Instrumental and sensory analyses of peptides created by the peptidolysis of plant sources

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Preliminary remark

The present work was carried out in the period from November 2015 to December 2018 at the Institute of Food Chemistry of the Leibniz University Hannover under the direction of Prof. Dr. Dr. R. G. Berger.

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Zusammenfassung

Umami ist neben süß, bitter, sauer und salzig eine der fünf Grundqualitäten des Geschmackssinnes. Dieser Geschmack ist charakteristisch für viele fermentierte Lebensmittel, wie Sojasauce. Umami-Geschmack wird durch die Aminosäureionen L-Glutamat sowie durch die Nukleinsäureionen Inosinat und Guanosinat ausgelöst. Da die Akzeptanz der Konsumenten für Natriumglutamat jedoch stetig sinkt, steigt das Interesse der Lebensmittelindustrie an natürlichen umami aktiven Zusatzstoffen kontinuierlich. Diese Arbeit erforschte den Umami-Geschmack von unterschiedlich produzierten Weizengluten-Hydrolysaten. Am Anfang dieses Projektes wurde der Umami-Geschmack der Hydrolysate untersucht und deren Peptid-Zusammensetzung bestimmt. Es wurden 197 verschiedene Komponenten identifiziert, unter ihnen verschiedene bekannte umami aktive Substanzen wie Ile-Glu, Val-Glu, Val-Asp, Ser-Glu, Glu-Gln-Glu, Val-Val, pGlu-Pro, pGlu-Gln, und pGlu-Gly. Eine derartig detaillierte Analyse der Peptid-Zusammensetzung von Weizengluten-Hydrolysaten, wurde bisher nicht beschrieben. Die Hydrolysate wurden fraktioniert mit dem Ziel, Fraktionen zu erzeugen, die weniger Substanzen enthalten, aber dennoch intensiven Umami Geschmack aufweisen. Eine Fraktionierung mittels Größenausschlusschromatographie (SEC) führte zu drei geschmacksaktiven Fraktionen. 93 % der Tester beschrieben eine fünffache Steigerung des Umami-Geschmacks. Die Peptide in diesen Fraktionen wurden genauer analysiert. Unter der Vielzahl der identifizierten Substanzen befanden sich einige, die ausschließlich in der einen oder anderen Fraktion detektiert wurden. Außerdem wurden die Umami aktiven Substanzen aus dem Ausgangshydrolysat erneut detektiert. Die große Anzahl der identifizierten Substanzen machte es zunächst unmöglich, eine einzelne umami aktive Substanz zu entdecken. Eine Subfraktionierung mittels präparativer HPLC führte nicht zu Subfraktionen mit intensiven Umami Geschmack, obwohl diese umami aktive Substanzen enthielten (Glu-Leu, Val-Glu, Val-Gly, Val-Asp, Pro-Glu, Pro-Gly, Pro-Thr, und diketo-Glu-Gln). Eine optimierte SEC wurde zur weiteren Subfraktionierung eingesetzt und geschmacksaktive Fraktionen generiert. Hier wurden 17 Substanzen identifiziert, von denen 2 bekannte Umami-Aktivität aufwiesen (Glu-Leu, diketo-Glu-Pro). Die 15 verbleibenden müssen einzeln sensorisch analysiert werden, um Substanzen zu identifizieren, die Umami Geschmack aufweisen, bisher aber noch nicht beschrieben wurden.

Schlagwörter: Umami, geschmacksaktive glutamyl-Peptide, Weizengluten- Hydrolysat, Fraktionierung, Größenausschluss-Chromatografie, präparative HPLC, sensorische Analyse, Massenspektrometrie

Abstract

Umami is one of the five basic tastes beside sweet, bitter, sour, and salty. It is well known in various fermented food preparations like soy sauce. Umami represents the taste of L-glutamate and 5'-ribonucleotides, such as guanosine and inosine monophosphate. Since the acceptance of the consumers for monosodium glutamate (MSG) is steadily declining, the interest of the food industry for umami active substitutes increased continuously. This study was based on the umami taste of wheat gluten hydrolysates. Differently produced hydrolysates were examined. At the beginning of this project, the overall umami taste of the samples and the peptide composition were determined. Up to 197 small biomolecules were identified, among them several umami active compounds like Ile-Glu, Val-Glu, Val-Asp, Ser-Glu, Glu-Gln-Glu, Val-Val, pGlu-Pro, pGlu-Gln, and pGlu-Gly. The composition of wheat gluten hydrolysate has not previously been described in such detail. A fractionation approach was performed to generate samples containing a lower number of substances while still imparting the umami taste.

Fractionation *via* Size Exclusion Chromatography (SEC) led to three fractions eliciting intense umami taste. A fivefold increase of the umami taste was described by 93 % of the panellists. As before, their peptide composition was determined by UPLC-HR-QTOF-MS/MS. Several peptides were identified, which made the sub-fractions unique, and the known umami active compounds of the starting material were found again. However, the multitude of identified substances made it impossible to discover a single substance that imparted the umami taste. Another fractionation was performed. Sub-fractionation *via* prepHPLC did not lead to samples showing a significant umami taste in the sensory analysis, even though known umami active substances were identified (Glu-Leu, Val-Glu, Val-Gly, Val-Asp, Pro-Glu, Pro-Gly, Pro-Thr, and diketo-Glu-Gln). A refined SEC sub-fractionation approach led to taste active sub-fractions. Only 17 substances were identified in the sub-fractions, whereas two (Glu-Leu, diketo-Glu-Pro) of them had known umami activities. Sensory analysis of the remaining 15 single compounds needs to be performed to discover a compound with umami activity, which was not described yet.

Keywords: Umami, taste active glutamyl peptides, wheat gluten hydrolysate, fractionation, Size Exclusion Chromatography, preparative HPLC, sensory analysis, mass spectrometry

Table of content

Abbreviations

ACE	Angiotensin I-converting enzyme
ACN	Acetonitrile
ADI	Acceptable daily intake
AFC	Alternative forced choice
Amp	Ampicillin
AMP	Adenosine monophosphate
ANS	Additives and Nutrient Sources added to Food
Boc	*tert*-butyloxycarbonyl
bp	Base pairs
BSTFA	*N,O*-bis(trimethylsilyl)trifluoroacetamide
cAMP	Cyclic adenosine monophosphate
CaSR	Calcium sensing receptors
CDS	Coding sequence
CID	Collision-induced dissociation
CNS	Central nervous system
DAG	Diacylglycerol
ddH_2O	Bidistilled water
dH_2O	Distilled water
DCM	Dichloromethane
DIN	*Deutsches Institut für Normung*
DKPs	2,5-Diketopiperazines
DLG e.V.	*Deutsche Landwirtschafts Gesellschaft eingetragener Verein*
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDC	1-Ethyl-3-(dimethylaminopropyl) carbodiimid
EDTA	Ethylendiaminetetraacetic acid
EFSA	European Food Safety Authority
EI	Electro ionisation
EIC	Extracted ion current
ESI	Electrospray ionisation
EtOH	Ethanol

eV	Electronvolt
FAO	Food and Agriculture Organization
FPLC	Fast Protein Liquid Chromatography
FTICR	Fourier Transform Ion Cyclotron Resonance
FZ	Flavourzyme
$GABA_BR$	γ-aminobutyric acid receptors B
GC-MS	Gas chromatography-mass spectrometry
GPCRs	G protein coupled receptors
gDNA	Genomic deoxyribonucleic acid
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
h	Hour
HILIC	Hydrophilic Interaction Liquid Chromatography
hPa	Hectopascal
HPLC	High Performance Liquid Chromatography
5-HT	5-hydroxytryptamine
i.d.	Inner diameter
IP_3	Inositol triphosphate
IT	Ion Trap
ITS	Internal transcribed spacer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JGI	Joint Genome Institute
kb	Kilo base
LB	Lysogeny broth
LC	Liquid Chromatography
LFGB	*Lebensmittel- und Futtermittelgesetzbuch*
MALDI	Matrix Assisted Laser Desorption/Ionisation
mAU*s	Milli absorbance units * second
MeOH	Methanol
mg	Milligrams
min	Minutes
mm	Millimetres
mGluR	Metabotropic glutamate receptors
mRNA	Messenger ribonucleic acid

MS	Mass spectrometry
MSG	Mono sodium glutamate
MW	“Molecular Weight”, means Molecular Mass
MWCO	Molecular Weight Cut-Off
m/*z*	Mass-to-charge ratio
NaAc	Sodium acetate
NCBI	National Center for Biotechnology Information
n.d.	Not detected
NE	Norepinephrine
ng	Nano gram
NMM	N-methylmorpholine
oPA	Ortho-phthaldialdehyde
p.a.	pro analysi
Panx1	Pannexin 1
PCI	Phenol/chloroform/isoamyl alcohol
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PIP_2	Phosphathidylinositol 4,5-bisphosphate
PLC	Phospholipase C
*p*NA	para-nitroaniline
ppm	parts per million
P6SD	Protease P “Amano” 6SD
Q	Quadrupole
rpm	Revolutions per Minute
RT	Room temperature
SCF	Scientific Committee on Food
sec	Seconds
SEC	Size Exclusion Chromatography
SNS	Standard nutrition solution
TAE	Tris Acetate Ethylenediaminetetraacetic acid
TIC	Total ion current
TLC	Thin layer chromatography
TMD	Transmembrane domain
TMCS	Trimethylchlorosilane

TOF	Time of Flight
TRIS	Tris(hydroxymethyl)aminomethane
T1R	Taste 1 receptor
U	Units
UV	Ultraviolet
V	Volt
VIS	Visible
VFT	Venus flytrap
WHO	World Health Organization
x-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Amino acids are abbreviated after the international one and three letter code.

1 Introduction

1.1 Fermentation, a thousands of years old food processing step

"La fermentation […] C'est la vie sans l'air, c'est la vie sans gaz oxygène libre" (Pasteur 1876) means, fermentation is life without air, it is life without free oxygen. That is how Louis Pasteur coined the term fermentation. Millennia ago, humans had begun using this technique by chance. At the present time, in biotechnology, all enzymatic and microbial processes leading to the conversion of organic substances, with or without oxygen, are summarised under the term fermentation (Spektrum Akademischer Verlag Heidelberg 2001).

When our earliest ancestors milked dairy cattle, they used to drink the milk within hours. Otherwise, an unknown reaction was leading to a curdled and sour product in those days. Almost accidentally, this process took place in raw material that was unpreserved. One could say that fermented food was very likely among the first processed foods consumed by humans. Like Prof. Keith H. Steinkraus from the Cornell University mentioned in 1993: "*The processes required for fermented foods were present on earth when man appeared on the scene… When we study these foods, we are in fact studying the most intimate relationship between man, microbes, and foods"* (Steinkraus 1993). Soured milk and cheese are not the only products created by fermentation. For instance, sweet fruit juices of grapes are known to remain sweet for some days before they become a pleasant wine-like drink which is mostly liked for its organoleptic characteristics (Hutkins 2006).

Asian people have been using this technique for centuries in a broad range of food preparations. These range from fermented vegetables, fish, and meat to fruits. Especially among the Chinese, Indians, Thais, Japanese, Korean, Vietnamese, and Taiwanese people, this is an integral part of their cuisine. These cultures are preparing fermented foods according to traditional methods to retain the taste impression they are used to (Sivamaruthi, Kesika et al. 2018). Well-known examples are the fermented fish sauces from Thailand, so-called *nampla*, *nuoc mam* (Vietnam) and *shottsuru* from Japan and the widely consumed fermented soybeans products soy sauce and miso (Nakano, Sato et al. 2018). In China, 5 million metric tons of soy sauce are produced annually, which equates to 50 % of the global production (Hoang, Ferng et al. 2016).

Fermentation also has a long tradition in the far north of Sweden. One of their national dishes is canned fermented herring called *Surströmming*. The fermentation lasts several month, for this the

fish is placed in brine and stored in barrels before it is bottled in cans without sterilization. Although the cans are sealed airtight, the fermentation progresses. This becomes visible through the bulging of the cans. The finished dish is known for its intense taste as well as for its intense smell (Kobayashi, Kimura et al. 2000). However, fermentation is popular all over the world and found in every culture.

1.2 Basics of fermentation

Any kind of fermentation is based on the use of microorganisms. Not only their major role in the production of alcoholic beverages and food, but also the application of several microbial fermentation products as additives in food raised the interest of the food industry. Nowadays, solid-state fermentation is the method of choice for industrial applications, because economic analyses indicated much higher enzyme titres or better product characteristics compared to submerged fermentation. Process conditions of fermentation like pH and temperature are limiting the number of usable microorganisms. Some bacteria, yeasts and fungi are suitable for this application. The most-well known representatives of the bacteria are *Lactobacillus* sp. (yoghurt production).

Ethanol production, however, is carried out by yeast (*Saccharomyces cerevisiae*). Fungi like *Penicilium roquefortii* are used for the production of cheese and miso, and soy sauce is produced by *Aspergillus oryzae*, for example. Soy sauce is very popular due to its intense umami taste.

Another commonality and at the same time an advantage over chemically synthesised products is the desire of consumers for natural foods (Couto and Sanromán 2006).

1.3 Umami: from “flavour enhancer” to the fifth basic taste

For centuries, humans believed that there were only four basic tastes: salty, sweet, bitter, and sour. Each of these tastes had its own function in humans. Foods rich in carbohydrates often taste sweet which helps to identify them as source of energy. Body functions, such as blood circulation or water balance are influenced by the absorption of sodium and other salts. Many individuals do not like the bitter taste of food. This is explained by the bitter taste of a variety of substances that are toxic or harmful for the human organism. To stabilize the acid-base balance of the body and to be safe from consuming spoiled food, the excessive intake of sour tasting foods is avoided.

In the course of time, mankind has learned that there are substances that, although they cause a “warning taste” (bitter or sour), can still be consumed almost without hesitation

(Chaudhari and Roper 2010). One of the best known examples might be the world's most popular beverage, coffee, which tastes bitter due to its caffeine content.

Professor Kikunae Ikeda is the discoverer of umami (/uˈmɑːmi/, from Japanese: うま味), which means savory and delicious. At the beginning of the 20th century, Prof. Ikeda conducted research at the Department of Chemistry of Tokyo Imperial University. He focused on seaweed broth and started analysing the composition of this broth after boiling tofu (*yudofu*) in it. Finally he succeeded in extracting monosodium L-glutamate and identified it as the real umami, since this compound is responsible for the umami flavour. In 1908, Ikeda applied for a patent "a manufacturing method for seasoning with glutamic acid as the key component", which was accepted the same year. Moreover, he proposed umami to be the fifth basic taste. Thus, he sparked a decade-long discussion among scientists (Ohkoshi 2018). Glutamate has a taste that is unlike any of the other four basic tastes. Many substances like 5'-inositate from dried bonito and 5'-guanylate from dried *Shiitake* mushrooms were also found, in the following years, to elicit umami taste. Ongoing research shows that umami substances are present in a variety of foods. Nevertheless, due to the weak umami taste caused by most of the umami substances, the umami taste was debated for a long time. On the contrary, the umami substances were classified as flavour enhancers. It took nearly 80 years until the first international umami symposium was held in Hawaii in 1985 to discuss the number of problems with umami. Until then, no systematic psychophysical data existed for umami. Electrophysiological studies performed with monosodium glutamate revealed that no single taste fibres which respond exclusively to monosodium glutamate (MSG) were determined. All taste fibres that responded to MSG also responded to sodium chloride. There was no indication for the presence of taste fibres that are exclusively stimulated by umami substances. However, more advanced recent psychophysical and electrophysiological studies showed that umami is, without a doubt, an independent basic taste (Kurihara 2015).

These data and the discovery of the umami taste receptors mGluR1, mGluR4, and T1R1 + T1R3 led to the international recognition of umami as the fifth basic taste (Kurihara 2015). One could say that the taste of L-glutamate and other amino acids indicates the protein content of food. All functions correlating with taste can be viewed as nutritional quality control mechanisms. Scientists even hypothesise that fat could be a sixth taste impression (Calvo and Egan 2015).

1.4 Human taste perception

1.4.1 Molecular pathway of tasting

The perception of man is composed of his five traditional senses hearing, sight, touch, smell, and taste. Food intake is significantly influenced by each one of these senses. Salivation is stimulated, hormone levels rise, temperature is evaluated, and consistency of the food determines the physical safety. All this information, gained during food intake, is transmitted to the central nervous system *via* cranial nerves. In the central nervous system, past experience and olfactory input are combined to generate a physiological, emotional, and sensory response. The latter is mainly evoked by the human taste organ, the tongue. Taste sensation is caused by taste papillae, which are spread over the tongue. They can be divided into three different types:

The circumvallate papillae are located in the middle and the back of the tongue. On the sides the foliate papillae are placed and the fungiform papillae, which settle two-third of the front surface (Figure 1.4-1) (Gravina, L Yep et al. 2013).

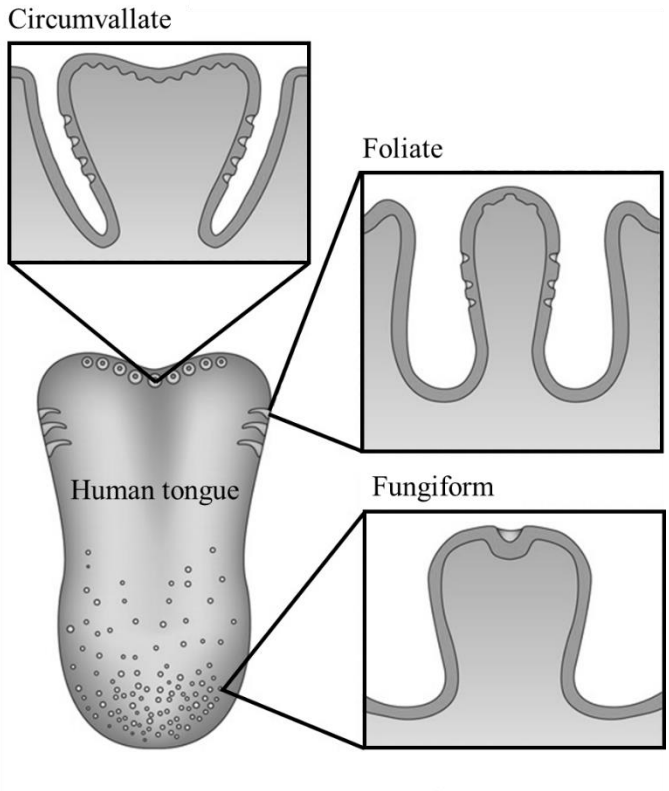


Figure 1.4-1: Schematic illustration of the human tongue and the localisation of the three different types of taste papillae. Fungiform are located in the middle and the front of the tongue, foliates at the sides of the tongue and the circumvallate in the back of the tongue (Calvo and Egan 2015).

On the soft palate, the upper reaches of the oesophagus, the larynx, and the nasopharynx the papillae are found as well. Each individual human papilla consists of three to five taste buds, which in turn contain the different taste receptor cells (Smith and Boughter 2007). Five types of tightly packed cylindrical cells of epithelial origin form a single taste bud. The data on the number of cells found in literature is diffuse; it varies between 50 and 150 (Cygankiewicz, Maslowska et al. 2014) and 150 and 300 (Gravina, L Yep et al. 2013). However, it is uniformly reported that the cells have some characteristics of neurons, such as the creation of synaptic connections and the ability to depolarise (Clapp, Medler et al. 2006) (Mombaerts 2004). Taste buds are composed of three different kind of cells: the glial-like cells (type I), the receptor cells (type II), which contain the G protein-coupled taste receptors (GPCR), and the presynaptic cells (type III) (Figure 1.4-2). Incoming chemicals get in contact with these cells and trigger the taste sensation.

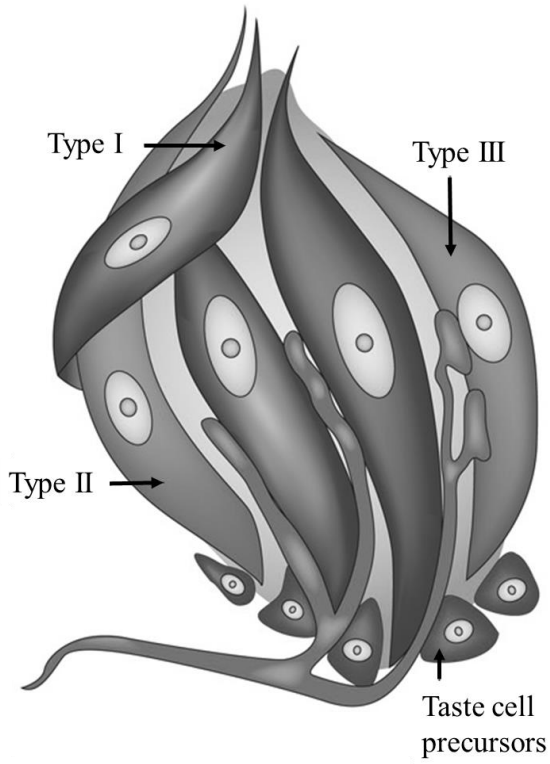


Figure 1.4-2: Schematic illustration of a human taste bud. Taste buds consist of four different cell types, the glia-like cells (type I), receptor cells containing G protein-coupled receptors (type II), presynaptic cells (type III), and the taste cell precursors. Afferent nerve fibres recognizable synapses with type III cells (Calvo and Egan 2015).

Type I cells are thought to transduce the salty taste; type II cells probably impart the bitter, sweet, and umami taste. Type III cells most likely mediate the sour taste and initiate cell communication and signalling *via* serotonin release to the afferent neurons (Gravina, L Yep et al. 2013).

1.4.2 Structure and activation of G protein-coupled receptors

Type II cells, which are responsible for the umami taste, contain GPCR. These receptors make up the largest family of membrane proteins found in the human genome. Due to the broad spectrum of structurally diverse ligands, receptors can be activated and modulate several specific signalling pathways. Over 800 different GPCR are known, and they all have seven hydrophobic transmembrane segments. The intracellular carboxyl- and the extracellular amino-terminus are characteristics of this structural element (Figure 1.4-3) (Kobilka 2007).

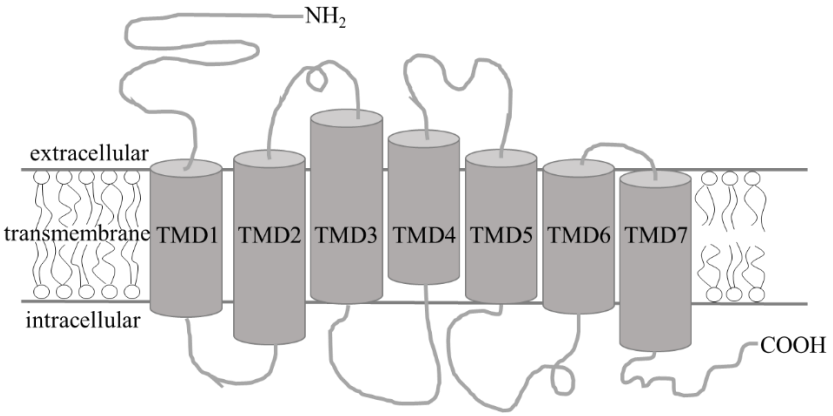


Figure 1.4-3: Schematic illustration of a G protein-coupled receptor. The receptor consists of seven transmembrane domains (TMD), which are embedded in the membrane. The amino-terminus is located in the extracellular and the carboxyl terminus in the intracellular space (Cygankiewicz, Maslowska et al. 2014).

Even though all GPCR have the 7 transmembrane domains (TMD), they can be divided into five subfamilies according to differences in the sequences of the TMDs. The frizzled/taste family involves 24 members, the glutamate family 15 members, the secretin family 15 members, the adhesion family 24 members, and the rhodopsin family is the largest one with 701 members (Fredriksson, Lagerström et al. 2003). GPCR are able to bind a variety of structurally diverse ligands. The largest molecules, which are bound by the GPCR are peptides and proteins. Small organic molecules, ions like H^+ and Ca^{2+}, and photons (subatomic particles) can also be recognised. Glutamate binds to large amino-terminal domains and thus leads to the activation of the receptor (Kobilka 2007). Most of the ligands do not enter the cell, but bind to the N-terminal extracellular part of the receptor. The interaction of ligand and receptor leads to a conformational change of the receptor. This in turn leads to the binding of a guanyl nucleotide to the cytoplasmic receptor domain and the activation of the G protein. The G protein is present as a heterotrimer, consisting of the three subunits α, β and γ when it is in its inactive state. Generally, the α- and γ-subunits are covalently bound to the membrane *via* fatty acids. The receptor/ligand complex catalyses the exchange of bound guanosine diphosphate (GDP) for guanosine-5`-triphosphate

(GTP). This reaction is triggered by interaction of the complex with the G protein. The nucleotide itself is bound to the α-subunit. Simultaneously with the GTP binding to the α-subunit, it dissociates from the $\beta\gamma$-dimer and transmits the signal of ligand binding to the receptor. One receptor/ligand complex is enough to cause the exchange of GDP to GTP on hundreds of α-subunits at the same time. This leads to a signal amplification (Stryer 2012). The activated G protein stimulates the enzyme adenylate-cyclase, which catalyses the conversion of adenosine monophosphate (AMP) to cyclic adenosine monophosphate (cAMP). Furthermore, the phospholipase C (PLC) is activated. Signal transduction triggered by PLC is described in section 1.4.4 (umami taste). The generated cAMP spreads out through the cell, whereas the G protein and the adenylate-cyclase remain membrane bound.

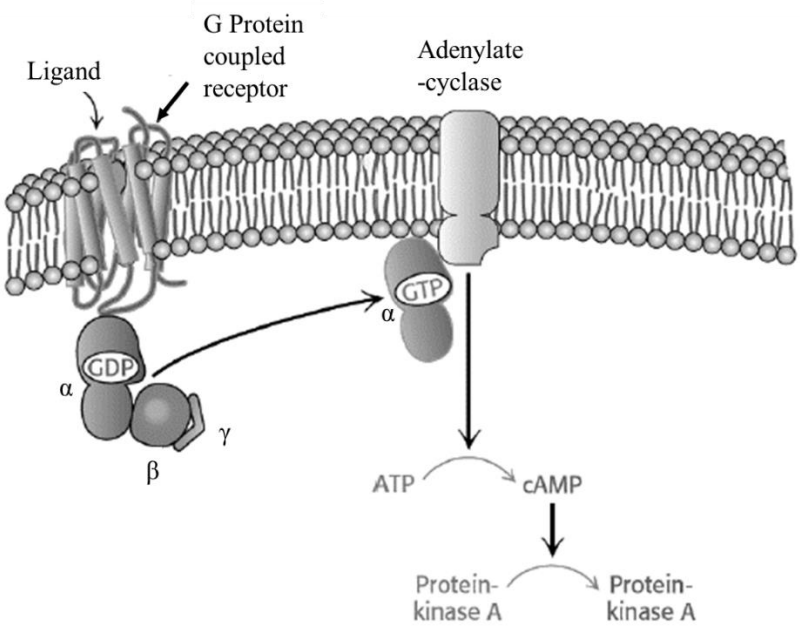


Figure 1.4-4: Signal transduction triggered by an activated G protein coupled receptor (GPCR). Ligand binding to the extracellular domains of the GPCR leads to a conformational change of the intracellular domain of the receptor and the binding of a guanylnucleotide to the α-subunit of the G protein. The G protein trimer dissociates and the α-subunit binds to the adenylate-cyclase and activates it (Stryer 2012).

In the next step, the intracellular concentration of second messenger increases, which results in the signal transduction in the cell. The most important second messengers are cAMP, cGMP, calcium ions, diacylglycerol and inositol-1,4,5-triphosphate. Protein kinase A is activated when the cyclic AMP binds to it. The main function of the protein kinase A is the specific phosphorylation of serine and tyrosine residues of target proteins which leads to a change of their activity. This leads to several different processes in the cell like gene expression or glycogen synthesis.

Each signal cascade has to be stopped after a while. G proteins are able to stop the signal transduction by themselves. The α-subunit has a GTPase activity, which allows the hydrolysis of GTP to GDP. After hydrolysis, the α-subunit/GDP complex binds again to the $\beta\gamma$-dimer and rebuilds the heterotrimer protein. Subsequently, the receptor also has to be inactivated to avoid the permanent activation of other G proteins.

Two inactivation routes are known. The first one is the dissociation of the ligand from the receptor, which returns the receptor to its initial inactive state. Secondly, a kinase is activated by the triggered signalling pathway. The kinase phosphorylates serine- and tyrosine-residues at the carboxyl term of the receptor. The receptor is thereby inactivated.

1.4.3 Salty taste mediated by type I cells

Type I cells are responsible for the maintenance of the taste bud structure. They represent around 50 % of the total number of taste bud cells Due to the expression of amiloride-sensitive sodium channel subunit α and the small voltage-gated inward Na^{2+} and outward K^{+} influx and outflux, respectively, they trigger the taste of salt. Until now, the downstream signalling mechanism caused by salt intake remains unknown. Nevertheless, type I cells play a role in cell signalling or cell communication. This function is based on the expression of a membrane bound ATPase, which degrades ATP in its surroundings. Furthermore, they probably have an influence on the control of the dissipation of cell signalling molecules throughout the taste bud and the isolation of ion fluctuations to specific areas (Calvo and Egan 2015)

1.4.4 Sweet, bitter, and umami taste mediated by type II cells

In 2010, only 3.8 % of German citizens were aware of the umami taste (Han, Mohebbi et al. 2018). However, all receptors that mediate one of this taste sensations belong to the same family, namely the taste receptor family (T1R). In 1999, two of the three family members (T1R1 and T1R2) were identified (Hoon, Adler et al. 1999). Three years later, in 2001, the third family member, T1R3, was identified in the human genome (Li 2009). The receptors are classified as class C GPCR based on the N-terminal Venus flytrap (VFT) domain. Other members of the T1R family are the metabotropic glutamate receptors (mGluRs), the γ-aminobutyric acid receptors B ($GABA_BR$), and the calcium sensing receptors (CaSR). The VFT consists of two subdomains, the lower lobe, and the upper lobe which are connected and represent the glutamate binding domain. Due to its bi-lobed architecture, the domain can be present in an open or closed conformation. Binding of

glutamate on one hand stabilises the dimer conformation of the receptor and on the other hand the closed conformation of the VFT (Zhang, Klebansky et al. 2008). As described in section 1.4.2, ligand binding, in this case glutamate, leads to the activation of the G protein-coupled receptor and the subsequent intracellular processes. First intracellular step of the umami taste sensing is the activation of a phospholipase *β*2 (PLC*β*2). Synthesis of inositol triphosphate (IP_3) is triggered by activated phospholipase. It hydrolyses the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG). IP_3 stimulates the opening of the ion channel IP_3R3. Open IP_3R3 channels enable Ca^{2+} release from the endoplasmic reticulum directly into the cytosol of the receptor cell. As a result, the intracellular concentration of Ca^{2+} increases and assumes two different functions there.

First, it ensures the opening of the taste-selective ion channel TRPM5, which is located in the membrane. Secondly, it influences a gap junction hemichannel consisting of pannexin (Panx1). In 2003, Liu and his colleagues found that Ca^{2+}- depended opening of TRPM5 leads to Na^+ influx in receptor cells, which in turn results in depolarisation of the cell (Liu and Liman 2003).

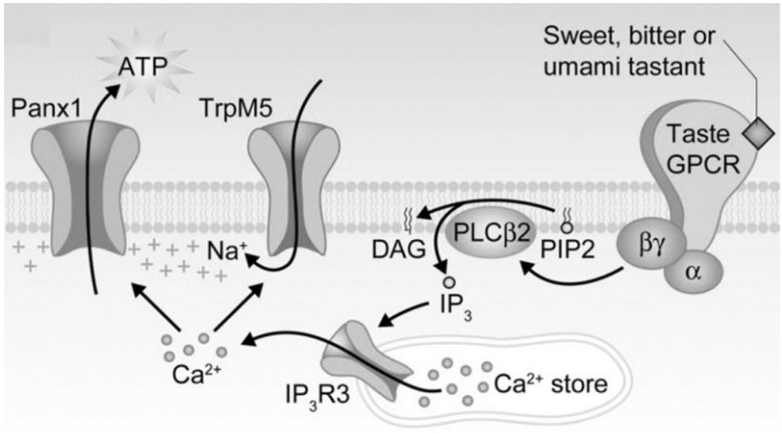


Figure 1.4-5: Taste transduction mechanism. Ligand binding to the receptor results in conformational change and the dissociation of the trimeric G protein. Activation of phospholipase *β*2 (PLC*β*2). The phospholipid PIP_2 is hydrolysed by PLC*β*2 to inositol triphosphate (IP_3) and diacylglycerol (DAG). Ca^{2+} release is provoked by activated IP_3 receptor (IP_3R) through IP_3. The increased intracellular Ca^{2+} concentration is responsible for the opening of the ion channel TRPM5, which allows a Na^+-influx that results in depolarization of the cell membrane (Chaudhari and Roper 2010).

The outcome of the opened Panx1 hemichannel is the secretion of ATP (Figure 1.4-5), the taste bud transmitter, into the extracellular space surrounding the activated receptor cell (Chaudhari and Roper 2010).

1.4.5 Cell-to-cell communication initiated by ATP secretion

After a receptor cell is stimulated by an appropriate ligand, ATP is released. To the present date, the neurotransmitter ATP is the only one known to be secreted by receptor cells. This chemical stimulus is transduced into signals by the taste buds, which represent specialised sensory organs. Finally, the signals are transmitted *via* primary gustatory afferent fibres into the central nervous system (CNS). Furthermore, Huang et al. (2009) hypothesised that ATP also affects adjacent cells in the taste bud. In detail, the ATP released by the taste receptor cells (type II) as response to a taste stimuli excites the presynaptic cells (type III). In turn, stimulation of these cells results in the secretion of the neurotransmitter 5-hydroxytryptamine (5-HT), better known as serotonin, and norepinephrine (NE). It is known that NE does not influence adjacent taste bud cells. However, its exact function has not been decrypted yet. In contrast, the mechanisms of action of ATP and 5-HT are known. Released 5-HT activates 5-HT_{1A} receptors which leads to the inhibition of the mobilization of intracellular Ca^{2+} in the receptor cells. That results in a reduced secretion of ATP. This process represents a negative paracrine feedback onto receptor cells. In contrast, released ATP has a positive autocrine feedback onto receptor cells (type II). It activates the P2Y1 receptor (purinoceptor) and enhances the secretion of ATP (Huang, Dando et al. 2009).

1.5 Biotechnological generation of bioactive peptides

Enzymes play the major role in biotechnological processes. Classification of enzymes is based on the chemical reaction they catalyse. This results in seven different enzyme classes, which can be distinguished by means of the enzyme commission number (EC number). EC 1 are the oxidoreductases, EC 2 transferases, EC 3 hydrolases, EC 4 lyases, EC 5 isomerases, EC 6 ligases, and EC 7 the translocases. Each of these classes is divided into subclasses. EC 3, the hydrolases, have 13 subclasses, of which subclass 4, the proteases or peptidases, encompasses the most important enzymes for protein hydrolysis in the food industry. They are able to hydrolyse peptide bonds. Depending on their cleavage site, they are further subdivided into endopeptidases that act internally and exopeptidases that act near the N- or C-terminus of the polypeptide chain. Further classifications into sub-subclasses (serine-, cysteine-, aspartic-, metallo-, and threonine-endopeptidases) are based on the catalytic mechanism. Due to their specificity proteases have a large number of applications. Proteins hydrolysed by specific proteases can have effects on the food product, like modification of the sensory quality, reduction of allergic compounds, improvement of antioxidative capability, or improved digestibility (Tavano 2013).

In addition to these effects, enzymatic hydrolysis has other obvious advantages over conventional acidic or alkaline hydrolysis. Peptidolysis of wheat gluten with peptidase mixture Flavourzyme (Novozymes, Copenhagen, Denmark) from *Aspergillus oryzae*, an ascomycete, can be carried out at 45 °C at pH 6 (Giesler, Linke et al. 2013). Enzymatic hydrolysis can increase the production of bioactive substances. Not all of them are desirable, as bitter tasting-peptides can be formed. Compared to acidic hydrolysis these are mild conditions e.g., while chemical processes are carried out under harsh conditions, e. g. using 6 M HCl at 110 °C for 24 h (Tsugita and Scheffler 1982), which can destroy tryptophan. Furthermore, this kind of treatment will affect nearly all other compounds of a food matrix and can end with in the production of harmful substances.

Nevertheless, soy sauce, which is widespread in Asia, is produced using the precursors of today´s biotechnological processes. Traditionally, a mixture of cooked soy beans and roasted wheat is fermented for 2 – 3 days below 40 °C. The so called koji fermentation is a two-step process and begins with the addition of a starter culture of *Aspergillus sojae*, *Aspergillus oryzae*, or, in some cases, *Aspergillus tamarii*. Varying the fermentation conditions leads to soy sauces with different characteristics like colour and taste intensity. During the first step (koji) the starter culture secretes peptidases and carbohydrase complexes. The different enzymes break down the proteins and carbohydrates of the substrates. In the first fermentation step, glutaminases are released, which convert glutamine into glutamic acid and thus increase its concentration (Soldo, Blank et al. 2003). Among other substances, glutamine and glutamic acid are released with glutamic acid as the most abundant amino acid in soybean protein (Van Etten, Hubbard et al. 1959) and wheat (Mossé, Huet et al. 1985). Since glutamic acid is known to taste like umami, it is not surprising that soy sauce has such an intense taste. A concentration of 1.5 mmol/L monosodium glutamate is claimed to be the sensory threshold concentration because it is sufficient to perceive the characteristic umami taste in sensory analysis (Soldo, Blank et al. 2003).

In addition to MSG, there are numerous other substances that contribute to the umami taste like the purine-5´-nucleotides adenosine-5´-monophosphate (AMP), guanosine-5´-monophosphate (GMP) and inosine-5´-monophosphate (IMP) (Maga 1983). Furthermore, several other substances are responsible for the umami taste of *Lentinus edodus* (Shiitake mushroom). The umami taste is significantly caused by ibotenic acid and trichlomic acid, derivatives of oxyglutamic acid (Solms 1969).

The second step is called moromi fermentation. Low molecular mass peptides, amino acids, and sugars, formed in the first koji step, are crucial for the subsequent brine fermentation step. For this, the brine solution is mixed with koji, from the first step, in equal amounts. The generated mixture

has a salt content of about 18 %, which is believed to prevent the growth of unwanted microorganisms. Various desirable microorganism are part of the second fermentation step. The lactic acid bacterium, *Pediococcus halophilus*, which leads to a pH drop of the moromi, a salt-tolerant yeast, *Saccharomyces rouxii*, for alcoholic fermentation in the middle stage, and a salt-resistant yeast, *Candida* sp., which produces phenolic compounds and contributes to the aroma of soy sauce (Lioe, Selamat et al. 2010). The moromi fermentation can last from three months to three years (Yokotsuka 1961).

Peptidolysis, which takes place during the fermentation, also releases peptides that confer umami taste. The intensity is usually much lower than that produced by MSG. Several di- or tripeptides with a molecular mass lower than 500 Da that are produced by the hydrolysis of fish protein and isolated from chromatographically generated fractions are responsible for the umami taste, among them Glu-Ser, Glu-Glu, Glu-Asp, Glu-Gln-Glu, Glu-Asp-Glu and Asp-Glu-Ser (Noguchi, Arai et al. 1975). The structure of umami active substances is very diverse. In addition to the di- and tripeptides mentioned before, tetrapeptides to octapeptides (Nakata, Takahashi et al. 1995) and cyclic peptides (2,5-diketopiperazines) (Chen, Dewis et al. 2009) have been described to contribute to this flavour. One can only hypothesise how many umami active substances have yet to be discovered.

1.6 Bioactivity of peptides and their condensation products

Countless natural sources contain high molecular mass proteins. One could name them parent proteins that can release various peptides by enzymatic hydrolysis. The peptide activity depends on its amino acid sequence. Known bioactive peptides are versatile. They can exercise regulatory functions and be used in functional food to prevent food degradation by microorganisms or food oxidation. In addition, bioactive peptides also can positively affect human health with regard to the nervous, immune, cardiovascular, endocrine, and digestive systems. Further applications are the treatment of various disorders and diseases (Sánchez and Vázquez 2017).

All these facts clarify why the scientific community has such an interest in bioactive peptides. Several working groups defined the influence of bioactive peptides on health and discovered a positive impact on body functions (Kitts and Weiler 2003). In 2014, more than 1500 different bioactive peptides were listed in a database called “Biopep” (University of Warmia and Mazury in Olsztyn 2003). Crucial to this activity is the amino acid composition and sequence by which they can be classified based on their mode of action. Thus, the hormone and drug-like peptides can confer antioxidative, antimicrobial, antihypertensive, antithrombotic, opioid, and

immunomodulatory effects. Studies showed that most of the bioactive peptides consist of 3-20 amino acids (Möller, Scholz-Ahrens et al. 2008). Although little is known about how the structure is related to the bioactivity, some structural similarities have been discovered, such as the presence of arginine, proline and lysine groups, as well as the general presence of hydrophobic amino acids (Kitts and Weiler 2003).

Bioactive peptides are one way to regulate derailed blood pressure, since hypertension negatively affects one quarter of the world´s population, bioactive peptides can be a feasible part of the treatment. Physiologically, the blood pressure is regulated by angiotensin I-converting enzyme (ACE). It catalyses the conversion of angiotensin I, a decapeptide, to angiotensin II. The former is converted to an octapeptide with vasoconstricting effects, which also has an influence on the fluid and salt balance in mammals. Several natural sources for the isolation of ACE inhibitory peptides are known. *Lactobacillus helveticus* is able to release the immunomodulatory and hypotensive peptides Ile-Pro-Pro and Val-Pro-Pro from β- and κ-casein. The peptides with hypotensive activity often carry polar amino acid residues, such as proline and are short chained (Hartmann and Meisel 2007).

In contrast, the structural diversity of antimicrobial peptides is much greater, especially those generated from animals or plants. Many peptides that have antimicrobial activity contain hydrophobic α-helices. The majority of these peptides is amphiphilic and cationic. The number of amino acids that make up antimicrobial peptides ranges from 12 to 45 with a high number of hydrophobic residues, but a positive net charge. An advantage of these peptides over more potent antimicrobials is that they often have a broader spectrum and are able to rapidly kill the target cells. As the bacteria multiply slower than they are killed by the peptides, the risk of resistance formation decreases. Since they are able to kill clinically relevant pathogens, they are qualified for the potential use as drugs. Their activity is directed against both gram positive and gram negative bacteria like *Escherichia coli*, *Enterococcus faecium*, *Salmonella* spp., and *Staphylococcus aureus*. The bacterial cytoplasmic membrane represents the main point of attack of hydrophobic and amphiphilic antimicrobial peptides. They can accumulate and form channels in the membrane (Minervini, Algaron et al. 2003). This affects the transmembrane electrochemical gradients and leads to cell swelling by increased water flow, osmolysis, and cell death (Bechinger 1997).

Condensation of dipeptides leads to the formation of cyclic dipeptides, 2,5-diketopiperazines (Figure 2.8-1), and their stereoisomers. They were discovered in 1924 and are found in a variety of organisms, such as animals, plants, bacteria, and fungi and are the smallest cyclic peptides. Fungi are the most important source for bioactive cyclic dipeptides (Wang, Li et al. 2017). Six

hundred thirty-five bioactive fungal cyclic dipeptides have been discovered, mainly from the genera of *Aspergillus* and *Penicillium*. The peptides encompass a variety of activities, for example cytotoxicity, phytotoxicity, insecticidal, vasodilatory, antituberculosis, antimicrobial, and antiviral. For decades, scientists suggested that these dipeptidyl cyclic ring closures can function as potent inhibitors of microbial growth, signal molecules, and that they reduce virulence-factor production. Their function can be attributed to vary side chains and their structural chirality, which make them an interesting basis for drug design. In 2017, scientists demonstrated that proline-based cyclic dipeptides (*cis*-cyclo(L-Leu-L-Pro); *cis*-cyclo(L-Phe-L-Pro); and *cis*-cyclo(L-Val-L-Pro)) have an inhibitory effect on the proliferation of influenza A virus as well as on plant and human pathogenic fungi (Liu, Kim et al. 2017). Beside these activities some cyclic dipeptides are sensory active and have been successfully isolated from different food. The popular stewed beef and dried aged beef are rich in volatile and semi volatile compounds, from which ten cyclic dipeptides have been identified. A study from 2008 found that their organoleptic properties are closely related with the concentration used for the sensory analysis. Authors showed that *cis*-cyclo(L-Pro-L-Val) has no taste at 10 and 200 ppm, but tasted bitter from 500 ppm on. In contrast, 10 ppm *cis*-cyclo(L-Leu-L-Pro) tastes like pineapple, glue, or ethyl acrylate and 100 ppm like rare beef, or green beans. However, the majority of the ten identified peptides tastes bitter at any of the tested concentrations. This elucidates that the taste of cyclic dipeptides is not only based on their sequence but also depended on the used concentration (Chen, Dewis et al. 2009). This variety of combinations of amino acids, concentrations and synergistic effects can lead to a multitude of effects that have yet to be fully discovered.

1.7 Sensory analysis

Sensory analyses are widely used in the field of food analysis. They represent a meaningful tool for the food industry, research, marketing, and quality assurance. There is a suitable approach for almost every imaginable question like determination of product accuracy, or the recognition of any deficiencies. Accordingly, numerous factors have to be considered to produce significant results. This chapter summarises different factors as well as the different methods and their fields of application. Most of the information in this chapter (1.7 to 1.7.3) relates to the worksheets of the DLG e.V. (*Deutsche Landwirtschafts-Gesellschaft*). This is the oldest institution of its kind in Germany and has been conducting quality inspections of food and beverages since 1885. Nowadays, official tests are carried out by accredited institutions according to DIN (*Deutsches Institut für Normung*) standards (Hildebrandt and Schneider 2009).

1.7.1 General information about sensory analysis

Since the human sense of taste is influenced by numerous factors, just as many have to be considered. These are important prerequisites for a successful and meaningful test. Probably the most important requirements are sample selection, sample preparation, sample neutralization, sample coding, and sample presentation.

Sample selection: The differences between the samples given to the examiners should be as small as possible. They should all be the same size and shape, if not samples have to be homogenised. This minimises the possibility that examiners make a biased assessment based on the nature of the sample. If homogenisation cannot be carried out, the whole food can be served. If the food is small, for example peanuts, a sufficient amount has to be provided to allow the examiner to re-taste and, if necessary, exclude outliers (Manthey-Karl and Oehlenschläger 2010).

Sample preparation: Sample preparation begins with the removal of the sample from the storage location. If the food to be examined has to be refrigerated, it must be brought to a sufficient temperature before the test in order to improve the mouthfeel. In contrast, if the samples have to be tested warm, they have to be warmed immediately before the test (Manthey-Karl and Oehlenschläger 2010).

Sample neutralisation and anonymisation: To avoid wrong conclusions it is necessary to make the samples unrecognisable. Since both the shape and the colour allow conclusions to be drawn, the samples should e.g., be crushed before the test. Furthermore, the dishes have to be served in neutral containers. If this is not possible, names of manufacturers must be pasted or painted over or the original container has to be covered (Manthey-Karl and Oehlenschläger 2010).

Sample coding: To ensure that the examiners will not be influenced by the sample name or description, each sample has to be coded with a four or five digit code consisting of numbers, letters, or a combination thereof. The code must be placed in such a way that it cannot be removed from the sample to prevent manipulation. Only the test leader may be aware of the decryption of the code (Manthey-Karl and Oehlenschläger 2010).

Sample presentation: Each container used for presentation of the samples has to be neutral. Moreover, they have to have the same colour, shape, and material. All containers that come in to contact with the food must be inert in terms of smell, taste, and dye ability. The order in which the samples are tested must be random and should vary from test to test (Manthey-Karl and Oehlenschläger 2010).

All these factors have to be taken into account while planning a reliable sensory analysis.

1.7.2 Discrimination of difference tests

The most common discrimination tests are the triangle test, duo-trio, “A”-“not A”, and two-out-of-five-test. These are used when two or more products have to be compared. The products have to be very similar, without obvious deviations. Discrimination tests do not give information about product quality. Every examiner has to take a decision during the tests even if no sensory difference has been recognised. Therefore, it is a so called “forced-choice” method. Individual uncertainties in the finding of results should be evenly distributed and not significantly influence the result.

Triangle test: In this test, the samples are submitted as groups of three samples, two of which are identical. It is a feasible method for the determination of marginal differences between two samples. In §64 of the LFGB (*Lebensmittel- und Futtermittelgesetzbuch*), examination regulations are published that contain significance tables from which the test can be evaluated. The result refers to the total number of correct answers relative to the number of examiners. Triangle tests must be carried out under constant experimental conditions and require a minimum number of examiners between five and seven. Based on this examination method, the following statements can be made: 1. There is a significant difference between the samples. Whether it is based on the odour, taste, or mouthfeel cannot be determined. 2. The examiners could not determine a significant difference between the samples. However, it cannot be ruled out that there is absolutely no difference (Oehlenschläger and Manthey-Karl 2010).

Duo-trio-test: This test is easier than the triangle test, because the examiners know the control sample. In this case, a sample pair always consists of one sample and the known control sample. Therefore, the likelihood of guessing the correct result is 50 %. Significance tables are published in DIN standards for this evaluation. As result, the test leader receives the statement whether a difference was detected (Oehlenschläger and Manthey-Karl 2010).

“A”- not ”A” test: This test should be applied, if neither the triangle nor the duo-trio-test are feasible. This is the case, if the samples have slight visual differences or contain strong taste and/or odour components. Therefore, the panellists receive a standard sample (A), which is used for training purpose. The samples are handed to the examiners and afterwards, based on the internal standard (A) a decision has to be made: “A” – not “A”, meaning a difference or no difference was determined compared with the standard. Since this is a demanding test, it is done with trained tasters that are able to memorise sensory impressions (Oehlenschläger and Manthey-Karl 2010).

Two-out-of-five-test: As the name suggests, sample pairs consisting of five samples are given, of which two and three are identical. Due to the possibility of placing the samples into the group, the likelihood of correct guesses is only 10 %. This leads to an efficient and powerful test. Since the workload is high in this case, however, it can lead to fatigue and memory effect of the panellists during the course of the test, which can affect the test result. For this reason, the test is only suitable for samples that have no intense, sharp, and lasting taste or odour. It is often used to study optical and tactile features. The work should only be carried out by trained tasters, who will recognise the two identical samples, from which the three identical ones automatically result (Oehlenschläger and Manthey-Karl 2010).

Alternative forced choice: In addition to the tests described, there are feature-related tests like the alternative forced choice (AFC) and the ranking test. Their field of application is the evaluation of only one attribute, for example, does sample A taste more salty than sample B. The AFC test is very specific and widely used for the determination of extremely small differences of the chosen attribute. No further information will be received. There are two variants of this test, the one-sided and the two-sided test.

In the one-sided test, the head of the examination group knows the difference between the samples and the correct answer (one-sided). Mostly, the questions are formulated in such a way that one can only answer yes or no. In the two-sided, test the head of the examination group knows the difference as well but he or she has no idea which effect it has and what the examiner has to decide correctly (two-sided) (Manthey-Karl and Oehlenschläger 2010).

Ranking test: The mentioned ranking test is an expansion of the described discrimination tests. It enables the comparison of several products and how they are related to each other in terms of additives, such as sugar. Thus, there is a quick classification according to the type and intensity of characteristics or a classification of popularity. Participants should arrange the samples in a row according to the severity of the asked attribute. This methodology is suitable for determining the influence of different raw materials or the evaluation of different treatment methods (Manthey-Karl and Oehlenschläger 2010).

1.7.3 Descriptive-, hedonic- and affective sensory tests

The goal of descriptive-, hedonic- and affective sensory tests is to capture and measure human perception and sensation in food consumption. Products are qualitatively and quantitatively described to create individual product profiles. Combined with data of hedonic tests, these profiles

are used to derive the product acceptance or rejection by consumers. On the basis of this data, products are further developed to e.g., increase their sales. The descriptive analyses are thus a link between product development, marketing, and market research. In addition, they serve to monitor the product profiles during storage and the minimum shelf life. Often, untrained consumers are used because they are less biased then experts. However, the consumers must be trained compulsorily. Recruiting consumers and training them is the first of three phases in descriptive tests. Phase two consists of finding and formulating terms for the odour, taste, and texture of the products by the trained consumers for the qualitative description of the samples. The third phase includes the quantitative description of the terms formulated in phase two.

Descriptive analyses are a wide field. The most well-known methods include the consensus profiling, conventional profiling, descriptive profiling, quantitative descriptive analysis, and the free choice profiling or flash profiling, which are not discussed in detail. For a thorough description refer to (*Freies Auswahlprofil* DIN 10967-3-2001). Last, but not least, affective and hedonic tests can be performed. The Latin word *affectus* means sensation and passion and the Greek word *hēdoné* stands for joy, pleasure, or *hēdonikós* pleasurable. The names indicate that the human emotions like joy and pleasure and their opposing disgust and displeasure are the main focus in these tests. As a result, these tests are largely based on unconscious and emotional assessment mechanisms. They are often used for new developments and modifications of products, but also in product optimization and quality assurance processes (Dürrschmid 2010).

1.8 Mass Spectrometry, a powerful tool for peptide and protein identification

Over the years, mass spectrometry has become one of the most important analytical approaches for peptide and protein identification. Due to the constant advancement of the devices and the thus increasing application possibilities, mass spectrometry has become an indispensable part of the laboratories of today. Probably the most important areas of application include the determination of the amino acid sequence of peptides, the characterization of post-translational modifications as well as the determination of the relative and absolute protein quantities. The identification and quantification of proteins from highly complex matrices is also possible. By contrast, Edman sequencing is unable to generate sequence information from complex peptide mixtures.

In routine applications, accuracies of measured molecular masses can be achieved that are 500 to 1000 times higher compared to estimated molecular masses obtained by SDS-PAGE. This high accuracy enables accurate identification of peptides, proteins, and other biomolecules.

In simple terms, the basic principle consists of the formation of gas-phase ions from intact and neutral molecules. These ions allow the determination of the molecular masses. For the realisation of this measurement, three components are essential, which are installed in all mass spectrometers: an ion source, a mass analyser, and a detector (Figure 1.8-1). The last two components usually are inside a high-vacuum chamber. Thus, the number of collisions of the formed ions with gas molecules is reduced during analysis.

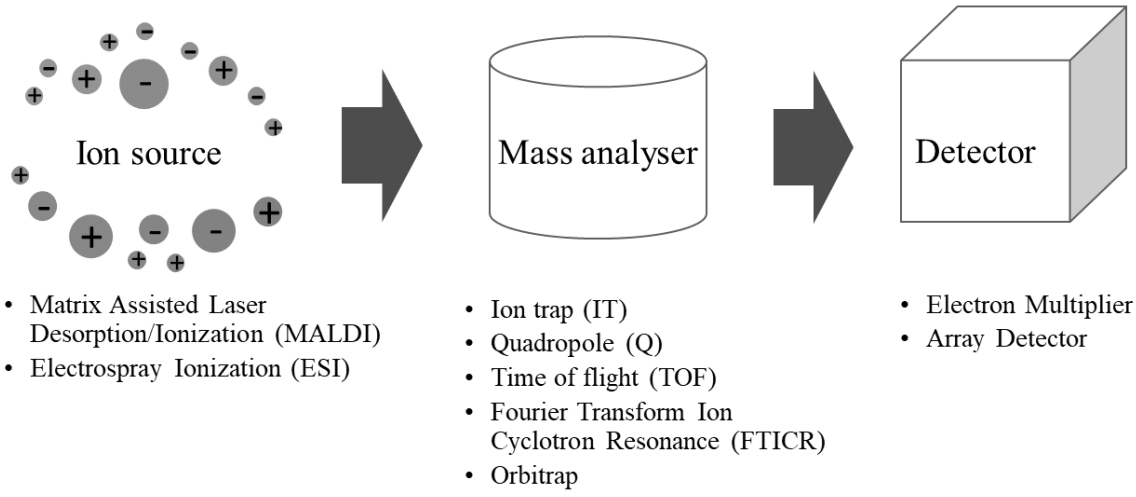


Figure 1.8-1: Schematic construction of a mass spectrometer. On the left side, the ion source is shown with its possible ionisation modes (MALDI; ESI), the middle shows the mass analyser and its variations (IT; Q; TOF; FTICR and Orbitrap). On the right side, possible detectors (electron multiplier and array detector) are shown.

All kinds of ion sources are responsible for the production of both positive and negative ions. Separation of the formed ions takes place in the mass analyser, based on their mass-to-charge (*m/z*) ratios. Finally, the ions are detected by a multichannel plate or an electron multiplier (Zhang, Annan et al. 2014).

1.9 Tandem Mass Spectrometry (MS/MS)

Tandem mass spectrometry, also called MS/MS, is typically used to generate sequence information from peptides. To achieve this, two mass analysers must be connected in series, each with a different task. First, precursor ions of a defined mass to charge ratio (*m/z*) are isolated. Subsequently, isolated precursor ions are fragmented and their product ions are mass-analysed. In

case of peptide analysis, the fragments are mainly formed by cleavage of the peptide bonds (Zhang, Annan et al. 2014).

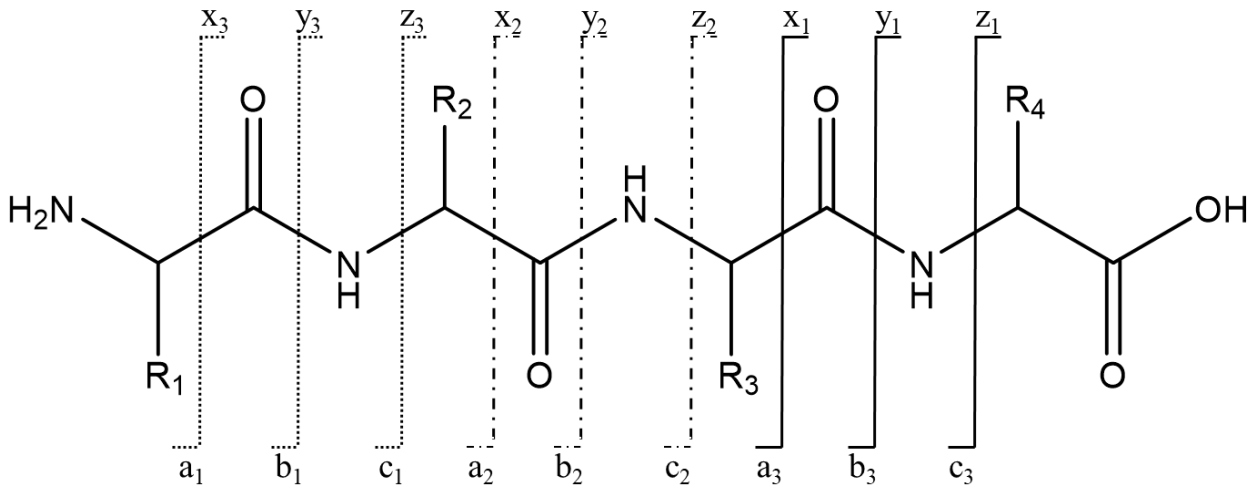


Figure 1.9-1: Ion fragmentation pattern of peptides and its nomenclature. Variable amino acid side chains are represented by R. Only ions carrying a charge can be detected. The fragment ions are named based on the position of the charge at the N- or C-terminus. If the ion carries the charge at the N-terminus, it is called a, b, or c. If the charge is at the C-terminus, the fragment ions are called x, y, or z. In addition, the number of residues of a fragment ion can be read of the subscripted letters. Depending on the position of the bond break, internal ions or immonium ions are formed (Biemann 1990).

The assignment of the most abundant and characteristic low-mass ions of the amino acids is based on Biemanns (Biemann 1990) nomenclature (Figure 1.9-1). Identified ions can be used to determine the amino acids in a sample.

1.10 Suitable “mild” ionisation techniques for peptide and protein analysis

In 2002, the Nobel Prize in chemistry was awarded to John B. Fenn and Koichi Tanaka for the revolutionary electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI) techniques invented in the 1980s (The Noble Prize 2002). The invention of these techniques simplifies the analysis of biomolecules many times over.

MALDI: The sample to be measured is mixed with a large molar excess of matrix. Of the mixture, up to 2 µL are applied onto the surface of a target plate and air dried. However, some requirements are placed on the matrix with respect to its behaviour under bombardment with photons of certain wavelength. Every commonly used matrix strongly absorbs the UV light it is exposed to. Intramolecular interactions are reduced by the separation of the analyte molecules by a large excess of the matrix. Furthermore, the matrix must be able to rapidly absorb a large proportion of the energy introduced by incoming photons, which ends up in an explosive collapse of the matrix-

analyte lattice. Thus, both analyte molecules and matrix molecules enter the gas phase. During laser irradiation, a dense cloud is formed above the target, in which gas-phase reactions are hypothesised to occur. In this reaction, protons are transferred from the matrix to the analyte molecules and lead to the analyte's ionisation. MALDI is most frequently applied with a time of flight (TOF) detector for the analysis of small proteins and peptides, which predominantly form single charged molecular ions. This combination is suitable for the analysis of unfractionated protein hydrolysate (Zhang, Annan et al. 2014).

ESI: An analyte solution is sprayed through the tip of a fine capillary at atmospheric pressure for ion formation. A fine droplet mist is created using a nebuliser gas. In addition, the droplet can only be formed and highly charged if a high voltage is applied between the spray tip and the counter-electrode. At this point, the peptide or protein molecules are still in the droplet. As the solvent evaporates, the molecules pick up protons from the solvent. This results in single to multiply charged ions, depending on how many possible points of attack are available for the protons. A rough derivation of the maximum charge state is thus possible. Furthermore, it was discovered that one proton can be deposited per approximately 1000 Da molecular mass. The release of the ions occurs after the drops, in which they are located, continue to shrink. The increasing charge density at the drop surface exceeds a critical point and the drops can no longer be held together. This phenomenon is called columbic repulsion. After ion release, they enter the high-vacuum part of the mass spectrometer to be analysed and detected (Figure 1.10-1).

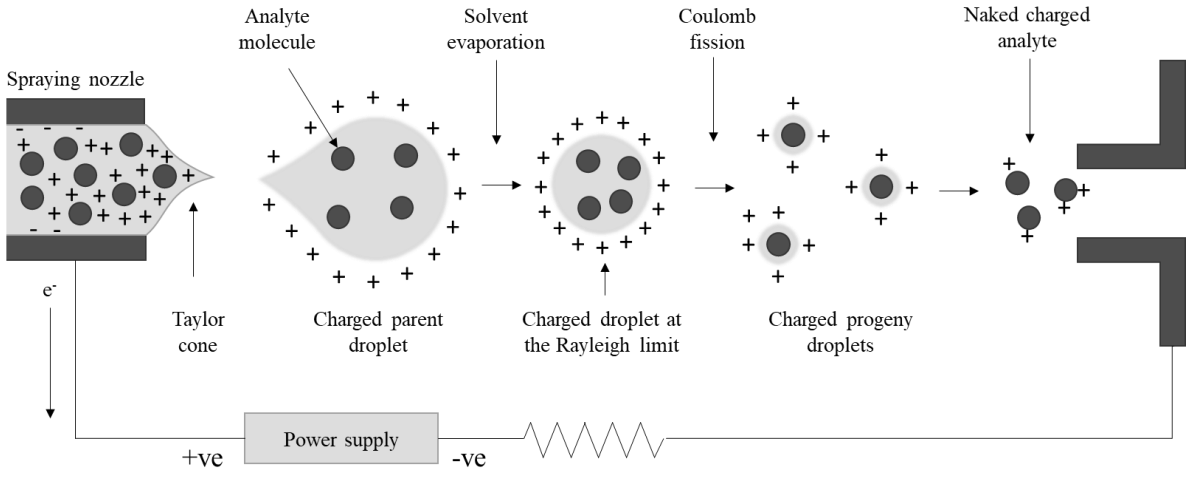


Figure 1.10-1: Schematic illustration of the electrospray ionisation process. Analyte solution is sprayed from the tip of the spraying nozzle through the Taylor cone. A positively charged parent droplet containing the analyte molecules is formed. Along with solvent evaporation, the droplet shrinks and the charge density on the surface of the droplet increases until it reaches a critical point. At this point, the force holding the droplet together is exceeded. Coulomb repulsion or coulomb fission ends up in naked charged analyte ions.

Usually a mixture of water and acetonitrile containing around 0.1 % formic acid is used for peptide analysis. The volatile acid promotes the ionisation of the molecules. However, only a little or none fragmentation of peptides is observed in normal ESI mode. In this work, an Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) combination was used. The complex protein mixture has been chromatographically separated before it entered the mass spectrometer. Combination of liquid chromatography and mass spectrometry has many advantages. The first one, is the mentioned separation of complex mixtures before they enter the mass analyser. Secondly, it safes precious and expensive instrument time and prevents sample losses during preparation. How frequently mass spectra are recorded can normally be chosen by the analyst.

One possibility is the operation in scan mode by time. Depending on the complexity of the sample it might be suitable to do a scan every second. For peptide analysis, a real-time decision operation mode is commonly used. An algorithm enables the system to select the fragments that require MS/MS analysis. Today´s technology opens up further possibilities for the analyst to switch between MS and MS/MS during a single run. The selection can be made based on the charge states of the precursor ions or a defined number of MS/MS can be set per cycle. From the data recorded during a run, different displays can be generated. Total Ion current (TIC) is a plot of the total number of ions detected during each mass spectrum scan versus time. Depending on the sample composition, this representation is often difficult to interpret. To simplify this, the so-called extracted ion current (EIC) can be created. It shows the ion current trace of a specific mass. The signal intensity-critical quantity produced by ESI is the concentration of the analyte rather than the total amount of it (Zhang, Annan et al. 2014).

1.11 Common Mass Analyzers in Mass Spectrometry

There are two main categories of mass analyser, the ion-beam and scanning types namely quadrupole (Q) and time of flight detector (TOF) and the trapping types ion trap (IT), fourier transform ion cyclotron resonance (FTICR), and orbitrap. Since these differ in their functional principles, they differ in resolution, MS/MS capability, mass accuracy, and sensitivity (Zhang, Annan et al. 2014). Although they are all suitable for peptide analysis, it is essential to choose the most efficient analyser for the given problem. This section deals with the quadrupole and time of flight detector, since these were exclusively used in this project.

Quadrupole: The main task of a quadrupole is the selection of defined mass to charge ratios. Mass selection is realised *via* the four electrodes arranged parallel to each other. These can be set under radiofrequency voltage and direct-current voltage.

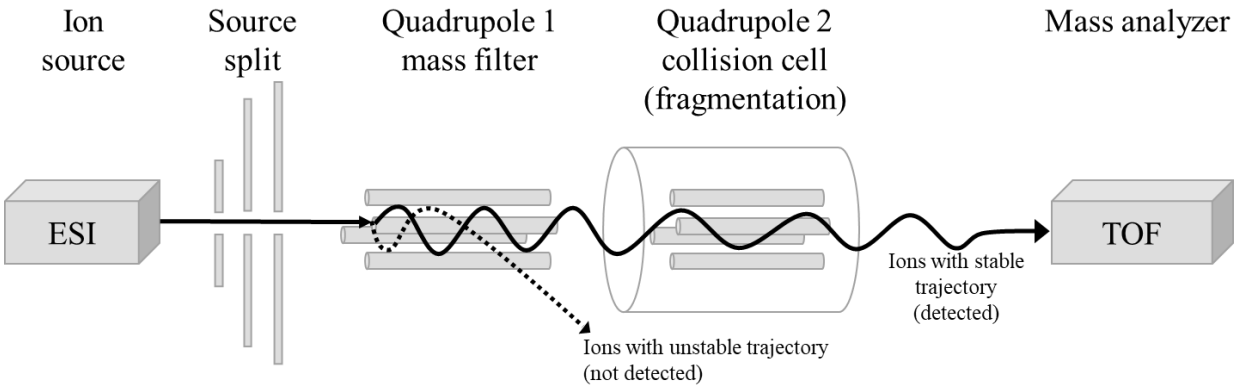


Figure 1.11-1: Schematic representation of a mass spectrometer including two quadrupoles. Electrospray ionisation (ESI) followed by source split and the first quadrupole for mass selection. The dotted line presents ions with unstable trajectory (not detectable) and the solid line shows ions selected by quadrupole 1, which are fragmented in the second quadrupole and detected in the time of flight detector (TOF).

Ions of a given *m/z* ratio can pass through the quadrupole unhindered. Unwanted ions are deflected from their trajectory, collide with the electrodes and are thus excluded from detection. If a scan mode is performed, the applied voltages change continuously and a variety of ions can be detected. Most applications combine the quadrupole with a second mass analyser like a second quadrupole or the time of flight detector (Figure 1.11-1). In this work, a combination of two quadrupoles and a TOF was mainly used. The second quadrupole serves as a collision cell, in which the ions are fragmented. Fragmented and charged ions enter the time of flight detector, where the mass to charge ratios are recorded. The settings on the quadrupoles can be adapted to almost any application.

Time of flight: Today´s time of flight detector, which is used by many laboratories, goes back to a design introduced by Wiley-McLaren in 1955. The first devices were characterised by low sensitivity and low resolution, which is why this technique did not prevail initially.

The method of ion production and the geometric construction of the ion source were the main limiting factors of the low resolution, which depends on the length of the produced ion packets. The second limitation factor was the acceleration method and the energy spread of an ion packet, which is due to the initial energy distribution. With the development of new ionisation techniques in the 1980`s, interest in TOF grew steadily. The operation principle of a linear time of flight mass spectrometer is quite simple. Ions with equal kinetic energy, but different mass to charge ratios are

separated according to their *m/z* ratios when entering an electric-field-free region. Mathematically, the separation is represented as a function of mass. Time of flight is calculated by:

$$t = l\sqrt{\frac{m}{2zeV}}$$

Whereby (l) is the fixed distance the ions travel, (z) is the ion charge, (e) is the electronic charge, and (V) the accelerating voltage. If the ion energy is constant, the flight time (t) is proportional to the square root of the ion mass. Heavy ions reach the detector after the light ions. The ion mass is determined by the measured flight time that is needed for the ions to move from the ion source to the detector (Li Gangqiang 1997). Beside the linear TOF-MS, a so-called reflectron TOF exists. It is equipped with a mirror and acts as an energy-focusing device by correcting the energy distribution. Reflectron voltage is set slightly higher than source-accelerating voltage. Thereby, ions are slowed down until they stop. Subsequently, ions turn around and get accelerated again until they reach a second detector. According to their kinetic energy and velocity, the time of flight differs. Thereby, resolution is improved as ions focused into packets have flight times that are close together. The TOF is very versatile and can be easily combined with different mass analysers and thus offers a versatile field of application (Zhang, Annan et al. 2014).

1.12 Aim of this thesis

As widely known a variety of edible hydrolysates of animal or vegetable origin are showing a highly intense umami taste. Precisely for this reason enzymatically generated hydrolysates of vegetable proteins from Nestlé were to be screened. A major goal was to identify biomolecules like small peptides and their cyclic compounds which were supposed to impart umami taste, or to be able to enhance the umami taste. Therefore concerted analytical approaches involving variants of FPLC and HPLC combined with extended mass spectrometry had to be developed and performed. Furthermore different processing steps had to be analytically characterised to describe precursors of the umami taste active substances and to track their reaction pathway caused by the processing conditions. Nevertheless the most taste active compounds should be identified by means of sensory analyses using a trained panel.

Finally, the sensory results should be correlated with the analytical results to clearly describe the compounds that are responsible for the intense umami taste appreciated by the consumers.

2 Material and methods

2.1 Chemicals

All chemicals and solvents of HPLC grade used were purchased from Carl Roth (Karlsruhe, Germany), Fluka (Neu-Ulm, Germany), Sigma-Aldrich (Steinheim, Germany) and VWR International (Darmstadt, Germany), respectively. The used peptides were delivered by Bachem (Weil am Rhein, Germany). Ultrapure water used for chromatography and spectroscopy was generated by a TKA GenPure system (Niederelbert, Germany).

Table 2.1-1: List of frequently used chemicals and substances.

Chemical	Quality	Supplier
Acetic acid	Pure, 100 %	Carl Roth
Acetonitrile	LC-MS grade	Carl Roth
Amino acid standard	Analytical standard	Sigma-Aldrich
Boric acid	*Puriss.*	Fluka
Ethanol	HPLC gradient grade, ≥ 99.9 %	Carl Roth
Formic acid	≥ 98 %, p.a.	Carl Roth
Methanol	HPLC grade	Carl Roth
3-Mercaptopropanic acid	≥ 99.9 %	Carl Roth
Monosodium glutamate	≥ 99 %	Ajinomoto
o-Phthaldialdehyde	≥ 99% for synthesis	Carl Roth
Sodium acetate	≥ 99 %, p.a.	Carl Roth
Triethylamine	≥ 99.5 % for synthesis	Carl Roth
Water with 0.1 % formic acid	LC-MS grade	Carl Roth

2.2 Frequently used Devices

Table 2.2-1: List of used devices

Device	Specification	Manufacturer
Magnetic stirrer	MR 3001	Heidolph (Kelheim)
pH-meter	FiveEasy	METTLER TOLEDO (Gießen)
Piston-stroke pipette	Transferpette®	Brand (Wertheim)
Ultra-Filtration system	Vivaspin 20, 3 kDa (polyethersulfone membrane)	Sartorius (Göttingen)
Ultrasonic cleaner	/	VWR International (Darmstadt)
Balance	SI-234	Denver Instrument (Göttingen)
Water conditioner	GenPure UV-TOC/UF	TKA (Niederelbert)
Centrifuge	Rotina 380R	Hettich (Lauenau)
Rotary evaporator	LABOROTA 4002- digital	Heidolph (Schwabach)
Freeze-dryer	VaCo 2	ZIRBUS technology (Bad Grund)

2.3 Ultra-Filtration

Stock solutions containing 100 mg mL^{-1} of all Nestlé samples were prepared and separated by the means of ultra-filtration. A Vivaspin 20 filtration system (Sartorius, Göttingen, Germany) with a cut-off membrane of 3 kDa was used. Centrifugation was carried out with a Rotina 380R centrifuge (Hettich, Lauenau, Germany) at 4 °C and 3500 rpm. For further analyses the flow through was used.

2.4 Solvent removal and freeze drying of sub-fractions

The ethanol fraction of each prepHPLC sub-fraction was distilled off under reduced pressure (80 hPa) and 50 °C bath temperature using a rotary evaporator. The remaining aqueous samples were transferred into large surface vacuum beakers and freeze dried (VaCo 2; ZIBRUS technology, Bad Grund, Germany). Spindle temperature was set to – 40 °C and plate temperature to – 11 °C.

2.5 Chromatographic Procedures

2.5.1 Fractionation of Hydrolysed Vegetable Proteins (HVP) using Size Exclusion Chromatography (SEC)

The ultra-filtered sample stock solutions were fractionated according to their molecular mass using a SEC "NGC Chromatography System" from Bio RAD (Hercules, California, USA). An isocratic separation at a flow rate of 0.5 mL min^{-1} was performed on a "Superdex Peptide 10/300 Gl" (GE Healthcare, Little Chalfont, United Kingdom) column. This column is usable in the separation in a range of 100 – 7000 Da. Peaks were detected by a UV detector at a wavelength of λ = 280 nm. Sodium acetate (25 mM) adjusted to pH 6.0 with acetic acid (1 M) was used as eluent. Injection volume was set to 250 µL. For every sample seven fractions of 5 mL were collected. A three point calibration was carried out with the tripeptide Val-Tyr-Val (379 Da), the dipeptide Tyr-Ala (252 Da) and the amino acid tyrosine (181 Da).

2.5.2 Sub-fractionation using High Performance Liquid Chromatography (prepHPLC)

The prepHPLC system (AZURA, Knauer, Germany) was equipped with a preparative column (NUCLEODUR C18 Pyramid, 5 µm; 16 * 250 mm; MACHEREY-NAGEL, Düren, Germany) without temperature control. Chromatographic runs were monitored at 210 nm and gradient elution was performed (Table 2.5-1). The injection volume was set to 200 µL. Fine- or sub-fractions were cut every ten minutes right from the start. With a total run time of 60 min this led to six sub-fractions of 80 mL per run at a flow rate of 8 mL min^{-1}. Separation was performed using a gradient with ddH_2O containing 0.1 % acetic acid (eluent A) and pure ethanol (eluent B).

Table 2.5-1: Gradient profile of the prepHPLC method for sample fractionation. Flow rate: 8 mL min^{-1}, preparative column (NUCLEODUR C18 Pyramid, 5 µm; 16 * 250 mm; MACHEREY-NAGEL, Düren, Germany), ddH_2O containing 0.1 % acetic acid (eluent A) and pure ethanol (eluent B).

Runtime [min]	Solvent A [%]	Solvent B [%]
0.0	90.0	10.0
15.0	90.0	10.0
40.0	40.0	60.0
45.0	0.0	100.0
50.0	0.0	100.0
55.0	90.0	10.0
60.0	90.0	10.0

2.5.3 Determination of oPA-derivatised free amino acid concentration by HPLC

The HPLC system consisted of an autosampler, Optimas Spark (TECHLAB, Braunschweig, Germany), a fluorescence detector RF-10AXL (Shimadzu, Kyoto, Japan), and a column thermostat Jetstream2Plus (Sigma-Aldrich, Steinheim Germany). Pump system PU-980, degaser DG-980-50 and a ternary gradient unit LG-980-02 from Jasco (Groß-Umstadt, Germany) were used. Amino acid standard solution containing 18 amino acids plus *β*-alanine as internal standard as well as all sample solutions were derivatised using the oPA-reagent. Before the derivatisation reaction, 10 µL of the respective sample filtrate was mixed with 110 µL potassium borate buffer (0.5 mM; pH 10). Derivatisation was automatically performed by the autosampler. Therefore, 20 µL of the oPA-reagent was added to the mixture which was allowed to react for 120 seconds and stopped by adding 50 µL of acetic acid (1 M).

Calibration of the system was carried out with eight standard solutions (10, 20, 30, 40, 50, 62.5, 75 and 100 µM) containing the proteinogenic amino acids with the exception of proline and cysteine. Separation was performed on an analytical NUCLEODUR C18 Pyramid column (250 mm * 4 mm, 5 µm) with a pre-column (EC 4/3, NUCLEODUR C18 Pyramid, 5 µm) (Macherey- Nagel, Düren, Germany). Excitation wavelength was set to λ = 330 nm and the emission was detected at λ = 460 nm. A binary elution gradient at a flow rate of 1.0 mL min^{-1} at 40 °C was applied (Table 2.5-2). Solvent A was a 0.1 M sodium acetate solution containing 0.044 % triethylamine at pH 6.5 adjusted with acetic acid (1 M). Solvent B

was methanol. Both eluents were membrane filtered (0.45 µm) and degassed with ultrasound for 15 min. Run time was set to 60 min.

Table 2.5-2: Gradient profile of the HPLC method for the determination of oPA-derivatised free amino acids. Flow rate: 1.0 mL min^{-1}, excitation wavelength λ = 330 nm, emission wavelength λ = 460 nm, oven temperature 40 °C, injection volume 20 µL, runtime 60 min, column: NUCLEODUR C18 Pyramid column, (250 mm * 4 mm, 5 µm), Solvent A: 0.1 M sodium acetate solution containing 0.044 % triethylamine at pH 6.5, Solvent B: methanol.

Runtime [min]	Solvent A [%]	Solvent B [%]
0.0	90.0	10.0
5.0	90.0	10.0
40.0	40.0	60.0
45.0	0.0	100.0
50.0	0.0	100.0
55.0	90.0	10.0
60.0	90.0	10.0

2.5.4 Peptide analysis using liquid chromatography-mass spectrometry (LC-MS)

For LC-MS analysis a VARIAN 320 Triple Quad LC-MS^2 (Palo Alto, California, USA) equipped with a NUCLEODUR C18 Gravity column (250 mm * 4 mm, 5 µm, 40 °C) was used. Elution was performed at 300 µL/min using a gradient (Table 2.5-3) of solvent A (water containing 0.1 % formic acid) and pure ethanol as solvent B.

Table 2.5-3: Gradient profile of the LC-MS method for peptide analysis. Flow rate: 300 µL min^{-1}, NUCLEODUR C18 Gravity column (250 mm * 4 mm, 5 µm, 40 °C), solvent A: water containing 0.1 % acetic acid, solvent B: pure ethanol.

Runtime [min]	Solvent A [%]	Solvent B [%]
0.0	95.0	5.0
15.0	95.0	5.0
20.0	40.0	60.0
25.0	40.0	60.0
30.0	20.0	80.0
35.0	20.0	80.0
40.0	95.0	5.0
45.0	95.0	5.0

A six port valve equipped with a 20 µL sample loop was used for manual injection. Detection was achieved spectrophotometrically at λ = 210 nm and subsequent MS analysis: Electrospray ionisation (ESI) in the positive and negative mode: capillary + 30 V/- 40 V; needle voltage 5000 V/- 4500 V; nebuliser gas pressure 379 kPa; drying gas 138 kPa at 200 °C.

2.5.5 Ultra-Performance Liquid Chromatography (UPLC) coupled with High-Resolution Mass-Spectrometry

A HILIC column (TOSOH BIOSCIENCE; TSKgel Amide; 3 µm; 4.6 * 150 mm) was installed in a Jasco XLC ULPC system. The column outlet was directly connected to the ESI interface of a QTOF device (MaXis Impact; Bruker). Elution was performed using a gradient (Table 2.5-4) with a flow rate of 0.3 mL min^{-1} (eluent A was ddH_2O and eluent B was acetonitrile, both containing 0.1 % formic acid). Injection volume was set to 5 µL and the column oven was set to 25 °C. Centroid mass spectra were recorded over a range of *m/z* 50-700. ESI parameters were 4500 V capillary voltage, 3 L min^{-1} dry gas at 180 °C. Tune parameters were set to get maximal ion yield in the *m/z* range from 150 to 300 at an average mass resolution of 25,000. CID was carried out at 31 eV.

Table 2.5-4: Gradient profile of the UPLC-HR-MS/MS method for peptide analysis. Flow rate: 300 µL min^{-1}, HILIC column (TOSOH BIOSCIENCE; TSKgel Amide; 3 µm; 4.6 * 150 mm), eluent A was acetonitrile and eluent B was ddH_2O, both containing 0.1 % formic acid.

Runtime [min]	Solvent A [%]	Solvent B [%]
0.0	95.0	5.0
5.0	95.0	5.0
30.0	40.0	60.0
32.0	40.0	60.0
40.0	95.0	5.0
45.0	95.0	5.0

2.5.6 Determination of peptide composition of sub-fractions generated by Size Exclusion Chromatography *via* Ultra-Performance Liquid Chromatography (UPLC) coupled with High-Resolution Mass-Spectrometry

A HILIC column (TOSOH BIOSCIENCE; TSKgel Amide-80; 3µm; 4.6 x 150 mm) was installed in a Jasco XLC device. The column outlet was directly connected to the ESI interface of the QTOF (MaXis Impact, Bruker). Isocratic elution was performed with a flow rate of 0.3 mL min^{-1} with a mixture of water and acetonitrile (30/70) with the addition of 0.1 % formic acid. The injection volume was set to 20 µL and the column was tempered at 25 °C. Centroid mass spectra were recorded over a range of *m/z* 50-700. ESI parameters were 4500 V capillary, 3 L min^{-1} dry gas at 180 °C. Tune parameters were set to get maximal ion yield in the *m/z* range from 150 to 300 CID was carried at 31 eV.

2.5.7 Liquid chromatography-mass spectrometry (LC-MS) analysis for identification of synthesised 2,5-diketopiperazines

Chemical synthesis (2.8) of several 2,5-diketopiperazines was verified. Therefore, moderately diluted products were directly injected into Varian Triple Quadrupole MS-system (Varian, Palo Alto, California, USA). Ionisation was realised by ESI and the most feasible system setting were used (Table 2.5-5).

Table 2.5-5: Method settings for MS device used for the verification of chemically synthesised 2,5-diketopiperazines.

Parameter	Setting
Detector Voltage	1200 V
Needle Voltage Negative	-4500 V
Spray Shield Voltage Negative	-600 V
Spray Chamber Temperature	50 °C
Drying Gas Temperature	350 °C
Nebulising Gas Pressure	379 kPa
Drying Gas Pressure	207 kPa
m/z Ratio (Quadrupole)	150 – 350
Capillary Voltage (Positive)	30 V
Capillary Voltage (Negative)	-40 V

2.5.8 Purification of synthesis products by flash chromatography

Chemically synthesised N-terminally-Boc protected dipeptide methyl ester had to be purified before cyclisation reaction took place. Therefore, flash chromatography of the intermediate products was performed (Tullberg, Grøtli et al. 2006). A 10 cm chromatography glass column with 1.5 cm diameter was wet packed with three grams of silica (Silica gel 60 (0.040-0.063 mm), E. Merck) as stationary phase. The sample was loaded onto the column with a flow rate of one to two drops per second. Elution was performed with three column volumes of a mixture of dichloromethane:methanol:hexane (6:1:5).

2.5.9 Gas Chromatography-Mass Spectrometry (GC-MS) analysis of derivatised 2,5-diketopiperazines

Prior to GC-MS measurement a derivatisation of the 2,5-diketopiperazines was necessary. Silylation was performed with SILYL-911 (Macherey-Nagel, Düren, Germany) consisting of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1 % trimethylchlorosilane (TMCS). This potent derivatisation reagent is suitable for the silylation of compounds which are difficult to silylate like secondary amines.

To 10 mg of the sample (cyclo(Leu-Pro), cyclo(Pro-Tyr) and cyclo(Glu-Glu)) 0.5 µL of the silylation reagent were added. The mixture was incubated for 3 h at 70 °C. After the silylation

reaction 0.5 µL were injected on-column into a VF-5ms column (30 m x, 0.25 mm i.d. x 0.25 µm; Agilent Technologies) installed in an Agilent-GC 7890B (Agilent, Waldbronn, Germany) coupled to an Agilent 5977A mass selective detector (MSD) operating in EI (70 eV) mode. Helium 5.0 was the carrier gas at a constant flow rate (1 mL min^{-1}). Oven temperature program: 40 °C held for 3 min; heating rate, 8 °C min^{-1} to 230 °C held for 3 min; heating rate, 25 °C min^{-1} to 325 °C held for 10 min. The temperatures of the transfer line, the source and the quadrupole were set to 330 °C, 230 °C and 150 °C, respectively.

The scan range of the MSD was set between *m/z* 71 to 700 with a scan speed of 1.562 scans sec^{-1}. Recording of ions started after a solvent delay of 12 min. Identification of the signals was achieved by comparison of the acquired mass spectra with the commercial mass spectral database NIST 14. Moreover, the plausibility of the results was verified by the comparison of the silylated masses of the commercial standards with the calculated *m/z* ratios for each possible silylated state of the 2,5-diketopiperazines.

2.6 Sensory analyses of sample stock solutions and SEC-fractions

A panel consisting of 15 healthy untrained subjects with no known taste or olfactory perception disorder was asked to evaluate umami taste enhancing properties of samples and fractions thereof. To calibrate the panel, three aqueous monosodium glutamate (MSG) standard solutions were offered (1, 10 and 50 mM). Each subject tasted the standard solutions to get an impression of the umami taste caused by different MSG concentrations. Due to the colouration of the sample stock solutions (100 mg mL^{-1}) the participants wore completely darkened glasses during the sensory analysis.

The sensory tests considered of two sample solutions and a standard solution (10 mM; MSG) or the other way round (A, A, B; A, B, B; duo-trio-test). Firstly each sample was evaluated for genuine umami taste. In a second test series the samples were rated with regard to umami taste enhancing qualities. Hence, according to the glutamic acid content analysed by HPLC, samples of lower MSG concentration were adjusted to 10 mM glutamic acid to exclude the impact of MSG. The subjects were asked to identify samples of equal/different umami taste impression and to rank them according to the MSG standard solution row. The sensory analyses of SEC-fractions was performed in the same manner, but because of the high genuine glutamic acid concentration in some of them, no MSG was added.

2.7 Identification approaches for detected mass to charge ratios

2.7.1 Evaluation of abundant signals

The evaluation of each sample was performed using four different software tools, three of them supplied by Bruker and an additional self-programmed VBA (Visual Basic for Application) Excel calculation program developed by M. Sc. Irina Santourian, Institute of Food Chemistry.

1. DataAnalysis 4.4 SR1 → accurate mass determination, elemental composition
2. ProfileAnalysis 2.3 → bucket table generation for data processing
3. MetaboScape Version 1.1.0 → elemental composition (accurate mass, isotopic pattern) and spectral library (SL) search
4. Excel VBA → accurate masses determined aligned against calculated masses of all proteinogenic di-, tri-, and tetrapeptides, pyroglutamyl dipeptides, and 2,5-diketo-piperazines

In order to confirm these preliminary identifications which based on accurate mass determination and biochemical/chemical plausibility, MS/MS analyses in the positive and negative mode were carried out and evaluated using software tools mentioned above. However, a few identifications succeeded only using automated routine evaluation methods. Most of the identifications required manual structure elucidation.

2.7.2 Identification using spectral library

Recorded MS/MS spectra were processed by the software tools 1-3 mentioned in 2.7.1. The last step included the automatic comparison of the detected MS/MS spectra with the spectra included in the commercial spectral library. If a recorded MS/MS spectrum matched the fragmentation pattern of a library spectrum it was automatically annotated as identified. However, spectral data had to fit several quality parameters (Table 2.7-1); otherwise they were not considered for the comparison.

Table 2.7-1: List of quality parameters used for the preselection of MS data.

Parameter	Narrow	Wide
Precursor *m/z*	2 mDa	5 mDa
Precursor mSigma	25	75
MS/MS	900	700

2.7.3 Identification by manual comparison with fragmentation patterns from literature

MS/MS experiments were performed in the positive and negative mode, and respective fragmentation patterns of potential pyroglutamyl-dipeptides were compared with published data (Frerot and Chen 2013).

2.7.4 Preliminary identification of peptides, pyroglutamyl dipeptides, and 2,5-diketopiperazines according to accurate mass determination and biochemical/chemical plausibility

Due to known processing of the sample with regard to hydrolysis conditions and the origin of the sample each result has been assessed if it was biochemical/chemical plausible. Additional parameter for the plausibility of the identified substances were the performed methods, for example the ultrafiltration with a 3 kDa cut-off and size-exclusion chromatography. Visual Basic for Applications (VBA) from Excel was used to calculate the accurate mass of all possible di-, tri- and tetrapeptides out of the 20 proteinogenic amino acids. Based on process parameters of gluten hydrolysis a formation of condensation products of released small peptides, such as diketopiperazines (cyclic dipeptides) and pyroglutamyl peptides was expected. Hence, data set of VBA search program was extended by calculated accurate masses of all possible 2,5-diketopiperazines and pyroglutamyl dipeptides out of the 20 proteinogenic amino acids.

2.8 Chemical synthesis of 2,5-diketopiperazines

2.8.1 Synthesis of 2,5-diketopiperazines performed with microwave assisted heating

The synthesis of the 2,5-diketopiperazines (DKPs) (Figure 2.8-1) was performed according to the method published by (Tullberg, Grøtli et al. 2006).

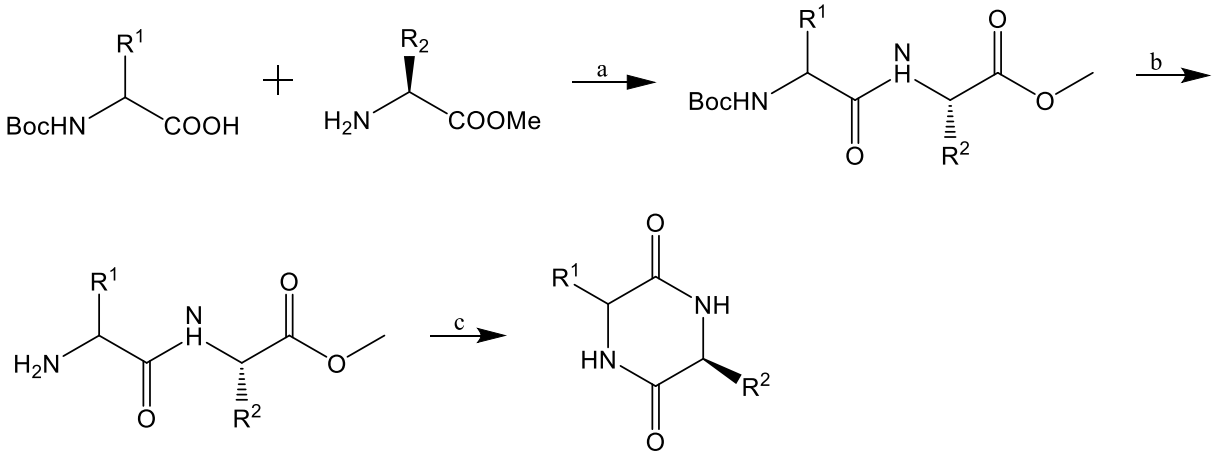


Figure 2.8-1: Chemical reaction scheme of the formation of 2,5-diketopiperazines. In the first step an N-terminal BOC-protected amino acid forms a peptide bound with a C-terminal amino acid methyl ester (condensation reaction). After deprotecting the N-BOC-dipeptide methyl ester a cyclisation reaction takes part. A: Coupling reaction of N-terminal Boc protected amino acid with C-terminal amino acid methyl ester dissolved in DCM and addition of 1 mmol of *N*-methylmorpholine and 1-Ethyl-3-(dimethylaminopropyl) carbodiimid, respectively. Reaction product is an N-Boc protected dipeptide methyl ester. B: Deprotection of the product using 10 % aqueous citric acid, results in dipeptide methyl ester. C: Cyclisation reaction for 10 min at 140 °C in the microwave.

One millimole of each C-terminal amino acid methyl ester was dissolved in 10 mL dry dichloromethane (DCM) and 1 mmol of *N*-methylmorpholine was added. During the reaction time of 40 min the mixture was stirred at 0 °C. Afterwards 1 mmol of *N*-Boc-Glu and 1-Ethyl-3-(dimethylaminopropyl) carbodiimid (EDC) were added. Thereafter, the reaction was stirred at 0 °C for 3 hours and overnight at room temperature. After dilution with 10 mL DCM the sample was washed three times with 10 % aqueous citric acid (10 mL) to remove the Boc protection group. The organic phase was dried over magnesium sulfate and concentrated using rotary evaporation. To purify the crude product a flash chromatography was performed (method 2.5.8). The second step of the synthesis was the cyclisation of the formed dipeptides. Around 50 mg of the deprotected dipeptide was dissolved in 3 mL of water and 2.5 equiv of triethylamine was added. All cyclisation reactions were carried out in the microwave Discover S-Class (CEM GmbH, Kamp-Lintfort, Germany) at 140 °C for 10 min (Tullberg, Grøtli et al. 2006). Subsequently the reaction mixture was again concentrated using rotary evaporation, and the precipitated product was finally dissolved in 750 µL of water.

2.9 Cultivation

2.9.1 Basidiomycota strains

Selected strains of *Laetiporus sulphureus* and *Fistulina hepatica* (Table 2.9-1) were either purchased from *Deutsche Stammsammlung für Mikroorganismen und Zellkulturen* (DSMZ, Braunschweig, Germany) or were self-isolated and identity verified by ITS sequencing.

Table 2.9-1: List of Basidiomycota strains used in this work including the names, abbreviations and internal strain numbers of the Institute of Food Chemistry as well as their origin.

Organism	Internal strain number	Origin
Laetiporus sulphureus	Lsul 235	DSMZ 11211
Laetiporus sulphureus	Lsu 279	Self-isolated (ITS verified)
Laetiporus sulphureus	Lsu 294	DSMZ 2785
Fistulina hepatica	Fhe 205	DSMZ 4987

2.9.2 Culture media

Cultivation was performed on standard nutrition solution agar plates. For this, a small piece of mycelium was transferred from tilted agar tubes onto the surface of a standard nutrition solution (SNS) agar plate. Plates were incubated at 24 °C until a sufficient amount of mycelia covered the surface of the SNS agar plate. When the fungi covered the whole surface, plates were stored at 4 °C.

Standard nutrition solution agar plates

Standard nutrition solution was prepared according to Sprecher (Sprecher 1959). Adjustment of the pH value to pH 6.0 was done using sodium hydroxide solution (1 M). The media was autoclaved for 20 min at 121 °C.

Table 2.9-2: Composition of standard nutrition solution for the preparation of agar plates.

Compound	Compound amount
D-(+)-Glucose × H_2O	30.0 g L^{-1}
L-Asparagine × H_2O	4.5 g L^{-1}
Yeast extract	3.0 g L^{-1}
KH_2PO_4	1.5 g L^{-1}
$MgSO_4$	0.5 g L^{-1}
Trace element solution (see below)	1.0 mL L^{-1}
Agar-Agar	20 g L^{-1}

Table 2.9-3: Composition of the trace element solution used for the preparation of standard nutrition solution.

Compound	Compound amount
$FeCl_3$ × 6 H_2O	0.08 g L^{-1}
$ZnSO_4$ × 7 H_2O	0.09 g L^{-1}
$MnSO_4$ × H_2O	0.03 g L^{-1}
$CuSO_4$ × 5 H_2O	0.005 g L^{-1}
EDTA	0.4 g L^{-1}

2.10 Molecular biological work

2.10.1 *In-silico* screening for glutamyl-specific peptidase genes in Basidiomycota

Several amino acid sequences of glutamyl endopeptidases were described in literature (Liu, Zhao et al. 2016). A glutamyl endopeptidase of *Thermoactinomyces* sp. (GenBank accession number WP_049719689) was thermostable and capable of hydrolysing proteins at high temperatures. Based on its accession number, the peptide sequence of the glutamyl endopeptidase was extracted. Subsequently, a Standard Protein BLAST at the National Center for Biotechnology Information (NCBI) against the taxis of the Basidiomycota was performed using the blastp algorithm.

2.10.2 Isolation of genomic DNA from fungal mycelium

The DNA was extracted with phenol/chloroform/isoamyl alcohol (PCI) and precipitated by adding ethanol. For harvesting, approximately 200 mg mycelium was scraped off from the top of the standard nutrition solution-agar plate and transferred into a reaction vessel containing glass beads, 400 µL digestion buffer and PCI, respectively. Cell disruption of the re-suspended pellet was accomplished according to the manufacturer´s instructions of the Precellys homogenizer (PEQLAB, Germany) (5,800 rpm; 3 times 20 sec with a 20 sec break between each step). The mixture was centrifuged at 17,000 *x* g for 5 min. Supernatant was transferred into a new reaction vial, mixed with 400 µL TRIS/EDTA (TE)-buffer and inverted 5 to 10 times. Centrifugation of the sample (17,000 *x* g, 5 min; 4 °C) led to the formation of two phases. The aqueous phase was transferred into a new vial, mixed with 200 µL PCI, inverted 5 to 10 times and centrifuged (17,000 *x* g, 5 min; 4 °C). Again, the aqueous phase was transferred into a new vial, mixed with 1 mL 99.5 % ethanol and inverted 5 to 10 times and centrifuged (17,000 *x* g; 10 min; room temperature). Supernatant was discarded and the pellet washed with 70 % ethanol. The ethanol was decanted and the pellet was dried in a thermoshaker at 50 °C. The pellet was re-suspended in 60 µL *aqua bidest* and DNA concentration was calculated from the absorbance measured at 260 nm using UV/VIS BioSpectrometer (Eppendorf, Germany).

2.10.3 PCR conditions for the amplification of glutamyl-specific peptidase gen

Primers for the amplification were designed with SnapGene® version 4.2.4 based on the annotated genomes of *Laetiporus sulphureus* (Lsu) and *Fistulina hepatica* (Fhe). For the amplification of the gene of interest the primers Lsu_start_fwd (5´ atggttaggaggaaattactccttcctgatgaag 3´) and Lsu_ende_rev (5´ tcaatttatagaatcctcgaaacagaagtcggtgaa 3´) were used as well as Fhe_start_fwd (5´ atggcgggcgccgatttcgaagattgg3 ´) and Fhe_ende_rev (5´ ttacgcaaagctgaacttgacgaactgaagtag 3´) for *Fistulina hepatica* and *Laetiporus sulphureus*, respectively. Polymerase chain reaction (PCR) was performed with Phusion® High-Fidelity DNA polymerase (Thermo Scientific, St. Leon-Rot, Germany) according to the manufacturer's instructions in a thermocycler (pegSTAR 2X Gradient Thermocycler, Peqlab, Erlangen, Germany). The protocol was as follows: Initiation: 98 °C for 30 s, denaturation at 98 °C for 30 s, annealing at 62 °C for 30 s, elongation at 72 °C for 60 s. Thirty cycles including

denaturation, annealing and elongation were performed and a final extension step for 5 min at 72 °C.

2.10.4 Verification of the amplification on agarose-gels

To verify the length of the amplified fragments, an agarose-gel (1 %) was performed. For this, 2.5 g agarose were dissolved in 250 mL boiling TRIS-Acetate-EDTA (TAE)-buffer, mixed with 12.5 µL rotisafe (Roth, Germany) and the gel was solidified. 20 µL of each sample was pipetted into one gel-pocket. The gel was run for 20 min at 100 V. As DNA-ladder, the O´GeneRuler™ 1 kb was used. Expected fragments were cut out of the gel and the DNA was extracted according to the standard protocol of innuPrep DOUBLEpure Kit (Analytik Jena, Germany).

2.10.5 Ligation of the peptidase genes into the vector (pUC57) and transformation of the constructs into *E. coli* Top 10

Ligation of the gene of interest was performed in a 0.2 mL reaction vial at 4 °C overnight. Components of the ligation mixture were as follows: 500 ng insert, 50 ng pUC57, 2 µL PEG4000, 2 µL 10 x T4 DNA ligase buffer, 5 U T4 DNA ligase ad 20 µL with water. 5 µL of the ligation mixture were added to 50 µL chemically competent *E. coli* cells. The mixture was incubated on ice for 15 min before the cells were heated to 42 °C for 45 to 60 s. Head shock was stopped on ice for 2 min. LB-medium (500 µL) was added to the reaction and the bacteria incubated at 37 °C for 30 to 45 min. After incubation, blue/white screening of the clones was performed on LB-Amp-x-Gal plates, which were incubated at 37 °C overnight.

2.10.6 Verifcation the peptidase genes

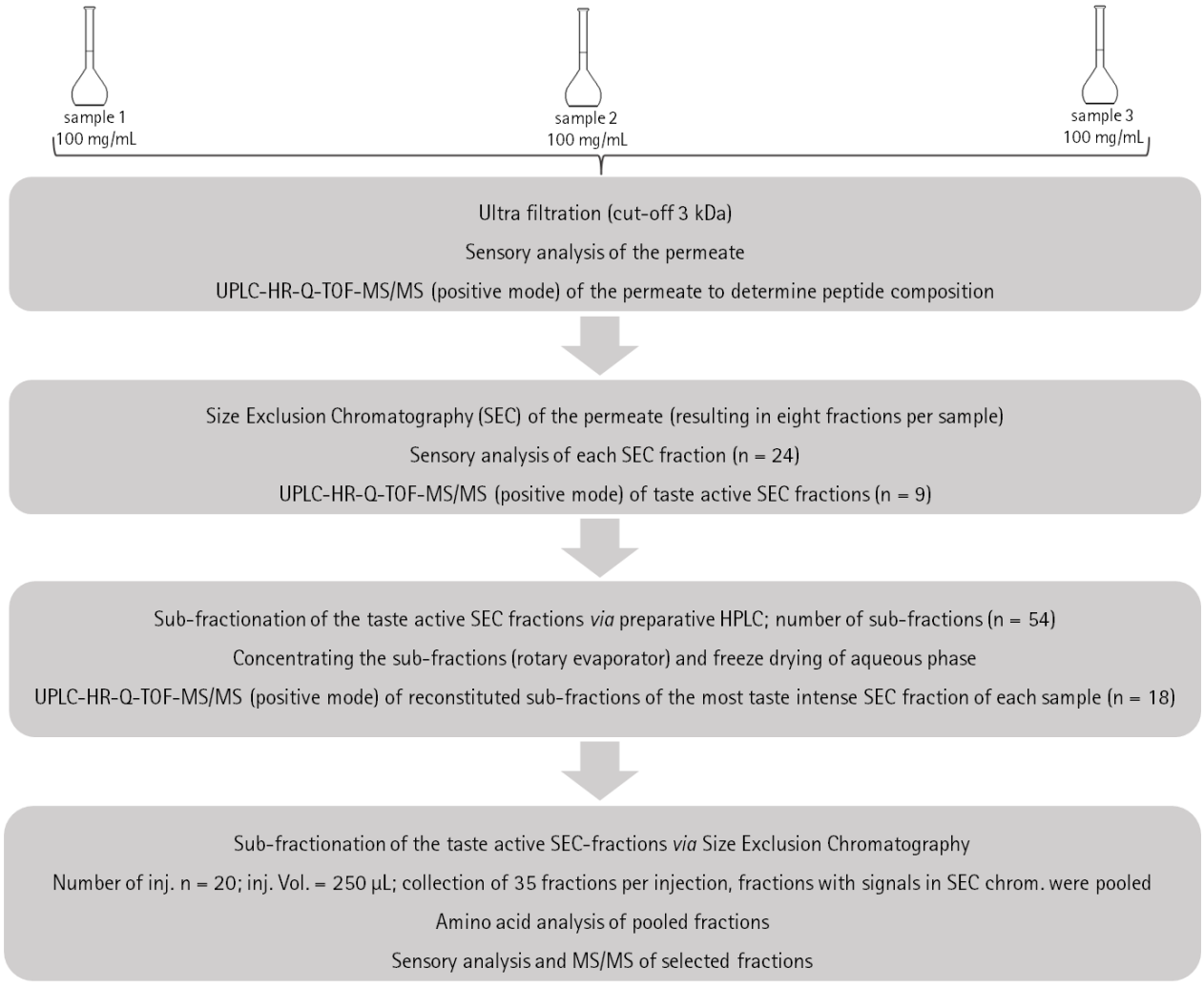
Clones were picked and used as template for a colony PCR. Colony PCR was performed according to the standard protocol for the Dream Taq DNA Polymerase (Thermo Scientific, St. Leon-Rot, Germany). PCR conditions were as follows: Initiation at 95 °C for 10 min; denaturation at 94 °C for 1 min; annealing at 55 °C for 1 min and elongation at 72 °C for 2 min. Thirty-five cycles of denaturation, annealing and elongation were performed and a final extension step for 10 min at 72 °C. Amplification of the expected fragments was examined using agarose-gel electrophoresis (see section 2.10.4). Clones containing the fragment of interest were transferred to overnight cultures. The next day, the plasmids were

isolated according to the standard protocol of the innuPREP Plasmid Mini Kit (Analytik Jena AG, Germany). Isolated plasmids were sent to Seqlab/Microsynth to verify the fragments by sequencing using M13 primers.

3 Results

Planned work-flow

Due to the complexity of the performed work and very similar sample description this work-flow chart was designed. It should enable the reader to follow the work easily. Each of the four fields (grey) shows the work packages performed at the different stages of sample treatment.



3.1 Evaluation of optical and olfactory properties of the raw material

The Nestlé Product Technology Center (*Lebensmittelforschung GmbH; Singen*) delivered three different samples to the Institute of Food Chemistry in Hannover. All samples were treated

differently (Table 3.1-1). Variations of the crystal structure and olfactory properties were estimated.

All samples were hydrolysed for 16 hours. Flavourzyme (FZ) was added to the samples 1 and 2 and Protease P "Amano" 6S (P6SD) was added to sample 3. Sample 1 acted as reference, whereas sample 2 was additionally treated for four hours with a glutaminase. FZ and P6SD are proteolytic enzyme preparations from different *Aspergillus* strains. Flavourzyme is from *Aspergillus oryzae* (Merz, Eisele et al. 2015) and P6SD from *Aspergillus melleus* (Amano-Enzyme 2003).

Table 3.1-1: Information received from Nestlé according to the treatment of the samples used for this work.

Sample No.	Hydrolysis	Additive	Additional Information
1	16 h	FZ	Reference
2	16 h	FZ	+ 4 h Protein Glutaminase
3	16 h	P6SD	/

Overall, the raw materials were inhomogeneous products that varied in terms of the particle size, and colour. The colour of the sample particles ranged from light orange over red to green. The colour of the samples produced under different thermal conditions varied from light yellow to an intensive orange. It became more intense with each additional thermal treatment step.

No difference in the odour of the samples was detected. A brothy and savoury odour was perceived for each sample.

3.2 HPLC analyses of the free amino acid content of the sample stock solutions

Samples were prepared according to section 2.3 (materials and methods). All data in this section were observed by the HPLC method 2.5.3. In this case, the system was calibrated with a five point calibration instead of eight point calibration, as described in the methods section. Calibration points were 10; 20; 62.5; 75 and 100 µM of each amino acid. An external calibration with linear regression was calculated using the linear equation ($y = mx + b$). The coefficient of determination varied between 0.9786 for lysine 2 to 0.9984 for lysine 1 (Figure 3.2-1). Due to the two amino functions of lysine, which both can react with the oPA reagent, lysine gave two different fluorescence signals.

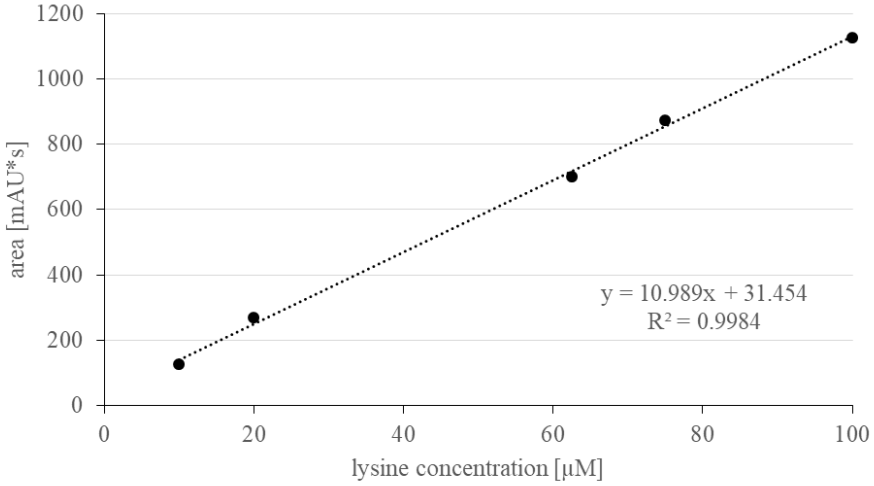


Figure 3.2-1: Exemplary presentation of a five point calibration curve of lysine 1 used for the calculation of the concentration of free lysine in the sample solution. Y-axis shows the peak area in mAU*s and the x-axis shows the lysine concentration [µM] of each calibration point. Linear equation and regression coefficient (R^2) are shown above.

Each amino acid was calculated (Table 3.2-1) with the linear equation of the associated calibration curve. Samples were diluted 1:10 and 1:100, respectively. Depending on evaluable peak areas either the areas of the 1:10 dilution or the areas of the 1:100 dilution were used for the calculation.

Table 3.2-1: Concentration [mM] and composition of free amino acids in samples 1 to 3 (delivered by Nestlé). Analysis was performed using permeate of the ultra-filtration of the sample stock solutions (100 mg mL^{-1}). Abbreviation n.d. means that no signal was detected at the expected retention time.

Amino acid (order of retention)	Sample 1 concentration [mM]	Sample 2 concentration [mM]	Sample 3 concentration [mM]
Aspartic acid (Asp)	0.6	0.6	1.0
Glutamic acid (Glu)	1.1	8.2	1.7
Asparagine (Asn)	1.1	0.7	1.6
Serine (Ser)	6.1	4.9	8.5
Glutamine (Gln)	21.1	2.5	23.7
Histidine (His)	0.9	0.8	1.3
Glycine (Gly)	1.0	0.5	2.4
Threonine (Thr)	1.9	1.9	3.7
Arginine (Arg)	1.4	1.6	3.6
Alanine (Ala)	2.4	2.5	4.6
Tyrosine (Tyr)	1.8	1.7	2.2
Methionine (Met)	0.7	0.9	1.7
Valine (Val)	5.1	5.2	8.1
Tryptophan (Trp)	n.d.	n.d.	n.d.
Phenylalanine (Phe)	3.3	2.4	4.6
Isoleucine (Ile)	3.5	4.0	5.7
Leucine (Leu)	9.3	9.6	12.0
Lysine 1 (Lys1)	0.2	0.2	0.5
Lysine 2 (Lys2)	3.4	0.4	1.0
Sum of total free amino acids	64.9	48.7	87.9

Except of tryptophan all amino acids were detected in each sample. The sum of total free amino acid concentration varied between 48.7 mM in sample 2 to 87.9 mM in sample 3 (Table 3.2-1).

3.3 Sensory analysis of the ultra-filtered sample stock solutions

At the beginning of the project the overall umami taste enhancing properties of the three samples delivered by Nestlé were reviewed. The samples were prepared each as a 100 mg mL^{-1} solution (stock solution). Afterwards ultrafiltration with a cut-off membrane of 3 kDa was performed. The free amino acid concentration (Table 3.2-1) of the ultra-filtrates was determined using HPLC. To mask the impact of free glutamic acid, the stock solution samples were adjusted to 10 mM with added monosodium glutamate (MSG).

The majority of the subjects ranked the umami taste of the samples (adjusted to 10 mM MSG) more intense than the 10 mM standard of MSG (Figure 3.3-1). For sample 1 and 2 72 % of the participants scored the umami taste of the ultra-filtered stock solutions more intense than a 50 mM MSG standard solution. In sample 3 at least 50 % had the impression that the umami taste was more intense than 50 mM. The impression that the stock solution tasted as strong as the 10 mM MSG solution or less was reported by two out of 14 panellists, only.

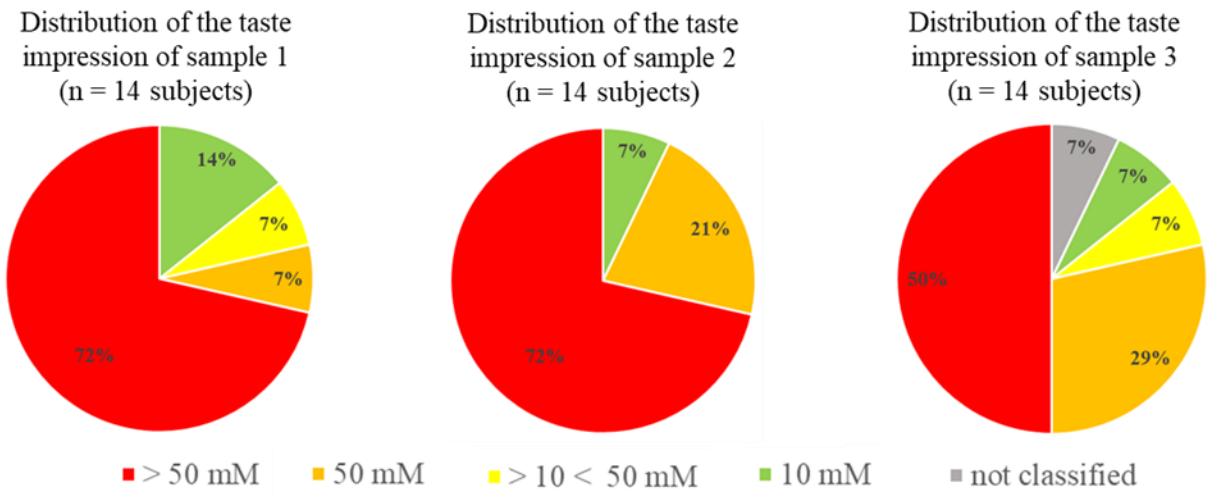


Figure 3.3-1: Pie chart of the taste distribution examined by sensory analysis of sample stock solutions (samples adjusted to 10 mM MSG). Each sample was tested by 14 subjects. Parts highlighted in red show a taste impression more intense than 50 mM (MSG), light orange shows taste impression of 50 mM (MSG), taste impression between 10 and 50 mM MSG is shown in yellow, green shows taste impression of 10 mM MSG and grey was not classified by the subjects.

Sensory evaluation clearly showed that all samples contained umami taste enhancing substances or substances which had an inherent umami taste.

3.4 Determination of the peptide composition of sample stock solutions by UPLC-HR-MS

Mass spectrometry (section 2.5.5) was performed with each ultra-filtered (3 kDa cut-off) sample stock solution (100 mg mL^{-1}). Most abundant signals were identified based on their mass to charge ratios and mass spectra, respectively.

The designed VBA program (2.7.1) calculated the exact masses of di-, tri-, and tetrapeptides, pyroglutamyl dipeptides, and 2,5-diketopiperazines, which were compared with the detected *m/z* ratios of the most abundant peaks (Figure 3.4-1). In addition, the biochemical and chemical plausibility of the proposed compounds was verified with regard to the known processing steps and conditions.

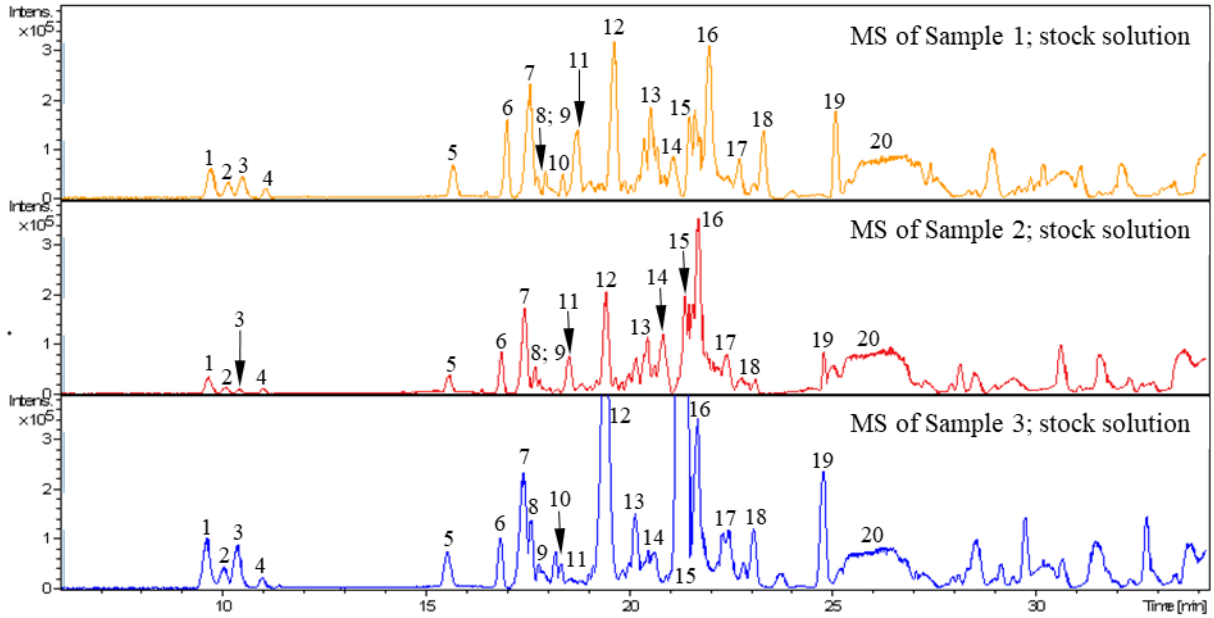


Figure 3.4-1: Base peak chromatogram of sample 1 (yellow), sample 2 (red) and sample 3 (blue). Numbers of peaks indicate the examined signals of each sample and correspond with the numbering in Table 3.7-1. Separation was performed on a HILIC column (2.5.5)

Using this calculation approach, the most abundant peaks turned out to be pyroglutamyl dipeptides and 2,5-diketopiperazines (Table 3.4-1) with the exception of the free amino acids Phe, Pro, and Tyr. In this evaluation only the most intense ions were considered.

Table 3.4-1: Calculated results of the most intense peaks. Peak numbers correlate with the peak numbering in Figure 3.4-1.

Peak number	Compound	Sample	Peak number	Compound	Sample
1	diketo(Ile-Pro)	1, 2, 3	11	diketo(Val-Tyr) Phe-Pro	1, 2
2	diketo(Ile-Pro)	1, 2, 3	12	pyro(Glu-Pro)	1, 2, 3
3	diketo(Phe-Pro)	1, 2, 3	13	diketo(His-Ala) diketo(Glu-Gly)	1, 2
4	diketo(Pro-Val)	1, 2, 3	14	Val-Pro-Leu	1, 2
5	diketo(Tyr-Pro)	1, 2, 3	15	Phe-Pro	1, 3
				Ile-Pro	1, 2, 3
6	diketo(Glu-Leu)	1, 2, 3	16	Phe	1, 2, 3
7	5-Oxo-L-proline	1, 2, 3	17	Pro	1, 2, 3
8	pyro(Glu-Ile-Pro)	1, 3	18	pyro(Glu-Gln)	1, 3
9	Phe	1, 3	19	Tyr	
10	Pro	1, 3	20	Formate clusters	1, 2, 3

For the complete analytical description of the compound composition of all samples, MS/MS analyses were performed afterwards.

3.5 Determination of the peptide composition of sample stock solutions by UPLC-HR-MS/MS

The sample solutions investigated in section 3.4 were also used for this analysis *via* HPLC-HR-MS/MS. Hydrolysed vegetable proteins often consist of a complex mixture of amino acids, small – to oligopeptides, as well as reaction products of the process like pyroglutamyl peptides and 2,5-diketopiperazines, for example. Identification of the signals was achieved by three different calculation approaches (2.7). The calculation approach ´Spectral Library` (SL) was an automatical approach, as well as ´SmartFormula` (SF). SmartFormula proposed the most plausible empirical formulas based on the accuracy of the detected *m/z* ratios. A manual approach

(LSc*, Lars Schmidt) included the calculation of exact masses by a VBA program and their comparison with detected *m/z* ratios. Additionally, detected ms/ms spectra were compared with published fragmentation pattern, and the chemical and biochemical plausibility of occurrence was evaluated. The amount of calculated signals varied between the samples. In total, 175 to 197 signals (Figure 3.5-1) were identified in each sample by at least one of the mentioned calculation approaches including the manual investigation of the signals, which were not identified by the automatic routine calculation.

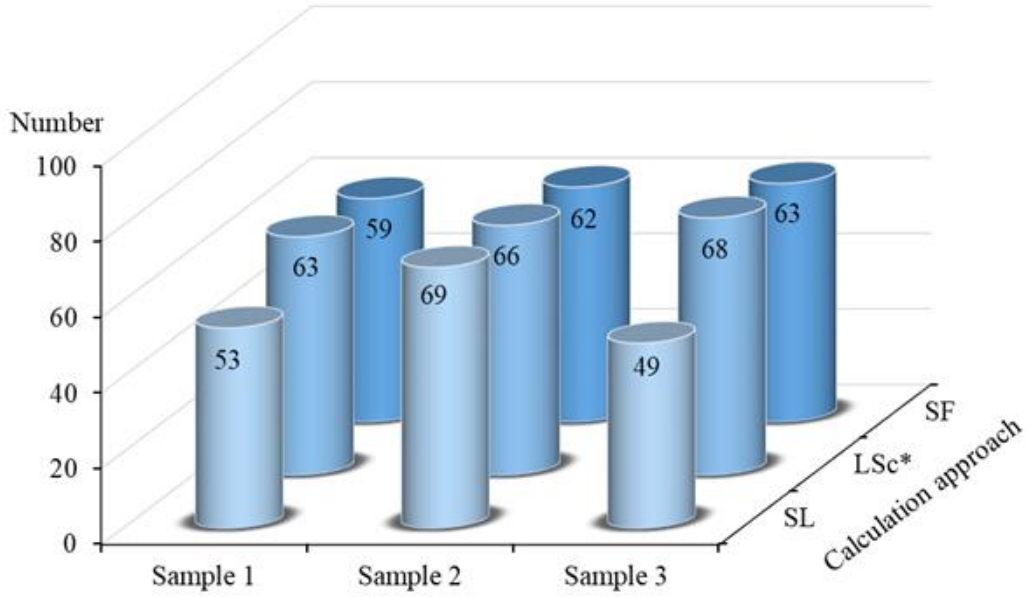


Figure 3.5-1: Bar chart of identified signals in sample stock solution of sample 1 to 3. The first row shows the number of identified substances in sample 1 by the three different calculation approaches. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue, and calculation approach 3 SmartFormula (SF) is shown in dark blue. Row 2 shows sample 2 and row 3 shows sample 3. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

Following the identification approaches, the samples were compared with each other. The aim was to present in detail, if potential umami substances were identified, which of them were exclusively detected in only one sample, in two of the samples or detected in all analysed samples. This strategy should outline the uniqueness of each sample. Moreover, it was possible to rapidly compare the exclusively detected substances with known umami active substances. Thereby, substances with known activity were excluded to rather focus on unique substances with unknown taste properties. The number of exclusively detected peptides (Figure 3.5-2) in sample 1 to 3 varied between two and seventeen.

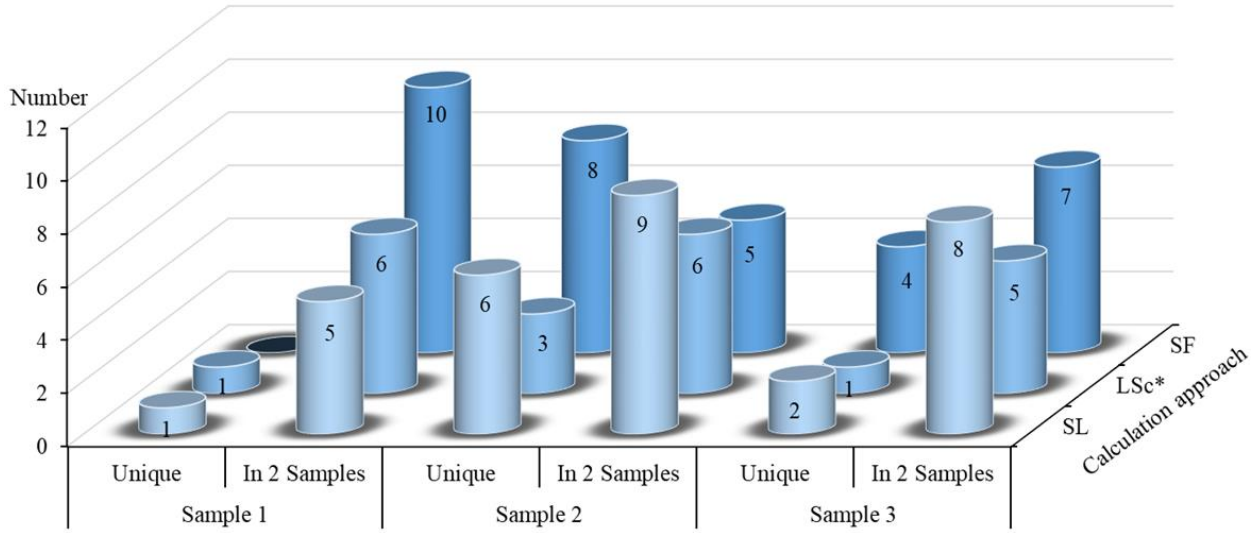


Figure 3.5-2: Bar chart of identified substances, which uniquely appeared in one sample, and substances which, were detected at least in two of the three samples. These results are based on the results of Figure 3.5-1. The first, third, and fifth row shows the number of uniquely identified substances by the three different calculation approaches in sample 1, 2, and 3. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue, and calculation approach 3 SmartFormula (SF) is shown in dark blue. Row two, four, and six shows substances detected in two of the three samples ranging from sample 1 to sample 3. Y-axis shows the number of identified substances, x-axis shows the sample name, and z-axis shows the different calculation approaches.

Uniqueness of the samples:

- Exclusively detected in sample 1:
  - Glu-Gly-Thr and Phe-Pro-Gln
- Exclusively detected in sample 2:
  - Acetyl-DL-Leucine; Thr-Gln-Gly; Ser-Gln-Gly; Val-Met; Ile-Pro-Glu; Pro-Gln; Glu-Ser; Glu-Glu-Gln; Met-Ser-Ser; *m/z* 287.12376; 505.26434; 485.18782; 292.10269; 310.11233; 234.09855; 472.16608; 454.15552
- Exclusively detected in sample 3:
  - Ala-Pro-Gln; Diketo-Ser-Gln; Gly-Gln-Gln; 489.24860; 325.17580; 314.13466; 269.99105

Based on the multitude of detected signals a fractionation approach needed to be performed. A successful fractionation led to lower signal density, and increase the likelihood of the identification of substances, which contribute to the umami taste by sensory analysis.

3.6 Size Exclusion Chromatography of sample stock solutions

Size Exclusion Chromatography was the beginning of the second work package.

Size exclusion chromatography (SEC) of the permeate (resulting in eight fractions per sample)
Sensory analysis of each SEC fraction (n = 24)
UPLC-HR-Q-TOF-MS/MS (positive mode) of taste active SEC fractions (n = 9)

Due to the variety of signals appearing in the sample stock solutions, a SEC was performed (Method 2.5.1). For this purpose, the permeate of the ultra-filtration (method 2.3) with a 3 kDa cut-off was used. The focus was on small compounds with molecular masses smaller than 3 kDa. A three-point calibration with Val-Tyr-Val (379 Da), Tyr-Ala (252 Da), and tyrosine (181 Da) was performed (Figure 3.6-1). Therefore, the partition coefficient (K_{av}) was plotted against the logarithm of the molecular mass (log_M).

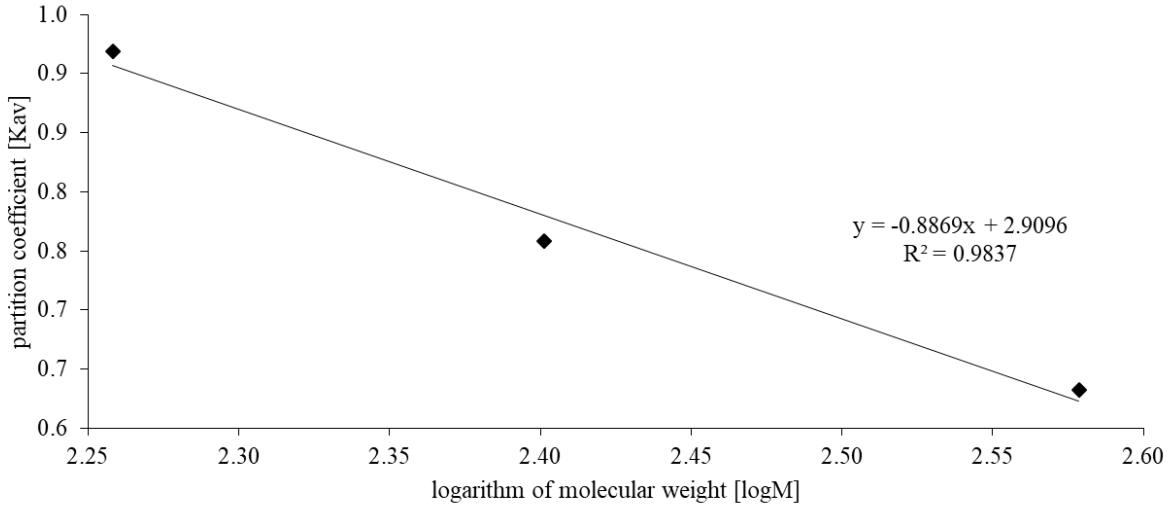


Figure 3.6-1: External calibration of SEC system using Val-Tyr-Val, Tyr-Ala, and tyrosine. Y-axis shows partition coefficient (K_{av}) and x-axis shows logarithm of molecular mass (log_M). Linear equation and coefficient of determination (R^2) are shown in the diagram.

Sample 1 to 3 were injected six times each with a volume of 250 µL per injection. Fractions were collected for five minutes and pooled, which resulted in the SEC-fractions A1/2 to A8 (Figure 3.6-2) with a total volume of 15 mL.

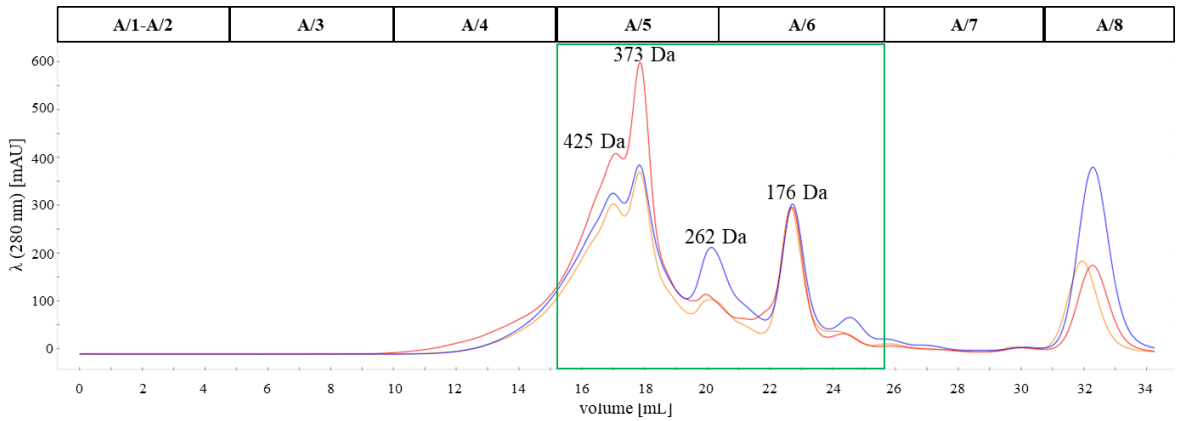


Figure 3.6-2: Chromatogram of Size-Exclusion Chromatography (SEC) of sample 1 (blue), sample 2 (red), and sample 3 (orange). Each fraction was collected for five minutes. Numbering of the fraction is shown on top of the figure (A/1 to A/8). Molecular masses of the most abundant peaks were calculated (425 – 176 Da), and corresponding fractions are framed in green.

Each sample showed the same elution pattern but had differences in the peak intensities. Detected peaks showed molecular masses between 425 to 176 Da. This indicated the presence of the expected di – and tripeptides, pyroglutamyl dipeptides and 2,5-diketopiperazines. Before a sensory analysis of the SEC-fractions was performed, the concentration of free glutamic acid of the fractions had to be measured. The glutamic acid concentration of the SEC-fractions, which were sensory analysed, was crucial for the experiment due to the inherent umami taste of glutamic acid.

3.7 HPLC analyses of the free glutamic acid content of the SEC-fractions

In this step a steeper gradient profile was used, which decreased the run time to 32 minutes. The method was calibrated in the range of 10 to 100 µM, and the corresponding coefficient of determination for glutamic acid was 0.9930.

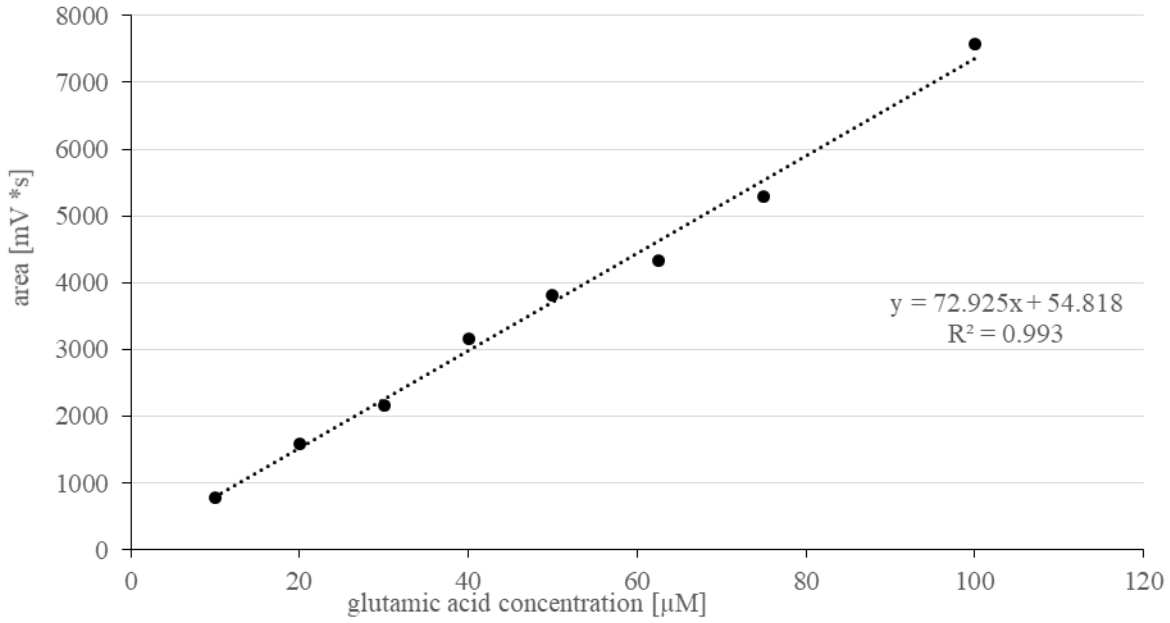


Figure 3.7-1: Exemplary presentation of a eight point calibration curve of glutamic acid used for the calculation of the concentration of free glutamic acid in SEC-fractions. Y-axis shows the peak are in mV*s and the x-axis shows the glutamic acid concentration [µM] of each calibration point. Linear equation and regression coefficient (R^2) are shown.

Since glutamic acid has an inherent umami taste, the concentration of glutamic acid was measured in the SEC-fractions of each sample Table 3.7-1. Based on these data the sensory analysis (2.6) of these fractions was planned.

Table 3.7-1: Glutamic acid concentration [mM] of SEC-fractions A/1 to A/8 of sample 1 to sample 3. Abbreviation n.d. means that no signal was detected at the expected retention time.

	SEC-fraction A1/2	SEC-fraction A3	SEC-fraction A4	SEC-fraction A5	SEC-fraction A6	SEC-fraction A7	SEC-fraction A8
Sample 1	n.d.	n.d.	n.d.	0.46 mM	0.01 mM	n.d.	n.d.
Sample 2	n.d.	n.d.	n.d.	4.31 mM	0.06 mM	n.d.	n.d.
Sample 3	n.d.	n.d.	n.d.	0.99 mM	0.02 mM	n.d.	n.d.

SEC-fractions A5 and A6 were the only fractions containing glutamic acid. However, the main portion of free glutamic acid was detected in SEC-fraction A5 (0.5 to 4.3 mM) of each sample. Based on these results the design of experiment for the sensory analysis of the SEC-fractions was done.

3.7.1 Sensory analysis of the SEC-fractions

To ensure that umami taste effects as well as umami taste enhancing activities were detected, samples without glutamic acid were adjusted to 10 mM with MSG.

All SEC-fractions of sample 1 to 3 were sensory evaluated by a panel of 14 (sample 1 and 3), or 15 (sample 2) untrained subjects. Except fractions A5 and A6, all fractions were adjusted to 10 mM with MSG. For all samples (Figure 3.7-2 to Figure 3.7-4), fractions A5 and A6 turned out as the most taste intensive ones. Eleven to fourteen subjects evaluated the fractions A5 and A6 with a higher umami taste compared to a 50 mM MSG standard solution even though these two fractions were not adjusted to a level of 10 mM MSG. Nevertheless, seven to eleven participants also rated SEC-fraction A4 of sample 1 to 3 as more taste intensive than 50 mM, although it was adjusted to 10 mM MSG, only.

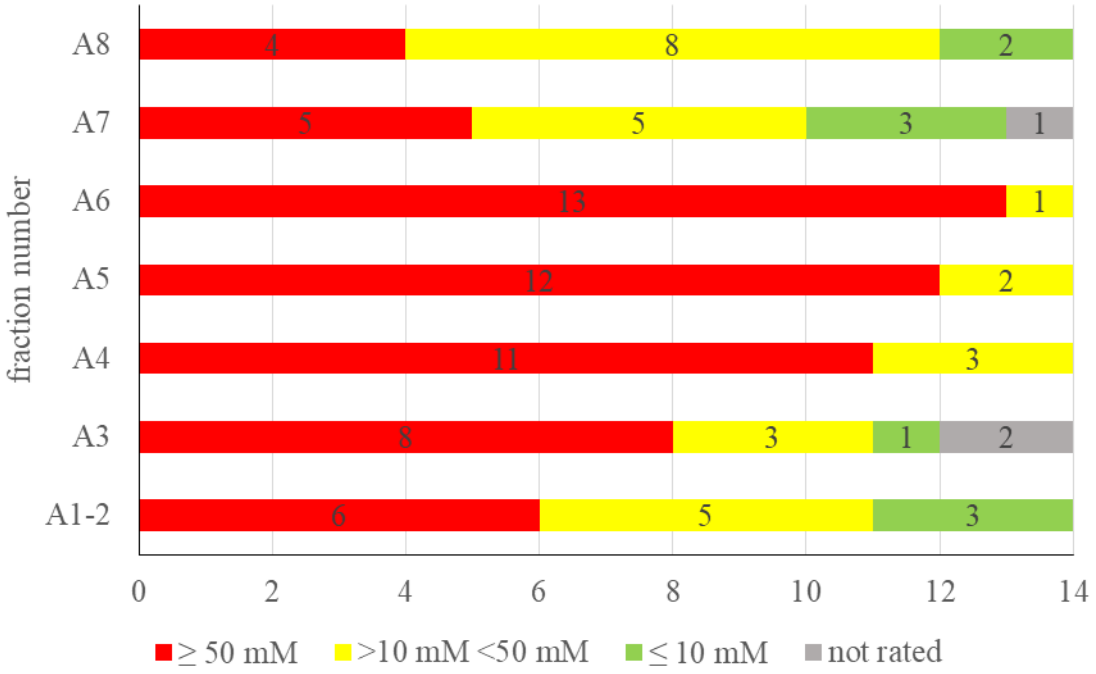


Figure 3.7-2: Bar chart of the results of sensory analysis of the SEC-fractions A1 to A8 of sample 1. Bars highlighted in red shows a taste impression, which is perceived more intense than a 50 mM mono sodium glutamate (MSG) solution. The yellow bars show taste impression between 10 and 50 mM MSG, the green bars show taste impression ≤ 10 mM and the grey bars show the number of participants, which were not able to rate the sample clearly (not rated). Y-Axis shows the fraction numbers and x-axis show the taste impression in mM compared with the MSG standard solutions.

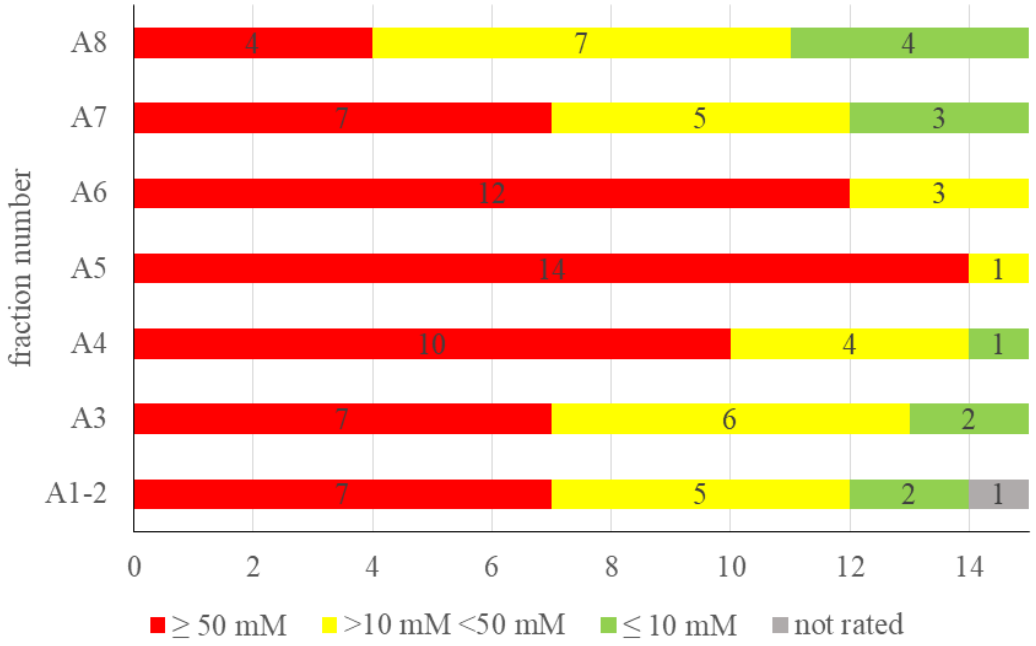


Figure 3.7-3: Bar chart of the results of sensory analysis of the SEC-fractions A1 to A8 of sample 2. Bars highlighted in red shows a taste impression, which is perceived more intense than a 50 mM mono sodium glutamate (MSG) solution. The yellow bars show taste impression between 10 and 50 mM MSG, the green bars show taste impression ≤ 10 mM and the grey bars show the number of participants, which were not able to rate the sample clearly (not rated). Y-Axis shows the fraction numbers and x-axis show the taste impression in mM compared with the MSG standard solutions.

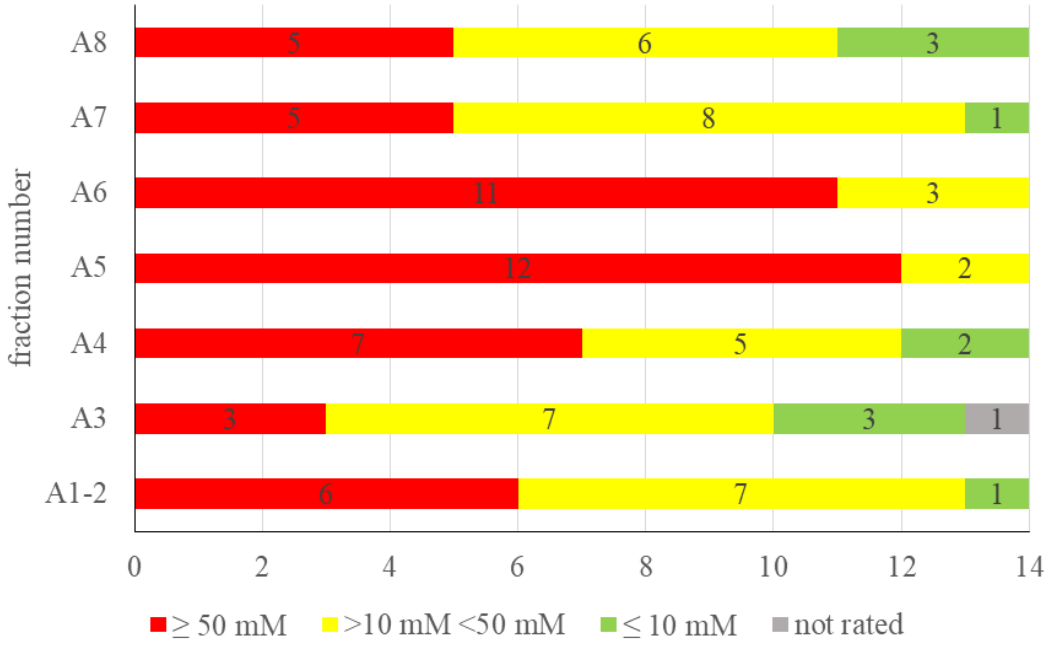


Figure 3.7-4: Bar chart of the results of sensory analysis of the SEC-fractions A1 to A8 of sample 3. Bars highlighted in red shows a taste impression, which is perceived more intense than a 50 mM mono sodium glutamate (MSG) solution. The yellow bars show taste impression between 10 and 50 mM MSG, the green bars show taste impression ≤ 10 mM and the grey bars show the number of participants, which were not able to rate the sample clearly (not rated). Y-Axis shows the fraction numbers and x-axis show the taste impression in mM compared with the MSG standard solutions.

These promising results of SEC-fractions A4, A5 and A6 of all three samples led to the exclusion of the other SEC-fractions for the subsequent experiments. The main focus was to identify the substance composition of the fractions of interest and potentially discover substances with inherent and yet unknown umami attributes.

3.8 Determination of the peptide composition of the SEC-fractions by UPLC-HR-MS/MS

The permeates of the sample stock solutions were fractionated by SEC (2.5.1) to separate the small molecules. This step facilitated the identification of the molecules and increased the likelihood of the detection of potential umami active substances. UPLC-HR-MS/MS was performed as before (2.5.5) as well as the identification based on the three mentioned calculation approaches (2.7). Additionally, MS/MS experiments in the negative ionisation mode were performed (Supplementary figure 4, 6, 8, 10, 12, 14, 16, 18, 20) to confirm the results of the positive MS/MS mode. Furthermore, more acidic substances became detectable in the negative mode. Only the most umami taste intense SEC-fractions A4 to A6 (3.7.1) of all samples were analysed *via* UPLC-HR-MS/MS. The number of identified peptides with at least one of the calculation approaches varied in each sample as well as in-between the SEC-fractions. In sample 1 SEC A4 a number of twelve peptides were identified, 15 peptides in sample 2 SEC A4 and eleven peptides in sample 3 SEC A4 (Figure 3.8-1), respectively.

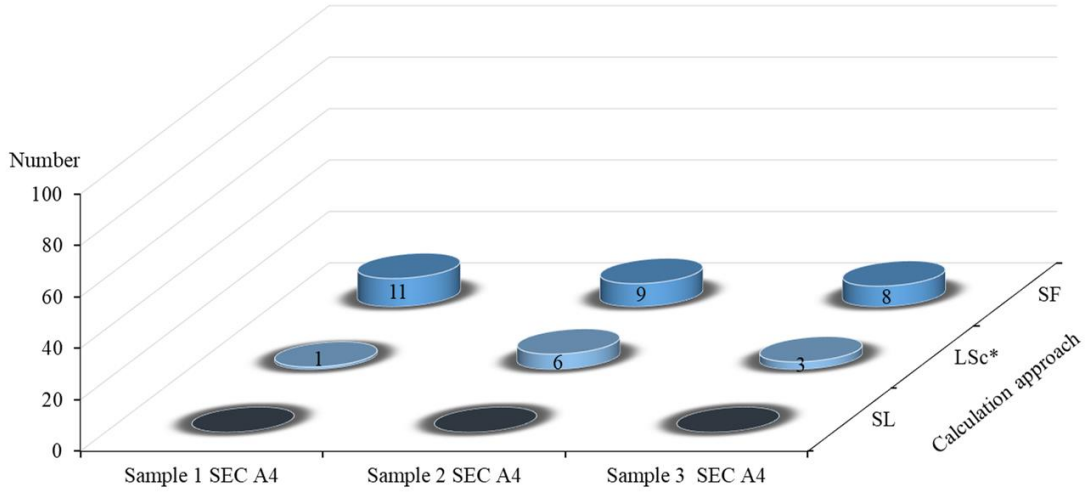


Figure 3.8-1: Bar chart of identified signals in SEC-fraction A4 of sample 1 to sample 3. The first row shows the number of identified substances by the three different calculation approaches in SEC-fraction A4 of sample 1. Calculation approach 1 spectral library (SL) is shown in black, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue and calculation approach 3 SmartFormula (SF) is shown in dark blue. Row 2 shows SEC-fraction A4 of sample 2 and row 3 shows SEC-fraction A4 of sample 3. Y-axis shows the number of identified substances, x-axis shows the sample name, and z-axis shows the different calculation approaches.

None of the twelve identified signals in sample 1 SEC A4 was a peptide exclusively occurring in this sample. In contrast, four of the signals identified in sample 2 SEC A4 were unique peptides in this sample and two unique peptides were detected in sample 3 SEC A4.

Uniqueness of SEC A4:

- Exclusively detected peptides in sample 1 SEC A4:
  - None
- Exclusively detected in sample 2:
  - Gln-Tyr-Lys; Gln-Arg-Ala; Ala-Thr-Arg-Arg; Glu-Lys-His-Ile
- Exclusively detected in sample 3:
  - Diketo-Pro-Pro; Gln-Lys-Ile

The same procedure was performed with SEC A5 and A6 of sample 1 to 3. The number of identified substances in SEC A5 varied from 91 in sample 1 to 141 in sample 3 (Figure 3.8-2).

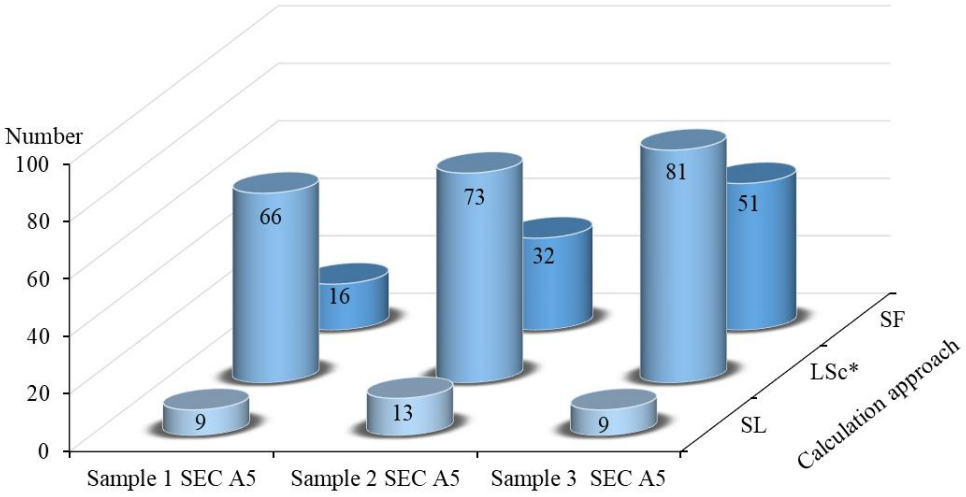


Figure 3.8-2: Bar chart of identified signals in SEC-fraction A5 of sample 1 to sample 3. The first row shows the number of identified substances by the three different calculation approaches in SEC-fraction A5 of sample 1. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue and calculation approach 3 SmartFormula (SF) is shown in dark blue. Row 2 shows SEC-fraction A5 of sample 2 and row 3 shows SEC-fraction A5 of sample 3. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

The fractions were compared with each other to determine their uniqueness. SEC A5 (sample 1) showed three unique peptides, SEC A5 (sample 2) 15 and SEC A5 (sample 3) 26 exclusively detected substances (Figure 3.8-3).

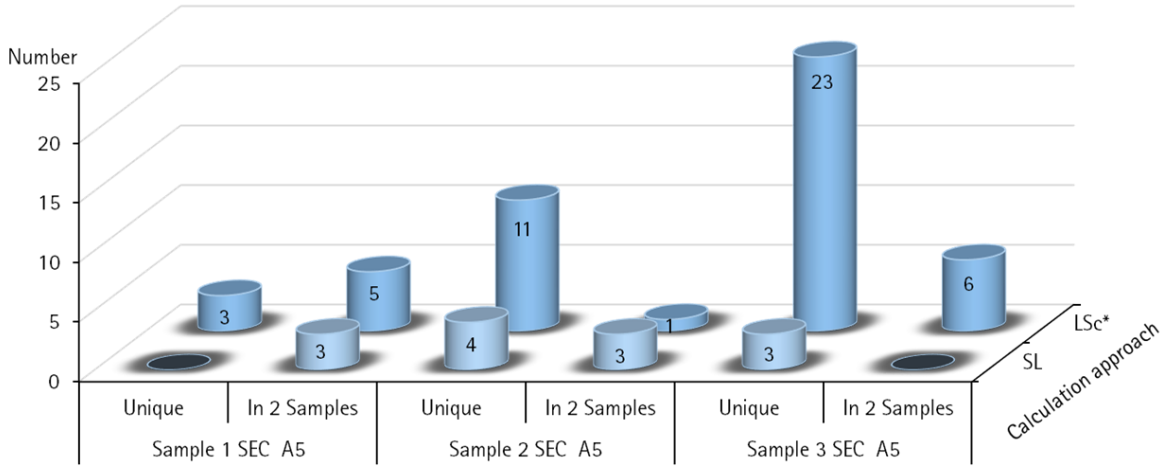


Figure 3.8-3: Bar chart of identified substances, which uniquely appeared in one sample and substances, which were detected at least in two of the three samples. These results are based on the results of Figure 3.8-2. The first, third and fifth row show the number of uniquely identified substances by two different calculation approaches in sample SEC-fraction A5 of 1, 2 and 3. Calculation approach 1 spectral library (SL) is shown in blue-grey and in case of no identified substance in black, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue, calculation approach three is not shown, because no substance was identified by this approach appearing uniquely. Row two, four and six show substances detected in two of the three samples ranging from SEC-fraction A5 of sample 1 to sample 3. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

Uniqueness of SEC A5:

- Exclusively detected in sample 1:
  - Diketo-Ala-Ala; Diketo-Glu-Gly; Gln-Ile
- Exclusively detected in sample 2:
  - Diketo-His-Cys; Diketo-Thr-Thr; Diketo-Thr-Phe; γ-Glu-Ile; L-Glutamate; Diketo-Tyr-Pro; Ile-Pro-Phe; Ile-Pro-Met; Pro-Phe-Ala; Diketo-Gln-Arg; Cys-Gln-Cys; Ile-Pro-Glu; Diketo-Asp-Tyr; Diketo-His-Pro; Diketo-Asn-His
- Exclusively detected in sample 3:
  - Diketo-Val-Val; Phe-Ala-Ser; Phe-Ala-Pro; Ile-Pro; Ile-Ala; Diketo-Asp-Thr; Diketo-Glu-Gln; Gln-Ser; Tyr-Arg-Met; Asn-Gln-Thr; Glu-Asn-Gln; Glu-Arg-Phe; Ile-Gly-Phe; Ile-Pro-Tyr; Ile-Pro-Pro; Glu-Gln-Leu; Tyr-Pro; Val-Pro-Pro; Pro-Ser-Val; Ile-Pro-Gln; Gln-Pro-Ser; Gln-Asn; Diketo-Glu-Ser; Ser-Ser-Gly; Gln-Gln-Gly; Gln-Asn-Ser

In SEC A6 of sample 1 36 peptides were identified, 40 peptides in SEC A6 of sample 2 and 45 peptides in SEC A6 of sample 3 (Figure 3.8-4).

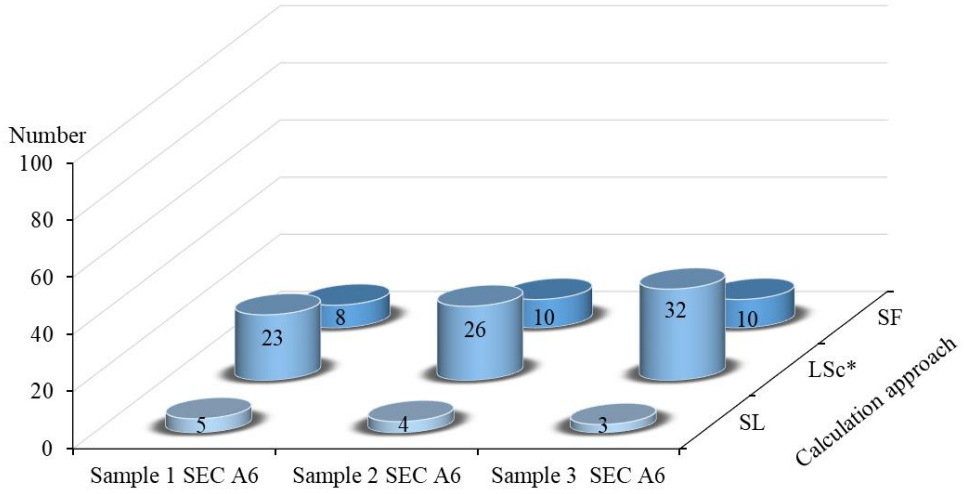


Figure 3.8-4: Bar chart of identified signals in SEC-fraction A6 of sample 1 to sample 3. The first row shows the number of identified substances by the three different calculation approaches in SEC-fraction A6 of sample 1. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue and calculation approach 3 SmartFormula (SF) is shown in dark blue. Row 2 shows SEC-fraction A6 of sample 2 and row 3 shows SEC-fraction A6 of sample 3. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

Two substances of the 36 identified in SEC A6 of sample 1 were unique for this sample, five substances were unique for SEC A6 of sample 2, and eight substances were exclusively detected in SEC A6 of sample 3 (Figure 3.8-5).

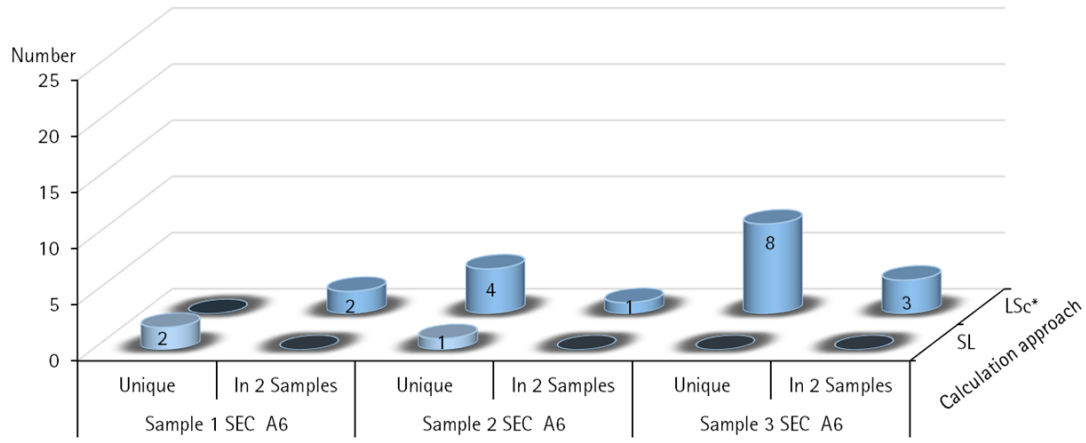


Figure 3.8-5: Bar chart of identified substances, which uniquely appeared in one sample and substances, which were detected at least in two of the three samples. These results are based on the results of Figure 3.8-4. The first, third and fifth row show the number of uniquely identified substances by two different calculation approaches in sample SEC-fraction A6 of 1, 2 and 3. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue, calculation approach three is not shown, because no substance was identified by this approach appearing uniquely. In case of no identified substances bars are shown in black. Row two, four and six show substances detected in two of the three samples ranging from SEC-fraction A6 of sample 1 to sample 3. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

Uniqueness of SEC A6:

- Exclusively detected in sample 1:
  - Gln-Phe; Tyr-Pro-Phe
- Exclusively detected in sample 2:
  - Tyr-Pro-Leu; Diketo-Ala-Ser; Cys-Met-Gly; Glu-Tyr; Diketo-Ala-Pro
- Exclusively detected in sample 3:
  - Trp-Arg-Gln; Diketo-Met-Pro; Diketo-Glu-Trp; Met-Met; Lys-Tyr-Cys; Diketo-His-Phe; Tyr-Asn-Gly; Met-Ser

Size exclusion chromatography was a promising tool for the fractionation of the sample stock solutions. Nonetheless, the multitude of detected and, to some extent, identified substances did not allow to draw any more specific conclusion about individual umami active substances. For this reason, a sub-fractionation method was developed and performed.

3.9 Sub-fractionation of the umami taste active SEC-fractions by preparative HPLC

The third work package started with the development of a “food-grade” sub-fractionation method.

Sub-fractionation of the taste active SEC fractions *via* preparative HPLC; number of sub-fractions (n = 54)
Concentrating the sub-fractions (rotary evaporator) and freeze drying of aqueous phase
UPLC-HR-Q-TOF-MS/MS (positive mode) of reconstituted sub-fractions of the most taste intense SEC fraction of each sample (n = 18)

Sub-fractionation of the taste active SEC-fractions on a preparative scale was considered in order to correlate compounds identified with umami taste activity. The practicability of this approach was reviewed with SEC-fraction A5 of sample 2 of the respective gluten hydrolysate. A reversed phase preparative HPLC-method was set up using “food-grade” equipment and solvents. The main goal was to keep the samples free from toxic and harmful ingredients. Sub-fractionation of the umami taste active SEC-fraction A5 of sample 2 was performed (2.5.2). Focus was laid on this sample because of the multitude of detected potential umami active substances.

An overlay of three consecutive runs of 200 µL sample injection at a time is shown in Figure 3.9-1 (fractionation pattern highlighted in red frames). Instantly after injection, sub-fractions were collected every 10 min, which resulted in sub-fractions of 80 mL. Respective fractions of the runs were pooled, concentrated and freeze dried (2.4), reconstituted with a volume (ddH_2O), which was equivalent with the total injection volume and finally analysed *via* UPLC-QTOF-HR-MS/MS (2.5.5).

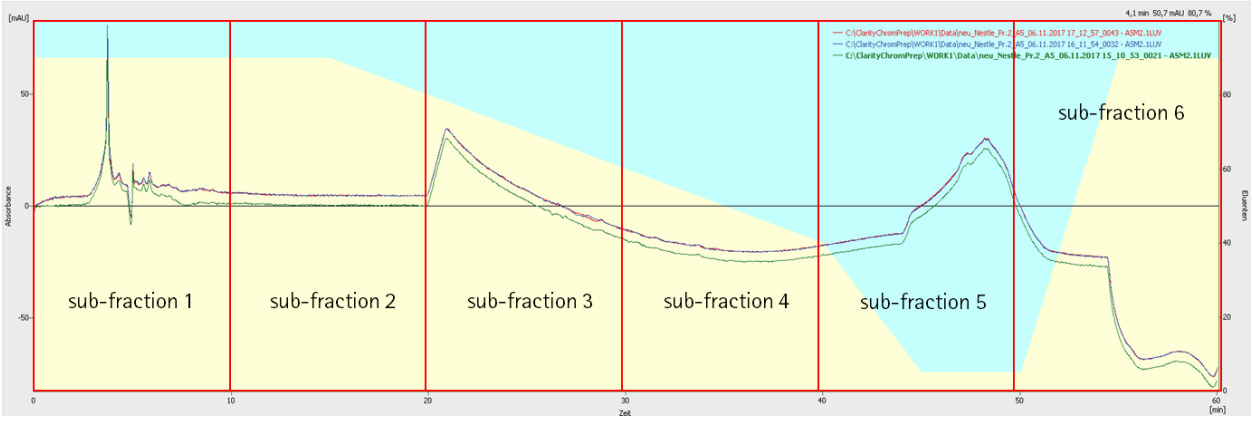


Figure 3.9-1: Typical chromatogram of sub-fractionation of SEC-fraction A5 of sample 2 *via* prepHPLC. Separation was performed on prepHPLC system AZURA (Knauer, Germany) on a preparative column (NUCLEODUR C18 Pyramid, 5 µm; 16 * 250 mm; MACHEREY-NAGEL, Düren, Germany). Gradient composition is shown in light blue and yellow. Fractions were cut every ten minutes. Single fractions are red framed from sub-fraction 1 to sub-fraction 6. SEC-fraction A5 of sample 1 is shown in green, sample 2 in blue and sample 3 in red.

Even though 210 nm is the most appropriate wavelength for the detection of peptides, in the UV spectra no significant peak was detected. The success of the fractionation was controlled afterwards using HR-MS.

3.10 HPLC analyses of the free glutamic acid content of the prepHPLC sub-fractions

HPLC was performed as before (method 2.5.3). In view of the ensuing sensory analyses, glutamic acid concentration was determined, a seven point calibration was performed (10 – 100 µM), correlation coefficient $R^2 = 0.9982$.

Table 3.10-1: Glutamic acid concentration [mM] of prepHPLC sub-fraction 1 from SEC A5 of sample 1 to sample 3.

Sample name	Glutamic acid concentration [mM]
prepHPLC sub-fraction 1 (sample 1; SEC A5)	0.01
prepHPLC sub-fraction 1 (sample 2; SEC A5)	0.10
prepHPLC sub-fraction 1 (sample 3; SEC A5)	0.03

According to the measured glutamic acid concentration (Table 3.10-1) of the prepHPLC sub-fraction 1 (SEC A5 of sample 1 to sample 3), all of them were adjusted to 10 mM MSG for the subsequent sensory analysis.

3.11 Sensory analysis of the prepHPLC sub-fractions

Of the 14 participants, two were taken out of the evaluation. Neither one of these two panellists tasted any test series correctly. In the performed triangle test the panel did not find a significant increase of the umami taste by any of the samples (Figure 3.11-1).

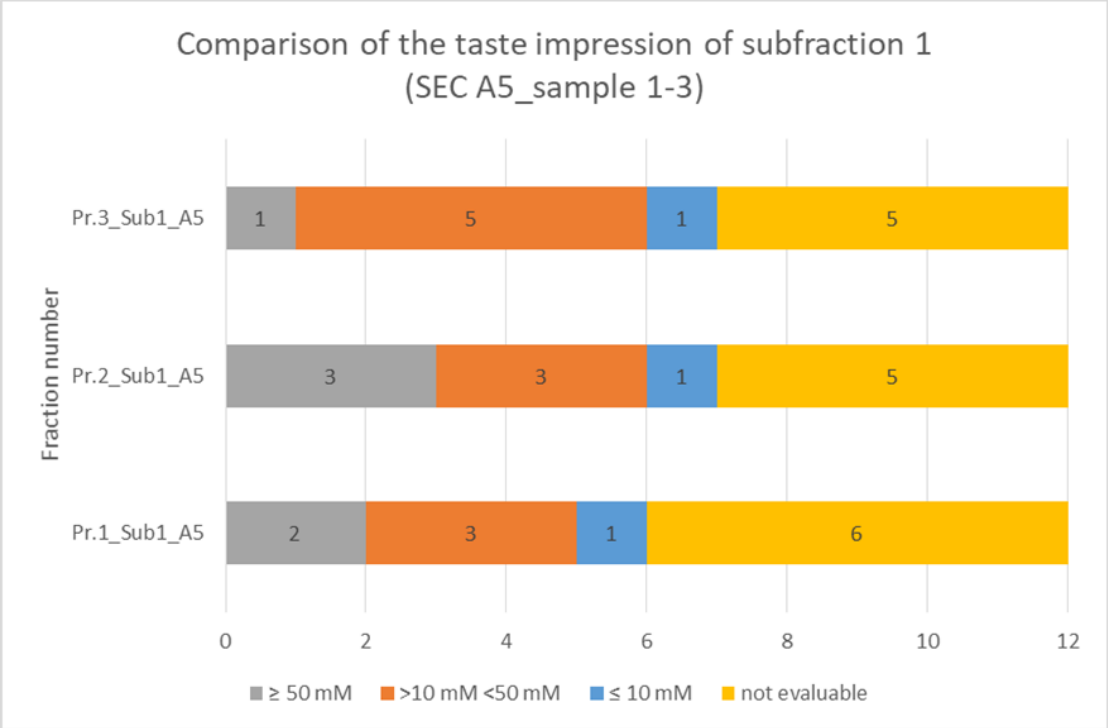


Figure 3.11-1: Triangle-test of the prep-HPLC sub-fraction 1 of each sample adjusted to 10 mM MSG. Taste impression higher than 50 mM monosodium glutamate (MSG) in grey, taste impression higher than 10 mM but lower than 50 mM in orange, taste impression below 10 mM in blue, and taste impression from candidates, who were not able to distinguish between sample, and standard solution are shown in yellow and were rated as not evaluated. The numbers in the bars show the number of given answers.

Around 50 % of the participants were able to taste a difference between the standard solution (10 mM MSG), and the sample solution. Since only 50 % had the impression of a strong umami taste, the enhancing effect was not strong enough to give the samples a taste impression clearly distinguishable from the standards.

3.12 Determination of the peptide composition of the prepHPLC sub-fractions from SEC-fraction A5 of all samples by UPLC-HR-MS/MS

The peptide composition of the sub-fractions was determined (2.5.6) (Figure 3.12-1 to Figure 3.12-3). Comparison of each constituent of all sub-fractions led to a specific overview of the separation feasibility of the preparative HPLC method. Sub-fraction 1 had

the highest number of peptides, which decreased from sub-fraction to sub-fraction. This distribution pattern was evident for all three samples.

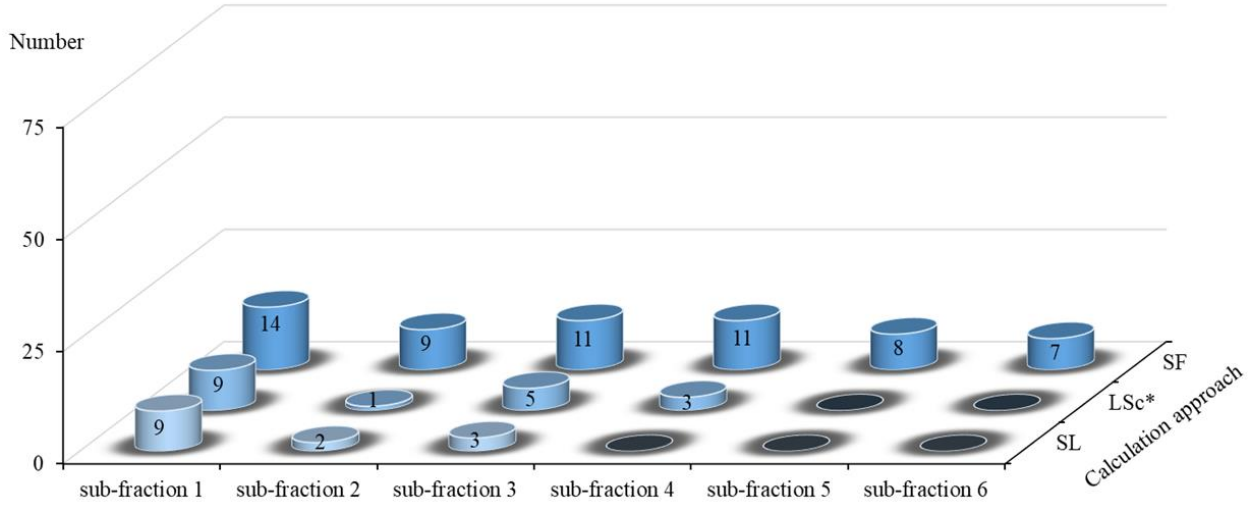


Figure 3.12-1: Bar chart of identified signals in sub-fraction 1 to sub-fraction 6 of sample 1. The first row shows the number of identified substances by the three different calculation approaches. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue, calculation approach 3 SmartFormula (SF) is shown in dark blue, and in case of no identified substance bars are shown in black. From row two to row six, sub-fractions two to six are shown. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

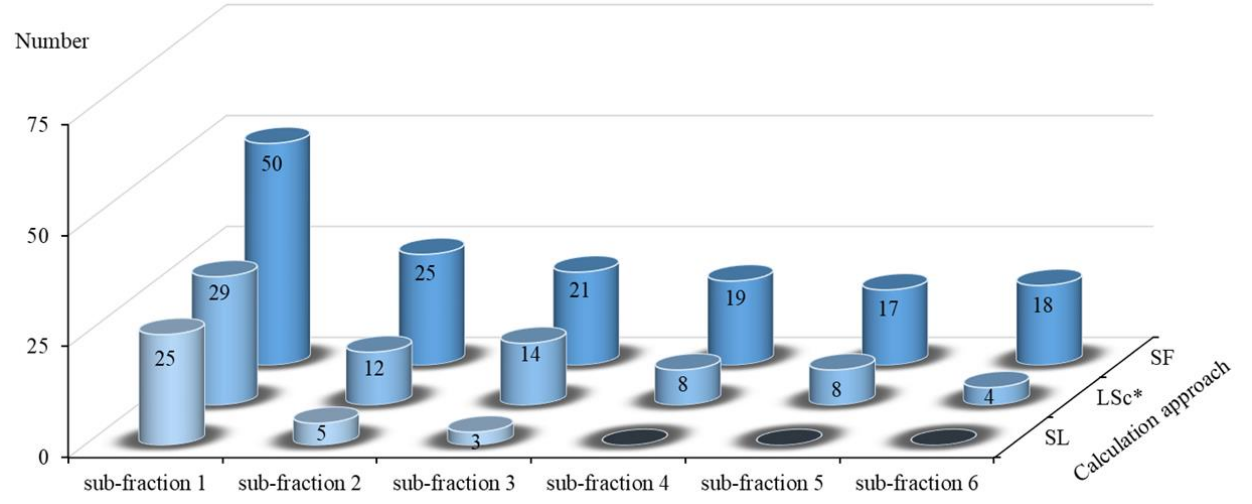


Figure 3.12-2: Bar chart of identified signals in sub-fraction 1 to sub-fraction 6 of sample 2. The first row shows the number of identified substances by the three different calculation approaches. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue, calculation approach 3 SmartFormula (SF) is shown in dark blue, and in case of no identified substance bars are shown in black. From row two to row six, sub-fractions two to six are shown. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

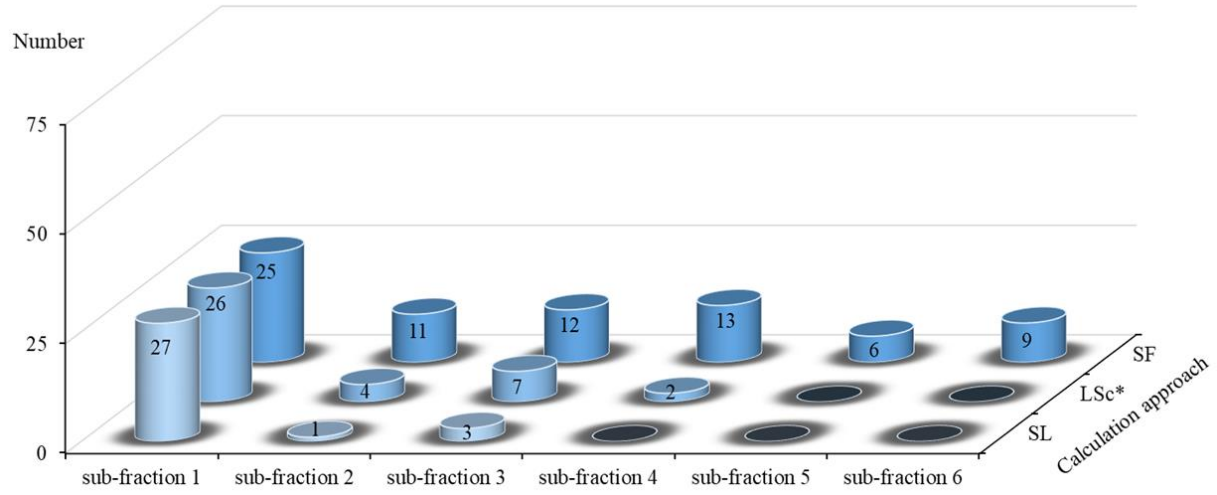


Figure 3.12-3: Bar chart of identified signals in sub-fraction 1 to sub-fraction 6 of sample 3. The first row shows the number of identified substances by the three different calculation approaches. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue, calculation approach 3 SmartFormula (SF) is shown in dark blue, and in case of no identified substance bars are shown in black. From row two to row six, sub-fractions two to six are shown. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

Due to the occurrence of the highest number of peptides in the sub-fraction one of each sample they were compared with each other to underline their uniqueness based on the exclusively detected substances (Figure 3.12-4).

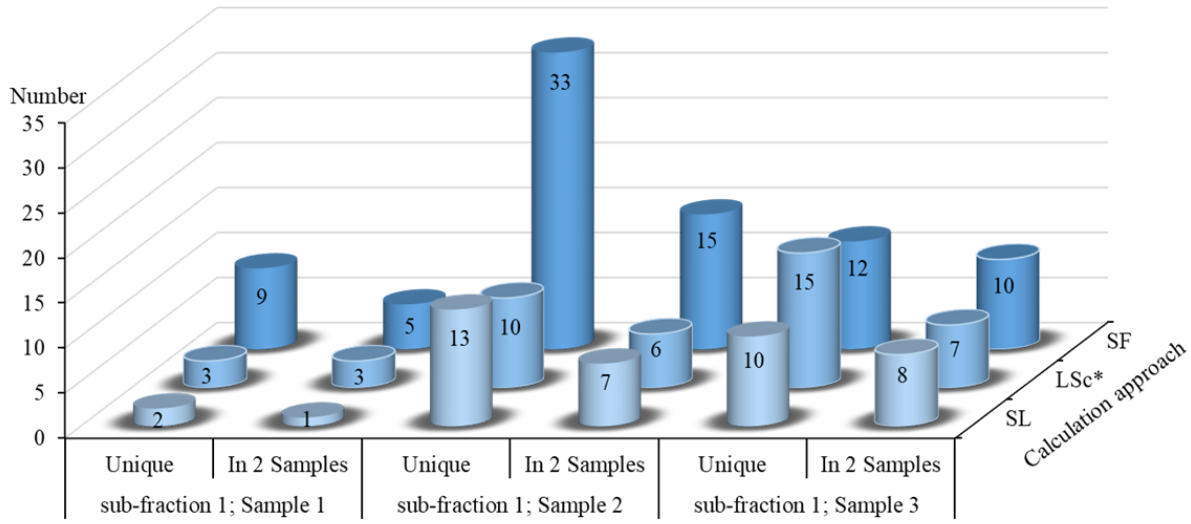


Figure 3.12-4: Bar chart of identified substances, which uniquely appeared in sub-fraction 1, and substances, which were detected at least in two of the three samples. These results are based on the results of Figure 3.12-3. The first, third and fifth row show the number of uniquely identified substances by the three different calculation approaches in sub-fraction 1 of sample 1 to 3. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue, calculation approach three is shown in dark blue. Row two, four and six shows substances detected in two of the three samples. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

- Exclusively detected in sub-fraction 1 of sample 1 SEC A5:
  - Ala-Arg-Ala; 295.21152; Ser-Val-Arg; 339.23773; 356.26293; 405.24561; 400.28781; 383.26260; 135.00115; 410.11538; L-Isoleucine; Thr-Cys-Gly; Pro-Gln; 266.11085
- Exclusively detected in sub-fraction 1 of sample 2 SEC A5:
  - 149.06002; 271.18820; 303.08381; Phe-Arg-Gly; Diketo-Trp-Lys; 257.13566; 233.07815; 229.14075; 505.33576; 447.29555; 194.11487; 365.19452; 267.12001; 299.14757; 259.15131; Diketo-His-Gly; 215.12510; 455.22621; Asp-Arg-Pro; 625.32119; 455.22786; Phe-Phe-Gln; 245.13566; Trp-Trp-Gln; 514.32218; 219.17434; Diketo-Tyr-Pro; 239.15025; 305.15813; 283.17647; 301.28495; Phe-Ile-Asn; 343.29552; Diketo-Ser-Pro; 249.08296; Ile-Ile; 281.07413; 328.22308; 203.13902; 157.13354; 285.18088; Glu-Leu; Ile-Glu; Ile-Pro-Glu; Diketo-Glu-Val; Val-Gly; Pro-Glu; 310.12985; Pro-Gly; Ser-Glu; Glu-Gln; L-Methionine S-oxide; 162.07608; 180.08665; 156.97976

- Exclusively detected in sub-fraction 1 of sample 3 SEC A5:
  - 304.29988; 468.41999; 388.13639; Val-Gln-Pro; Diketo-Gln-Pro; Diketo-Glu-Gly; 231.17032; Diketo-His-Cys; Diketo-Pro-Asn; Thr-Gly-Phe; Diketo-Glu-Thr; 251.13633; Diketo-Pro-Ile; Pro-Pro-Ile; Pro-Ile-Ala; 355.16255; 240.09788; Tyr-Pro; 355.16121; Pro-Pro-Val; 133.03178; L-Methionine; Ile-Asp; Gly-Leu-Ala; Pro-Ala; Ile-Pro-Gln; Diketo-Arg-Pro; Diketo-Glu-Thr; Diketo-Lys-Pro; Pro-Ser; 244.13052; 293.14556; Gln-Gln; Ser-Gln; Diketo-Glu-Ser; 273.10945; Gly-Gln-Gln

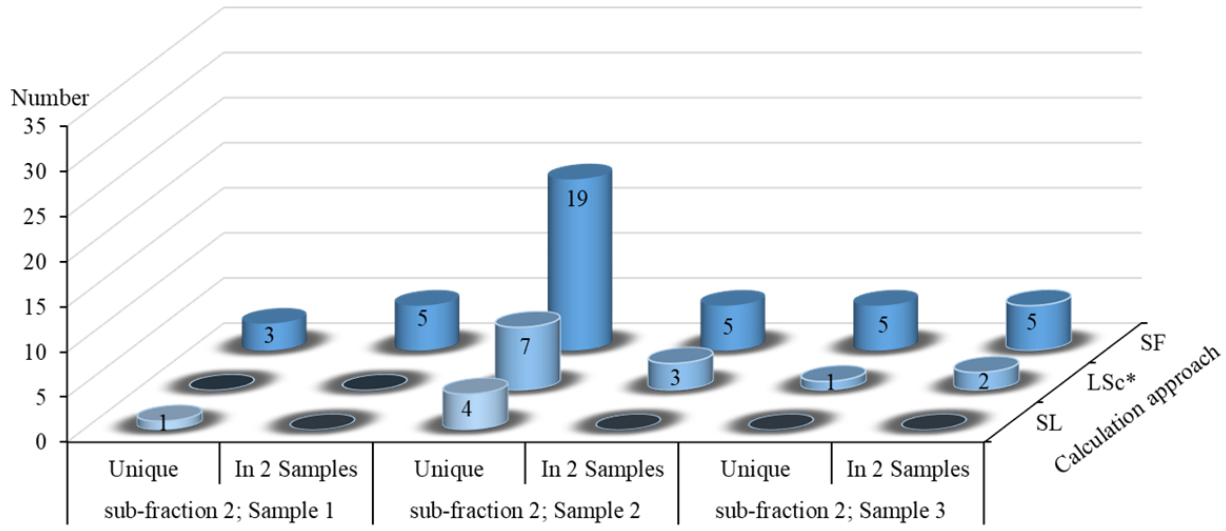


Figure 3.12-5: Bar chart of identified substances, which uniquely appeared in sub-fraction 2 and substances, which were detected at least in two of the three samples. These results are based on the results of Figure 3.12-3. The first, third and fifth row show the number of uniquely identified substances by the three different calculation approaches in sub-fraction 2 of sample 1 to 3. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue, calculation approach three is shown in dark blue. If none of the calculation approaches led to identification of a substance bars are shown in black. Row two, four and six shows substances detected in two of the three samples. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

- Exclusively detected in sub-fraction 2 of sample 1 SEC A5:
  - 251.18664; 249.08430; Pro-Val-Val; 156.97976
- Exclusively detected in sub-fraction 2 of sample 2 SEC A5:
  - 149.05971; 337.10436; 381.13192; 233.07815; 229.14075; Glu-Pro-Asn; Glu-Asp-Ile; 215.12779; 625.32319; 603.33946; 620.36504; 611.30620; 597.28817; Diketo-Met-Val; 198.14886; Diketo-Phe-His; Diketo-Ile-Cys; 130.04987; 343.29552; Arg-Tyr-Asp; Ile-Ile; Val-Pro-Leu; Pro-Ile-Tyr; Pro-Ala-Phe; pyro-Glu-Phe; 485.18697; 145.04954; 163.06010; 365.10784; 155.97499
- Exclusively detected in sub-fraction 2 of sample 3 SEC A5:
  - 468.41999; 209.09207; 453.19830; 475.18131; Thr-Phe-Gly; 120.08078

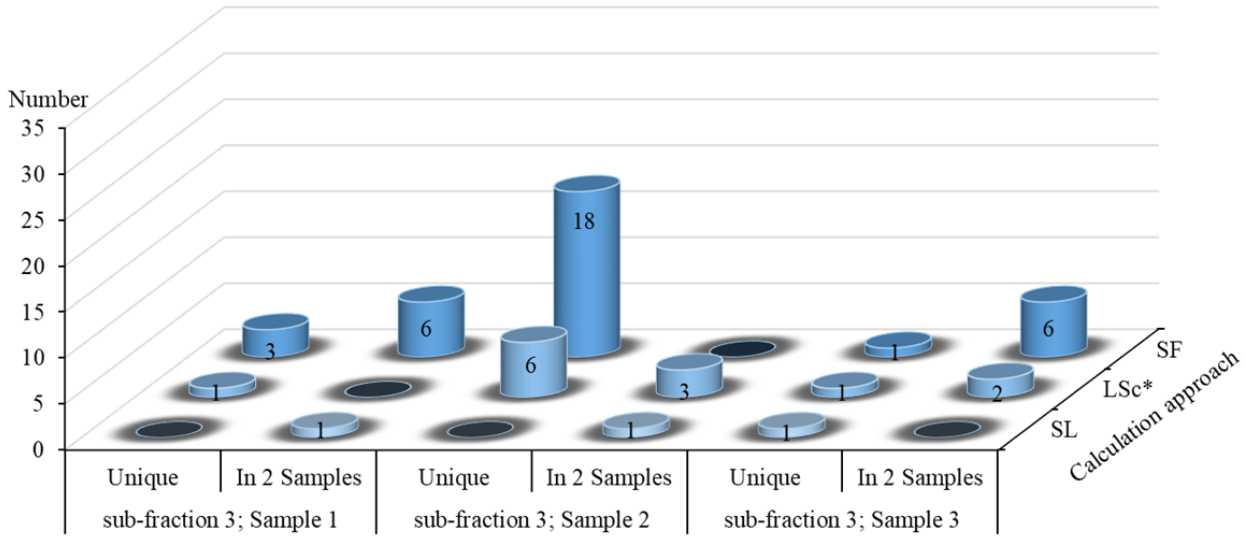


Figure 3.12-6: Bar chart of identified substances, which uniquely appeared in sub-fraction 3 and substances, which were detected at least in two of the three samples. These results are based on the results of Figure 3.12-3. The first, third and fifth row show the number of uniquely identified substances by the three different calculation approaches in sub-fraction 3 of sample 1 to 3. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue, calculation approach three is shown in dark blue. If none of the calculation approaches led to identification of a substance bars are shown in black. Row two, four and six shows substances detected in two of the three samples. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

- Exclusively detected in sub-fraction 3 of sample 1 SEC A5:
  - 493.35102; 185.11454; 267.12001; Arg-Tyr-Phe
- Exclusively detected in sub-fraction 3 of sample 2 SEC A5:
  - 149.05971; 337,10436; Gln-Met-Cys; 271.18770; 257.13566; 233.07815; 207.15909; 229.14075; Glu-Asp-Ile; 251.18664; 273.16696; 365.19452; 308.18697; 259.15131; 215.12510; Diketo-Met-Val; 210.10978; Diketo-Ile-Cys; Diketo-Tyr-Pro; Ile-Pro; 598.29704; 505.26483; 489.23303; 145.05087
- Exclusively detected in sub-fraction 3 of sample 3 SEC A5:
  - Gln-Gln-Ser; 326.16836; Leu-Pro-Phe

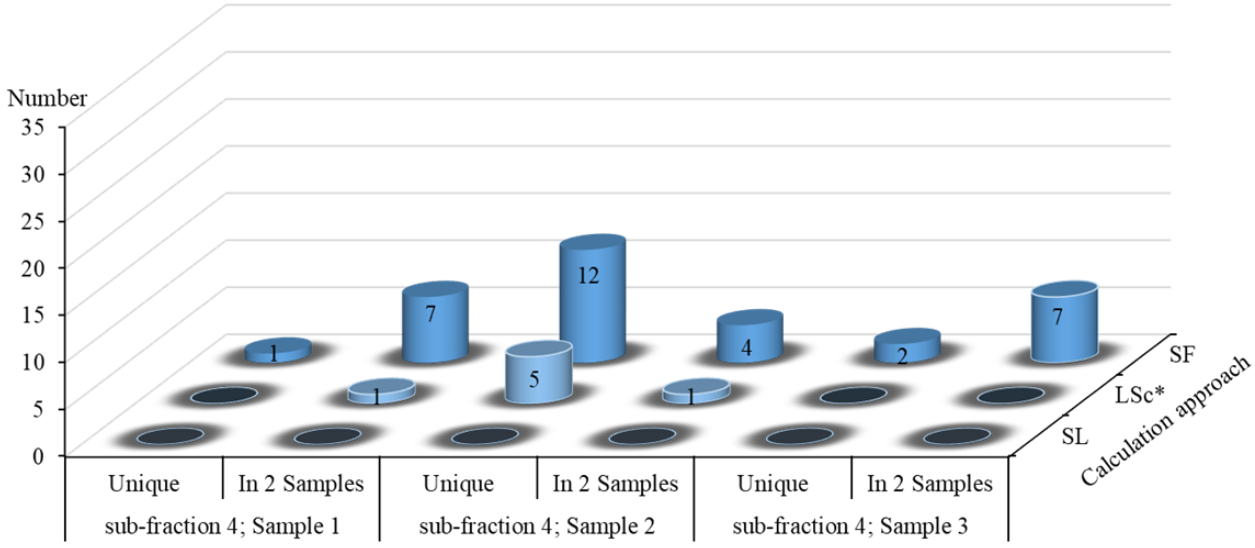


Figure 3.12-7: Bar chart of identified substances which, uniquely appeared in sub-fraction 4 and substances, which were detected at least in two of the three samples. These results are based on the results of Figure 3.12-3. The first, third and fifth row show the number of uniquely identified substances by the three different calculation approaches in sub-fraction 4 of sample 1 to 3. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue, calculation approach three is shown in dark blue. If none of the calculation approaches led to identification of a substance bars are shown in black. Row two, four and six shows substances detected in two of the three samples. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

- Exclusively detected in sub-fraction 4 of sample 1 SEC A5:
  - 155.97499
- Exclusively detected in sub-fraction 4 of sample 2 SEC A5:
  - 149.05971; 337,10436; Gln-Met-Cys; 233.07815; Diketo-Asp-Tyr; 273.16696; 251.18664; Diketo-His-Gly; 215.12510; 144.98550; Diketo-Phe-Pro; 210.10978; Diketo-Ile-Cys; 365.10649; 145.04954; 163.06144
- Exclusively detected in sub-fraction 4 of sample 3 SEC A5:
  - 468.41999; 340.18803

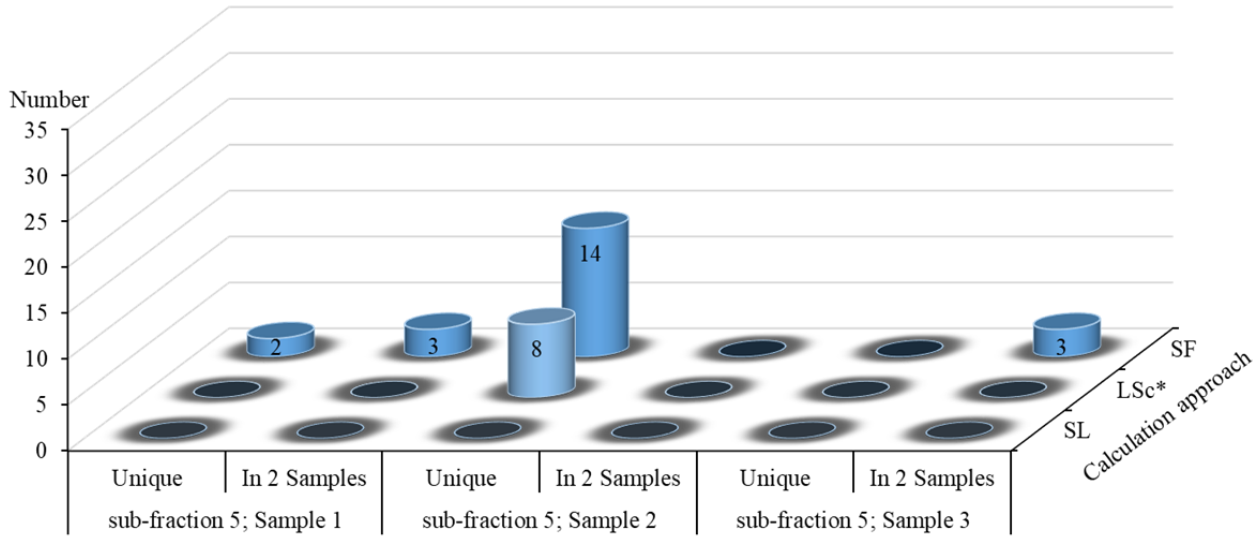


Figure 3.12-8: Bar chart of identified substances which, uniquely appeared in sub-fraction 5 and substances, which were detected at least in two of the three samples. These results are based on the results of Figure 3.12-3. The first, third and fifth row show the number of uniquely identified substances by the three different calculation approaches in sub-fraction 5 of sample 1 to 3. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue, calculation approach three is shown in dark blue. If none of the calculation approaches led to identification of a substance bars are shown in black. Row two, four and six shows substances detected in two of the three samples. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

- Exclusively detected in sub-fraction 5 of sample 1 SEC A5:
  - 332.33118; 155.97499
- Exclusively detected in sub-fraction 5 of sample 2 SEC A5:
  - 323.14587; 271.18770; Diketo-Asp-Tyr; 228.19581; 250.17747; Gln-Met-Cys; Glu-Pro-Asn; Glu-Asp-Ile; 273.16696; Diketo-His-Gly; 215.12510; 226.18016; 266.17238; 212.16451; Diketo-Met-Ile; 201.10945; 174.05495; Diketo-Ile-Cys; 130.04987; 316.21319; 180,13829; Phenylalanine

- Exclusively detected in sub-fraction 5 of sample 3 SEC A5:
  - None

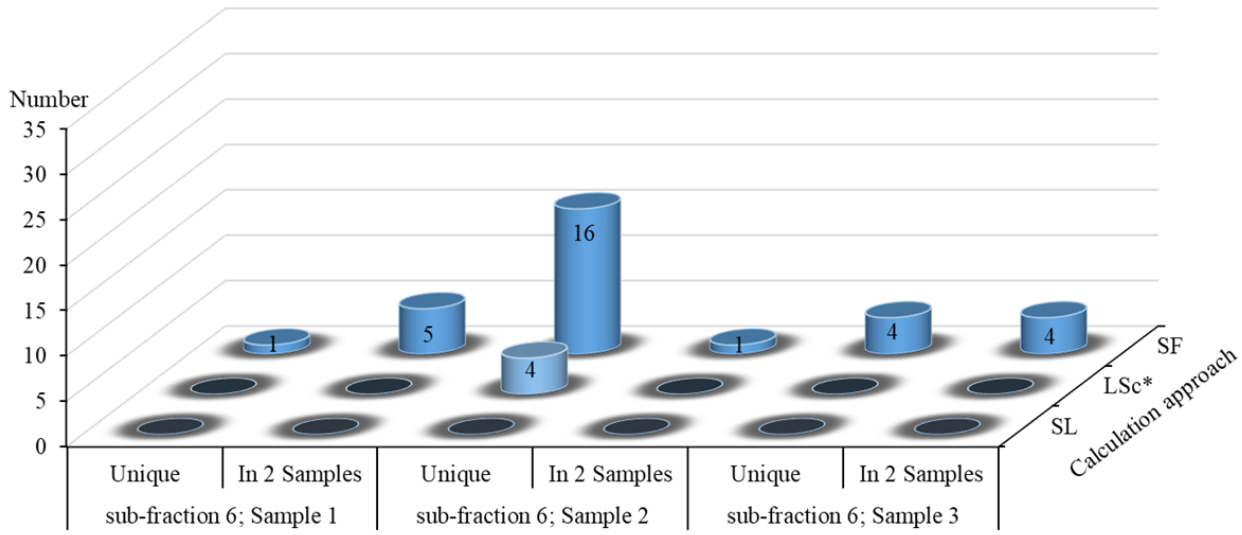


Figure 3.12-9: Bar chart of identified substances, which uniquely appeared in sub-fraction 6 and substances, which were detected at least in two of the three samples. These results are based on the results of Figure 3.12-3. The first, third and fifth row show the number of uniquely identified substances by the three different calculation approaches in sub-fraction 6 of sample 1 to 3. Calculation approach 1 spectral library (SL) is shown in blue-grey, calculation approach 2 Lars Schmidt (LSc*) is shown in light blue, calculation approach three is shown in dark blue. If none of the calculation approaches led to identification of a substance bars are shown in black. Row two, four and six shows substances detected in two of the three samples. Y-axis shows the number of identified substances, x-axis shows the sample name and z-axis shows the different calculation approaches.

- Exclusively detected in sub-fraction 6 of sample 1 SEC A5:
  - 521.37964
- Exclusively detected in sub-fraction 6 of sample 2 SEC A5:
  - 149.05971; 337.10436; 233.07815; 229.14075; Glu-Pro-Asn; Glu-Glu-Val; Gln-Met-Cys; 215.12510; 266.17238; 226.18016; 188.12812; 170.11756; 210.10978; Diketo-Ile-Cys; 283.17647; 197.16484: 167.11789; 145.04954; 365.10783; 156.98550
- Exclusively detected in sub-fraction 6 of sample 3 SEC A5:
  - 343.29552; 240.23219: 158.96813; 226.94673

This section summarises biomolecules identified so far, and the sub-fraction in which they were found. Besides biomolecules which occurred exclusively in one of the six sub-fractions, there were biomolecules detected in two consecutive sub-fractions, and some molecules detected in each sub-fraction (Table 3.12-1 and Table 3.12-2).

Results are presented in ascending order. The detailed evaluation of the samples including MS/MS analyses in the ESI-positive mode with all detected *m/z* ratios of the compounds identified are presented in the appendix (Supplementary figure 28 to Supplementary figure 33 and corresponding Supplementary table 28 to Supplementary table 33).

Listed data were received in the positive ionisation (ESI) MS/MS mode. Each molecule was annotated with SmartFormula (SF). If SF and LSc are mentioned in the same row, both approaches led to the same molecular formula. ´Origin unknown` means that the identified substance was not identified in the starting material. In some cases, identified substances were detected in each of the sub-fractions, but were not detected in SEC A5, which was used to generate these sub-fractions. One can hypothesise that newly detected biomolecules became visible due to the additional fractionation step. If these substances were co-eluting with others in the SEC-fractions, they possibly were hidden by higher concentrated substances.

Moreover, literature research was done for the exclusively detected molecules to figure out, whether they had known umami attributes or not. All results in this section were based on the SEC-fraction A5 of sample 2 that exhibits the strongest umami taste.

Sub-fraction 1 contained eight different known umami taste active biomolecules (Table 3.12-1) Glu-Leu (Ohyama, Ishibashi et al. 1988), Val-Glu; Pro-Glu (Dang, Gao et al. 2015), Val-Asp; Val-Gly (Ishibashi, Ono et al. 1988), Glu-Ser (Arai, Yamashita et al. 1972), Pro-Gly and Pro-Thr (Yamamoto, Shiga et al. 2014).

Table 3.12-1: Biomolecules detected and identified by operator (LSc), spectral library (SL), or SmartFormula (SF). Each of the four sections (separated by a massive black line) of the table contains biomolecules exclusively in one sub-fraction. Unknown means not detected in respective donor fraction.

	Sub-fraction	Origin	RT [min]	Name	Molecular Formula	*m/z* calc.	Annotations
1	1	unknown	6.42	Phe-Arg-Gly	$C_{17}H_{26}N_6O_4$	379.20951	LSc
2	1	unknown	6.79	Diketo-Trp-Lys	$C_{17}H_{22}N_4O_2$	315.18212	LSc
3	1	unknown	10.34	Asp-Arg-Pro	$C_{15}H_{26}N_6O_6$	387.19935	LSc
4	1	unknown	10.74	Phe-Phe-Gln	$C_{23}H_{28}N_4O_5$	441.21393	LSc
5	1	unknown	10.87	Trp-Trp-Gln	$C_{27}H_{30}N_6O_5$	519.27212	LSc
6	1	unknown	17.51	Phe-Ile-Asn	$C_{19}H_{28}N_4O_5$	393.21393	LSc
7	1	SEC-A5	19.07	diketo(Ser-Pro)	$C_8H_{12}N_2O_3$	185.09263	SF/LSc
8	1	SEC-A5 also SEC-A6	20.37	pyro(Glu-Glu)	$C_{10}H_{14}N_2O_6$	259.09322	LSc

	Sub-fraction	Origin	RT [min]	Name	Molecular Formula	*m/z* calc.	Annotations
9	1	SEC-A5	21.37	Pro-Val	$C_{10}H_{18}N_2O_3$	215.13902	SF/SL
10	1	SEC-A5	21.63	Val-Pro-Val	$C_{15}H_{27}N_3O_4$	314.20743	SF/SL
11	1	SEC-A5	21.71	Ala-Leu	$C_9H_{18}N_2O_3$	203.13092	SF/SL
12	1	unknown	21.98	L-Norleucine	$C_6H_{13}NO_2$	132.10191	SF/SL
13	1	SEC-A5	22.19	Glu-Leu	$C_{11}H_{20}N_2O_5$	261.14450	SF/SL
14	1	unknown	22.27	Gly-Ile	$C_8H_{16}N_2O_3$	189.12337	SF/SL
15	1	SEC-A5	22.57	diketo(Thr-Ile)	$C_{10}H_{18}N_2O_3$	215.1396	LSc
16	1	SEC-A5	22.96	Glutamic acid	$C_5H_9NO_4$	148.05651	LSc
17	1	SEC-A5	22.96	Ile-Glu	$C_{11}H_{20}N_2O_5$	261.14450	SF/SL
18	1	SEC-A5	23.30	Ile-Pro-Glu	$C_{16}H_{27}N_3O_6$	358.19726	SF/SL
19	1	SEC-A5	23.34	Ile-Glu	$C_{11}H_{20}N_2O_5$	261.14450	SF/SL
20	1	SEC-A5	23.35	Pro-Val	$C_{10}H_{18}N_2O_3$	215.13902	SF
21	1	SEC-A5	23.81	Ile-Ser	$C_9H_{18}N_2O_4$	219.13461	LSc
22	1	SEC-A5	23.84	Leu-Pro-Thr	$C_{15}H_{27}N_3O_5$	330.20235	SF/SL
23	1	SEC-A5	24.55	Glutamic acid	$C_5H_9NO_4$	148.05651	LSc
24	1	SEC-A5	24.57	Val-Glu	$C_{10}H_{18}N_2O_5$	247.12885	SF/SL
25	1	SEC-A5	24.81	Val-Gly	$C_7H_{14}N_2O_3$	175.10772	SF/SL
26	1	SEC-A5	25.24	Pro-Glu	$C_{10}H_{16}N_2O_5$	245.11320	SF/SL
27	1	SEC-A5	25.31	Val-Asp	$C_9H_{16}N_2O_5$	233.11320	SF/SL
28	1	SEC-A5	25.53	diketo(His-Pro)	$C_{11}H_{14}N_4O_2$	235.11951	LSc
29	1	SEC-A5	25.89	Pro-Gly	$C_7H_{12}N_2O_3$	173.09207	SF/SL
30	1	SEC-A5	25.97	Pro-Thr	$C_9H_{16}N_2O_4$	217.11828	SF/SL
31	1	SEC-A5	26.11	Val-Ser	$C_8H_{16}N_2O_4$	205.11828	SF/SL
32	1	SEC-A5	26.13	diketo(Glu-Ala)	$C_8H_{12}N_2O_4$	201.08755	LSc
33	1	SEC-A5 Also SEC-A6	26.64	pyro(Glu-Glu)	$C_{10}H_{14}N_2O_6$	259.09322	LSc
34	1	SEC-A5	27.92	Glutamic acid	$C_5H_9NO_4$	148.05651	LSc
35	1	SEC-A5	27.92	Ser-Glu	$C_8H_{14}N_2O_6$	235.09246	SF/SL
36	1	SEC-A5 also SEC-A6	28.34	Glutamine	$C_5H_{10}N_2O_3$	147.07250	LSc
37	1	SEC-A5	28.36	diketo(Glu-Gln) (pyro-Glu-Gln)	$C_{10}H_{15}N_3O_5$	258.10901	LSc
38	1	SEC-A5	28.36	Glu-Gln Ala-Asp-Ala	$C_{10}H_{17}N_3O_6$ $C_{10}H_{17}N_3O_6$	276.11901 276.1197	SF/SL LSc
39	1	SEC-A5	28.75	L-Methionine s-oxide	$C_5H_{11}NO_3S$	166.05324	SF/SL
40	1	SEC-A5	31.32	Lysine	$C_6H_{14}N_2O_2$	147.10888	LSc
41	1	SEC-A5 also SEC-A6	31.31	Arginine	$C_6H_{14}N_4O_2$	175.11895	SF/SL
42	1	SEC-A5 also SEC-A6	31.41	Histidine	$C_6H_9N_3O_2$	156.07283	LSc
43	2	unknown	11.03	diketo(Phe-His)	$C_{15}H_{16}N_4O_2$	285.13516	LSc
44	2	unknown	19.38	Arg-Tyr-Asp	$C_{19}H_{28}N_6O_7$	453.20991	LSc
45	2	SEC-A6	21.29	Pro-Ile-Tyr	$C_{20}H_{29}N_3O_5$	392.21800	SF/SL
46	2	SEC-A5	22.08	Pro-Ala-Phe	$C_{17}H_{23}N_3O_4$	334.17666	SF/SL
47	2	SEC-A5 also SEC-A6	22.40	pyro(Glu-Phe)	$C_{11}H_{20}N_2O_3$	227.11828	LSc
48	3	SEC-A5	9.70	diketo(Pro-Ile)	$C_{11}H_{18}N_2O_2$	211.14467	SF/LSc
49	3	SEC-A5	10.12	diketo(Pro-Ile)	$C_{11}H_{18}N_2O_2$	211.14467	SF/LSc
50	3	SEC-A5	10.81	diketo(Pro-Val)	$C_{10}H_{16}N_2O_2$	197.12902	SF/LSc
51	3	SEC-A5	16.68	diketo(Pro-Val)	$C_{10}H_{16}N_2O_2$	197.12902	SF/LSc

	Sub-fraction	Origin	RT [min]	Name	Molecular Formula	*m/z* calc.	Annotations
52	3	SEC-A5	16.68	diketo(Glu-Ile)	$C_{11}H_{18}N_2O_4$	243.1345	SF/LSc
53	3	unknown	17.76	Leu; Ile	$C_6H_{13}NO_2$	132.09798	SF/LSc
54	3	SEC-A6	18.95	Tyrosine	$C_9H_{11}NO_3$	182.07725	SF/LSc
55	3	unknown	18.96	Glu-Gln-Asn	$C_{14}H_{23}N_5O_8$	390.16262	LSc
56	3	SEC-A5	20.00	Leu-Pro-Ile	$C_{17}H_{31}N_3O_4$	342.23873	SF/SL
57	3	SEC-A5	20.63	Val-Pro-Phe	$C_{19}H_{27}N_3O_4$	362.20743	SF/SL
58	5	unknown	10.97	diketo(Met-Ile)	$C_{11}H_{20}N_2O_2S$	245.13239	LSc

Moreover, the diketo- or pyro(Glu-Gln) dipeptide was detected, which is also known to enhance the umami taste (Kiyono, Hirooka et al. 2013). This highly potent substance was exclusively detected in sub-fraction 1, which made this sub-fraction the most interesting one. The sub-fractions 4 and 6 did not exhibit biomolecules detected exclusively in one of these fractions.

Other small biomolecules were detected in more than one sub-fraction. Generally, they eluted in 2 consecutive sub-fractions. Taken into account that sub-fractionation was done according to the time and not by detected signals it was conceivable that substances were part of two consecutive fractions. Very few substances eluted in more than four consecutive sub-fractions.

Table 3.12-2: Biomolecules detected and identified by the operator (LSc), spectral library (SL), or SmartFormula (SF). The table is seperated into three sections by massive black lines. Section one includes biomolecules that were detected in two consecutive sub-fractions with the exception of number 6 that were detected in sub-fraction 1, and 3. Section two includes biomolecules detectd in more than two not consecutive sub-fractions. Section three includes biomolecules detected in sub-fractions 1 to 4 and 1 to 6, respectively.

	Sub-fraction	Origin	RT [min]	Name	Molecular Formula	*m/z* calc.	Annotations
1	1; 2	SEC-A5	17.52	diketo(Glu-Pro)	$C_{10}H_{14}N_2O_4$	227.1032	LSc
2	1; 2	SEC-A5	19.36 19.38	diketo(Glu-Pro) (pyro-Glu-Pro)	$C_{10}H_{14}N_2O_4$	227.1032	LSc
3	1; 2	SEC-A5	19.66 19.65	Ile-Ile	$C_{12}H_{24}N_2O_3$	245.18597	SF/SL
4	1; 2	SEC-A5	20.99 20.84	Ile-Pro-Val	$C_{16}H_{29}N_3O_4$	328.22308	SF/SL
5	1; 2	unknown	23.82 17.27	diketo(Glu-Val)	$C_{10}H_{16}N_2O_4$	229.11845	LSc
6	1; 3	SEC-A5	16.65 16.81	diketo(Tyr-Pro)	$C_{14}H_{16}N_2O_3$	261.1239	LSc
7	2; 3	unknown	10.88 10.85	diketo(Met-Val)	$C_{10}H_{18}N_2O_2S$	231.11674	LSc
8	4; 5	SEC-A5	7.48 7.53	diketo(Asp-Tyr)	$C_{13}H_{14}N_2O_5$	279.0981	LSc
9	4; 5	SEC-A6	17.69 22.34	Phenylalanine	$C_9H_{11}NO_2$	166.08233	SF/LSc
10	2; 5; 6	unknown	7.94 7.93 8.04	Glu-Pro-Asn	$C_{14}H_{22}N_4O_7$	359.15681	LSc
11	1; 4; 5	SEC-A5	10.05 10.10 10.14	diketo(His-Gly)	$C_8H_{10}N_4O_2$	195.0882	LSc
12	2; 3; 5; 6	unknown	7.94	Asp-Glu-Leu	$C_{15}H_{25}N_3O_8$	376.17213	LSc

	Sub-fraction	Origin	RT [min]	Name	Molecular Formula	*m/z* calc.	Annotations
			7.93 7.93 8.04				
13	1-4	SEC-A5	20.18 21.73 21.68 18.44	Pro-Phe	$C_{14}H_{18}N_2O_3$	263.13902	SF/SL
14	1-4	SEC-A5	20.34 21.93 19.00 18.72	Pro-Ile	$C_{11}H_{20}N_2O_3$	229.15467	SF/SL LSc LSc LSc
15	1-6	unknown	7.86 7.94 5.82 5.78 7.92 8.04	Gln-Met-Cys	$C_{13}H_{24}N_4O_5S_2$	381.12677	LSc
16	1-6	SEC-A4	16.07 16.38 16.35 16.30 16.38 16.49	diketo(Ile-Cys)	$C_9H_{16}N_2O_5S$	217.10487	LSc

The umami active substance pyro(Glu-Pro) (Schlichtherle-Cerny and Amadò 2002) was detected in sub-fractions 1 and 2. Furthermore, the umami active tripeptide Asp-Glu-Leu (Dang, Gao et al. 2015) was detected in the sub-fractions 2, 3, 5 and 6. At least one known umami active substance was detected in each sub-fraction, except of sub-fraction 4.

Fractionation based on elution time segments did not lead to the separation of the majority of small biomolecules. Most of the substances eluted in sub-fraction one, or two, respectively. The majority of known umami taste active biomolecules was solely present in sub-fraction 1. In addition, this fraction contained a myriad of substances compared to the five other sub-fractions (Supplementary figure 28 - Supplementary figure 33).

Based on the results of the prepHPLC, the corresponding MS/MS results and the results of the sensory analysis of the prepHPLC sub-fractions, it was concluded that the performed sub-fractionation method was not a suitable sub-fractionation approach for the taste intense SEC-fractions. Obviously, a better sub-fractionation method had to be performed, taking again into consideration that the resulting samples had to be “food-safe”.

3.13 Sub-fractionating of the most taste intense SEC-fraction A5 and A6 of sample 2 *via* refined Size Exclusion Chromatography

A refined SEC was the starting point of the fourth work package.

Sub-fractionation of the taste active SEC-fractions *via* Size Exclusion Chromatography
Number of inj. n = 20; inj. Vol. = 250 µL; collection of 35 fractions per injection, fractions with signals in SEC chrom. were pooled
Amino acid analysis of pooled fractions
Sensory analysis and MS/MS of selected fractions

Based on results of the sub-fractionation *via* prepHPLC another sub-fractionation method was applied. Sub-fractionation of taste intense SEC-fractions was performed with a refined SEC. Fraction A5 and A6 of sample 2 were sub-fractionated, and sub-fractions were collected every minute, which resulted in 35 sub-fractions per sample (Figure 3.13-1).

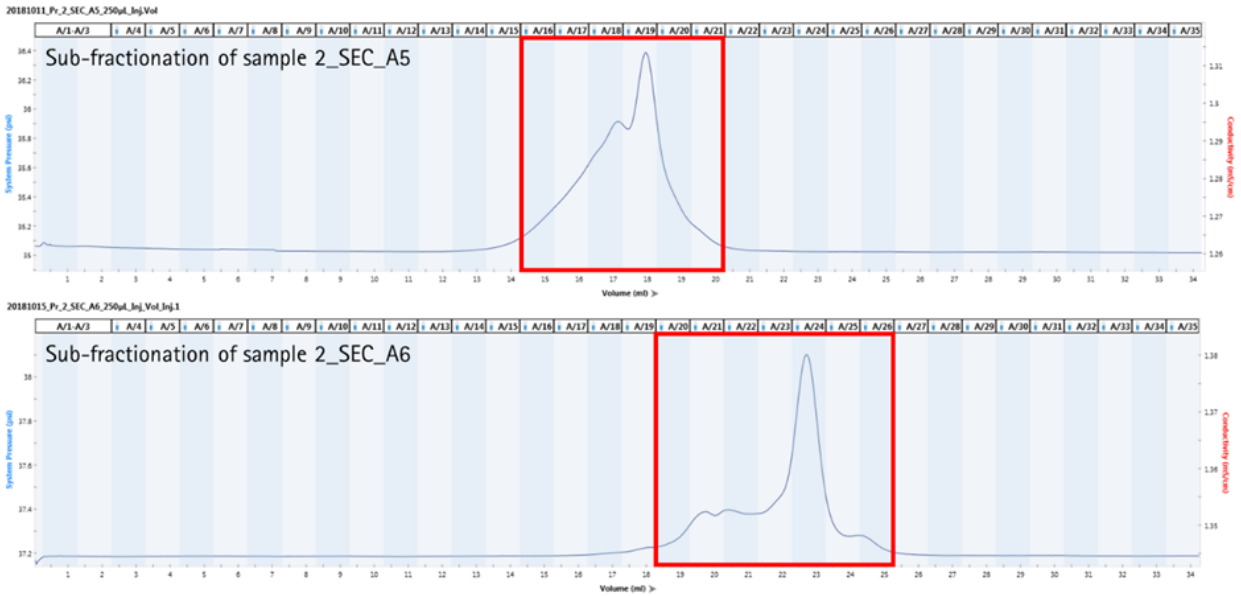


Figure 3.13-1: SEC chromatograms of sub-fractionation of SEC-A5 of sample 2 (on top of the figure) and SEC-A6 of sample 2 (bottom). Sub-fractions were cut every minute ($n_{sub\text{-}fractions}$ = 35). Red framed sub-fractions are the fractions showing a signal in the chromatogram (λ = 280 nm).

Only few sub-fractions gave signals in the SEC chromatogram, which are highlighted in red frames (Figure 3.13-1). SEC A5 showed six, and SEC A6 seven interesting sub-fractions. Respective sub-fractions were pooled from 20 injections (total volume of one sub-fraction = 20 mL), freeze-dried, reconstituted in 5 mL ddH_2O (total injection volume), and analysed *via* amino acid HPLC, UPLC-QTOF-MS/MS, and sensorally evaluated.

3.14 Determination of the free amino acid content of SEC-sub-fractions

Calibration was performed at eight calibration points (10; 20; 30; 40; 50; 62.5; 75; 100 µM). Each standard solution contained all amino acids but proline and cysteine. *β*-alanine was used as internal standard. Sub-fractions A16 to 21 of SEC A5, and sub-fractions A20-A26 of SEC A6 (sample 2) were analysed. These were the only sub-fractions showing signals in SEC, so it was expected to detect free amino acids in these fractions. Surprisingly, in none of these analysed sub-fractions (A16 – A21 of SEC A5, sample 2, and A20 – A26 of SEC A6, sample 2) amino acids were detected with concentrations above 10 µM (data not shown). Since 10 µM was equivalent to the lowest standard concentration, no quantification of concentrations below this level was possible.

This information was crucial for the sensory analysis design. Usually, the detected concentration of free glutamic acid had to be taken into account for the triangle test. As mentioned before, each solution was spiked with MSG to a concentration of 10 mM before sensory analysis. More information on the effect of the absence of free glutamic acid in the SEC sub-fractions concerning sensory analysis can be found in the section sensory analysis of SEC sub-fractions (3.15).

The combination of the SEC sub-fractionation approach, and HPLC results of the sub-fractions (free amino acids) led to a new question. If the signals detected in the SEC sub-fractions were generated from the most taste intense SEC-fractions A5, and A6 of sample 2, devoid of amino acids, what kind of compounds were they? According to the SEC procedure, it was surmised that these were smaller molecules with umami attributes. The most promising sub-fractions were A19 of SEC A5, sample 2, and A24 of SEC A6, sample 2. These two fractions gave the highest SEC signals, but did not contain free amino acids with a concentration in the calibration range (10 to 100 µM). These sub-fractions (A19 of SEC A5 and A24 of SEC A6, both from sample 2) were sensorial analysed as well as evaluated using the UPLC-HR-QTOF-MS/MS.

3.15 Sensory analysis of SEC sub-fractions

Sensory analysis was performed with the most promising SEC sub-fractions A19 of SEC A5, sample 2 and A24 of SEC A6, sample 2. Triangle tests were performed as before with one exception. In this series of experiments, the samples were tasted twice. Once with the addition of 10 mM MSG, because no glutamic acid was detected, as described above, and one without the

addition of MSG. The aim was to figure out, whether the sub-fractions contained compounds that had an umami taste enhancing effect, or whether they contained compounds with an own umami taste.

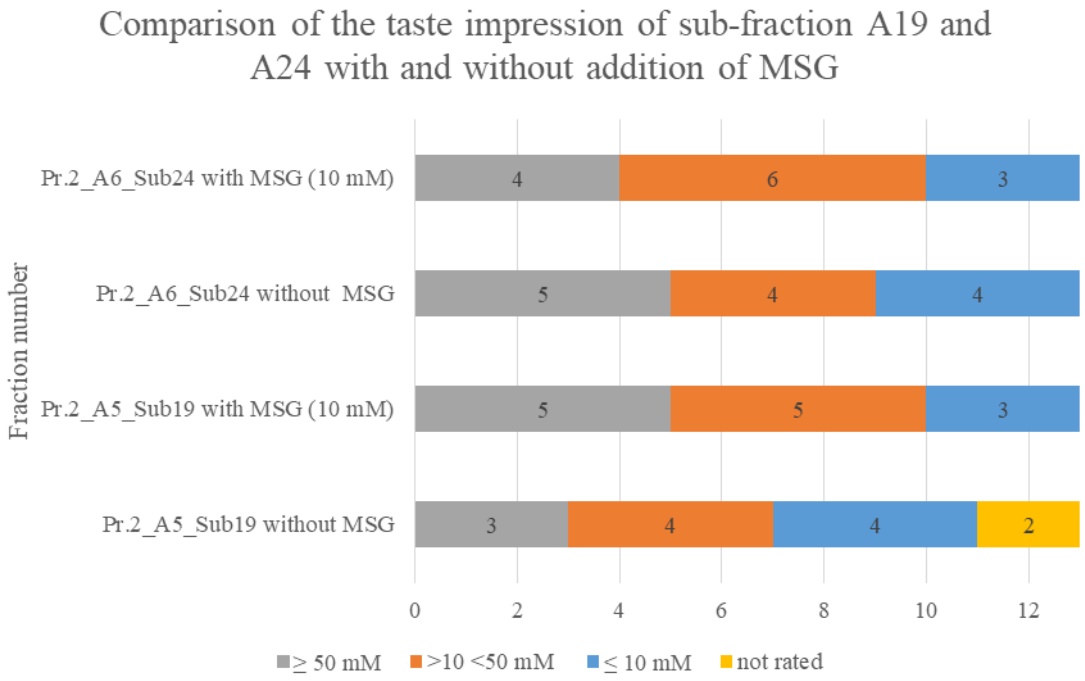


Figure 3.15-1: Triangle test of the SEC sub-fraction A19 of SEC A5, sample 2 with and without the addition of mono sodium glutamate (MSG), and SEC sub-fraction A24 of SEC A6, sample 2 with and without the addition of MSG. Taste impression higher than 50 mM MSG is shown in grey, taste impression higher than 10 mM but lower than 50 mM orange, taste impression below 10 mM in blue, and taste impression from candidates who were not able to distinguish between sample and standard solution are yellow and were rated as not evaluated. The numbers in the bars show the number of given answers.

The bar at the bottom (Figure 3.15-1) shows the results of sub-fraction A19 without the addition of 10 mM MSG. 54 % of the participants had the impression that the taste of this sub-fraction was more intense or equal to 10 mM MSG, 43 % out of the 54 % even had the impression that the taste intensity was above 50 mM MSG. Since no MSG was present, it was assumed that this sub-fraction contained compounds possessing umami taste. Furthermore, the taste impression of this sub-fraction increased with the addition of MSG. For this sample, 77 % of the panellists rated the taste more intense than 10 mM MSG. Of these 77 %, even 50 % of the subjects had the impression that the taste was more intense than 50 mM. It was concluded that sub-fraction A19 not only contained compounds possessing umami taste, but also compounds with umami taste enhancing properties.

Sub-fraction A24 of SEC A6, sample 2 was evaluated in the same way. Without the addition of 10 mM MSG 70 % of the participants had the impression that the taste was more intense than 10 mM MSG. Of these 70 %, even 55 % had the impression that sub-fraction A24 without MSG tasted more intense than 50 mM MSG. With the addition of 10 mM MSG to sub-fraction A24 the percentage of participants who rated the sample more intense than 10 mM MSG increased to 77 %. This suggested that it contained umami active compounds, but none that enhanced the umami taste, since the addition of MSG did not cause a significant increase in taste perception. To identify the compounds in these highly promising sub-fractions (A19 of SEC A5 and A24 or SEC A6, both from sample 2) in depth UPLC-HR-QTOF-MS/MS analyses were performed.

3.16 Identification of potential umami active compounds by UPLC-HR-QTOF-MS/MS

Analyses were performed as described in section 2.5.6. Detailed information regarding the procedure for evaluating the signals can be found in section 2.7. Base peak chromatograms were recorded, and the most abundant signals were evaluated (Figure 3.16-1).

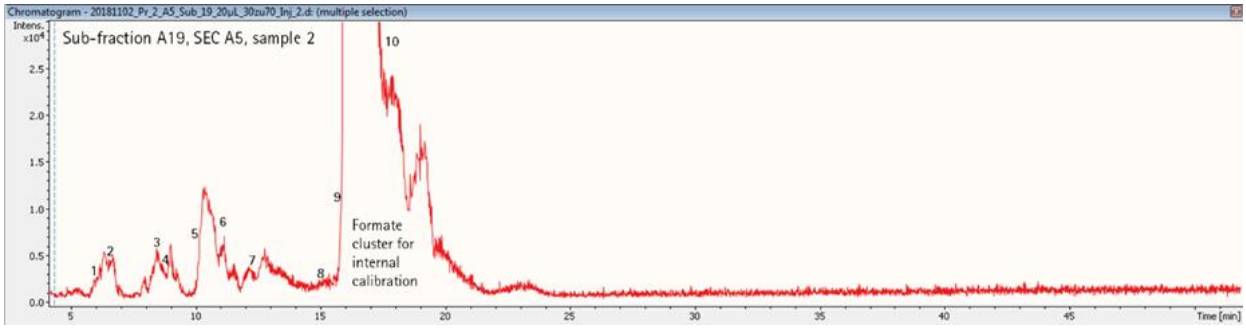


Figure 3.16-1: Base Peak chromatogram (positive MS/MS mode) of reconstituted sub-fraction A19 of SEC A5 from sample 2. The numbering of the peaks correlates with the numbering in Table 3.16-1.

In sub-fraction A19 of SEC A5, originating from sample 2, 17 dipeptides and their condensation products were detected (Table 3.16-1). All of them were already found in at least one of the SEC-fractions, which showed that the sample pre-treatment, freeze-drying and following analyses were reproducible, and did not result in significant losses or chemical alterations.

Table 3.16-1: Identification of the most abundant signals of sub-fraction A19 of SEC-fraction A5, sample 2. Annotation was done by two of the three calculation approaches (SF = SmartFormula, and LSc* = manually by operator). Numbering in the table correlates with the numbering of the signals in Figure 3.16-1.

	RT [min]	m/z meas.	m/z calc.	Name	Molecular Formula	MS/ MS	IΔm/zI [mDa]	Anno-tations
1	6.35	243.13314	243.13393	/	$C_{11}H_{18}N_2O_4$	/	0.79558	SF
			243.1345	Diketo-Glu-Ile	equivalent to SF	/	1.36	LSc*
2	6.69	263.13804	263.13902	/	$C_{14}H_{18}N_2O_3$	/	0.98140	SF
			263.13902	Pro-Phe	equivalent to SF	/	0.98140	LSc*
3	8.51	259.09152	259.09246	/	$C_{10}H_{14}N_2O_6$	/	0.94307	SF
			259.09303	Diketo-Glu-Glu	equivalent to SF	/	1.51	LSc*
3	8.59	227.10164	227.10263	/	$C_{10}H_{14}N_2O_4$	/	0.99072	SF
			227.1032	Diketo-Glu-Pro	equivalent to SF	/	1.56	LSc*
3	8.71	231.16938	231.17032	/	$C_{11}H_{22}N_2O_3$	/	0.94358	SF
			231.17032	Val-Ile	equivalent to SF	/	0.94358	LSc*
4	8.83	152.14267	152.14338	/	$C_{10}H_{17}N$	/	0.70771	SF
4	9.09	328.22177	328.22308	/	$C_{16}H_{29}N_3O_4$	/	1.31375	SF
			328.22308	Pro-Leu-Val	equivalent to SF	/	1.31375	LSc*
4	9.28	229.15367	229.15467	/	$C_{11}H_{20}N_2O_3$	/	1.00072	SF
			229.15467	Pro-Leu	equivalent to SF	/	1.00072	LSc*
4	9.79	314.20635	314.20743	/	$C_{15}H_{27}N_3O_4$	/	0.38509	SF
			314.20743	Val-Pro-Val	equivalent to SF	/	0.38509	LSc*
5	10.39	215.13808	215.13902	/	$C_{10}H_{18}N_2O_3$	/	0.94211	SF
			215.13902	Pro-Val	equivalent to SF	/	0.94211	LSc*
5	10.59	203.13835	203.13902	/	$C_9H_{18}N_2O_3$	/	0.67039	SF
			203.1397	Ile-Ala	equivalent to SF	/	1.35	LSc*
5	10.64	132.10147	132.10191	/	$C_6H_{13}NO_2$	/	0.43814	SF
			132.10191	L-Norleucine	equivalent to SF	/	0.43814	LSc*
5	10.76	243.13306	243.13393	/	$C_{11}H_{18}N_2O_4$	/	0.87400	SF
			243.1345	Diketo-Glu-Ile	equivalent to SF	/	1.44	LSc*
5	10.77	197.12768	197.12845	/	$C_{10}H_{16}N_2O_2$	/	0.77184	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	/	1.34	LSc*
6	11.55	261.14331	261.14450	/	$C_{11}H_{20}N_2O_5$	/	1.18858	SF
			261.11450	Glu-Leu	equivalent to SF	/	1.18858	LSc*
7	12.24	229.11752	229.11828	/	$C_{10}H_{16}N_2O_4$	/	0.76221	SF
			229.11885	Diketo-Glu-Val	equivalent to SF	/	1.33	LSc*
8	15.71	254.15903	254.15981	/	$C_{10}H_{23}NO_6$	/	0.78697	SF
			254.16172	Diketo-Pro-Arg	$C_{11}H_{19}N_5O_2$	/	2.69	LSc*
9	15.97	210.13284	210.13360	/	$C_8H_{19}NO_5$	/	0.75977	SF
9	16.03	232.11503	232.11526	/	$C_6H_{13}N_7O_3$	/	0.22847	SF
			232.11199	Diketo-Cys-Lys	$C_9H_{17}N_2O_2S$	/	3.04	LSc*

Annotation of the signal was done by SmartFormula (SF) and the operator (LSc*). The third calculation approach by spectral library (SL) was not applicable due to the low signal intensity of CID mass spectra. Two known umami active compounds were among the 17 identified compounds. The umami taste of Glu-Leu was described by Ohyama et al. 1988, and the taste of pyro(Glu-Pro) was described by Kiyono et al. 2013. It was likely that these compounds contributed to the intense umami taste of sub-fraction A19 of SEC A5, sample 2.

Several other dipeptides with one amino acid being Glu, Val or Pro were detected. Many of the previously described umami active peptides carried one of these amino acids in their sequence, for example Val-Glu, Pro-Glu (Dang, Gao et al. 2015), Val-Asp, Val-Gly (Ishibashi, Ono et al. 1988), Pro-Gly or Pro-Thr (Yamamoto, Shiga et al. 2014).

Sub-fraction A24, SEC A6, sample 2 was evaluated the same way. The numbering in Figure 3.16-2 correlates with the numbering in Table 3.16-2.

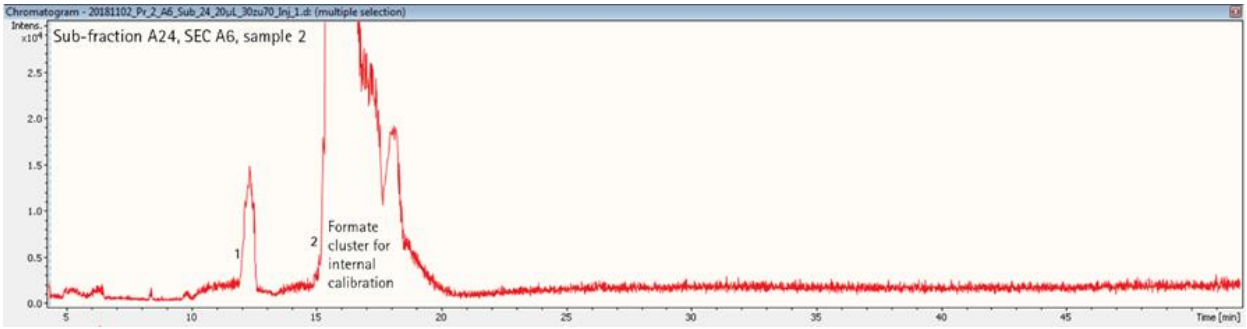


Figure 3.16-2: Base Peak chromatogram (positive MS/MS mode) of reconstituted sub-fraction A24 of SEC A6 from sample 2. The numbering of the peaks correlates with the numbering in Table 3.16-2.

One compound was identified in sub-fraction A24, but it was not known to evoke umami taste. Four further compounds of different *m/z* ratios were measured but were not identified by one of the three calculation approaches.

Table 3.16-2: Identification of the most abundant signals of sub-fraction A24 of SEC-fraction A6, sample 2. Annotation was done by two of the three calculation approaches (SF = SmartFormula, and LSc* = done by operator). Numbering in the table correlates with the numbering of the signals in Figure 3.16-2: Base Peak chromatogram (positive MS/MS mode) of reconstituted sub-fraction A24 of SEC A6 from sample 2. The numbering of the peaks correlates with the numbering in Table 3.16-2.

	RT [min]	m/z meas.	m/z calc.	Name	Molecular Formula	MS/ MS	IΔm/zI [mDa]	Anno-tations
1	12.40	165.05409	165.05462	/	$C_9H_8O_3$	/	0.53063	SF
1	12.41	182.08056	182.08117	/	$C_9H_{11}NO_3$	/	0.60968	SF
1	12.42	136.07518	136.07569	/	C_8H_9NO	/	0.51058	SF
2	15.36	210.13294	210.13494	/	$C_9H_{15}N_5O$	/	1.99969	SF
2	15.48	232.11510	232.11526	/	$C_6H_{13}N_7O_3$	/	0.15944	SF
			232.11199	Diketo-Cys-Lys	$C_9H_{17}N_2O_2S$	/	3.11	LSc*

The umami taste of sub-fraction A24 could not be explained at present by the detected compound. It is still questionable, if one of the detected, but unidentified compound ion molecules stands for a potential umami active compound. Di- or tripeptides did not fit to the suggested molecular

formula. Another spectroscopy, preferably NMR, would be needed to shed more light into this mystery.

3.17 Determination of the peptide composition of samples from different processing steps by UPLC-HR-MS/MS

Since it was hypothesised that thermal treatment during the process favours the formation of 2,5-diketo- and pyro-glutamylpeptides new samples were analysed. Samples were taken after each of four consecutive processing steps. Moreover, their treatment was different compared to the samples discussed above.

The influence of different process steps on the taste of gluten hydrolysate was examined.

- Sample 1: not pasteurised
- Sample 2: pasteurised
- Sample 3: pasteurised and evaporated
- Sample 4: pasteurised, evaporated and treated in vacuum oven

Sample preparation was done as described (2.3). Thereafter, the samples were analysed *via* UPLC-HR-Q-TOF-MS/MS (2.5.5) in the positive ionisation mode. The overlay (Figure 3.17-1) of the BPC of the four samples showed nearly identical elution pattern. With a few exceptions, even the signal intensities were comparable. Only sample 4 (pasteurised, evaporated and treated in vacuum oven) showed signals that were not detected in any other sample. Both, the different signal intensities, and the exclusively occurring signals are highlighted with red frames in Figure 3.17-1.

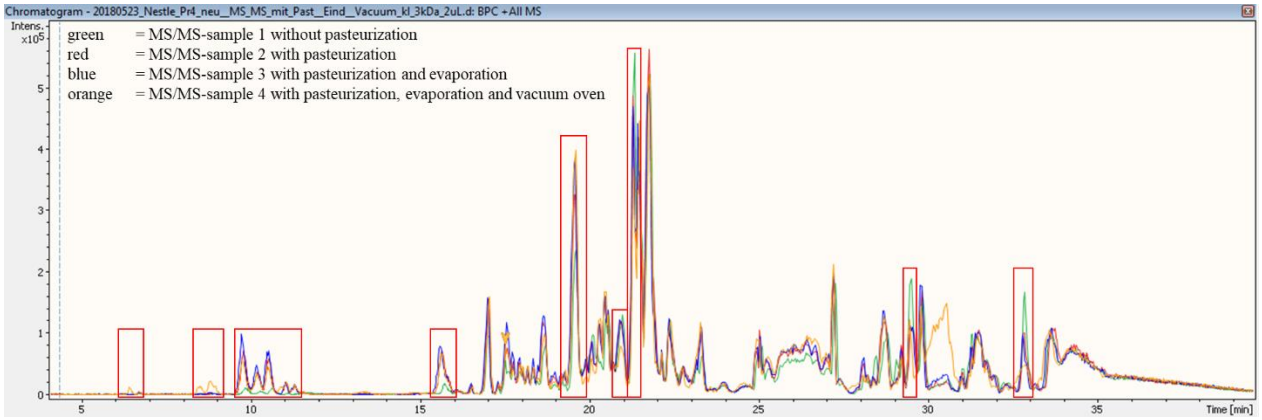


Figure 3.17-1: Overlay of the BPC (positive MS/MS mode) of the four differently treated samples. Different signal intensities and exclusively occuring signals are highlighted with red frames.

As chromatograms of sample 1 to 3 looked similar, only sample 1 and 4 were compared. These two samples differed most in their respective processing steps, and it was assumed that they differ in their composition, especially in thermally formed condensation products. (Figure 3.17-2).

Most abundant signals were calculated with the three different calculation approaches (2.7).

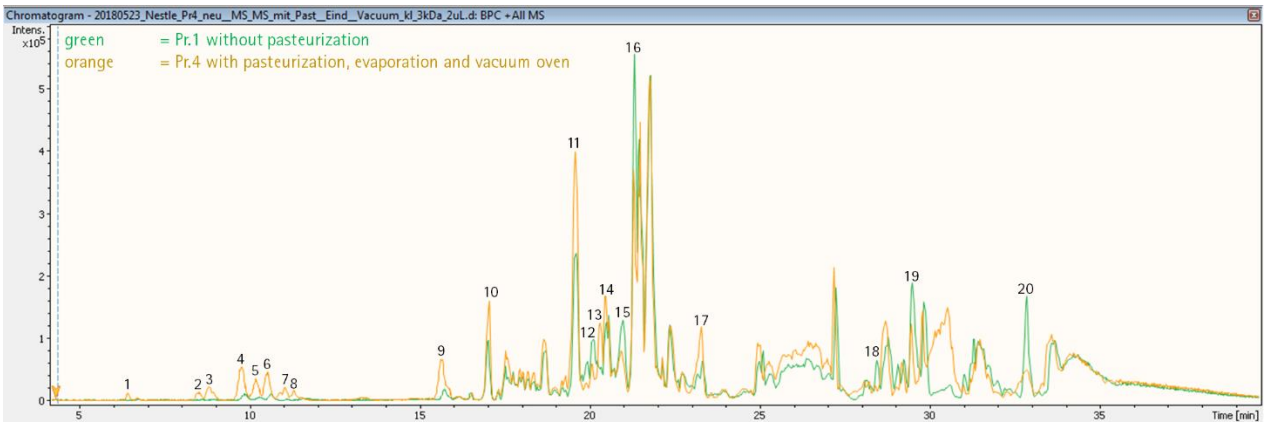


Figure 3.17-2: Overlay of the BPC (positive MS/MS mode) of sample 1 (shown in green) and sample 4 (shown in orange). Numbering of the signals indicate the signals which were calculated and correlate with the numbering in Table 3.17-1.

In total, 27 substances were identified in sample 1 (Table 3.17-1). Among them, several peptides and cyclic peptides, containing glutamic acid, valine or proline were detected. All of them are possible candidates for umami taste or umami taste enhancing characters.

Table 3.17-1: Results of the three calculation approaches for the most abundant signals in sample stock solution (100 mg mL^{-1}) of sample 1 (not pasteurised). Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc), calculation approach 3 is the automatic approach by Spectral Library (SL) and not detected (n.d.) indicates signals that were not identified in sample 1 but in sample 4. A hook in the MS/MS column indicates that an ms/ms spectrum was recorded, and a slash that no ms/ms spectrum was recorded. "Equivalent to SF" means that proposed molecular formula by operator is the same like the molecular formula calculated by SF. Numbering in the table correlates with the numbering of the corresponding Figure 3.17-2.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	I$\Delta m/z$I [mDa]	Anno-tations
1	6.45	282.27850	/	Oleamide	$C_{18}H_{35}NO$	/	/	n.d.
2	8.62	182.11704	182.11756	/	$C_{10}H_{15}NO_2$	√	0.51101	SF
3	8.83	216.10137	/	/	/	/	/	n.d.
3	8.83	262.10669	/	/	$C_{14}H_{15}NO_4$	/	/	n.d.
			262.10404	Glu-Asn	$C_9H_{15}N_3O_6$	/	/	n.d.
			262.10403	Glu-Gly-Gly	$C_9H_{15}N_3O_6$	/	/	n.d.
3	8.96	284.08893	284.08905	/	$C_{12}H_9N_7O_2$	/	0.12308	SF
4	9.88	211.14356	211.14410	/	$C_{11}H_{18}N_2O_2$	√	0.54568	SF
			211.14467	Diketo-Pro-Leu	equivalent to SF	√	0.95	LSc
5	10.32	211.14372	211.14410	/	$C_{11}H_{18}N_2O_2$	/	0.63158	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
			211.14467	Diketo-Pro-Leu	equivalent to SF	/	0.95	LSc
6	10.66	245.12799	245.12845	/	$C_{14}H_{16}N_2O_2$	√	0.46868	SF
			245.12902	Diketo-Pro-Phe	equivalent to SF	√	1.03	LSc
7	11.12	197.12789	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.56068	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	1.13	LSc
7	11.19	333.14147	333.14181	/	$C_{13}H_{16}N_8O_3$	√	0.34546	SF
			333.14115	Glu-Gly-Gln	$C_{12}H_{20}N_4O_7$	√	0.32	LSc
			333.14115	Glu-Asn-Ala	$C_{12}H_{20}N_4O_7$	√	0.32	LSc
7	11.21	212.12749	/	/	$C_{11}H_{17}NO_3$	/	/	n.d.
8	11.28	246.11188	/	/	$C_{14}H_{15}NO_3$	/	/	n.d.
8	11.33	232.09637	/	/	$C_{13}H_{13}NO_3$	/	/	n.d.
9	15.75	261.12300	261.12337	/	$C_{14}H_{16}N_2O_3$	√	0.36706	SF
			261.12393	Diketo-Tyr-Pro	equivalent to SF	√	0.93	LSc
9	15.84	169.09663	/	/		/	/	n.d.
			169.09772	Diketo-Ala-Pro	$C_8H_{12}N_2O_2$	/	/	n.d.
10	17.05	197.12817	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.28780	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	0.85	LSc
10	17.03	243.13392	243.13393	/	$C_{11}H_{18}N_2O_4$	√	0.01293	SF
			243.1345	Diketo-Glu-Ile	equivalent to SF	√	0.58	LSc
10	17.03	485.26041	485.25925	/	$C_{21}H_{40}O_{12}$	√	1.15491	SF
11	19.61	227.10266	227.10429	/	$C_8H_{19}O_5P$	√	1.62703	SF
			227.1032	Diketo-Glu-Pro	$C_{10}H_{14}N_2O_4$	√	0.54	LSc
11	19.62	453.19803	453.19696	/	$C_{14}H_{29}N_8O_7P$	√	1.07115	SF
11	19.61	679.29291	679.29201	/	$C_{29}H_{46}N_2O_{16}$	√	0.90387	SF
12	20.14	342.23867	342.23873	Leu-Pro-Ile	$C_{17}H_{31}N_3O_4$	√	0.05875	SF/SL
13	20.33	181.09679	/	/	$C_9H_{12}N_2O_2$	/	/	n.d.
13	20.35	209.09178	209.09372	/	$C_8H_{17}O_4P$	√	1.9444	SF
13	20.34	451.22964	451.22861	/	$C_{17}H_{22}N_{16}$	/	1.02608	SF
14	20.50	263.13916	263.13902	Pro-Phe	$C_{14}H_{18}N_2O_3$	√	0.13800	SF/SL
14	20.51	187.07127	187.07133	/	$C_7H_{10}N_2O_4$	√	0.06455	SF
			187.07189	Diketo-Glu-Gly	equivalent to SF	√	0.62	LSc
14	20.60	229.15455	229.15467	Pro-Ile	$C_{11}H_{20}N_2O_3$	√	0.12117	SF/SL
14	20.67	362.20719	362.20743	Val-Pro-Phe	$C_{19}H_{27}N_3O_4$	√	0.24031	SF/SL
15	20.98	328.22316	328.22308	Ile-Pro-Val	$C_{16}H_{29}N_3O_4$	√	0.07498	SF/SL
15	21.01	392.21815	392.21800	Leu-Pro-Tyr	$C_{20}H_{29}N_3O_5$	√	0.15686	SF/SL
15	21.02	215.13873	215.13902	Pro-Val	$C_{10}H_{18}N_2O_3$	√	0.28420	SF/SL
16	21.34	120.08071	120.08078	/	C_8H_9N	/	0.06546	SF
16	21.35	263.13933	263.13902	Phe-Pro	$C_{14}H_{18}N_2O_3$	√	0.31568	SF/SL
16	21.36	525.27048	525.26942	/	$C_{27}H_{40}O_{10}$	√	1.05474	SF
17	23.34	258.10819	258.11010	/	$C_8H_{20}NO_6P$	√	1.90665	SF
			258.10901	Diketo-Glu-Gln	$C_{10}H_{15}N_3O_5$	√	0.82	LSc
17	23.33	515.20901	515.20828	/	$C_{19}H_{34}N_2O_{14}$	√	0.72690	SF
17	23.37	241.08157	241.08190	/	$C_{10}H_{12}N_2O_5$	√	0.32539	SF
			241.07593	Diketo-His-Cys	$C_9H_{12}N_4O_2S$	√	5.64	LSc
18	28.47	187.07134	187.07133	/	$C_7H_{10}N_2O_4$	√	0.00767	SF
			187.07189	Diketo-Glu-Gly	equivalent to SF	√	0.55	LSc
18	28.47	204.09774	204.09788	Gln-Gly	$C_7H_{13}N_3O_4$	√	0.14653	SF/SL
18	28.79	147.07639	147.07642	D-Glutamine	$C_5H_{10}N_2O_3$	√	0.03076	SF/SL
19	29.52	275.1505	275.13500	Gln-Gln	$C_{10}H_{18}N_4O_5$	√	0.05906	SF/SL
20	32.87	241.03114	241.03113	L-Cysteine	$C_6H_{12}N_2O_4S_2$	√	0.01237	SF/SL
20	32.94	262.15096	262.15098	Arg-Ser	$C_9H_{19}N_5O_4$	√	0.10224	SF/SL
20	32.91	337.17138	337.17178	/	$C_{12}H_{24}N_4O_7$	√	0.39178	SF
20	32.90	318.12913	318.12958	/	$C_{12}H_{19}N_3O_7$	/	0.44260	SF
			318.12764	Diketo-Met-Trp	$C_{16}H_{19}N_3O_2S$	/	1.49	LSc

Sample 4 was evaluated in the same way, and identified substances were compared with the substances identified in sample 1. The aim was to clarify if the different processing steps had an influence on the composition of the samples.

Table 3.17-2: Results of the three calculation approaches for the most abundant signals in sample stock solution (100 mg mL^{-1}) of sample 4 (pasteurised, evaporated, and treated in vacuum oven). Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc), calculation approach 3 is the automatic approach by Spectral Library (SL) and not detected (n.d.) indicates signals that were not identified in sample 1 but in sample 4. A hook in the MS/MS column indicates that an ms/ms spectrum was recorded, and a slash that no ms/ms spectrum was recorded. "Equivalent to SF" means that proposed molecular formula by operator is the same like the molecular formula calculated by SF. Numbering in the table correlates with the numbering of the corresponding Figure 3.17-2.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
1	6.45	282.27850	282.27914	Oleamide	$C_{18}H_{35}NO$	√	0.63644	SF/SL
2	8.52	182.11714	182.11756	/	$C_{10}H_{15}NO_2$	√	0.41139	SF
3	8.83	216.10137	216.10191	/	$C_{13}H_{13}NO_2$	√	0.53325	SF
3	8.83	262.10669	262.10738	/	$C_{14}H_{15}NO_4$	√	0.69831	SF
			262.10404	Glu-Asn	$C_9H_{15}N_3O_6$	√	2.65	LSc
			262.10403	Glu-Gly-Gly	$C_9H_{15}N_3O_6$	√	2.65	LSc
3	8.83	284.08853	284.08905	/	$C_{12}H_9N_7O_2$	√	0.52058	SF
4	9.78	211.14372	211.14410	/	$C_{11}H_{18}N_2O_2$	√	0.38816	SF
			211.14467	Diketo-Pro-Leu	equivalent to SF	√	0.95	LSc
5	10.21	211.14372	211.14410	/	$C_{11}H_{18}N_2O_2$	√	0.38744	SF
			211.14467	Diketo-Pro-Leu	equivalent to SF	√	0.95	LSc
6	10.54	245.12797	245.12845	/	$C_{14}H_{16}N_2O_2$	√	0.47918	SF
			245.12902	Diketo-Pro-Phe	equivalent to SF	√	1.05	LSc
7	11.06	197.12795	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.49904	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	1.07	LSc
7	11.14	333.14166	333.14181	/	$C_{13}H_{16}N_8O_3$	√	0.15295	SF
			333.14115	Glu-Gly-Gln	$C_{12}H_{20}N_4O_7$	√	0.51	LSc
			333.14115	Glu-Asn-Ala	$C_{12}H_{20}N_4O_7$	√	0.51	LSc
7	11.21	212.12749	212.12812	/	$C_{11}H_{17}NO_3$	√	0.63419	SF
8	11.28	246.11188	246.11247	/	$C_{14}H_{15}NO_3$	√	0.59228	SF
8	11.33	232.09637	232.09682	/	$C_{13}H_{13}NO_3$	√	0.45384	SF
9	15.66	261.12315	261.12502	/	$C_{12}H_{21}O_4P$	√	1.87362	SF
			261.12393	Diketo-Tyr-Pro	$C_{14}H_{16}N_2O_3$	√	0.78	LSc
9	15.84	169.09663	169.09715	/	$C_8H_{12}N_2O_2$	√	0.52414	SF
			169.09772	Diketo-Ala-Pro	equivalent to SF	√	1.09	LSc
10	17.05	197.12811	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.34801	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	1.07	LSc
10	17.05	243.13400	243.13393	/	$C_{11}H_{18}N_2O_4$	√	0.07143	SF
			243.1345	Diketo-Glu-Ile	equivalent to SF	√	0.5	LSc
10	17.05	485.26065	485.25956	/	$C_{16}H_{37}N_8O_7P$	√	1.08952	SF
11	19.60	227.10263	227.10429	/	$C_8H_{19}O_5P$	√	1.65703	SF
			227.1032	Diketo-Glu-Pro	$C_{10}H_{14}N_2O_4$	√	0.57	LSc
11	19.60	453.19800	453.19861	/	$C_{12}H_{34}N_6O_8P_2$	√	0.60738	SF
11	19.60	679.29259	679.29335	/	$C_{30}H_{42}N_6O_{12}$	√	0.75681	SF
12	20.12	342.23826	342.23873	Leu-Pro-Ile	$C_{17}H_{31}N_3O_4$	√	0.47471	SF/SL
13	20.33	181.09679	181.09715	/	$C_9H_{12}N_2O_2$	√	0.35930	SF
13	20.33	209.09192	209.09372	/	$C_8H_{17}O_4P$	√	1.80042	SF
13	20.33	451.22923	451.22996	/	$C_{20}H_{30}N_6O_6$	√	0.73002	SF
14	20.47	263.13895	263.13902	Pro-Phe	$C_{14}H_{18}N_2O_3$	√	0.06942	SF/SL
14	20.49	187.07120	187.07133	/	$C_7H_{10}N_2O_4$	√	0.13111	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
			187.07189	Diketo-Glu-Gly	equivalent to SF	√	0.69	LSc
14	20.57	229.15451	229.15467	Pro-Leu	$C_{11}H_{20}N_2O_3$	√	0.16217	SF
14	20.66	362.20711	362.20743	Val-Pro-Phe	$C_{19}H_{27}N_3O_4$	√	0.31784	SF/SL
15	20.95	328.22299	328.22308	Ile-Pro-Val	$C_{16}H_{29}N_3O_4$	√	0.08973	SF/SL
15	20.99	392.21767	392.21800	Leu-Pro-Tyr	$C_{20}H_{29}N_3O_5$	√	0.32788	SF/SL
16	21.33	120.08065	120.08078	/	C_8H_9N	√	0.12970	SF
16	21.33	263.13918	263.13902	Phe-Pro	$C_{14}H_{18}N_2O_3$	√	0.16150	SF/SL
16	21.34	525.27089	525.26973	/	$C_{22}H_{37}N_8O_5P$	√	1.15927	SF
17	23.29	258.10844	258.11010	/	$C_8H_{20}NO_6P$	√	1.66387	SF
			258.10901	Diketo-Glu-Gln	$C_{10}H_{15}N_3O_5$	√	0.57	LSc
17	23.29	515.20930	515.21127	/	$C_{18}H_{35}N_4O_{11}P$	√	1.96614	SF
17	23.32	241.08162	241.08190	/	$C_{10}H_{12}N_2O_5$	√	0.27583	SF
			241.07593	Diketo-His-Cys	$C_9H_{12}N_4O_2S$	√	5.69	LSc
18	28.42	187.07113	187.07133	/	$C_7H_{10}N_2O_4$	√	0.20776	SF
			187.07189	Diketo-Glu-Gly	equivalent to SF	√	0.76	LSc
18	28.71	147.07634	147.07642	D-Glutamine	$C_5H_{10}N_2O_3$	√	0.08099	SF/SL
19	29.48	275.13498	275.13500	Gln-Gln	$C_{10}H_{18}N_4O_5$	√	0.01781	SF/SL
20	32.82	241.03069	241.03113	L-Cysteine	$C_6H_{12}N_2O_4S_2$	√	0.43513	SF/SL
20	32.86	262.15070	262.15098	Arg-Ser	$C_9H_{19}N_5O_4$	√	0.27706	SF/SL
20	32.86	337.17169	337.17178	/	$C_{12}H_{24}N_4O_7$	√	0.08542	SF
20	32.87	318.12944	318.13123	/	$C_{10}H_{24}NO_8P$	√	1.78573	SF
			318.12764	Diketo-Met-Trp	$C_{16}H_{19}N_3O_2S$	√	1.8	LSc

A total of 29 different amino acids, di- or tripeptides as well as their condensation products were detected. Just as like in sample 1 almost all identified substances contained either glutamic acid, valine or proline. These amino acids are known to occur in the group of umami active peptides.

The comparison of sample 1 and sample 4 illustrates that the different processing steps had no significant influence on the composition of the sample. Only three substances (oleamide, Glu-Asn and diketo-Ala-Pro) were detected in sample 4, which were not detected in sample 1.

3.18 Gas chromatography-mass spectrometry (GC-MS) results of derivatised 2,5-diketopiperazines standard solutions

To facilitate the identification of 2,5-diketopiperazines a derivatisation by silylation was performed. The derivatised products were measured *via* GC-MS (2.5.9). This analysis should show, if there were specific and characteristic fragmentation patterns of 2,5-diketopiperazines. If so, the identification of these molecule class could be easily done in crude sample solutions containing multitude signals. Therefore cyclo(Leu-Pro), cyclo(Pro-Tyr) and cyclo(Glu-Glu) were silylated.

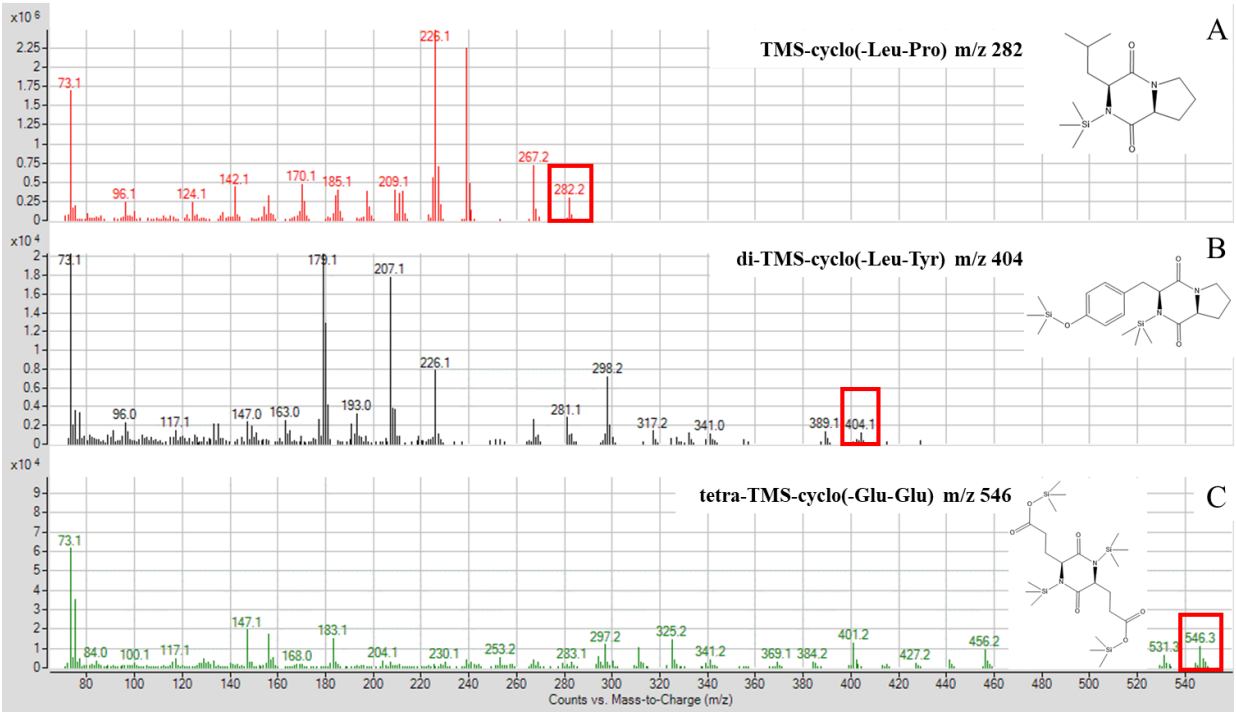


Figure 3.18-1: Gas chromatographic mass spectra of three different cyclic dipeptides. A: Mass spectrum (red) of silylated cyclo(-Leu-Pro); *m/z* 282; B: Mass spectrum (black) of di-silylated cyclo(-Leu-Tyr); *m/z* 404; C: Mass spectrum (green) of tetra-silylated cyclo(-Glu-Glu); *m/z* 546. Molecule ions are red framed.

Each 2,5-diketo compound was silylated successfully and the intact molecule ions were detected. Only the mass spectra of the highly silylated forms are shown (Figure 3.18-1). Expected fragments from silylated compounds were detected, such as *m/z* 73 (trimethylsilyl group), or fragments with a loss of *m/z* 15 (methyl group). Characteristic fragmentation pattern for 2,5-diketo compounds were not detected. No conclusion could be drawn for specific fragmentation patterns, which do allow the operator to easily identify 2,5-diketo compounds in complex matrices.

3.19 Liquid chromatography-mass spectrometry (LC-MS) data of synthesised 2,5-diketopiperazines

Three different 2,5-diketopiperazines were synthesised using the microwave assisted technology (2.8.1).

1. 2,5-diketo-Glu-Gly exact mass 186.06406
2. 2,5-diketo-Glu-Leu exact mass 242.12666
3. 2,5-diketo-Glu-Pro exact mass 226.09536

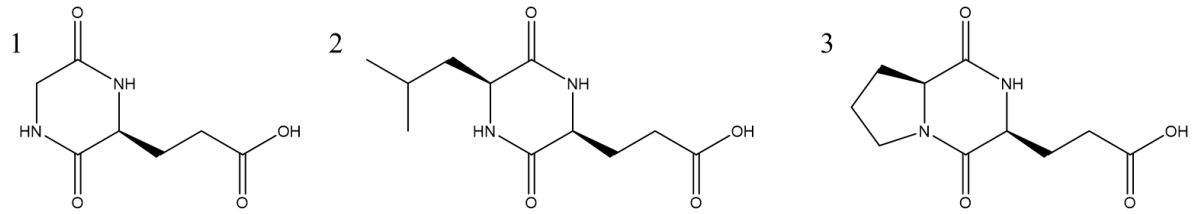


Figure 3.19-1: Structural formulas of the three cyclic dipeptides synthesised. 1: 2,5-diketo-Glu-Gly; 2: 2,5-diketo-Glu-Leu; 3: 2,5-diketo-Glu-Pro.

To analyse, if the synthesis of the 2,5-diketopiperazines (Figure 3.19-1) was successful, a LC-MS was performed (2.5.7) in the ESI positive and negative mode.

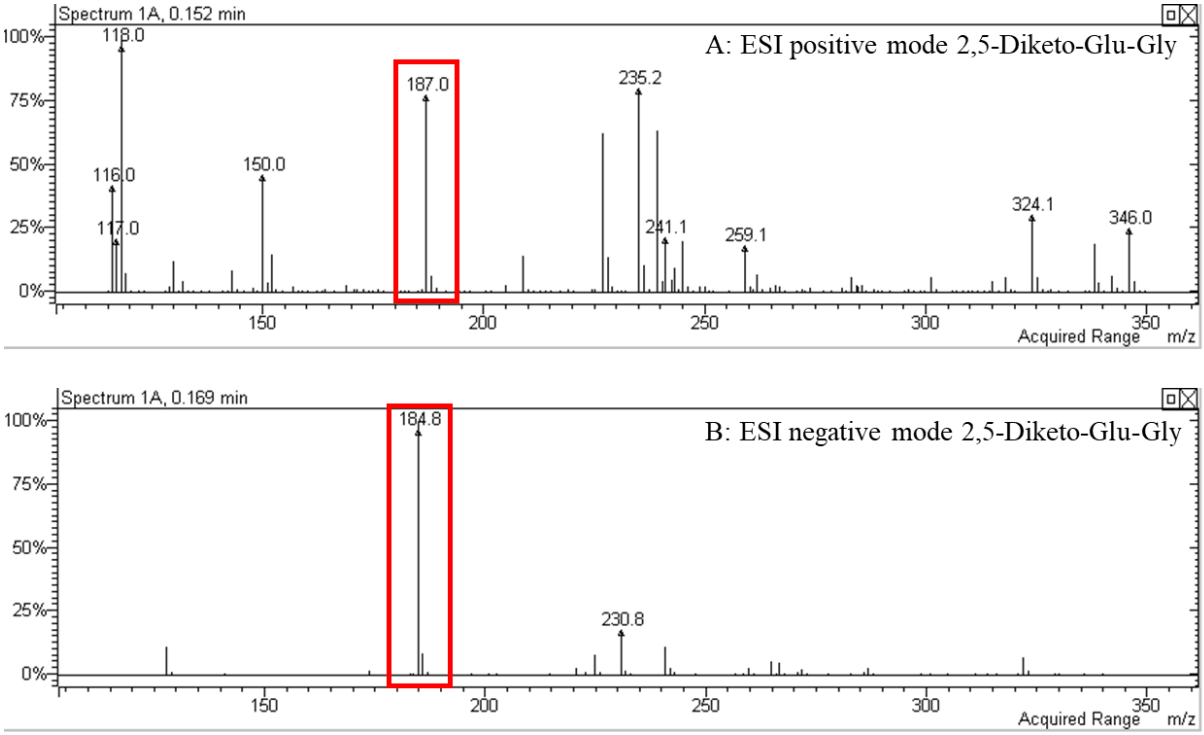


Figure 3.19-2: In positive MS mode (A) the spectrum of 2,5-diketo-Glu-Gly is shown. Spectrum B shows the 2,5-diketo-Glu-Gly in the negative mode. Molecule ions of the products are framed in red.

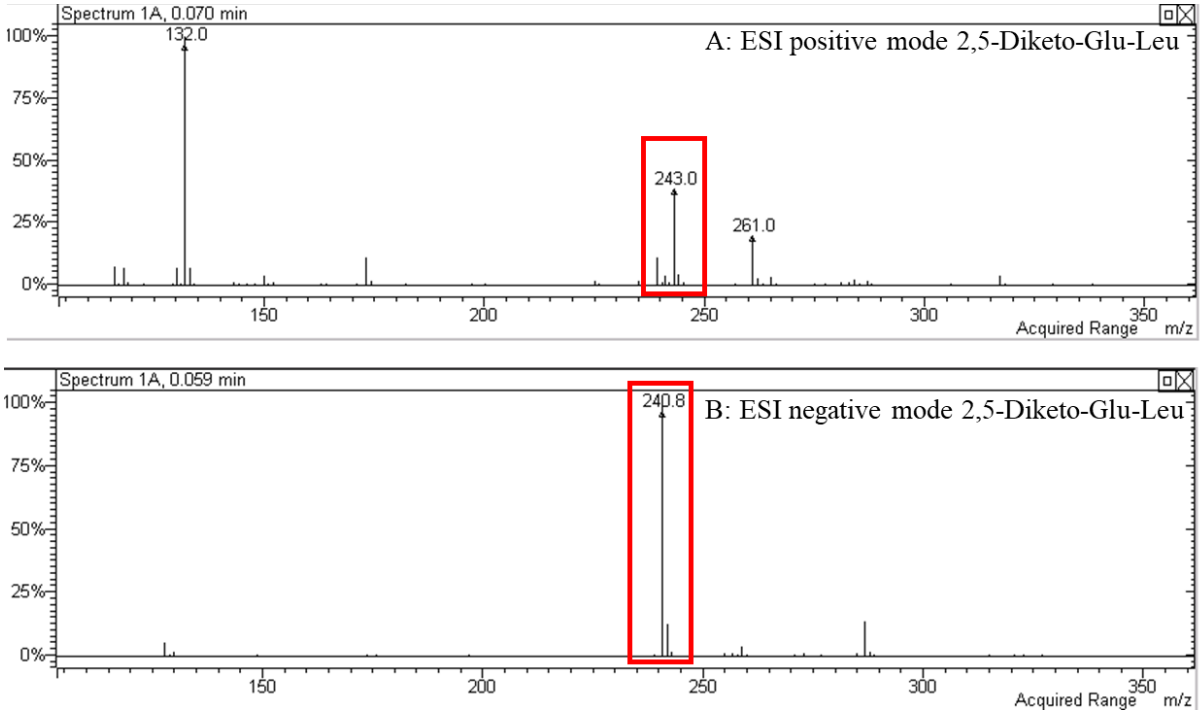


Figure 3.19-3: In positive MS mode (A) the spectrum of 2,5-diketo-Glu-Leu is shown. Spectrum B shows the 2,5-diketo-Glu-Leu in the negative mode. Molecule ions of the products are framed in red.

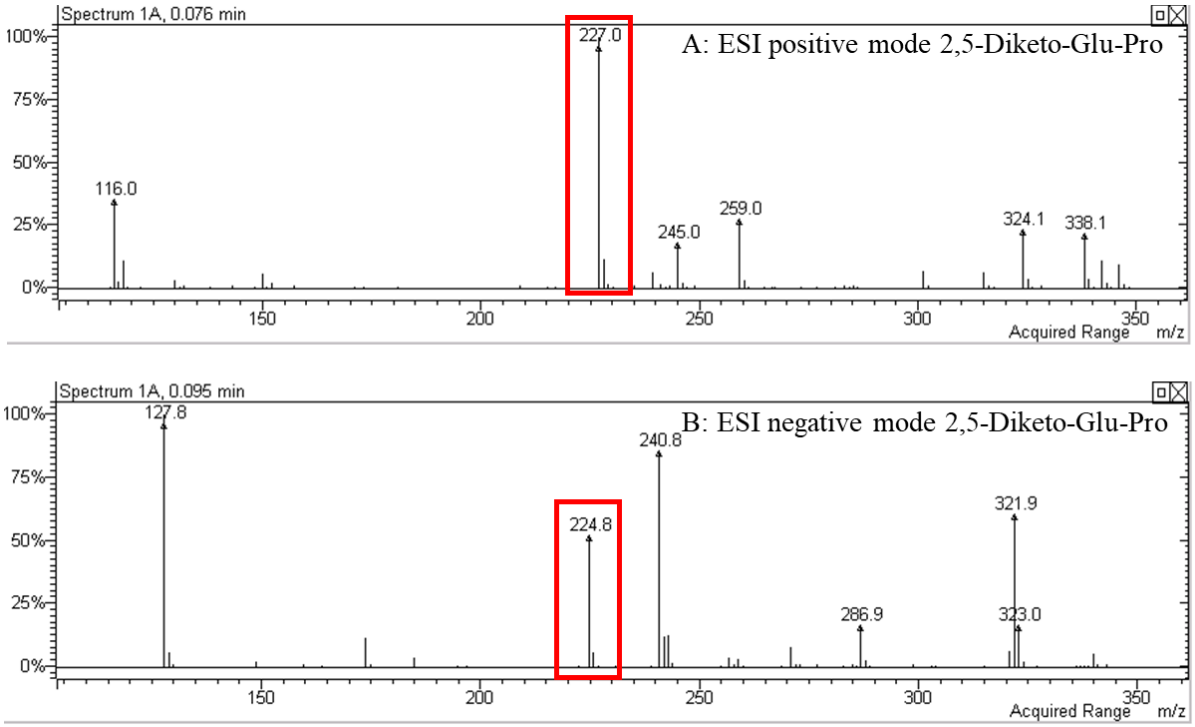


Figure 3.19-4: In positive MS mode (A) the spectrum of 2,5-diketo-Glu-Pro is shown. Spectrum B shows the 2,5-diketo-Glu-Pro in the negative mode. Molecule ions of the products are framed in red.

The synthesis of all 2,5-diketopiperazines was successful. The molecule ions were detected in both the positive and negative mode at the expected *m/z* ratio. As shown by these results this reaction was a powerful and fast tool to generate cyclic dipeptides, which could potentially have umami enhancing properties. A sufficient purification strategy was developed to remove unwanted remainders of the reaction before sensory analysis.

3.20 *In-silico* screening for glutamyl-specific peptidase genes in Basidiomycota

The majority of umami active compounds is generated by fermentation processes and hydrolysis of food constituents. In both cases glutamyl-specific peptidases could be responsible for the formation of umami taste imparting glutamyl peptides. The targeted use of such peptidases could increase the degree of hydrolysis of vegetable protein sources and might lead to an increased formation of umami active compounds. Since Basidiomycota express a variety of peptidases *in-silico* screening for glutamyl-specific peptidase genes has been performed based on published sequences.

Thermoactinomyces sp. exhibit a serine protease (accession number: WP_049719689), which has a V8-like Glu-specific endopeptidase region. It is a member of the trypsin superfamily. The protein sequence has been taken in FASTA format and the blastp search on NCBI resulted in two hits in the taxa of Basidiomycota. Both, *Laetiporus sulphureus* and *Fistulina hepatica* showed a hypothetical protein (Table 3.20-1).

Table 3.20-1: Results of the blastp search on NCBI. The main scores and values as well as the published accession numbers are listed.

Organism	Total score	Query cover	E value	Ident	Accession
Laetiporus sulphureus	41.6	38 %	0.033	32 %	KZT11210.1
Fistulina hepatica	37.0	40 %	0.95	25 %	KIY45975.1

Reported sequences were blasted on Joint Genome Institute (JGI) against the annotated genomes of Lsu and Fhe. This resulted in protein sequences and corresponding potential mRNA sequences, which were used to design specific primers for the start and the end of the potential coding sequences (CDS).

Amplification of the possible glutamyl-specific peptidase genes was verified on a 1 % agarose-gel (data not shown). The purified and from gel eluted DNA was ligated into the pUC57 vector and successfully transformed into *E.coli* TOP 10. Colony PCR of the positive clones (blue/white screening) showed the presence of the amplified fragments, which were verified by sequencing (Seqlab/Microsynth; Göttingen; Germany) using M13 primers.

3.21 Sequencing results of Lsul 235, Lsu 279, Lsu 294 and Fhe 205

The amplification of the glutamyl-specific peptidase genes of all four selected strains was successful. The four tested strains were sequenced using M13 primer pairs. Resulting sequence information on gDNA level was aligned against glutamyl-specific peptidase genes of four different Basidiomycota. The results showed that the sequence identity was below 100 %, which was explained by the presence of introns on gDNA level. For each of the tested strains the whole gDNA of the hypothetical glutamyl-specific peptidase gene was sequenced.

Number of base pairs (bp) of the sequenced genes (gDNA level) for Fhe 205 was 1392, 1251 for Lsul235, and Lsu294, and 1332 for Lsu279, which corresponds to 464, 417, 417 and 444 amino acids, respectively.

>Fhe205 hypothetical glutamyl-specific peptidase CDS
ATGAAAGATGGCAAAGAAAAAACCGGCAGCGGCTTTTTTGTGGATATTGATCTGCCGACC
GATTATGTGATTCTGACCGCGGCGCATAACCTGTGGAGCCTGGAAAGCGATACCCCGAGC
ACCGATATTAAAATTGAATATCCGAGCAGCACCGGCAGCGGCATTGAAATGGCGGGCGCG
GATTTTGAAGATTGGATGGATCCGAGCTGGGATGATGATGCGGAATATACCGTGCCGCCG
GGCCTGATTTATGTGGATGTGCTGGCGATGCGCGGCTGGAGCGTGAAATTTACCTTTATG
AAAGATGGCAAAGAAAAAACCGGCAGCGGCTTTTTTGTGGATATTGATCTGCCGACCGAT
TATGTGATTCTGACCGCGGCGCATAACCTGTGGAGCCTGGAAAGCGATACCCCGAGCACC
GATATTAAAATTGAATATCCGAGCAGCACCGGCAGCGGCATTGAAAGCTATACCATTGCG
CAGCAGACCCAGGATAGCGTGTATGTGAACAAACCGTATCATGAAAAAAGCAGCCAGCAG
CGCGGCGAACCGGAAGTGGATTATGGCTTTCTGCGCATTCCGCGCAAACCGGGCGAACCG
CGCCGCGGCTTTGGCCTGAGCCTGAAACTGGCGTATGCGGATTTTTTTACCGGCGATATG
CATATTCAGGGCTTTCAGGATAAAAGCAAACCGGGCCAGCCGATTCAGAGCAGCGGCGCG
TGCGTGGAATGCTATCCGGCGCGCGTGGAATATAGCATTAAAACCCAGCCGGGCATTAGC
GGCAGCGTGGTGTGGGTGGAATTTGCGGGCAGCCCGTTTGCGGTGGCGATTCAGAGCGTG
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TATGCGGGCGGCGGCAGCCGCGGCGCGCGCATTACCGAAGCGATGATGCGCGAAGTGTTT
AAATGGCTGGGCGATGCGAAACTGAAAGAAAACGTGCGCCTGCAGGTGGTGGATCTGCGC
CGCCAGGCGCGCCCGGGCCAGCCGAGCCTGGCGCCGCCGAACGGCCTGTTTCTGAGCTTT
GAAACCAGCTTTGGCTTTGGCCGCGTGCGCCTGGGCACCGGCACCAGCTTTGATCTGATT
CCGGCGCAGCTGCTGAACGGCCCGGAAGAACTGTATGTGCTGAAAGCGCATGCGAGCGGC
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GTGGTGCCGGAACGCGATCCGAACATGGATAAACTGAAAGGCGAAAGCTATGTGCTGCGC
ATGGAAGCGAACTATCTGAGCCTGACCGAACCGGAAGCGGAAAGCAGCGAAGTGAGCCTG
GTGCGCTATCCGACCCAGGATCTGGTGAGCCGCTGCCAGACCATGAGCTGCTTTAAAGTG
CTGCTGCTGTTTAGCGCGGATTTTAGCCTGCTGCAGTTTGTGAAATT**TAG**

>Fhe205 hypothetical glutamyl-specific peptidase protein sequence

MAGADFEDWMDPSWDDDAEYTVPPGLIYVDVLAMRGWSVKFTFMKDGKEKTGSGFFVDID
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SSQQRGEPEVDYGFLRIPRKPGEPRRGFGLSLKLAYADFFTGDMHIQGFQDKSKPGQPIQ
SSGACVECYPARVEYSIKTQPGISGSVVWVEFAGSPFAVAIQSVSCFCFLFMSDSLASSN
NGPEYAGGGSRGARITEAMMREVFKWLGDAKLKENVRLQVVDLRRQARPGQPSLAPPNGL
FLSFETSFGFGRVRLGTGTSFDLIPAQLLNGPEELYVLKAHASGKWLKFEPMKNRVVLED
KWNDNCTFRMDTSSPTAKQPWGSLVVPERDPNMDKLKGESYVLRMEANYLSLTEPEAESS
EVSLVRYPTQDLVSRCQTMSCFKVLLLFSADFSLLQFVKFSFA*

>Lsu235 hypothetical glutamyl-specific peptidase CDS

ATGGTGCGCCGCAAACTGCTGCTGCCGGATGAAGTGAAACATGATGGCTGGGCGGGCCGC
CTGCAGACCCCGCTGGCGAACCTGGATGGCCGCCGCGCGTTTCATGATGCGAGCTTTGAA
GCGCGCAACGTGATTAAACTGGTGTTTTATAGCGGCCCGGGCCGCAACCTGACCTATGGC
AGCGGCTTTATTCTGCGCCCGCCGGATGTGACCGGCGTGGTGATTCTGACCGCGGCGCAT
AACCTGCTGCCGATTCTGGATGCGGCGGATGAACCGACCGGCCAGCAGAGCAACAGCACC
CAGCCGAGCGTGAACATTCTGGGCGTGGAACATCCGATTACCAAAGATAACTGCCGCGTG
AGCGATACCTATCGCACCGGCGATAAACCGCCGGAAGCGGATTATGGCGCGATTATTCTG
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GTGGTGGCGATTCAGATGGAAGTGTTTTATTGGCTGGGCATTGGCGATTTTGGCATGCGC
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>Lsu235 hypothetical glutamyl-specific peptidase protein sequence

MVRRKLLLPDEVKHDGWAGRLQTPLANLDGRRAFHDASFEARNVIKLVFYSGPGRNLTYG
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SDTYRTGDKPPEADYGAIILSSLPVGDLEGFGFSICLGYEQSLPGELCVTGYRATDVAGQ
PKTSTGHCMGCYTRRLEYDAQTEQGISGSPVWMYHRGCPTVVAIQMEVFYWLGIGDFGMR
IRACASKHAALGTLPPRGLYLNFSKHFSFARVRVGNGTRFNILPAERRKDNVTLYTLQVA
DAEFLNKWLVFDVVKNEIQLDDKLSTEGLFSYRNKKKNTFKIVIERISPSEVQLGCQCKR
IEEIDGEDAESSEVSLVPYPIGEKYAVRWSVLPNNSITYLTPIKFTDFCFEDSIN*

>Lsu294 hypothetical glutamyl-specific peptidase CDS

ATGGTGCGCCGCAAACTGCTGCTGCCGGATGAAGTGAAACATGATGGCTGGGCGGGCCGC
CCGCAGGCGCCGCTGGCGAACCTGGATGGCCGCCGCACCTTTCATGATGCGAGCTTTGAA
GCGCGCAACGTGATTAAACTGGTGTTTTATAGCGGCCCGGGCCATAACCTGACCTATGGC
AGCGGCTTTATTTTTCGCCCGCCGGATGTGACCGGCGTGGTGATTCTGACCGCGGCGCAT
AACCTGCTGCCGATTCTGGATGCGGCGAACGATCCGACCGATCAGCCGAGCAACAGCACC
TATCCGTATGTGAACATTCTGGGCGTGGAACATCCGATTACCAAAGATAACTGCCGCGTG
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CGCATTCAGGAAATTGATGGCGAAGATGCGGAAAGCAGCGAAGTGAGCCTGGTGCCGTAT
CCGATTGGCGAAAAATATGCGGTGCGCTGGAGCGTGCTGCCGAACAACCCGATTGCGTAT
CTGACCCCGATTAAATTTACCGATTTTTGCTTTGAAGATAGCATTAAC**TAA**

>Lsu294 hypothetical glutamyl-specific peptidase protein sequence

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SDAYCRGDRTPDADYGAIILSSLAVGDLEGFGFSICVGYEESLPGELCVTGYRAADVAGQ
PKTSTGHCMGCYTRLLEYDAQTEQGISGSPVWMYHRGCPTVVAIQMEVFSWLDIGDFGRR
IRACASKKHAALGTLPPRGLYLNFSKHFSFARVRVGNGTRFNILPAERRKDNVTLYALQV
ADAEFLNKWLVFDVVKNEIQLDDKLSTEGLFSFRNKKKNTFKIVIERISPSEVQLGCQCK
RIQEIDGEDAESSEVSLVPYPIGEKYAVRWSVLPNNPIAYLTPIKFTDFCFEDSIN*

>Lsu279 hypothetical glutamyl-specific peptidase CDS

ATGGTGCGCCGCAAACTGCTGCTGCCGGATGAAGTGAAACATGATGGCTGGGCGGGCCGC
CCGCAGGCGCCGCTGGCGAACCTGGATGGCCGCCGCGCGTTTCATGATGCGAGCTTTGAA
GCGCGCAACGTGGTGAAACTGGTGTTTTATACCGATCCGGGCCGCAACGTGACCCATGGC
AGCGGCTTTATTTTTCGCCCGCCGGATGTGACCGGCGTGGTGATTCTGACCGCGGCGCAT
AACCTGCTGCCGATTCTGGATGTGGCGGATGATCCGACCGGCGATAGCAGCAACAGCCCG
TATCCGAGCGTGAACATTCCGGGCGCGGAACATCCGATTACCAAAGATAACTGCCGCATT
AGCGATGCGTATCGCCGCGGCGATCGCAGCGCGGAAGCGGATTATGGCGCGATTATTGTG
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GGCGCGAAAACCGAACAGGGCATTAGCGGCAGCCCGGTGTGGATGTATCATCGCGGCTTT
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GGCGGCAGCCGCGGCGCGCGCATTACCCTGGCGCTGCTGATGGAAGTGTTTAGCTGGCTG
GGCATTGGCAAATTTGGCAAAAGCATTCGCGCGTGCACCAGCAAAAAACATGCGGCGCTG
GGCACCCTGCCGCCGCGCGGCCTGTATCTGAACTTTAGCAAACATTTTAGCTTTGCGCGC
GTGCGCGTGGGCAGCGGCACCCGCTTTAACGTGTTTCCGGCGGAAGTGACCAAAGATAAA
GTGCTGTATGCGCTGCAGATTGCGAACGCGGAATTTCTGAACAAATGGCTGGTGTTTGAT
GTGGTGAAAAACGAAATTCAGCTGGATGATAAACTGAGCACCGAAGGCCTGTTTAGCTAT
GGCAACAAAAAAAAAAACAGCTTTAAAATTGTGATGGAACGCACCTTTCCGAGCGAAGTG
CAGCTGGGCTGCCAGTGCAAACGCATTGATGAAATTGATGGCGAAGATGCGGAAAGCAGC
GAAGTGAGCCTGGTGCCGTATCCGGCGGGCGAAAAATATGTGGTGCGCTTTAGCGTGCTG
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GAAGA**TAG**

>Lsu279 hypothetical glutamyl-specific peptidase protein sequence

MVRRKLLLPDEVKHDGWAGRPQAPLANLDGRRAFHDASFEARNVVKLVFYTDPGRNVTHG
SGFIFRPPDVTGVVILTAAHNLLPILDVADDPTGDSSNSPYPSVNIPGAEHPITKDNCRI
SDAYRRGDRSAEADYGAIIVSSPLHAFDDIEGFGFSICLGYEQSLPGELSVTGYRAADLA
GQPKTSTGNCVGCYTRWLEYGAKTEQGISGSPVWMYHRGFPTVVAIQMPNSSNNGPAGRR
GGSRGARITLALLMEVFSWLGIGKFGKSIRACTSKKHAALGTLPPRGLYLNFSKHFSFAR
VRVGSGTRFNVFPAEVTKDKVLYALQIANAEFLNKWLVFDVVKNEIQLDDKLSTEGLFSY
GNKKKNSFKIVMERTFPSEVQLGCQCKRIDEIDGEDAESSEVSLVPYPAGEKYVVRFSVL
SSPDNLTAYSTSIKFTDFCFEDSI*

Protein sequence analysis and classification was done on the webpage of the European Bioinformatics Institute and showed that the sequenced genes belonged to the peptidase family S1 (clan PA). This family includes cysteine and serine peptidases (European Bioinformatics Institute 2018). The superfamily was predicted to be trypsin-like serine peptidases based on the amino acid sequences 35 to 222 (Fhe 205), 20 to 233 (Lsul 235), 22 to 214 (Lsu279), and 20 to 232 (Lsu294). The theoretical molecular mass of 21 kDa, 25 kDa, 20 kDa, and 23 kDa was calculated by (ExPASy Bioinformatics Resource Portal n.d.) for Fhe 205, Lsul 235, Lsu 279, and Lsu 294, respectively.

Lsul 235 shared 84 % sequence identity with Lsu 279, and 93 % with Lsu 294, whereas Lsu 279, and Lsu 294 shared 84 % sequence identity (European Bioinformatics Institute 2018).

4 Discussion

In the present work, small biomolecules imparting or enhancing the umami taste of wheat gluten hydrolysates should be identified. For this purpose, the Nestlé Product Technology Center, *Lebensmittelforschung* GmbH (Singen, Germany) kindly provided wheat gluten hydrolysates. The hydrolysates were produced using different peptidase preparations. The composition of the hydrolysates was investigated, and they were sensorially analysed. To identify the substances eliciting the umami taste, hydrolysates were fractionated with Size Exclusion Chromatography and preparative HPLC, respectively. The composition of promising fractions and sub-fractions thereof and the sensory relevance were investigated. The aim was to find out which of the substances identified in the sub-fractions contributed to the umami taste.

4.1 Optical properties and odour of enzymatically hydrolysed wheat gluten

Wheat gluten is a protein rich vegetable source, whose hydrolysates are widely used as seasoning of culinary products due to their umami taste (Schlichtherle-Cerny and Amadò 2002). Since wheat gluten is a by-product of wheat starch production it is easily obtained (Hardt, Janssen et al. 2014), and used in industrial applications.

The difference of the hydrolysates in term of particle size and colour (result section 3.1) is not explainable by the use of different enzyme preparations in the manufacturing process. Since the other process conditions remained unchanged, there is no indication which factors influenced the outcome of the optical properties.

In addition, samples from different processing steps were analysed to compare the impact of temperature and drying on the composition of peptides and temperature-induced condensation products thereof. The variation in terms of the colour of the samples from different processing steps is partially explainable by the process parameter in combination with the enzyme preparation used. Flavourzyme contains an α-Amylase (Merz, Eisele et al. 2015) that degrades the starch of wheat gluten. Thereby maltose is generated (Whan, Dielen et al. 2014) that can undergo Maillard reactions with amine compounds under thermal conditions (Kanzler, Schestkowa et al. 2017). The differences in the thermal treatment and the resulting degree of Maillard reactions explains the different colouration of the samples.

4.2 Influence of peptidase preparation on the outcome of wheat gluten hydrolysis

It is widely known that the enzymatic hydrolysis of plant proteins leads to products showing better functional properties compared to the original isolate. Thus, the water absorption, oil-holding, and foaming capacity as well as emulsion activity can be improved, which however, depends on the degree of hydrolysis and the used enzyme preparation (Vioque, Sánchez-Vioque et al. 2000). This work is focused on the released umami active substances. They include a variety of amino acids and oligopeptides (Su, Cui et al. 2012) as well as pyro- and cyclic peptides (Chen, Dewis et al. 2009). Soy sauce contains, among others, Asp-Ala (Oka and Nagata 1974) which has an intense umami taste. Glu-Ser (Noguchi, Arai et al. 1975) is contained in fish protein hydrolysate, which also imparts umami flavour. Condensation products of di- or tripeptides, e.g. pGlu-Pro; pGlu-Pro-Gln; pGlu-Pro-Glu and pGlu-Pro-Ser, which taste monosodium glutamate-like, were found in deaminated wheat gluten hydrolysate. The sequences of their precursor amino acids are very common motives of glutenin and gliadin, the main components of wheat gluten (Schlichtherle-Cerny and Amadò 2002). It is not surprising that pGlu-Pro was identified in the latest umami tasting sub-fractions.

Various studies regarding the enzymatic hydrolysis of wheat gluten are published. Almost all publications concern the improvement of the degree of hydrolysis by changing process parameters, and the enzyme preparation used for hydrolysis. Examinations of quite a few enzyme preparations or technical enzymes, such as Alcalase, Flavourzyme, Protamex (Koo, Bae et al. 2014), papain (Li, Yu et al. 2016), Debitrase HYW20, Corolase PP (Nongonierma, Hennemann et al. 2017), Validase FP concentrate, and Pronase (Widyarani, Sari et al. 2016) show that the selection of the enzyme preparation has a great influence on the outcome of the hydrolysis. However, none of these enzymatic hydrolyses has the same efficiency compared to acid-catalysed chemical hydrolysis.

Hydrolysis of the samples for this work was either performed with Flavourzyme, a mixture of Flavourzyme and Glutaminase, or P6SD, which most likely stands for Protease P “Amano” 6 (Table 3.1-1). These different hydrolysis approaches led to differences in the peptide composition and taste, which was disclosed in this work.

Protease P “Amano” 6 has not been used before to hydrolyse wheat gluten. Only a working group from Reykjavík, Iceland, published hydrolysis studies of fish protein with Protease P (Halldorsdottir, Kristinsson et al. 2013, Halldorsdottir, Sveinsdottir et al. 2014). The manufacturer

indicates that this proteolytic enzyme preparation has its pH-optimum at pH 8, a temperature optimum at 45 °C, a residual activity over 50 % in the pH range of 5 to 9, and in the temperature range of 10 to 45 °C. Furthermore, the manufacturer claims that this proteolytic enzyme preparation manufactured by fermentation with *Aspergillus mellu*s can be used for flavour improvement of meat extract and fish juice (Amano-Enzyme 2003). Due to its broad application possibilities Protease P "Amano" 6 was a promising tool for the hydrolysis of wheat gluten. At best, the use of this enzyme preparation led to an increased yield of umami active substances like glutamyl-dipeptides or cyclic-dipeptides compared with Flavourzyme.

4.3 The umami peptides of the sample stock solutions

The varying concentration of free amino acids in the three samples is explainable by the use of the different enzyme preparations for the wheat gluten hydrolysis. Samples treated with glutaminase showed a lower concentration of glutamine than the other samples. Since glutaminase belongs to the enzyme group of amidases it catalyses the conversion of L-glutamine to L-glutamic acid (Nanga, DeBrosse et al. 2014). Approximately 60 % of L-glutamic acid in soy sauce fermented with *Aspergillus sojae*, can be produced by a glutaminase reaction (Ito, Koyama et al. 2013). This reaction explains the high concentration of L-glutamic acid in sample 2. It can be hypothesised that the taste intensity of the samples correlates with the concentration of free glutamic acid (Table 3.2-1). The differences in the absolute values of Gln and Glu between the samples is explainable by the process conditions of sample preparation. The glutamine in sample 2 is partially converted to glutamic acid by the added glutaminase. Thermal treatment of aqueous solutions containing glutamic acid can lead to the condensation reaction of equimolar proportions of glutamic acid to pyroglutamic acid (Harada and Fox 1958). However, no pyroglutamic acid was detected in the sample stock solutions. This was due to the overloaded chromatogram and the myriad of detected MS signals. The measurement of the subsequently generated sub-fractions A5 in the negative ESI mode, however, confirmed the hypothesis of pyroglutamic acid formation (Supplementary figure 6, Supplementary figure 8, Supplementary figure 12, and Supplementary figure 18).

The degree of hydrolysis, as determined by the sample treatment, is the cause of the release of amino acids. The higher the degree of hydrolysis the higher the concentration of free amino acids should be (Giesler, Linke et al. 2013). In 2016 it was shown that with increasing casein hydrolysis

in ripened Parmesan cheese, the concentration of taste-active enzymatically formed γ-glutamyl dipeptides increased (Hillmann, Behr et al. 2016). Accordingly, effective enzymatic protein hydrolysis should result in a more flavourful hydrolysate.

Umami taste of sample stock solutions: Different standardised sensory tests (2.6) were combined, which led to the highest possible information content of the sample taste. The test included parts of the triangle-tests, alternative forced choice test, and the ranking test.

Although glutamic acid is primarily responsible for umami taste (Yamaguchi and Ninomiya 2000), the intense umami taste of the samples cannot be explained by the detected concentration. Beside glutamic acid, various substances are known, which evoke an umami taste sensation, like p(Glu-Pro-Ser), p(Glu-Pro), Asp-Glu-Ser and Thr-Glu (Suess, Festring et al. 2015). The composition of wheat gluten, which consists of 80 to 85 % protein, and is rich in proline and glutamine (Van Der Borght, Goesaert et al. 2005), indicates that such substances are produced during sample processing using enzyme preparations and thermal treatment. Since a more than fivefold increase (Figure 3.3-1) in the taste strength was detected, umami taste-enhancing or modulating compounds must also be present in the samples. To confirm this assumption the peptide composition of the sample stock solutions was analysed.

Determination of peptide composition of the sample stock solutions *via* UPLC-HR-Q-TOF-MS/MS: Successful separation (Supplementary figure 1, Supplementary figure 2, and Supplementary figure 3) using the most feasible conditions for the complex matrices was achieved by method 2.5.5. The MSG-like tasting substances Glu-Ser and Glu-Gln-Glu (Noguchi, Arai et al. 1975) were solely present in sample stock solution 2 and explained its umami taste. The solely detected substances in the other samples are not known to evoke the umami taste. However, umami taste is elicited by Glu-Leu; Val-Asp (Ohyama, Ishibashi et al. 1988); pGlu-Pro (Schlichtherle-Cerny and Amadò 2002); Pro-Thr (Yamamoto, Shiga et al. 2014); pGlu-Gln; pGlu-Gly (Kaneko, Kumazawa et al. 2011), Val-Val; Val-Glu (Ishibashi, Ono et al. 1988); Glu-Tyr, Pro-Ala-Gln (Dang, Gao et al. 2015) which were present in the samples. The presence of monosodium glutamate and umami peptides may lead to synergistic effects, which increase the umami taste. MSG has three different effects on the umami taste receptor. It enables the small peptides to bind to the T1R3 part of the receptor. The binding cavity of the T1R1/T1R3 receptor is enlarged by MSG, and binding residues are increased, which are important for the hydrogen bonding (Dang, Hao et al. 2019).

Beside the umami active compounds identified in the samples, the umami taste activity of 165, 167, and 163 substances in sample 1, 2, and 3 is not known. Among them there might be substances whose umami taste or enhancing effect has not previously been described.

A fractionation was performed to increase the likelihood of the identification of not described umami active substances. It was necessary to generate fractions containing less substances, but still possessing a distinct umami taste.

4.4 Fractionation of samples stock solutions

Size Exclusion Chromatography (method 2.5.1.) is a common technique for the separation of molecules according to their size. It is feasible, especially if the samples are very complex with molecules of different size. SEC was already successfully used for the separation of umami active peptides (Su, Cui et al. 2012). The majority of umami active substances are di- to tetrapeptides with masses up to 450 Da (Zhang, Venkitasamy et al. 2017). The distinct umami taste of Korean soy sauce is evoked by small substances of less than 500 Da (Kim, Kim et al. 2017). Exactly in this range (170 to 430 Da) the separation of the molecules occurred (Figure 3.6-2).

Since the used SEC "NGC Chromatography System" from Bio RAD (Hercules, California, USA) was equipped with a fixed wavelength detector with two different selectable wavelength the most suitable wavelength (λ = 280 nm) for this approach was chosen (Schlichtherle-Cerny and Amadò 2002). At this wavelength the aromatic amino acids Phe, Trp, Tyr, and His are detectable. However, the almost identical elution patters of the SEC chromatograms suggested that the samples did not differ much in composition, although they were produced differently.

4.5 Umami taste of SEC fractions from sample stock solutions

Determination of glutamic acid concentration in SEC-fractions: The concentration of free glutamic acid (Table 3.7-1) was determined with a rapid HPLC method. The ratio of the detected free glutamic acid in the SEC-fractions of the different samples was the same as in the sample stock solutions. Comparable ratios of glutamic acid concentration in wheat gluten hydrolysates treated with and without glutaminase are described in literature (Schlichtherle-Cerny and Amadò 2002).

Even though the initial concentration of glutamic acid was not fully recovered, the separation was successful. The "Superdex Peptide 10/300 Gl" column (GE Healthcare, Little Chalfont, United Kingdom) was able to separate proteins, peptides and other small biomolecules in a range of 100 to 7000 Da, like glutamic acid with a mass of 147 Da, dipeptides and their condensation products. Since glutamic acid was only present in two of the eight SEC-fractions a carryover during the chromatography can be precluded.

Peptide composition and umami taste of SEC-fractions: Most commonly peptides are identified using databases and libraries. The identification of peptides using an automatic approach is limited, since it is affected by the size of the search space of the used databases (Shanmugam and Nesvizhskii 2015). To significantly increase the number of identified or predicted peptides, a manual approach is necessary. However, the order of the amino acids in the peptides remains unknown. In order to improve the sequence prediction, an alignment with the protein sequence of the protein, which was used for the hydrolysis, can be carried out. A complete *de novo* sequencing requires a MS/MS analysis of every single peptide (Standing 2003) and the manual interpretation of the complete ion series.

For the first time, the peptide composition of SEC-fractions of hydrolysed wheat gluten samples is described here in such a detail. A number of 91, 118, and 141 small biomolecules were identified in samples 1, 2, and 3 (SEC A5), respectively. Their umami taste, which is five fold higher (sample 1; Figure 3.7-2, sample 2; Figure 3.7-3, and sample 3; Figure 3.7-4) compared with the standard solution is evoked by several known umami active compounds like Glu-Leu, Val-Asp (Ohyama, Ishibashi et al. 1988), Glu-Ser (Noguchi, Arai et al. 1975) Glu-Val (Maehashi, Matsuzaki et al. 1999), Pro-Glu (Dang, Gao et al. 2015), Pro-Thr (Yamamoto, Shiga et al. 2014), diketo-, or pyro(Glu-Gln), diketo-, or pyro(Glu-Gly) (Kaneko, Kumazawa et al. 2011), and pyro(Glu-Pro) (Schlichtherle-Cerny and Amadò 2002). In contrast, one umami active substance Glu-Tyr (Dang, Gao et al. 2015) is present in SEC A6 of sample 2. The two other samples do not

exhibit known umami active compounds. Moreover it has been hypothesised that SEC A6 contains more substances with umami attributes or substances having a higher umami taste enhancing potential than SEC A5 because SEC A6 contained less glutamic acid but evoked a more intense umami taste. Remarkably, these solutions have an intense umami taste even though no MSG was added.

The taste strength of SEC A4 was also increased by a factor of five. This fraction was adjusted to 10 mM MSG. Due to the lower percentage of panellists who have perceived the intense umami taste, it is considered that these fractions contain substances with lower umami activity compared with SEC A5 and A6, respectively.

Instead of conducting sensory analyses with all identified substances with unknown umami attributes, it was decided to generate sub-fractions showing a lower number of small molecules but still possessing an intense umami taste.

4.6 Composition of prepHPLC sub-fractions and how they taste

Preparative HPLC is a common LC method for the isolation of non-volatile compounds from complex food preparations, as well as High Speed Countercurrent Chromatography, or Fast Centrifugal Partition Chromatography. With regard to subsequent sensory analysis, the use of solvents that are harmful and toxic for humans (Reichelt, Peter et al. 2010) has to be renounced in order not to violate ethical standards for human consumption. The use of “food-grade” solvents has another advantage. The implementation of time consuming procedures that remove the toxic solvents, such as thermal processes or extractions, which could lead to extreme stress for the peptides and may alter them (New Hope NETWORK an informa business 2005), is not necessary. Peptide bonds can be detected at 205 nm. Due to the UV cut off of the ethanol used in the gradient the detection wavelength was set to 210 nm. At this wavelength, the absorption of the peptide bonds is still sufficiently high and it is far enough away from the ethanol cut off. The success of fractionation *via* prepHPLC was examined using the UPLC-HR-MS/MS, because no statement about the composition of the sub-fractions could be derived from the UV chromatograms from the prepHPLC. The composition of the sub-fractions will be discussed below.

The performed gradient and the reversed phase C18 column led to an early elution of polar substances (Schlichtherle-Cerny and Amadò 2002). Since the majority of the taste active molecules elute early, it is assumed that they are comparatively polar. Furthermore, the

fractionation time of 10 min resulted in a poor separation of the molecules. To optimise the result, the fractions could have been cut at shorter time intervals. However, without the use of toxic solvents the chromatographic options for the separation of small peptides by reversed phase chromatography are limited. Chromatographic approaches using nontoxic solvents, such as water and ethanol are called “green chromatography” (Płotka, Tobiszewski et al. 2013).

The German flavour company Symrise (Holzminden) developed the so called LC Taste® method, which is comparable to gas chromatography-olfactometry. Unfortunately, this method is barely described in literature. Nevertheless, this approach is a combination of High Temperature Liquid Chromatography with non-toxic eluents and sensory analysis. High temperatures up to 200 °C are necessary to change the physicochemical properties of ethanol and water. These changes make the “green” solvents a proper alternative for the separation of complex natural products (Reichelt, Peter et al. 2010). This technique has not been used for the analysis of hydrolysed wheat gluten. Since it requires relatively high temperatures, which in turn require special equipment and rarely available temperature stable stationary phases, it is excluded from further consideration.

Determination of free glutamic acid concentration in prepHPLC sub-fractions and sensory analysis: Taken into account that glutamic acid is the major compound eliciting umami taste, its concentration was determined by HPLC (2.5.3). The total concentration of glutamic acid in the prepHPLC sub-fractions was lower than in the SEC-fraction used for the sub fractionation. A dilution effect can be excluded, since each prepHPLC sub-fraction was freeze dried and reconstituted to the initial volume. The “food-safe” solvents used for the elution of the compounds might be an explanation for the discrepancy in the glutamic acid concentration.

Both the detected diketo- or p(Glu-Gln) which are known to increase the umami taste of Japanese soy sauce by one fifth (Kaneko, Kumazawa et al. 2011) and the added MSG (10 mM) are not sufficient to generate the intense umami taste as in SEC-fraction A5. The intrinsic taste of p(Glu-Gln) is not strong enough to give the sub-fractions the typical umami taste or its concentration is below the taste threshold. Based on their results, Kaneko et al. claimed in 2011 that there must be an optimum of the ratio of umami compounds and their enhancers. This hypothesis indicates that the presence of an umami enhancing compound and MSG did not necessarily lead to an intense enhancement of the umami taste. A concentration dependency was shown for MSG and IMP mixtures. The enhancement of the umami taste was clearly related to the added amount of IMP. To find the mixture with the most intense umami taste, 47 different solutions were sensory analysed (Yamaguchi 1967). Although an enlargement of the size of the binding cavity of the umami receptor by MSG was described, no synergistic effect with

p(Glu-Gln) was observed. It can be assumed that the conformation of p(Glu-Gln) leads to steric hindrance at the receptor, which prevents more molecules from binding to it. This hypothesis is confirmed by the fact that it is known that the addition of MSG only promotes an improved binding of small peptides to the receptor (Dang, Hao et al. 2019).

In order to further optimise the accuracy of the sensory results, the training period and frequency for the panellists could be increased (Mittermeier, Dunkel et al. 2018). These trainings enable the panellist to describe their taste impressions of aqueous reference solutions even more clearly. (Ottinger and Hofmann 2003).

Determination of the peptide composition of prepHPLC sub-fractions: The number of identified substances was highest in sub-fraction 1 and decreased from sub-fraction 2 to sub-fraction 6. This separation pattern was similar for all three samples. Furthermore, the total number of detected molecules was highest in sample 2 (258 molecules) (Figure 3.12-2), followed by sample 3 (146 molecules) (Figure 3.12-3), and sample 1 (92 molecules) (Figure 3.12-1). Detailed information of all identified substances can be found in Supplementary figure 22 to Supplementary figure 39 and Supplementary table 22 to Supplementary table 39. Compared with the total number of identified substances in the SEC-fractions of the same samples, the number of identified substances decreased by 47 for sample 1, and 51 for sample 3, but increased for sample 2 by 85.

Among the multitude of identified substances in sub-fraction one of sample 2, eight substances, namely (Glu-Leu (Ohyama, Ishibashi et al. 1988); Val-Glu; Pro-Glu (Dang, Gao et al. 2015); Val-Asp; Val-Gly (Ishibashi, Ono et al. 1988); Glu-Ser (Arai, Yamashita et al. 1972); Pro-Gly and Pro-Thr (Yamamoto, Shiga et al. 2014)) showing umami attributes as well as one diketopiperazine (diketo(Glu-Gln) (Kiyono, Hirooka et al. 2013) were present (Table 3.12-1). The sub-fractions 2, 3, 5, and 6 showed one umami active substance (Table 3.12-2).

4.7 Umami taste of SEC sub-fractions

The formerly performed sub-fractionation *via* prepHPLC did not lead to sub-fractions, which elicited an intense umami taste. For this reason a refined SEC was performed. Since the two sub-fractionation methods are based on different separation principles, they are not directly comparable. In the reversed phase prepHPLC the compounds are separated according to their polarity and their behaviour on the reversed phase column. In contrast, SEC is based on the

separation by the size of the molecules. Also, the detection in the two different chromatographic methods could not be compared, since both were carried out at different wavelengths due to selectable system settings.

The refined SEC method led to a better separation of the small molecules. The sub-fractions were collected every minute instantly after injection (n = 35). Based on the UV trace, six sub-fractions were taken from SEC-A5 and seven from SEC-A6 of sample 2 (Figure 3.13-1) for further sensory analysis.

Determination of free amino acid concentration in SEC sub-fractions and sensory analysis: The SEC chromatograms of sub-fraction A19 of SEC A5 and sub-fraction A26 of SEC A6 showed signals at λ = 280 nm. However, according to the HPLC analysis, these were no quantifiable free amino acids. It is obvious that these sub-fractions had to be analysed further to describe their composition and to identify potentially unknown umami active substances.

The umami taste of both sub-fractions was fivefold higher compared with the MSG standard solution (10 mM), according to 23 to 38% of the panellists, although the sub-fractions did not contain MSG. Because of this, it must be assumed that these sub-fractions contained substances that have an intrinsic and intense umami taste. Another series of experiments with the addition of MSG confirmed this assumption. Again, a significant enhancement of taste was found. This was due to the presence of two umami active compounds. UPLC-HR-QTOF-MS/MS shows the presence of diketo-Glu-Pro, which is known to enhance the umami taste in Japanese rice wine (Kiyono, Hirooka et al. 2013), and the dipeptide Glu-Leu that elicits umami taste (Ohyama, Ishibashi et al. 1988) as well in sub-fraction A19 of SEC-A5 (sample 2).

However, the intense umami taste of sub-fraction A26 of SEC A6 of sample 2, without 10 mM MSG added, remains unexplained, since it contained no known umami compounds. If this sub-fraction contained substances with umami enhancing attributes, which are still unknown, they had to be very powerful due to the intense umami taste they evoked without clear UPLC-HR-QTOF-MS/MS signals.

Since the umami taste sensing is highly complex and has a myriad of substances that can contribute to the umami taste (Suess, Festring et al. 2015), an electronic-tongue could be a possible alternative to the conventional sensory analysis (Liu, Zhu et al. 2017). Since this system was developed to distinguish between the five basic tastes, it could also be used as a complementary method. Compared to the traditional sensory analysis this system would not suffer from fatigue during the tests and be independent on daily physical conditions like the human sensory system. Some studies show possible applications for this system. Thus, the sensory quality of apple juice was assessed

(Bleibaum, Stone et al. 2002), and the taste properties of brown rice were analysed (Uyen Tran, Suzuki et al. 2004). Furthermore, the umami taste of edible mushrooms was differentiated (Phat, Moon et al. 2016). The use of the electronic tongue in the field of umami analysis has risen in recent years. Different working principles build the core part of the system. Soluble components and global characteristic response signals of the taste substances are measured potentiometrically, voltammetrcally, and *via* impedance spectroscopy sensors. Their recognition threshold is much lower than human threshold perception and can be used for discrimination of the five basic tastes, astringency quantification, and the evaluation of binary interactions of basic tastes (Jiang, Zhang et al. 2018). The optimisation of the sensor is ongoing to enable the electronic tongue to mimic the human sense of taste. Nano-vesicles that carry the human umami taste receptor T1R1/T1R3 on their membranes were immobilised on the micropatterned graphene surface of the latest sensor generation. These sensors were used for the detection of umami taste (Ahn, An et al. 2016).

4.8 How thermal treatment influences the composition of wheat gluten hydrolysates

Samples of four different processing procedures were analysed to describe the influence of a thermal treatment on the composition of wheat gluten hydrolysates. The base peak chromatogram of the sample treated most harshly varied the most from the sample without thermal treatment. The evaluation of the most intense signals shows that the majority of the identified substances were proline-containing diketo-compounds (diketo-Leu-Pro, diketo-Pro-Phe, diketo-Pro-Val, and diketo-Glu-Pro for example) with increasing signal intensity from sample 1 to sample 4. Thermal treatment for 1 hour at 130 °C is known to be responsible for the increase of proline based diketopiperazines in chicken essence (Chen, Liou et al. 2004). This observation is confirmed in this work, according to the increased signal intensity of the detected diketo-Pro-compounds after additional thermal treatment steps.

Moreover, it is described in literature that roasting of fermented cocoa beans led to the generation of diketopiperazines from hydrophobic amino acids (Stark and Hofmann 2005). Also a comparison of cocking time for stewed beef and dry aged grilled beef showed that a prolonged cocking time generates a number of diketopiperazines and their concentration increased, respectively (Chen, Dewis et al. 2009). This is not surprising, since their major route of formation are chemical

reactions of peptides and proteins during thermal processing (Borthwick and Da Costa 2017). But obviously, the different thermal treatment of the hydrolysed wheat gluten did not lead to the generation of various diketo-compounds. Only sample 4 (most thermally treated) contained one additional diketopiperazine (diketo-Ala-Pro) compared with sample 1. It can be concluded that the use of different enzyme preparations for the hydrolysis of wheat gluten had a greater influence on the composition of peptides and their condensation products than the different thermal treatment steps.

4.9 The umami taste of hydrolysed wheat gluten

The analytical and sensory analyses performed in this work used state of the art methods and instrumentation. Several actual studies concerning the identification of umami compounds in different food preparations have followed a similar procedure. One of the key points is the fractionation of the highly complex food matrices. Most commonly, as in this work, ultrafiltration is used, followed by gel filtration chromatography (Xu, Xu et al. 2019). Fine fractionation of the ultra-filtrated and chromatographically fractionated food preparations by preparative HPLC has also been performed by some research groups (Kong, Yang et al. 2017) (Charve, Manganiello et al. 2018) (Shibata, Hirotsuka et al. 2017). For a few years, more and more research groups are using high-resolution mass spectrometry for the identification of taste modulating compounds (Zhang, Ayed et al. 2019) (Yang, Sun-Waterhouse et al. 2017) (Yu, Zhang et al. 2017) (Yu, Jiang et al. 2018). Due to its high selectivity, low-concentration compounds in complex matrices can be analysed better. The use of a Q-TOF HR-MS method has led to reliable results on a higher level compared with results gained by LC-MS methods used in several publications from the last decade. The application of simple LC-MS methods continues to decrease in this field of research for several reasons. Compared to the HR-MS-techniques the resolution and the mass accuracy of MS/MS methods is relatively low. Looking at this method, it must be assumed, that it can hardly be further optimised, since the boundaries defined by the laws of physics are nearly reached (Kaufmann 2012).

The high number of recent publications illustrates the great scientific interest in the identification of umami taste modulating compounds that are so far unknown. Researchers have focused on the identification of umami active substances in fermented foods like corn sauce (Charve, Manganiello et al. 2018), *Tianyou*, a traditional fermented wheat flour condiment (Gao, Zhang et al. 2018), modernized Korean soy sauce (Kim, Kim et al. 2017), protein hydrolysates of mung beans (Sonklin, Laohakunjit et al. 2018) bovine muscle, porcine plasma

(Fu, Liu et al. 2018), wheat gluten (Liu, Zhu et al. 2017) (Wang, Xu et al. 2016), peanuts (Zhang, Zhao et al. 2019), silkworm pupa (Yu, Jiang et al. 2018) and mushrooms like *Volvariella volvacea* (Xu, Xu et al. 2019), *Agaricus bisporus* (Tao Feng, Yang Wu et al. 2019), and *Pleurotus geesteranus* (Zhang, Zhang et al. 2019). Furthermore, the taste modulating substances in meat and fish products like chicken soup (Kong, Yang et al. 2017), pork meat (Ngapo and Vachon 2016), dry-cured ham (Paolella, Prandi et al. 2018) and *Takifugu obscurus* (Zhang, Ayed et al. 2019) were studied.

Often the umami active compounds are not described in detail, but instead a mass range is specified in which they were found e. g. 1 – 5 kDa (Gao, Zhang et al. 2018). The most promising umami taste modulating substances (n = 17), identified in this work, including sequence information were found in a mass range between 0.2 and 0.3 kDa. In 2017, it was shown that a fraction of Korean soy sauce elicited a distinct umami taste. Its taste was attributed to the presence of free amino acids and Glu-enriched oligopeptides of less than 0.5 kDa. However, the umami taste was not attributed to any single substance. The umami taste might have correlated with the compounds of the fraction that had the ability of bitter masking, which in turn could possibly have evoked a strong umami taste by lowering the bitter taste (Kim, Kim et al. 2017). Wheat gluten hydrolysates showed a stronger umami taste the higher the degree of hydrolysis, the concentration of free amino acids and the protein content was. Again, the umami taste could not be assigned to any single substance (Wang, Xu et al. 2016). Most of the umami eliciting peptides, clearly identified in the past five years consisted of 7 – 8 amino acids, like Ala-Ser-Asn-Met-Ser-Asp-Leu, Tyr-Tyr-Gly-Ser-Asn-Ser-Ala, Leu-Gln-Pro-Leu-Asn-Ala-His (Xu, Xu et al. 2019), Pro-Val-Ala-Arg-Met-Cys-Arg, Tyr-Gly-Gly-Thr-Pro-Pro-Phe-Val (Zhang, Ayed et al. 2019). We focused on sensory guided fractionation of amino acids and di – tripeptides. The sub-fraction A19 of sample 2, which had an intense umami taste, contained 17 small molecules, among them amino acids, di – tripeptides, and their condensation products. Diketo-Glu-Pro and Glu-Leu partially explain the intense umami taste, however the taste activity of Diketo-Glu-Ile, Diketo-Glu-Glu, Diketo-Glu-Ile, Diketo-Pro-Val, Diketo-Glu-Val, Diketo-Pro-Arg, Diketo-Cys-Lys, Pro-Phe, Val-Ile, Pro-Leu, Pro-Val, Ile-Ala, Pro-Leu-Val, and Val-Pro-Val remains unknown. Until now, there is little information about the composition of wheat gluten hydrolysates, fractionated twice and still imparting an intense umami taste. Of particular note is the detailed description of the small molecules in the mass range ≤ 0.3 kDa. This might be the basis for the development of deeper flavour analysis of wheat gluten hydrolysates.

Since a myriad of umami active compounds are known but no reports of a full reconstitution of the taste using combinations of these compounds, more research needs to be done.

4.10 Mass spectrometric analysis of 2,5-diketopiperazines

In this work, no specific fragmentation patterns of silylated 2,5-diketopiperazines were found. Although a specific fragmentation pattern after cleavage of the side chains and the rupture of the 2,5-diketopiperazine ring using EI mode is described in literature (Szafranek, Palacz et al. 1976), it could not be confirmed. It was hypothesised that fragments could be detected that, without a doubt, would allow the identification of an existing diketopiperazines ring. But specific diketopiperazines fragmentation was not observed (Nagarajan, Occolowitz et al. 1969). The only recurrent fragments were (*m/z* -15) after the cleavage of CH_3 and the fragment of the trimethylsilyl group (*m/z* 73) (Figure 3.18-1). In general, silylation of organic compounds is a powerful tool to gain structural information about the position of functional groups. Furthermore, functional groups can be identified by the use of different silylation reagents with different abilities of derivatising functional groups (Halket and Zaikin 2003). However, beside the expected ions of silylated compounds, no fragment, characteristic for the rupture of the 2,5-diketopiperazine was found. Not much topical is known about the specific fragmentation of the diketopiperazine ring in the EI mode, and a literature search showed only results published decades ago.

Researchers focus on the fragmentation of diketopiperazines using the ESI mode. Different studies in positive (Furtado, Vessecchi et al. 2007) and negative ESI mode (Guo, Cao et al. 2009) showed that there is no specific fragment of a 2,5-diketopiperazine, which enabled the operator to clearly predict the presence of a diketo compound. Several fragmentation pathways are described. In some cases, elimination of both amino acid residues leads to a specific fragment. However, this was not the case for the tested compounds. To date, diketo compounds can only be identified by automated methods or by the manual evaluation of individual spectra. It should be noted that almost any change in ionisation settings can influence the spectra. Based on that it was summarised that the generated results did not lead to a calculation approach for a reliable prediction of the presence of 2,5-diketopiperazines in complex food matrices.

Verification of chemically synthesised diketopiperazines: In this work, three different 2,5-diketopiperazines were chemically synthesised. To verify the identity of the products LC-MS was performed (section 2.5.7.). The success of the chemical synthesis was confirmed by the presence of the parent ions in positive and negative ionisation mode (Figure 3.19-2 to Figure 3.19-4). Additional verification steps were not performed but could have

been the determination of the melting point or structural identification *via* nuclear magnetic resonance spectrometry or HPLC analysis (Tullberg, Grøtli et al. 2006, Tullberg, Luthman et al. 2006).

4.11 Molecular biological findings

Since a variety of umami active compounds contain glutamic acid, a glutamyl-specific peptidase was sought after in this work. Specific glutamyl endopeptidases from various microorganism, which hydrolyse peptide bonds formed by α-carboxyl groups of Glu and Asp residues are described in literature. The majority of this enzymes was found in bacterial strains, such as *Staphylococcus epidermidis* (Ohara-Nemoto, Ikeda et al. 2002), *Bacillus amyloliquefaciens* (Balaban, Mardanova et al. 2008), *Enterococcus faecalis* (Kawalec, Potempa et al. 2005), *Streptomyces fradiae* (Kitadokoro, Nakamura et al. 1993), or *Thermoactinomyces* sp. (Liu, Zhao et al. 2016). Based on the published sequence of a glutamyl endopeptidase from *Thermoactinomyces* with the molecular mass of 26 kDa hypothetical proteins from *Fistulina hepatica* (Fhe) and *Laetiporus sulphureus* (Lsu) were found *in silico*. The gene of interest was successfully amplified and sequenced from one Fhe strain and three different Lsu strains.

In silico protein sequence analysis shows that all sequenced genes of Fhe 205, Lsul 235, Lsu 279, and Lsu 294 contain a trypsin-like serine peptidase domain like the gene of *Thermoactinomyces* sp. The theoretical molecular mass of the potential glutamyl endopeptidases from Lsu und Fhe are in the same order of magnitude as the molecular mass published for the glutamyl endopeptidase of *Thermoactinomyces*, of which-glutamyl-specific activity was shown (Liu, Zhao et al. 2016). According to this results, it was assumed that the amplified genes might have the same glutamyl-specific hydrolysation activity. However, for this purpose they have to be expressed in a suitable expression host, such as *E. coli* or *Komagatella phaffii*, purified, and characterised. Such an enzyme would be highly applicable in the food industry. Vegetable proteins could be pre-hydrolysed by specific glutamyl endopeptidases to increase the yield of glutamyl-peptides and the precursors of cyclic peptides.

4.12 Glutamate as food additive

The amino acid glutamic acid naturally occurs in all foods from living cells. Glutamic acid and its salts (glutamic acid E 620; sodium glutamate E 621; potassium glutamate E 622; calcium glutamate E 623; ammonium glutamate E 624; and magnesium glutamate E 625) are particularly high in tomatoes, soy sauce, and long-ripened cheeses. These are potent umami active substances, authorised food additives in the European Union, and listed in Annex II of Regulation (EC) No 1333/2008. Glutamates are widely used as taste enhancers in several food preparations to evoke the typical umami taste. In 1990 the Scientific Committee on Food (SCF) published that the acceptable daily intake (ADI) for glutamates was not specified. This was confirmed in 2006 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), since glutamic acid and its salts possess low acute toxicity. However, negative effects in humans were described associated with glutamate, such as headache (85.8 mg/kg body weight per day), blood pressure increase (150 mg/kg body weight per day) and insulin increase (> 143 mg/kg body weight per day). The ADI status (not specified) was re-evaluated by the European Food Safety Authority (EFSA) Panel on Food Additives and Nutrient Sources added to Food (ANS) in 2017 (Mortensen, Aguilar et al. 2017). This evaluation led to an ADI of 30 mg/kg body weight per day for each of the six different glutamate additives. The value was based on the highest dose that did not lead to an adverse effect in humans. At present, the maximum permitted level of glutamate in the EU, which may be added to food preparations, is 10 g per kg food.

Glutamate and some umami active peptides can be a solution for two research fields. It is known that these compounds not only evoke umami taste, but also can help to reduce the salt content of processed food. According to reports of the World Health Organization, the recommended daily intake of salt (2 g per day) is exceeded worldwide (World Health Organization 2012). Thus, the daily intake of sodium chloride represents a significant health risk. A variety of diseases, such as coronary heart disease, cardiovascular diseases, stroke and increased blood pressure are thereby promoted. Vegetable soup tastes more pleasant and saltier if 1 % MSG is added, compared to soup without the addition of MSG (van Stokkom, de Graaf et al. 2018). White mushrooms (*Agaricus bisporus*) do evoke umami taste due to the presence of 5´-ribonucleotides, aspartic and glutamic acids. It can be used for the substitution of up to 80 % of meat in a meat-based dish like beef taco blend. The flavour profile of the resulting dish was not dramatically altered, even though the salt content was reduced by 25 %. The obtained increased saltiness, based on the use of white mushrooms containing umami active compounds, can be a “healthy flavour” principle (Myrdal Miller, Mills et al. 2014). However, the utilization of umami peptides for salt reduction

of food preparations is not straightforward. Although peptides are known which either enhance the umami taste or the saltiness in various hydrolysed food, no synergistic effects are known which enhances the umami taste and allows the reduction of added sodium chloride to food products. This might be due to their low concentration in the used hydrolysates (Hoppu, Hopia et al. 2017). Flavour profiles of food products are evoked by a myriad of different taste active compounds. Reducing saltiness by enhancing the umami taste might lead to an increased bitterness of food preparations. The number of possible combinations of taste-active substances in highly complex foods is almost limitless. Taste-enhancing properties of a substance in a model broth do not necessarily lead to an intensification of the desired taste in a food which contains innumerable taste-active compounds. Much more work has to be done to evaluate synergistic effects that evoke the typical flavour profiles of foods accepted by consumers.

4.13 Awareness of umami in the European population

World's population is familiar with the four basic tastes salty, bitter, sour, and sweet, but what about the fifth basic taste, umami? Since umami means savoury, and delicious and describes a pleasant mouthfeel it might be differently interpreted by different populations. However, natural glutamate is a big part of the daily diet due to its presence in different foods like seaweed, tea, vegetables, beans, potatoes, mushrooms, seafood, eggs and meats, dairy products and fermented products (Umami Information Center 2017). Research has shown that the awareness of the umami taste is low in the European population. A survey published in 2010 by Singh and co-workers compared the umami taste perception in the German and Norwegian population. They found out that only 3.8 % of the German participants and 10.3 % of the Norwegian participants were familiar with the umami taste. In addition, they claimed that the participants were sceptic to MSG. It was concluded that is essential to educate people about the umami taste and MSG (Singh, Schuster et al. 2010). A survey published in 2019 maintains that public recognition of the umami taste increased only recently. Participants for this study were from Finland, Germany, and Italy. Even though the majority of the European participants were sensitive to MSG, only a few were familiar with this taste (15 % of the Finnish group and 2 % of Germans and Italians, respectively) or were able to describe their taste perception correctly. Participants used up to 106 different classes of verbal descriptors trying to describe the umami taste in their own words. Often the umami taste is described as savoury, meaty, soupy or brothy by the Europeans or even reduced to salty, sour and sweet. Both studies showed results in the same order of magnitude, but still the

umami taste is not well known yet in the European culture (Paola, Antti et al. 2019). Many consumers are unaware that HVP seasonings like the famous MAGGI® are an umami product and part of many convenience foods, which are bought because of their pleasant flavour. Foods that taste like umami are often eaten, without the consumers being able to describe the taste sensation or being aware that the taste they prefer is umami (Paola, Antti et al. 2019).

5 Conclusion

Contrary to the state of the art techniques, which all require toxic solvents (Wang, Zhao et al. 2007, Deng, Wang et al. 2016, Wang, Xu et al. 2016, Liu, Zhu et al. 2017), in this work all fractions were successfully produced "food-safe". Sensory analysis of fractions and sub-fractions from wheat gluten hydrolysates showed intense umami taste in some specific fractions and sub-fractions, respectively. For the first time the composition of umami active sub-fractions is described in detail with regard to small peptides and their condensation products. Several substance classes are known to enhance the umami taste. So it was not surprising, that substances already described in literature to evoke the umami taste were among them. Besides them, a large number of additional substances were identified. However, it has not been possible to clearly establish which individual components contribute to the umami taste and were not described yet.

The smallest sub-fraction, which showed umami taste contained only 17 substances. Two of them are already known to evoke umami taste (diketo-Glu-Pro and Glu-Leu). It is very likely, that there are more umami active compounds among the 15 remaining substances (L-Norleucine; Pro-Phe; Val-Ile; Pro-Leu; Pro-Val; Ile-Ala; Pro-Leu-Val; Val-Pro-Val; diketo(Glu-Ile); diketo(Glu-Glu); diketo(Glu-Ile); diketo(Pro-Val); diketo(Glu-Val); diketo(Pro-Arg), and diketo(Cys-Lys). Most of the known umami active peptides have Glu, Val, or Pro in their sequence. The composition of the identified peptides and their condensation products, supports this hypothesis. The results presented may contribute to identify to date unknown umami active substances.

6 Outlook

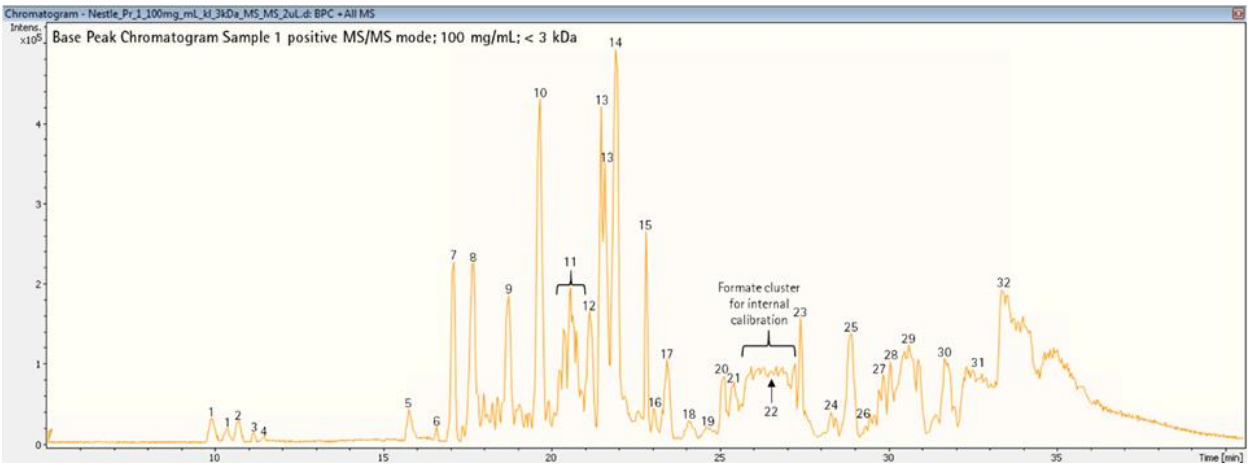
The sensory activity of the 15 substances identified in the latest SEC-fractions can now be tested. The probability of discovering a hitherto unknown umami active peptide is high due to the presence of Glu, Val, or Pro in the identified sequences. After the identification of substances the umami activity of which is undescribed yet, studies can be performed regarding the synergistic effects of MSG and umami active compounds. Synergistic effects could be analysed by mixing the substances in different concentrations before sensory analysis. On one hand, it could be examined, which substances show synergistic effects, and on the other hand, the concentrations with the highest umami enhancing potential could be determined. Small target molecules with umami taste may be accessible through a more concerted hydrolysis. The aim would be to increase the yield of the umami active substances. One possibility would be to optimise the performed hydrolysis process. Another approach would be the use of representative peptidase cocktails from the very versatile edible Basidiomycota or from other microorganisms. It would be also conceivable to screen the Basidiomycota for glutamyl-specific peptidases. These peptidases could be heterologously produced as described above in first steps. This biotechnological approach could possibly complement the traditional production process of vegetable plant source hydrolysis or even replace it, if the obtained degree of hydrolysis is sufficient.

To generate more reliable results in sensory analysis the panellists could be trained more frequently over a long period of time, to increase the significance of their taste impressions.

Moreover, the electronic tongue system could be used to increase the reproducibility as well as the detectable taste thresholds, since the umami taste is hardly describable.

The food industry is interested in substances with umami properties, as these could reduce the use of MSG in food preparations, since MSG is not appreciated by some consumers. Many consumers enjoy the taste of umami, but are not aware of it.

7 Attachment



Supplementary figure 1: Base Peak chromatogram (BPC) of the positive MS/MS mode of sample 1. The numbering of the peaks correlates with the numbering in Supplementary table 1.

Supplementary table 1: Results of the three calculation approaches for the most abundant signals in sample stock solution (100 mg mL^{-1}) of sample 1. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 1.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	I$\Delta m/z$I [mDa]	Anno-tations
1	9.93	211.14399	211.14410	/	$C_{11}H_{18}N_2O_2$	√	0.11355	SF
			211.14467	Diketo-Pro-Ile	equivalent to SF	√	0.68	LSc*
1	9.93	549.41263	549.41362	/	$C_{30}H_{48}N_{10}$	√	0.98397	SF
1	9.94	493.34975	493.35102	/	$C_{26}H_{40}N_{10}$	√	1.26293	SF
1	9.94	521.38101	521.38232	/	$C_{21}H_{49}N_{10}O_3P$	√	1.05699	SF
2	10.72	245.12831	245.12845	/	$C_{14}H_{16}N_2O_2$	√	0.14582	SF
			245.12902	Diketo-Pro-Phe	equivalent to SF	√	0.71	LSc*
2	10.72	267.11014	267.11012	/	$C_{12}H_{10}N_8$	√	0.01883	SF
			267.10934	Diketo-Glu-His	$C_{11}H_{14}N_4O_4$	√	0.8	LSc*
3	11.17	197.12812	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.33097	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	0.9	LSc*
3	11.20	381.14255	381.14181	/	$C_{17}H_{16}N_8O_3$	/	0.73619	SF
3	11.22	296.08930	296.08905	/	$C_{13}H_9N_7O_2$	√	0.25267	SF
			296.09176	Ser-Cys-Ser	$C_9H_{17}N_3O_6S$	√	2.46	LSc*
4	11.46	326.37782	326.37813	/	$C_{22}H_{47}N$	√	0.30367	SF
4	11.50	284.13875	284.13935	/	$C_{16}H_{17}N_3O_2$	√	0.60113	SF
			284.13992	Diketo-Pro-Trp	equivalent to SF	√	1.17	LSc*
4	11.50	306.12157	306.12102	/	$C_{14}H_{11}N_9$	√	0.54871	SF
			306.1125	Ser-Pro-Cys	$C_{11}H_{19}N_3O_5S$	√	9.07	LSc*
			306.13025	Glu-Gly-Thr	$C_{11}H_{19}N_3O_7$	√	8.68	LSc*
			306.13026	Ala-Asp-Thr	$C_{11}H_{19}N_3O_7$	√	8.69	LSc*

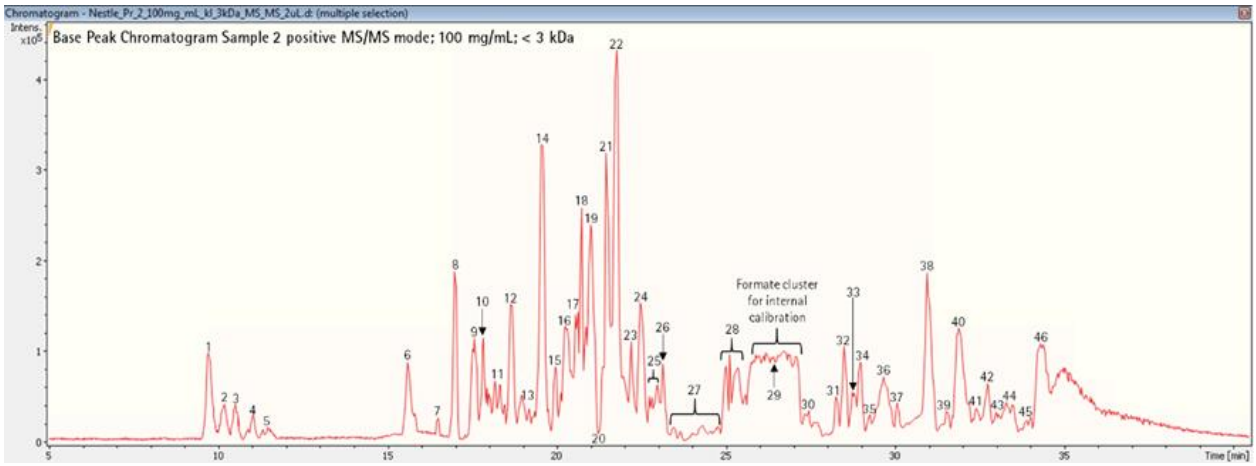
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
5	15.80	261.12300	261.12337	/	$C_{14}H_{16}N_2O_3$	√	0.36362	SF
			261.12393	Diketo-Tyr-Pro	equivalent to SF	√	0.93	LSc*
5	15.80	283.10502	283.10503	/	$C_{12}H_{10}N_8O$	√	0.01499	SF
5	15.80	521.23873	521.23946	/	$C_{28}H_{32}N_4O_6$	√	0.73487	SF
5	15.96	169.09667	169.09715	/	$C_8H_{12}N_2O_2$	√	0.48502	SF
			169.09772	Diketo-Ala-Pro	equivalent to SF	√	1.05	LSc*
6	16.60	378.19960	378.19832	/	$C_{14}H_{27}N_5O_7$	√	1.27384	SF
			378.20303	Val-Tyr-Pro	$C_{19}H_{27}N_3O_5$	√	3.43	LSc*
6	16.61	132.10162	132.10191	L-Norleucine	$C_6H_{13}NO_2$	√	0.28863	SF/LSc *
6	16.61	356.21744	356.21800	/	$C_{17}H_{29}N_3O_5$	√	0.55746	SF
7	17.06	277.11788	277.11828	/	$C_{14}H_{16}N_2O_4$	√	0.40761	SF
			277.11885	Diketo-Glu-Phe	equivalent to SF	√	0.97	LSc*
7	17.10	197.12791	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.54509	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	1.11	LSc*
7	17.10	203.13381	243.13393	/	$C_{11}H_{18}N_2O_4$	√	0.12467	SF
			243.1345	Diketo-Glu-Ile	equivalent to SF	√	0.69	LSc*
7	17.10	485.26033	485.26224	/	$C_{20}H_{41}N_2O_9P$	√	1.91232	SF
8	17.68	130.04970	130.04987	(R)-(+)-2-Pyrrolidone-5-carboxyylic acid	$C_5H_7NO_3$	√	0.17281	SF/SL
8	17.68	388.13448	388.13506	/	$C_{15}H_{21}N_3O_9$	√	0.57457	SF
			388.12921	His-Glu-Cys	$C_{14}H_{21}N_5O_6S$	√	5.27	LSc*
8	17.69	259.09199	259.09246	/	$C_{10}H_{14}N_2O_6$	√	0.46916	SF
			259.09303	Diketo-Glu-Glu	equivalent to SF	√	1.04	LSc*
8	17.83	340.18609	340.18670	/	$C_{16}H_{25}N_3O_5$	√	0.60816	SF
8	17.93	371.22660	371.22756	/	$C_{16}H_{34}O_9$	√	0.96341	SF
8	17.98	231.16970	231.17032	/	$C_{11}H_{22}N_2O_3$	/	0.61888	SF
			231.16982	Val-Ile	equivalent to SF	/	0.12	LSc*
8	18.00	374.17035	374.17002	/	$C_{13}H_{24}N_7O_4P$	√	0.33756	SF
8	18.03	209.09144	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.62334	SF
9	18.26	476.30563	476.30684	/	$C_{15}H_{42}N_9O_6P$	√	1.21797	SF
9	18.26	459.27897	459.27730	/	$C_{16}H_{38}N_6O_9$	√	1.67149	SF
			459.27933	Arg-Arg-Gln	$C_{17}H_{34}N_{10}O_5$	√	0.36	LSc*
9	18.27	481.26098	481.26196	/	$C_{13}H_{33}N_{14}O_4P$	/	0.97718	SF
9	18.40	503.30502	503.30620	/	$C_{22}H_{46}O_{12}$	/	1.18221	SF
9	18.40	520.33192	520.33306	/	$C_{17}H_{46}N_9O_7P$	√	1.13680	SF
9	18.40	293.11225	293.11320	/	$C_{14}H_{16}N_2O_5$	√	0.94308	SF
			293.11376	Diketo-Glu-Tyr	equivalent to SF	√	1.51	LSc*
9	18.42	326.17034	326.17105	/	$C_{15}H_{23}N_3O_5$	√	0.70590	SF
9	18.48	213.08638	213.08832	/	$C_{10}H_8N_6$	/	1.94501	SF
			213.08755	Diketo-Asp-Pro	$C_9H_{12}N_2O_4$	/	1.17	LSc*
9	18.50	564.35765	564.35628	/	$C_{20}H_{49}N_7O_{11}$	√	1.36948	SF
9	18.66	493.20442	493.20414	/	$C_{21}H_{28}N_6O_8$	√	0.28581	SF
9	18.72	471.22291	471.22278	/	$C_{18}H_{31}N_8O_5P$	√	0.12972	SF
9	18.73	263.13860	263.13902	Pro-Phe	$C_{14}H_{18}N_2O_3$	√	0.41705	SF/SL
10	19.00	437.23851	437.23678	/	$C_{17}H_{28}N_{10}O_4$	√	1.72962	SF
10	19.03	459.22026	459.21979	/	$C_{18}H_{30}N_6O_8$	√	0.46715	SF
10	19.25	148.06033	148.06043	/	$C_5H_9NO_4$	/	0.10054	SF
			148.05651	Glutamic acid	equivalent to SF	/	3.82	LSc*
10	19.26	390.16506	390.16328	/	$C_{15}H_{19}N_9O_4$	√	1.78722	SF
			390.16262	Gln-Gln-Asp	$C_{14}H_{23}N_5O_8$	√	2.44	LSc*
10	19.66	227.10246	227.10429	/	$C_8H_{19}O_5P$	√	1.82336	SF
			227.1032	Diketo-Glu-Pro	$C_{10}H_{14}N_2O_4$	√	0.74	LSc
10	19.67	453.19775	453.19696	/	$C_{14}H_{29}N_8O_7P$	√	0.78776	SF
10	19.76	279.16939	279.17032	Phe-Ile	$C_{15}H_{22}N_2O_3$	√	0.92628	SF/SL
10	19.89	410.20660	410.20743	Phe-Pro-Phe	$C_{23}H_{27}N_3O_4$	√	0.83021	SF/SL

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
10	19.94	245.18532	245.18597	NH-DVal(NMe)-Val-OMe	$C_{12}H_{24}N_2O_3$	√	0.64751	SF/SL
11	20.09	279.13323	279.13393	/	$C_{14}H_{18}N_2O_4$	√	0.70298	SF
			279.13461	Tyr-Pro	equivalent to SF	√	1.38	LSc*
11	20.09	405.17559	405.17551	/	$C_{16}H_{16}N_{14}$	√	0.07990	SF
11	20.11	376.22229	376.22308	Leu-Pro-Phe	$C_{20}H_{29}N_3O_4$	√	0.79728	SF/SL
11	20.23	415.23284	415.23398	Leu-Pro-Trp	$C_{22}H_{30}N_4O_4$	√	1.14321	SF/SL
11	20.28	342.23801	342.23873	Leu-Pro-Ile	$C_{17}H_{31}N_3O_4$	√	0.72238	SF/SL
11	20.40	248.10001	248.09894	/	$C_7H_{13}N_5O_5$	√	1.06281	SF
11	20.41	209.09159	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.48291	SF
11	20.41	226.11811	226.11862	/	$C_{10}H_{15}N_3O_3$	√	0.50493	SF
			226.11918	Diketo-Gln-Pro	equivalent to SF	√	1.07	LSc*
11	20.58	187.07096	187.07133	/	$C_7H_{10}N_2O_4$	√	0.36884	SF
			187.07189	Diketo-Glu-Gly	equivalent to SF	√	0.93	LSc*
11	20.58	373.13438	373.13539	/	$C_{14}H_{20}N_4O_8$	√	1.01147	SF
			373.12954	Asn-His-Cys	$C_{13}H_{20}N_6O_5S$	√	4.84	LSc*
11	20.66	263.13853	263.13902	Pro-Phe	$C_{14}H_{18}N_2O_3$	√	0.48954	SF/SL
11	20.71	259.09158	259.09246	/	$C_{10}H_{14}N_2O_6$	√	0.88387	SF
			259.09303	Diketo-Glu-Glu	equivalent to SF	√	1.45	LSc*
			259.08661	His-Cys	$C_9H_{14}N_4O_3S$	√	4.97	LSc*
11	20.77	229.15401	229.15467	Pro-Ile	$C_{11}H_{20}N_2O_3$	√	0.65795	SF/SL
11	20.79	195.07552	195.07642	/	$C_9H_{10}N_2O_3$	√	0.90165	SF
11	20.81	324.15427	324.15271	/	$C_{11}H_{17}N_9O_3$	√	1.56234	SF
			324.15607	Phe-Thr-Gly	$C_{15}H_{21}N_3O_5$	√	1.8	LSc*
			324.15608	Ala-Tyr-Ala	$C_{15}H_{21}N_3O_5$	√	1.81	LSc*
11	20.84	346.13612	346.13706	/	$C_{13}H_{15}N_9O_3$	√	0.93824	SF
11	20.84	295.16403	295.16523	/	$C_{15}H_{22}N_2O_4$	/	1.20535	SF
			295.16591	Tyr-Leu	equivalent to SF	/	1.88	LSc*
11	20.88	362.20632	362.20743	Val-Pro-Phe	$C_{19}H_{27}N_3O_4$	√	1.11399	SF/SL
12	21.00	360.19269	360.19178	Pro-Pro-Phe	$C_{19}H_{25}N_3O_4$	√	0.90868	SF/SL
12	21.16	328.22235	328.22308	Ile-Pro-Val	$C_{16}H_{29}N_3O_4$	√	0.73495	SF/SL
12	21.20	392.21675	392.21800	Leu-Pro-Tyr	$C_{20}H_{29}N_3O_5$	√	1.24436	SF/SL
13	21.45	217.15381	217.15467	Val-Val	$C_{10}H_{20}N_2O_3$	√	0.86305	SF/SL
13	21.49	120.08046	120.08078	/	C_8H_9N	√	0.31346	SF
13	21.49	263.13859	263.13902	Phe-Pro	$C_{14}H_{18}N_2O_3$	√	0.42646	SF/SL
13	21.57	215.13849	215.13902	Pro-Val	$C_{10}H_{18}N_2O_3$	√	0.52391	SF/SL
13	21.61	188.07019	188.07060	/	$C_{11}H_9NO_2$	√	0.41779	SF
13	21.61	205.09668	205.09715	D-Tryptophan	$C_{11}H_{12}N_2O_2$	√	0.47256	SF/SL
13	21.64	314.20675	314.20743	Val-Pro-Val	$C_{15}H_{27}N_3O_4$	√	0.68566	SF/SL
13	21.68	203.13851	203.13902	/	$C_9H_{18}N_2O_3$	√	0.50937	SF
			203.1397	Ile-Ala	equivalent to SF	√	1.19	LSc*
13	21.76	132.10167	132.10191	L-Norleucine	$C_6H_{13}NO_2$	√	0.23623	SF/SL
14	21.90	331.16464	331.16420	/	$C_{12}H_{23}N_6O_3P$	√	0.44130	SF
			331.16189	Ser-Pro-Gln	$C_{13}H_{22}N_4O_6$	√	2.75	LSc*
14	21.92	166.08597	166.08626	L-Phenylalanine	$C_9H_{11}NO_2$	√	0.28909	SF/SL
14	22.01	300.19096	300.19178	Leu-Pro-Ala	$C_{14}H_{25}N_3O_4$	√	0.82435	SF/SL
14	22.01	187.10693	187.10772	/	$C_8H_{14}N_2O_3$	√	0.79324	SF
			187.10828	Diketo-Val-Ser	equivalent to SF	√	1.35	LSc*
			187.1084	Pro-Ala	equivalent to SF	√	1.47	LSc*
14	22.02	223.10698	223.10772	/	$C_{11}H_{14}N_2O_3$	/	0.74188	SF
			223.10726	Phe-Gly	equivalent to SF	/	0.28	LSc*
14	22.22	132.10152	132.10191	L-Norleucine	$C_6H_{13}NO_2$	√	0.37988	SF/SL
14	22.39	326.20680	326.20743	/	$C_{16}H_{27}N_3O_4$	√	0.51450	SF
			326.20812	Pro-Pro-Ile	equivalent to SF	√	1.32	LSc*

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
15	22.60	189.12276	189.12337	Ile-Gly	$C_8H_{16}N_2O_3$	√	0.60471	SF/SL
15	22.63	261.14355	261.14450	Leu-Glu	$C_{11}H_{20}N_2O_5$	√	0.94859	SF/SL
15	22.81	279.13312	279.13125	/	$C_{10}H_{14}N_8O_2$	√	1.87174	SF
			279.13461	Tyr-Pro	equivalent to SF	√	1.49	LSc*
15	22.83	316.18590	316.18670	/	$C_{14}H_{25}N_3O_5$	/	0.89462	SF
			316.18568	Pro-Ser-Leu	equivalent to SF	/	0.22	LSc*
15	22.83	215.13869	215.13902	Pro-Val	$C_{10}H_{18}N_2O_3$	√	0.32775	SF/SL
15	22.88	312.19086	312.19178	Pro-Pro-Val	$C_{15}H_{25}N_3O_4$	√	0.92283	SF/SL
16	22.93	213.12280	213.12337	/	$C_{10}H_{16}N_2O_3$	√	0.57213	SF
			213.12405	Pro-Pro	equivalent to SF	√	1.25	LSc*
16	23.01	286.17534	286.17613	/	$C_{13}H_{23}N_3O_4$	√	0.79215	SF
			286.17681	Pro-Ile-Gly	equivalent to SF	√	1.1	LSc*
			286.17682	Val-Ala-Pro	equivalent to SF	√	1.11	LSc*
16	23.07	173.12777	173.12845	/	$C_8H_{16}N_2O_2$	√	0.68442	SF
16	23.07	219.13330	219.13393	Leu-Ser	$C_9H_{18}N_2O_4$	√	0.63403	SF/SL
16	23.11	229.11744	229.11828	/	$C_{10}H_{16}N_2O_4$	√	0.84068	SF
			229.11885	Diketo-Glu-Val	equivalent to SF	√	1.41	LSc*
16	23.13	330.20146	330.20235	Leu-Pro-Thr	$C_{15}H_{27}N_3O_5$	√	0.88748	SF/SL
17	23.31	182.08047	182.08117	L-Tyrosine	$C_9H_{11}NO_3$	√	0.69753	SF/SL
17	23.36	150.05771	150.05833	L-Methionine	$C_5H_{11}NO_2S$	√	0.61379	SF/SL
17	23.44	280.08954	280.08877	/	$C_7H_{13}N_5O_7$	√	0.77096	SF
17	23.45	258.10787	258.10845	/	$C_{10}H_{15}N_3O_5$	√	0.58056	SF
			258.10901	Diketo-Glu-Gln	equivalent to SF	√	1.14	LSc*
17	23.48	241.08136	241.08190	/	$C_{10}H_{12}N_2O_5$	√	0.53943	SF
			241.07593	Diketo-His-Cys	$C_9H_{12}N_4O_2S$	√	5.43	LSc*
17	23.73	217.11743	217.11828	Pro-Thr	$C_9H_{16}N_2O_4$	√	0.85671	SF/SL
17	23.78	391.19706	391.19760	Phe-Pro-Gln	$C_{19}H_{26}N_4O_5$	√	0.53292	SF/SL
18	24.11	355.16027	355.15853	/	$C_{11}H_{18}N_{10}O_4$	√	1.74036	SF
18	24.12	147.07570	147.07642	D-Glutamine	$C_5H_{10}N_2O_3$	√	0.71967	SF/SL
18	24.24	254.16035	254.16115	/	$C_{11}H_{19}N_5O_2$	√	0.79948	SF
			254.16172	Diketo-Arg-Pro	equivalent to SF	√	1.37	LSc*
19	24.65	182.08037	182.08117	L-Tyrosine	$C_9H_{11}NO_3$	√	0.79882	SF/SL
19	24.67	165.05391	165.05462	/	$C_9H_8O_3$	√	0.70844	SF
19	24.70	136.07524	136.07569	/	C_8H_9NO	√	0.44953	SF
20	25.25	226.04424	226.04585	/	$C_8H_7N_3O_5$	√	1.60469	SF
20	25.36	314.08397	314.08569	/	$C_{10}H_7N_{11}O_2$	/	1.72153	SF
21	25.34	357.21224	357.21325	Ile-Pro-Gln	$C_{16}H_{28}N_4O_5$	√	1.00888	SF/SL
21	25.41	257.10023	257.10062	/	$C_9H_8N_{10}$	/	0.38988	SF
21	25.42	235.11813	235.11761	/	$C_{10}H_{18}O_6$	√	0.51908	SF
			235.11951	Diketo-His-Pro	$C_{11}H_{14}N_4O_2$	√	1.38	LSc*
21	25.63	233.11230	233.11320	/	$C_9H_{16}N_2O_5$	√	0.89437	SF
			233.11388	Val-Asp	equivalent to SF	√	1.58	LSc*
21	25.64	162.04949	162.05093	/	$C_4H_7N_3O_4$	√	1.44575	SF
21	25.64	298.02399	298.02400	/	$C_8H_{13}NO_7P$	√	0.00905	SF
22	26.51	205.11737	205.11828	Val-Ser	$C_8H_{16}N_2O_4$	√	0.91731	SF/SL
23	27.41	147.07573	147.07642	D-Glutamine	$C_5H_{10}N_2O_3$	√	0.68727	SF/SL
23	27.41	244.12857	244.12918	Pro-Ala-Gly	$C_{10}H_{17}N_3O_4$	√	0.61092	SF/SL
23	27.41	266.10993	266.11085	/	$C_8H_{11}N_9O_2$	√	0.91246	SF
23	27.52	203.10176	203.10263		$C_8H_{14}N_2O_4$	√	0.86937	SF
			203.10331	Pro-Ser	equivalent to SF	√	1.55	LSc*
24	28.32	364.15980	364.16021	/	$C_{15}H_{25}NO_9$	√	0.40762	SF
			364.15437	Ile-Glu-Cys	$C_{14}H_{25}N_3O_6S$	√	5.43	LSc*
			364.15437	Asp-Met-Val	$C_{14}H_{25}N_3O_6S$	√	5.43	LSc*
			364.1656	Asn-Lys-Cys	$C_{13}H_{25}N_5O_5S$	√	5.8	LSc*
24	28.46	249.09712	249.09822	/	$C_{11}H_{12}N_4O_3$	√	1.09999	SF
24	28.46	267.10742	267.10744	/	$C_{10}H_{18}O_8$	√	0.01960	SF
			267.10934	Diketo-Glu-His	$C_{11}H_{14}N_4O_4$	√	1.92	LSc*

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
25	28.69	204.09654	204.09788	Gln-Gly	$C_7H_{13}N_3O_4$	√	1.33775	SF/SL
25	28.83	276.11808	276.11901	/	$C_{10}H_{17}N_3O_6$	/	0.93541	SF
			276.11901	Glu-Gln	equivalent to SF	/	0.93	LSc*
25	28.89	293.14456	293.14556	/	$C_{10}H_{20}N_4O_6$	√	0.99811	SF
25	28.90	147.07588	147.07642	D-Glutamine	$C_5H_{10}N_2O_3$	√	0.53369	SF/SL
25	29.12	375.14922	375.14835	/	$C_{10}H_{18}N_{10}O_6$	√	0.86745	SF
25	29.20	166.05227	166.05324	/	$C_5H_{11}NO_3S$	√	0.96657	SF
			166.05324	DL-Methionine sulfoxide	equivalent to SF	√	0.97	LSc*
25	29.25	207.09659	207.09755	/	$C_7H_{14}N_2O_5$	√	0.95402	SF
			207.09822	Ser-Thr	equivalent to SF	√	1.63	LSc*
26	29.33	331.15932	331.16121	Ser-Pro-Gln	$C_{13}H_{22}N_4O_6$	√	1.89428	SF/SL
26	29.34	265.11278	265.11426	/	$C_8H_{16}N_4O_6$	√	1.48142	SF
			265.11884	Diketo-Tyr-Thr	$C_{13}H_{16}N_2O_4$	√	6.06	LSc*
26	29.35	133.06009	133.06077	/	$C_4H_8N_2O_3$	√	0.68194	SF
			133.05685	Asparagine	equivalent to SF	√	3.24	LSc*
26	29.49	170.04398	170.04478	/	$C_7H_7NO_4$	√	0.80638	SF
27	29.76	275.13394	275.13500	Gln-Gln	$C_{10}H_{18}N_4O_5$	√	1.05737	SF/SL
28	30.02	193.08101	193.08190	Ser-Ser	$C_6H_{12}N_2O_5$	√	0.88353	SF/SL
28	30.07	234.10670	234.10845	Ser-Gln	$C_8H_{15}N_3O_5$	√	1.74799	SF/SL
28	30.08	147.07579	147.07642	D-Glutamine	$C_5H_{10}N_2O_3$	√	0.63052	SF/SL
28	30.08	217.08098	217.08190	/	$C_8H_{12}N_2O_5$	√	0.91385	SF
			217.08246	Diketo-Glu-Ser	equivalent to SF	√	1.48	LSc*
28	30.27	424.17964	424.18133	/	$C_{15}H_{17}N_{15}O$	/	1.68765	SF
			424.17682	His-His-Met	$C_{17}H_{25}N_7O_4S$	/	2.82	LSc*
29	30.57	291.11780	291.11599	/	$C_7H_{14}N_8O_5$	√	1.81326	SF
29	30.62	273.10718	273.10543	/	$C_7H_{12}N_8O_4$	√	1.74972	SF
29	30.63	309.12825	309.12656	/	$C_7H_{16}N_8O_6$	√	1.69096	SF
29	30.75	250.09105	250.09213	/	$C_9H_{15}NO_7$	√	1.07322	SF
			250.08625	Ala-Cys-Gly	$C_8H_{15}N_3O_4S$	√	4.77	LSc*
29	30.93	162.07543	162.07608	/	$C_6H_{11}NO_4$	√	0.65838	SF
29	30.93	359.16440	359.16602	/	$C_{12}H_{26}N_2O_{10}$	√	1.62184	SF
			359.15681	Asn-Glu-Pro	$C_{14}H_{22}N_4O_7$	√	7.59	LSc*
			359.15681	Asp-Gln-Pro	$C_{14}H_{22}N_4O_7$	√	7.59	LSc*
29	30.94	180.08586	180.08665	/	$C_6H_{13}NO_5$	√	0.78958	SF
29	30.94	319.14843	319.14863	/	$C_{10}H_{14}N_{12}O$	√	0.20331	SF
29	31.08	295.11251	295.11359	/	$C_{10}H_{18}N_2O_8$	/	1.08546	SF
29	31.11	274.18639	274.18737	/	$C_{11}H_{23}N_5O_3$	/	0.96640	SF
			274.18636	Val-Arg	equivalent to SF	/	0.03	LSc*
30	31.58	253.12846	253.12952	/	$C_{11}H_{16}N_4O_3$	√	1.06070	SF
			253.13019	Pro-His	equivalent to SF	√	1.73	LSc*
30	31.61	272.17116	272.17172	Pro-Arg	$C_{11}H_{21}N_5O_3$	√	0.70332	SF/SL
30	31.67	246.15511	246.15607	Arg-Ala	$C_9H_{19}N_5O_3$	√	0.96017	SF/SL
30	31.74	349.22943	349.23063	/	$C_{12}H_{28}N_8O_4$	√	1.20114	SF
30	31.76	175.11835	175.11895	/	$C_6H_{14}N_4O_2$	√	0.60569	SF
			175.11503	Arginine	equivalent to SF	√	3.32	LSc*
30	31.88	330.18692	330.18843	/	$C_{12}H_{23}N_7O_4$	√	1.51261	SF
30	31.96	311.14487	311.14489	/	$C_{11}H_{22}N_2O_8$	√	0.01935	SF
			311.13958	Diketo-Tyr-Phe	$C_{18}H_{18}N_2O_3$	√	5.29	LSc*
30	31.98	156.07603	156.07675	L-Histidine	$C_6H_9N_3O_2$	√	0.72089	SF/SL
30	31.99	304.16041	304.16155	/	$C_{11}H_{21}N_5O_5$	√	1.13531	SF
			304.16074	Arg-Glu	equivalent to SF	√	0.33	LSc*
30	32.09	147.11231	147.11280	L-Lysine	$C_6H_{14}N_2O_2$	√	0.58422	SF/SL
31	32.61	232.13943	232.14042	/	$C_8H_{17}N_5O_3$	√	1.01295	SF
			232.14109	Arg-Gly	equivalent to SF	√	1.66	LSc*

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
31	32.67	213.09728	213.09822	His-Gly	$C_8H_{12}N_4O_3$	√	0.94704	SF/SL
31	33.04	318.12825	318.12689	/	$C_8H_{15}N_9O_5$	√	1.35467	SF
			318.12764	Diketo-Trp-Met	$C_{16}H_{19}N_3O_2S$	√	0.61	LSc*
31	33.05	262.15010	262.15098	Arg-Ser	$C_9H_{19}N_5O_4$	√	0.89233	SF/SL
31	33.24	289.16090	289.16188	Asn-Arg	$C_{10}H_{20}N_6O_4$	√	0.97743	SF/SL
32	33.46	159.96853	159.96991	/	C_4HNO_4S	√	1.37577	SF
32	33.73	309.16461	309.16563	/	$C_{12}H_{24}N_2O_7$	/	1.01473	SF
32	34.74	182.98422	182.98417	/	$C_7H_3O_3P$	√	0.05170	SF



Supplementary figure 2: Base Peak chromatogram (BPC) of the positive MS/MS mode of sample 2. The numbering of the peaks correlates with the numbering in Supplementary table 2.

Supplementary table 2: Results of the three calculation approaches for the most abundant signals in sample stock solution (100 mg mL^{-1}) of sample 2. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 2.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
1	9.72	521.38004	521.37830	/	$C_{23}H_{44}N_{12}O_2$	√	1.74269	SF
1	9.72	549.41135	549.41010	/	$C_{30}H_{61}O_4PS$	√	1.25781	SF
1	9.74	211.14353	211.14410	/	$C_{11}H_{18}N_2O_2$	√	0.57001	SF
			211.14467	Diketo-Pro-Ile	equivalent to SF	√	0.68	LSc*
2	10.20	231.11210	231.11280	/	$C_{13}H_{14}N_2O_2$	√	0.70439	SF
			231.11674	Diketo-Met-Val	$C_{10}H_{18}N_2O_2S$	√	4.64	LSc*
3	10.52	245.12763	245.12845	/	$C_{14}H_{16}N_2O_2$	√	0.82448	SF
			245.12902	Diketo-Pro-Phe	equivalent to SF	√	1.39	LSc*
4	11.04	197.12781	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.64446	SF
			197.12902	Diketo-Val-Pro	equivalent to SF	√	1.21	LSc*
4	11.07	174.11206	174.11247	Acetyl-DL-Leucine	$C_8H_{15}NO_3$	√	0.41072	SF/SL
5	11.33	284.13854	284.13935	/	$C_{16}H_{17}N_3O_2$	√	0.81588	SF
			284.13992	Diketo-Pro-Trp	equivalent to SF	√	1.38	LSc*
5	11.33	326.37747	326.37813	/	$C_{22}H_{47}N$	√	0.65484	SF
5	11.34	305.14850	305.14690	/	$C_{12}H_{16}N_8O_2$	√	1.60517	SF
			305.14623	Thr-Gln-Gly	$C_{11}H_{20}N_4O_6$	√	2.27	LSc*

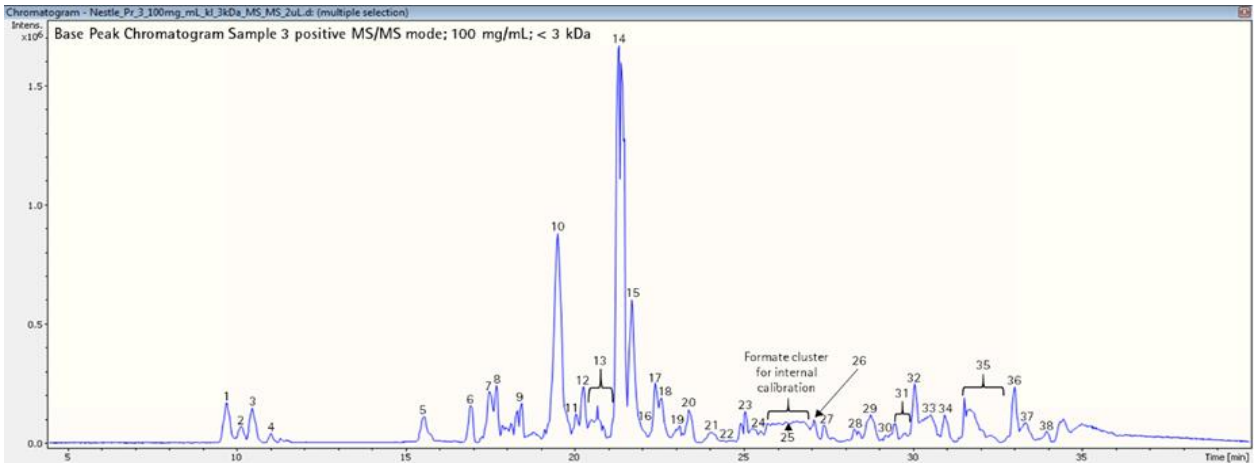
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
			305.14624	Ser-Gln-Ala	$C_{11}H_{20}N_4O_6$	√	2.27	LSc*
5	11.57	291.13322	291.13393	/	$C_{15}H_{18}N_2O_4$	√	0.71333	SF
			291.13058	Ser-Gln-Gly	$C_{10}H_{18}N_4O_6$	√	2.64	LSc*
			291.13059	Ala-Asn-Ser	$C_{15}H_{18}N_2O_4$	√	2.64	LSc*
6	15.57	124.03895	124.03930	/	$C_6H_5NO_2$	√	0.35632	SF
6	15.61	283.10427	283.10503	/	$C_{12}H_{10}N_8O$	/	0.76407	SF
6	15.63	521.23842	521.23696	/	$C_{32}H_{32}N_4OS$	√	1.46107	SF
6	15.63	261.12310	261.12502	/	$C_{12}H_{21}O_4P$	√	1.91731	SF
			261.12393	Diketo-Tyr-Pro	$C_{14}H_{16}N_2O_3$	√	0.83	LSc*
6	15.83	169.09642	169.09715	/	$C_8H_{12}N_2O_2$	√	0.73701	SF
			169.09772	Diketo-Ala-Pro	equivalent to SF	√	1.05	LSc*
7	16.51	132.10161	132.10191	Norleucine	$C_6H_{13}NO_2$	/	0.29687	SF/LSc *
7	16.51	356.21738	356.21800	/	$C_{17}H_{29}N_3O_5$	√	0.61862	SF
7	16.51	378.19937	378.19966	/	$C_{15}H_{23}N_9O_3$	√	0.29163	SF
			378.20303	Val-Tyr-Pro	$C_{19}H_{27}N_3O_5$	√	3.66	LSc*
8	16.98	277.11753	277.11828	/	$C_{14}H_{16}N_2O_4$	√	0.74831	SF
			277.11885	Diketo-Glu-Phe	equivalent to SF	√	1.32	LSc*
8	17.01	197.12794	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.51290	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	1.08	LSc*
8	17.01	243.13367	243.13393	/	$C_{11}H_{18}N_2O_4$	√	0.26041	SF
			243.1345	Diketo-Glu-Ile	equivalent to SF	√	0.83	LSc*
8	17.01	485.26014	485.25925	/	$C_{21}H_{40}O_{12}$	√	0.89153	SF
9	17.58	130.04966	130.04987	(R)-(+)-2-Pyrrolidone-5-carboxyylic acid	$C_5H_7NO_3$	√	0.21260	SF/SL
9	17.58	259.09199	259.09246	/	$C_{10}H_{14}N_2O_6$	√	0.47174	SF
			259.09303	Diketo-Glu-Glu	equivalent to SF	√	1.04	LSc*
9	17.58	388.13458	388.13371	/	$C_{12}H_{13}N_{13}O_3$	√	0.86935	SF
			388.12921	His-Glu-Cys	$C_{14}H_{21}N_5O_6S$	√	5.37	LSc*
10	17.84	227.10236	227.10263	/	$C_{10}H_{14}N_2O_4$	√	0.27276	SF
			227.1032	Diketo-Glu-Pro	equivalent to SF	√	0.84	LSc*
10	17.85	371.22690	371.22786	/	$C_{11}H_{31}N_8O_4P$	√	0.96271	SF
10	17.86	209.09155	209.09207	/	$C_{10}H_{21}N_2O_3$	√	0.52189	SF
10	17.95	374.17013	374.16836	/	$C_{15}H_{19}N_9O_3$	√	1.76739	SF
10	18.03	340.18596	340.18670	/	$C_{16}H_{25}N_3O_5$	√	0.73459	SF
11	18.19	476.30593	476.30684	/	$C_{15}H_{42}N_9O_6P$	√	0.90882	SF
11	18.20	459.27906	459.27730	/	$C_{16}H_{38}N_6O_9$	√	1.75718	SF
			459.27933	Arg-Arg-Gln	$C_{17}H_{34}N_{10}O_5$	√	0.27	LSc*
11	18.20	481.26114	481.26165	/	$C_{18}H_{36}N_6O_9$	/	0.51025	SF
11	18.34	503.30540	503.30620	/	$C_{22}H_{46}O_{12}$	√	0.80003	SF
11	18.34	520.33194	520.33306	/	$C_{17}H_{46}N_9O_7P$	√	1.12119	SF
11	18.35	293.11319	293.11051	/	$C_{10}H_{12}N_8O_3$	√	1.91348	SF
			293.11376	Diketo-Glu-Tyr	$C_{14}H_{16}N_2O_5$	√	0.57	LSc*
11	18.41	213.08633	213.08698	/	$C_9H_{12}N_2O_4$	√	0.65678	SF
			213.08755	Diketo-Asp-Pro	equivalent to SF	√	1.22	LSc*
11	18.47	564.35813	564.35927	/	$C_{19}H_{50}N_9O_8P$	√	1.14699	SF
12	18.66	493.20481	493.20548	/	$C_{22}H_{24}N_{10}O_4$	/	0.66946	SF
12	18.68	263.13892	263.14067	/	$C_{12}H_{23}O_4P$	√	1.74940	SF
			263.13958	Diketo-Tyr-Val	$C_{14}H_{18}N_2O_3$	√	0.66	LSc*
			263.1397	Phe-Pro	$C_{14}H_{18}N_2O_3$	√	0.78	LSc*
12	18.68	471.22343	471.22278	/	$C_{18}H_{31}N_8O_5P$	√	0.65168	SF
12	18.76	326.17041	326.17105	/	$C_{15}H_{23}N_3O_5$	√	0.63350	SF
13	18.97	459.22032	459.22113	/	$C_{19}H_{26}N_{10}O_4$	√	0.80193	SF
13	18.98	437.23898	437.23812	/	$C_{20}H_{36}O_{10}$	√	0.85414	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
13	18.99	229.15427	229.15467	Pro-Leu	$C_{11}H_{20}N_2O_3$	√	0.39691	SF/SL
13	19.19	148.05993	148.06177	/	$C_6H_5N_5$	/	1.84369	SF
			148.05651	Glutamic acid	$C_5H_9NO_4$	/	3.42	LSc*
13	19.19	182.08085	182.08117	L-Tyrosine	$C_9H_{11}NO_3$	√	0.31673	SF/SL
13	19.20	390.16524	390.16596	/	$C_{19}H_{23}N_3O_6$	√	0.72174	SF
			390.16262	Gln-Gln-Asp	$C_{14}H_{23}N_5O_8$	√	2.62	LSc*
14	19.59	227.10255	227.10429	/	$C_8H_{19}O_5P$	√	1.73821	SF
			227.1032	Diketo-Glu-Pro	$C_{10}H_{14}N_2O_4$	√	0.65	LSc*
14	19.59	453.19757	453.19696	/	$C_{14}H_{29}N_8O_7P$	√	0.61096	SF
14	19.77	279.16946	279.17032	Leu-Phe	$C_{15}H_{22}N_2O_3$	√	0.86190	SF/SL
14	19.85	287.12305	287.12376	/	$C_{12}H_{18}N_2O_6$	√	0.71718	SF
15	19.89	410.20576	410.20743	/	$C_{23}H_{27}N_3O_4$	/	1.67689	SF
			410.20660	Phe-Pro-Phe	equivalent to SF	/	0.84	LSc*
15	19.99	245.18567	245.18597	NH-DVal(NMe)-Val-OMe	$C_{12}H_{24}N_2O_3$	√	0.29610	SF/SL
15	20.03	279.13351	279.13527	/	$C_{15}H_{14}N_6$	√	1.76168	SF
			279.13461	Tyr-Pro	$C_{14}H_{18}N_2O_4$	√	1.1	LSc*
15	20.12	376.22263	376.22308	Ile-Pro-Phe	$C_{20}H_{29}N_3O_4$	√	0.44889	SF/SL
16	20.21	415.23360	415.23398	Leu-Pro-Trp	$C_{22}H_{30}N_4O_4$	√	0.38271	SF/SL
16	20.29	342.23849	342.23873	Leu-Pro-Ile	$C_{17}H_{31}N_3O_4$	√	0.24010	SF/SL
16	20.33	209.09178	209.09372	/	$C_8H_{17}O_4P$	√	1.93976	SF
16	20.33	248.09993	248.10028	/	$C_8H_9N_9O$	/	0.35164	SF
16	20.33	226.11822	226.11862	/	$C_{10}H_{15}N_3O_3$	√	0.39756	SF
			226.11918	Diketo-Gln-Pro	equivalent to SF	√	0.96	LSc*
17	20.49	187.07099	187.07133	/	$C_7H_{10}N_2O_4$	√	0.34676	SF
			187.07189	Diketo-Glu-Gly	equivalent to SF	√	0.9	LSc*
17	20.52	346.13697	346.13706	/	$C_{13}H_{15}N_9O_3$	/	0.08855	SF
17	20.60	259.09223	259.09412	/	$C_8H_{19}O_7P$	√	1.88441	SF
			259.09303	Diketo-Glu-Glu	$C_{10}H_{14}N_2O_6$	√	0.8	LSc*
17	20.60	241.08141	241.08190	/	$C_{10}H_{21}N_2O_5$	√	0.48590	SF
			241.07593	Diketo-His-Cys	$C_9H_{12}N_4O_2S$	√	5.48	LSc*
17	20.64	231.16982	231.17032	Val-Ile	$C_{11}H_{22}N_2O_3$	√	0.49654	SF/SL
17	20.64	263.13875	263.13902	Pro-Phe	$C_{14}H_{18}N_2O_3$	√	0.26366	SF/SL
18	20.71	195.07601	195.07642	/	$C_9H_{10}N_2O_3$	√	0.40759	SF
18	20.73	324.15477	324.15540	/	$C_{15}H_{21}N_3O_5$	√	0.6328	SF
			324.15607	Phe-Thr-Gly	equivalent to SF	√	2	LSc*
			324.15608	Ala-Tyr-Ala	equivalent to SF	√	1.3	LSc*
							1.3	
18	20.76	229.15447	229.15467	Pro-Leu	$C_{11}H_{20}N_2O_3$	√	0.20203	SF/SL
18	20.79	362.20699	362.20743	Val-Pro-Phe	$C_{19}H_{27}N_3O_4$	√	0.44567	SF/SL
18	20.85	360.19443	360.19178	Pro-Pro-Phe	$C_{19}H_{25}N_3O_4$	√	2.65157	SF/SL
18	20.86	295.16481	295.16420	/	$C_9H_{23}N_6O_3P$	/	0.60592	SF
			295.16591	Tyr-Leu	$C_{15}H_{22}N_2O_4$	/	1.1	LSc*
19	21.03	328.22306	328.22308	Pro-Leu-Val	$C_{16}H_{29}N_3O_4$	√	0.02608	SF/SL
19	21.06	392.21746	392.21800	Ile-Pro-Tyr	$C_{20}H_{29}N_3O_5$	√	0.53835	SF/SL
19	21.09	505.26523	505.26434	/	$C_{24}H_{40}O_{11}$	√	0.89608	SF
20	21.27	249.12586	249.12674	Val-Met	$C_{10}H_{20}N_2O_3S$	√	0.87738	SF/SL
20	21.34	217.15422	217.15467	Val-Val	$C_{10}H_{20}N_2O_3$	√	0.44680	SF/SL
20	21.37	263.13883	263.13902	Phe-Pro	$C_{14}H_{18}N_2O_3$	√	0.19215	SF/SL
21	21.46	215.13891	215.13902	Pro-Val	$C_{10}H_{18}N_2O_3$	√	0.11160	SF/SL
21	21.49	188.07059	188.07060	/	$C_{11}H_9NO_2$	√	0.01567	SF
21	21.49	205.09707	205.09715	D-Tryptophan	$C_{11}H_{12}N_2O_2$	√	0.08833	SF/SL
21	21.50	229.15471	229.15467	Pro-Leu	$C_{11}H_{20}N_2O_3$	√	0.03818	SF/SL
21	21.53	314.20723	314.20743	Val-Pro-Val	$C_{15}H_{27}N_3O_4$	√	0.19886	SF/SL
21	21.57	203.13882	203.13902	/	$C_9H_{18}N_2O_3$	√	0.19512	SF
			203.1397	Ile-Ala	equivalent to SF	√	0.88	LSc*

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
21	21.63	132.10171	132.10191	L-Norleucine	$C_6H_{13}NO_2$	√	0.19678	SF/SL
22	21.76	331.16472	331.16523	/	$C_{18}H_{22}N_2O_4$	√	0.51455	SF
			331.16189	Ser-Pro-Gln	$C_{13}H_{22}N_4O_6$	√	2.83	LSc*
22	21.78	120.08066	120.08078	/	C_8H_9N	√	0.11877	SF
22	21.78	166.08613	166.08626	L-Phenylalanine	$C_9H_{11}NO_2$	√	0.12588	SF/SL
22	21.86	223.10726	223.10772	Phe-Gly	$C_{11}H_{14}N_2O_3$	√	0.46207	SF/SL
22	21.88	300.19144	300.19178	Leu-Pro-Ala	$C_{14}H_{25}N_3O_4$	√	0.34378	SF/SL
22	21.89	326.20688	326.20743	/	$C_{16}H_{27}N_3O_4$	/	0.55547	SF
			326.20812	Pro-Pro-Ile	equivalent to SF	/	1.24	LSc*
23	22.27	189.12299	189.12337	Ile-Gly	$C_8H_{16}N_2O_3$	√	0.38273	SF/SL
24	22.52	215.13891	215.13902	Pro-Val	$C_{10}H_{18}N_2O_3$	√	0.10630	SF/SL
24	22.59	279.13339	279.13393	/	$C_{14}H_{18}N_2O_4$	√	0.54488	SF
			279.13461	Tyr-Pro	equivalent to SF	√	1.22	LSc*
24	22.61	350.17041	350.17105	Ser-Pro-Phe	$C_{17}H_{23}N_3O_5$	√	0.63950	SF/SL
24	22.62	189.12285	189.12337	Ile-Gly	$C_8H_{16}N_2O_3$	√	0.51368	SF/SL
24	22.62	261.14385	261.14450	Ile-Glu	$C_{11}H_{20}N_2O_5$	√	0.64331	SF/SL
24	22.65	316.18568	316.18670	Pro-Ser-Leu	$C_{14}H_{25}N_3O_5$	√	1.01908	SF/SL
24	22.68	358.19639	358.19726	Ile-Pro-Glu	$C_{16}H_{27}N_3O_6$	√	0.87077	SF/SL
24	22.70	312.19122	312.19178	Pro-Pro-Val	$C_{15}H_{25}N_3O_4$	√	0.56692	SF/SL
25	22.74	286.17558	286.17613	/	$C_{13}H_{23}N_3O_4$	√	0.55500	SF
			286.17681	Pro-Ile-Gly	equivalent to SF	√	1.1	LSc*
			286.17682	Val-Ala-Pro	equivalent to SF	√	1.11	LSc*
25	22.75	213.12283	213.12337	/	$C_{10}H_{16}N_2O_3$	√	0.54108	SF
			213.12405	Pro-Pro	equivalent to SF	√	1.22	LSc*
25	22.82	187.10720	187.10772	/	$C_8H_{14}N_2O_3$	√	0.51659	SF
			187.10828	Diketo-Val-Ser	equivalent to SF	√	1.08	LSc*
			187.1084	Pro-Ala	equivalent to SF	√	1.2	LSc*
25	22.84	150.05797	150.05833	L-Methionine	$C_5H_{11}NO_2S$	√	0.35219	SF/SL
25	22.87	485.18725	485.18782	/	$C_{20}H_{28}N_4O_{10}$	√	0.57223	SF
25	22.96	219.13357	219.13393	Leu-Ser	$C_9H_{18}N_2O_4$	√	0.36336	SF/SL
25	22.96	173.12786	173.12845	/	$C_8H_{16}N_2O_2$	√	0.59530	SF
25	23.01	229.11782	229.11828	/	$C_{10}H_{16}N_2O_4$	√	0.46721	SF
			229.11885	Diketo-Glu-Val	equivalent to SF	√	1.03	LSc*
25	23.01	247.12823	247.12885	Glu-Val	$C_{10}H_{18}N_2O_5$	√	0.62111	SF/SL
25	23.05	330.20179	330.20235	Leu-Pro-Thr	$C_{15}H_{27}N_3O_5$	√	0.55613	SF/SL
26	23.15	136.07528	136.07569	/	C_8H_9NO	√	0.40694	SF
26	23.15	165.05422	165.05462	/	$C_9H_8O_3$	√	0.39796	SF
26	23.16	182.08088	182.08117	L-Tyrosine	$C_9H_{11}NO_3$	√	0.29041	SF/SL
26	23.24	150.05784	150.05833	L-Methionine	$C_5H_{11}NO_2S$	√	0.48880	SF/SL
27	23.48	241.08129	241.08190	/	$C_{10}H_{12}N_2O_5$	√	0.60744	SF
			241.07593	Diketo-His-Cys	$C_9H_{12}N_4O_2S$	√	5.36	LSc*
27	23.48	258.10798	258.10845	/	$C_{10}H_{15}N_3O_5$	√	0.46575	SF
			258.10901	Diketo-Glu-Gln	equivalent to SF	√	1.03	LSc*
27	23.67	217.11761	217.11828	Pro-Thr	$C_9H_{16}N_2O_4$	√	0.67336	SF/SL
27	24.07	247.12811	247.12885	Val-Glu	$C_{10}H_{18}N_2O_5$	√	0.73635	SF/SL
27	24.19	311.12359	311.12376	Tyr-Glu	$C_{14}H_{18}N_2O_6$	√	0.17097	SF/SL
27	24.32	254.16051	254.16115	/	$C_{11}H_{19}N_5O_2$	√	0.64360	SF
			254.16172	Diketo-Arg-Pro	equivalent to SF	√	1.21	LSc*
28	25.15	182.08059	182.08117	DL-o-Tyrosine	$C_9H_{11}NO_3$	√	0.58038	SF/SL
28	25.15	226.04436	226.04585	/	$C_8H_7N_3O_5$	√	1.48390	SF
28	25.39	235.11822	235.11895	/	$C_{11}H_{14}N_4O_2$	√	0.73585	SF
			235.11951	Diketo-His-Pro	equivalent to SF	√	1.29	LSc*
28	25.38	257.10003	257.10062	/	$C_9H_8N_{10}$	√	0.58540	SF
28	25.57	162.04958	162.05093	/	$C_4H_7N_3O_4$	√	1.35110	SF
28	25.57	298.02408	298.02400	/	$C_8H_{13}NO_7P$	√	0.08248	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
28	25.60	233.11249	233.11320	/	$C_9H_{16}N_2O_5$	/	0.70456	SF
		233.11388	Val-Asp	equivalent to SF		/	1.39	LSc*
29	26.40	217.11750	217.11828	Pro-Thr	$C_9H_{16}N_2O_4$	√	0.78399	SF/SL
29	26.47	205.11754	205.11828	/	$C_8H_{16}N_2O_4$	/	0.74757	SF
		205.11737	Val-Ser	equivalent to SF		/	0.17	LSc*
30	27.42	148.05966	148.06043	L-Glutamate	$C_5H_9NO_4$	√	0.76933	SF/SL
30	27.42	244.12813	244.12918	Pro-Gln	$C_{10}H_{17}N_3O_4$	√	1.04985	SF/SL
30	27.42	295.11224	295.11359	/	$C_{10}H_{18}N_2O_8$	√	1.35564	SF
30	27.47	266.11016	266.11085	/	$C_8H_{11}N_9O_2$	/	0.68494	SF
30	27.58	203.10163	203.10263		$C_8H_{14}N_2O_4$	√	0.47572	SF
		203.10331	Pro-Ser	equivalent to SF		√	1.68	LSc*
31	28.19	258.14378	258.14483	/	$C_{11}H_{19}N_3O_4$	/	1.05001	SF
		258.1454	Diketo-Glu-Lys	equivalent to SF		/	1.62	LSc*
31	28.29	364.16009	364.15886	/	$C_{12}H_{17}N_{11}O_3$	√	1.22662	SF
		364.15437	Asp-Met-Val	$C_{14}H_{25}N_3O_6S$		√	5.72	LSc*
		364.15437	Ile-Glu-Cys	$C_{14}H_{25}N_3O_6S$		√	5.72	LSc*
		364.1656	Asn-Lys-Cys	$C_{13}H_{25}N_5O_5S$		√	5.51	LSc*
31	28.30	156.07606	156.07675	L-Histidine	$C_6H_9N_3O_2$	√	0.69730	SF/SL
31	28.41	267.10748	267.10878	/	$C_{11}H_{14}N_4O_4$	√	1.30331	SF
		267.10934	Diketo-Glu-His	equivalent to SF		√	1.86	LSc*
31	28.42	249.09711	249.09822	/	$C_{11}H_{12}N_4O_3$	/	1.10667	SF
32	28.52	235.09177	235.09246	Ser-Glu	$C_8H_{14}N_2O_6$	√	0.69081	SF/SL
32	28.52	148.05981	148.06043	L-Glutamate	$C_5H_9NO_4$	√	0.62124	SF/SL
32	28.53	217.08098	217.08190	/	$C_8H_{12}N_2O_5$	√	0.91690	SF
		217.08246	Diketo-Glu-Ser	equivalent to SF		√	1.48	LSc*
33	28.78	293.14468	293.14556	/	$C_{10}H_{20}N_4O_6$	√	0.87619	SF
33	28.80	147.07582	147.07642	L-Glutamine	$C_5H_{10}N_2O_3$	√	0.59821	SF/SL
33	28.97	276.11802	276.11901	Glu-Gln	$C_{10}H_{17}N_3O_6$	√	0.99080	SF/SL
33	28.98	258.10758	258.10845	/	$C_{10}H_{15}N_3O_5$	√	0.86923	SF
		258.10901	Diketo-Glu-Gln	equivalent to SF		√	1.43	LSc*
33	28.98	241.08106	241.08190	/	$C_{10}H_{12}N_2O_5$	√	0.84024	SF
		241.07593	Diketo-His-Cys	$C_9H_{12}N_4O_2S$		√	5.13	LSc*
34	29.12	166.05259	166.05324	DL-Methionine sulfoxide	$C_5H_{11}NO_3S$	√	0.65141	SF/SL
34	29.14	375.14985	375.14969	/	$C_{11}H_{14}N_{14}O_2$	√	0.15875	SF
35	29.25	265.11327	265.11426	/	$C_7H_{14}N_2O_5$	√	0.98629	SF
		265.11884	Diketo-Tyr-Thr	$C_{13}H_{16}N_2O_4$		√	5.57	LSc*
35	29.27	133.06025	133.06077	L-Asparagine	$C_4H_8N_2O_3$	√	0.51965	SF/SL
35	29.27	207.09669	207.09755	/	$C_7H_{14}N_2O_5$	√	0.85311	SF
		207.09822	Ser-Thr	equivalent to SF		√	1.53	LSc*
36	29.54	405.15992	405.16160	Glu-Glu-Gln	$C_{15}H_{24}N_4O_9$	√	1.68011	SF/SL
37	29.55	204.09692	204.09788	/	$C_7H_{13}N_3O_4$	/	0.96092	SF
		204.09855	Gln-Gly	equivalent to SF		/	1.63	LSc*
		204.09855	Gly-Ala-Gly	equivalent to SF		/	1.63	LSc*
37	29.55	170.04402	170.04478	/	$C_7H_7NO_4$	/	0.76078	SF
36	29.69	292.10211	292.10269	/	$C_{11}H_{17}NO_8$	√	0.58751	SF
36	29.69	310.11233	310.11057	/	$C_7H_{15}N_7O_7$	√	1.75853	SF
37	29.85	275.13418	275.13500	/	$C_{10}H_{18}N_4O_5$	/	0.82050	SF
		275.13394	Gln-Gln	equivalent to SF		/	0.24	LSc*
37	30.02	234.09782	234.09855	/	$C_{10}H_{11}N_5O_2$	√	0.73114	SF
37	30.09	193.08114	193.08190	Ser-Ser	$C_6H_{12}N_2O_5$	√	0.76024	SF/SL
37	30.20	424.17972	424.18134	/	$C_{17}H_{29}NO_{11}$	√	1.61632	SF
		424.17682	His-His-Met	$C_{17}H_{25}N_7O_4S$		√	2.9	LSc*
37	30.38	291.11874	291.12001	/	$C_{12}H_{14}N_6O_3$	/	1.26963	SF
38	30.40	180.08585	180.08665	/	$C_6H_{13}NO_5$	√	0.79745	SF
38	30.47	273.10750	273.10945	/	$C_{12}H_{12}N_6O_2$	/	1.95133	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
38	30.54	309.12797	309.12924	/	$C_{11}H_{20}N_2O_8$	√	1.26875	SF
38	30.68	250.09112	250.09213	/	$C_9H_{15}NO_7$	√	1.00775	SF
			250.08625	Ala-Cys-Gly	$C_8H_{15}N_3O_4S$	√	4.87	LSc*
38	30.98	359.16472	359.16602	/	$C_{12}H_{26}N_2O_{10}$	√	1.30026	SF
			359.15681	Asn-Glu-Pro	$C_{14}H_{22}N_4O_7$	√	7.91	LSc*
			359.15681	Asp-Gln-Pro	$C_{14}H_{22}N_4O_7$	√	7.91	LSc*
38	30.99	162.07557	162.07608	/	$C_6H_{11}NO_4$	√	0.51227	SF
38	31.04	319.14889	319.14863	/	$C_{10}H_{14}N_{12}O$	√	0.26283	SF
39	31.38	274.18636	274.18737	Val-Arg	$C_{11}H_{23}N_5O_3$	√	1.00976	SF/SL
39	31.46	472.16519	472.16608	/	$C_{17}H_{29}NO_{14}$	√	0.89555	SF
39	31.48	454.15463	454.15552	/	$C_{17}H_{27}NO_{13}$	√	0.88208	SF
40	31.75	253.12868	253.12952	Pro-His	$C_{11}H_{16}N_4O_3$	√	0.83412	SF/SL
40	31.78	272.17102	272.17172	Pro-Arg	$C_{11}H_{21}N_5O_3$	√	0.83572	SF/SL
40	31.87	246.15522	246.15607	Arg-Ala	$C_9H_{19}N_5O_3$	√	0.85006	SF/SL
40	31.90	349.22961	349.23063	/	$C_{12}H_{28}N_8O_4$	√	1.01510	SF
40	31.92	175.11852	175.11895	/	$C_6H_{14}N_4O_2$	√	0.43019	SF
			175.11503	Arginine	equivalent to SF	√	3.49	LSc*
40	32.05	330.18729	330.18843	/	$C_{12}H_{23}N_7O_4$	√	1.13840	SF
40	32.15	311.14480	311.14623	/	$C_{12}H_{18}N_6O_4$	√	1.42903	SF
			311.13958	Diketo-Tyr-Phe	$C_{18}H_{18}N_2O_3$	√	5.22	LSc*
40	32.16	156.07614	156.07675	L-Histidine	$C_6H_9N_3O_2$	√	0.61130	SF/SL
40	32.32	147.11239	147.11280	L-Lysine	$C_6H_{14}N_2O_2$	√	0.50284	SF/SL
41	32.44	304.16074	304.16155	Arg-Glu	$C_{11}H_{21}N_5O_5$	√	0.91619	SF/SL
42	32.74	324.12808	324.12622	/	$C_8H_{17}N_7O_7$	√	1.85707	SF
			324.12306	Met-Ser-Ser	$C_{11}H_{21}N_3O_6S$	√	5.02	LSc*
42	32.93	232.13972	232.14042	/	$C_8H_{17}N_5O_3$	√	0.77233	SF
			232.14109	Arg-Gly	equivalent to SF	√	0.96	LSc*
42	32.99	213.09726	213.09822	His-Gly	$C_8H_{12}N_4O_3$	√	0.96009	SF/SL
43	33.00	241.03039	241.03113	L-Cystine	$C_6H_{12}N_2O_4S_2$	√	0.73889	SF/SL
44	33.28	243.10809	243.10878	His-Ser	$C_9H_{14}N_4O_4$	√	0.88146	SF/SL
44	33.36	300.11828	300.11901	/	$C_{12}H_{17}N_3O_6$	√	0.90993	SF
44	33.36	318.12873	318.12689	/	$C_8H_{15}N_9O_5$	√	1.84221	SF
			318.12764	Diketo-Trp-Met	$C_{16}H_{19}N_3O_2S$	√	1.09	LSc*
44	33.44	234.14409	234.14483	/	$C_9H_{19}N_3O_4$	/	1.28243	SF
			234.14551	Ser-Lys	equivalent to SF	/	1.42	LSc*
44	33.50	262.15021	262.15098	Arg-Ser	$C_9H_{19}N_5O_4$	√	0.84210	SF/SL
44	33.79	289.16084	289.16188	Arg-Asn	$C_{10}H_{20}N_6O_4$	√	1.03603	SF/SL
45	33.88	309.16466	309.16563	/	$C_{12}H_{24}N_2O_7$	√	0.98684	SF
46	34.34	159.96857	159.96991	/	C_4HNO_4S	√	1.33947	SF
46	34.35	182.98441	182.98417	/	$C_7H_3O_3P$	√	0.23405	SF



Supplementary figure 3: Base Peak chromatogram (BPC) of the positive MS/MS mode of sample 3. The numbering of the peaks correlates with the numbering in Supplementary table 3.

Supplementary table 3: Results of the three calculation approaches for the most abundant signals in sample stock solution (100 mg mL^{-1}) of sample 3. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 3.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
1	9.74	211.14382	211.14410	/	$C_{11}H_{18}N_2O_2$	√	0.28202	SF
			211.14467	Diketo-Pro-Ile	equivalent to SF	√	0.85	LSc*
1	9.80	521.38027	521.37879	/	$C_{28}H_{57}O_4PS$	√	1.47659	SF
1	9.80	549.41162	549.41228	/	$C_{29}H_{52}N_6O_4$	√	0.66021	SF
2	10.17	231.11273	231.11280	/	$C_{13}H_{14}N_2O_2$	√	0.07104	SF
			231.11674	Diketo-Met-Val	$C_{10}H_{18}N_2O_2S$	√	4.01	LSc*
2	10.17	493.34901	493.34968	/	$C_{25}H_{44}N_6O_4$	√	0.66742	SF
3	10.49	245.12831	245.13011	/	$C_{12}H_{21}O_3P$	√	1.80218	SF
			245.12902	Diketo-Phe-Pro	$C_{14}H_{16}N_2O_2$	√	0.71	LSc*
3	10.50	489.24911	489.24860	/	$C_{22}H_{33}N_8O_3P$	√	0.51010	SF
3	10.69	325.17502	325.17580	/	$C_{16}H_{24}N_2O_5$	√	0.78174	SF
4	11.03	197.12786	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.59151	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	1.16	LSc*
4	11.03	296.08915	296.08905	/	$C_{17}H_{16}N_8O_3$	/	0.09936	SF
			296.09176	Ser-Cys-Ser	$C_9H_{17}N_3O_6S$	/	2.61	LSc*
4	11.04	381.14121	381.14181	/	$C_{17}H_{16}N_8O_3$	√	0.60393	SF
4	11.09	359.15931	359.16015	/	$C_{19}H_{22}N_2O_5$	√	0.84035	SF
			359.15681	Asn-Glu-Pro	$C_{14}H_{22}N_4O_7$	√	2.5	LSc*
			359.15681	Asp-Gln-Pro	$C_{14}H_{22}N_4O_7$	√	2.5	LSc*
4	11.33	326.37765	326.37813	/	$C_{22}H_{47}N$	√	0.47172	SF
4	11.33	284.13863	284.13935	/	$C_{16}H_{17}N_3O_2$	√	0.72233	SF
			284.13992	Diketo-Pro-Trp	equivalent to SF	√	1.29	LSc*
5	15.55	521.23953	521.23946	/	$C_{28}H_{32}N_4O_6$	√	0.07197	SF
5	15.58	124.03899	124.03930	/	$C_6H_5NO_2$	√	0.31540	SF
5	15.56	261.12334	261.12502	/	$C_{12}H_{21}O_4P$	√	1.68150	SF
			261.12393	Diketo-Tyr-Pro	$C_{14}H_{16}N_2O_3$	√	0.59	LSc*
5	15.77	169.09667	169.09715	/	$C_8H_{12}N_2O_2$	√	0.48502	SF
			169.09772	Diketo-Ala-Pro	equivalent to SF	√	1.05	LSc*
6	16.46	132.10148	132.10191	Norleucine	$C_6H_{13}NO_2$	√	0.42630	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
								LSc*
6	16.45	356.21776	356.21933	/	$C_{18}H_{25}N_7O$	√	1.57345	SF
6	16.93	277.11827	277.11994	/	$C_{12}H_{21}O_5P$	√	1.67121	SF
			277.11885	Diketo-Glu-Phe	$C_{14}H_{16}N_2O_4$	√	0.58	LSc*
6	16.94	243.13383	243.13393	/	$C_{11}H_{18}N_2O_4$	√	0.10028	SF
			243.1345	Diketo-Glu-Ile	equivalent to SF	√	0.67	LSc*
6	16.95	485.26050	485.25956	/	$C_{16}H_{37}N_8O_7P$	√	0.94143	SF
6	16.95	197.12803	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.42282	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	0.99	LSc*
7	17.50	259.09228	259.09412	/	$C_8H_{19}O_7P$	√	1.83317	SF
			259.09303	Diketo-Glu-Glu	$C_{10}H_{14}N_2O_6$	√	0.75	LSc*
7	17.50	388.13467	388.13536	/	$C_{10}H_{18}N_{11}O_4P$	√	0.68759	SF
			388.12921	His-Glu-Cys	$C_{14}H_{21}N_5O_6S$	√	5.46	LSc*
7	17.51	130.04973	130.04987	(R)-(+)-2-Pyrrolidone-5-carboxylic acid	$C_5H_7NO_3$	√	0.14179	SF/SL
7	17.54	374.17079	374.17270	/	$C_{17}H_{28}NO_6P$	√	1.91018	SF
8	17.70	340.18666	340.18567	/	$C_{10}H_{26}N_7O_4P$	√	0.99906	SF
8	17.80	371.22718	371.22756	/	$C_{16}H_{34}O_9$	√	0.37743	SF
8	17.92	209.09163	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.43591	SF
8	18.14	476.30654	476.30684	/	$C_{15}H_{42}N_9O_6P$	√	0.30753	SF
8	18.14	459.27994	459.28029	/	$C_{15}H_{39}N_8O_6P$	√	0.35832	SF
			459.27933	Arg-Arg-Gln	$C_{17}H_{34}N_{10}O_5$	√	0.61	LSc*
8	18.14	481.26110	481.26299	/	$C_{19}H_{32}N_{10}O_5$	/	1.89382	SF
9	18.27	503.30592	503.30651	/	$C_{17}H_{43}N_8O_7P$	/	0.58399	SF
9	18.27	520.33284	520.33306	/	$C_{17}H_{46}N_9O_7P$	√	0.21982	SF
9	18.27	293.11276	293.11320	/	$C_{14}H_{16}N_2O_5$	√	0.44231	SF
			293.11376	Diketo-Glu-Tyr	equivalent to SF	√	1.0	LSc*
9	18.35	213.08641	213.08832	/	$C_{10}H_8N_6$	/	1.90625	SF
			213.08755	Diketo-Asp-Pro	$C_9H_{12}N_2O_4$	/	1.14	LSc*
9	18.40	564.35862	564.35927	/	$C_{19}H_{50}N_9O_8P$	√	0.64840	SF
9	18.44	471.22375	471.22278	/	$C_{18}H_{31}N_8O_5P$	√	0.96631	SF
9	18.46	493.20477	493.20548	/	$C_{22}H_{24}N_{10}O_4$	√	0.70097	SF
9	18.48	263.13863	263.13902	Pro-Phe	$C_{14}H_{18}N_2O_3$	√	0.38362	SF
9	18.68	326.17063	326.17105	/	$C_{15}H_{23}N_3O_5$	√	0.41977	SF
9	18.81	437.23922	437.24111	/	$C_{19}H_{37}N_2O_7P$	√	1.89033	SF
9	18.87	459.22087	459.22246	/	$C_{20}H_{22}N_{14}$	√	1.59157	SF
10	19.14	390.16538	390.16493	/	$C_{13}H_{24}N_7O_5P$	√	0.45132	SF
			390.16262	Gln-Gln-Asp	$C_{14}H_{23}N_5O_8$	√	2.76	LSc*
10	19.52	227.10267	227.10429	/	$C_8H_{19}O_5P$	√	1.61554	SF
			227.1032	Diketo-Glu-Pro	$C_{10}H_{14}N_2O_4$	√	0.53	LSc*
10	19.52	453.19833	453.19861	/	$C_{12}H_{34}N_6O_8P_2$	√	0.28595	SF
10	19.72	279.16975	279.17032	Leu-Phe	$C_{15}H_{22}N_2O_3$	√	0.57100	SF/SL
10	19.84	410.20685	410.20743	Phe-Pro-Phe	$C_{23}H_{27}N_3O_4$	√	0.57931	SF/SL
10	19.90	215.13839	215.13902	/	$C_{10}H_{18}N_2O_3$	√	0.62730	SF
			215.1397	Pro-Val	equivalent to SF	√	1.31	LSc*
10	19.90	415.23311	415.23264	/	$C_{21}H_{34}O_8$	/	0.47091	SF
			415.23284	Leu-Pro-Trp	$C_{22}H_{30}N_4O_4$	/	0.27	LSc*
10	19.92	245.18520	245.18597	NH-DVal(NMe)-Val-OMe	$C_{12}H_{24}N_2O_3$	√	0.76653	SF/SL
11	19.96	405.17549	405.17551	/	$C_{16}H_{16}N_{14}$	√	0.02240	SF
11	20.06	376.22313	376.22308	Leu-Pro-Phe	$C_{20}H_{29}N_3O_4$	√	0.05075	SF/SL
12	20.26	226.11868	226.12027	/	$C_8H_{20}NO_4P$	√	1.59473	SF
			226.11918	Diketo-Gln-Pro	$C_{10}H_{15}N_3O_3$	√	0.5	LSc*

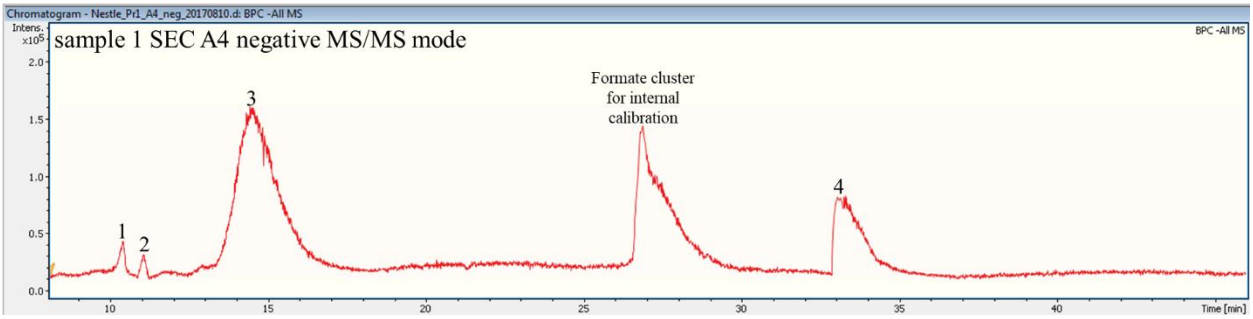
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
12	20.26	342.23873	342.23873	Leu-Pro-Ile	$C_{17}H_{31}N_3O_4$	√	0.00012	SF/SL
12	20.28	209.09198	209.09372	/	$C_8H_{17}O_4P$	√	1.73731	SF
12	20.44	373.13504	373.13691	/	$C_{23}H_{20}N_2OP$	/	1.87427	SF
			373.12954	Asn-His-Gly	$C_{13}H_{20}N_6O_5S$	/	5.5	LSc*
12	20.49	346.13690	346.13706	/	$C_{13}H_{15}N_9O_3$	/	0.16364	SF
13	20.56	259.09215	259.09246	/	$C_{10}H_{14}N_2O_6$	√	0.33124	SF
			259.09303	Diketo-Glu-Glu	equivalent to SF	√	0.88	LSc*
13	20.58	231.16970	231.17032	/	$C_{11}H_{22}N_2O_3$	/	0.62312	SF
			231.16982	Val-Ile	equivalent to SF	/	0.12	LSc*
13	20.59	263.13893	263.13902	Pro-Phe	$C_{14}H_{18}N_2O_3$	√	0.08467	SF/SL
13	20.61	362.20693	362.20877	/	$C_{20}H_{23}N_7$	/	1.84322	SF
			362.20632	Val-Pro-Phe	$C_{19}H_{27}N_3O_4$	/	0.61	LSc*
13	20.65	195.07597	195.07642	/	$C_9H_{10}N_2O_3$	√	0.45042	SF
13	20.66	324.15522	324.15705	/	$C_{13}H_{26}NO_6P$	√	1.83356	SF
			324.15607	Thr-Phe-Gly	$C_{15}H_{21}N_3O_5$	√	0.85	LSc*
			324.15608	Ala-Tyr-Ala	$C_{15}H_{21}N_3O_5$	√	0.86	LSc*
13	20.70	229.15454	229.15467	Pro-Leu	$C_{11}H_{20}N_2O_3$	√	0.12663	SF/SL
13	20.81	295.16461	295.16523	Tyr-Leu	$C_{15}H_{22}N_2O_4$	√	0.62207	SF/SL
13	20.87	328.22259	328.22308	Ile-Pro-Val	$C_{16}H_{29}N_3O_4$	√	0.48978	SF/SL
13	20.99	229.11722	229.11828	/	$C_{10}H_{16}N_2O_4$	/	1.06646	SF
			229.11885	Diketo-Glu-Val	equivalent to SF	/	1.63	LSc*
13	21.01	392.21743	392.21800	Ile-Pro-Tyr	$C_{20}H_{29}N_3O_5$	√	0.56649	SF/SL
13	21.15	360.19217	360.19344	/	$C_{17}H_{30}NO_5P$	/	1.26647	SF
			360.19269	Pro-Pro-Phe	$C_{19}H_{25}N_3O_4$	/	0.52	LSc*
14	21.30	120.08065	120.08078	/	C_8H_9N	√	0.13017	SF
14	21.30	263.13915	263.13902	Phe-Pro	$C_{14}H_{18}N_2O_3$	√	0.13523	SF/SL
14	21.43	188.07038	188.07060	/	$C_{11}H_9NO_2$	√	0.22205	SF
14	21.43	205.09689	205.09715	D-Tryptophan	$C_{11}H_{12}N_2O_2$	√	0.26417	SF/SL
14	21.44	229.15462	229.15467	/	$C_{11}H_{20}N_2O_3$	√	0.05145	SF
			229.15535	Pro-Leu	equivalent to SF	√	0.73	LSc*
14	21.48	214.20712	314.20743	/	$C_{15}H_{27}N_3O_4$	/	0.31636	SF
			314.20723	Val-Pro-Val	equivalent to SF	/	0.11	LSc*
14	21.48	203.13862	203.13902	/	$C_9H_{18}N_2O_3$	/	0.39714	SF
			203.1397	Ile-Ala	equivalent to SF	/	1.08	LSc*
14	22.54	350.17042	350.17105	/	$C_{17}H_{23}N_3O_5$	√	0.63080	SF
			350.17041	Ser-Pro-Phe	equivalent to SF	√	0.01	LSc*
14	21.56	326.20692	326.20743	Pro-Pro-Ile	$C_{16}H_{27}N_3O_4$	√	0.51450	SF/SL
14	21.59	132.10167	132.10191	L-Norleucine	$C_6H_{13}NO_2$	√	0.22978	SF/SL
15	21.70	331.16474	331.16420	/	$C_{12}H_{23}N_6O_3P$	√	0.53725	SF
			331.16189	Ser-Pro-Gln	$C_{13}H_{22}N_4O_6$	√	2.85	LSc*
15	21.71	166.08604	166.08626	L-Phenylalanine	$C_9H_{11}NO_2$	√	0.21552	SF/SL
15	21.79	223.10706	223.10772	/	$C_{11}H_{14}N_2O_3$	√	0.65567	SF
			223.10726	Phe-Gly	equivalent to SF	√	0.20	LSc*
15	21.81	300.19115	300.19178	/	$C_{14}H_{25}N_3O_4$	√	0.63662	SF
			300.19096	Leu-Pro-Ala	equivalent to SF	√	0.19	LSc*
16	21.96	132.10167	132.10191	L-Norleucine	$C_6H_{13}NO_2$	√	0.23398	SF/SL
16	22.20	286.17571	286.17613	/	$C_{13}H_{23}N_3O_4$	√	0.42471	SF
			286.17681	Pro-Ile-Gly	equivalent to SF	√	1.1	LSc*
			286.17682	Val-Ala-Pro	equivalent to SF	√	1.11	LSc*
16	22.22	189.12296	189.12337	Ile-Gly	$C_8H_{16}N_2O_3$	√	0.40962	SF/SL
17	22.42	215.13887	215.13902	/	$C_{10}H_{18}N_2O_3$	√	0.15305	SF
			215.13869	Pro-Val	equivalent to SF	√	0.18	LSc*
17	22.59	279.13380	279.13559	/	$C_{12}H_{23}O_5P$	√	1.78410	SF
			279.13461	Tyr-Pro	$C_{14}H_{18}N_2O_4$	√	0.81	LSc*
18	22.63	261.14399	261.14450	Ile-Glu	$C_{11}H_{20}N_2O_5$	√	0.50708	SF/SL
18	22.64	316.18601	316.18670	/	$C_{14}H_{25}N_3O_5$	√	0.68565	SF
			316.18738	Pro-Ser-Leu	equivalent to SF	√	1.37	LSc*

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
18	22.69	312.19105	312.19178	Pro-Pro-Val	$C_{15}H_{25}N_3O_4$	√	0.73072	SF/SL
18	22.71	213.12287	213.12337	/	$C_{10}H_{16}N_2O_3$	√	0.49773	SF
			213.12405	Pro-Pro	equivalent to SF	√	1.18	LSc*
18	22.79	187.10714	187.10772	/	$C_8H_{14}N_2O_3$	√	0.57803	SF
			187.10828	Diketo-Val-Ser	equivalent to SF	√	1.14	LSc*
			187.1084	Pro-Ala	equivalent to SF	√	1.26	LSc*
18	22.80	150.05781	150.05833	L-Methionine	$C_5H_{11}NO_2S$	√	0.51536	SF/SL
18	22.93	219.13333	219.13393	/	$C_9H_{18}N_2O_4$	√	0.60626	SF
			219.13461	Leu-Ser	equivalent to SF	√	1.28	LSc*
18	22.94	173.12806	173.12845	/	$C_8H_{16}N_2O_2$	/	0.39782	SF
18	23.02	330.20175	330.20235	Leu-Pro-Thr	$C_{15}H_{27}N_3O_5$	√	0.59911	SF/SL
19	23.11	165.05414	165.05462	/	$C_9H_8O_3$	√	0.47723	SF
19	23.11	182.08079	182.08117	L-Tyrosine	$C_9H_{11}NO_3$	√	0.37992	SF/SL
19	23.19	314.13397	314.13466	/	$C_{13}H_{19}N_3O_6$	√	0.69562	SF
19	23.23	150.05796	150.05833	L-Methionine	$C_5H_{11}NO_2S$	√	0.36948	SF/SL
20	23.38	280.09010	280.09011	/	$C_8H_9N_9O_3$	/	0.00936	SF
20	23.41	258.10823	258.11010	/	$C_8H_{20}NO_6P$	√	1.86819	SF
			258.10901	Diketo-Glu-Gln	$C_{10}H_{15}N_3O_5$	√	0.78	LSc*
20	23.42	241.08156	241.08190	/	$C_{10}H_{12}N_2O_5$	√	0.33749	SF
			241.07593	Diketo-His-Cys	$C_9H_{14}N_4O_3S$	√	5.63	LSc*
21	24.07	147.07601	147.07642	/	$C_5H_{10}N_2O_3$	√	0.40415	SF
			147.07570	Glutamine	equivalent to SF	√	0.31	LSc*
21	24.07	355.16060	355.15987	/	$C_{14}H_{26}O_{10}$	√	0.72602	SF
21	24.17	247.12847	247.12885	Val-Glu	$C_{10}H_{18}N_2O_5$	√	0.37816	SF/SL
21	24.25	311.12320	311.12376	Tyr-Glu	$C_{14}H_{18}N_2O_6$	√	0.56295	SF/SL
21	24.34	254.16038	254.16115	/	$C_{11}H_{19}N_5O_2$,	√	0.77265	SF
			254.16172	Diketo-Arg-Pro	equivalent to SF	√	1.34	LSc*
22	24.67	182.08053	182.08117	DL-o-Tyrosine	$C_9H_{11}NO_3$	√	0.64266	SF/SL
22	24.69	136.07536	136.07569	/	C_8H_9NO	√	0.33262	SF
23	25.08	165.05410	165.05462	/	$C_9H_8O_3$	√	0.51626	SF
23	25.08	182.08070	182.08117	L-Tyrosine	$C_9H_{11}NO_3$	√	0.46844	SF/SL
23	25.09	226.04448	226.04585	/	$C_8H_7N_3O_5$	√	1.36530	SF
23	25.23	314.08406	314.08569	/	$C_{10}H_7N_{11}O_2$	/	1.63776	SF
23	25.24	357.21243	357.21325	Leu-Pro-Gln	$C_{16}H_{28}N_4O_5$	√	0.81127	SF/SL
23	25.36	235.11833	235.11761	/	$C_{10}H_{18}O_6$	√	0.71185	SF
			235.11951	Diketo-His-Pro	$C_{11}H_{14}N_4O_2$	√	1.18	LSc*
23	25.38	257.10009	257.10062	/	$C_9H_8N_{10}$	/	0.52648	SF
24	25.52	162.04965	162.05093	/	$C_4H_7N_3O_4$	√	1.28116	SF
24	25.52	298.02425	298.02400	/	$C_8H_{13}NO_7P_2$	√	0.24764	SF
24	25.58	233.11249	233.11320	Val-Asp	$C_9H_{16}N_2O_5$	√	0.70896	SF/SL
25	26.46	205.11779	205.11828	Val-Ser	$C_8H_{16}N_2O_4$	√	0.49510	SF/SL
25	26.79	217.11740	217.11828	/	$C_9H_{16}N_2O_4$	√	0.88165	SF
			217.11896	Pro-Thr	equivalent to SF	√	1.56	LSc*
26	27.21	203.10216	203.10263	Pro-Ser	$C_8H_{14}N_2O_4$	√	0.47572	SF/SL
26	27.72	269.99286	269.99105	/	$C_8H_4N_3O_6P$	√	1.81545	SF
27	27.39	266.11045	266.11085	/	$C_8H_{11}N_9O_2$	√	0.39552	SF
27	27.41	244.12872	244.12918	Pro-Ala-Gly	$C_{10}H_{17}N_3O_4$	√	0.46603	SF/SL
27	28.19	258.14413	258.14483	/	$C_{11}H_{19}N_3O_4$	√	0.70702	SF
			258.1454	Diketo-Glu-Lys	equivalent to SF	√	1.27	LSc*
28	28.30	364.16107	364.16021	/	$C_{15}H_{25}NO_9$	√	0.85769	SF
			364.15437	Asp-Met-Val	$C_{14}H_{25}N_3O_6S$	√	6.7	LSc*
			364.15437	Ile-Glu-Cys	$C_{14}H_{25}N_3O_6S$	√	6.7	LSc*
			364.1656	Lys-Asn-Cys	$C_{13}H_{25}N_5O_5S$	√	4.53	LSc*
28	28.42	267.10836	267.10744	/	$C_{10}H_{18}O_8$	√	0.91283	SF
			267.10934	Diketo-Glu-His	$C_{11}H_{14}N_4O_4$	√	0.98	LSc*

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	IΔ*m/z*I [mDa]	Anno-tations
28	28.42	249.09753	249.09822	/	$C_{11}H_{12}N_4O_3$	√	0.68186	SF
28	28.46	315.16570	315.16630	Ala-Pro-Gln	$C_{13}H_{22}N_4O_5$	√	0.59437	SF/SL
29	28.77	147.07611	147.07642	D-Glutamine	$C_5H_{10}N_2O_3$	√	0.31220	SF/SL
29	28.78	276.11817	276.11901	/	$C_{10}H_{17}N_3O_6$	/	0.83958	SF
		276.11901	Glu-Gln	equivalent to SF	/	0.83	LSc*	
29	28.79	293.14520	293.14556	/	$C_{10}H_{20}N_4O_6$	√	0.35845	SF
29	29.09	166.05239	166.05324	DL-Methionine sulfoxide	$C_5H_{11}NO_3S$	√	0.85171	SF/SL
29	29.10	216.09681	216.09788	/	$C_8H_{13}N_3O_4$	√	1.07592	SF
		216.09844	Diketo-Ser-Gln	equivalent to SF	√	1.63	LSc*	
30	29.24	133.06030	133.06077	L-Asparagine	$C_4H_8N_2O_3$	√	0.47156	SF/SL
30	29.24	207.09678	207.09755	/	$C_7H_{14}N_2O_5$	√	0.76522	SF
		207.09822	Ser-Thr	equivalent to SF	√	1.44	LSc*	
30	29.25	265.11340	265.11426	/	$C_8H_{16}N_4O_6$	√	0.85700	SF
		265.11884	Diketo-Tyr-Thr	$C_{13}H_{16}N_2O_4$	√	5.44	LSc*	
31	29.49	204.09732	204.09788	/	$C_7H_{13}N_3O_4$	√	0.56572	SF
		204.09855	Gln-Gly	equivalent to SF	√	1.23	LSc*	
		204.09855	Gly-Ala-Gly	equivalent to SF	√	1.23	LSc*	
31	29.50	170.04421	170.04478	/	$C_7H_7NO_4$	√	0.57521	SF
31	29.50	187.07082	187.07133	/	$C_7H_{10}N_2O_4$	√	0.50897	SF
		187.07189	Diketo-Glu-Gly	equivalent to SF	√	1.07	LSc*	
31	29.79	275.13418	275.13500	Gln-Gln	$C_{10}H_{18}N_4O_5$	√	0.73566	SF/SL
31	30.03	193.08145	193.08190	/	$C_6H_{12}N_2O_5$	√	0.44254	SF
		193.08257	Ser-Ser	equivalent to SF	√	1.12	LSc*	
32	30.08	217.08158	217.08190	/	$C_8H_{12}N_2O_5$	√	0.31698	SF
		217.08246	Diketo-Glu-Ser	equivalent to SF	√	0.88	LSc*	
32	30.08	234.10786	234.10845	Ser-Gln	$C_8H_{15}N_3O_5$	√	0.59089	SF/SL
33	30.47	332.15617	332.15646	Gly-Gln-Gln	$C_{12}H_{21}N_5O_6$	√	0.29324	SF/SL
33	30.50	291.11973	291.11868	/	$C_{11}H_{18}N_2O_7$	√	1.05712	SF
33	30.52	309.12903	309.12924	/	$C_{11}H_{20}N_2O_8$	√	0.21306	SF
33	30.53	273.10811	273.10811	/	$C_{11}H_{16}N_2O_6$	√	0.00008	SF
33	30.70	250.09164	250.09213	/	$C_9H_{15}NO_7$	√	0.49160	SF
		250.08625	Ala-Cys-Gly	$C_8H_{15}N_3O_4S$	√	5.39	LSc*	
34	30.82	295.11276	295.11359	/	$C_{10}H_{18}N_2O_8$	/	0.83444	SF
34	30.95	319.14918	319.14863	/	$C_{10}H_{14}N_{12}O$	√	0.54632	SF
34	30.98	162.07570	162.07608	/	$C_6H_{11}NO_4$	√	0.38095	SF
34	30.99	180.08625	180.08665	/	$C_6H_{13}NO_5$	√	0.39835	SF
35	31.38	232.13920	232.14042	/	$C_8H_{17}N_5O_3$	/	1.21995	SF
		232.14109	Arg-Gly	equivalent to SF	/	1.89	LSc*	
35	31.51	253.12902	253.12952	/	$C_{11}H_{16}N_4O_3$	/	0.49267	SF
		253.13019	Pro-His	equivalent to SF	/	1.17	LSc*	
35	31.55	272.17161	272.17172	Pro-Arg	$C_{11}H_{23}N_5O_3$	√	0.16485	SF/SL
35	31.63	246.15559	246.15607	/	$C_9H_{19}N_5O_3$	√	0.47053	SF
		246.15522	Arg-Ala	equivalent to SF	√	0.37	LSc*	
35	31.69	349.22966	349.23063	/	$C_{12}H_{28}N_8O_4$	√	0.96700	SF
35	31.71	175.11854	175.11895	/	$C_6H_{14}N_4O_2$	√	0.40861	SF
		175.11503	Arginine	equivalent to SF	√	3.51	LSc*	
35	31.92	330.18762	330.18709	/	$C_{11}H_{27}N_3O_8$	√	0.52458	SF
35	32.03	311.14567	311.14623	/	$C_{12}H_{18}N_6O_4$	√	0.56220	SF
		311.13958	Diketo-Tyr-Phe	$C_{18}H_{18}N_2O_3$	√	6.09	LSc*	
35	32.05	156.07632	156.07675	L-Histidine	$C_6H_9N_3O_2$	√	0.43492	SF/SL
35	32.32	147.11250	147.11280	L-Lysine	$C_6H_{14}N_2O_2$	√	0.42306	SF/SL
35	32.34	304.16092	304.16155	Arg-Glu	$C_{11}H_{21}N_5O_5$	√	0.62087	SF/SL
35	32.62	159.96851	159.96991	/	C_4HNO_4S	/	1.39052	SF
36	33.03	213.09771	213.09822	Gly-His	$C_8H_{12}N_4O_3$	√	0.50784	SF/SL
36	33.03	241.03093	241.03113	L-Cystine	$C_6H_{12}N_2O_4S_2$	√	0.19185	SF/SL
37	33.40	318.12915	318.12958	/	$C_{12}H_{19}N_3O_7$	√	0.42222	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/ MS	I Δ*m/z* I [mDa]	Anno-tations
			318.12764	Diketo-Trp-Met	$C_{16}H_{19}N_3O_2S_2$	√	1.51	LSc*
37	33.40	300.11856	300.11901	/	$C_{12}H_{17}N_3O_6$	√	0.57153	SF
37	33.48	243.10835	243.10878	Ser-His	$C_9H_{14}N_4O_4$	√	0.70815	SF/SL
37	33.49	262.15053	262.15098	Ser-Arg	$C_9H_{19}N_5O_4$	√	0.45036	SF/SL
37	33.51	234.14394	234.14483	Ser-Lys	$C_9H_{19}N_3O_4$	√	0.88931	SF/SL
38	33.97	309.16511	309.16563	/	$C_{12}H_{24}N_2O_7$	√	0.52108	SF
38	34.44	182.98456	182.98417	/	$C_7H_3O_4P$	/	0.38574	SF

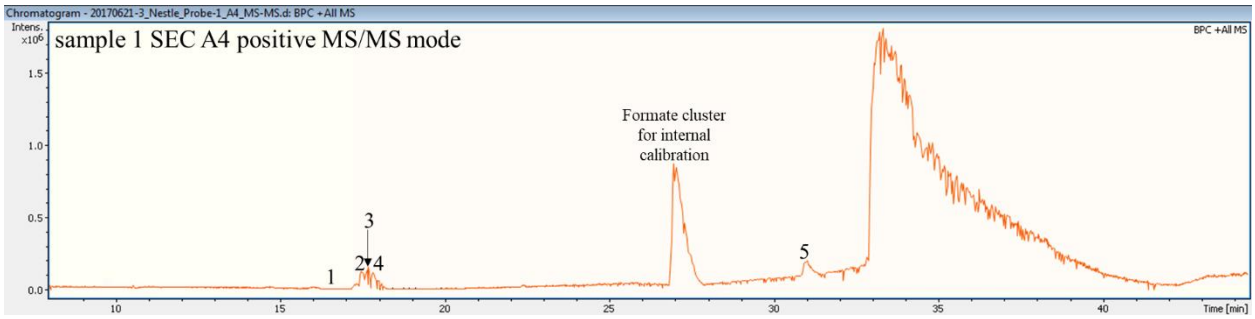
Chromatograms and tables of SEC-fractions A4 to A6 of all three samples.



Supplementary figure 4: Base Peak chromatogram (BPC) of the negative MS/MS mode of SEC-fraction A4 of sample 1. The numbering of the peaks correlates with the numbering in Supplementary table 4.

Supplementary table 4: Results of the three calculation approaches for the most abundant signals (negative mode) in SEC-fraction A4 of sample 1. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 4.

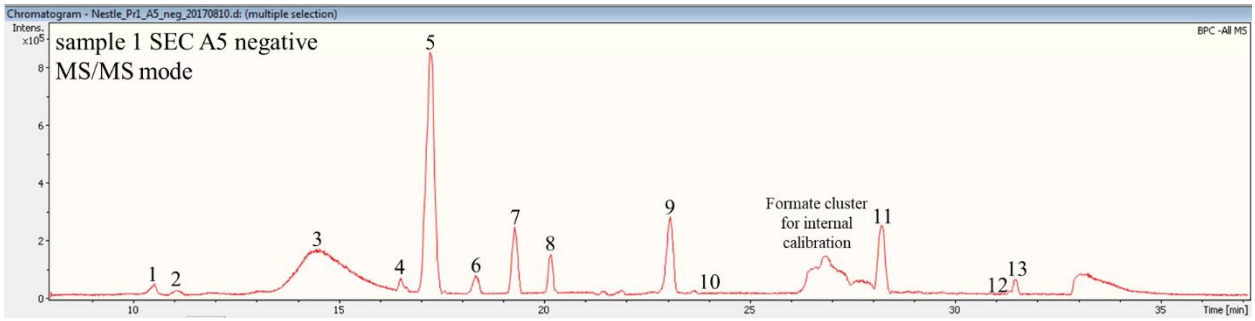
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	I Δ*m/z* I [mDa]	Anno-tations
1	10.41	112.98509	112.98858	/	$C_4H_2O_4$	/	2.94466	SF
1	10.41	130.99226	130.99262	/	$C_3H_4N_2O_2S$	/	0.18296	SF
1	10.41	68.99469	68.99875	/	$C_3H_2O_2$	/	3.51696	SF
2	11.10	146.96553	146.96978	/	$C_3H_4N_2OS_2$	/	3.69812	SF
3	14.51	112.98501	112.98858	/	$C_4H_2O_4$	/	3.01790	SF
3	14.52	68.99461	68.99875	/	$C_3H_2O_2$	/	3.59186	SF
3	14.55	130.99224	130.99262	/	$C_3H_4N_2OS_2$	/	0.16959	SF
4	33.25	190.92793	190.93062	/	$C_5H_4O_2S_3$	/	2.13928	SF



Supplementary figure 5: Base Peak chromatogram (BPC) of the positive MS/MS mode of SEC-fraction A4 of sample 1. The numbering of the peaks correlates with the numbering in Supplementary table 5.

Supplementary table 5: Results of the three calculation approaches for the most abundant signals (positive mode) in SEC-fraction A4 of sample 1. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 5.

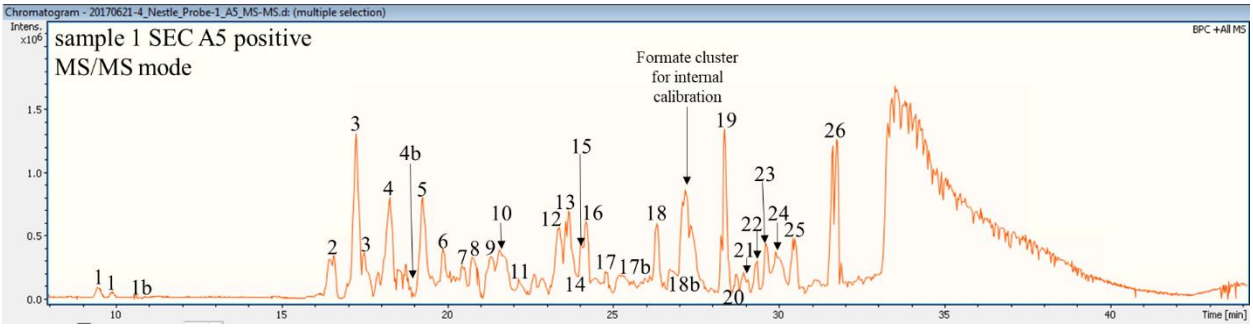
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
1	16.0	217.1050	217.10109	Diketo-Ile-Cys	$C_9H_{16}N_2O_2S$	/	3.91	LSc*
2	17.50	550.32001	550.31931	/	$C_{32}H_{44}N_3O_3P$	√	0.70493	SF
3	17.71	481.26027	481.26165	/	$C_{18}H_{36}N_6O_9$	/	1.37999	SF
4	17.84	520.33165	520.33324 520.3249	/ Val-Val-Arg-Phe	$C_{25}H_{50}N_3O_4PS$	√	1.59587 6.75	SF LSc*
5	31.02	200.97084	/	/	/	√	/	/



Supplementary figure 6: Base Peak chromatogram (BPC) of the negative MS/MS mode of SEC-fraction A5 of sample 1. The numbering of the peaks correlates with the numbering in Supplementary table 6.

Supplementary table 6: Results of the three calculation approaches for the most abundant signals (negative mode) in SEC-fraction A5 of sample 1. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 6.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
1	10.53	68.99466	68.99875	/	$C_3H_2O_2$	/	3.53911	SF
2	11.01	121.02887	121.03005	/	$C_7H_6NO_2$	√	0.63139	SF
3	14.41	117.01879	117.01988	/	$C_4H_6O_4$	/	0.54189	SF
4	16.54	241.11805	241.11724	/	$C_7H_{14}N_8O_2$	√	1.35750	SF
			241.12115	diketo(Glu-Ile)	$C_{11}H_{18}N_2O_4$	√	3.1	LSc*
4	16.5	275.1013	275.1016	pyro(Glu-Phe)	$C_{14}H_{16}N_2O_4$	/	0.0003	LSc*
5	17.25	128.03491	128.03587	pyroglutamic acid	$C_5H_7NO_3$	√	0.40646	SF/SL
5	17.25	257.0764	257.08051	pyro(Glu-Glu)	$C_{10}H_{14}N_2O_6$	/	4.11	LSc*
6	18.36	469.20652	469.20609	/	$C_{14}H_{27}N_{14}O_3P$	√	0.97436	SF
6	18.42	324.15438	324.15601	/	$C_9H_{24}N_7O_4P$	/	1.08589	SF
7	19.31	181.09756	181.09880	/	$C_9H_{14}N_2O_2$	√	0.69188	SF
7	19.31	225.08706	225.08863	/	$C_{10}H_{14}N_2O_4$	√	1.01951	SF
			225.091	pyro(Glu-Pro)	equivalent to SF	√	3.94	LSc*
8	20.17	141.06636	141.0665	diketo(Ala-Ala)	$C_6H_{10}N_2O_3$	√	0.14	LSc*
8	20.17	185.05618	185.0653	diketo(Glu-Gly)	$C_{17}H_{10}N_2O_4$	√	9.12	LSc*
9	23.09	238.08193	238.08388	/	$C_{10}H_{13}N_3O_4$	√	1.40356	SF
9	23.08	513.19237	513.19458	/	$C_{14}H_{31}N_{10}O_9P$	√	1.66183	SF
10	23.82	148.04309	148.04432	L-Methionine	$C_5H_{11}NO_2S$	√	0.68032	SF/SL
11	28.23	145.06152	145.06608	Glutamine	$C_5H_{10}N_2O_3$	√	0.00456	SF
12	31.34	173.10370	173.10495	L-Arginine	$C_6H_{14}N_4O_2$	√	0.70451	SF/SL
12	31.48	154.06162	154.06275	L-Histidine	$C_6H_9N_3O_2$	√	0.58290	SF/SL



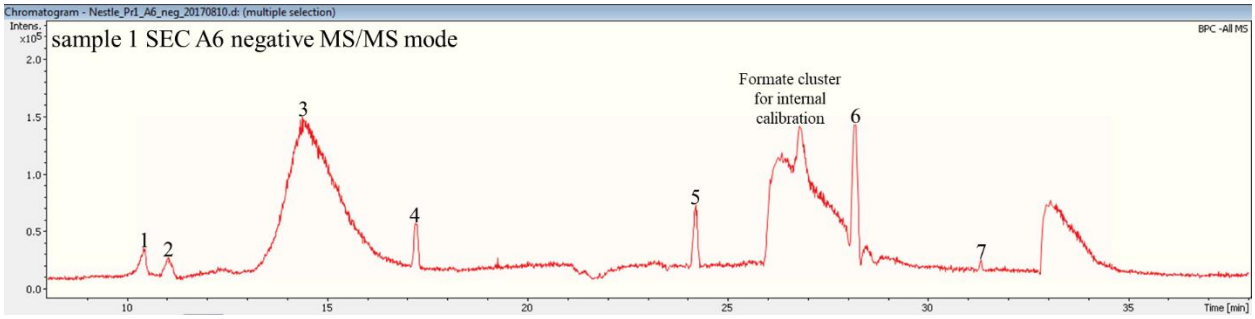
Supplementary figure 7: Base Peak chromatogram (BPC) of the positive MS/MS mode of SEC-fraction A5 of sample 1. The numbering of the peaks correlates with the numbering in Supplementary table 7.

Supplementary table 7: Results of the three calculation approaches for the most abundant signals (positive mode) in SEC-fraction A5 of sample 1. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 7.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	I Δ*m/z* I [mDa]	Anno-tations
1	9.47	211.14413	211.14410	/	$C_{11}H_{18}N_2O_2$	√	0.02346	SF
			211.14467	diketo(Pro-Ile)	equivalent to SF	√	0.54	LSc*
1b	10.6	197.1280	197.12902	diketo(Pro-Val)	$C_{10}H_{16}N_2O_2$	/	1.02	LSc*
2	16.58	277.11829	277.11828	/	$C_{14}H_{16}N_2O_4$	√	0.00999	SF
			277.11885	diketo(Glu-Phe)	equivalent to SF	√	0.56	LSc*
2	16.60	243.13411	243.13393	/	$C_{11}H_{18}N_2O_4$	√	0.17995	SF
			243.1345	diketo(Glu-Ile)	equivalent to SF	√	0.89	LSc*
3	17.27	259.09260	259.09380	/	$C_{11}H_{10}N_6O_2$	√	1.20418	SF
			259.09303	diketo(Glu-Glu)	$C_{10}H_{14}N_2O_6$	√	0.43	LSc*
			259.09303	pyro(Glu-Glu)	$C_{10}H_{14}N_2O_6$	√	0.43	LSc*
3	17.51	374.17101	374.17105	/	$C_{19}H_{23}N_3O_5$	√	0.03751	SF
3	17.59	340.18604	340.18670	/	$C_{16}H_{25}N_3O_5$	√	0.65729	SF
			340.19401	Ile-His-Ala	$C_{15}H_{25}N_5O_4$	√	7.97	LSc*
4	17.99	213.08654	213.08698	/	$C_9H_{12}N_2O_4$	√	0.44054	SF
			213.08755	diketo(Pro-Asp)	equivalent to SF	√	0.57	LSc*
4	18.3	263.1386	263.1396	diketo(Val-Tyr)	$C_{14}H_{18}N_2O_3$	/	1.0	LSc*
4b	18.89	185.09136	185.09207	/	$C_8H_{12}N_2O_3$	√	0.71271	SF
			185.09263	diketo(Ser-Pro)	equivalent to SF	√	0.56	LSc*
4b	18.9	201.0866	201.08755	diketo(Glu-Ala)	$C_8H_{12}N_2O_4$	/	0.95	LSc*
5	19.28	227.10267	227.10263	/	$C_{10}H_{14}N_2O_4$	√	0.04082	SF
			227.1032	pyro(Glu-Pro)	equivalent to SF	√	0.53	LSc*
6	19.8	245.1864	245.18665	Ile-Ile	$C_{12}H_{24}N_2O_3$	/	0.25	LSc*
				Ile-Leu	equivalent to SF			
				Leu-Leu	equivalent to SF			
6	19.90	209.09189	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.17530	SF
			209.10386	diketo(His-Ala)	$C_9H_{12}N_4O_2$	√	11.97	LSc*
6	19.90	226.11862	226.11862	/	$C_{10}H_{15}N_3O_3$	√	0.00090	SF
			226.11918	diketo(Gln-Pro)	equivalent to SF	√	0.56	LSc*
6	19.95	342.23810	342.23873	Leu-Pro-Ile	$C_{17}H_{31}N_3O_4$	√	0.63608	SF/SL
7	20.2	187.0709	187.07189	diketo(Glu-Gly)	$C_7H_{10}N_2O_4$	/	0.99	LSc*
			187.0719	diketo(Asp-Ala)	$C_7H_{10}N_2O_4$	/	1.0	LSc*
7	20.27	212.10271	212.10297	/	$C_9H_{13}N_3O_3$	√	0.25837	SF
			212.10353	diketo(Pro-Asn)	equivalent to SF	√	0.82	LSc*
7	20.3	195.0758	195.0882	diketo(His-Gly)	$C_8H_{10}N_4O_2$	/	12.4	LSc*
7	20.46	263.13907	263.13902	/	$C_{14}H_{18}N_2O_3$	√	0.04999	SF
			263.13958	diketo(Tyr-Val)	equivalent to SF	√	0.51	LSc*
			263.1397	Pro-Phe	equivalent to SF		0.63	LSc*
			263.14307	Met-Ile	$C_{11}H_{22}N_2O_3S$		4.0	LSc*
7	20.55	362.20710	362.20743	Val-Pro-Phe	$C_{19}H_{27}N_3O_4$	√	0.33628	SF/SL
8	20.8	231.0977	231.09811	diketo(Glu-Thr)	$C_9H_{14}N_2O_5$	/	0.41	LSc*
8	20.79	328.22277	328.22308	Val-Pro-Leu	$C_{16}H_{29}N_3O_4$	√	0.31742	SF/SL
9	21.2	324.1549	324.15607	Thr-Gly-Phe	$C_{15}H_{21}N_3O_5$	/	1.17	LSc*
9	21.3	215.1386	215.13958	diketo(Thr-Ile)	$C_{10}H_{18}N_2O_3$	/	0.98	LSc*
			215.1397	Pro-Val	$C_{10}H_{18}N_2O_3$	/	1.1	LSc*
9	21.3	229.1544	229.15535	Ile-Pro	$C_{11}H_{20}N_2O_3$	/	0.95	LSc*
9	21.34	263.13924	263.13902	/	$C_{14}H_{18}N_2O_3$	√	0.22450	SF
			263.13958	Diketo-Tyr-Val	equivalent to SF	√	0.34	LSc*
			263.1397	Phe-Pro	equivalent to SF	√	0.46	LSc*
10	21.6	203.1388	203.1397	Ile-Ala	$C_9H_{18}N_2O_3$	/	0.9	LSc*

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
10	21.61	229.15488	229.15467	/	$C_{11}H_{20}N_2O_3$	√	0.21103	SF
			229.15535	Ile-Pro	equivalent to SF	√	0.47	LSc*
10	21.64	314.20697	314.20743	Val-Pro-Val	$C_{15}H_{27}N_3O_4$	√	0.00046	SF/SL
11	22.19	263.19654	263.19653	/	$C_{12}H_{26}N_2O_4$	√	0.01068	SF
12	22.97	261.14468	261.14450	/	$C_{11}H_{20}N_2O_5$	√	0.18124	SF
			261.14058	γ-Glu-Leu	equivalent to SF	√	4.1	LSc*
12	23.38	258.10852	258.10845	/	$C_{10}H_{15}N_3O_5$	√	0.07481	SF
			258.10901	Diketo-Glu-Gln	equivalent to SF	√	0.49	LSc*
13	23.69	215.1386	215.13902	/	$C_{10}H_{18}N_2O_3$	√	0.01039	SF
			215.13958	Diketo-Thr-Ile	equivalent to SF	√	0.98	LSc*
			215.1397	Pro-Val	equivalent to SF	√	1.1	LSc*
14	23.9	189.1227	189.12405	Val-Ala	$C_8H_{16}N_2O_3$	/	1.35	LSc*
14	23.98	330.20186	330.20235	Ile-Pro-Thr	$C_{15}H_{27}N_3O_5$	√	0.48333	SF/SL
15	24.05	260.16044	260.16048	/	$C_{11}H_{21}N_3O_4$	√	0.03812	SF
			260.16116	Gln-Ile	equivalent to SF	√	0.72	LSc*
15	24.07	219.13394	219.13393	/	$C_9H_{18}N_2O_4$	√	0.00392	SF
			219.13461	Ile-Ser	equivalent to SF	√	0.67	LSc*
16	24.12	150.05738	150.05833	/	$C_5H_{11}NO_2S$	√	0.92447	SF
			150.05440	Methionine	equivalent to SF	√	2.98	LSc*
16	24.14	247.12889	247.12885	/	$C_{10}H_{18}N_2O_5$	√	0.04109	SF
			247.12953	Glu-Val	equivalent to SF	√	0.64	LSc*
17	24.82	187.10721	187.10772	/	$C_8H_{14}N_2O_3$	√	0.50549	SF
			187.10828	Diketo-Ser-Val	equivalent to SF	√	1.07	LSc*
			187.1084	Pro-Ala	equivalent to SF	√	1.19	LSc*
17b	25.1	213.1230	213.12405	Pro-Pro	$C_{10}H_{16}N_2O_3$	/	1.05	LSc*
17b	25.17	175.10662	175.10772	/	$C_7H_{14}N_2O_3$	√	1.10195	SF
			175.11503	Arginine	$C_6H_{14}N_4O_2$	√	8.41	LSc*
17b	25.6	233.1130	233.11388	Asp-Val	$C_9H_{16}N_2O_5$	√	0.88	LSc*
17b	25.61	245.11307	245.11320	/	$C_{10}H_{16}N_2O_5$	√	0.13130	SF
			245.11388	Glu-Pro	equivalent to SF	√	0.81	LSc*
18	26.2	161.0925	261.09275	Ala-Ala	$C_6H_{12}N_2O_3$	/	0.25	LSc*
18	26.34	173.09103	173.09207	/	$C_7H_{12}N_2O_3$	√	1.04221	SF
			173.09263	Diketo-Ala-Thr	equivalent to SF	√	1.6	LSc*
			173.09274	Pro-Gly	equivalent to SF	√	1.71	LSc*
18	26.35	217.11847	217.11828	/	$C_9H_{16}N_2O_4$	√	0.19032	SF
			217.11896	Thr-Pro	equivalent to SF	√	0.49	LSc*
18	26.40	205.11827	205.11962	/	$C_9H_{12}N_6$	√	1.35222	SF
			205.11896	Val-Ser	$C_8H_{16}N_2O_4$	√	0.69	LSc*
18b	26.57	246.14533	246.14483	/	$C_{10}H_{19}N_3O_4$	√	0.49662	SF
			246.14551	Gln-Val	equivalent to SF	√	0.18	LSc*
			246.1455	Gly-Ile-Gly	equivalent to SF	√	0.17	LSc*
			246.14551	Ala-Val-Gly	equivalent to SF	√	0.18	LSc*
19	28.1	235.0921	235.09314	Glu-Ser	$C_8H_{14}N_2O_6$	/	1.04	LSc*
			235.09314	Asp-Thr	$C_8H_{14}N_2O_6$	/	1.04	LSc*
19	28.12	177.08576	177.08698	/	$C_6H_{12}N_2O_4$	/	1.21821	SF
			177.08765	Thr-Gly	equivalent to SF	/	1.89	LSc*
			177.08765	Ser-Ala	equivalent to SF	/	1.89	LSc*
19	28.25	218.11328	218.11353	/	$C_8H_{15}N_3O$	√	0.25316	SF
			218.11421	Gln-Ala	equivalent to SF	√	0.93	LSc*
			218.11421	Ala-Ala-Gly	equivalent to SF	√	0.93	LSc*
19	28.3	191.0394	191.04905	Diketo-Ser-Cys	$C_6H_{10}N_2O_3S$	/	9.65	LSc*
19	28.38	147.07522	147.07642	/	$C_5H_{10}N_2O_3$	√	1.20184	SF
			147.07250	Glutamine	equivalent to SF	√	2.72	LSc*

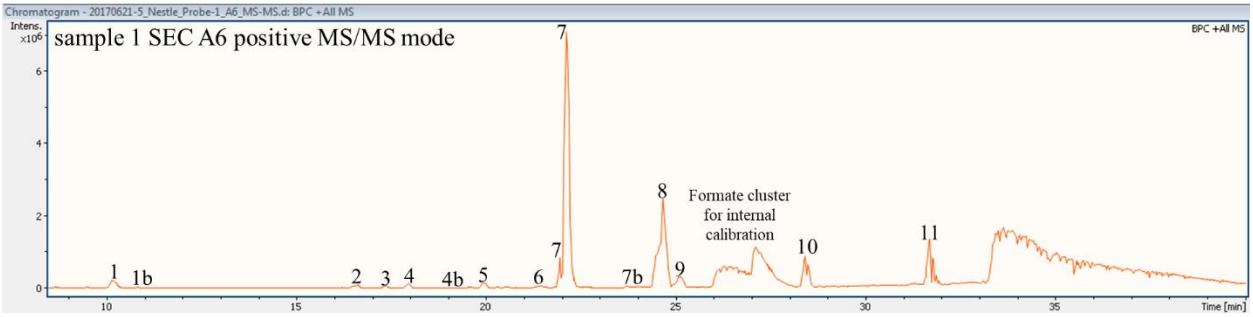
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
20	28.6	276.1190	276.11969	Glu-Gln	$C_{10}H_{17}N_3O_6$	/	0.69	LSc*
20	28.75	166.05360	166.05324	/	$C_5H_{11}NO_3S$	√	0.35539	SF
21	28.9	207.0970	207.09822	Thr-Ser	$C_7H_{14}N_2O_5$	/	1.22	LSc*
21	29.0	163.0723	163.072	Ser-Gly	$C_5H_{10}N_2O_4$	/	0.3	LSc*
21	29.06	204.09767	204.09922	/	$C_8H_9N_7$	√	1.55261	SF
			204.09855	Gln-Gly	$C_7H_{13}N_3O_4$	√	0.88	LSc*
22	29.34	275.13500	275.13500	/	$C_{10}H_{18}N_4O_5$	√	0.00803	SF
			275.13567	Gln-Gln	equivalent to SF	√	0.67	LSc*
23	29.60	193.08126	193.08190	/	$C_6H_{12}N_2O_5$	√	0.63536	SF
			193.08257	Ser-Ser	equivalent to SF		1.31	LSc*
23	29.63	234.10842	234.10845	/	$C_8H_{15}N_3O_5$	√	0.03118	SF
			234.10912	Gln-Ser	equivalent to SF	√	0.7	LSc*
			234.10912	Asn-Thr	equivalent to SF	√	0.7	LSc*
			234.10912	Ala-Ser-Gly	equivalent to SF	√	0.7	LSc*
			234.10911	Gly-Thr-Gly	equivalent to SF	√	0.69	LSc*
24	29.87	291.12761	291.12607	/	$C_{13}H_{22}O_5S$	√	1.53991	SF
			291.13058	Asn-Thr-Gly	$C_{10}H_{18}N_4O_6$	√	2.97	LSc*
			291.13058	Ser-Gln-Gly	$C_{10}H_{18}N_4O_6$	√	2.97	LSc*
24	29.95	309.12908	309.12924	/	$C_{11}H_{20}N_2O_8$	√	0.15794	SF
25	30.40	250.09229	250.09213	/	$C_9H_{15}NO_7$	√	0.16254	SF
25	30.52	162.07696	162.07608	/	$C_6H_{11}NO_4$	/	0.87510	SF
25	30.52	180.08582	180.08665	/	$C_6H_{13}NO_5$	√	0.82983	SF
26	31.8	147.1113	147.10888	Lysine	$C_6H_{14}N_2O_2$	/	2.4	LSc*
26	31.84	156.07667	156.07675	/	$C_6H_9N_3O_2$	√	0.02097	SF
			156.07283	Histidine	equivalent to SF	√	3.84	LSc*
26	31.64	175.11806	175.11895	/	$C_6H_{14}N_4O_2$	√	0.88700	SF
			175.11503	Arginine	equivalent to SF	√	3.03	LSc*



Supplementary figure 8: Base Peak chromatogram (BPC) of the negative MS/MS mode of SEC-fraction A6 of sample 1. The numbering of the peaks correlates with the numbering in Supplementary table 8.

Supplementary table 8: Results of the three calculation approaches for the most abundant signals (negative mode) in SEC-fraction A6 of sample 1. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 8.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	I Δ*m/z* I [mDa]	Anno-tations
1	10.46	112.98519	112.98858	/	$C_4H_2O_4$	/	2.84023	SF
1	10.41	68.99466	68.99875	/	$C_3H_2O_2$	/	3.54378	SF
2	11.06	146.96569	146.96978	/	$C_3H_4N_2OS_2$	/	3.54296	SF
3	14.45	112.98521	112.98858	/	$C_4H_2O_4$	/	2.82333	SF
4	17.25	128.03502	128.03587	/	$C_5H_7NO_3$	/	0.29284	
5	24.22	180.06600	180.06717	L-Tyrosine	$C_9H_{11}NO_3$	√	0.61812	SF/SL
6	28.20	127.05115	127.05185	/	$C_5H_8N_2O_2$	√	0.15287	SF
			127.05185	Diketo-Gly-Ala	equivalent to SF	√	0.7	LSc*
6	28.20	145.06185	145.06241	/	$C_5H_{10}N_2O_3$	√	0.01826	SF
			145.06186	Glutamine	equivalent to SF	√	0.01	LSc*
7	31.34	131.08218	131.08315	/	$C_5H_{12}N_2O_2$	√	0.42234	SF
7	31.34	173.10398	173.10495	L-Arginine	$C_6H_{14}N_4O_2$	√	0.42442	SF/SL
7	31.48	154.06178	154.06275	L-Histidine	$C_6H_9N_3O_2$	√	0.42382	SF/SL

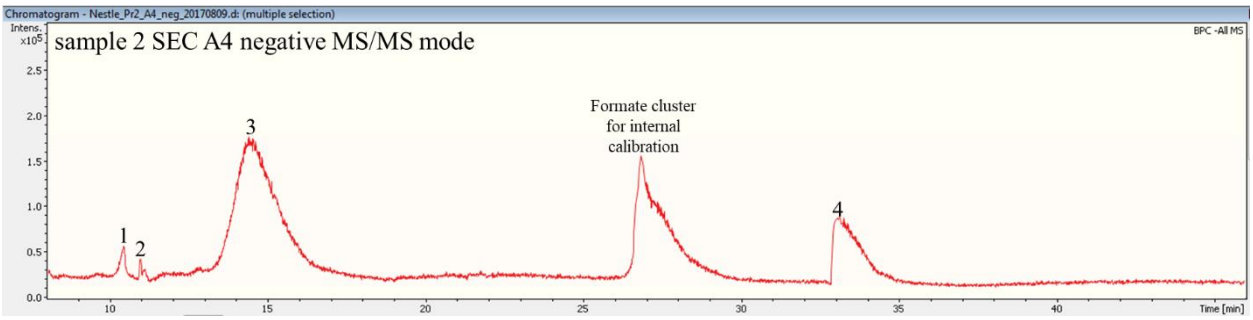


Supplementary figure 9: Base Peak chromatogram (BPC) of the positive MS/MS mode of SEC-fraction A6 of sample 1. The numbering of the peaks correlates with the numbering in Supplementary table 9.

Supplementary table 9: Results of the three calculation approaches for the most abundant signals (positive mode) in SEC-fraction A6 of sample 1. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 9.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	I Δ*m/z* I [mDa]	Anno-tations
1	10.19	245.12888	245.12845	/	$C_{14}H_{16}N_2O_2$	√	0.42689	SF
			245.12902	Diketo-Pro-Phe	equivalent to SF	√	0.14	LSc*
1	10.19	267.11061	267.11012	/	$C_{12}H_{10}N_8$	√	0.48831	SF
			267.10934	Diketo-Glu-His	$C_{11}H_{14}N_4O_4$	√	1.27	LSc*
1b	10.70	231.12020	231.12001	/	$C_7H_{14}N_6O_3$	√	0.18167	SF
			231.11674	Diketo-Met-Val	$C_{10}H_{18}N_2O_2S$	√	3.46	LSc*
2	16.65	277.11818	277.11828	/	$C_{14}H_{16}N_2O_4$	√	0.10130	SF
			277.1143	pyro-Glu-Phe	equivalent to SF	√	3.88	LSc*
			277.11493	Ser-Asn-Gly	$C_9H_{16}N_4O_6$	√	3.25	LSc*
3	17.36	259.09274	259.09246	/	$C_{10}H_{14}N_2O_6$	√	0.27713	SF
			259.09322	pyro-Glu-Glu	equivalent to SF	√	0.48	LSc*
4	17.99	293.11338	293.13320	/	$C_{14}H_{16}N_2O_5$	√	0.18337	SF
			293.11376	Diketo-Glu-Tyr	equivalent to SF	√	0.38	LSc*
4b	19.0	185.0913	185.09263	Diketo-Ser-Pro	$C_8H_{12}N_2O_3$	/	1.33	LSc*
5	19.94	209.09175	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.32316	SF
5	19.96	226.11866	226.11862	/	$C_{10}H_{15}N_3O_3$	√	0.03785	SF
			226.11918	Diketo-Gln-Pro	equivalent to SF	√	0.52	LSc*
5	20.34	212.10288	212.10699	/	$C_{14}H_{13}NO$	√	4.10848	SF
			212.10353	Diketo-Pro-Asn	$C_9H_{13}N_3O_3$	√	0.65	LSc*
5	20.54	426.20140	426.20235	Tyr-Pro-Phe	$C_{23}H_{27}N_3O_5$	√	0.94263	SF/SL
6	21.45	263.13912	263.13902	/	$C_{14}H_{18}N_2O_3$	√	0.10152	SF
			263.13958	Diketo-Tyr-Val	equivalent to SF	√	0.46	LSc*
			263.1397	Pro-Phe	equivalent to SF	√	0.58	LSc*
7	22.06	166.08759	166.08626	/	$C_9H_{11}NO_2$	√	1.44882	SF
			166.08233	Phenylalanine	equivalent to SF	√	5.26	LSc*
7b	23.73	279.13378	279.13393	/	$C_{14}H_{18}N_2O_4$	√	0.14993	SF
			279.13461	Tyr-Pro	equivalent to SF	√	0.83	LSc*
7b	23.74	294.14494	294.14483	Gln-Phe	$C_{14}H_{19}N_3O_4$	√	0.11083	SF/SL
7b	24.01	253.11838	253.11828	/	$C_{12}H_{16}N_2O_4$	√	0.09293	SF
			253.11896	Tyr-Ala	equivalent to SF	√	0.58	LSc*
			253.11896	Ser-Phe	equivalent to SF	√	0.58	LSc*
8	24.7	147.0425	147.07250	Glutamine	$C_5H_{10}N_2O_3$	/	30.0	LSc*
8	24.69	182.08079	182.08117	/	$C_9H_{11}NO_3$	√	0.38285	SF
			182.07725	Tyrosine	equivalent to SF	√	3.54	LSc*

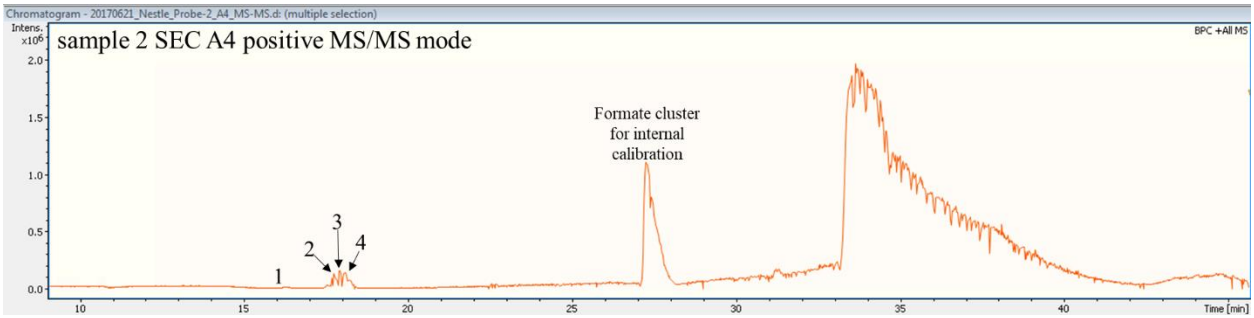
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
8	24.7	221.0920	221.0926	Diketo-Tyr-Gly	$C_{11}H_{17}N_3O_5$	/	0.6	LSc*
			221.09612	Met-Ala	$C_8H_{16}N_2O_3S$	/	4.12	LSc*
			221.09612	Cys-Val	$C_8H_{16}N_2O_3S$	/	4.12	LSc*
9	25.13	254.16155	254.16115	/	$C_{11}H_{19}N_5O_2$	√	0.40268	SF
			254.16172	Diketo-Pro-Arg	equivalent to SF	√	0.17	LSc*
10	28.4	147.0753	147.07250	Glutamine	$C_5H_{10}N_2O_3$	/	2.8	LSc*
10	28.41	169.05766	169.05291	/	$C_5H_{12}O_4S$	√	4.75245	SF
10	28.44	191.03965	191.03859	/	$C_8H_6N_4S$	/	1.02360	SF
			191.04905	Diketo-Cys-Ser	$C_6H_{10}N_2O_3S$	/	9.4	LSc*
11	31.7	156.0764	156.07283	Histidine	$C_6H_9N_3O_2$	/	3.57	LSc*
11	31.75	175.11829	175.11895	/	$C_6H_{14}N_4O_2$	√	0.66498	SF
			175.11503	Arginine	equivalent to SF	√	3.26	LSc*



Supplementary figure 10: Base Peak chromatogram (BPC) of the negative MS/MS mode of SEC-fraction A4 of sample 2. The numbering of the peaks correlates with the numbering in Supplementary table 10.

Supplementary table 10: Results of the three calculation approaches for the most abundant signals (negative mode) in SEC-fraction A4 of sample 2. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 10.

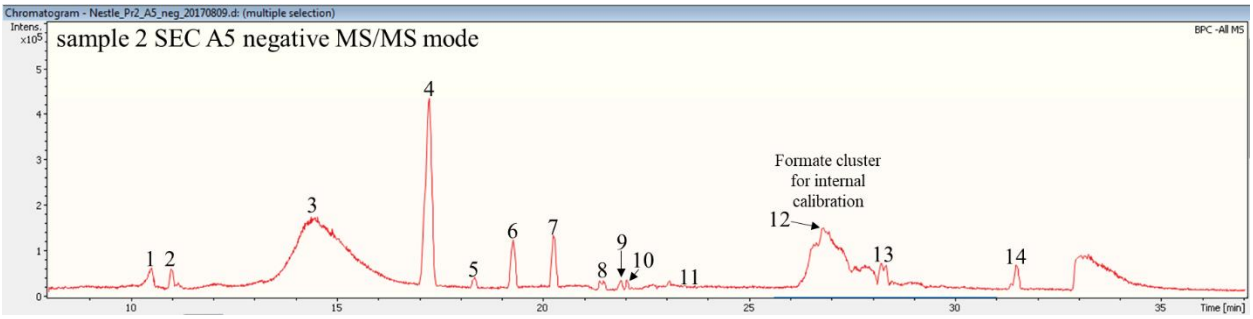
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
1	10.45	68.99485	68.99875	/	$C_3H_2O_2$	/	3.35671	SF
1	10.45	112.98515	112.98858	/	$C_4H_2O_4$	/	2.88355	SF
2	11.03	121.02911	121.03005	/	$C_7H_6O_2$	√	0.39385	SF
2	11.03	165.01912	165.01988	/	$C_8H_6O_4$	√	0.21352	SF
3	14.45	68.99479	68.99875	/	$C_3H_2O_2$	/	3.41042	SF
3	14.45	112.98509	112.98858	/	$C_4H_2O_4$	/	2.94539	SF
4	33.20	190.92809	190.93062	/	$C_5H_4O_2S_3$	√	1.97747	SF



Supplementary figure 11: Base Peak chromatogram (BPC) of the positive MS/MS mode of SEC-fraction A4 of sample 2. The numbering of the peaks correlates with the numbering in Supplementary table 11.

Supplementary table 11: Results of the three calculation approaches for the most abundant signals (positive mode) in SEC-fraction A4 of sample 2. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 11.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
1	16.2	217.1041	217.10109	Diketo-Ile-Cys	$C_9H_{16}N_2O_2S$	/	3.01	LSc*
2	17.78	432.28071	432.27898	/	$C_{15}H_{33}N_{11}O_4$	√	1.73828	SF
2	17.84	438.2398	438.23539	Gln-Tyr-Lys	$C_{20}H_{21}N_4O_6$	/	4.41	LSc*
3	18.0	459.2797	459.27933	Gln-Arg-Arg	$C_{17}H_{30}N_6O_3$	/	3.7	LSc*
3	17.95	476.3068	476.30788	/	$C_{21}H_{41}N_5O_7$	√	1.24476	SF
4	18.1	503.3068	503.30555	Ala-Thr-Arg-Arg	$C_{19}H_{38}N_{10}O_6$	/	1.25	LSc*
4	18.10	520.33316	520.33409 520.3249	/ Val-Val-Arg-Phe	$C_{23}H_{45}N_5O_8$ $C_{25}H_{31}N_7O_7$	/ /	0.92655 8.26	SF LSc*
4	18.1	526.2913	526.29906	Glu-Lys-His-Ile	$C_{23}H_{39}N_7O_7$	/	7.76	LSc*

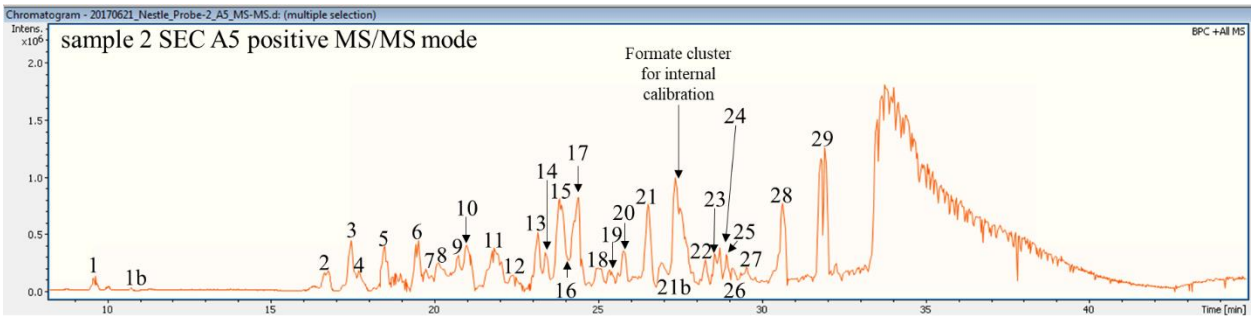


Supplementary figure 12: Base Peak chromatogram (BPC) of the negative MS/MS mode of SEC-fraction A5 of sample 2. The numbering of the peaks correlates with the numbering in Supplementary table 12.

Supplementary table 12: Results of the three calculation approaches for the most abundant signals (negative mode) in SEC-fraction A5 of sample 2. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 12.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
1	10.50	68.99647	68.99875	/	$C_3H_2O_2$	/	1.73816	SF
1	10.50	112.98571	112.98858	/	$C_4H_2O_4$	/	2.32611	SF
2	11.04	121.02963	121.03005	/	$C_7H_6O_2$	√	0.12672	SF
2	11.03	165.01931	165.01988	/	$C_8H_6O_4$	√	0.02570	SF
3	14.46	68.99644	68.99875	/	$C_3H_2O_2$	/	1.76617	SF
3	14.46	112.98565	112.98858	/	$C_4H_2O_4$	/	2.38512	SF
3	14.55	89.02472	89.02497	/	$C_3H_6O_3$	/	0.29753	SF
4	17.28	128.03537	128.03587	Pyroglutamic acid	$C_5H_7NO_3$	√	0.04825	SF/SL
4	17.28	257.07811	257.08011	/	$C_8H_{19}O_7P$	√	1.45506	SF
			257.07847	pyro-Glu-Glu	$C_{10}H_{14}N_2O_6$	√	0.36	LSc*
5	18.37	469.21034	469.21146	/	$C_{22}H_{35}N_2O_7P$	√	0.57737	SF
5	18.42	324.15666	324.15870	/	$C_{13}H_{28}NO_6P$	/	1.49170	SF
6	19.32	225.08783	225.09028	/	$C_8H_{19}O_5P$	√	1.90306	SF
			225.0876	pyro-Glu-Pro	$C_{10}H_{14}N_2O_4$	√	0.23	LSc*
6	19.33	181.09807	181.10045	/	$C_7H_{19}O_3P$	√	1.83870	SF
7	20.32	128.03536	128.03587	/	$C_5H_7NO_3$	√	0.04649	SF
7	20.32	239.06705	239.06789	/	$C_{10}H_{12}N_2O_5$	√	0.29465	SF
			239.0603	Diketo-His-Cys	$C_9H_{12}N_4O_2S$	√	6.75	LSc*
7	20.32	257.07780	257.08011	/	$C_8H_{19}O_7P$	√	1.76513	SF
			257.0774	Diketo-Glu-Glu	$C_{10}H_{14}N_2O_6$	√	0.4	LSc*
8	21.47	201.12421	201.12501	/	$C_9H_{18}N_2O_3$	√	0.25653	SF
			201.08863	Diketo-Thr-Thr	$C_8H_{14}N_2O_4$	√	35.58	LSc*
8	21.48	237.10110	237.10152	/	$C_8H_{19}N_2O_4P$	/	0.13334	SF
			237.1083	Diketo-Thr-Phe	$C_{15}H_{16}N_2O_3$	/	7.2	LSc*
9	21.89	166.06397	166.06440	/	$C_5H_{14}NO_3P$	√	0.11134	SF
10	22.07	259.12956	259.13049	/	$C_{11}H_{20}N_2O_5$	√	0.38356	SF
			259.1287	γ-Glu-Ile	$C_{11}H_{20}N_2O_5$	√	0.86	LSc*
11	23.78	148.04360	148.04432	L-Methionine	$C_5H_{11}NO_2S$	√	0.17149	SF/SL
12	26.87	146.04568	146.04643	L-Glutamate	$C_5H_9NO_4$	√	0.20251	SF/SL
13	28.21	127.05128	127.05185	/	$C_5H_8N_2O_2$	√	0.02514	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
13	28.21	145.06170	145.06241	/	$C_5H_{10}N_2O_3$	√	0.16891	SF
			145.06186	Glutamine	equivalent to SF	√	0.16	LSc*
13	28.35	200.02309	200.02360	/	$C_6H_8N_3O_3P$	√	0.03655	SF
13	28.40	172.02223	172.02319	/	$C_{10}H_7NS$	√	0.41561	SF
14	31.35	173.10423	173.10495	L-Arginine	$C_6H_{14}N_4O_2$	√	0.17286	SF/SL
14	31.54	154.06200	154.06275	L-Histidine	$C_6H_9N_3O_2$	√	0.19602	SF/SL



Supplementary figure 13: Base Peak chromatogram (BPC) of the positive MS/MS mode of SEC-fraction A5 of sample 2. The numbering of the peaks correlates with the numbering in Supplementary table 13.

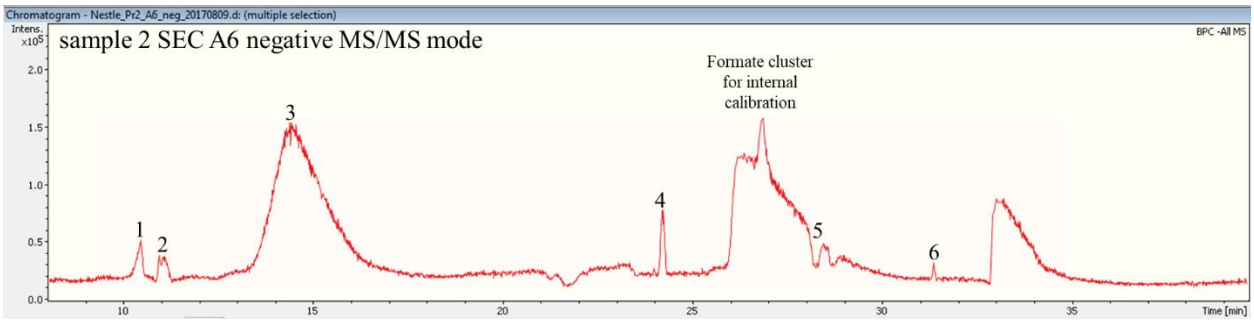
Supplementary table 13: Results of the three calculation approaches for the most abundant signals (positive mode) in SEC-fraction A5 of sample 2. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 13.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
1	9.61	421.28001	421.28093	/	$C_{22}H_{36}N_4O_4$	√	0.92054	SF
1	9.66	211.14409	211.14410	/	$C_{11}H_{18}N_2O_2$	√	0.01529	SF
			211.14467	Diketo-Pro-Ile	equivalent to SF	√	0.58	LSc*
1b	10.7	197.1277	197.12902	Diketo-Pro-Val	$C_{16}H_{16}N_2O_2$	/	1.32	LSc*
2	16.77	243.13427	243.13393	/	$C_{11}H_{18}N_2O_4$	/	0.33560	SF
			243.1345	Diketo-Glu-Ile	equivalent to SF	/	0.23	LSc*
2	16.8	261.1316	261.1239	Diketo-Tyr-Pro	$C_{14}H_{16}N_2O_3$	/	7.7	LSc*
2	16.8	277.1192	277.1189	Diketo-Glu-Phe	$C_{14}H_{16}N_2O_4$	/	0.3	LSc*
3	17.58	277.10257	277.10263	/	$C_{10}H_{14}N_2O_4$	√	0.05805	SF
3	17.47	259.09268	259.09246	/	$C_{10}H_{14}N_2O_6$	√	0.22274	SF
			259.09322	pyro-Glu-Glu	equivalent to SF	√	0.54	LSc*
4	17.76	340.18615	340.18670	/	$C_{16}H_{25}N_3O_5$	√	0.07679	SF
			340.19401	Ile-His-Ala	$C_{15}H_{25}N_5O_4$	√	7.86	LSc*
4	17.72	374.17105	374.17105	/	$C_{19}H_{23}N_3O_5$	√	0.07679	SF
5	18.2	195.0757	195.0882	Diketo-His-Gly	$C_8H_{10}N_4O_2$	/	12.5	LSc*
5	18.19	213.08683	213.08698	/	$C_9H_{12}N_2O_4$	√	0.15346	SF
			213.08755	Diketo-Pro-Asp	equivalent to SF	√	0.72	LSc*
5	18.56	326.17052	326.17105	/	$C_{15}H_{23}N_3O_5$	√	0.52666	SF
6	19.1	185.0916	185.09263	Diketo-Ser-Pro	$C_8H_{12}N_2O_3$	/	1.03	LSc*
6	19.13	201.08699	201.08832	/	$C_9H_8N_6$	√	1.33133	SF
			201.08755	Diketo-Glu-Ala	$C_8H_{12}N_2O_4$	√	0.56	LSc*
6	19.53	227.10291	227.10263	/	$C_{10}H_{14}N_2O_4$	√	0.27606	SF
			227.1032	Diketo-Glu-Pro	equivalent to SF	√	0.29	LSc*

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
7	19.9	245.1862	245.18665	Ile-Ile	$C_{12}H_{24}N_2O_3$	√	0.45	LSc*
				Leu-Ile	$C_{12}H_{24}N_2O_3$	√	0.45	LSc*
				Leu-Leu	$C_{12}H_{24}N_2O_3$	√	0.45	LSc*
7	19.98	376.22310	376.22308	Ile-Pro-Phe	$C_{20}H_{29}N_3O_4$	√	0.01506	SF/SL
8	20.09	209.09195	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.11479	SF
			209.10386	Diketo-His-Ala	$C_9H_{12}N_4O_2$	√	11.91	LSc*
8	20.10	226.11863	226.11862	/	$C_{10}H_{15}N_3O_3$	√	0.01270	SF
			226.1192	Diketo-Pro-Gln	equivalent to SF	√	0.57	LSc*
8	20.12	248.10081	248.10462	/	$C_{10}H_{18}NO_4P$	√	3.80985	SF
8	20.15	342.23815	342.23873	Leu-Pro-Ile	$C_{17}H_{31}N_3O_4$	√	0.58172	SF/SL
9	20.4	187.0702	187.0719	Diketo-Asp-Ala	$C_7H_{10}N_2O_4$	/	1.7	LSc*
			187.0719	Diketo-Glu-Gly	$C_7H_{10}N_2O_4$	/	1.7	LSc*
9	20.46	212.10276	212.10297	/	$C_9H_{13}N_3O_3$	√	0.20506	SF
			212.10353	Diketo-Pro-Asn	equivalent to SF	√	0.77	LSc*
9	20.67	324.15453	324.15540	/	$C_{15}H_{21}N_3O_5$	√	0.87036	SF
			324.15607	Thr-Gly-Phe	equivalent to SF	√	1.54	LSc*
9	20.67	263.13919	263.13902	/	$C_{14}H_{18}N_2O_3$	√	0.16869	SF
			263.13958	Diketo-Val-Tyr	equivalent to SF	√	0.39	LSc*
			263.1397	Pro-Phe	equivalent to SF	√	0.51	LSc*
9	20.73	229.15454	229.15535	Ile-Pro	$C_{11}H_{20}N_2O_3$	√	0.00081	LSc*
9	20.78	259.09244	259.09246	/	$C_{10}H_{14}N_2O_6$	√	0.02167	SF
			259.09322	pyro-Glu-Glu	equivalent to SF	√	0.78	LSc*
9	20.75	362.20692	362.20743	Val-Pro-Phe	$C_{19}H_{27}N_3O_4$	√	0.51349	SF/SL
9	20.83	360.19446	360.19515	Ile-Pro-Met	$C_{16}H_{29}N_3O_4S$	√	0.69239	SF/SL
10	21.00	328.22251	328.22308	Val-Pro-Leu	$C_{16}H_{29}N_3O_4$	√	0.56902	SF/SL
11	21.9	203.1381	203.1032	Diketo-Thr-Thr	$C_8H_{14}N_2O_4$	/	0.0349	LSc*
			203.1397	Ile-Ala	$C_9H_{18}N_2O_3$	/	0.0016	LSc*
11	21.87	229.1548	229.15467	/	$C_{11}H_{20}N_2O_3$	√	0.15597	SF
			229.15535	Ile-Pro	equivalent to SF	√	0.55	LSc*
11	21.87	314.20709	314.20743	Val-Pro-Val	$C_{15}H_{27}N_3O_4$	√	0.33811	SF/SL
11	21.9	334.1767	334.17682	Pro-Phe-Ala	$C_{17}H_{23}N_3O_4$	/	0.12	LSc*
12	22.40	263.19670	263.19653	/	$C_{12}H_{26}N_2O_4$	√	0.16440	SF
12	22.4	285.1788	285.1675	Diketo-Gln-Arg	$C_{11}H_{20}N_6O_3$	/	11.3	LSc*
12	22.4	353.0930	353.09547	Cys-Gln-Cys	$C_{11}H_{13}N_4O_3S$	/	2.47	LSc*
13	23.2	197.1278	197.12902	Diketo-Pro-Val	$C_{10}H_{16}N_2O_2$	/	1.22	LSc*
13	23.18	243.13398	243.13393	/	$C_{11}H_{18}N_2O_4$	√	0.04580	SF
			243.1345	Diketo-Glu-Ile	equivalent to SF	√	0.52	LSc*
13	23.18	261.14465	261.14450	γ-Glu-Leu	$C_{11}H_{20}N_2O_5$	√	0.15433	LSc*
14	23.4	189.1229	189.12405	Val-Ala	$C_8H_{16}N_2O_3$	/	1.15	LSc*
14	23.43	261.14482	261.14450	/	$C_{11}H_{20}N_2O_5$	√	0.32213	SF
			261.14518	Glu-Leu	equivalent to SF	√	0.36	LSc*
14	23.41	314.13444	314.13466	/	$C_{13}H_{19}N_3O_6$	√	0.22442	SF
15	23.72	358.19700	358.19726	Ile-Pro-Glu	$C_{16}H_{27}N_3O_6$	√	0.25997	SF/SL
15	23.81	215.13910	215.13902	/	$C_{10}H_{18}N_2O_3$	√	0.07824	SF
			215.1396	Diketo-Thr-Ile	equivalent to SF	√	0.5	LSc*
			215.1397	Val-Pro	equivalent to SF	√	0.6	LSc*
15	23.8	247.1289	247.12953	Glu-Val	$C_{10}H_{18}N_2O_5$	/	0.63	LSc*
16	24.17	330.20198	330.20235	Ile-Pro-Thr	$C_{15}H_{27}N_3O_5$	√	0.36261	SF/SL
17	24.40	150.05749	150.05495	/	$C_8H_7NO_2$	√	2.53754	SF
			150.06609	Methionine	$C_5H_{11}NO_2S$	√	8.6	LSc*
17	24.49	279.10086	279.10092	/	$C_{10}H_{18}N_2O_5S$	√	0.05417	SF
			279.0981	Diketo-Asp-Tyr	$C_{13}H_{14}N_2O_5$	√	2.76	LSc*
			279.1016	Glu-Met	equivalent to SF	√	0.74	LSc*
18	25.07	187.10707	187.10772	/	$C_8H_{14}N_2O_3$	√	0.57766	SF
			187.10828	Diketo-Ser-Val		√	1.21	LSc*

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
			187.1084	Pro-Ala	equivalent to SF	√	1.33	LSc*
					equivalent to SF			
18	25.09	247.12903	247.12885	Glu-Val	$C_{10}H_{18}N_2O_5$	√	0.18275	LSc*
19	25.33	213.12318	213.12337	/	$C_{10}H_{16}N_2O_3$	√	0.18352	SF
			213.12405	Pro-Pro	equivalent to SF	√	0.87	LSc*
19	25.35	175.10661	175.10772	/	$C_7H_{14}N_2O_3$	√	1.10950	SF
			175.11503	Arginine	$C_6H_{14}N_4O_2$	√	8.42	LSc*
19	25.4	219.1338	219.13461	Ile-Ser	$C_9H_{18}N_2O_4$	/	0.81	LSc*
19	25.43	254.16135	254.16115	/	$C_{11}H_{19}N_5O_2$	√	0.20051	SF
			254.1617	Diketo-Pro-Arg	equivalent to SF	√	0.35	LSc*
20	25.7	233.1132	233.11388	Asp-Val	$C_9H_{16}N_2O_5$	/	0.68	LSc*
20	25.8	235.1191	235.1195	Diketo-His-Pro	$C_{11}H_{14}N_4O_2$	/	0.4	LSc*
			235.1118	Leu-Cys	$C_9H_{18}N_2O_3S$	/	7.3	LSc*
20	25.81	245.11362	245.11320	/	$C_{10}H_{16}N_2O_5$	√	0.42041	SF
			245.11388	Glu-Pro	equivalent to SF	√	0.26	LSc*
21	26.4	161.0928	161.09275	Ala-Ala	$C_6H_{12}N_2O_3$	/	0.05	LSc*
21	26.5	173.0913	173.0926	Diketo-Ala-Thr	$C_7H_{12}N_2O_3$	/	1.3	LSc*
			173.0927	Pro-Gly	$C_7H_{12}N_2O_3$	/	1.4	LSc*
21	26.65	205.11806	205.11828	/	$C_8H_{16}N_2O_4$	√	0.22119	SF
			205.11896	Val-Ser	equivalent to SF	√	0.9	LSc*
21	26.55	217.11840	217.11962	/	$C_{10}H_{12}N_6$	√	1.21990	SF
			217.11896	Thr-Pro	$C_9H_{16}N_2O_4$	√	0.56	LSc*
21	26.75	246.14504	246.14483	/	$C_{10}H_{19}N_3O_4$	√	0.20435	SF
			246.14551	Gln-Val	equivalent to SF	√	0.47	LSc*
			246.1455	Gly-Ile-Gly	equivalent to SF	√	0.46	LSc*
21	27.22	148.05909	148.06043	/	$C_5H_9NO_4$	√	1.34674	SF
b			148.05990	Glutamic acid	equivalent to SF	√	0.81	LSc*
22	28.33	177.08589	177.08698	/	$C_6H_{12}N_2O_4$	√	1.09651	SF
			177.08765	Thr-Gly	equivalent to SF	√	1.76	LSc*
			177.08766	Ser-Ala	equivalent to SF	√	1.77	LSc*
22	28.30	235.09247	235.09246	/	$C_8H_{14}N_2O_6$	√	0.00287	SF
			235.09314	Glu-Ser	equivalent to SF	√	0.67	LSc*
			235.09314	Asp-Thr	equivalent to SF	√	0.67	LSc*
22	28.29	257.07430	257.07431	/	$C_{14}H_{12}N_2OS$	√	0.01105	SF
23	28.4	218.1130	218.11421	Gln-Ala	$C_8H_{15}N_3O_4$	/	1.21	LSc*
			218.11421	Ala-Ala-Gly	$C_8H_{15}N_3O_4$	/	1.21	LSc*
23	28.5	191.0392	191.04905	Diketo-Ser-Cys	$C_6H_{10}N_2O_3S$	/	9.85	LSc*
23	28.59	147.07518	147.07642	/	$C_5H_{10}N_2O_3$	√	1.23821	SF
			147.07642	Glutamine	equivalent to SF	√	1.24	LSc*
23	28.72	276.11909	276.11901	/	$C_{10}H_{17}N_3O_6$	√	0.07809	SF
			276.11969	Glu-Gln	equivalent to SF	√	0.6	LSc*
24	28.71	258.10859	258.10845	/	$C_{10}H_{15}N_3O_5$	√	0.13979	SF
			258.10901	Diketo-Glu-Gln	equivalent to SF	√	0.42	LSc*
24	28.82	456.20672	456.20755	/	$C_{18}H_{33}NO_{12}$	√	0.83391	SF
25	28.95	166.05398	166.05324	/	$C_5H_{11}NO_3S$	√	0.73803	SF
25	29.01	207.09730	207.09755	/	$C_7H_{14}N_2O_5$	√	0.24944	SF
			207.09822	Thr-Ser	equivalent to SF	√	0.92	LSc*
26	29.13	163.07190	163.07133	/	$C_5H_{10}N_2O_4$	√	0.56750	SF
			163.0720	Gly-Ser	equivalent to SF	√	0.1	LSc*
26	29.3	204.0974	204.09855	Gln-Gly	$C_7H_{13}N_3O_4$	/	1.15	LSc*
			204.09855	Gly-Ala-Gly	$C_7H_{13}N_3O_4$	/	1.15	LSc*
26	29.22	365.10531	365.10515	/	$C_{10}H_{16}N_6O_9$	√	0.16028	SF
27	29.59	252.10779	252.10778	/	$C_9H_{17}NO_7$	√	0.00814	SF
			252.1097	Diketo-Asn-His	$C_{10}H_{13}N_5O_3$	√	1.91	LSc*
27	29.57	282.11804	282.11834	/	$C_{10}H_{19}NO_8$	√	0.30174	SF
27	29.56	310.11273	310.11326	/	$C_{11}H_{19}NO_9$	√	0.52982	SF
27	29.8	193.0812	193.08257	Ser-Ser	$C_6H_{12}N_2O_5$	/	1.37	LSc*
27	29.8	234.1068	234.10912	Gln-Ser	$C_8H_{15}N_3O_5$	/	2.32	LSc*

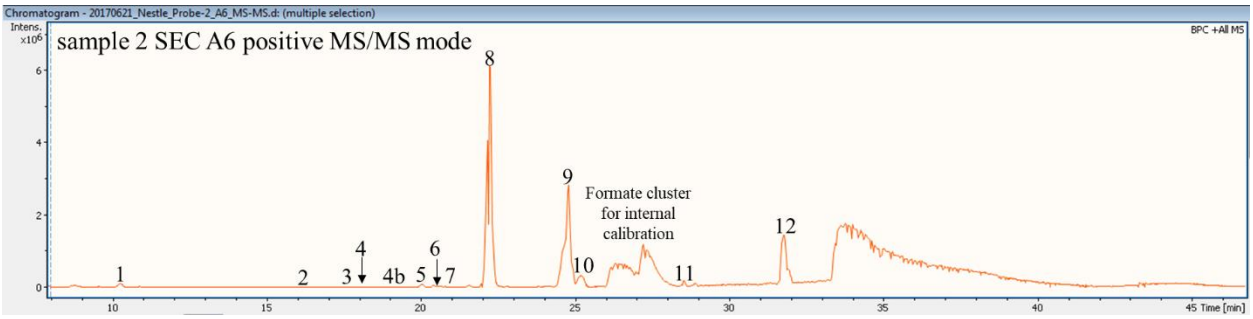
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
			234.10912	Ala-Ser-Gly	$C_8H_{15}N_3O_5$	/	2.32	LSc*
			234.10911	Gly-Thr-Gly	$C_8H_{15}N_3O_5$	/	2.31	LSc*
28	30.64	202.06799	202.06850	/	$C_{12}H_{11}NS$	√	0.50627	SF
28	30.56	268.10268	268.10269	/	$C_9H_{17}NO_8$	√	0.00756	SF
28	30.66	359.16517	359.16517	/	$C_{14}H_{31}O_6PS$	√	0.00166	SF
29	31.8	147.1118	147.11281	Lysine	$C_6H_{14}N_2O_2$	/	1.01	LSc*
29	31.8	156.0772	156.07676	Histidine	$C_6H_9N_3O_2$	/	0.44	LSc*
29	31.82	175.11818	175.11895	/	$C_6H_{14}N_4O_2$	√	0.76756	SF
			175.11503	Arginine	equivalent to SF	√	3.15	LSc*



Supplementary figure 14: Base Peak chromatogram (BPC) of the negative MS/MS mode of SEC-fraction A6 of sample 2. The numbering of the peaks correlates with the numbering in Supplementary table 14.

Supplementary table 14: Results of the three calculation approaches for the most abundant signals (negative mode) in SEC-fraction A6 of sample 2. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 14.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
1	10.46	68.99484	68.99875	/	$C_3H_2O_2$	/	3.36455	SF
1	10.46	112.98518	112.98858	/	$C_4H_2O_4$	/	2.84867	SF
2	11.00	121.02908	121.03005	/	$C_7H_6O_2$	/	0.41910	SF
2	11.00	165.01900	165.01988	/	$C_8H_6O_4$	/	0.33629	SF
3	13.99	311.16713	311.16900	/	$C_9H_{24}N_6O_6$	/	1.33014	SF
3	14.19	297.15141	297.15335	/	$C_8H_{22}N_6O_6$	/	1.39596	SF
4	24.23	180.06598	180.06717	L-Tyrosine	$C_9H_{11}NO_3$	√	0.63838	SF/SL
5	28.1	127.0503	127.0508	Diketo-Gly-Ala	$C_5H_8N_2O_2$	/	0.5	LSc*
5	28.21	145.06155	145.06241	/	$C_5H_{10}N_2O_3$	/	0.31764	SF
			145.06186	Glutamine	equivalent to SF	/	0.31	LSc*
6	31.38	173.10403	173.10495	L-Arginine	$C_6H_{14}N_4O_2$	√	0.33811	SF/SL
6	31.55	154.06169	154.06275	L-Histidine	$C_6H_9N_3O_2$	√	0.50710	SF/SL

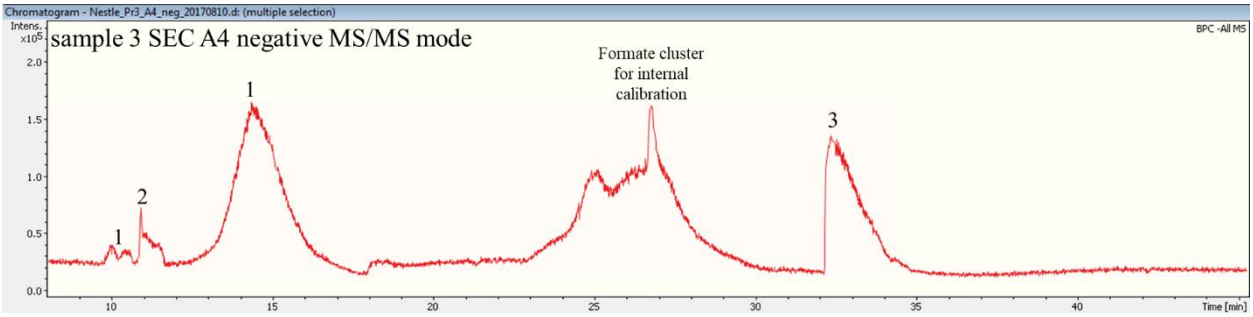


Supplementary figure 15: Base Peak chromatogram (BPC) of the positive MS/MS mode of SEC-fraction A6 of sample 2. The numbering of the peaks correlates with the numbering in Supplementary table 15.

Supplementary table 15: Results of the three calculation approaches for the most abundant signals (positive mode) in SEC-fraction A6 of sample 2. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 15.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
1	10.27	245.12878	245.12845	/	$C_{14}H_{16}N_2O_2$	√	0.32483	SF
			245.12902	Diketo-Phe-Pro	equivalent to SF	√	0.24	LSc*
1	10.3	267.1113	267.1093	Diketo-Glu-His	$C_{11}H_{14}N_4O_4$	/	2.0	LSc*
2	16.4	277.1197	277.11828	pyro-Glu-Phe	$C_{14}H_{16}N_2O_4$	/	1.42	LSc*
3	17.6	259.0923	259.09246	pyro-Glu-Glu	$C_{10}H_{14}N_2O_6$	/	0.16	LSc*
4	18.07	293.11337	293.11320	/	$C_{14}H_{16}N_2O_5$	√	0.16877	SF
			293.11376	Diketo-Glu-Tyr	equivalent to SF	√	0.39	LSc*
4b	19.1	185.0916	185.09263	Diketo-Ser-Pro	$C_8H_{12}N_2O_3$	/	1.03	LSc*
5	20.07	209.09212	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.04759	SF
5	20.06	226.11902	226.11862	/	$C_{10}H_{15}N_3O_3$	√	0.03785	SF
			226.11918	Diketo-Gln-Pro	equivalent to SF	√	0.16	LSc*
5	20.07	248.10100	248.10028	/	$C_8H_9N_9O$	√	0.71390	SF
6	20.44	212.10315	212.10297	/	$C_9H_{13}N_3O_3$	√	0.18616	SF
			212.10353	Diketo-Asn-Pro	equivalent to SF	√	0.38	LSc*
6	20.85	392.21786	392.21800	Tyr-Pro-Leu	$C_{20}H_{29}N_3O_5$	√	0.14057	SF/SL
7	21.6	159.0512	159.07698	Diketo-Ala-Ser	$C_6H_{10}N_2O_4$	/	25.78	LSc*
			159.07697	Diketo-Thr-Gly	$C_6H_{10}N_2O_4$	/	25.77	LSc*
7	21.60	288.10770	288.10778	/	$C_{12}H_{17}NO_7$	√	0.07588	SF
7	21.58	310.08935	310.08963	/	$C_{18}H_{15}NO_2S$	√	0.27657	SF
			310.08965	Cys-Met-Gly	$C_{10}H_{19}N_3O_4S_2$	√	0.3	LSc*
8	21.98	166.08674	166.08626	Phenylalanine	$C_9H_{11}NO_2$	√	0.48752	SF/LSc*
8	22.05	279.13382	279.13393	/	$C_{14}H_{18}N_2O_4$	√	0.11056	SF
			279.13461	Tyr-Pro	equivalent to SF	√	0.79	LSc*
8	22.26	331.16525	331.16523	/	$C_{18}H_{22}N_2O_4$	√	0.01863	SF
			331.16189	Gln-Pro-Ser	$C_{13}H_{22}N_4O_6$	√	3.36	LSc*
9	24.1	253.1190	253.11896	Tyr-Ala	$C_{12}H_{16}N_2O_4$	/	0.04	LSc*
			253.11896	Ser-Phe	$C_{12}H_{16}N_2O_4$	/	0.04	LSc*
9	24.8	147.0423	147.07642	Glutamine	$C_5H_{10}N_2O_3$	/	0.03412	LSc
9	24.80	165.05582	165.05462	/	$C_9H_8O_3$	√	1.20118	SF
9	24.80	182.08074	182.08117	Tyrosine	$C_9H_{11}NO_3$	√	0.43075	SF/LSc
9	25.09	311.12353	311.12376	/	$C_{14}H_{18}N_2O_6$	√	0.23706	SF
			311.12444	Glu-Tyr	equivalent to SF	√	0.91	LSc*
9	24.9	221.0917	221.0926	Diketo-Tyr-Gly	$C_{11}H_{17}N_3O_5$	/	0.9	LSc*

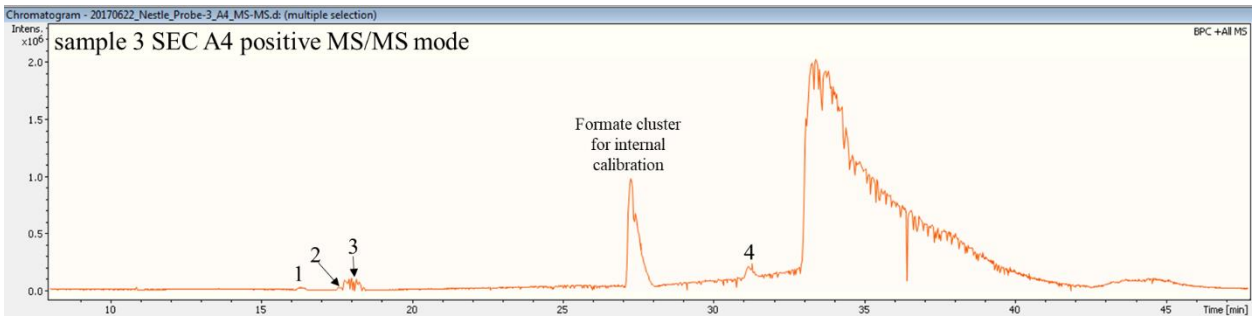
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
			221.09612	Met-Ala	$C_8H_{16}N_2O_3S$	/	4.42	LSc*
			221.09612	Cys-Val	$C_8H_{16}N_2O_3S$	/	4.42	LSc*
10	25.22	254.16152	254.16115	/	$C_{11}H_{19}N_5O_2$	√	0.36591	SF
			254.16172	Diketo-Pro-Arg	equivalent to SF	√	0.2	LSc*
11	28.55	191.03951	191.03859	/	$C_8H_6N_4S$	√	0.91985	SF
			191.04905	Diketo-Cys-Ser	$C_6H_{10}N_2O_3S$	√	9.54	LSc*
11	28.57	147.07525	147.07642	/	$C_5H_{10}N_2O_3$	√	1.16472	SF
			147.07642	Glutamine	equivalent to SF	√	11.7	LSc*
11	28.6	169.0576	169.09772	Diketo-Pro-Ala	$C_8H_{12}N_2O_2$	/	40.12	LSc*
12	31.95	156.07687	156.07675	/	$C_6H_9N_3O_2$	√	0.11918	SF
			156.07283	Histidine	equivalent to SF	√	4.04	LSc*
12	31.79	175.11824	175.11895	/	$C_6H_{14}N_4O_2$	√	0.71630	SF
			175.11503	Arginine	equivalent to SF	√	3.21	LSc*



Supplementary figure 16 Base Peak chromatogram (BPC) of the negative MS/MS mode of SEC-fraction A4 of sample 3. The numbering of the peaks correlates with the numbering in Supplementary table 16Supplementary table 10.

Supplementary table 16: Results of the three calculation approaches for the most abundant signals (negative mode) in SEC-fraction A4 of sample 3. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 16.

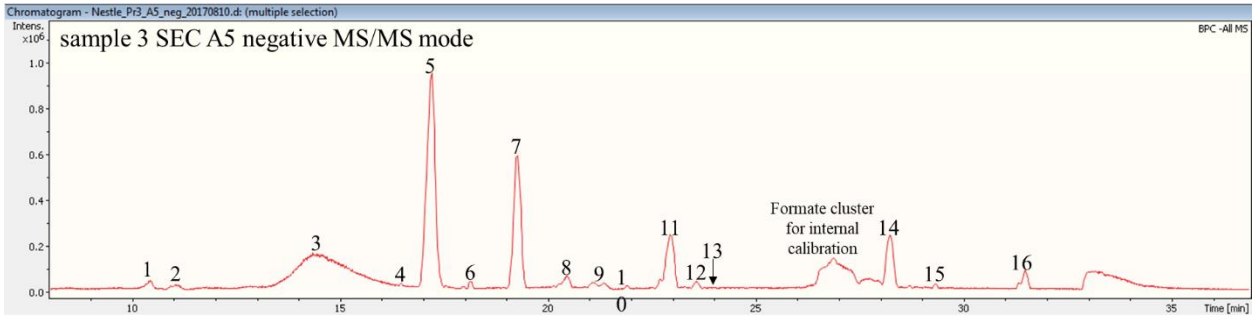
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
1	10.02	68.99466	68.99875	/	$C_3H_2O_2$	/	3.54186	SF
1	10.02	112.98495	112.98858	/	$C_4H_2O_4$	/	3.08734	SF
2	10.98	121.02878	121.03005	/	$C_7H_6O_2$	/	0.72514	SF
3	32.48	146.93777	146.96978	/	$C_4H_4S_3$	/	0.03201	SF
3	32.48	190.92763	190.93062	/	$C_5H_4O_2S_3$	/	0.00299	SF



Supplementary figure 17: Base Peak chromatogram (BPC) of the positive MS/MS mode of SEC-fraction A4 of sample 3. The numbering of the peaks correlates with the numbering in Supplementary table 17Supplementary table 10.

Supplementary table 17: Results of the three calculation approaches for the most abundant signals (positive mode) in SEC-fraction A4 of sample 3. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 17.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
1	16.33	217.10444	217.10436	/	$C_6H_{12}N_6O_3$	√	0.07752	SF
			217.10109	Diketo-Cys-Ile	$C_9H_{16}N_2O_2S$	√	3.35	LSc*
1	16.4	195.1217	195.11337	Diketo-Pro-Pro	$C_{10}H_{14}N_2O_2$	/	8.33	LSc*
2	17.6	388.2534	388.25613	Gln-Lys-Ile	$C_{17}H_{33}N_5O_5$	/	2.73	LSc*
2	17.59	393.20918	393.20922	/	$C_{14}H_{28}N_6O_7$	√	0.03979	SF
3	17.80	432.27940	432.28031	/	$C_{16}H_{29}N_{15}$	√	0.91452	SF
4	31.22	154.98982	154.98926	/	$C_6H_3N_3P$	/	0.56082	SF

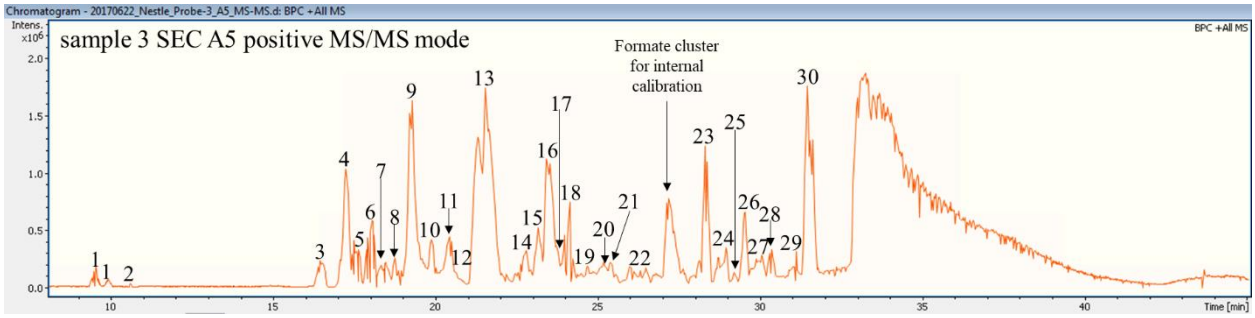


Supplementary figure 18: Base Peak chromatogram (BPC) of the negative MS/MS mode of SEC-fraction A5 of sample 3. The numbering of the peaks correlates with the numbering in Supplementary table 18.

Supplementary table 18: Results of the three calculation approaches for the most abundant signals (negative mode) in SEC-fraction A5 of sample 3. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 18.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
1	10.46	68.99459	68.99875	/	$C_3H_2O_2$	/	3.61508	SF
1	10.46	112.98498	112.98858	/	$C_4H_2O_4$	/	3.05269	SF
1	10.61	89.02367	89.02497	/	$C_3H_6O_3$	/	0.74971	SF
2	11.03	117.01876	117.01988	/	$C_4H_6O_4$	/	0.57535	SF
2	10.96	165.01874	165.01988	/	$C_8H_6O_4$	√	0.59623	SF
3	13.96	325.18219	325.18465	/	$C_{10}H_{26}N_6O_6$	/	1.92102	SF
3	13.98	311.16675	311.16900	/	$C_9H_{24}N_6O_6$	/	1.70181	SF
3	14.05	297.15109	297.15017	/	$C_{19}H_{22}O_3$	/	1.47448	SF
3	14.43	117.01884	117.01988	/	$C_4H_6O_4$	/	0.49473	SF
4	16.48	197.12804	197.13010	/	$C_{10}H_{18}N_2O_2$	/	1.50940	SF
			197.13011	Diketo-Val-Val	equivalent to SF	/	2.07	LSc*
4	16.48	275.10199	275.10325	/	$C_8H_{17}N_6O_3P$	/	0.71128	SF
			275.1016	pyro-Glu-Phe	$C_{14}H_{16}N_2O_4$	/	0.39	LSc*
4	16.55	241.11801	241.11724	/	$C_7H_{14}N_8O_2$	√	1.29871	SF
			241.11994	Diketo-Glu-Ile	$C_{11}H_{18}N_2O_4$	√	1.93	LSc*
5	17.18	372.15406	372.15467	/	$C_{12}H_{28}N_3O_8P$	√	0.06764	SF
5	17.22	128.03483	128.03587	pyro-Glutamic acid	$C_5H_7NO_3$	√	0.49088	SF/SL
5	17.22	257.07655	257.07846	/	$C_{10}H_{14}N_2O_6$	√	1.35568	SF
			257.08051	pyro-Glu-Glu	equivalent to SF	√	3.96	LSc*
			257.07847	Diketo-Glu-Glu	equivalent to SF	√	1.92	LSc*
			257.07205	His-Cys	$C_9H_{14}N_4O_3S$	√	4.5	LSc*
5	17.35	338.17021	338.17166	/	$C_{10}H_{26}N_7O_4P$	√	0.90695	SF
6	18.16	469.20652	469.20878	/	$C_{18}H_{31}N_8O_5P$	√	1.70986	SF
7	19.29	181.09754	181.09880	/	$C_9H_{14}N_2O_2$	√	0.71525	SF
7	19.29	225.08710	225.08863	/	$C_{10}H_{14}N_2O_4$	√	0.97848	SF
			225.08864	Diketo-Glu-Pro	equivalent to SF	√	1.54	LSc*
			225.0910	pyro-Glu-Pro	equivalent to SF	√	3.9	LSc*
7	19.28	451.18075	451.18295	/	$C_{14}H_{29}N_8O_7P$	√	1.66002	SF
8	20.48	322.13873	322.14036	/	$C_9H_{22}N_7O_4P$	√	1.08499	SF
			322.14152	Phe-Ala-Ser	$C_{15}H_{21}N_3O_5$	√	2.79	LSc*
9	21.10	261.12300	261.12501	Phe-Ala-Pro	$C_{14}H_{18}N_2O_3$	√	1.46686	SF/SL
9	21.37	227.13874	227.13798	/	$C_7H_{16}N_8O$	√	1.31124	SF
			227.14079	Ile-Pro	$C_{11}H_{20}N_2O_3$	√	2.05	LSc*
9	21.41	201.12348	201.12501	/	$C_9H_{18}N_2O_3$	/	0.98386	SF
			201.12514	Ile-Ala	equivalent to SF	/	1.66	LSc*
10	21.95	166.06336	166.06440	/	$C_5H_{14}NO_3P$	√	0.49271	SF
10	21.93	215.06609	215.06789	/	$C_8H_{12}N_2O_5$	√	1.25612	SF
			215.0679	Diketo-Asp-Thr	equivalent to SF	√	1.81	LSc*
10	21.93	251.04243	251.04440	/	$C_7H_{13}N_2O_6P$	/	1.41762	SF
11	22.71	353.14441	353.14617	/	$C_9H_{23}N_8O_5P$	√	1.22122	SF
11	22.82	312.11791	312.11797	/	$C_9H_{15}N_9O_4$	/	0.48188	SF
11	22.98	256.09239	256.09444	/	$C_{10}H_{15}N_3O_5$	√	1.50932	SF
			256.09445	Diketo-Glu-Gln	equivalent to SF	√	2.06	LSc*
11	22.98	513.19282	513.19324	/	$C_{13}H_{35}N_6O_{13}P$	√	0.12052	SF
11	22.99	238.08190	238.08388	/	$C_{10}H_{13}N_3O_4$	√	1.42733	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
12	23.60	353.14436	353.14617	/	$C_9H_{23}N_8O_5P$	√	1.26478	SF
13	23.79	148.04313	148.04432	L-Methionine	$C_5H_{11}NO_2S$	√	0.63952	SF/SL
13.	22.79	200.05546	200.05699	/	$C_8H_{11}NO_5$	/	0.98215	SF
13	22.78	290.08624	290.08600	/	$C_7H_{13}N_7O_6$	/	0.78461	SF
14	28.24	145.06155	145.06241	/	$C_5H_{10}N_2O_3$	√	0.31531	SF
			145.06186	Glutamine	equivalent to SF	√	0.31	LSc*
14	28.3	313.1107	313.11188	/	$C_7H_{18}N_6O_8$	√	0.58295	SF
15	29.34	232.09261	232.09444	/	$C_8H_{15}N_3O_5$	√	1.28177	SF
			232.09456	Gln-Ser	equivalent to SF	√	1.95	LSc*
			232.09455	Gly-Thr-Gly	equivalent to SF	√	1.94	LSc*
16	31.36	173.10385	173.10495	L-Arginine	$C_6H_{14}N_4O_2$	√	0.54845	SF/SL
16	31.50	154.06158	154.06275	L-Histidine	$C_6H_9N_3O_2$	√	0.61926	SF/SL



Supplementary figure 19: Base Peak chromatogram (BPC) of the positive MS/MS mode of SEC-fraction A5 of sample 3. The numbering of the peaks correlates with the numbering in Supplementary table 19Supplementary table 17Supplementary table 10.

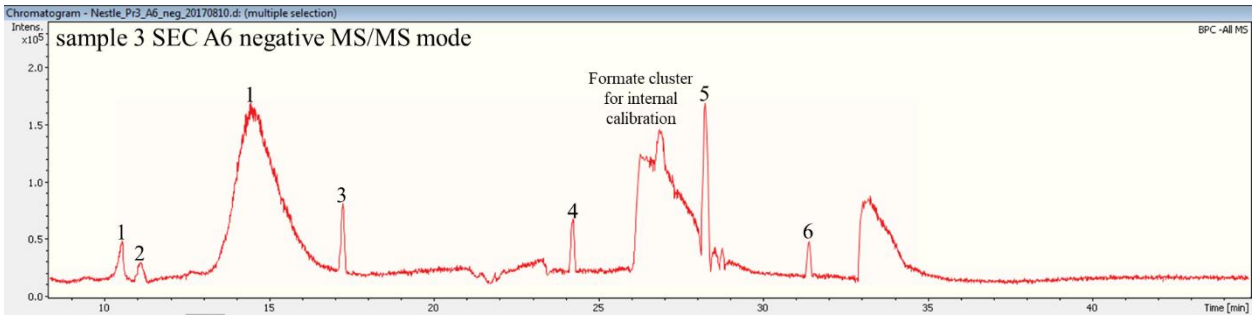
Supplementary table 19: Results of the three calculation approaches for the most abundant signals (positive mode) in SEC-fraction A5 of sample 3. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 19.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
1	9.53	211.14405	211.14410	/	$C_{11}H_{18}N_2O_2$	√	0.11356	SF
			211.14467	Diketo-Pro-Ile	equivalent to SF	√	0.62	LSc*
1	9.53	421.28046	421.28093	/	$C_{22}H_{36}N_4O_4$	√	0.47541	SF
2	10.63	197.12797	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.48649	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	1.05	LSc*
2	10.63	311.15874	311.15746	/	$C_{11}H_{18}N_8O_3$	√	1.27366	SF
3	16.50	243.13424	243.13393	/	$C_{11}H_{18}N_2O_4$	√	0.31106	SF
			243.1345	Diketo-Glu-Ile	equivalent to SF	√	0.26	LSc*
3	16.49	277.11818	277.11828	/	$C_{14}H_{16}N_2O_4$	√	0.10513	SF
			277.1189	Diketo-Glu-Phe	equivalent to SF	√	0.72	LSc*
3	16.48	485.26029	485.26012	/	$C_{22}H_{44}O_7S_2$	√	0.17214	SF
4	17.27	259.09254	259.09246	/	$C_{10}H_{14}N_2O_6$	√	0.07859	SF
			259.09303	Diketo-Glu-Glu	equivalent to SF	√	0.49	LSc*
			259.09303	pyro-Glu-Glu	equivalent to SF	√	0.49	LSc*
4	17.27	469.22929	469.22795	/	$C_{20}H_{36}O_{12}$	√	1.33871	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
			469.22345	Tyr-Arg-Met	$C_{20}H_{32}N_6O_5S$	√	5.84	LSc*
4	17.50	374.17116	374.17105	/	$C_{19}H_{23}N_3O_5$	√	0.29847	SF
4	17.53	396.15356	396.15303	/	$C_{14}H_{26}N_3O_8P$	√	0.53238	SF
4	17.56	340.18638	340.18803	/	$C_{17}H_{21}N_7O$	√	1.65852	SF
			340.19401	Ile-His-Ala	$C_{15}H_{25}N_5O_4$	√		LSc*
5	17.59	362.16785	362.16836	/	$C_{14}H_{19}N_9O_3$	√	0.51024	SF
			362.1677	Asn-Gln-Thr	$C_{13}H_{23}N_5O_7$	√	0.15	LSc*
6	17.92	326.17034	326.17105	/	$C_{15}H_{23}N_3O_5$	√	0.63253	SF
6	18.0	213.0861	213.08755	Diketo-Pro-Asp	$C_9H_{12}N_2O_4$	/	1.45	LSc*
6	18.10	493.20556	493.20414	/	$C_{21}H_{28}N_6O_8$	√	1.64085	SF
6	18.19	471.22384	471.22246	/	$C_{21}H_{22}N_{14}$	√	1.37837	SF
7	18.34	326.17048	326.17105	/	$C_{15}H_{23}N_3O_5$	/	0.56776	SF
7	18.28	358.14254	358.14312	/	$C_{15}H_{23}N_3O_5S$	√	0.58030	SF
7	18.46	437.23924	437.23946	/	$C_{21}H_{32}N_4O_6$	√	0.00445	SF
8	18.77	390.16613	390.16596	/	$C_{19}H_{23}N_3O_6$	√	0.17089	SF
			390.16262	Glu-Asn-Gln	$C_{14}H_{23}N_5O_8$	√	3.51	LSc*
8	18.90	201.08658	201.08698	/	$C_8H_{12}N_2O_4$	√	0.40432	SF
			201.08755	Diketo-Glu-Ala	$C_8H_{13}N_3O_3$	√	0.97	LSc*
8	18.98	185.09159	185.09207	/	$C_8H_{12}N_2O_3$	√	0.47939	SF
			185.09263	Diketo-Ser-Pro	equivalent to SF	√	1.04	LSc*
9	19.30	227.10285	227.10397	/	$C_{11}H_{10}N_6$	√	1.12342	SF
			227.1032	Diketo-Glu-Pro	$C_{10}H_{14}N_2O_4$	√	0.35	LSc*
			227.1032	pyro-Glu-Pro	$C_{10}H_{14}N_2O_4$	√	0.35	LSc*
9	19.50	245.1857	245.18665	Ile-Ile	$C_{12}H_{24}N_2O_3$	√	0.95	LSc*
			245.18665	Leu-Ile	$C_{12}H_{24}N_2O_3$	√	0.95	LSc*
			245.18665	Leu-Leu	$C_{12}H_{24}N_2O_3$	√	0.95	LSc*
10	19.86	342.23815	342.23873	Leu-Pro-Ile	$C_{17}H_{31}N_3O_4$	√	0.58529	SF/SL
10	19.92	209.09188	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.18645	SF
			209.10386	Diketo-His-Ala	$C_9H_{12}N_4O_2$	√	11.98	LSc*
10	19.91	226.11871	226.11862	/	$C_{10}H_{15}N_3O_3$	√	0.09560	SF
			226.11918	Diketo-Pro-Gln	equivalent to SF	√	0.47	LSc*
10	19.90	451.22971	451.23130	/	$C_{21}H_{26}N_{10}O_2$	√	1.58638	SF
			451.23065	Glu-Arg-Phe	$C_{20}H_{30}N_6O_6$	√	0.94	LSc*
11	20.1	187.0704	187.0719	Diketo-Asp-Ala	$C_7H_{10}N_2O_4$	/	1.5	LSc*
			187.0719	Diketo-Glu-Gly	$C_7H_{10}N_2O_4$	/	1.5	LSc*
11	20.29	195.07562	195.07642	/	$C_9H_{10}N_2O_3$	√	0.79456	SF
			195.0882	Diketo-His-Gly	$C_8H_{10}N_4O_2$	√	12.58	LSc*
11	20.28	212.10252	212.10297	/	$C_9H_{13}N_3O_3$	√	0.44995	SF
			212.10353	Diketo-Pro-Asn	equivalent to SF	√	1.01	LSc*
11	20.43	324.15498	324.15540	/	$C_{15}H_{21}N_3O_5$	√	0.41346	SF
			324.15607	Thr-Gly-Phe	equivalent to SF	√	1.09	LSc*
11	20.44	336.19086	336.19178	/	$C_{17}H_{25}N_3O_4$	√	0.92354	SF
			336.19246	Ile-Gly-Phe	equivalent to SF	√	1.6	LSc*
12	20.77	231.09772	231.09755	/	$C_9H_{14}N_2O_5$	√	0.16825	SF
			231.09811	Diketo-Glu-Thr	equivalent to SF	√	0.39	LSc*
12	20.88	392.21798	392.21800	Ile-Pro-Tyr	$C_{20}H_{29}N_3O_5$	√	0.04529	SF/SL
13	21.34	263.13922	263.13902	/	$C_{14}H_{18}N_2O_3$	√	0.19718	SF
			263.13958	Diketo-Val-Tyr	equivalent to SF	√	0.36	LSc*
			263.1397	Pro-Phe	equivalent to SF	√	0.48	LSc*
13	21.57	229.15487	229.15467	/	$C_{11}H_{20}N_2O_3$	√	0.20179	SF
			229.15535	Ile-Pro	equivalent to SF	√	0.48	LSc*
13	21.8	203.1387	203.1397	Ile-Ala	$C_9H_{18}N_2O_3$	/	1.0	LSc*
13	21.86	326.20705	326.20743	/	$C_{16}H_{27}N_3O_4$	√	0.38130	SF
			326.20812	Ile-Pro-Pro	equivalent to SF	√	1.07	LSc*
14	22.81	355.16126	355.16121	/	$C_{15}H_{22}N_4O_6$	√	0.05327	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
14	22.81	486.25584	486.25584	/	$C_{21}H_{35}N_5O_8$	√	0.00267	SF
15	23.19	258.10882	258.10845	/	$C_{10}H_{15}N_3O_5$	√	0.37578	SF
			258.10901	Diketo-Glu-Gln	equivalent to SF	√	0.19	LSc*
15	23.23	389.20393	389.20308	/	$C_{16}H_{28}N_4O_7$	√	0.85843	SF
			389.20376	Glu-Gln-Leu	equivalent to SF	√	0.17	LSc*
16	23.41	279.13387	279.13393	/	$C_{14}H_{18}N_2O_4$	√	0.06334	SF
			279.13461	Tyr-Pro	equivalent to SF	√	0.74	LSc*
16	23.47	215.13908	215.13902	/	$C_{10}H_{18}N_2O_3$	√	0.06091	SF
			215.13958	Diketo-Ile-Thr	equivalent to SF	√	0.5	LSc*
			215.1397	Pro-Val	equivalent to SF	√	0.62	LSc*
16	23.49	261.14436	261.14450	/	$C_{11}H_{20}N_2O_5$	√	0.14138	SF
			261.14518	γ-Glu-Leu	equivalent to SF	√	0.82	LSc*
17	23.6	189.1227	189.12405	Val-Ala	$C_8H_{16}N_2O_2$	/	1.35	LSc*
17	23.76	355.16106	355.16121	/	$C_{15}H_{22}N_4O_6$	√	0.14862	SF
17	23.89	312.19144	312.19178	/	$C_{15}H_{25}N_3O_4$	√	0.34703	SF
			312.19247	Val-Pro-Pro	equivalent to SF	√	1.03	LSc*
17	23.89	330.20177	330.20235	Ile-Pro-Thr	$C_{15}H_{27}N_3O_5$	√	0.57525	SF/SL
18	24.06	150.05733	150.05833	/	$C_5H_{11}NO_2S$	√	0.88993	SF
			150.05440	Methionine	equivalent to SF	√	2.93	LSc*
18	24.39	274.09200	274.09213	/	$C_{11}H_{15}NO_7$	√	0.13129	SF
18	24.3	175.1064	175.11503	Arginine	$C_6H_{14}N_4O_2$	/	8.63	LSc*
19	24.71	247.12910	247.12885	/	$C_{10}H_{18}N_2O_5$	√	0.25066	SF
			247.12953	Glu-Val	equivalent to SF	√	0.43	LSc*
19	24.90	302.17076	302.17105	/	$C_{13}H_{23}N_3O_5$	√	0.29175	SF
			302.17173	Pro-Ser-Val	equivalent to SF	√	0.97	LSc*
19	25.0	219.1346	219.13461	Ile-Ser	$C_9H_{18}N_2O_4$	/	0.01	LSc*
20	25.16	254.16138	254.16115	/	$C_{11}H_{19}N_5O_2$	√	0.23237	SF
			254.16172	Diketo-Arg-Pro	equivalent to SF	√	0.34	LSc*
20	25.21	357.21294	357.21325	Ile-Pro-Gln	$C_{16}H_{28}N_4O_5$	√	0.30444	SF/SL
20	25.28	205.11770	205.11828	/	$C_8H_{16}N_2O_4$	√	0.58261	SF
			205.11896	Val-Ser	equivalent to SF	√	1.26	LSc*
21	25.42	233.11347	233.11320	/	$C_9H_{16}N_2O_5$	√	0.27425	SF
			233.11388	Asp-Val	equivalent to SF	√	0.41	LSc*
21	25.49	580.27238	580.27121	/	$C_{24}H_{41}N_3O_{13}$	√	1.16163	SF
22	25.37	187.10679	187.10772	/	$C_8H_{14}N_2O_3$	/	0.93249	SF
			187.10828	Diketo-Ser-Val	equivalent to SF	/	1.49	LSc*
			187.1084	Pro-Ala	equivalent to SF	/	1.61	LSc*
22	26.0	161.0924	161.09275	Ala-Ala	$C_6H_{12}N_2O_3$	/	0.35	LSc*
22	26.1	245.1136	245.11388	Glu-Pro	$C_{10}H_{16}N_2O_5$	/	0.28	LSc*
22	26.5	217.1180	217.11896	Thr-Pro	$C_9H_{16}N_2O_4$	/	0.96	LSc*
22	26.5	246.1449	246.14551	Gln-Val	$C_{10}H_{19}N_3O_4$	/	0.61	LSc*
			246.14551	Ala-Val-Gly	$C_{10}H_{19}N_3O_4$	/	0.61	LSc*
			246.1455	Gly-Ile-Gly	$C_{10}H_{19}N_3O_4$	/	0.6	LSc*
22	27.00	173.09115	173.09207	/	$C_7H_{12}N_2O_3$	/	0.92044	SF
			173.09263	Diketo-Ala-Thr	equivalent to SF	/	1.48	LSc*
			173.09274	Pro-Gly	equivalent to SF	/	1.59	LSc*
22	27.02	148.05893	148.06043	/	$C_5H_9NO_4$	√	1.50062	SF
			148.05651	Glutamic acid	equivalent to SF	√	2.42	LSc*
23	27.99	177.08592	177.08698	/	$C_6H_{12}N_2O_4$	√	1.10601	SF
			177.08765	Thr-Gly	equivalent to SF	√	1.73	LSc*
			177.08766	Ser-Ala	equivalent to SF	√	1.74	LSc*
23	28.0	235.0928	235.09314	Glu-Ser	$C_8H_{14}N_2O_6$	/	0.34	LSc*
			235.09314	Asp-Thr	$C_8H_{14}N_2O_6$	/	0.34	LSc*
23	28.15	218.11342	218.11353	/	$C_8H_{15}N_3O_4$	√	0.11293	SF
			218.11421	Gln-Ala	equivalent to SF	√	0.79	LSc*
			218.11421	Ala-Ala-Gly	equivalent to SF	√	0.79	LSc*
23	28.32	147.07506	147.07642	/	$C_5H_{10}N_2O_3$	√	1.36078	SF
			147.07709	Ala-Gly	equivalent to SF	√	2.03	LSc*

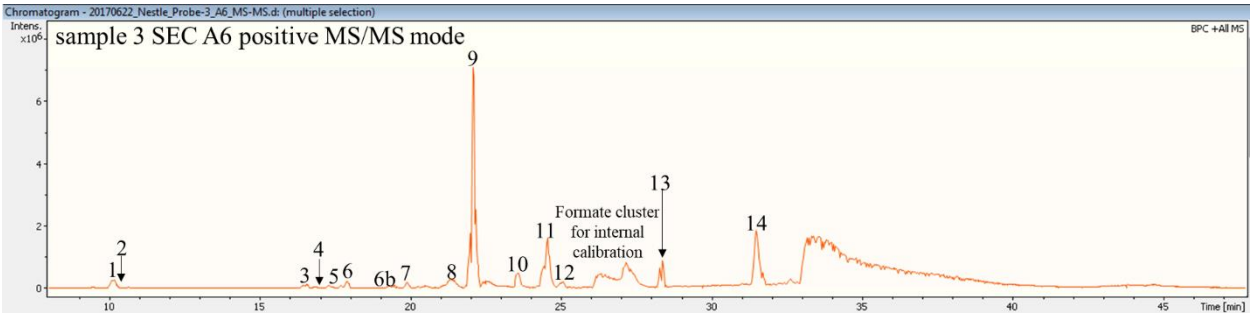
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
			147.07642	Glutamine	equivalent to SF	√	1.36	LSc*
23	28.31	169.05735	169.06077	/	$C_7H_8N_2O_3$	√	3.42071	SF
23	28.36	293.14534	293.14556	/	$C_{10}H_{20}N_4O_6$	√	0.22269	SF
23	28.47	276.11882	276.11901	/	$C_{10}H_{17}N_3O_6$	√	0.19051	SF
			276.11969	Glu-Gln	equivalent to SF	√	0.87	LSc*
			276.11969	Ala-Glu-Gly	equivalent to SF	√	0.87	LSc*
24	28.82	331.16043	331.16121	/	$C_{13}H_{22}N_4O_6$	√	0.78472	SF
			331.16189	Gln-Pro-Ser	equivalent to SF	√	1.46	LSc*
24	28.8	207.0970	207.09822	Thr-Ser	$C_7H_{14}N_2O_5$	/	1.22	LSc*
24	28.9	163.0719	163.072	Gly-Ser	$C_5H_{10}N_2O_4$	/	0.1	LSc*
24	28.98	204.09742	204.09788	/	$C_7H_{13}N_3O_4$	√	0.46264	SF
			204.09855	Gln-Gly	equivalent to SF	√	1.13	LSc*
25	29.2	261.1200	261.12002	Gln-Asn	$C_9H_{16}N_4O_5$	/	0.02	LSc*
25	29.23	275.13514	275.13500	/	$C_{10}H_{18}N_4O_5$	√	0.14756	SF
			275.13567	Gln-Gln	equivalent to SF	√	0.53	LSc*
			275.13567	Ala-Gln-Gly	equivalent to SF	√	0.53	LSc*
26	29.49	193.08140	193.08190	/	$C_6H_{12}N_2O_5$	√	0.49290	SF
			193.08257	Ser-Ser	equivalent to SF	√	1.17	LSc*
26	29.55	234.10850	234.10845	/	$C_8H_{15}N_3O_5$	√	0.05716	SF
			234.10912	Gln-Ser	equivalent to SF	√	0.62	LSc*
			234.10912	Ala-Ser-Gly	equivalent to SF	√	0.62	LSc*
			234.10912	Gly-Thr-Gly	equivalent to SF	√	0.62	LSc*
26	29.57	217.08182	217.08324	/	$C_9H_8N_6O$	√	1.41006	SF
			217.08246	Diketo-Glu-Ser	$C_8H_{12}N_2O_5$	√	0.64	LSc*
			217.08246	Diketo-Thr-Asp	$C_8H_{12}N_2O_5$	√	0.64	LSc*
27	29.77	291.12922	291.12991	/	$C_{10}H_{18}N_4O_6$	√	0.68808	SF
			291.13058	Asn-Thr-Gly	equivalent to SF	√	1.36	LSc*
			291.13058	Ser-Gln-Gly	equivalent to SF	√	1.36	LSc*
27	29.93	250.10263	250.10336	/	$C_8H_{15}N_3O_6$	√	0.73526	SF
			250.10403	Ser-Ser-Gly	equivalent to SF	√	1.4	LSc*
27	29.94	332.15591	332.15646	/	$C_{12}H_{21}N_5O_6$	√	0.54590	SF
			332.15713	Gln-Gln-Gly	equivalent to SF	√	1.22	LSc*
27	30.18	220.08174	220.08290	/	$C_9H_9N_5O_2$	√	1.16214	SF
27	30.37	268.10274	268.10269	/	$C_9H_{17}NO_8$	√	0.04728	SF
28	30.31	162.07671	162.07608	/	$C_6H_{11}NO_4$	/	0.62471	SF
28	30.34	348.15075	348.14887	/	$C_{16}H_{21}N_5O_2S$	√	1.87933	SF
			348.15205	Gln-Asn-Ser	$C_{12}H_{21}N_5O_7$	√	1.3	LSc*
28	30.40	180.08572	180.08665	/	$C_6H_{13}NO_5$	√	0.92752	SF
29	30.94	200.97186	200.97130	/	$C_4N_4O_4S$	√	0.62554	SF
30	31.48	175.11811	175.11895	/	$C_6H_{14}N_4O_2$	√	0.84382	SF
			175.11503	Arginine	equivalent to SF	√	3.08	LSc*
30	31.53	147.11146	147.11280	/	$C_6H_{14}N_2O_2$	√	1.34812	SF
			147.10888	Lysine	equivalent to SF	√	2.58	LSc*
30	31.62	156.07685	156.07675	/	$C_6H_9N_3O_2$	√	0.09272	SF
			156.07283	Histidine	equivalent to SF	√	4.02	LSc*



Supplementary figure 20: Base Peak chromatogram (BPC) of the negative MS/MS mode of SEC-fraction A6 of sample 3. The numbering of the peaks correlates with the numbering in Supplementary table 20Supplementary table 10.

Supplementary table 20: Results of the three calculation approaches for the most abundant signals (negative mode) in SEC-fraction A6 of sample 3. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 20.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	I Δ*m/z* I [mDa]	Anno-tations
1	10.55	68.99485	68.99875	/	$C_3H_2O_2$	/	3.35674	SF
1	10.63	112.98516	112.98858	/	$C_4H_2O_4$	/	2.87179	SF
2	11.12	61.98729		/	/	/	/	/
3	17.26	128.03489	128.05387	/	$C_5H_7NO_3$	/	0.00452	SF
4	24.25	180.06602	180.06717	Tyrosine	$C_9H_{11}NO_3$	/	0.59443	SF/SL
5	28.27	127.05115	127.05185	/	$C_5H_8N_2O_2$	√	0.15240	SF
			127.06641	Diketo-Gly-Ala	equivalent to SF	√	15.26	LSc*
5	28.27	145.06200	145.06241	/	$C_5H_{10}N_2O_3$	/	0.31764	SF
			145.06186	Glutamine	equivalent to SF	/	0.14	LSc*
6	31.42	173.10399	173.10495	Arginine	$C_6H_{14}N_4O_2$	√	0.41470	SF/SL
6	31.58	154.06190	154.06275	Histidine	$C_6H_9N_3O_2$	√	0.29882	SF/SL



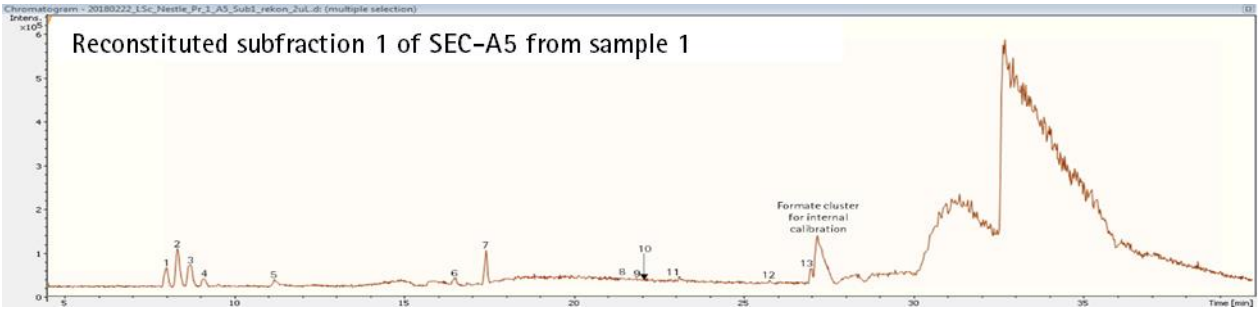
Supplementary figure 21: Base Peak chromatogram (BPC) of the positive MS/MS mode of SEC-fraction A6 of sample 3. The numbering of the peaks correlates with the numbering in Supplementary table 21Supplementary table 17Supplementary table 10.

Supplementary table 21: Results of the three calculation approaches for the most abundant signals (positive mode) in SEC-fraction A6 of sample 3. Calculation approach 1 is SmartFormula (SF); calculation approach 2 is done by operator Lars Schmidt (LSc) and calculation approach 3 is the automatic approach by Spectral Library (SL). The hook in the MS/MS column indicates that a mass spectrum was recorded, the strokes indicates signals without recorded mass spectrum. Equivalent to SF means that calculated molecular formula by operator is the same like the molecular formula calculated by SF. Substances detected in all three samples are highlighted in light green, substances detected in two of the three samples are highlighted in light orange and exclusively detected substances are highlighted in light blue. Numbering in the table correlates with the numbering of the corresponding Supplementary figure 21.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
1	10.17	245.12890	245.12845	/	$C_{14}H_{16}N_2O_2$	√	0.44192	SF
			245.12902	Diketo-Pro-Phe	equivalent to SF	√	0.12	LSc*
1	10.16	267.11081	267.11280	/	$C_{16}H_{14}N_2O_2$	√	1.99419	SF
			267.10934	Diketo-Glu-His	$C_{11}H_{14}N_4O_4$	√	1.47	LSc*
1	10.16	489.24972	489.24829	/	$C_{27}H_{36}O_8$	√	1.42481	SF
			489.25753	Trp-Arg-Gln	$C_{22}H_{32}N_8O_5$	√	7.81	LSc*
1	10.16	511.23155	511.23261	/	$C_{29}H_{34}O_8$	√	1.09050	SF
2	10.67	229.10102	229.10053	/	$C_{10}H_{16}N_2O_2S$	√	0.49607	SF
			229.10109	Diketo-Met-Pro	equivalent to SF	√	0.07	LSc*
3	16.59	277.11852	277.11828	/	$C_{14}H_{16}N_2O_4$	√	0.23736	SF
			277.11615	Pyro-Glu-Phe	equivalent to SF	√	2.37	LSc*
4	16.8	231.1142	231.11674	Diketo-Met-Val	$C_{10}H_{18}N_2O_2S$	/	2.54	LSc*
4	16.88	316.12918	316.12918	/	$C_{16}H_{17}N_3O_4$	√	0.00207	SF
			316.12975	Diketo-Glu-Trp	equivalent to SF	√	0.57	LSc*
5	17.31	259.09270	259.09246	/	$C_{10}H_{14}N_2O_6$	√	0.23592	SF
			259.09507	Pyro-Glu-Glu	equivalent to SF	√	2.37	LSc*
5	17.31	281.07445	281.07413	/	$C_8H_8N_8O_4$	√	0.32068	SF
			281.09949	Met-Met	$C_{10}H_{20}N_2O_3S_2$	√	25.04	LSc*
5	17.32	539.15926	539.15943	/	$C_{25}H_{32}O_9P_2$	√	0.17504	SF
6	17.72	413.18189	413.18195	/	$C_{21}H_{24}N_4O_5$	√	0.06064	SF
			413.186	Lys-Tyr-Cys	$C_{18}H_{28}N_4O_5S$	√	4.11	LSc*
6	17.93	293.11359	293.11320	/	$C_{14}H_{16}N_2O_5$	√	0.39455	SF
			293.11376	Diketo-/	equivalent to SF	√	0.17	LSc*
6	17.94	585.21869	585.21912	/	$C_{28}H_{32}N_4O_{10}$	√	0.42514	SF
6b	19.0	185.0919	185.09263	Diketo-Ser-Pro	$C_8H_{12}N_2O_3$	/	0.73	LSc*
7	19.94	209.09211	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.04511	SF
7	19.92	226.11940	226.11862	/	$C_{10}H_{15}N_3O_3$	√	0.78357	SF
			226.11918	Diketo-Gln-Pro	equivalent to SF	√	0.22	LSc*
7	19.93	248.10113	248.10028	/	$C_8H_9N_9O$	√	0.84565	SF
7	20.29	212.10323	212.10297	/	$C_9H_{13}N_3O_3$	√	0.26347	SF
			212.10353	Diketo-Pro-Asn	equivalent to SF	√	0.3	LSc*

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Anno-tations
8	21.38	263.13931	263.13902	/	$C_{14}H_{18}N_2O_3$	√	0.29292	SF
			263.13958	Diketo-Tyr-Val	equivalent to SF	√	0.27	LSc*
			263.1397	Pro-Phe	equivalent to SF	√	0.39	LSc*
8	21.38	285.12151	285.12068	/	$C_{12}H_{12}N_8O$	√	0.82910	SF
			285.13516	Diketo-His-Phe	$C_{15}H_{16}N_4O_2$	√	13.65	LSc*
			285.12002	Glu-His	$C_{11}H_{16}N_4O_5$	√	1.49	LSc*
9	22.06	166.08774	166.08626	/	$C_9H_{11}NO_2$	√	1.64081	SF
			166.08233	Phenylalanine	equivalent to SF	√	5.41	LSc*
9	22.12	331.16507	331.16523	/	$C_{18}H_{22}N_2O_4$	√	0.16774	SF
			331.16189	Gln-Pro-Ser	$C_{13}H_{22}N_4O_6$	√	3.18	LSc*
			331.16189	Thr-Asn-Pro	$C_{13}H_{22}N_4O_6$	√	3.18	LSc*
9	22.11	353.14694	353.14690	/	$C_{16}H_{16}N_8O_2$	√	0.04358	SF
			353.14623	Tyr-Asn-Gly	$C_{15}H_{20}N_4O_6$	√	0.71	LSc*
10	23.55	253.11867	253.11828	/	$C_{12}H_{16}N_2O_4$	√	0.38357	SF
			253.11896	Tyr-Ala	equivalent to SF	√	0.29	LSc*
			253.11896	Phe-Ser	equivalent to SF	√	0.29	LSc*
10	23.59	279.13413	279.13393	/	$C_{14}H_{18}N_2O_4$	√	0.19349	SF
			279.13461	Tyr-Pro	equivalent to SF	√	0.48	LSc*
11	24.6	147.0426	147.07250	Glutamine	$C_5H_{10}N_2O_3$	/	29.9	LSc*
11	24.57	165.05573	165.05462	/	$C_9H_8O_3$	√	1.10529	SF
11	24.57	182.08063	182.08117	/	$C_9H_{11}NO_3$	√	0.53901	SF
			182.07725	Tyrosine	equivalent to SF	√	3.38	LSc*
11	24.66	221.09181	221.09207	/	$C_{11}H_{12}N_2O_3$	√	0.25940	SF
			221.0926	Diketo-Tyr-Gly	$C_{11}H_{17}N_3O_5$	√	0.79	LSc*
			221.09612	Met-Ala	$C_8H_{16}N_2O_3S$	√	4.31	LSc*
			221.09612	Cys-Val	$C_8H_{16}N_2O_3S$	√	4.31	LSc*
12	25.1	237.0870	237.09103	Met-Ser	$C_8H_{16}N_2O_4S$	/	4.03	LSc*
12	25.08	254.16163	254.16115	/	$C_{11}H_{19}N_5O_2$	√	0.48166	SF
			254.16115	Diketo-Pro-Arg	equivalent to SF	√	0.48	LSc*
13	28.3	191.0399	191.04905	Diketo-Cys-Ser	$C_6H_{10}N_2O_3S$	/	9.15	LSc*
13	28.4	147.0752	147.07638	Glutamine	$C_5H_{10}N_2O_3$	/	1.18	LSc*
13	28.40	169.05766	169.06242	/	$C_5H_{13}O_4P$	√	4.76644	SF
14	31.52	175.11807	175.11895	/	$C_6H_{14}N_4O_2$	√	0.87710	SF
			175.11503	Arginine	equivalent to SF	√	3.86	LSc*
14	31.72	156.07669	156.07675	/	$C_6H_9N_3O_2$	√	0.05813	SF
			156.07283	Histidine	equivalent to SF	√	3.86	LSc*

All following results are based on the evaluated UPLC-HR-MS method (positive MS/MS mode). Measured sub-fractions were generated by the preparative HPLC. Each sub-fraction was reduced by the rotary evaporator resulting in an aqueous and an alcoholic phase. The aqueous phases were freeze dried and reconstituted with 600 µL ddH_2O. Generated sample were analysed by the UPLC-HRMS and measured in the positive MS and MS/MS mode. The goal was to determine if the developed prepHPLC method is feasible for the sub-fractionation of the SEC-fractions.

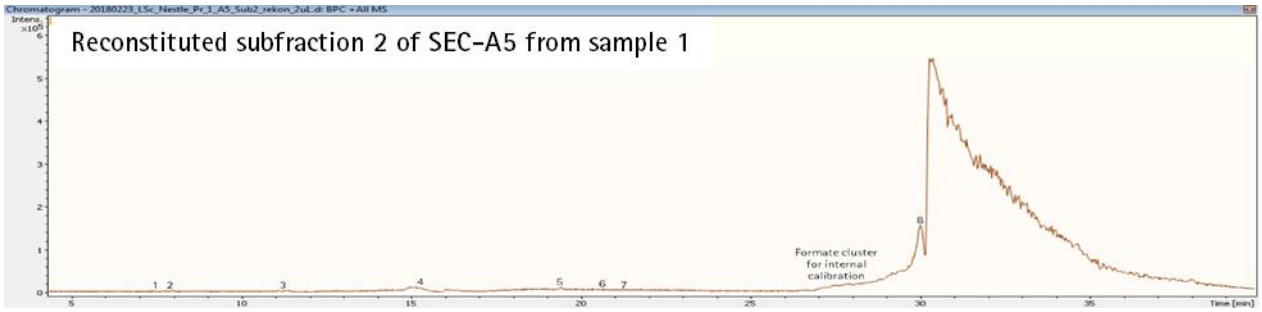


Supplementary figure 22: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 1 of SEC-A5 from sample 1. The numbering of the peaks correlates with the numbering in Supplementary table 22.

Supplementary table 22: Detected mass to charge ratios in sub-fraction 1 of SEC-A5 sample 1. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 22Supplementary figure 28. Results highlighted in light green were also detected in the starting material. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	7.94	381.12877	381.12739	/	$C_{29}H_{16}O$	√	1.38211	SF
			381.12677	Gln-Met-Cys	$C_{13}H_{24}N_4O_5S_2$	√	2.0	LSc
1	8.02	273.16579	273.16696	/	$C_{10}H_{20}N_6O_3$	√	1.17009	SF
1	8.03	251.18371	251.18530	/	$C_{12}H_{26}O_5$	√	1.59264	SF
2	8.36	317.19198	317.19318	/	$C_{12}H_{24}N_6O_4$	√	1.19561	SF
			317.19387	Ala-Arg-Ala	equivalent to SF	√	1.89	LSc
2	8.37	295.20987	295.21152	/	$C_{14}H_{30}O_6$	√	1.64479	SF
3	8.73	361.21794	361.21939	/	$C_{14}H_{28}N_6O_5$	√	1.45060	SF
			361.22008	Ser-Val-Arg	equivalent to SF	√	2.14	LSc
3	8.73	339.23588	339.23773	/	$C_{16}H_{34}O_7$	√	1.85334	SF
3	8.73	356.26262	356.26293	/	$C_{13}H_{29}N_{11}O$	√	0.30965	SF
4	9.13	405.24370	405.24561	/	$C_{16}H_{32}N_6O_6$	√	1.90609	SF
4	9.14	400.28831	400.28781	/	$C_{14}H_{37}N_7O_6$	√	0.49855	SF
4	9.14	383.26171	383.26260	/	$C_{15}H_{30}N_{10}O_2$	√	0.88290	SF
5	11.22	326.37665	326.37813	/	$C_{22}H_{47}N$	√	1.47774	SF
5	11.40	135.00195	135.00115		$C_6H_2N_2S$	√	0.80373	SF
6	16.51	217.10314	217.10436	/	$C_6H_{12}N_6O_3$	√	1.22710	SF
			217.10109	Diketo-Cys-Ile	$C_9H_{16}N_2O_2S$	√	2.05	LSc
7	17.43	410.11515	410.11538	/	$C_{12}H_{19}N_5O_{11}$	√	0.23675	SF
7	17.43	539.15798	539.15798	/	$C_{17}H_{26}N_6O_{14}$	√	1.04925	SF
7	17.44	130.04902	130.04987	(R)-(+)-2-Pyrrolidone-5-carboxylic acid	$C_5H_7NO_3$	√	0.84704	SF/SL
8	21.40	215.13763	215.13902	Pro-Val	$C_{10}H_{18}N_2O_3$	√	1.38808	SF/SL
9	21.59	229.15322	229.15467	/	$C_{11}H_{20}N_2O_3$	√	1.44380	SF
			229.15535	Ile-Pro	equivalent to SF	√	1.45	LSc
9	21.68	314.20572	314.20743	Val-Pro-Val	$C_{15}H_{27}N_3O_4$	√	1.71060	SF/SL
10	21.98	132.10099	132.10191	L-Norleucine	$C_6H_{13}NO_2$	√	0.91550	SF/SL
10	22.26	132.10099	132.10191	L-Norleucine	$C_6H_{13}NO_2$	√	0.91836	SF/SL
11	23.00	132.10093	132.10191	L-Isoleucine	$C_6H_{13}NO_2$	√	0.97033	SF/SL
11	23.12	280.08879	280.09011	/	$C_8H_9N_9O_3$	√	1.32119	SF
			280.09684	Thr-Cys-Gly	$C_9H_{17}N_3O_5S$	√	8.05	LSc
11	23.13	258.10695	258.10845	/	$C_{10}H_{15}N_3O_5$	√	1.49794	SF
			258.10901	Diketo-Glu-Gln	equivalent to SF	√	2.06	LSc
12	25.77	235.11718	235.11895	/	$C_{11}H_{14}N_4O_2$	√	1.77416	SF

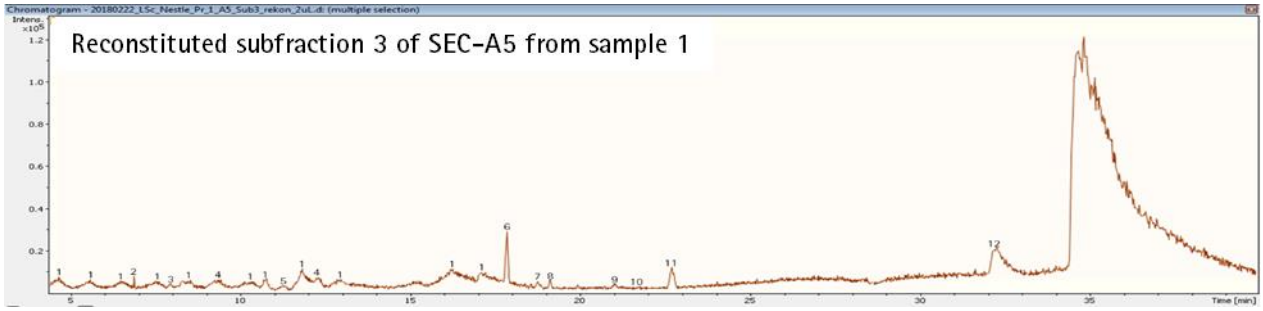
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
			235.11951	Diketo-Pro-His	equivalent to SF	√	2.33	LSc
12	25.80	217.11653	217.11828	Pro-Thr	$C_9H_{18}N_2O_4$	√	1.74827	SF/SL
12	26.06	205.11669	205.11828	Val-Ser	$C_8H_{16}N_2O_4$	√	1.59484	SF/SL
12	26.08	200.97075	200.96958	/	$C_5HN_2O_5P$	√	1.16893	SF
13	27.00	147.07491	147.07642	/	$C_5H_{10}N_2O_3$	√	1.51317	SF
			147.07250	Glutamine	equivalent to SF	√	2.41	LSc
13	27.00	244.12733	244.12918	Pro-Gln	$C_{10}H_{17}N_3O_4$	√	1.85222	SF/SL
13	27.00	266.10936	266.11085	/	$C_8H_{11}N_9O_2$	√	1.49124	SF



Supplementary figure 23: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 2 of SEC-A5 from sample 1. The numbering of the peaks correlates with the numbering in Supplementary table 23.

Supplementary table 23: Detected mass to charge ratios in sub-fraction 2 of SEC-A5 sample 1. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 23 Supplementary figure 28. Results highlighted in light green were also detected in the starting material. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

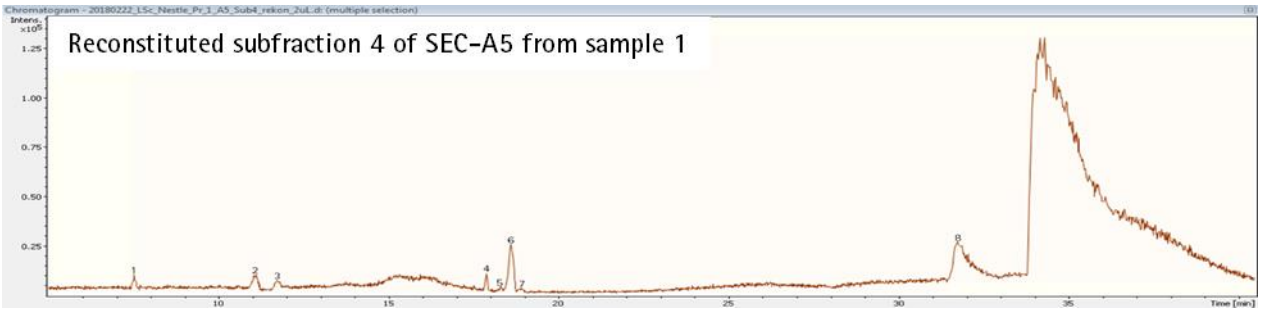
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	7.48	185.11431	185.11454	/	$C_6H_{12}N_6O$	√	0.22367	SF
2	7.96	251.18478	251.18664	/	$C_{13}H_{22}N_4O$	√	1.85700	SF
2	7.98	273.16666	273.16696	/	$C_{10}H_{20}N_6O_3$	√	0.30730	SF
3	11.34	326.37766	326.37813	/	$C_{22}H_{47}N$	√	0.46367	SF
4	15.41	304.29929	304.29988	/	$C_{21}H_{37}N$	√	0.59070	SF
5	19.43	227.10159	227.10263	/	$C_{10}H_{14}N_2O_4$	√	1.04553	SF
			227.1032	Diketo-Glu-Pro	equivalent to SF	√	1.61	LSc
5	19.43	249.08365	249.08430	/	$C_8H_8N_8O_2$	√	0.64433	SF
6	20.60	328.22206	328.22308	Pro-Val-Val	$C_{16}H_{29}N_3O_4$	√	1.02310	SF/SL
7	21.24	263.13783	263.13902	L-phenylalanyl-L-Proline	$C_{14}H_{18}N_2O_3$	√	1.19093	SF/SL
8	30.02	156.98132	156.97976	/	$C_4HN_2O_3P$	√	1.56794	SF
8	30.02	200.97158	200.97130	/	$C_4H_4O_4S$	√	0.27552	SF
8	30.04	182.96118	182.95690	/	$C_7H_2O_2S_2$	√	4.28051	SF



Supplementary figure 24: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 3 of SEC-A5 from sample 1. The numbering of the peaks correlates with the numbering in Supplementary table 24.

Supplementary table 24: Detected mass to charge ratios in sub-fraction 3 of SEC-A5 sample 1. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 24 Supplementary figure 28. Results highlighted in light green were also detected in the starting material. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

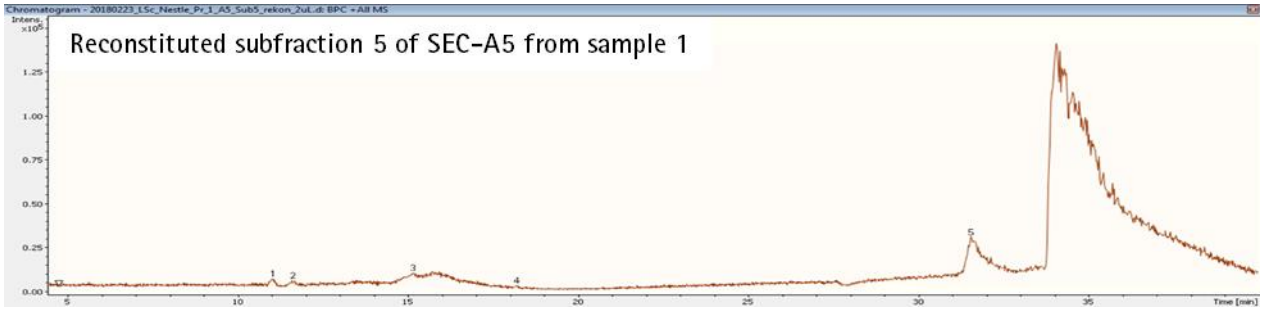
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	4.67	326.37770	326.37813	/	$C_{22}H_{47}N$	√	0.42374	SF
2	6.67	493.34906	493.35102	/	$C_{26}H_{40}N_{10}$	√	1.96060	SF
2	6.89	521.38006	521.38116	/	$C_{35}H_{52}OS$	√	1.10285	SF
3	7.96	185.11422	185.11454	/	$C_6H_{12}N_6O$	√	0.31923	SF
4	9.26	304.29899	304.29988	/	$C_{21}H_{37}N$	√	0.89050	SF
5	11.26	267.11970	267.12001	/	$C_{10}H_{14}N_6O_3$	√	0.31658	SF
6	17.86	243.13322	243.13393	/	$C_{11}H_{18}N_2O_4$	√	0.70937	SF
			243.1345	Diketo-Glu-Ile	equivalent to SF	√	1.28	LSc
6	17.86	485.25949	485.25924	/	$C_{19}H_{28}N_{14}O_2$	√	0.24566	SF
			485.25138	Arg-Tyr-Phe	$C_{24}H_{32}N_6O_5$	√	8.11	LSc
6	17.87	197.12783	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.62445	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	1.19	LSc
7	18.76	340.18605	340.18567	/	$C_{10}H_{26}N_7O_4P$	√	0.38047	SF
			340.19401	Ile-His-Ala	$C_{15}H_{25}N_5O_4$	√	7.96	LSc
7	18.80	132.10158	132.10191	/	$C_6H_{13}NO_2$	√	0.32315	SF
			132.09798	Isoleucine	equivalent to SF	√	3.6	LSc
8	19.11	343.29496	343.29552	/	$C_{19}H_{38}N_2O_3$	√	0.56391	SF
8	19.12	240.23167	240.23219	/	$C_{15}H_{29}NO$	√	0.52096	SF
9	21.22	342.23827	342.23873	Leu-Pro-Ile	$C_{17}H_{31}N_3O_4$	√	0.46036	SF/SL
10	21.74	362.20701	362.20743	Val-Pro-Phe	$C_{19}H_{27}N_3O_4$	√	0.42331	SF/SL
11	22.71	120.08052	120.08078	/	C_8H_9N	√	0.25330	SF
11	22.72	263.13838	263.13902	L-phenylalanyl-L-proline	$C_{14}H_{18}N_2O_3$	√	0.63977	SF/SL
12	32.22	182.96139	182.96027	/	$C_4H_6O_2S_3$	√	1.12496	SF
12	32.27	200.97183	200.97130	/	$C_4N_4O_4S$	√	0.52696	SF



Supplementary figure 25: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 4 of SEC-A5 from sample 1. The numbering of the peaks correlates with the numbering in Supplementary table 25.

Supplementary table 25: Detected mass to charge ratios in sub-fraction 4 of SEC-A5 sample 1. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 25 Supplementary figure 28. Results highlighted in light green were also detected in the starting material. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

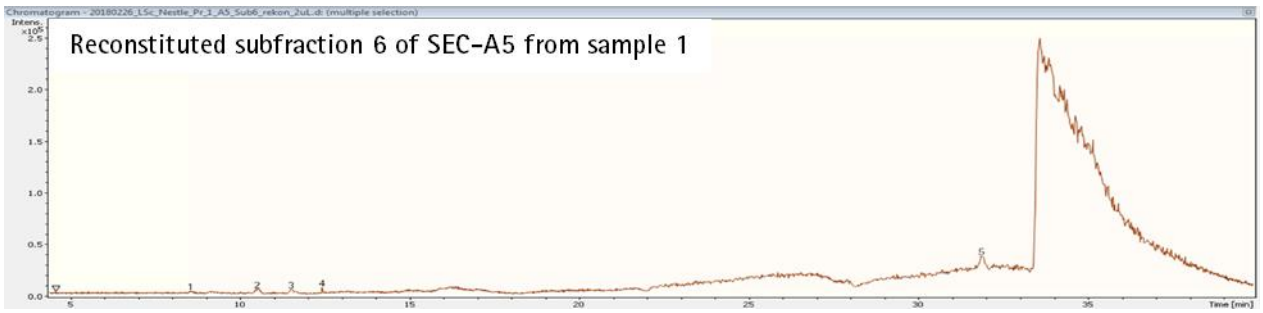
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	7.54	185.11428	185.11454	/	$C_6H_{12}N_6O$	√	0.25569	SF
2	11.10	326.37784	326.37813	/	$C_{22}H_{47}N$	√	0.28552	SF
3	11.76	304.29953	304.29988	/	$C_{21}H_{37}N$	√	0.34965	SF
4	17.89	166.08592	166.08626	/	$C_9H_{11}NO_2$	√	0.33271	SF
			166.08233	Phenylalanine	equivalent to SF	√	3.59	LSc
4	17.89	209.09146	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.60821	SF
4	17.89	374.17104	374.17105	/	$C_{19}H_{23}N_3O_5$	√	0.01011	SF
5	18.30	240.23205	240.23219	/	$C_{15}H_{29}NO$	√	0.14239	SF
6	18.63	263.13865	263.13902	/	$C_{14}H_{18}N_2O_3$	√	0.36873	SF
			263.13958	Diketo-Tyr-Val	equivalent to SF	√	0.93	LSc
6	18.63	471.22370	471.22381	/	$C_{24}H_{30}N_4O_6$	√	0.11084	SF
6	18.63	493.20540	493.20681	/	$C_{23}H_{20}N_{14}$	√	1.41627	SF
7	18.88	229.15413	229.15467	/	$C_{11}H_{20}N_2O_3$	√	0.53406	SF
			229.15535	Ile-Pro	equivalent to SF	√	1.22	LSc
8	31.74	155.97414	155.97499	/	C_5HNO_3S	√	0.84926	SF
8	31.77	182.96162	182.95690	/	$C_7H_2O_2S_2$	√	4.72405	SF
8	31.78	200.97201	200.97130	/	$C_4N_4O_4S$	√	0.71245	SF



Supplementary figure 26: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 5 of SEC-A5 from sample 1. The numbering of the peaks correlates with the numbering in Supplementary table 26.

Supplementary table 26: Detected mass to charge ratios in sub-fraction 5 of SEC-A5 sample 1. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 26 Supplementary figure 28.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	11.04	326.37774	326.37813	/	$C_{22}H_{47}N$	√	0.38821	SF
2	11.63	304.29930	304.29988	/	$C_{21}H_{37}N$	√	0.58034	SF
3	15.24	332.33055	332.33118	/	$C_{23}H_{41}N$	√	0.62879	SF
4	18.21	240.23159	240.23219	/	$C_{15}H_{29}NO$	√	0.60102	SF
4	18.22	343.29481	343.29552	/	$C_{19}H_{38}N_2O_3$	√	0.71222	SF
5	31.60	182.96127	182.95690	/	$C_7H_2O_2S_2$	√	4.36767	SF
5	31.60	200.97169	182.97130	/	$C_4N_4O_4S$	√	0.39209	SF
5	31.74	155.97370	155.97499	/	C_5HNO_3S	√	1.29290	SF

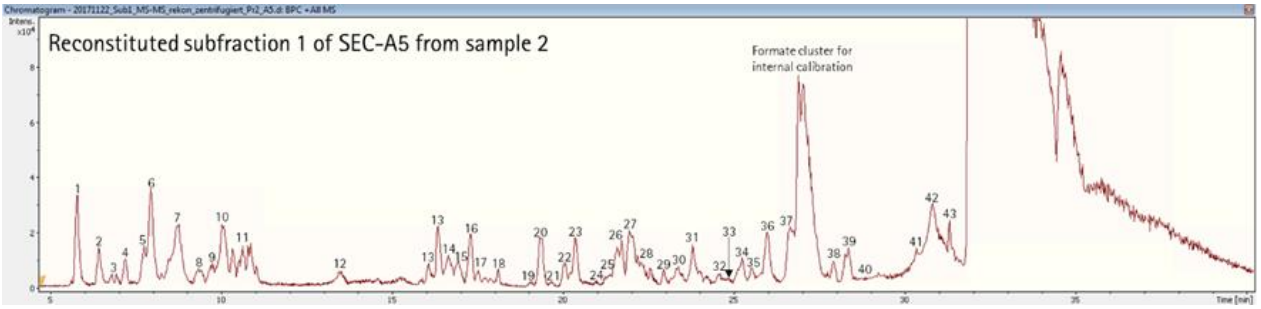


Supplementary figure 27: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 6 of SEC-A5 from sample 1. The numbering of the peaks correlates with the numbering in Supplementary table 27.

Supplementary table 27: Detected mass to charge ratios in sub-fraction 6 of SEC-A5 sample 1. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 27 Supplementary figure 28. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	8.59	273.16639	273.16696	/	$C_{10}H_{20}N_6O_3$	√	0.26998	SF
2	10.55	326.37751	326.37813	/	$C_{22}H_{47}N$	√	0.61859	SF
3	11.52	304.29942	304.29988	/	$C_{21}H_{37}N$	√	0.45389	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
3	11.67	468.41927	468.41999	/	$C_{32}H_{53}NO$	√	0.72545	SF
4	12.50	521.38021	521.37964	/	$C_{26}H_{52}N_2O_8$	√	0.56719	SF
5	31.90	200.97152	200.96958	/	$C_5HN_2O_5P$	√	1.93433	SF
5	31.91	182.96117	182.95690	/	$C_7H_2O_2S_2$	√	4.27727	SF



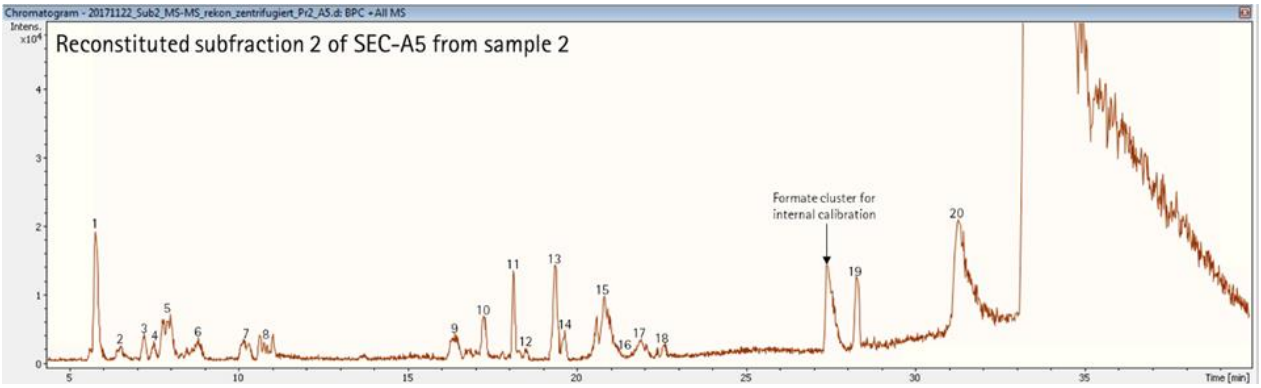
Supplementary figure 28: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 1 of SEC-A5 from sample 2. The numbering of the peaks correlates with the numbering in Supplementary table 28.

Supplementary table 28: Detected mass to charge ratios in sub-fraction 1 of SEC-A5 sample 2. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 28. Results highlighted in light green were also detected in the starting material. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	5.78	149.06002	149.05971	/	$C_9H_8O_2$	√	0.31122	SF
2	6.42	271.18820	271.18770	/	$C_{11}H_{22}N_6O_2$	√	0.49628	SF
2	6.45	303.08477	303.08381	/	$C_{20}H_{14}OS$	√	0.95740	SF
2	6.42	379.21029	379.21017	/	$C_{18}H_{22}N_{10}$	√	0.12690	SF
			379.20951	Phe-Arg-Gly	$C_{17}H_{26}N_6O_4$	√	0.66	LSc
3	6.79	315.17790	315.17887	/	$C_{13}H_{18}N_{10}$	√	0.96948	SF
			315.18212	Diketo-Trp-Lys	$C_{17}H_{22}N_4O_2$	√	3.25	LSc
3	6.94	257.13613	257.13566	/	$C_9H_{16}N_6O_3$	√	0.46688	SF
4	7.18	233.07849	233.07815	/	$C_9H_8N_6O_2$	√	0.34299	SF
5	7.73	229.14131	229.14075	/	$C_8H_{16}N_6O_2$	√	0.56378	SF
5	7.86	381.13138	381.13326	/	$C_{22}H_{20}O_6$	√	1.88445	SF
			381.12677	Gln-Met-Cys	$C_{13}H_{24}N_4O_5S_2$	√	6.49	LSc
5	7.73	505.33681	505.33576	/	$C_{23}H_{40}N_{10}O_3$	√	1.04825	SF
6	7.95	251.18591	251.18530	/	$C_{12}H_{26}O_5$	√	0.61377	SF
6	7.95	273.16767	273.16696	/	$C_{10}H_{20}N_6O_3$	√	0.70592	SF
6	8.00	447.29370	447.29555	/	$C_{18}H_{39}N_8O_3P$	√	1.85008	SF
7	8.79	194.11551	194.11487	/	$C_7H_{11}N_7$	√	0.64093	SF
7	8.73	273.16769	273.16696	/	$C_{10}H_{20}N_6O_3$	√	0.72698	SF
7	8.73	365.19409	365.19452	/	$C_{17}H_{20}N_{10}$	√	0.42946	SF
8	9.34	267.12069	267.12001	/	$C_{10}H_{14}N_6O_3$	√	0.67872	SF
8	9.41	299.14664	299.14757	/	$C_{12}H_{14}N_{10}$	√	0.92623	SF
9	9.73	259.15195	259.15131	/	$C_9H_{18}N_6O_3$	√	0.63096	SF
10	10.05	195.08777	195.08765	/	$C_8H_{10}N_4O_2$	√	0.12161	SF
			195.0882	Diketo-His-Gly	equivalent to SF	√	0.43	LSc
10	10.05	215.12555	215.12510	/	$C_7H_{14}N_6O_2$	√	0.45025	SF
10	10.15	455.22543	455.22621	/	$C_{20}H_{26}N_{10}O_3$	√	0.78227	SF
11	10.34	387.19927	387.20000	/	$C_{16}H_{22}N_{10}O_2$	√	0.73003	SF
			387.19935	Asp-Arg-Pro	$C_{15}H_{26}N_6O_6$	√	0.08	LSc

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
11	10.53	625.32065	625.32119	/	$C_{22}H_{40}N_{16}O_4S$	√	0.53724	SF
11	10.54	455.22957	455.22786	/	$C_{18}H_{31}N_8O_4P$	√	1.89730	SF
11	10.74	441.21098	441.21056	/	$C_{19}H_{24}N_{10}O_3$	/	0.42105	SF
			441.21393	Phe-Phe-Gln	$C_{23}H_{28}N_4O_5$	/	2.95	LSc
11	10.88	245.13585	245.13566	/	$C_8H_{16}N_6O_3$	√	0.18127	SF
11	10.87	519.27794	519.27646	/	$C_{23}H_{43}N_4O_5PS$	√	1.48236	SF
			519.27212	Trp-Trp-Gln	$C_{27}H_{30}N_6O_5$	√	5.82	LSc
11	10.88	514.32284	514.32218	/	$C_{21}H_{35}N_{15}O$	√	0.66321	SF
12	13.46	219.17443	219.17434	/	$C_{15}H_{22}O$	√	0.08738	SF
13	16.07	217.10487	217.10436	/	$C_6H_{12}N_6O_3$	√	0.50301	SF
			217.10109	Diketo-Ile-Cys	$C_9H_{16}N_2O_2S$	√	3.78	LSc
14	16.65	261.13107	261.13192	/	$C_9H_{12}N_{10}$	√	0.84816	SF
			261.1239	Diketo-Tyr-Pro	$C_{14}H_{16}N_2O_3$	√	7.17	LSc
14	16.72	239.14946	239.15025	/	$C_{11}H_{18}N_4O_2$	√	0.79344	SF
15	16.92	305.15749	305.15813	/	$C_{11}H_{16}N_{10}O$	√	0.63935	SF
15	16.93	283.17561	283.17647	/	$C_{13}H_{22}N_4O_3$	√	0.85346	SF
15	17.02	301.28550	301.28495	/	$C_{17}H_{36}N_2O_2$	√	0.54306	SF
16	17.30	130.05010	130.04987	/	$C_5H_7NO_3$	√	0.23091	SF
16	17.30	539.16103	539.16066	/	$C_{21}H_{30}O_{16}$	√	0.37092	SF
17	17.51	393.21076	393.20922	/	$C_{14}H_{28}N_6O_7$	√	1.54124	SF
			393.21393	Phe-Ile-Asn	$C_{19}H_{28}N_4O_5$	√	3.17	LSc
17	17.52	209.09245	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.38642	SF
17	17.52	227.10300	227.10397	/	$C_{11}H_{10}N_6$	√	0.96910	SF
			227.1032	Diketo-Glu-Pro	$C_{10}H_{14}N_2O_4$	√	0.2	LSc
18	18.10	240.23248	240.23219	/	$C_{15}H_{29}NO$	√	0.29343	SF
18	18.10	343.29622	343.29552	/	$C_{19}H_{38}N_2O_3$	√	0.69996	SF
18	18.12	195.07707	195.07642	/	$C_9H_{10}N_2O_3$	√	0.65371	SF
19	19.07	185.09239	185.09207	/	$C_8H_{12}N_2O_3$	√	0.32417	SF
			185.09263	Diketo-Ser-Pro	equivalent to SF	√	0.24	LSc
20	19.35	453.19946	453.20067	/	$C_{22}H_{20}N_{12}$	√	1.20103	SF
20	19.36	227.10310	227.10263	/	$C_{10}H_{14}N_2O_4$	√	0.46748	SF
			227.1032	Diketo-Glu-Pro	equivalent to SF	√	0.1	LSc
20	19.37	249.08490	249.08296	/	$C_7H_{12}N_4O_6$	√	1.94404	SF
21	19.66	245.18626	245.18597	Ile-Ile	$C_{12}H_{24}N_2O_3$	√	0.29593	SF/SL
22	20.05	209.09236	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.28929	SF
22	20.05	248.10100	248.10297	/	$C_{12}H_{13}N_3O_3$	√	1.96783	SF
22	20.18	263.13939	263.13902	Pro-Phe	$C_{14}H_{18}N_2O_3$	√	0.36982	SF/SL
23	20.34	229.15476	229.15467	Pro-Ile	$C_{11}H_{20}N_2O_3$	√	0.08682	SF/SL
23	20.37	259.09289	259.09380	/	$C_{11}H_{10}N_6O_2$	√	0.92597	SF
			259.09322	pyro-Glu-Glu	$C_{10}H_{14}N_2O_6$	√	0.33	LSc
23	20.38	281.07422	281.07413	/	$C_8H_8N_8O_4$	√	0.09364	SF
24	20.99	328.22410	328.22308	Ile-Pro-Val	$C_{16}H_{29}N_3O_4$	√	1.01373	SF/SL
25	21.37	215.13921	215.13902	Pro-Val	$C_{10}H_{18}N_2O_3$	√	0.18799	SF/SL
26	21.59	229.15491	229.15467	/	$C_{11}H_{20}N_2O_3$	√	0.24212	SF
26	21.63	314.20782	314.20743	Val-Pro-Val	$C_{15}H_{27}N_3O_4$	√	0.38509	SF/SL
26	21.71	203.13926	203.13902	Ala-dl-Leu	$C_9H_{18}N_2O_3$	√	0.24286	SF/SL
26	21.71	157.13387	157.13354	/	$C_8H_{16}N_2O$	√	0.33293	SF
27	21.98	132.10214	132.10191	L-Norleucine	$C_6H_{13}NO_2$	√	0.23805	SF/SL
27	21.98	263.19726	263.19653	/	$C_{12}H_{26}N_2O_4$	√	0.72811	SF
27	22.00	285.17930	285.18088	/	$C_{14}H_{24}N_2O_4$	√	1.58048	SF
28	22.19	261.14444	261.14450	Glu-Leu	$C_{11}H_{20}N_2O_5$	√	0.05816	SF/SL
28	22.27	189.12356	189.12337	Gly-Ile	$C_8H_{16}N_2O_3$	√	0.18746	SF/SL
			189.12405	Val-Ala	$C_8H_{16}N_2O_3$	√	0.49	LSc
28	22.57	215.13925	215.13902	/	$C_{10}H_{18}N_2O_3$	√	0.23497	SF
			215.1396	Diketo-Thr-Ile	equivalent to SF	√	0.35	LSc

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
29	22.96	148.06084	148.06043	/	$C_5H_9NO_4$	√	0.40922	SF
			148.05651	Glutamic acid	equivalent to SF	√	4.33	LSc
29	22.96	261.14465	261.14450	Ile-Glu	$C_{11}H_{20}N_2O_5$	√	0.14726	SF/SL
30	23.30	358.19784	358.19726	Ile-Pro-Glu	$C_{16}H_{27}N_3O_6$	√	0.57516	SF/SL
30	23.34	261.14429	261.14450	Ile-Glu	$C_{11}H_{20}N_2O_5$	√	0.21098	SF/SL
30	23.35	215.13908	215.13902	/	$C_{10}H_{18}N_2O_3$	√	0.05839	SF
				Pro-Val	detected twice.			
31	23.81	219.13422	219.13527	/	$C_{10}H_{14}N_6$	√	1.04695	SF
			219.13461	Ile-Ser	$C_9H_{18}N_2O_4$	√	0.39	LSc
31	23.82	229.11845	229.11962	/	$C_{11}H_{12}N_6$	√	1.17109	SF
			229.11885	Diketo-Glu-Val	$C_{10}H_{16}N_2O_4$	√	0.4	LSC
31	23.84	330.20286	330.20235	Leu-Pro-Thr	$C_{15}H_{27}N_3O_5$	√	0.50886	SF/SL
32	24.55	148.06077	148.06043	/	$C_5H_9NO_4$	√	0.33221	SF
			148.05651	Glutamic acid	equivalent to SF	√	4.26	LSc
32	24.57	247.12925	247.12885	Val-Glu	$C_{10}H_{18}N_2O_5$	√	0.40426	SF/SL
33	24.81	175.10779	175.10772	Val-Gly	$C_7H_{14}N_2O_3$	√	0.07312	SF/SL
				Detected in SEC but not identified				
34	25.24	245.11350	245.11320	Pro-Glu	$C_{10}H_{16}N_2O_5$	√	0.30692	SF/SL
34	25.31	233.11334	233.11320	Val-Asp	$C_9H_{16}N_2O_5$	√	0.14622	SF/SL
35	25.53	235.11895	235.11895	/	$C_{11}H_{14}N_4O_2$	√	0.00099	SF
			235.11951	Diketo-His-Pro	equivalent to SF	√	0.56	LSc
35	25.67	310.12898	310.12985	/	$C_{16}H_{15}N_5O_2$	√	0.86938	SF
36	25.89	173.09271	173.09207	Pro-Gly	$C_7H_{12}N_2O_3$	√	0.63690	SF/SL
36	25.97	217.11847	217.11828	Pro-Thr	$C_9H_{16}N_2O_4$	√	0.18504	SF/SL
36	26.01	276.14455	276.14550	/	$C_{13}H_{17}N_5O_2$	√	0.95078	SF
36	26.11	205.11852	205.11828	Val-Ser	$C_8H_{16}N_2O_4$	√	0.24072	SF/SL
36	26.13	201.08715	201.08698	/	$C_8H_{12}N_2O_4$	√	0.17031	SF
			201.08755	Diketo-Glu-Ala	equivalent to SF	√	0.4	LSc
37	26.64	259.09299	259.09380	/	$C_{11}H_{10}N_6O_2$	√	0.81134	SF
			259.09322	pyro-Glu-Glu	$C_{10}H_{14}N_2O_6$	√	0.23	LSc
38	27.92	148.06067	148.06043	/	$C_5H_9NO_4$	√	0.23210	SF
			148.05651	Glutamic acid	equivalent to SF	√	4.16	LSc
38	27.92	235.09269	235.09246	Ser-Glu	$C_8H_{14}N_2O_6$	√	0.22953	SF/SL
39	28.34	147.07665	147.07642	/	$C_5H_{10}N_2O_3$	√	0.23229	SF
			147.07250	Glutamine	equivalent to SF	√	4.05	LSc
39	28.36	258.10860	258.10978	/	$C_{11}H_{11}N_7O$	√	1.17972	SF
			258.10901	Diketo-Glu-Gln	$C_{10}H_{15}N_3O_5$	√	0.41	LSc
39	28.36	276.11939	276.11901	Glu-Gln	$C_{10}H_{17}N_3O_6$	√	0.37877	SF/SL
			276.1197	Ala-Asp-Ala	$C_{10}H_{17}N_3O_6$	√	0.31	LSc
40	28.75	166.05324	166.05324	L-Methionine S-oxide	$C_5H_{11}NO_3S$	√	0.00266	SF/SL
41	30.35	162.07614	162.07608	/	$C_6H_{11}NO_4$	√	0.05486	SF
41	30.36	180.08675	180.08665	/	$C_6H_{13}NO_5$	√	0.10078	SF
42	30.82	200.97242	200.97130	/	$C_4N_4O_4S$	√	1.12019	SF
42	30.82	156.98229	156.97976	/	$C_4HN_2O_3P$	√	2.53130	SF
42	30.82	182.96188	182.95690	/	$C_7H_2O_2S_2$	√	4.98544	SF
43	31.32	147.11284	147.11280	/	$C_6H_{14}N_2O_2$	√	0.03383	SF
			147.10888	Lysine	equivalent to SF	√	3.96	LSc
43	31.31	175.11894	175.11895	L-Arginine	$C_6H_{14}N_4O_2$	√	0.01493	SF/SL
43	31.41	156.07675	156.07675	/	$C_6H_9N_3O_2$	√	0.00343	SF
			156.07283	Histidine	equivalent to SF	√	3.92	LSc

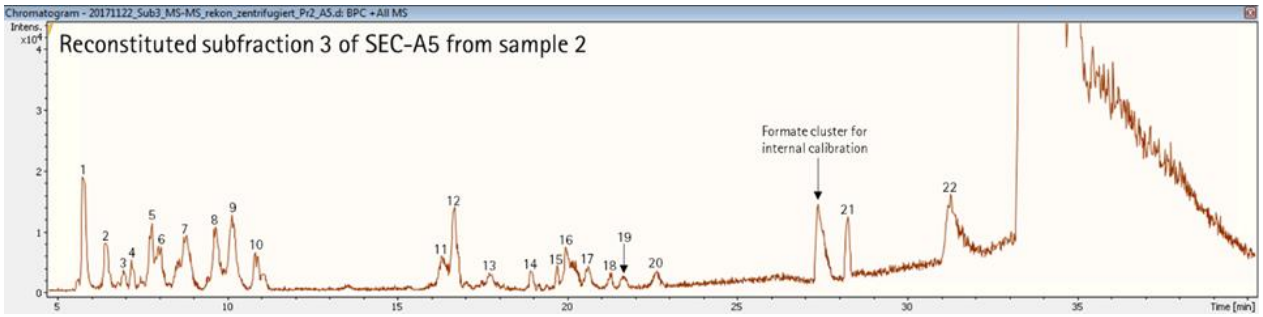


Supplementary figure 29: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 2 of SEC-A5 from sample 2. The numbering of the peaks correlates with the numbering in Supplementary table 29.

Supplementary table 29: Detected mass to charge ratios in sub-fraction 2 of SEC-A5 sample 2. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 29. Results highlighted in light green were also detected in the starting material. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	5.82	149.05997	149.05971	/	$C_9H_8O_2$	√	0.26635	SF
1	5.82	337.10471	337.10436	/	$C_{16}H_{12}N_6O_3$	√	0.34431	SF
1	5.83	381.13222	381.13192	/	$C_{19}H_{12}N_{10}$	√	0.30393	SF
2	6.6	261.1105	/	/	/	/	/	/
3	7.23	233.07866	233.07815	/	$C_9H_8N_6O_2$	√	0.51410	SF
4	7.53	185.11528	185.11722	/	$C_{10}H_{16}O_3$	√	1.93999	SF
5	7.79	229.14124	229.14075	/	$C_8H_{16}N_6O_2$	√	0.48992	SF
5	7.94	359.14948	359.15025	/	$C_{21}H_{18}N_4O_2$	√	0.76759	SF
			359.15681	Glu-Pro-Asn	$C_{14}H_{22}N_4O_7$	√	6.56	LSc
5	7.94	376.17558	376.17680	/	$C_{21}H_{21}N_5O_2$	√	1.22472	SF
			376.17213	Glu-Asp-Ile	$C_{15}H_{25}N_3O_8$	√	4.67	LSc
5	7.94	381.13104	381.13192	/	$C_{19}H_{21}N_{10}$	√	0.87829	SF
			381.12677	Gln-Met-Cys	$C_{13}H_{24}N_4O_5S_2$	√	5.15	LSc
5	8.03	273.16775	273.16696	/	$C_{10}H_{20}N_6O_3$	√	0.78176	SF
6	8.83	273.16775	273.16696	/	$C_{10}H_{20}N_6O_3$	√	0.62387	SF
7	10.18	215.12601	215.12779	/	$C_{11}H_{18}O_4$	√	1.77698	SF
8	10.65	625.32130	625.32319	/	$C_{33}H_{44}N_4O_8$	√	1.89136	SF
8	10.66	603.33919	603.33946	/	$C_{23}H_{52}N_6O_8P_2$	√	0.27080	SF
8	10.66	620.36545	620.36504	/	$C_{18}H_{50}N_{15}O_5PS$	√	0.40545	SF
8	10.78	611.30479	611.30620	/	$C_{31}H_{46}O_{12}$	√	1.41231	SF
8	10.84	597.28971	597.28817	/	$C_{21}H_{37}N_{14}O_5P$	√	1.53324	SF
8	10.88	231.12110	231.12270	/	$C_{11}H_{18}O_5$	√	1.60199	SF
			231.11674	Diketo-Met-Val	$C_{10}H_{18}N_2O_2S$	√	4.36	LSc
8	10.91	198.14922	198.14886	/	$C_{11}H_{19}NO_2$	√	0.36461	SF
8	11.03	285.13146	285.13192	/	$C_{11}H_{12}N_{10}$	√	0.45328	SF
			285.13516	Diketo-Phe-His	$C_{15}H_{16}N_4O_2$	√	3.7	LSc
9	16.38	217.10515	217.10705	/	$C_{10}H_{16}O_5$	√	1.89880	SF
			217.10109	Diketo-Ile-Cys	$C_9H_{16}N_2O_2S$	√	4.03	LSc
10	17.27	229.11885	229.11828	/	$C_{10}H_{16}N_2O_4$	√	0.56697	SF
			229.11885	Diketo-Glu-Val	equivalent to SF	√	0.00	LSc
10	17.32	130.05044	130.04987	/	$C_5H_7NO_3$	√	0.57359	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
11	18.16	240.23290	240.23219	/	$C_{15}H_{29}NO$	√	0.70760	SF
11	18.16	343.29626	343.29552	/	$C_{19}H_{38}N_2O_3$	√	0.74333	SF
12	18.51	326.17238	326.17238	/	$C_{16}H_{19}N_7O$	√	0.52933	SF
13	19.38	227.10310	227.10397	/	$C_{11}H_{10}N_6$	√	0.86928	SF
			227.1032	Diketo-Glu-Pro	$C_{10}H_{14}N_2O_4$	√	0.1	LSc
13	19.38	453.20120	453.20270	/	$C_{18}H_{28}N_8O_4S$	√	1.49366	SF
			453.20991	Arg-Tyr-Asp	$C_{19}H_{28}N_6O_7$	√	8.71	LSc
14	19.65	245.18644	245.18597	Ile-Ile	$C_{12}H_{24}N_2O_3$	√	0.46971	SF/SL
15	20.84	328.22372	328.22308	Val-Pro-Leu	$C_{16}H_{29}N_3O_4$	√	0.63431	SF/SL
15	20.85	229.15500	229.15467	/	$C_{11}H_{20}N_2O_3$	√	0.33186	SF
			229.15535	Ile-Pro	equivalent to SF	√	0.35	LSc
16	21.29	392.21910	392.21800	Pro-Ile-Tyr	$C_{20}H_{29}N_3O_5$	√	1.10544	SF/SL
16	21.73	263.13934	263.13902	Phe-Pro	$C_{14}H_{18}N_2O_3$	√	0.32508	SF/SL
17	21.92	229.15510	229.15467	/	$C_{11}H_{20}N_2O_3$	√	0.43523	SF
			229.15535	Ile-Pro	equivalent to SF	√	0.25	LSc
17	22.08	334.17666	334.17613	Pro-Ala-Phe	$C_{17}H_{23}N_3O_4$	√	0.52434	SF/SL
18	22.40	277.11830	277.11962	/	$C_{15}H_{12}N_6$	√	1.31718	SF
			227.11828	pyro-Glu-Phe	$C_{11}H_{20}N_2O_3$	√	0.02	LSc
18	22.60	485.18894	285.18697	/	$C_{22}H_{32}N_2O_6PS$	√	1.96847	SF
19	28.29	145.04997	145.04954	/	$C_6H_8O_4$	√	0.43282	SF
19	28.30	163.06042	163.06010	/	$C_6H_{10}O_5$	/	0.32340	SF
19	28.30	365.10618	365.10784	/	$C_{14}H_{20}O_{11}$	√	1.66170	SF
20	31.31	200.97273	200.97130	/	$C_4N_4O_4S$	√	1.42960	SF
20	31.32	155.97479	155.97499	/	C_5HNO_3S	√	0.20345	SF

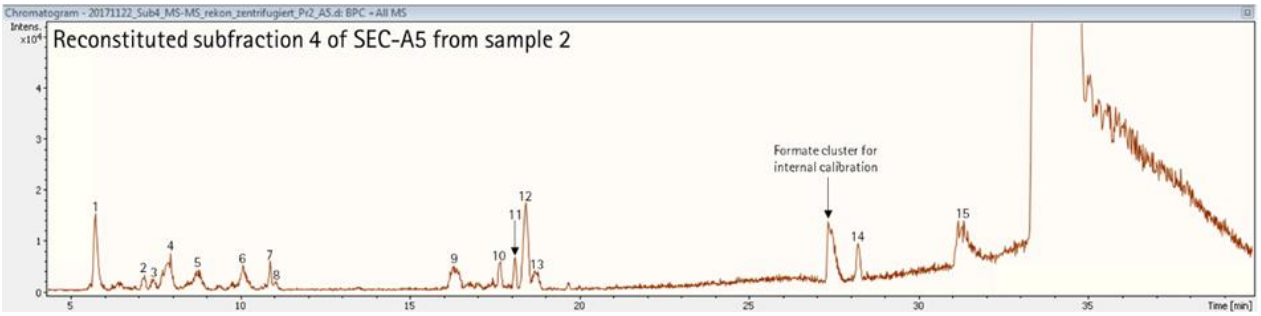


Supplementary figure 30: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 3 of SEC-A5 from sample 2. The numbering of the peaks correlates with the numbering in Supplementary table 30.

Supplementary table 30: Detected mass to charge ratios in sub-fraction 3 of SEC-A5 sample 2. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 30. Results highlighted in light green were also detected in the starting material. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	5.81	149.05942	149.05971	/	$C_9H_8O_2$	√	0.28767	SF
1	5.81	337.10400	337.10436	/	$C_{16}H_{12}N_6O_3$	√	0.36305	SF
1	5.82	381.12991	381.13058	/	$C_{18}H_{16}N_6O_4$	√	0.66496	SF
			381.12677	Gln-Met-Cys	$C_{13}H_{24}N_4O_5S_2$	√	3.14	LSc
2	6.44	271.18746	271.18770	/	$C_{11}H_{22}N_6O_2$	√	0.23807	SF
3	6.98	257.13569	257.13566	/	$C_9H_{16}N_6O_3$	√	0.02196	SF
4	7.22	233.07791	233.07815	/	$C_9H_8N_6O_2$	√	0.24368	SF
5	7.77	207.15869	207.15909	/	$C_{10}H_{22}O_4$	√	0.39391	SF

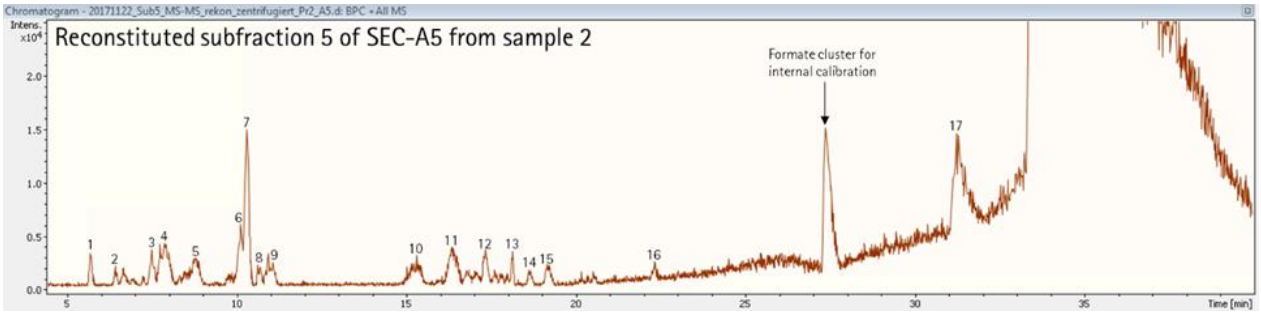
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
5	7.77	229.14066	229.14075	/	$C_8H_{16}N_6O_2$	√	0.09315	SF
5	7.93	376.17485	376.17296	/	$C_{24}H_{25}NOS$	√	1.88609	SF
			376.17213	Glu-Asp-Ile	$C_{25}H_{25}N_3O_8$	√	0.83	LSc
6	8.02	251.18527	251.18664	/	$C_{13}H_{22}N_4O$	√	1.36385	SF
6	8.03	273.16685	273.16696	/	$C_{10}H_{20}N_6O_3$	√	0.11741	SF
7	8.86	365.19260	365.19452	/	$C_{17}H_{20}N_{10}$	√	1.91997	SF
7	8.90	308.18565	308.18697	/	$C_{18}H_{21}N_5$	√	1.32050	SF
8	9.70	211.14357	211.14410	/	$C_{11}H_{18}N_2O_2$	√	0.53592	SF
			211.14467	Diketo-Pro-Ile	equivalent to SF	√	1.1	LSc
8	9.81	259.15097	259.15131	/	$C_9H_{18}N_6O_3$	√	0.34483	SF
9	10.12	211.14358	211.14410	/	$C_{11}H_{18}N_2O_2$	√	0.52269	SF
			211.14467	Diketo-Pro-Ile	equivalent to SF	√	1.09	LSc
9	10.17	215.12485	215.12510	/	$C_7H_{14}N_6O_2$	√	0.24533	SF
10	10.81	197.12822	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.23433	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	0.8	LSc
10	10.85	231.11976	231.12001	/	$C_7H_{14}N_6O_3$	√	0.25006	SF
			231.11674	Diketo-Met-Val	$C_{10}H_{18}N_2O_2S$	√	3.02	LSc
10	11.14	210.10962	210.10978	/	$C_7H_{11}N_7O$	√	0.16698	SF
11	16.35	217.10432	217.10436	/	$C_6H_{12}N_6O_3$	√	0.04125	SF
			217.10109	Diketo-Ile-Cys	$C_9H_{16}N_2O_2S$	√	3.23	LSc
12	16.68	197.12798	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.46944	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	1.04	LSc
12	16.68	243.1335	243.13393	/	$C_{11}H_{18}N_2O_4$	√	0.41593	SF
			243.1345	Diketo-Glu-Ile	equivalent to SF	√	1.0	LSc
12	16.81	261.13049	261.13192	/	$C_9H_{12}N_{10}$	√	1.42744	SF
			261.1239	Diketo-Tyr-Pro	$C_{14}H_{16}N_2O_3$	√	6.59	LSc
13	17.74	340.18683	340.18803	/	$C_{17}H_{33}N_7O$	√	1.20393	SF
13	17.76	132.10184	132.10191	/	$C_6H_{13}NO_2$	√	0.06444	SF
			132.09798	Leu; Ile	equivalent to SF	√	3.86	LSc
14	18.95	182.08112	182.08117	/	$C_9H_{11}NO_3$	√	0.05125	SF
			182.07725	Tyr	equivalent to SF	√	3.87	LSc
14	18.96	390.16529	390.16461	/	$C_{16}H_{15}N_{13}$	√	0.67492	SF
			390.16262	Glu-Gln-Asn	$C_{14}H_{23}N_5O_8$	√	1.37	LSc
14	19.00	229.15398	229.15467	/	$C_{11}H_{20}N_2O_3$	√	0.09315	SF
			229.15535	Ile-Pro	equivalent to SF	√	1.37	LSc
15	19.72	598.29833	598.29704	/	$C_{28}H_{43}N_3O_{11}$	√	1.29309	SF
16	20.00	342.23837	342.23873	Leu-Pro-Ile	$C_{17}H_{31}N_3O_4$	√	0.36617	SF/SL
17	20.63	362.20722	362.20743	Val-Pro-Phe	$C_{19}H_{27}N_3O_4$	√	0.21176	SF/SL
18	21.29	505.26584	505.26483	/	$C_{27}H_{41}N_2O_3PS$	√	1.01485	SF
19	21.68	263.13883	263.13902	Phe-Pro	$C_{14}H_{18}N_2O_3$	√	0.18533	SF/SL
20	22.64	489.23412	489.23303	/	$C_{21}H_{24}N_{14}O$	√	1.09457	SF
21	28.29	145.04933	145.05087	/	$C_7H_4N_4$	√	1.54012	SF
22	31.29	182.96171	182.95690	/	$C_7H_2O_2S_2$	√	4.81172	SF
22	31.30	200.97214	200.97130	/	$C_4H_4O_4S$	√	0.84178	SF



Supplementary figure 31: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 4 of SEC-A5 from sample 2. The numbering of the peaks correlates with the numbering in Supplementary table 31.

Supplementary table 31: Detected mass to charge ratios in sub-fraction 3 of SEC-A5 sample 2. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 31. Results highlighted in light green were also detected in the starting material. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	5.77	149.05932	149.05971	/	$C_9H_8O_2$	√	0.38324	SF
1	5.77	337.10395	337.10436	/	$C_{16}H_{12}N_6O_3$	√	0.41349	SF
1	5.78	381.13037	381.13058	/	$C_{18}H_{16}N_6O_4$	√	0.20949	SF
			381.12677	Gln-Met-Cys	$C_{13}H_{24}N_4O_5S_2$	√	3.6	LSc
2	7.18	233.07784	233.07815	/	$C_9H_8N_6O_2$	√	0.30616	SF
3	7.45	185.11449	185.11454	/	$C_6H_{12}N_6O$	√	0.04509	SF
3	7.48	279.09309	279.09167	/	$C_{20}H_{10}N_2$	√	1.41931	SF
			279.0981	Diketo-Asp-Tyr	$C_{13}H_{14}N_2O_5$	√	5.01	LSc
4	7.97	273.16657	273.16696	/	$C_{10}H_{20}N_6O_3$	√	0.39090	SF
4	7.98	251.18478	251.18664	/	$C_{13}H_{22}N_4O$	√	1.85310	SF
5	8.80	273.16653	273.16696	/	$C_{10}H_{20}N_6O_3$	√	0.43808	SF
6	10.10	195.08693	195.08765	/	$C_8H_{10}N_4O_2$	√	0.71915	SF
			195.0882	Diketo-His-Gly	equivalent to SF	√	1.27	LSc
6	10.11	215.12477	215.12510	/	$C_7H_{14}N_6O_2$	√	0.32502	SF
7	10.90	121.96594	/	/	/	√	/	/
7	10.91	144.98174	144.98550	/	C_7N_2S	√	3.75294	SF
7	10.93	245.13610	245.13566	/	$C_8H_{16}N_6O_3$	√	0.43925	SF
			245.12902	/	$C_{14}H_{16}N_2O_2$	√	7.08	LSc
8	11.10	210.10985	210.10978	/	$C_7H_{11}N_7O$	√	0.06274	SF
9	16.30	217.10443	217.10436	/	$C_6H_{12}N_6O_3$	√	0.06580	SF
			217.10109	Diketo-Ile-Cys	$C_9H_{16}N_2O_2S$	√	3.34	LSc
10	17.69	166.08618	166.08626	/	$C_9H_{11}NO_2$	√	0.07751	SF
			166.08233	Phe	equivalent to SF	√	3.85	LSc
10	17.69	374.17073	374.17238	/	$C_{20}H_{19}N_7O$	√	1.65560	SF
11	18.12	240.23161	240.23219	/	$C_{15}H_{29}NO$	√	0.57856	SF
11	18.12	343.29544	343.29552	/	$C_{19}H_{38}N_2O_3$	√	0.07428	SF
12	18.44	263.13901	263.13902	/	$C_{14}H_{18}N_2O_3$	√	0.00468	SF
			263.13958	Diketo-Tyr-Val	equivalent to SF	√	0.57	LSc
			263.1397	Pro-Phe	equivalent to SF	√	0.69	LSc
12	18.44	471.22390	471.22515	/	$C_{25}H_{26}N_8O_2$	√	1.24686	SF
13	18.72	229.15444	229.15467	/	$C_{11}H_{20}N_2O_3$	√	0.22872	SF
			229.15535	Ile-Pro	equivalent to SF	√	0.91	LSc
14	28.24	365.10500	365.10649	/	$C_{11}H_{12}N_{10}O_5$	√	1.49350	SF
14	28.25	145.04936	145.04954	/	$C_6H_8O_4$	/	0.17785	SF
14	28.25	163.05988	163.06144	/	$C_7H_6N_4O$	√	1.55522	SF
15	31.25	156.98222	156.97976	/	$C_4HN_2O_3P$	/	2.46804	SF
15	31.27	182.96165	182.95690	/	$C_7H_2O_2S_2$	√	4.75169	SF

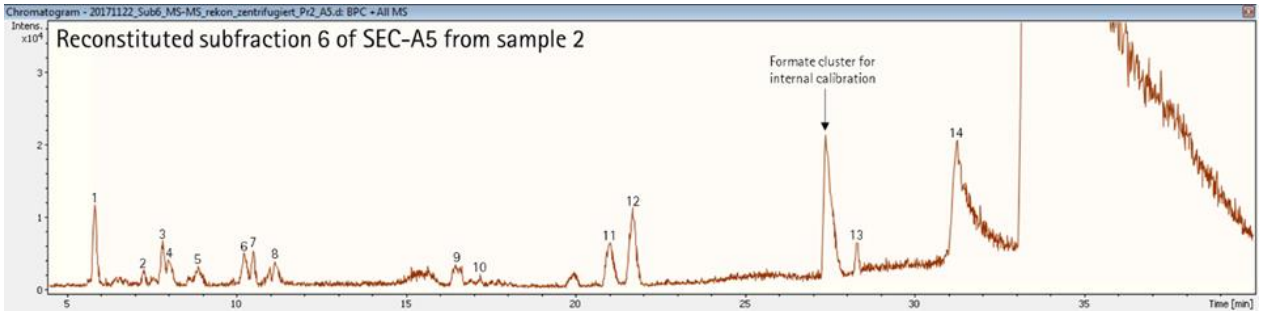


Supplementary figure 32: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 5 of SEC-A5 from sample 2. The numbering of the peaks correlates with the numbering in Supplementary table 32.

Supplementary table 32: Detected mass to charge ratios in sub-fraction 3 of SEC-A5 sample 2. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 32. Results highlighted in light green were also detected in the starting material. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	5.72	323.14587	323.14623	/	$C_{13}H_{18}N_6O_4$	√	0.36372	SF
2	6.46	271.18742	271.18770	/	$C_{11}H_{22}N_6O_2$	√	0.28360	SF
3	7.53	279.09251	279.09167	/	$C_{20}H_{10}N_2$	√	0.83185	SF
			279.0981	Diketo-Asp-Tyr	$C_{13}H_{14}N_2O_5$	√	5.59	LSc
3	7.54	228.19528	228.19581	/	$C_{13}H_{25}NO_2$	√	0.52365	SF
3	7.55	250.17747	250.17747	/	$C_{11}H_{19}N_7$	√	0.00373	SF
4	7.92	381.12985	381.13058	/	$C_{18}H_{16}N_6O_4$	√	0.72680	SF
			381.12677	Gln-Met-Cys	$C_{13}H_{24}N_4O_5S_2$	√	3.08	LSc
4	7.93	359.14816	359.14623	/	$C_{16}H_{18}N_6O_4$	√	1.93416	SF
			359.15681	Glu-Pro-Asn	$C_{14}H_{22}N_4O_7$	√	8.65	LSc
4	7.93	376.17406	376.17546	/	$C_{20}H_{25}NO_6$	√	1.40369	SF
			376.17213	Glu-Asp-Ile	$C_{15}H_{25}N_3O_8$	√	1.93	LSc
5	8.81	273.16670	273.16696	/	$C_{10}H_{20}N_6O_3$	√	0.26724	SF
6	10.14	195.08749	195.08765	/	$C_8H_{10}N_4O_2$	√	0.16485	SF
			195.0882	Diketo-His-Gly	equivalent to SF	√	0.71	LSc
6	10.15	215.12479	215.12510	/	$C_7H_{14}N_6O_2$	√	0.31372	SF
7	10.34	226.17967	226.18016	/	$C_{13}H_{23}NO_2$	√	0.48358	SF
7	10.34	266.17208	266.17238	/	$C_{11}H_{19}N_7O$	√	0.30843	SF
8	10.73	212.16403	212.16451	/	$C_{12}H_{21}NO_2$	√	0.47507	SF
9	10.97	245.13594	245.13585	/	$C_{16}H_{20}S$	√	0.09487	SF
			245.13239	Diketo-Met-Ile	$C_{11}H_{20}N_2O_2S$	√	3.55	LSc
9	11.00	201.10929	201.10945	/	$C_6H_{12}N_6O_2$	√	0.16368	SF
10	15.35	174.05468	174.05495	/	$C_{10}H_7NO_2$	√	0.26996	SF
11	16.38	217.10434	217.10436	/	$C_6H_{12}N_6O_3$	√	0.02660	SF
			217.10109	Diketo-Ile-Cys	$C_9H_{16}N_2O_2S$	√	3.25	LSc
12	17.36	130.04990	130.04987	/	$C_5H_7NO_3$	√	0.03023	SF
13	18.16	240.23215	240.23219	/	$C_{15}H_{29}NO$	√	0.04396	SF
14	18.66	316.21207	316.21319	/	$C_{17}H_{25}N_5O$	√	1.11254	SF
15	19.21	180.13816	180.13829	/	$C_{11}H_{17}NO$	√	0.13008	SF
16	22.34	166.08628	166.08626	/	$C_9H_{11}NO_2$	√	0.02382	SF
			166.08233	Phe	equivalent to SF	√	3.95	LSc
17	31.30	200.97227	200.97130	/	$C_4H_4O_4S$	√	0.96633	SF

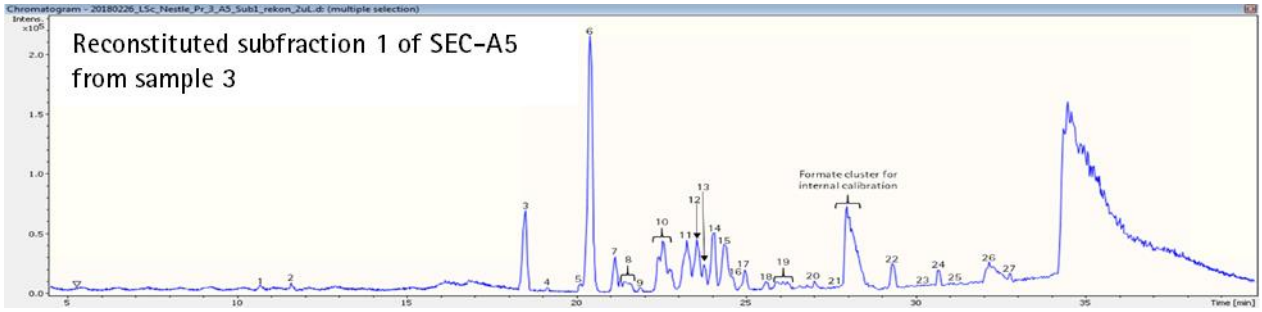
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
17	31.32	182.96165	182.96027	/	$C_4H_6O_2S_3$	√	1.38018	SF



Supplementary figure 33: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 5 of SEC-A5 from sample 2. The numbering of the peaks correlates with the numbering in Supplementary table 33.

Supplementary table 33: Detected mass to charge ratios in sub-fraction 3 of SEC-A5 sample 2. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 33. Results highlighted in light green were also detected in the starting material. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	5.86	149.05944	149.05971	/	$C_9H_8O_2$	√	0.27011	SF
1	5.86	337.10419	337.10436	/	$C_{16}H_{12}N_6O_3$	√	0.17098	SF
2	7.29	233.07816	233.07815	/	$C_9H_8N_6O_2$	√	0.01185	SF
3	7.85	229.14069	229.14075	/	$C_8H_{16}N_6O_2$	√	0.05876	SF
4	8.04	359.14842	359.15025	/	$C_{21}H_{18}N_4O_2$	√	1.82948	SF
			359.15681	Glu-Pro-Asn	$C_{14}H_{22}N_4O_7$	√	8.39	LSc*
4	8.04	376.17547	376.17412	/	$C_{17}H_{17}N_{11}$	√	1.35109	SF
			376.17213	Glu-Glu-Val	$C_{15}H_{25}N_3O_8$	√	3.34	LSc*
			376.17213	Glu-Asp-Ile	$C_{15}H_{25}N_3O_8$	√	3.34	LSc*
4	8.04	381.13033	381.12839	/	$C_{19}H_{25}O_4PS$	√	1.93695	SF
			381.12677	Gln-Met-Cys	$C_{13}H_{24}N_4O_5PS_2$	√	3.56	LSc*
5	8.90	273.16693	273.16696	/	$C_{10}H_{20}N_6O_3$	√	0.03192	SF
6	10.27	215.12511	215.12510	/	$C_7H_{14}N_6O_2$	√	0.00810	SF
7	10.51	266.17269	266.17238	/	$C_{11}H_{19}N_7O$	√	0.30665	SF
7	10.52	226.17979	226.18016	/	$C_{13}H_{23}NO_2$	√	0.36287	SF
8	11.17	188.12823	188.12812	/	$C_9H_{17}NO_3$	√	0.11051	SF
8	11.18	170.11753	170.11756	/	$C_9H_{15}NO_2$	√	0.02027	SF
8	11.21	210.10981	210.10978	/	$C_7H_{11}N_7O$	√	0.03069	SF
9	16.49	217.10469	217.10436	/	$C_6H_{12}N_6O_3$	√	0.32356	SF
			217.10109	Diketo-Ile-Cys	$C_9H_{16}N_2O_2S$	√	3.6	LSc*
10	17.20	283.17572	283.17647	/	$C_{13}H_{22}N_4O_3$	√	0.74431	SF
11	21.04	197.16507	197.16484	/	$C_{11}H_{20}N_2O$	√	0.22612	SF
12	21.71	167.11815	167.11789	/	$C_9H_{14}N_2O$	√	0.25725	SF
13	28.33	145.04979	145.04954	/	$C_6H_8O_4$	√	0.25635	SF
13	28.33	365.10597	365.10783	/	$C_{12}H_8N_{14}O$	√	1.85215	SF
14	31.29	156.98264	156.98550	/	C_8N_2S	√	2.85925	SF
14	31.29	200.97268	200.97130	/	$C_4N_4O_4S$	√	1.38147	SF



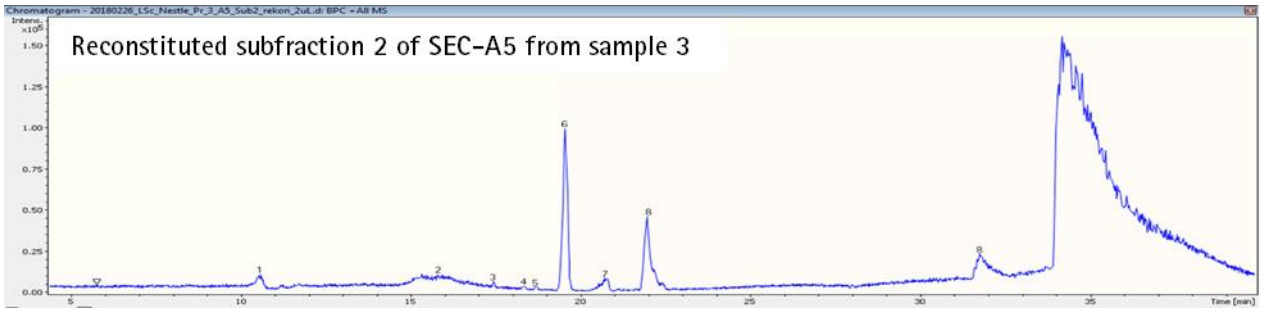
Supplementary figure 34: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 1 of SEC-A5 from sample 3. The numbering of the peaks correlates with the numbering in Supplementary table 34.

Supplementary table 34: Detected mass to charge ratios in sub-fraction 1 of SEC-A5 sample 3. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 34. Results highlighted in light green were also detected in the starting material. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	I Δ*m/z* I [mDa]	Annotations
1	10.71	326.37754	326.37813	/	$C_{22}H_{47}N$	√	0.58807	SF
2	11.63	304.29956	304.29988	/	$C_{21}H_{37}N$	√	0.31490	SF
2	11.68	468.41919	468.41999	/	$C_{32}H_{53}NO$	√	0.80327	SF
3	18.50	130.04962	130.04987	(R)-(+)-Pyrrolidone-5-carboxylic acid	$C_5H_7NO_3$	√	0.24669	SF/SL
3	18.50	259.09209	259.09246	/	$C_{10}H_{14}N_2O_6$	√	0.37230	SF
			259.09303	Diketo-Glu-Glu	equivalent to SF	√	0.94	LSc
3	18.50	388.13469	388.13639	/	$C_{16}H_{17}N_7O_5$	√	1.70215	SF
4	19.17	240.23194	240.23219	/	$C_{15}H_{29}NO$	√	0.25253	SF
4	19.17	343.19702	343.19893	/	$C_{19}H_{38}N_2O_3$	√	0.69228	SF
			343.19828	Val-Gln-Pro	$C_{15}H_{26}N_4O_5$	√	1.26	LSc
5	20.12	201.08632	201.08832	/	$C_9H_8N_6$	√	1.99942	SF
			201.08755	Diketo-Glu-Ala	$C_8H_{12}N_2O_3$	√	1.23	LSc
6	20.43	227.10233	227.10429	/	$C_8H_{19}O_5P$	√	1.95784	SF
			227.1032	Diketo-Glu-Pro	$C_{10}H_{14}N_2O_4$	√	0.87	LSc
6	20.43	453.19753	453.19830	/	$C_{15}H_{25}N_{12}O_3P$	√	0.76709	SF
7	21.16	209.09158	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.49294	SF
7	21.16	248.09984	248.10028	/	$C_8H_9N_9O$	√	0.44010	SF
7	21.17	226.11791	226.11862	/	$C_{10}H_{15}N_3O_3$	√	0.70488	SF
			226.11918	Diketo-Gln-Pro	equivalent to SF	√	1.27	LSc
8	21.31	187.07095	187.07267	/	$C_8H_6N_6$	√	1.72128	SF
			187.0719	Diketo-Asp-Ala	$C_7H_{10}N_2O_4$	√	0.95	LSc
			187.0719	Diketo-Glu-Gly	$C_7H_{10}N_2O_4$	√	0.95	LSc
8	21.38	263.13841	263.13902	Pro-Phe	$C_{14}H_{18}N_2O_3$	√	0.60743	SF/SL
8	21.38	231.16932	231.17032	/	$C_{11}H_{22}N_2O_3$	√	0.99597	SF
8	21.42	259.09195	259.09246	/	$C_{10}H_{14}N_2O_6$	√	0.51392	SF
			259.09303	Diketo-Glu-Glu	equivalent to SF	√	1.08	LSc
8	21.42	241.08154	241.08324	/	$C_{11}H_8N_6O$	√	1.69540	SF
			241.07593	Diketo-His-Cys	$C_9H_{12}N_4O_2S$	√	5.61	LSc
8	21.50	229.15420	229.15467	Pro-Leu	$C_{11}H_{20}N_2O_3$	√	0.46582	SF/SL
8	21.55	195.07580	195.07642	/	$C_9H_{10}N_2O_3$	√	0.62246	SF
8	21.55	212.10262	212.10297	/	$C_9H_{13}N_3O_3$	√	0.34302	SF
			212.10353	Diketo-Pro-Asn	equivalent to SF	√	0.91	LSc
8	21.59	324.15459	324.15290	/	$C_{19}H_{21}N_3S$	√	1.69029	SF

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
			324.15608	Thr-Gly-Phe	$C_{15}H_{21}N_3O_5$	√	1.49	LSc
			324.15608	Ser-Phe-Ala	$C_{15}H_{21}N_3O_5$	√	1.49	LSc
9	21.90	231.09679	231.09755	/	$C_9H_{14}N_2O_5$	√	0.75815	SF
			231.09811	Diketo-Glu-Thr	equivalent to SF	√	1.32	LSc
10	22.56	251.13609	251.13633	/	$C_9H_{14}N_8O$	√	0.24368	SF
10	22.57	229.15419	229.15467	/	$C_{11}H_{20}N_2O_3$	√	0.47410	SF
			229.15535	Ile-Pro	equivalent to SF	√	1.16	LSc
10	22.77	263.19563	263.19653	/	$C_{12}H_{26}N_2O_4$	√	0.90763	SF
10	22.78	132.10165	132.10191	L-Norleucine	$C_6H_{13}NO_2$	√	0.25130	SF/SL
11	23.27	229.15425	229.15467	Pro-Leu	$C_{11}H_{20}N_2O_3$	√	0.46582	SF/LSc
11	23.35	211.14361	211.14410	/	$C_{11}H_{18}N_2O_2$	√	0.49333	SF
			211.14467	Diketo-Pro-Ile	equivalent to SF	√	1.06	LSc
11	23.37	326.20671	326.20743	Pro-Pro-Ile	$C_{16}H_{27}N_3O_4$	√	0.72521	SF/SL
12	23.58	132.10159	132.10191	L-Norleucine	$C_6H_{13}NO_2$	√	0.31130	SF/SL
12	23.58	263.19578	263.19653	/	$C_{12}H_{26}N_2O_4$	√	0.75699	SF
12	23.64	300.19136	300.19178	/	$C_{14}H_{25}N_3O_4$	√	0.42437	SF
			300.19247	Pro-Ile-Ala	equivalent to SF	√	1.11	LSc
13	23.78	355.16067	355.16255	/	$C_{16}H_{18}N_8O_2$	√	1.87534	SF
13	23.79	240.09727	240.09788	/	$C_{10}H_{13}N_3O_4$	√	0.61432	SF
14	24.02	132.10162	132.10191	L-Norleucine	$C_6H_{13}NO_2$	√	0.28529	SF/SL
14	24.05	258.10819	258.10845	/	$C_{10}H_{15}N_3O_5$	√	0.25694	SF
			258.10901	Diketo-Glu-Gln	equivalent to SF	√	0.82	LSc
14	24.06	241.08148	241.08190	/	$C_{10}H_{12}N_2O_5$	√	0.41710	SF
15	24.39	215.13857	215.13902	/	$C_{10}H_{18}N_2O_3$	√	0.45212	SF
			215.13958	Diketo-Thr-Ile	equivalent to SF	√	1.01	LSc
			215.1397	Val-Pro	equivalent to SF	√	1.13	LSc
15	24.41	279.13323	279.13393	/	$C_{14}H_{18}N_2O_4$	√	0.69903	SF
			279.13461	Tyr-Pro	equivalent to SF	√	1.38	LSc
15	24.48	189.12281	189.12337	/	$C_8H_{16}N_2O_3$	√	0.56191	SF
			189.12404	Ile-Gly	equivalent to SF	√	1.23	LSc
			189.12404	Val-Ala	equivalent to SF	√	1.23	LSc
16	24.59	355.16058	355.16121	/	$C_{15}H_{22}N_4O_6$	√	0.62823	SF
16	24.60	147.07594	147.07642	/	$C_5H_{10}N_2O_3$	√	0.47857	SF
			147.07250	Glutamine	equivalent to SF	√	3.44	LSc
17	24.83	312.19176	312.19178	Pro-Pro-Val	$C_{15}H_{25}N_3O_4$	√	0.02404	SF/SL
17	24.97	330.20167	330.20235	Leu-Pro-Thr	$C_{15}H_{27}N_3O_5$	√	0.67446	SF/SL
17	24.98	133.03129	133.03178	/	$C_5H_8O_2S$	/	0.48933	SF
17	24.99	150.05791	150.05833	L-Methionine	$C_5H_{11}NO_2S$	√	0.51125	SF/SL
17	25.10	247.12786	217.12885	Ile-Asp	$C_{10}H_{18}N_2O_5$	√	0.99091	SF/SL
18	25.60	247.12829	247.12885	Val-Glu	$C_{10}H_{18}N_2O_5$	√	0.55889	SF/SL
18	25.61	148.05997	148.06177	/	$C_6H_5N_5$	√	1.79727	SF
			148.05651	Glutamic acid	$C_5H_9NO_4$	√	3.46	LSc
19	25.89	219.13365	219.13393	Ile-Ser	$C_9H_{18}N_2O_4$	√	0.28506	SF/SL
19	25.91	260.16013	260.16048	Gly-Leu-Ala	$C_{11}H_{21}N_3O_4$	√	0.35623	SF/SL
19	25.97	187.10718	187.10772	/	$C_8H_{14}N_2O_3$	√	0.53396	SF
			187.1084	Pro-Ala	equivalent to SF	√	1.22	LSc
19	26.11	357.21254	357.21325	Ile-Pro-Gln	$C_{16}H_{28}N_4O_5$	√	0.70561	SF/SL
19	26.13	205.11789	205.11962	Val-Ser	$C_8H_{16}N_2O_4$	√	1.73035	SF/SL
19	26.25	233.11292	233.11320	Val-Asp	$C_9H_{16}N_2O_5$	√	0.28221	SF/SL
19	26.36	254.16094	254.16115	/	$C_{11}H_{19}N_5O_2$	√	0.20607	SF
			254.16172	Diketo-Arg-Pro	equivalent to SF	√	0.78	LSc
20	26.84	217.11771	217.11828	Pro-Thr	$C_9H_{16}N_2O_4$	√	0.57726	SF/SL
20	26.91	231.09743	231.09889	/	$C_{10}H_{10}N_6O$	√	1.45676	SF
			231.09811	Diketo-Glu-Thr	$C_9H_{14}N_2O_5$	√	0.68	LSc
20	27.00	226.15450	226.15500	/	$C_{11}H_{19}N_3O_2$	√	0.49947	SF
			226.15557	Diketo-Lys-Pro	equivalent to SF	√	1.07	LSc
20	27.05	205.11787	205.11828	Val-Ser	$C_8H_{16}N_2O_4$	√	0.41380	SF/SL

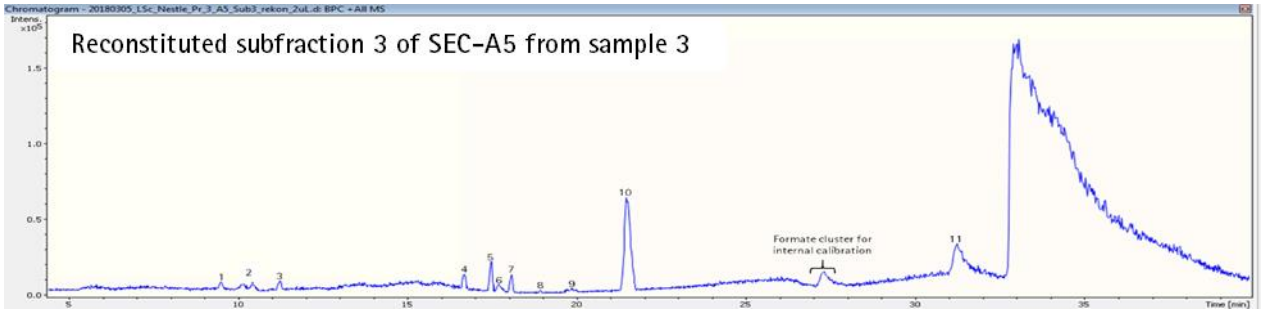
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
20	27.07	276.14349	276.14282	/	$C_9H_{13}N_{11}$	√	0.67071	SF
21	27.68	203.10200	203.10263	Pro-Ser	$C_8H_{14}N_2O_4$	√	0.66489	SF/SL
21	27.83	148.06021	148.06043	L-Glutamate	$C_5H_9NO_4$	√	0.22046	SF/SL
21	27.89	244.12873	244.13052	/	$C_{11}H_{13}N_7$	√	1.79366	SF
22	29.34	130.04956	130.04987	/	$C_5H_7NO_3$	√	0.31391	SF
22	29.35	147.07592	147.07642	D-Glutamine	$C_5H_{10}N_2O_3$	√	0.49918	SF/SL
22	29.37	293.14508	293.14556	/	$C_{10}H_{20}N_4O_6$	√	0.48161	SF
23	30.39	275.13415	275.13500	Gln-Gln	$C_{10}H_{18}N_4O_5$	√	0.84986	SF/SL
24	30.70	234.10726	234.10845	Ser-Gln	$C_8H_{15}N_3O_5$	√	1.18248	SF/SL
24	30.71	217.08161	217.08190	/	$C_8H_{12}N_2O_5$	√	0.28657	SF
			217.08246	Diketo-Glu-Ser	equivalent to SF	√	0.85	LSc
25	31.08	273.10812	273.10945	/	$C_{12}H_{12}N_6O_2$	√	1.32765	SF
25	31.14	332.15589	332.15646	Gly-Gln-Gln	$C_{12}H_{21}N_5O_6$	√	0.57193	SF/SL
26	32.23	182.96141	182.95690	/	$C_7H_2O_2S_2$	√	4.51040	SF
27	32.79	175.11845	175.11895	L-Arginine	$C_6H_{14}N_4O_2$	√	0.55057	SF/SL
27	32.87	147.11278	147.11280	/	$C_6H_{14}N_2O_2$	√	0.41281	SF
27	32.87	156.07654	156.07675	L-Histidine	$C_6H_9N_3O_2$	√	0.20987	SF/SL



Supplementary figure 35: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 2 of SEC-A5 from sample 3. The numbering of the peaks correlates with the numbering in Supplementary table 35.

Supplementary table 35: Detected mass to charge ratios in sub-fraction 2 of SEC-A5 sample 3. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 35. Results highlighted in light green were also detected in the starting material.

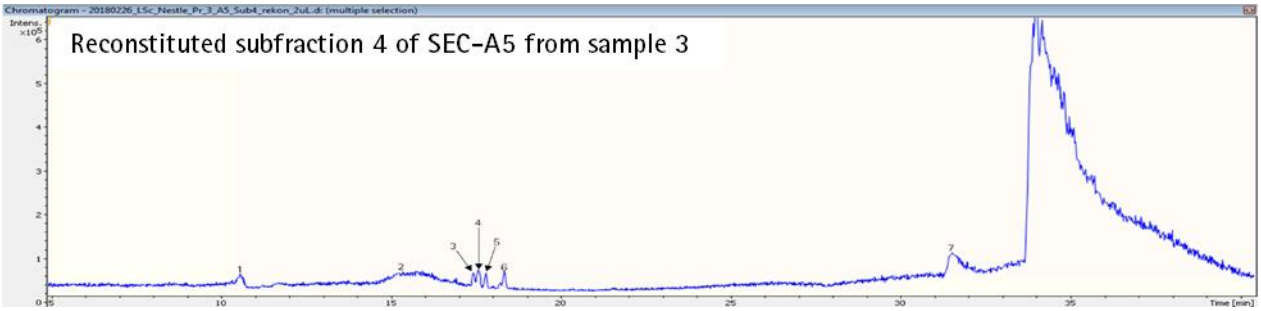
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	I Δ*m/z* I [mDa]	Annotations
1	10.56	326.37770	326.37813	/	$C_{22}H_{47}N$	√	0.42842	SF
2	15.95	304.29933	304.29988	/	$C_{21}H_{37}N$	√	0.54683	SF
2	15.95	468.41907	468.41999	/	$C_{32}H_{53}NO$	√	0.92182	SF
3	17.47	229.11759	229.11828	/	$C_{10}H_{16}N_2O_4$	√	0.68804	SF
			229.11885	Diketo-Asp-Ile	equivalent to SF	√	1.26	LSc
4	18.36	240.23203	240.23219	/	$C_{15}H_{29}NO$	√	0.15988	SF
5	18.72	209.09188	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.18801	SF
5	18.72	326.17062	326.17105	/	$C_{15}H_{23}N_3O_5$	√	0.42332	SF
6	19.57	227.10236	227.10263	/	$C_{10}H_{14}N_2O_4$	√	0.26942	SF
			227.1032	Diketo-Glu-Pro	equivalent to SF	√	0.84	LSc
6	19.57	453.19741	453.19830	/	$C_{15}H_{25}N_{12}O_3P$	√	0.88402	SF
6	19.57	475.17933	475.18131	/	$C_{16}H_{27}N_8O_7P$	√	1.97366	SF
7	20.76	324.15478	324.15540	/	$C_{15}H_{21}N_3O_5$	√	0.62110	SF
			324.15607	Thr-Phe-Gly	equivalent to SF	√	1.29	LSc
			324.15607	Ala-Tyr-Ala	equivalent to SF	√	1.29	LSc
8	21.97	120.08044	120.08078	/	C_8H_9N	√	0.33086	SF
8	21.97	263.13863	263.13902	L-phenylalanyl-L-proline	$C_{14}H_{18}N_2O_3$	√	0.39222	SF/SL
8	22.19	229.15415	229.15467	/	$C_{11}H_{20}N_2O_3$	√	0.51618	SF
			229.15535	Ile-Pro	equivalent to SF	√	1.2	LSc
9	31.79	200.97184	200.97130	/	$C_4N_4O_4S$	√	0.54000	SF
9	31.83	182.96132	182.95690	/	$C_7H_2O_2S_2$	√	4.42038	SF



Supplementary figure 36: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 3 of SEC-A5 from sample 3. The numbering of the peaks correlates with the numbering in Supplementary table 36.

Supplementary table 36: Detected mass to charge ratios in sub-fraction 3 of SEC-A5 sample 3. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 36. Results highlighted in light green were also detected in the starting material. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

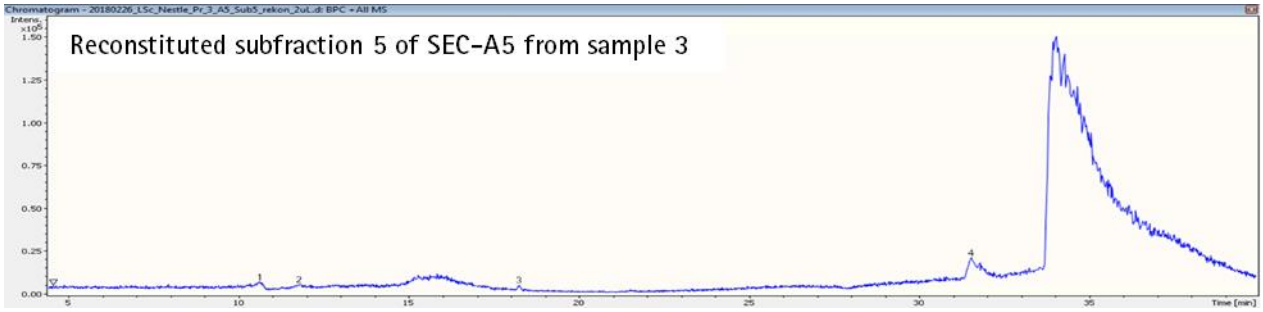
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	9.51	211.14306	211.14410	/	$C_{11}H_{18}N_2O_2$	√	1.04280	SF
			211.14467	Diketo-Pro-Ile	equivalent to SF	√	1.61	LSc
1	9.54	521.37963	521.37830	/	$C_{23}H_{44}N_{12}O_2$	√	1.33237	SF
2	10.16	326.37732	326.37813	/	$C_{22}H_{47}N$	√	0.80296	SF
3	11.26	304.29895	304.29988	/	$C_{21}H_{37}N$	√	0.92570	SF
4	16.69	243.13297	243.13393	/	$C_{11}H_{18}N_2O_4$	√	0.96772	SF
			243.1345	Diketo-Glu-Ile	equivalent to SF	√	1.53	LSc
4	16.73	197.12723	197.12845	/	$C_{10}H_{16}N_2O_2$	√	1.22332	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	1.79	LSc
5	17.49	340.18571	340.18670	/	$C_{16}H_{25}N_3O_5$	√	0.98997	SF
5	17.49	362.16751	362.16836	/	$C_{14}H_{19}N_9O_3$	√	0.85513	SF
			362.1677	Gln-Gln-Ser	$C_{13}H_{23}N_5O_7$	√	0.19	LSc
			362.1677	Asn-Gln-Thr	$C_{13}H_{23}N_5O_7$	√	0.19	LSc
5	17.52	197.12747	197.12845	/	$C_{10}H_{16}N_2O_2$	√	0.98833	SF
			197.12902	Diketo-Pro-Val	equivalent to SF	√	1.55	LSc
6	17.73	132.10132	132.10191	/	$C_6H_{13}NO_2$	√	0.58078	SF
6	17.73	340.18581	340.18670	/	$C_{16}H_{25}N_3O_5$	√	0.88260	SF
7	18.09	326.16995	326.16836	/	$C_{11}H_{19}N_9O_3$	√	1.58990	SF
7	18.11	343.29432	343.29552	/	$C_{19}H_{38}N_2O_3$	√	1.19745	SF
7	18.12	240.23096	240.23219	/	$C_{15}H_{29}NO$	√	1.23473	SF
8	18.95	390.16454	390.16346	/	$C_{23}H_{23}N_3OS$	√	1.08072	SF
			390.16262	Gln-Glu-Asn	$C_{14}H_{23}N_5O_8$	√	1.92	LSc
8	18.96	182.08045	182.08117	/	$C_9H_{11}NO_3$	√	0.72298	SF
			182.07725	Tyrosine	equivalent to SF	√	3.2	LSc
9	19.75	376.22161	376.22308	Leu-Pro-Phe	$C_{20}H_{29}N_3O_4$	√	1.47069	SF/SL
9	19.88	342.23745	342.23873	Leu-Pro-Ile	$C_{17}H_{31}N_3O_4$	√	1.27770	SF/SL
10	21.52	120.08031	120.08078	/	C_8H_9N	√	0.46199	SF
10	21.52	263.13828	263.13902	L-phenylalanyl-L-proline	$C_{14}H_{18}N_2O_3$	√	0.74250	SF/SL
11	31.26	182.96102	182.96027	/	$C_4H_6O_2S_3$	√	0.74985	SF
11	31.27	200.97142	200.96958	/	$C_5HN_2O_5P$	√	1.83722	SF



Supplementary figure 37: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 4 of SEC-A5 from sample 3. The numbering of the peaks correlates with the numbering in Supplementary table 37.

Supplementary table 37: Detected mass to charge ratios in sub-fraction 4 of SEC-A5 sample 3. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 37. Results highlighted in light green were also detected in the starting material. Results highlighted in light yellow were not detected in the sub-fractionated SEC-fraction but at least in one of the SEC-fractions.

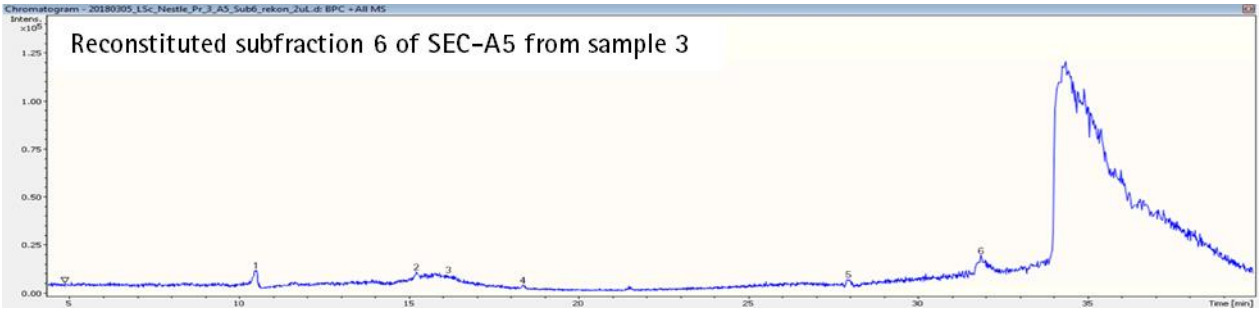
	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	10.59	326.37774	326.37813	/	$C_{22}H_{47}N$	√	0.38793	SF
2	15.37	468.42005	468.41999	/	$C_{32}H_{53}NO$	√	0.06142	SF
2	16.09	304.29983	304.29988	/	$C_{21}H_{37}N$	√	0.04263	SF
3	17.45	374.17100	374.17105	/	$C_{19}H_{23}N_3O_5$	√	0.04506	SF
4	17.60	340.18659	340.18803	/	$C_{17}H_{21}N_7O$	√	1.44000	SF
5	17.80	166.08609	166.08626	/	$C_9H_{11}NO_2$	√	0.16408	SF
			166.08233	Phenylalanine	equivalent to SF	√	3.76	LSc
5	17.80	209.09183	209.09207	/	$C_{10}H_{12}N_2O_3$	√	0.24319	SF
5	17.80	374.17096	374.17238	/	$C_{20}H_{19}N_7O$	√	1.42207	SF
6	18.23	240.23203	240.23219	/	$C_{15}H_{29}NO$	√	0.15690	SF
6	18.24	343.29599	343.29552	/	$C_{19}H_{38}N_2O_3$	√	0.47171	SF
6	18.37	471.22424	471.22381	/	$C_{24}H_{30}N_4O_6$	√	0.43303	SF
6	18.38	493.20577	493.20681	/	$C_{23}H_{20}N_{14}$	√	1.04117	SF
6	18.47	263.13898	263.13902	/	$C_{14}H_{18}N_2O_3$	√	0.03503	SF
			263.1397	Phe-Pro	equivalent to SF	√	0.72	LSc
7	31.56	200.97220	200.97130	/	$C_4N_4O_4S$	√	0.90322	SF
7	31.58	156.98194	156.97976	/	$C_4HN_2O_3P$	√	2.18598	SF



Supplementary figure 38: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 5 of SEC-A5 from sample 3. The numbering of the peaks correlates with the numbering in Supplementary table 38.

Supplementary table 38: Detected mass to charge ratios in sub-fraction 5 of SEC-A5 sample 3. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 38. Results highlighted in light green were also detected in the starting material.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	10.63	326.37786	326.37813	/	$C_{22}H_{47}N$	√	0.26875	SF
2	11.81	304.29965	304.29988	/	$C_{21}H_{37}N$	√	0.22565	SF
3	18.27	240.23217	240.23219	/	$C_{15}H_{29}NO$	√	0.01588	SF
3	18.27	343.29546	343.29552	/	$C_{19}H_{38}N_2O_3$	√	0.05782	SF
4	31.57	200.97201	200.97130	/	$C_4N_4O_4S$	√	0.70673	SF
4	31.58	182.96151	182.95690	/	$C_7H_2O_2S_2$	√	4.59609	SF



Supplementary figure 39: Base Peak chromatogram (BPC) of the ESI positive MS/MS mode of the reconstituted sub-fraction 6 of SEC-A5 from sample 3. The numbering of the peaks correlates with the numbering in Supplementary table 39.

Supplementary table 39: Detected mass to charge ratios in sub-fraction 6 of SEC-A5 sample 3. Indicated names of the substances were proposed by the operator (LSc) or calculated and identified by the spectral library (SL). Numbering in this table corresponds to the numbering in Supplementary figure 39. Results highlighted in light green were also detected in the starting material.

	RT [min]	*m/z* meas.	*m/z* calc.	Name	Molecular Formula	MS/MS	IΔ*m/z*I [mDa]	Annotations
1	10.51	326.37777	326.37813	/	$C_{22}H_{47}N$	√	0.35460	SF
2	15.37	468.41969	468.41999	/	$C_{32}H_{53}NO$	√	0.30243	SF
3	16.20	304.29960	304.29988	/	$C_{21}H_{37}N$	√	0.27441	SF
4	18.39	343.29530	343.29552	/	$C_{19}H_{38}N_2O_3$	√	0.22200	SF
4	18.41	240.23207	240.23219	/	$C_{15}H_{29}NO$	√	0.12509	SF
5	27.98	158.96409	158.96813	/	$C_4H_2N_2OS$	√	4.03651	SF
5	27.98	226.95094	226.94673	/	$C_8H_2O_4S_2$	√	4.20961	SF
6	31.87	182.96165	182.95690	/	$C_7H_2O_2S_2$	√	4.74804	SF
7	31.88	200.97201	200.97130	/	C4H4O4S	√	0.71101	SF

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WEITERER WERDEGANG

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