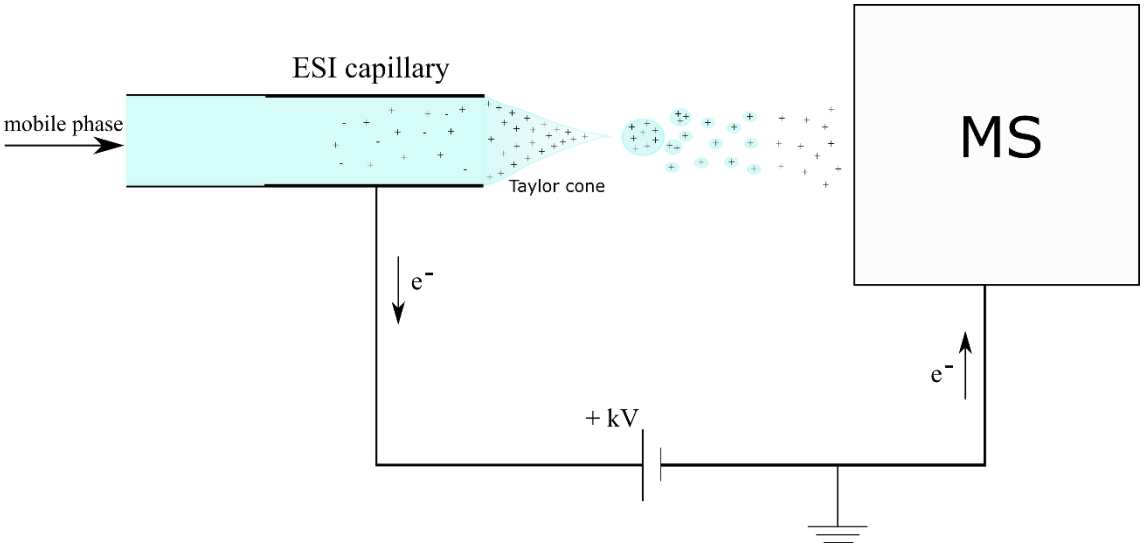

Fig. 9 Electrospray ionization in positive ion mode

In the positive ion mode, where the capillary potential is positive vs ground, the droplets have an excessive positive charge. The positive charge of droplets is mainly caused by protons generated at the surface of the metal capillary by oxidation of water, according to **Equation 3**. The ESI source acts as an electrochemical cell in which the electric current is mediated by ions in the spray and by electrons that flow from the capillary tip (anode) through the wiring to the mass spectrometer (cathode).

$$2H_2O \longrightarrow 4H^+ + O_2 + 4e^- \quad (3)$$

As shown in **Fig. 9**, the solvent from the Taylor cone is readily evaporated. Most liquid-phase separation methods use mixtures of water and an organic solvent, but the organic solvent evaporates faster than water; thus, the water content of the droplets gradually increases. As the droplet size decreases due to evaporation, the droplet charge density increases until the surface tension is offset by Coulombic repulsion. Such balance was termed a Rayleigh limit, as defined in **Equation 4**.

$$z_R = \frac{8\pi}{e}\sqrt{\varepsilon_0 \gamma R^3} \quad (4),$$

where z_R represents the charge number of elementary charges e, ε_0 is the permittivity of vacuum, γ is the surface tension and R is the radius of a droplet.

The droplet size decreases until the Rayleigh limit, producing smaller droplets of a few nanometers. From this point, gaseous ions are formed from highly charged nanodroplets. Three different models have been proposed for this process: the charged residue model (CRM), the chain ejection model (CEM), and the ion evaporation model (IEM).

On one hand, CRM and CEM describe the ionization of proteins in either globular (CRM) or unfolded (CEM) states. According to CRM, globular proteins remain in nanodroplets until the solvent is evaporated together with smaller ions. Conversely, CEM can be applicable in LC-MS separations of proteins because the mostly acidic mobile phase of LC causes protein unfolding. Consequently, hydrophobic chains of unfolded proteins migrate to the droplet surface and eject from Raleigh-charged nanodroplets.

On the other hand, IEM is applicable to small molecules/ions that enter ESI already in a charged state, usually due to analyte protonation. The IEM theory suggests that sufficiently high electric field of Rayleigh-charged nanodroplets causes the ejection of small solvated ions from the nanodroplet surface [109]. After the formation of gaseous ions, some of them pass through the orifice and then through the heated capillary until reaching the MS analyzer [110].

The ESI requires a constant flow of liquid, which helps to maintain the stability of the spray. In LC-ESI-MS, the spray is stabilized by the flow of the liquid mobile phase. However, in SFC-ESI-MS, the mobile phase predominantly consists of CO_2, which evaporates after passing through the back-pressure regulator. Thus, precipitation can occur when using a low percentage of co-solvent (<5 %); moreover, gradient co-solvent addition does not provide a constant flow of liquid through the ESI probe, thus requiring the addition of a post-column make-up solvent [111]. In *Study II*, we applied a T-junction for mixing the column effluent with 0.1% formic acid in methanol using an isocratic solvent manager. The flowrate of the solvent was kept at 0.2 mL/min, which ensured sufficient electrospray stability and method sensitivity.

5.3.2 Triple quadrupole analyzer

Generally, mass analyzers serve as ion filters that transmit only ions of selected *m*/*z*. In electrospray ionization, gaseous ions produced in the ion source are then

transported via ion optics to the coupled mass analyzer. In this research, we used a quadruple analyzer; thus, we will specifically focus on this type of mass analyzers.

The first quadrupole analyzer was described in the 1950s. Although some changes have been introduced since then, the main principles of the functioning of these setups have remained unchanged over time. Such quadruple analyzers consist of four parallel electrical rods; in this setup, a DC potential is applied to two of the rods whilst the other two rods are linked to an alternating radio-frequency potential. Under these conditions, potential, polarity and frequency change at fast rates. When ions are guided into a quadrupole analyzer, some ions gain unstable trajectories and collide with one of the rods. However, ions of selected *m*/*z* follow stable trajectories and pass through the analyzer until reaching the detector [112].

In a single quadrupole analyzer, ions within a *m*/*z* range can be filtered using a full scan (FS) mode. Alternatively, only ions of a specific *m*/*z* value can be analyzed using selected ion monitoring (SIM). Nevertheless, tandem mass spectrometry (MS/MS) should be used when detection selectivity and sensitivity are low. MS/MS combines several serially connected mass analyzers. For example, combining three quadrupoles in a series improves the sensitivity and selectivity of the method primarily by enabling a new mode of operation – multiple reaction monitoring (MRM). In MRM, first, the quadrupole filters the selected ion (precursor); then, the precursor is fragmented in the second quadrupole, and the product ions are filtered through the third quadrupole. Importantly, the fragmentation process is controlled by collision-induced dissociation (CID) in the second, RF-only quadrupole. During CID, the precursors gain an excess of internal energy, which leads to their fragmentation, through collision with neutral molecules (N_2 or Ar) in the second quadrupole, which serves as a collision cell [113].

In *Study II*, we used a Waters Xevo TQ-S mass spectrometer with a triple quadrupole analyzer (QQQ). When ions are sampled into a mass analyzer from an atmospheric pressure ion source, a significant amount of gas containing neutral molecules also enters the vacuum system. However, the Waters Xevo TQ-S mass spectrometer uses off-axis StepWave technology to filter ions from neutrals before they enter the first quadrupole, which helps to focus the ions and improves the signal-to-noise ratio of the scanned ions. After the ions are scanned through the first quadrupole, they enter the RF-only quadrupole – collision cell – where ions are fragmented and transported to the third

quadrupole using traveling wave ion guides that help to maintain sensitivity even at shorter dwell times. Lastly, product ions are scanned in the third quadrupole, which is constructed similarly to the first quadrupole.

5.3.3 Detector

Historically, the first MS instruments used photographic plates as ion detectors. Once the ions pass through the MS analyzer, they impact on the photographic plate. All ions of the same *m*/*z* impact on the same spot of a photographic plate, which causes the spot to darken, and the intensity of a specific *m*/*z* is proportional to the darkness of the spot.

Currently, 3 types of detectors are commonly used in MS instruments: Faraday cup, electron multiplier and photomultiplier. The Faraday cup detector consists of a hollow electrode on which ions strike, thus inducing an electric current that is amplified and measured. Faraday cup detectors are used when measuring high ion currents. However, electron multiplier and photomultiplier detectors are used to detect lower ion currents. Electron multiplier detectors contain either a known number of serially connected dynodes or a continuous dynode. In principle, ions from an MS analyzer hit the dynode surface, which causes secondary electron emission from the dynode surface. The electrons multiply by repeated impacts on the dynode surface until they reach the output electrode [114].

In *Study II*, a photomultiplier detector was used. This detector follows principles similar to those of electron multiplier detection. The ions pass through an MS analyzer and hit a dynode, which emits electrons. Subsequently, the electrons are deflected onto a phosphorous screen that emits photons, which are then multiplied in a photomultiplier tube. In contrast to an electron multiplier, the photomultiplier tube can remain sealed under vacuum, which prevents detector contamination and extends its lifetime [115].

STUDY II - A novel sensitive supercritical fluid chromatography-tandem mass spectrometry method for analysis of proteinogenic amino acids

Hodek O, Bergquist J, Ubhayasekera K.

Manuscript

A novel sensitive supercritical fluid chromatography-tandem mass spectrometry method for analysis of proteinogenic amino acids

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Abstract

Ultra-performance supercritical fluid chromatography-tandem mass spectrometry (UPSFC–MS/MS) is an alternative method for proteinogenic amino acid (AA) analysis. Moreover, AA profiling is a powerful bioanalytical technique in biomedical research. In the present study, we developed an analytical method for AA analysis in 6 min. The carboxyl group of AA was derivatized with 1-chlorobutane to AA butyl ester, which increased AA hydrophobicity and basicity. Derivatized AAs were analyzed by UPSFC coupled with electrospray ionization tandem mass spectrometry (ESI^+-MS/MS). The method was robust, selective and adequately sensitive after derivatization of AAs to butyl esters in 10 μL human plasma/blood. Under optimized conditions, the AAs were determined by multiple reaction monitoring (MRM). The linearity of the method was acceptable, with correlation coefficients (R^2) ranging from 0.9954 to 1.0000 and with calibration ranging from 5 to 7,000 nmol/L. The precision of the method, expressed as coefficient of variation (CV), was lower than 15 % for all amino acids. Analyte recovery was higher than 80 %. The novel method was successfully applied for the determination of AAs within 6 minutes. Therefore, this method can be used for both research and routine healthcare practices.

Keywords: Amino acids, derivatization, mass spectrometry, supercritical fluid chromatography

Introduction

Amino acids (AAs) play an irreplaceable role as the main building blocks in proteins and peptides as well as in purine and pyrimidine synthesis. While only 20 AAs are found in proteins, more than 100 AAs occur in body fluids [1]. In fact, some hormones are also derived from AAs such as tyrosine is converted to epinephrine, and tryptophan acts as a precursor to the neurotransmitter serotonin, the hormone melatonin and vitamin B3 [2]. Recent studies have shown the positive association between increased circulating branched chain AAs (valine, leucine, and isoleucine) and insulin resistance providing prognosis for the onset and progress of type

2 diabetes [3]. Moreover, sulphur-containing amino acids such as cysteine help to maintain the redox potential in the cellular environment and also participate in stabilizing the tertiary structure of the protein molecule by forming disulphide bridges [4]. AAs have been implicated in a number of diseases such as neurodegenerative, metabolic and endocrine diseases and implicated in many physiological effects. Therefore, there has been a considerable importance to develop analytical methods for identification and quantification of AAs in the clinical environment.

AAs have been previously separated by capillary electrophoresis (CE), gas chromatography (GC) and liquid chromatography (LC) [5-11]. The separation techniques are well established without or with derivatization of AAs [12]. In fact, several chemical derivatization methods have been developed to improve the sensitivity of the MS detection response. Uutela et al. have reported that after comparing three different derivatization methods of AAs by LC–MS/MS, the butyl and propyl chloroformate AA derivatization methods were the best among them, even at lower concentrations in nM levels, and provided detection limits of more than 2 to 10-fold lower than underivatized AAs [13].

Supercritical fluid chromatography (SFC) with packed columns has been used even in the early 90's as an alternative separation technique to LC or GC analysis. In recent past, supercritical CO_2 ($scCO_2$: CO_2 above its critical temperature and pressure) is used as the nonpolar mobile phase A, where the second mobile phase (B) needs to be a polar organic solvent such as methanol with polar additives (acid and/or base) to obtain a better resolution for polar AAs. The major benefits of $scCO_2$ are low viscosity and high diffusivity, that facilitate advanced throughput and higher resolution across a wide polarity range with faster elution. Thus, resistance to mass transfer and generated back pressure are substantially lower in SFC than in LC separations. Therefore, the same separation efficiency can be expected in SFC at higher flow rates than in LC [14]. Analysis of rather polar or even ionic analytes such as AAs by SFC requires a polar mobile phase and a polar stationary phase for obtaining the best separation efficiency and selectivity. Addition of water to a co-solvent up to 5% (*v*/*v*) enhances the overall solvating power of the mobile phase and its hydrogen-bonding capacity, which improves peak symmetry [15]. Water as an additive proved to be suitable for separation of polar nucleobases and amino acids because water enhances solubility of polar or ionic analytes

in the mobile phase [16,17]. Moreover, addition of water improves peak shape of such compounds – hydrogen bond acceptors. Although, supercritical fluid chromatography with tandem mass spectrometry (SFC–MS/MS) was already used for separation of underivatized amino acids [18]; validation parameters such as linear dynamic range, limit of detection (LOD) and limit of quantification (LOQ) have not been studied.

The goal of this study was to find a fast screening method to analyze free *L*-AAs which are incorporated in human protein synthesis. Therefore, we have developed an SFC and positive electrospray ionisation tandem mass spectrometry (ESI^+-MS/MS) method for the analysis of AAs after their derivatization into butyl esters (**Figure 1**). Derivatized AAs were quantified using a suitable derivatized isotopically labeled internal standard mixture of AAs.

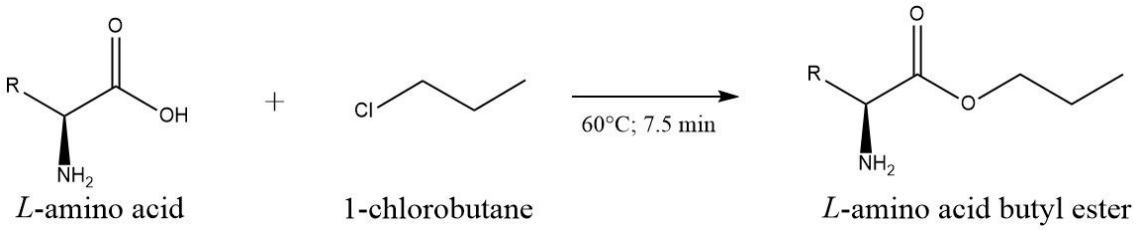

Fig. 1 Derivatization of *L*-amino acid with 1-chlorobutane to coreresponding AA butyl ester

Materials and methods

Reagents and chemicals

L-Alanine (*L*-Ala), *L*-Arginine (*L*-Arg), *L*-Asparagine (*L*-Asn), *L*-Aspartic acid (*L*-Asp), *L*-Glutamic acid (*L*-Glu), *L*-Glutamine (*L*-Gln), *L*-Histidine (*L*-His), *L*-Isoleucine (*L*-Ile), *L*-Phenylalanine (*L*-Phe), *L*-Proline (*L*-Pro), *L*-Serine (*L*-Ser), *L*-Threonine (*L*-Thr), *L*-Tryptophan (*L*-Trp), *L*-Tyrosine (*L*-Tyr) and *L*-Valine (*L*-Val), *L*-Cysteine (*L*-Cys), *L*-Homocysteine (*L*-Homocys), Glycine (Gly), *L*-Leucine (*L*-Leu), *L*-Lysine (*L*-Lys), *L*-Methionine (*L*-Met), 3 M HCl in *n*-butanol and ammonium formate (LC–MS grade) were obtained from Sigma-Aldrich (Stockholm, Sweden). *L*-Met-D3, *L*-Ala-D4 and *L*-Gln-D5 were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). All solvents and reagents were of LC–MS grade and purchased

from Fisher Scientific (Gothenburg, Sweden) unless otherwise stated. Deionized water was supplied by a Milli-Q purification system (Millipore, Bedford, MA, USA). Phosphate buffered saline (PBS), which consisted of 12 mM phosphate buffer, 2.7 mM potassium chloride and 137 sodium chloride of pH 7.4, was purchased from VWR (Stockholm, Sweden). Carbon dioxide (99.99%) was supplied by AGA, industrial gasses (Lindingö, Sweden)

The human plasma sample from a healthy blood donor (healthy control plasma) was obtained from the Academic Hospital ("blodcentralen UAS"), Uppsala, Sweden. Blood was collected from each participant by venepuncture into EDTA vacutainer tubes and centrifuged at 3,500g for 15 min. The study was conducted in accordance with the Declaration of Helsinki. Human plasma samples were included in this study, which was approved by the Regional Ethical Review Board of Uppsala, Sweden, and undertaken with the written consent of the individual donors.

Preparation of stock solutions

All *L*-amino acid standards were dissolved in 1 mM hydrochloric acid to obtain 5 mM solutions. Moreover, the *L*-Cys solution contained 3 mM *L*-ascorbic acid to prevent its auto-oxidation. All stock solutions were stored at −80°C until further use.

Sample preparation

The human plasma (10 µL) samples were mixed with 200 µL MeOH and 50 µL of deuterated IS mixture (100 ng mL^{-1} in MeOH). It was then gently vortexed for 5 min and allowed to stand for 10 min at room temperature. The samples were centrifuged at 12,000g for 3 min at 4 °C. The MeOH extracts were dried under nitrogen and reconstituted in 50 µL of MeOH.

Derivatization of AAs

The extracted AAs were derivatized to amino acid butyl esters [19]. In brief, 100 µL of 3 M HCl in *n*-butanol was added to the dried sample and incubated at 60 °C for 7.5 minutes. Eventually, the incubated mixture was dried under nitrogen and redissolved in 50 µL of MeOH prior to the SFC–MS/MS analysis.

Separation of AAs by supercritical fluid chromatography (SFC)

This study was conducted using an Acquity supercritical fluid chromatography system (UPC^2) (Waters, Milford, MA, USA), equipped with a binary solvent delivery pump, an autosampler, and a column oven. SFC was connected with the mass spectrometer by the commercial interface kit (Waters) composed of two T-pieces enabling the back pressure control and post column infusion of a make-up solvent.

The preliminary column screening was performed using fixed initial chromatographic conditions, where CO_2 (99.99%) was the mobile phase A and MeOH with 10 mM ammonium acetate was the mobile phase B. Gradient elution was started at 5% B with a flow rate of 1.5 mL min^{-1} (except for the DIOL and 1-AA columns where the flow rate was set at 1.2 mL min^{-1}). Mobile phase B was increased linearly to 30% over 0.2–7.5 min before returning to the initial conditions in 8 min. The column oven, back-pressure, and make-up flow were set at 40 °C, 1,500 psi (10.3 MPa), and 0.2 mL min^{-1} respectively.

We studied six different column materials to find the best stationary phase for the separation of AAs: HSS C18 SB column (3 mm × 100 mm, 1.8 µm), BEH column (3 mm × 150 mm, 1.7 µm), DIOL column (3 mm × 150 mm, 1.7 µm), 2-PIC column (3 mm × 150 mm, 1.7 µm), FP column (2.1 mm × 100 mm, 1.7 µm) and 1-AA column (2.1 mm × 100 mm, 1.7 µm). All columns were purchased from Waters (Milford, MA, USA).

The optimized quantitative analysis was performed using a BEH column at 40 °C. The mobile phase consisted of (A) carbon dioxide and (B) 20 mM ammonium formate in methanol:water (95:5, *v*/*v*). The separation process was conducted at a flow rate of 1.2 mL min^{-1} with the following gradient: 0–3 min, 15–40% B; 3–5 min 40% B; 5–6 min 40–15% B. The back pressure was kept constant at 1,600 psi (11 MPa). The make-up

solvent consisted of 0.1% (*v*/*v*) formic acid in methanol and was mixed with column eluate at 0.2 mL min^{-1} and held for 1.0 min for equilibration before the next injection.

Identification and quantification of AAs by tandem mass spectrometry (XEVO® TQ-S)

Derivatized AAs were identified by using a Waters Xevo® TQ-S mass spectrometer (Milford, MA, USA). The data acquisition was in the positive electrospray ionisation (ESI^+) mode with unit mass resolution. The desolvation gas was nitrogen, and the collision gas was argon (0.17 mL min^{-1}). The data acquisition range was *m*/*z* 50–500. The capillary voltage was 2.3 kV and the source offset was 30 V. The source temperature was 150 °C and the desolvation temperature was 400 °C with the desolvation gas flow rate of 800 L h^{-1}. The collision energy and cone voltage values were optimized for each compound to generate the most abundant product ions to construct the multiple reaction monitoring (MRM) method (**Table 1**). The cone gas flow rate was 150.0 L h^{-1}. The nebulizer gas flow pressure was maintained at 7.0 bar. Data were acquired and analyzed with Waters MassLynx™ 4.1 software (Waters, Milford, MA, USA).

Table 1 Retention times (t_R), collision energy (CE, in eV) values in brackets, and cone voltage (CV) for derivatized amino acids

Amino acid	t_R [min]	Precursor	Quantifier (CE)	Qualifier (CE)	CV [V]
L-Gly	1.5	132	76 (13)	-	25
L-Ala	1.4	146	44 (8)	57 (10)	30
L-Arg	4.7	231	70 (15)	214 (15)	25
L-Asn	2.4	189	144 (10)	87 (10)	20
L-Asp	3.5	190	88 (10)	144 (10)	40
L-Cys	1.3	178	82 (15)	132 (10)	20
L-Glu	5.1	204.4	84 (15)	130 (5)	40
L-Gln	2.6	203	130 (10)	84 (10)	25
L-His	3.9	212	110 (15)	156 (10)	40
L-Ile	1.0	188	86 (10)	-	30
L-Leu	1.0	188	86 (10)	-	30
L-Lys	2.6	203	84 (10)	186 (10)	25
L-Met	1.1	206	104 (10)	56 (10)	25
L-Phe	1.1	222	120 (5)	166 (10)	25
L-Pro	1.3	172	70 (15)	57 (15)	25
L-Ser	1.9	162	60 (10)	106 (10)	25
L-Thr	1.6	176	74 (10)	102 (10)	25
L-Trp	2.0	261	244 (10)	159 (15)	25
L-Tyr	1.9	238	136 (10)	221 (10)	25
L-Val	1.0	174	72 (10)	-	25
L-Ala-D4	1.4	150	48 (8)	57 (10)	30
L-Met-D3	1.1	209	107 (10)	56 (10)	25
L-Gln-D5	2.6	208	135 (10)	89 (10)	25

Method validation

The method was evaluated for limit of quantification (LOQ), selectivity, sensitivity, precision, linearity and recovery. Slightly modified guidelines of the EU Commission Decision/657/EEC were implemented during method validation. Phosphate buffered saline (PBS) was used as matrix for calibration and quality control (QC) samples owing to the unavailability of AA free plasma. A total of seven calibrators were prepared in PBS with AA concentrations ranging from 5–7,000 nM. The concentration of internal standards after sample dilution was as follows: 15 nM for *L*-Met-D3, 100 nM for *L*-Ala-D4 and 30 nM for *L*-Gln-D5. Blank and double blank (blank without IS) were also

included while preparing the calibrators. The linearity was evaluated using calibration curve. Linear regression with $1/x^2$ weights and precision were evaluated by running a batch of QC samples. Precision and recovery of the method were evaluated at three concentration levels – low, medium and high, which corresponded to concentration at LOQ, at 50% of the calibrated concentration range and at 80% of the upper calibration curve range, respectively. The precision was expressed as coefficient of variation (CV %) of measurement, which should be within ± 15%. Recovery experiments were performed by comparing the analytical results for extracted samples from PBS at three concentrations with unextracted standards that represent 100% recovery.

Results and discussion

Derivatization of AAs

AAs were derivatized into their butyl esters to increase the detection sensitivity [19]. The butyl esters of AAs are required to force a cationic character upon the amino acids in order to obtain similar ionization efficiencies for all the AAs whether they are acidic, neutral or basic. The optimized conditions for derivatization are listed in the *Materials and methods* section. The carboxyl functional groups of amino acids were derivatized with 1-chlorobutane prior to analysis in order to decrease the polarity of AAs (**Figure 1**).

As a result, the separation efficiency and method sensitivity improved for all studied AAs by giving the greatest response to any amino acid that shows hydrophobicity. The butylation of the acidic group attached to AA allows for an initial formation of the 86+ product ions. The MS/MS fragmentation of underivatized amino acids usually forms smaller ions, which cannot be used to differentiate between the amino acids. However, the derivatization reagent, which was mixed with the sample directly, provides more abundant characteristic ions.

SFC–MS/MS method development for derivatized AAs

The initial effort was to develop an SFC–MS/MS method to determine AAs. The most important parameter affecting the retention of the analytes was the chemistry of the stationary phase and organic co-solvent. Different SFC columns, with known chemistry of the stationary phases, were available for selecting the best column for the present study. The overall chromatographic resolution and separation efficiency of the derivatized *L*-AAs provided a basis for the selection of column. In this study, the tested columns were: HSS C18 SB, BEH, DIOL, 2-PIC, FP and 1-AA column. The chemistry of the selected stationary phases is shown in **Figure 2**.

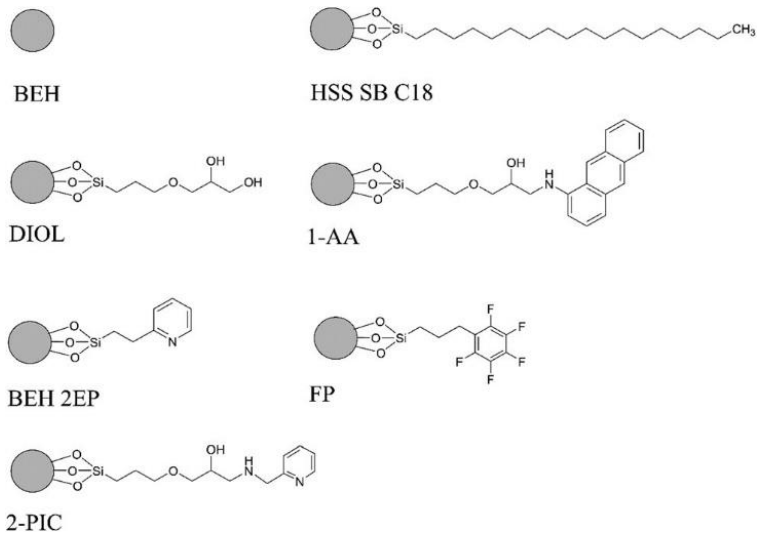

Fig. 2 The different stationary phases tested on supercritical fluid chromatography (SFC)

After stationary phase screening for derivatized *L*-AAs, the best overall separation efficiencies were obtained with the BEH column, providing substantial retention for all AAs and following HILIC-like mode. Leu and Ile were co-eluting and weak sensitivity was observed for His. The other tested columns produced a similar elution order to the selected BEH. The lowest retention of AAs was observed on the HSS C18 SB column which indicates the need of electrostatic attraction between carboxylic/amine groups of AAs and the functional group of the stationary phase for the retention. Nevertheless, all tested columns (except for the DIOL, FP and BEH) could provide suitable separation of

Leu and Ile. However, the highest separation efficiency for all amino acids and the shortest analytical time were achieved by using the BEH column.

When using the BEH column, the separation of derivatized AAs is governed by their polarity, i.e., retention times are higher for more polar AAs. By definition, amino acids contain an amino group and a carboxyl group, and often contain another functional group (e.g., sulfhydryl, hydroxyl, or secondary amino or carboxyl group). In fact, the polarity of those AAs changes based on additional functional group. Separation selectivity of AAs is given by the nature of used mobile phase starting from normal-phase-like conditions of nonpolar carbon dioxide with very low concentration of the polar modifier at the beginning of the gradient (15%) changing up to HILIC-like conditions with high concentration of the modifier (40%) containing 5% of water and 20 mM ammonium formate.

The retention pattern of AAs is also affected by a charge of the AA polar group demonstrated by poor separation efficiency of AA containing a very polar group, which is retained well on the SFC column. The retention order of the AA was as follows: Ile+Leu, Val, Phe, Met, Pro, Ala, Gly, Thr, Ser, Tyr, Trp, Asn, Gln, Lys, Asp, His, Arg, Glu. The basic amino acids (Lys and Arg) were the two positively charged amino acids in the AA mixture when separated in CO_2/MeOH/H_2O with addition of 0.1% formic acid and 20 mM ammonium formate. Between these two compounds, the high pK_a (13.8) of Arg results from delocalization of the positive charge within the π-bond of the side chain; the Arg side chain remains protonated and eluted with a split peak. This contrasts with Lys, where the charge is largely focused on the terminal aliphatic amino group and the side chain is readily deprotonated and resulted in a symmetric peak. Aromatic amino acids are amino acids that include an aromatic ring. Trp, Phe and Tyr were eluted between 1-2 min and have slightly better separation efficiency because they contain an aromatic ring of low polarity that can be easily protonated in the ESI mode compared to the aliphatic AAs, thus sensitivity is also higher in contrast to aliphatic AAs. The concentrations of additives (acids or bases) in the co-solvent increase the solubility of analytes and provide coverage of the active sites on the stationary phase, resulting in a beneficial effect on the peak shape as well as mass spectrometric sensitivity.

Therefore, we tested different gradient programs and additives (0.1%, 0.2% and 0.5% formic acid, 5% H_2O as well as 10 mM and 20 mM ammonium formate) to improve

the on-column separation and peak shape of the analytes. We observed that 5% H_2O together with 20 mM ammonium formate as modifier in MeOH enhanced the chromatographic separation (**Figure 3**). Moreover, derivatization of individual amino acids increased their hydrophobicity, thus enhancing retention on a BEH column. Analysis of derivatized AAs resulted in chromatograms with a stable baseline, sufficient peak resolution, and excellent peak symmetry for all AAs except for those eluting last (**Figure 3**). The optimized method has been described in more detail in the *Materials and methods* section.

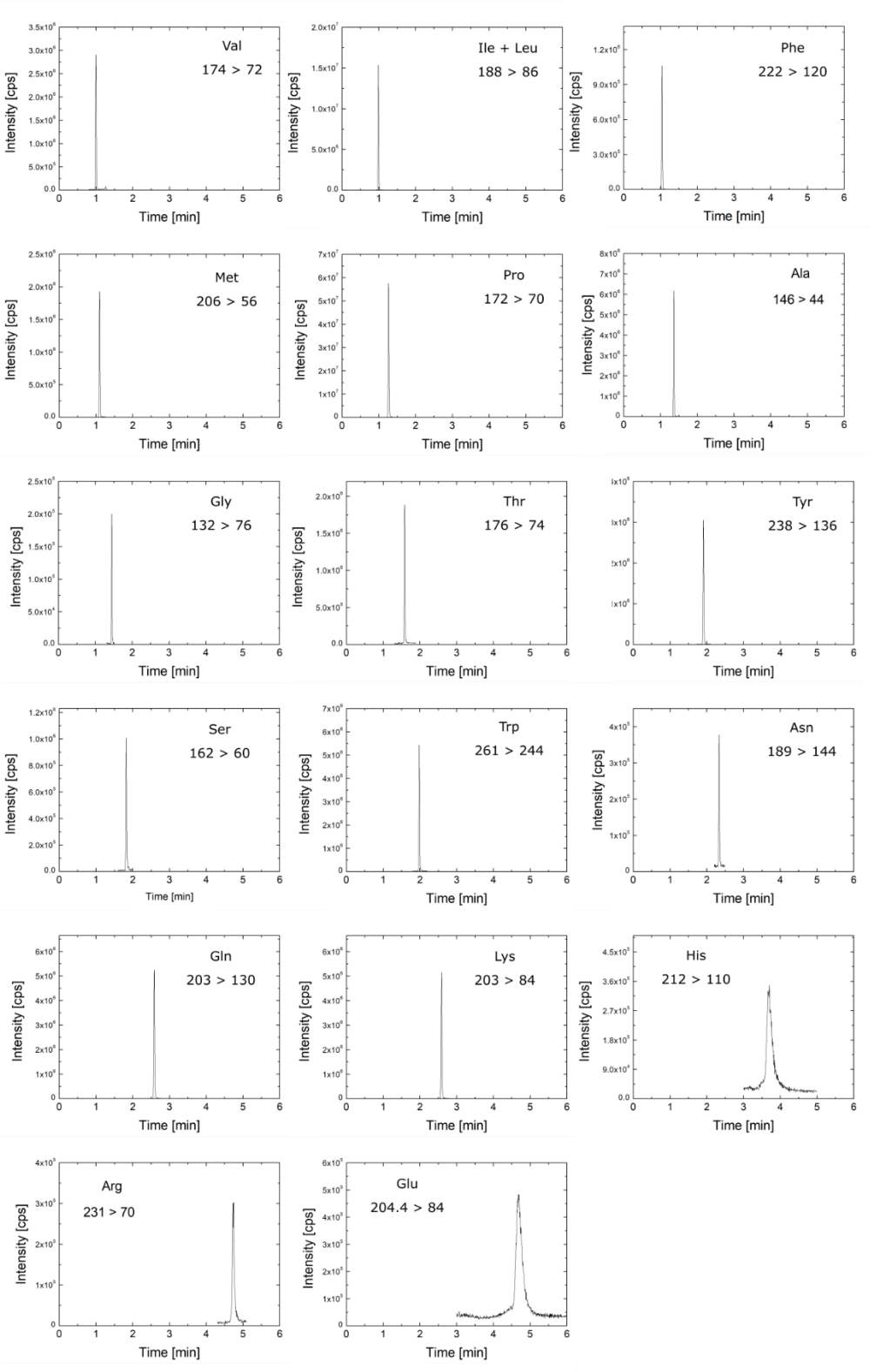

Fig. 3 Chromatograms of *L*-amino acid butyl esters extracted from human plasma. Separation conducted using a BEH column under optimized conditions. Mobile phase: CO_2 + MeOH:H_2O (50/50 v/v) with 0.1% formc acid and 20 mM ammonium formate

All the AAs contain a carboxylic group, where derivatization occurs. Then, abundant protonated ions $[M + H]^+$ are formed during the positive electrospray ionization (ESI^+) step. The ions enter the analyzer and once inside the analyzer, each amino acid's molecular ions are selected with the first mass analyser (Q1), fragmented in the Q2 region and the product (fragment) ions are analyzed by the final mass analyser (Q3). During fragmentation the charge generally resides on the moiety with the highest proton affinity; therefore, the free amino group(s) will retain the charge and fragmentation will proceed losing stable neutrals. Most amino acids, and certainly all neutral and acidic ones, fragment losing butyl formate, representing a "neutral loss" of 102 Da. The major fragmentation pathways involve the loss of fragments from the butyl ester end of the molecular ion. The MRM approach is the most commonly used mode in the quantification of targeted derivatized AAs. To select a proper transition for the MS/MS detection of an analyte, two factors should be considered: first, the transition should be specific to this compound; second, the product ion should be abundant enough for sensitive detection. In order to enhance the selectivity of the target analyte, product ions were chosen with the most abundant product ion selected as the quantifier and the other ion as the qualifier by direct infusion of each of the AA analytes and by investigation of the product mass spectra of the 20 AAs under positive ion mode.

Method validation

Method validation results are listed in **Table 2**. The optimized method was validated for different parameters. The linearity, LOQ, precision and recovery of the method were determined. The calibration curve comprised of seven points prepared in triplicates. The linearity was determined by linear regression analysis. The correlation coefficients (R^2) of the calibration curves were >0.9940. The back calculated concentration of the calibration samples was within ±12% of the nominal value.

Table 2 Validation parameters for derivatized AAs

Amino acid	LOQ [nM]	Linear range [nM]	Recovery [%]	Precision [CV %]		
				low	medium	high
L-Gly	10.0	70-7000	87.3	10.0	4.7	8.0
L-Ala	4.0	70-7000	92.4	5.0	3.6	1.3
L-Arg	70.0	70-7000	82.6	7.1	5.4	5.4
L-Asn	1.5	5-900	92.1	5.8	8.3	9.3
L-Asp	15.0	70-7000	80.2	9.2	10.9	14.1
L-Glu	12.0	70-7000	83.7	2.9	14.5	8.8
L-Gln	1.5	5-900	80.1	8.8	6.4	12.6
L-His	12.0	70-7000	90.6	3.3	6.2	4.3
L-Ile + *L*-Leu	1.0	5-1000	-	4.0	10.1	8.1
L-Lys	1.8	5-900	85.8	3.3	12.8	2.9
L-Met	1.0	5-1000	88.4	12.0	5.0	1.3
L-Phe	0.8	70-7000	90.4	6.7	4.8	3.6
L-Pro	1.0	5-900	93.4	4.3	6.6	4.7
L-Ser	3.5	70-7000	92.3	5.1	5.6	3.6
L-Thr	1.0	70-7000	83.5	5.4	11.4	2.8
L-Trp	1.0	5-1000	92.4	9.6	1.7	3.0
L-Tyr	1.0	5-1000	88.1	7.4	3.7	9.8
L-Val	2.5	70-7000	90.4	2.6	9.7	0.9

The linearity range obtained from this study was comparable to already published LC-MS results. LOQs ranged from 5 to 70 nM for all derivatized AAs, which are comparable or better as compared to already existing LC-MS/MS methods for similar analytes (**Table S1**). Precision was assessed by replicate analysis ($n = 4$) of spiked plasma samples at three different concentrations and data are presented in **Table 2**. The precisions were between 0.9% and 14.5% for all the AAs. The recovery was determined based on comparison of extracted vs unextracted AAs from spiked PBS solution, where unextracted AAs were considered as 100 % recovery. The recovery of all AAs ranged from 80.1 to 93.4%.

Conclusions

We have developed a novel, simple, rapid, and sensitive UPSFC–MS/MS method for profiling of intracellular and extracellular free *L*-amino acids (AAs). To the best of our knowledge, there are no previously published SFC–MS/MS data for comparison to our method. AAs are amphoteric in nature and their ionization efficiencies by ESI are relatively low. The analytical method applied in this study provided satisfactory results for simultaneous determination of free AAs in smaller or scarce amounts of plasma after derivatizing the AAs into butyl esters. This method provided better sensitivity and comparably shorter analytical time (6 min). Currently, the only known negative characteristic of this technique is the inability to obtain an adequate resolution for Leu and Ile when compared with published method [20]. The problem with the methodology is the fact that isomeric amino acids, such as leucine and isoleucine, often fragment very similarly, thus determination of Leu and Ile requires their chromatographic separation. Moreover, determination of *L*-Cys provided poor repeatability and recovery because of its oxidation during sample extraction and derivatization.

Nevertheless, the separation capacity of UPSFC is much higher than other chromatographic techniques due to the high diffusivity of the supercritical CO_2. According to the Van Deemter curve, supercritical CO_2 maintains a low height equivalent to a theoretical plate (HETP) at a relatively high flow rate; therefore, the analysis time can be shortened. Thus, the addition of an organic solvent as a modifier (e.g., methanol) expands the selection ability for elution.

Amino acids were successfully derivatized by reaction with 1-chlorobutane, which formed butylated amino acids with the butyl group bonded to the carboxylic groups of the amino acids. Then, optimal separation peak shapes and sensitivities for all amino acids were achieved using the Viridis BEH as a stationary phase and methanol:water 95/5 (*v*/*v*) with 20 mM ammonium formate as a co-solvent. Subsequently, co-solvent gradient was optimized to shorten the analysis time, resulting in a 6-minute run. Lastly, method validation parameters, namely, LOQ, linearity, precision and recovery were determined using the developed method.

Results from method validation demonstrate that this method is sensitive enough to perform comprehensive analysis of AAs in large sample sets in clinical studies with limited sample volumes. Future work will be focused on using the method on a larger

human cohort and also on expansion of the method to analyze *L* and *D*-amino acids in biological samples at once.

Conflicts of interest

There are no conflicts to declare.

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Supporting material

A novel sensitive supercritical fluid chromatography-tandem mass spectrometry method for analysis of proteinogenic amino acids

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Table S1 Comparison of LOQ [μM] of the deveoped SFC method to conventional LC-MS methods

technique	New SFC method	LC-MS/MS [19]	LC-MS/MS [21]	LC-MS/MS [22]	LC-MS/MS [23]	LC-MS/MS [24]	LC-MS/MS [25]
Amino acid	derivatized	derivatized	derivatized	derivatized	underivatized	underivatized	underivatized
L-Ala	0.004		0.51	7.55	33.7		0.03
L-Arg	0.07		2.13	2.06	-		0.15
L-Asn	0.0015		0.69	0.84	-		0.67
L-Asp	0.015		0.33	-	22.6		0.26
L-Glu	0.012		1.52	1.49	20.4		0.35
L-Gln	0.0015		2.97	4.56	-		0.20
L-Gly	0.01		1.12	0.84	40.0		0.15
L-His	0.012		0.33	6.2	-		0.28
L-Ile +	0.001		0.17	0.39	-		0.05
L-Leu		> 1 μM	0.16	1.67	22.9	0.001-0.36	0.05
L-Lys	0.0018		0.39	5.14	-		0.09
L-Met	0.001		0.11	0.46	20.1		0.08
L-Phe	0.0008		0.21	1.73	-		0.05
L-Pro	0.001		0.77	2.44	-		0.11
L-Ser	0.0035		0.94	4.59	28.6		0.17
L-Thr	0.001		0.92	2.12	-		0.12
L-Trp	0.001		0.17	1.48	14.7		0.07
L-Tyr	0.001		0.14	3.39	16.6		0.09
L-Val	0.0025		0.09	3.26	-		0.07
Stationary phase	BEH	C8	C18	C18 Amino	HILIC	BEH amide	BEH C18
Run time (min)	6	16	9	8	4	18	12

6 COMPARISON OF CAPILLARY ELECTROPHORESIS AND SUPERCRITICAL FLUID CHROMATOGRAPHY IN DETERMINATION OF AMINO ACIDS

In this thesis, we compared capillary electrophoresis with supercritical fluid chromatography in the determination of proteinogenic amino acids. For this purpose, we used method performance characteristics such as separation efficiency and limits of quantification. We also compared the methods from economic point of view, i.e., the acquisition and operating costs, amount of time and work required.

6.1 Separation efficiency

The separation efficiency of the two methods was compared using height equivalent to a theoretical plate (*H*) as a parameter of separation efficiency. Initially, the number of theoretical plates (*N*) was calculated according to **Equation 5**.

$$N = 16\frac{t_R^2}{w^2} \quad (5),$$

where t_R is the retention or migration time of an analyte and *w* is baseline peak width. Following this, *H* was calculated using **Equation 6**.

$$H = \frac{L}{N} \quad (6),$$

where *L* refers to the column or capillary length. *H* was calculated for the first and last separated *L*-amino acids at concentrations corresponding to approx. 35% of the highest calibration point. In CE-C^4D separation, *L*-Lys was the fastest amino acid, whereas *L*-Asp migrated last. In UPSFC-MS/MS separation, the first amino acid to elute was *L*-Val, whereas the last amino acid to elute was *L*-Glu, which was strongly retained on the

stationary phase. Retention/migration times, together with *w*, *N* and *H* values, are listed in **Table 2**.

Table 2 Separation efficiency parameters of CE-C^{4}D and UPSFC-MS/MS

Technique	*L* [cm]	*L*-amino acid	t_R [min]	*w* [min]	*N*	*H* [μm]
CE-C^{4}D	66.5	Lys	9.87	0.139	80,535	8.3
		Asp	33.99	0.436	97,240	6.8
UPSFC-MS/MS	10.0	Val	1.00	0.042	9,070	11.0
		Glu	5.10	0.500	1,665	60.0

Table 2 shows that the CE method provides a higher number of theoretical plates due to the higher retention-time-to-peak-width ratio of the amino acid. However, when considering column length and calculating *H*, the separation efficiency of SFC becomes similar to that of CE for the first amino acid to elute. As the last amino acid to elute in SFC, the separation efficiency of *L*-Glu is compromised by exceptionally wide peak.

Unlike UPSFC-MS/MS, the CE-C^{4}D method enables baseline separation of all proteinogenic amino acids using simple instrumental setup but the analysis time reaches 40 minutes. However, in SFC, the separation efficiency does not play such a key role because MRM enables the detection of co-eluting analytes. Moreover, the UPSFC-MS/MS method is approx. 7× faster than the CE-C^{4}D method.

6.2 Limit of quantification

The LOQ values for amino acids achieved by CE-C^{4}D and UPSFC-MS/MS are compared in **Table 3**.

Table 3 Limits of quantification (LOQ) with respective migration, retention times and RSD of the migration/retention times of amino acids. LOQ values are listed in µmol/L for CE-C^4D and in nmol/L for UPSFC-MS/MS

	CE-C^4D			**UPSFC-MS/MS**		
L-amino acid	t_{mig} [min]	RSD [%]	LOQ [µmol/L]	t_R [min]	RSD [%]	LOQ [nmol/L]
L-Ala	17.2	0.60	100	1.4	0.20	4.0
L-Arg	10.4	0.34	70	4.7	0.13	70.0
L-Asn	25.0	0.86	120	2.4	1.12	1.5
L-Asp	34.0	1.04	230	3.5	1.69	15.0
L-Glu	26.8	0.91	110	5.1	1.53	12.0
L-Gln	26.0	0.88	150	2.6	0.80	1.5
L-Gly	15.1	0.53	100	1.5	0.91	10.0
L-His	10.6	0.35	60	3.9	1.07	12.0
L-Ile	21.3	0.76	80	1.0	0.78	1.0
L-Leu	21.9	0.78	90	1.0	0.78	1.0
L-Lys	9.9	0.31	60	2.6	0.98	1.8
L-Met	25.4	0.90	150	1.1	0.19	1.0
L-Phe	27.4	0.91	110	1.1	1.06	0.8
L-Pro	29.2	0.96	180	1.3	1.01	1.0
L-Ser	22.3	0.80	130	1.9	0.33	3.5
L-Thr	24.6	0.87	150	1.6	0.43	1.0
L-Trp	25.7	0.89	90	2.0	0.14	1.0
L-Tyr	28.5	0.95	100	1.9	0.30	1.0
L-Val	21.1	0.75	120	1.0	0.23	2.5

The UPSFC-MS/MS method enables the determination of amino acids in the nmol/L range. *L*-Phe and *L*-Arg represent the amino acids with the lowest and the highest LOQ values, respectively. In CE-C^4D, the LOQ values range from tens to hundreds of µmol/L. *L*-His and *L*-Lys represent the amino acids with the lowest LOQ values; on the contrary, *L*-Asp has the highest LOQ value.

The UPSFC-MS/MS method allows the determination of amino acids in concentrations four or five orders of magnitude lower than CE-C^4D.

6.3 Operating costs

All chemicals used for mobile phase preparation in UPSFC-MS/MS were of LC-MS grade, whilst those for CE-C^4D were of analytical grade. Prices of all chemicals such as acetic acid, HEC, methanol, ammonium formate and formic acid were provided by Sigma-Aldrich (St. Louis, MO, USA). Price of CO_2 of supercritical fluid grade was provided by Linde Gas a.s. (Prague, Czech Republic). Prices together with consumed amount of respective items are listed in **Table 4**.

Table 4 Chemicals with respective prices per unit used for BGE and mobile phase preparation. Amount consumed was calculated for 3,000 runs. Prices in CZK excluding VAT

Technique	Item	Price per unit	Amount consumed
CE-C^4D	acetic acid	1,380 per 1 L	125 mL
	hydroxyethyl cellulose	2,180 per 500 g	120 g
UPSFC-MS/MS	methanol	574 per L	7.1 L
	CO_2	8,200 per 40 L	14.8 L
	ammonium formate	2,380 per 25 g	8.6 g
	formic acid	3,000 per 50 mL	4.9 mL

Acquisition and operating costs are compared in **Table 5**. The operating costs were calculated as consumption of BGE, mobile phase constituents and gases needed for analysis of 1,000 samples, assuming that each sample was measured three times. Therefore, total number of runs considered was 3,000.

Nitrogen generator was included in the acquisition costs of UPSFC-MS/MS because of high nitrogen consumption in MS/MS. Using gas cylinders would be approx. twice as expensive than using nitrogen generator. Electricity and water consumption were not included in the calculations of operating costs.

Table 5 Acquisition and operating costs of CE-C^{4}D and UPSFC-MS/MS. The operating costs for 3,000 runs were calculated. Prices are listed in CZK excluding VAT

Costs	**CE-C^{4}D**		**UPSFC-MS/MS**	
acquisition costs including software	2,040,000		9,000,000	
capillary/column	3,520 (fused silica, 50 µm ID)*		20,700 (Viridis BEH)	
Nitrogen generator	-		387,000	
total acquisition costs	2,043,520		9,407,700	
operating costs	**BGE**		**Mobile phase**	
	HEC	520	methanol	4,070
	Acetic acid	170	CO_2	3,000
			ammonium formate	800
			formic acid	300
Total operating costs	690		8,170	

* one fused silica capillary of 80 cm was considered to last for 300 runs, therefore the price of 8 m capillary was calculated at a price of 440 CZK per m

Concluded from **Table 5**, initial costs of CE-C^{4}D are 88% lower than those of UPSFC-MS/MS. Similarly, CE-C^{4}D operates at significantly lower costs than UPSFC-MS/MS. Besides being economically less demanding, CE-C^{4}D can be operated by a less skilled operator. However, CE-C^{4}D requires 2,000 hours of instrumental time compared to 300 hours needed for measurement of 3,000 runs at UPSFC/MS/MS.

Unlike CE-C^{4}D, the UPSFC-MS/MS produces large amount of heated gas, thus operating this technique requires air conditioning of the laboratory environment.

7 CONCLUSIONS

This thesis describes method development of two different separation techniques and compares their separation efficiency, LOQ values and operating costs in determination of proteinogenic amino acids. The first study has been previously published in *Analytical and Bioanalytical Chemistry*. The second study was inserted in this thesis as a manuscript and it will be soon submitted.

The first study focuses on optimization of extraction step using DoE to increase extraction yield of amino acids prior to their determination by CE-C^4D in tobacco plants. The amino acids were separated using 0.1% HEC in 1.8 M acetic acid as BGE. The addition of HEC suppresses EOF and eliminates adsorption of analytes to capillary wall, therefore, it improves the separation resolution of amino acids. Thus, this method enables baseline separation of all proteinogenic amino acids within a 40-minute run. Moreover, the method enables determination of free amino acids in the plant extract in concentrations approx. above 10^{-4} mol/L. Because free proteinogenic amino acids belong among highly abundant metabolites, relatively high LOQ values of the method suffice for their determination.

The second study aims at development of a UPSFC-MS/MS method for determination of free proteinogenic amino acids in human plasma. Because the amino acids are highly polar/ionic metabolites, their derivatization is required prior to analysis by SFC in order to decrease their polarity thus increasing their solubility in CO_2-rich mobile phase. The amino acids were derivatized by 1-chlorobutane, which binds to their carboxyl groups. Therefore, the derivatization not only decreases their polarity but also eliminates negative charge of the amino acids. Subsequently, the amino acids from human plasma were separated using a mixture of CO_2 with methanol/water (95/5) and 20 mM ammonium formate. Then, the amino acids were determined using tandem mass spectrometry within a 6-minute run. Such conditions enabled determination of free proteinogenic amino acids in concentrations approx. above 10^{-9} mol/L.

The UPSFC-MS/MS method enables faster and more sensitive determination of amino acids than CE-C^4D, even with the derivatization step. However, CE-C^4D consists of less expensive and easy-to-use setup compared to UPSFC-MS/MS; moreover, CE-C^4D provides substantially higher separation efficiency than UPSFC-MS/MS. Therefore, in

analyses of materials rich in free proteinogenic amino acids, CE-C^4D becomes an analytical method of choice. On the contrary, UPSFC-MS/MS can be used in cases when determination of low concentrations ($> 10^{-9}$ mol/L) of amino acids is required.

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2. Taraba L, Křížek T, **Hodek O**, Kalíková K, Coufal P. Characterization of polyaniline-coated stationary phases by using the linear solvation energy relationship in the hydrophilic interaction liquid chromatography mode using capillary liquid chromatography. *J Sep Sci*. 2017;40:677-687.

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In preparation

7. Hodek O, Bergquist J, Ubhayasekera. A novel sensitive supercritical fluid chromatography-tandem mass spectrometry method for analysis of proteinogenic amino acids.

8. Hodek O, Křížek T. Determination of short-chain fatty acids in feces by capillary electrophoresis with indirect UV-VIS detection.

LIST OF CONFERENCE CONTRIBUTIONS

1. Hodek O, Bosáková Z, Křížek T, Coufal P, Benkovics G. *Application of self-assembling α-cyclodextrin derivatives in capillary electrophoresis*. HPLC 2015, Rožnov pod Radhoštěm, Czech Republic. Oral presentation
2. Hodek O, Křížek T. *Determination of biogenic amino acids in tobacco plant leaves by capillary electrophoresis*. Cycle of Lectures for Collaborating Czech Universities "Perspektivy v analytické chemii 2016". University of Chemistry and Technology, Prague, Czech Republic. Oral presentation
3. Hodek O, Křížek T, Coufal P, Ryšlavá H. *Design of experiments for amino acid extraction from nicotiana tabacum and their subsequent determination by capillary zone electrophoresis*. ITP 2016, Minneapolis, USA. Poster presentation
4. Hodek O, Křížek T, Ryšlavá H, Coufal P. *Enzymatic reaction of α-amylase with starch and its monitoring by an online capillary electrophoresis method.* 13th International Students Conference "Modern Analytical Chemistry", 2017, Prague, Czech Republic. Oral presentation
5. Hodek O, Křížek T, Ryšlavá H, Coufal P. *An online electrophoresis method for monitoring of potential α-amylase inhibitors.* 2nd International Caparica Christmas Congress on Translational Chemistry IC$_3$TC, 2017, Caparica, Portugal. Poster presentation

DECLARATION OF CO-AUTHORS

On behalf of the co-authors I declare that Mgr. Ondřej Hodek contributed substantially to paper 3 entitled *Design of experiments for amino acid extraction from tobacco leaves and their subsequent determination by capillary zone electrophoresis*.

His share was 90 %.

On behalf of the co-authors I declare that Mgr. Ondřej Hodek contributed substantially to paper 4 entitled *Online screening of alpha-amylase inhibitors by capillary electrophoresis*.

His share was 90 %.

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