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„Mass Spectrometry based Multicomponent Analysis in Impurity Profiling and Metabolic Approaches “

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1. Objective

The present PhD thesis deals with multicomponent analysis in the context of pharmaceutical applications. Two such applications of multicomponent analysis were treated herein. The one application was addressing the establishment of qualitative and quantitative impurity profiles in stress test samples of an amino acid/dipeptide infusion solution and the other was focusing on the development of methods for metabolic profiling in fermentation broths from β-lactam antibiotics production. Basically, such applications require highly selective analysis methods in order to obtain accurate and reliable results. Nowadays, the analysis technique of choice constitutes liquid chromatography coupled to tandem mass spectrometry (MS) because of its wide applicability and flexibility. Nevertheless, also this very powerful combination has its shortcomings and pitfalls and thus, can not solve every analytical problem. Careful optimization was therefore necessary to avoid erroneous results.

In general, two strategies can be differentiated in multicomponent analyses that respond to different analytical tasks. The **comprehensive approach** aims at the elucidation of the entire composition of a sample. The aim is to give a comprehensive quantitative picture of the sample and to make sure that no compound is missed. In the **targeted approach** the focus lies on the quantification of a predefined set of analytes. All other compounds in the sample need not to be investigated but might interfere somehow with determinations of target compounds.

In the present work, both approaches were employed to cope with two different analytical problems, impurity profiling of a complex new drug product and metabolic profiling in fermentation broths, which are both related to analysis of multicomponent mixtures.

The first project with Fresenius Kabi was dealing with the establishment of a quantitative and qualitative impurity profile of a newly formulated nutritional infusion solution containing amino acids and dipeptides.

According to the guidelines of the International Conference on Harmonization (ICH) the impurity profile is considered to be a summary of all impurities that are present in a new drug substance independent, whether they are identified or not. Impurities in drug products may exhibit unwanted side effects and may pose a risk to patient's health. Thus, detailed knowledge of all impurities that may emerge during storage is of high importance and builds the basis for risk assessment and high drug product quality.

The task of the project was to unveil all relevant impurities within a stressed infusion solution, which was kept under forced degradation conditions, and to provide quantitative data that allow a classification of identified impurities according to the ICH guidelines into those that need to be reported, identified and qualified (vide infra).

Pharmaceutical impurity profiling of single compound drug products is quite straight forward as opposed to impurity profiling of multi compound drug products that may need a comprehensive analysis approach in order to not overlook any relevant impurities. In this context, one challenge is to adequately separate and detect all constituents in the infusion solution in order to assure that all (relevant) impurities are found. Thereby, the focus lies on the separation of low abundant impurities from active agents, present as bulk compounds, in order to avoid masking of the former ones. In this context the separation of impurities such as pairs of constitutional isomers and stereoisomers which can not be differentiated by MS and thus need to be adequately separated can represent a particular challenge. Moreover, identification and quantification of unknown impurities in stressed formulations are critical tasks. Concerning the purpose of quantification of unknown compounds the major problem is that response factors of most detectors are compound specific and may show strong compound to compound variations. For this reason calibration is usually performed with corresponding authentic standards. However, in all stages of impurity profiling standards are often not available as most compounds are unknown. Quantification relative to a structurally similar compound is then common practice in pharmaceutical impurity studies, although it involves the risk of severe under- or overestimation of impurities if response factors are significantly different.

One solution to this dilemma constitutes employment of universal detectors with consistent detector responses for structurally different species permitting use of unified calibration function. In this application a multidimensional RPLC-HILIC method with complementary detection systems such as Ion Trap Mass Spectrometry (IT-MS) for substance identification and Charged Aerosol Detector (CAD) for quantification was employed to establish comprehensive impurity profiles in stressed infusion solutions.

In the second project with Sandoz the objective was to develop LC-MS/MS methods intended for process optimization and process control of the biotechnological production of β–lactam antibiotics.

The world market for antibiotics is huge (23 billion dollar in 1996) and over half of it can be ascribed to β lactam antibiotics [1]. Thus, it is not surprising that pharmaceutical companies seek at the optimization and improvement of production processes. β–Lactam antibiotics like cephalosporin and penicillin derivatives are mainly produced biotechnologically. Biosynthetic pathways for these drugs are quite well known and genes encoding for involved enzymes have already been investigated and cloned. Such fermentation process may take place in huge scale fermenters with volume capacities up to 400 cubic meters. Thus, processes of such

dimensions need to be controlled carefully. Temperature, pH, dissolved CO_2, ammonia, sugar, content of precursor compounds etc. need to be continuously monitored to keep the process under control.

Basically, there are several strategies available to optimize fermentation production efficiency. One strategy is related to genetic modification of microorganism strains and the second one to the optimization of process conditions. Concerning the second approach detailed knowledge of the metabolism of the microorganisms plays an important role. It is essential to know under which conditions biosynthetic efficiency is improved, how to amplify viable biochemical pathways and when to supplement precursor compounds. Another way to improve productivity is optimization of down stream processing.

In this context, the main purpose of the project with Sandoz was to develop methods for targeted metabolic profiling. Metabolic profiling aims at the quantification of a predefined set of metabolites and thus, was treated with a targeted approach of multicomponent analysis.

The analysis technique of choice for quantification of multiple analytes in a complex matrix constitutes LC-MS/MS because of its high combined selectivity from chromatographic separation and detection specificity. Thus, the goal was to develop LC-MS/MS methods and detection in the MRM (multiple reaction monitoring) mode employing a hybrid triple quadrupole/linear ion trap (Q-Trap) for quantification of primary metabolites like amino acids and organic acids, secondary metabolites like penicillin and its biosynthetic intermediates but also nutrients like fatty acids and vitamins.

In contrast to the former discussed application, calibration standards were available and compound specific MRM transitions could be readily acquired. The major challenge constitutes accurate and reliable quantification of the analytes in the very complex matrix of fermentation extracts. Coelution of isobaric compounds, interferences, crosstalk and matrix effects are critical points to consider in method development. Furthermore, wide linear ranges are needed in order to cover the highly different concentrations of metabolites in the samples.

The characteristics and challenges of the two strategies which were employed to cope with multicomponent analyses in pharmaceutical applications and metabolomics are summarized in Figure 1.

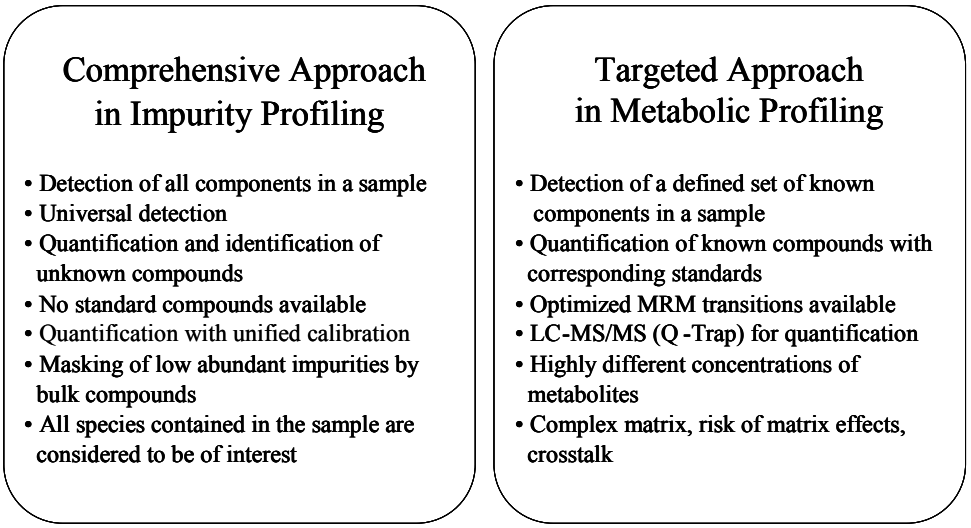

Figure 1: Comparison of the characteristics of the comprehensive approach for impurity profiling and the targeted approach for metabolic profiling.

2. Introduction

Multicomponent analysis is not a discipline of its own but is rather an issue reflecting the growing demands on today's analysis techniques. Multicomponent analysis can be found in many scientific fields e.g. in pesticide analysis, environmental analysis, metabolomics or proteomics. The number of analytes to monitor is growing as e.g. new environmental pollutants enter into the focus or new pesticides are developed. Complex plant extracts are investigated seeking for new potential herbal drug compounds. Ambitious new goals have been formulated like in metabolic research, where metabolomics studies target at the identification and quantification of the whole metabolome of a biological system (global approach). This is quite a challenging task thinking about the human metabolome which is estimated to comprise about 2 000 more or less structurally different compounds.

In various "omics" fields biomarker research plays an important role and constitutes a very ambitious aim, which seeks to identify one specific protein, sugar molecule, metabolite or a pattern of molecules indicating abnormal conditions in complex biological systems.

To cope with this kind of analysis highly potent separation techniques in combination with highly selective detection is required. Although the classical separation techniques liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE) have experienced enormous advance in the last centuries it is obvious that one dimensional analysis methods alone will not be sufficient to handle this kind of analytical tasks. The limiting factors constitute peak capacity and selectivity, as chromatographic methods provide a limited separation space i.e. only a defined number of peaks fit into the chromatogram.

The development of mass spectrometry (MS) was a milestone in analytical chemistry and analysts nowadays can hardly imagine life without mass spectrometry. The great benefit of MS arises due to its ability to function as an additional separation dimension as MS differentiates molecules according to their mass to charge ratios (m/z) allowing their specific detection. The logic step was to combine this highly selective new detector technology with separation techniques. Doing so complete separation of all compounds of a sample was claimed to be not obligatory anymore, as specific m/z could be independently detected adding a second dimension to the separation (although this is not always valid).

First combinations of MS and chromatographic techniques were hyphenations of GC and MS. The most frequently used ionization technique was electron impact ionization. Employing this "hard" ionization technique analyte molecules are strongly fragmented due to collisions with high energetic electrons (20 to 70 keV). The advantage of this analysis technique constitutes

its high reproducibility of the fragmentation pattern for specific ionization energies, which builds the basis for the construction of highly comprehensive structure libraries allowing fast and easy structure identification. One considerable pitfall of GC represents its limited application range, as GC is only suitable for volatile and rather hydrophobic compounds. Compounds containing polar functional groups require derivatization before they can be analyzed, which is associated with additional operational efforts and sources of errors.

The development of the electrospray ionization (ESI) interface was the breakthrough that made it possible to easily combine HPLC with mass spectrometry. For its development work on ESI-MS Thomas Fenn was awarded in 2002 with a share of the Nobel Prize of Chemistry, which underlines the importance of this new invention.

Further developments were brought about by MS/MS techniques through coupling of mass analyzers, which again opened up new avenues. In this manner, it was not only possible to separate ions according to their m/z ratio but also to distinguish them according to their specific fragmentation pattern.

Further advances of the MS technology aim at increasing selectivity and sensitivity. With mass analyzers like Time-of-Flight (TOF), Orbitrap or the Fourier Transform Ion Cyclotron Resonance (FT-ICR) extremely high mass accuracy and mass resolution can be achieved contributing enormously to the gain in selectivity.

Despite of all benefits that were provided by the steady technical advance in the last years MS alone is, by far, not capable to solve all analytical questions reliably that are raised in multicomponent analysis. For instance matrix effects arising due to modulation of ionization efficiencies by coeluting non detected matrix compounds and separation of isobaric and stereoisomeric compounds constitute still impairment that must be compensated for by powerful separation techniques.

On the sector of separation techniques also new ideas and techniques evolved in particular under the header of “Multidimensionality”. Instrumental set-ups for multidimensional separations in LC x LC and GC x GC are already commercially available. A powerful combination frequently used for the separation of proteins constitutes the combination of isoelectric focusing (IEF) and gelelectrophoresis (GE), where in the first dimension proteins are separated according to their isoelectric point and in the second dimension according to their molecular weight (2-D PAGE) [2]. However multidimensional separation can even go further by combining different separation techniques like LC x GC, LC x CE and LC x TLC (Thin Layer Chromatography) [3,4]. The key is to combine orthogonal methods that respond to different separation mechanisms.

Future efforts in the development of multidimensional separation techniques will aim at increasing automation and techniques that are compatible with MS.

2.1. Multicomponent analysis

Multicomponent analysis can be found in many fields like pesticide analysis, environmental analysis, toxicological analysis, pharmaceutical analysis, food chemistry, biomarker research, biological analysis like proteomics and metabolomics and in many more. Two fields in which multicomponent analysis is widely used are impurity profiling, especially of multicomponent drug products, and metabolic profiling. They are discussed in the following.

2.2.1. Pharmaceutical applications – Impurity profiling

In the course of the development of new drug products potential impurities originating from different sources have to be detected and identified. According to the ICH guidelines (Q3A: Impurities in new drug substances and Q3B: Impurities in new drug products) [5,6] they can be classified into organic impurities (process- and drug-related), inorganic impurities and residual solvents as summarized in Table 1.

Table 1: Classification of impurities in new drug substances (excluded are extraneous contaminants, polymorphic forms and enantiomeric impurities).

Organic Impurities	Inorganic Impurities	Residual Solvent
• Starting materials • Intermediates • Reagents • Ligands • Catalysts • By-products • Degradation products	• Reagents • Ligands • Catalysts • Heavy metals or other metals • Inorganic salts • Other materials (e.g. charcoal)	• Organic liquids • Inorganic liquids

Impurity profiling is of high priority throughout all stages of drug development. In the early phases of research of new drug compounds knowledge of compound stability and main degradation products of the drug substance supports selection of potential drug candidates for pharmacologic screenings and toxicologic tests. Furthermore, organic chemists need at this early stage of research information on key impurities in order to optimize synthesis strategies of target compounds [7].

During up-scaling of drug synthesis and process optimization continuous investigation and control of impurity profiles is obligatory in order to identify potential new impurities and to monitor possible changes in impurity contents. Early investigation of the stability of drug compounds and their susceptibility to transformation (e.g. oxidation) also provides valuable information for formulation research. The final composition of the drug product i.e. also its formulation has a tremendous influence on drug stability and thus on the quality of the drug product. Even at a late stage of drug development reformulation would be necessary in the case that a stability problem is discovered. The consequences in such a case are unpleasant, as additional costs come up and development time is increased [8].

In the guidelines of the International Conference on Harmonization of Clinical Requirements for Registration of Pharmaceuticals for Human Use (ICH) impurity profile is defined as "A description of the identified and unidentified impurities present in a drug product" [5,6] .

Every impurity, be it a degradation product or a side product, poses a risk of unwanted biologic activity and additional side effects, jeopardizing patient's health and well-being. Thus registration authorities demand detailed investigation of all impurities that may be contained in the final drug products and that may emerge during storage. In this context stability testing is performed to identify possible degradation products and degradation pathways [9]. In the course of stability testing the influence of different environmental factors like heat, light, pH or humidity on the stability of the active drug compound or the complete drug formulation is investigated in order to establish optimal storage conditions and shelf lives.

Degradation related impurities (DRI) found during stability testing can be classified according to the thresholds established by the ICH into those that need to be reported, identified and qualified (Table 2). For the establishment of a first qualitative and quantitative impurity profile, drug formulations stored under heavily forced degradation conditions are investigated employing orthogonal analysis methods. Based on the results of these first screening runs, stability indicating methods are developed that provide a comprehensive quantitative picture of all relevant degradation products formed during stress testing [10]. To ensure method reliability detailed validation of these methods has to be performed e.g. as proposed by the ICH [11].

Table 2: Thresholds for reporting, identification and qualification of degradation related impurities in new drug products [5,6] .

Classification	Maximum daily dose	% relative to the precursor compound	Action when thresholds are exceeded
Reporting threshold	≤ 1 g > 1 g	0.1 % 0.05 %	Impurity must be reported with the analytical procedure indicated
Identification threshold	< 1 mg 1 mg – 10 mg 10 mg – 2 g > 2 g	1.0 % or 5 µg, * 0.5 % or 20 µg, * 0.2 % or 2 mg, * 0.1 %	Structure elucidation of the found impurity
Qualification threshold	< 10 mg 10 mg – 100 mg 100 mg – 2 g > 2 g	1.0 % or 5 µg, * 0.5 % or 20 µg, * 0.2 % or 2 mg, * 0.1 %	Investigation of the biological safety of the respective impurity

** whichever is lower*

From an analytical point of view, the establishment of impurity profiles is a quite challenging task especially in drug products with multiple active compounds. In the analysis of stressed samples an unknown number of impurities with partly unknown structures are to be detected and quantified. Thus, there is a need for highly selective separation methods and for detectors that are capable of detecting all impurities in a stressed sample.

2.2.1.1. Complementary detectors for impurity profiling

As discussed above the aim of impurity profiling is to uncover all impurities that may be formed in drug products. In the course of stability testing the main focus lies on degradation related impurities, which can be classified according to the thresholds as proposed by the ICH (Table 2). This classification is based on determinations of the impurities' content relative to their respective parent compound. Thus, quantitative data of all impurities, known as well as unknown are required. Moreover, the concentration of the parent compound has to be quantified too. In this context the problem is that no standards are available for calibration if impurities are unknown. Widely used detectors like UV or MS exhibit compound specific detector responses and may in the worst case fail to detect compounds lacking a chromophor or an ionizable functionality. As a consequence quantification relative to structurally similar compounds or relative to supposed precursor compounds, may lead to over- or underestimation of impurities [7]. A solution to this dilemma provides the use of detectors

which exhibit consistent detector responses for distinct structures and thus permit universal calibration.

Four detectors are available that are considered to provide relatively consistent detector responses independent on analyte properties: Refractive Index (RI), Evaporative Light Scattering Detector (ELSD), Chemiluminescence Nitrogen Detectors (CLND) and Charged Aerosol Detectors (CAD) [12-14].

RI detectors measure changes in the refractive index, which occur when a compound is eluted from the column and enters into the detector cell. A drawback of the detector is that it exhibits low sensitivity for compounds with refractive indices similar to that of the mobile phase. Such compounds may in the worst case become invisible and are not detected.

In ELSD detectors the incoming column effluent is first nebulized and adherent solvent is evaporized. Passing an irradiated drift tube analyte particles cause scattering of the light which is detected. The measured signal is related to the absolute quantity of the analyte. Unfortunately, this detector is reported to exhibit limitation in precision, linearity and sensitivity [15].

CLND is limited to the detection of nitrogen containing compounds. The mechanism of this detector is based on combustion of the HPLC effluent (mobile phase all together with contained analytes) in a furnace, whereby nitrogen in analyte molecules is converted to nitric oxide in the oxygen rich environment and is further reacted with ozone to yield excited-state nitrogen dioxide [16]. Excited molecules relax by release of excess energy in form of chemiluminescent light (photons), which is finally detected. The amount of light is proportional to the nitrogen content in the analyte molecule. As a consequence of the operation principal of CLND the detector is not compatible with nitrogen containing mobile phases like acetonitrile due to high background signals.

The operation principle of the CAD is illustrated in Figure 2. The first two steps, nebulization and evaporization, are the same as for ELSD. Hence, the effluent coming from the HPLC is nebulized with a stream of nitrogen gas. In the next step aerosol droplets are directed into a drying tube where the solvent is evaporated to generate a fine aerosol of analyte particles. As in the drying tube the complete solvent is removed and only analyte particles remain, the original analyte concentration plays no role providing the basis for mass sensitive (mass-flow dependent) detection. The resulting stream of analyte particles collides with a stream of positively charged nitrogen gas ($N_2^{+\cdot}$, [17]) produced by corona discharges. During this collision events charge transfer takes place whereby the number of imparted charges depends on the particle size [18]. This way positively charged aerosol particles are produced that

transfer their charges to an electrometer for signal transduction [15]. Thus, the signal of the CAD is based on the diameter of the particles and their individual charges as well as on the number of charged particles [17].

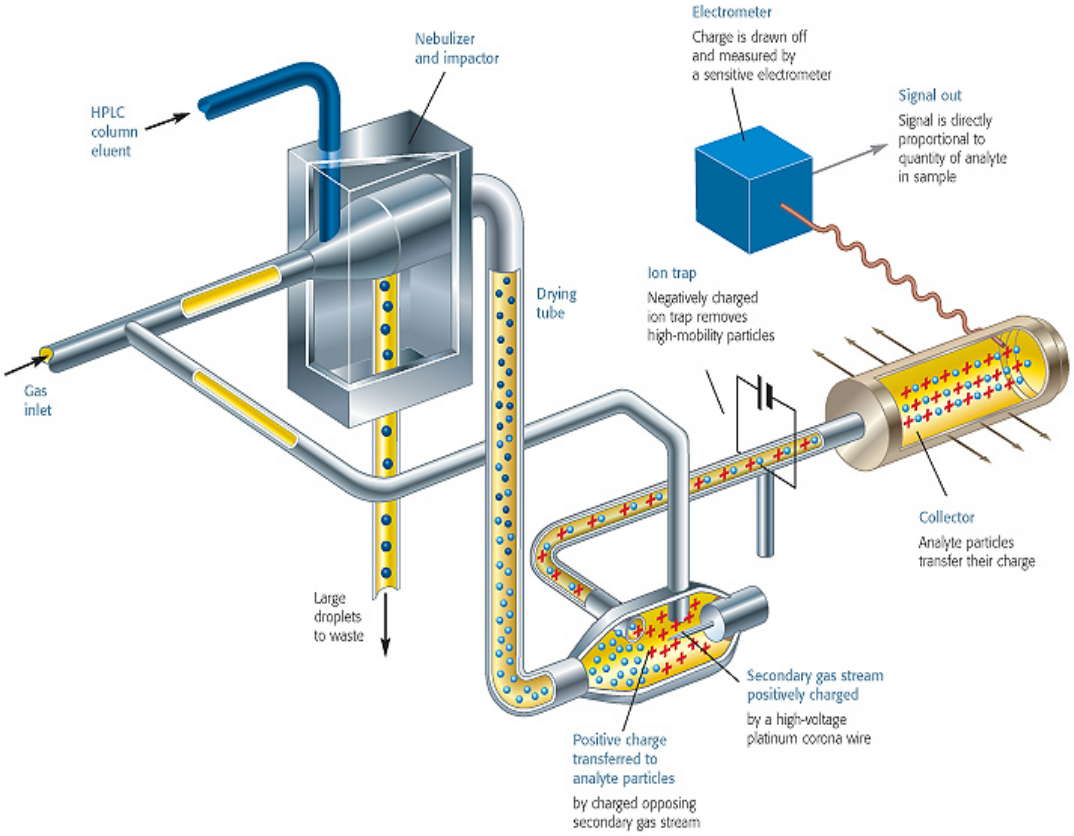

Figure 2: Illustration of the operation principle of the CAD.
Adopted from http://coronacad.com/CAD_Overview.htm on 20.03.09.

CAD detector response is not linear and fits to the equation (1), whereby y is the signal intensity (area or height); A is the response factor; x is the analyte mass and b corresponds to sensitivity.

$$y = Ax^b \qquad (1)$$

The non-linearity of detector response can be explained by the fact, that aerosol charging is not directly related to the aerosol mass [18]. Nevertheless, for a lower concentration range linearity is fulfilled over a range of two orders of magnitude (1 – 100 mg/L, [19]).
The CAD is considered to provide advantages in precision and sensitivity, compatibility with chromatography and ease of use as compared to the other already discussed types of detectors. Moreover, the CAD is well suited for the detection of all non-volatile compounds and thus, may be considered as complementary detector to UV and MS, which permits detection of compounds that fail to be detected with UV or MS.

Nevertheless similar to ELSD and IR, the CAD has also only limited compatibility with gradient elution as detector response significantly depends on solvent composition. The reason for these observations is that the transport efficiency of the nebulizer is improved at higher organic contents.

However, a method to overcome this obstacle is reported [12,19]. In this context, the eluent of a gradient run is mixed with a second eluent of an exactly inverse gradient delivered by a second pump before entering the CAD. Doing so mobile phase composition entering the CAD can be kept constant providing a constant detector response throughout the whole LC run.

In the literature several applications using the CAD as alternative detector in pharmaceutical analysis can be found, which confirm the utility of the CAD for impurity profiling [13,20-22].

2.2.2. Metabolomics – Metabolic profiling

A modern direction in life science research is system biology. It attempts to look at cells or organisms in a global and comprehensive way. Such investigations of biological systems though can take place at different levels. The supreme level constitutes the genome which deals with genetic information. The transcriptome translates the genetic information which is used as construction plan for the proteome. The expression of the proteome decides on which biochemical processes are actually activated. The result is reflected in the entirety of the metabolites termed metabolome. This so-called “omics” cascade [23] is outlined in Figure 3 and summarizes all analytical streams that deal with the characterization of the response of a biological system to an impulse, which may constitute a disease, a genetic or an environmental perturbation. With each level of the cascade the response is amplified and can finally be most accurately measured at the level of the metabolome, which is located “downstream” in view of this cascade and thus is considered to be most predictive of the phenotype.

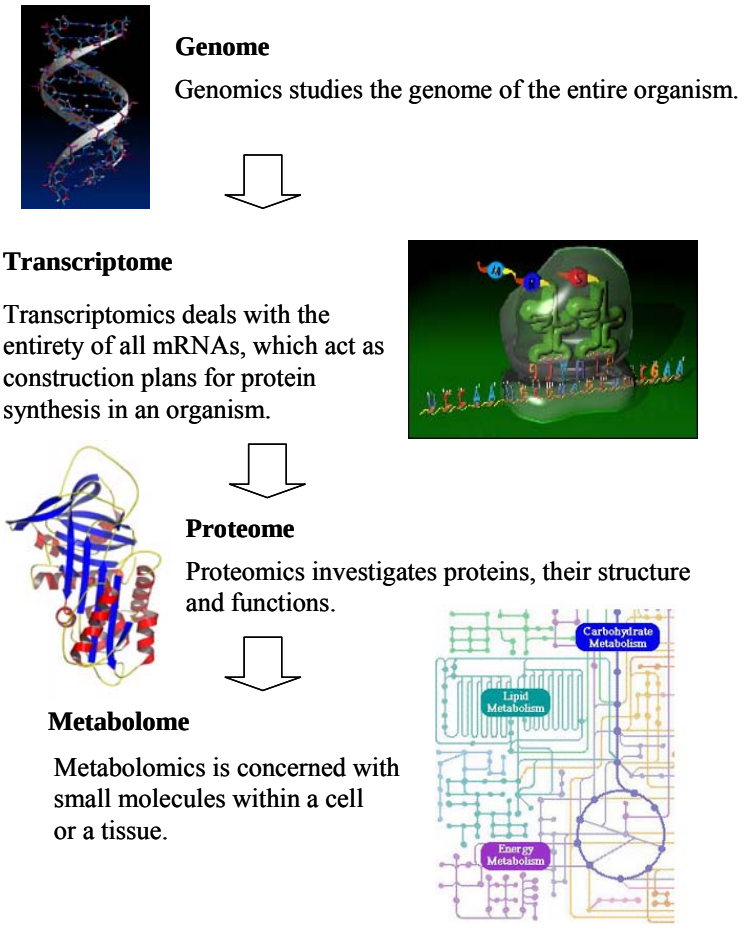

Figure 3: Schematic illustration of the "omics" cascade.

The metabolome constitutes the entirety of small compounds with a low molecular weight (usually < 1000 Da), that take part in metabolic reactions in a biological system. These reactions are involved in biochemical processes that aim at the biosynthesis of precursor compounds and substrates and on the degradation of compounds for the generation of energy, respectively. This interplay of catabolism (degradation) and anabolism (buildup) allows maintenance of cell functions and growth.

The metabolome is composed of a multitude of diverse compounds including amino acids, organic acids, carbohydrates, nucleotides, lipids, hormones, antioxidants and so on. Yeast (*Saccharomyces cervisiae*), for example, is supposed to contain about 600 metabolites and the human metabolome is estimated to be even more profound containing more than 2 000 different metabolites. The plant metabolome is considered to be even more complex exceeding a number of 200 000 primary and secondary metabolites [24].

Strategies employed for metabolic analysis can be classified into two groups depending on the focus of the intended application, as is outlined in Table 3 [23,25].

1) *Quantitative approaches* deal with the identification and quantification of the complete set of all metabolites (global approach) or a limited number of metabolites, depending on the objective of the study.
2) In *qualitative or comparative approaches* the identification of each peak found is not necessary, as emphasis is put on the entirety of peaks and on the recognition of characteristic molecular patterns.

Table 3: Characteristics of quantitative and comparative approaches in metabolomics.

Quantitative Approaches	Comparative Approaches
Metabolomics Global approach, seeks to identify and quantify the complete set of metabolites occurring in a system **Metabolic Profiling** Quantification of a set of pre-defined analytes **Targeted Analysis** Quantification of distinct metabolites related to an enzyme e.g. a substrate or product	**Metabolic Fingerprinting** Analysis of all metabolites within a cell corresponding to the endo-metabolome (intracellular metabolites) **Metabolic Footprinting** Analysis of all metabolites in the medium outside the cell corresponding to the exo-metabolome (extracellular metabolites)

2.2. Techniques in multicomponent analysis

The success of multicomponent analysis is decisively linked to a proper choice of methodology which strongly depends on the types of samples and the group of compounds to be analyzed. In the following, benefits and drawbacks of different analysis techniques frequently used in multicomponent analysis as well as performance and limits of state of the art MS techniques are discussed.

2.2.1. Hyphenation of separation techniques with MS

As already mentioned MS was first hyphenated with GC in the 1960 [26]. The first step in GC separations constitutes evaporization of analyte molecules, which then migrate in the gas phase through the capillary column with rates depending on their boiling points and interactions with the stationary phase. Thus, to introduce the molecules into the MS no evaporization or nebulization step is necessary. As a consequence, the column effluent can be directly transferred to the MS. Ionization is typically achieved by electron impact or chemical ionization. GC exhibits very high separation efficiencies providing high peak capacities and is thus still a widely used and very powerful technique, provided that sample characteristics are suitable. GC-MS analysis is limited to rather hydrophobic, thermally stable and unpolar compounds which are sufficiently volatile. In this context, GC-MS offers the possibility to directly analyze volatile compounds. For example, terpenoid emission of Arabidopsis flowers could be investigated employing headspace-GC-MS [27].

For compounds exhibiting polar functional groups pre-column derivatization (esterification of carboxylic acids, silylation of alcohols, etc.) is a prerequisite for their ability to be analyzed by GC. It constitutes a considerable drawback of GC, because additional operational efforts are associated with increased total analysis times, extra costs and the risk of sample decomposition.

Therefore, today LC-MS is considered to be the method of choice for multicomponent analysis of samples with high polarity. Another advantage is its high degree of flexibility. LC can be connected to MS by several API (Atmospheric Pressure Ionization) interfaces. The advent of API sources made it possible to introduce the analyte molecules into the mass analyzer without compromising its high vacuum environment inside. The API ionization process starts with nebulization of the HPLC effluent. In the next sequential steps analyte molecules are ionized and released from solvent molecules and are transferred into the gas

phase under atmospheric pressure. Finally only ionized species can be tracked by the electric field to enter into the mass analyzer which is under vacuum.

Therefore the success of the ionization process decides whether an analyte is detectable by MS or not. Different types of ion sources are available with distinct efficiencies for analytes depending on their respective molecular weights and polarity as illustrated in Figure 4. API sources provide an operational range that covers nearly the complete range of polarities. The most popular ionization source constitutes ESI (ElectroSpray Ionization) which exhibits the broadest applicability followed by APCI (Atmospheric Pressure Chemical Ionization). Considering the analysis of highly polar compounds ESI is most suitable. APCI, APPI (Atmospheric Pressure Photoionization) and APLI (Atmospheric Pressure Laser Ionization) exhibit good ionization efficiencies for more apolar compounds.

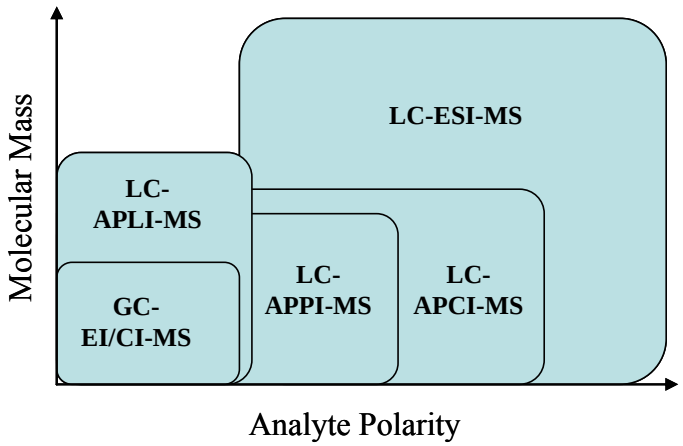

Figure 4: Illustration of application ranges of various ionization interfaces.

Coupling of CE (Capillary Electrophoresis) with ESI constitutes a more challenging task. The critical point is the combination of the two high voltage circuits of CE and API. However, further developments of interface techniques made the combination of CE and MS recently possible. CE is limited to charged molecules as separation is based on distinct electrophoretic mobilities of analytes which thus migrate with different velocities in an electric field.

To provide separation of neutrals a more advanced operation mode Micellar Electrokinetic Chromatography (MEKC) can be employed [28]. In this mode charged surfactants are added to the background buffer at concentrations above the critical micelle concentration to provoke the formation of micelles. Micelles carrying charges also migrate in the electric field. As a consequence, analytes partition in and out of the micelles depending on the strength of their interactions. When portioned into the micelle the solutes migrate with the velocity of the

micelle. Outside the micelles analytes are transported with the electroosmotic flow. Based on distinct distribution equilibria of analytes between the buffer and the micelles separation is achieved. One drawback of MEKC is that compatibility with MS detection is compromised by the presence of the charged surfactants in the buffer, since these cause ion suppression and contamination of ESI interfaces.

On the whole CE-MS is considered to be a less significant technique in multicomponent analysis as compared to GC-MS and LC-MS.

2.2.2. Mass analyzers: Benefits and limits

Single Quadrupols (Q) and **triple Quadrupols** (QqQ) are very robust and exhibit a wide mass range (m/z 2 - 4 000). These detectors are considered to be of low mass resolution. Nevertheless, they are most frequently used for quantitative analyses in complex samples as they provide a wide dynamic range and good detection sensitivity. QqQ instruments are composed of three quadrupols instrumentally arranged in a series. The special feature is that the second quadrupole works as collision cell in which pre-selected molecules are fragmented. This setup allows analysts to exploit alternative scan modes like precursor ion scan, neutral loss scan, product ion scan or multiple reaction monitoring (MRM), which provide additional structural information and increase method selectivity. For quantitative analysis use of the MRM mode is preferred as it permits highly selective detection of compounds by their corresponding specific fragmentation transitions, simultaneously reducing the background signal leading to improved sensitivity and clearer chromatograms.

Ion traps are often used for qualitative analysis and structure elucidation.

Due to their operational mode ion traps are capable of performing MS^n analysis and thus provide an outstanding feature for structure elucidation. In the trap a certain number of ions is accumulated and finally scanned out. In MS^n analyses defined fragment ions are isolated, further fragmented and scanned out according to increasing m/z. Several cycles of isolation, fragmentation and scanning out can be performed illuminating step by step molecular structures. Unfortunately with each step of fragmentation the absolute number of individual fragments available for further fragmentation decreases and hence lowers sensitivity.

A shortcoming of ion traps constitutes the lack of linearity of detector response which compromises their utility for quantification. Furthermore, combination of ion traps with LC is limited due to their susceptibility to be contaminated by column bleeding or buffer additives.

The result is a very high background that strongly declines sensitivity and masks low abundant compounds.

High mass resolution MS instruments provide high selectivity and improved sensitivity as interferences of background compounds are suppressed. With regard to the identification of compounds highly accurate mass detection allows calculation of the atomic composition providing a reduction of possible structures. The higher the achievable mass accuracy, the lesser the number of possible structures that are suggested.

TOF (Time of Flight) analyzers, **Orbitrap** and **Fourier Transform Ion Cyclotron Resonance** (FT-ICR) are considered to be high resolution mass analyzers providing top mass accuracies of 1-2 ppm and FWHM (Full Width Half Maximum) of 100 000 up to > 500 000 (FT-ICR) [29]. These high resolution detectors are optimal for qualitative and comparative analyses, protein identification and structure elucidation. Because of their rather narrow dynamic range they are only of limited applicability for quantification.

In Direct Injection (DI) analysis samples are directly injected or infused into the MS without previous chromatographic separation. It is a very fast method allowing a high throughput screening of samples. The DI technique is predominantly combined with ESI ionization and high mass resolution analyzers like TOF and FTICR [23]. It is particular attractive for metabolic fingerprinting or footprinting, which are untargeted approaches that aim at sample classification by the recognition of metabolite patterns. Unfortunately, this approach is not quite suitable for quantitative purposes as it is extremely susceptible to suffer from matrix effects especially when biological samples often containing high salt concentrations are to be analyzed.

Combinations of different mass analyzers, so called hybrid mass spectrometers, like **Q-TOF** or **Q-Trap** instruments, offer additional features. For example, the Q-Trap 4000 (Applied Biosystems) is composed of three quadrupols, whereupon the third quadrupole can be alternatively used as linear ion trap, which again provides new operational possibilities.

2.2.3. Pitfalls of ESI-MS in multicomponent analysis

MS is the unrivalled detector for multicomponent analysis. However despite of increasing selectivity of MS detectors combination with powerful chromatographic separation techniques is still obligatory. In our studies we made use of ESI-MS, either ESI-IT-MS or ESI-Q-Trap.

A characteristic of the ESI ionization mechanism (explained in detail later) is that only a limited number of charged species can be produced depending on the applied ESI voltage and

flow rate. Thus, only a limited number of species can be resolved and detected simultaneously which again underlines the importance of the combination with chromatographic separation for multicomponent analysis.

A further consequence that may arise due to the ESI mechanism is that highly abundant compounds suppress ionization of less abundant ones simulating their absence. This circumstance is of special importance in impurity profiling of pharmaceutical products, where the intact active agent present as bulk compound may mask low abundant impurities [30]. For this reason it is advisable to chromatographically isolate bulk compounds from possible impurities.

Another issue arising from the limited ionization capacity constitutes the narrowing of the linear range. When the concentration is increased above a certain limit analyte molecules start to impede their own ionization. In the presence of coeluting compounds with higher ionization efficiencies linear dynamic range of analyte compounds can be adversely affected by further reduction of the linear range [31]. As a consequence linear relation between signal intensity and concentration diminishes and calibration curves stagnate. This condition constitutes a special matter in the analysis of biological samples and in metabolomic profiling approaches, as the concentrations at which metabolites are present in biological systems are spread over a wide range. Eventually multiple sample analysis using different dilutions would be required to ensure that concentrations of all analytes fall within their corresponding linear ranges. These additional efforts are unsatisfactory. A possibility to increase the linear dynamic range constitutes reduction of the incoming flow rate. This can easily be achieved by reducing column diameters from e.g. 4 mm to 2 mm. At a reduced flow rate ionization efficiency is improved as smaller charged droplets are produced during the spraying process.

Considering quantitative analysis coeluting matrix components may suppress analyte signals by competing for ionization sources. The mechanism that works in matrix effects is discussed in detail later. Matrix effects may cause an enormous adulteration of quantitative data and may lead to misinterpretation.

The method of choice for the quantification of compounds in complex matrices constitutes the MRM mode provided by triple quadrupole or Q-Trap mass analyzers, which provide a wide dynamic range and high sensitivity. In the MRM mode the first quadrupole selects a specific precursor ion which is subsequently fragmented in the second quadrupole. The third quadrupole is set to the m/z of a specific fragment ion, which is finally detected at the secondary electron multiplier (SEV). Thus, specific transitions are monitored which improves selectivity and peak capacity. Nevertheless, signal interferences due to other compounds,

especially such with similar substructures, may occur. Hence, coeluting compounds should be tested with regard to possible interactive influences on analyte signals.

A matter of concern in LC-MS constitutes detection of isobaric compounds, which cannot be differentiated by their respective m/z but sometimes they may be distinguished in the MRM mode provided that they exhibit specific fragmentation transitions. The situation is even more complex regarding stereoselective compounds which can not be differentiated by their MRM transitions due to their characteristics to exhibit the same molecular constitution. Consequently baseline separation of stereoisomers must be accomplished chromatographically. Determination of stereoisomeric or more precisely enantiomeric impurities plays an important role in pharmaceutical applications, as stereoisomers may exhibit distinct bioactivities. Furthermore, measuring in the MRM mode also exhibits some drawbacks compared to full scan modes [32]:

1. A portion of absolute intensity is irretrievably lost since intact analyte molecules are fragmented.
2. Smaller molecules may be difficult to fragment and most often fragments are non-specific due to losses of H_2O or CO_2.
3. The number of adjustable MRM transitions is limited, thus also the number of simultaneously detectable analytes. For each MRM scan a certain amount of time is needed (dwell time 1 – 100 ms). When the number of transitions measured during one run increases, less data points per peak can be recorded for each individual MRM transition. The consequence is that for each peak only a reduced number of data points are available which may compromise reliability of quantification [29].

ABI/Sciex developed a new software version Analyst 1.5. to improve the situation in simultaneous measurement of multitple MRM transitions. Analyst 1.5. is capable of measuring scheduled MRM transitions. Scheduled MRM, as illustrated in Figure 5, monitors specific MRM transitions only during predefined time intervals matching elution time of the respective compounds. Thus, the number of MRM transitions that is simultaneously scanned is drastically reduced in multicomponent analyses. Similar software is also available from other suppliers e.g. from Agilent (Dynamic MRM).

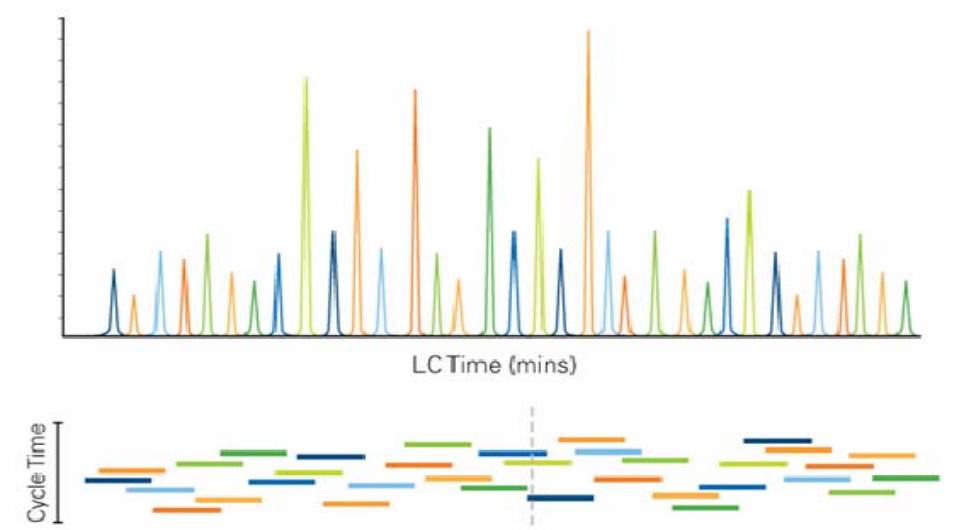

Figure 5: Illustration of the Scheduled MRM Algorithm.
Adopted from
http://www3.appliedbiosystems.com/cms/groups/psm_marketing/documents/generaldocuments/cms_053911.pdf on 10.03.09.

Unfortunately, the new software which is still in its infancy suffers from some problems as polarity switching during one run is unfortunately not possible. Furthermore, the application of Scheduled MRM affords strict control of chromatographic conditions and highly reproducible retention times in order to prevent analytes from escaping their specific detection time windows.

Nevertheless, tandem MS in the MRM mode is widely employed in various fields of multicomponent analysis like in targeted metabolomics [33,34], for clinical investigations e.g. newborn screening [35], toxicologic applications [36,37], food chemistry [38] and many more.

2.2.4. Matrix effects: Causes and strategies

Matrix effects constitute an inherent issue in quantitative MS based assays and are deemed to be the Archilles heel of quantitative LC-ESI-MS analysis [39]. In the following chapters the ESI ionization mechanism and the mechanisms by which matrix effects evolve are discussed. Furthermore, strategies to assess and to overcome or compensate matrix effects in quantitative analysis assays are reviewed.

2.2.4.1. Mechanism of ESI

The liquid flow enters a high voltage capillary tip. In the high electric field at the end of the tip the liquid is pulled out against surface tension to form a Taylor cone (a characteristic liquid conus), that finally breaks up at the top to release fine droplets with a diameter of a few µm. This process is pneumatically supported by a stream of nitrogen gas in ion spray sources. Depending on the adjusted potential charged species are separated in the liquid cone. As a consequence excess charge enters into the droplets when the liquid film breaks up. In the following step a heated stream of nitrogen, the drying gas, causes evaporation of more volatile solvent molecules. Thus, droplets shrink causing concentration of excess charge at the droplet surface, thereby ion pairs, neutrals and low surface activity compounds are banished into the interior of the droplets [40].

When the charge density of the droplets reaches the Reyleigh limit repulsive Coulomb forces increase and outbalance the forces of surface tension that stabilize the droplets [41]. Hence, coulomb explosions and fission processes take place yielding smaller diameter (nm range) offspring droplets.

Formation of gas phase ions is explained by two accepted theories. The first one, the ion evaporation model by Irbane and Thompson, is based on the assumption that ions desorb from the droplet surface when repulsive forces increase. The second one, the charged residue model also known as the single ion in droplet theory of Dole and Röllgen, assumes that fission processes continue until very small droplets, containing just one analyte, are left. Once ions are released into the gas phase they may undergo gas phase reactions like proton transfer, charge transfer or charge neutralization.

The final gas phase ions are tracked by the electric field and transferred into the mass analyzer. A consequence of the described ESI mechanism is that compounds that failed to be

ionized and transferred into the gas phase do not arrive at the interior of the mass analyzer and thus can not be detected.

2.2.4.2. How matrix effects emerge

Matrix effects are caused by coeluting components in the matrix that either suppress or less frequently enhance ionization of compounds. Thus, analysis results of analytes embedded in complex matrices like in biological samples (blood, urine, fermentation extracts, etc.) are jeopardized to be invalidated and corrupted by matrix effects. Furthermore, investigations of Mei et. al. [42] revealed that also exogenous sources like plasticizer and polymers extracted from storage vials may contribute to matrix effects.

Matrix effects can fully be ascribed to processes in the ion source during the ionization process and result most often in ionization suppression.

In general, ESI sources are considered to be more sensitive to matrix effects than APCI sources. This may be explained by the different ionization mechanisms of APCI and ESI. In APCI ionization occurs due to gas phase reactions of ionized solvent and buffer molecules with analyte molecules. As ionization mediators are in high excess the influence of matrix components on ionization efficiency is less pronounced. In ESI analyte ions and matrix components compete for ionization resources as will be discussed in the following chapters. Nevertheless also with APCI matrix effects have been detected. Furthermore, it was shown that susceptibility of ion sources to matrix effects also depends on the source design and the source manufacturer [42].

There are several ways in which matrix compounds may sabotage analyte ionization and in this manner cause ion suppression. However, the exact mechanisms are most often unclear.

A characteristic of ESI is that only a limited number of excess charges can be formed in the Taylor cone depending on the applied voltage and the flow rate. In other words the ESI current and thus, the number of detectable ions, is limited for specific experimental parameters (flow rate and adjusted potential) [43]. As a consequence the presence of electrolytes or other compounds with higher ionization efficiencies would strongly reduce the number of analyte ions produced in the Taylor cone.

After the formation of droplets analyte ions have to compete with matrix compounds for access to the droplet surface from which they can be transferred into the gas phase [40]. Surface active matrix compounds are more likely to reside at the surface and thus would force low surface active analyte ions into the interior of the droplets, where they have no chance to

escape into the gas phase. In the case that ion pairing reagents like trifluoroacetic acid are present in the mobile phase ion pairs may be formed with basic analytes, which would also be trapped inside the droplet and thus would be hindered from desorption.
Involatile salts, like phosphate and sulfate, may cause increase of the solutions boiling point and changes of volatility, viscosity and conductivity of the sprayed solution. In this way unfavorable conditions for the formation of offspring droplets are created that hinder gas phase desorption of analytes [44].
Another mechanism by which the ionization efficiency is compromised constitutes the competition for protons in the gas phase. Once an ion is desorbed from the droplet surface it may undergo several further chemical reactions in the gas phase. Among others proton transfer reactions take place that contribute to ionization efficiency.

2.2.4.3. Strategies to assess matrix effects in quantitative analysis

Biological samples show high variability and equal matrices will not exhibit the same composition. For instance, in clinical applications congeneric matrices like blood or urine will show patient to patient variability depending on the patient's metabolism and his physiologic state. Thus, matrix effects will also show up differently in these samples. This condition is termed as relative matrix effect. Differently to relative matrix effects, absolute matrix effects refer to signal variabilities between equal amounts of analytes spiked to a solution lacking a matrix (e.g. pure solvent) and to a solution of a blank matrix.
For the above discussed reasons it is essential to investigate and assess matrix effects in order to make sure that generated calibration functions and validation data are valid for the intended analysis samples [39].
One strategy to elucidate matrix effects is based on the determination of signal differences obtained for equal amounts of a standard compound in a pure, matrix-free solution and in a matrix-burdened solution. The absolute difference of the responses divided by the response obtained in pure solution is a quantitative measure of the matrix effect.
The postcolumn infusion method allows to visualize enhancement or suppression of analyte signals due to matrix effects across the whole chromatogram [44]. Performing this experiment a blank matrix (sample not containing the analyte) is injected and analyzed under the chromatographic conditions of the intended analysis method. At the same time a solution, containing the analyte at similar concentration levels as found in the samples, is constantly infused postcolumn via a T piece. The united liquid streams, the one from the column and the

postcolumn stream, enter the detector. For the case that no matrix effects are present the detector would record a steady, constant signal. For the case that compounds of the matrix cause signal suppression, the analyte signal would decline as long as the respective matrix compound is eluting from the column and would then increase again to the original value as shown in Figure 6. A LC-ESI-MS/MS method was developed and validated by Bicker et al. [45] to study the potential ethanol consumption marker ethylglucoronide, ethylphosphate and ethylsulfate in urine. In Figure 6 the chromatograms of postcolumn infusion experiments intended for the evaluation of matrix effects on ethyl sulfate are shown. The chromatograms reveal strong ionization suppression at the beginning of the chromatogram and for pooled urine samples signal enhancement after about 1.8 min. At the elution time of ethylsulfate (indicated by the arrow) a stable signal was obtained for water and 1:1000 diluted urine indicating absence of disturbing matrix effects. Not quite unexpected the signal of the 1:20 urine injection fluctuated quite strongly across the chromatogram due to higher concentrations of matrix components. However, postcolumn infusion experiments allow only semi-quantitative evaluation of the extent of matrix effects.

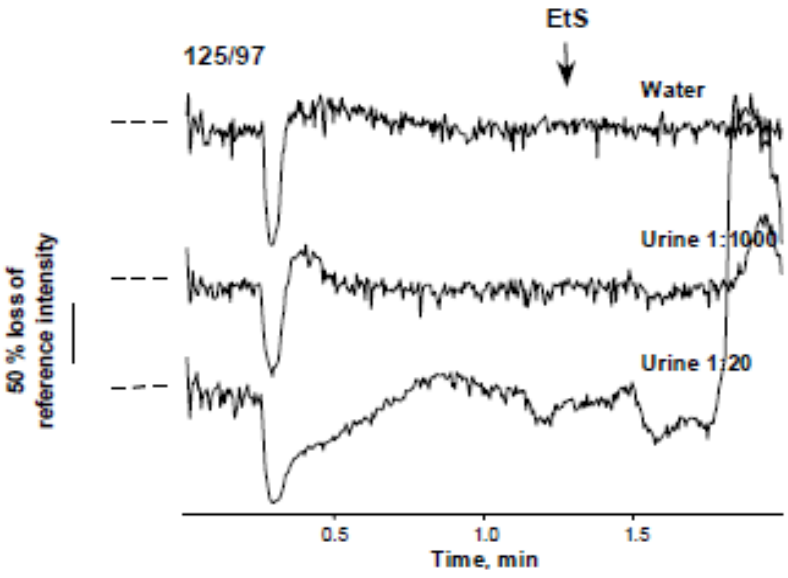

Figure 6: Postcolumn infusion experiment for the determination of matrix effects in different solutions. Water and pooled blank urine samples diluted 1:20 and 1:1000 were injected while infusing etylsulfate.

Adopted with permission of the American Chemical Society.

Matuszewski et. al. [46] have suggested an alternative method to assess matrix effects in biofluids. Employing this method standard line slopes generated by spiking distinct amounts of standards to matrix-free solutions and to different batches of matrix samples are compared [47-49] . As a criterion for a method to be considered as free of matrix effects they proposed that the deviation of slopes of calibration functions prepared in five different biofluid lots should fall within 3 to 4%.

2.2.4.4. Strategies to overcome matrix effects in quantitative analysis

As already discussed matrix effects may negatively affect method reliability in terms of accuracy and precision and thus may distort analysis results.
The main problem in quantitative LC-ESI-MS(MS) analysis related to matrix effects is that calibrants in neat standard solutions exhibit different signal intensities compared to the respective analytes in the sample invalidating calibration. Hence, there are two basic strategies to handle this problem:

1. Getting rid of matrix compounds by sample preparation or chromatographic separation
2. Compensation of matrix effects by standard addition, matrix-matched calibration or by involving (isotope labeled) internal standards subjected to the same matrix effect

Concerning the first strategy, sample clean-up is a very effective method to get rid of matrix compounds.
Several sample preparation techniques like solid-phase extraction (SPE), liquid-liquid extraction, protein precipitation, (micro)dialysis and so on can be exploited to reduce the number of disturbing species in complex samples.
Unfortunately, sample preparation suffers from drawbacks like additional operational efforts, elongation of total analysis times, possible introduction of contaminants and the risk of losses of analyte. Thus, efforts are made to automate preparation procedures, for instance the use of column switching devices permits automation of sample preparation by integration of SPE into the chromatographic process (on-line-SPE-HPLC).
Another possibility to prevent matrix effects constitutes the separation of matrix compounds from analytes by chromatographic techniques so that they are not present during the ESI process. However, for very complex multicomponent samples one-dimensional (1-D) chromatographic separations may be insufficient in terms of peak capacity and selectivity. A

more effective approach constitutes application of two-dimensional (2-D) chromatography which provides high selectivity and high peak capacity separations [50].
An on-line 2-D LC-MS approach effectively used to remove matrix compounds from the sample is column back-flushing [51]. In doing so analytes are trapped on the first column while unretained matrix species are eluted. Finally trapped analyte species are eluted by reversal of the mobile phase flow from the first column and thus are transferred onto the second column where they are chromatographically separated. This method is quite useful removing matrix components but it only exploits a portion of achievable chromatographic selectivity in 2-D LC.

What the second approach is concerned, there are basically two strategies to compensate for matrix effects in quantitative analysis assays, which are on the one hand standard addition and on the other hand use of internal standards.
The methods are useful in terms of correcting for matrix effects but it has to be noticed that losses of sensitivity due to ionization suppression can not be compensated [52]. Standard addition and matrix-matched calibration are aimed at creating equal detection conditions for standard compounds and analytes.
Under this rationale **standard addition** is performed by spiking distinct amounts of standards to the sample. Thus calibrants experience the same molecular environment and matrix effects as the analytes. Measured signals are plotted versus spiked quantities to yield a calibration curve as illustrated in Figure 7A. Analyte concentrations are determined by extrapolation of the curve to $y = 0$.
This strategy may be of high value for singular samples but is too time consuming to be employed for a multitude of samples, as for each sample an individual calibration curve has to be set up.
A very similar method constitutes **matrix-matched calibration**. In this manner standard addition is performed in one sample batch and effective analyte concentration is represented as the sum of spiked quantities and determined intrinsic quantities. A corrected calibration function can be obtained by plotting measured signals vs effective analyte quantities as shown in Figure 7B. Thus, a calibration function that matches a certain sample matrix is generated and can be used for reliable quantification provided that relative matrix effects are absent.

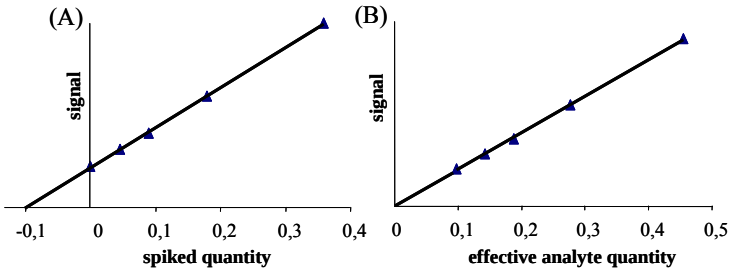

Figure 7: Calibration function using (A) standard addition and (B) corrected matrix-matched calibration functions, respectively.

The most prominent strategy to compensate for matrix effects is to employ **internal standards**. Doing so, internal standards are added at constant concentrations to the sample. Calibration functions are set up by plotting the quotient of the detected signals of the analyte and the internal standard versus analyte concentration. The key is that the internal standard is subjected to the same signal increase or decrease as the analyte, thus formation of the quotient of the signals would cancel out matrix effects. The consequence is that the signal relation for two constant analyte and internal standard concentrations would be the same in a standard solution as well as in a complex matrix.

As already mentioned a prerequisite of internal standards is to behave similarly in terms of ionization efficiency to the analyte in order to compensate matrix related effects correctly.

Since matrix effects evolve due to matrix component elution at a certain retention time within the chromatogram, internal standards should elute at similar retention times as the analytes, in the best case they coelute. Furthermore, it is essential that the mechanisms by which internal standards are ionized are similar to those of the analytes, in order to be subjected to the same ionization variation by the matrix as the respective analytes. The use of structurally similar compounds as internal standards has often been reported [36,53]. Depending on how good they match the properties of the analyte they will compensate for matrix effects to a greater or lesser extent. Thus, it is undoubted that stable isotope labeled compounds are the best choice as internal standards, since they exhibit almost the same properties as their respective analytes and coelute with them. Stable isotope labeled internal standards are very often employed and are considered to be the most reliable strategy to compensate for matrix effects which is also reflected by the many publications on this issue [54-57].

However, also this method exhibits pitfalls which should be considered during application.

1. Isotope purity of internal standards is an important issue. Isotope labeled compounds may contain unlabeled species that may be introduced into the sample and hence, would contribute to the analyte signal.
2. Molecular masses of internal standards and analytes should differ at least by 3 Da in order to prevent crosstalk and other signal interferences of the coeluting isotope labeled compounds.
3. Most often D, ^{13}C, ^{15}N or ^{17}O are used as isotope labels.
   The use of deuterated standards harbors several risks. Deuterium exchange may take place at acidic positions for example in the phenolic hydroxyl group of tyrosine. Furthermore concerning reversed phase separations a deuterium effect was recognized causing slight differences in elution times of analytes and internal standards. Wang et al. [58] reported that due to a retention time difference of only 0.02 minutes the deuterated internal standard of derivatized (S)-Carvedilol experienced a different matrix related ionization suppression than derivatized (S)-Carvedilol. As the analyte to internal standard signal ratio was changed this way, the internal standard could not accurately compensate for matrix effects anymore.
4. Isotope labeled internal standards are expensive and very often not available for the analytes of interest. Availability of internal standards is a critical issue often encountered in pharmaceutical applications, e.g. in ADME studies, impurity profiling as the actually investigated target analytes may represent new structural identity.

In comparative metabolomics studies and metabolic profiling a great number of compounds is analyzed simultaneously. The dilemma on how to provide such a multitude of isotope labeled individual internal standards is solved by employment of completely ^{13}C labeled metabolomes [32,34,59,60]. ^{13}C labeled metaboloms are prepared by growing microorganisms like yeast on media containing uniformly labeled ^{13}C glucose. After certain incubation time the whole metabolome is converted into a ^{13}C labeled form. To avoid influences by culture to culture variability one preparation should be used within a study.

The presented methods to compensate matrix effects are also very useful in terms of correcting for instrumental fluctuations caused by contamination of the ion source or by retention time shifts. Another aspect of internal standards constitutes compensation of sample manipulation or analyte losses that might occur during sample clean-up procedures. In this

manner internal standards are added to the crude sample before sample preparation. Thus, internal standards will be subjected to the same manipulations as the analytes and thus allow for corrections of the calibration function and sample results.

2.3. 2-D LC Analysis assays

2.3.1. Introduction

In the last decades chromatographic separation techniques have experienced enormous advance and development, allowing to solve almost every separation problem.

In liquid chromatography new techniques have evolved like Ultra Performance Liquid Chromatography (UPLC) [61], monoliths [62,63]. High Temperature Liquid Chromatography (HTLC) [64] or nano/chip LC [65]. 1-D separation techniques like Hydrophilic Interaction Chromatography (HILIC), which were already investigated by Alpert in the 1990's [66] have gained increasing popularity. Stationary phases combining different separation mechanisms like mixed mode Reversed Phase Ion Exchangers (RP-IEX) are currently investigated. Ingenious innovations have also emerged in mass spectrometry in the last years like high-resolution MS (Orbitrap [67], FT-ICR MS [68]), Nanospray ESI [69] and many more advancements. Turbo Ionspray sources now afford splitless introduction of flow rates up to 2 ml/min and thus facilitated hyphenation to LC.

In combination with LC, MS constitutes an additional dimension, as detection relies on the separation of compounds according to their proprietary mass to charge ratios (m/z).

As already discussed in detail, MS exhibits shortcomings in the differentiation of isobaric compounds like constitutional isomers as well as even more delicate stereoisomers. Furthermore, MS can only resolve a limited number of coeluting compounds and matrix effects may cause severe distortion of quantitative analysis results. Thus, for many analytical problems powerful chromatographic separation is still a prerequisite. It is the combination that makes LC-MS/MS an extremely powerful and highly selective analytical technique.

For this reason future approaches will target at liquid chromatographic methods allowing coupling to MS.

However, demanding new separation problems have evolved, that seek for even more powerful and highly capable techniques. Emerging applications like proteomics and metabolomics require new methodologies that are characterized by a high selectivity and high peak capacity. In this context, one has to deal with hundreds of highly variable compounds in extremely complex matrices which are to be separated and analyzed accurately and reliably.

2.3.2. Reasons for 2-D LC

The first 2-D approach was undertaken in 1944 by Martin and Coworkers [70], who employed simple paper chromatography, using two different mobile phases. The practical set-up was simple: First, the paper was run with the first mobile phase. In the second dimension, the paper was turned by 90° and the second mobile phase was used for the development in a run direction rectangular to the first one. The result was that the sample compounds were distributed over a two-dimensional space, an area, instead of being aligned one after another along the run direction. This implied an enormous gain in separation space and thus in peak capacity.

The peak capacity n_c under isocratic conditions is described by expression (1) and depends on the column efficiency N and the retention factors of the first eluting k_1 and the latest eluting peak k_n. Thereby, it is assumed that all peaks have the same plate number, while it is not the case in practice. The peak capacity under the conditions of gradient elution is calculated according to equation (2). $t_{R,G,n}$ and $t_{R,G,1}$ are the retention times of the last eluting and the least retained peak.

$$n_c = 1 + \frac{\sqrt{N}}{4} \ln \frac{1 + k_n}{1 + k_1} \qquad (1)$$

$$n_c = 1 + \frac{\sqrt{N}}{4} \left(\frac{t_{R,G,n}}{t_{R,G,1}} - 1 \right) \qquad (2)$$

After several transformations (2) translates into the simplified expression (3) describing the peak capacity as function of the gradient time t_G (duration of a gradient). t_0 represents the void time of the column.

$$n_c = \frac{\sqrt{N}}{4} \frac{t_G}{t_0} \qquad (3)$$

Basically, there are three approaches for the optimization of peak capacity in 1-D LC:

1) Longer columns

   An increase of the column length L with a simultaneous, proportional increase of gradient time (in the case of gradient elution) is associated with an increase of the efficiency N. Unfortunately, as can be deduced from equations 1-3 peak capacity increases with the square root of N. Thus, by doubling column length an increase of peak capacity by a factor of $\sqrt{2}$ can be achieved on the one side but on the other side a twofold increase of analysis time has to be accepted.

2) Smaller adsorbent particles

   Smaller particle diameters reduce the contributions of Eddy diffusion and mass transfer kinetics to band broadening and thus increase column efficiency. Unfortunately this approach is limited by the drastic increase of the column back pressure caused by smaller particle diameters. Using UHPLC, which tolerates backpressures up to 1000 bar, may provide an extended working range allowing the routine use of sub 2 µm particles at conventional column lengths.

3) Elongation of the gradient time

   The elongation of the gradient time is associated with the flattening of the gradient slope. In other words, the rate of increase of the proportion of the stronger eluent is reduced. Thus, retention times of later eluting compounds are shifted backwards causing an extension of the retention time window. This approach is unfortunately counteracted by peak broadening effects, which partly destroy the gain in peak capacity.

Generally, employing conventional 1-D chromatographic methods considerable peak capacities of about 1000-2000 [4] can be obtained within one day, when gradient elution is performed with very long gradient times and for the case that the analytes are spread over a wide retention time window.

Nevertheless, the achievable efficiencies and peak capacities are limited and must be paid off by accepting longer analysis times and higher back pressures.

Hence, the most effective way to increase peak capacity is implementation of multidimensional chromatography.

2.3.3. Theoretical background

To assess the performance of a 2-D separation system the concept of peak capacity is used [4]. The peak capacity defines the number of peaks that fit into the available separation space at a constant peak width and a fixed resolution. The separation space is defined as the space between the first eluting peak (or alternatively the peak of an unretained tracer) and the strongest retained, latest eluting peak. In the case of gradient elution it is most convenient to define the end of the gradient as the upper limit of the separation space.
For 2-D LC the peak capacity ^2n_c is defined as the product of the peak capacities of the first $^1n_{c,1}$ and the second dimension $^1n_{c,2}$ (4).

$$^2n_c = {}^1n_{c,1}\,{}^1n_{c,2} \qquad (4)$$

Unfortunately this concept constitutes a vague approximation since the concept behind the expression (4) is based on the following assumptions:

1) The peaks are uniformly distributed over the separation space at a constant peak width and at a given resolution (usually Rs = 1).
2) The two separation mechanisms implemented in the 2-D assay are completely independent and orthogonal, in other words they show no correlation.
3) No loss of peak capacity achieved in the first dimension occurs in the course of the fraction transfer to the second dimension.

Unfortunately, ^2n_c is just an "ideal" number that expresses the maximum number of resolvable peaks that would fit into a given separation space. The true peak capacity is much lower and can by far not hold what the theoretical peak capacity promises. The cause for this overestimation of peak capacity lies in the approximations that were used to formulate expression (4) as discussed in the following.

Ad 1) Distribution of peaks across the separation space
In the statement of (1) it is assumed that the peaks are stringed one after another as shown in Figure 8A. The reality is that peaks are randomly distributed across the separation space, which results in zones were no peaks are found and other zones, where several peaks cluster

together. Giddings et al. developed the statistical theory of band overlap that allows to estimate the number of singlet, duplet and triplet peaks for a given 2-D chromatographic system [71,72]. According to these statistical considerations only 37% of the theoretically available separation space can be exploited and the number of single compound peaks will not exceed 18% of the theoretical peak capacity [73].

Ad 2) Orthogonality

In (2) orthogonality of the two separation methods is postulated. Two separation methods are claimed to be orthogonal, when they are independent and do not exhibit any correlations. Strict orthogonality is hardly achievable even not for the case that the implemented separation methods separate according to different mechanisms like in IEX and RP separations. Thus, in most cases two separation methods will correlate to some extent. The consequence is that losses of separation space and thus losses of peak capacity have to be expected depending on the extent of overlap or degree of correlation of the two individual separation spaces of the two dimensions as illustrated in Figure 8B.

Ad 3) Fraction transfer

During the fraction transfer from the first dimension to the second dimension backmixing occurs, diminishing separation achieved in the first separtion. Thus, it is favorable to keep fractionation intervals low. Optimal sampling periods were determined by Murphy et al. [74] to be three to four fractions across the peak. Consequently $4x^1n_{c,1}$ fractions, but a minimum of $3x^1n_{c,1}$, are recommended to be transferred to the second dimension.

In the case that separation is mainly achieved in the first dimension the choice of an inappropriate sampling interval is fatal. Resolution already achieved in the first dimension is lost and can not be retrieved by the separation in the second dimension. Overall 2-D resolution would considerably decrease. Considering the contrary situation that second dimension separation mainly contributes to 2-D resolution, the effect of undersampling is by far less drastic and may occasionally be acceptable, potentially leading to a reduction of the analysis time.

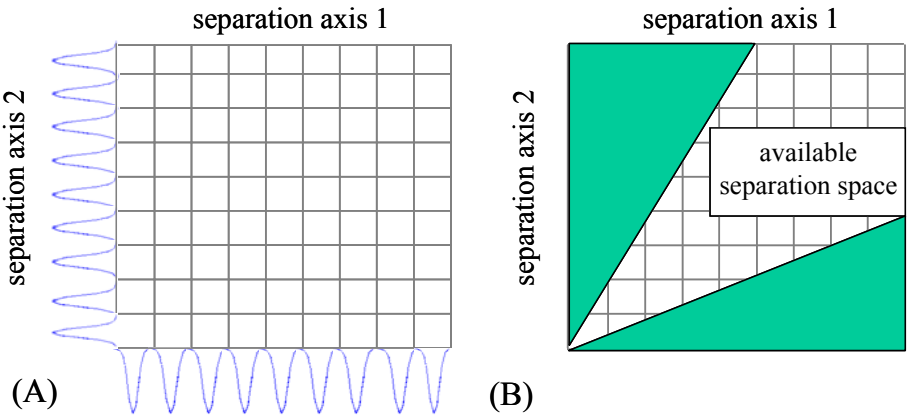

Figure 8: In scheme (A) on the left side the separation space of an idealized 2-D separation system is shown. Peaks are lined up one after the other at constant resolution.
In (B) parts of the separation space are not accessible because of losses due to correlation of separation mechanisms.

To account for the above mentioned restrictions e.g. random peak distribution, correlation of separation mechanisms, loss of resolution due to undersampling, that markedly reduce the practical accessible peak capacity, two factors can be introduced to correct for the theoretical approach [75]:

$$^{2}n_c = {}^{1}n_{c,1}\,{}^{1}n_{c,2}\,n_F \cos\beta_{1,2}$$

Whereby n_F constitutes the Nobuo factor, which corrects for peak capacity losses due to backmixing and $\cos\beta_1$ accounts for the losses of separation power due to employment of non orthogonal retention mechanisms.

2.3.4. Implementation of 2-D LC

2.3.4.1. Instrumental set-up

There are three approaches commonly used for the practical implementation of 2-D separation methods [4]:

1) On-line approach

Using the on-line approach fractions are collected from the eluent of the first column, stored in the sample loop and directly injected via a switching valve onto the second column. Thus, analysis time on the second column is limited to a time interval equal to the duration of fractions collection.

Considering the optimal sampling interval of three to four fractions a peak, as proposed by Murphy et.al., it becomes clear that the separation in the second dimension must be executed very fast.

2) Stop-and-go approach

Using the stop-and-go approach the run is interrupted after the collection of a fraction is finished. The fraction is subsequently injected onto the second column and analysis is performed. Thereafter, when the analysis on the second column has finished, the run on the first column continues.

3) Off-line approach

Fractions are collected manually or automated from the first run and are stored for further analysis, which is done independently of the runs on the second column.

Furthermore, it can be distinguished between heart-cut and comprehensive 2-D separations. In heart-cut separations specific fractions from the first dimension are submitted to a second dimension separation. In comprehensive separations the entire sample i.e. all fractions collected from the first dimension separation are transferred onto the second dimension column, thereby maintaining resolution achieved in the first separation [76,77].

2.3.4.2. Choice of phase systems

As discussed above, orthogonality of the employed separation systems is a pivotal factor for a functional and successful 2-D LC assay.

During the development of a 2-D method the two individual separation systems have to be chosen carefully. It is recommended to compare combinations of different phase systems with regard to their extent of correlation. Several approaches to assess and illustrate the degree of orthogonality of two separation mechanisms have been published in the literature.

In the simplest approach, 1-D normalized retention times of the analytes in the two respective phase systems are plotted [78,79] and analyzed visually: In the case that the data points are randomly distributed across the space as illustrated in Figure 9A, a high degree of orthogonality can be deduced but in the contrary case, that the data points are most frequently situated along the x = y curve as shown in Figure 9B, a high degree of correlation can be estimated. The choice of highly correlated phase systems for 2-D LC is not reasonable as no significant gain in selectivity can be expected. Most often the situation illustrated in Figure 9C will be encountered, where the two methods show good complementarity but also show correlation to a certain extent.

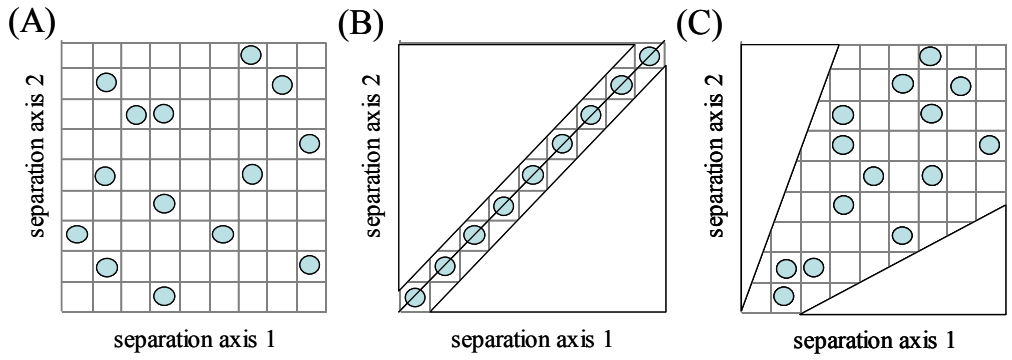

Figure 9: Illustration of the influence of orthogonality/correlation of two separation mechanisms employed in a 2-D chromatographic method on the accessibility of potential separation space. In (A) the theoretical case of orthogonality is shown. Peaks are randomly distributed across the space. In (B) the case of complete correlation of two methods is illustrated. Figure (C) displays the most frequent case of two methods that exhibit good complementarity, but that also show correlation to some extent.

Gilar et al. [80] tested several phase systems with regard to their orthogonality using a set of 196 tryptic peptides. The retention time plots revealed, that the separation mechanisms of tryptic peptides on a RP-18 phase and on a phenyl-phase are very similar, thus they exhibited a high degree of correlation. On the contrary, separation of the peptides using a strong cation exchanger and a reversed phase column resulted in almost orthogonal retention time characteristics, making eventually a combination of these two separation mechanisms favorable for the analysis of tryptic peptides.
Other methods that allow the assessment of the degree of orthogonality include Principal Component Analysis (PCA), Information theory or Factor analysis.

For the implementation of 2-D LC solvent compatibility of the two separation methods plays an important role, which is discussed later. Moreover, the column format of the stationary phases should be selected carefully. Especially for the on-line approach the use of rather short columns sustaining high flow rates is recommended in the second dimension. Thus, monoliths are widely used in the second dimension, as they exhibit a high porosity, which affords high flow rates at low backpressures and short analysis times.
Another criterion which should be considered especially for the on-line and stop-and-go approach is the column diameter. Usually collected fractions are completely transferred onto the second dimension column. Thus, from large column diameters in the first dimension large injection volumes result which may lead to an overload of the second dimension column and problems with solvent compatibility. For this reason it is recommended to employ a smaller column diameter in the first dimension and a larger diameter in the second dimension.

2.3.4.3. Solvent compatibility

Most often the two individual columns in 2-D separations require different mobile phase systems for optimal sample retention and separation characteristics. This circumstance is problematic with regard to solvent compatibility and principally concerns all three practical approaches of 2-D LC separations.
The matter of solvent compatibility is strongly depending on the miscibility of the mobile phases, but also on the injection volume and on the elution strength of the first mobile phase in the second dimension.
Solvent compatibility is notably critical in the case of 2-D separation with highly different mobile phase systems e.g. for combinations of RP, HILIC or IEX employing mainly aqueous

solutions of ACN and MeOH with e.g. NP (Normal Phase) or SEC (Size Exclusion Chomatography) using mainly organic solvents like hexane. The effects can be very troublesome and may hinder appropriate separation in the second dimension by distortion of the peak shapes.

Miscibility of the solvents

Injection of an organic solvent typically used in SEC or NP into a system operated under aqueous conditions brings about serious consequences.

As the two solvents are not miscible the injected volume does not or only insufficiently wet the stationary phase and it may not enter into the pores of the particles. As a consequence sample components may not arrive at interaction sites and thus are not retained. Hence, they are transported within the injected plaque through the column.

Another scenario that may occur is that the injected bolus may be dispersed into smaller droplets that become dispersed within the mobile phase. In either case the obtained chromatograms are useless.

Differences in viscosity

In the case of different viscosities of the sample solvent and the mobile phase mixing may not be fast enough. This is particularly a matter of concern in preparative approaches. Viscous fingers [81] may form that cause band broadening which is associated with a loss of efficiency and resolution. These fingers evolve at the interface where two solvents of different viscosity converge. In the case that the low viscous solvent is upstream and thus pushes the higher viscous solvent across the column, the interface may collapse and fingers may form as illustrated in Figure 10.

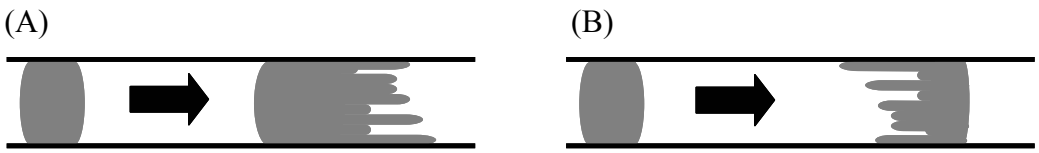

Figure 10: Demonstration of the band distortions due to the formation of viscous fingers. In (A) a plaque of lower viscosity solvent was injected. In (B) the introduced plaque exhibited a higher viscosity compared to the mobile phase.

Solvent of first dimension acts as strong eluent in the second dimension

Sample compounds that are well dissolved in the mobile phase of the first separation step may be transported within the injection plug through the column without being retained, whereas another fraction may show retention to some extent. This phenomenon may occur when e.g. RP chromatography is combined with HILIC chromatography, as the separation mechanisms underlying these two methods are complementary. Thus, in the RP mode the aqueous phase constitutes the weak eluting mobile phase, whereas in the HILIC mode the aqueous phase acts as a strong eluent. This may result in peak splitting.

2.3.4.4. Comparison of the approaches

In on-line and stop-and-go 2-D LC usually the complete sample fraction collected from the first column is transferred onto the second column making the above discussed issues, mobile phase miscibility and compatibility, especially important for these approaches. Nevertheless, troubles arising due to the change of mobile phase conditions can be minimized by the use of narrow bore columns in the first dimension, which would provide minimal fraction volumes and which would also reduce sample dilution [82].

Furthermore, the extent of dilution can also be minimized by the implementation of an intermediate processing step like reversed osmosis, partial evaporation or compound trapping. Concerning on-line 2-D LC and stop-and-go 2-D LC the use of a vacuum-evaporation loop-type interface has already been published [83]. Trapping the collected fractions on an appropriate sorbent packed directly into the sample loop [4] or trapping analytes on the head of the second column also provides focusing and concentration of the sample.

Concerning the off-line approach the case is different as a flexible volume of the fraction may be injected onto the second column. The remaining parts of the collected fractions may be stored and submitted to further experiments. Moreover, additional sample preparation steps like evaporation of solvent can be easily conducted. Another advantage of the off-line approach constitutes the simplicity of implementation. However, a disadvantage of the off-line approach constitutes the additional efforts due to manual sample handling and the risk of analyte losses and introduction of contaminants.

2.3.5. Application of 2-D LC

2-D LC finds wide application in fields where multicomponent analysis in complex matrices is demanded.

A prominent example, where 2-D LC techniques are widely-used, constitutes proteomics. In the bottom-up proteomics approaches proteins are identified by means of characteristic peptides. Thus, protein samples are submitted to enzymatic digestion producing lots of peptides in a complex sample matrix. For reliable analysis and separation of these peptides high selectivities and high peak capacities are required.

For complex separation problems multidimensional chromatographic methods combined with MS and MS/MS detection, respectively, provide the necessary separation space and selectivity, as reflected by numerous reports on the application of multidimensional separations for e.g. the analysis of protein digests [75,84-88].

An interesting approach in proteomics constitutes Mudpit (Multidimensional Protein Identification Technology), which was developed by Yates et al. [73,89]. In Mudpit a biphasic column is used that is filled with a strong cation exchange (SCX) material followed by a RP-18 packing material in one column. The samples are injected and peptides are retained due to electrostatic interactions (ion exchange mechanism) on the SCX material located in the heading part of the column. Peptides are stepwise eluted from the SCX material according to their charge state driven by injection of salt plugs. Arriving in the second dimension of the separation peptides are further resolved according to their hydrophobicity on the RP phase which is located in the terminating end of the column.

Analysis of tryptic peptides with Mudpit in combination with MS allows identification of over 1.000 proteins in one sample [3].

Further applications of 2-D LC include analysis of plant extracts [90], analysis of Chinese patent medicine [91], metabolomic research [92], pharmaceutical applications like impurity profiling [79,93] and polymer analysis, where numerous applications have been reported [94-97]. Polymers constitute a very heterogeneous class of compounds. Most often they exhibit a distribution of molar masses coupled with a distribution with respect to functionality. For this reason typical 2-D chromatography protocols for the analysis of polymers employ a combination of two different separation mechanisms that separate polymers into chemical classes. For example, SEC provides separation according to the molecular weight and with NP or RP a separation according to the respective functionality distributions is achieved [94,95,97].

3. Results and Discussion

As already outlined in the objective, the work of the present thesis was carried out in the course of two industry projects. Even though both projects were focused on multicomponent analysis for pharmaceutical applications, they differed strongly in their aims and thus, required distinct analysis strategies.

The practical work and results of the two projects are described in form of four scientific papers that can be found in Appendix I, II, III and IV. The manuscripts are planed to be submitted once permission for publication is granted by the industry partners.

The aim of the first project with Fresenius Kabi was to establish a qualitative and quantitative impurity profile of a nutritional infusion solution.

The working flow can be roughly divided into two main steps:

In step 1 a preliminary study using a multidimensional analysis approach was carried out to unveil impurities in an infusion solution which was kept under degradation enforced conditions (40°C for 12 months). The work and the results of this first study are discussed in detail in Appendix I (Manuscript: “Comprehensive impurity profiling of multicomponent nutritional infusion solutions for amino acid supplementation by a multidimensional analysis assay using off-line RPLCxHILIC - Ion trap MS and Charged Aerosol Detection with universal calibration”).

In step 2 relevant impurities, which were selected on the basis of the results of step 1, were submitted to further quantitative analysis by LC-MS/MS. In Appendix II (manuscript “Quantitative LC-MS/MS impurity profiling methods for the analysis of parenteral infusion solutions for amino acid supplementation containing L-Alanyl-L-Glutamine”) challenges in method development, method validation and results are summarized.

In the second project with Sandoz the goal was to develop LC-MS/MS methods for the quantification of about 70 extracellular metabolites and nutrients in extracts of fermentation broths. Several preliminary experiments revealed that it was not possible to accomplish simultaneous analysis of all 70 compounds due to their distinct molecular characteristics. For this reason, two LC-MS/MS methods were developed.

To examine appropriate chromatographic conditions for more polar compounds (amino acids, organic acids, β-lactams, vitamins and biogenic amines), a column screening was carried out involving different stationary/mobile phase conditions. Finally, a ZIC HILIC column from Merck SeQuant was found to provide reasonable elution characteristics for the majority of investigated analytes and adequate selectivity for critical pairs. Then, the finally optimized

LC-MS/MS method was validated paying special attention to the evaluation of matrix effects and the effect of distinct calibration types on accuracies. Experimental conditions, problems and results are discussed in detail in Appendix III (manuscript "Multi-target metabolic profiling of hydrophilic metabolites in fermentation broths of β-lactam antibiotics production by HILIC-ESI-MS/MS"). For apolar compounds (fatty acids, α-tocopherol, penicillin V and its degradation products) a RP-LC method was developed using a X-Bridge C18 column from Waters and validated. The results can be found in Appendix IV (manuscript "Quantitative LC-ESI-MS/MS metabolic profiling method for fatty acids and lipophilic metabolites in fermentation broths from β-lactam antibiotics production").

3.1. Impurity profiling in nutritional infusion solutions

3.1.1. Preliminary study

As already outlined in the objective the goal of the project with Fresenius Kabi was to establish a qualitative and quantitative impurity profile.

In impurity profiling the main focus lies on the comprehensiveness of the analysis assays, as it is important to detect all impurities and degradation products within the pharmaceutical formulation [5-7]. In this context a preliminary impurity profile was established for an infusion solution which was stored under degradation enforced conditions (40°C/12 months). The artificially increased impurity concentrations significantly facilitate the detection of low concentrated impurities and minimize the risk of overlooking relevant compounds [9,10].

A goal was to differentiate between relevant and non-relevant impurities (below the reporting threshold) and to classify impurities into those that need to be reported, identified and qualified according to the thresholds of the ICH [5,6]. Thus, an analysis strategy had to be developed that fulfilled three major demands, namely 1) quantification of unknown compounds without standards, 2) sufficient chromatographic separation of main components of the infusion solution and impurities and 3) identification of relevant impurities.

Commonly used detectors like UV and MS detectors exhibit compound specific detector sensitivities [7]. Thus, for quantification calibration with authentic standards is required. Furthermore, compounds lacking a chromophoric group or ionization properties may be invisible for UV and MS detectors, respectively. Hence, a detector with universal detection characteristics was required. The charged aerosol detector (CAD) is deemed to be such a detector providing consistent detector responses for non-volatile compounds [12,14,15]. Several experiments were carried out to examine the characteristics of this detector and its capability to provide compound independent detector sensitivity.

In Figure 11 two chromatograms are shown which were obtained by use of an UV detector and a CAD in series. A solution containing equal amounts of Glu, Leu, cyclo(AlaGlu) and $(AcCys)_2$ was injected.

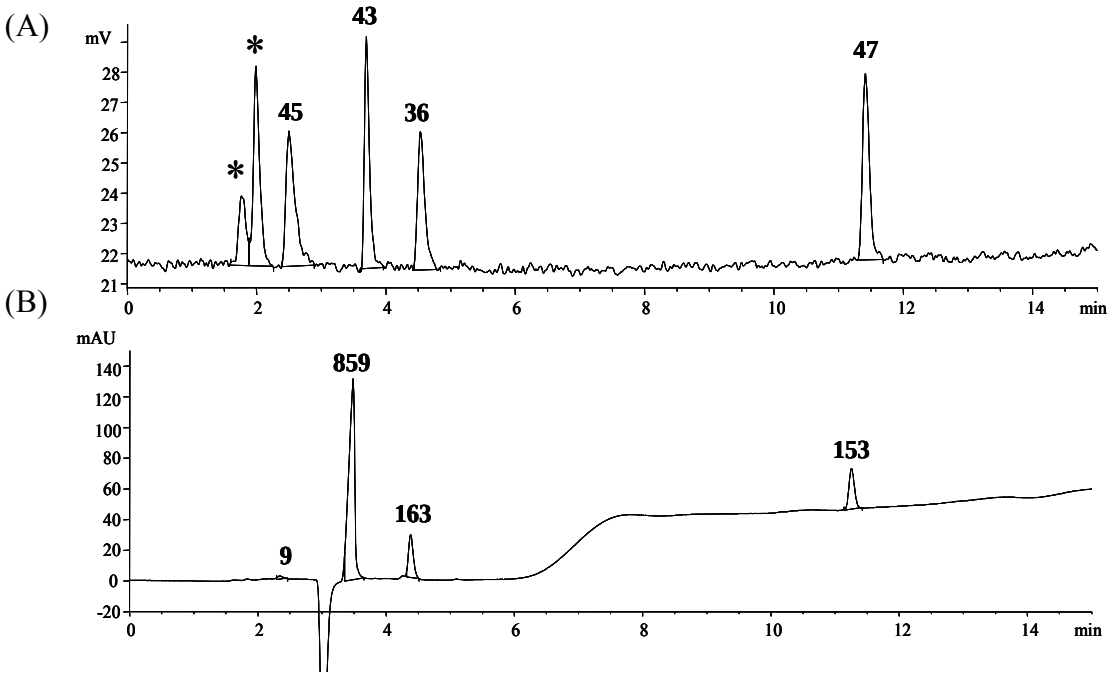

Figure 11: Chromatograms obtained with (A) CAD and (B) UV detector.
*Equal amounts of analytes were injected into the column (100 ng). Analytes eluted in the following order: Glu, Leu, cyclo(AlaGlu) and $(AcCys)_2$. Denotation above the peaks corresponds to the peak area. * System peaks*
Chromatographic conditions: Gemini C18 from Phenomenex; (A) 0.1% formic acid in water; (B) 0.1% formic acid in ACN; gradient elution: 5% (B) to 52.5% (B) in 30 minutes; flow rate 0.3 ml/min

The peak areas in the CAD trace are quite similar for identical injected amounts of structurally different non-volatile solutes (36-47) reflecting similar detector responses. In sharp contrast, the peak areas in the UV trace differed drastically (9-859). Furthermore, in the UV trace the first peak corresponding to Glu is hardly recognizable and bears a high risk to be overlooked, whereas in the CAD chromatogram Glu yields a well defined peak. Thus, the CAD can be considered as complementary to UV and MS detectors, respectively, allowing to detect compounds that would otherwise fail to be detected.
Moreover, calibration functions with authentic standards were established for eight different compounds (Trp, Phe, Leu, GlyTyr, LeuTrpMetArg, cyclo(AlaGlu), Glu and $(AcCys)_2$). The slopes of the resulting calibration functions which indicate the detector sensitivity for the respective compounds were compared and deviated only slightly for the individual compounds (21% RSD) confirming the suitability of the CAD for universal calibration.

Nevertheless, a strong dependency of the detector response on the mobile phase composition was recognized, which arises due to an improvement of the transport efficiency of the CAD sprayer with increasing organic modifier content in the mobile phase. Plotting slopes of compound–specific calibration functions versus % organic modifier in the mobile phase provides a linear relation which can be used to calculate slopes specific for a defined retention time (Figure 12). Further results are discussed in detail in Appendix I.

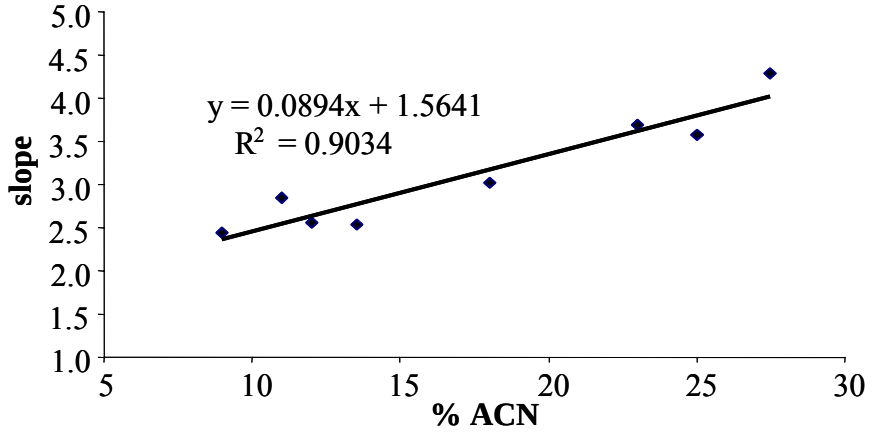

Figure 12: Slopes of a set of eight compound-specific calibration functions plotted versus the % of ACN at the time of elution.

Concerning identification of relevant impurities an ion trap mass spectrometer (IT-MS) was considered to be appropriate because of its capability to automatically produce MS^2 spectra which can be exploited for structure elucidation and confirmation.

Nevertheless, separation of the complex mixture of ingredients, mainly consisting of amino acids, dipeptides and additives like citric acid and taurine, and impurities was necessary.

In the first place several RP phases like Chromolith RP18e from Merck, Gemini C18 and Synergi Fusion-RP 80 from Phenomenex in combination with different mobile phase additives e.g. 0.1% formic acid (FA), trifluoracetic acid (TFA) and heptafluorobutyric acid (HFBA) were tested and qualitatively evaluated.

As illustrated in Figure 13, Gemini C18 provided better resolution and efficiency as compared to Chromolith Performance Si. Synergi Fusion-RP, a polar embedded C18 phase, exhibited similar separation characteristics to Gemini C18. The use of ion pair reagents in order to improve retentivity of polar compounds on the C18 phase was partly successful and retention times increased in the order of FA < TFA < HFBA. However, TFA and HFBA were avoided as mobile phase additives as they were considered to be not ideally compatible with IT-MS detection.

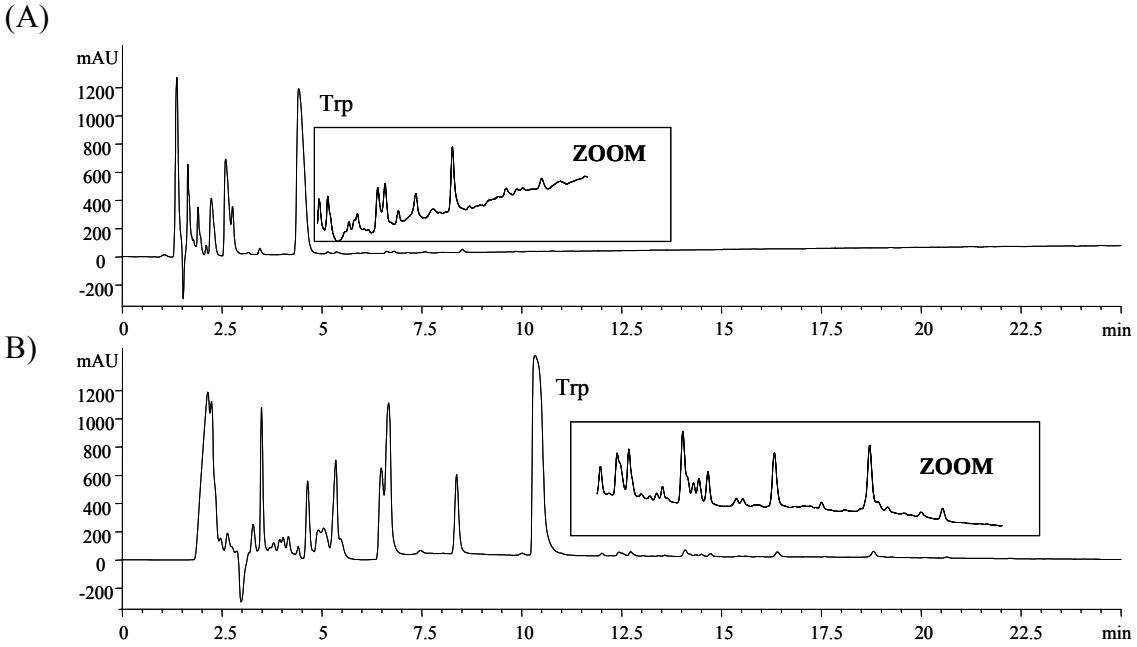

Figure 13: UV-chromatograms of a stressed infusion solution (stored at 60°C for 9 months) on (A) Chromolith RP18e and on (B) Gemini C18.
Chromatographic conditions: (A) 0.1% formic acid in water; (B) 0.1% formic acid in ACN; gradient elution: 5% (B) to 52.5% (B) in 30 minutes; flow rate 1 ml/min (Chromolith) and 0.3 ml/min (Gemini C18)

In both chromatograms shown in Figure 13 highly polar and hydrophilic compounds were hardly retained and eluted unresolved at the front of the chromatogram. However, in the late eluting part of the chromatograms, after Trp the latest eluting ingredient of the infusion solution, several minor peaks can be found. These peaks were of special interest as they were assumed to correspond to peptide-like impurities. In the course of impurity profiling of the stressed infusion solution emphasis was put on the investigation of the presence or absence of larger peptides (> two amino acids) which may be formed by condensation reactions of amino acids and dipeptides. Examination of such peptides is of utmost importance since they may exhibit bioactivity and may potentially cause side effects.

Not unexpected, more polar compounds of the infusion solution were not sufficiently retained and resolved under RP conditions. Hence, further experiments were performed under HILIC conditions using more polar stationary phases like Chromolith Performance Si from Merck, mixed-mode RP-WAX (housemade) and Polysulfoethyl A from PolyLC. Using CAD and IT-MS compatible mobile phase conditions no acceptable resolution of the polar compounds

could be achieved. Furthermore, RP-WAX and Polysulfoethyl A suffered to some extent from column bleeding which substantially decreased sensitivity of the IT-MS detector and caused high background signals in the CAD chromatogram.

Sufficient resolution could not be achieved using one-dimensional (1-D) chromatographic separation techniques but a two-dimensional (2-D) LC method combining the complementary separation mechanisms of HILIC and RP provided improved selectivity and peak capacity as demonstrated in Figure 14.

In Figure 14 A 1-D retention time plots reveal that in the chromatograms of three separation systems (described in detail in Appendix I), gradient RPLC on Gemini C18 (H_2O/ACN, 0.1% FA as mobile phase additive), gradient RPLC on tandem Gemini C18/Synergi Fusion-RP (H_2O/ACN, 0.1% TFA as mobile phase additive) and gradient elution HILIC on Chromolith Performance Si (ACN/H_2O, 3 mM AcOH adjusted to pH 5.5 with NH_3 as buffer), several peaks are unresolved and cluster together. It is obvious that the best peak capacity is provided by the tandem Gemini C18/ Synergi Fusion-RP column (coupled in series) as the peaks are spread over a wider retention time window. In 1-D LC peaks may be represented as a lines as shown in Figure 14A whereas in 2-D LC illustrations peaks are distributed over an area, which is associated with a substantial gain in peak capacity and selectivity as shown in Figure 14B and C, where retention times on Chromolith Peformance Si are plotted versus retention times on Gemini C18 and on the tandem column Gemini C18/Synergy Fusion-RP, respectively. The combination of tandem Gemini C18/Synergi Fusion-RP column in the RPLC mode and Chromolith Performance Si in the HILIC mode provided superior resolution of peaks.

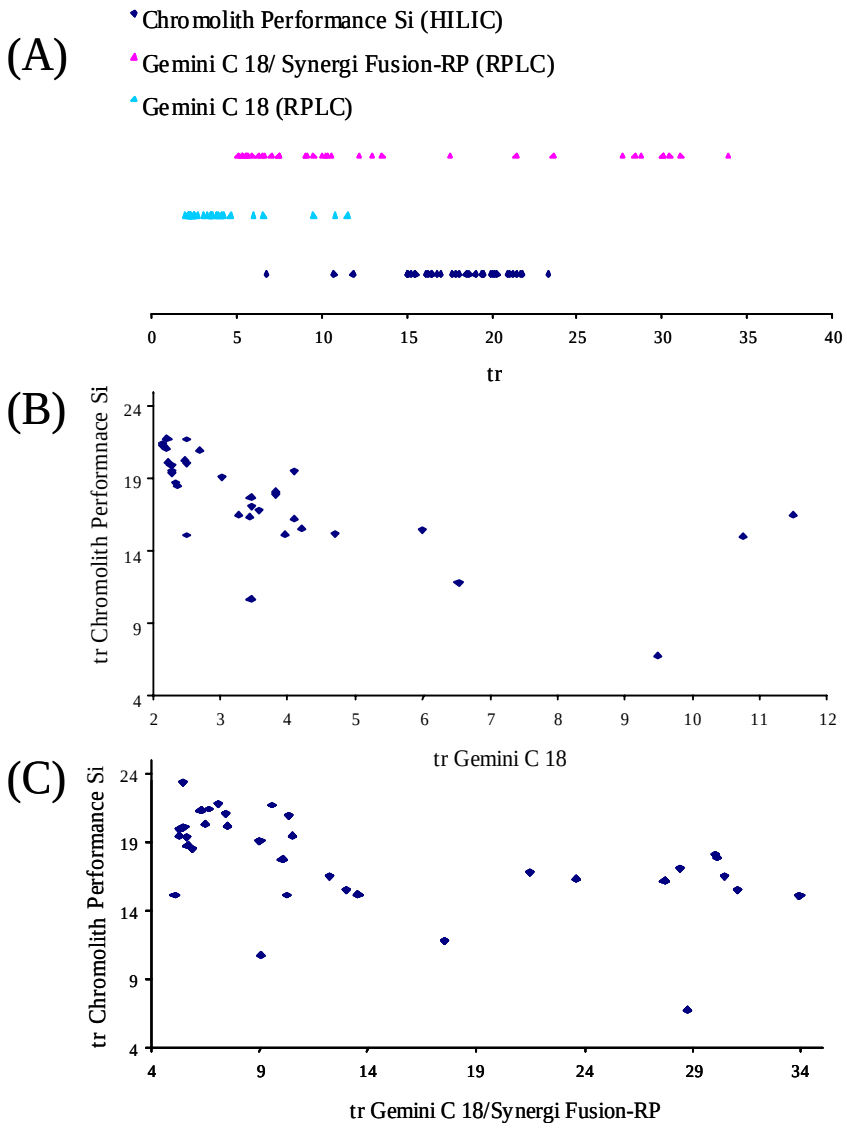

Figure 14: (A) 1-D plots of retention times on Gemini C18, tandem Gemini C18/Synergi Fusion-RP80 and Chromolith Performance Si. Complementarity plots of retention times on Chromolith Performance Si versus retention times on (B) Gemini C18 and on (C) tandem Gemini C18/Synergi Fusion-RP, respectively.

Finally, distinct separation mechanisms, RP and HILIC, and complementary detector principles, CAD for quantification and IT-MS for identification, were combined offline to yield one multidimensional analysis assay. The working flow of the multidimensional analysis assay is based on three steps as shown in Figure 15 and is described in more detail in Appendix I.

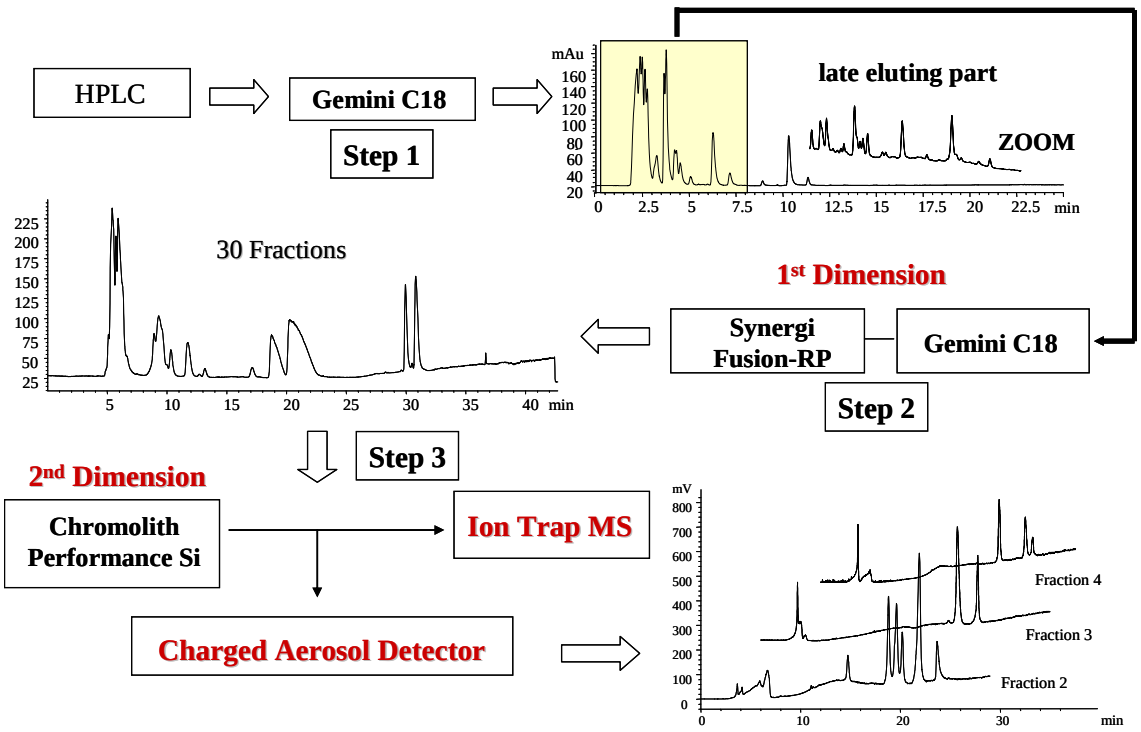

Figure 15: Schematic illustration of the work flow of the developed multidimensional analysis assay.

In step 1 a Gemini C18 column was employed. The early eluting part containing polar compounds was collected into one fraction. The late eluting part exhibiting minor peaks which were assumed to correspond to peptide-like impurities was directly analyzed using CAD and IT-MS detection.

The polar fraction (highlighted in yellow in Figure 15) was submitted to further separation by 2-D LC in step 2 and 3.

In step 2 a combination of Gemini C18 and Synergi Fusion-RP in series was used as first dimension for the separation of the polar fraction and provided a remarkable extension of the retention time window allowing to collect 30 fractions. In step 3 (2^{nd} dimension) separation of the 30 fractions was performed on Chromolith Performance Si.

Unfortunately, the combination of tandem Gemini C18 and Synergi Fusion-RP and Chromolith Performance Si was not optimal since full separation power of the system could not be exploited. The problem was that the retention time window on the silica monolith was rather narrow and several peaks were not fully resolved, thus it is very probable that resolution already achieved in the first dimension on tandem Gemini C18/Synergi-Fusion-RP was irretrievably lost due to backmixing caused by undersampling during fraction collection. The fraction collection intervals were selected in order to keep the number of fractions in the

second dimension reasonable. In this context, peak capacity and selectivity can be further improved by selecting narrower fraction collection time intervals. Nevertheless, complementarity plots (Figure 14) confirm that the 2-D LC strategy provided an extended separation space as compared to 1-D analysis on each of the three single columns.

All 30 fractions from the 1st dimension (step 2) were injected three times into the 2nd dimension HILIC system (step 3): 2 µl and 20 µl (low and high load) injection volumes employing the CAD and 10 µl using IT-MS detection.

Peaks found in the CAD chromatogram were quantified using unified calibration functions. Comparison with the IT-MS chromatograms allowed identification of peaks with the help of scan spectra and fragmentation spectra. The results are summarized in Table 4 and 5.

In fractions 1 and 2 of the multidimensional analysis assay a peak with considerable area was found (compare Table 5; tr = 20.2 min). Further analysis of the fractions using the ion trap did not reveal the identity of the compound(s) of the peak. The position of the peak in the chromatogram and the concentration calculated via universal calibration using the CAD indicated that the peak may stem from coeluting Glycine and Alanine, two known ingredients of the infusion solution. These two amino acids exhibit very low molecular masses (75 and 89 g/mol, respectively) for which the ion trap may show insufficient detection sensitivity and non-characteristic fragmentation. This may be the reason why the peaks could not have been identified with the IT-MS. To finally ensure this assumption the molecular weight of the two amino acids was increased by derivatisation with Sanger's reagent which selectively reacts with the amino function. The derivatisation procedure can be found in Appendix II. The in this way introduced 2,4-dinitrophenyl group increases strongly hydrophobicity of the molecules, which enables reasonable chromatography of the derivatized amino acids on RP-18 columns for further analysis.

The described approach was also used for further identification and analysis of unknown compounds exhibiting an amino group or verification, whether the respective compounds of particular peaks contain a derivatizable amino group.

This way, it was possible to detect Ala and Gly (in form of their corresponding 2,4-dinitrophenyl derivatives) which in underivatized form were indeed detected by the CAD but not by the IT-MS in the course of the multidimensional analysis. Thus, corresponding unidentified peak could be assigned to Gly and Ala.

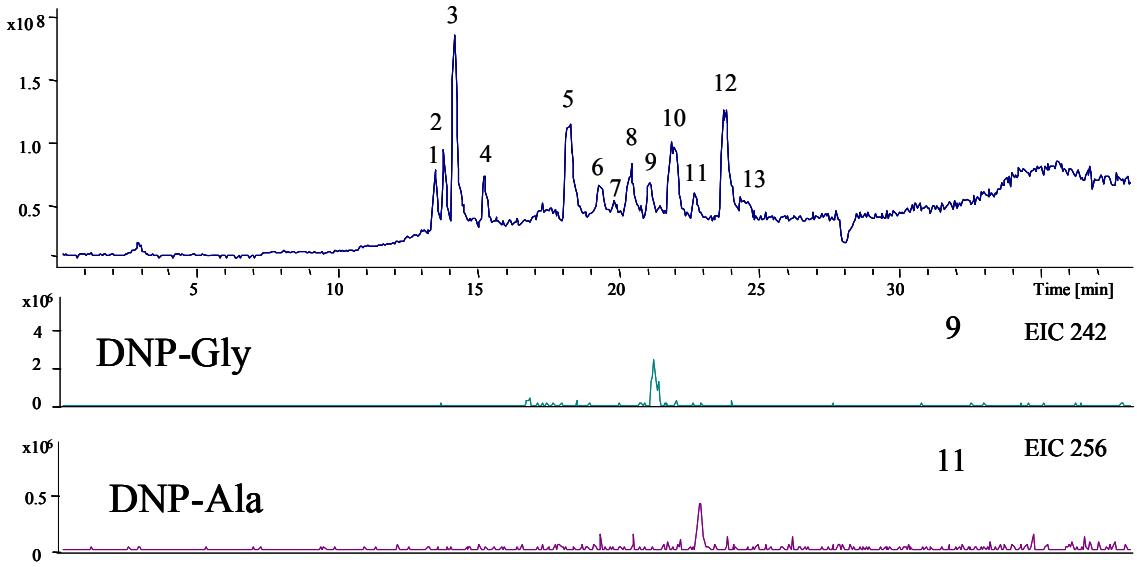

Figure 16: Chromatogram of fraction 2 of the tandem RP column derivatized with Sanger's reagent (2,4-dinitrofluorobenzene).

Peak annotation: 1 DNP-His ; 2 unidentified; 3 DNP-Arg; 4 DNP-Lys; 5 DNP-AlaGln; 6 DNP-Ser; 7 unidentified; 8 DNP-Thr; 9 DNP-Gly; 10 DNP-Pro; 11 DNP-Ala; 12 DNP_2-His; 13 unidentified

Chromatographic conditions: Gemini C18 from Phenomenex; (A) 0.1% FA in H_2O and (B) 0.1% in ACN; gradient: 5% (B) to 100% (B) in 30 min; 0.3 ml/min

In Table 4 results for the late eluting part of step 1, which included more apolar compounds, can be found and in Table 5 results for the polar fraction which was submitted to 2-D RP x HILIC are presented. Qunatitative results obtained with the multidimensional analysis assay agreed quite well with the results obtained with validated UV methods and validated LC-MS/MS methods (see Appendix II). Several peaks detected with the CAD failed to be detected with the IT-MS and could not be identified for this reason. However, most of these unidentified peaks exhibited concentrations below the reporting threshold and for this reason were considered to be not of relevance. Relevant peaks which could not be identified after a number of additional experiments had been performed e.g. derivatisation with Sanger's reagent, rechromatography with alternative separation systems, were assumed to originate from contaminations introduced during 2-D LC fractionation.

However, several degradation products of AlaGln, which constitutes the main component of the infusion solution, could be detected and identified employing the multidimensional analysis assay. In Figure 17 identified degradation products of AlaGln are shown. No peptides composed of more than four amino acids were found.

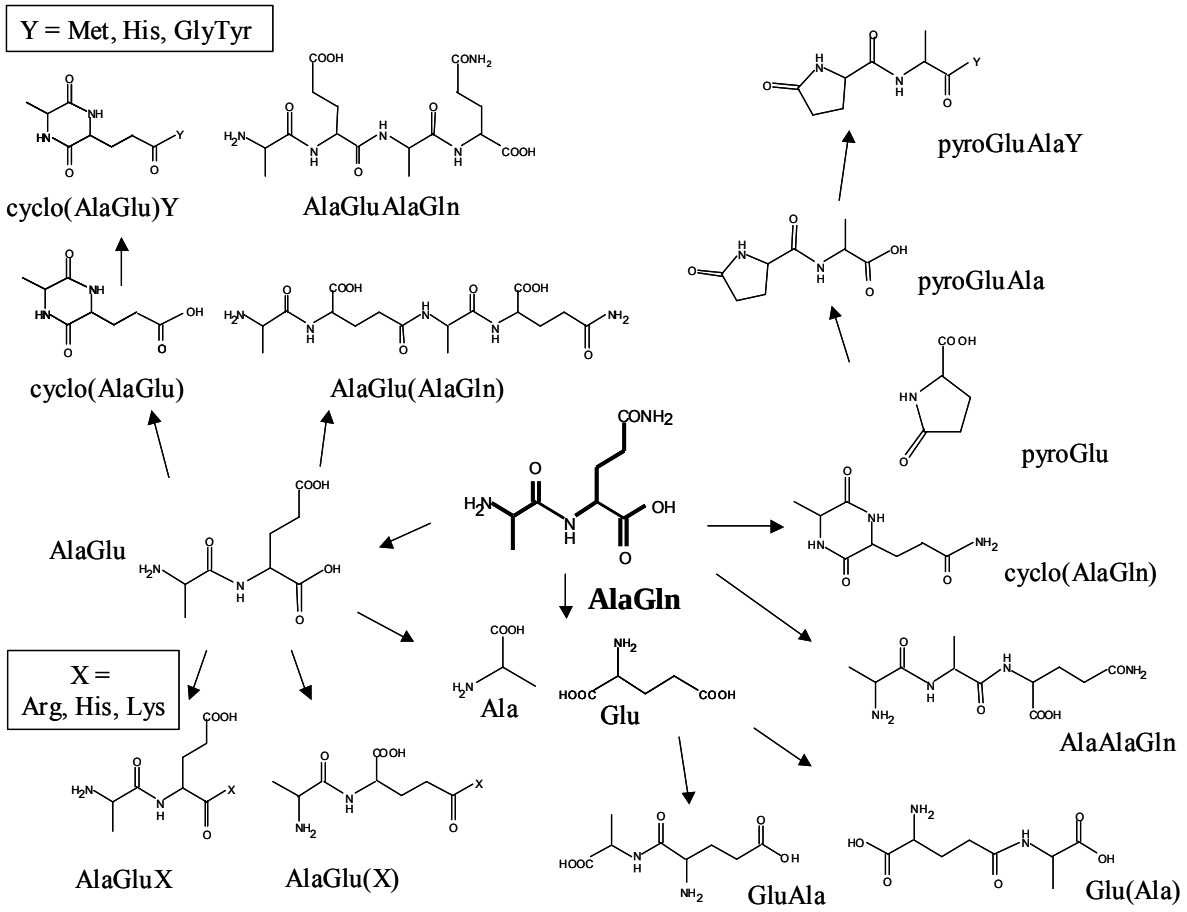

Figure 17: Degradation pathways of AlaGln in nutritional infusion solutions.

Table 4: List of impurities detected in the late eluting part of step 1 and their corresponding quantification results using the RP-CAD method, validated LC-UV methods and a validated LC-MS/MS methods (Appendix I and II).

retention time [98]	found m/z	name	RPLC - CAD [µg/ml]	LC-UV [µg/ml]	LC-MS/MS [µg/ml]	comment
5.1	**201**	**pyroGluAla**[1]	**260.6**	**226.6**	**309.7**	**> qualification threshold**
7.2	164	N-AcCys[2]	546.6	690	n.a.[4]	ingredient
8.9	**221**	**cyclo(GlyTyr)**	**85.0**	**108**	**n.a.**	**> qualification threshold**
9.6	n.f.[3]	n.a.	2.6	n.a.	n.a.	< reporting threshold
10.0	n.f.	n.a.	6.1	n.a.	n.a.	< reporting threshold
10.3	205	Trp	1993.9	2180	n.a.	ingredient
11.2	**325**	**$(AcCys)_2$**[2]	**131.8**	**235**	**n.a.**	**> qualification threshold**
12.0	n.f.	n.a.	3.4	n.a.	n.a.	< reporting threshold
12.3	n.f.	n.a.	3.3	n.a.	n.a.	< reporting threshold
12.5	*n.f.*	*n.a.*	*17.2*	*n.a.*	*n.a.*	*> reporting threshold*
12.7	281	n.a.	7.7	n.a.	n.a.	< reporting threshold
12.9	n.f.	n.a.	5.9	n.a.	n.a.	< reporting threshold
13.3	**421,332**	**cyclo(AlaGlu)GlyTyr,cyclo(AlaGlu)Met pyroGluAlaGlyTyr,pyroGluAlaMet**	**30.8**	**n.a.**	**n.a.**	**further investigations necessary**
14.0	n.f.	n.a.	1.3	n.a.	n.a.	< reporting threshold
14.8	*231*	*n.a.*	*13.9*	*n.a.*	*n.a.*	*> reporting threshold*
14.9	*395*	*n.a.*	*15.4*	*n.a.*	*n.a.*	*> reporting threshold*
15.2	n.f.	n.a.	2.8	n.a.	n.a.	< reporting threshold
15.6	n.f.	n.a.	2.7	n.a.	n.a.	< reporting threshold
15.8	n.f.	n.a.	2.6	n.a.	n.a.	< reporting threshold
15.9	n.f.	n.a.	5.1	n.a.	n.a.	< reporting threshold
16.3	n.f.	n.a.	3.5	n.a.	n.a.	< reporting threshold
16.4	n.f.	n.a.	4.0	n.a.	n.a.	< reporting threshold
16.8	n.f.	n.a.	1.7	n.a.	n.a.	< reporting threshold
17.0	n.f.	n.a.	5.2	n.a.	n.a.	< reporting threshold
17.5	n.f.	n.a.	2.4	n.a.	n.a.	< reporting threshold
17.5	n.f.	n.a.	1.4	n.a.	n.a.	< reporting threshold
18.7	n.f.	n.a.	0.9	n.a.	n.a.	< reporting threshold
19.0	n.f.	n.a.	1.3	n.a.	n.a.	< reporting threshold
19.3	n.f.	n.a.	2.8	n.a.	n.a.	< reporting threshold
19.9	n.f.	n.a.	6.1	n.a.	n.a.	< reporting threshold
20.2	275	n.a.	8.0	n.a.	n.a.	< reporting threshold
20.6	n.f.	n.a.	0.5	n.a.	n.a.	< reporting threshold
20.8	n.f.	n.a.	3.0	n.a.	n.a.	< reporting threshold
22.0	n.f.	n.a.	1.9	n.a.	n.a.	< reporting threshold
22.5	n.f.	n.a.	4.2	n.a.	n.a.	< reporting threshold
23.2	n.f.	n.a.	0.8	n.a.	n.a.	< reporting threshold
23.5	n.f.	n.a.	0.5	n.a.	n.a.	< reporting threshold

[1] pyroGluAla eluted as isolated peak in the polar fraction and thus, could also be quantified with the RP method of step 1.
[2] $(AcCys)_2$ is the dimer of N-AcCys and emerges due to disulfide bond formation
[3] n.f. not found
[4] n.a. not available
in bold letters: impurities determined to be above the identification and the qualification threshold, respectively
in italic letters: impurities determined to be above the reporting threshold

Table 5: List of impurities detected and quantified in the course of the multidimensional analysis assay (RPLC x HILIC – CAD). Furthermore, quantification was performed with validated LC-UV methods and validated LC-MS/MS methods (Appendix I and II).

Retention time [min]	Fraction	m/z	Name	RPLC-HILIC - CAD [µg/ml]	LC-UV [µg/ml]	LC-MS/MS [µg/ml]	Comment
10.6	**7/8/9/10/11/12**	**200**	**cyclo(AlaGln)**	**1247.0**	**1144**	**n.a.**	**> qualification threshold**
11.1	8	n.f.[4]	n.a.	6.1	n.a.[5]	n.a.	< reporting threshold
11.4	10	n.f.	n.a.	0.9	n.a.	n.a.	< reporting threshold
11.6	10/11	n.f.	n.a.	5.2	n.a.	n.a.	< reporting threshold
11.7	6	n.f.	n.a.	5.3	n.a.	n.a.	< reporting threshold
11.8	17	164	N-AcCys[1]	97.0	690	n.a.	ingredient
11.9	19/20	n.f.	n.a.	3.7	n.a.	n.a.	< reporting threshold
12.6	7	n.f.	n.a.	2.7	n.a.	n.a.	< reporting threshold
12.8	28	n.f.	n.a.	3.2	n.a.	n.a.	< reporting threshold
13.7	*9/10*	*n.f.*	*n.a.*	*10.4*	*n.a.*	*n.a.*	*> reporting threshold*
13.9	6	n.f.	n.a.	2.5	n.a.	n.a.	< reporting threshold
14.3	16	n.f.	n.a.	7.3	n.a.	n.a.	< reporting threshold
14.4	19/20/21	n.f.	n.a.	9.7	n.a.	n.a.	< reporting threshold
14.4	24/25/26	n.f.	n.a.	9.0	n.a.	n.a.	< reporting threshold
14.5	29/30	n.f.	n.a.	7.3	n.a.	n.a.	< reporting threshold
14.6	**10**	**n.f.**	**n.a.**	**68.1**	**n.a.**	**n.a.**	**> qualification threshold**
14.7	1/2	126	Taurine	1222.6	980	n.a.	ingredient
14.8	**12/13**	**201**	**cyclo(AlaGlu)/pyroGluAla[2]**	**333**	**275**	**386**	**> qualification threshold**
15.2	**8**	**430**	**n.a.**	**30.2**	**n.a.**	**n.a.**	**> qualification threshold**
15.2	**9/10/11**	**n.f.**	**n.a.**	**125.6**	**n.a.**	**n.a.**	**> qualification threshold**
15.2	**15**	**n.f.**	**n.a.**	**194.8**	**n.a.**	**n.a.**	**> qualification threshold**
15.5	**17**	**201**	**n.a.**	**26.0**	**n.a.**	**n.a.**	**> identification threshold**
15.6	11	n.f.	n.a.	2.7	n.a.	n.a.	< reporting threshold
15.6	28/29/30	166	Phe	2271.9	3580	n.a.	ingredient
16.2	17/18/19/20/21	132	Leu	6937.2	10640	n.a.	ingredient
16.4	*22/23/24/25*	*n.f.*	*n.a.*	*18.2*	*n.a.*	*n.a.*	*> reporting threshold*
16.4	**27/28**	**325**	**$(AcCys)_2$[1]**	**265.7**	**235**	**n.a.**	**> qualification threshold**
16.5	9/10/11/12	150	Met	1686.6	1990	n.a.	ingredient
16.6	8	n.f.	n.a.	4.9	n.a.	n.a.	< reporting threshold
16.8	15/16/17/18	132	Ile	3590.5	4780	n.a.	ingredient
17.0	24/25	n.f.	TyrGly	7.7	13.5	n.a.	< reporting threshold
17.2	*17*	*n.f.*	*n.a.*	*13.5*	*n.a.*	*n.a.*	*> reporting threshold*
17.4	8	n.f.	n.a.	1.1	n.a.	n.a.	< reporting threshold

Retention time [min]	Fraction	m/z	Name	RPLC-HILIC - CAD [µg/ml]	LC-UV [µg/ml]	LC-MS/MS [µg/ml]	Comment
17.4	22	n.f.	n.a.	0.6	n.a.	n.a.	< reporting threshold
17.4	27	n.f.	n.a.	2.1	n.a.	n.a.	< reporting threshold
17.5	**15**	**303**	**n.a.**	**315.8**	**n.a.**	**n.a.**	**> qualification threshold**
17.7	**7/8/9/10/11**	**n.f.**	**n.a.**	**100.3**	**n.a.**	**n.a.**	**> qualification threshold**
17.8	4/5/6	118	Val	5092.0	5460	n.a.	ingredient
17.8	11	n.f.	n.a.	1.8	n.a.	n.a.	< reporting threshold
18.1	**8**	**283**	**n.a.**	**51.0**	**n.a.**	**n.a.**	**> qualification threshold**
18.3	**13**	**n.f.**	**n.a.**	**23.3**	**n.a.**	**n.a.**	**> identification threshold**
18.3	27/28	239	GlyTyr	1976.0	2550	n.a.	ingredient
18.8	2/3	120	Thr	3739.1	5440	n.a.	ingredient
18.9	**3/4/5/6/7**	**n.f.**	**n.a.**	**160.7**	**n.a.**	**n.a.**	**> qualification threshold**
19.0	*28*	*439*	*n.a.*	*15.3*	*n.a.*	*n.a.*	*> reporting threshold*
19.7	1/2/3/4/5/6	106, 116	Ser,Pro	12182.1	14020	n.a.	ingredient
19.7	**5**	**n.f.**	**n.a.**	**37.8**	**n.a.**	**n.a.**	**> qualification threshold**
19.7	12	n.f.	n.a.	6.9	n.a.	n.a.	< reporting threshold
19.7	**26/27**	**n.f.**	**n.a.**	**54.0**	**n.a.**	**n.a.**	**> qualification threshold**
19.8	**9/10**	**193, 338**	**Citric acid; cyclo(AlaGlu)His/pyroGluAlaHis**	**1131.6**	**n.a.**	**n.a.**	**further investigations necessary**
20.2	1/2	n.f.	Gly, Ala	2644.1	3100	n.a.	ingredient
20.2	*7/8*	*n.f.*	*n.a.*	*14.4*	*n.a.*	*n.a.*	*> reporting threshold*
20.3	12	n.f.	n.a.	7.8	n.a.	n.a.	< reporting threshold
20.5	7/8	n.f.	n.a.	7.5	n.a.	n.a.	< reporting threshold
20.9	4	n.f.	n.a.	2.7	n.a.	n.a.	< reporting threshold
21.5	**7/8**	**418**	**AlaGlu(AlaGln)**	**21.8**	**39.6**	**37.8**	**> identification threshold**
21.7	2/3/4/5/6/7	218	AlaGln/AlaGlu[3]	14615.8	19290	n.a.	Ingredient/impurity
21.7	**7/8**	**211**	**n.a.**	**51.1**	**n.a.**	**n.a.**	**> qualification threshold**
21.7	*10*	*n.f.*	*n.a.*	*11.8*	*n.a.*	*n.a.*	*> reporting threshold*
22.3	*6*	*418*	*AlaGluAlaGln*	*13.3*	*22*	*16.9*	*> reporting threshold*
22.4	*4*	*289*	*AlaAlaGln*	*18.1*	*31*	*23.0*	*> reporting threshold*
22.8	*3*	*218*	*n.a.*	*11.9*	*n.a.*	*n.a.*	*> reporting threshold*
23.6	2/3	156	His	3763.6	3030	n.a.	ingredient
25.0	**2/3/4**	**356,375**	**"AlaGluHis/AlaGluArg"-isomers**	**41.5**	**n.a.**	**n.a.**	**further investigations necessary**
27.1	**4**	**356**	**"AlaGluHis"-isomers**	**18.8**	**n.a.**	**n.a.**	**further investigations necessary**
27.8	**4**	**375**	**"AlaGluArg"-isomers**	**14.6**	**n.a.**	**n.a.**	**further investigations necessary**

[1] $(AcCys)_2$ is the dimer of N-Ac-Cys and is formed via disulfide bonding. Considering the retention time of $(AcCys)_2$ in the RP mode in step 1 (10.2 min), it was not expected to detect it in the polar part (< 8 min). Thus, it is assumed that $(AcCys)_2$ is formed during intermediate sample processing in the course of the 2-D assay due to disulfide bonding of N-AcCys, which was expected to be contained in the polar part.
[2] As cyclo(AlaGlu) and pyroGluAla could not be reliably distinguished, the combined concentration is given. Both compounds were accurately quantified using a validated LC-MS/MS method (Appendix II).
[3] AlaGln and AlaGlu could not be resolved during the multidimensional analysis assay. Thus, individual quantification was not possible. Yet the combined concentration is given. AlaGlu was accurately quantified using a validated LC-MS/MS method (Appendix II).
[4] n.f. not found
[5] n.a. not available
in **bold** letters: impurities determined to be above the identification and the qualification threshold, respectively
in *italic* letters: impurities determined to be above the reporting threshold

3.1.2. Quantification of relevant impurities

Based on the results of the preliminary impurity profile LC-MS/MS methods were developed for more accurate quantification of relevant impurities (Table 4 and 5, impurities highlighted in yellow) in the course of stability testing. Chromatographic methods are described in detail in Appendix II.

The main component of the infusion solution constituted L-Ala-L-Gln, which is a chiral compound containing two chiral centers. During stress testing AlaGln may undergo epimerization at one or at both stereogenic centers yielding diastereomeric (LD, DL) and enantiomeric (DD) impurities, respectively. Since stereoisomeric compounds may exhibit distinct bioactivity it is important to also investigate potential conversion of L-Ala-L-Gln into its stereoisomeric forms in the course of stability testing. As stereoisomers can not be differentiated by MS, analysis relies on chromatographic separation. The multidimensional analysis assay discussed above did not provide stereoselective separation power and thus potential stereoisomeric forms of L-Ala-L-Gln could not be detected.

Preliminary experiments were focused on the separation of the diastereomers (DL,LD) of L-Ala-L-Gln , which were more likely to be formed. Furthermore, separation of AlaGln from its degradation product AlaGlu and AlaGluAlaGln and AlaGlu(AlaGln), which were detected in the course of the multidimensional analysis assay (see Table 5), was necessary in order to avoid signal interferences.

Employing RP conditions the above mentioned compounds were not sufficiently retained and resolved, respectively. Further experiments were targeted at increasing retention times in the RP mode. In this context, several fluorinated ion pair reagents TFA, HFBA, nonafluoropentanoic acid (NFPA) and tridecafluoroheptanoic acid (TDFHA) were tested as mobile phase additives in a concentration range of 2 – 20 mM using a Gemini C18 column from Phenomenex. As expected retention times were significantly increased in the order of TFA < HFBA < NFBA < TDFPA. Resolution of all critical pairs could be achieved with 2 mM NFPA as well as with 2 mM TDFHA as mobile phase additives employing isocratic elution conditions as illustrated in Figure 18. Nevertheless, use of ion pair reagent was associated with several severe drawbacks, since the methods suffered from poor retention time reproducibility, very long equilibration times and very broad peaks. Furthermore, ion pair reagents are not very well suitable for MS detection as contamination of the ion source and ionization suppression are likely to occur.

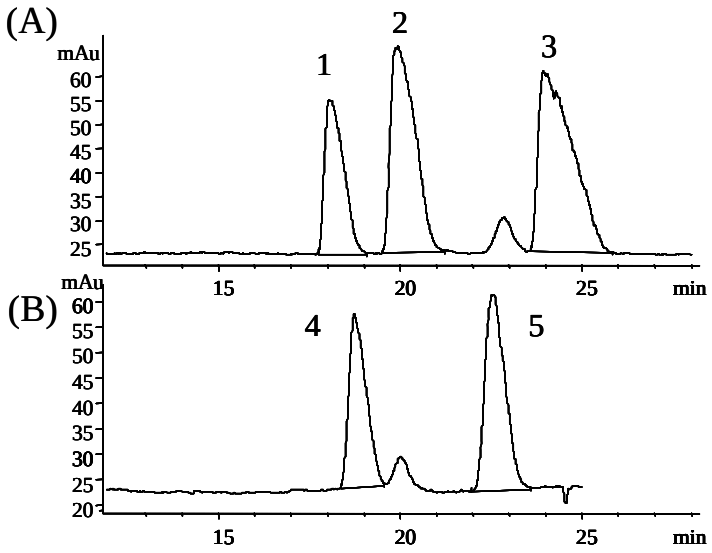

Figure 18: Chromatograms of the separation of 1 DL/LD AlaGln; 2 LL/DD AlaGln; 3 AlaGlu; 4 AlaGlu(AlaGln) and 5 AlaGluAlaGln.
Chromatographic conditions: Gemini C18; 2 mM NFPA in H_2O/ACN (90/10, v/v) isocratic elution; 0.3 ml/min

Further experiments were carried out on a Chiralpak QN-AX column from Chiral Technologies (Illkirch, France). The stationary phase exhibits a chiral selector, tert-butylcarbamoylquinine, which is known to provide selectivity for the separation of N-derivatized amino acids and peptides. Employing derivatization with Sanger's reagent separation of the four stereoisomeric forms of AlaGln dipeptide was accomplished and revealed that the D-Ala-D-Gln, the enantiomeric form of the main component L-Ala-L-Gln, was only present in non-relevant quantities in the stressed infusion solution. Thus, a diastereoselective method, which does not require derivatisation of the terminal amino group, was deemed to be adequate providing separation of LD/DL and LL/DD. Employing the Chiralpak QN-AX column also the critical pairs AlaGln and AlaGlu, and AlaGlu(AlaGln) and AlaGluAlaGln could be separated.

The tripeptidic isobaric impurities AEX and AE(X) formed by condensation reactions of AlaGlu with Arg, Lys, His as well as pyroGluAlaHis and cyclo(AlaGlu)His had to be separated as well. Since these peptides exhibit basic groups in their side chains Polysulfoethyl A, a strong cation exchanger, constituted an adequate stationary phase for separation.

The less polar peptides cyclo(AlaGlu)GlyTyr, pyroGluAlaGlyTyr, cyclo(AlaGlu)Met and pyroGluAlaMet were well separated on Gemini C18 employing a very flat RP gradient.

Several other impurities, e.g. TyrGly, cyclo(GlyTyr), cyclo(AlaGln) could be easily integrated into these three developed methods.

In the course of validation of these three methods LOQs, linear range, intra-assay as well as interday precision and accuracy were determined. With the only exception of $(AcCys)_2$, which is known to be prone to undergo redox-reactions, validation results were satisfactory for all compounds and proved suitability of the three developed methods for the purpose of quantitative analysis in the course of stability testing. The results are presented and discussed in detail in Appendix II.

Overall, the combination of different separation mechanisms and complementary detection principles allowed to uncover a multitude of unknown impurities that were formed in the infusion solutions during stress testing. Furthermore, distinction between relevant (> 0.05% relative to the parent compound) and non-relevant impurities could be achieved exploiting the universal detection characteristics of the CAD. Based on these preliminary results, quantitative LC-MS/MS methods were developed and validated that provided reliable quantitative data for relevant impurities.

3.2. Metabolic profiling in fermentation broths

The project with Sandoz was focused on the development of LC-MS/MS methods for the quantitative determination of extracellular metabolites and nutritional compounds in fermentation broths. In this context, two LC-MS/MS methods, a HILIC method for polar compounds and a RPLC method for unpolar compounds, were developed and validated.
Furthermore, emphasis was put on the evaluation of matrix effects that might easily occur in complex matrices like fermentation broths. Both methods as well as their corresponding validation results are discussed in detail in Appendix III and IV.
Herein, a procedure for the establishment of a multicomponent LC-MS/MS method (in particular also for metabolite quantification) is proposed taking into account the experiences made during method development and the obtained validation results.

3.2.1. Procedure for the development and validation of a multicomponent analysis method

Table 6: Steps in the development and validation of multicomponent analysis methods.

Steps in multicomponent LC-MS/MS method development	**Comment**
1. Optimization of MRM transitions	"Quantitative optimization" tool of the Analyst software
2. Critical pairs	Evaluation of potential signal interferences
3. Screening of chromatographic conditions	Investigation of different stationary/mobile phase combinations Complementary separation methods
4. Method fine tuning	Further optimization of the most promising stationary/mobile phase combination
5. Preliminary method validation	Includes calibration with neat standard solutions, evaluation of accuracy and precision Gives a first insight into method performance Provides data that allow to design the final method validation procedure
6. Stability of compounds	Measurement of quality control samples (stored under experimental conditions, e.g. in the autosampler at 5°C, refrigerator at -20°C) on different days, quantification using freshly prepared calibration function
7. Final method validation	Before each sequence a system suitability test should be performed.
7.1. LLOQ, ULOQ, calibration function	Investigation of LLOQ and ULOQ using neat standard solutions, linear range
7.2. Calibration (extended)	Evaluation of the most suitable calibration procedure: • External calibration with neat standard solutions • Matrix-matched calibration • Standard addition • Use of internal standards
7.3. Matrix effects	Evaluation of absolute and relative matrix effects: • Comparison of peak areas of analytes in spiked to different matrices • Comparison of slopes generated in different matrices by standard addition (note, overlay of concentration ranges of calibrants in different matrices is required otherwise results may not be reliable) • Postcolumn infusion experiments
7.4. Intra-assay accuracy and precision	Multiple (n = 3 or more) consecutive measurements of quality control samples (standards spiked to matrix at three distinct levels, near to LLOQ, intermediate range, near to ULOQ)
7.5. Interday accuracy and precision	Measurement of quality control samples (freshly prepared) on different days, quantification using the calibration function established on day 1

3.2.1.1. Optimization of MRM transitions

Compound specific fragmentation parameters are optimized using the „Quantitative optimization" (or "Compound optimization") tool of the Analyst software. Herein, mixtures of compounds that differ in their molecular weights by a minimum of three mass units are prepared in mobile phase (or similar medium) and infused into the MS with a syringe pump. In the following the two most intensive transitions are tested using an appropriate LC-MS/MS method.

3.2.1.2. Evaluation of critical pairs

Critical pairs are analytes that need to be separated chromatographically as their signals give interferences in analyte detection. Especially isobars (like Leu and Ile, Figure A), compounds that differ in their molecular weights by only one mass unit or compounds with similar substructures (like nicotinic acid and pyridoxine, Figure B,C) are prone to cause interferences, which would lead to false quantitative results, if not separated.

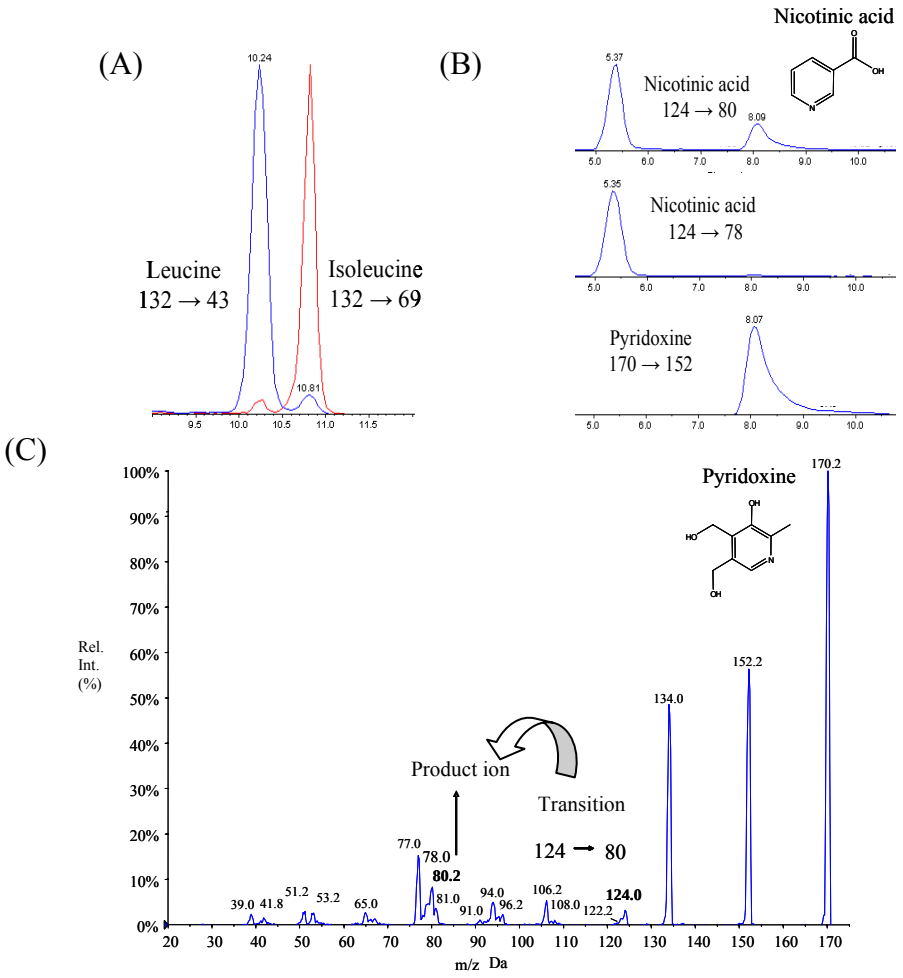

Figure 19: Critical pairs
(A) Separation of Leu and Ile
(B) Separation of nicotinic acid and pyridoxine
(C) Fragmentation spectrum of pyridoxine
Nicotinic acid exhibits a transition 124 → 80. Both masses, precursor and product ion, can be found in the fragmentation spectrum of pyridoxine.

In Figure 19A peaks of Leu and Ile are shown. Two different transitions were chosen which show distinct intensities for the two compounds. Nevertheless, these transitions were not absolutely specific, leaving chromatographic separation of Leu and Ile a prerequisite for reliable quantification. Figure 19B illustrates the interference of pyridoxine in the signal of one of two MRM traces of nicotinic acid. In the MRM transition 124 → 78 no peak of pyridoxine can be found which thus can be considered to be specific. A possible explanation for the interference of pyridoxine on the nicotinic acid trace constitutes in-source decay. In this context, decomposition of pyridoxine would take place in the ion source to yield a fragment with a m/z of 124.
If a critical pair can not be separated, specific transitions must be found that allow unbiased quantification.
To evaluate possible signal interferences, compounds have to be analyzed individually with appropriate LC-MS/MS methods. Then the MRM traces of the remaining analytes are checked for peaks at the retention time of the tested compound.

3.2.1.3. Screening of chromatographic conditions

In multicomponent methods, especially in the case of metabolic studies, one has to cope with a multitude of analytes with strongly differing polarities and structural attributes. With regards to an improvement of method specificity and robustness it is important to find chromatographic conditions under which 1) compounds are adequately retained so that they are shifted away from the early eluting zone which often suffers from matrix effects; 2) all compounds are eluted; 3) critical pairs are resolved; 4) compounds are spread over a wide elution time window.

- Choice of stationary phases

The chromatographic screening should comprise different stationary phases that follow different retention mechanisms (RP, HILIC and ion exchange) in order to reveal which type of stationary phase is most suitable for which type of analytes.

- Linear velocity

In order to compensate for different column formats (diameter, porosity) equal linear flow velocities (v) are adjusted. For this purpose, void times (t_0, time that is needed by the solvent to pass through the column) are determined at distinct flow rates. The linear flow velocity (cm/min), calculated as the quotient of the column length and t_0, is plotted versus flow rate (ml/min) as shown in Figure 20. The resulting linear equation can be used to calculate the corresponding flow rate.

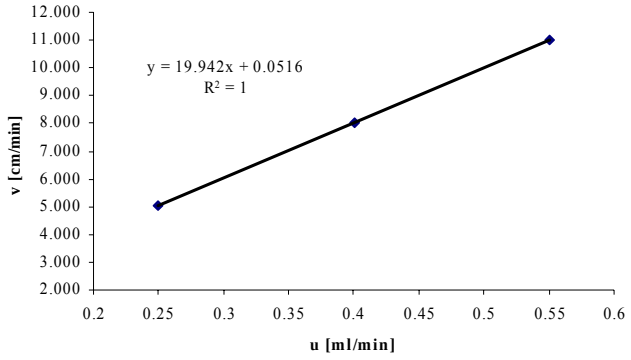

Figure 20: Plot of linear velocity versus flow rate on Luna Amino (150 x 3 mm; 5 µm) using acetone as void time marker.

- Choice of mobile phase conditions

The set of different mobile phases should include conditions with varying buffer pH, ion strength and solvent content. For the screening a gradient with a simple time program (e.g. 100% (A) => 20% (A) in 30 min) should be adjusted.

- Evaluation of screening results
  - Retentivity of compounds
  - Eluability of compounds
  - Separation of critical pairs
  - Compounds distributed over a wide elution time window
  - Peak shape, peak asymmetry

3.2.1.4. Method fine tuning

Further method fine tuning is done for the two most promising stationary/mobile phase systems. Method fine tuning includes optimization of the flow rate, mobile phase (buffer pH, ion strength, solvent) and gradient time program.

3.2.1.5. Preliminary method validation

Preliminary method validation is necessary to uncover possible problems like interferences which were not uncovered yet or insufficient separation at an early stage and to provide data that serve as basis for the design of the final validation procedure.
Calibration is done for a reasonable concentration range with pure standard solutions using the finally optimized LC-MS/MS method. Accuracy and precision are assessed for three concentrations ranging in the low, medium and high region of the calibration function using a minimum of three determinations per concentration. Acceptance criteria for validation of bioanalytical methods can be found in the guidelines of the FDA [99]. If preliminary validation results are not acceptable, further method optimization is necessary.

3.2.1.6. Stability

To evaluate compound stability a quality control sample prepared on day one is stored under experimental conditions (e.g. in the autosampler at 5°C) and is measured in triplicate over a period of six days using freshly prepared calibration functions.

3.2.1.7. Final method validation [11,99]

- Lower and upper limit of quantification (LLOQ and ULOQ), linear range

LLOQ is determined by dilution of neat (matrix-free) standard solutions to a concentration yielding a signal to noise ratio of 10:1. In terms of an equation the signal intensity of the LLOQ calculates to the background signal plus 10 times the noise.
ULOQ is determined with neat standard solutions. As it is rather difficult to prepare multicomponent solutions at high concentration levels, analytes can be divided into groups. The ULOQ constitutes the highest concentration that is still within the linear range.
Thus, the linear range spans concentrations from the LLOQ to the ULOQ.

A satisfactory linear range covers a range of three to four orders of magnitude.

- Calibration

There are several strategies to generate calibration functions:

➢ Calibration with neat standard solutions

External calibration with neat standard solutions is straightforward and the simplest, fastest and cheapest calibration method. A severe drawback of this calibration method is that the compounds in the standard solution experience a completely different environment as compared to the analytes in their corresponding matrix. Thus, when matrix effects play a role, accuracy of quantitative results may be strongly compromised.

➢ Calibration by standard addition

Standard addition is performed by spiking distinct amounts of standards to the sample, which thus, experience the same environment and the same matrix effects as the analyte in the sample. The resulting calibration function is intersected with the x axis to yield the intrinsic concentration of the analyte in the sample (y = 0).

To perform reliable standard addition some knowledge on the analyte concentrations is necessary in order to estimate appropriate spiking concentrations, as these should be in the range of the analyte concentration. This calibration strategy is quite elaborate and time consuming as several runs are necessary to quantify analyte concentration in only one sample. Although matrix effects are effectively compensated using standard addition, it is not a feasible strategy for multi-component analysis.

➢ Matrix-matched calibration

Matrix-matched calibration is carried out by spiking distinct amounts of standards to a blank matrix or, if not available as e.g. in metabolic approaches, to a sample solution [36,48,49,100]. The resulting calibration function must be corrected for intrinsic analyte concentrations if no blank matrix is available. In the absence of relative matrix effects, this calibration strategy is quite useful as absolute matrix effects are compensated this way.

➢ Calibration with peak area normalization (use of internal standards)

Use of internal standards for peak area normalization is an effective strategy to compensate for matrix effects as well as for instrumental fluctuations. Optimal results are obtained using

isotope labeled internal standards which behave equally to their non-labeled analogues. However, isotope labeled internal standards are expensive and not always available. In multi-component analysis it is almost impossible to provide isotope labeled internal standards for every analyte. For example, in the study presented in Appendix III secondary metabolites of penicillin and cephalosporin biosynthesis were investigated for which no isotope labeled internal standards were available.

In metabolomics studies fully ^{13}C labeled cell extracts are often used to provide a multitude of internal standards.

To select an appropriate calibration method, accuracy is a proper parameter to assess suitability of the calibration method for reliable quantification. Inaccuracies, when determined with a quality control sample (spiked sample matrix), may also indicate the presence of absolute and relative matrix effects, which is a critical point concerning the choice of an appropriate calibration strategy. However, necessary operational efforts in calibration also depend on the required acceptance criteria concerning accuracy and precision.

In the LC-MS/MS method presented in Appendix, 87% of all accuracy values (determined in spiked extract 12 at three concentration levels) were within the acceptance limit of ± 20%, when external calibration with neat standard solutions was employed. Using matrix-matched calibration in extract 12 even 98% of all accuracy values were within the acceptance limit of ± 20%. Thus, for the purpose of process control calibration with neat standards would provide acceptable results.

However, evaluation of intraday accuracy on different days using the calibration function set up on day 1 and interday accuracy reveals which compounds are prone to instrumental fluctuations. In the study of Appendix III especially compounds measured in the negative mode (e.g. organic acid) strongly suffered from instrumental fluctuations, decreasing sensitivity by a factor of two within three days. For malonic acid and succinic acid ^{13}C labeled internal standards were available that successfully compensated for these instrumental variations. Thus, for the residual organic acids isotope labeled internal standards should also be employed.

- Matrix effects

Matrix effects constitute an inherent problem in quantitative mass spectrometric analysis [40]. Matrix effects are caused by coeluting not detected compounds of the matrix and may lead to signal suppression or enhancement and erroneous quantitative analysis results. Especially biologic samples like cell extracts, blood, tissue or urine exhibit very complex matrices with

high batch-to-batch variability. Thus, concerning biologic samples not only absolute matrix effects have to be considered but also relative matrix effects i.e. lot-to-lot variability of matrix effects. There are several strategies to evaluate matrix effects:

- Variability of peak area in different matrices

To assess matrix effects peak areas of equal amounts of a compound spiked to different blank matrices are compared to those of a neat standard solution [101-103]. If no blank matrix is available, as it is usually the case in metabolic studies, the peak area of the spiked sample must be corrected by subtraction of the peak area of the unspiked matrix.

$$\%\text{absolute matrix effect} = 100 * \frac{\text{peak area in matrix}}{\text{peak area in neat solution}} \qquad (5)$$

- Comparison of slopes in different matrices

Coeluting compounds of the matrix most often cause suppression of the ionization of analytes. Thus, sensitivity is reduced which effects a decrease of slopes of calibration functions. Comparison of slopes obtained with neat, matrix free standard solutions with those generated by spiking matrices allows to estimate absolute matrix effects.

$$\%\text{absolute matrix effect} = 100 * \frac{\text{slope in matrix}}{\text{slope in neat solution}} \qquad (6)$$

The coefficient of variation (CV) of slopes in different matrices is indicative for a relative matrix effect [46].

In order to account for method variability values for absolute and relative matrix effects are compared with the precision of slopes obtained by repeated analysis with neat standard solutions or in one matrix.

Unfortunately, several critical points must be considered in the practical assessment of matrix effects by slope comparison.

- Intrinsic concentrations of analytes in sample matrix

As already mentioned above, blank matrices are usually not available in metabolic studies.

Thus, spiking sample matrices the intrinsic concentrations of analytes in the sample must be considered. As a consequence calibrated concentration ranges in matrix and neat solutions may only partly or not at all overlap, which may cause inaccuracies of such slope

comparisons. Furthermore, concentrations in spiked matrices may easily be shifted beyond the linear range. In Table 7 slopes of phenoxyacetic acid obtained with neat standard solutions and in different matrices are shown. In extract 1 and 5 phenoxcyacetic acid could not be detected, whereas in extract 12 and 15 relatively high amounts (9.86 and 5.36 mg/L) were found. Values for slopes generated with neat solutions and in extract 1 and 5 agree well in contrast to slopes in extract 12 and 15, where high intrinsic concentrations of phenoxyacetic acid were found. In this case slopes of calibration functions can not be compared in order to assess matrix effects.

Table 7: Slopes of phenoxyacetic acid in different matrices.

Phenoxyacetic acid	Neat solution	Extract 12[a]	Extract 15[a]	Extract 1[b]	Extract 5[b]
slopes	1,96E+07	5,57E+06	6,07E+06	2,00E+07	1,92E+07

[a] *Extract from penicillin synthesis.*

[b] *Extract from cephalosporin synthesis.*

- Instrumental fluctuations

Instrumental fluctuations due to e.g. contamination of the ESI spray may cause changes in sensitivity. In the study presented in Appendix III calibration was carried out with neat standard solutions and in 6 different matrices, which took about three days. Obtained data show that instrumental performance was not stable over the period of three days for several compounds. Interestingly, especially the negative mode was affected by a decrease of sensitivity as illustrated in Figure 21, where a steady decline of calibration slopes can be recognized. From day one to day three, sensitivity was halved for many compounds measured in the negative mode.

Peak area normalization with internal standard could compensate for these fluctuations but only for those compounds, for which isotope labeled internal standards were available (malonic acid, succinic acid). Thus, if no isotope labeled internal standards are available only calibration functions set up within a short time period should be compared. Concerning studies similar to the one presented in Appendix III (method length 39 min; 7 to 8 data points per calibration) and considering the results of this study the number of calibrations carried out one after another in the positive mode should be limited to 5. Whereas in the negative mode only three calibration functions set up in a series should be compared. To evaluate absolute matrix effects calibrations should be carried out in the following order:

Positive mode: matrix 1- matrix 2 – neat solution – matrix 3 – matrix 4

Negative mode: matrix 1 - neat solution - matrix 2; matrix 3 - neat solution - matrix 4

Nevertheless, it also should be emphasized that in the study presented in Appendix III there was also a great number of compounds for which method performance was almost constant over the time period of three days and that for these compounds comparison of slopes of 7 calibration functions generated in one series was possible.

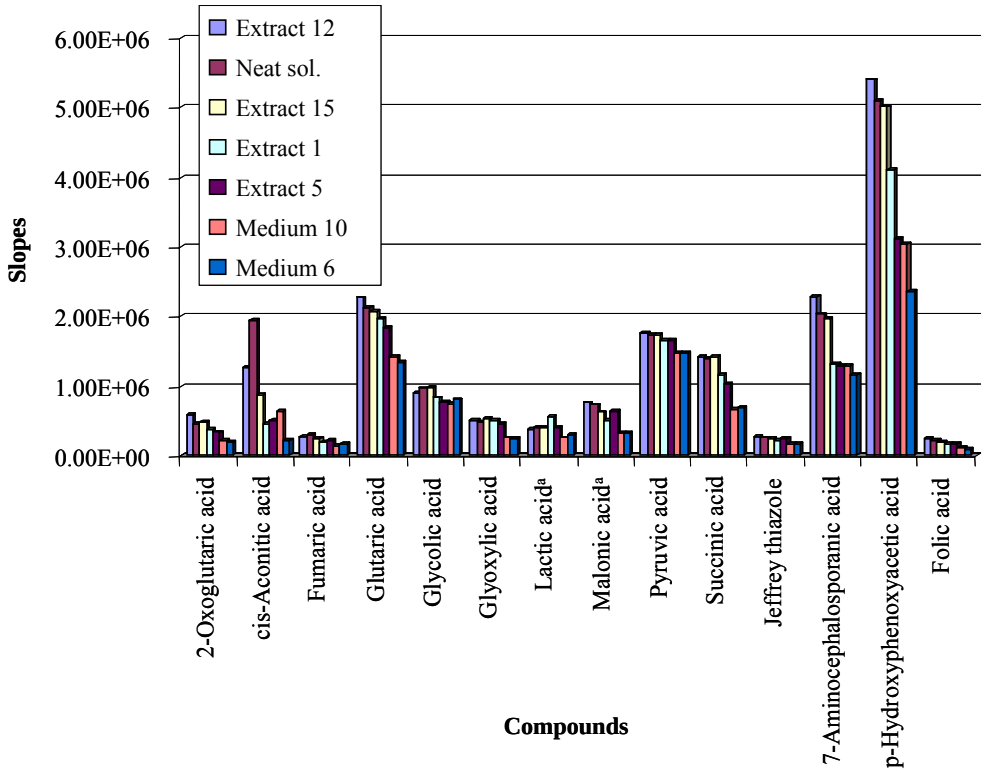

Figure 21: Slopes of calibration functions obtained with neat standard solutions and in different matrices. Calibration was performed in the order as listed above and lasted over a period of three days. All compounds were measured in the negative mode.

[a] Slopes of lactic and malonic acid were multiplied by 10 for the sake of illustration.

- Postcolumn infusion

The experimental set-up involves the postcolumn infusion of a solution containing the investigated analytes via a T-piece, while a blank matrix is injected and analyzed. The analyte signal is monitored and a decrease or an increase of the signal would uncover positions in the chromatogram where matrix effects occur. Unfortunately, this strategy to assess matrix effects is not feasible for metabolic studies in cell extracts because of the lack of blank matrices [44,45].

- Accuracy and precision

Accuracy and precision are evaluated at three concentration levels: in the low, middle and high region of the calibration range. For this purpose quality control samples (spiked sample matrix) are prepared and repeated (n = 3 - 5) measurements are performed.
Accuracy is calculated as the percentage of the averaged (n = 3 - 5) found concentration relative to the theoretical concentration. Precision corresponds to the % relative standard deviation of the determined concentrations found in the quality control sample.

- Interday accuracy and precision

To determine interday accuracy and precision quality control samples prepared on day one and stored under appropriate conditions are measured in triplicate on three to four consecutive days. For quantification the calibration function set up on day one is used.

Detailed descriptions of method development and validation along with results for metabolic profiling of extracellular primary and secondary metabolites in fermentation broths from β-lactam antibiotics production (penicillins and cephalosporins) are presented in Appendix III and IV. No further discussion is therefore given here.

To sum up, it is to say that the two LC-MS/MS methods (presented in Appendix III and IV) complement one another very well and allow quantitative analysis of a broad spectrum of analytes. All together 69 metabolites were successfully quantified in spiked extracts of fermentation broths using one or the other LC-MS/MS method. However, concerning quantification of fatty acids employing the RPLC-MS/MS method, several validation results were not optimal for some fatty acids (in particular stearic acid and palmitic acid). For these compounds use of conventional GC-MS procedures may be more advantageous.

In various fields of modern sciences, the number of chemical parameters that need to be analyzed simultaneously is steadily growing and analytical techniques are continuously adjusted to cope with this need. Such multicomponent analysis relies strongly on highly selective analysis techniques. Advances in chromatographic separation sciences (e.g. 2-D LC) have led to high selectivity and high peak capacity of separation methods. Nevertheless, it is the combination of robust liquid chromatography with mass spectrometry that opens up tremendous possibilities in multicomponent analysis. In LC-MS(MS) analysis the detector

provides an additional dimension of selectivity, mitigating the need for comprehensive chromatographic separation. Progress of MS technology leads to further improvement of mass accuracy and sensitivity as well as on speeds that can cope with new chromatographic strategies like UPLC allowing to reliably analyze and differentiate increasing numbers of compounds. Thus, nowadays LC-MS(MS) is amongst the most powerful methodologies for multicomponent analysis of more or less polar, non volatile compounds and is widely used in different fields of science and becoming more and more state of the art.

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Appendix I

Manuscript

Comprehensive impurity profiling of multicomponent nutritional infusion solutions for amino acid supplementation by a multidimensional analysis assay using off-line RPLCxHILIC – ion trap MS and charged aerosol detection with universal calibration

Comprehensive impurity profiling of multicomponent nutritional infusion solutions for amino acid supplementation by a multidimensional analysis assay using off-line RPLCxHILIC – ion trap MS and charged aerosol detection with universal calibration

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Abstract

A new analysis strategy was employed for the establishment of a comprehensive qualitative and quantitative impurity profile of a stressed multicompound pharmaceutical drug formulation, namely a nutritional infusion solution composed of amino acids and dipeptides.
To deal with the highly complex samples a multidimensional analysis approach was developed which made use of an off-line two-dimensional separation, reversed-phase liquid chromatography (RPLC) x hydrophilic interaction liquid chromatography (HILIC) and the combination of complementary detection involving ion trap mass spectrometry (IT-MS) and a charged aerosol detector (CAD).
The CAD is a mass-sensitive universal detector for non-volatile compounds with relatively consistent detector response. A universal calibration function was set up with a set of standards. This universal calibration function was then employed to quantify unknown impurities allowing their classification into those that need to be reported (> 0.05% relative to the precursor compound), identified (> 0.1%), and quantified (> 0.15%). The dilemma of unavailability of authentic standards at this stage of research for quantification could thereby be circumvented. Relevant impurities above the reporting threshold were identified by IT-MS. As typical impurities di-, tri- and tetrapeptides, cyclic dipeptides (diketopiperazines), pyroglutamic acid derivatives and condensation products were found. Cross-validation with HPLC-MS/MS methods using synthesized authentic standards largely confirmed the results obtained by the presented multidimensional analysis assay.

Keywords: impurity profiling, amino acids, peptides, off-line two dimensional liquid chromatography, charged aerosol detector, unified calibration function

1. Introduction

Comprehensive impurity profiling is an integral step in the development of new drug products, since impurities play a major role in the assessment of the quality and innocuousness of pharmaceutical products. Detailed knowledge on all impurities that might emerge during production and storage provides the basis for a comprehensive risk assessment as required by drug regulation authorities [1].

Besides known process impurities from raw material production, forced degradation of the drug substances and drug product in the course of stability testing is an adequate means to generate impurities that are likely to be formed during the production process and storage. Stability testing is thus, a generally used strategy for providing necessary information on the stability of drugs and shelf lives of pharmaceutical products [2,3].

For the purpose of stability testing the active agents or complete pharmaceutical formulations are kept under stress conditions (e.g. elevated temperature, humidity, pH, UV-irradiation). Hence, the formation of degradation products is enhanced and concentrations are elevated facilitating the establishment of impurity profiles. The goal is to reveal all relevant degradation products and to quantify the extent to which they are formed using a so called stability indicating analysis method [4,5].

The International Conference on Harmonization of Clinical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has established thresholds for impurities which originated due to degradation processes. Considering these thresholds, that are based on the relative content to the parent compound, classification of impurities into such that need to be reported, identified and qualified becomes possible with a reliable quantitative analysis assay [6,7].

For single constituent drug products the impurity profiling process may be relatively straight forward. However, stressed multicomponent formulations or drug products, often constitute very complex mixtures containing many unknown minor impurities besides major compounds that are present at concentration levels of two to three orders of magnitude higher. Because of the ability to separate compounds according to their m/z, implementation of MS may be helpful as it can be considered as an additional separation dimension increasing the selectivity and the capacity of the whole analysis method [8-11]. Nevertheless, two major difficulties arise. First, when the selectivity and peak capacity of a one-dimensional (1-D) LC assay is insufficient to resolve all sample compounds low abundance impurities may easily be masked by high quantity ingredients and thus remain undetected, even if highly specific mass

spectrometric detection is employed. Second, considering the separation of isomeric or more generally isobaric compounds, MS selectivity is also insufficient and appropriate detection depends on prior chromatographic separation. For this reason, highly selective separation methods exhibiting extended peak capacities are required and multidimensional separation systems may become methods of first choice in this regard. Moreover, universal detectors would be needed in order to minimize the risk that relevant impurities are not detected. Since such a detector is currently not available, combinations of different detection principles, like UV detection, mass spectrometry, evaporative light scattering detection (ELSD) and chemiluminescent nitrogen detection (CLND) are frequently utilized [12].

Another major difficulty is related to the accurate quantification of the impurities being a prerequisite for their correct classification. Usually, the structure of many impurities is not known at the early stage of impurity testing. Therefore, authentic standards for accurate calibration and unequivocal quantification are not available. For this reason quantitative information on detected degradation products is derived relative to their parent compound assuming an identical or similar response factor, although this bears a high risk for strong over- or underestimation of impurities' contents [13].

While the above outlined problems can be more or less swiftly solved in case of single active component drug products, they constitute a serious dilemma for a multicomponent pharmaceutical formulation with a multitude of reactive ingredients like in the presently examined infusion solutions. To overcome the described obstacles we propose herein a methodology for the comprehensive determination of impurities employing a multidimensional analysis approach that combines complementary separation and detection methods.

Through the use of a multidimensional LC separation the above selectivity and peak capacity problem for the complex sample should be relieved as theory predicts that the peak capacity is the product of the respective peak capacities of the one-dimensional (1-D) methods [14,15]. Unfortunately, this is only valid for the combination of strictly orthogonal separation mechanisms and under the circumstance that no backmixing or loss of resolution occurs during fraction transfer from the first separation dimension to the second one. Two methods are considered to be orthogonal when they follow different mechanistic principles and when separation is achieved independently [16-18]. Pharmaceutical applications of two-dimensional (2-D) LC methods have been reported in the literature [19-21]. Huidobro et al. [22] employed RPLCxRPLC hyphenated to an ion trap for stability and stress test studies of alprazolam tablets.

To establish a comprehensive impurity profile of a stressed nutritional infusion solution containing mainly amino acids and dipeptides we used herein a combination of RP and hydrophilic interaction liquid chromatography (HILIC), which may exhibit a significant degree of orthogonality [23] and excellent capability to retain and resolve the highly hydrophilic compounds of the stressed infusion solutions. An ion trap mass spectrometer (IT-MS) was used for peak identification according to the monitored m/z and for structure elucidation based on fragmentation spectra generated in the automated MS^2 mode.

The problem of quantification of unknown compounds with unknown detector responses was attempted to be solved by the use of charged aerosol detector (CAD) which is considered to be a universal detector for non-volatile compounds for which it provides a relatively consistent detector response independent of their structures and physicochemical attributes [24-26]. Thus, calibration with individual standards would not be necessary and quantification would be carried out by use of a unified calibration function. Comparison between the CAD and ELSD (evaporative light scattering detector), which is a well established universal detector for non-volatile compounds, revealed that the CAD may provide even better performance in terms of sensitivity, precision and dynamic range [27,28]. Several applications reporting on the successful employment of the CAD as detector can be found in the literature [29-32]. In the field of pharmaceutical chemistry, application of the CAD detector in various stability indicating methods [33,34] and for analysis of drug mixtures has been described [35]. It is demonstrated herein that reliable quantification of impurities with unknown structures can be achieved using a CAD employing a universal calibration function. The generated quantitative results allowed classification of the compounds as proposed by ICH guidelines and a sorting of impurities according to their relevance. Impurities above the critical identification threshold were identified by structure elucidation with IT-MS. Subsequently, authentic standards were organized of these compounds and the quantitative results of the RPLCxHILIC-CAD method with the unified calibration function were cross-validated by HPLC-MS/MS [36] employing reference compounds for calibration.

2. Experimental

2.1. Chemicals

The investigated drug formulation was a parenteral solution for supplementation of amino acids. It contained as active metabolites N-Acetyl-L-Cysteine, L-Alanine, L-Alanyl-L-

Glutamine, L-Arginine, Glycine, Glycyl-L-Tyrosine, L-Histidine, L-Isoleucine, L-Leucine, L-Lysine acetate , L-Methionine, L-Phenylalanine, L-Proline, L-Serine, Taurine, L-Threonine, L-Tryptophan, L-Valine. A stressed parenteral infusion solution was obtained by storage at 40°C for 12 months.

AlaGlu 98%, GlyTyr 99%, cyclo(AlaGlu) 99%, LeuTrpMetArg (LWMR) 71.6%, N,N'-Diacetylcystin ($(AcCys)_2$) 98% and cyclo(AlaGln) 99% were from Bachem (Bubendorf, Switzerland). L-Glu 98% and L-Phe 99% were purchased from Sigma-Aldrich (Vienna, Austria). L-Trp 99.5%, L-Leu 99% and L-pyroglutamic acid (pyroGlu) 99% were obtained from Fluka (Buchs, Switzerland).

Acetonitrile (ACN) was of HPLC grade and from VWR (Vienna, Austria). Ammonium hydroxide solution (NH_4OH) 25% in water, acetic acid (AcOH) 99.8% and trifluoroacetic acid (TFA) 99.5% were obtained from Fluka and formic acid (FA) 98-100% from Riedel-de Haën (Seelze, Germany). The employed water was purified with a Millipore water filtration system (Elze, Germany).

2.2. Instrumentation

Separations were performed on two 1100 LC-systems from Agilent (Waldbronn, Germany), which both were equipped with an autosampler, an UV detector, a binary pump as well as a thermostatted column compartment. One of the systems was connected to a Corona charged aerosol detector (CAD) from ESA Analytical (Villiers Le Bel, France), whereas the other one was attached to a series 1100 LC MSD ion trap from Agilent (Waldbronn, Germany).

The nitrogen flow of the CAD was adjusted to 35 psi.

The scan range of the ion trap was set from m/z 103 to 800 with a target mass of m/z 300. Furthermore the automated MS^2 mode was activated, which automatically fragmented the most abundant precursor ions in the range of m/z 103 to 600.

The parameters of the ESI sprayer were adjusted as follows: flow rate of the dry gas at 10 L/min, dry temperature at 350°C and nebulizer gas pressure at 60 psi.

2.3. Multidimensional liquid chromatography approach

The scheme in Figure 1 illustrates the general workflow of the comprehensive analysis of the stressed infusion solutions. Tentatively hydrophobic impurities were directly analyzed by RPLC with CAD and IT-MS and the entire polar fraction from 0-8 minutes was collected into

one fraction which was subjected to off-line 2-D RPLC x HILIC separation, whereby the second dimension separations were performed once by coupling to a CAD and once by hyphenation to IT-MS.

RPLC separation of the hydrophobic compounds (step 1)

In the first step 100 µl of the stressed infusion solution were injected into a Gemini C18 column (150 x 3.0 mm; 3 µm) equipped with a guard column (4.0 x 3.0 mm) from Phenomenex (Aschaffenburg, Germany). Channel (A) contained as mobile phase 0.1 % FA in water and channel (B) 0.1 % FA in ACN. The employed gradient elution time program is specified in Table 1 (a). The column effluent was collected into a single fraction (polar fraction) between 0 and 8 minutes. Subsequently, the mobile phase of the collected fraction was evaporated to dryness under a stream of nitrogen and the residue was reconstituted in 100 µl of water containing 0.1% TFA (start conditions of the following 2D-LC).

RPLC prefractionation of the polar fraction (step 2)

A tandem column consisting of Gemini C18 (150 x 3.0 mm; 3 µm) equipped with a guard column (4.0 x 3.0 mm) coupled in series with a polar embedded Synergi Fusion-RP (150 x 3.0 mm; 3 µm) column, both from Phenomenex was employed as stationary phase in the second step. 25 µl of the polar fraction obtained from step 1 separation were injected. Mobile phase conditions were as follows. Channel (A) contained 0.1% TFA in water and in channel (B) 0.1% TFA in ACN. The employed gradient program is summarized in Table 1b. 30 fractions were collected into Eppendorf vials with sampling intervals as indicated in Figure 4. The same run was repeated. The corresponding fractions from the two injections were combined and evaporated to dryness.

2nd dimension HILIC separation (step 3)

The fractions collected from the second separation step were reconstituted in 100 µl of a solution composed of 50% (v/v) (A) and 50% (v/v) (B). Thereby the mobile phase (A) consisted of 1.5% (v/v) buffer in water and (B) of 1.0% (v/v) water and 1.5% (v/v) buffer in ACN. The mobile phase buffer contained 200 mM AcOH adjusted with ammonium hydroxide solution to a pH of 5.5. A plain silica monolith Chromolith Performance Si (100 x

4.6 mm) from Merck (Darmstadt, Germany) was employed as column. It was run using two different gradients, which are specified in detail in Table 1 (c) and 1 (d).
Each fraction collected from the RPLC separation with the tandem column of the second step was analyzed by three chromatographic runs. A low and high volume injection of 2 and 20 µl, respectively, were carried out on the chromatographic system connected to the CAD. Furthermore, a third run was conducted with an injection volume of 10 µl on the system connected to the IT-MS.
Only early fractions (1-10) of step 2 that were expected to contain highly hydrophilic basic Arg and Lys or other unknown basic compounds, were analyzed using the gradient with higher elution strength specified in Table 1 (d) as well.

2.4. Calibration

Calibration of the RP method, which was used for the separation in step one, was performed for Trp, Phe, Leu, GlyTyr, LeuTrpMetArg, cyclo(AlaGlu), Glu and $(AcCys)_2$ using the following concentrations 1, 5, 10, 50, 100, 500, 1000 µg/ml in 50% mobile phase (A) and 50% mobile phase (B) of the RP method employed in step 1.
For the calibration of the HILIC method used as second separation dimension for the polar fraction collected in step 1, calibration functions were constructed for cyclo(AlaGln), GlyTyr, AlaGlu, Glu, Gln, Trp, Leu and pyroGlu using mixed calibration standards at concentrations of 1, 5, 10, 50, 100, 500, 1000 µg/ml in 50% H_2O and 50% mobile phase (B) of the HILIC method employed in step 3.
Precision and accuracy were determined for both methods. For the RP method (step 1) six consecutive runs with quality control standards (QC) at a concentration level of 10 µg/ml were performed and for the HILIC method (step 3) five runs with QC standards at a concentration of 50 µg/ml. The LOQ of the RP and the HILIC method was determined as the concentration which yielded a signal to noise ratio of 5:1.

3. Results and discussion

3.1. Multidimensional analysis assay

The stressed pharmaceutical formulation under investigation was composed of small polar compounds, namely amino acids, two dipeptides and a few other constituents. This infusion

solution was stored at 40°C for 12 months to enforce impurity formation. Inital experiments using RPLC indicated the formation of minor peaks. As expected, the majority of the polar ingredients eluted close to the front and it was safe to assume that several polar degradation products were hidden beneath the major compound peaks.

Attempts to increase retention by lowering the organic content and adopting flatter gradients as well as addition of fluorinated ion-pair agents such as trifluoroacetic acid (TFA) and heptafluorobutyric acid were all unsuccessful or only partially successful. Since tested HILIC methods with Polysulfoethyl A and mixed mode RPWAX [37-39], respectively, yielded also chromatograms with strongly overlapped peaks, an off-line 2-D separation using a combination of RP and HILIC was envisioned to solve this problem of insufficient retentivity of hydrophilic compounds in the first separation step and inadequate peak capacity for the complex mixture of the stressed sample.

While the late eluting compounds from the RPLC column were directly analyzed, the early eluting part of the RPLC chromatogram (< 8 min) with the unresolved peaks was reinjected into a Gemini C18 column coupled in series with a more polar RP column i.e. Synergi Fusion-RP. Thereby the hydrophilic compounds were spread over a wider retention time window facilitating separation in the HILIC separation dimension. This specific tandem column combination appears uncommon, but yielded a better separation than either one of the two single columns alone. Thus, 30 fractions were collected at variable time intervals that were reinjected into the 2^{nd} dimension column. It is well known that separation of polar analytes can be most probably accomplished on polar stationary phases in the HILIC mode. A critical factor was the selection of the type of HILIC column. Many bonded HILIC phases suffer from continuous bleeding of the chemical bonded selector which is incompatible with the IT-MS, but also with the CAD [40]. Besides loosing sensitivity due to filling of the ion trap with ions from the bleed, ions in the mass spectra stemming from the column bleed would complicate the MS spectra which might be puzzling during structure elucidation of unknown impurities. For this reason, a bare silica monolith column Chromolith Performance Si from Merck, was selected.

Blank injections showed that the background signal obtained with the silica monolith was acceptable and that a combination with the CAD and the IT-MS, respectively, was possible. Every fraction from tandem Gemini C18/Synergi Fusion-RP column was injected into the monolithic HILIC separation system using the gradient program shown in Table 1c.

Although RP and HILIC are both separating by hydrophobicity/hydrophilicity differences and are to some extent negatively correlated, the correlation is usually not perfect i.e. the data are

not lying on a -45°C regression line [41]. This is supported for the given case by complementarity plots i.e. retention times on the Chromolith Performance Si plotted versus retention times on the Gemini as well as on the tandem column (Gemini C18 and Synergi Fusion-RP) (Figure 2). Retention times on the two distinct phase systems in fact exhibited only minor correlation. Hence, sufficient complementarity in the two modes was existing to minimize peak overlap. The column and separation conditions were further chosen such that the risk of trapping compounds on the stationary phase and hence, missing relevant impurities was minimized. The RP method in the first separation step had weak retentivity so that one can be sure everything was eluted. Likewise, in HILIC separation more strongly retentive ion-exchangers were avoided for this reason.

For multidimensional LC an off-line strategy was deemed to be preferable over an on-line approach in the given application for several reasons. First, it is more straightforward to implement. The fractions from the first dimension can be concentrated by solvent evaporation before injection into the 2nd dimension which may be of importance to allow determination of minor impurities. In contrast, in the on-line mode fractions are collected from the first separation and directly injected into the second dimension. In the course of the separation, a dilution of the injected sample mass will result so that considerable volume has to be transferred into the second dimension. Hence, the risk of column overload (volume overload) and peak shape problems due to mobile phase incompatibility may be a serious problem since the effluent fractions from the RP column are representing strong eluents in HILIC. Employing the off-line approach the handling of the two complementary modes was not at all critical. Besides lack of compatibility issues and feasibility of pre-concentration before reinjection, there are fewer restrictions in terms of fractionation volume and injection volume in the 2nd dimension. Moreover, which was of prime importance in the presented application, mobile phases incompatible with the 2nd dimension can be removed and the samples reconstituted in an appropriate solvent. Further, distinct experiments can be undertaken with collected 1st dimension fractions, here IT-MS and CAD, and the non-consumed sample may be stored for later additional experiments. This was of particular interest in view of structure elucidation of impurities determined to be above the identification threshold.

Thus, the stressed parenteral solution was injected into a Gemini C18 column. While the effluent from the entire polar part of the chromatogram (0-8 min) was collected into a single fraction (Figure 3, part A), the more hydrophobic components (> 8min) were directly analyzed (Figure 3, part B). All active ingredients were eluted before 11 minutes and no impurities were eluting after 24 minutes.

The effluent from part A (Figure 3) was then pre-fractionated on the Gemini C18/Synergi Fusion-RP tandem column and 30 fractions were collected (sampling times are indicated in Figure 4). In the early eluting part a sample was taken every half minute, while this sampling period was slightly extended in the later eluting part of the chromatogram. Thereby, the number of fractions to be analyzed in the 2nd dimension was kept reasonable at expense of a slight undersampling. All 30 fractions were then injected three times into the 2nd dimension HILIC system: 2 µl and 20 µl (low and high load) injection volumes employing CAD and 10 µl for IT-MS detection. The obtained chromatograms of all 30 fractions are depicted in Figure 4.

Early eluting fractions from the tandem Gemini C18/Synergi Fusion-RP columns were expected to contain the basic amino acids Arg and Lys as well as other basic compounds. As they might be strongly retained on the silica monolith under the employed mobile phase conditions due to ionic interactions between negatively charged dissociated silanol groups of the monolith and positively charged Arg and Lys, a second run for each of these fractions was performed with a stronger eluting gradient program outlined in Table 1d. A representative chromatogram will be discussed later.

3.2. Calibration

The vast majority of analytical detectors exhibits signals which are first of all proportional to the concentration of the compound and secondly depend on the specific detector responses of individual analytes. Thus, for the purpose of accurate quantification knowledge of the structures of the analyte and calibration with individual standards is required. In opposition to that, the CAD is believed to exhibit a mass-sensitive signal, which is largely independent of the analyte structure allowing quantification of unknown compounds relative to known standards. Thus, suitability of the CAD for the intended application as universal detector was tested in the course of the validation of the RP method (step 1) and the HILIC method (step 3).

Calibration functions for eight structurally different compounds were set up using the RP method of the first separation step. Concentrations of calibrants ranged from 1 to 1000 µg/ml. As expected [33,42] the obtained calibration data better fit quadratic than linear functions as illustrated in Figure 5, in which the calibration data of three different compounds Glu, cyclo(AlaGlu) and GlyTyr are superimposed. However, in the low concentration range from 1 to 100 µg/ml the curves show a linear trend, which can be used as calibration function for

quantification (see Figure 5B). Alternatively, double logarithmic plots can be set up. This way linear calibration functions can be obtained over the full concentration range (Figure 5C).

It becomes also evident from Figure 5 that the data points for the distinct compounds are at equal concentrations nearly perfectly overlapping. This consistent detector response indicates the utility of the CAD for unified calibration for Glu, cyclo(AlaGlu) and GlyTyr - three structurally quite different compounds. Linear calibration data of the complete set of analytes (8 compounds) are presented in Table 2. Due to a relatively consistent detector response the slopes of the calibration functions for the distinct compounds show only minor deviations, as expected. A relative standard deviation of 21% was calculated for the slopes, which was considered to be within acceptable limits allowing the construction of a unified calibration function by averaging over individual slope and intercept values. However, detailed evaluation of the calibration data presented in Table 2 reveals that individual slopes vary systematically. A significant trend towards larger slopes with increasing retention times, which correlates with an increase of the relative fraction of organic phase at the time of elution, was detected (Figure 6). As already reported by other groups, the response of the CAD depends on the mobile phase composition. Organic solvents improve transport efficiency of the CAD nebulizer, and hence, lead to increasing sensitivity [25,42]. Several strategies were developed to eliminate the influence of the mobile phase composition on detector response. Gorecki et al. [42] reported that changes in the mobile phase composition during gradient elution were successfully compensated by the implementation of an exactly reverse gradient, which was combined via a T-piece with the flow of the analysis column before entering the detector. As the CAD is mass-sensitive no loss of sensitivity upon dilution of the column effluent was to be expected. This procedure made it possible to keep solvent composition constant and provided a constant detector response. A technical more simple and straight forward strategy is to use a correction function to calculate calibration slopes specific for a certain retention time. In Figure 6A individual slopes of calibration functions constructed for the RP method are plotted versus % of organic modifier at the elution time. It is seen that a linear relation ship with an acceptable correlation coefficient of $R^2 = 0.903$ can be obtained. This way it is possible to calculate corrected slopes specific for each compound in dependency of individual retention times.

A similar behavior was also noticed for the HILIC method (Table 2). The slopes deviated within the set of distinct compounds by 14% RSD, which was considered to be still acceptable for a preliminary quantification via a unified calibration function obtained by the average of the slopes and intercepts of the individual standards. As for the RP method, there

was again a trend for larger slopes with higher percentage of ACN, which allowed for correction of slopes in dependence of elution times.

3.3. Validation

In the course of the present work the RP method of step 1 (for more hydrophobic impurities) and the HILIC method (step 3) were preliminary validated according to the ICH guidelines. In the course of this preliminary validation unified calibration and slope-corrected calibration were compared with regard to the capability of the latter to correct for variations of the detector response due to gradient elution.

For the RP method, the LOQ (S:N = 5:1) of the investigated compounds was determined to be around 10 ng on column, corresponding to concentrations of 1 µg/ml (injection volume of 10 µl). Linearity was determined to range from 1 – 100 µg/ml. Precision for the RP method was determined for six consecutive runs with a quality control standard at a concentration level of 10 µg/ml (Table 3). The %RSD values ranged between 5% (Glu) and 12% (GlyTyr). Accuracy was determined for six runs over five days and was assessed by three distinct ways using calibration functions which were constructed with authentic standards (compound-specific calibration), a unified calibration function obtained as mean of the calibration functions of eight standard compounds and a slope-corrected unified calibration function exploiting the linear relation between the organic content at the elution times of compounds and their respective slopes (Figure 6). As expected, the best results for accuracy were obtained (for most compounds) using the compound-specific calibration functions. Nevertheless, accuracies determined for the use of unified calibration functions (from mean of individual calibration functions and slope-corrected calibration functions) were, except for the one or the other outlier, mostly within acceptable ranges 75-130%. The use of slope-corrected calibration functions provided slightly improved results as compared to the unified calibration functions averaged over all employed standards.

Validation was also performed for the HILIC method. As for the RP method, calibration functions for four different compounds were constructed with corresponding standards. Obtained results are summarized in Table 2. The linear range was determined to be within 5 and 100 µg/ml. The LOQ of the examined compounds using a signal-to-noise ratio of 5:1 was found to be 5 µg/ml. Thus, the RP method exhibited a higher sensitivity as compared to the HILIC method. Precision and accuracy were determined for four different compounds (Trp, Leu, Glu, Gln) performing five consecutive runs at a concentration level of 50 µg/ml (Table

3). Precision was found to be ≤ 6% for the four compounds. Accuracy was determined using three differently established calibration functions (compound-specific, unified and slope-corrected calibration functions). Similar to the results of the RP method improved accuracy was obtained using compound-specific calibration functions. However, both unified calibration function as well as slope-corrected calibration functions again provided mostly accuracies within an acceptable range 80-123%.

Thus, for the purpose of quantification of unknown compounds relative to known standards employing gradient elution, unified as well as slope-corrected calibration functions yield adequate accuracies allowing a preliminary estimation of impurity contents, the latter being preferred and therefore employed in the present study.

3.4. Evaluation of results

3.4.1. Quantitative Analysis

For the purpose of quantification two runs, employing injection volumes of 2 and 20 µl, respectively, corresponding to low and high sample load were carried out using the CAD as detector and slope-corrected calibration functions were utilized for calculation of results. Different injection volumes were employed in order to assure that the detected concentrations fall within the linear range of the CAD. The run with high sample load further ascertained that also minor impurities were detectable above the LOQ.

The chromatogram of the RP-run was already shown in Figure 3 and those of the 30 fractions from the tandem RP column analyzed by the HILIC method have been depicted in Figure 4.

The components that eluted in the RP method (Figure 3) after 8 min were directly analyzed by this method (one injection of 20µl undiluted sample). Additionally, the isolated peaks at 5.08 min (corresponding to pyroglutamyl-alanine) and at 7.2 min (corresponding to *N*-acetyl-cysteine) were included in this method as well. The results of the quantitative analysis by the RPLC-CAD method are summarized in Table 4 along with respective retention times and m/z of the respective components. Several of the impurity peaks were coeluting or partially overlapping (see Figure 3, insert). Completely overlapping peaks were quantified as sum of the coeluted impurities. If the combined concentration was below the reporting threshold, also the individual components were irrelevant as impurities. Peaks that were partially overlapping were quantified individually using integration by peak splitting in the valley. Even if such quantification might be less accurate than in the case of fully baseline separated peaks, it was

deemed to be adequate for a preliminary quantification and classification of the detected impurities. As can be seen from Table 4, a large majority of the peaks detected by the CAD were present at concentrations below the reporting threshold and no further considerations were taken into account for these components at this stage. They were regarded to be irrelevant as impurities. It is also notable that many of these minor peaks did not yield a reasonable signal in the IT-MS run pointing towards less important impurities because it may be assumed that they are not of peptidic nature. A number of relevant impurities above the reporting threshold remained (see Table 4, marked in italic) as well as above the identification threshold (Table 4, marked in bold), which needed to be identified (vide infra) or were classified as being present above the qualification threshold. Besides, the peak corresponding to Trp was collected and re-chromatographed by a complementary RPLC method (using pH 5.5 instead of 2.7) in order to check for minor impurities that might be coeluted under this main constituent. There were no relevant impurities found with this second complementary analysis method and hence the peak was assumed to be pure.

A similar procedure was pursued for the polar components of the stressed sample mixture employing the HILIC-CAD chromatograms from the 30 fractions of the tandem-RPLC run (Figure 4). The complete list of components detected in the 30 chromatograms is presented in Table 5. Many of the detected peaks were found in several fractions and the final concentration was calculated from the combined quantities in these fractions. The splitting of peaks into different fractions in the 2D-HPLC method may have been accompanied by minor losses of sample during fraction transfer. Peaks quantified with HILIC-CAD to be below the reporting threshold were not further treated. Peaks that were well detected by CAD above the reporting threshold but did not provide a reasonable signal by IT-MS, i.e. a characteristic m/z, were further investigated. For example, an aliquot of the respective fractions (1-4) was subjected to derivatization with Sanger's reagent (2,4-dinitrofluorobenzene) with consecutive analysis on a Gemini C-18 in order to elucidate whether the specific component has an amino function suitable for derivatization. This way, small amino acids like Gly (m/z 76) and Ala (m/z 90) could be unequivocally assigned to specific peaks in the HILIC chromatograms. Several relevant peaks could be identified by MS scan spectra and MS^2 fragmentation spectra, respectively, as discussed in detail below. Overall, quite a number of impurities above the reporting and identification/qualification thresholds could be detected and they are indicated in Table 5 in italic and bold, respectively. Particular attempts were undertaken to elucidate the structures of those impurity peaks that were present above the identification and qualification threshold values.

3.4.2. Identification of relevant impurities

All samples were also analyzed by the same separation methods but hyphenated to an IT-MS (injection volume 10 µl) instead of CAD for identification of the detected peaks. While typically high-resolution MS instrumentation would be advantageous for this application, IT-MS allowed for identification of most of the relevant peaks. The peaks that have been identified are specified in Table 4 and 5. Table 6 provides a list with characteristic fragment ions of the identified relevant impurities being present in the stressed sample above the identification and qualification threshold, respectively. A few of these impurities are dealt with in more detail in the following. Verification of these structures by authentic standards and HPLC-MS/MS analysis is reported in detail elsewhere [36].

First of all, peaks corresponding to (active) ingredients were readily identified by a set of informations comprising concentrations as determined by RPLC-CAD and HILIC-CAD, respectively, MS(MS) data of specific peaks in native form or after derivatisation of respective fractions with Sanger's reagent as well as standards to determine retention times of known ingredients of the stressed infusion solution for the RPLC run and the HILIC separation, respectively, in order to support peak assignment.

In Figure 7 an exemplary evaluation of the chromatogram of fraction 2 which contains several active constituents is illustrated. Trace (A) constitutes the CAD signal and trace (B) the signal of the IT-MS. Complications were encountered due to the formation of associates and adducts in the ionization source. The formation of Na and K adducts, dimer and multimer complexes during the ionization process gives rise to higher mass signals (Table 6) and compromises sensitivity as the analyte signal intensity is split over several ions. A compound being particularly prone to adduct formation turned out to be taurine (see Figure 7, spectrum of peak 1). The primary focus of the study was not the identification of the (active) constituents, hence no further details are discussed concerning this point. Instead the major task was to find and identify impurity peaks, especially those representing peptides that might show bioactivities.

A critical issue in this context is that low abundance impurities may easily be masked by main compounds. To discriminate between signals obtained from real sample compounds and background signals extracted ion chromatograms (EIC) were created and chromatograms of blank injections were compared in order to figure out system peaks and background signals. In this context a compound with a specific m/z would give a peak in the EIC as opposed to a signal stemming from the background. For example, in fraction 6 at 22.3 min a peak was found in the CAD chromatogram as shown in Figure 8A. In the corresponding spectrum, m/z

418 was found to be the dominant mass beside several others (Figure 8B). An extracted ion chromatogram (Figure 8C) was created in which clearly two peaks can be recognized. Thus, the found m/z of 418 were considered to originate from isobaric components in the sample. Examination of the fragmentation spectra provided structural information, which allowed to identify the compounds as AlaGlu(AlaGln) (Figure 8D) and AlaGluAlaGln (Figure 8E), respectively. Structure identification was confirmed with the help of standard compounds.

Similarly, at the end of the upper chromatogram in Figure 9 three low abundance impurities can be found in the CAD trace, as well as in the TIC of the IT-MS. For each peak a useful mass spectrum could be obtained at the corresponding retention times, revealing the m/z of the impurities as well as providing fragmentation spectra.

Examination of fragmentation spectra revealed that the peptidic impurities were formed by condensation reactions of AlaGlu with Arg, His and Lys. Respective y1 ions could be clearly identified in the fragmentation spectra (Figure 9). Unfortunately, obtained fragmentation pattern were not unequivocal as it was not posible to differentiate between linear peptides formed by condensation at the C-terminal carboxylic group and such peptides that were linked by the carboxylic group positioned in the side chain of Glu, e.g. AlaGluHis and AlaGlu(His). Further elucidation of these structures with synthesized standards of these isomeric forms could clarify this matter [36].

As shown in Table 4 and 5, a considerable number of impurities was found, quantified and identified including cyclo(AlaGln), cyclo(AlaGlu), AlaAlaGln and several others. Extracted ion chromatograms (EIC) and fragmentation spectra are shown in Figure 10. For several impurities, structural information achieved by the determined m/z and the fragmentation pattern was not sufficient for unequivocal identification, Thus, for AlaGluX (X = Arg, His, Lys), cyclo(AlaGlu)His and pyro(AlaGlu)His, further investigations were conducted using standard compounds and alternative analysis techniques [36].

Several fractions for which peaks were found in the CAD chromatogram but for which no peaks were obtained in the chromatogram of the IT-MS were further investigated employing derivatisation with Sanger's reagent and complementary chromatographic conditions. Unfortunately, many of these unknown peaks in the CAD chromatograms remained unidentified and, since no spectra were available with and without derivatisation, were assumed to be low molecular contaminations (< 100 Da) from 2D-HPLC fraction transfer.

There were only a few impurities above the identification/qualification threshold which could be assigned to a m/z, but which could not be identified (Table 5). Hence, structure elucidation of these compounds needs still to be performed. Several identified and unidentified impurities

were determined to be above the qualification threshold (30 µg/ml) and thus, need to be examined with regard to potential bioactivity.

In the spectrum of the peak at 13.2 min of the RP chromatogram (step 1), which exhibited an area reflecting a content above the identification threshold, two m/z, 332 and 421, were detected. The corresponding fragmentation spectra provided valuable structure information but did not allow unequivocal identification [36]. For each of the two masses two isobaric structures were found to match the fragmentation spectrum. For 421 the structures pyroGluAlaGlyTyr or cyclo(AlaGlu)GlyTyr were suggested and for 332 pyroGluAlaMet and cyclo(AlaGlu)Met, respectively (Figure 10). The compounds could be identified after standards have been supplied as described in detail elsewhere [36].

3.4.3. Cross-validation

Quantitative results of compunds in the infusion solution determined with the multidimensional analysis assay were compared with those obtained with a validated LC-UV method and with three validated LC-MS/MS methods [36] which were developed to provide accurate quantitative data of identified impurities. The results are in good agreement confirming the validity of the developed multidimensional analysis assay (see Table 7). However, it was striking that quantitative results obtained with 2-D chromatography with CAD detection were for the majority of cases lower than those obtained by the LC-UV and LC-MS/MS methods. This outcome may be explained by possible sample losses due to peak splitting and intermediate sample treatment. Thus, further optimization of the reported assay should be possible by on-line hyphenation of multidimensional LC as well as of the IT-MS and the CAD.

4. Conclusion

The reported multidimensional analysis assay was successfully employed to establish a preliminary qualitative and quantitative impurity profile of a stressed multicomponent infusion solution.

A combination of two complementary separation mechanisms, RP and HILIC in an off-line multidimensional LC approach, provided the selectivity and peak capacity necessary for the separation of the multiple compounds in the infusion solution. Detection was accomplished with an IT-MS and a CAD, two detectors that respond to different detection principles.

Spectra obtained with IT-MS allowed peak identification and to some extent structure elucidation of new impurities. The use of the CAD as universal detector for non-volatile compounds with relatively consistent detector response allowed to determine contents of unknown impurities. Two distinct strategies for calibration, namely by a unified calibration function obtained from the mean of a set of compound specific calibration functions and by slope-corrected calibration functions, which compensate for changes in the detector response due to different organic modifier content at the elution time of the compounds, were evaluated. Both strategies can be considered to provide acceptable accuracy for preliminary quantification. Accuracies were determined and ranged mostly between 75 and 130%.

Based on the results of this preliminary quantification a differentiation between relevant and non-relevant impurities was possible. Moreover, impurities that demand further investigations such as structural identification or biological safety tests could be figured out.

In a follow up study, quantitative and qualitative confirmation of several of the found impurities was furnished by virtue of synthesized authentic standards of these impurities [36]. Thus, the presented multidimensional analysis approach may be regarded a powerful strategy for the establishment of comprehensive impurity profiles of complex pharmaceutical formulations.

Acknowledgements

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Figure Legends

Figure 1: Scheme of the workflow of the multidimensional analysis assay for establishing the impurity profile of a stressed nutritional infusion solution.

Figure 2: Orthogonality plots of retention times on Chromolith Performance Si versus (A) Gemini C18 and (B) tandem Gemini C18/Synergi Fusion-RP.

Figure 3: RPLC chromatogram of the stressed formulation recorded with the CAD detector.
A Gemini C18 column was employed utilizing the chromatographic conditions specified in Table 1a.
Part (A) contains polar hydrophilic compounds, which were investigated in detail using the multidimensional analysis assay.
Part (B) of the chromatogram was directly analyzed using the RPLC method with CAD detection and unified calibration.
Peaks denoted with an asterisk are spikes.

Figure 4: Representative 2^{nd} dimension chromatograms of the 30 collected fractions from the 1^{st} dimension tandem RP column (gradient conditions as specified in Table 1c). The chromatograms were recorded with the CAD.
The injection volume was 2 µl for fractions 1, 2, 16, 18 and 20 µl for the remaining fractions.
* fraction collection time intervals

Figure 5: Entire range (A), linear calibration function (B), double logarithmic over full concentration range (C) of the three compounds Glu, cyclo(AlaGlu) and GlyTyr using the RP method of the first separation step (Gemini C18)

Figure 6: In the presented graph the slopes of calibration functions constructed with individual standards using (a) the RP method and (b) the HILIC method are plotted versus the content of organic phase (B) at the time of elution.

Figure 7: Representative chromatograms and spectra of a selected fraction from 2D-LC.
Top: Chromatograms of fraction 2 (gradient conditions see Table 1d). Trace (A) was recorded using the CAD and trace (B) using the ion trap.

Bottom: Spectra of the identified peaks are shown.
Peak annotation: 1 Taurine; 2 Thr, 3/4 Ser/Pro; 5 AlaGln; 6 His; 7/8 Lys/Arg

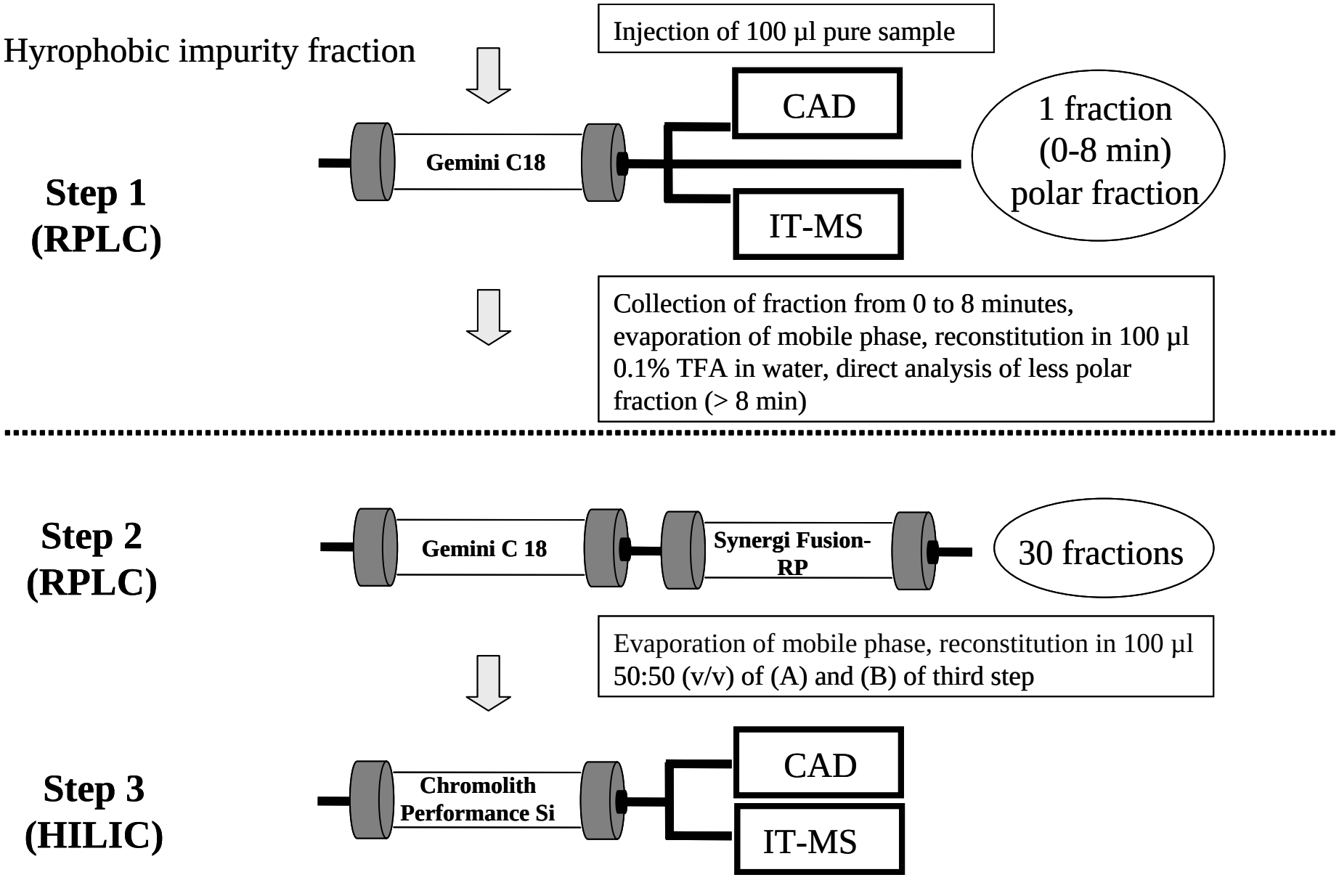
Figure 8: Evaluation of fraction 6 of tandem Gemini C18/Synergi Fusion-RP column:
(A) HILIC-CAD chromatogram of fraction 6
(B) Scan spectrum of peak 6.
(C) Extracted ion chromatogram of m/z 418. 1 AlaGlu(AlaGln); 2 AlaGluAlaGln
(D) Fragmentation spectrum of AlaGlu(AlaGln)
(E) Fragmentation spectrum of AlaGluAlaGln
Peak annotation: 1 Val, 2 unknown, 3 unknown, 4 Pro, 5 AlaGln/6 AlaGlu(AlaGln),
7 AlaGluAlaGln

Figure 9: Evaluation of fraction 4 of tandem Gemini C18/Synergi Fusion-RP column:
At the top: Chromatogram of fraction 4 (A) trace of the CAD and (B) trace of the ion trap.
Below: Spectra of peaks 7 to 11 and corresponding fragmentation spectra (below).
Peak annotation: 1 Val; 2 m/z 369; 4 no specific m/z found; 5 AlaGln; 6 AlaAlaGln; 7/8 m/z 356; 9/10 m/z 347; 10/11 m/z 375

Figure 10: On the left side EIC of identified impurities which are eventually marked by an asterisk are shown and on the right side their corresponding fragmentation spectra.

[1] AlaGlu was detected with a slightly different 2-D RPLC approach. In step 1 the infusion solution was injected on a Gemini C18. The early eluting part corresponding to the polar fraction was collected between 0 and 4 minutes and rechromatographed on tandem Gemini C18/Synergi Fusion-RP in step 2. The same chromatographic conditions were used as described in Table 1a and 1b.

Figure 1 **stressed parenteral infusion solution**



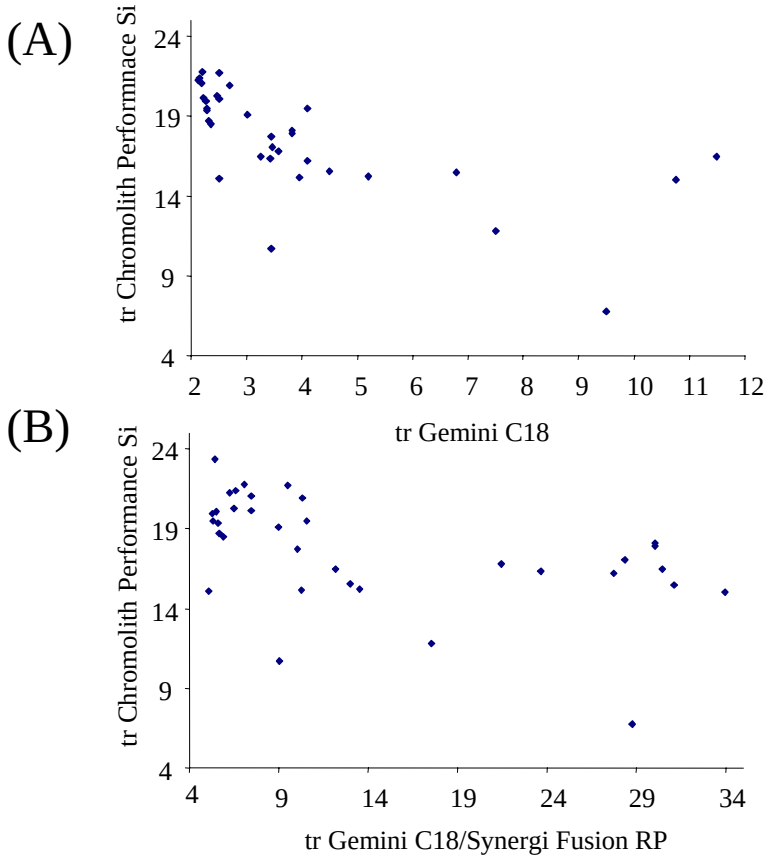

(A)
tr Chromolith Performnace Si
24
19
14
9
4
2
3
4
5
6
7
8
9
10
11
12
tr Gemini C18
(B)
tr Chromolith Performance Si
24
19
14
9
4
4
9
14
19
24
29
34
tr Gemini C18/Synergi Fusion RP

Figure 2

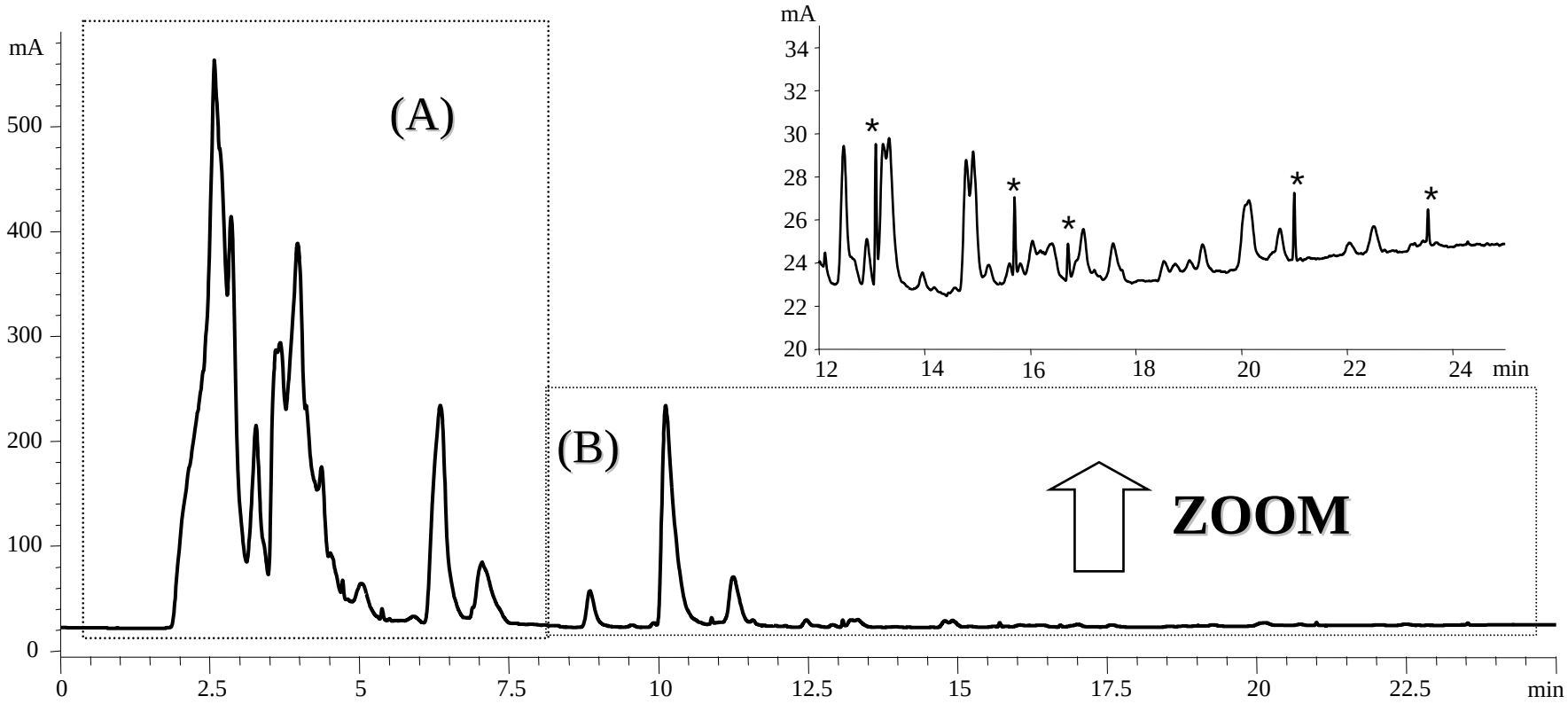
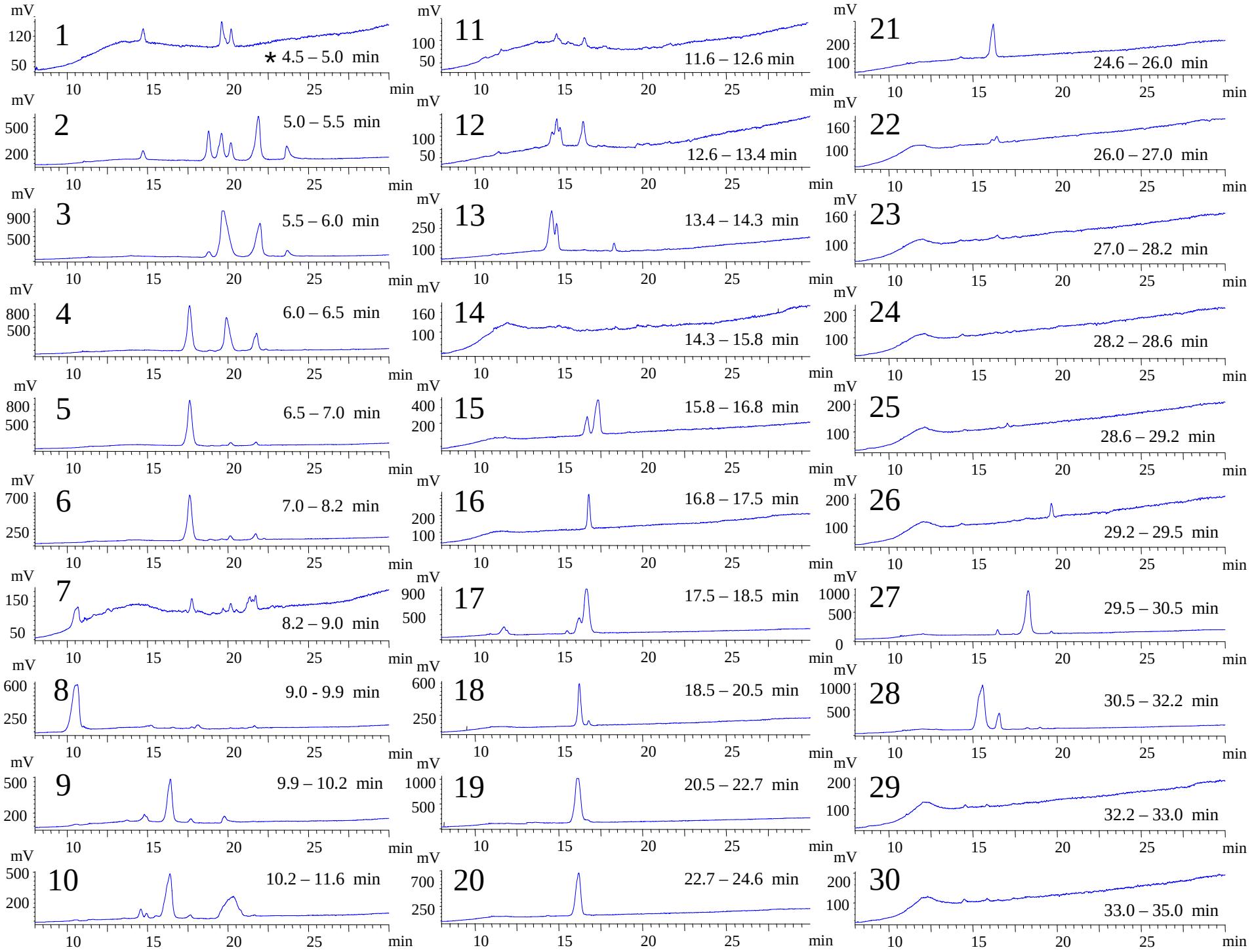

mA
500
400
300
200
100
0
(A)
(B)
ZOOM
0
2.5
5
7.5
10
12.5
15
17.5
20
22.5
min
mA
34
32
30
28
26
24
22
20
12
14
16
18
20
22
24
min
*
*
*
*

Figure 3

Figure 4


1
* 4.5 – 5.0 min
2
5.0 – 5.5 min
3
5.5 – 6.0 min
4
6.0 – 6.5 min
5
6.5 – 7.0 min
6
7.0 – 8.2 min
7
8.2 – 9.0 min
8
9.0 - 9.9 min
9
9.9 – 10.2 min
10
10.2 – 11.6 min
11
11.6 – 12.6 min
12
12.6 – 13.4 min
13
13.4 – 14.3 min
14
14.3 – 15.8 min
15
15.8 – 16.8 min
16
16.8 – 17.5 min
17
17.5 – 18.5 min
18
18.5 – 20.5 min
19
20.5 – 22.7 min
20
22.7 – 24.6 min
21
24.6 – 26.0 min
22
26.0 – 27.0 min
23
27.0 – 28.2 min
24
28.2 – 28.6 min
25
28.6 – 29.2 min
26
29.2 – 29.5 min
27
29.5 – 30.5 min
28
30.5 – 32.2 min
29
32.2 – 33.0 min
30
33.0 – 35.0 min
mV
min

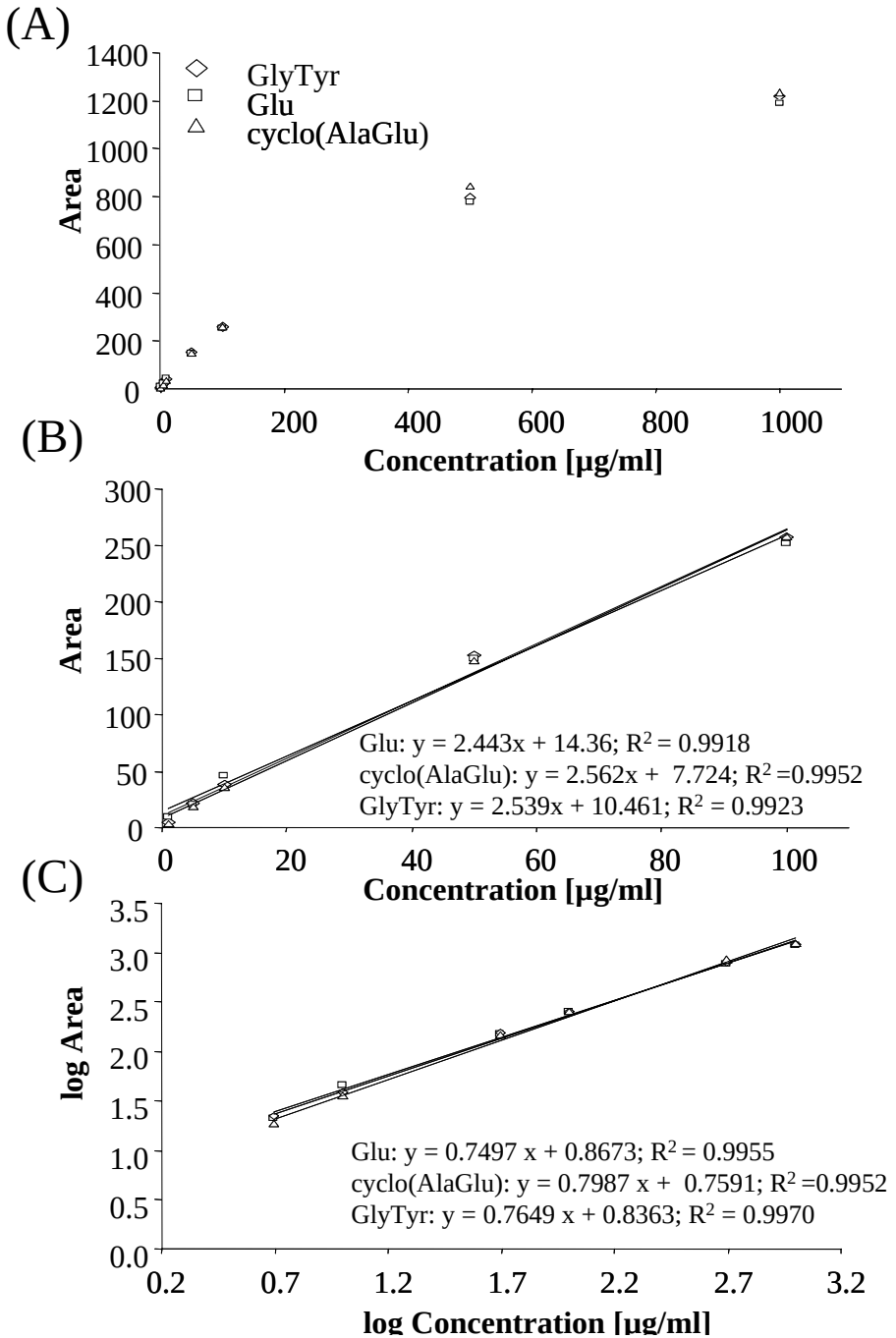
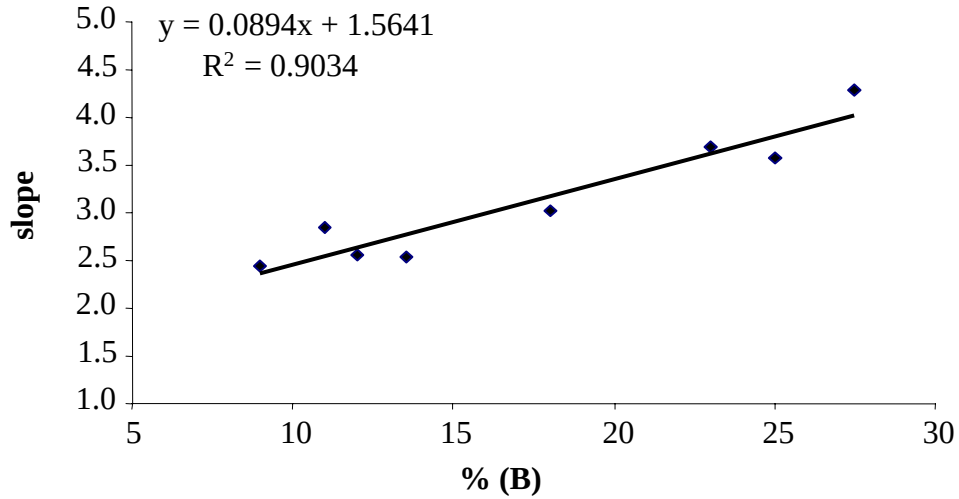

(A)
GlyTyr
Glu
cyclo(AlaGlu)
Area
Concentration [µg/ml]
(B)
Area
Glu: y = 2.443x + 14.36; R2 = 0.9918
cyclo(AlaGlu): y = 2.562x + 7.724; R2 =0.9952
GlyTyr: y = 2.539x + 10.461; R2 = 0.9923
Concentration [µg/ml]
(C)
log Area
Glu: y = 0.7497 x + 0.8673; R2 = 0.9955
cyclo(AlaGlu): y = 0.7987 x + 0.7591; R2 =0.9952
GlyTyr: y = 0.7649 x + 0.8363; R2 = 0.9970
log Concentration [µg/ml]

Figure 5

(A)


y = 0.0894x + 1.5641
R2 = 0.9034
slope
% (B)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
5
10
15
20
25
30

(B)

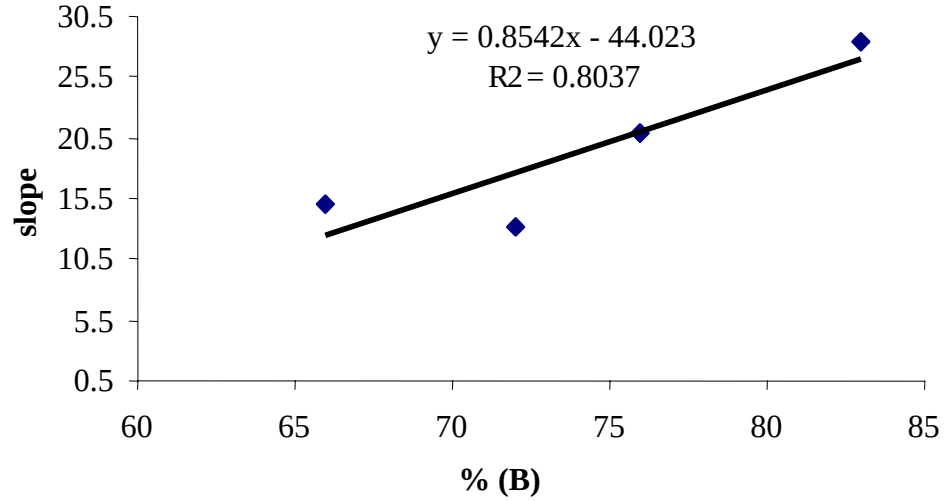
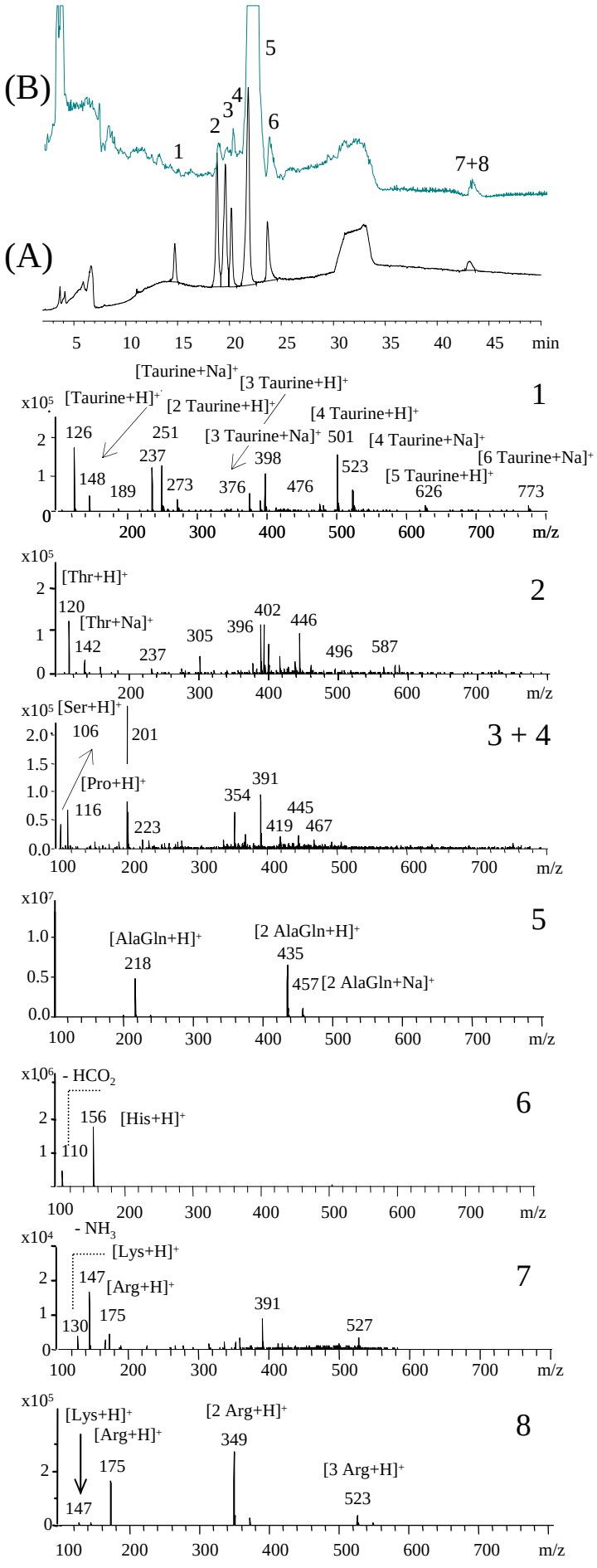

y = 0.8542x - 44.023
R2 = 0.8037
slope
% (B)
30.5
25.5
20.5
15.5
10.5
5.5
0.5
60
65
70
75
80
85

Figure 6

Figure 7


(B)
(A)
1
2
3
4
5
6
7+8
5
10
15
20
25
30
35
40
45
min
1
[Taurine+Na]+
[3 Taurine+H]+
x10^5
[Taurine+H]+
[2 Taurine+H]+
[4 Taurine+H]+
126
251
[3 Taurine+Na]+
501
[4 Taurine+Na]+
237
398
[6 Taurine+Na]+
523
148
189
273
376
476
[5 Taurine+H]+
626
773
200
300
400
500
600
700
m/z
2
x10^5
[Thr+H]+
120
[Thr+Na]+
402
396
446
142
305
237
496
587
3 + 4
x10^5
[Ser+H]+
106
201
[Pro+H]+
391
354
116
445
223
419
467
5
x10^7
[AlaGln+H]+
[2 AlaGln+H]+
218
435
457
[2 AlaGln+Na]+
6
x10^6
- HCO2
156
[His+H]+
110
7
x10^4
- NH3
[Lys+H]+
147
[Arg+H]+
391
130
175
527
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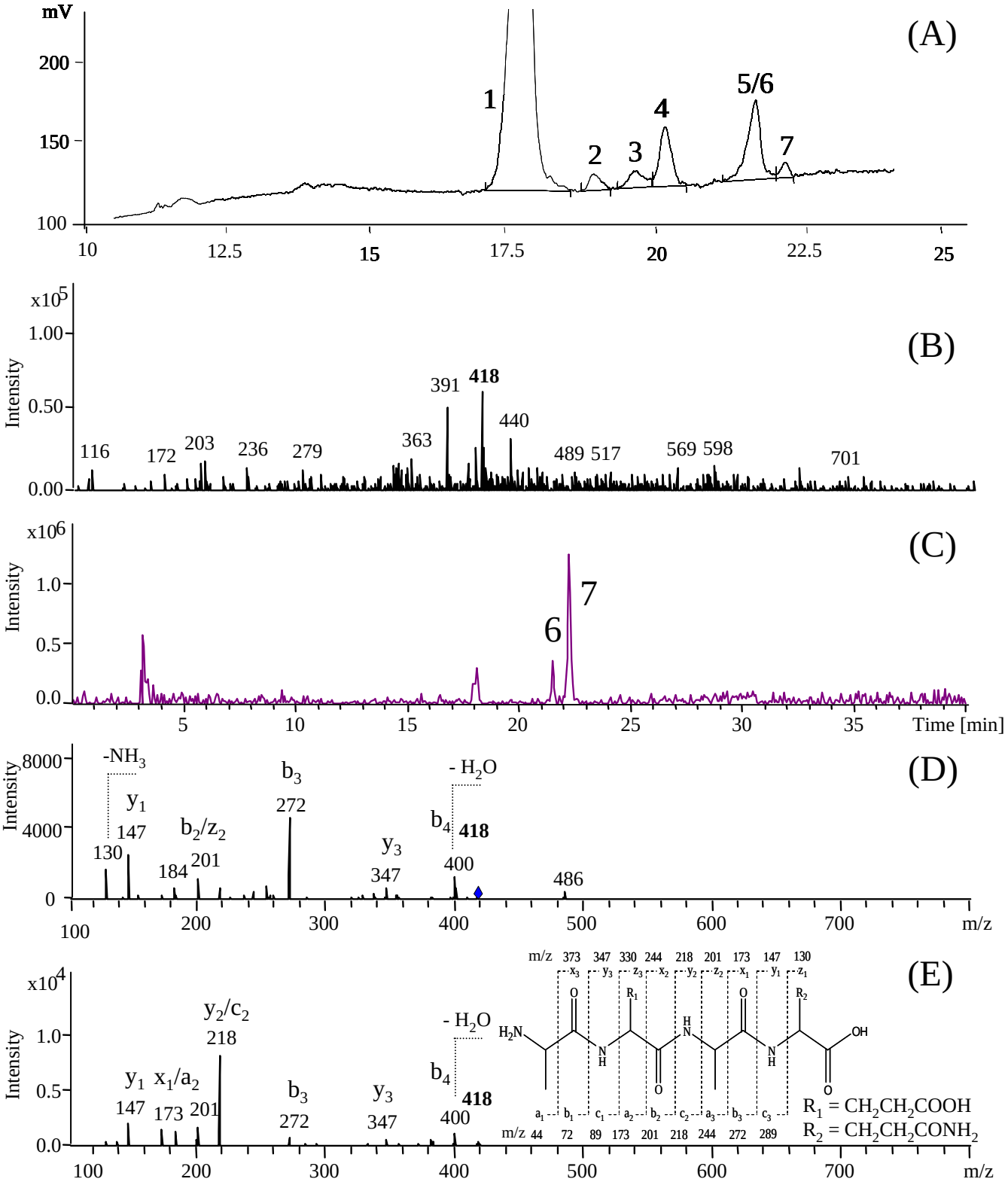
x10^5
[Lys+H]+
[2 Arg+H]+
[Arg+H]+
349
175
[3 Arg+H]+
147
523

Figure 8


(A)
mV
200
150
100
10
12.5
15
17.5
20
22.5
25
1
2
3
4
5/6
7
(B)
x10^5
1.00
0.50
0.00
Intensity
116
172
203
236
279
363
391
418
440
489 517
569 598
701
(C)
x10^6
1.0
0.5
0.0
Intensity
6
7
5
10
15
20
25
30
35
Time [min]
(D)
8000
4000
0
Intensity
-NH3
y1
147
130
b2/z2
184
201
b3
272
y3
347
- H2O
b4
418
400
486
100
200
300
400
500
600
700
m/z
(E)
x10^4
1.0
0.5
0.0
Intensity
y2/c2
218
y1
147
x1/a2
173
201
b3
272
y3
347
- H2O
b4
418
400
m/z
373 347 330 244 218 201 173 147 130
x3 y3 z3 x2 y2 z2 x1 y1 z1
a1 b1 c1 a2 b2 c2 a3 b3 c3
m/z 44 72 89 173 201 218 244 272 289
R1 = CH2CH2COOH
R2 = CH2CH2CONH2
100
200
300
400
500
600
700
m/z

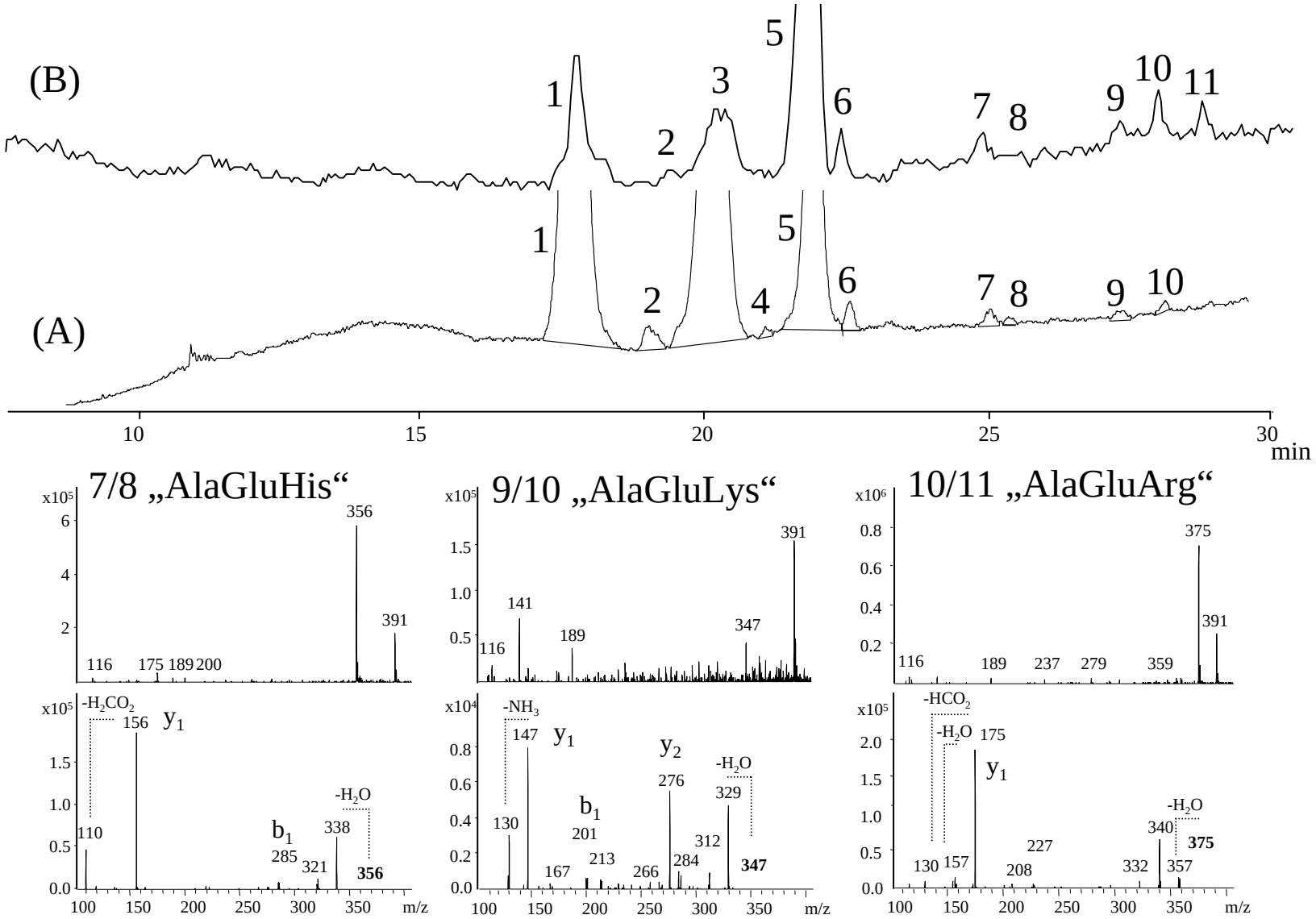
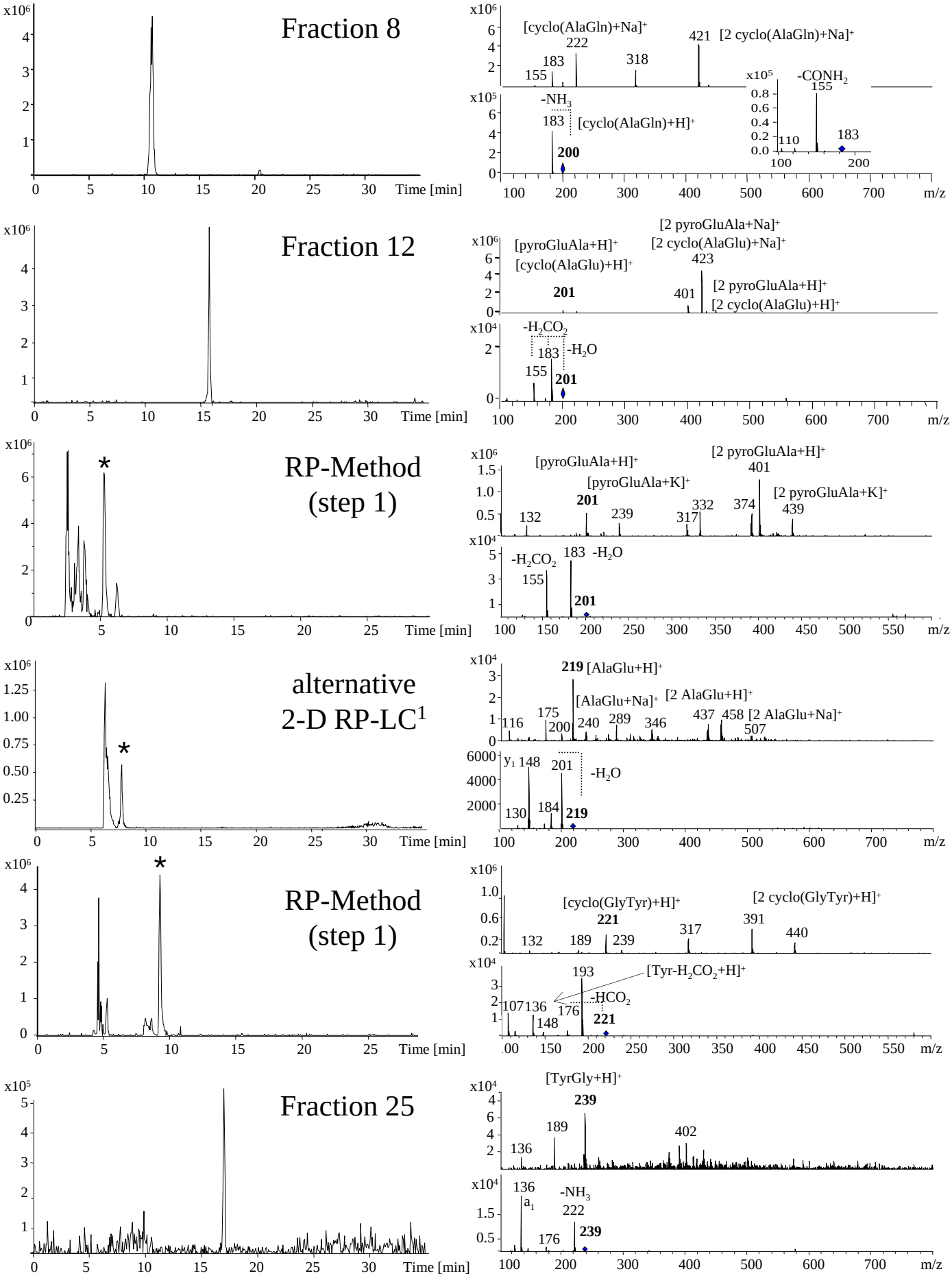
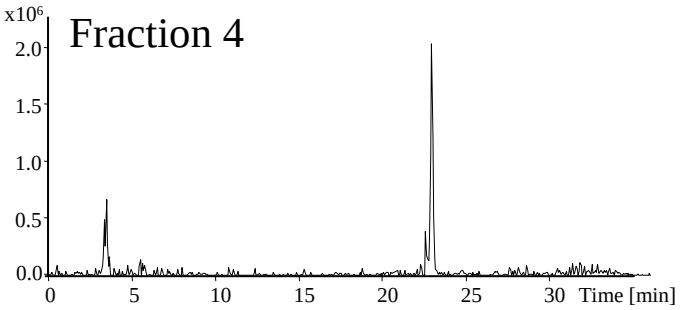
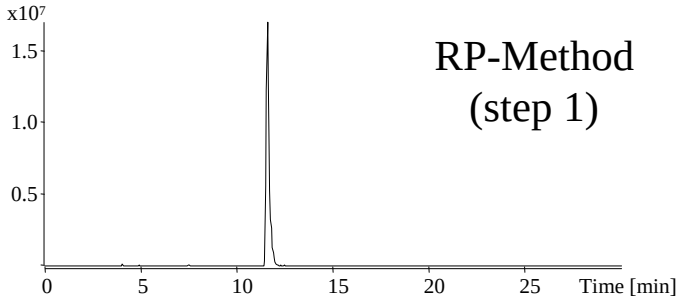

(B)
(A)
1
2
3
4
5
6
7
8
9
10
11
10
15
20
25
30
min
7/8 „AlaGluHis“
9/10 „AlaGluLys“
10/11 „AlaGluArg“
m/z
-H2CO2
-NH3
-HCO2
-H2O
y1
y2
b1

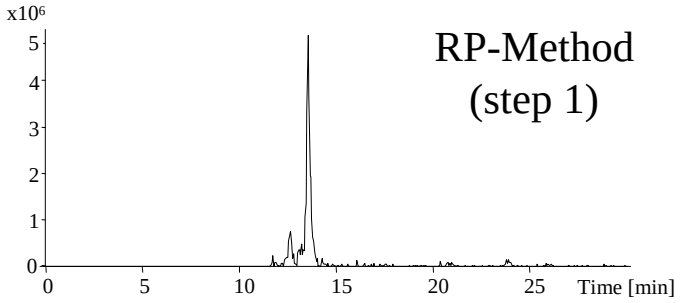
Figure 9

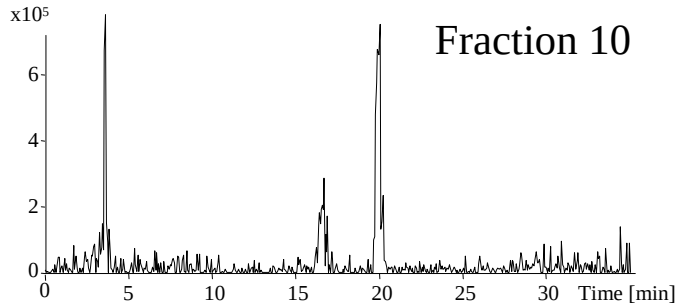
Figure 10

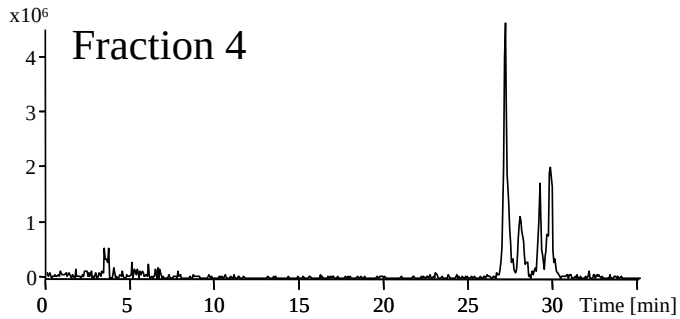

Fraction 8
[cyclo(AlaGln)+Na]+
[2 cyclo(AlaGln)+Na]+
-NH3
[cyclo(AlaGln)+H]+
-CONH2
Fraction 12
[pyroGluAla+H]+
[cyclo(AlaGlu)+H]+
[2 pyroGluAla+Na]+
[2 cyclo(AlaGlu)+Na]+
[2 pyroGluAla+H]+
[2 cyclo(AlaGlu)+H]+
-H2CO2
-H2O
RP-Method (step 1)
[pyroGluAla+K]+
[2 pyroGluAla+K]+
alternative 2-D RP-LC1
[AlaGlu+H]+
[AlaGlu+Na]+
[2 AlaGlu+H]+
[2 AlaGlu+Na]+
RP-Method (step 1)
[cyclo(GlyTyr)+H]+
[2 cyclo(GlyTyr)+H]+
[Tyr-H2CO2+H]+
-HCO2
Fraction 25
[TyrGly+H]+
-NH3
Time [min]
m/z

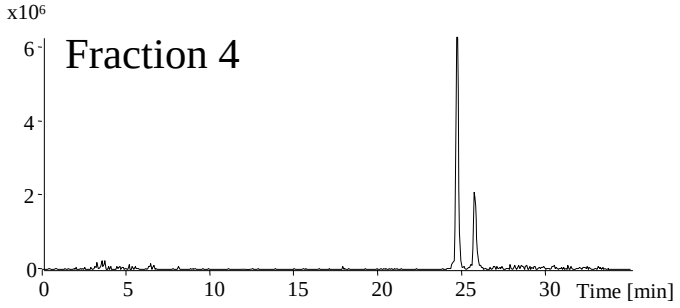

Fraction 4
x10^6
Time [min]

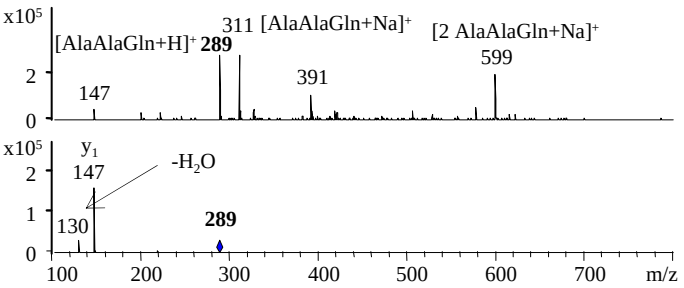

RP-Method
(step 1)
x10^7
Time [min]

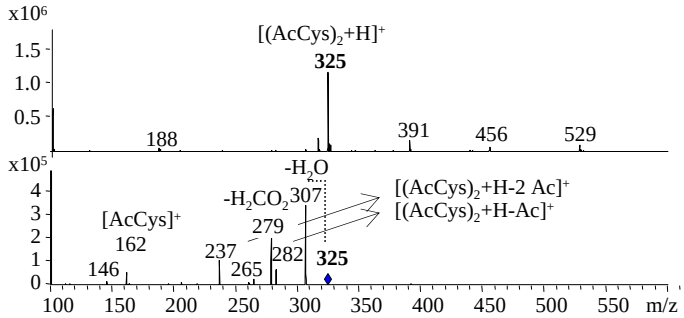

RP-Method
(step 1)
x10^6
Time [min]

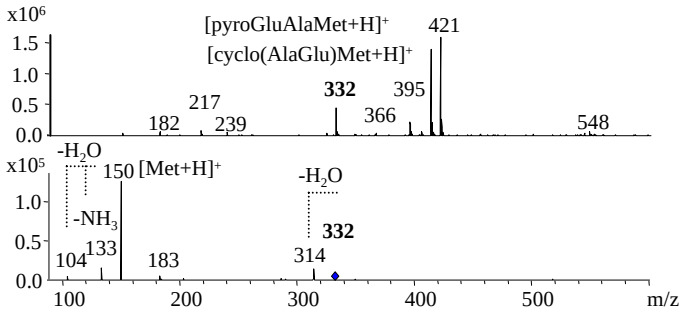

Fraction 10
x10^5
Time [min]

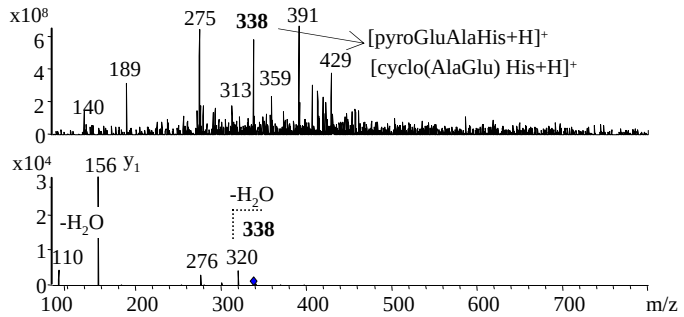

Fraction 4
x10^6
Time [min]

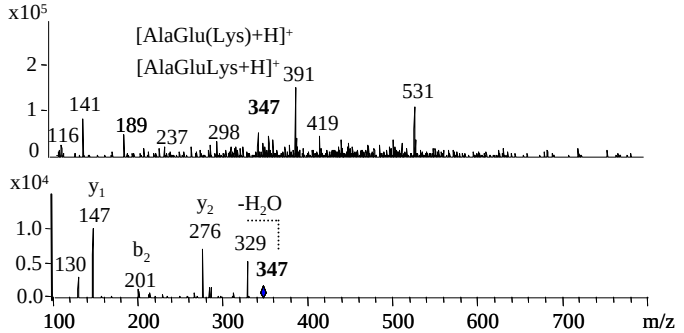

Fraction 4
x10^6
Time [min]

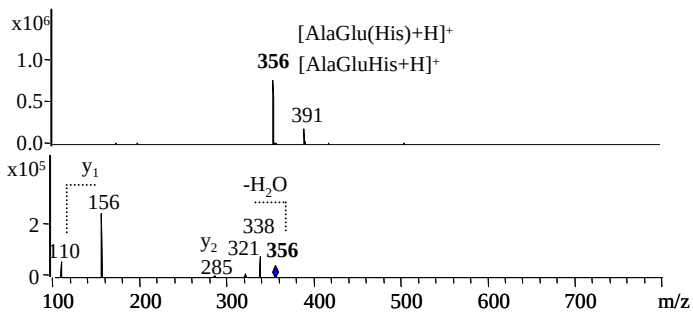

[AlaAlaGln+H]+
289
311 [AlaAlaGln+Na]+
[2 AlaAlaGln+Na]+
599
391
147
y1
-H2O
130
289
m/z

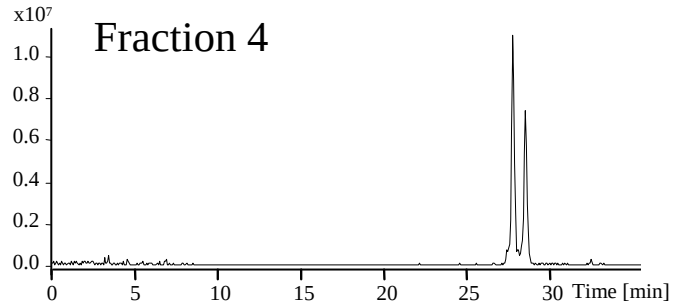

[(AcCys)2+H]+
325
188
391
456
529
-H2O
-H2CO2
307
[(AcCys)2+H-2 Ac]+
[(AcCys)2+H-Ac]+
[AcCys]+
162
279
237
282
325
146
265
m/z

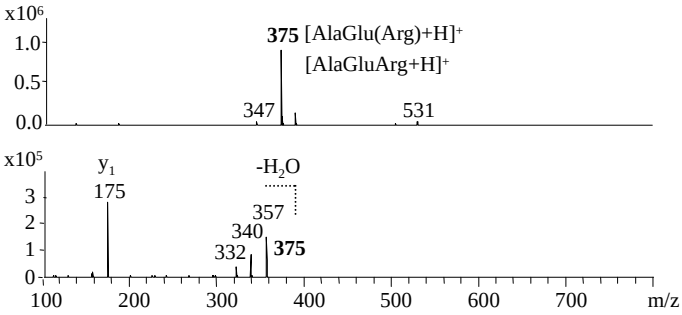

[pyroGluAlaMet+H]+
[cyclo(AlaGlu)Met+H]+
421
332
395
217
182
239
366
548
-H2O
150 [Met+H]+
-H2O
-NH3
332
104
133
183
314
m/z

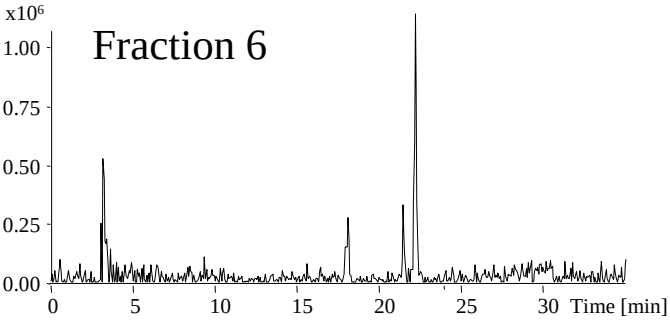

275
338
391
[pyroGluAlaHis+H]+
[cyclo(AlaGlu) His+H]+
429
189
313
359
140
156 y1
-H2O
-H2O
338
110
276
320
m/z

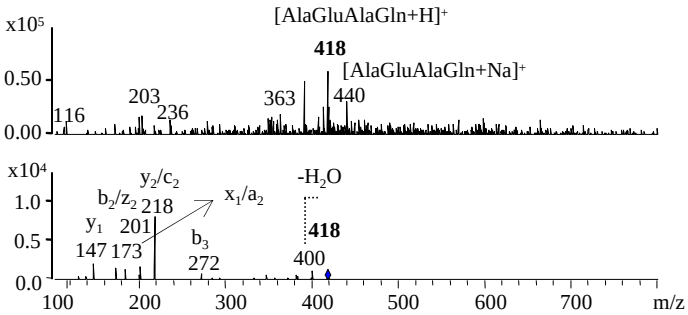

[AlaGlu(Lys)+H]+
[AlaGluLys+H]+
391
531
141
347
189
237
298
419
116
y1
147
y2
-H2O
276
329
b2
130
201
347
m/z

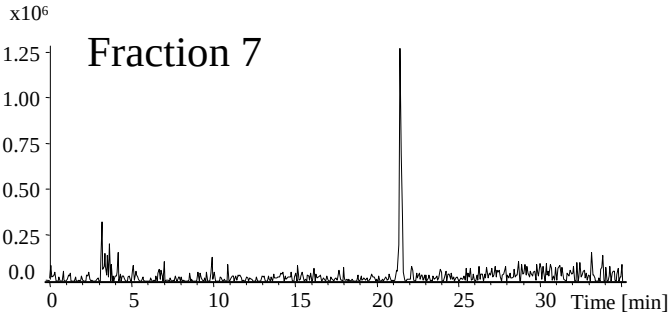

[AlaGlu(His)+H]+
356
[AlaGluHis+H]+
391
y1
156
-H2O
338
110
y2
321
356
285
m/z

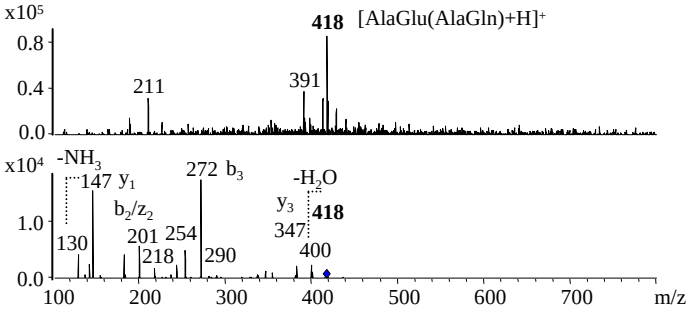

x10^7
Fraction 4
Time [min]

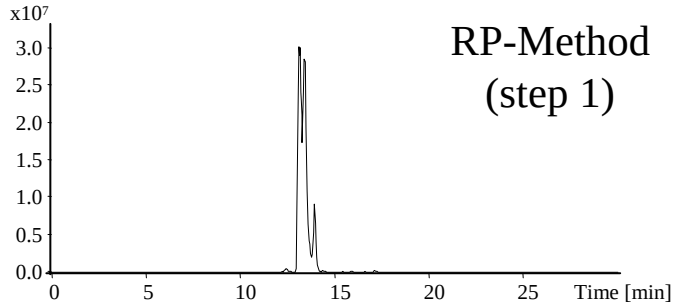

x10^6
375 [AlaGlu(Arg)+H]+
[AlaGluArg+H]+
347
531
x10^5
y1
175
-H2O
357
340
332
375
m/z


x10^6
Fraction 6
Time [min]


[AlaGluAlaGln+H]+
x10^5
418
[AlaGluAlaGln+Na]+
116
203
236
363
440
x10^4
y2/c2
b2/z2
218
x1/a2
-H2O
y1
201
418
147
173
b3
272
400
m/z


x10^6
Fraction 7
Time [min]


x10^5
418 [AlaGlu(AlaGln)+H]+
211
391
x10^4
-NH3
147
y1
272
b3
-H2O
b2/z2
y3
418
130
201
254
218
290
347
400
m/z


x10^7
RP-Method
(step 1)
Time [min]

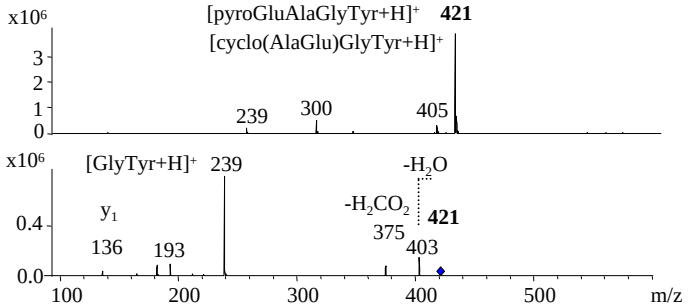

x10^6
[pyroGluAlaGlyTyr+H]+ 421
[cyclo(AlaGlu)GlyTyr+H]+
239
300
405
[GlyTyr+H]+ 239
-H2O
y1
-H2CO2
421
375
136
193
403
m/z

Table 1: Gradient elution time programs of

(a) RPLC separation of hydrophobic fraction in step 1; (A) 0.1 % FA in water; (B) 0.1 % FA in ACN

(b) RPLC prefractionation of polar fraction of step 1; (A) 0.1 % TFA in water; (B) 0.1 % TFA in ACN

(c) 2nd dimension HILIC separation with standard gradient; (A) 1.5 % (v/v) buffer in water; (B) 1% (v/v) water, 1.5% (v/v) buffer in ACN
buffer: 200 mM AcOH adjusted with ammonium hydroxide solution to pH of 5.5

(d) 2nd dimension HILIC separation with high elution strength gradient; (A) and (B) as in (c)

(a)

time [min]	% (A)	% (B)	flow rate [µl/min]
0	95	5	300
30	47.5	52.5	300
31	95	5	300
45	95	5	300

(b)

time [min]	% (A)	% (B)	flow rate [µl/min]
0	98	2	300
15	98	2	300
35	49	51	300
36	98	2	300
45	98	2	300

(c)

time [min]	% (A)	% (B)	flow rate [µl/min]
0	0	100	500
30	47.5	52.5	500
31	0	100	1000
40	0	100	500

(d)

time [min]	% (A)	% (B)	flow rate [µl/min]
0	0	100	500
30	47.5	52.5	500
31	92.5	7.5	1000
50	92.5	7.5	1000
51	0	100	1000
60	0	100	500

Table 2: Linear calibration functions for individual standards.

Compound	%ACN[1]	Slope	Intercept	R^2	Corrected slopes[3]
RP-Method					
Glu	9	2.44	14.33	0.992	2.37
Leu	11	2.84	10.84	0.994	2.55
Cyclo(AlaGlu)	12	2.56	7.72	0.995	2.64
GlyTyr	14	2.54	10.46	0.992	2.77
Phe	18	3.02	8.82	0.993	3.17
(AcCys)2	23	3.69	7.40	0.997	3.62
Trp	25	3.57	15.60	0.990	3.80
LeuTrpMetArg	28	4.28	8.90	0.994	4.02
Mean[2]		3.12	10.51		
Stdandard dev.		0.7	3.0		
%RSD		21.3	28.7		
HILIC-Method					
Gln	66	12.54	29.58	0.996	15.76
Glu	70	15.47	18.71	0.987	14.05
Leu	75	14.09	-54.19	0.995	19.60
Trp	77	17.58	-13.34	1.000	21.31
Mean[2]		14.92	-4.81		
Standard dev.		2.1	37.6		
%RSD		14.3	-782.1		

[1] Relative content of ACN in the mobile phase at individual elution times.
[2] unified calibration function
[3] Calculated using linear functions established by plotting slopes versus % ACN as shown in Figure 6

Table 3: Validation results of the RP method (n = 6) and the HILIC method (n = 5) at a concentration level of 10 µg/ml and 50 µg/ml, respectively[1].

Compound	intraday Precision	Accuracy compound-specific calibration function	Accuracy unified calibration function	Accuracy slope-corrected calibration function[2]
RP-Method				
Glu	5	131	115	151
Leu	10	125	115	141
Cyclo(AlaGlu)	9	123	92	109
GlyTyr	12	93	75	85
Phe	8	92	84	83
(AcCys)2	7	118	129	111
Trp	8	98	129	106
LeuTrpMetArg	8	94	121	94
HILIC-Method				
Gln	6	106	93	99
Glu	4	115	123	116
Leu	2	92	80	61
Trp	3	100	117	82

[1] Linear range: RP-method: 1-100 µg/ml; HILIC method: 5-100 µg/ml
LOQ (s:n = 5:1): RP-method: 10 ng on column (1 µg/ml; injection volume 10 µl);
HILIC method: 50 ng on column (1 µg/ml; injection volume 10 µl)
[2] Slopes of calibration functions were calculated for each compound using the linear relation ship established in Figure 6. The same intercept as for unified calibration functions were used (see Table 2)

Table 4: List of detected impurities and their corresponding quantification results using the RP-CAD method, a validated LC-UV method and a validated LC-MS/MS method.

retention time [min]	found m/z	name	RPLC - CAD [µg/ml]	LC-UV [µg/ml]	LC-MS/MS [µg/ml]	comment
5.1	**201**	**pyroGluAla**[1]	**260.6**	**226.6**	**309.7**	**> qualification threshold**
7.2	164	N-AcCys[2]	546.6	690	n.a.[4]	ingredient
8.9	**221**	**cyclo(GlyTyr)**	**85.0**	**108**	**n.a.**	**> qualification threshold**
9.6	n.f.[3]	n.a.	2.6	n.a.	n.a.	< reporting threshold
10.0	n.f.	n.a.	6.1	n.a.	n.a.	< reporting threshold
10.3	205	Trp	1993.9	2180	n.a.	ingredient
11.2	**325**	**$(AcCys)_2$**[2]	**131.8**	**235**	**n.a.**	**> qualification threshold**
12.0	n.f.	n.a.	3.4	n.a.	n.a.	< reporting threshold
12.3	n.f.	n.a.	3.3	n.a.	n.a.	< reporting threshold
12.5	*n.f.*	*n.a.*	*17.2*	*n.a.*	*n.a.*	> *reporting threshold*
12.7	281	n.a.	7.7	n.a.	n.a.	< reporting threshold
12.9	n.f.	n.a.	5.9	n.a.	n.a.	< reporting threshold
13.3	**421,332**	**cyclo(AlaGlu)GlyTyr,cyclo(AlaGlu)Met pyroGluAlaGlyTyr,pyroGluAlaMet**	**30.8**	**n.a.**	**n.a.**	**further investigations necessary**
14.0	n.f.	n.a.	1.3	n.a.	n.a.	< reporting threshold
14.8	*231*	*n.a.*	*13.9*	*n.a.*	*n.a.*	> *reporting threshold*
14.9	*395*	*n.a.*	*15.4*	*n.a.*	*n.a.*	> *reporting threshold*
15.2	n.f.	n.a.	2.8	n.a.	n.a.	< reporting threshold
15.6	n.f.	n.a.	2.7	n.a.	n.a.	< reporting threshold
15.8	n.f.	n.a.	2.6	n.a.	n.a.	< reporting threshold
15.9	n.f.	n.a.	5.1	n.a.	n.a.	< reporting threshold
16.3	n.f.	n.a.	3.5	n.a.	n.a.	< reporting threshold
16.4	n.f.	n.a.	4.0	n.a.	n.a.	< reporting threshold
16.8	n.f.	n.a.	1.7	n.a.	n.a.	< reporting threshold
17.0	n.f.	n.a.	5.2	n.a.	n.a.	< reporting threshold
17.5	n.f.	n.a.	2.4	n.a.	n.a.	< reporting threshold
17.5	n.f.	n.a.	1.4	n.a.	n.a.	< reporting threshold
18.7	n.f.	n.a.	0.9	n.a.	n.a.	< reporting threshold
19.0	n.f.	n.a.	1.3	n.a.	n.a.	< reporting threshold
19.3	n.f.	n.a.	2.8	n.a.	n.a.	< reporting threshold
19.9	n.f.	n.a.	6.1	n.a.	n.a.	< reporting threshold
20.2	275	n.a.	8.0	n.a.	n.a.	< reporting threshold
20.6	n.f.	n.a.	0.5	n.a.	n.a.	< reporting threshold
20.8	n.f.	n.a.	3.0	n.a.	n.a.	< reporting threshold
22.0	n.f.	n.a.	1.9	n.a.	n.a.	< reporting threshold
22.5	n.f.	n.a.	4.2	n.a.	n.a.	< reporting threshold
23.2	n.f.	n.a.	0.8	n.a.	n.a.	< reporting threshold
23.5	n.f.	n.a.	0.5	n.a.	n.a.	< reporting threshold

[1] pyroGluAla eluted as isolated peak in the polar fraction and thus, could also be quantified with the RP method of step 1.
[2] $(AcCys)_2$ is the dimer of N-AcCys and emerges due to disulfide bond formation
[3] n.f. not found
[4] n.a. not available
in bold letters: impurities determined to be above the identification and the qualification threshold, respectively
in italic letters: impurities determined to be above the reporting threshold

Table 5: List of impurities detected and quantified in the course of the multidimensional analysis assay (RPLC x HILIC – CAD). Furthermore, quantification was performed with a validated LC-UV method and a validated LC-MS/MS method [36].

Retention time [min]	Fraction	m/z	Name	RPLC-HILIC - CAD [µg/ml]	LC-UV [µg/ml]	LC-MS/MS [µg/ml]	Comment
10.6	**7/8/9/10/11/12**	**200**	**cyclo(AlaGln)**	**1247.0**	**1144**	**n.a.**	**> qualification threshold**
11.1	8	n.f.[4]	n.a.[5]	6.1	n.a.	n.a.	< reporting threshold
11.4	10	n.f.	n.a.	0.9	n.a.	n.a.	< reporting threshold
11.6	10/11	n.f.	n.a.	5.2	n.a.	n.a.	< reporting threshold
11.7	6	n.f.	n.a.	5.3	n.a.	n.a.	< reporting threshold
11.8	17	164	N-AcCys[1]	97.0	690	n.a.	ingredient
11.9	19/20	n.f.	n.a.	3.7	n.a.	n.a.	< reporting threshold
12.6	7	n.f.	n.a.	2.7	n.a.	n.a.	< reporting threshold
12.8	28	n.f.	n.a.	3.2	n.a.	n.a.	< reporting threshold
13.7	*9/10*	*n.f.*	*n.a.*	*10.4*	*n.a.*	*n.a.*	*> reporting threshold*
13.9	6	n.f.	n.a.	2.5	n.a.	n.a.	< reporting threshold
14.3	16	n.f.	n.a.	7.3	n.a.	n.a.	< reporting threshold
14.4	19/20/21	n.f.	n.a.	9.7	n.a.	n.a.	< reporting threshold
14.4	24/25/26	n.f.	n.a.	9.0	n.a.	n.a.	< reporting threshold
14.5	29/30	n.f.	n.a.	7.3	n.a.	n.a.	< reporting threshold
14.6	**10**	**n.f.**	**n.a.**	**68.1**	**n.a.**	**n.a.**	**> qualification threshold**
14.7	1/2	126	Taurine	1222.6	980	n.a.	ingredient
14.8	**12/13**	**201**	**cyclo(AlaGlu)/pyroGluAla[2]**	**333**	**275**	**386**	**> qualification threshold**
15.2	**8**	**430**	**n.a.**	**30.2**	**n.a.**	**n.a.**	**> qualification threshold**
15.2	**9/10/11**	**n.f.**	**n.a.**	**125.6**	**n.a.**	**n.a.**	**> qualification threshold**
15.2	**15**	**n.f.**	**n.a.**	**194.8**	**n.a.**	**n.a.**	**> qualification threshold**
15.5	**17**	**201**	**n.a.**	**26.0**	**n.a.**	**n.a.**	**> identification threshold**
15.6	11	n.f.	n.a.	2.7	n.a.	n.a.	< reporting threshold
15.6	28/29/30	166	Phe	2271.9	3580	n.a.	ingredient
16.2	17/18/19/20/21	132	Leu	6937.2	10640	n.a.	ingredient
16.4	*22/23/24/25*	*n.f.*	*n.a.*	*18.2*	*n.a.*	*n.a.*	*> reporting threshold*
16.4	**27/28**	**325**	**$(AcCys)_2$[1]**	**265.7**	**235**	**n.a.**	**> qualification threshold**
16.5	9/10/11/12	150	Met	1686.6	1990	n.a.	ingredient
16.6	8	n.f.	n.a.	4.9	n.a.	n.a.	< reporting threshold
16.8	15/16/17/18	132	Ile	3590.5	4780	n.a.	ingredient
17.0	24/25	239	TyrGly	7.7	13.5	n.a.	< reporting threshold
17.2	*17*	*n.f.*	*n.a.*	*13.5*	*n.a.*	*n.a.*	*> reporting threshold*
17.4	8	n.f.	n.a.	1.1	n.a.	n.a.	< reporting threshold

Retention time [min]	Fraction	m/z	Name	RPLC-HILIC - CAD [µg/ml]	LC-UV [µg/ml]	LC-MS/MS [µg/ml]	Comment
17.4	22	n.f.	n.a.	0.6	n.a.	n.a.	< reporting threshold
17.4	27	n.f.	n.a.	2.1	n.a.	n.a.	< reporting threshold
17.5	**15**	**303**	**n.a.**	**315.8**	**n.a.**	**n.a.**	**> qualification threshold**
17.7	**7/8/9/10/11**	**n.f.**	**n.a.**	**100.3**	**n.a.**	**n.a.**	**> qualification threshold**
17.8	4/5/6	118	Val	5092.0	5460	n.a.	ingredient
17.8	11	n.f.	n.a.	1.8	n.a.	n.a.	< reporting threshold
18.1	**8**	**283**	**n.a.**	**51.0**	**n.a.**	**n.a.**	**> qualification threshold**
18.3	**13**	**n.f.**	**n.a.**	**23.3**	**n.a.**	**n.a.**	**> identification threshold**
18.3	27/28	239	GlyTyr	1976.0	2550	n.a.	ingredient
18.8	2/3	120	Thr	3739.1	5440	n.a.	ingredient
18.9	**3/4/5/6/7**	**n.f.**	**n.a.**	**160.7**	**n.a.**	**n.a.**	**> qualification threshold**
19.0	*28*	*439*	*n.a.*	*15.3*	*n.a.*	*n.a.*	*> reporting threshold*
19.7	1/2/3/4/5/6	106, 116	Ser,Pro	12182.1	14020	n.a.	ingredient
19.7	**5**	**n.f.**	**n.a.**	**37.8**	**n.a.**	**n.a.**	**> qualification threshold**
19.7	12	n.f.	n.a.	6.9	n.a.	n.a.	< reporting threshold
19.7	**26/27**	**n.f.**	**n.a.**	**54.0**	**n.a.**	**n.a.**	**> qualification threshold**
19.8	**9/10**	**193, 338**	**Citric acid; cyclo(AlaGlu)His/pyroGluAlaHis**	**1131.6**	**n.a.**	**n.a.**	**further investigations necessary**
20.2	1/2	n.f.	Gly, Ala	2644.1	3100	n.a.	ingredient
20.2	*7/8*	*n.f.*	*n.a.*	*14.4*	*n.a.*	*n.a.*	*> reporting threshold*
20.3	12	n.f.	n.a.	7.8	n.a.	n.a.	< reporting threshold
20.5	7/8	n.f.	n.a.	7.5	n.a.	n.a.	< reporting threshold
20.9	4	n.f.	n.a.	2.7	n.a.	n.a.	< reporting threshold
21.5	**7/8**	**418**	**AlaGlu(AlaGln)**	**21.8**	**39.6**	**37.8**	**> identification threshold**
21.7	2/3/4/5/6/7	218	AlaGln/AlaGlu[3]	14615.8	19290	n.a.	Ingredient/impurity
21.7	**7/8**	**211**	**n.a.**	**51.1**	**n.a.**	**n.a.**	**> qualification threshold**
21.7	*10*	*n.f.*	*n.a.*	*11.8*	*n.a.*	*n.a.*	*> reporting threshold*
22.3	*6*	*418*	*AlaGluAlaGln*	*13.3*	*22*	*16.9*	*> reporting threshold*
22.4	*4*	*289*	*AlaAlaGln*	*18.1*	*31*	*23.0*	*> reporting threshold*
22.8	*3*	*218*	*n.a.*	*11.9*	*n.a.*	*n.a.*	*> reporting threshold*
23.6	2/3	156	His	3763.6	3030	n.a.	ingredient
25.0	**2/3/4**	**356,375**	**"AlaGluHis/AlaGluArg"-isomers**	**41.5**	**n.a.**	**n.a.**	**further investigations necessary**
27.1	**4**	**356**	**"AlaGluHis"-isomers**	**18.8**	**n.a.**	**n.a.**	**further investigations necessary**
27.8	**4**	**375**	**"AlaGluArg"-isomers**	**14.6**	**n.a.**	**n.a.**	**further investigations necessary**

[1] $(AcCys)_2$ is the dimer of N-Ac-Cys and is formed via disulfide bonding. Considering the retention time of $(AcCys)_2$ in the RP mode in step 1 (10.2 min), it was not expected to detect it in the polar part (< 8 min). Thus, it is assumed that $(AcCys)_2$ is formed during intermediate sample processing in the course of the 2-D assay due to disulfide bonding of N-AcCys, which was expected to be contained in the polar part.
[2] As cyclo(AlaGlu) and pyroGluAla could not be reliably distinguished, the combined concentration is given. Both compounds were accurately quantified using a validated LC-MS/MS method [36].
[3] AlaGln and AlaGlu could not be resolved during the multidimensional analysis assay. Thus, individual quantification was not possible. Yet the combined concentration is given. AlaGlu was accurately quantified using a validated LC-MS/MS method [36].
[4] n.f. not found
[5] n.a. not available
in **bold** letters: impurities determined to be above the identification and the qualification threshold, respectively
in *italic* letters: impurities determined to be above the reporting threshold

Table 6: Compounds detected in the course of the multidimensional analysis assay. For many compounds besides the protonated molecular ion several m/z of charged Na and K adducts, dimers and multimers were found. Furthermore, fragment ions found in the MS^2 mode are listed as well.

Compound	m/z	Associates and Adducts	Fragments
Ingredients			
Ser	106		
Pro	116	138, 384,406	
Val	118	140, 279, 301, 440, 579, 718	
Thr	120	142	
Taurine	126	148, 237, 251, 376, 398, 501, 523, 626, 773	108
Ileu	132	154, 176, 432, 454, 482	84
Leu	132	154, 176, 432, 454, 482	84
Lys	147		84,130
Met	150	337, 486,508	104, 133
His	156		110
N-AcCys	164	186, 208, 349, 371, 578, 763	122, 146
Phe	166	331,634	120
Arg	175	349,523	116,130,140,157
Citrate	193	215,407,423,614	129, 147, 175
Trp	205	409	188
AlaGln	218	240, 435, 457	89, 130, 147, 173, 184, 201
GlyTyr	239	261, 477, 498	136, 182, 193, 221
Impurities			
cyclo(AlaGln)	200	222, 421, 612	110,155,183
cyclo(AlaGlu)	201	401, 423	155,183
pyroGluAla	201	401	90,155,183
cyclo(GlyTyr)	222	421	204, 205
AlaAlaGln	289	311, 599	130, 147
$(AcCys)_2$	325	347, 363	162, 209, 237, 279, 283, 307
cyclo(AlaGlu)Met/pyroGluAlaMet	332		104,133, 150, 183, 314
cyclo(AlaGlu)His/pyroGluAlaHis	339		110, 156, 276, 320
"AlaGluLys" isomers	347		130, 147, 200, 276, 329
"AlaGluHis" isomers	356		110, 156, 285, 321, 338
"AlaGluArg" isomers	375		175, 357, 340, 332
AlaGlu(AlaGln)/AlagluAlaGln	418	440	130, 147, 173, 201, 218, 272, 347, 400
cyclo(AlaGlu)GlyTyr/pyroGluAlaGlyTyr	421		126, 165, 182, 193, 221

Table 7: Cross validation of RPLC-CAD and HILIC-CAD results with a validated LC-UV method and validated LC-MS/MS methods [36]

Retention time [min]	m/z	Name	RPLC-HILIC-CAD [µg/ml]	LC-UV [µg/ml]	LC-MS/MS [µg/ml]
HILIC-CAD					
10.6	**200**	**cyclo(AlaGln)**	**1247.0**	**1144**	**n.a.[2]**
14.8	**201**	**cyclo(AlaGlu)/pyroGluAla**	**332.5[1]**	**275[1]**	**386.4[1]**
17.0	**239**	**TyrGly**	**7.7**	**13.5**	**n.a.**
21.5	**418**	**AlaGlu(AlaGln)**	**21.8**	**39.6**	**37.8**
22.3	**418**	**AlaGluAlaGln**	**13.3**	**22**	**16.9**
22.4	**289**	**AlaAlaGln**	**18.1**	**31**	**23.0**
RPLC-CAD					
5.1	**201**	**pyroGluAla**	**260.6**	**226.6**	**309.7**
8.9	**221**	**cyclo(GlyTyr)**	**85.0**	**108**	**n.a.**
11.2	**325**	**$(AcCys)_2$**	**131.8**	**235**	**n.a.**
13.3	**332/421**	**cyclo(AlaGlu)GlyTyr/cyclo(AlaGlu)Met pyroGluAlaGlyTyr/pyroGluAlaMet**	**30.8[1]**	**n.a.**	**4.5[1]**

[1] Determined as the sum of the indicated compounds.
[2] not available

Appendix II

Manuscript

Quantitative LC-MS/MS impurity profiling methods for the analysis of parenteral infusion solutions for amino acid supplementation containing L-Alanyl-L-Glutamine

Quantitative LC-MS/MS impurity profiling methods for the analysis of parenteral infusion solutions for amino acid supplementation containing L-Alanyl-L-Glutamine

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Abstract

Potential impurities in a parenteral infusion solution for amino acid supplementation with alanylglutamine (AlaGln) and glycyltyrosine (GlyTyr) as peptide constituents have been determined. Such complex multicomponent pharmaceutical formulations with reactive ingredients may provide a multitude of impurities in stress testing samples. Thus, three stability indicating LC-ESI-MS/MS methods for the establishment of quantitative impurity profiles employing a Chiralpak QN-AX and a Polysulfoethyl A stationary phase in HILIC mode as well as a Gemini C18 stationary phase in gradient RPLC mode were developed to separate isobaric compounds (stereoisomers, constitutional isomers, retro-peptides) and to provide quantitative data of impurities identified in stressed nutritional infusion solutions. The optimized methods were calibrated by standard addition in the samples and validated according to the ICH guidelines. The methods were then applied for the analysis of stressed sample solutions stored under different conditions.

Keywords: stability indicating methods, stereoisomeric impurities, peptides, LC-MS/MS

1. Introduction

The establishment of qualitative and quantitative impurity profiles of drug substances and drug products is a crucial part in the course of the development of new pharmaceutical formulations. It provides a basis to assure quality and innocuousness of the drug products. In this context, stability testing with forced degradation is considered to be an important tool to uncover degradation processes and unwanted side reactions [1]. Part of such investigations are studies on the influence of environmental factors on the stability of drug substances and the evaluation of different storage conditions.

The International Conference on Harmonization of Clinical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has published a set of quality documents that provide guidelines on various aspects of impurity profiling and stability testing. Amongst others these guidelines state that drug developers should summarize the degradation products that can be observed during manufacture and/or stability studies of new products. Further, these impurities have to be quantified reliably by validated assays to enable classification into those that need to be reported (< 0.05% relative to the precursor compound at daily dosage > 1g), identified (0.2% above 10 mg daily dose and 0.1% above 2 g) and qualified (i.e. by assessment of their biological safety) (0.2% and 0.15% above 100 mg and 2 g, respectively) [2-4]. Such strict demands should help to preserve product safety. Hence, analytical assays that allow for the accurate and reliable quantification of all detected impurities are required. The present report deals with such methods that have been developed for identified impurities in a parenteral infusion solution for amino acid supplementation.

Glutamine (Gln) is considered to be the most abundant amino acid in the body and serves as important nitrogen source for many biosynthetic pathways. During hypercatabolic states like traumata, infection or injury depletion of the intracellular nitrogen content is likely to occur and may have a detrimental impact on protein synthesis. Thus, the benefits of reconstitution of the glutamine level in the body are obvious. However, the beneficial health effect of glutamine in nutritional infusion solutions has turned out to be not reliable because of the limited stability of the free amino acid during storage or heat sterilization [5]. A solution to this dilemma provides the administration of Gln in form of the AlaGln dipeptide. Several clinical studies revealed that AlaGln is quickly hydrolyzed in the extracellular space and thus the free amino acids Ala and Gln are set free and can be absorbed quickly [6,7].

The object of the present study was the examination of a pharmaceutical nutritional infusion solution that contained AlaGln as main component, but also GlyTyr and various amino acids

as well as other constituents. As high quantities of AlaGln are administered, it was of special importance to uncover degradation pathways and side products of AlaGln. The impurity pattern of the multicomponent formulation with its various reactive ingredients was expected to be quite complex. Hence, in a first step a qualitative impurity profile was established for a severely stressed infusion solution (40°C for 12 months) by using a multidimensional analysis approach consisting of offline two-dimensional HPLC combined with ion-trap (IT) MS and Charged Aerosol Detector (CAD) detection [8]. Due to its relatively universal and consistent detector response (for non-volatile compounds) the latter detector allowed a preliminary quantification of the impurities that were identified by MS^2 before, via use of a universal calibration function. Herein, we present accurate quantitative LC-MS/MS assays which were developed for the quantification of previously identified impurities employing calibration with authentic standards to confirm the validity of the results obtained with HPLC-CAD.

These methods were used to investigate the characteristics of degradation processes in detail by analyzing several stressed samples in order to monitor changes over time under defined storage conditions.

LC-MS/MS with multiple reaction monitoring (MRM) mode for specific detection nowadays constitutes the method of choice for the analysis of compounds in complex matrices, which is confirmed by the increasing number of publications in this sector [9-13]. It is especially valuable for multi-target component analysis assays like the present application because detection selectivity may reduce demands on the separation. Yet, MS- based methods also exhibit some shortcomings. For example, major coeluting undetected constituents may influence ionization efficiencies and thus quantitative results, which may be of particular concern for minor impurities overlapping with ingredients. Furthermore, in the present application a number of isomeric or isobaric impurities were expected that cannot be distinguished by MS emphasizing the importance of selective chromatographic separation. Due to the hydrophilicity of the target compounds this constitutes a major challenge.

In the presented work three LC-MS/MS methods are described that afforded unequivocal quantification and classification of several impurities identified in stressed nutritional infusion solutions. Validation was performed according to the ICH guidelines [14,15]. Unknown new degradation products of AlaGln formed in nutritional infusion solutions have been identified.

2. Experimental

2.1. Chemicals

The investigated drug formulation was a parenteral solution for supplementation of amino acids. It contained N-Acetyl-L-Cysteine, L-Alanine, L-Alanyl-L-Glutamine, L-Arginine, Glycine, Glycyl-L-Tyrosine, L-Histidine, L-Isoleucine, L-Leucine, L-Lysine acetate, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, Taurine, L-Threonine, L-Tryptophan and L-Valine as active metabolites.

Preliminary experiments and method development were carried out with three preparations of nutritional infusion solutions which were subjected to different treatments. One was kept under optimal storage conditions (< -20°C) whereas the two other solutions were stressed at increased temperatures of 40°C for 12 months and at 60°C for 9 months, respectively. In the course of stress testing further preparations of infusion solutions were kept under various stress conditions and finally analyzed employing the developed methods.

L-Ala-L-Gln and 2,4-dinitrofluorobenzene 99% were purchased from Sigma-Aldrich (Vienna, Austria). D-Ala-L-Gln 99%, L-Tyr-L-Gly 99%, N,N'-Diacetylcystine ($(AcCys)_2$) 98%, AlaAlaGln 98%, AlaGlu 98%, cyclo(AlaGlu) 99%, cyclo(AlaGln) 99% and pyroGluAla 99% were supplied by Bachem (Bubendorf, Switzerland). Cyclo(GlyTyr) and the two structural isomeric tetrapeptides AlaGluAlaGln 74.8% and AlaGlu(AlaGln) 76.0% were provided from Fresenius Kabi (Graz, Austria). The standards of cyclo(AlaGlu)His (TFA salt) 90%, pyroGluAlaHis (TFA salt) 90%, AlaGlu(His) (TFA salt) 90%, AlaGlu(Arg) (TFA salt) 90%, AlaGlu(Lys) (TFA salt) 90%, AlaGluLys (TFA salt) 90%, cyclo(AlaGlu)Met 95%, pyroGluAlaMet 95%, cyclo(AlaGlu)GlyTyr 95% were custom synthesized by piChem (Graz, Austria). Standards of pyroGluAlaGlyTyr 96.8%, AlaGluHis 91.2% and AlaGluArg 96.4% were obtained from GenScript Corporation (New Jersey, USA). Aqueous ammonium hydroxide (NH_4OH) 25%, acetic acid (AcOH) 99.8%, trifluoroacetic acid (TFA) 99.5%, ammonium acetate 97% and sodium carbonate anhydrous 98% were purchased from Fluka, formic acid (FA) 98-100% and sodium bicarbonate 99% were from Riedel-de Haën (Seelze, Germany). Acetonitrile (ACN) of HPLC grade was from VWR (Vienna, Austria). The employed water was purified with a Millipore (Elze, Germany) water filtration system.

2.2. Mass spectrometry

The instrumental set-up consisted of an Agilent 1200 HPLC system (Waldbronn, Germany) composed of a thermostatted autosampler, a binary pump and a column thermostat, hyphenated to a Q-Trap 4000 from Applied Biosystems/MDS SCIEX (Thornhill, Canada). For the quantification of degradation products of AlaGln and other identified impurities measurements were performed in the MRM (multiple reaction monitoring) mode. Compound specific parameters like declustering potential (DP), collision energy (CE) and cell exit potential (CXP) were determined using the "Quantitative Optimization" tool of the Analyst software (version 1.4.2). For this purpose standard solutions of the compounds were prepared at concentrations of 250 µg/L using solvents with a composition similar to the mobile phase conditions during detection. The standard solutions were infused with a syringe pump at a flow rate of 30 µL/min. For each analyte two specific transitions were monitored, one of which served for quantification (quantifier) and the other as identifier (qualifier) minimizing the risk of false peak assignment. Optimized values for MS parameters of all impurities investigated in the study can be found in Table 1.

A turbo ion spray (TIS) was employed as ion source. TIS voltage was adjusted to + 4500 V in the positive mode and to –4 300 V in the negative mode. Source temperature was set to 600°C and the flow of curtain, nebulizer and heater gas were kept at 10, 50 and 60 psi, respectively. Pressure of the collision gas was adjusted to medium and a dwell time of 100 ms was utilized. The chromatograms were separated into periods and only the transitions of compounds eluting within this time frame were measured.

2.3. Chromatography

Stereoselective analysis of L-Ala-L-Gln stereoisomeric impurities

20 µL of nutritional infusion solutions or standard solution were combined with 500 µL of carbonate buffer, which was prepared by mixing 0.1 M $NaHCO_3$ and 0.1 M Na_2CO_3 to yield a pH 9.5. After addition of 200 µL of Sanger's reagent (5% *2,4*-dinitrofluorobenzene in ACN, w/v) the reaction mixture was incubated on a shaker at room temperature for 60 minutes. To remove apolar side products of the derivatisation, reaction solutions were extracted twice with 500 µL diethylether. In the next step mobile phase (vide infra) was added to the derivatized samples to yield a volume of 1 ml and further 1:10 dilution was prepared.

The samples were analyzed on the LC-MS/MS (Q-Trap) system in the negative ionization mode recording two specific MRM transitions (382 => 192; DP -65; CE -22; CXP -11; 382 => 162; DP -65; CE -32; CXP -9). 20 µL of the derivatized samples were injected onto a Chiralpak QN-AX column (150 x 4.0 mm, 5 µm) from Chiral Technologies (Illkirch, France) and eluted under isocratic conditions employing 20 mM aqueous ammonium acetate (adjusted to pH 4.5 with AcOH) / ACN 60:40 (v/v) at a flow rate of 1 ml/min.

Chiralpak QN-AX HILIC method

In this method a Chiralpak QN-AX column was employed as stationary phase. The mobile phase was composed of 10% (v/v) buffer in water (channel A) and 10% (v/v) buffer in ACN (channel B), respectively. The utilized buffer contained 100 mM formic acid in water adjusted to pH 3.5 with NH_4OH solution. Linear gradient elution from 100 % (B) to 65% (B) in 20 min was carried out at a flow rate of 1 ml/min. The column was then reequilibrated with 100% (B) for 13 minutes.

Polysulfoethyl A HILIC method

In this method a Polysulfoethyl A (150 x 4.6 mm, 5 µm) column from PolyLC (Columbia, USA) was used as stationary phase. Mobile phase conditions were as follows: (A) 10% buffer in water and (B) 10 % buffer in ACN. The buffer consisted of 100 mM NH_4OH, pH adjusted to 5.0 with AcOH.
A linear gradient from 100 % (B) to 100 % (A) in 30 min at a flow rate of 500 µL/min was applied. The column was reequilibrated with 100% (B) for 13 min, thereby starting with a flow rate of 1 ml/min which continuously decreased to 0.5 ml/min until the end of the run.
Between 19.0 and 21.0 minutes of the gradient run the effluent was directed to waste in order to avoid contamination of the ion source.

Gemini C18 RP method

A Gemini C18 column (150 x 3.0 mm, 3 µm) from Phenomenex (Aschaffenburg, Germany) was used as stationary phase. The column was protected with a guard column (4.0 x 3.0 mm) containing the same stationary phase. Mobile phase conditions were as follows: (A) 0.1 % FA in water and (B) 0.1 % FA in ACN. A linear gradient from 5% (B) to 15 % (B) in 30 minutes

at a flow rate of 300 µL/min was applied. After the gradient was finished, the system was allowed to reequilibrate with starting conditions (95% (A); 5% (B)) for 10 minutes. The effluent from the column was directed to waste during the first 10 minutes of the run in order to avoid contamination of the ion source.

2.4. Preparation of standard solutions

Standard addition was performed by adding 100 µL multicomponent spiking standard to 1 ml diluted sample solution. Concentration increments of spiking standards were chosen to match expected intrinsic concentrations of individual analytes in the sample.

Preparation of calibrants for HILIC method with Chiralpak QN-AX:

The sample (unstressed or stressed infusion solutions) was diluted 1:50 with mobile phase (B). The concentrations of spiking standards were 0.5; 1.0; 2.0; 5.0; 10; 25; 50 µg/ml for AlaGln epimers (DL and LD); AlaAlaGln; cyclo(AlaGlu); AlaGlu; AlaGluAlaGln; AlaGlu(AlaGln) and 4; 8; 16; 40; 80; 200; 400 µg/ml for pyroGluAla.

Preparation of calibrants for the HILIC method with Polysulfoethyl A:

The sample was diluted 1:20 with mobile phase (B). The concentrations of spiking standards were 0.2; 0.5; 1.0; 2.0; 5.0; 10; 20 µg/ml for TyrGly; AlaGluArg; AlaGlu(Arg); AlaGluHis; AlaGlu(His); AlaGluLys; AlaGlu(Lys) and 20; 50; 100; 200; 500; 1000; 2000 µg/ml for cyclo(GlyTyr) and cyclo(AlaGln).

Preparation of calibrants for the RP method with Gemini C18:

The sample was diluted 1:5 with mobile phase (A). The concentrations of spiking standards were 0.2; 0.5; 1.0; 2.0; 5.0; 10; 15 µg/ml for pyroGluMet; cyclo(AlaGlu)Met; pyroGluGlyTyr; cyclo(AlaGlu)GlyTyr and 10; 25; 50; 100; 250; 500; 750 µg/ml for $(AcCys)_2$.

2.5. Validation

The three reported methods were validated according to the ICH guidelines (Q2 R1) [14,15]. Linearity, intra- and interday precision and accuracy as well as LOQ were determined. Standard addition was performed by spiking defined amounts of standard compounds to stressed sample solutions. Thus, matrix matched calibration functions were obtained by correcting for analyte contents already present in the samples. Standards were individually spiked and respective amounts can be found in Tables 2, 4 and 6. Accuracy and precision

were determined for three different concentration levels (low, middle, high; see Tables 3, 5, 7) in spiked sample solutions (quality control samples) by triplicate analysis. Interday precision and accuracy were determined on three consecutive days using freshly generated calibration functions. The LOQ was defined as the concentration at which the qualifier transition of the analyte yields a signal to noise ratio of at least 3 and the quantifier of at least 10.

3. Results and discussion

3.1. Degradation and side reactions of AlaGln

AlaGln represents the most abundant active constituent of the investigated undiluted infusion solution and is preferred as more stable administration form of Gln. Nevertheless it has been shown in a previous qualitative impurity profiling study, that also AlaGln is prone to undergo various degradation reactions (amide and peptide bond hydrolysis followed by condensation reactions), especially during forced degradation and stability testing [8]. Furthermore, in nutritional solutions containing nearly all native amino acids in a free form, additional side reactions such as condensation with various amino acids may be expected in the course of stress tests.

The complex pattern of the relevant, but non abundant impurities in stressed infusion solutions as identified by the previous qualitative impurity profiling investigations [8] is illustrated in Figure 1.

Some degradation products of AlaGln were well known from literature. In this context, it has been reported that the main degradation products of AlaGln are formed by side chain and main chain hydrolysis leading to AlaGlu as well as to the free amino acids Ala and Glu [16]. As Glu is not stable, it is rapidly converted into pyroglutamic acid (pyroGlu). Besides these primary AlaGln degradation products, a number of secondary AlaGln related impurities have been identified in the course of the previous qualitative impurity profiling. The susceptibility of AlaGlu and free Glu for condensation reactions is reflected in a number of peptide and peptide-like impurities such as GluAla, Glu(Ala), AlaGluAlaGln, AlaGlu(AlaGln), cyclo(AlaGlu) and pyroGluAla (Figure 1). Moreover, in the stressed nutritional infusion solutions several of the aforementioned impurities underwent condensation reactions with other amino acids or dipeptides. Such tertiary impurities derived from AlaGlu, pyroGluAla or cyclo(AlaGlu) were identified to be AlaGluX, AlaGlu(X), cyclo(AlaGlu)Y and pyroGluAlaY whereby X stands for Arg, His, Lys, AlaGln and Y for GlyTyr, His or Met. It is striking that

condensation reactions of AlaGlu preferentially took place with amino acids containing basic functional groups like Arg, His and Lys, which seem to be more susceptible for peptide formation maybe due to self-catalytic activity of the side chain.
The previous preliminary quantitative 2-D-HPLC-CAD/MS screening assay suggested that these impurities are above or close to the reporting threshold and thus demand accurate assays for their reliable quantification which was realized by the herein presented three different HPLC-MS/MS methods.

3.2. General aspects

Several challenges have to be met in the course of the development of stability indicating methods employing LC-MS and LC-MS/MS methods, respectively. A fact to be considered is that the active pharmaceutical ingredients (APIs) constitute bulk components, whereas the impurities, which must be accurately and reliably quantified, are usually present at very low levels. This brings about, that only limited sample dilutions can be tolerated in order to obtain signals above the LOQ for impurities. The consequence is that high quantities of the bulk compounds are introduced into the MS which may easily lead to contamination of the ion source as well as matrix effects such as ion suppression. To minimize contamination the HPLC effluent was directed to waste during the elution time of the main compound L-Ala-L-Gln. Unfortunately, this was only possible for the methods using the RP-18 Gemini and the Polysulfoethyl A column. Diverting of the eluting main compound L-Ala-L-Gln was not feasible for the method using the Chiralpak QN-AX column because of coelution with AlaAlaGln and cyclo(AlaGlu), which had to be determined.
The reporting threshold for impurities proposed by the ICH for maximal daily doses of the active agent exceeding 2g is 0.05% relative to the parent compound. Hence, the LOQ of the employed analysis method should reach at least a concentration level of 0.05% of the impurity related to the parent compound. Moreover, the assay must provide a linear range that covers the concentrations of the proposed ICH thresholds.
In order to be able to accurately and reliably analyze degradation-related impurities in the sub-percentage range related to the parent compound, it is usually necessary to fully or at least partly separate them from parent compounds. This is advised for stability indicating methods even with highly specific detection such as tandem MS, because low abundant impurities may be easily masked by highly abundant ingredients (main constituents) through suppression of the ionization [17,18]. Moreover, another common problem is the structural similarity

between ingredients and their degradation products which may compromise the specificity of MRM transitions and cause erroneous quantitative results due to interferences and/or crosstalk.

This may be a particular problem in the present applications, where similar substructure sequences are for instance present in parent compounds and condensation products. A careful validation of assay specificity is hence of utmost importance.

In the presented studies matrix-matched calibration by standard addition was carried out in each of the three methods by spiking distinct amounts of standard to the sample solutions. This assures similar conditions during calibration and measurement of the samples alleviating the problem of errors from distinct ionization efficiencies of analytes in plain standard solutions and complex stressed infusion solutions. This method is supposed to produce more accurate results because several calibrants are utilized in a narrow relevant concentration range.

3.3. Preliminary study on stereoisomeric impurities of L-Ala-L-Gln

L-Ala-L-Gln is the most abundant component in the parenteral solution. Its stereoisomeric forms are conceivable as potential impurities being void of any changes in atomic composition.

L-Ala-L-Gln has multiple chiral centers and therefore the monitoring of diastereomers appears to be more important at first place than the analysis of its enantiomeric form.

Diastereomeric D-Ala-L-Gln and L-Ala-D-Gln may be formed by single step epimerization at the stereogenic centers of N-terminal Ala or C-terminal Gln amino acids. In contrast, racemization, if any, more likely occurs via a two step epimerization as indicated in Figure 2, while simultaneous inversion of both stereogenic centers of the dipeptide, which yields the enantiomeric impurity D-Ala-D-Gln, is unlikely. Hence, D-Ala-D-Gln is expected to be present at lower concentration than the epimers.

Nevertheless, regulatory agencies suggest to assess whether enantiomers of such compounds with multiple chiral centers are a realistic impurity or not. For this purpose we developed a stereoselective assay.

As stereoisomeric compounds can not be differentiated by mass spectrometry, chiral separation techniques are required. Based on a reported chromatographic method for the separation of stereosiomers of AlaAsn [19], preliminary experiments were performed on a Chirobiotic T (Teicoplanin, 250 x 4.0 mm, 5 µm) column from Astec (Whippany, NJ, USA).

Using isocratic elution with MeOH/H_2O (90/10; v/v) at a flow rate of 0.5 ml/min separation of the four stereoisomers of AlaGln could be accomplished. The stereoisomers eluted in the order of D-Ala-L-Gln < L-Ala-L-Gln < L-Ala-D-Gln < D-Ala-D-Gln.

In spite of a successful separation of all four stereoisomers, the method was not very well suited for the intended quantitative analysis of minor stereoisomeric impurities of L-Ala-L-Gln in presence of the parent compound. Unfortunately, the DL- and the LL-AlaGln isomers were not sufficiently resolved anymore on the Chirobiotic T column when a real sample with high percentage of LL-form and trace amounts of the other form (LD, DL, DD) was injected. Furthermore, under such conditions the late eluting LD- and DD-isomers suffered from severe peak broadening effects preventing an accurate sensitive detection of trace levels (< 1%) of these isomers. For these limitations the method using Chirobiotic T was considered to be not practical.

Thus, further experiments were carried out on a Chiralpak QN-AX column, which is a tert-butylcarbamoylquinine based chiral stationary phase known to exhibit stereoselectivity for N-derivatized amino acids and peptides according to an anion-exchange retention principle [20,21]. Hence, AlaGln dipeptides were derivatized with Sanger's reagent and injected into the Chiralpak QN-AX column. Employing isocratic elution conditions reasonable separation of the N-dinitrophenyl-AlaGln (DNP-AlaGln) isomers could be accomplished with acceptable peak shapes (Figure 3A).

Three infusion solutions subjected to different treatments, i.e. not stressed (Figure 3B), stressed at 40°C for 12 months (Figure 3C) and stressed at 60°C for 9 months (Figure 3D), were analyzed. The relative peak areas of the four stereoisomeric forms are summarized in Table 2.

The LD- and DL-stereoisomers could be detected at levels above the reporting threshold in each of the three infusion solutions, while the DD-enantiomer was always present at levels below the reporting threshold of 0.05% except for the harshly stressed solution stored at 60°C for 9 months (0.078%).

As the reporting threshold of DD-isomer was not exceeded in the reference solution (40°C/12 month), the DD-isomer was excluded from further considerations as a relevant impurity. Even if a slightly different detector response is taken into account for the distinct stereoisomers, it is safe to assume that quantification of the DD-isomer is less important and a non-enantioselective but diastereoselective assay for the quantitative determination of epimeric forms of L-Ala-L-Gln is adequate.

3.4. Chiralpak QN-AX HILIC method

The majority of impurities listed in Table 1 are hydrophilic peptide (-like) impurities that elute unresolved in the polar bulk of RPLC, i.e. in the early eluting part of the chromatogram which is extremely susceptible for ion suppression and matrix effects on the one hand and is also associated with a low detection sensitivity due to the high water content of the eluent resulting in high surface tension and poor ionization efficiency on the other hand [22]. Hence, a HILIC separation mode was considered as first choice. The above employed Chiralpak QN-AX column provides the required diastereoselectivity for the epimers (DL, LD isomers) of L-Ala-L-Gln without derivatisation when a HILIC elution mode with a negative ACN gradient was employed. It allows the combined determination of the sum of both epimers which is sufficient for the present purpose. A chromatogram of a spiked sample is shown in Figure 4 A. The combined epimeric impurities (DL and LD) coelute as minor impurity peak in front of the major ingredient compound L-Ala-L-Gln. This facilitates accurate peak integration and provides a lower LOQ than for the case where it elutes on the tailing edge of the main component.

Besides the epimers of L-Ala-L-Gln, a number of other impurities were analyzed with the HILIC method developed on the Chiralpak QN-AX column (see Table 1). For example other critical solute pairs that demanded separation owing to their isobaric nature were the constitutional isomers cyclo(AlaGlu) and pyroGluAla as well as AlaGluAlaGln and AlaGlu(AlaGln). Successful separation of these compounds was also achieved as illustrated in Figure 4. The tripeptide AlaAlaGln and cyclo(AlaGlu) coeluted with L-Ala-L-Gln. To examine the specificity of the employed MRM transitions a single standard of L-Ala-L-Gln at a content corresponding to that in the formulation was injected and the MRM transitions of AlaAlaGln and cyclo(AlaGlu) were monitored. In the MRM traces of AlaAlaGln and cyclo(AlaGlu) no peak could be found at the retention time corresponding to L-Ala-L-Gln which demonstrated that the employed transitions were specific for the individual compounds and allowed distinction from the bulk compound. Thus, significant interference of L-Ala-L-Gln on the signals of AlaAlaGln and cyclo(AlaGlu) was not to be expected.

In preliminary investigations it was demonstrated that besides AlaGlu also structural analogues namely GluAla and Glu(Ala) were formed in harshly stressed solutions (e.g. 60°C/9 months). The selectivity of the present method for these potential impurities was examined, in order to exclude interference of isobaric GluAla and Glu(Ala) with the

quantification of AlaGlu. It becomes evident from Figure 5 that neither GluAla nor Glu(Ala) interfered with the determination of AlaGlu because both are adequately resolved.

After validation of assay specificity, other validation parameters have been assessed including linear range, LOQ, intra- and inter-assay precision and accuracy and the results are summarized in Table 3 and Table 4.

Calibration functions have initially been constructed with plain standard solutions in the range of 0.005 – 5.0 µg/ml. Linearity with $r^2 > 0.9938$ was observed for all target solutes in the range specified in Table 3. From these dilution series, LOQs could be determined for the individual compounds as concentrations at which the signal-to-noise ratio was 10:1 (see Table 3). Except for pyroGluAla and cyclo(AlaGlu), the LOQs were adequate and allowed the determination of all impurities below the reporting threshold. For pyroGluAla and cyclo(AlaGlu) the LOQ was above the reporting threshold. However, this was of no concern because pyroGluAla was always present in the investigated infusion solutions at concentration levels significantly above the LOQ. Concentrations of cyclo(AlaGln) were near the determined LOQ in the infusion solutions. However, intra and interday precision and accuracy results where acceptable at the lowest concentration (Table 4). Thus, the applicability of the method was not compromised by the lower sensitivity for these compounds.

Furthermore, calibration functions have also been set up in a narrower but more relevant concentration range by standard addition into an unstressed infusion solution. Slopes of these calibration curves were compared with those obtained by neat standard solutions (i.e. standards spiked into a L-Ala-L-Gln solution with a concentration level matching the one in infusion solutions), in order to assess whether the MS responses in the sample solutions are affected by potentially coeluting non-detected sample constituents. As can be seen in Figure 6 the matrix matched calibration function was completely overlapping with that in neat standard solution for AlaGlu(AlaGln) and only minor deviations were found for the other compounds. This indicates that matrix effects are insignificant and potentially coeluting compounds do not affect the ionization efficiency and the quantitative results for these solutes. Although slopes of calibration curves generated in the different matrices differed only to a minor extent, matrix-matched calibration by a standard addition procedure was considered to be more accurate and more reliable, and was thus further employed for the validation process and the analysis of the samples from stress testing.

Intra- and interday precision and accuracy have been determined by three replicate injections

of quality control standards (stressed sample solution spiked at three concentration levels; low, middle and high) on three different days.

Considering established acceptance criteria of 10% for intra- and interday precision, excellent results were obtained for all analytes at the tested concentration levels, with RSD values mostly lower than 2% but never above 7% for intraday precision. Interday precision measured on three different days mainly ranged between 1 and 5% and was always lower than 10%. Accuracy was assessed by % recoveries of spiked sample solutions after correction of the intrinsic impurity content of the utilized infusion solutions. The acceptance criterion for accuracy was set to a range of 95 to 105%. As can be seen from Table 4, both intra- and interday accuracies at the middle and the high concentration levels were always within the acceptance range. There were only a few values outside the accepted interval in the low concentration level which was found to be still acceptable.

3.5. Polysulfoethyl A HILIC method

In the course of ongoing investigations a number of other impurities could be identified. Since they could not be simultaneously analyzed by the above method without major adaptations and further experimentation, a quick screening of a selected set of stationary phase/mobile phase combinations including HILIC and RP conditions was performed. A Polysulfoethyl A column operated under HILIC conditions appeared to be promising and finally allowed for adequate separation and analysis of the majority of remaining impurities.

Table 1 provides MS acquisition data and retention time information on the compounds analyzed in the present study using the Polysulfoethyl A stationary phase.

Polysulfoethyl A is a strong cation exchange stationary phase that is also well suited for HILIC separations of hydrophilic peptides. The column is widely used for the separation of peptides in the course of protein characterization [23,24]. Most of the target solutes are net positively charged under the employed conditions and highly hydrophilic. Hence, it is no surprise that this stationary phase is applicable to solve selectivity issues of dipeptides to separate isobaric compounds. Most importantly, the corresponding pairs of structural isomers such as AlaGluX and AlaGlu(X) (X = Arg, His, Lys) and cyclo(AlaGlu)His as well as pyroAlaGluHis had to be separated (Figure 7) in order to allow unequivocal quantification. In this context, it is worthwhile to mention that this chromatographic phase system allows also separation of AlaGluX/AlaGlu(X) pair from the corresponding peptide with retro-sequence GluAlaX. For example, the three peptides AlaGluArg/AlaGlu(Arg)/GluAlaArg were baseline

resolved (see Figure 8) and the corresponding set with His replacing Arg showed a similar separation. Preliminary experiments, however, proved that the retro-sequence GluAlaX is of no relevance and below the reporting threshold value or not at all found.

These peptides exhibit basic functional entities in their amino acid side chains that provide, in addition to their terminal amino groups, sites for ion exchange interactions with the negatively charged sulfonic acid group of the stationary phase. Thus, a mixed mode SCX/HILIC mechanism may be at work which may be the key for the intriguing selectivity of this separation system for this delicate separation problem. However, also cyclic peptides such as cyclo(AlaGln) and cyclo(GlyTyr) lacking an free primary amine are well retained on this phase and well resolved from the parent dipeptides in accordance to a HILIC retention mechanism. Cyclo(GlyTyr) is stemming from the parent GlyTyr constituent which is another peptide ingredient for Tyr supplementation. Since Tyr exhibits poor solubility in physiological solutions it is supplemented in infusion solutions in form of the synthetic dipeptide GlyTyr. GlyTyr is, similarly to AlaGln, rapidly hydrolyzed to free Gly and Tyr in the plasma as shown by previous studies [6]. Moreover, again the retro-peptide TyrGly was well resolved from GlyTyr as well as from cyclo(GlyTyr). In this case preliminary experiments suggested that TyrGly should be of relevance as impurity being present in the stressed samples presumably above the reporting threshold value.

Calibration (with neat standard solutions over extended range and standard addition to an infusion solution, respectively) results as well as sensitivity data (LOQ) were determined as described above for the HILIC method with the Chiralpak QN-AX column. The results can be found in Table 5. Acceptable correlation coefficients > 0.994 were obtained for the calibration functions in any case. LOQ (signal-to-noise = 10:1) of 0.05 µg/ml or lower for all analytes confirm appropriate method sensitivity for assessing concentrations at reporting threshold levels.

For all analytes intraday precision at the three tested concentration levels (n=3) was always lower than the acceptance criterion of 10% RSD (Table 6). Similar results were obtained for interday precision, which ranged between 0.6 to 8.3% RSD for all analytes, except for AlaGlu(Arg) for which %RSD values of 14.9 and 12.7% were determined at the spiking levels of 0.02 and 0.1 µg, respectively. Intra- and interday accuracy values were mostly within the acceptance interval of 95 to 105% and always between 80 – 120% even at the lower concentration level confirming applicability of the method.

3.6. RP method employing Gemini C18

A few of the remaining impurities which were less polar were investigated using the Gemini C18 phase. Thus, the pairs of constitutional isomers cyclo(AlaGlu)Met and pyro(AlaGlu)Met as well as cyclo(AlaGlu)GlyTyr and pyro(AlaGlu)GlyTyr could be separated according to hydrophobicity differences as illustrated in Figure 9 and were finally accurately quantified by this method.

The validation results for this method are summarized in Tables 7 and 8. As for the other methods, also with the RP-method preliminary calibration functions were set up with neat standard solutions. Correlation coefficients > 0.999 were obtained for the investigated extended calibration range. LOQs (signal-to-noise ratio > 10:1) were determined by dilution of standard solutions and ranged between 0.005 and 0.05 µg/ml (see Table 7) being in any case below the reporting threshold of the respective impurities.

Results for intraday precision (n=3) were always better than the acceptance limit of 10% RSD. The same was valid for interday precisions at the medium and high concentration levels. However, interday precision for pyroGluAlaMet and cyclo(AlaGlu)Met was slightly above the limit of 10% at the lowest concentration level. Accuracies, both intra-assay as well as interday, were quite acceptable (see Table 8) except for the lowest QC level.

Hence the applicability has to be restricted to a higher concentration range.

Stronger variations at the lowest concentration levels might be attributed to ESI spray instabilities caused by the high water content in the RP mode. In this context, it is striking that above HILIC elution modes seem to offer an advantage since the high organic content in the HILIC mode provides better sprayer efficiency and stability due to lower surface tension [22].

A particular problem represented $(AcCys)_2$. Several significant outliers were found with regard to both precision and accuracy (see Table 8). $(AcCys)_2$ is formed by disulfide bonding of N-Acetyl-Cysteine, which is contained as active compound in the infusion solutions. Thus, inconsistencies of $(AcCys)_2$ levels at the lower concentration levels might be attributed to instability of the compound and the susceptibility of this disulfide compound to uncontrolled and irreproducible redox-reactions may be an explanation for the unacceptable precision and accuracy at the lowest examined QC level. Considering middle and high concentration levels of $(AcCys)_2$ accuracy and precision were again within an acceptable range.

3.7. Application

The three validated methods were employed for the quantification of the discussed impurities in differently stressed infusion solutions. In Table 9 the results of three solutions stored at 40°C for different time spans (3, 6 and 12 months) are presented.

Quantitative results reveal that cyclo(AlaGln) (diketopiperazine derivative of AlaGln) is the most abundant degradation product of AlaGln. It is present at concentrations far above the qualification threshold of 0.15%. The same is true for cyclo(GlyTyr) which is the main degradation product of GlyTyr. Furthermore, it may be surprising that the impurity TyrGly was also found in concentrations above the qualification limit. Moreover, high concentrations of $(AcCys)_2$ were found in stressed sample solutions as expected due to the redox-instability of N-Acetyl-Cysteine. Its concentration was also determined to be above the qualification threshold of 0.15%.

Several isobaric peptide-like impurities were formed by condensation reactions with AlaGlu during storage at elevated temperatures. Thereby, it is striking that condensation reactions with the carboxylic function in the side chain seem to be preferred over condensation at the C terminal end as indicated by the analysis results (see Table 9). Furthermore, higher contents of cyclo(AlaGln)Y were found compared to pyroGluAlaY, which was unexpected because higher concentrations of the pyroGluAla precursor were detected in the solutions.

However, most of these condensation products were found in concentrations below the reporting threshold (0.05%) except for AlaGlu(His), AlaGlu(AlaGln) and AlaGluAlaGln for which a content higher than the reporting threshold was determined.

The obtained quantitative data allow unambiguous classification of impurities and provide a basis for shelf life estimation as well as for assessment of long-term stability.

4. Conclusion

Three analysis methods based on HPLC-ESI-MS/MS using different stationary phases (Chiralpak QN-AX and Polysulfoethyl A under HILIC elution conditions and RPLC with a Gemini C18) were developed. Different separation mechanisms (HILIC, ion-exchange and hydrophobic interactions) were exploited for the separation and quantitative analysis of several impurities formed during stress testing of nutritional infusion solutions. Critical pairs like stereoisomers, constitutional isomers or other isobaric compounds that cannot be distinguished by specific MRM transisitions, could be chromatographically separated and

thus accurately quantified. Validation of the assays according to the ICH guidelines was performed. Obtained validation results confirmed the applicability of the methods for the purpose of impurity profiling. Furthermore investigation of stressed samples revealed that not only deamidation of AlaGln to AlaGlu and peptide based hydrolysis of AlaGln occur, but also cyclization (diketopiperazine formation) and other condensation reactions take place, preferably with constituents present at higher concentration levels such as AlaGln and GlyTyr or amino acids carrying basic functional groups (Arg, His, Lys).

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Figure Legends

Figure 1: Scheme of the degradation pathways of AlaGln and its follow-up reactions.

Figure 2: Stereochemical relationship of AlaGln stereoisomers.

Figure 3: Stereoselective separation of DNP- derivatized AlaGln isomers in parenteral infusion solutions on Chiralpak QN-AX under isocratic conditions. DNP-derivatives elute in the order DD < LD < LL < DL. MRM transition: 382 => 192 (DP -65; CE -22; CXP -11)
(A) standard solution of the four stereoisomers of AlaGln; (B) nutritional infusion solutions (not stressed); (C) stressed nutritional infusion solution stored for 12 months at 40°C; (D) stressed nutritional infusion solution stored for 9 months at 60°C

Figure 4: HILIC-ESI-MS/MS chromatograms of the separation of AlaGln degradation products on Chiralpak QN-AX. Peak annotation: (A) 1 AlaGln epimers (DL,LD); 2 L-Ala-L-Gln; (B) 3 AlaAlaGln; (C) 4 cyclo(AlaGlu); (D) 5 AlaGlu; (E) 6 AlaGluAlaGln; (F) 7 pyroGluAla ; (G) 8 AlaGlu(AlaGln)

Figure 5: TIC chromatogram illustrating the separation of 1 AlaGlu; 2 GluAla; 3 Glu(Ala) on Chiralpak QN-AX.

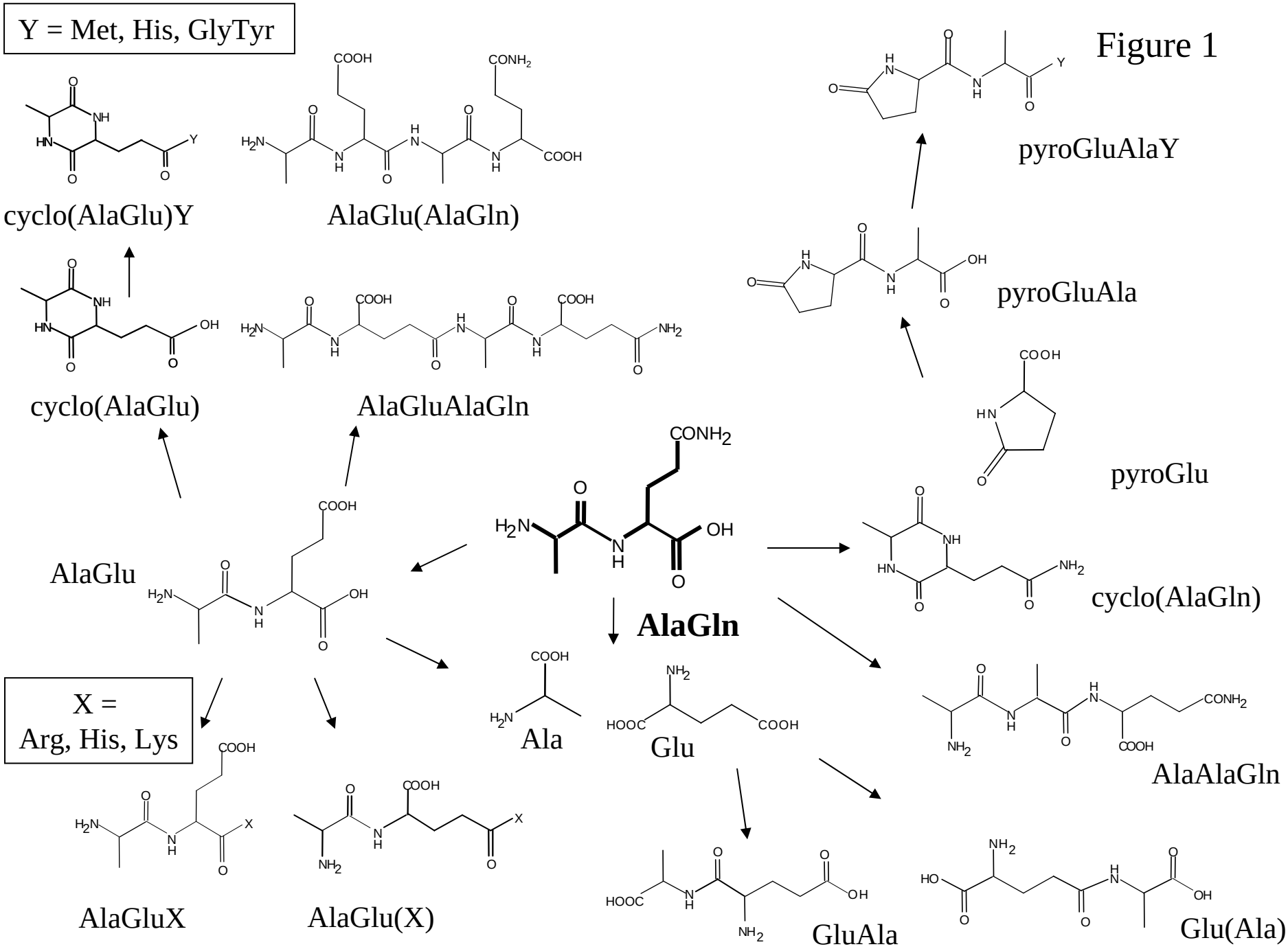
Figure 6: Calibration curves obtained by standard addition to a non-stressed infusion solution (solid line) (note, examined impurities are already present in non-stressed infusion solution) and to a neat solution (dotted line) of L-Ala-L-Gln with a similar concentration
as in infusion solutions. (A) AlaGlu(AlaGln); (B) AlaGlu; (C) AlaGluAlaGln; (D) AlaGln epimers (DL,LD)

Figure 7: HILIC-ESI-MS/MS chromatograms of the separation of degradation products on Polysulfoethyl A column. Peak annotation: (A) overlaid MRM traces of 1 cyclo(GlyTyr) (221 → 107); 2 cyclo(AlaGln) (200 → 155); 3 TyrGly (239 → 136); 4 pGluAlaHis (338 → 156); 5 cyclo(AlaGlu)His (338 → 156); (B) 6 AlaGlu(Arg); 7 AlaGluArg; (C) 8 AlaGlu(His); 9 AlaGluHis; (D) 10 AlaGlu(Lys); 11 AlaGluLys

Figure 8: HILIC-CAD (Charged Aerosol Detector) chromatogram illustrating separation of 1 GluAlaArg; 2 AlaGlu(Arg) ; 3 AlaGluArg in a standard solution on Polysulfoethyl A employing mobile phase conditions as specified in the Experimental section.

Figure 9: RPLC-ESI-MS/MS chromatograms of the separation of degradation products on a Gemini C18 column. Peak annotation: (A) 1 $(AcCys)_2$; (B) 2 pyroGluAlaMet; 3 cyclo(AlaGlu)Met; (C) 4 cyclo(AlaGlu)GlyTyr; 5 pyroGluAlaGlyTyr

Figure 1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pyroGluAlaY
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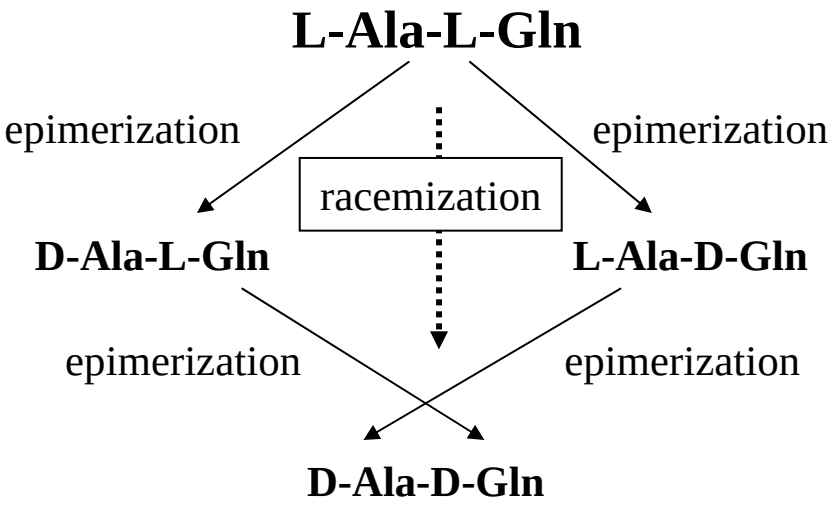
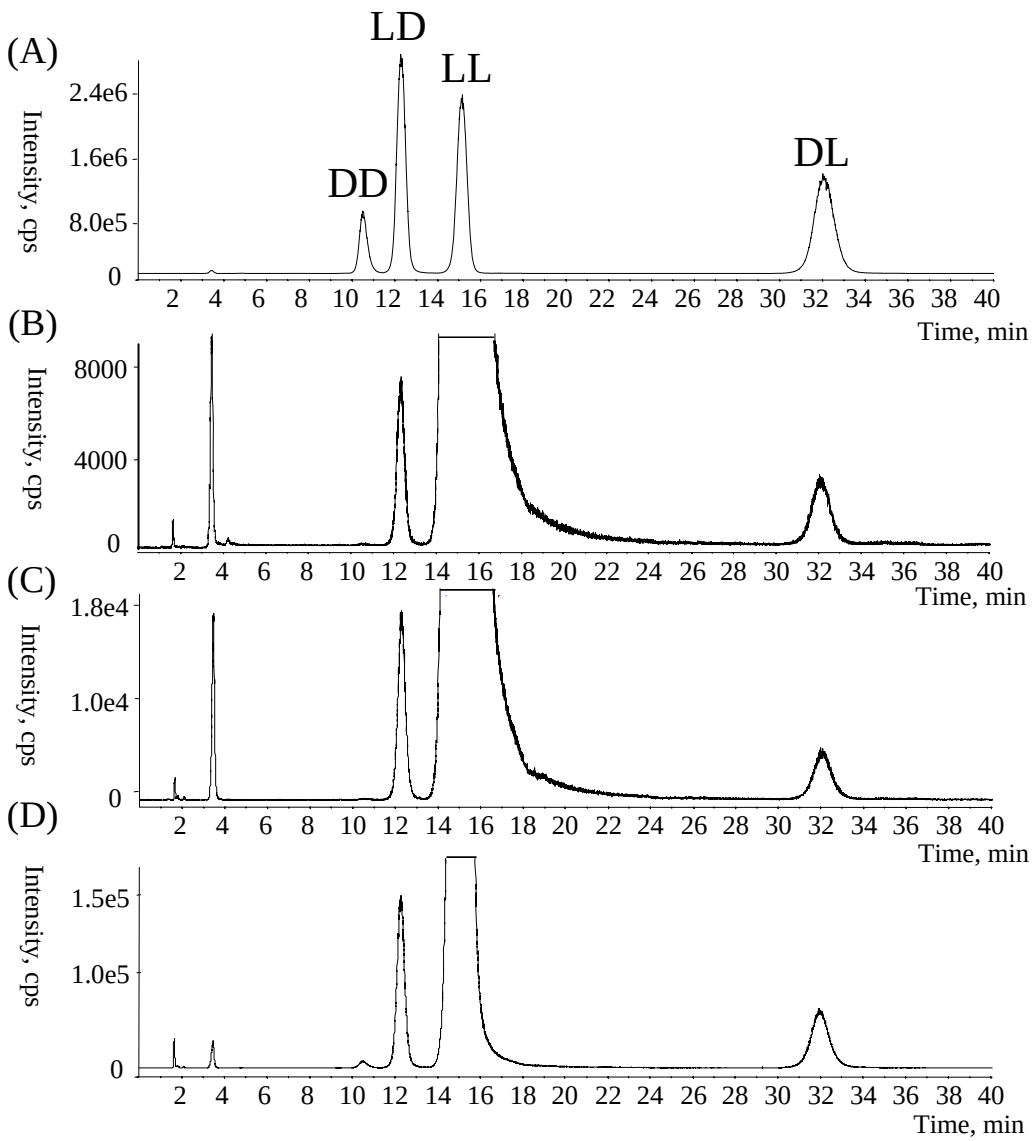

L-Ala-L-Gln
epimerization
racemization
epimerization
D-Ala-L-Gln
L-Ala-D-Gln
epimerization
epimerization
D-Ala-D-Gln

Figure 2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DD
DL
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1.6e6
8.0e5
0
Intensity, cps
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Time, min
(B)
8000
4000
0
Intensity, cps
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Time, min
(C)
1.8e4
1.0e4
0
Intensity, cps
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Time, min
(D)
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1.0e5
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Intensity, cps
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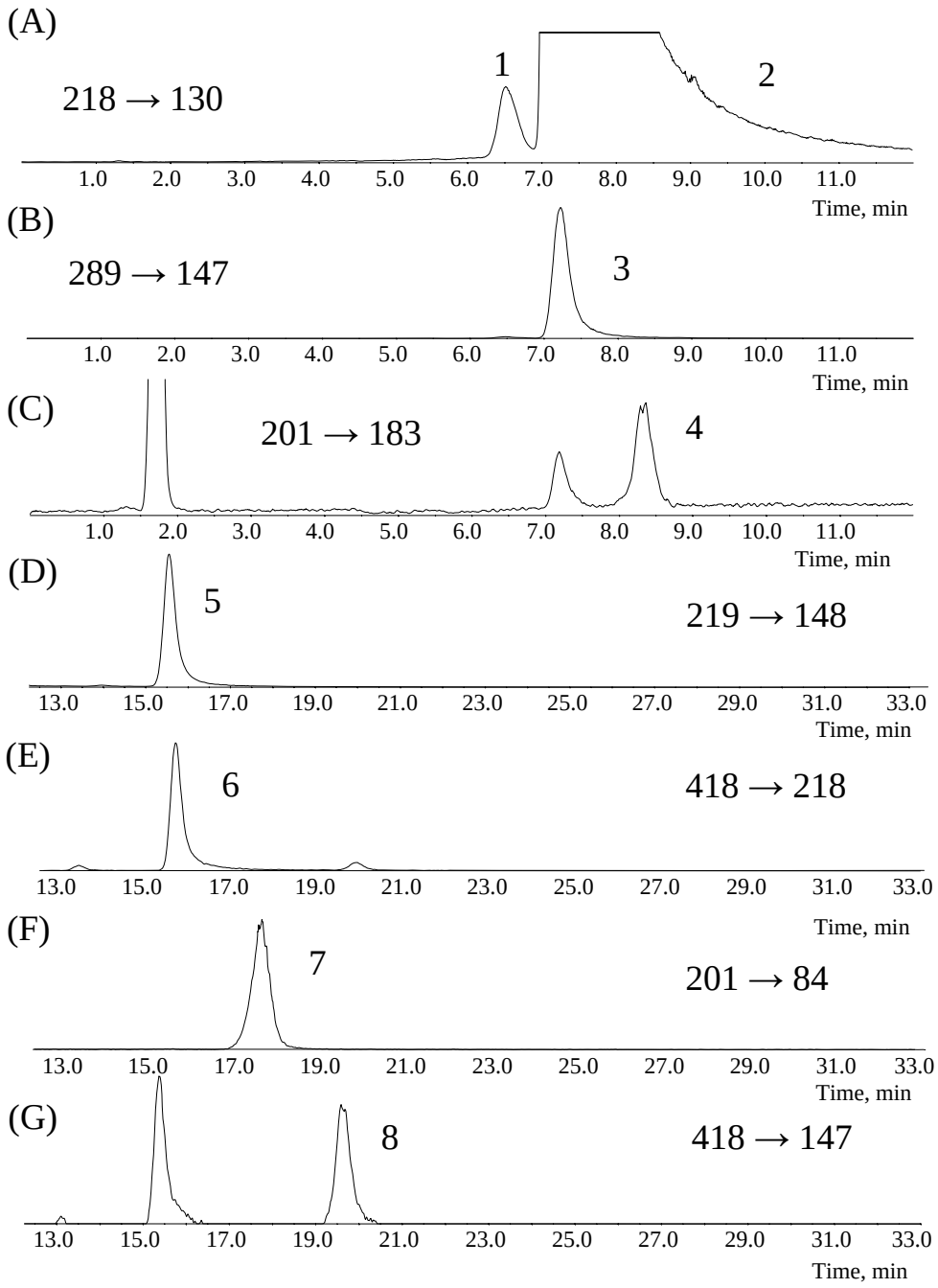
Time, min

Figure 3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2
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Time, min
(B)
289 → 147
3
1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0
Time, min
(C)
201 → 183
4
1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0
Time, min
(D)
5
219 → 148
13.0 15.0 17.0 19.0 21.0 23.0 25.0 27.0 29.0 31.0 33.0
Time, min
(E)
6
418 → 218
13.0 15.0 17.0 19.0 21.0 23.0 25.0 27.0 29.0 31.0 33.0
Time, min
(F)
7
201 → 84
13.0 15.0 17.0 19.0 21.0 23.0 25.0 27.0 29.0 31.0 33.0
Time, min
(G)
8
418 → 147
13.0 15.0 17.0 19.0 21.0 23.0 25.0 27.0 29.0 31.0 33.0
Time, min

Figure 4

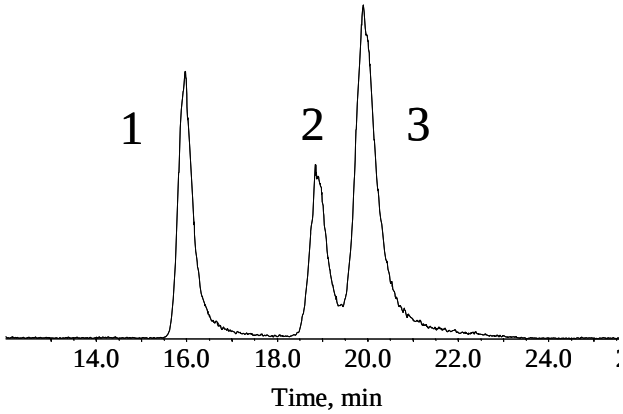

Figure 5

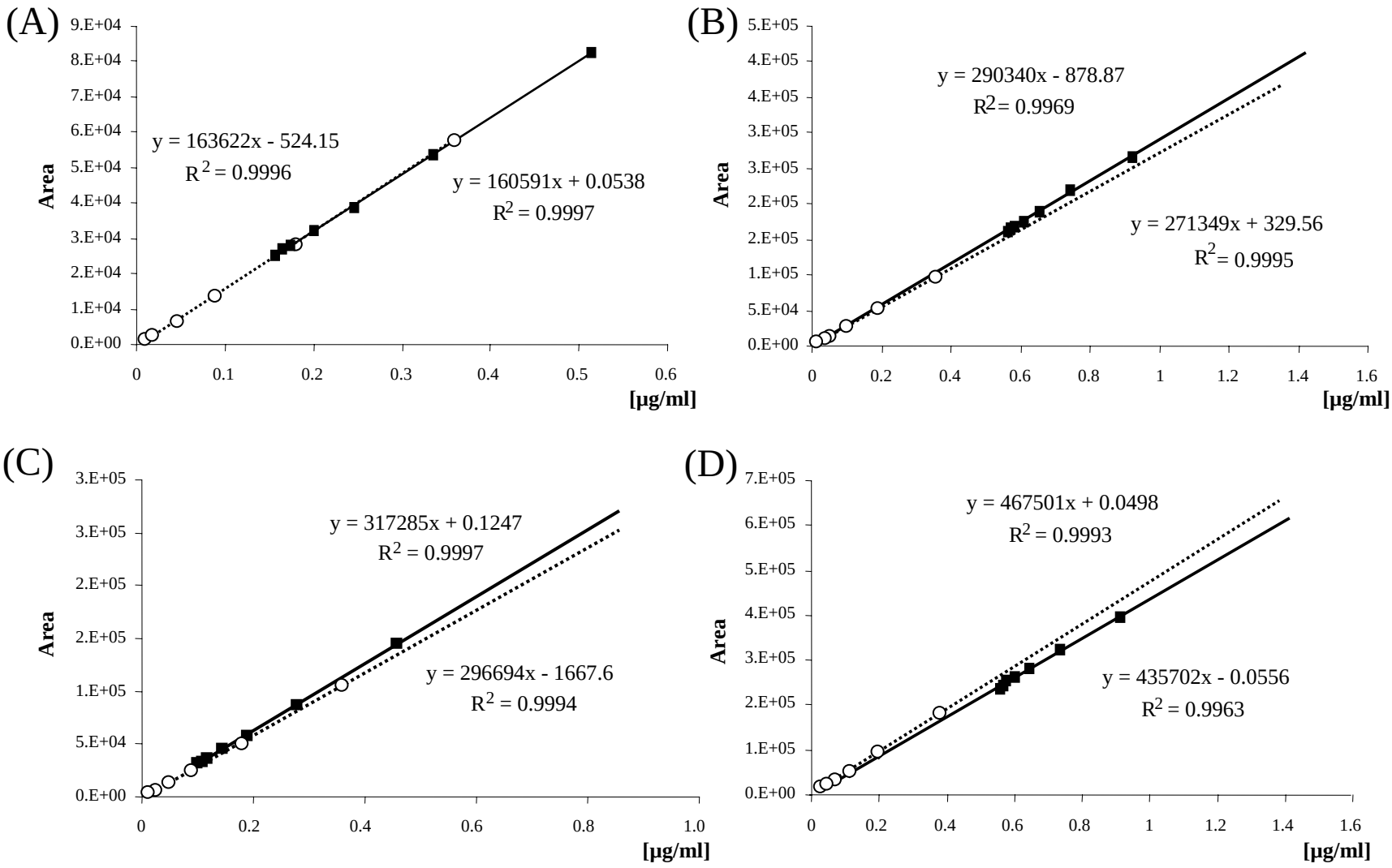
(A)
y = 163622x - 524.15
R 2 = 0.9996
y = 160591x + 0.0538
R2 = 0.9997
Area
[µg/ml]
(B)
y = 290340x - 878.87
R2= 0.9969
y = 271349x + 329.56
R2= 0.9995
Area
[µg/ml]
(C)
y = 317285x + 0.1247
R2 = 0.9997
y = 296694x - 1667.6
R2 = 0.9994
Area
[µg/ml]
(D)
y = 467501x + 0.0498
R2 = 0.9993
y = 435702x - 0.0556
R2 = 0.9963
Area
[µg/ml]

Figure 6

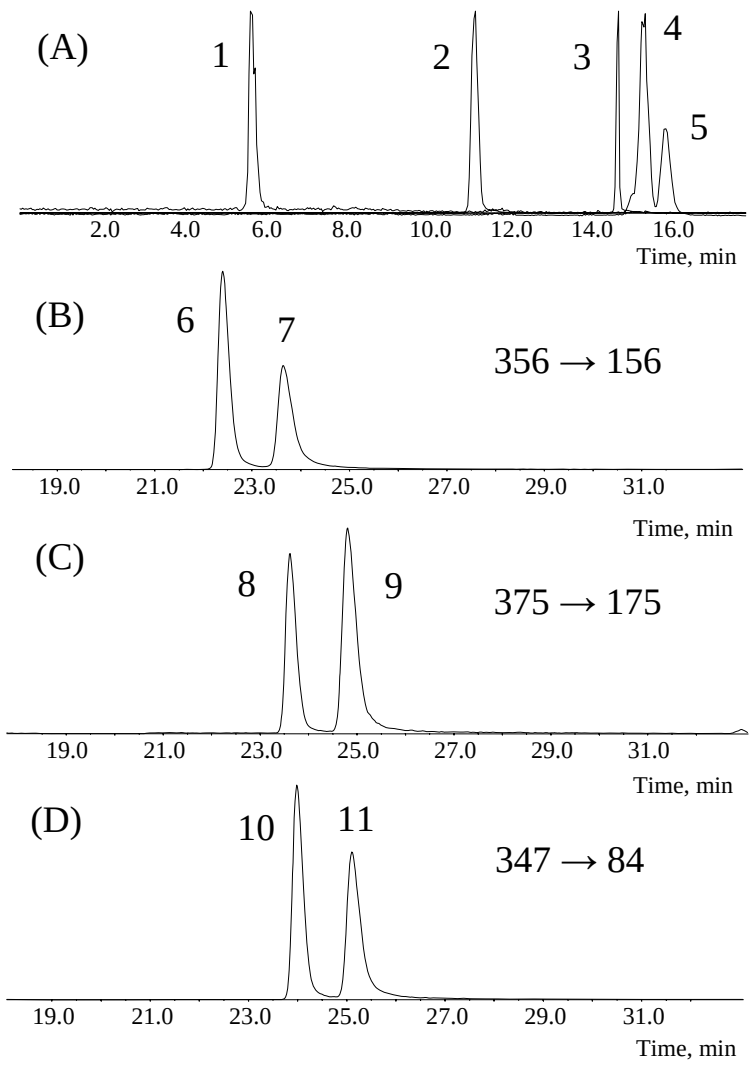
(A)
1
2
3
4
5
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
Time, min
(B)
6
7
356 → 156
19.0
21.0
23.0
25.0
27.0
29.0
31.0
Time, min
(C)
8
9
375 → 175
19.0
21.0
23.0
25.0
27.0
29.0
31.0
Time, min
(D)
10
11
347 → 84
19.0
21.0
23.0
25.0
27.0
29.0
31.0
Time, min

Figure 7

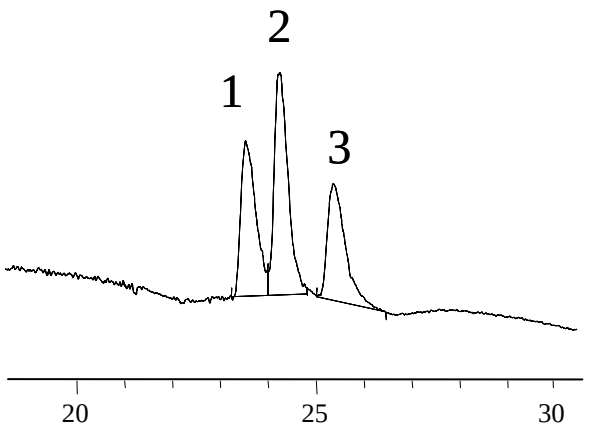

Figure 8

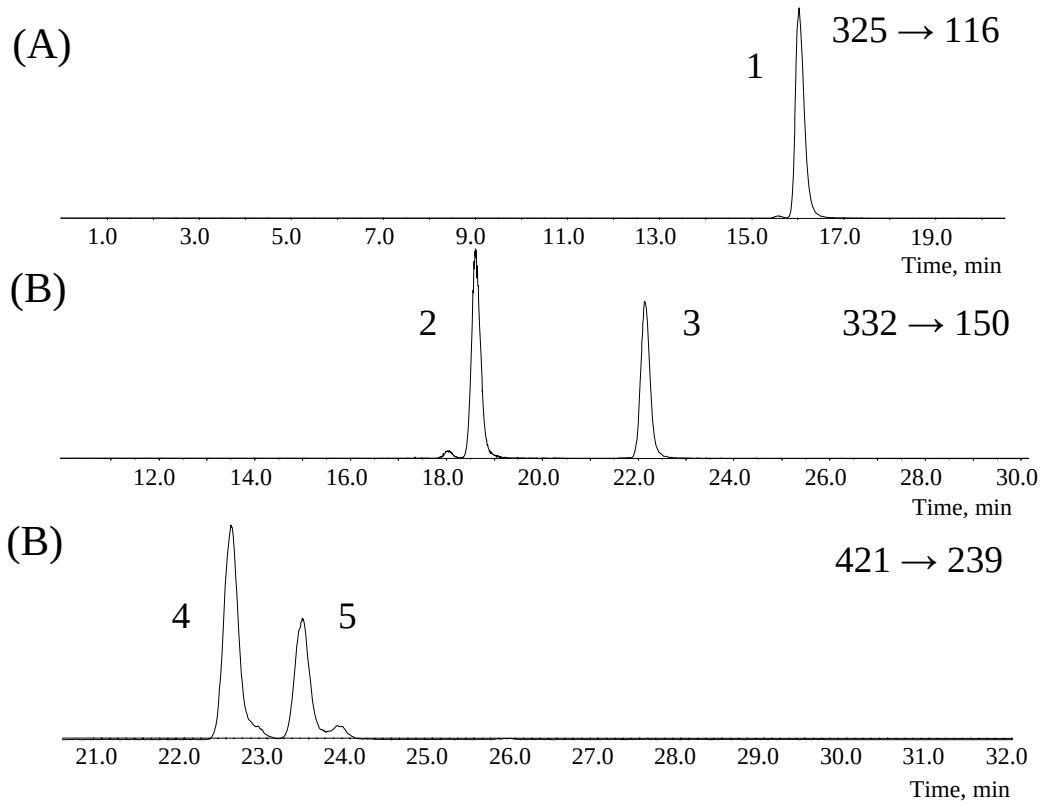
(A)
325 → 116
1
1.0
3.0
5.0
7.0
9.0
11.0
13.0
15.0
17.0
19.0
Time, min
(B)
2
3
332 → 150
12.0
14.0
16.0
18.0
20.0
22.0
24.0
26.0
28.0
30.0
Time, min
(B)
421 → 239
4
5
21.0
22.0
23.0
24.0
25.0
26.0
27.0
28.0
29.0
30.0
31.0
32.0
Time, min

Figure 9

Table 1: List of investigated compounds along with their respective analysis methods and specific MS-parameters.
With only one exception (qualifier of cyclo(GlyTyr)) all transitions were measure in the positive polarity mode. Fragments in italic were used as quantifier transitions.

Analyte	m/z Precursor	m/z Product Ion	DP [V]	CE [V]	CXP [V]	tr [min]	Method	Period
AlaGln epimers (DL/LD)	218.1	*130.0* 84.2	51	*27* 41	*8* 4	7.2	Chiralpak QN-AX	1
AlaAlaGln	289.1	*147.2* 130.1	46	*19* 33	*8* 6	7.4	Chiralpak QN-AX	1
cyclo(AlaGlu)	201.1	*183.0* 155.0	46	*15* 21	*10* 8	8.4	Chiralpak QN-AX	1
pyroGluAla	201.2	*84.1* 90.1	56	*29* 17	*14* 14	17.6	Chiralpak QN-AX	2
AlaGlu	219.1	*148.1* 84.1	56	*19* 41	*8* 14	15.5	Chiralpak QN-AX	2
AlaGluAlaGln	418.3	*218.1* 147.3	71	*23* 27	*12* 8	15.4	Chiralpak QN-AX	2
AlaGlu(AlaGln)	418.3	*147.3* 201.0	71	*27* 35	*8* 10	19.7	Chiralpak QN-AX	2
cyclo(GlyTyr)	221.1 219.1	*107.1* 113.0	61 -90	*31* -20	*6* -7	5.5	Polysulfoethyl A	1
cyclo(AlaGln)	200.0	*155.2* 183.1	46	*23* 15	*8* 10	11.0	Polysulfoethyl A	2
cyclo(AlaGlu)His	338.1	*156.1* 110.1	56	*23* 45	*8* 6	15.8	Polysulfoethyl A	2
pyroGluAlaHis	338.1	*156.1* 110.1	56	*23* 45	*8* 6	15.2	Polysulfoethyl A	2
TyrGly	239.1	*136.0* 91.0	36	*23* 51	*8* 14	14.3	Polysulfoethyl A	2
AlaGlu(His)	356.1	*156.1* 110.1	56	*23* 47	*8* 6	22.4	Polysulfoethyl A	3
AlaGluHis	356.1	*156.1* 110.1	56	*23* 47	*8* 6	23.7	Polysulfoethyl A	3
AlaGlu(Arg)	375.2	*175.1* 70.0	81	*31* 69	*10* 10	23.8	Polysulfoethyl A	3
AlaGluArg	375.2	*175.1* 70.0	81	*31* 69	*10* 10	25.1	Polysulfoethyl A	3
AlaGlu(Lys)	347.2	*84.1* 130.2	66	*67* 35	*14* 8	24.0	Polysulfoethyl A	3
AlaGluLys	347.2	*84.1* 130.2	66	67 35	*14* 8	25.1	Polysulfoethyl A	3
(AcCys)2	325.0	*116.1* 162.1	41	*49* 27	*6* 8	16.0	RP-18 Gemini	1
pyroGluAlaMet	332.3	*150.1* 104.1	41	*17* 29	*8* 6	18.6	RP-18 Gemini	1
cyclo(AlaGlu)Met	332.3	*150.1* 104.1	41	*17* 29	*8* 6	22.2	RP-18 Gemini	2

cyclo(AlaGlu)-GlyTyr	421.2	*239.0* 136.2	66	*19* 47	*14* 6	22.6	RP-18 Gemini	2
pyroGluAla-GlyTyr	421.2	*239.0* 136.2	66	*19* 47	*14* 6	23.4	RP-18 Gemini	2

[1] Declustering potential
[2] Collision energy
[3] Cell exit potential
[4] Retention time

Table 2: Relative peak areas (%) of isomeric forms of AlaGln determined for three differently treated infusion solutions.

Infusion solution	DD [%]	LD [%]	LL [%]	DL [%]
non-stressed	< 0.01	0.14	99.73	0.13
stored at 40°C/12 months	< 0.01	0.21	99.66	0.13
stored at 60°C/9 months	0.08	1.93	96.47	1.53

Table 3: Calibration functions, linear range, and LOQ of impurities determined by the optimized HILIC method using a Chiralpak QN-AX column.

Compound	Linear range[1] [µg/ml]	LOQ[2] [µg/ml]	Spiked quantity[3] [µg]	Measurement range[4] [µg/ml]	Corrected calibration function[5]		
					Slope	Intercept	R^2
AlaGln epimers (DL/LD)	0.005 - 5.00	0.005	0.05 - 5.00	0.55 - 5.05	4.25E+05	98.17	0.9999
AlaAlaGln	n.d. [6]	0.005	0.05 - 5.00	0.28 - 4.78	8.76E+05	-54.68	1.0000
cyclo(AlaGlu)	0.50 - 5.00	0.500	0.05 - 5.00	0.16 - 4.66	8.10E+04	-15.90	0.9995
pyroGluAla	0.50 - 5.00	0.500	0.40 - 40.00	2.01 - 38.01	6.15E+04	0.89	0.9999
AlaGlu	0.025 - 5.00	0.025	0.05 - 5.00	1.26 - 5.76	4.08E+05	-187.46	0.9998
AlaGluAlaGln	0.025 - 5.00	0.025	0.05 - 5.00	0.14 - 4.64	4.59E+05	104.03	0.9986
AlaGlu(AlaGln)	0.10 - 5.00	0.100	0.05 - 5.00	0.21 - 4.71	1.41E+05	-3.14	0.9998

[1] The linear range was determined in preliminary calibration experiments using neat standard solutions.
[2] LOQ was determined with standard solutions using an injection volume of 10 µl. The LOQ was defined as the concentration where the quantifier yields a signal to noise ratio of 10:1 and the qualifier at least 3:1.
[3] 100 µl of spiking standard were added to 1 ml of 1:50 diluted sample.
[4] The measurement range constitutes the concentration range that was effectively measured. It is calculated as the sum of the spiked quantity and the concentration of the analyte already present in the sample solution.
[5] Calibration was accomplished using standard addition. Generated calibration functions were corrected for the concentration of the analyte already present in the sample.
[6] n.d. not determined

Table 4: Intra- and interday precision and accuracy determined for the HILIC method employing a Chiralpak QN-AX column. Experiments were carried out on three different days using three concentration levels (n=3).

	Precision [%RSD]				**Accuracy [%]**			
Spiking level [µg]	**intra-assay**			**inter-assay**	**intra-assay**			**inter-assay**
AlaGln epimers (DL/LD)	**day 1**	**day 2**	**day 3**	**interday**	**day 1**	**day 2**	**day 3**	**interday**
0.05	1.8	6.1	1.4	3.7	100.0	99.6	100.2	99.9
0.5	0.9	1.0	1.0	1.9	98.9	100.6	98.4	99.3
2.5	0.8	1.3	0.9	1.1	100.7	99.4	100.9	100.3
AlaAlaGln								
0.05	1.6	6.8	0.5	9.4	101.8	99.4	101.9	101.1
0.5	0.4	0.5	0.9	4.9	98.7	99.7	98.8	99.1
2.5	1.0	0.5	0.9	1.9	100.3	101.5	99.9	100.5
cyclo(AlaGlu)								
0.05	2.3	2.8	6.2	6.4	102.3	102.4	110.6	105.1
0.5	0.8	1.7	1.5	3.3	100.3	97.7	96.9	98.3
2.5	2.5	1.2	1.9	3.0	97.4	101.1	100.2	99.5
pyroGluAla								
0.05	2.6	5.6	0.0	8.2	100.8	87.6	100.3	96.2
0.5	0.8	1.7	1.9	7.8	100.3	103.4	99.5	101.0
2.5	0.7	3.5	0.7	3.7	102.1	104.8	100.2	102.4
AlaGlu								
0.05	0.2	4.0	0.4	1.5	99.3	99.8	100.4	99.8
0.5	0.4	0.6	1.0	0.6	98.7	100.7	99.6	99.7
2.5	1.0	1.0	0.4	1.4	97.4	101.2	99.9	99.5
AlaGluAlaGln								
0.05	0.5	1.3	0.7	6.9	109.0	127.6	103.4	113.3
0.5	1.1	0.8	0.8	1.9	99.2	104.3	97.9	100.5
2.5	0.5	0.5	1.4	1.6	99.4	102.9	100.1	100.8
AlaGlu(AlaGln)								
0.05	2.1	3.1	0.3	6.7	105.3	101.7	116.4	107.8
0.5	0.4	1.1	0.6	3.9	96.4	98.1	99.2	97.9
2.5	0.9	0.7	0.3	2.4	98.3	101.3	99.4	99.6

Table 5: Calibration functions, linear range and LOQ of impurities determined by the optimized HILIC method using Polysulfoethyl A column.

Impurity	Linear range[1] [µg/ml]	LOQ[2] [µg/ml]	Spiked quantity[3] [µg]	Measurement range[4] [µg/ml]	Corrected calibration function[5]		
					Slope	Intercept	R^2
cyclo(GlyTyr)	0.05 - 5.0	0.05	0.05 - 2.0	3.79 - 47.43	2.66E+05	663.35	0.9987
cyclo(AlaGln)	0.013 - 5.0	0.013	0.013 - 2.0	34.74 - 51.10	6.75E+05	1.00E+07	0.9897
TyrGly	0.025 - 5.0	0.025	0.025 - 2.0	1.49 - 3.29	8.81E+04	-0.23	0.9950
AlaGluHis	0.025 - 5.0	0.025	0.025 - 2.0	0.10 - 1.90	3.62E+05	-0.37	0.9995
AlaGlu(His)	0.025 - 5.0	0.025	0.025 - 2.0	0.67 - 2.47	4.97E+05	-0.80	0.9998
AlaGluArg	0.05 - 5.0	0.05	0.05 - 2.0	0.09 - 1.89	2.07E+05	0.34	0.9991
AlaGlu(Arg)	0.05 - 5.0	0.05	0.05 - 2.0	0.22 - 2.02	1.41E+05	-0.03	0.9996
AlaGluLys	0.05 - 5.0	0.05	0.05 - 2.0	0.11 -1.91	2.58E+05	0.28	0.9992
AlaGlu(Lys)	0.05 - 5.0	0.05	0.05 - 2.0	0.17 - 1.97	3.86E+05	-0.32	0.9995

[1] The linear range was determined in preliminary calibration experiments using neat standard solutions.
[2] LOQ was determined with standard solutions using an injection volume of 10 µl. The LOQ was defined as the concentration where the quantifier yields a signal to noise ratio of 10:1 and the qualifier at least 3:1.
[3] 100 µl of spiking standard were added to 1 ml of 1:20 diluted sample.
[4] The measurement range constitutes the concentration range that was effectively measured. It is calculated as the sum of the spiked quantity and the concentration of the analyte already present in the sample solution.
[5] Calibration was accomplished using standard addition. Generated calibration functions were corrected for the concentration of the analyte already present in the sample.

Table 6: Intra- and interday precision and accuracy for the HILIC method on Polysulfoethyl A determined at three concentration levels performing three consecutive runs on three different days.

	Precision [%RSD]				Accuracy [%]			
Spiking level [µg]	**intra-assay**			**inter-assay**	**intra-assay**			**inter-assay**
cyclo(GlyTyr)	**day 1**	**day 2**	**day 3**	**interday**	**day 1**	**day 2**	**day 3**	**interday**
2.0	9.7	6.0	2.7	4.0	96.7	106.9	101.1	101.6
10.0	9.3	6.7	1.7	3.1	94.7	96.6	98.4	96.6
100.0	2.8	3.4	8.1	1.1	109.3	108.8	110.5	109.6
cyclo(AlaGlu)								
2.0	1.1	0.8	2.2	7.0	103.1	100.1	119.0	107.4
10.0	3.6	0.4	2.2	5.2	103.2	99.5	116.4	106.4
100.0	0.1	0.6	1.4	4.7	88.0	81.0	95.8	88.3
TyrGly								
0.02	7.3	3.7	3.5	1.9	100.4	101.2	106.4	102.6
0.10	3.2	1.6	1.4	4.4	98.3	96.8	97.0	97.4
1.00	4.1	1.8	2.0	3.3	101.8	100.3	100.0	100.7
AlaGluHis								
0.02	1.8	0.7	1.6	8.3	103.4	103.6	99.8	102.3
0.10	0.6	1.4	1.6	6.3	93.4	99.0	98.8	97.1
1.00	0.3	1.4	1.2	1.3	101.3	102.5	101.7	101.8
AlaGlu(His)								
0.02	0.6	0.6	1.2	6.8	100.0	101.1	100.3	100.5
0.10	1.0	1.3	0.9	4.8	100.3	98.1	98.6	99.0
1.00	0.4	0.5	0.4	3.2	100.4	101.1	100.1	100.5
AlaGluArg								
0.02	5.3	1.1	4.1	3.1	109.8	98.7	111.5	106.7
0.10	1.1	1.5	3.3	6.0	99.2	102.2	103.5	101.7
1.00	1.3	1.1	4.2	2.7	101.1	103.2	99.4	101.2
AlaGlu(Arg)								
0.02	0.2	1.3	2.5	14.9	100.0	100.2	98.8	99.7
0.10	0.4	0.3	1.6	12.7	100.4	100.4	96.8	99.2
1.00	0.9	1.8	0.5	2.1	102.5	101.5	102.8	102.3
AlaGluLys								
0.02	2.4	2.3	1.3	5.5	105.1	103.0	100.4	102.8
0.10	1.0	1.8	2.3	3.8	91.5	97.7	99.5	96.2
1.00	1.3	1.1	1.9	0.6	101.7	103.5	104.7	103.3
AlaGlu(Lys)								
0.02	0.7	1.0	0.4	4.0	99.6	97.5	94.5	97.2
0.10	0.8	1.0	0.8	4.6	96.7	99.7	97.6	98.0
1.00	1.0	0.8	0.9	1.9	102.8	103.4	105.9	104.1

Table 7: Calibration functions, linear range and LOQ of impurities determined by the optimized RP method using a Gemini C18.

Impurity	Linear range[1] [µg/ml]	LOQ[2] [µg/ml]	Spiked quantity[3] [µg]	Measurement range[4] [µg/ml]	Corrected calibration function[5]		
					Slope	Intercept	R^2
$(AcCys)_2$	0.05 - 2.00	0.050	10.0 - 750.0	33.2 - 705.9	8.18E+03	0.87	0.9989
cyclo(GluAla)GlyTyr	0.025 - 2.00	0.025	0.02 - 1.50	0.09 - 1.43	3.30E+05	0.02	0.9925
pyroGluAlaGlyTyr	0.025 - 2.00	0.025	0.02 - 1.50	0.05 - 1.40	2.02E+05	0.0543	0.9929
cyclo(AlaGlu)Met	0.005 - 2.00	0.005	0.02 - 1.50	0.03 - 1.37	6.31E+05	-0.0385	0.9940
pyroGluAlaMet	0.005 - 2.00	0.005	0.02 - 1.50	0.02 - 1.36	8.52E+05	0.054	0.9929

[1] The linear range was determined in preliminary calibration experiments using neat standard solutions.
[2] LOQ was determined with standard solutions using an injection volume of 10 µl. The LOQ was defined as the concentration where the quantifier yields a signal to noise ratio of 10:1 and the qualifier at least 3:1.
[3] 100 µl of spiking standard were added to 1 ml of 1:20 diluted sample.
[4] The measurement range constitutes the concentration range that was effectively measured. It is calculated as the sum of the spiked quantity and the concentration of the analyte already present in the sample solution.
[5] Calibration was accomplished using standard addition. Generated calibration functions were corrected for the concentration of the analyte already present in the sample.

Table 8: Intra- and interday precision and accuracy obtained for the analysis method employing a Gemini C-18 column. Precision and accuracy were determined for three concentration levels performing three consecutive runs on three different days.

	Precision [%RSD]				**Accuracy [%]**			
Spiking level [µg]	**intra-assay**			**inter-assay**	**intra-assay**			**inter-assay**
$(AcCys)_2$	**day 1**	**day 2**	**day 3**	**interday**	**day 1**	**day 2**	**day 3**	**Interday**
10	1.7	1.6	1.9	39.9	91.0	139.3	231.6	153.9
50	5.9	1.3	0.9	10.9	97.1	85.8	88.8	90.6
500	1.4	0.8	0.4	13.1	100.3	102.2	120.8	107.8
c(AE)GY								
0.02	1.9	6.2	1.0	12.8	79.3	101.1	94.2	91.5
0.1	4.7	1.1	1.9	5.9	93.0	99.3	99.8	97.4
1	8.6	1.0	0.0	2.8	103.9	98.8	101.5	101.4
pEAGY								
0.02	0.9	3.3	0.9	14.9	62.4	95.5	88.4	82.1
0.1	5.2	5.1	0.3	4.3	91.3	100.8	100.9	97.7
1	9.8	0.7	1.3	3.4	105.3	100.1	99.9	101.7
c(AE)M								
0.02	2.8	1.6	3.2	9.7	58.0	97.3	91.7	82.3
0.1	1.0	2.2	0.4	4.2	86.9	98.6	102.0	95.8
1	7.8	0.5	0.2	2.5	103.2	99.8	99.9	101.0
pEAM								
0.02	1.6	1.1	1.0	6.1	58.9	193.2	91.2	114.4
0.1	0.7	3.8	1.0	3.3	90.8	118.9	103.7	104.5
1	5.4	1.3	5.2	4.1	103.6	107.8	99.6	103.7

Table 9: Concentration, standard deviation and % concentration of impurities relative to main precursor compounds identified and quantified in infusion solutions stored at 40°C for different time spans (3,6 and 12 months).
bold letters: > reporting threshold; *italic*: > identification threshold; <u>underlined</u>: > qualification threshold

	Solution 40°C/3 months			Solution 40°C/6 months			Solution 40°C/12 months		
Impurity	**[µg/ml]**	**Std.dev. [µg/ml]**	**%[1]**	**[µg/ml]**	**Std.dev. [µg/ml]**	**%[1]**	**[µg/ml]**	**Std.dev. [µg/ml]**	**%[1]**
AlaGln epimers (DL/LD)	*31.7*	0.6	*0.144*	<u>38.0</u>	n.d.[3]	<u>0.173</u>	<u>78.2</u>	3.3	<u>0.355</u>
AlaAlaGln	**16.7**	0.3	**0.076**	**17.5**	n.d.	**0.080**	*23.0*	1.0	*0.104*
cyclo(AlaGlu)	9.2	0.4	0.042	**15.6**	n.d.	**0.071**	<u>76.7</u>	4.9	<u>0.348</u>
AlaGluAlaGln	6.9	0.3	0.031	7.7	n.d.	0.035	**16.9**	0.5	**0.077**
AlaGlu(AlaGln)	**11.5**	0.3	**0.052**	**17.4**	n.d.	**0.079**	<u>37.8</u>	0.7	<u>0.172</u>
AlaGlu	<u>63.6</u>	1.2	<u>0.289</u>	<u>116.5</u>	n.d.	<u>0.530</u>	<u>328.9</u>	7.2	<u>1.495</u>
pyroGluAla	<u>111.7</u>	5.7	<u>0.508</u>	<u>121.9</u>	n.d.	<u>0.554</u>	<u>309.7</u>	8.3	<u>1.408</u>
cyclo(GlyTyr)	<u>45.1</u>	4.4	<u>1.670</u>	<u>60.2</u>	11.5	<u>2.228</u>	n.d.[2]	n.d.[2]	n.d.[2]
cyclo(AlaGln)	<u>752.3</u>	101.2	<u>3.419</u>	<u>807.6</u>	58.9	<u>3.671</u>	n.d.	n.d.	n.d.
TyrGly	<u>31.1</u>	9.9	<u>1.150</u>	<u>27.4</u>	9.8	<u>1.016</u>	n.d.	n.d.	n.d.
AlaGlu(His)	9.2	0.6	0.042	**11.9**	0.5	**0.054**	n.d.	n.d.	n.d.
AlaGluHis	1.1	0.1	0.005	1.3	0.2	0.006	n.d.	n.d.	n.d.
AlaGlu(Arg)	2.9	0.1	0.013	2.9	0.2	0.013	n.d.	n.d.	n.d.
AlaGluArg	1.0	0.2	0.004	0.9	0.2	0.004	n.d.	n.d.	n.d.
AlaGlu(Lys)	2.0	0.1	0.009	2.1	0.1	0.010	n.d.	n.d.	n.d.
AlaGluLys	1.0	0.1	0.005	1.0	0.2	0.004	n.d.	n.d.	n.d.
(AcCys)2	<u>147.8</u>	21.5	<u>14.778</u>	<u>239.0</u>	n.d.[3]	<u>23.897</u>	<u>4151.7</u>	212.0	<u>415.168</u>
cyclo(AlaGlu)GlyTyr	0.39	0.03	0.002	0.78	n.d.	0.004	3.25	0.23	0.015
pyroGluAlaGlyTyr	0.16	0.03	0.001	0.38	n.d.	0.002	1.14	0.05	0.005
cyclo(AlaGlu)Met	0.02	0.01	0.0001	0.03	n.d.	0.0001	0.13	0.005	0.001
pyroGluAlaMet	< LOQ	< LOQ	< LOQ	0.005	n.d.	0.00002	0.02	0.01	0.0001

[1] % concentration relative to the precursor compound present at higher concentrations in the infusion solutions
[2] n.d. - not determined

Appendix III

Manuscript

Multi-target metabolic profiling of hydrophilic metabolites in fermentation broths of β-lactam antibiotics production by HILIC-ESI-MS/MS

Multi-target metabolic profiling of hydrophilic metabolites in fermentation broths of β-lactam antibiotics production by HILIC-ESI-MS/MS

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Abstract

The presented work deals with the development and comprehensive validation of a quantitative LC-ESI-MS/MS method using a triple quadrupole in the MRM mode for the metabolic profiling of amino acids, organic acids, vitamins, some biogenic amines, secondary metabolites of β-lactam antibiotics biosynthesis as well as their intermediates and degradation products in fermentation broths of β-lactam antibiotics production (in total 57 polar, hydrophilic compounds). A great number of chromatographic systems (22 different stationary phase/mobile phase conditions) were screened for their adequate chromatographic selectivity to cope with isobaric compounds and other critical analyte pairs. Finally, a HILIC method employing a zwitterionic ZIC-HILIC column was selected. Particular focus was given on the elucidation of absolute and relative matrix effects via comparison of slopes of calibration functions of spiked matrix and standard solutions. These data as well as precision and accuracy data confirm suitability of the HILIC-MS/MS assay for metabolic profiling studies in fermentation samples. Detailed comprehensive data sets are presented which should illustrate critical issues, problems and challenges of multi-target quantitative metabolic profiling, and should outline possible strategies to circumvent pitfalls and overcome common problems.

Keywords: microbial metabolomics, fermentation, process analysis technology (PAT), process control, β–lactam antibiotics, amino acids, organic acids, HILIC, HPLC-MS/MS, matrix effects, matrix-matched calibration

1. Introduction

Despite growing problems with resistances β-lactam antibiotics nowadays still represent the world's most important antibiotics class with a share of about 65% in sales of the total world market for antibiotics [1]. Amongst β-lactam antibiotics penicillin V and cephalosporin C are major products which are produced on industrial scale by fermentation processes that have been optimized over more than 50 years or so. In fact, penicillin and cephalosporin production are examples of success stories of biotechnology. There are many factors that have contributed to the exceptional productivity (including selection of efficient strains of microorganisms, their specific genetical engineering, dedicated optimization of various fermentation process parameters) which have lead to harvest titers with > 40 g/L penicillin as compared to about 0.5 – 1.0 g/L 50 years ago [1]. Last but not least downstream processing steps have undergone also significant changes and were optimized to reach recovery yields of more than 90% so that the costs of penicillins and cephalosporins could be greatly reduced.
During the fermentation process, which takes place in reactors encompassing capacities of up to 300 000 liters, microorganisms must be provided with sufficient energy and precursor compounds for biosynthesis. Thus, intensive monitoring of various compounds in the fermentation broth is necessary to keep the complete fermentation process under control and to generate optimal conditions for high production efficiency. While the major goal of process analysis technology (PAT) is to enhance understanding and control of the manufacturing process, it may thereby lead to further optimization. In view of this, metabolomics approaches are evolving and are becoming more popular nowadays in the context of PAT and process optimization in biotechnology [2]. The information gained from such metabolomics studies may help to identify substrate shortages as well as limiting and inhibiting biosynthesis steps, respectively. Furthermore, extended metabolic network modelling of biosynthesis pathways requires besides biological information (K_m, V_{max}, K_i) reliable experimental data on enzyme activities as well as substrate and product concentrations, which need to be measured accurately and reliably. Eventually these metabolomics data reveal metabolic engineering targets e.g. for strain and process optimization.

From an analytical point of view, metabolomics deals with the qualitative and quantitative analysis of intra- and extracellular metabolites, usually with the goal of a comprehensive strategy. In practice, however, it may be distinguished between different methodologies:

(1) *Metabolic fingerprinting* performs comparative-semiquantitative or qualitative studies of the whole set of intracellular metabolites (global screening approach) [3,4] . The aim of this strategy is not to identify every peak detected but rather to uncover peak clusters and patterns in which samples of different biological status or origin differ. Acquired data are evaluated with the help of statistical methods like principal component analysis (PCA) or partial least squares discriminant analysis (PLS-DA). This type of analysis finds wide application e.g. in biomarker discovery [5,6] or as tool for bacterial characterization.

(2) Similarly, *metabolic footprinting* is a comprehensive metabolic profiling methodology that actually follows the same principles as metabolic fingerprinting with the difference that footprinting is focused on the analysis of extracellular metabolites [7,8]. It is of special importance for cell strain development in biotechnological productions [2,9,10].

(3) *Metabolic profiling* aims on quantitative analysis of a predefined set of analytes, which may be involved in selected biochemical pathways or which belong to certain metabolite classes [2,3,11].

(4) The last approach, *targeted metabolic profiling*, is the most specific one and deals with the quantiation of only a few metabolites like substrate and/or product metabolites of a target protein [2,3].

In spite of great advances in the last years metabolic studies are still facing several problems and there is actually no single analysis technique that could ideally cope with the comprehensive analysis of the entirety of metabolites in a biological system. The difficulties are in the first place related to the nature and complexity of the samples. For example, the metabolome of saccharomyces cervisiae is estimated to comprise about 600 metabolites and the human genome is estimated to encompass more than 2,000 major metabolites [3]. Furthermore, metabolites strongly differ in their chemical and physicochemical attributes, as well as in their relative abundances. Thus, there is a strong demand for analytical methods with adequate selectivity, high peak capacity and wide dynamic range. Analysis of such a multitude of compounds usually requires combination of highly selective chromatographic separation methods like gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE) with detection techniques that afford effective discrimination of compound structures like mass spectrometry (MS), Fourier transform infrared (FT-IR)

spectroscopy or nuclear magnetic resonance (NMR) spectroscopy, thus adding an additional dimension of selectivity and information, respectively [4].

Application of GC is most suitable for apolar, hydrophobic, thermally stable compounds. Hence, the analysis of polar metabolites by GC-MS requires derivatisation in order to increase compound volatility and improve thermal stability [12,13]. It is frequently employed nowadays for metabolic studies [3,14-16] and is becoming more powerful as GCxGC-MS/MS is routinely implemented [17-19]. On contrary, CE is a separation technique that is highly suitable for the separation of ionic polar compounds. Unfortunately, CE suffers from poor migration time reproducibility, low sensitivity and difficulties encountered in interfacing CE with MS detectors and is thus of limited applicability [20,21].

In the past years, LC methods coupled to MS have gained much importance and advanced to methods of first choice for metabolomic applications. This is also due to the development of highly efficient electrospray interface technology in the early 1990s that acts as robust and versatile ionization source exhibiting wide applicability. Moreover various mass analyzers have proven their suitability for metabolic studies [14]. After all, the choice of the mass analyzer is mainly governed by the purpose of application. Considering targeted quantitative analysis of a predefined set of analytes, as in the presented work, mass analyzers like triple quadrupole or hybrid triple quadrupole/linear ion trap (Q-Trap) exploiting the multiple reaction monitoring (MRM) mode are widely used and seem to be preferable, as they are very robust and provide a wide dynamic range. For direct infusion experiments with the purpose of e.g. metabolic fingerprinting high resolution and mass accuracy are essential, which can be provided e.g. by fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) as well as by Orbitrap [22-24].

Despite all benefits implementation of ESI brought with, there is still the inherent problem of matrix effects caused by coeluting non-detected compounds occurring in biological matrices that may interfere with analyte detection through ionization suppression or enhancement.

One approach to get rid of matrix effects aims at the reduction of the sample complexity, which can be achieved by applying most often laborious sample preparation techniques like protein precipitation or analyte preconcentration using solid phase extraction (SPE) or liquid liquid extraction (LLE). Unfortunately, these procedures are prone to analyte losses as well as to sample modifications and are thus usually not applicable for metabolic studies [3,25].

Improvement of chromatographic separations has also high potential to reduce the risk of matrix effects. High efficiency, selectivity and peak capacity contribute to better separation of compounds, eventually preventing coelution with matrix compounds. One strategy to achieve

this is by reducing particle diameters. Ultra performance liquid chromatography (UPLC) uses small particle diameters (< 3.0 μm) and tolerates high backpressures (up to 100 MPa). This way a reduction of peak broadening can be effected which is accompanied by an increase of sensitivity. Peak widths of about 1-2 seconds can be achieved, which consequently reduces the probability for coelution with matrix compounds but at the same time increases requirements on the speed of data acquisition in order to provide enough data points for reliable peak description [24,26] .

Another strategy to compensate for matrix effects represents the use of isotope labeled internal standards for each solute which coelute with the target compound and are subjected to the same ionization suppression/enhancement effects [27-31]. Besides, reliability and robustness of methods may be improved thereby by compensation for instrumental fluctuations. However, isotope labeled internal standards are not always available and are expensive minimizing their applicability especially if a large number of analytes must be measured such as in metabolic studies. One approach to overcome these barriers constitutes the use of ^{13}C labeled extracts, which can be obtained from cultivations of cells on ^{13}C labeled substrates e.g. uniformly ^{13}C labeled glucose. ^{13}C labeled extracts have already been successfully utilized for metabolomic studies [32-34].

Last but not least matrix-matched calibration by standard addition may also constitute a useful approach to compensate for matrix effects [35-39]. Thereby distinct amounts of standards are spiked to the sample matrix. Thus, the standard compounds experience the same impairment through the matrix as the analyte in the corresponding sample, while the slope (indicative for the detector response) in this matrix is different from a plain standard solution.

Along this line, the objective of the present work was to develop a metabolic profiling strategy for the accurate and reliable quantitative analysis of diverse extracellular primary and secondary metabolites as well as nutritional compounds in fermentation broths from β-lactam antibiotics production. The broad set of compounds encompassed amino acids, organic acids, vitamins, fatty acids and secondary metabolites of the β–lactam biosynthesis. Due to the huge lipophilicity range that is spanned by these compounds (with log D values from – 6 to + 12) the initial idea of employing a single analysis method was quickly dropped and the target analyte set was divided into hydrophilic and lipophilic compounds. The results of the RPLC method for the latter analyte set will be described separately elsewhere [40].

Herein, we report on the method development and validation for the multi-target quantitative analysis of the hydrophilic metabolites by hydrophilic interaction liquid chromatography (HILIC)-MS/MS. Like other metabolites, penicillins and cephalosporins are excreted into the

medium during the fermentation [1] and can be analyzed in the extracellular space together with extracellular metabolites, nutrients, and intermediates as well as degradation products of the β–lactam antibiotics after whole broth extraction. The complete list of target solutes addressed herein is summarized in Table 1. This multi-target metabolic profiling assay of extracellular metabolites was supposed to be applied for the process control aiming in particular at the elucidation of substrate shortages in the fermentation process. Due to the relatively low mass resolution of the employed Q-trap mass spectrometer, specific attention was paid to a careful optimization of liquid chromatographic separation of critical peaks (including isobaric compounds and such that constitute potential interferences on other target analytes). From an initial screening, a HILIC method employing a zwitterionic bonded silica column emerged as best compromise for the given analyte set. Recently, HILIC chromatography has gained enormous popularity, as it enables separation of polar compounds like amino acids without previous derivatisation and furthermore shows excellent compatibility with MS detection [41-46]. It circumvents the problem of insufficient retention of hydrophilic compounds on RP materials and alleviates the necessity to use ion pair reagents like trifluoroacetic acid, tributylamine, hexylamine, octylammonium acetate, dibutylammonium acetate or perfluorinated carboxylic acids that have also been frequently tested in metabolomic assays [28,33,47-50]. Distinctive drawbacks of ion-pair RPLC comprise problems with reproducibilities, ionization suppression due to the ion pair reagents and faster ESI source contaminations due to salt deposits in the interface. Particular attention was paid to an extensive validation of parameters such as assay specificity, relative matrix effects, precision and accuracy. In particular, we wanted to evaluate whether precision and accuracies may be improved by use of internal standards and matrix-matched calibration by standard addition as compared to calibration with neat standard solutions.

2. Experimental

2.1. Chemicals

Amino acid standards purchased from Fluka (Buchs, Switzerland) were all L-configurated and > 99% purity (except lysine which was > 98%). Isoleucine (> 98%) was obtained from Sigma-Aldrich (Vienna, Austria). Ornithine (Fluka) was provided as hydrochloride salt. Succinic acid was from Riedel-de-Haën (Seelze, Germany). Pyruvic acid 98%, glutaric acid 99%, glycolic acid 99%, glyoxylic acid 98% (monohydrate), lactic acid 97% (lithium salt), 2-

aminoadipic acid 98%, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) 95.5%, para-hydroxyphenoxyacetic acid 97.8%, pantothenic acid (hemicalcium salt) 97%, pyridoxine 98%, folic acid 98% and cobalamine 99% were purchased from Sigma-Aldrich. Fumaric acid 99.5%, malonic acid 98%, 2–oxoglutaric acid 99%, cis-aconitic acid 90%, putrescine 98%, ethanolamine 99%, riboflavin 98%, nicotinic acid 99% and biotin 99% were from Fluka.

6-Aminopenicillanic acid 99%, 6-aminopenicilloic acid 73%, 8-hydroxypenillic acid 75%, penicillin V (potassium salt) 99.4%, phenoxyacetic acid 99.8%, phenoxymethylpenicilloic acid 92%, phenoxymethylpenillic acid 91%, para-hydroxypenicillin (potassium salt) 94.1%, penicillamine disulfide 97.8%, cephalosporin C (sodium salt) 88%, desacetylcephalosporin (sodium salt) 85.6%, desacetoxycephalosporin (sodium salt) 88%, 2-amino-5-(4-carboxy-2-thiazolyl)-valeric acid (Jeffrey thiazole) 98.9%, 7-aminocephalosporanic acid 96.4%, cephalosporin C lactone 83.2% were provided by Sandoz (Kundl, Austria). Structures of these β-lactam antibiotics, intermediates and degradation products, respectively, are depicted in Figure 1.

Uniformly ^{13}C labeled internal standards of succinic acid and malonic acid (99 atom %^{13}C) and ethanol-1,1,2,2-d_4-amine were obtained from Isotec (Tulln-Staasdorf, Austria). “Cell free” amino acid mix (U-^{13}C, 98%; U-^{15}N, 98%) (see supporting information) was from Cambridge Isotope Laboratories (Andover, MA, USA).

For LC-MS/MS analysis Chromasolv Plus ultra pure water for HPLC from Sigma-Aldrich and HPLC grade acetonitrile (ACN) from VWR (Vienna, Austria) were used. Ammonium hydroxide solution 25.0% in water and acetic acid 99.8% were obtained from Fluka and formic acid 98-100% from Riedel-de Haën.

2.2. LC-MS/MS instrumentation

Experiments were performed on an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany) coupled to a Q-Trap 4000 (Applied Biosystems/MDS Sciex, ON, Canada). The HPLC system was equipped with a thermostatted autosampler, which allowed cooling of samples at 5°C, a binary pump and a column thermostat. The use of a turboionspray interface allowed splitless hyphenation of HPLC (0.7 ml/min) with MS. Data were processed using the analyst 1.4.1. software from MDS Sciex (San Francisco, CA).

2.3. Optimization of chromatographic conditions

To find optimal chromatographic conditions 10 stationary phases were tested in the HILIC mode: ZIC-HILIC (150 x 4.6 mm; 5 µm) from Merck SeQuant (Marl, Germany), Luna Amino (150 x 3.0 mm; 5 µm) and Luna HILIC (150 x 4.6 mm; 5 µm) from Phenomenex (Aschaffenburg, Germany), Chromolith Performance Si (100 x 4.6 mm) from Merck (Darmstadt, Germany), TSKgel Amide-80 (150 x 2.0 mm, 5 µm) from Tosoh (Stuttgart, Germany), Acclaim Mixed-Mode WAX-1 (150 x 4.6 mm; 5 µm) from Dionex (Vienna, Austria), Obelisk N and Obelisk R (150 x 4.6 mm; 5 µm) from Sielc (Prospect Heights, IL USA), Chiralpak QN-AX (150 x 4.0 mm, 5 µm) from Chiral Technologies (Illkirch, France) and Biobasic AX (150 x 3.0 mm; 5 µm) from Thermo (Waltham, MA, USA). Furthermore, one stationary phase was tested in the RP mode: Synergi Fusion-RP 80 (150 x 3.0 mm, 4 µm) from Phenomenex (Aschaffenburg, Germany).
Mobile phases were composed of 10% (v/v) buffer dissolved in water (A) and ACN (B). Different buffers were tested: 800 mM and 200 mM formic acid adjusted to pH 3.0 with ammonium hydroxide solution and 200 mM acetic acid adjusted to pH 5.0 using also ammonium hydroxide solution. Gradient elution was carried out from 100% (B) to 20% (B) in 30 minutes, changing in one minute to the starting conditions followed by reequilibration for 14 minutes. Standard mixtures containing all analytes in detectable amounts were used for qualitative measurements.

2.4. Optimized LC-MS/MS method

Various stationary phases and mobile phase conditions were tested. Finally ZIC–HILIC was chosen for further considerations. The analysis column was equipped with a guard column (ZIC-HILIC Guard 20 x 2.1 mm; 5 µm).
The optimized mobile phase conditions were as follows: channel (A) 10% (v/v) buffer in water and channel (B) 10% (v/v) buffer in ACN. The buffer consisted of 200 mM formic acid adjusted to pH 4.0 with ammonium hydroxide solution. A gradient from 100% (B) to 35% (B) in 25 minutes was applied followed by an increase to the starting conditions in one minute and reequilibration for 13 minutes. The flow rate was 700 µl/min and the column was thermostatted at 25°. Temperature in the autosampler was kept at 5°C. The injector needle was washed after each sample injection by dipping into a vial containing water/ACN (50:50, v/v).

MRM transitions were measured in the positive mode as well as in the negative mode by polarity switching. Ion source parameters were adjusted as follows: turboionspray voltage +/- 4300 V, temperature 600°C, curtain gas 10 psi, turbogas 60 psi, nebulizer gas 50 psi, cell entrance potential +/- 10 V. Nitrogen was used as collision gas and its pressure (CAD) was set to "high". Dwell time was adjusted to 10 ms for each MRM transition. Declustering potential (DP), collision energy (CE) and cell exit potential (CXP) of the individual compounds were optimized using the Fragmentation Optimization tool of the software and adjusted accordingly in the analysis method (Table 1).

2.5. Preparation of standards

2.5.1. Preparation of standard stock solution

Individual standard solutions were prepared by weighing 0.8-1.0 mg of all compounds except organic acids and Gly into eppendorf vials and subsequently dissolving in 800-1000 µl of solvent to yield a concentration of 1 mg/ml. For most compounds a mixture of water/ACN (1:1, v/v) was used as solvent. A few compounds needed other solvent compositions (see the supplementary information section). Riboflavin, cystine, 7-aminocephalosporanic acid and folic acid standards were prepared at concentrations of 0.5 mg/ml because of limited solubility.

The compounds under investigation exhibited different linear ranges and sensitivities. Therefore, the different compounds were assorted into four calibration groups with distinct calibration ranges (see also Table 1). A mixed standard solution 1 was prepared by mixing the following volumina of individual standards with concentration of 0.5 and 1 mg/ml respectively: 50 µl pyridoxine (calibration group 1), 100 µl Arg, Asn, Asp, Cit, Glu, Gln, His, Ile, Leu, Lys, Met, Orn, Phe, Pro, Ser, Thr, Trp, Val, putrescine, ethanolamine, biotin, panthothenic acid and 200 µl riboflavin, cystine (group 2) and 200 µl Ala, 2-aminoadipic acid, 6-aminopenicillanic acid, 6-aminopenicilloic acid, 8-hydroxypenillic acid, para-hydroxyphenoxyacetic acid, phenoxyacetic acid, phenoxymethylpenicilloic acid, phenoxy-methylpenillic acid, penicillamine disulfide, cephalosporin C, desacetoxy-cephalosporin, desacetylcephalosporin, Jeffrey thiazole, cephalosporin-C-lactone, ACV, cobalamine, nicotinic acid and 400 µl 7-aminocephalosporanic acid and folic acid (group 3). The mixture was filled up with 2,450 µl standard solvent (mobile phase (A):(B), 2:8; v/v) to give a volume of 10 ml. Depending on the pipetted volume the concentrations were 5.0 mg/L (group 1); 10.0

mg/L (group 2) and 20.0 mg/L (group 3) in standard solution 1. For the preparation of standard solution 2 1.0 mg of Gly, succinic -, fumaric -, glutaric -, glyoxylic -, glycolic -, lactic -, pyruvic -, malonic -, 2-oxoglutaric- and cis-aconitic acid (group 4) were accurately weighed into a 20 ml glass vial and dissolved in 10 ml water/ACN 1:1 (v/v).
The resulting concentration was 0.1 mg/ml for all compounds. Mixing 2 ml of standard 1, 1 ml of standard 2 and 1 ml of standard solvent (mobile phase (A):(B), 2:8; v/v) yielded the standard stock solution, which contained all analytes (2.5 mg/L of group 1; 5.0 mg/L of group 2; 10 mg/L of group 3; 25 mg/L of group 4) and was used to prepare all working standard solutions.

2.5.2. Preparation of spiking standard solutions

For matrix-matched calibration using standard addition, standards were prepared by spiking distinct amounts of analytes to selected samples (methanol extracts of fermentation broths and nutrition media). These spiking standards were obtained by diluting the above standard stock solution with standard solvent (mobile phase (A):(B), 2:8; v/v), which was used for all further dilutions if not stated otherwise. The concentrations of the spiking standards were for group 1 0.25, 0.5, 1.25, 2.5 mg/L; for group 2 0.5, 1.0, 2.5, 5.0 mg/L; for group 3 1.0; 2.0; 5.0; 10.0 mg/L and for group 4 2.5; 5.0; 12.5; 25.0 mg/L.

2.5.3. Preparation of internal standard stock solution

1 mg U-^{13}C-malonic acid, U-^{13}C-succinic acid, ethanol-1,1,2,2-d_4-amine and U-^{15}N,^{13}C-“cell free” amino acid mix were each weighed into an eppendorf vial and dissolved in 1 ml water/ACN (1:1,v/v). Ethanol-1,1,2,2-d_4-amine was further diluted 1:5 to give a concentration of 0.2 mg/ml. 500 µl of U-^{13}C-malonic acid and U-^{13}C-succinic acid were mixed to result in a mixed standard solution with a concentration of 0.5 mg/ml. 100 µl of the mixed standard of U-^{13}C-malonic acid and U-^{13}C-succinic acid, 100 µl of 0.2 mg/ml ethanol-1,1,2,2-d_4-amine and 500 µl of the U-^{15}N,^{13}C-“cell free” amino acid mix (1 mg/ml) were combined into a glass vial and diluted with 9.3 ml standard solvent to yield a final volume of 10 ml.

2.5.4. Sample preparation

The sample extracts provided by Sandoz were prepared by extraction of the fermentation broth with methanol. Furthermore the extracts were diluted 1:10 with water/ACN (2:8, v/v). The samples intended for LC-MS/MS analysis were prepared by mixing 100 µl 1:10 diluted extract with 100 µl spiking standard, 100 µl internal standard solution and 700 µl diluent (standard solvent, mobile phase (A):(B); 2:8; v/v) corresponding to concentrations of 0.025, 0.05, 0.125, 0.25, 0.375, 0.5 mg/L of group 1; 0.05, 0.1, 0.25, 0.5, 0.75, 1.0 mg/L of group 2; 0.1, 0.2, 0.5, 1.0, 1.5, 2.0 mg/L of group 3; 0.25, 0.5, 1.25, 2.5, 3.75, 5.0 mg/L of group 4; 5.0 mg/L of U-^{15}N,^{13}C-"cell free" amino acid mix; 0.5 mg/L of U-^{13}C-malonic acid and U-^{13}C-succinic acid and 0.2 mg/L of ethanol-1,1,2,2-d_4-amine. Spiked standards with concentrations higher than 0.25 (group 1), 0.5 (group 2), 1.0 (group 3), 2.5 (group 4) mg/L were prepared using larger volumes of the standard stock solution and lower volumes of diluent.

2.5.5. Preparation of neat standard solutions for analysis

Neat standard solutions were prepared by diluting the standard stock solution to the corresponding concentration, whereupon 100 µl of the standard solvent were displaced by internal standard solution.

2.6. Calibration and validation

Calibration was performed applying two different approaches: In the first, matrix-free standard solutions (standards dissolved in the corresponding solvent) were used for calibration. In the second approach, matrix-matched calibration, standards applied for the construction of calibration functions were prepared by spiking distinct amounts of the compounds to the sample. It is once more emphasized that each calibrant contained all compounds (calibration group 1 - 4) in a mixture.

Four different concentration ranges were covered for the distinct compounds depending on the sensitivity of the assay and the concentrations levels in the samples (see Table 1).

Concentrations of neat standards and spiked standards were as follows: 0.025, 0.05, 0.125, 0.25, 0.375, 0.5 mg/L of group 1; 0.05, 0.1, 0.25, 0.5, 0.75, 1.0 mg/L of group 2; 0.1, 0.2, 0.5, 1.0, 1.5, 2.0 mg/L of group 3; 0.25, 0.5, 1.25, 2.5, 3.75, 5.0 mg/L of group 4.

For calibration using neat standard solutions two additional concentration levels 0.0375 and 1.0 (group 1); 0.075 and 2.0 (group 2); 0.15 and 4.0 (group 3); 0.375 and 10.0 mg/L (group 4) were prepared and measured.

Matrix-matched calibration was performed using six different matrices: two different nutrition media (medium 10 and medium 6) as well as methanol extracts of distinct fermentation broths from penicillin synthesis (extract 12 and extract 15) and cephalosporin synthesis (extract 1 and extract 5). Calibration functions were constructed using internal standards (plotting the ratio of analyte peak area and internal standard peak area versus concentration) as well as without use of internal standards (plotting the analyte area vs concentration). Linear regression functions were calculated with the analyst software and to improve accuracy for lower concentrations $1/x^2$ weighted linear regression was employed, if the resulting sum of relative errors decreased.

Intra-assay precision and accuracy were determined at three concentration levels 0.025, 0.25, 0.5 mg/L for group 1; 0.05, 0.5, 1.0 mg/L for group 2; 0.1, 1.0, 2.0 mg/L for group 3 and 0.25, 2.5, 5.0 mg/L for group 4 by analyzing quality control samples (spiked extract 12) in triplicate. Interday precision and accuracy were determined at an intermediate concentration level (0.25 mg/L for group 1, 0.5 mg/L for group 2, 1.0 mg/L for group 3 and 2.5 mg/L for group 4) by analysis of quality control standards at four different occasions (embedded into the first calibration series and three, four and six days afterwards).

Matrix effects were examined by comparing the slopes of calibration functions obtained with matrix-free neat standard solutions and those from standard addition experiments i.e. matrix-matched calibrations.

3. Results and discussions

3.1. Optimization of chromatographic conditions

Due to the wide range of physicochemical attributes of the metabolites, metabolic profiling studies usually employ an array of different methods, preferentially GC-MS/MS and LC-MS/MS with RP-type separations. In the present study, GC-MS/MS was not a viable option mainly due to inavailability of such instrumentation. Moreover, it requires an additional (more or less time consuming and moisture sensitive) derivatisation step which is attempted to be avoided in process analysis, if possible. On the other hand, LC-MS/MS with RPLC, although often utilized, performs greatly suboptimal for the current hydrophilic solutes due to

retentivity problems, clustering within a narrow elution window close to the void volume and thus, susceptibility to matrix effects when solutes elute in this primary ion suppression zone [11,32].

Hence, in a first method development step the goal was to find appropriate chromatographic conditions for the extended analyte set i.e. all of the compounds of Table 1 complemented by the set of fatty acids (arachidonic -, lauric -, linoleic -, linolenic -, myristic -, oleic -, palmitic- and stearic acid), some less hydrophilic β-lactam derivatives (penicillin V, para-hydroxypenicillin, phenoxymethylpenilloic acid, cephalosporin P1) and α-tocopherole that were finally analyzed by RPLC-MS/MS [40]. This analyte set was subjected to an extensive screening procedure comprising 11 distinct stationary phases and 4 different elution conditions yielding 22 specific phase systems i.e. stationary/mobile phase combinations which are specified in Table 2.

Due to the hydrophilic nature of most analytes a number of polar stationary phases were evaluated in the HILIC mode [43] and in addition a polar embedded RP phase (Synergi Fursion-RP 80) was tested under weakly eluting RP gradient conditions as well (see Table 2). For structures of surface chemistries interested readers are referred to reference [51] or the webpages of the column suppliers. Owing to different internal diameters of the evaluated columns, the flow rate was adjusted to obtain similar linear velocities.

In this screening it became rapidly evident that a single assay will not be appropriate for all solutes. Hence, the analyte set was divided into two groups covering hydrophilic compounds (with log D roughly < 0) including 22 amino acids, 10 organic acids, vitamins and biogenic amines (9 compounds) as well as β-lactams and derivatives (16 compounds) that are dealt with herein (Table 1) and another group for RPLC (with log D > 0) [40]. The screening strategy followed similar considerations as presented recently by [32]. The quality of the achieved chromatographic separations was assessed in terms of i.) sufficient retardation (with t_R of at least 2 x t_0) to shift the solutes away from the primary ion suppression zone near t_0, ii.) adequate separation of isobaric compounds that lack specific MRM transitions and other critical peak pairs, iii) peak performance with regard to narrow peaks and minimal tailing as to provide better peak capacity and maximal peak heights (i.e. sensitivity) as well as facilitate correct integration, iv.) eluability and detectability of as many target metabolites as possible (i.e. the number of detected compounds), v.) (equal) spreading of the analytes over the entire or a wide elution window (as opposed to clustering of the solutes in a narrow time window of the chromatogram) in order to minimize the risk of (mutual) effects on ionization efficiencies and of non-detected matrix components as well as to reduce problems with interferences of

less specific MRM traces, and vi.) particular focus was of course also given to suitable ESI-MS/MS compatibility (volatile eluents, avoidance of ion-pairing effects, low column bleed). Speed of the separations was not a primary focus and was kept nearly constant by running always about 30 min gradients. The quality of the obtained separations was assessed in a semi-quantitative manner and some key findings are summarized in Table 2.

The results of the RP runs on Synergi Fusion-RP 80 under acidic conditions (pH 2.7) revealed that a great portion of the target solutes were not sufficiently retained. A large number of the solutes (especially amino acids and organic acids) were clustered together in the first part of the chromatogram (2-5 min) so that assay specificity might be compromised. For this reason RP conditions were not further considered (except for the solutes finally analyzed by RP as reported elsewhere [40]. A few amino phases (Luna Amino) and anion-exchangers (Biobasic AX, Chiralpak QN-AX), respectively, that were included in the screening with typical RP elution conditions i.e. aqueous-rich hydroorganic eluents showed satisfactory retention for a large number of solutes, however, completely failed for amino acids. Similar observations were found for mixed-mode phases with both ion-exchanger groups and alkyl moieties (i.e. weak anion exchange type Acclaim Mixed Mode WAX-1 and zwitterionic type Obelisk R).

To this end it became clear that a HILIC mode would be most promising for the majority of the solutes and a large number of polar stationary phases thus was screened under HILIC conditions (i.e. negative acetonitrile gradients) employing distinct pH and ionic strengths as further variables. On the amino- and anion-exchange type columns, respectively, (especially Luna Amino, Biobasic AX) organic acids (except fatty acids) were strongly retained by ionic interactions and, in particular under less acidic conditions such as pH of 5.0, several acidic compounds did not elute at all employing HILIC conditions. The silica monolith (Chromolith Performance Si) showed reasonable retention (except for fatty acids) and acceptable peak shapes for the majority of compounds, yet suffered from a narrow elution window (especially for organic acids and cephalosporins) which increased the risk for interferences of isobaric compounds and solutes with less specific MRM transitions. Amongst the zwitterionic phases that were tested in the HILIC mode (ZIC-HILIC, Obelisk R and Obelisk N) a higher number of compounds could not be eluted on the Obelisk N and the Obelisk R columns under the tested conditions and a larger number of compounds showed poor peak shape on these stationary phases as compared to the zwitterionic ZIC-HILIC column. As neutral HILIC columns, i.e. silica-based stationary phases with neutral bonding, TSKgel Amide-80 (with polyacrylamide surface layer) and Luna HILIC (with crosslinked diol surface) were incorporated in the screening. The retention behavior of TSKgel Amide-80 was largely

satisfactory (especially at pH 5.0), yet peak performance with strong tailing and splitted peaks was observed for quite a few compounds. In general, Luna HILIC performed better with the constraint that amino acids, regardless of the mobile phase conditions, eluted within a relatively narrow retention time window.

Amongst all screened phase systems ZIC-HILIC turned out to be the best compromise for all compound classes with regard to the intended application and the criteria defined above. Also Luna HILIC gave quite acceptable results and could be an alternative for the ZIC-HILIC phase. Next, the HILIC method with the ZIC-HILIC column was further subjected to a fine-tuning of the mobile phase conditions (pH and ionic strength) and the gradient profile (steepness). For example, the effect of buffer was investigated at pH 3.0, 4.0 and 5.0. The results revealed that for amino acids and most β-lactams a change in buffer pH from 3.0 to 4.0 altered retention times just minimal, whereas the pH shift had a more relevant effect on the retention times of organic acids. At a pH of 4.0, which exceeded the pK_a of most organic acids, retention times of these solutes were markedly increased but selectivities declined at this pH, as most organic acids eluted between 12 and 14 minutes. Decreasing the ionic strength of the buffer from 800 mM to 200 mM (tantamount with a total buffer concentration of 80 and 20 mM, respectively) in the hydroorganic mixture caused a slight decrease of retention times of acidic compounds. An explanation, especially for organic acids, may be involved electrostatics: The ZIC-HILIC has a slight negative net charge so that electrostatic repulsion may play a role which is stronger at low ionic strength i.e. c_{tot} of 20 mM. The situation is quite different regarding compounds with basic functional groups like putrescine, which were slightly stronger retained at lower ionic strength. For these components ionic interactions seem to play a crucial role which is in agreement with electrostatics.

Finally, an optimized method resulted from these experiments, which is described in detail in the experimental section. It makes use of 10% (v/v) of a buffer containing 200 mM formic acid adjusted with ammonium hydroxide to pH 4.0 (corresponding to a total buffer concentration of 20 mM which is well compatible with ESI-MS) and running a negative acetonitrile gradient.

The chromatographic results (retention times, peak widths, peak asymmetries) are summarized in Table 3, along with potential interferences in individual MRM traces that gave additional peaks in neat standard solutions and in extracts of fermentation broths. As can be seen from Figure 2, for the majority of analyzed target metabolites the peak shapes were quite acceptable and the peaks were relatively uniformly distributed over the elution window between 3 and 25 min thereby minimizing the risk for interferences. Only few compounds

showed a stronger tailing (e.g. putrescine, succinic acid, His, pyridoxine). Later it was figured out that a further significant improvement is possible if the eluent pH is reduced to pH 3.5 (for chromatographic data see supporting information). Overall, the optimized chromatographic separation on the ZIC-HILIC column at pH 4 was, however, assessed to be suitable for the intended purpose. This method was then validated.

3.2. Mass spectrometric detection and assay specificity

The final optimized LC-MS/MS method was acquired to allow the analysis of 57 different hydrophilic compounds within a single run employing the MRM scan mode. Since the complete separation of all compounds under investigation within an acceptable time span is hardly possible and quadrupole mass spectrometry has not enough mass resolution power to enable accurate quantification of isobaric compounds, particular attention was paid to a careful selection of specific MRM transitions and/or to a dedicated optimization of liquid chromatography for critical peak pairs such as isobaric compounds as stressed above. The corresponding compound specific MS parameters are presented in Table 1.

A particular limitation in view of MS/MS detection is the low molecular mass of several metabolites which is associated with non-characterisitic fragmentations and low signal intensities yielding, in some cases, no intense transition at all. In such cases, these analytes have been detected by "pseudo-molecular" MRM transitions with Q1 and Q3 monitoring the same m/z, namely the pseudo-molecular ion, without fragmentation in Q2 (collision cell). Especially for small organic acids like glyoxylic acid, glycolic acid and pyruvic acid as well as the amino acid Gly this kind of MRM transition was chosen to allow for sufficiently sensitive quantitative measurements.

Metabolites with a nominal difference in their m/z of 1 mass unit as well as structurally similar compounds releasing the same fragment ions were separated to avoid potential signal interferences. Asn, Asp, Leu, Ile, Orn and glutaric acid have a nominal molecular mass ranging from 131 to 133 being prone to interferences from isotopomer peaks. The same holds true for instance for Lys, Gln, Glu. The former two are isobaric and exhibit identical MRM transitions, the letter differing by only 1 mass unit. Moreover, Met is isobaric with penicillamine, which is a degradation product of penicillin and may be present in extracts of fermentation broths, and does not exhibit specific MS/MS fragmentation pathways. Several such potentially interfering matrix compounds that are detected but not analyzed have been found in the MRM traces and are listed in Table 3. For example, maleic acid (m/z = 115) and

trans-aconitic acid (m/z = 173) which were of no interest as analytes are isobaric compounds of fumaric acid and cis-aconitic acid, respectively, and give rise to the same product ions. It was thus assured that these organic acids are chromatographically separated. Similarly, a number of other potential interferences showed up in various MRM traces. Glutaric acid (131 → 87) caused an additional peak in the monitored trace of pyruvic acid (87→ 87). Likewise, succinic acid (117→ 73) was detected in the channel acquired for glyoxylic acid (73 → 73) and malic acid (133 → 115) interfered with the transition of fumaric acid (115 → 71). Further, pyridoxine (m/z 170 → 152) caused an additional peak in the trace of nicotinic acids (124 → 78). These findings can probably be attributed to in-source decay i.e. fragmentation in the ESI source to derivatives that possess the same precursor ions and product ions as other analyzed metabolites. In all these cases an adequate chromatographic separation was of utmost importance, because otherwise accurate quantification would be seriously compromised. Specific care was taken in the course of the screening and method development, respectively, to end up with an LC method that overcomes these specificity problems of MS/MS detection by adequate chromatographic resolution. As can be seen from Table 3 the present optimized method provides satisfactory chromatographic selectivities to alleviate many of the MS/MS specificity shortcomings.

3.3. Validation results

Reliability and applicability of the optimized method were extensively evaluated in the course of the validation process, by investigating besides assay specificity (*vide supra*) the lower and upper limits of quantiation (LLOQ and ULOQ, respectively), matrix effects, as well as inter-assay and intraday precision and accuracies. The lack of specificity of certain utilized MRM transitions of some specific analytes could be overcome by adequate chromatographic reolution (see above Table 3) and will not be further discussed here in more detail.

3.3.1. Calibration, linearity and sensitivity

Calibration functions were set up with standard solutions by two distinct ways, without and with internal standards. The results along with correlation coefficients, LLOQ and ULOQ are presented in Table 4. ULOQ and LLOQ were examined with neat standard solutions.
For most analytes linearity ranges over two to three orders of magnitude and R^2 determined in the present study were mostly larger than 0.99. The linear range (between LLOQ and

determined ULOQ) covers over three orders of magnitude for 49% and over two orders of magnitude for 44% of the investigated compounds. Only few compounds showed a slightly narrower linear range.

LLOQs were determined by dilution of standard solutions to a concentration yielding a signal to noise ratio of 1:10 and ranged between 0.005 and 0.1 mg/L except for organic acids for which the assay sensitivity was lower (LLOQs between 0.15 and 1.25 mg/L) as well as the amino acid Gly (0.5 mg/L) and the cephalosporin degradation product Jeffrey thiazole (0.15 mg/mL). LLOQs of all analytes are illustrated in Figure 3. In this context it is worth noting that a few analytes such as Lys, Arg, Orn and putrescine showed a minor memory effect so that the actually applicable LLOQ for these compounds had to be slightly raised. This memory effect was materialized as minor peaks which were measurable in blank runs at the corresponding retention times of the compounds even after excessive washing of injector needle, injection of plaques (100 µl) containing displacing agents or flushing with strong eluting solvents. However, in the specified measurement range for these compounds, accuracies were not significantly compromised.

3.3.2. Matrix effects

Matrix effects, which occur when non-detected compounds from the matrix that coelute with the analyte alter the ionization efficiency of the ESI-interface [52], are a major source for inaccuracies and unreliability in quantitative metabolic studies using ESI-MS [37,53]. The exact mechanism is not fully understood, although a number of studies attempted to shed light on the fundamental basis of this phenomenon [54-57]. The most frequently proposed explanations for the occurrence of matrix effects are competition of coeluting compounds for limited charges (charge competition) [57,58], competition of surface active compounds and analytes for access to the droplet surface for transfer to the gas phase [58,59], ion pairing [58], incomplete evaporation due to excessive non-volatile matrix compounds (droplet solution properties) [55,58]. Such matrix effects may be effectively eliminated by reducing the sample complexity and the extent of coelution, respectively. Improvements in sample preparation, e.g. by solid phase extraction or liquid-liquid extraction, or chromatographic separations are effective strategies to achieve this goal. If this is not successful, normalization using isotope-labeled internal standards can reliably correct for matrix effects [30,31,60]. In metabolic studies, the former is not a viable route because sample preparation has to be minimized as far as possible (e.g. to cold solvent extraction) to avoid analyte losses [25]. Moreover, due to

resultant sample complexity the chromatographic separation is incomplete as resolution and peak capacity are limited. Hence, matrix effects are hard to be completely overcome.

The most common strategies to investigate matrix effects are postextraction addition [37,53] and postcolumn infusion [53,61]. Another option, particularly suitable for samples where there is no blank matrix available such as in assays of endogenic compounds like metabolomic studies, is standard addition at distinct levels to the specific matrix and comparison of slopes of calibration functions with those obtained in a standard solution [35,39,62]. Significant deviations of slopes of calibration lines generated using matrix-free standards and spiked sample extracts would indicate an absolute matrix effect. If this strategy is utilized for different lots of matrix, relative matrix effects may be assessed by the relative standard deviations (RSD) of the slopes in theses different matrices [63].

Thus, in the course of the present work calibration was performed using matrix-free (neat) standard solutions and by standard addition using different sample matrices, four sample extracts obtained from two penicillin (extract 12 and 15) and two cephalosporin (extract 1 and 5) fermentation batches and two nutrition media (medium 6 and 10). The complete calibration data for all solutions (standard solutions, matrices and media) with and without use of internal standards can be found in the supporting information. Table 5 summarizes absolute and relative matrix effects which were calculated by the ratio of slopes in matrix and standard solution multiplied by 100 and as the relative standard deviation from the mean in the 4 extracts and two nutrition media, respectively.

It can be seen from Table 5 that the majority of determined values for absolute matrix effects (given as % sensitivity in the respective matrix relative to a standard solution) in the extracts of the fermentation broths show a bias of less than 20%. Values ranging outside (> 20% bias) are indicated in bold and mostly have some explainable reason. Some of the compounds appeared to be not fully stable over the measurement time frame. Calibration standards were collectively prepared on one day and stored at 5°C in the autosampler. The prepared calibration standards (neat standard soluions and spiked extracts and media, respectively) were analyzed within a time window of about three days, which also led to deviations of calibration functions of compounds with limited stability when respective calibration functions were not analyzed on the same day. Moreover, a number of compounds was present in the extracts in high abundance so that even after 1:100 dilution the concentration was either outside the linear range (missing values in Table 5) or at the upper range of the calibration function (i.e. no overlapping concentration ranges in neat standards and spiked extracts and media, respectively) which made the comparison of slopes less accurate.

For a representative assessment such cases, like compounds that are not stable, must be excluded and the resulting frequency distribution of observations yields quite a satisfactory result. The findings in the media are similar. With regards of absolute matrix effects values of extract 12 (and extract 15) which were measured directly before and after the plain standard solutions are most representative. The worse results for absolute matrix effects have their origin in the high intrinsic concentrations so that actually a further dilution is required. The relative matrix effects after removal of those cases with stability or linearity problems is on average 11% for the four extracts and 5% for the two media.

It is also worth mentioning that the use of internal standards, while providing better values for absolute matrix effects for certain compounds, did on average not yield better results (for data see supporting information). Possible explanations may be problems with IS due to cross-talk between IS and analyte channels, cross-contaminations, isotopic stability (isotope exchange) and inadequate concentration levels of IS. For example, for amino acids, U-^{13}C, ^{15}N labeled internal standards were used, which were available as homogeneous solid mixture, containing the compounds in different proportions. Consequently the resulting concentrations in the samples differed and were for some compounds rather high e.g. leucine and isoleucine and for others e.g. serine at the edge of the LOQ, where the precision of measurements is usually inferior and thus it is inappropriately compensated for matrix effects. Moreover it is pointed out that stable isotope-labeled internal standards were just available for a small number of analytes including most amino acids, ethanolamine, malonic and succinic acid. For the remaining compounds the closest eluting isotope-labeled compound was applied as internal standard (see Table 1). Due to completely different molecular structure and physicochemical attributes they may show a divergent behavior than the corresponding analytes, when disturbances by coeluting matrix compounds or instrumental fluctuations occur. Thus, these internal standards may not correct appropriately for matrix effects as well as introduce fluctuations provoking an increase of imprecision (*vide infra*).

Overall, the results in terms of matrix effects were acceptable for the majority of compounds for the intended application, yet care has to be taken that samples are adequately diluted for highly abundant analytes.

3.3.3. Precision

Intra-assay and inter-day precision were determined with quality control (QC) samples obtained by spiking extract E12 with solutes at three different concentration levels (low,

middle, high) across the measurement range and the results are given as percentage of relative standard deviation (% RSD). The detailed results for use with and without internal standards as well as individual data on the distinct concentration levels are summarized in the supporting information along with the employed concentrations of the QC samples.

For intraday precision the prepared QC samples were analyzed in triplicate. Figure 4 illustrates the frequency distributions of all %RSD values regardless of the three concentration levels classified into 5 categories, i.e. 0-5%, 5-10%, 10-15%, 15-20% and >20%. It can be seen that intra-assay precision conforms with typical acceptance criteria of bioanalytical assay guidelines [64] as the majority of % RSD values were less than 10% and only 4 observations (about 2% of all cases) were above 20% RSD (Figure 4A). The use of internal standards had no beneficial effect on intra-assay precision (Figure 4B). The reason for that may be the same as explained above for compensation of matrix effects. For interday precision, which was measured at an intermediate concentration level only, the frequency distribution of %RSD values is slightly shifted to higher values, yet still about 90% of the measured %RSD values were below 15% and one single observation with RSD > 20% was found. The use of internal standards slightly improved interday precisions, probably due to compensation for instrumental fluctuations.

3.3.4. Intra-assay accuracy

Intra-assay accuracies were measured with QC samples prepared by spiking standard solutions at three distinct concentration levels to extract 12 (group 1: 0.025, 0.25, 0.5 mg/L; group 2: 0.05, 0.5, 1.0 mg/L; group 3: 0.1, 1.0, 2.0 mg/L and group 4: 0.25, 2.5, 5.0 mg/L).

Accuracy values expressed as the percentage of calculated concentrations relative to nominal concentrations (% recovery), corrected for the intrinsic analyte concentrations in extract 12, were determined by use of four different calibration functions: i.) calibration from neat standard solutions without and ii.) with internal standards, iii.) calibrations from standard addition experiments in different matrices (extract 12, extract 1, and medium 10) without and iv.) with use of internal standards for area normalization. The use of such matrix-matched calibration by standard addition is quite uncommon in targeted metabolomic studies. However, the idea was to elucidate whether such matrix-matched calibration by standard addition in extracts from fermentation broths and generation of corrected matrix-matched calibration functions would offer significant advantages in terms of accuracy as it might partly correct for matrix effects. The detailed results are presented in the supporting information.

Figure 5 shows a graphical representation of the accuracy values obtained for the different analytes in the spiked QC sample (extract 12) at an intermediate concentration level, calculated by use of calibration functions from neat standard solutions. It becomes evident that at this intermediate concentration level the majority of accuracy values fall within 90-110% (about 82% of all cases) and only 1 observation (less than 2% of cases) was made with bias larger than 20% (phenylalanine). Frequency distributions of accuracy values including all four concentration levels are presented in Figure 6A. All in all, with internal standards accurracies were not much better (Figure 6B), probably for the same reasons as discussed above. However, for some specific compounds that provided bias > ±20% without use of IS such as Gln, Phe, Trp and Tyr accuracies could be significantly improved by the use of isotope labeled internal standards (see also supporting information for corresponding values). On the other hand, accuracies can be significantly improved if matrix-matched calibration by standard addition in extracts of fermentation broths is employed for the analysis (Figure 6 C). For example, if only the intermediate concentration range is considered, about 90% of the measurements furnished accuracies within 90-110% and only 2 cases (corresponding to about 3% of all observations) were below 80% (nicotinic acid and pyridoxine, each 79% recovery) when matrix-matched calibration in extract 12 without internal standards was used. Once again, the additional use of an internal standard for matrix-matched calibration by standard addition could improve the accuracy for the two critical solutes (from 79% to 96% for nicotinic acid and to 92% for pyridoxine) but did not provide better accuracies in general (Figure 6D).

When all the accuracy values from intermediate, low and high concentration levels were included in this frequency distributions (Figure 6) a similar picture results, however, the number of observations with bias > ±20% is increased, not surprisingly, mostly at the low concentration levels (see supporting information).

3.3.5. Intra- and interday accuracy and stability of compounds

A quality control sample (extract 12 spiked at intermediate concentration levels 0.25 mg/L for group 1, 0.5 mg/L for group 2, 1.0 mg/L for group 3 and 2.5 mg/L for group 4) was analyzed in triplicate at four different days within a week (day 1, 3, 4 and 6). The sample was stored in the autosampler tray at 5°C over the entire period. For calculations of intra- and interday accuracies calibration functions generated in extract 12 at the first day without and with area

normalization by internal standards were used. For the evaluation of compound stability the above mentioned quality control sample and freshly prepared calibration functions were used. The data were supposed to indirectly provide knowledge about compound and also instrumental stability, but also information about the necessity to employ internal standards as well as on how frequently calibration is required.

The data of the intraday accuracies (n = 3) on the individual days and mean accuracy values from each day as well as interday precision are summarized in Table 6. It is obvious that the various compound classes and/or specific compounds behave quite differently in terms of interday accuracy. In general, intra-assay accuracies conform with acceptance criteria (< ±20% bias) for all analytes except for nicotinic acid and pyridoxine (both 79%) on the first day. For about 70% of amino acids also interday accuracies were quite acceptable when no internal standards were used and stable-isotope labeled internal standards adequately corrected for inaccuracies except for Glu, Gln, Orn and Tyr. Serious problems concerning interday accuracies are noticed for organic acids and internal standards could improve the situation only for malic and succinic acid, the only solutes of this class for which isotope-labeled internal standards were available. For both β-lactams and vitamins, about 50% of the compounds revealed inadequate interday accuracies for both types of calibration without and with internal standards.

Detailed inspection of the trends in intra- and interday accuracy values allowed to classify compounds into five groups as outlined in Table 7.

The first group (38% of the compounds) encompasses analytes that are stable and for which acceptable and comparable results were obtained for interday accuracy and precision independent of the use of internal standards.

For the second group (20% of all compounds) a decrease of accuracy was observed with every measurement cycle when calibration functions obtained without peak normalization by internal standards were used for the calculation of concentrations. As these compounds are considered to be stable, this trend indicates instrumental fluctuations e.g. a loss of sensitivity eventually due to contaminations of the ion-source, which were readily compensated for by the use of internal standards.

Compounds of the third group (14% of all compounds) exhibited relatively constant interday accuracy values when no internal standards were used. However, when internal standards were employed, an enormous increase of accuracies values (partly over 200%) was observed for each analysis cycle (from day to day) except for putrescine, for which a slight decrease of values was found. These results show that the employed internal standards were not

appropriate to correct for inaccuracies. An explanation is that these internal standards were prone to instrumental fluctuations which strongly decreased sensitivity and thus caused a severe rise of accuracy values.

The fourth group (16% of all compounds) of analytes included compounds that showed a significant decrease of intraday accuracy (as calculated without internal standards) with every analysis cycle and for which the employed stable isotope-labeled were not able to compensate these decreases. Both, analytes and internal standards, were seriously affected by instrumental fluctuations that caused significant decrease of sensitivity and accuracy. Especially organic acids but also other compounds that were measured in the negative mode can be found in this group. Furthermore, detailed examination of intraday accuracy values on each day revealed that for a great portion of compounds of group 4 rather selective compound-dependent perturbations took place between the first and the third day which then remained constant over day 4 to 6.

Compounds of the fifth group (14% of all compounds) were not stable at the storage conditions of 5°C over the measurement period of six days, which was evaluated with freshly prepared calibration functions. Hence, results for intraday accuracies on each day, as well as interday accuracies were not acceptable. The use of internal standards (no isotope labeled internal standard) did not provide any improvement.

Hence, it can be concluded that 72% of all compounds can be reliably analyzed either with or without use of internal standards over a time period of four to six days without the need to prepare fresh calibration functions.

For 16% of all compounds (group 4) serious instrumental fluctuations were encountered during the measurement period of six days. Likewise unstable compounds (14%, group 5) were problematic. In both cases employed internal standards were not appropriate and stable internal isotope standards were not available. Thus, for the compounds of group 4 and 5 either preparation of calibration functions on a daily basis would be necessary or use of isotope labeled internal standards, which eventually would compensate for losses in sensitivity and compound decomposition.

These data sets now permit to design a better assay in terms of optimal calibration as it becomes evident for which compounds no internal standard is necessary and for which compounds isotope-labeled standards are absolutely required or other compounds might be used as internal standard. The alternative (regarding improvements of interday accuracies) to the use of internal standards is a daily calibration (at least with a limited set of calibrants) at expense of instrument time.

3.4. Application

To test the applicability of the method several sample extracts and nutrition media were analyzed after dilution by a factor of 1:100 and 1:500, respectively. The results are shown in Table 8. Most amino acids were readily detectable at concentrations above the LLOQ in both extracts as well as in the nutrition medium. Moreover, several β-lactams were found in the analyzed extract and the profile clearly indicated the type of fermentation broth the two extracts were stemming from, cephalosporin production (extract 5) or penicillin production (extract 15). In extract 5 2-aminoadipinic acid, 2-amino-5-(4-carboxy-2-thiazolyl)-valeric acid, cephalosporin C, cephalosporin C lactone, desacetoxycephalosporin, desacetyl-cephalosporin, phenoxymethylpenicilloic acid and in extract 15 2-aminoadipic acid, 6-aminopenicilloic acid, 8-Hydroxypenillic acid, phenoxyacetic acid, phenoxymethylpenicilloic acid, p-hydroxyphenoxyacetic acid, δ-(L-α-aminoadipoyl)-L-Cys-D-Val were found to be present in amounts above the LLOQ. Organic acids were present at concentrations below the LLOQ except for succinic acid and lactic acid. Besides, also a few vitamins and biogenic amines (riboflavin, panthothenic acid, ethanolamine and putrescine) were quantified.
At this point it is unclear whether the concentrations of the non-detected compounds were present in the extracts really below the LLOQ or whether the employed extraction procedure was inadequate or whether some of the metabolites are simply not excreted into the extracellular space in reasonable amounts. Nevertheless, all compounds under investigation were successfully quantified when they were spiked to sample extracts, proving this way applicability of the developed method in the specified concentration ranges.

4. Conclusions

A quantitative HPLC-MS/MS-assay that makes use of hydrophilic interaction chromatography employing a ZIC-HILIC column for metabolic profiling of amino acids, organic acids, β-lactam antibiotics (intermediates and degradation products), vitamins and some endogenic amines in fermentation broths of β-lactam antibiotics (penicillins and chephalosporins) is proposed. Method development including an extended column screening and final mobile phase fine tuning as well as method validation comprising parameters such as assay specificity, linearity, sensitivity (LLOQ), matrix effects, intra-assay and interday precision and accuracy as well as compound stability are described in great detail. From screening of 11 columns with 4 mobile phases (in total 22 different stationary phase/mobile

phase systems) the ZIC-HILIC column was selected as the best compromise with regard to sufficient retentivity for the hydrophilic solutes, peak efficiencies and symmetries, chromatographic selectivities for isobaric compounds and other critical pairs and more equal spreading of solutes over the elution window.

A mobile phase fine tuning led to mobile phase conditions with 20 mM total buffer concentration at pH 4.0 (or 3.5) with a negative linear ACN gradient. The combined power of chromatographic selectivities and MRM specificities provided adequate assay specificity for the investigated compounds. Particular attention was paid to the evaluation of absolute and relative matrix effects (lot-to-lot variation) via comparison of sensitivities (i.e. slopes) of calibration functions in spiked extracts of fermentation broths and standard solutions. For a large number of solutes both absolute and relative matrix effects were in an acceptable range, while for another group of compounds no safe conclusion was possible due to problems with compound stabilities (calibration functions in matrix and standard solutions measured on different days) or mismatched calibration ranges (i.e. concentration close to ULOQ in spiked matrix) due to high endogenic concentrations. Precision (both intra-assay and interday) as well as intra-assay accuracies were mostly quite acceptable conforming to suggestions of bioanalytical assay validation guidelines [64]. For several compounds for which inaccuracies were noticed stable isotope labeled internal standards adequately corrected for this bias. Information on compound stabilities could be derived from interday accuracy evaluations. Overall, the present method illustrates critical issues, problems and challenges of multi-target quantitative metabolic profiling and allows to draw conclusions on how to avoid problems and circumvent pitfalls as well as on how to design better even more accurate and reliable assays e.g. by implementation of matrix-matched calibration via standard addition, stable isotope labeled internal standards for specific critical compounds, adjustment of storage times of samples/calibrants in the autosampler as well as adjustment of frequencies of calibration.

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Figure captions:

Figure 1: Structures of β-lactam antibiotics (7, 12, 14), intermediates of biosynthesis (1, 2, 3, 4, 5, 6, 11, 13) and degradation products (8, 9, 10, 15, 16, 17, 18, 19, 20, 21, 22).
1 Val, 2 Cys, 3 2-Aminoadipic acid, 4 δ-(L-α-Aminoadipoyl)-L-Cys-D-Val, 5 Desacetoxycephalosporin, 6 Desacetylcephalosporin, 7 Cephalosporin C, 8 7-Aminocephalosporanic acid, 9 Cephalosporin-C-lactone, 10 2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid (Jeffrey thiazole), 11 Phenoxyacetic acid, 12 Penicillin V, 13 para-Hydroxyphenoxyacetic acid, 14 para-Hydroxypenicillin V, 15 Phenoxymethylpenicilloic acid, 16 Phenoxymethylpenilloic acid, 17 6-Aminopenicilloic acid, 18 6-Aminopenicillanic acid, 19 Phenoxymethylpenillic acid, 20 8-Hydroxypenillic acid, 21 Penicillamine, 22 Penicillamine disulfide

Figure 2: Chromatograms of optimized HILIC method: Overlaid MRM traces normalized to 100% of amino acids (A), organic acids (B), β-lactams (C) and vitamins/biogenic amines (D). Experimental conditions: Column, ZIC-HILIC, 5 µm (150 x 4.6 mm ID); eluent, channel A: 10 % (v/v) buffer in water; channel B: 10 % (v/v) buffer in acetonitrile; buffer, 200 mM formic acid, adjusted to pH 4.0 with ammonia; gradient, 100% B to 35% B in 25 min; flow rate, 0.7 mL/min; temperature, 25°C.

Figure 3: LLOQs of investigated analytes, determined by dilution of neat standard solutions to concentrations that yield a signal to noise ratio of about 10:1.

Figure 4: Frequency distributions of intra-assay precision values (A and B, n = 3) as well as interday precisions, (C and D) (n = 4) in terms of % RSD determined for QC samples prepared by spiking extract 12 at three distinct concentration levels (group 1: 0.025, 0.25, 0.5 mg/L; group 2: 0.05, 0.5, 1.0 mg/L; group 3: 0.1, 1.0, 2.0 mg/L and group 4 0.25, 2.5, 5.0 mg/L). For interday precisions QC samples were prepared at one concentration level positioned in the middle of the calibration range (group 1: 0.25 mg/L; group 2: 0.5 mg/L; group 3: 1.0 mg/L; group 4: 2.5 mg/L). Concentrations were calculated by matrix-matched calibration in extract 12 (A) and (C) without use of internal standards and (B) and (D) using internal standards. Values that were distorted due to instrumental fluctuations or due to limited compound stability were excluded from the determination of interday precision.

Figure 5: Accuracy values in % recovery of all compounds at a medium concentration level (group 1: 0.25; group 2: 0.5 mg/L; group 3: 1.0 and group 4 2.5 mg/L) using calibration with neat standard solutions without internal standards.

Figure 6: Frequency distribution of intra-assay accuracy values in % recovery for different calibration approaches. Accuracy was determined for QC samples prepared by spiking extract 12 at three distinct concentration levels (group 1: 0.025, 0.25, 0.5 mg/L; group 2: 0.05, 0.5, 1.0 mg/L; group 3: 0.1, 1.0, 2.0 mg/L and group 4: 0.25, 2.5, 5.0 mg/L).
(A) calibration with neat standard solutions without internal standards
(B) calibration with neat standard solutions with internal standards
(C) matrix-matched calibration in extract 12 without internal standards
(D) matrix-matched calibration in extract 12 with internal standards

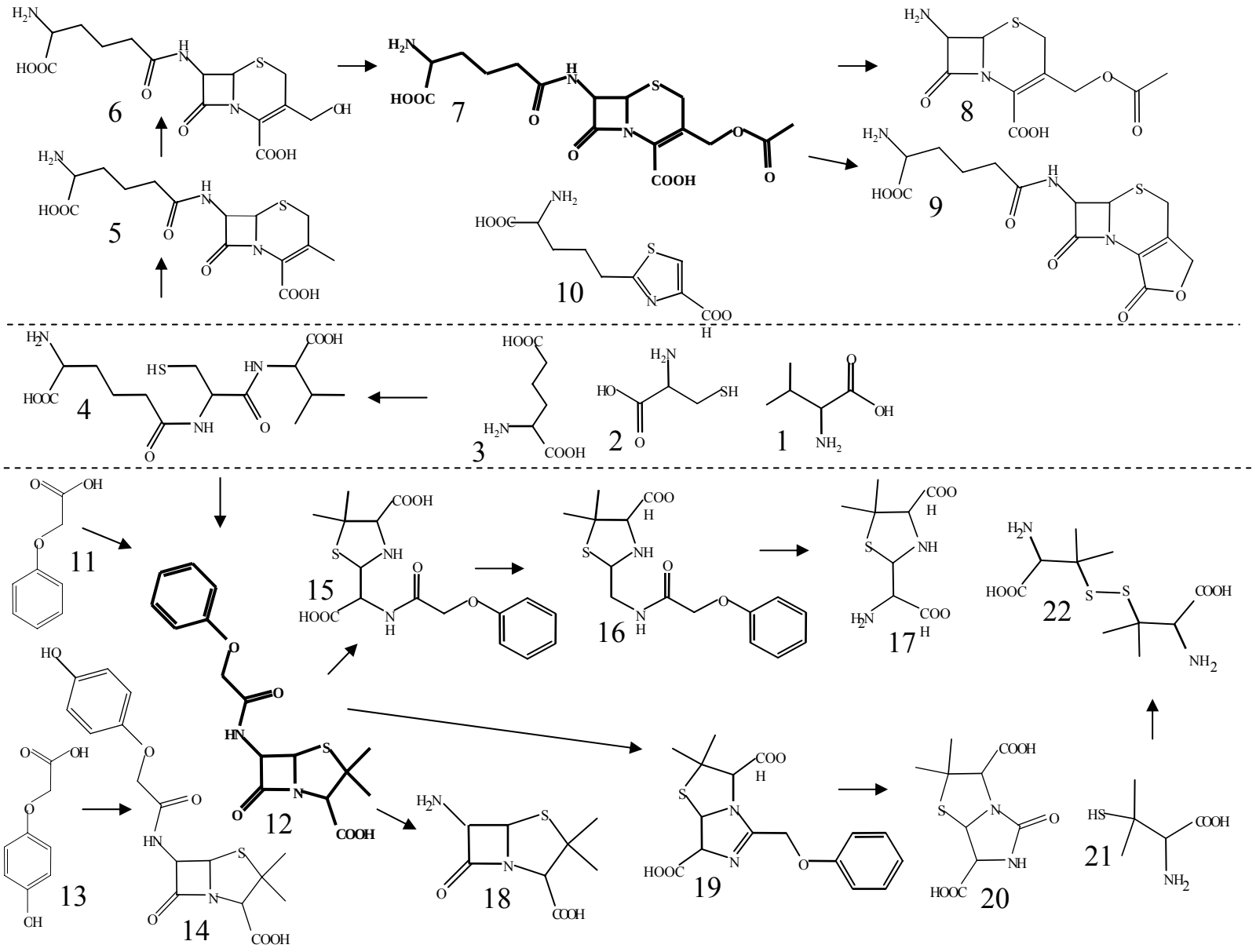

Figure 1

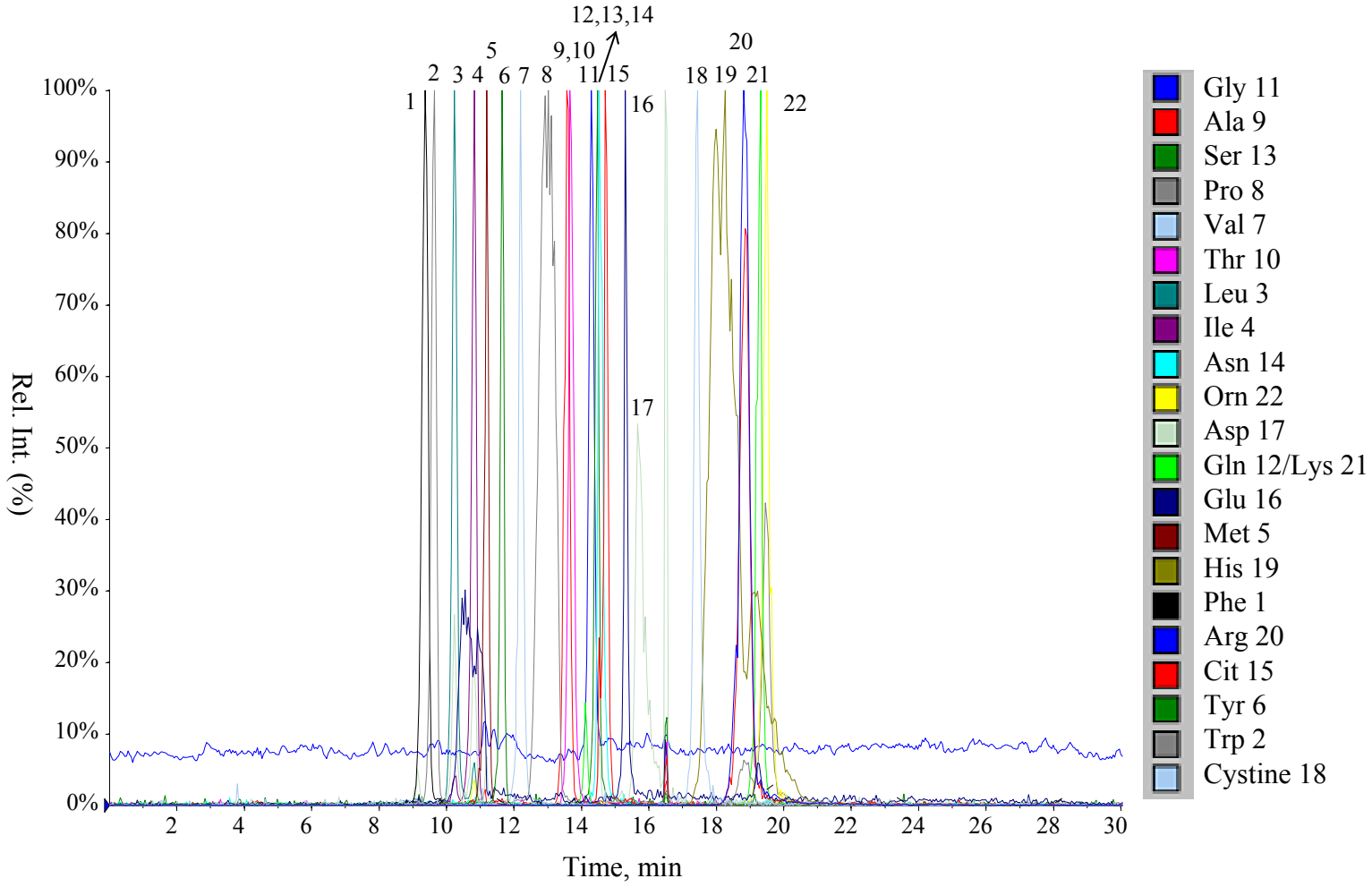
12,13,14
5
9,10
20
2 3 4 6 7 8 11/15 18 19 21
1
16
22
17
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Rel. Int. (%)
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Time, min
Gly 11
Ala 9
Ser 13
Pro 8
Val 7
Thr 10
Leu 3
Ile 4
Asn 14
Orn 22
Asp 17
Gln 12/Lys 21
Glu 16
Met 5
His 19
Phe 1
Arg 20
Cit 15
Tyr 6
Trp 2
Cystine 18

Figure 2A

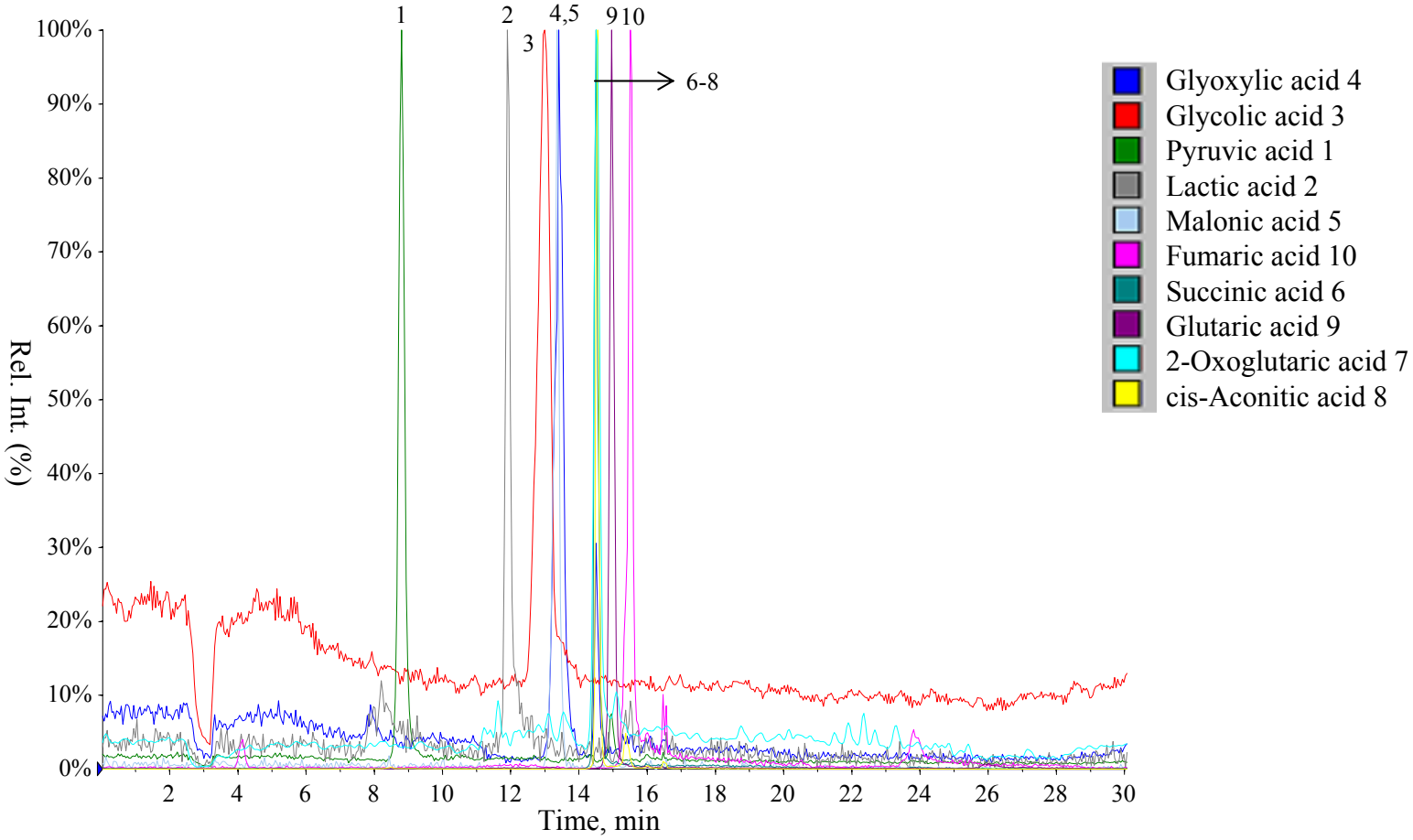
1
2
3
4,5
9
10
6-8
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Rel. Int. (%)
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Time, min
Glyoxylic acid 4
Glycolic acid 3
Pyruvic acid 1
Lactic acid 2
Malonic acid 5
Fumaric acid 10
Succinic acid 6
Glutaric acid 9
2-Oxoglutaric acid 7
cis-Aconitic acid 8

Figure 2B

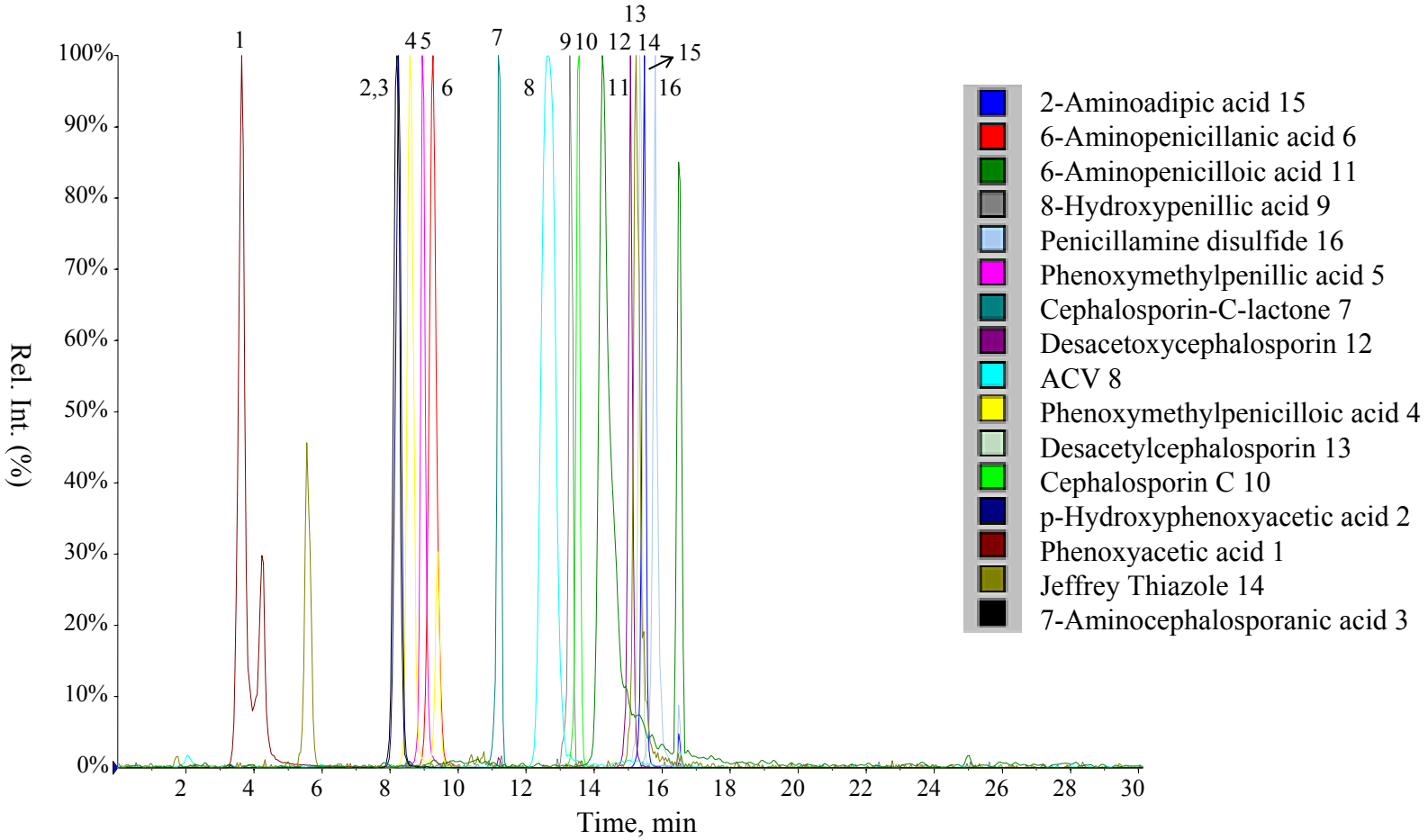
1
4 5
7
9 10
12 14
13
15
2,3
6
8
11
16
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Rel. Int. (%)
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Time, min
2-Aminoadipic acid 15
6-Aminopenicillanic acid 6
6-Aminopenicilloic acid 11
8-Hydroxypenillic acid 9
Penicillamine disulfide 16
Phenoxymethylpenillic acid 5
Cephalosporin-C-lactone 7
Desacetoxycephalosporin 12
ACV 8
Phenoxymethylpenicilloic acid 4
Desacetylcephalosporin 13
Cephalosporin C 10
p-Hydroxyphenoxyacetic acid 2
Phenoxyacetic acid 1
Jeffrey Thiazole 14
7-Aminocephalosporanic acid 3

Figure 2C

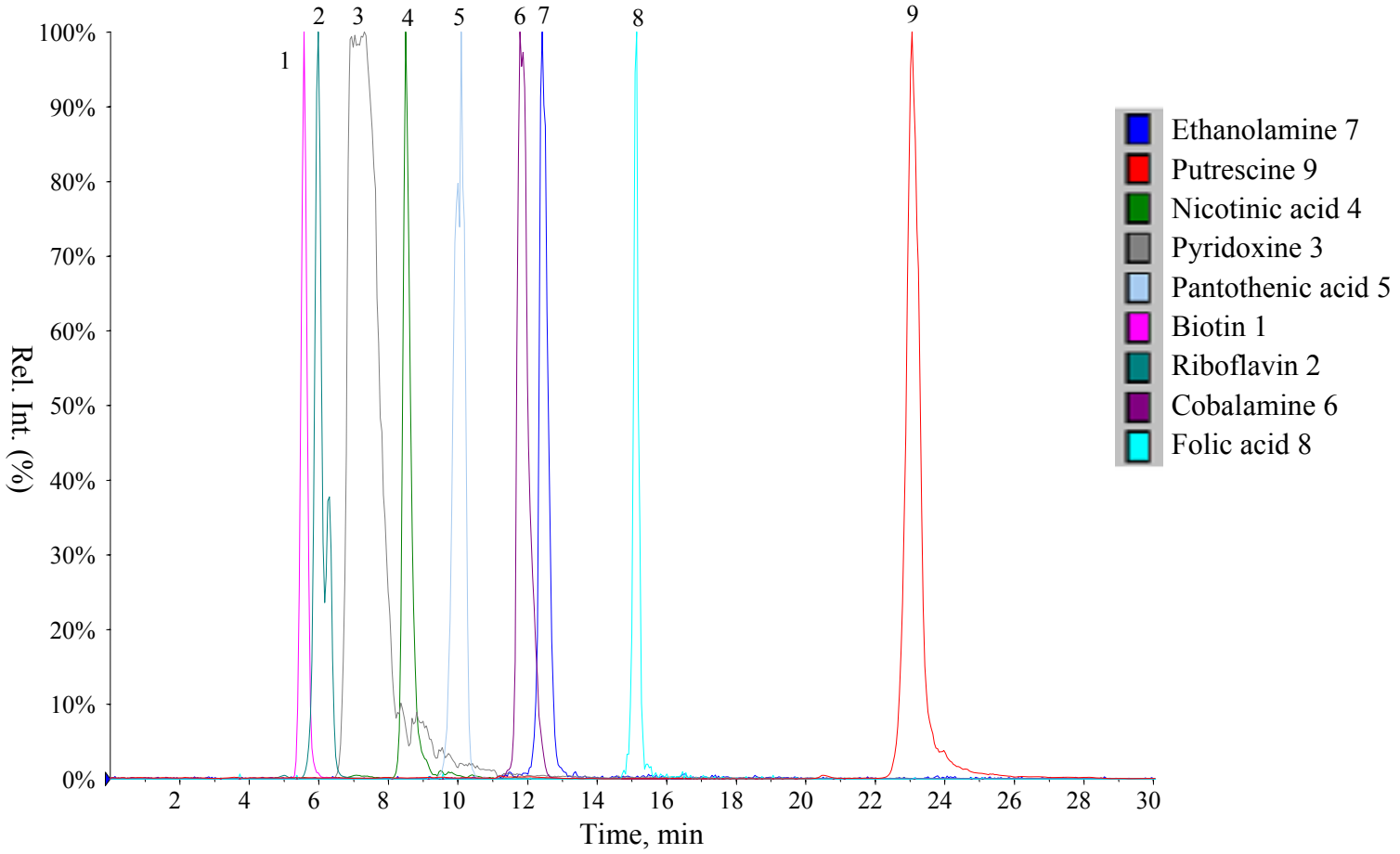
1
2
3
4
5
6
7
8
9
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Rel. Int. (%)
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Time, min
Ethanolamine 7
Putrescine 9
Nicotinic acid 4
Pyridoxine 3
Pantothenic acid 5
Biotin 1
Riboflavin 2
Cobalamine 6
Folic acid 8

Figure 2D

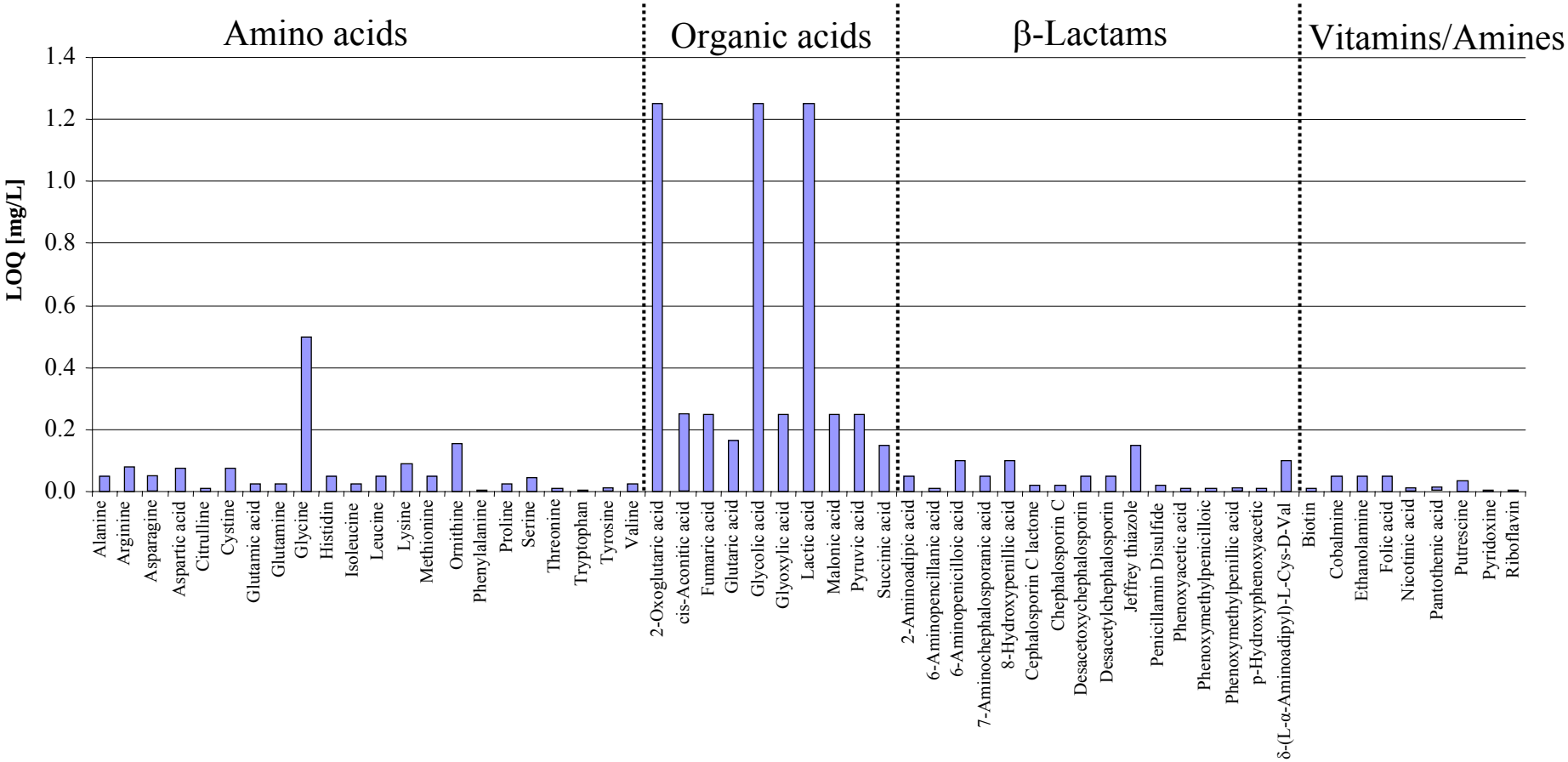
Amino acids
Organic acids
β-Lactams
Vitamins/Amines
LOQ [mg/L]
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Alanine
Arginine
Asparagine
Aspartic acid
Citrulline
Cystine
Glutamic acid
Glutamine
Glycine
Histidin
Isoleucine
Leucine
Lysine
Methionine
Ornithine
Phenylalanine
Proline
Serine
Threonine
Tryptophan
Tyrosine
Valine
2-Oxoglutaric acid
cis-Aconitic acid
Fumaric acid
Glutaric acid
Glycolic acid
Glyoxylic acid
Lactic acid
Malonic acid
Pyruvic acid
Succinic acid
2-Aminoadipic acid
6-Aminopencillanic acid
6-Aminopenicilloic acid
7-Aminochephalosporanic acid
8-Hydroxypenillic acid
Cephalosporin C lactone
Chephalosporin C
Desacetoxychephalosporin
Desacetylchephalosporin
Jeffrey thiazole
Penicillamin Disulfide
Phenoxyacetic acid
Phenoxymethylpenicilloic
Phenoxymethylpenillic acid
p-Hydroxyphenoxyacetic
δ-(L-α-Aminoadipyl)-L-Cys-D-Val
Biotin
Cobalmine
Ethanolamine
Folic acid
Nicotinic acid
Pantothenic acid
Putrescine
Pyridoxine
Riboflavin

Figure 3

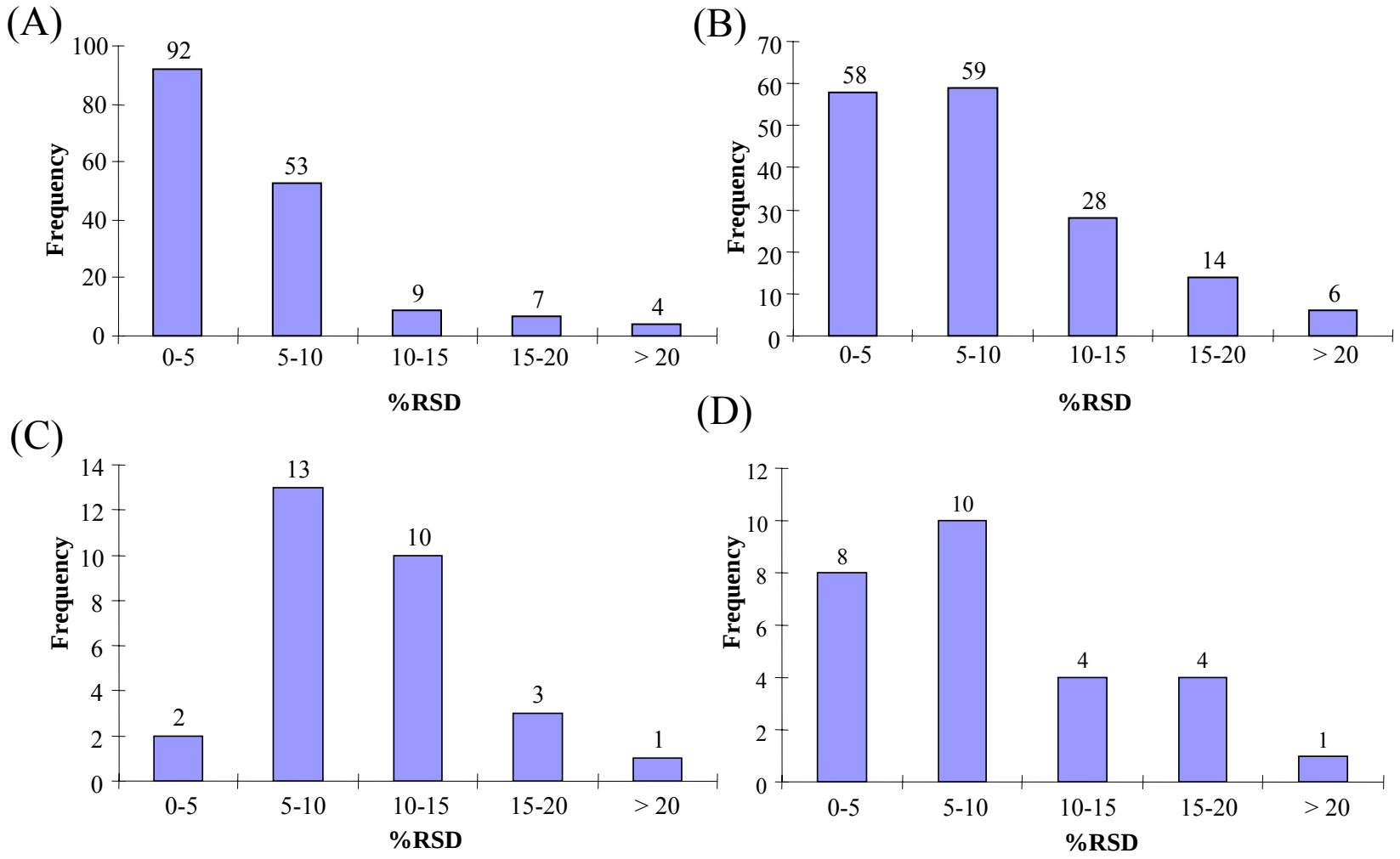
(A)
Frequency
100
80
60
40
20
0
92
53
9
7
4
0-5
5-10
10-15
15-20
> 20
%RSD
(B)
Frequency
70
60
50
40
30
20
10
0
58
59
28
14
6
0-5
5-10
10-15
15-20
> 20
%RSD
(C)
Frequency
14
12
10
8
6
4
2
0
2
13
10
3
1
0-5
5-10
10-15
15-20
> 20
%RSD
(D)
Frequency
12
10
8
6
4
2
0
8
10
4
4
1
0-5
5-10
10-15
15-20
> 20
%RSD

Figure 4

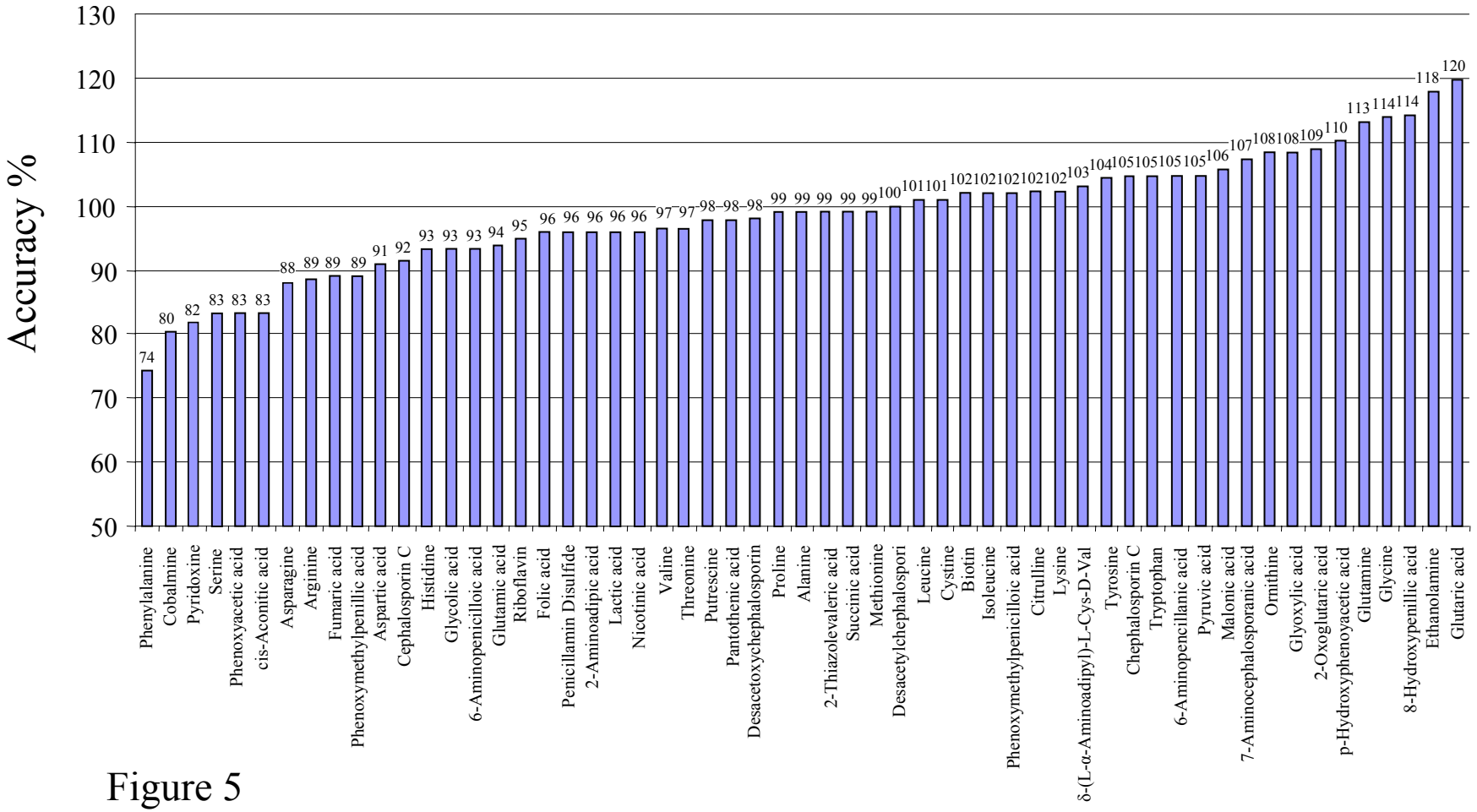
Accuracy %
130
120
110
100
90
80
70
60
50
74 80 82 83 83 83 88 89 89 89 91 92 93 93 93 94 95 96 96 96 96 96 97 97 98 98 98 99 99 99 99 99 100 101 101 102 102 102 102 102 103 104 105 105 105 105 106 107 108 108 109 110 113 114 114 118 120
Phenylalanine
Cobalmine
Pyridoxine
Serine
Phenoxyacetic acid
cis-Aconitic acid
Asparagine
Arginine
Fumaric acid
Phenoxymethylpenillic acid
Aspartic acid
Cephalosporin C
Histidine
Glycolic acid
6-Aminopenicilloic acid
Glutamic acid
Riboflavin
Folic acid
Penicillamin Disulfide
2-Aminoadipic acid
Lactic acid
Nicotinic acid
Valine
Threonine
Putrescine
Pantothenic acid
Desacetoxychephalosporin
Proline
Alanine
2-Thiazolevaleric acid
Succinic acid
Methionine
Desacetylchephalospori
Leucine
Cystine
Biotin
Isoleucine
Phenoxymethylpenicilloic acid
Citrulline
Lysine
δ-(L-α-Aminoadipyl)-L-Cys-D-Val
Tyrosine
Chephalosporin C
Tryptophan
6-Aminopencillanic acid
Pyruvic acid
Malonic acid
7-Aminocephalosporanic acid
Ornithine
Glyoxylic acid
2-Oxoglutaric acid
p-Hydroxyphenoyacetic acid
Glutamine
Glycine
8-Hydroxypenillic acid
Ethanolamine
Glutaric acid

Figure 5

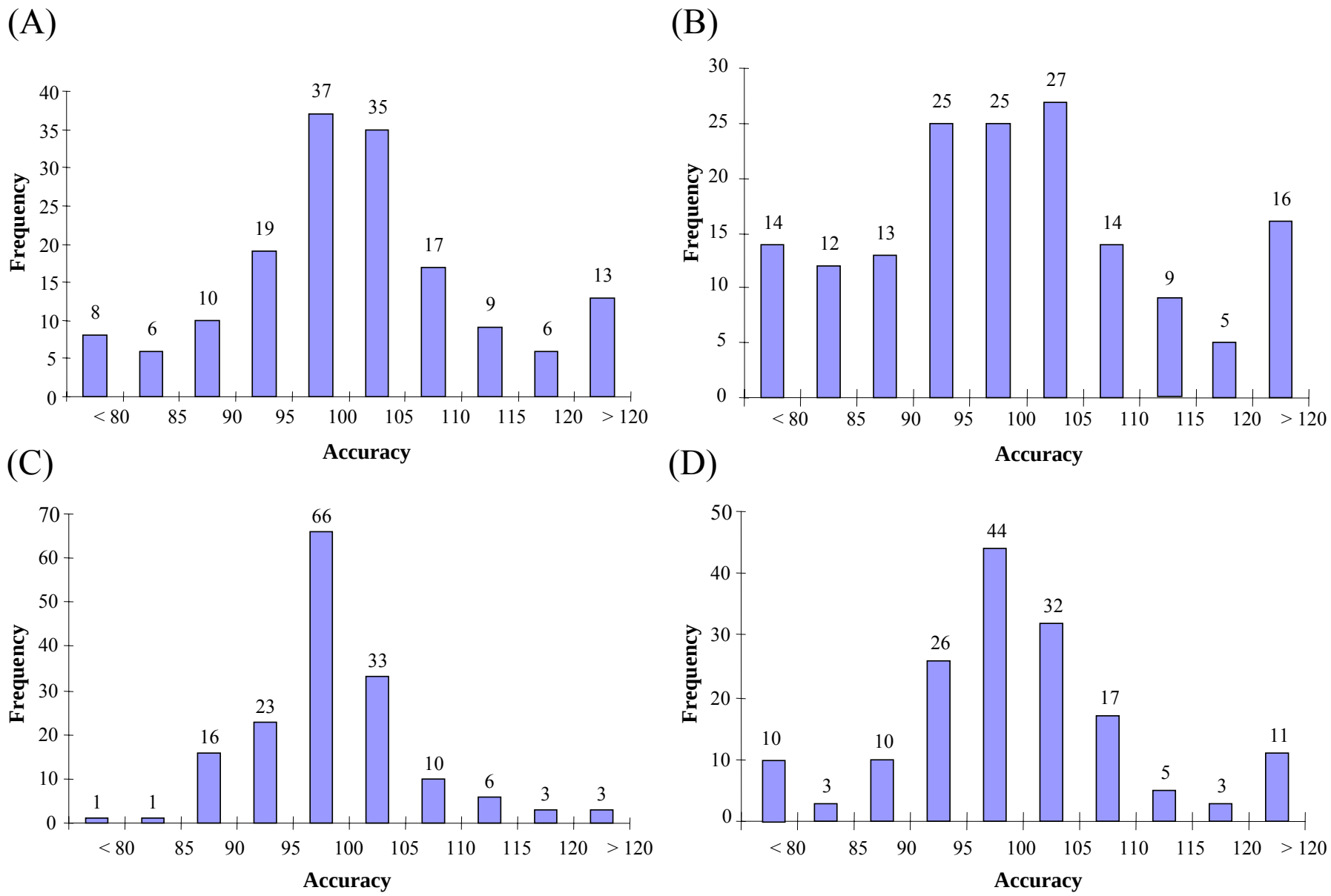
(A)
Frequency
Accuracy
< 80
85
90
95
100
105
110
115
120
> 120
8
6
10
19
37
35
17
9
6
13
(B)
Frequency
Accuracy
14
12
13
25
25
27
14
9
5
16
(C)
Frequency
Accuracy
1
1
16
23
66
33
10
6
3
3
(D)
Frequency
Accuracy
10
3
10
26
44
32
17
5
3
11

Figure 6

Table 1: List of investigated analytes along with their compound specific MS/MS parameters as well as associated internal standards.

Compound	Cal. group[a]	Molecular weight	Polarity	Precursor Ion	Product Ion	DP [V][b]	CE [V][c]	CXP [V][d]	Internal standard[e]
Amino acids									
Alanine	3	89	pos.	90	44	26	21	8	U-^{13}C,^{15}N-Ala
Arginine	2	174	pos.	175	116	46	19	6	U-^{13}C,^{15}N-Arg
Asparagine	2	132	pos.	133	74	41	21	2	U-^{13}C,^{15}N-Gln
Aspartic acid	2	133	pos.	134	88	41	15	14	U-^{13}C,^{15}N-Glu
Citrulline	2	175	pos.	176	70	36	37	12	U-^{13}C,^{15}N-Gln
Cystine	2	240	pos.	241	74	46	37	4	U-^{13}C,^{15}N-Cystine
Glutamic acid	2	147	pos.	148	130	31	12	22	U-^{13}C,^{15}N-Glu
Glutamine	2	146	pos.	147	84	36	29	4	U-^{13}C,^{15}N-Gln
Glycine	4	75	pos.	76	76	16	5	12	U-^{13}C,^{15}N-Ala
Histidine	2	155	pos.	156	110	46	21	18	U-^{13}C,^{15}N-His
Isoleucine	2	131	pos.	132	69	46	25	12	U-^{13}C,^{15}N-Ile
Leucine	2	131	pos.	132	43	46	39	6	U-^{13}C,^{15}N-Leu
Lysine	2	146	pos.	147	84	36	25	0	U-^{13}C,^{15}N-Lys
Methionine	2	149	pos.	150	133	56	15	8	U-^{13}C,^{15}N-Met
Ornithine	2	132	pos.	133	70	36	31	10	U-^{13}C,^{15}N-Arg
Phenylalanine	2	165	pos.	166	120	51	21	20	U-^{13}C,^{15}N-Phe
Proline	2	115	pos.	116	70	26	25	12	U-^{13}C,^{15}N-Pro
Serine	2	105	pos.	106	60	16	15	6	U-^{13}C,^{15}N-Ser
Threonine	2	119	pos.	120	74	36	17	4	U-^{13}C,^{15}N-Thr
Tryptophan	2	204	pos.	205	188	51	17	10	U-^{13}C,^{15}N-Trp
Tyrosine	2	181	pos.	182	136	46	21	6	U-^{13}C,^{15}N-Tyr
Valine	2	117	pos.	118	72	41	19	10	U-^{13}C,^{15}N-Val
Organic acids									
2-Oxoglutaric acid	4	146	neg.	145	101	-20	-12	-7	U-^{13}C-Succinic acid
cis-Aconitic acid	4	174	neg.	173	85	-25	-18	-5	U-^{13}C-Succinic acid
Fumaric acid	4	116	neg.	115	71	-40	-10	-5	U-^{13}C-Succinic acid
Glutaric acid	4	132	neg.	131	87	-25	-18	-7	U-^{13}C-Succinic acid
Glycolic acid	4	76	neg.	75	75	-30	-6	-3	U-^{13}C-Malonic acid
Glyoxylic acid	4	74	neg.	73	73	-30	-6	-3	U-^{13}C-Malonic acid
Lactic acid	4	90	neg.	89	43	-45	-18	-5	U-^{13}C-Malonic acid
Malonic acid	4	104	neg.	103	41	-25	-36	-3	U-^{13}C-Malonic acid
Pyruvic acid	4	88	neg.	87	87	-30	-6	-3	U-^{13}C-Malonic acid
Succinic acid	4	118	neg.	117	73	-35	-6	-7	U-^{13}C-Succinic acid
β-Lactam antibiotics, intermediates of biosynthesis and degradation products									
2-Aminoadipic acid	3	161	pos.	162	98	36	23	8	U-^{13}C,^{15}N-Ser
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	3	244	neg.	243	199	-50	-14	-15	U-^{13}C-Succinic acid
6-Aminopenicillanic acid	3	216	pos.	217	189	51	11	12	U-^{13}C,^{15}N-Phe
6-Aminopenicilloic acid	3	234	pos.	235	128	51	29	10	U-^{13}C,^{15}N-Ser
7-Aminocephalosporanic acid	3	272	neg.	271	211	-25	-8	-1	U-^{13}C-Malonic acid
8-Hydroxypenillic acid	3	260	pos.	261	114	76	33	18	Ethanol-1,1,2,2-D_4-amine
Cephalosporin C	3	415	pos.	416	143	51	27	14	U-^{13}C,^{15}N-Ala
Cephalosporin C lactone	3	355	pos.	356	98	51	49	16	U-^{13}C,^{15}N-Tyr
Desacetoxycephalosporin	3	357	pos.	358	201	56	23	10	U-^{13}C,^{15}N-Glu
Desacetylcephalosporin	3	373	pos.	374	143	51	23	6	U-^{13}C,^{15}N-Glu
Penicillamine disulfide	3	296	pos.	297	180	41	21	16	U-^{13}C,^{15}N-Glu
Phenoxyacetic acid	3	152	neg.	151	93	-40	-20	-3	U-^{13}C-Malonic acid
Phenoxymethylpenicilloic acid	3	368	pos.	369	160	41	23	8	U-^{13}C,^{15}N-Phe
Phenoxymethylpenillic acid	3	350	pos.	351	213	71	37	10	U-^{13}C,^{15}N-Phe
p-Hydroxyphenoxyacetic acid	3	168	neg.	167	109	-40	-16	-5	U-^{13}C-Malonic acid
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	3	363	pos.	364	55	51	49	16	U-^{13}C,^{15}N-Val
Vitamins and biogenic amines									
Biotin	2	244	pos.	245	97	46	43	8	U-^{13}C,^{15}N-Phe
Cobalamine	3	1355	pos.	678.5	147	66	69	8	U-^{13}C,^{15}N-Trp
Ethanolamine	2	61	pos.	62	45	31	21	6	Ethanol-1,1,2,2-D_4-amine
Folic acid	3	441	neg.	440	132	-80	-62	-5	U-^{13}C-Succinic acid
Nicotinic acid	3	123	pos.	124	78	81	35	12	U-^{13}C,^{15}N-Phe
Pantothenic acid	2	219	pos.	220	90	41	21	6	U-^{13}C,^{15}N-Trp
Putrescine	2	88	pos.	89	72	31	13	10	U-^{13}C,^{15}N-Arg
Pyridoxine	1	169	pos.	170	152	41	19	12	U-^{13}C,^{15}N-Phe
Riboflavine	2	376	pos.	377	243	71	35	14	U-^{13}C,^{15}N-Phe
Internal standards									
U-^{13}C,^{15}N-Ala		93	94	47	pos	31	17	6	
U-^{13}C,^{15}N-Arg		184	185	75	pos	56	33	12	
U-^{13}C,^{15}N-Cystine		248	249	156	pos	36	19	8	
U-^{13}C,^{15}N-Gln		153	154	136	pos	36	15	8	
U-^{13}C,^{15}N-Glu		153	154	136	pos	36	15	8	
U-^{13}C,^{15}N-His		164	165	118	pos	36	21	6	
U-^{13}C,^{15}N-Ile		138	139	92	pos	41	17	14	
U-^{13}C,^{15}N-Leu		138	139	92	pos	41	17	14	
U-^{13}C,^{15}N-Lys		154	155	90	pos	36	25	14	
U-^{13}C,^{15}N-Met		155	156	109	pos	41	15	18	
U-^{13}C,^{15}N-Phe		175	176	129	pos	26	21	8	
U-^{13}C,^{15}N-Pro		121	122	75	pos	36	23	12	
U-^{13}C,^{15}N-Ser		109	110	63	pos	51	17	15	
U-^{13}C,^{15}N-Thr		124	125	78	pos	31	17	12	
U-^{13}C,^{15}N-Trp		217	218	200	pos	36	15	10	
U-^{13}C,^{15}N-Tyr		191	192	174	pos	26	15	8	
U-^{13}C,^{15}N-Val		123	124	77	pos	36	17	12	
Ethanol-1,1,2,2-d_4-amine		65	66	48	pos	36	15	6	
U-^{13}C Succinic acid		122	121	76	neg	-35	-16	-5	
U-^{13}C Malonic acid		107	106	61	neg	-30	-14	-1	

[a] For calibration with neat standard solutions analytes were divided into four calibration groups: group 1 was calibrated in a range of 0.025 - 1.0 mg/L; group 2 in a range of 0.05 to 2.0 mg/L; group 3 in a range of 0.1 to 4.0 mg/L and group 4 in a range of 0.25 to 10 mg/L. Standard addition was carried out by spiking 0.025 - 0.5 mg/L of group 1; 0.05 - 1.0 mg/L of group 2; 0.1 - 2.0 mg/L of group 3 and 0.25 - 5.0 mg/L of group 4 to diluted samples (fermentation extracts 1:100; nutrition media 1:500).

[b] Declustering potential

[c] Collision energy

[d] Cell exit potential

[e] Internal standard used for peak area normalization

Table 2: Results of the screening of various chromatographic conditions.

Method	Stationary Phase	Buffer[1]	flow rate	retentivity[2] (tr > 2 t_0)				peak shape problems[3]				not detected[4]				unresolved critical pairs[5]
			[µl/min]	aa[6]	oa[7]	βl[8]	vit[9]	aa	oa	βl	vit	aa	oa	βl	vit	
				[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]					
RP																
1	Synergi Fusion-RP 80	0.1% formic acid	300	5	0	43	n.a.[10]	23	43	29	n.a.	0	2	2	n.a.	9
HILIC																
2	Acclaim Mixed-Mode	200 mM acetic acid/NH_3 pH 5.0	800	100	100	100	n.a.	15	0	0	n.a.	2	9	14	n.a.	1
3	Acclaim Mixed-Mode	800 mM formic acid/NH_3 pH 3.0	800	100	100	100	n.a.	0	100	10	n.a.	0	6	4	n.a.	1
4	Chiralpak QN-AX	200 mM acetic acid/NH_3 pH 5.0	700	100	100	100	n.a.	73	83	100	n.a.	0	3	3	n.a.	5
5	Chiralpak QN-AX	800 mM formic acid/NH_3 pH 3.0	700	100	100	100	n.a.	91	100	38	n.a.	0	2	1	n.a.	7
6	Luna Amino	200 mM acetic acid/NH_3 pH 5.0	350	100	100	100	100	19	0	40	63	1	8	2	0	1
7	Luna Amino	800 mM formic acid/NH_3 pH 3.0	350	100	100	100	100	24	20	33	38	1	4	3	0	0
8	Biobasic AX	200 mM acetic acid/NH_3 pH 5.0	410	100	80	100	75	45	20	14	38	0	4	1	0	2
9	Biobasic AX	800 mM formic acid/NH_3 pH 3.0	410	100	100	79	63	27	22	7	25	0	1	1	0	2
10	Biobasic AX	200 mM formic acid/NH3 pH 3.0	410	100	100	100	50	32	0	9	50	0	4	4	0	1
11	Chromolith Si	200 mM acetic acid/NH_3 pH 5.0	1010	100	100	50	78	23	38	57	22	0	1	0	0	3
12	ZIC-HILIC	200 mM acetic acid/NH_3 pH 5.0	700	100	100	73	100	9	0	18	20	0	0	0	0	1
13	ZIC-HILIC	200 mM formic acid/NH3 pH 3.0	700	100	91	67	78	0	45	7	33	0	0	0	0	2
14	ZIC-HILIC	800 mM formic acid/NH_3 pH 3.0	700	100	89	67	75	9	33	0	25	0	0	0	0	1
15	Obelsik R	800 mM formic acid/NH_3 pH 3.0	900	100	100	100	75	23	86	31	13	0	3	1	0	1
16	Obelsik N	800 mM formic acid/NH_3 pH 3.0	870	100	86	82	88	33	71	65	75	1	4	0	0	3
17	TSKgel Amide-80	200 mM acetic acid/NH_3 pH 5.0	170	100	100	67	100	36	25	20	63	0	1	0	0	1
18	TSKgel Amide-80	800 mM formic acid/NH_3 pH 3.0	170	100	75	60	75	27	50	27	38	0	1	0	0	1
19	TSKgel Amide-80	200 mM formic acid/NH3 pH 3.0	170	100	73	71	78	59	55	71	67	0	0	0	0	1
20	Luna HILIC	200 mM acetic acid/NH_3 pH 5.0	820	100	100	80	88	29	0	20	13	1	1	2	0	3
21	Luna HILIC	800 mM formic acid/NH_3 pH 3.0	820	100	91	76	75	19	9	6	25	1	0	0	0	2
22	Luna HILIC	200 mM formic acid/NH3 pH 3.0	820	100	50	71	67	18	20	18	56	0	1	0	0	2

[1] elution conditions:
RP method: (A) 0.1% formic acid in H_2O; (B) 0.1% formic acid in ACN; 0-10 min 100% (A); 10 - 30 min 100% (A) to 100% (B); 0.3 ml/min
HILIC methods: (A) 10% buffer in H_2O; (B) 10% buffer in ACN; 0-30 min 100% (B) to 20% (B), 0.7 ml/min
[2] percentage of analytes with retention times greater than twice the dead volume
[3] percentage of analytes with peak shape problems including peak asymmetry, tailing, peak splitting, peak width > 2 min
[4] number of analytes injected but not detected
[5] critical pairs: isobars, compounds that differ in their molecular weight by 1 Da only, compounds with signal interference
[6] aa: amino acids
[7] oa: organic acids
[8] βl: β-lactam antibiotics and related compounds
[9] vit: vitamins and biogenic amines
[10] n.a. not available

Table 3: Retention times of investigated analytes along with some potential signal interferences in neat standard solutions and spiked fermentation extracts, respectively.[a]

Compound	pH 4.0[a] tr [min]	Peak width at 50% height	Peak Asymmetry	neat standard solution Interferences tr [min]	spiked extract 12 Interferences tr [min]
Amino acids					
Alanine	13.6	0.224	1.090	11.2	16.7
Arginine	19.0	0.254	1.070		15.5
Asparagine	14.6	0.218	1.100		16.7
Aspartic acid	15.8	0.420	2.290	10.2 (Leu); 10.8 (Ile); 11.2 (Met); 14.7 (Gln)	10.5 (Leu); 11.0 (Ile); 16.8
Citrulline	14.8	0.182	1.140	19.0 (Arg)	16.2; 16.7;19.1 (Arg)
Cystine	17.5	0.224	1.660		
Glutamic acid	15.3	0.177	1.050	11.1	10.7; 16.7
Glutamine	14.2	0.177	0.632	19.5 (Lys)	19.5 (Lys); 16.7
Glycine	14.3	0.171	0.630	11.2	11.4
Histidine	18.8	0.967	1.290		11.7
Isoleucine	10.8	0.196	0.881	10.2 (Leu)	8.0-10.0; 10.8 (Leu); 15.0
Leucine	10.2	0.176	0.738	10.8 (Ile)	8.0-10.0;11.1 (Ile)
Lysine	19.5	0.152	0.720	14.2 (Gln)	14.3 (Gln); 16.7
Methionine	11.2	0.200	1.160		
Ornithine	19.6	0.261	0.817	10.2 (Leu); 10.8 (Ile)	11.10
Phenylalanine	9.4	0.184	0.812		7.5; 16.8
Proline	13.0	0.424	0.803	19.0 (Arg); 19.7 (Orn)	16.7;19.1 (Arg); 19.7(Orn)
Serine	14.5	0.169	0.646		16.7
Threonine	13.7	0.207	1.420		16.7
Tryptophan	9.7	0.185	1.580		
Tyrosine	11.7	0.175	0.748		16.7
Valine	12.2	0.238	0.765		10.9;16.7
Organic acids					
2-Oxoglutaric acid	14.5	0.184	1.000		
cis-Aconitic acid	14.5	0.144	0.696	15.4 (trans Aconitic acid)[b]; 16.5	15.6 (trans-Aconitic acid)[b];16.7
Fumaric acid	15.5	0.161	0.857	4.0; 16.5; 23.9	4.4; 16.8; 24.1
Glutaric acid	15.0	0.176	0.928	26.0	13.6; 26.2
Glycolic acid	13.0	0.367	0.708		
Glyoxylic acid	13.5	0.289	0.576	14.5 (Succinic acid)	14.7 (Succinic acid)
Lactic acid	11.9	0.201	1.070		
Malonic acid	13.4	0.223	1.020		
Pyruvic acid	8.8	0.210	1.900	15.0 (Glutaric acid)	15.2 (Glutaric acid)
Succinic acid	14.5	0.168	1.460		
β-Lactam antibiotics, intermediates of biosynthesis and degradation products					
2-Aminoadipic acid	15.5	0.170	1.320		
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	15.3	0.288	1.840	5.6 (Biotin)	5.8 (Biotin)
6-Aminopenicillanic acid	9.2	0.207	1.230		
6-Aminopenicilloic acid	14.4	0.402	3.000		16.7
7-Aminocephalosporanic acid	8.2	0.183	1.580		
8-Hydroxypenillic acid	13.3	0.181	1.700		
Cephalosporin C	13.5	0.172	1.390		
Cephalosporin C lactone	11.2	0.174	0.943		
Desacetoxycephalosporin	15.1	0.188	1.230	11.2	
Desacetylcephalosporin	15.3	0.185	0.940		
Penicillamine disulfide	15.8	0.223	2.310		16.7
Phenoxyacetic acid	4.0	0.223	6.280		
Phenoxymethylpenicilloic acid	8.6	0.188	0.889	9.4 (degradation product or epimer)	3.1; 3.3; 3.8; 4.3[c]; 9.4
Phenoxymethylpenillic acid	9.0	0.181	2.36	8.7 (Phenoxymethylpenicilloic acid)	2.8; 3.3; 3.8[c]
p-Hydroxyphenoxyacetic acid	8.3	0.201	1.400		4.1; 4.5; 7.5
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	12.7	0.459	0.514		15.0
Vitamins and biogenic amines					
Biotin	5.6	0.211	2.600		
Cobalamine	11.9	0.438	1.570		
Ethanolamine	12.5	0.312	1.440		
Folic acid	15.2	0.213	0.724		
Nicotinic acid	8.5	0.224	2.720	7.0	
Pantothenic acid	10.0	0.306	1.330		
Putrescine	23.1	0.355	2.090	11.3; 21.0	20.6
Pyridoxine	7.1	0.517	4.820		
Riboflavin	6.2	0.276	1.900	5.3	5.5

[a] Chromatographic conditions: (A) 10% buffer in H_2O; (B) 10% buffer in ACN; buffer: 200 mM formic acid adjusted with NH_4OH solution to pH 4.0.
Gradient: 100% (B) to 35% (B) in 25 minutes; flow rate 700 µl/min

[b] Trans-aconitic acid was not a target solute, however, it was contained in the employed standard of cis-aconitic acid.

[c] In extracts of fermentation broths considerable amounts of penicillin (isobaric to phenoxymethylpenillic acid) and related degradation products are contained that may interfere with the detection of phenoxymethylpenillic acid and phenoxymethylpenicilloic acid.

Table 4: LLOQ, ULOQ, calibration functions and corrsponding correlation coefficients obtained for the compounds under investigation. Calibration functions were constructed with pure matrix-free standard solutions with and without use of internal standards.

			without use of internal standards			with use of internal standards		
	LLOQ [mg/L]	ULOQ [mg/L]	slope	intercept	R^2	slope	intercept	R^2
Amino acids								
Alanine	0.050	100	6.50E+05	4.70E+04	0.9976	2.040	0.117	0.9977
Arginine	0.081[a]	5	9.40E+06	2.00E+06	0.9395	0.783	0.020	0.9885
Asparagine	0.050	100[b]	5.35E+05	-1.97E+04	0.9981	2.650	-0.030	0.9934
Aspartic acid	0.075	100	3.66E+05	3.36E+02	0.9902	1.370	0.054	0.9975
Citrulline	0.010	65	1.09E+06	3.95E+04	0.9956	5.560	0.103	0.9930
Cystine	0.075	75[b]	3.10E+05	-3.66E+02	0.9943	9.090	0.055	0.9931
Glutamic acid	0.025	150[b]	5.00E+05	2.51E+06	0.9725	1.750	0.087	0.9899
Glutamine	0.025	150[b]	5.75E+05	1.65E+04	0.9973	2.990	0.045	0.9966
Glycine	0.500	150[b]	3.68E+05	-7.44E+03	0.9969	1.080	0.183	0.9960
Histidine	0.052[a]	30	8.10E+07	6.07E+06	0.9987	15.100	0.586	0.9982
Isoleucine	0.025	15	5.65E+06	5.39E+04	0.9979	0.596	0.001	0.9976
Leucine	0.050	12	3.59E+06	1.33E+05	0.9946	0.178	0.001	0.9976
Lysine	0.089[a]	65	5.85E+06	7.70E+05	0.9952	2.760	0.068	0.9943
Methionine	0.050	30	1.95E+06	-1.90E+04	0.9995	6.490	-0.001	0.9981
Ornithine	0.156[a]	40	7.04E+06	3.32E+06	0.9860	0.664	0.062	0.9845
Phenylalanine	0.005	2	8.95E+07	3.62E+06	0.9976	7.260	0.084	0.9973
Proline	0.003	9	1.56E+07	2.28E+05	0.9992	5.260	0.091	0.9982
Serine	0.045[a]	100[b]	5.55E+05	8.92E+04	0.9938	11.500	1.370	0.9898
Threonine	0.011[a]	100	1.14E+06	3.90E+04	0.9955	4.050	0.101	0.9986
Tryptophan	0.005	5	3.24E+07	7.22E+06	0.9933	6.780	0.098	0.9808
Tyrosine	0.010	80	1.10E+06	4.40E+03	0.9975	14.900	-0.009	0.9926
Valine	0.025	15	3.64E+06	-2.78E+04	0.9956	2.290	0.010	0.9906
Organic acids								
2-Oxoglutaric acid	1.250	150	4.47E+05	1.41E+05	0.9966	0.649	0.023	0.9910
cis-Aconitic acid	0.250	50[b]	1.94E+06	-8.34E+05	0.9646	2.120	-0.518	0.9842
Fumaric acid	0.250	50[b]	2.96E+05	-1.10E+04	0.9760	0.474	-0.017	0.9543
Glutaric acid	0.167[a]	100[b]	2.13E+06	1.23E+06	0.9962	2.830	1.080	0.9990
Glycolic acid	1.250	150[b]	9.65E+05	4.59E+05	0.9975	0.779	0.803	0.9865
Glyoxylic acid	0.250	100[b]	4.72E+05	3.27E+05	0.9792	0.560	0.119	0.9805
Lactic acid	1.250	100[b]	4.05E+04	2.53E+04	0.9968	0.034	0.035	0.9980
Malonic acid	0.250	20	7.30E+04	2.69E+03	0.9922	0.078	0.001	0.9974
Pyruvic acid	0.250	100[b]	1.73E+06	8.25E+04	0.9985	1.800	0.053	0.9891
Succinic acid	0.150[a]	100[b]	1.41E+06	1.34E+04	0.9969	1.810	0.092	0.9943
β-Lactam antibiotics, intermediates of biosynthesis and degradation products								
2-Aminoadipic acid	0.050	100[b]	1.04E+06	1.16E+04	0.9944	26.700	0.518	0.9947
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	0.150	50	2.58E+05	4.55E+03	0.9934	0.352	0.008	0.9946
6-Aminopenicillanic acid	0.010	15	1.42E+07	2.68E+06	0.9898	1.310	0.060	0.9923
6-Aminopenicilloic acid	0.100	40	2.08E+05	2.16E+04	0.9940	5.285	0.428	0.9935
7-Aminocephalosporanic acid	0.050	50	2.04E+06	1.28E+04	0.9834	2.540	-0.051	0.9854
8-Hydroxypenillic acid	0.100	30	4.20E+04	-7.80E+01	0.9948	0.020	-0.001	0.9980
Cephalosporin C	0.020	40	8.27E+05	-2.73E+04	0.9997	2.671	-0.010	0.9994
Cephalosporin C lactone	0.020	40	1.58E+06	-5.76E+04	0.9976	21.200	-0.427	0.9960
Desacetoxycephalosporin	0.050	50	6.60E+05	-1.58E+04	0.9986	2.382	0.059	0.9963
Desacetylcephalosporin	0.050	40	2.13E+05	-1.52E+02	0.9854	0.767	0.032	0.9870
Penicillamine disulfide	0.020	65	2.11E+06	-4.96E+04	0.9988	7.830	0.110	0.9966
Phenoxyacetic acid	0.010	2	1.96E+07	6.99E+05	0.9981	26.400	-0.430	0.9997
Phenoxymethylpenicilloic acid	0.010	20	2.68E+07	-9.77E+05	0.9816	2.360	-0.091	0.9952
Phenoxymethylpenillic acid	0.010	10	2.19E+06	1.21E+05	0.8415	0.388	-0.018	0.9526
p-Hydroxyphenoxyacetic acid	0.010	20	5.10E+06	3.57E+04	0.9976	6.410	-0.126	0.9926
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	0.100	100[b]	5.70E+05	2.37E+04	0.9964	0.376	0.008	0.9841
Vitamins and biogenic amines								
Biotin	0.010	10	1.06E+07	1.69E+05	0.9944	1.110	0.000	0.9887
Cobalamine	0.050	65	2.33E+06	-3.53E+04	0.9843	0.376	-0.017	0.9907
Ethanolamine	0.050	6.5	1.98E+06	8.64E+04	0.9884	0.926	0.005	0.9968
Folic acid	0.050	75	2.09E+05	6.50E+04	0.9190	0.278	0.060	0.9977
Nicotinic acid	0.010	2	1.23E+07	1.92E+06	0.9623	0.928	0.060	0.9804
Pantothenic acid	0.010	30	5.40E+06	-5.98E+04	0.9931	0.915	-0.023	0.9930
Putrescine	0.037[a]	2	4.11E+07	1.63E+06	0.9936	2.290	0.062	0.9995
Pyridoxine	0.003	0.25	9.35E+08	2.50E+07	0.9936	72.300	0.768	0.9918
Riboflavine	0.005	7	2.04E+07	-4.75E+05	0.9982	2.020	-0.053	0.9984

[a] LLOQ calculated from standard deviation of memory peak areas of blank runs: 3 x standard deviation of memory peak area (n = 5)/slope of calibration function obtained with neat standard solutions without use of internal standards.

[b] ULOQ may be underestimated since no higher concentration was measured.

Table 5: Assessment of matrix effects by comparison of the slopes of calibration functions (without IS) in standard solution and of standard addition in 4 different extracts and two nutrition media.[h]

	Extracts of fermentation broths					Nutrition media		
	Absolute matrix effect (%) [a]				Relative matrix effect [b]	Absolute matrix effect (%) [a]		Relative matrix effect [b]
	Extract 12[c]	Extract 15[c]	Extract 1[d]	Extract 5[d]	RSD (%)	Medium 6	Medium 10	RSD (%)
measured on	**day 1**	**day1**	**day 2**	**day 2**		**day 3**	**day 3**	
Amino acids								
Alanine	100	102	97	107	4.1	78	100	15.7
Arginine	89	**77**	117	n.a.[f]	**20.6**	**45**[e]	**41**[e]	2.9
Asparagine	91	87	82	92	4.5	94	81	9.0
Aspartic acid	93	86	89	**123**	16.9	84	93	7.0
Citrulline	95	102	92	101	4.8	83	85	1.8
Cystine	95	97	97	91	2.7	101	101	0.0
Glutamic acid	91	96	81	**158**	**34.9**	87	90	2.1
Glutamine	**124**	109	105	115	8.5	**140**	**149**	6.3
Glycine	111	103	106	113	4.6	106	**131**	17.1
Histidine	100	97	**78**[e]	**63**[e]	17.1	**28**[e]	**31**[e]	2.4
Isoleucine	100	96	93	86	6.0	87	99	8.3
Leucine	102	92	**71**	85	13.0	**69**	**77**	5.7
Lysine	100	87	**79**[e]	**78**[e]	10.1	**57**[e]	**37**[e]	14.1
Methionine	106	116	90	84	14.9	78	113	**24.3**
Ornithine	98	89	**70**	82	11.7	**43**[e]	**40**[e]	2.0
Phenylalanine	**70**[f]	**46**[f]	**31**[f]	**23**[f]	**20.7**[f]	**31**[f]	**40**[f]	5.8
Proline	97	104	90	87	7.7	85	104	13.6
Serine	90	89	89	88	1.0	81	**70**	7.9
Threonine	**122**	106	105	111	8.1	87	90	1.7
Tryptophan	**127**	**140**	109	80	**25.8**	**68**[e]	81	8.7
Tyrosine	102	90	85	85	8.0	88	102	10.0
Valine	104	106	**127**	112	10.2	104	105	1.0
Organic acids								
2-Oxoglutaric acid	**131**	105	84	**76**[e]	**24.4**[e]	**46**[e]	**47**[e]	1.0
cis-Aconitic acid	**66**[e]	**45**[e]	**24**[e]	**26**[e]	19.5	**11**[e]	**33**[e]	15.3
Fumaric acid	89	82	**69**[e]	**76**[e]	8.4	**55**[e]	**47**[e]	5.3
Glutaric acid	107	98	92	86	8.7	**63**[e]	**66**[e]	2.0
Glycolic acid	94	102	87	80	9.7	84	**77**[e]	5.0
Glyoxylic acid	109	113	108	96	7.4	**52**[e]	**55**[e]	1.8
Lactic acid	92	97	**139**	98	**21.6**	**72**[e]	**64**[e]	5.9
Malonic acid	104	85	**59**[e]	88	14.0	**44**[e]	**43**[e]	0.8
Pyruvic acid	102	100	96	95	3.3	85	86	0.4
Succinic acid	101	101	82	**73**[e]	14.3	**48**[e]	**47**[e]	0.8
β-Lactam antibiotics, intermediates of biosynthesis and degradation products								
2-Aminoadipic acid	**121**	116	97	104	11.1	91	98	4.9
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	102	97	84	92	7.7	**63**[e]	**66**[e]	2.2
6-Aminopenicillanic acid	106	102	**55**[e]	**56**[e]	**28.1**	**65**[e]	**68**[e]	2.4
6-Aminopenicilloic acid	108	82	87	**77**	13.6	92	101	6.4
7-Aminocephalosporanic acid	113	97	**64**[e]	**63**[e]	**24.6**[c]	**57**[e]	**63**[e]	4.2
8-Hydroxypenillic acid	103	108	95	99	5.4	105	113	5.8
Cephalosporin C	103	104	94	n.a.[f]	5.5	95	98	2.2
Cephalosporin C lactone	92	92	**78**	87	6.4	106	113	4.9
Desacetoxycephalosporin	97	99	81	93	8.3	94	97	1.7
Desacetylcephalosporin	111	101	90	n.a.[f]	10.6	106	104	1.1
Penicillamine disulfide	97	94	96	98	1.7	105	110	3.7
Phenoxyacetic acid	**28**[f]	**31**[f]	102	98	**40.7**[f]	84	91	4.7
Phenoxymethylpenicilloic acid	103	93	**70**[g]	**58**[g]	**21**[g]	**48**[g]	**55**[g]	5.0
Phenoxymethylpenillic acid	88	n.a.[g]	n.a.[g]	n.a.[g]	n.a.[g]	n.a.[g]	n.a.[g]	n.a.[g]
p-Hydroxyphenoxyacetic acid	106	98	81	**61**[e]	19.9	**46**[e]	**60**[e]	9.4
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	112	99	82	**22**[g]	**39.8**[g]	**22**[g]	**73**[g]	**35.6**[g]
Vitamins and biogenic amines								
Biotin	105	107	97	101	4.6	84	89	3.8
Cobalamine	82	88	100	**78**	9.4	91	101	6.7
Ethanolamine	113	108	115	83	14.7	96	95	0.7
Folic acid	119	94	81	85	17.1	**41**[g]	**53**[g]	8.2
Nicotinic acid	**124**[f]	115	**133**[f]	94	16.7	**54**[g]	**53**[g]	0.3
Pantothenic acid	96	89	95	89	3.8	90	90	0.0
Putrescine	87	105	**37**[e]	**54**[e]	**30.7**[c]	**56**[e]	**65**[e]	6.4
Pyridoxine	113	111	**125**[f]	102	9.5	**72**[e]	**63**[e]	6.4
Riboflavin	99	96	82	**36**[g]	**28.9**[g]	**26**[g]	94[g]	**47.8**[g]
Mean	104	118	104	102	20	117	115	10
Standard deviation	32	17	54	39	23	40	36	11
Mean (without assigned outliers)	103	99	94	95	11	92	99	5
Standard deviation	11	11	14	15	7	13	15	5

[a] calculated by ratio of slopes in matrix and standard solutions multiplied by 100
[b] calculated as the relative standard deviation from the mean in the 4 extracts and two nutrition media, respectively
[c] E 12 and E 15 are extracts from penicillin synthesis.
[d] E 1 and E 5 are extracts from cephalosporin synthesis.
[e] problems due to instrumental fluctuations (loss of sensitivity)
[f] linearity problems (endogenic concentration in extract extremely high so that 1:100 dilution is still outside linear range)
[g] problems due to limited compound stability
[h] values with a bias above +/- 20% in bold

Table 6: Intraday accuracy on day 1, 3, 4, 6; interday accuracy (n=4) and interday precision of quality control sample (extract 12 spiked with 0.25 mg/L of group 1, 0.5 mg/L of group 2, 1.0 mg/L of group 3 and 2.5 mg/L of group 4).

Accuracy	Calibration in extract 12[a] without internal standards							Calibration in extract 12[a] with internal standards						
measured on	day 1	day 3	day 4	day 6	mean	std. dev.	interday	day 1	day 3	day 4	day 6	mean	std. dev.	interday
Amino acids	intraday				interday		precision[b]	intraday				interday		precision[b]
Alanine	100	88	88	88	91	6	7	102	95	96	95	97	3	4
Arginine [c]	101	101	**44**	**43**	**40**	**33**	**51**	97	107	98	97	100	5	5
Asparagine	94	91	80	85	88	6	7	102	109	108	107	107	3	3
Aspartic acid	94	92	86	105	94	8	9	107	109	107	**125**	112	9	8
Citrulline	104	82	**65**	**69**	**80**	17	**22**	112	101	96	94	101	8	8
Cystine	102	86	83	**72**	86	12	14	**126**	105	103	100	108	12	11
Glutamic acid	96	97	97	87	94	5	5	97	**134**	**142**	116	**122**	20	16
Glutamine	98	**120**	93	**80**	98	17	17	104	**143**	**124**	98	117	**20**	17
Glycine	96	101	97	83	94	8	8	100	107	105	86	100	10	10
Histidine [c]	98	**28**	**29**	**18**	**43**	**37**	**85**	97	111	105	**120**	108	10	9
Isoleucine	100	86	92	105	96	8	9	99	93	94	94	95	3	3
Leucine	100	**77**	**77**	**79**	83	11	13	96	98	98	100	98	2	2
Lysine [c]	93	**48**	**54**	**39**	**59**	**24**	**41**	105	110	**124**	110	112	8	8
Methionine	96	99	114	**125**	108	14	13	99	101	110	95	101	6	6
Ornithine [c]	114	**59**	**62**	**53**	**72**	**28**	**39**	98	**71**	**70**	**64**	**76**	15	20
Phenylalanine [c]	97	**62**	**62**	**51**	**68**	20	**29**	96	102	106	100	101	4	4
Proline	100	98	93	95	96	3	3	102	104	100	103	102	2	2
Serine	91	87	**76**	102	89	11	12	**80**	93	82	116	93	17	18
Threonine	87	88	**79**	85	85	4	5	91	96	92	92	93	2	3
Tryptophan [c]	104	**62**	**62**	**42**	**67**	**26**	**39**	103	104	106	103	104	2	2
Tyrosine	100	98	90	106	98	7	7	112	**125**	116	**129**	**121**	8	7
Valine	98	107	101	103	102	4	4	98	105	107	107	104	4	4
Organic acids														
2-Oxoglutaric acid [c]	97	**32**	**32**	**26**	**47**	**34**	**72**	109	109	118	**134**	118	12	10
cis-Aconitic acid [c]	97	**17**	**16**	**12**	**36**	**41**	**116**	93	**57**	**59**	**62**	**68**	17	**25**
Fumaric acid [c]	110	**59**	**52**	**43**	**66**	**30**	**46**	118	**53**	**61**	**77**	**77**	**29**	**37**
Glutaric acid [c]	109	**49**	**50**	**34**	**60**	**33**	**55**	**120**	**179**	**198**	**198**	**174**	**37**	**21**
Glycolic acid [c]	101	**67**	**68**	**78**	**78**	16	**20**	87	**197**	**209**	**335**	**207**	**101**	**49**
Glyoxylic acid [c]	100	**48**	**50**	**46**	**61**	**26**	**42**	95	**134**	**144**	**184**	**139**	**37**	**26**
Lactic acid [d]	97	**76**	84	89	86	9	11	91	**190**	**218**	**316**	**204**	**93**	**45**
Malonic acid [c]	92	**32**	**27**	**17**	**42**	**34**	**81**	90	107	97	100	99	7	7
Pyruvic acid [d]	96	**79**	**80**	84	85	8	9	94	**215**	**230**	**322**	**215**	**94**	**43**
Succinic acid [c]	98	**29**	**29**	**20**	**44**	**36**	**83**	110	111	116	**120**	114	5	4
β-Lactam antibiotics, intermediates of biosynthesis and degradation products														
2-Aminoadipic acid [c]	86	**75**	**75**	**72**	**77**	6	8	89	84	82	84	85	3	4
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid [c]	97	**52**	**52**	**53**	**64**	**23**	**36**	105	**195**	**210**	**318**	**207**	**87**	**42**
6-Aminopenicillanic acid [c]	99	**63**	**69**	**53**	**71**	20	**28**	103	103	117	116	110	8	7
6-Aminopenicilloic acid [c]	91	**73**	**64**	**46**	**68**	19	**27**	88	**78**	**70**	**53**	**72**	15	**20**
7-Aminocephalosporanic acid [c]	99	**56**	**58**	**52**	**66**	**22**	**34**	98	**140**	**150**	**180**	**142**	**34**	**24**
8-Hydroxypenillic acid	103	102	95	**76**	94	13	13	97	**138**	**123**	112	118	18	15
Cephalosporin C	101	93	88	**75**	89	11	12	98	100	97	81	94	9	9
Cephalosporin C lactone [d]	96	115	113	110	108	9	8	100	**134**	**130**	**121**	**121**	15	12
Desacetoxycephalosporin	99	96	90	**78**	91	9	10	104	116	116	97	108	9	9
Desacetylcephalosporin	91	92	88	**73**	86	9	10	95	111	113	91	103	11	11
Penicillamine disulfide	96	102	92	88	95	6	6	100	**124**	117	108	112	10	9
Phenoxyacetic acid [d]	96	84	86	90	89	5	6	95	**212**	**227**	**320**	**213**	**93**	**43**
Phenoxymethylpenicilloic acid [e]	100	**41**	**30**	**20**	**48**	**36**	**75**	99	**70**	**56**	**44**	**67**	**24**	**35**
p-Hydroxyphenoxyacetic acid [c]	98	**56**	**56**	**54**	**66**	**21**	**32**	98	**146**	**152**	**199**	**149**	**41**	**28**
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val [e]	95	**46**	**31**	**10**	**46**	**36**	**79**	99	**45**	**32**	**9**	**46**	**38**	**82**
Vitamins and biogenic amines														
Biotin [d]	100	82	81	82	86	9	11	97	**131**	**134**	**155**	**129**	**24**	18
Cobalamine [d]	95	114	107	97	104	9	9	81	**208**	**200**	**263**	**188**	**77**	**41**
Ethanolamine	101	**79**	83	**76**	85	11	13	98	94	94	99	96	3	3
Folic acid [e]	97	**30**	**23**	**16**	**42**	**38**	**90**	107	113	93	97	103	9	9
Nicotinic acid [c]	**79**	**40**	**45**	**33**	**49**	**20**	**41**	96	116	**136**	114	115	17	14
Pantothenic acid [d]	100	96	96	**141**	108	**22**	**20**	96	**168**	**172**	**360**	**199**	**113**	**57**
Putrescine [d]	102	100	92	**80**	93	10	11	90	**34**	**43**	**56**	**56**	**25**	**44**
Pyridoxine [c]	**79**	**50**	**57**	**61**	**62**	12	**20**	92	90	107	**132**	105	19	18
Riboflavin [e]	96	**52**	**43**	**17**	**52**	**33**	**63**	87	**79**	**68**	**31**	**66**	**25**	**37**

[a] Calibration was performed by standard addition to extract 12 on day 1 with and without internal standards (IS). Samples were stored at 5°C in the autosampler.
[b] Interday precision was determined as %RSD of concentrations determined for quality control sample with matrix-matched calibration in extract 12.
[c] problems due to instrumental fluctuations (loss of sensitivity)
[d] problems due to inappropriate internal standard
[e] problems due to limited compound stability
[f] Accuracy values above +/- 20% are in bold.

Table 7: Classification of compounds with respect to their interday precision and accuracy.

	Group 1	Group 2	Group 3	Group 4	Group 5
compound	**stable**	**stable**	**stable**	**stable**	**unstable**
internal standard	**stable**	**stable**	**stable**	**stable**	**stable**
	Alanine Asparagine Aspartic acid Cystine Glutamine Glutamic acid Glycine Leucine Isoleucine Methionine Proline Serine Threonine Tyrosine Valine 8-Hydroxy-Penillic acid Penicillamine disulfide Cephalosporin C Desacetoxycephalosporin Desacetylcephalosporin Ethanolamine	Arginine Citrulline Histidine Lysine Phenylalanine Tryptophan 2-Oxoglutaric acid Malonic acid Succinic acid 2-Aminoadipic acid 6-Aminopencillanic acid Pyridoxine	Lactic acid Pyruvic acid Cephalosporin C lactone Phenoxyacetic acid Pantothenic acid Biotin Cobalamine Putrescine	Ornithine cis-Aconitic acid Fumaric acid Glutaric acid Glycolic acid Glyoxylic acid 2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid 7-Aminocephalosporanic acid p-Hydroxyphenoxyacetic acid	6-Aminopenicilloic acid Phenoxymethylpenillic acid Phenoxymethylpenicilloic acid δ-(L-α-Aminoadipyl)-L-Cys-D-Val Folic acid Nicotinic acid Riboflavin

Table 8: Analysis results of diluted extract 5 from cephalosporin production, extract 15 from penicillin production and medium 10 (nutrition medium).[a]

	Extract 5[b]	Extract15[b]	Medium 10[c]
Amino acids	**[mg/L]**	**[mg/L]**	**[mg/L]**
Alanine	2.180	0.534	4.680
Arginine	1.762	0.634	0.402
Asparagine	0.862	0.135	< LOQ
Aspartic acid	2.240	0.234	0.087
Citrulline	< LOQ	< LOQ	< LOQ
Cystine	< LOQ	< LOQ	0.294
Glutamic acid	2.560	1.752	0.132
Glutamine	3.440	0.444	2.560
Glycine	0.942	< LOQ	0.790
Histidine	0.212	0.133	< LOQ
Isoleucine	0.900	0.206	1.002
Leucine	2.320	0.620	1.200
Lysine	2.140	0.614	0.346
Methionine	0.836	0.179	0.376
Ornithine	< LOQ	< LOQ	< LOQ
Phenylalanine	0.648	0.284	0.208
Proline	2.120	0.173	1.258
Serine	0.964	0.204	1.206
Threonine	0.882	0.278	0.678
Tryptophan	0.368	< LOQ	0.140
Tyrosine	1.308	0.230	0.440
Valine	1.592	0.282	1.122
Organic acids			
2-Oxoglutaric acid	< LOQ	< LOQ	< LOQ
cis-Aconitic acid	< LOQ	< LOQ	< LOQ
Fumaric acid	< LOQ	< LOQ	< LOQ
Glutaric acid	< LOQ	< LOQ	< LOQ
Glycolic acid	< LOQ	< LOQ	< LOQ
Glyoxylic acid	< LOQ	< LOQ	< LOQ
Lactic acid	3.620	< LOQ	< LOQ
Malonic acid	< LOQ	< LOQ	< LOQ
Pyruvic acid	< LOQ	< LOQ	< LOQ
Succinic acid	0.508	0.196	< LOQ
Vitamins and biogenic amines			
Biotin	< LOQ	< LOQ	< LOQ
Cobalamine	< LOQ	< LOQ	< LOQ
Ethanolamine	0.074	< LOQ	< LOQ
Folic acid	< LOQ	< LOQ	< LOQ
Nicotinic acid	< LOQ	< LOQ	< LOQ
Pantothenic acid	0.055	0.054	0.012
Putrescine	0.592	0.103	0.252
Pyridoxine	< LOQ	< LOQ	< LOQ
Riboflavin	0.038	0.030	< LOQ

[a] Analysis results were obtained from calibration with neat standard solutions without use of internal standards.
[b] For concentrations in undiluted extracts values must be multiplied by a factor of 100.
[c] For concentrations in undiluted extracts values must be multiplied by a factor of 500.

Multi-target metabolic profiling of hydrophilic metabolites in fermentation broths of β-lactam antibiotics production by HILIC-ESI-MS/MS

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Supporting Information

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Figure S 9: Accuracy values in % along with corresponding frequency analysis of all compounds at a medium concentration level using different calibration approaches.
(A) matrix-matched calibration in extract 12 without internal standards
(B) matrix-matched calibration in extract 12 with internal standards
(C) calibration with neat standard solutions with internal standards

Table S 10: Intra-assay precision determined for QC samples at three distinct concentration levels using matrix-matched calibration in extract 12 with and without internal standards.

Table S 11: Intra- and interday precision (n = 3) determined for a QC sample at a medium concentration level using matrix-matched calibration in extract 12 with and without internal standards.

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Table S 1: Amino acid profile for "cell free" U-^{13}C,^{15}N labeled amino acid mix.

Amino Acid	Molecular weight	Molar %	Weight%	Comment
Alanine	93	13.29	9.35	
Arginine	184	4.90	6.78	
Asparagine	138	4.34	4.56	problems with detection, no adequate MRM transition found
Aspartic acid	138	7.84	8.25	problems with detection, no adequate MRM transition found
Cystine	248	not determined	not determined	low signal (near LOQ)
Glutamic acid	153	10.04	11.67	
Glutamine	153	4.43	5.15	
Glycine	78	9.33	5.53	not employed due to too low signal intensity (< LOQ)
Histidine	164	0.41	0.51	
Isoleucine	138	4.71	4.88	
Leucine	138	8.66	8.97	
Lysine	154	3.98	4.60	
Methionine	155	1.63	1.92	
Phenylalanine	175	2.41	3.15	
Proline	121	3.90	3.55	
Serine	109	4.26	3.53	low signal (near LOQ)
Threonine	124	4.77	4.48	
Tryptophan	217	1.81	2.92	
Tyrosine	191	2.13	3.04	low signal (near LOQ)
Valine	123	6.53	6.04	

Table S 2: Solvents of single analyte standards.

Amino acids	**Solvent**
Alanine	water/ACN 1:1 (v/v)
Arginine	water/ACN 1:1 (v/v)
Asparagine	water/ACN 1:1 (v/v)
Aspartic acid	1% TFA in water/ACN 8:2 (v/v)
Citrulline	water/ACN 1:1 (v/v)
Cystine	1% TFA in water/ACN 8:2 (v/v)
Glutamic acid	1% TFA in water/ACN 8:2 (v/v)
Glutamine	water/ACN 1:1 (v/v)
Glycine	water/ACN 1:1 (v/v)
Histidine	water/ACN 1:1 (v/v)
Isoleucine	water/ACN 1:1 (v/v)
Leucine	water/ACN 1:1 (v/v)
Lysine	water/ACN 1:1 (v/v)
Methionine	water/ACN 1:1 (v/v)
Ornithine	water/ACN 1:1 (v/v)
Phenylalanine	water/ACN 1:1 (v/v)
Proline	water/ACN 1:1 (v/v)
Serine	water/ACN 1:1 (v/v)
Threonine	water/ACN 1:1 (v/v)
Tryptophan	water/ACN 1:1 (v/v)
Tyrosine	1% TFA in water/ACN 8:2 (v/v)
Valine	water/ACN 1:1 (v/v)
Organic acids	
2-Oxoglutaric acid	water/ACN 1:1 (v/v)
cis-Aconitic acid	water/ACN 1:1 (v/v)
Fumaric acid	water/ACN 1:1 (v/v)
Glutaric acid	water/ACN 1:1 (v/v)
Glycolic acid	water/ACN 1:1 (v/v)
Glyoxylic acid	water/ACN 1:1 (v/v)
Lactic acid	water/ACN 1:1 (v/v)
Malonic acid	water/ACN 1:1 (v/v)
Pyruvic acid	water/ACN 1:1 (v/v)
Succinic acid	water/ACN 1:1 (v/v)
β-Lactam antibiotics, intermediates of biosynthesis and degradation products	
2-Aminoadipic acid	1% TFA in water/ACN 8:2 (v/v)
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	1% TFA in water/ACN 8:2 (v/v)
6-Aminopenicillanic acid	1% TFA in water/ACN 8:2 (v/v)
6-Aminopenicilloic acid	mobile phase (A)/(B) 2:8 (v/v)
7-Aminocephalosporanic acid	1% TFA in water/ACN 8:2 (v/v)
8-Hydroxypenillic acid	mobile phase (A)/(B) 2:8 (v/v)
Cephalosporin C	mobile phase (A)/(B) 2:8 (v/v)
Cephalosporin C lactone	mobile phase (A)/(B) 2:8 (v/v)
Desacetoxycephalosporin	mobile phase (A)/(B) 2:8 (v/v)
Desacetylcephalosporin	mobile phase (A)/(B) 2:8 (v/v)
Penicillamine disulfide	1% TFA in water/ACN 8:2 (v/v)
Phenoxyacetic acid	water/ACN 1:1 (v/v)
Phenoxymethylpenicilloic acid	mobile phase (A)/(B) 2:8 (v/v)
Phenoxymethylpenillic acid	mobile phase (A)/(B) 2:8 (v/v)
p-Hydroxyphenoxyacetic acid	water/ACN 1:1 (v/v)
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	water/ACN 1:1 (v/v)
Vitamins and biogenic amines	
Biotin	water/ACN 1:1 (v/v)
Cobalamine	water/ACN 1:1 (v/v)
Ethanolamine	water/ACN 1:1 (v/v)
Folic acid	water/ACN 8:2 (v/v)
Nicotinic acid	water/ACN 1:1 (v/v)
Pantothenic acid	water/ACN 1:1 (v/v)
Putrescine	water/ACN 1:1 (v/v)
Pyridoxine	water/ACN 1:1 (v/v)
Riboflavin	water/ACN 8:2 (v/v)

Table S 3: Retention times and log D values (pH 3.5) of investigated analytes along with some potential signal interferences in neat standard solutions and spiked fermentation extracts, respectively[a]

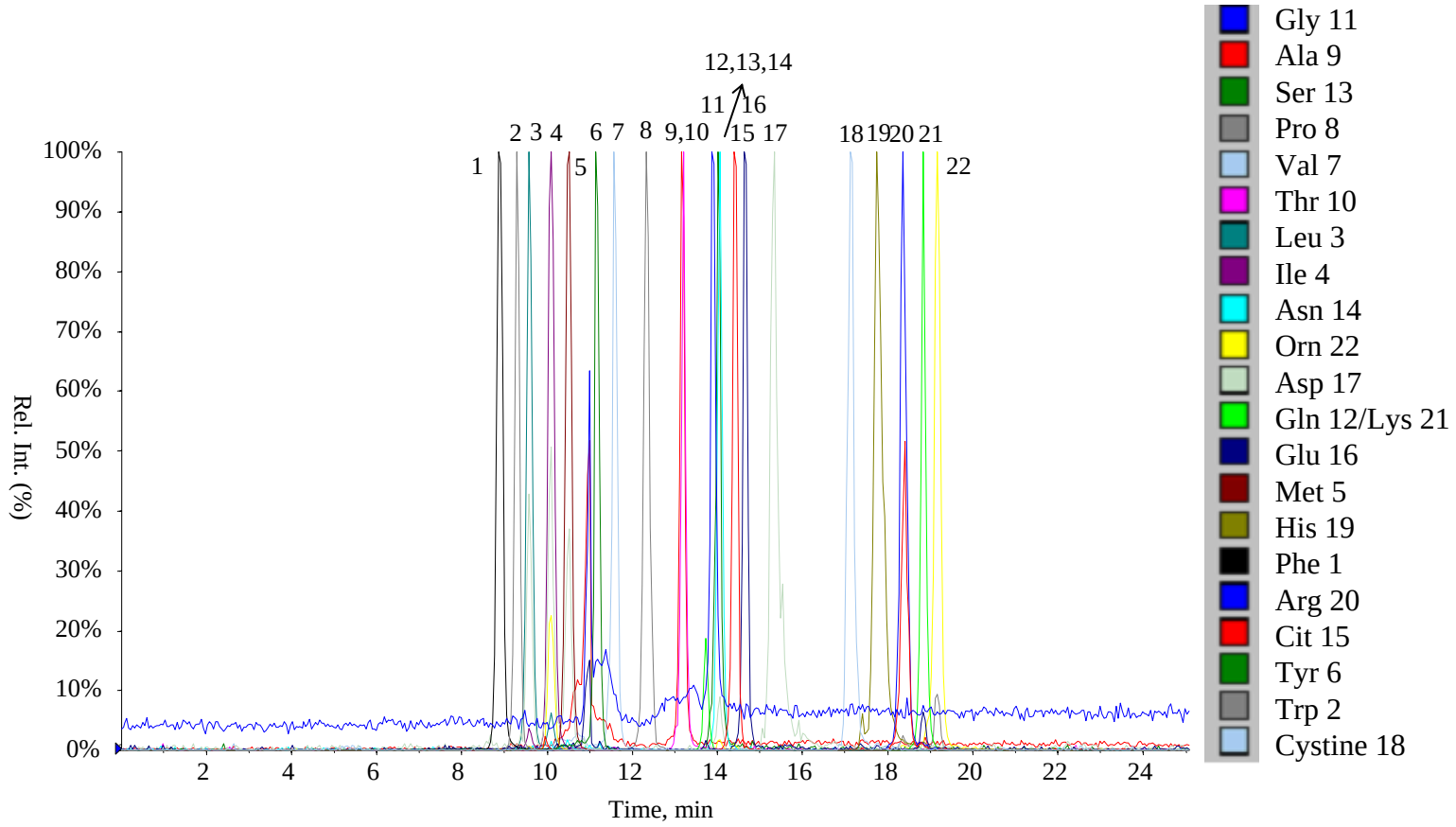
	neat standard solution				spiked extract 12			neat standard solution				
Compound	pH 4.0[a] tr [min]	Interferences tr [min]	Peak width at 50% height	Peak Asymmetry	Interferences tr [min]	Peak width at 50% height	Peak Asymmetry	pH 3.5[a] tr [min]	Interferences [min]	Peak width at 50% height	Peak Asymmetry	pH 3.5 log D
Amino acids												
Alanine	13.6	11.2	0.224	1.090	16.7	0.209	1.400	13.2	11.0	0.109	1.300	-3.20
Arginine	19.0		0.254	1.070	15.5	0.271	1.090	18.3	15.2	0.145	0.922	-5.32
Asparagine	14.6		0.218	1.100	16.7	0.209	1.260	13.9	11.0	0.129	1.040	-4.02
Aspartic acid	15.8	10.2 (Leu); 10.8 (Ile); 11.2 (Met); 14.7 (Gln)	0.420	2.290	10.5 (Leu); 11.0 (Ile); 16.8	0.368	1.820	15.3	9.6 (Leu);10.2 (Ile); 13.9 (Asn)	0.147	1.920	-3.30
Citrulline	14.8	19.0 (Arg)	0.182	1.140	16.2; 16.7;19.1 (Arg)	0.200	1.200	14.4	18.4 (Arg)	0.124	1.150	-4.06
Cystine	17.5		0.224	1.660		0.214	1.580	17.1		0.153	0.825	-2.65
Glutamic acid	15.3	11.1	0.177	1.050	10.7; 16.7	0.170	1.260	14.7	11.0	0.106	1.010	-3.98
Glutamine	14.2	19.5 (Lys)	0.177	0.632	19.5 (Lys); 16.7	0.178	0.742	13.7	18.8 (Lys)	0.131	0.995	-4.19
Glycine	14.3	11.2	0.171	0.630	11.4	0.172	1.010	13.9	11.1	0.138	0.970	-3.55
Histidine	18.8		0.967	1.290	11.7	1.240	2.250	17.8		0.225	1.200	-4.77
Isoleucine	10.8	10.2 (Leu)	0.196	0.881	8.0-10.0; 10.8 (Leu); 15.0	0.192	0.690	10.1	9.6 (Leu)	0.130	0.921	-1.80
Leucine	10.2	10.8 (Ile)	0.176	0.738	8.0-10.0;11.1 (Ile)	0.174	0.730	9.6	10.1 (Ile)	0.118	1.010	-1.80
Lysine	19.5	14.2 (Gln)	0.152	0.720	14.3 (Gln); 16.7	0.091	0.827	18.8	13.7 (Gln)	0.167	1.430	-4.57
Methionine	11.2		0.200	1.160		0.204	0.949	10.5		0.134	0.733	-2.14
Ornithine	19.6	10.2 (Leu); 10.8 (Ile)	0.261	0.817	11.10	0.244	1.150	19.2	10.1 (Ile)	0.168	0.354	-4.49
Phenylalanine	9.4		0.184	0.812	7.5; 16.8	0.192	1.140	8.9		0.128	1.020	-1.40
Proline	13.0	19.0 (Arg); 19.7 (Orn)	0.424	0.803	16.7;19.1 (Arg); 19.7(Orn)	0.446	0.766	12.3		0.131	1.270	-3.09
Serine	14.5		0.169	0.646	16.7	0.180	1.350	14.0		0.135	0.625	-4.09
Threonine	13.7		0.207	1.420	16.7	0.209	1.590	13.2		0.120	0.639	-3.75
Tryptophan	9.7		0.185	1.580		0.179	0.947	9.3		0.117	0.672	-1.48
Tyrosine	11.7		0.175	0.748	16.7	0.161	0.805	11.2		0.107	0.694	-2.14
Valine	12.2		0.238	0.765	10.9;16.7	0.223	1.100	11.6	6.0-11.0	0.133	0.947	-2.32
Organic acids												
2-Oxoglutaric acid	14.5		0.184	1.000		0.195	2.760	12.7		0.076	0.944	-2.60
cis-Aconitic acid	14.5	15.4 (trans Aconitic acid[b]); 16.5	0.144	0.696	15.6 (trans-Aconitic acid[b]);16.7	0.166	1.260	12.7	13.7 (trans Aconitic acid[b])	0.069	0.718	-0.06
Fumaric acid	15.5	4.0; 16.5; 23.9	0.161	0.857	4.4; 16.8; 24.1	0.182	0.828	14.1	3.5	0.120	1.500	-0.36
Glutaric acid	15.0	26.0	0.176	0.928	13.6;26.2	0.180	0.982	7.0	5.1; 12.8	0.336	1.920	-1.07
Glycolic acid	13.0		0.367	0.708		0.377	0.702	12.1		0.170	1.810	-1.24
Glyoxylic acid	13.5	14.5 (Succinic acid)	0.289	0.576	14.7 (Succinic acid)	0.305	1.840	13.1	8.8 (Succinic acid)	0.233	1.060	-1.87
Lactic acid	11.9		0.201	1.070		0.202	0.938	8.9		0.399	1.260	-0.84
Malonic acid	13.4		0.223	1.020		0.242	1.290	7.5		0.294	1.990	-1.02
Pyruvic acid	8.8	15.0 (Glutaric acid)	0.210	1.900	15.2 (Glutaric acid)	0.204	1.450	7.7		0.161	0.953	-2.15
Succinic acid	14.5		0.168	1.460		0.179	1.580	8.8	12.7 (cis-Aconitic acid)	0.436	1.630	-0.63
β-Lactam antibiotics, intermediates of biosynthesis and degradation products												
2-Aminoadipic acid	15.5		0.170	1.320		0.186	0.735	14.1		0.127	1.190	-3.1
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	15.3	5.6 (Biotin)	0.288	1.840	5.8 (Biotin)	0.254	1.370	14.8	3.9 (Biotin)	0.199	1.700	-2.24
6-Aminopenicillanic acid	9.2		0.207	1.230		0.211	1.890	8.1		0.131	1.610	-2.73
6-Aminopenicilloic acid	14.4		0.402	3.000	16.7	0.422	2.360	13.8		0.138	1.050	-2.73
7-Aminocephalosporanic acid	8.2		0.183	1.580		0.198	1.570	7.3	8.8	0.184	0.943	-3.03
8-Hydroxypenillic acid	13.3		0.181	1.700		0.171	0.956	13.1		0.127	1.030	0.57
Cephalosporin C	13.5		0.172	1.390		0.174	0.957	13.2		0.132	4.320	-3.25
Cephalosporin C lactone	11.2		0.174	0.943		0.177	0.688	10.8		0.137	1.020	-5.91
Desacetoxycephalosporin	15.1	11.2	0.188	1.230		0.173	0.796	14.8	10.3	0.138	0.833	-3.2
Desacetylcephalosporin	15.3		0.185	0.940		0.173	0.664	15.2	13.6	0.137	0.732	-3.7
Penicillamine disulfide	15.8		0.223	2.310	16.7	0.212	1.020	15.8		0.165	1.250	0.09
Phenoxyacetic acid	4		0.223	6.280		0.523	10.300	3.3		0.254	2.300	0.84
Phenoxymethylpenicilloic acid	8.6	9.4 (degradation product or epimer)	0.188	0.889	3.1; 3.3; 3.8; 4.3[c]; 9.4	0.188	0.987	6.7	8.3 (degradation product or epimer)	0.146	0.832	-0.14
Phenoxymethylpenillic acid	9.0	8.7 (Phenoxymethylpenicilloic acid)	0.181	2.36	2.8; 3.3; 3.8[c]	0.193	1.290	n.a.		n.a.	n.a.	0.83
p-Hydroxyphenoxyacetic acid	8.3		0.201	1.400	4.1; 4.5; 7.5	0.191	1.290	6.5		0.176	1.580	0.09
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	12.7		0.459	0.514	15.0	0.431	1.050	12.2		0.110	0.600	-2.15
Vitamins and biogenic amines												
Biotin	5.6		0.211	2.600		0.205	1.230	3.9		0.182	1.010	0.09
Cobalamine	11.9		0.438	1.570		0.338	1.500	11.9		0.177	1.200	n.a.
Ethanolamine	12.5		0.312	1.440		0.292	0.794	20.1		0.140	1.010	-4.41
Folic acid	15.2		0.213	0.724		0.198	0.993	13.1		0.161	0.813	-2.64
Nicotinic acid	8.5	7.0	0.224	2.720		0.239	1.820	6.8		0.228	3.390	-1.21
Pantothenic acid	10.0		0.306	1.330		0.334	1.090	5.5		0.157	1.860	-0.91
Putrescine	23.1	11.3; 21.0	0.355	2.090	20.6	0.351	1.110	23.7		0.315	1.430	-4.82
Pyridoxine	7.1		0.517	4.820		0.458	2.560	8.1		0.658	4.780	-2.63
Riboflavin	6.2	5.3	0.276	1.900	5.5	0.246	1.230	5.9		0.191	1.760	-2.07

[a] Chromatographic conditions: (A) 10% buffer in H_2O; (B) 10% buffer in ACN; buffer: 200 mM formic acid adjusted with NH_3 to pH 4.0 and 3.5, respectively
Gradient: 100% (B) to 35% (B) in 25 minutes; flow rate 700 µl/min

[b] Trans-aconitic acid was not a target solute, however, it was contained in the employed standard of cis-aconitic acid.

[c] In extracts of fermentation broths considerable amounts of penicillin (isobaric to phenoxymethylpenillic acid) and related degradation products are contained that may interfere with the detection of phenoxymethylpenillic acid and phenoxymethylpenicilloic acid.

Figure S 4: Chromatograms of optimized HILIC method: Overlaid MRM traces normalized to 100% of amino acids (A), organic acids (B), β-lactams (C) and vitamins/biogenic amines (D). Experimental conditions: Column, ZIC-HILIC, 5 µm (150 x 4.6 mm ID); eluent, channel A, 10 % (v/v) buffer in water; channel B, 10 % (v/v) buffer in acetonitrile; buffer, 200 mM formic acid, adjusted to pH 3.5 with ammonia; gradient, 100% B to 35% B in 25 min; flow rate, 0.7 mL/min; temperature, 25°C.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Rel. Int. (%)
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Time, min
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6 7
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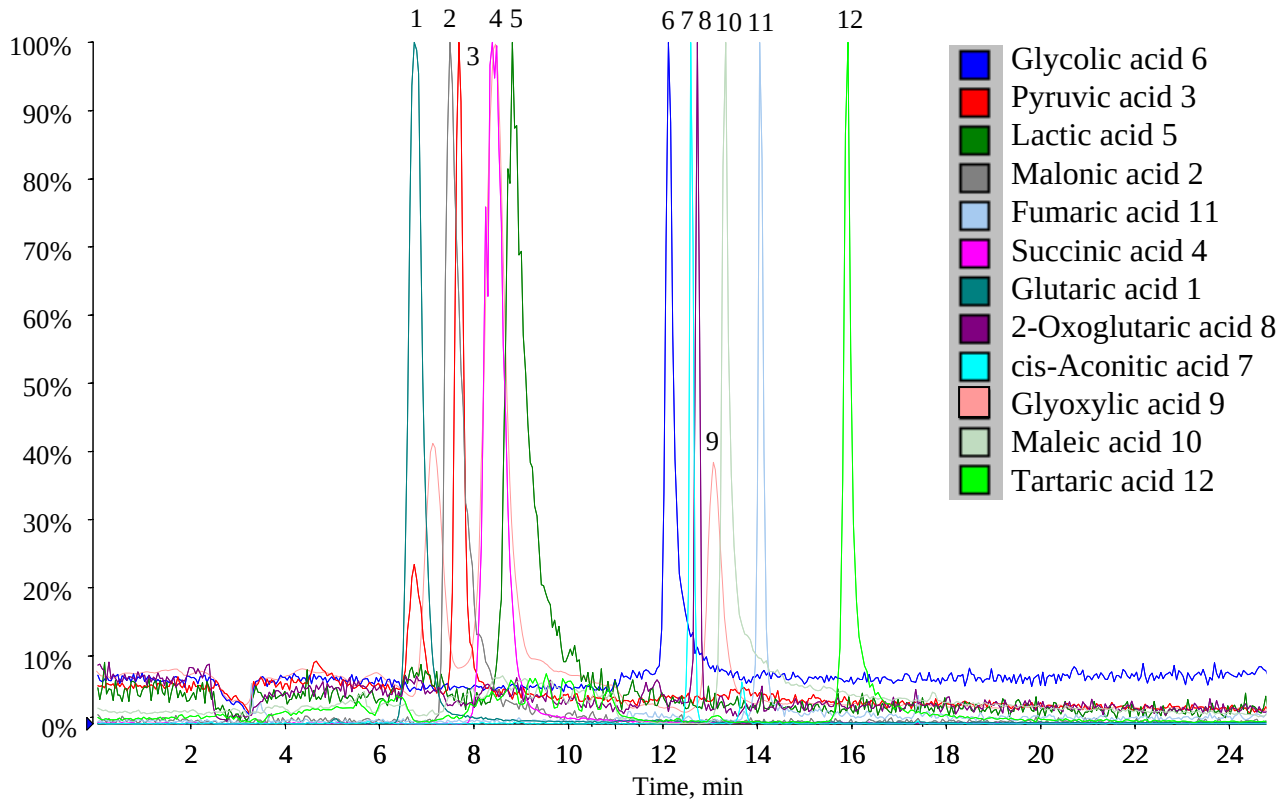
9,10
11
12,13,14
15 16 17
18 19 20 21
22
Gly 11
Ala 9
Ser 13
Pro 8
Val 7
Thr 10
Leu 3
Ile 4
Asn 14
Orn 22
Asp 17
Gln 12/Lys 21
Glu 16
Met 5
His 19
Phe 1
Arg 20
Cit 15
Tyr 6
Trp 2
Cystine 18

Figure S 4A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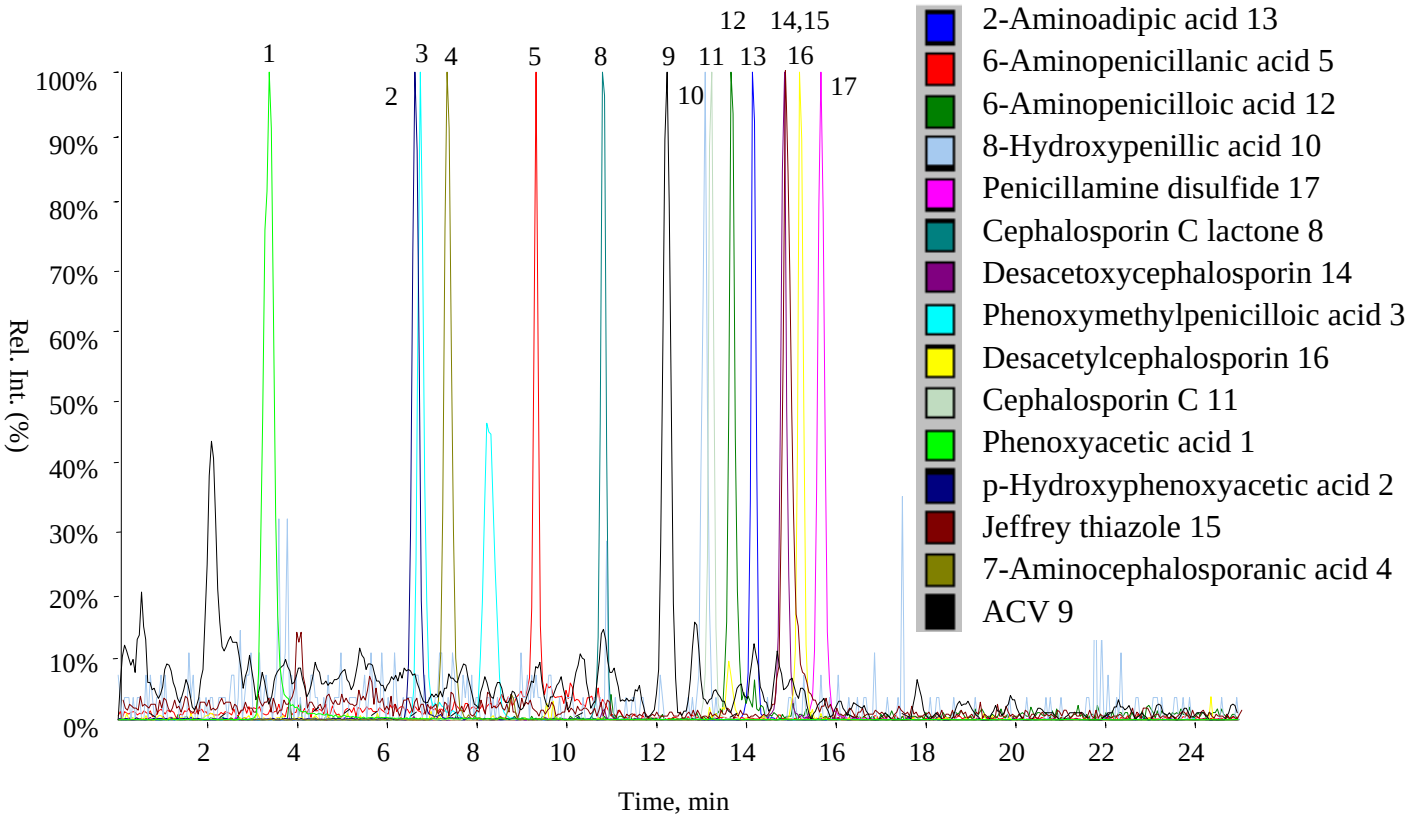
Time, min
Glycolic acid 6
Pyruvic acid 3
Lactic acid 5
Malonic acid 2
Fumaric acid 11
Succinic acid 4
Glutaric acid 1
2-Oxoglutaric acid 8
cis-Aconitic acid 7
Glyoxylic acid 9
Maleic acid 10
Tartaric acid 12

Figure S 4B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Rel. Int. (%)
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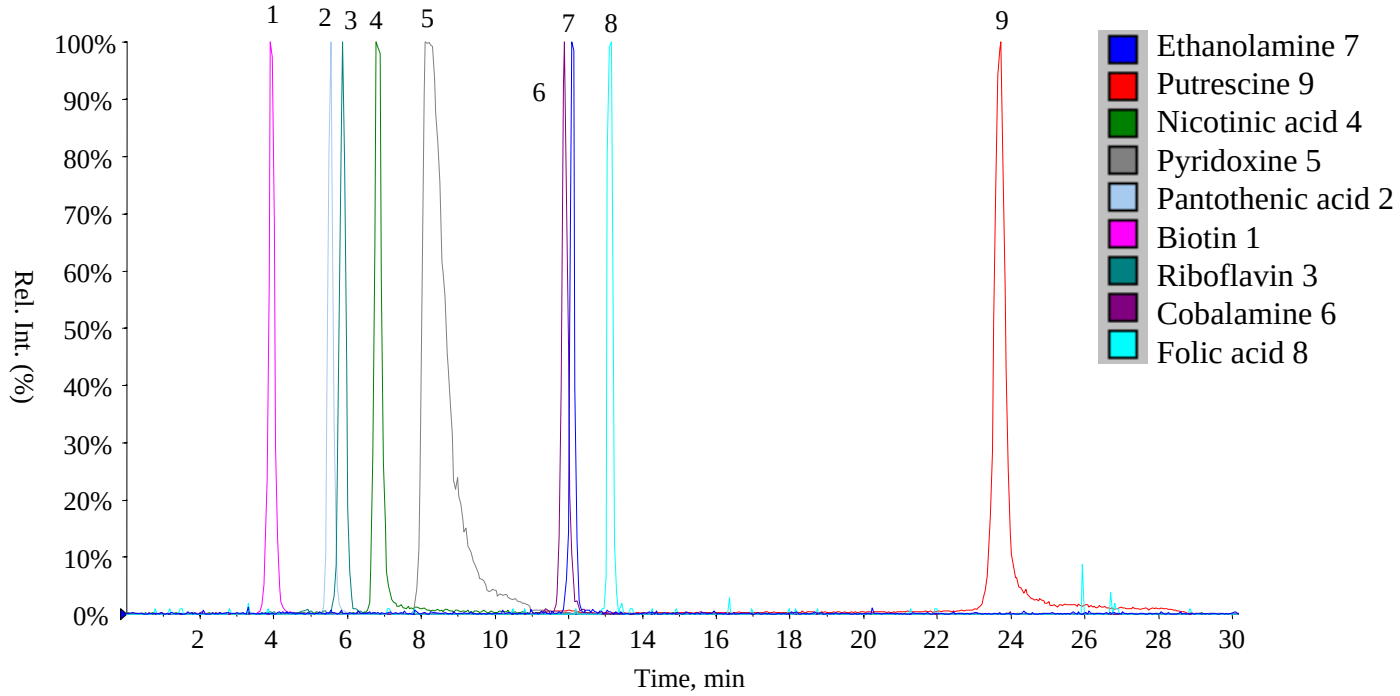
Time, min
2-Aminoadipic acid 13
6-Aminopenicillanic acid 5
6-Aminopenicilloic acid 12
8-Hydroxypenillic acid 10
Penicillamine disulfide 17
Cephalosporin C lactone 8
Desacetoxycephalosporin 14
Phenoxymethylpenicilloic acid 3
Desacetylcephalosporin 16
Cephalosporin C 11
Phenoxyacetic acid 1
p-Hydroxyphenoxyacetic acid 2
Jeffrey thiazole 15
7-Aminocephalosporanic acid 4
ACV 9

Figure S 4C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Rel. Int. (%)
2
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14
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18
20
22
24
26
28
30
Time, min
Ethanolamine 7
Putrescine 9
Nicotinic acid 4
Pyridoxine 5
Pantothenic acid 2
Biotin 1
Riboflavin 3
Cobalamine 6
Folic acid 8

Figure S 4D

Table S 5 : LLOQ (determined at a signal to noise ratio of 10:1), ULOQ (both determined with neat standard solutions), calibration functions and corresponding correlation coefficients obtained for the compounds under investigation. Calibration functions were constructed with pure matrix-free standard solutions, as well as by standard addition to different sample matrices. Both calibration approaches were performed (A) without and (B) with internal standards for peak area normalization.

			matrix-free neat solutions			spiked extract 12			spiked extract 15		
(A) without internal standards	**LLOQ [mg/L]**	**ULOQ [mg/L]**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**
measured on				**day 1**			**day 1**			**day 1**	
Amino acids											
Alanine	0.050	100	6.50E+05	4.70E+04	0.9976	6.52E+05	5.27E+05	0.9981	6.60E+05	4.08E+05	0.9994
Arginine	0.081[a]	5	9.40E+06	2.00E+06	0.9395	8.41E+06	5.78E+06	0.9875	7.22E+06	8.82E+06	0.9467
Asparagine	0.050	100[b]	5.35E+05	-1.97E+04	0.9981	4.86E+05	7.95E+03	0.9958	4.67E+05	5.62E+04	0.9988
Aspartic acid	0.075	100	3.66E+05	3.36E+02	0.9902	3.42E+05	9.38E+04	0.9964	3.14E+05	1.09E+05	0.9783
Citrulline	0.010	65	1.09E+06	3.95E+04	0.9956	1.04E+06	4.82E+04	0.9988	1.11E+06	2.29E+04	0.9990
Cystine	0.075	75[b]	3.10E+05	-3.66E+02	0.9943	2.93E+05	6.89E+03	0.9920	3.00E+05	-2.55E+03	0.9950
Glutamic acid	0.025	150[b]	5.00E+05	2.51E+04	0.9725	4.53E+05	6.25E+05	0.9915	4.80E+05	8.85E+05	0.9443
Glutamine	0.025	150[b]	5.75E+05	1.65E+04	0.9973	7.14E+05	1.63E+05	0.9917	6.26E+05	3.05E+05	0.9925
Glycine	0.500	150[b]	3.68E+05	-7.44E+03	0.9969	4.08E+05	6.68E+04	0.9977	3.79E+05	1.15E+05	0.9980
Histidine	0.052[a]	30	8.10E+07	6.07E+06	0.9987	8.06E+07	1.25E+07	0.9983	7.84E+07	1.57E+07	0.9992
Isoleucine	0.025	15	5.65E+06	5.39E+04	0.9979	5.67E+06	1.69E+06	0.9998	5.42E+06	1.07E+06	0.9951
Leucine	0.050	12	3.59E+06	1.33E+05	0.9946	3.66E+06	2.53E+06	0.9909	3.31E+06	2.37E+06	0.9732
Lysine	0.089[a]	65	5.85E+06	7.70E+05	0.9952	5.83E+06	1.93E+06	0.9751	5.07E+06	4.09E+06	0.9838
Methionine	0.050	30	1.95E+06	-1.90E+04	0.9995	2.06E+06	3.03E+05	0.9971	2.27E+06	3.26E+05	0.9904
Ornithine	0.156[a]	40	7.04E+06	3.32E+06	0.9860	6.92E+06	2.72E+06	0.9786	7.79E+06	2.59E+06	0.9897
Phenylalanine	0.005	2	8.95E+07	3.62E+06	0.9976	6.23E+07	2.08E+07	0.9421	4.14E+07	3.05E+07	0.9958
Proline	0.003	9	1.56E+07	2.28E+05	0.9992	1.52E+07	2.03E+06	0.9992	1.63E+07	2.82E+06	0.9989
Serine	0.045[a]	100[b]	5.55E+05	8.92E+04	0.9938	5.00E+05	2.44E+05	0.9931	4.93E+05	2.00E+05	0.9926
Threonine	0.011[a]	100	1.14E+06	3.90E+04	0.9955	1.39E+06	4.61E+05	0.9587	1.20E+06	3.39E+05	0.9993
Tryptophan	0.005	5	3.24E+07	7.22E+06	0.9933	4.10E+07	4.95E+06	0.9868	4.53E+07	3.69E+06	0.9965
Tyrosine	0.010	80	1.10E+06	4.40E+03	0.9975	1.12E+06	1.72E+05	0.9987	9.87E+05	2.96E+05	0.9954
Valine	0.025	15	3.64E+06	-2.78E+04	0.9956	3.78E+06	1.53E+06	0.9821	3.85E+06	9.99E+05	0.9985
Organic acids											
2-Oxoglutaric acid	1.250	150	4.47E+05	1.41E+05	0.9966	5.83E+05	-6.66E+04	0.9980	4.71E+05	-4.57E+04	0.9946
cis-Aconitic acid	0.250	50[b]	1.94E+06	-8.34E+05	0.9646	1.27E+06	-5.02E+04	0.9667	8.71E+05	-1.04E+05	0.9774
Fumaric acid	0.250	50[b]	2.96E+05	-1.10E+04	0.9760	2.64E+05	9.61E+03	0.9803	2.42E+05	3.32E+04	0.9809
Glutaric acid	0.167[a]	100[b]	2.13E+06	1.23E+06	0.9962	2.27E+06	1.29E+06	0.9975	2.08E+06	1.08E+06	0.9946
Glycolic acid	1.250	150[b]	9.65E+05	4.59E+05	0.9975	9.09E+05	4.05E+05	0.9992	9.87E+05	2.60E+05	0.9971
Glyoxylic acid	0.250	100[b]	4.72E+05	3.27E+05	0.9792	5.13E+05	3.21E+05	0.9894	5.32E+05	1.56E+05	0.9846
Lactic acid	0.250	100[b]	4.05E+04	2.53E+04	0.9968	3.73E+04	1.75E+05	0.8463	3.94E+04	6.87E+02	0.9889
Malonic acid	0.250	20	7.30E+04	2.69E+03	0.9922	7.56E+04	2.28E+04	0.9965	6.24E+04	1.06E+04	0.9979
Pyruvic acid	0.250	100[b]	1.73E+06	8.25E+04	0.9985	1.77E+06	2.95E+05	0.9978	1.73E+06	8.46E+04	0.9998
Succinic acid	0.150[a]	100[b]	1.41E+06	1.34E+04	0.9969	1.42E+06	7.45E+05	0.9979	1.42E+06	2.51E+05	0.9996
β-Lactam antibiotics, intermediates of biosynthesis and degradation products											
2-Aminoadipic acid	0.050	100[b]	1.04E+06	1.16E+04	0.9944	1.26E+06	2.41E+05	0.9929	1.21E+06	4.02E+05	0.9954
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	0.150	50	2.58E+05	4.55E+03	0.9934	2.64E+05	2.99E+03	0.9928	2.49E+05	6.48E+03	0.9955
6-Aminopenicillanic acid	0.010	15	1.42E+07	2.68E+06	0.9898	1.50E+07	2.72E+06	0.9846	1.45E+07	9.41E+05	0.9982
6-Aminopenicilloic acid	0.100	40	2.08E+05	2.16E+04	0.9940	2.25E+05	8.87E+03	0.9993	1.71E+05	1.24E+05	0.9992
7-Aminocephalosporanic acid	0.050	50	2.04E+06	1.28E+04	0.9834	2.29E+06	-8.42E+04	0.9959	1.97E+06	-7.65E+04	0.9962
8-Hydroxypenillic acid	0.100	30	4.20E+04	-7.80E+01	0.9948	4.31E+04	1.04E+04	0.9996	4.52E+04	7.92E+04	0.9816
Cephalosporin C	0.020	40	8.27E+05	-2.73E+04	0.9997	8.48E+05	-1.48E+04	0.9987	8.57E+05	-1.21E+04	0.9973
Cephalosporin C lactone	0.020	40	1.58E+06	-5.76E+04	0.9976	1.46E+06	-1.11E+04	0.9981	1.45E+06	-2.75E+04	0.9962
Desacetoxycephalosporin	0.050	50	6.60E+05	-1.58E+04	0.9986	6.43E+05	-3.71E+03	0.9957	6.52E+05	-2.31E+02	0.9973
Desacetylcephalosporin	0.050	40	2.13E+05	-1.52E+02	0.9854	2.37E+05	-2.53E+03	0.9990	2.15E+05	5.71E+03	0.9949
Penicillamine disulfide	0.020	65	2.11E+06	-4.96E+04	0.9988	2.05E+06	-5.42E+03	0.9998	1.98E+06	5.43E+04	0.9987
Phenoxyacetic acid	0.010	2	1.96E+07	6.99E+05	0.9981	5.57E+06	1.97E+08	0.8449	6.07E+06	1.05E+08	0.9544
Phenoxymethylpenicilloic acid	0.010	20	2.68E+07	-9.77E+05	0.9816	2.76E+07	9.08E+05	0.9969	2.50E+07	1.11E+07	0.9876
Phenoxymethylpenillic acid	0.010	10	2.19E+06	1.21E+05	0.8415	1.92E+06	2.06E+05	0.9973	n.a.[c]	n.a.[c]	n.a.[c]
p-Hydroxyphenoxyacetic acid	0.010	20	5.10E+06	3.57E+04	0.9976	5.40E+06	1.36E+05	0.9882	5.01E+06	6.32E+05	0.9990
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	0.100	100[b]	5.70E+05	2.37E+04	0.9964	6.40E+05	3.15E+03	0.9937	5.64E+05	2.16E+04	0.9850
Vitamins and biogenic amines											
Biotin	0.010	10	1.06E+07	1.69E+05	0.9944	1.11E+07	-1.62E+04	0.9967	1.13E+07	-3.58E+03	0.9995
Cobalamine	0.050	65	2.33E+06	-3.53E+04	0.9843	1.90E+06	2.94E+04	0.9949	2.04E+06	-2.65E+04	0.9977
Ethanolamine	0.050	6.5	1.98E+06	8.64E+04	0.9884	2.23E+06	1.28E+05	0.9953	2.14E+06	9.20E+04	0.9996
Folic acid	0.050	75	2.09E+05	6.50E+04	0.9190	2.49E+05	2.33E+04	0.9956	1.96E+05	2.34E+04	0.9973
Nicotinic acid	0.010	2	1.23E+07	1.92E+06	0.9623	1.53E+07	2.01E+06	0.9998	1.42E+07	2.15E+06	0.9980
Pantothenic acid	0.010	30	5.40E+06	-5.98E+04	0.9931	5.16E+06	1.77E+04	0.9981	4.78E+06	2.30E+05	0.9960
Putrescine	0.037[a]	2	4.11E+07	1.63E+06	0.9936	3.56E+07	5.94E+06	0.9974	4.31E+07	6.14E+06	0.9944
Pyridoxine	0.003	0.25	9.35E+08	2.50E+07	0.9936	1.06E+09	8.24E+06	0.9942	1.04E+09	1.29E+07	0.9936
Riboflavin	0.005	7	2.04E+07	-4.75E+05	0.9982	2.01E+07	1.17E+05	0.9990	1.95E+07	-1.76E+05	0.9992

[a] LLOQ calculated from standard deviation of memory peak areas of blank runs: 3 x standard deviation of memory peak area (n = 5)/slope of calibration function obtained with neat standard solutions without use of internal standards
[b] ULOQ may be underestimated since no higher concentrations were measured.
[c] n.a. not available due to stability reasons

	spiked extract 1			spiked extract 5			spiked medium 6			spiked medium 10		
(A) without internal standards	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**
measured on	**day 2**			**day 2**			**day 3**			**day 3**		
Amino acids												
Alanine	6.76E+05	2.21E+06	0.9977	6.96E+05	1.47E+06	0.9921	5.07E+05	2.03E+06	0.9237	6.51E+05	2.98E+06	0.9606
Arginine	1.09E+07	4.75E+06	0.9981	n.a.[b]	n.a.[b]	n.a.[b]	4.22E+06	7.12E+06	0.9963	3.83E+06	5.87E+06	0.9364
Asparagine	4.41E+05	1.28E+05	0.9977	4.91E+05	4.12E+05	0.9843	5.01E+05	9.50E+04	0.9917	4.33E+05	1.25E+04	0.9979
Aspartic acid	3.27E+05	6.90E+04	0.9970	4.49E+05	7.96E+05	0.9795	3.06E+05	3.67E+05	0.9956	3.42E+05	2.89E+04	0.9981
Citrulline	9.91E+05	6.85E+04	0.9968	1.10E+06	3.78E+04	0.9973	9.01E+05	1.54E+04	0.9992	9.29E+05	1.68E+04	0.9940
Cystine	3.00E+05	-6.94E+03	0.9949	2.82E+05	-1.56E+03	0.9989	3.13E+05	1.08E+05	0.9966	3.13E+05	8.25E+04	0.9982
Glutamic acid	4.97E+05	2.59E+05	0.9932	7.90E+05	1.38E+06	0.9790	4.33E+05	2.96E+05	0.9946	4.48E+05	8.84E+04	0.9944
Glutamine	6.43E+05	1.74E+05	0.9984	6.63E+05	1.91E+06	0.9036	8.06E+05	1.61E+06	0.9924	8.57E+05	1.49E+06	0.9855
Glycine	3.90E+05	1.36E+05	0.9939	4.16E+05	3.17E+05	0.9976	3.91E+05	3.22E+05	0.9901	4.80E+05	9.25E+04	0.9992
Histidine	6.75E+07	1.00E+07	0.9984	5.12E+07	2.36E+07	0.9994	2.25E+07	9.67E+06	0.9724	2.52E+07	6.60E+06	0.9983
Isoleucine	5.26E+06	3.84E+06	0.9938	4.86E+06	4.47E+06	0.9919	4.94E+06	7.39E+06	0.9830	5.60E+06	4.96E+05	0.9908
Leucine	3.03E+06	8.60E+06	0.9958	3.03E+06	8.70E+06	0.9692	2.46E+06	5.72E+06	0.9838	2.75E+06	4.13E+06	0.9835
Lysine	5.30E+06	2.28E+06	0.9678	4.55E+06	1.36E+07	0.9565	3.34E+06	3.44E+06	0.9759	2.17E+06	2.79E+06	0.9871
Methionine	1.76E+06	1.05E+06	0.9915	1.63E+06	1.71E+06	0.9830	1.53E+06	1.01E+06	0.9980	2.20E+06	7.23E+05	0.9961
Ornithine	8.73E+06	4.19E+06	0.9825	5.75E+06	3.67E+06	0.9798	3.04E+06	7.55E+05	0.9980	2.84E+06	6.74E+05	0.9860
Phenylalanine	2.74E+07	3.36E+07	0.9972	2.02E+07	6.12E+07	0.9907	2.80E+07	3.11E+07	0.9800	3.54E+07	2.14E+07	0.9971
Proline	1.41E+07	3.19E+07	0.9923	1.36E+07	3.54E+07	0.9579	1.33E+07	2.53E+07	0.9969	1.63E+07	1.98E+07	0.9957
Serine	4.92E+05	3.78E+05	0.9914	4.86E+05	6.28E+05	0.9996	4.51E+05	8.42E+05	0.9610	3.89E+05	7.43E+05	0.9869
Threonine	1.19E+06	8.96E+05	0.9989	1.26E+06	1.14E+06	0.9897	9.92E+05	1.15E+06	0.9944	1.02E+06	7.43E+05	0.9839
Tryptophan	3.25E+07	1.55E+06	0.9985	2.60E+07	1.76E+07	0.9761	2.21E+07	1.20E+07	0.9982	2.61E+07	8.59E+06	0.9944
Tyrosine	9.35E+05	2.15E+05	0.9934	9.33E+05	1.41E+06	0.9964	9.65E+05	6.31E+05	0.9627	1.12E+06	4.71E+05	0.9889
Valine	3.62E+06	5.17E+06	0.9930	4.07E+06	5.79E+06	0.9700	3.78E+06	5.54E+06	0.9922	3.83E+06	3.81E+06	0.9817
Organic acids												
2-Oxoglutaric acid	3.77E+05	-1.53E+05	0.9904	3.39E+05	-9.45E+04	0.9941	2.04E+05	-2.21E+04	0.9896	2.10E+05	-1.65E+04	0.9932
cis-Aconitic acid	4.55E+05	-6.54E+04	0.9887	5.09E+05	-3.04E+04	0.9757	2.17E+05	2.86E+03	0.9771	6.37E+05	-1.23E+04	0.9969
Fumaric acid	2.05E+05	8.60E+02	0.9992	2.26E+05	4.48E+03	0.9827	1.62E+05	-2.22E+03	0.9868	1.40E+05	-1.10E+03	0.9952
Glutaric acid	1.97E+06	4.09E+05	0.9994	1.83E+06	6.24E+05	0.9985	1.35E+06	2.13E+05	0.9895	1.41E+06	2.41E+05	0.9915
Glycolic acid	8.35E+05	2.43E+05	0.9975	7.71E+05	6.71E+05	0.9999	8.08E+05	2.88E+05	0.9971	7.40E+05	3.37E+05	0.9945
Glyoxylic acid	5.10E+05	1.25E+05	0.9992	4.51E+05	5.80E+04	0.9840	2.46E+05	5.43E+04	0.9935	2.58E+05	1.20E+05	0.9983
Lactic acid	5.62E+04	1.33E+06	0.9899	3.97E+04	1.72E+05	0.9846	2.92E+04	1.17E+05	0.9646	2.58E+04	5.44E+04	0.9778
Malonic acid	5.06E+04	8.10E+03	0.9963	6.39E+04	8.21E+03	0.9909	3.24E+04	6.18E+03	0.9957	3.16E+04	6.83E+03	0.9849
Pyruvic acid	1.66E+06	2.81E+05	0.9991	1.65E+06	-1.91E+04	0.9994	1.47E+06	4.69E+05	0.9988	1.48E+06	2.00E+05	0.9999
Succinic acid	1.15E+06	3.22E+05	0.9932	1.02E+06	5.58E+05	0.9970	6.79E+05	-4.06E+04	0.9964	6.64E+05	-5.35E+04	0.9929
β-Lactam antibiotics, intermediates of biosynthesis and degradation products												
2-Aminoadipic acid	1.01E+06	1.03E+03	0.9977	1.08E+06	6.32E+05	0.9942	9.48E+05	4.03E+02	0.9956	1.02E+06	-1.70E+04	0.9989
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	2.17E+05	1.67E+03	0.9992	2.38E+05	4.46E+05	0.9955	1.62E+05	1.17E+03	0.9930	1.70E+05	-7.73E+03	0.9936
6-Aminopenicillanic acid	7.76E+06	-7.39E+04	0.9966	7.98E+06	8.80E+04	0.9988	9.19E+06	1.18E+05	0.9990	9.67E+06	5.87E+04	0.9991
6-Aminopenicilloic acid	1.81E+05	1.45E+04	0.9917	1.61E+05	4.29E+04	0.9946	1.92E+05	2.86E+04	0.9977	2.11E+05	1.01E+04	0.9941
7-Aminocephalosporanic acid	1.31E+06	-7.36E+03	0.9959	1.28E+06	-3.47E+03	0.9981	1.16E+06	-2.30E+04	0.9985	1.28E+06	-5.22E+04	0.9985
8-Hydroxypenillic acid	4.00E+04	1.07E+03	0.9866	4.14E+04	1.65E+03	0.9985	4.40E+04	1.29E+03	0.9886	4.75E+04	-1.35E+03	0.9901
Cephalosporin C	7.74E+05	6.39E+05	0.9989	n.a.[b]	n.a.[b]	n.a.[b]	7.82E+05	9.73E+03	0.9997	8.07E+05	-6.51E+03	0.9955
Cephalosporin C lactone	1.24E+06	1.33E+04	0.9927	1.37E+06	5.43E+04	0.9987	1.67E+06	-2.85E+03	0.9985	1.78E+06	-2.25E+04	0.9990
Desacetoxycephalosporin	5.31E+05	1.35E+04	0.9951	6.13E+05	2.96E+05	0.9993	6.22E+05	2.13E+03	0.9995	6.37E+05	6.39E+02	0.9974
Desacetylcephalosporin	1.92E+05	1.46E+04	0.9866	n.a.[b]	n.a.[b]	n.a.[b]	2.26E+05	6.91E+02	0.9965	2.23E+05	1.18E+02	0.9991
Penicillamine disulfide	2.03E+06	-2.88E+03	0.9937	2.06E+06	1.08E+04	0.9993	2.21E+06	6.42E+03	0.9984	2.32E+06	-4.03E+04	0.9990
Phenoxyacetic acid	2.00E+07	3.29E+05	0.9986	1.92E+07	4.24E+05	0.9983	1.65E+07	4.34E+07	997.0000	1.78E+07	1.34E+05	0.9994
Phenoxymethylpenicilloic acid	1.87E+07	-1.51E+06	0.9990	1.54E+07	-4.07E+05	0.9933	1.29E+07	-4.20E+05	0.9924	1.48E+07	-1.36E+06	0.998
Phenoxymethylpenillic acid	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]
p-Hydroxyphenoxyacetic acid	4.12E+06	7.54E+04	0.9939	3.12E+06	1.51E+07	0.9910	2.37E+06	2.15E+05	0.9922	3.05E+06	1.14E+05	0.9971
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	4.67E+05	1.20E+04	0.8960	1.26E+05	3.44E+04	0.9925	1.28E+05	1.09E+05	0.9902	4.15E+05	2.06E+04	0.9906
Vitamins and biogenic amines												
Biotin	1.02E+07	5.35E+03	0.9997	1.07E+07	9.43E+03	0.9999	8.87E+06	7.66E+04	0.9985	9.44E+06	1.28E+04	0.9992
Cobalamine	2.32E+06	-4.31E+04	0.9900	1.82E+06	-1.12E+04	0.9939	2.12E+06	1.31E+04	0.9993	2.34E+06	-2.22E+04	0.9927
Ethanolamine	2.27E+06	1.31E+05	0.9970	1.64E+06	2.48E+05	0.9981	1.88E+06	1.90E+05	0.9994	1.90E+06	1.05E+05	0.9995
Folic acid	1.69E+05	8.31E+02	0.9888	1.78E+05	3.06E+02	0.9898	8.58E+04	6.38E+04	0.9934	1.10E+05	-1.83E+04	1.0000
Nicotinic acid	1.64E+07	2.89E+05	0.9959	1.16E+07	1.61E+06	0.9960	6.54E+06	3.91E+05	0.9950	6.60E+06	3.46E+05	0.9934
Pantothenic acid	5.11E+06	1.28E+04	0.9984	4.78E+06	2.30E+05	0.9960	4.87E+06	2.33E+04	0.9993	4.87E+06	-1.73E+04	0.9995
Putrescine	1.53E+07	4.39E+07	0.9564	2.20E+07	2.75E+07	0.9951	2.32E+07	1.53E+07	0.9925	2.69E+07	1.21E+07	0.9903
Pyridoxine	1.17E+09	5.55E+05	0.9999	9.54E+08	1.36E+07	0.9954	6.74E+08	5.91E+06	0.9990	5.90E+08	7.35E+06	0.9916
Riboflavin	1.67E+07	-4.94E+05	0.9927	7.37E+06	3.04E+05	0.9943	5.30E+06	2.85E+05	0.9871	1.91E+07	-1.92E+06	0.9781

[a] n.a. not available due to stability reasons

[b] n.a. not available due to too high intrinsic concentration of the compound in the corresponding sample matrix shifting concentrations beyond the linear range

	Matrix-free neat solutions			spiked extract 12			spiked extract 15		
(B) with internal standard	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**
measured on	**day 1**			**day 1**			**day 1**		
Amino acids									
Alanine	2.040	0.117	0.9977	2.140	1.890	0.9966	2.060	1.620	0.9927
Arginine	0.783	0.020	0.9885	0.726	0.246	0.9692	0.740	0.451	0.9729
Asparagine	2.650	-0.030	0.9934	1.990	0.037	0.9978	2.140	0.299	0.9947
Aspartic acid	1.370	0.054	0.9975	1.070	0.447	0.9884	1.310	0.484	0.9954
Citrulline	5.560	0.103	0.9930	4.310	0.198	0.9970	5.340	0.103	0.9991
Cystine	9.090	0.055	0.9931	8.030	0.250	0.9925	9.280	-0.017	0.9991
Glutamic acid	1.750	0.087	0.9899	1.400	2.760	0.9947	0.961	4.160	0.9465
Glutamine	2.990	0.045	0.9966	2.990	0.658	0.9955	2.820	1.400	0.9896
Glycine	1.080	0.183	0.9960	1.350	0.478	0.9917	1.130	0.746	0.9961
Histidine	15.10	0.586	0.9982	15.40	2.350	0.9991	15.50	3.220	0.9989
Isoleucine	0.596	0.001	0.9976	0.596	0.168	0.9987	0.569	0.122	0.9964
Leucine	0.178	0.001	0.9976	0.164	0.113	0.9975	0.176	0.109	0.9903
Lysine	2.760	0.068	0.9943	3.130	0.579	0.9956	2.930	1.560	0.9819
Methionine	6.490	-0.001	0.9981	6.390	1.180	0.9984	6.840	1.300	0.9974
Ornithine	0.664	0.062	0.9845	0.693	0.073	0.9781	0.521	0.141	0.9987
Phenylalanine	7.260	0.084	0.9973	5.970	2.140	0.9992	5.100	2.540	0.9959
Proline	5.260	0.091	0.9982	5.150	0.837	0.9933	5.710	1.100	0.9991
Serine	11.50	1.370	0.9898	16.60	6.740	0.9724	11.60	5.540	0.9972
Threonine	4.050	0.101	0.9986	4.190	1.600	0.9921	4.660	1.190	0.9991
Tryptophan	6.780	0.098	0.9808	6.730	0.650	0.9935	7.780	0.571	0.9519
Tyrosine	14.90	-0.009	0.9926	12.40	2.850	0.9966	11.70	4.340	0.9809
Valine	2.290	0.010	0.9906	2.350	0.967	0.9939	2.100	0.581	0.9891
Organic acids									
2-Oxoglutaric acid	0.649	0.023	0.9910	0.651	-0.054	0.9970	0.648	0.176	0.9850
cis-Aconitic acid	2.120	-0.518	0.9842	1.880	-0.549	0.9922	1.320	-0.132	0.9789
Fumaric acid	0.474	-0.017	0.9543	0.294	0.000	0.9969	0.425	0.037	0.9919
Glutaric acid	2.830	1.080	0.9990	2.790	1.230	0.9952	3.030	2.060	0.9920
Glycolic acid	0.779	0.803	0.9865	0.858	0.748	0.9983	1.030	0.742	0.0989
Glyoxylic acid	0.560	0.119	0.9805	0.548	0.155	0.9805	0.614	0.277	0.9883
Lactic acid	0.034	0.035	0.9980	0.069	0.105	0.9830	0.037	0.028	0.9806
Malonic acid	0.078	0.001	0.9974	0.076	0.015	0.9994	0.077	0.006	0.9962
Pyruvic acid	1.800	0.053	0.9891	1.830	0.064	0.9976	2.140	0.008	0.9982
Succinic acid	1.810	0.092	0.9943	1.710	0.700	0.9966	2.090	0.560	0.9984
β-Lactam antibiotics, intermediates of biosynthesis and degradation products									
2-Aminoadipic acid	26.70	0.518	0.9947	31.40	7.200	0.9977	30.10	12.200	0.9819
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	0.352	0.008	0.9946	0.319	-0.009	0.9996	0.370	0.022	0.9971
6-Aminopenicillanic acid	1.310	0.060	0.9923	1.360	0.301	0.9936	1.540	0.036	0.9986
6-Aminopenicilloic acid	5.285	0.428	0.9935	6.044	0.243	0.9981	3.774	3.320	0.9646
7-Aminocephalosporanic acid	2.540	-0.051	0.9854	2.300	-0.131	0.9963	2.520	-0.135	0.9936
8-Hydroxypenillic acid	0.020	-0.001	0.9980	0.019	0.003	0.9990	0.022	0.029	0.9846
Cephalosporin C	2.671	-0.010	0.9994	3.074	-0.077	0.9956	2.688	0.052	0.9998
Cephalosporin C lactone	21.20	-0.427	0.9960	19.50	0.018	0.9993	19.20	-0.455	0.9926
Desacetoxycephalosporin	2.382	0.059	0.9963	2.482	0.030	0.9921	2.913	-0.041	0.9965
Desacetylcephalosporin	0.767	0.032	0.9870	0.923	0.004	0.9990	0.859	0.028	0.9954
Penicillamine disulfide	7.830	0.110	0.9966	8.090	0.110	0.9985	8.140	0.205	0.9969
Phenoxyacetic acid	26.40	-0.430	0.9997	75.20	132.00	0.9915	n.a.[b]	n.a.[b]	n.a.[b]
Phenoxymethylpenicilloic acid	2.360	-0.091	0.9952	2.650	0.152	0.8987	2.630	0.844	0.9935
Phenoxymethylpenillic acid	0.388	-0.018	0.9526	0.186	0.022	0.9932	n.a.[a]	n.a.[a]	n.a.[a]
p-Hydroxyphenoxyacetic acid	6.410	-0.126	0.9926	5.700	-0.091	0.9993	6.450	0.623	0.9995
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	0.376	0.008	0.9841	0.376	0.008	0.9841	0.294	0.060	0.8743
Vitamins and biogenic amines									
Biotin	1.110	0.000	0.9887	1.140	-0.019	0.9965	1.120	-0.009	0.9983
Cobalamine	0.376	-0.017	0.9907	0.302	-0.001	0.9935	0.385	-0.011	0.9900
Ethanolamine	0.926	0.005	0.9968	0.964	0.035	0.9989	0.937	0.030	0.9984
Folic acid	0.278	0.060	0.9977	0.296	0.018	0.9965	0.286	0.045	0.9987
Nicotinic acid	0.928	0.060	0.9804	1.120	0.271	0.9783	1.430	0.172	0.9804
Pantothenic acid	0.915	-0.023	0.9930	0.865	-0.014	0.9980	1.040	0.003	0.9943
Putrescine	2.290	0.062	0.9995	2.970	0.155	0.9983	2.880	0.294	0.9981
Pyridoxine	72.30	0.768	0.9918	82.60	2.180	0.9938	84.70	1.520	0.9941
Riboflavin	2.020	-0.053	0.9984	2.170	0.002	0.9964	2.130	-0.107	0.9970

[a] n.a. not available due to stability reasons

[b] n.a. not available due to too high intrinsic concentration of the compound in the corresponding sample matrix shifting concentrations beyond the linear range

	spiked extract 1			spiked extract 5			spiked medium 6			spiked medium 10		
(B) with internal standard	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**
measured on	**day 2**			**day 2**			**day 3**			**day 3**		
Amino acids												
Alanine	1.940	7.570	0.9877	1.750	4.230	0.9696	2.290	6.660	0.9571	2.580	10.700	0.8875
Arginine	0.750	0.265	0.9888	0.602	1.850	0.9572	1.130	1.090	0.9933	0.612	1.090	0.7896
Asparagine	1.930	0.717	0.9946	2.680	1.810	0.9974	2.630	0.454	0.9994	1.930	0.088	0.9982
Aspartic acid	1.320	0.308	0.9970	1.390	3.180	0.9768	1.340	1.620	0.9867	1.370	0.254	0.9959
Citrulline	4.600	0.419	0.9987	5.110	0.217	0.9938	4.780	0.030	0.9980	4.420	0.096	0.9877
Cystine	10.20	-0.416	0.9760	8.660	0.135	0.9996	10.20	3.280	0.9936	10.40	2.390	0.9918
Glutamic acid	2.000	1.120	0.9935	2.060	5.900	0.9477	1.810	1.340	0.9932	1.800	0.438	0.9934
Glutamine	2.840	0.988	0.9621	3.800	8.910	0.9133	4.690	8.040	0.9711	4.410	6.960	0.9424
Glycine	1.360	0.419	0.9890	1.080	1.140	0.9842	1.500	0.819	0.9997	1.650	0.913	0.9905
Histidine	15.50	2.720	0.9971	13.80	6.880	0.9996	14.50	6.660	0.9544	15.20	4.970	0.9971
Isoleucine	0.546	0.456	0.9961	0.511	0.595	0.9546	0.609	0.829	0.9674	0.572	0.659	0.9764
Leucine	0.121	0.485	0.9670	0.175	0.504	0.9852	0.229	0.328	0.9900	0.151	0.240	0.9564
Lysine	3.180	0.958	0.9993	3.490	7.200	0.9207	3.210	3.070	0.9896	2.550	2.320	0.9809
Methionine	6.120	3.790	0.9939	5.440	6.280	0.9811	6.380	3.330	0.9878	6.270	2.370	0.9896
Ornithine	0.532	0.212	0.9909	0.645	0.396	0.9818	0.600	0.096	0.9862	0.553	0.107	0.9882
Phenylalanine	4.180	7.380	0.9520	2.640	11.200	0.9650	5.120	5.110	0.9855	5.210	3.370	0.9932
Proline	5.250	11.700	0.9832	5.240	12.900	0.9808	4.800	8.720	0.9920	5.620	7.310	0.9814
Serine	17.80	9.760	0.9954	9.740	17.900	0.6869	10.20	21.500	0.9215	n.a.[b]	n.a.[b]	n.a.[b]
Threonine	4.280	3.620	0.9967	4.110	4.370	0.9807	4.830	4.280	0.9660	4.200	2.910	0.9757
Tryptophan	8.500	0.318	0.9803	6.450	4.610	0.9584	7.560	3.270	0.9775	6.570	2.780	0.9947
Tyrosine	11.30	3.330	0.9891	10.70	22.600	0.9221	14.00	8.620	0.9867	14.30	6.340	0.9848
Valine	2.380	3.350	0.9994	1.970	3.630	0.9670	2.650	3.010	0.9893	2.170	2.310	0.9694
Organic acids												
2-Oxoglutaric acid	0.656	0.003	0.9963	0.538	0.321	0.9834	0.656	0.064	0.9916	0.558	0.166	0.9958
cis-Aconitic acid	1.080	-0.086	0.9892	1.030	-0.040	0.9946	0.795	0.045	0.9922	0.902	0.005	0.9965
Fumaric acid	0.441	0.062	0.9895	0.444	0.066	0.9942	0.545	0.032	0.9960	0.492	0.017	0.9897
Glutaric acid	3.920	1.760	0.9874	3.220	2.380	0.9818	3.850	2.250	0.9911	3.460	2.560	0.9812
Glycolic acid	1.360	0.430	1.0000	1.080	0.533	0.9858	1.710	1.400	0.9997	1.710	0.721	0.9900
Glyoxylic acid	0.808	0.254	0.9820	0.574	0.031	0.9956	0.614	0.148	0.9945	0.540	0.366	0.8878
Lactic acid	0.081	2.090	0.9107	0.062	0.154	0.9956	0.064	0.304	0.9850	0.058	0.153	0.9920
Malonic acid	0.074	0.033	0.9969	0.072	0.029	0.9971	0.086	-0.005	0.9948	0.079	-0.002	0.9850
Pyruvic acid	2.620	0.496	0.9991	2.130	-0.105	0.9963	3.640	1.230	0.9932	3.350	0.527	0.9989
Succinic acid	1.730	1.730	0.9960	1.740	1.960	0.9946	1.990	0.221	0.9961	1.980	0.153	0.9920
β-Lactam antibiotics, intermediates of biosynthesis and degradation products												
2-Aminoadipic acid	30.10	0.290	0.9915	25.00	16.400	0.9904	24.40	-0.233	0.9942	27.10	-0.582	0.9940
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	0.370	0.067	0.9945	n.a.[b]	n.a.[b]	n.a.[b]	0.470	0.139	0.9934	0.363	0.168	0.9947
6-Aminopenicillanic acid	1.440	-0.005	0.9992	1.400	0.023	0.9966	1.600	0.000	0.9990	1.470	0.027	0.9934
6-Aminopenicilloic acid	5.716	0.208	0.9923	4.818	0.619	0.9973	5.679	0.327	0.9974	4.957	0.263	0.9999
7-Aminocephalosporanic acid	2.120	-0.017	0.9942	1.670	-0.050	0.9927	2.830	-0.028	0.9988	3.010	-0.123	0.9910
8-Hydroxypenillic acid	0.018	-0.001	0.9869	0.025	0.000	0.9950	0.020	0.000	0.9918	0.024	-0.003	0.9993
Cephalosporin C	2.680	2.200	0.9970	n.a.[b]	n.a.[b]	n.a.[b]	2.797	0.005	0.9969	2.982	-0.043	0.9973
Cephalosporin C lactone	18.200	0.053	0.9950	19.600	1.160	0.9866	24.500	-0.395	0.9955	24.400	-0.492	0.9949
Desacetoxycephalosporin	2.390	0.003	0.9966	2.357	1.220	0.9998	2.639	-0.006	0.9981	2.764	0.028	0.9974
Desacetylcephalosporin	0.806	0.001	0.9862	n.a.[b]	n.a.[b]	n.a.[b]	1.004	-0.006	0.9963	0.972	-0.001	0.9974
Penicillamine disulfide	8.490	0.023	0.9952	7.790	0.102	0.9962	9.900	-0.031	0.9987	10.200	-0.128	0.9965
Phenoxyacetic acid	32.50	0.375	0.9965	25.10	-0.268	0.9982	38.90	2.570	0.9964	42.70	0.364	0.9957
Phenoxymethylpenicilloic acid	3.350	-0.108	0.9955	2.670	-0.058	0.9963	2.220	-0.091	0.9895	2.060	-0.064	0.9937
Phenoxymethylpenillic acid	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]
p-Hydroxyphenoxyacetic acid	6.710	0.103	0.9919	3.690	0.094	0.9965	6.040	0.608	0.9827	7.160	0.255	0.9978
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	0.308	0.005	0.9940	0.060	0.031	0.9691	0.097	0.053	0.9941	0.239	0.014	0.9880
Vitamins and biogenic amines												
Biotin	1.900	0.037	0.9973	1.820	0.017	0.9999	1.540	0.004	0.9984	1.480	0.007	0.9944
Cobalamine	0.553	-0.011	0.9680	0.454	-0.005	0.9975	0.683	-0.001	0.9949	0.655	-0.002	0.9924
Ethanolamine	0.964	0.049	0.9960	0.922	0.126	0.9988	0.929	0.075	0.9995	0.900	0.037	0.9990
Folic acid	0.236	0.095	0.9717	0.373	0.016	0.9758	0.276	0.037	0.9984	0.253	0.045	0.9895
Nicotinic acid	3.390	0.072	0.9997	2.110	0.303	0.9907	1.180	0.043	0.9977	1.050	0.066	0.9799
Pantothenic acid	1.250	0.003	0.9915	1.220	0.058	0.9966	1.490	0.006	0.9925	1.370	0.002	0.9972
Putrescine	2.560	2.290	0.9965	3.430	2.640	0.9956	6.130	2.460	0.9997	5.530	1.890	0.9949
Pyridoxine	236.0	0.523	0.9989	174.0	2.550	0.9930	99.70	1.080	0.9884	91.40	2.120	0.9950
Riboflavin	3.100	-0.088	0.9966	1.310	0.055	0.9973	1.070	0.027	0.9718	2.830	-0.263	0.9828

[a] n.a. not available due to stability reasons

[b] n.a. not available due to too high intrinsic concentration of the compound in the corresponding sample matrix shifting concentrations beyond the linear range

Table S 6: Assessment of matrix effects by comparison of the slopes of calibration functions (with IS) in standard solution and of standard addition in 4 different extracts and two nutrition media, respectively.[i]

	Extracts of fermentation broths					Nutrition media		
	Absolute matrix effect (%) [a]				Relative matrix effect [b]	Absolute matrix effect (%) [a]		Relative matrix effect [b]
Amino acids	**Extract 12[c]**	**Extract 15[c]**	**Extract 1[d]**	**Extract 5[a]**	**RSD (%)**	**Medium 6**	**Medium 10**	**RSD (%)**
Alanine	105	101	95	86	8.3	112	**126**	10.1
Arginine	93	95	96	**77**	8.8	**144**	**78**	**46.8**
Asparagine	**75**	81	**73**	101	12.9	99	**73**	18.7
Aspartic acid	**78**	96	96	101	10.2	98	100	1.5
Citrulline	**78**	96	83	92	8.4	86	**79**	4.6
Cystine	88	102	112	95	10.2	112	114	1.6
Glutamic acid	80	**55**	114	118	**29.9**	103	103	0.4
Glutamine	100	94	95	**127**	15.5	**157**	**147**	6.6
Glycine	**125**	105	**126**	100	13.5	119	**153**	**24.2**
Histidine	102	103	103	91	5.5	96	101	3.3
Isoleucine	100	95	92	86	6.0	102	96	4.4
Leucine	92	99	68	98	14.6	**129**	85	**31.0**
Lysine	113	106	115	**126**	8.4	116	92	16.9
Methionine	98	105	94	84	9.0	98	97	1.2
Ornithine	104	**78**	80	97	12.7	90	83	5.0
Phenylalanine	82	**70[e]**	**58[e]**	**36[e]**	19.6	**71[e]**	**72[e]**	0.9
Proline	98	109	100	100	4.8	91	107	11.0
Serine	**144**	101	**155**	85	**33.7**	89	n.a.[c,h]	n.a.[c]
Threonine	103	115	106	101	6.0	119	104	11.0
Tryptophan	99	115	**125**	95	14.0	112	97	10.3
Tyrosine	83	**79**	**76**	**72**	4.8	94	96	1.4
Valine	103	92	104	86	8.7	116	95	14.8
Organic acids								
2-Oxoglutaric acid	100	100	101	83	8.8	101	86	10.7
cis-Aconitic acid	89	**62[f]**	**51[f]**	**49[f]**	18.4	**38[f]**	**42[f]**	3.6
Fumaric acid	**62**	90	93	94	15.2	115	104	7.9
Glutaric acid	99	107	**139**	114	17.2	**136[f]**	**122[f]**	9.7
Glycolic acid	110	**132[f]**	**175[f]**	**139[f]**	**26,7**[f]	**220[f]**	**220[f]**	0.0
Glyoxylic acid	98	110	**144**	102	**21.1**	110	96	9.3
Lactic acid	**207[f]**	110	**243[f]**	**184[f]**	**56,1**[f]	**190[f]**	**173[f]**	12.5
Malonic acid	97	99	96	93	2.6	111	102	6.4
Pyruvic acid	102	119	**146[f]**	118	18.2	**202[f]**	**186[f]**	11.4
Succinic acid	94	115	96	96	10.1	110	109	0.4
β-Lactam antibiotics, intermediates of biosynthesis and degradation products								
2-Aminoadipic acid	118	113	113	94	10.6	91	101	7.2
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	91	105	105	n.a.[c]	8.4	**134**	103	**21.5**
6-Aminopenicillanic acid	104	118	110	107	5.9	**122**	112	7.0
6-Aminopenicilloic acid	114	**71**	108	91	19.3	107	94	9.7
7-Aminocephalosporanic acid	91	99	83	**66**	14.2	111	119	5.0
8-Hydroxypenillic acid	98	110	92	**125**	14.7	104	**121**	11.9
Cephalosporin C	115	101	100	n.a.[c]	8.4	105	112	4.9
Cephalosporin C lactone	92	91	86	92	3.0	116	115	0.3
Desacetoxycephalosporin	104	**122**	100	99	10.8	111	116	3.7
Desacetylcephalosporin	120	112	105	n.a.[c]	7.7	**131**	**127**	3.0
Penicillamine disulfide	103	104	108	99	3.7	**126**	**130**	2.7
Phenoxyacetic acid	**285[e,f]**	n.a.[c]	**123[e,f]**	95	**102,4**[e,f]	**147[e,f]**	**162[e,f]**	10.2
Phenoxymethylpenicilloic acid	112	111	**142**	113	14.8	94	87	4.8
Phenoxymethylpenillic acid	**48[g]**	n.a.[g]	n.a.[g]	n.a.[g]	n.a.[g]	n.a.[g]	n.a.[g]	n.a.[g]
p-Hydroxyphenoxyacetic acid	89	101	105	**58[f]**	**21,3**[f]	94	112	12.4
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	100	**78[g]**	82	**16[g]**	**36,6**[g]	**26[g]**	**64[g]**	**26,7**[g]
Vitamins and biogenic amines								
Biotin	103	101	**171[f]**	**164[f]**	**38,1**[f]	**139[f]**	**133[f]**	3.8
Cobalamine	80	102	**147[f]**	**121[f]**	**28.3**	**182[f]**	**174[f]**	5.3
Ethanolamine	104	101	104	100	2.3	100	97	2.2
Folic acid	106	103	85	**134**	**20.4**	99	91	5.9
Nicotinic acid	**121[e,f]**	**154[e,f]**	**365[e,f]**	**227[e,f]**	**108,5**[e,f]	**127[e,f]**	113	9.9
Pantothenic acid	95	114	**137[f]**	**133[f]**	19.5	**163[f]**	**150[f]**	9.3
Putrescine	**130[f]**	**126[f]**	112	**150[f]**	15.7	**268[f]**	**241[f]**	18.5
Pyridoxine	114	117	**326[e,f]**	**241[e,f]**	**103,0**[e,f]	**138[e,f]**	**126[e,f]**	8.1
Riboflavin	107	105	**153[f,g]**	**65[f,g]**	**36,2**[f,g]	**53[f,g]**	**140[f,g]**	**61,6**[f,g]
Mean	99	101	103	98	12	109	104	9
Standard deviation	32	17	54	39	23	40	36	11
Mean (without assigned outliers)	99	101	103	98	12	109	104	9
Standard deviation	14	13	19	15	7	15	17	8

[a] calculated by ratio of slopes in matrix and standard solution multiplied by 100
[b] calculated as the relative standard deviation from the mean in the 4 extracts and two nutrition media, respectively
[c] E 12 and E 15 are extracts from penicillin synthesis.
[d] E1 and E5 are extracts from cephalosporin synthesis.
[e] linearity problems (endogenic concentration in extract extremely high so that 1:100 dilution is still outside linear range)
[f] problems due to instrumental fluctuations and/or inappropriate internal standard
[g] problems due to limited compound stability
[h] n.a. not available
[i] values above +/- 20% in bold

Figure S 7: Plots of slopes in different matrices for assessment of matrix effects.
(A) slopes of extracts 12 and 15 versus slopes in neat solutions
(B) slopes of extracts 1 and 5 versus slopes in neat solutions
(C) slopes of medium 10 versus slopes in medium 6
[a] problems due to instrumental fluctuations
[b] linearity problems (endogenic concentration in extract extremely high shifting concentrations outside the linear range)
[c] stability problems

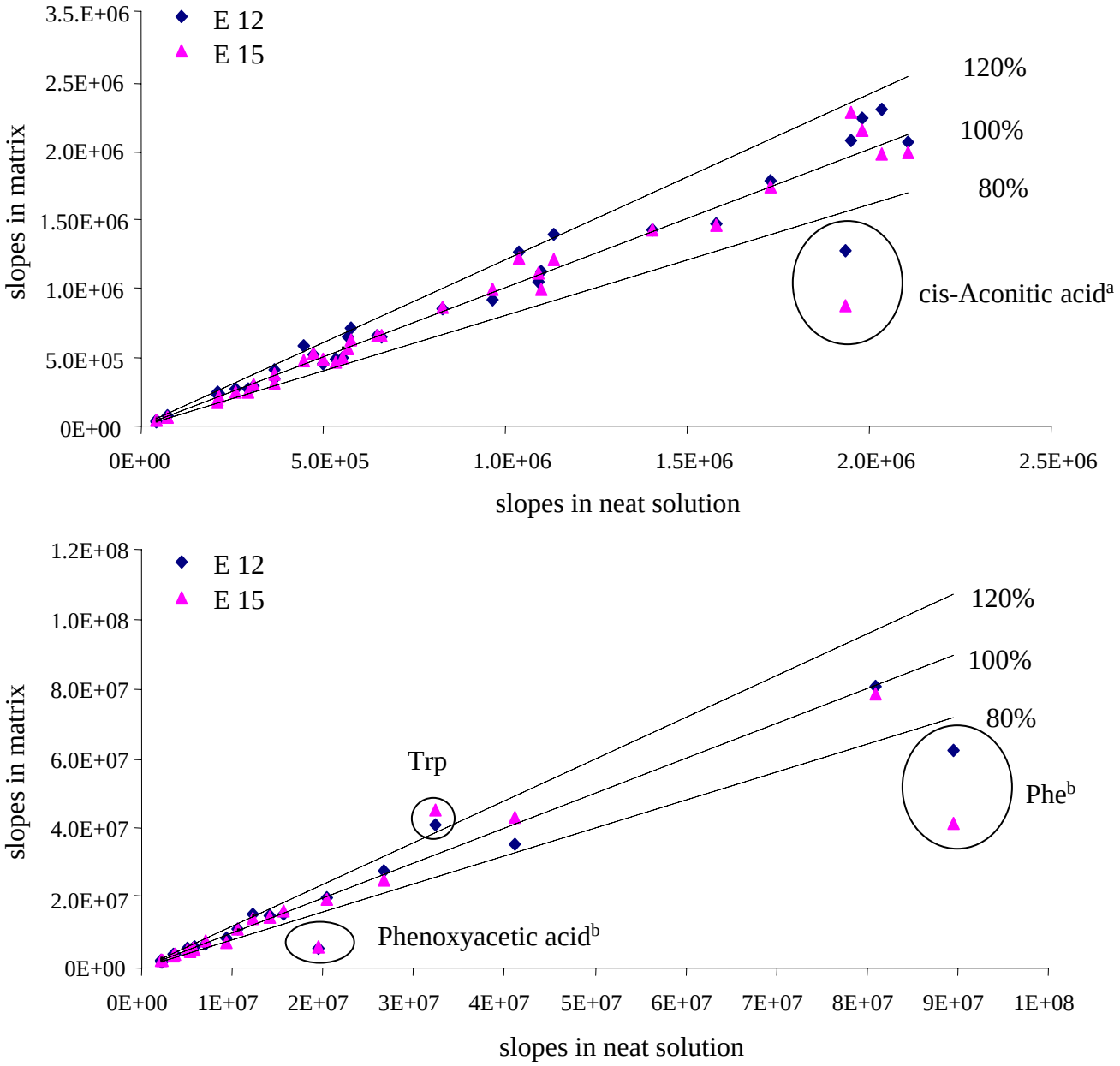
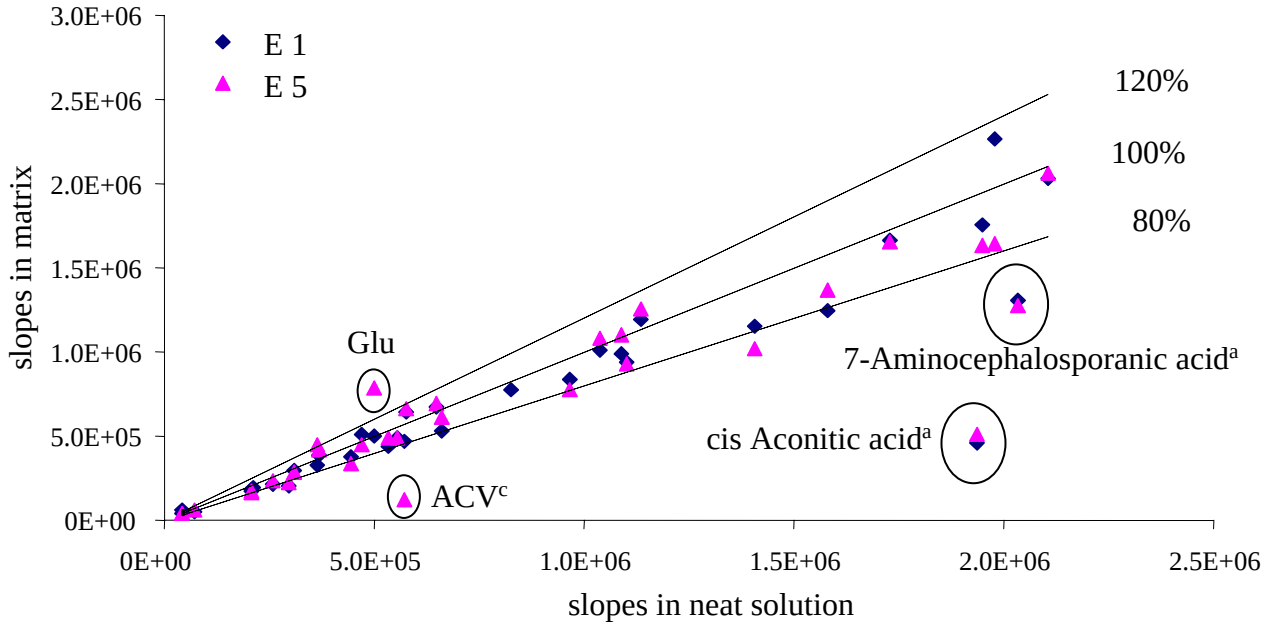

E 12
E 15
120%
100%
80%
cis-Aconitic acid[a]
slopes in matrix
slopes in neat solution
3.5.E+06
2.5E+06
2.0E+06
1.50E+06
1.0E+06
5.0E+05
0E+00
0E+00
5.0E+05
1.0E+06
1.5E+06
2.0E+06
2.5E+06
E 12
E 15
120%
100%
80%
Trp
Phe[b]
Phenoxyacetic acid[b]
slopes in matrix
slopes in neat solution
1.2E+08
1.0E+08
8.0E+07
6.0E+07
4.0E+07
2.0E+07
0E+00
0E+00
1E+07
2E+07
3E+07
4E+07
5E+07
6E+07
7E+07
8E+07
9E+07
1E+08

Figure S 7A


E 1
E 5
120%
100%
80%
Glu
7-Aminocephalosporanic acid[a]
cis Aconitic acid[a]
ACV[c]
slopes in matrix
slopes in neat solution
3.0E+06
2.5E+06
2.0E+06
1.5E+06
1.0E+06
5.0E+05
0E+00

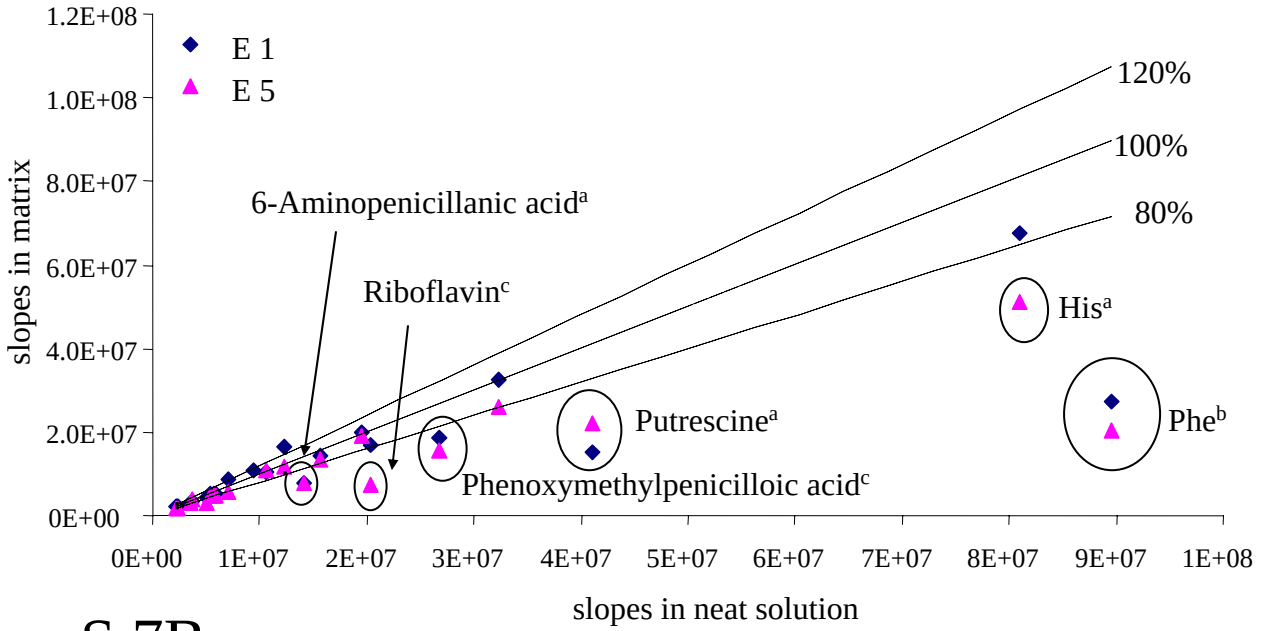
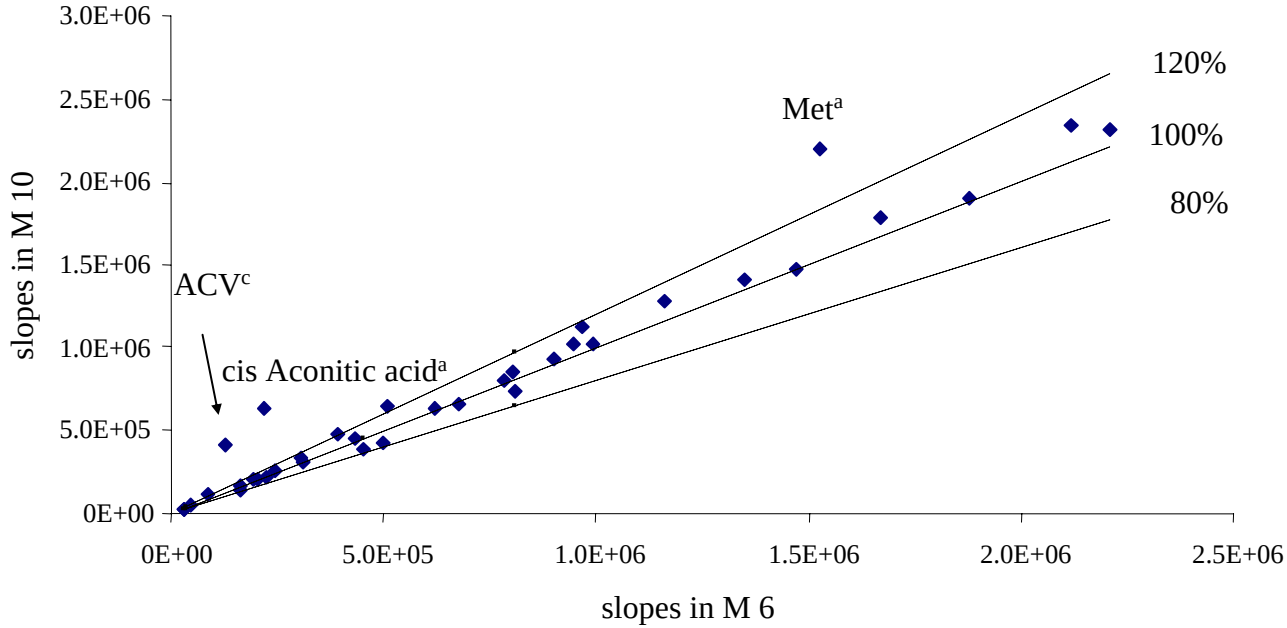

E 1
E 5
120%
100%
80%
6-Aminopenicillanic acid[a]
Riboflavin[c]
His[a]
Putrescine[a]
Phe[b]
Phenoxymethylpenicilloic acid[c]
slopes in matrix
slopes in neat solution
1.2E+08
1.0E+08
8.0E+07
6.0E+07
4.0E+07
2.0E+07
0E+00
1E+07
2E+07
3E+07
4E+07
5E+07
6E+07
7E+07
8E+07
9E+07
1E+08

Figure S 7B


3.0E+06
2.5E+06
2.0E+06
1.5E+06
1.0E+06
5.0E+05
0E+00
slopes in M 10
0E+00
5.0E+05
1.0E+06
1.5E+06
2.0E+06
2.5E+06
slopes in M 6
120%
100%
80%
Met[a]
ACV[c]
cis Aconitic acid[a]

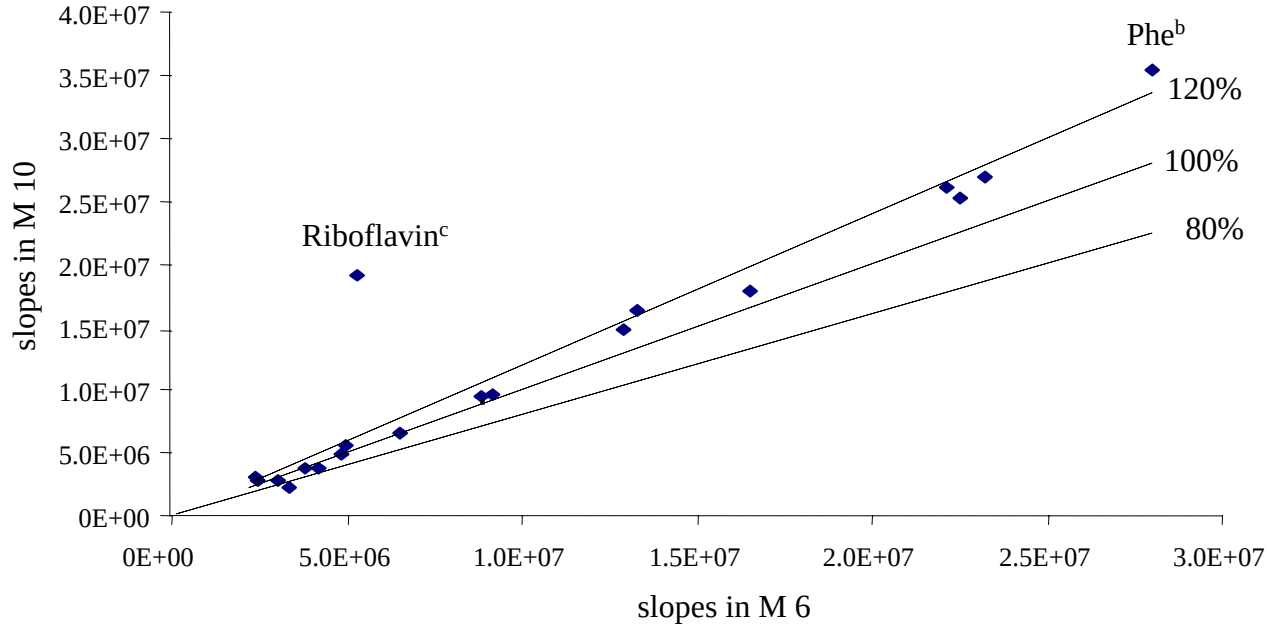

4.0E+07
3.5E+07
3.0E+07
2.5E+07
2.0E+07
1.5E+07
1.0E+07
5.0E+06
0E+00
slopes in M 10
0E+00
5.0E+06
1.0E+07
1.5E+07
2.0E+07
2.5E+07
3.0E+07
slopes in M 6
120%
100%
80%
Phe[b]
Riboflavin[c]

Figure S 7C

Table S 8: Intra-assay accuracy determined for QC samples prepared by spiking extract 12 at three distinct concentration levels
(group 1: 0.025, 0.25, 0.5 mg/L; group 2: 0.05, 0.5, 1.0 mg/L; group 3: 0.1, 1.0, 2.0 mg/L and group 4: 0.25, 2.5, 5.0 mg/L) (A) without and (B) with use of internal standards. [h]

(A) without internal standards

Calibration standards	Calibration	matrix-free neat solutions			spiked extract 12[a]			spiked extract 1[b]			spiked medium 10		
Analyte	group	low	middle	high	low	middle	high	low	middle	high	low	middle	high
measured on		day 1			day 1			day 2			day 3		
Amino acids													
Alanine	3	100	99	99	99	100	101	105	100	101	106	102	104
Arginine	2	83	89	99	98	101	110	85	83	89	89	118	**150**
Asparagine	2	113	88	99	112	94	108	**174**[c]	109	**121**	**188**[c]	118	**132**
Aspartic acid	2	105	91	94	102	94	99	89	90	98	89	88	96
Citrulline	2	100	102	104	94	104	106	99	112	115	102	117	**121**
Cystine	2	< LOQ	101	96	< LOQ	102	99	< LOQ	107	99	< LOQ	98	93
Glutamic acid	2	99	94	101	99	96	106	92	91	95	93	94	99
Glutamine	2	114	113	**124**	109	98	104	109	105	112	100	83	82
Glycine	4	< LOQ	114	118	< LOQ	96	102	< LOQ	97	103	< LOQ	84	87
Histidine	2	89	93	100	105	98	104	96	106	117	108	**189**[f]	**240**[f]
Isoleucine	2	102	102	99	98	100	97	101	105	104	103	100	96
Leucine	2	96	101	98	96	100	96	98	105	106	97	108	110
Lysine	2	103	102	99	87	93	92	96	104	99	102	**150**[f]	**165**[f]
Methionine	2	103	99	102	104	96	97	102	106	110	108	93	93
Ornithine	2	110	108	95	118	114	102	89	112	111	93	**150**[f]	**167**[f]
Phenylalanine	2	89	**74**[c]	**61**[c]	98	97	83	81	**122**[c]	**126**[c]	82	113	111
Proline	2	104	99	98	100	100	100	103	107	108	99	95	94
Serine	2	103	83	93	104	91	101	99	88	100	97	96	115
Threonine	2	105	97	113	103	87	99	105	93	108	107	102	**120**
Tryptophan	2	**64**	105	93	94	104	92	81	116	108	**78**[f]	**131**[f]	**127**[f]
Tyrosine	2	**142**	104	95	**126**	100	91	**131**	114	107	**141**	102	93
Valine	2	92	97	104	98	98	103	91	96	104	92	94	101
Organic acids													
2-Oxoglutaric acid	4	< LOQ	109	107	< LOQ	97	89	< LOQ	**160**[f]	**143**[f]	< LOQ	**256**[f]	**240**[f]
cis-Aconitic acid	4	**220**[c]	83	105	95	97	**139**[f]	**280**[f]	**272**[f]	**390**[f]	**170**[f]	**185**[f]	**272**[f]
Fumaric acid	4	115	89	**139**[f]	94	110	**177**[f]	**142**[f]	**126**[f]	**198**[f]	**217**[f]	**190**[f]	**290**[f]
Glutaric acid	4	80	120	106	89	109	99	**253**[f]	**146**[f]	**123**[f]	119	**167**[f]	**154**[f]
Glycolic acid	4	< LOQ	93	98	< LOQ	101	105	< LOQ	118	118	< LOQ	**128**[f]	**130**[f]
Glyoxylic acid	4	90	108	100	95	100	92	**210**[c]	113	98	**422**[f]	**214**[f]	**192**[f]
Lactic acid	4	100	96	104	97	97	107	95	83	85	103	109	119
Malonic acid	4	**191**[c]	106	106	90	92	97	**253**[f]	**148**[f]	**150**[f]	**370**[f]	**235**[f]	**241**[f]
Pyruvic acid	4	**139**	105	104	92	96	97	**158**	109	108	**189**[f]	**123**[f]	**121**[f]
Succinic acid	4	89	99	102	87	98	101	93	117	**123**	101	**172**[f]	**189**[f]
β-Lactam antibiotics, intermediates of biosynthesis and degradation products													
2-Aminoadipic acid	3	93	96	118	101	86	102	95	99	**121**	93	97	115
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	3	< LOQ	99	104	< LOQ	97	102	< LOQ	119	**124**[f]	< LOQ	**156**[f]	**161**[f]
6-Aminopenicillanic acid	3	91	105	90	94	99	86	**71**	**139**	**138**	**72**	**124**	118
6-Aminopenicilloic acid	3	**67**	93	90	103	91	86	105	110	105	106	97	97
7-Aminocephalosporanic acid	3	**62**	107	99	97	99	90	112	**168**[f]	**154**[f]	**148**[f]	**175**[f]	**159**[f]
8-Hydroxypenillic acid	3	**131**[c]	114	99	86	103	93	**241**[c]	**129**	108	85	94	84
Cephalosporin C	3	113	105	102	97	101	99	91	109	107	93	104	103
Cephalosporin C lactone	3	**127**	92	91	106	96	96	105	111	113	92	**79**	**79**
Desacetoxycephalosporin	3	113	98	104	100	99	106	109	115	**124**	93	98	106
Desacetylcephalosporin	3	101	100	108	99	91	98	113	116	**126**	95	94	103
Penicillamine disulfide	3	**128**	96	102	110	96	103	110	97	104	109	86	92
Phenoxyacetic acid	3	101	83	**70**[c]	101	96	90	101	83	**69**[c]	104	97	89
Phenoxymethylpenicilloic acid	3	101	102	99	104	100	97	86	**130**[d]	**134**[d]	81	**151**[d]	**161**[d]
Phenoxymethylpenillic acid	3	120	89	**79**	92	97	88	n.a.[d,g]	n.a.[d]	n.a.[d]	n.a.[d]	n.a.[d]	n.a.[d]
p-Hydroxyphenoxyacetic acid	3	**124**	110	97	115	98	86	**144**	**135**	119	**67**	**154**	**148**
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	3	**58**	103	105	85	95	95	97	**129**[b]	**130**[b]	**39**	**141**[b]	**143**[b]
Vitamins and biogenic amines													
Biotin	2	**70**	102	108	100	100	105	105	108	114	111	116	**122**
Cobalamine	3	97	80	96	86	95	116	100	81	97	91	**79**	95
Ethanolamine	2	**145**	118	106	96	101	93	87	99	90	**131**	120	109
Folic acid	3	n.a.[e]	96	95	84	97	88	**252**[f]	**151**[f]	**135**[f]	**562**[f]	**250**[f]	**217**[f]
Nicotinic acid	3	**132**	96	**71**[c]	100	**79**[c]	**60**[c]	105	**75**[c]	**56**[c]	81	**133**[c]	116
Pantothenic acid	2	**130**	98	94	106	100	97	109	101	98	**127**	106	103
Putrescine	2	118	98	86	90	102	94	n.a.[f]	**197**[f]	**197**[f]	**125**[f]	**136**[f]	**125**[f]
Pyridoxine	1	**60**	82	**57**[c]	117	**79**[c]	**53**[c]	**132**	**74**[c]	**50**[c]	**204**[c]	**141**[c]	96
Riboflavin	2	94	95	100	93	96	102	**154**	103	106	97	100	106

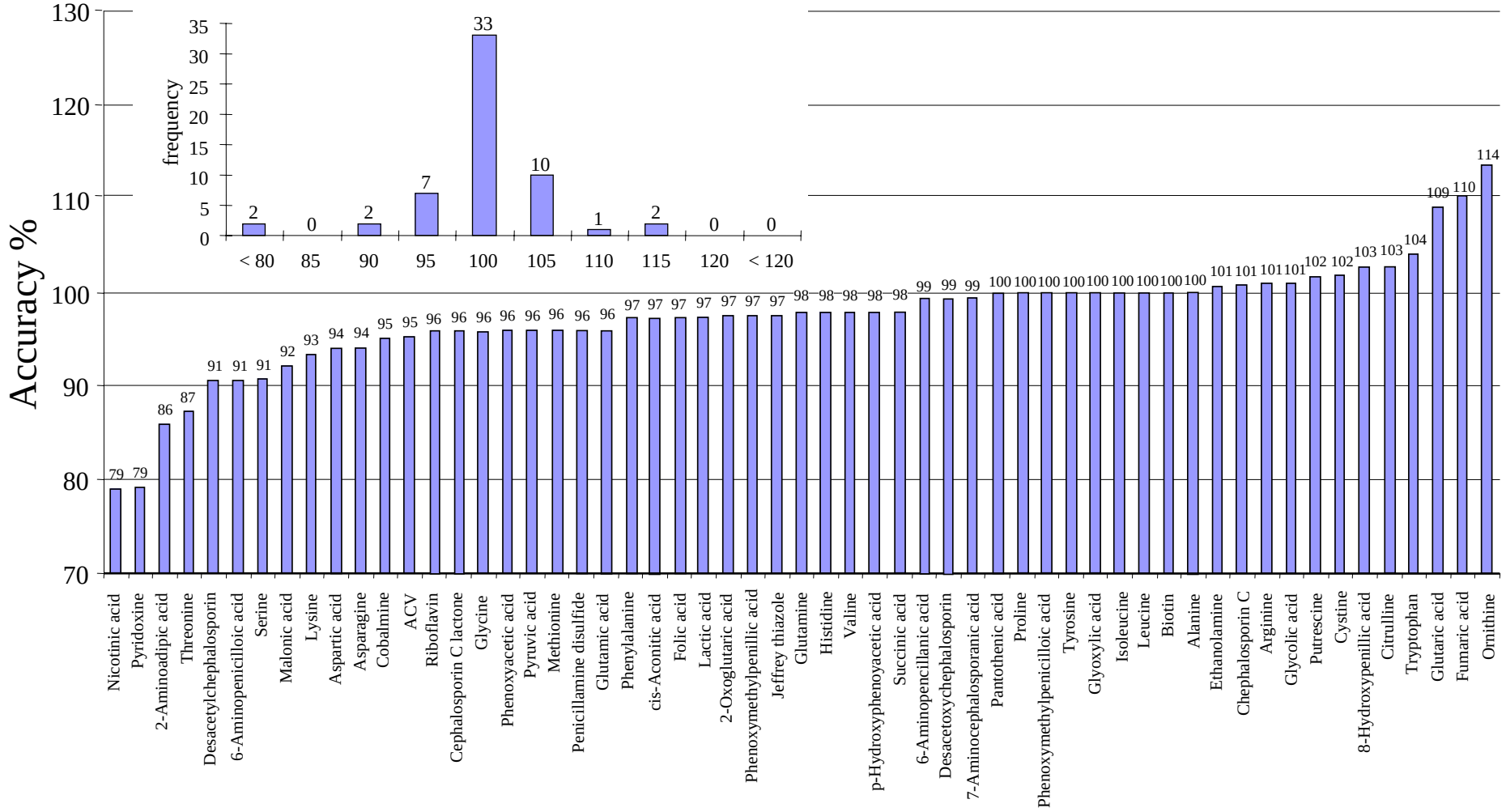
[a] E 12 is a extract from penicillin synthesis.
[b] E1 is a extract from cephalosporin synthesis.
[c] linearity problems (endogenic concentration in extract extremely high so that 1:100 dilution is still outside linear range)
[d] problems due to limited analyte stability
[e] concentration near the LLOQ
[f] problems due to instrumental fluctuations (loss of sensitivity)
[g] n.a. not availble
[h] Accuracy values above +/- 20% are in bold.

(B) with internal standards

Calibration standards	Calibration	matrix-free neat solutions			spiked extract 12 [a]			spiked extract 1 [b]			spiked medium 10		
Analyte	group	low	middle	high	low	middle	high	low	middle	high	low	middle	high
measured on			day 1			day 1			day 2			day 3	
Amino acids													
Alanine	3	96	105	99	105	102	96	107	108	102	103	92	84
Arginine	2	**79**	86	96	86	97	106	83	89	99	85	99	114
Asparagine	2	**130**[c]	**79**	83	**127**[c]	102	110	**170**[c]	110	116	**172**[c]	109	115
Aspartic acid	2	90	91	91	96	107	109	94	95	93	93	92	90
Citrulline	2	**79**	87	87	98	112	109	95	105	102	98	110	107
Cystine	2	< LOQ	111	90	< LOQ	**126**	102	< LOQ	112	87	< LOQ	102	81
Glutamic acid	2	100	102	102	102	97	91	101	94	88	102	97	92
Glutamine	2	101	97	104	114	104	103	108	105	106	120	**76**	**71**
Glycine	4	< LOQ	**129**	**126**	< LOQ	100	95	< LOQ	101	95	< LOQ	**76**	**74**
Histidine	2	83	93	99	97	97	100	85	92	97	85	93	98
Isoleucine	2	118	104	100	105	99	98	104	103	104	81	90	93
Leucine	2	98	95	95	102	96	97	99	106	115	100	100	103
Lysine	2	108	104	84	**135**	105	**80**	100	93	**74**	103	107	88
Methionine	2	116	101	106	103	99	107	101	100	109	117	104	110
Ornithine	2	**248**[f]	102	92	**146**[f]	98	87	**78**[f]	96	97	**78**[f]	94	94
Phenylalanine	2	101	86	**73**[c]	99	96	84	98	113	106	105	106	94
Proline	2	96	99	99	95	102	102	94	100	100	93	95	94
Serine	2	94	88	82	102	**80**	**71**	112	81	**69**	n.a.[c,g]	n.a.[c]	n.a.[c]
Threonine	2	92	87	94	104	91	95	114	94	96	94	86	92
Tryptophan	2	118	102	98	119	103	101	110	87	83	**120**	105	103
Tyrosine	2	111	100	90	111	112	107	114	120	115	108	102	95
Valine	2	106	102	104	101	98	102	103	88	102	104	103	109
Organic acids													
2-Oxoglutaric acid	4	< LOQ	105	96	< LOQ	109	98	< LOQ	105	95	< LOQ	**123**	112
cis-Aconitic acid	4	**144**[c]	82	107	**169**[c]	93	**121**	**123**[f]	**145**[f]	**202**[f]	106	**169**[f]	**239**[f]
Fumaric acid	4	**75**	**75**	108	97	118	**172**	**8**[f]	**73**[f]	112	**44**[f]	**69**[f]	102
Glutaric acid	4	**71**	**124**	101	81	**120**	101	**181**[f]	99	**77**[f]	84	102	84
Glycolic acid	4	< LOQ	95	93	< LOQ	87	109	< LOQ	**64**[f]	**73**[f]	< LOQ	**44**[f]	**55**[f]
Glyoxylic acid	4	116	95	93	116	95	93	**27**[f]	**60**[f]	**62**[f]	n.a.[f]	84	89
Lactic acid	4	88	**137**	**149**	108	91	86	96	**79**[f]	**74**[f]	101	98	96
Malonic acid	4	**127**[c]	95	105	**56**[c]	90	104	n.a.[c]	**79**	97	**140**[c]	95	104
Pyruvic acid	4	94	96	103	90	94	101	**76**[f]	**66**[f]	**70**[f]	**57**[f]	**52**[f]	**56**[f]
Succinic acid	4	**75**	104	100	80	110	106	**79**	100	96	**68**	96	92
β-Lactam antibiotics, intermediates of biosynthesis and degradation products													
2-Aminoadipic acid	3	105	102	83	100	89	**72**	101	92	**74**	104	100	82
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	3	< LOQ	90	93	< LOQ	105	105	< LOQ	**70**[f]	80	< LOQ	**44**[f]	**68**[f]
6-Aminopenicillanic acid	3	93	106	94	94	103	92	97	99	88	**125**	101	86
6-Aminopenicilloic acid	3	97	103	82	106	88	**69**	117	98	**77**	**127**	112	88
7-Aminocephalosporanic acid	3	**58**	86	85	99	98	95	**54**	101	100	**73**[f]	**75**[f]	**72**[f]
8-Hydroxypenillic acid	3	**206**[e]	109	96	**66**[e]	97	91	**231**[e]	115	103	**237**[e]	96	82
Cephalosporin C	3	94	111	99	100	98	86	90	110	98	93	100	89
Cephalosporin C lactone	3	113	95	93	100	100	99	105	107	106	101	82	80
Desacetoxycephalosporin	3	87	107	111	93	104	107	106	109	112	84	94	96
Desacetylcephalosporin	3	83	111	114	94	95	98	110	109	110	93	90	92
Penicillamine disulfide	3	101	103	105	98	100	102	104	96	98	101	82	82
Phenoxyacetic acid	3	108	**127**[c]	118	102	95	**76**[c]	108	**122**	111	107	115	100
Phenoxymethylpenicilloic acid	3	**75**	103	106	94	99	98	**149**	86	81	105	**123**[e]	**124**[e]
Phenoxymethylpenillic acid	3	**149**[f]	**56**[f]	**48**[f]	93	96	88	n.a.[d]	n.a.[d]	n.a.[d]	n.a.[d]	n.a.[d]	n.a.[d]
p-Hydroxyphenoxyacetic acid	3	93	87	82	98	98	93	**54**[f]	80	**77**[f]	**30**[f]	**73**[f]	**71**[f]
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	3	**48**[e]	99	98	**48**[e]	99	98	**67**[f]	**122**[f]	**119**[f]	**50**[f]	**153**[f]	**152**[f]
Vitamins and biogenic amines													
Biotin	2	**131**	98	102	**131**	97	102	**20**[f]	**53**[f]	**58**[f]	**66**[f]	**72**[f]	**77**[f]
Cobalamine	3	113	95	**127**	**134**	81	104	80	**54**[f]	**70**[f]	**54**[f]	**44**[f]	**59**[f]
Ethanolamine	2	**168**	109	102	99	98	95	**71**	95	94	101	105	101
Folic acid	3	n.a.[f]	98	91	**78**	107	93	n.a.[f]	101	100	n.a.[f]	114	103
Nicotinic acid	3	**80**	104	87	97	96	**77**[c,f]	88	**37**[c,f]	**27**[c,f]	117	92	**72**[c,f]
Pantothenic acid	2	**165**	93	96	**153**	96	101	**78**[f]	**64**[f]	**68**[f]	**73**[f]	**58**[f]	**62**[f]
Putrescine	2	110	113	106	104	90	84	101	103	95	**74**[f]	**50**[f]	**44**[f]
Pyridoxine	1	83	101	**74**[c,f]	92	92	**66**[c,f]	**57**[f]	**35**[c,f]	**24**[c,f]	**78**[f]	82	**58**[c,f]
Riboflavin	2	**66**	83	96	96	87	93	**126**[f]	**66**[f]	**68**[f]	**121**[f]	**76**[f]	**77**[f]

[a] E 12 is a extract from penicillin synthesis.
[b] E1 is a extract from cephalosporin synthesis.
[c] linearity problems (endogenic concentration in extract extremely high so that 1:100 dilution is still outside linear range)
[d] problems due to limited analyte stability
[e] concentration near the LLOQ
[f] problems due to instrumental fluctations and/or inappropriate internal standard
[g] n.a. not availble
[h] Accuracy values above +/- 20% are in bold.

Figure S 9 : Accuracy values in % along with corresponding frequency analysis of all compounds at a medium concentration level (extract 12 spiked with 0.25 mg/L of group 1; 0.5 mg/L of group 2; 1.0 mg/L of group 3; 2.0 mg/L of group 4) using different calibration approaches:
(A) matrix matched calibration in extract 12 without internal standards
(B) matrix matched calibration in extract 12 with internal standards
(C) calibration with neat standard solutions with internal standards


Accuracy %
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120
110
100
90
80
70
frequency
35
30
25
20
15
10
5
0
2
0
2
7
33
10
1
2
0
0
< 80
85
90
95
100
105
110
115
120
< 120
79 79 86 87 91 91 91 92 93 94 94 95 95 96 96 96 96 96 96 96 97 97 97 97 97 97 97 98 98 98 98 98 99 99 99 100 100 100 100 100 100 100 100 100 101 101 101 101 102 102 103 103 104 109 110 114
Nicotinic acid
Pyridoxine
2-Aminoadipic acid
Threonine
Desacetylchephalosporin
6-Aminopenicilloic acid
Serine
Malonic acid
Lysine
Aspartic acid
Asparagine
Cobalmine
ACV
Riboflavin
Cephalosporin C lactone
Glycine
Phenoxyacetic acid
Pyruvic acid
Methionine
Penicillamine disulfide
Glutamic acid
Phenylalanine
cis-Aconitic acid
Folic acid
Lactic acid
2-Oxoglutaric acid
Phenoxymethylpenillic acid
Jeffrey thiazole
Glutamine
Histidine
Valine
p-Hydroxyphenoyacetic acid
Succinic acid
6-Aminopencillanic acid
Desacetoxychephalosporin
7-Aminocephalosporanic acid
Pantothenic acid
Proline
Phenoxymethylpenicilloic acid
Tyrosine
Glyoxylic acid
Isoleucine
Leucine
Biotin
Alanine
Ethanolamine
Chephalosporin C
Arginine
Glycolic acid
Putrescine
Cystine
8-Hydroxypenillic acid
Citrulline
Tryptophan
Glutaric acid
Fumaric acid
Ornithine

Figure S 9A

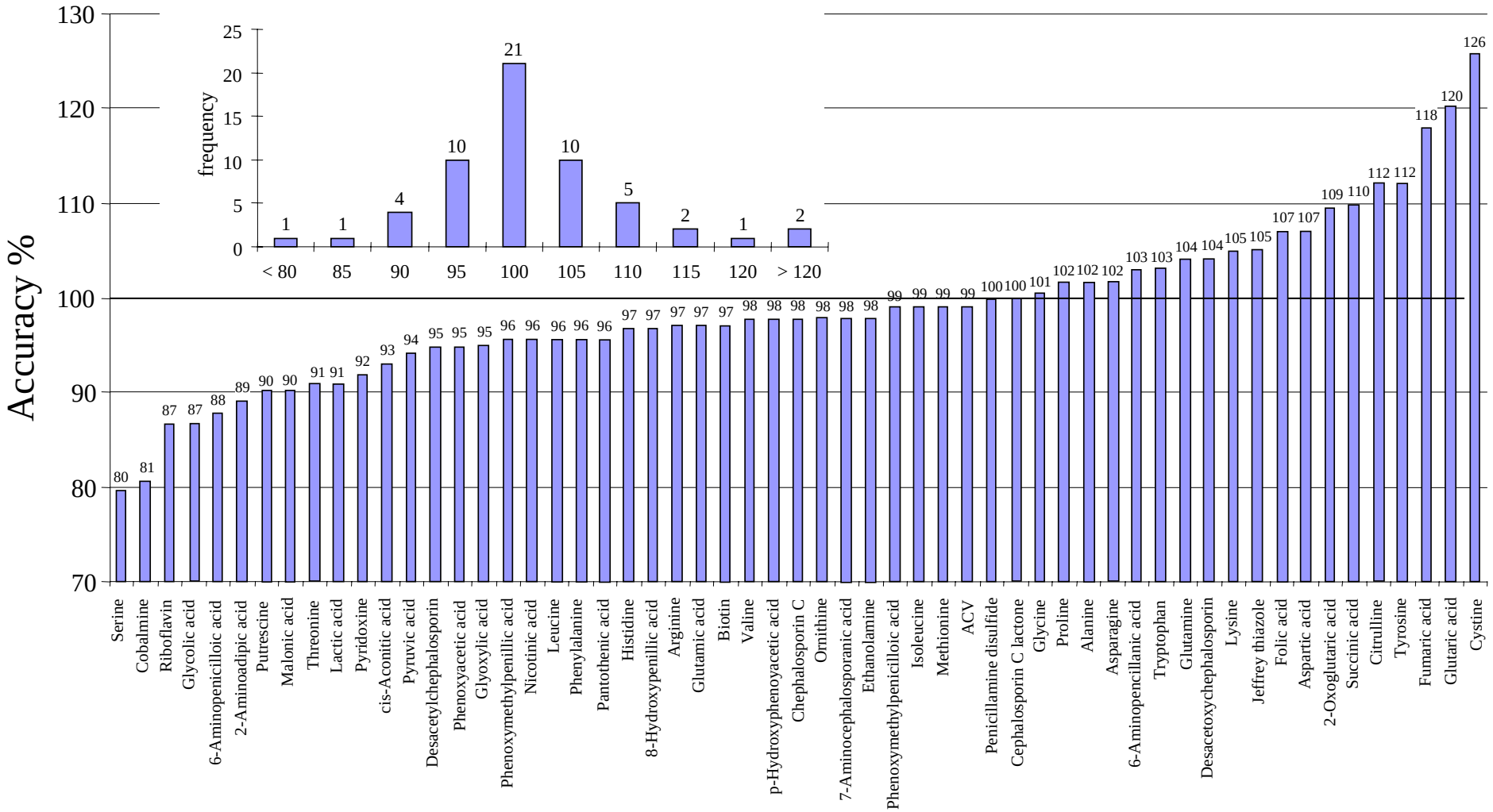
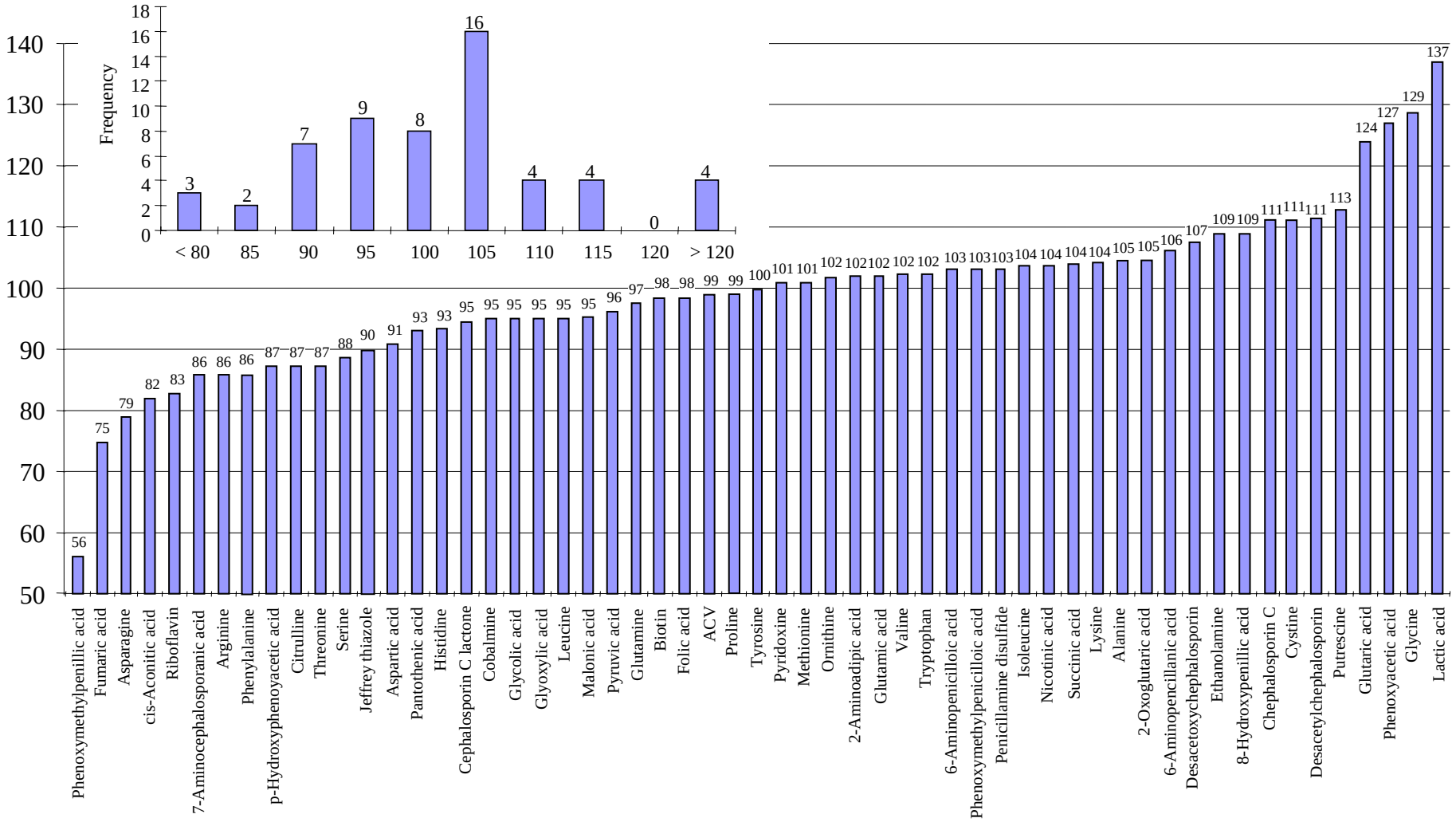

Accuracy %
130
120
110
100
90
80
70
frequency
25
20
15
10
5
0
1
1
4
10
21
10
5
2
1
2
< 80
85
90
95
100
105
110
115
120
> 120
80 81 87 87 88 89 90 90 91 91 92 93 94 95 95 95 96 96 96 96 96 97 97 97 97 97 98 98 98 98 98 98 99 99 99 99 100 100 101 102 102 102 103 103 104 104 105 105 107 107 109 110 112 112 118 120 126
Serine
Cobalmine
Riboflavin
Glycolic acid
6-Aminopenicilloic acid
2-Aminoadipic acid
Putrescine
Malonic acid
Threonine
Lactic acid
Pyridoxine
cis-Aconitic acid
Pyruvic acid
Desacetylchephalosporin
Phenoxyacetic acid
Glyoxylic acid
Phenoxymethylpenillic acid
Nicotinic acid
Leucine
Phenylalanine
Pantothenic acid
Histidine
8-Hydroxypenillic acid
Arginine
Glutamic acid
Biotin
Valine
p-Hydroxyphenoyacetic acid
Chephalosporin C
Ornithine
7-Aminocephalosporanic acid
Ethanolamine
Phenoxymethylpenicilloic acid
Isoleucine
Methionine
ACV
Penicillamine disulfide
Cephalosporin C lactone
Glycine
Proline
Alanine
Asparagine
6-Aminopencillanic acid
Tryptophan
Glutamine
Desacetoxychephalosporin
Lysine
Jeffrey thiazole
Folic acid
Aspartic acid
2-Oxoglutaric acid
Succinic acid
Citrulline
Tyrosine
Fumaric acid
Glutaric acid
Cystine

Figure S 9B


Frequency
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16
14
12
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8
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4
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0
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0
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< 80
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105
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> 120
140
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90
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113
124
127
129
137
Phenoxymethylpenillic acid
Fumaric acid
Asparagine
cis-Aconitic acid
Riboflavin
7-Aminocephalosporanic acid
Arginine
Phenylalanine
p-Hydroxyphenoyacetic acid
Citrulline
Threonine
Serine
Jeffrey thiazole
Aspartic acid
Pantothenic acid
Histidine
Cephalosporin C lactone
Cobalmine
Glycolic acid
Glyoxylic acid
Leucine
Malonic acid
Pyruvic acid
Glutamine
Biotin
Folic acid
ACV
Proline
Tyrosine
Pyridoxine
Methionine
Ornithine
2-Aminoadipic acid
Glutamic acid
Valine
Tryptophan
6-Aminopenicilloic acid
Phenoxymethylpenicilloic acid
Penicillamine disulfide
Isoleucine
Nicotinic acid
Succinic acid
Lysine
Alanine
2-Oxoglutaric acid
6-Aminopencillanic acid
Desacetoxychephalosporin
Ethanolamine
8-Hydroxypenillic acid
Chephalosporin C
Cystine
Desacetylchephalosporin
Putrescine
Glutaric acid
Phenoxyacetic acid
Glycine
Lactic acid

Figure S 9C

Table S 10: Intra-assay precision determined for QC samples prepared by spiking extract 12 at three distinct concentration levels (group 1: 0.025, 0.25, 0.5 mg/L; group 2: 0.05, 0.5, 1.0 mg/L; group 3: 0.1, 1.0, 2.0 mg/L and group 4: 0.25, 2.5, 5.0 mg/L) using calibration in extract 12 without and with use of internal standards.

Calibration standards	without internals standards			with internal standards		
Analyte	**low**	**middle**	**high**	**low**	**middle**	**high**
Amino acids						
Alanine	4	7	2	13	11	1
Arginine	7	6	13	1	8	**18**
Asparagine	**24**[b]	4	3	**19**[b]	9	10
Aspartic acid	5	4	1	2	4	8
Citrulline	8	7	8	9	8	15
Cystine	< LOQ	13	3	< LOQ	7	9
Glutamic acid	2	6	4	3	3	9
Glutamine	6	8	1	15	8	7
Glycine	< LOQ	5	3	< LOQ	12	1
Histidine	3	1	1	4	5	1
Isoleucine	3	3	7	3	0	9
Leucine	5	2	1	6	3	5
Lysine	3	3	7	**23**	1	10
Methionine	9	3	3	4	7	6
Ornithine	5	10	5	**19**	11	15
Phenylalanine	6	2	1	7	9	3
Proline	5	4	3	2	1	4
Serine	9	5	5	11	14	**23**
Threonine	9	0	4	15	4	5
Tryptophan	5	2	1	**19**	5	2
Tyrosine	8	4	4	12	**17**	9
Valine	0	8	5	1	13	7
Organic acids						
2-Oxoglutaric acid	< LOQ	5	8	< LOQ	3	5
cis-Aconitic acid	5	7	1	3	5	2
Fumaric acid	6	6	4	3	11	13
Glutaric acid	**16**	3	3	**18**	6	1
Glycolic acid	< LOQ	9	3	< LOQ	13	5
Glyoxylic acid	9	5	4	**16**[b]	11	3
Lactic acid	2	7	6	11	13	9
Malonic acid	**15**[b]	9	6	**16**[b]	14	5
Pyruvic acid	12	3	2	13	4	2
Succinic acid	14	5	2	**17**	6	5
β-Lactam antibiotics, intermediates of biosynthesis and degradation products						
2-Aminoadipic acid	6	4	8	4	10	6
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	< LOQ	3	4	< LOQ	9	2
6-Aminopenicillanic acid	5	5	4	8	11	3
6-Aminopenicilloic acid	6	1	2	3	8	**20**
7-Aminocephalosporanic acid	3	2	8	3	1	5
8-Hydroxypenillic acid	**21**[b]	3	4	**51**	7	5
Cephalosporin C	2	10	3	4	15	3
Cephalosporin C lactone	8	4	1	**17**	15	6
Desacetoxycephalosporin	2	7	1	12	8	3
Desacetylcephalosporin	5	12	**20**	3	13	**19**
Penicillamine disulfide	9	3	3	9	5	5
Phenoxyacetic acid	2	2	1	6	3	1
Phenoxymethylpenicilloic acid	4	2	1	4	10	2
Phenoxymethylpenillic acid	4	3	8	12	5	11
p-Hydroxyphenoxyacetic acid	4	3	3	1	4	1
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	11	6	5	**20**[b]	9	3
Vitamins and biogenic amines						
Biotin	3	6	5	5	8	2
Cobalamine	**16**	11	3	5	6	6
Ethanolamine	2	2	2	7	6	4
Folic acid	**38**	6	7	**37**[b]	12	5
Nicotinic acid	2	4	2	5	9	6
Pantothenic acid	9	3	1	13	2	3
Putrescine	4	1	1	12	8	6
Pyridoxine	1	1	1	7	7	4
Riboflavin	5	7	5	9	6	6

[a] problems due to limited compound stability
[b] analyte concentration near the LLOQ
[c] n.a. not available
Values above 15% RSD are in bold.

Table S 11: Intraday precision (n=3) of quality control sample (extract 12 spiked with 0.25 mg/L of group 1; 0.5 mg/L of group 2, 1.0 mg/L of group 3 and 2.5 mg/L of group 4) on four different days.[a] Calibration was performed by standard addition to extract 12 on day 1[b].

	Calibration in extract 12 without internal standards					Calibration in extract 12 with internal standards				
measured on	**day 1**	**day 3**	**day 4**	**day 6**	**interday[c]**	**day 1**	**day 3**	**day 4**	**day 6**	**interday[c]**
Amino acids										
Alanine	7	2	6	5	7	11	11	12	7	4
Arginine	6	7	12	7	**51**[d]	8	15	21	2	5
Asparagine	4	10	11	8	7	9	11	4	6	3
Aspartic acid	4	6	6	7	9	4	10	6	3	8
Citrulline	7	7	2	3	**22**	8	11	7	5	8
Cystine	13	14	12	11	14	7	16	13	7	11
Glutamic acid	6	8	8	5	5	3	**27**	**22**	15	16
Glutamine	8	4	6	6	17	8	5	13	5	17
Glycine	5	1	11	9	8	12	10	20	5	10
Histidine	1	1	2	5	**85**[d]	5	0	1	4	9
Isoleucine	3	7	3	2	9	0	15	7	2	3
Leucine	2	4	3	2	13	3	2	5	1	2
Lysine	3	6	11	11	**41**[d]	1	13	**28**	10	8
Methionine	3	11	7	3	13	7	7	7	5	6
Ornithine	10	5	6	12	**39**[d]	11	8	17	18	20
Phenylalanine	2	2	3	4	**29**[d]	9	7	5	6	4
Proline	4	2	5	2	3	1	3	5	3	2
Serine	5	5	7	5	12	14	17	**23**	16	18
Threonine	0	7	6	2	5	4	3	5	5	3
Tryptophan	2	3	6	4	**39**[d]	5	8	10	4	2
Tyrosine	4	9	5	4	7	17	8	14	5	7
Valine	8	7	3	5	4	13	8	6	5	4
Organic acids										
2-Oxoglutaric acid	5	8	10	16	**72**[d]	3	6	4	14	10
cis-Aconitic acid	7	6	12	7	**116**[d]	5	6	12	10	**25**[e]
Fumaric acid	6	3	7	9	**46**[d]	11	7	14	12	**37**[e]
Glutaric acid	3	5	4	3	**55**[d]	6	7	9	8	**21**[e]
Glycolic acid	9	9	13	13	**20**[d]	13	3	12	12	**49**[e]
Glyoxylic acid	5	6	8	1	**42**[d]	11	11	12	6	**26**[e]
Lactic acid	7	1	8	5	11	13	7	11	9	**45**[e]
Malonic acid	9	4	16	13	**81**[d]	14	8	16	9	7
Pyruvic acid	3	0	3	2	9	4	9	6	5	**43**[e]
Succinic acid	5	2	4	5	**83**[d]	6	4	5	9	4
β-Lactam antibiotics, intermediates of biosynthesis and degradation products[e]										
2-Aminoadipic acid	4	8	6	3	8	10	17	19	15	4
2-Amino-5-(4-carboxy-2-thiazolyl)-valeric acid	3	5	6	5	**36**[d]	9	8	12	3	**42**[e]
6-Aminopenicillanic acid	5	6	6	5	**28**[d]	11	14	7	10	7
6-Aminopenicilloic acid	1	6	5	6	**27**[d,f]	8	15	12	14	**20**[e,f]
7-Aminocephalosporanic acid	2	1	2	5	**34**[d]	1	6	4	3	**24**[e]
8-Hydroxypenillic acid	3	13	3	4	13	7	11	2	4	15
Cephalosporin C	10	7	1	1	12	15	6	7	5	9
Cephalosporin C lactone	4	4	2	3	8	15	11	17	3	12
Desacetoxycephalosporin	7	1	4	3	10	8	9	14	9	9
Desacetylcephalosporin	12	7	2	4	10	13	16	8	3	11
Penicillamine disulfide	3	1	3	2	6	5	13	9	7	9
Phenoxyacetic acid	2	1	0	1	6	3	6	3	6	**43**[e]
Phenoxymethylpenicilloic acid	2	2	5	3	**75**[f]	10	10	6	4	**35**[f]
p-Hydroxyphenoxyacetic acid	3	2	1	2	**32**[d]	4	4	3	6	**28**[e]
δ-(L-α-Aminoadipoyl)-L-Cys-D-Val	6	2	9	6	**79**[f]	9	4	2	7	**82**[f]
Vitamins and biogenic amines										
Biotin	6	2	3	6	11	8	10	4	7	18
Cobalamine	11	5	10	8	9	6	3	5	11	**41**[e]
Ethanolamine	2	3	2	1	13	6	3	1	1	3
Folic acid	6	12	15	6	**90**[d,f]	12	15	12	3	9
Nicotinic acid	4	2	3	4	**41**[d,f]	9	10	4	5	14
Pantothenic acid	3	5	3	2	20	2	10	3	5	**57**[e]
Putrescine	1	5	2	2	11	8	12	13	5	**44**[e]
Pyridoxine	1	2	0	2	20	7	11	3	4	18
Riboflavin	7	2	3	3	**63**[d,f]	6	9	2	1	**37**[e,f]

[a] values above 20% in bold

[b] Phenoxymethylpenillic acid was excluded because of its limited stability.

[c] Interday precision was calculated as the %RSD of concentrations determined on the four different days.

[d] problems due to instrumental fluctuations

[e] problems due to instrumental fluctuations and/or inappropriate internal standard

[f] problems due to limited compound stability

Appendix IV

Manuscript

Quantitative LC-ESI-MS/MS metabolic profiling method for fatty acids and lipophilic metabolites in fermentation broths from β-lactam antibiotics production

Quantitative LC-ESI-MS/MS metabolic profiling method for fatty acids and lipophilic metabolites in fermentation broths from β-lactam antibiotics production

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Abstract

In the present paper we report on the development of a straightforward RP-LC-ESI-MS/MS method for the determination of most abundant fatty acids, α-tocopherol and cephalosporin P1 in fermentation broths. Using this method fatty acids could be successfully determined in extracts of fermentation broths from penicillin and cephalosporin production without prior derivatisation. Matrix effects were investigated in detail and various kinds of calibrations (i.e. by use of neat standard solutions with and without internal standards as well as by matrix-matched calibration employing standard addition with and without internal standards) were comparatively assessed. The optimized and validated method was employed for the analysis of extracts of fermentation broths and nutrition media.

Keywords: fatty acids, α-tocopherol, β-lactam antibiotics, penicillin V, HPLC-ESI-MS/MS, metabolomics, metabolic profiling

1. Introduction

Metabolomics is an emerging field in systems biology. It also is gaining increasing popularity in biotechnology as a tool to advance the understanding of metabolic pathways occurring for instance during fermentation processes. In this context, metabolic profiling studies have been implemented, in which arrays of analysis methods measure concentrations of various groups

of metabolites. Amongst them, analysis of free fatty acids is an integral part of any extended metabolic profiling study.

In living cells fatty acids are stored in form of triglycerides and if required, are catabolized to acetylcoenzyme A in the course of β-oxidation in order to serve as energy provider. In form of phospholipids they build up cell membranes. In industrial fermentation processes oils are frequently used as ingredients of nutrition media to partly replace the more expensive glucose and by this way fatty acids are introduced as energy source.

Gas chromatography (GC) is the most widely used separation technique for the analysis of fatty acids because of its high specificity, sensitivity and good reproducibility. Separation of fatty acids by GC requires prior derivatisation to increase compound volatility and thermal stability. Most often, this is accomplished by esterification resulting in methyl [1], or trimethylsilyl and pentafluorobenzyl esters [2,3]. Commonly employed detectors are flame ionization detectors (FID) and in metabolomics related studies primarily mass spectrometers. Thus, GC-MS has become the method of first choice for metabolic studies of free fatty acids.

Relatively rarely HPLC-MS/MS is employed in metabolomics for the analysis of free fatty acids, although it circumvents derivatisation steps. Nevertheless, a number of applications has been reported in other context, where analysis of free fatty acids was successfully achieved by LC-MS [2] [4-9].

Lack of sensitivity for cis/trans isomers of fatty acids has been alleviated by silver ion chromatography, which allows separation of cis and trans isomers of unsaturated fatty acids, but most often it was employed with the intention to fractionate complex mixtures, which were then further analyzed employing GC-MS [10,11].

One of the major problems of LC-MS/MS of fatty acids is their non-ideal fragmentation behavior. Under low energy collision induced dissociation (CID) conditions fragmentation hardly occurs. Most prominent losses originate from elimination of water (Δm -18) from the carboxylic group as well as loss of CO_2 (Δm -44). Fatty acids exhibiting double bonds show to some extent structure specific fragmentation but the intensities are rather weak for the purpose of MRM (multiple reaction monitoring) measurements furnishing poor sensitivity. Recently Zehethofer et al. [12] determined fatty acids in plasma using postcolumn infusion of a barium ion solution, thereby enhancing detection sensitivity of fatty acids by the formation of positive charged adduct ions and at the same time promoting fragmentation reactions. Other cationizing agents including alkaline and alkaline earth metals or copper ions also proved to be suitable to improve detection sensitivity of fatty acids in the MRM mode [13,14]. Another strategy to improve ionization efficiency of fatty acids constitutes stable

isotope coding. Thereby, an easily ionizable group is introduced by derivatisation [15], which unfortunately necessitates additional sample preparation steps. Even higher signal intensities could be obtained by the incorporation of immanently positively charged groups like quaternary amines e.g. trimethylaminoethyl ester moiety [16-18].

Along with fatty acids, fat-soluble vitamins such as in particular α-tocopherol (vitamin E) and its analogues (β-, γ-, δ-) might be simultaneously analyzed by HPLC-MS/MS as they are of similar physico-chemical characteristics and of relevance in metabolomics studies of fermentation processes as well.

They are typically separated on RP stationary phases like C18 [19-21] but also on C 30 stationary phases under strong eluting conditions using acetone [22].

Again MS detection may be problematic. Regarding MS detection of α-tocopherol it was reported that its complexation with silver ions, achieved by postcolumn infusion of $AgClO_4$ solution, affected fragmentation, as additional fragments appeared [22].

The goal of the present work was to develop a LC-ESI-MS/MS analysis method allowing simultaneous monitoring of various lipophilic metabolites and nutritional compounds in fermentation broths from production of β-lactam antibiotics. This method is supposed to complement our LC-MS based metabolic profiling platform which comprises a small array of HILIC-MS/MS and RPLC-MS/MS methods for the quantitative analysis of extracellular metabolites (including amino acids, organic acids, water-soluble vitamins, secondary metabolites i.e. β-lactams) [23] as well as intracellular metabolites (such as nuclobases, nucleosides, nucleotides and other phosphorylated compounds, sugars and sugar acids) [24].

This paper reports on the development and validation of a method for the determination of apolar compounds including fatty acids, cephalosporin P1 and α-tocopherol. A straightforward RP-LC-ESI-MS/MS method was developed, affording analysis of most relevant fatty acids without derivatization or postcolumn addition of complexing agents. "Pseudo molecular" MRM transitions of most analytes were measured to alleviate the problem of limited sensitivity. Furthermore, much emphasis was put on the evaluation of calibration efforts and strategies to deal with matrix effects in the complex sample matrices. Different calibration approaches were comparatively examined and evaluated with regard to their possible routine application.

An extension of the method which additionally allows determination of less hydrophilic β-lactam derivatives (Penicillin V and degradation products) is suggested.

2. Experimental Section

2.1. Chemicals and Reagents

Standards of myristic acid 99.0%, tridecanoic acid 98.0%, pentadecanoic acid 99.0% and heneicosanoic acid 99.0% were supplied from Sigma-Aldrich (Vienna, Austria). Arachidonic acid 98.5%, nonanoic acid 99.5%, heptadecanoic acid 99.0%, linolenic acid 98.5%, linoleic acid 99.0%, (+)-α tocopherol 99.0% were from Fluka (Sigma-Aldrich). Uniformly ^{13}C labeled palmitic acid (99 atom % ^{13}C) and stearic acid (99 atom % ^{13}C) were purchased from Sigma-Aldrich. Lauric acid 99.6%, palmitic acid 99.3%, stearic acid 99.9%, oleic acid 99.4%, cephalosporin P1 93.8 %, p-hydroxypenicillin 94.1% (potassium salt), penicillin V 99.4% (potassium salt), phenoxymethylpenillic acid 91.0%, phenoxymethylpenilloic acid 99.7%, phenoxymethylpenicilloic acid 92.0% were provided by Sandoz Austria (Kundl, Austria). For LC-MS/MS analysis Chromasolv Plus ultra pure water from Sigma Aldrich, HPLC grade acetonitrile (ACN) from VWR (Leuven, Belgium), HPLC grade isopropanol (IPA) and ethylacetate (EtOAc) from Roth (Karlsruhe, Germany) were used. Acetic acid 99.8% and ammonium hydroxide solution (NH_4OH) 25.0% in water were obtained from Fluka (Sigma-Aldrich).

Methanolic extracts of fermentation broths stemming from the production of β-lactam antibiotics as well as various nutrition media were provided by Sandoz Austria.

2.2. LC-MS/MS instrumentation

Experiments were performed on an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany) coupled to a Q-Trap 4000 (Applied Biosystems/MDS Sciex, ON, Canada). The HPLC system was equipped with a thermostatted autosampler, which allowed cooling of the samples to 5°C, a binary pump and a column thermostat. A turboionspray (TIS) source was used as ESI-interface. Data were processed using the Analyst 1.4.1. software.

2.3. Optimization of MS – parameters

Detection was carried out in the MRM mode (multiple reaction monitoring) with positive and negative polarity depending on the solute (see Table 1).

Compound-dependent fragmentation parameters were optimized using the quantitative optimization tool of the Analyst software. For this purpose standard solutions were prepared with concentrations ranging from 0.5-5.0 mg/L in a mixture of ACN and buffer (20 mM acetic acid adjusted to pH 5.0 with ammonium hydroxide solution) (50:50; v/v) and then introduced into the MS by continuous infusion using a syringe pump. The flow rate was set to 30 µl/min. The resultant optimized MS parameters for the target solutes are summarized in Table 1. Further, MS parameters were optimized by performing LC-MS/MS runs applying different TIS temperatures (550°C, 600°C and 650°C) and TIS voltages (4300 V and 4000 V).

2.4. Optimization of chromatographic conditions

A mixed standard solution of eight fatty acids (Table 1) was analyzed using two stationary phases, namely Synergi Fusion-RP 80 (150 x 3.0 mm; 4 µm particles) from Phenomenex (Aschaffenburg, Germany) and X-Bridge C18 (150 x 3.0 mm; 3.5 µm particles) from Waters (Vienna, Austria) using a mobile phase pH of 5.0. Additionally the X-Bridge C18 stationary phase was also tested at a buffer pH of 9.5. Both columns were equipped with dedicated precolumns (Synergi Fusion-RP 5 µm, 4.0 x 3.0 mm and X-Bridge^{TM} C18 3.5 µm, 10 x 2.1 mm, respectively).
Mobile phase conditions were as follows: eluent (A) containing 10% (v/v) buffer in H_2O and eluent (B) 10% (v/v) buffer in ACN, whereupon the buffer consisted of 50 mM acetic acid, pH adjusted with ammonium hydroxide solution to 5.0 and 9.5, respectively. Solutes were eluted by linear mobile phase gradients either starting from 50 or 70% (B) and increasing to 100% (B) in 20 minutes at a flow rate of 0.3 ml/min. Finally, mobile phase conditions were changed within one minute to gradient starting conditions and the stationary phase was allowed to reequilibrate for 13 minutes.

2.5. Final LC-MS/MS method

The set of analytes comprised besides the eight mentioned fatty acids also α-tocopherol and cephalosporin P1 (see Table 1). Finally, optimized mobile phase conditions were as follows: eluent (A) contained 5% (v/v) buffer in water and eluent (B) 5% (v/v) buffer in 55% (v/v) ACN and 40% (v/v) IPA. The employed buffer consisted of 100 mM acetic acid adjusted to pH 5.0 with ammonium hydroxide solution.

The corresponding optimized gradient profile is outlined in Table 2. The LC run was divided into three time periods corresponding to retention time windows of the analytes. Only MRM transitions of compounds that eluted within the respective time windows were measured (Table 1). Ion source parameters were adjusted as following: turboionspray voltage ± 4000 V, temperature 550°C, curtain gas 10 psi, turbogas 50 psi, nebulizer gas 50 psi, cell entrance potential ± 10 V. CAD (collision gas pressure) was set to high. DP and CE were adjusted according to the results of the fragmentation optimization experiments (see Table 1) and dwell time was set to 100 ms for each MRM transition.

The injector needle was washed after each sample injection by dipping into a vial containing EtOAc.

2.6 Preparation of solutions

0.8 to 1.0 mg of each compound were weighed into Eppendorf vials and diluted with EtOAc/IPA (50:50, v/v) to yield a concentration of 1.0 mg/ml. These individual standard solutions were used to prepare mixed stock solutions of spiking standards intended for standard addition (0.50; 0.75; 1.0; 2.5; 5.0; 7.5; 10.0; 20.0 mg/L) and internal standards (1.0 mg/L and 5.0 mg/L, respectively). Cold methanolic sample extracts of fermentation broths and nutrition media were diluted 1:10 with the standard diluent water/ACN (80:20; v/v).

Each calibration sample for analysis was prepared by mixing of 100 µl 1:10 diluted sample (of extracts or nutrition media), 100 µl spiking solution and 100 µl internal standard solution with 700 µl diluent.

15 methanol extracts stemming from two different fermentation lots, one from penicillin synthesis the other one from cephalosporin synthesis, sampled at different time intervals of the fermentation process and eight different nutrition media were analyzed. Sample preparation included mixing of 100 µl internal standard and 100 µl of 1:10 diluted extracts which after addition of 800 µl diluent (water:ACN, 80:20, v/v) yielded a final dilution factor of 1:100.

2.7. Validation of the final LC-MS/MS method

2.7.1. Calibration

Four different approaches for calibration were evaluated: (A) calibration with neat standard solutions (matrix-free solutions of standard compounds in water:ACN, 80:20; v/v) with and without use of internal standards for peak area normalization and (B) matrix-matched calibration using standard addition, also performed with and without use of internal standards.
Fatty acids with uneven carbon number (nonanoic acid, tridecanoic acid, pentadecanoic acid, heptadecanoic acid and heneicosanoic acid) as well as uniformly ^{13}C-labeled palmitic acid and stearic acid were employed as internal standards.
Neat standard solutions of seven different concentrations (0.05; 0.075; 0.1; 0.5; 0.75; 1.0; 2.0; 4.0 mg/L) in water:ACN (80:20; v/v) were employed for generation of calibration functions.
For matrix-matched calibration using standard addition, distinct amounts of standards were spiked to three extracts (extract 11, 16 and 4), stemming from two different fermentation lots (extract 11 and 16 from penicillin production, extract 4 from cephalosporin production), and to two nutrition media (medium 10 and medium 16). The calibration set covered eight different concentration levels. The concentrations of the corresponding spiking standard solutions were 0; 0.5; 0.75; 1.0; 5.0; 7.5; 10.0 and 20.0 mg/L. 100 µl of spiking solution were added to the 1:10 diluted extracts and nutrition media.
Calibration functions were constructed by 1) plotting peak area vs concentration and 2) by plotting the ratio of analyte peak and internal standard peak area (normalized area) vs concentration. Linear regression was performed using the Analyst software. $1/x^2$ was introduced as weighting factor if accuracy of calibration functions in the low concentration range could be improved by that. The sum of relative errors served as goodness-of-fit parameter.
Calibration functions resulting from standard additions were corrected for the endogenic analyte concentrations in the sample.

2.7.2. Precision, Accuracy and LOQ

Method precisions and accuracies were determined by measuring quality control samples obtained by spiking a sample extract at three concentration levels (Extract 11 spiked with concentrations of 0.075; 0.5; 1.0 mg/L) in triplicate. Accuracies were calculated utilizing calibration functions constructed from different calibration procedures with and without internal standards.
To elucidate interday precisions and accuracies calibrations were performed on three different days. Interday precisions and accuracies were determined for calibration via standard addition

using extract 11. For that purpose one quality control sample (extract 11 spiked with a concentration of 0.5 mg/L) was stored at 5°C and analyzed at three different days in triplicate using freshly constructed calibration functions.

LOQ (limit of quantitation) was determined using neat standard solutions and was considered as the concentration for which the ratio of signal/noise was greater than 10. For linoleic acid, oleic acid, palmitic acid and stearic acid LOQ was calculated from the standard deviation of the memory peak areas. Thus, standard deviations of peak areas of the respective compounds in blank runs were determined (n = 3). LOQ was then calculated for these compounds according to equation (1):

$$\text{LOQ} = \frac{10^* \text{ standard deviation of peak area + mean of peak area}}{\text{slope of calibration function in neat standard solutions}} \quad (1)$$

3. Results and Discussion

3.1. Optimization of MS/MS parameters

Optimization of the compound specific fragmentation parameters was performed for each individual analyte using the quantitative optimization tool of the Analyst software. While cephalosporin P1 and arachidonic acid revealed characteristic fragmentations, other fatty acids and α-tocopherol were more or less resistant to strong specific fragmentation under the relatively soft ESI conditions. In fact, especially saturated fatty acids do not readily fragment. Thus, fragmentation completely failed for oleic acid and stearic acid, for which no detectable MRM transitions could be obtained. For this reason, "pseudo-MRM" transitions were optimized, where the pseudomolecular ion was selected as precursor ion in Q1 and as product ion in Q3 as well, while no fragmentation occurred in Q2. This allowed to detect these analytes with sufficient sensitivity as compared to regular MRM transitions with more specific fragments as product ions.

Results of optimization of the fragmentation of α-tocopherol showed an interesting abnormality. α-Tocopherol was detected in the positive mode. Typically positive ionization in the ESI process occurs by the formation of proton adducts. Thereby the molecular mass is increased by one mass unit. In case of α-tocopherol, which exhibits a molecular mass of 430.7 g/mol, higher signal intensities were obtained for m/z (mass/charge ratio) 430.5 (molecular ion) than for 431.5. This may indicate the formation of a radical cation. As a result transition 430.5 → 165, which may be employed as an alternative to the "pseudo-MRM" transition,

yielded a more intense signal than the transition 431.5 → 165. This phenomenon was also observed by other groups [21,25] which reported to find a constant ratio of m/z 430 to 431 using an APCI ion source. The exact mechanism of positive ionization of α-tocopherol is not completely clear, but it is assumed that a radical cation is generated.
Overall, reasonable intensities could be obtained with the selected optimized MS parameters which are summarized in Table 1.

3.2. Optimization of chromatographic conditions

Fatty acids completely differ in their physico-chemical characteristics from other target analytes of the present extended metabolic profiling study, which mostly comprised rather small hydrophilic compounds like amino acids, organic acids, β-lactams or vitamins [23]. Because of the wide range of polarities it was not possible to find chromatographic conditions that were suitable for all compounds of interest. For this reason analytes were separated into two groups: 1) metabolites and other polar compounds that can be retained on polar stationary phases in the HILIC mode, which are dealt with elsewhere [23], 2) fatty acids and other hydrophobic compounds, which can be separated by reversed-phase chromatography.
Concerning analysis of fatty acids it was supposed that higher pH values of the mobile phase would increase deprotonation of fatty acids and thus would have a positive effect on ESI ionization efficiency [26]. For this reason a buffer pH of 9.5 was tested initially using X-Bridge C18, which is claimed to be stable in a pH range of 1-12. Usually silica based stationary phases are prone to undergo destruction at higher pH values, as siloxane groups are readily hydrolyzed under these conditions. X-Bridge C18 of Waters is a hybrid organic-inorganic material that incorporates ethylene bridges into the siloxane matrix which provide more stability. Moreover mobile phase conditions with a buffer pH of 5.0 were also tested on X-Bridge C18 as well as on Synergi Fusion-RP 80.
In Figure 1 total ion chromatograms (TIC) of standard solutions of fatty acids obtained on X-Bridge at a pH 9.5 and at a pH 5.0 are shown. As expected at pH 5.0 retention was markedly increased for all compounds in comparison to pH 9.5. Saturated fatty acids were stronger affected than unsaturated ones, causing a change in the elution order. A possible explanation is that unsaturated fatty acids exhibit lower pKa values than saturated ones, when adsorbed to a surface [27]. At a pH of 5.0 the degree of deprotonation would be higher for unsaturated than for saturated fatty acids, causing shorter retention times of unsaturated fatty acids. Baseline separation of all fatty acids could be achieved at pH 9.5, whereas at pH 5.0 palmitic

acid and oleic acid were not fully separated anymore and stearic acid was not eluted within 30 minutes.

Since X-Bridge C18 provided better peak efficiencies and selectivities for fatty acids than Synergi-Fusion, this stationary phase was chosen for further method optimization.

Cephalosporin P1 and α-tocopherol being also very apolar hydrophobic compounds, could be analyzed under RP conditions along with fatty acids. Cephalosporin P1 can be easily integrated in the aforementioned RP methods. However, α-tocopherol exhibited very strong interactions with the stationary phase. To decrease retention time elution strength of the mobile phase had to be increased, which was achieved by partly substituting ACN by IPA in the organic phase. The effect of higher elution strength with a mixture of ACN/IPA/buffer (50/40/10, v/v/v) in channel B is confirmed by the chromatograms of lauric acid, linolenic acid and linoleic acid presented in Figure 2. Finally, we ended up with a reasonably fast LC method using X-Bridge and a mobile phase buffer pH of 5.0, as α-tocopherol could not be analyzed using a buffer at pH 9.5. A lower pH (< 7.0) was also favorable in view of the possibility to include some β-lactam derivatives which are less stable at high pH values. Under such optimized conditions compounds differing in their m/z by only two mass units such as oleic acid (m/z 281) and stearic acid (m/z 283) were readily separated, which was of importance in order to avoid interferences.

The chromatograms of selected fatty acids at equal concentrations (1 mg/L) presented in Figure 2, reveal that higher signal intensities were obtained at a pH of 5.0 and that signal intensities were further improved by addition of IPA revealing a positive effect on ionization efficiency of fatty acids. This stands in contrast to the assumption, which was stated before, that higher mobile phase pH would provide better ionization efficiency. Mobile phase conditions specified in Figure 2B have been adopted as optimized conditions and the corresponding chromatogram of all analytes is depicted in Figure 3.

3.3. Memory effects

In blank runs (injection of pure ACN) peaks for nearly all fatty acids could be found at their corresponding retention times. Even after excessive washing of column and injection needle as well as running of several blank runs the peaks did not disappear. So called memory effects could be found for nearly all fatty acids, except for arachidonic acid. For lauric acid, linolenic acid and myristic acid the effect was negligible and mostly much lower than the LOQ. For linoleic acid, oleic acid, palmitic acid and stearic acid the effect was more pronounced and

required to elevate the LOQs. Several experiments were carried out including flushing with 100% organic phase and injection of several 100 µl plaques of EtOAc to eliminate such memory effects without ground breaking success. These memory effects are supposed to have a noteworthy influence on furnished validation results especially for palmitic acid, oleic acid and stearic acid at low concentrations. Precision and accuracy of these compounds were always worse compared to the results obtained for the other compounds and LOQ was thus much higher. LOQ of linoleic acid, oleic acid, palmitic acid and stearic acid were therefore differently determined as given by equation 1. Above these obtained LOQs the method showed to be suitable despite this memory effect.

3.4. Validation of final LC-MS/MS method

3.4.1. Calibration, linearity and LOQ

Calibration was performed applying four different calibration protocols: calibration using neat standard solutions with and without internal standards and matrix-matched calibration via standard addition in extracts, again, with and without internal standards. Structural analogs of fatty acids with an uneven number of carbon atoms were employed as internal standards, as they were not expected to be present in the sample extracts of the fermentation broths. Moreover, they exhibit similar structures and retention times and thus should also respond similarly to disturbances caused by the matrix or instrumental fluctuations. Additionally, ^{13}C labeled stearic and palmitic acid were tested as internal standards.

Linearity was evaluated by establishment of calibration functions obtained by analysis of neat standard solutions with and without peak area normalization and ranged over two orders of magnitude for linoleic, oleic, palmitic, stearic acid and tocopherol and over three orders of magnitude for arachidonic acid, chepalosporin P1, lauric, linolenic and myristic acid. The corresponding calibration curves exhibited correlation coefficients $R^2 > 0.98$. Weighted linear regression was performed using $1/x^2$ as weighting factor, in case where accuracy could be improved this way.

Calibrants for matrix-matched calibration by standard addition were prepared by spiking distinct amounts of standard to three different fermentation extracts (extracts 11, 16 and 4) and two nutrition media (medium 10 and medium 16).

The correlation coefficient R^2 obtained with matrix matched calibration was also > 0.98 for the majority of analytes. For oleic acid, palmitic acid and stearic acid R^2 was somewhat lower

especially using extract 4. This can be explained by high endogenic concentration levels of these analytes in the extract, which caused deviations in the low concentration range.
Detailed data on calibration functions established in the different matrices (extracts and media) can be found in the supporting information.
LOQ was defined as the concentration yielding a signal to noise ratio of 10:1 and was determined by dilution of neat standard solutions, except for linoleic, oleic, palmitic and stearic acid. Since these compounds exhibited memory effects, a different strategy for the evaluation of LOQ was selected (see equation 1). As can be seen in Table 3, LOQs ranged between 0.005 and 0.05 mg/L for compounds without memory effects and between 0.05 and 0.137 mg/L for compounds exhibiting a memory effect.

3.4.2. Matrix effects

Matrix effects are one of the main reasons for the failure of quantitative HPLC-MS/MS bioassays [28]. Constituents of the matrix may coelute with analytes of interest and may cause signal enhancement or more frequently suppression through modulation of ionization efficiencies in the ESI process. Absolute matrix effects refer to the situation that there are significant differences in signal intensities obtained in neat standard solutions and spiked blank samples. Moreover, the composition of various lots of biological samples may show strong variation. Thus, the type and extent of matrix effect in different lots of samples may not be uniform, which is referred to as relative matrix effects. Since quantitative results may be severely biased by matrix effects, it is necessary to investigate the possible influence of the matrix, especially when complex samples are analyzed without sample pretreatment like in the present metabolic profiling application.
There are several approaches to investigate matrix effects such as post-extraction addition and postcolumn infusion experiments. Another one involves comparison of slopes of calibration functions obtained from calibration with neat standards with the slopes from matrix-matched calibration obtained by standard addition [29]. In Table 4 results of the evaluation of absolute and relative matrix effects by comparison of slopes of calibration functions, which were obtained without use of internal standards, is presented. The acceptance criterion for absolute matrix effects was established at 80-120% relative to slopes obtained in neat solutions. Except for palmitic and stearic acid no strong absolute matrix effects were found in the investigated sample matrices. Furthermore, no relative matrix effects (calculated as the relative standard

deviation of the mean in 6 different matrices) were found as %RSD values of slopes obtained in different matrices were consistently lower than the established acceptance criterion of 20%.
In Figure 4 slopes of calibration functions obtained in various matrices without use of internal standards are plotted against each other. Significant deviations of data points from the 45° parity line (corresponding to equal slopes and thus equal sensitivities in neat standard solutions and in matrix) would indicate the presence of matrix effects. As illustrated in Figure 4 slopes of most fatty acids lie within the acceptance interval of 80-120% relative to the slopes obtained in neat standard solutions, indicating absence of significant matrix effects, except for palmitic acid and stearic acid. The disagreement for the latter analytes could be interpreted as an absolute matrix effect. Use of internal standards did not provide improvement concerning absolute matrix effects.

3.4.3. Precision and accuracy

Intra-assay precision was determined by repetitively (n = 3) analyzing spiked standards of one extract (extract 11) at three concentration levels (0.075, 0.5, 1.0 mg/L). The % relative standard deviation (RSD) of resultant concentrations calculated by corresponding calibration functions was always lower than 7% for analysis with internal standards (Table 3) and lower than 5% for analysis without internal standards.
Interday precision was determined for one spiking level (0.5 mg/L) using matrix-matched calibration in extract 11 with and without internal standards. Without use of internals standards interday precision ranged within 20% and 30% for all analytes. Interday precision was improved when internal standards were employed and ranged between 3% and 16% RSD for all analytes, except for stearic acid for which it was 22% when heptadecanoic acid and 31% when the ^{13}C-labeled internal standard was utilized for normalization (compare Table 3). On each day, new calibration curves were established using freshly prepared solutions.
Accuracies that were furnished by the different calibration protocols were determined with quality control samples prepared by spiking of extract 11 and are given as % recovery.
Detailed data of accuracies for these QC samples calculated with corrected matrix-matched calibration functions from extract 11 and other extracts/media are given in the supporting information.
From the data in the supporting information it can be deduced that matrix-matched calibration was more accurate than calibration with neat standards. It is not surprising that the best results for accuracy were obtained with corrected matrix-matched calibration in extract 11. Table 3

shows intraday accuracy of QC samples based on extract 11 spiked at three concentration levels and calculated with corrected matrix-matched calibration functions obtained by standard addition to the same extract 11. It is evident that the majority of accuracies were within 93 and 116% except for stearic acid for which accuracy at the highest level was 71%. Utilization of internal standards did not generally improve intra-assay accuracies, yet that for stearic acid could be increased to about 80%.

This is quite acceptable and in agreement with common acceptance limits as proposed by bioanalytical method validation guidelines [30].

Calibration of stearic acid and palmitic acid was performed using two different types of internal standards, namely a structurally related compound heptadecanoic acid and uniformly ^{13}C labeled standards. No significant differences concerning accuracy could be determined for the two different internal standards which were more or less equivalent in terms of correcting for inaccuracies (compare Table 3).

Figure 5 illustrates that the bias of accuracies (averaged for three concentration levels) employing different calibration approaches (with and without internal standards, calibration in different matrices) were for all compounds, except for stearic acid, lower than the acceptance limit of 20% with and without use of internal standards.

Interday accuracies were determined for a QC sample prepared from extract 11 (intermediate level only) and for calculations a corrected matrix-matched calibration function in the same extract 11 was utilized. Detailed results as obtained with and without internal standards are given in the supporting information. Briefly, without internal standards accuracies scattered between 71% and 127% and could be significantly improved when internal standards were employed for data analysis, as expected. Table 3 depicts the values afforded with internal standards which ranged between 88 and 111%, which was considered to be quite tolerable. Instrumental fluctuations may be compensated for by use of internal standards.

Interday precision and accuracy values for cephalosporin P1 and α-tocopherol were not considered because they were severely biased by their limited compound stabilities.

3.4.4. Discussion of method validation results

Calibrations were performed over a period of four days using neat standard solutions and matrix-matched calibration by standard addition in five different matrices.

Within one sequence slopes of calibration functions (in neat solution, in extract 11, 4, 16 and in media 10, 16) showed quite good agreement for the different calibration approaches.

Without use of internal standards %RSD of slopes in the above mentioned distinct matrices was < 9% for cephalosporin P1, lauric acid, linolenic acid, myrisitc acid and arachidonic acid. For linoleic acid, oleic acid, palmitic acid and stearic acid significantly higher %RSD values were found (12.2 – 21.1%).

Using internal standards %RSD values of slopes of the calibration functions were even lower and were < 7% for lauric acid, linolenic acid, linoleic acid, myrisitc acid and arachidonic acid. Concerning oleic acid, palmitic acid and stearic acid the slope for calibration with neat standard solutions using internal standards was increased by a factor of 2, which may be attributed to an absolute matrix effect.

Comparing the slopes of calibration functions generated over a period of 14 days without use of internal standards a significant decrease in steepness of slopes was recognized, whereas the slopes of calibration functions generated using internal standards agreed quite well (data not shown). An explanation may be a loss of detection sensitivity, which may have been caused by accumulation of impurities in the ESI sprayer or by contamination stemming from other sources. Internal standards can correct for such changes in detector sensitivity and can thus enable prolonged calibration intervals.

Concerning the situation within individual assays for oleic, palmitic and stearic acid, calibration without internal standards often provided better results for accuracy and better agreement of slopes of calibration functions generated by spiking different matrices. Nevertheless, for routine use it is recommended to utilize some kind of internal standards and to regularly analyze quality standards between series of measurements, which should indicate changes in instrument sensitivity.

Validation results suggest that the structurally similar fatty acids with uneven carbon number are appropriate to be used as internal standards for fatty acids. Surprisingly it turned out that these structurally similar internal standards sometimes corrected even better for changes in experimental conditions for palmitic acid and stearic acid than expensive ^{13}C-labeled internal standards.

Overall, it is stressed that matrix-matched calibration with averaged equations (from different lots of matrix) corrected for endogenic analyte concentrations is recommended for routine use and the work load to do so is only slightly higher than with neat standard solutions.

3.5. Application of the developed method

Samples originating from two different fermentation batches of penicillin as well as cephalosporin synthesis were taken and methanolic extracts of extracellular metabolites were prepared from these fermentation broths. Afterwards these sample extracts were analyzed employing the presented method together with further samples stemming from different nutrition media. Linoleic, linolenic, oleic, palmitic and stearic acid and cephalosporin P1 were successfully detected and quantified in extract 4 and 11. The results are summarized in Table 5 and confirm the applicability of the developed assay for fatty acids.

3.6. Extension of the method

We recently proposed a HILIC-MS/MS assay for hydrophilic metabolites including besides amino acids, organic acids, a variety of β-lactam derivatives, vitamins and biogenic amines. Amongst them a few β-lactams performed suboptimal under such conditions and were thus attempted to be included in the present RPLC-MS/MS assay.

The set of compounds comprised penicillin V and its degradation products para-hydroxypenicillin, phenoxymethylpenillic acid, phenoxymethylpenilloic acid and phenoxymethylpenicilloic acid. Penicillin V and its degradation products exhibit rather similar structures, which can easily lead to problems with detection interferences, if not sufficiently separated. Thus, chromatographic separation of these compounds is necessary.

As the few analytes are by far less hydrophobic than the fatty acids, the gradient profile of the method had to be adjusted starting the gradient with lower elution strength. The chromatographic conditions and the applied gradient program are described in detail in Table 6. The adapted method starts with a highly aqueous content (only 5% B) and exhibits two linear increases in the gradient: the first for eluting less hydrophobic β-lactam analytes and the second one to elute fatty acids and α-tocopherol. A chromatogram of the proposed set of analytes is shown in Figure 6.

4. Conclusions

A RPLC-ESI-MS/MS method was developed for the quantitative analysis of free underivatised fatty acids and some other apolar compounds (α-tocopherol, cephalosporin P1)

in extracts of fermentation broths and nutritional media. Although no sample clean up or other sample treatment was performed no significant matrix effects were detected except for stearic acid.

Comprehensive investigation of various approaches for calibration including calibration using matrix-free standard solutions and calibration via standard addition in five different matrices, revealed, that matrix-matched calibration combined with the use of internal standards performs best with regard to routine applications, as longer intervals for calibration can be tolerated. The use of structurally similar fatty acids with an uneven number of carbon atoms as internal standards for fatty acids proved to be a good alternative to expensive ^{13}C or other isotopic labeled standards.

Memory effects that appeared for most fatty acids could not be entirely eliminated. These memory effects seemed to be constant and for the concentration range of interest no intolerable negative influences on accuracy and precision were observed.

The method was originally intended for the analysis of fatty acids. Extension of the analyte set to other relatively apolar, hydrophobic metabolites appeared to be possible. Penicillin V and some of its degradation products could be separated and integrated in the existing analysis method, by simply changing the time program of the gradient. The present RPLC-ESI-MS/MS method represents a useful complement to our LC-MS based metabolic profiling platform that consists of a small set of HILIC and RPLC ESI-MS/MS methods.

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Figure Legends

Figure 1: Total ion chromatograms (TIC) of fatty acids on X-Bridge C18 obtained with eluents at pH 9.5 (A) and pH 5.0 (B).
1 Lauric acid, 2 Myristic acid 3 Linolenic acid 4 Linoleic acid 5 Arachidonic acid 6 Palmitic acid 7 Oleic acid 8 Stearic acid
Mobile phase conditions: (A) 10% (v/v) buffer in water; (B) 10% (v/v) buffer in ACN. Gradient elution from 50% (B) to 100% (B) in 20 minutes, followed by reequilibration with starting conditions for 13 minutes
(A) buffer: 50 mM acetic acid adjusted with ammonium solution to pH 9.5 and (B) buffer: 50 mM acetic acid adjusted with ammonium solution to pH 5.0.

Figure 2: Overlaid extracted ion chromatograms (XIC) of (1) lauric acid, (2) linolenic acid, (3) linoleic acid in standard mixtures with a defined concentration of 1 mg/L employing different mobile phase conditions. The first eluting peak (A) corresponds to a buffer pH 9.5, the second one (B) to a buffer pH 5.0 containing 40% (v/v) IPA in the organic phase and the third (C) to a buffer pH 5.0 containing solely ACN as organic modifier.

Figure 3: Chromatogram obtained employing the optimized RPLC-MS/MS method: Overlaid MRM traces normalized to 100% of fatty acids (investigated analytes as well as internal standards), cephalosporin P1 and α-tocopherol.
Experimental conditions: Column: X-Bridge C18 from Waters (150 x 3.0 mm ID); eluent: channel A, 5 % (v/v) buffer in water; channel B, 5 % (v/v) buffer in 55% (v/v) ACN and 40% (v/v) IPA; buffer: 100 mM acetic acid, adjusted to pH 5.0 with ammonia; gradient as depicted in Table 2

Figure 4: Slopes of calibration functions in different matrices (extract 4, 11, 16 and Media 10, 16) versus slopes in neat solutions. α-Tocopherol was excluded because of its limited stability.
1 Arachidonic acid; 2 Cephalosporin P1; 3 Stearic acid; 4 Linoleic acid; 5 Myristic acid; 6 Palmitic acid; 7 Lauric acid; 8 Oleic acid; 9 Linolenic acid

Figure 5: Bias of accuracies for QC samples averaged over three concentration levels (extract 11 spiked at 0.075, 0.5 and 1.0 mg/L) calculated employing calibration with neat standard

solutions and various corrected matrix-matched calibration functions. (A) with and (B) without internal standard. Detailed information can be found in the supplementary information.

Figure 6: Chromatogram of the proposed expanded set of analytes on X-Bridge C18. (1) Phenoxymethlpenillic acid; (2) p-Hydropxypenicillin; (3) Phenoxymethylpenicilloic acid; (4) Phenoymethylpenilloic acid; (5) Penicillin V; (6) Cephalosporin P1; (7) Lauric acid; (8) Linolenic acid; (9) Myristic acid; (10) Arachidonic acid; (11) Linoleic acid; (12) Palmitic acid; (13) Oleic acid; (14) Stearic acid

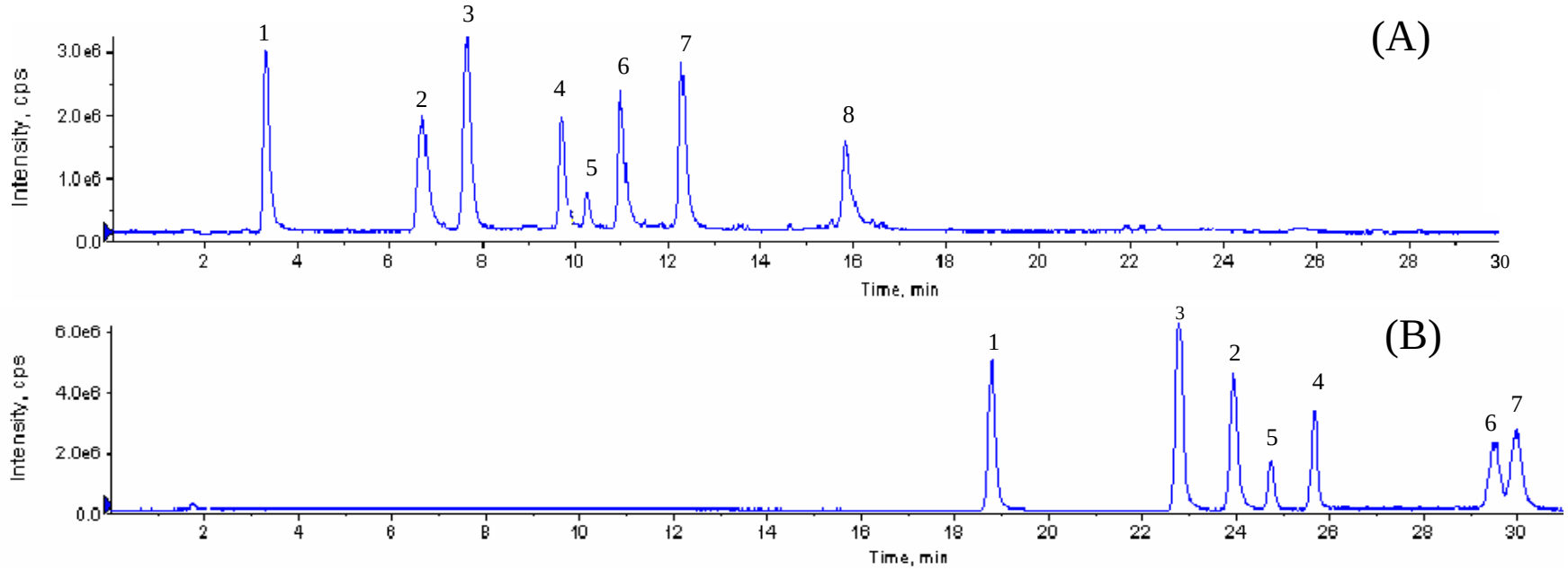
(A)
Intensity, cps
3.0e6
2.0e6
1.0e6
0.0
1
2
3
4
5
6
7
8
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Time, min
(B)
Intensity, cps
6.0e6
4.0e6
2.0e6
0.0
1
2
3
4
5
6
7
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Time, min

Figure 1

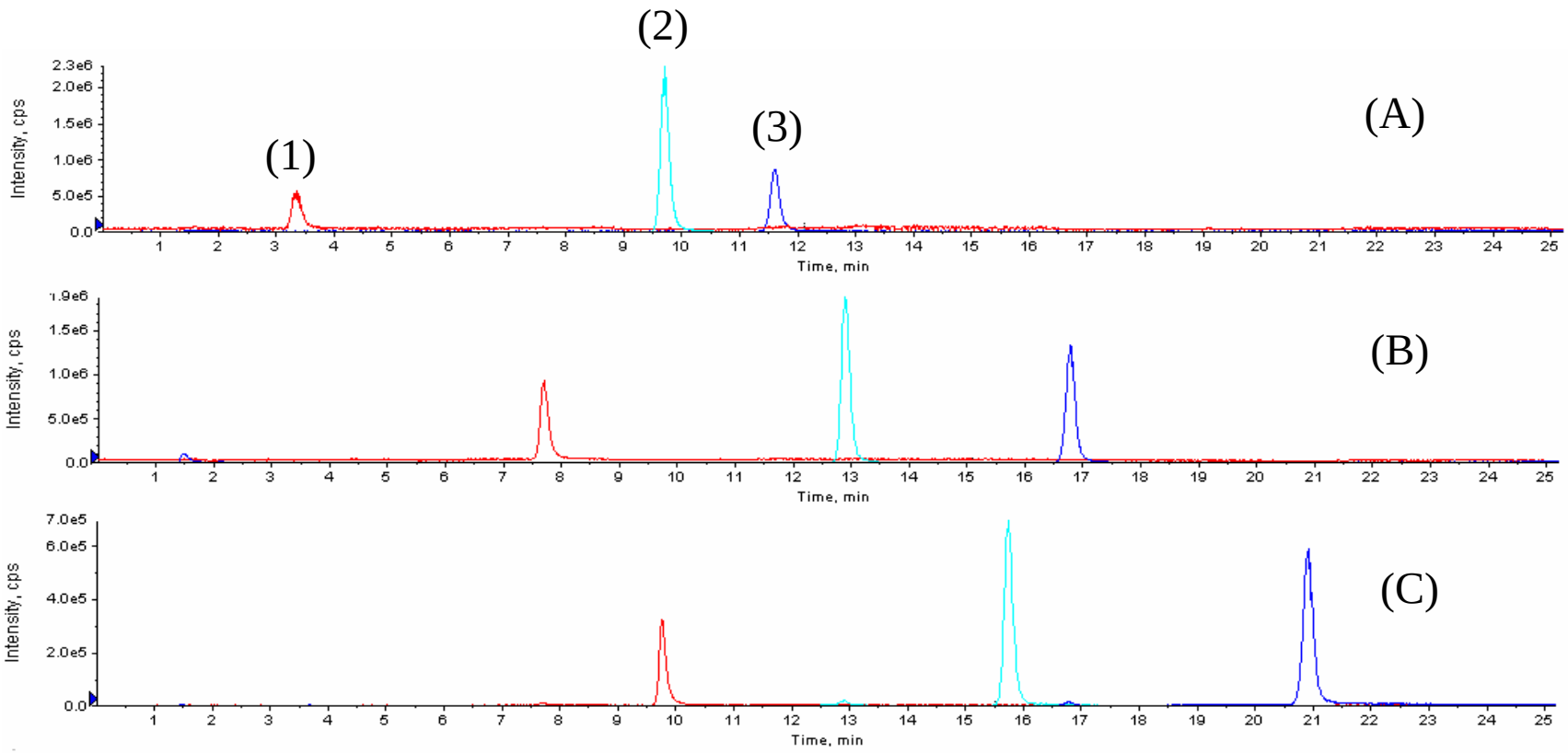

Figure 2

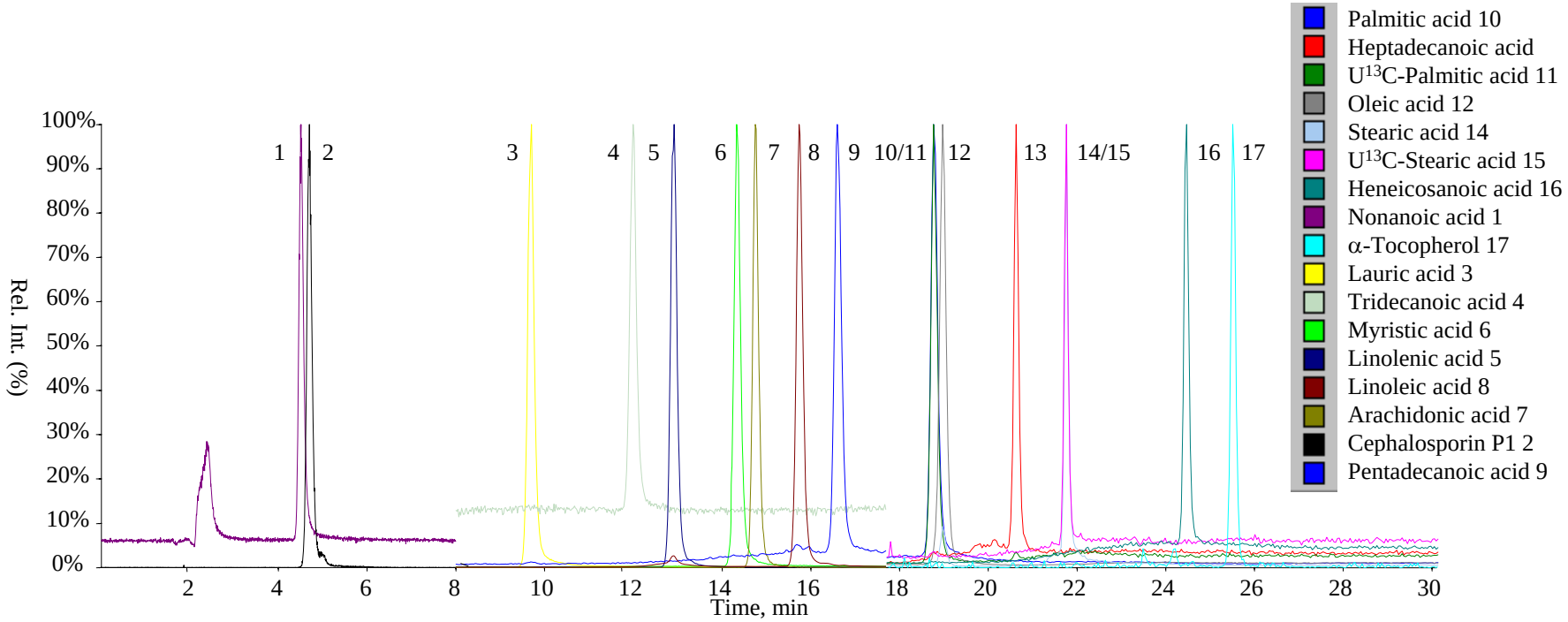
Rel. Int. (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1
2
3
4
5
6
7
8
9
10/11
12
13
14/15
16
17
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Time, min
Palmitic acid 10
Heptadecanoic acid
U13C-Palmitic acid 11
Oleic acid 12
Stearic acid 14
U13C-Stearic acid 15
Heneicosanoic acid 16
Nonanoic acid 1
α-Tocopherol 17
Lauric acid 3
Tridecanoic acid 4
Myristic acid 6
Linolenic acid 5
Linoleic acid 8
Arachidonic acid 7
Cephalosporin P1 2
Pentadecanoic acid 9

Figure 3

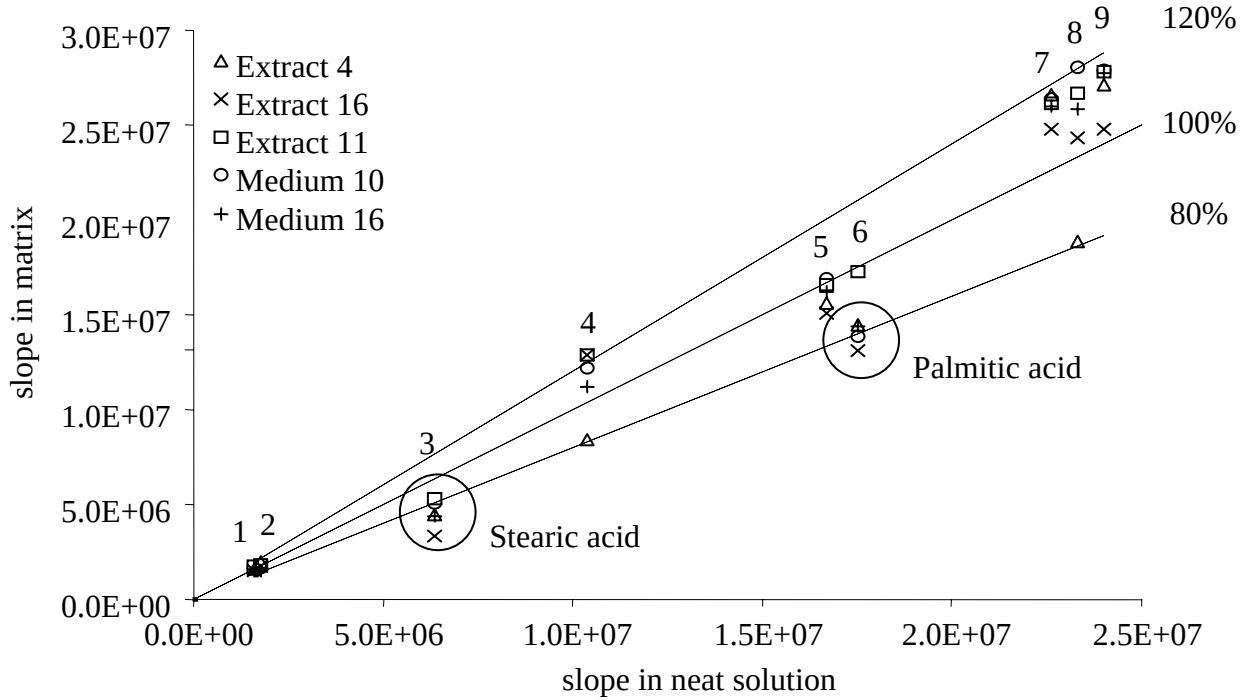
3.0E+07
2.5E+07
2.0E+07
1.5E+07
1.0E+07
5.0E+06
0.0E+00
slope in matrix
0.0E+00
5.0E+06
1.0E+07
1.5E+07
2.0E+07
2.5E+07
slope in neat solution
Extract 4
Extract 16
Extract 11
Medium 10
Medium 16
120%
100%
80%
1
2
3
4
5
6
7
8
9
Stearic acid
Palmitic acid

Figure 4

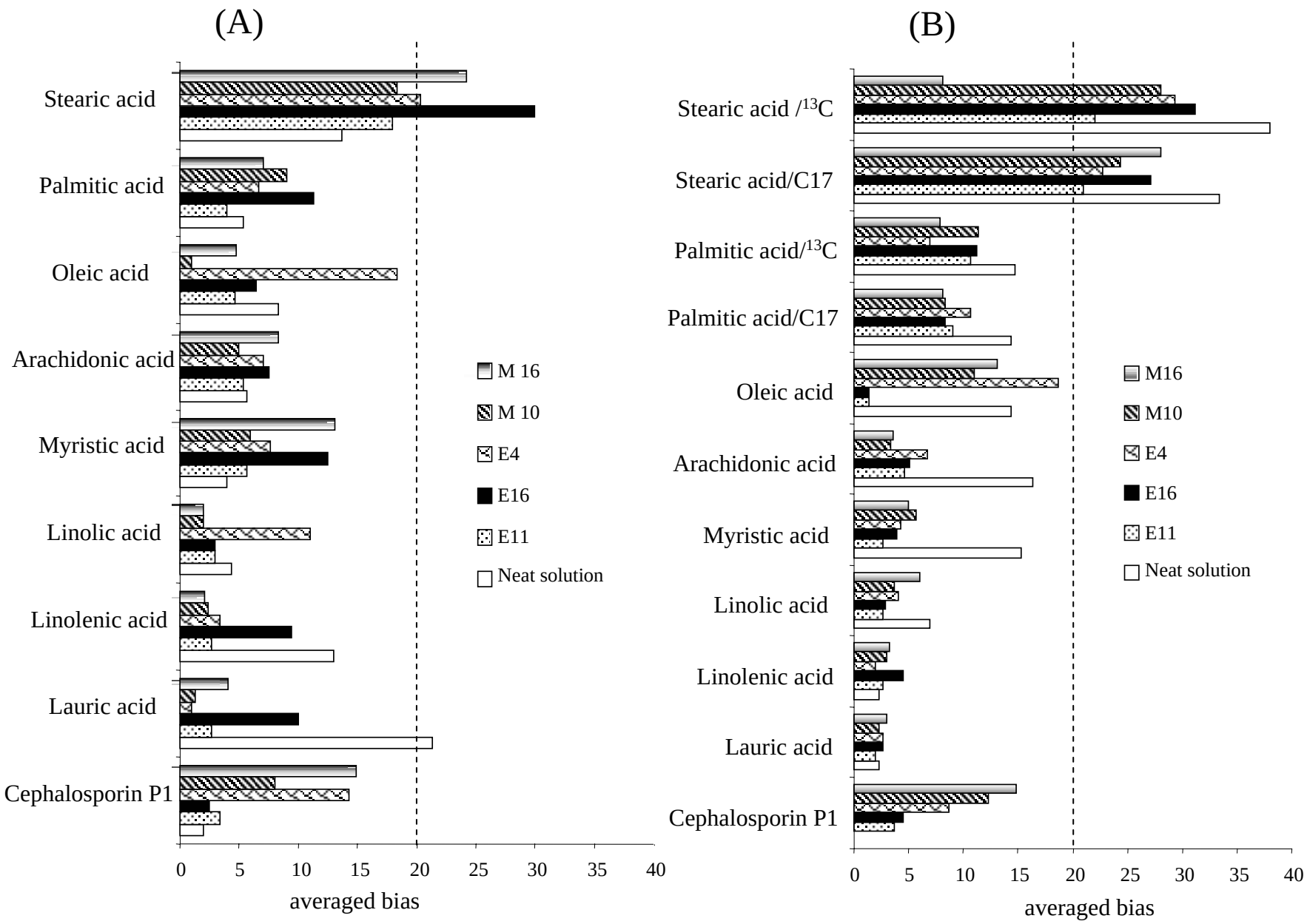
(A)
Stearic acid
Palmitic acid
Oleic acid
Arachidonic acid
Myristic acid
Linolic acid
Linolenic acid
Lauric acid
Cephalosporin P1
M 16
M 10
E4
E16
E11
Neat solution
0
5
10
15
20
25
30
35
40
averaged bias
(B)
Stearic acid /13C
Stearic acid/C17
Palmitic acid/13C
Palmitic acid/C17
Oleic acid
Arachidonic acid
Myristic acid
Linolic acid
Linolenic acid
Lauric acid
Cephalosporin P1
M16
M10
E4
E16
E11
Neat solution
0
5
10
15
20
25
30
35
40
averaged bias

Figure 5

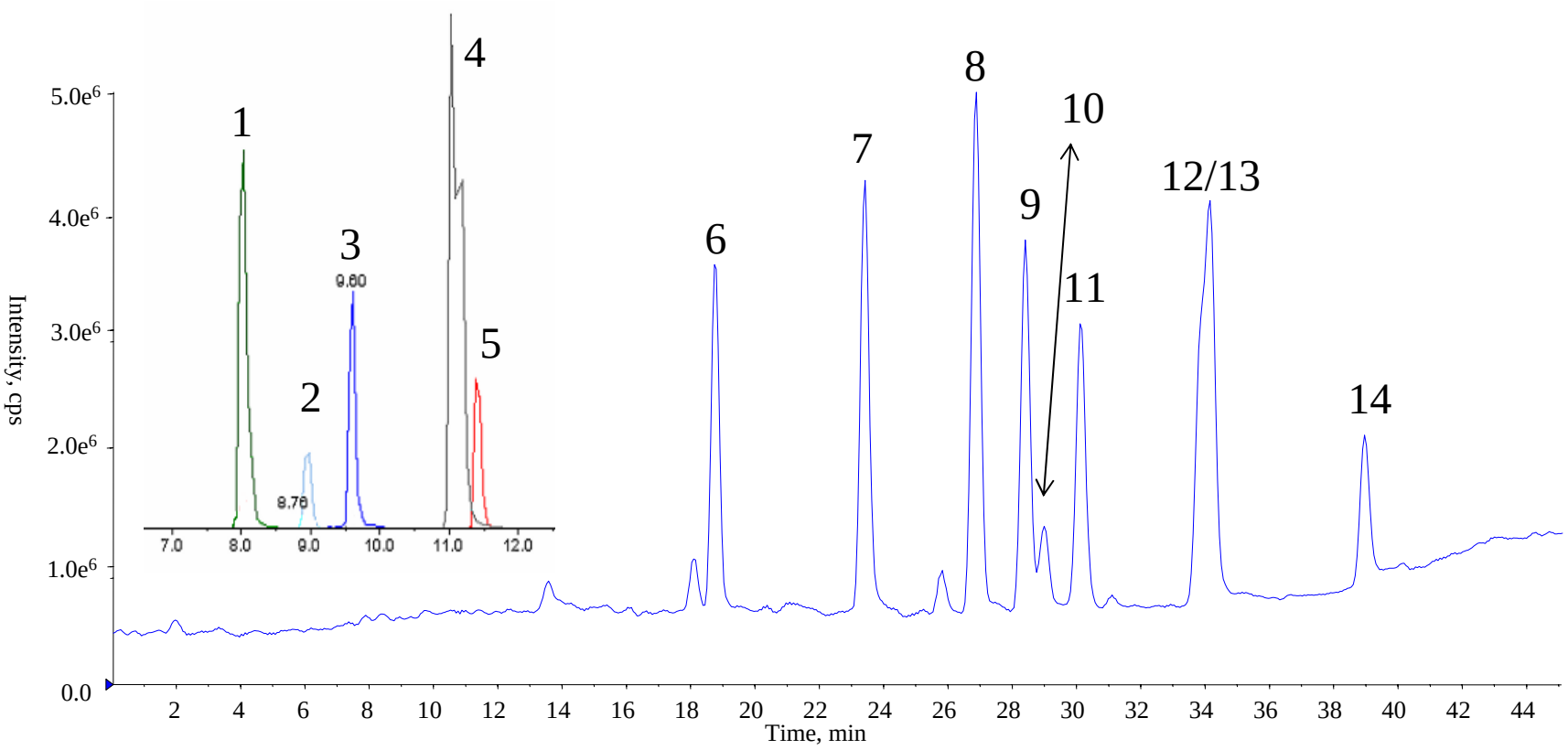
Intensity, cps
5.0e6
4.0e6
3.0e6
2.0e6
1.0e6
0.0
Time, min
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
1
2
3
4
5
6
7
8
9
10
11
12/13
14
9.60
9.76
7.0
8.0
9.0
10.0
11.0
12.0

Figure 6

Table 1: Investigated compounds along with their specific MS/MS parameters and their corresponding internal standards.

Analyte	Molecular weight	Precursor ion	Product ion	Polarity	DP [V][a]	CE [V][b]	CXP [V][c]	periode[d]	internal standard[e]
Arachidonic acid	304	303	259	neg	-70	-18	-15	2	Pentadecanoic acid
Cephalosporin P1	574	573	513	neg	-95	-30	-15	1	Nonanoic acid
Lauric acid	200	199	199	neg	-50	-6	-3	2	Tridecanoic acid
Linoleic acid	280	279	279	neg	-85	-10	-39	2	Pentadecanoic acid
Linolenic acid	278	277	277	neg	-70	-10	-7	2	Tridecanoic acid
Myristic acid	228	227	227	neg	-75	-8	-15	2	Pentadecanoic acid
Oleic acid	282	281	281	neg	-30	-10	-5	3	Heptadecanoic acid
Palmitic acid	256	255	255	neg	-70	-10	-7	3	Heptadecanoic acid/U-^{13}C Palmitic acid
Stearic acid	284	283	283	neg	-50	-20	-19	3	Heptadecanoic acid/U-^{13}C Stearic acid
α-Tocopherol	430.5	430.5	430.5	pos	91	13	24	3	Heneicosanoic acid
Internal Standards									
Nonanoic acid	158	157	157	neg	-25	-6	-9	1	
Tridecanoic acid	214	213	213	neg	-55	-6	-3	2	
Pentadecanoic acid	242	241	241	neg	-70	-6	-7	2	
Heptadecanoic acid	270	269	269	neg	-80	-10	-7	3	
Heneicosanoic acid	326	325	325	neg	-85	-6	-9	3	
U-^{13}C Palmitic acid	272	271	271	neg	-70	-8	-7	3	
U-^{13}C Stearic acid	302	301	301	neg	-20	-6	-9	3	

[a] Declustering potential
[b] Collision energy
[c] Cell exit potential
[d] Time periode during which the analyte transitions were measured.
period 1 = 0-8 min; period 2 = 8-18 min; period 3 = 18-39 min
[e] Internal standard which was employed for peak area normalization.

Table 2: Optimized gradient profile of the final LC-MS/MS method.

time [min]	% Eluent (A)	% Eluent (B)	flow rate [µl/min]
0	30	70	300
20	0	100	300
21	0	100	500
26	0	100	500
27	30	70	300
40	30	70	300

Table 3: Validation results[a]

						intraday												interday[d]			
						without internal standards						with internal standards						without internal standards		with internal standards	
						Precision (%RSD) Spiking level [mg/L]			Accuracy (%) Spiking level [mg/L]			Precision (%RSD) Spiking level [mg/L]			Accuracy (%) Spiking level [mg/L]			Precision (%RSD) Spiking level [mg/L]	Accuracy(%) Spiking level [mg/L]	Precision (%RSD) Spiking level [mg/L]	Accuracy(%) Spiking level [mg/L]
Analyte	**tr [min]**	**internal Standard**	**tr [min]**	**LOQ[b] [mg/L]**	**slopes[c]**	**0.075**	**0.5**	**1.0**	**0.075**	**0.5**	**1.0**	**0.075**	**0.5**	**1.0**	**0.075**	**0.5**	**1.0**	**0.5**	**0.5**	**0.5**	**0.5**
Arachidonic acid	14.7	Pentadecanoic acid	16.6	0.005	1.71E+06	0.5	0.6	0.8	95	94	105	1.6	0.9	1.5	95	96	105	28	71	11	88
Cephalosporin P1	4.7	Nonanoic acid	4.5	0.005	1.79E+06	1.5	0.2	1.2	105	97	98	2.0	0.9	2.4	107	98	98	n.a.[e]	n.a.[e]	n.a.[e]	n.a.[e]
Lauric acid	9.7	Tridecanoic acid	12.0	0.005	2.61E+07	2.4	0.9	1.7	103	101	104	3.1	1.4	1.4	98	96	100	16	85	4	99
Linoleic acid	15.7	Pentadecanoic acid	16.6	0.050	1.23E+07	1.7	0.8	0.5	98	97	101	0.7	2.0	2.1	97	98	103	2	87	16	93
Linolenic acid	12.9	Tridecanoic acid	12.0	0.003	2.78E+07	1.3	0.9	0.6	98	98	104	3.3	0.7	0.4	96	96	102	22	79	6	95
Myristic acid	14.3	Pentadecanoic acid	16.6	0.010	1.66E+07	1.1	0.9	0.3	95	99	111	0.6	1.7	2.1	96	98	108	27	76	7	93
Oleic acid	19.0	Heptadecanoic acid	20.6	0.026	2.67E+07	3.4	1.0	2.9	95	97	106	0.2	3.2	1.4	100	99	103	26	85	8	101
Palmitic acid	18.8	Heptadecanoic acid	20.6	0.086	1.73E+07	2.4	3.2	2.4	103	102	93	3.7	1.8	2.7	113	106	92	23	78	14	90
Palmitic acid	18.8	U-^{13}C Palmitic acid	18.8	0.086	1.73E+07	2.4	3.2	2.4	103	102	93	4.9	2.6	1.5	111	96	83	23	78	3	94
Stearic acid	21.8	Heptadecanoic acid	20.6	0.137	5.33E+06	0.8	5.2	2.7	109	116	71	3.3	3.4	6.6	110	136	83	25	127	22	111
Stearic acid	21.8	U-^{13}C Stearic acid	21.8	0.137	5.33E+06	0.8	5.2	2.7	109	116	71	3.5	2.2	4.6	115	129	78	25	127	31	98
α-Tocopherol	25.5	Heneicosanoic acid	24.5	n.a.	1.45E+05	< LOQ	2.9	1.7	< LOQ	97	102	< LOQ	3.4	1.1	< LOQ	93	99	n.a.[e]	n.a.[e]	n.a.[e]	n.a.[e]

[a] Intraday (n = 3) and interday (n = 3) precision and accuracy were determined using extract 11 spiked at three concentration levels. For calculations calibration functions of matrix-matched calibration in extract 11 generated without and with use of internal standards were used.

[b] LOQ was determined as the concentration where the signal is higher than 10 times the standard deviation of noise. For linoleic, oleic, palmitic and stearic acid LOQ was calculated from the standard deviation (s) of the blank peak area (memory effect): LOQ = (10 x s + average of blank peak)/slope of calibration function in neat solution

[c] Slopes of calibration functions were obtained by spiking extract 11 without use of internal standards.

[d] Interday precision and accuracy were determined using freshly prepared calibration functions.

[e] not available due to stability reasons

Table 4: Evaluation of matrix effects by comparison of the slopes of calibration functions (without IS) in standard solution and of standard addition in 3 different extracts and two nutrition media.

	Absolute matrix effect (%) [a]					Relative matrix effect [b]
without IS	**Extract 11**	**Extract 16**	**Extract 4**	**Medium 10**	**Medium 16**	**RSD (%)**
mesured on	3	2	3	4	4	
Analytes						
Arachidonic acid	106	94	98	105	99	5
Cephalosporin P1	100	98	111	92	85	10
Lauric acid	115	110	118	117	115	3
Linolenic acid	116	103	113	116	115	5
Linoleic acid	118	**124**[e]	81	117	108	16
Myristic acid	99	90	93	101	97	5
Oleic acid	115	104	81	120	111	14
Palmitic acid	99	**75**	83	**79**	82	11
Stearic acid	84	**52**	**70**	**79**	**69**	17
α-Tocopherol[c]	**16**	**51**	**34**	**34**	**51**	**39**
Mean[d]	106	95	94	103	98	9
Standard deviation[d]	11	21	17	16	16	5

[a] calculated by ratio of slopes in matrix and standard solutions multiplied by 100; 100% means absence of matrix effects; <100% ion suppression; >100% signal enhancement due to matrix

[b] calculated as the relative standard deviation from the mean in the four extracts and two nutrition media, respectively

[c] problems due to limited stability

[d] α-Tocopherol was excluded.

[e] values with a bias above ± 20% are in bold

Table 5: Quantification results of extract 4 and 11 using calibration with neat standard solutions without internal standards.

Analytes	Extract 4 [mg/L]	Extract 11 [mg/L]
Lauric acid	< LOQ[a]	< LOQ
Linoleic acid	550.8	106.1
Linolenic acid	60.4	12.9
Myristic acid	< LOQ	< LOQ
Arachidonic acid	< LOQ	< LOQ
Oleic acid	275.9	46.1
Palmitic acid	276.5	55.8
Stearic acid	190.3	11.5
α-Tocopherol	< LOQ	< LOQ

[a] < LOQ in 1:100 diluted sample extracts.

Table 6: Gradient profile of the extended RPLC-MS/MS method.

time [min]	% Eluent (A)[a]	% Eluent (B)[b]	flow rate [µl/min]
0	95	5	300
12	70	30	300
40	0	100	300
40.1	0	100	500
45	0	100	500
46	95	5	500
56	95	5	500
57	95	5	300
58	95	5	300

[a] 5% (v/v) buffer in water

[b] 5% (v/v) buffer; 55% (v/v) ACN; 40% (v/v) IPA

Buffer: 100 mM acetic acid adjusted to pH 5.0 with ammonium hydroxide solution

Quantitative LC-ESI-MS/MS metabolic profiling method for fatty acids and lipophilic metabolites in fermentation broths from β-lactam antibiotics production

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Supporting Information

Table of contents

Table S 1 : Lower limit of quantitation (LLOQ), upper limit of quantitation (ULOQ), calibration functions[a] and correlation factors determined for the compounds under investigation.

(A)

	linear range		matrix-free neat soloutions			spiked extract 16			spiked extract 11			spiked extract 4			spiked medium 10			spiked medium 16		
measured on			day 1			day 2			day 3			day 3			day 4			day 4		
Analyte	**LLOQ [mg/L]**	**ULOQ [mg/L]**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**
Arachidonic acid	0.010	10[c]	1.61E+06	-2.09E+04	0.9944	1.52E+06	-1.99E+04	0.9952	1.71E+06	-2.77E+04	0.9934	1.58E+06	-1.49E+04	0.9943	1.69E+06	-3.83E+04	0.9982	1.59E+06	-3.60E+04	0.9965
Cephalosporin P1	0.005	4[c]	1.79E+06	-3.50E+03	0.9979	1.76E+06	-8.58E+03	0.9999	1.79E+06	-1.07E+04	0.9980	1.98E+06	3.83E+06	0.9900	1.64E+06	-7.45E+03	0.9994	1.53E+06	-5.00E+03	0.9991
Lauric acid	0.005	10	2.26E+07	-1.58E+04	0.9992	2.48E+07	-9.42E+03	0.9997	2.61E+07	7.40E+04	0.9991	2.66E+07	9.83E+04	0.9996	2.64E+07	9.75E+04	0.9930	2.60E+07	3.07E+04	0.9986
Linoleic acid	0.051[b]	5	1.04E+07	-5.27E+04	0.9934	1.29E+07	2.28E+07	0.9944	1.23E+07	9.86E+06	0.9831	8.39E+06	5.14E+07	0.9800	1.22E+07	-2.96E+05	0.9993	1.12E+07	-1.57E+05	0.9966
Linolenic acid	0.005	10	2.40E+07	4.95E+04	0.9966	2.48E+07	6.45E+05	0.9973	2.78E+07	2.99E+06	0.9985	2.71E+07	1.37E+07	0.9962	2.79E+07	-1.84E+04	0.9999	2.77E+07	-2.19E+05	0.9987
Myristic acid	0.005	10	1.67E+07	8.62E+04	0.9988	1.51E+07	1.52E+05	0.9962	1.66E+07	1.85E+05	0.9963	1.56E+07	1.83E+05	0.9991	1.69E+07	-1.60E+05	0.9998	1.62E+07	-9.26E+04	0.9975
Oleic acid	0.026[b]	10	2.33E+07	-1.57E+05	0.9912	2.43E+07	2.84E+06	0.9981	2.67E+07	7.24E+06	0.9990	1.89E+07	5.44E+07	0.9936	2.80E+07	-5.21E+05	0.9990	2.58E+07	-5.31E+05	0.9984
Palmitic acid	0.086[b]	10[c]	1.75E+07	6.20E+04	0.9935	1.31E+07	8.94E+06	0.9945	1.73E+07	6.82E+06	0.9909	1.45E+07	3.55E+07	0.8576	1.39E+07	5.40E+05	0.9960	1.44E+07	4.62E+05	0.9889
Stearic acid	0.137[b]	10[c]	6.36E+06	1.23E+05	0.9834	3.33E+06	7.42E+05	0.9981	5.33E+06	7.72E+05	0.9778	4.47E+06	1.22E+07	0.8858	5.05E+06	1.39E+05	0.9808	4.41E+06	2.24E+05	0.9776
α-Tocopherol	0.050	4	8.96E+05	-6.78E+03	0.9977	4.59E+05	-3.56E+04	0.9977	1.45E+05	-3.30E+03	1.0000	3.04E+05	-9.34E+03	0.9934	3.09E+05	-4.46E+04	0.9927	4.59E+05	-3.56E+04	0.9977

(B)

	matrix-free neat soloutions			spiked extract 16			spiked extract 11			spiked extract 4			spiked medium 10			spiked medium 16		
measured on	day 1			day 2			day 3			day 3			day 4			day 4		
Analyte	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**	**R^2**
Arachidonic acid	0.929	-0.014	0.9917	0.766	-0.010	0.9994	0.766	-0.010	0.9963	0.760	-0.007	0.9951	0.762	-0.014	0.9988	0.793	-0.017	0.9987
Cephalosporin P1	0.154	0.000	0.9995	0.227	-0.001	0.9995	0.217	-0.001	0.9994	0.191	0.447	0.9232	0.195	-0.001	0.9999	0.180	0.000	0.9993
Lauric acid	2.250	-0.005	0.9989	2.290	0.007	0.9995	2.280	0.007	0.9995	2.260	0.010	0.9994	2.230	0.011	0.9993	2.300	0.007	0.9990
Linoleic acid	6.100	-0.042	0.9868	4.860	12.100	0.9823	5.010	4.740	0.9896	5.500	24.200	0.9804	5.500	-0.049	0.9987	5.810	-0.062	0.9986
Linolenic acid	2.400	-0.001	0.9943	2.460	0.039	0.9991	2.340	0.258	0.9986	2.180	1.170	0.9875	2.230	0.006	0.9997	2.420	-0.018	0.9981
Myristic acid	9.580	0.011	0.9960	7.630	0.080	0.9974	7.650	0.092	0.9988	7.600	0.092	0.9993	7.820	-0.005	0.9991	8.130	-0.020	0.9993
Oleic acid	19.700	-0.141	0.9840	15.200	2.000	0.9980	15.000	3.710	0.9982	11.400	27.500	0.9769	18.600	-0.325	0.9980	19.700	-0.218	0.9920
Palmitic acid	14.200	0.117	0.9918	9.090	5.520	0.9985	9.680	3.330	0.9947	8.250	18.100	0.9375	9.210	0.453	0.9976	9.290	0.478	0.9957
Palmitic acid/^{13}C[d]	8.410	0.046	0.9941	5.100	3.800	0.9926	7.330	2.310	0.9735	5.970	12.900	0.9332	5.250	0.229	0.9939	5.520	0.198	0.9948
Stearic acid	5.320	0.092	0.9927	2.310	0.448	0.9939	2.340	0.465	0.9869	2.610	6.200	0.8592	2.510	0.336	0.9680	2.230	0.356	0.9749
Stearic acid/^{13}C[d]	10.000	-0.041	0.9915	3.680	0.762	0.9973	4.370	0.676	0.9840	4.050	11.800	0.8721	3.700	0.490	0.9653	3.740	0.368	0.9820
α-Tocopherol	0.367	-0.002	0.9931	0.149	-0.002	0.9950	0.055	-0.001	0.9997	0.040	-0.001	0.9912	0.047	-0.001	0.9996	0.153	-0.035	0.9990

[a] Calibration functions were generated with pure, matrix-free standard solutions, as well as by spiking different sample matrices (A) without and (B) with internal standards for peak area normalization.

[b] Because of memory effects the LOQ was determined as the concentration yielding a peak area greater than ten times the standard deviation of the memory peak (n = 3).

[c] ULOQ not reached at this concentration level.

[d] ^{13}C labeled structure analogues internal standards were employed for peak area normalization.

Table S 2: Evaluation of matrix effects by comparison of the slopes of calibration functions (with IS) in standard solution and of standard addition in 3 different extracts and two nutrition media.

	Absolute matrix effect (%)[a]					Relative matrix effect[b]
with IS	**Extract 16**	**Extract 11**	**Extract 4**	**Medium 10**	**Medium 16**	**RSD (%)**
mesured on day	2	3	3	4	4	
Analytes						
Arachidonic acid	82	82	82	82	85	2
Cephalosporin P1	**147**	**141**	**124**	**127**	117	10
Lauric acid	102	101	100	99	102	1
Linolenic acid	103	98	91	93	101	5
Linoleic acid	80	82	90	90	95	7
Myristic acid	80	80	**79**	82	85	3
Oleic acid	**77**	**76**	**58**	94	100	**21**
Palmitic acid	**64**	**68**	**58**	**65**	**65**	6
Palmitic acid/^{13}C	**61**	87	**71**	**62**	**66**	15
Stearic acid	**43**	**44**	**49**	**47**	**42**	6
Stearic acid/^{13}C	**37**	**44**	**41**	**37**	**37**	8
α-Tocopherol[c]	**41**	**15**	**11**	**13**	**42**	**64**
Mean[d]	80	82	77	80	81	8
Standard deviation[d]	31	27	25	25	26	6

[a] calculated by ratio of slopes in matrix and standard solutions multiplied by 100

[b] calculated as the relative standard deviation from the mean in the 4 extracts and two nutrition media, respectively

[c] problems due to limited stability

[d] α-Tocopherol was excluded.

[e] values with a bias above ± 20% are in bold

Table S 3: Intraday accuracies of QC samples prepared by spiking of extract 11 at three concentration levels (low 0.075 mg/L, medium 0.5 mg/L, high 1.0 mg/L) obtained with different functions, i.e. with neat standard solutions and matrix-matched calibration functions obtained by standard addition to various extracts/media (A) without and (B) with internal standards.

(A)

without internal standards	calculation with calibration functions from: matrix-free neat soloutions			spiked extract 11			spiked extract 16			spiked extract 4			spiked medium 16			spiked medium 10		
Analyte	**low**	**medium**	**high**	**low**	**medium**	**high**	**low**	**medium**	**high**	**low**	**medium**	**high**	**low**	**medium**	**high**	**low**	**medium**	**high**
Arachidonic acid	95	99	111	95	94	105	100	105	118	92	100	113	109	102	114	104	96	107
Cephalosporin P1	100	96	98	105	97	98	105	98	100	83	86	88	118	112	114	112	105	107
Lauric acid	124	118	122	103	101	104	113	107	110	100	99	102	106	102	105	101	100	103
Linoleic acid	100	103	110	98	97	101	98	95	98	101	110	122	99	99	103	98	97	101
Linolenic acid	108	112	119	98	98	104	104	108	116	101	101	108	99	99	104	99	98	104
Myristic acid	101	100	111	95	99	111	106	109	122	100	105	118	119	105	115	94	97	109
Oleic acid	95	104	116	95	97	106	96	102	113	99	118	136	95	98	108	99	98	100
Palmitic acid	97	97	90	103	102	93	106	116	112	105	110	105	105	111	105	106	113	108
Stearic acid	101	100	60	109	116	71	123	162	105	113	131	83	117	138	83	109	120	74
α-Tocopherol[a]	< LOQ	16	17	< LOQ	97	102	< LOQ	45	39	< LOQ	50	51	< LOQ	45	39	< LOQ	72	61

(B)

with internal standards	calculation with calibration functions from: matrix-free neat soloutions			spiked extract 11			spiked extract 16			spiked extract 4			spiked medium 16			spiked medium 10		
Analyte	**low**	**medium**	**high**	**low**	**medium**	**high**	**low**	**medium**	**high**	**low**	**medium**	**high**	**low**	**medium**	**high**	**low**	**medium**	**high**
Arachidonic acid	84	80	87	95	96	105	94	96	105	90	96	106	102	94	102	101	97	106
Cephalosporin P1	107	98	98	107	98	98	100	93	93	107	109	110	119	117	117	119	109	109
Lauric acid	94	101	98	98	96	100	97	95	100	97	96	101	97	95	99	98	97	102
Linoleic acid	96	91	92	97	98	103	96	99	103	95	95	98	95	93	94	96	95	98
Linolenic acid	99	95	101	96	96	102	96	93	98	100	100	106	97	94	100	98	100	107
Myristic acid	87	80	87	96	98	108	97	99	108	96	99	108	108	95	103	109	99	107
Oleic acid	93	82	82	100	99	103	101	99	102	108	120	128	95	83	83	95	86	86
Palmitic acid	99	81	67	113	106	92	112	109	96	114	115	103	112	108	95	112	108	95
Palmitic acid/^{13}C[b]	104	86	74	111	96	83	112	115	106	110	106	95	112	111	101	114	115	105
Stearic acid	88	72	40	110	136	83	124	143	86	116	129	77	126	147	89	119	134	80
Stearic acid/^{13}C[b]	83	66	37	115	129	78	134	153	93	131	142	85	112	108	95	126	149	91
α-Tocopherol[a]	< LOQ	14	15	< LOQ	93	99	< LOQ	35	37	< LOQ	124	134	< LOQ	77	58	< LOQ	124	134

[a] α-Tocopherol is not stable over the investigated time periode and thus data obtained for accuracy are not representative.
[b] ^{13}C labeled structure analog internal standards were employed for peak area normalization of marked analytes.

Table S 4: Intraday and interday precision (n = 3) determined for a medium concentration level (quality control sample, extract 11 spiked with 0.5 mg/L, stored at 5°C) at three different days using freshly established calibration functions.

	without internal standards			Precision	with internal standards			Precision
Analyte	**day 1**	**day 8**	**day 11**	**interday**	**day 1**	**day 8**	**day 11**	**interday**
Arachidonic acid	0.6	0.5	1.9	28.0	0.9	1.9	2.1	11.1
Cephalosporin P1	0.2	2.3	2.4	49.0	0.9	2.9	2.7	42.4
Lauric acid	0.9	1.6	2.2	16.3	1.4	2.4	3.3	4.2
Linoleic acid	0.8	0.3	0.7	20.7	2.0	1.8	3.8	16.4
Linolenic acid	0.9	0.9	0.8	22.1	0.7	1.9	1.9	5.8
Myristic acid	0.9	0.7	2.0	27.3	1.7	2.1	2.2	7.1
Oleic acid	1.0	0.5	1.8	25.6	3.1	1.7	2.5	8.0
Palmitic acid	3.7	3.1	4.6	23.0	1.8	2.0	6.0	14.3
Palmitic acid/^{13}C[a]	-	-	-	-	2.9	5.0	3.3	2.6
Stearic acid	5.2	8.3	2.1	25.3	3.4	7.6	0.8	21.9
Stearic acid/^{13}C[a]	-	-	-	-	2.2	4.2	3.8	30.9

[a] ^{13}C-labeled structural analogs were employed as internal standards for peak area normalization of denoted analytes.
Corrected matrix-matched calibration funtions were obtained by standard addition in extract 11.
α–Tocopherol was excluded because of its limited stability.

Table S 5: Intraday and interday precision (n = 3) determined for a medium concentration level (quality control sample, extract 11 spiked with 0.5 mg/L, stored at 5°C) at three different days using calibration functions set up on day 1.

	without internal standards			Precision	with internal standards			Precision
Analyte	**day 1**	**day 8**	**day 11**	**interday**	**day 1**	**day 8**	**day 11**	**interday**
Arachidonic acid	0.6	0.4	1.4	93.2	0.9	1.9	2.1	35.8
Cephalosporin P1	0.2	2.2	1.9	117.8	0.9	2.6	2.5	78.7
Lauric acid	0.9	1.7	2.2	64.3	1.4	2.4	3.3	1.7
Linoleic acid	0.7	0.9	1.1	99.7	2.0	1.8	3.8	22.1
Linolenic acid	0.9	0.9	0.8	76.4	0.7	1.9	1.9	15.7
Myristic acid	0.9	0.7	2.3	91.5	1.7	2.1	2.2	20.3
Oleic acid	1.0	0.5	1.4	49.7	3.1	1.7	2.5	17.9
Palmitic acid	3.7	3.1	4.6	71.5	1.8	2.0	6.0	25.9
Palmitic acid/^{13}C[a]	-	-	-	-	2.9	5.0	3.3	30.1
Stearic acid	5.2	7.2	2.1	62.8	3.4	7.6	0.8	19.0
Stearic acid/^{13}C[a]	-	-	-	-	2.2	4.2	3.8	13.9

[a] ^{13}C-labeled structural analogs were employed as internal standards for peak area normalization of denoted analytes.

Corrected matrix-matched calibration funtions were obtained by standard addition in extract 11.

α-Tocopherol was excluded because of its limited stability.

Table S 6: Intraday and interday accuracy (n = 3) determined for a medium concentration level (quality control sample, extract 11 spiked wit 0.5 mg/L, stored at 5°C) at three different days using freshly established calibration functions.

	without internal standards			Accuracy	with internal standards			Accuracy
Analyte	**day 1**	**day 8**	**day 11**	**interday**	**day 1**	**day 8**	**day 11**	**interday**
Arachidonic acid	94	57	63	71	96	90	77	88
Cephalosporin P1	97	36	56	63	98	41	63	68
Lauric acid	101	74	81	85	96	104	98	99
Linoleic acid	97	93	72	87	98	97	85	93
Linolenic acid	98	67	71	79	96	100	89	95
Myristic acid	99	60	68	76	98	95	86	93
Oleic acid	97	66	93	85	99	104	98	101
Palmitic acid	101	61	73	78	106	84	80	90
Palmitic acid/^{13}C[a]	-	-	-	-	96	94	91	94
Stearic acid	116	175	90	127	136	96	103	111
Stearic acid/^{13}C[a]	-	-	-	-	129	89	74	98

[a] ^{13}C-labeled structural analogs were employed as internal standards for peak area normalization of denoted analytes.
Corrected matrix-matched calibration funtions were obtained by standard addition in extract 11.
α-Tocopherol was excluded because of its limited stability.

Table S 7: Intraday and interday accuracy (n = 3) determined for a medium concentration level (quality control sample, extract 11 spiked wit 0.5 mg/L, stored at 5°C) at three different days using calibration functions set up on day 1.

	without internal standards			Accuracy	with internal standards			Accuracy
Analyte	**day 1**	**day 8**	**day 11**	**interday**	**day 1**	**day 8**	**day 11**	**interday**
Arachidonic acid	94	32	12	46	96	69	46	70
Cephalosporin P1	97	21	6	41	98	38	20	52
Lauric acid	101	51	26	59	96	98	94	96
Linoleic acid	95	32	8	45	98	84	62	83
Linolenic acid	98	43	19	53	96	83	70	82
Myristic acid	99	36	12	49	98	85	65	83
Oleic acid	97	56	36	63	100	79	114	98
Palmitic acid	101	48	21	57	106	69	70	82
Palmitic acid/^{13}C[a]	-	-	-		106	186	193	162
Stearic acid	116	57	32	68	136	92	118	115
Stearic acid/^{13}C[a]	-	-	-	-	129	113	98	113

[a] ^{13}C-labeled structural analogs were employed as internal standards for peak area normalization of denoted analytes.
Corrected matrix-matched calibration funtions were obtained by standard addition in extract 11.
α-Tocopherol was excluded because of its limited stability.

Summary

The main topic of the PhD thesis was the development of multicomponent analysis assays for pharmaceutical applications. Two distinct applications of LC-MS/MS based multicomponent analysis were treated herein, the one related to impurity profiling of stressed infusion solutions composed of 19 ingredients and an unknown number of impurities, and the other one related to metabolic profiling i.e. the quantitative analysis of 70 target analytes in extracts of fermentation broths.

Two strategies can be differentiated in multicomponent analysis to satisfy distinct analytical demands. The "comprehensive approach" is targeted at unveiling and quantifying the entirety of compounds in a sample. In this context, it is crucial to detect all compounds in a sample. Considering the "targeted approach", the focus lies on the detection and quantification of a predefined set of analytes. Other sample constituents are not detected but may interfere with the analysis of target compounds.

Both approaches were employed in the course of two industry projects.

The goal of the first project with Fresenius Kabi Austria (Graz, Styria) was to establish a comprehensive qualitative and quantitative impurity profile of a newly formulated infusion solution intended for parenteral supplementation of nutrients.

Detailed information on the stability of drug compounds and potential degradation products provides the basis for the quality and risk management of pharmaceutical products.

In this context stability tests are performed. The aim of such tests is to study the influence of environmental factors like temperature, humidity, light, pH on the stability of the drug compound as well as on the whole pharmaceutical formulation. Furthermore, optimal storage conditions and shelf lives can be deduced from the results of such tests.

Since each impurity in a pharmaceutical product may potentially cause side effects, it is of utmost importance to detect all relevant impurities and degradation products.

The establishment of impurity profiles of pharmaceutical products containing only one active agent and a small number of additives may be straightforward, as opposed to such products that are composed of a mixture of drug compounds like nutritional infusion solutions as in the given application.

The infusion solution under investigation mainly contained amino acids and dipeptides but also several additives like citric acid and taurine.

In the first place a preliminary impurity profile was established for a stressed infusion solution (storage at 40°C for 12 months). Thereby a challenge was to achieve best possible separation

of an unknown number of low abundant impurities from highly concentrated main components of the infusion solution and to detect them. The ingredients of the infusion solution are highly polar small compounds that are hardly retained under conventional reversed-phase (RP) conditions.

Common detectors like UV and mass spectrometer (MS) exhibit compound specific detector responses. Thus, for accurate quantification calibration with authentic standards is required. In impurity profiling most often authentic standards are not available for unidentified impurities in the early stage of research. Hence, quantification and classification (vide infra) constitutes a serious problem. Furthermore, compounds lacking a chromophoric group or ionizable functionalities may not be detected by UV or MS detectors.

For the outlined reasons a multidimensional analysis assay was developed. The infusion solution was submitted to multidimensional analysis using an off-line combination of RP Liquid Chromatography (LC) and Hydrophilic Interaction Liquid Chromatography (HILIC) for separation and two complementary detectors, a Charged Aerosol Detector (CAD) exhibiting a relatively consistent detector response for quantification and an Ion Trap MS (IT-MS) for identification. Due to the characteristic of the CAD as universal detector for non-volatile compounds and its structure independent signal sensitivity it was possible to quantify unknown compounds with unified calibration functions. Thus, differentiation between relevant and non-relevant impurities as well as classification of impurities into those that need to be reported, identified and quantified could be accomplished. The ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) has established guidelines for such classifications.

Scan spectra and fragmentation spectra provided by IT-MS^n allowed identification of peaks detected with the CAD.

Employing the described multidimensional analysis assay a preliminary impurity profile was successfully established. The majority of identified impurities were formed by 1) peptide condensation (di-, tri- and tetrapeptides), 2) hydrolysis of the glutamine side chain and the peptide bond, respectively, of a main ingredient 3) cyclization reactions of dipeptides (diketopiperazine derivatives) as well as 4) cyclization of glutamic acid to pyroglutamic acid followed by condensation reactions with amino groups or peptides.

Three LC-MS/MS methods were finally developed using the Multiple Reaction Monitoring (MRM) mode providing more accurate and reliable quantification of identified relevant impurities in stressed infusion solutions.

In the second project with Sandoz (Kundl, Austria) the aim was to develop and validate LC-MS/MS methods (MRM mode) intended to be used for process optimization and –control of the biotechnological production of β-lactam antibiotics. β-lactam antibiotics are produced on industrial scale in huge fermentors with volume capacities exceeding 200 m^3. Thus, continuous surveillance of the process is of utmost importance.

Target analytes comprised 70 metabolites (amino acids, organic acids, vitamins, fatty acids, biogenic amines as well as penicillin and cephalosporin and their corresponding intermediates of biosynthesis) i.e. this metabolic profiling involved a targeted analysis approach.

The target analytes were characterized by quite different molecular features and distinct polarities. The majority of compounds were considered to be rather polar and hydrophilic. Thus, in the first instance a column screening using HILIC phases (ZIC-HILIC from Merck Sequant AB, Luna Amino and Luna HILIC from Phenomenex, Biobasic AX from Thermo Scientific, Obelisk N and Obelisk R from Sielc Technologies, TSK-Gel Amide 80 from Tosoh, Acclaim Mixed-mode WAX from Dionex as well as Chromolith Performance Si from Merck) was conducted. Since β-lactam antibiotics and their respective intermediates are not stable under basic conditions, only acidic (pH 3-5) mobile phase buffers were tested in the course of the column screening.

With such a high number of target analytes complete resolution of all compounds is almost impossible when 1-D chromatographic techniques are employed. Thus, the final chromatographic analysis conditions were selected with regard to separation of critical pairs (isobaric analytes, compounds with similar structures). Selectivity of the MS detector utilizing the MRM mode was exploited for the analysis of coeluting non-isobaric compounds.

Finally, the ZIC HILIC column from Merck Sequant AB was selected for further fine-tuning of chromatographic conditions.

Fatty acids and several other more apolar compounds were not sufficiently retained under HILIC conditions. Hence, for the analysis of apolar compounds a second method using RP conditions was developed on a X-Bridge C18 column from Waters.

A common problem encountered in quantitative analysis in biological matrices constitute matrix effects. Matrix effects arise due to non-detected coeluting compounds of the matrix that cause fluctuation of the ionization efficiency which may cause severe distortion of quantitative results. For this reason several strategies to overcome matrix effects like matrix-matched calibration with standard addition or use of stable isotope labeled internal standards or structurally similar internal standards were investigated and evaluated in the course of method development.

Precision of the HILIC method for three concentration levels (low, middle, and high) was found to be in 90% of cases lower than 10% RSD for 57 analytes when no internal standards were employed. For the case that internal standards were used, precision was surprisingly slightly worse and in 70% of cases lower than 10% RSD. Accuracy at a medium concentration level determined for external calibration with neat standard solutions was within an acceptable interval of 80-120%, except for only a few outliers. Even better results were obtained for matrix-matched calibration (without internal standards) as accuracy ranged mostly between 95-105%. Satisfying validation results were obtained for all calibration approaches. Use of internal standards (stable isotope labeled and structure analogous) did not provide significant improvement of the validation results. Nevertheless, results of interday accuracy and precision indicate that internal standards are capable of compensating for instrumental fluctuations.

Concerning validation of the RP method, a major problem was encountered. While fatty acids were well separated under RP conditions, quantitative analysis was compromised by strong memory effects especially of oleic acid, palmitic acid and stearic acid. Unless analysis at low levels near the LOQ is necessary, these memory effects were acceptable in terms of accuracies. Since fatty acids were present at higher concentration levels serious problems should not exist.

Several experiments were performed to examine memory effects. The results indicate that these effects originate from chromatographic conditions and are not caused by sample carry over during injection.

For most compounds linear ranges were determined to range over two to three orders of magnitude. Since the target compounds are present at quite different concentration levels in the fermentation extract, both, the strong deviations of compound specific detector responses and the limited linear range may become problematic. In order to have concentrations of all analytes above the LOQ and within the linear range, analysis of several sample dilutions may be necessary.

However, process control aims at high throughput and manifold analysis runs of the same sample are to be avoided - if possible. Nevertheless, the methods are suitable in terms of monitoring defined analyte groups that are present in the fermentation extract at similar concentration levels like amino acids.

Accomplishment of multicomponent analysis nowadays is a current issue in many scientific fields like metabolomics or proteomics. Advances in the field of MS contributed enormously

to the development of multicomponent analysis allowing to simultaneously analyze an increasing number of analytes.

Nevertheless, coupling to chromatographic techniques is still essential. The power of LC-MS/MS arises due to the combination of chromatographic selectivity and mass spectrometric selectivity allowing to differentiate molecules according to their mass and thus, providing a powerful analysis system, which is state of the art for many modern analytical tasks.

Zusammenfassung

Der Fokus der Doktorarbeit lag auf der Erstellung von Methoden zur Multikomponentenanalyse für pharmazeutische Fragestellungen. Im Zuge der Arbeiten wurden zwei verschiedene Fragestellungen behandelt. Zum einen wurde ein Verunreinigungsprofil einer Infusionslösung mit 19 Inhaltsstoffen (Aminosäuren und Dipeptide) und einer unbekannten Zahl an Verunreinigungen erstellt, und zum anderen wurden zwei LC-MS/MS Methoden zur Analyse von ca. 70 Metaboliten und Nährstoffen in Fermentationsextrakten aus der biotechnologischen Produktion von β-Lactam Antibiotika entwickelt.

In der Multikomponentenanalyse kristallisieren sich zwei Strategien zur Bearbeitung unterschiedlich gelagerter Fragestellungen heraus. Beim umfassenden Analysenansatz (Comprehensive Approach) steht die Analyse der Gesamtheit aller Inhaltsstoffe einer Probe im Vordergrund. Wichtig dabei ist, alle Analyte zu erfassen. Im gezielten Analysenansatz (Targeted Approach) liegt der Fokus darauf, eine vordefinierte Zahl an Analyten in sehr komplexen Proben zu analysieren. Diverse Matrixinhaltsstoffe werden dabei nicht mitanalysiert, können aber die richtige und zuverlässliche Analyse der Zielverbindungen beeinträchtigen.

Beide Analysenansätze wurden im Zuge zweier Industrieprojekte angewendet.

Das Ziel des ersten Projekts mit der Firma Fresenius Kabi Austria (Graz, Österreich) war die Erstellung eines Verunreinigungsprofils einer neu entwickelten Infusionslösung zur parenteralen Nährstoffzufuhr.

Um die Qualität und Unbedenklichkeit von pharmazeutischen Produkten gewährleisten zu können, werden umfangreiche Information über die Stabilität von Wirkstoffen und die Entstehung möglicher Abbauprodukte benötigt. In diesem Zusammenhang werden Stabilitätstests durchgeführt. Das Ziel derartiger Tests ist, den Einfluss verschiedener Umweltgrößen wie Temperatur, Feuchtigkeit, Licht, pH auf die Stabilität des Wirkstoffes bzw. der gesamten Formulierung zu untersuchen. Aus den so gewonnenen Daten können im Folgenden optimale Lagerungsbedingungen und das Verfallsdatum abgeleitet werden.

Da jede Verunreinigung in einem pharmazeutischen Produkt potentiell Nebenwirkungen verursachen kann, ist es von höchster Wichtigkeit bei der Erstellung von Verunreinigungsprofilen alle Verunreinigungen bzw. Abbauprodukte zu erfassen. Bei pharmazeutischen Produkten mit nur einem Wirkstoff und wenigen Inhaltsstoffen sind

Verunreinigungsprofile zumeist überschaubar, im Gegensatz zu solchen Produkten, bei denen ein Wirkstoffgemisch vorliegt, wie z.B. im Fall von Nährstofflösungen.

Die Inhaltsstoffe der zu untersuchenden Infusionslösung umfassten zum größten Teil Aminosäuren und Peptide, aber auch Hilfsstoffe wie Zitronensäure und Taurin. Zunächst wurde ein qualitatives Verunreinigungsprofil einer gestressten Infusionslösung (Lagerung 12 Monate bei 40°C) erstellt. Die Schwierigkeit bestand darin, eine unbekannte Zahl an niedrig konzentrierten Verunreinigungen bestmöglich von den Inhaltsstoffen, die in der Lösung in vergleichsweise hoher Konzentration vorliegen, aufzutrennen und zu detektieren. Da diese Verbindungen sehr polar sind, können diese mit konventionellen Reversed-Phase (RP) Methoden kaum retardiert werden. Eine weitere Herausforderung bestand darin, die gefundenen Verbindungen, für die größtenteils keine Standards verfügbar waren, zu quantifizieren. Die üblicherweise verwendeten Detektoren wie z.B. UV Detektoren oder Massenspektrometer (MS) weisen eine verbindungsspezifische Detektorsensitivität auf. Deshalb ist es nötig mit authentischen Standards zu kalibrieren. Außerdem besteht bei Verbindungen ohne chromophore Gruppen bzw. solchen Verbindungen ohne ionisierbare funktionelle Gruppen die Gefahr, dass diese mit UV bzw. MS Detektoren nicht erfasst werden können und daher übersehen werden.

Aus diesen Gründen wurde ein umfassender multidimensionaler Analysenansatz entwickelt.

Die Infusionslösung wurde mittels multidimensionaler Chromatographie unter Verwendung einer off-line Kombination aus RP Flüssigchromatographie (Liquid Chromatography, LC) und Hydrophiler Interaktionschromatographie (Hydrophilic Interaction Liquid Chromatography, HILIC) aufgetrennt und mit zwei komplementären Detektoren, einem Charged Aerosol Detector (CAD) mit substanzunabhängiger Signalempfindlichkeit zur Quantifizierung und einem Ionenfallen Massenspektrometer (IT-MS) zur Identifizierung, detektiert. Auf Grund der Eigenschaft des CAD Detektors als universeller Detektor für nicht-flüchtige Verbindungen und seiner relativ strukturunabhängigen Signalempfindlichkeit war es möglich, unbekannte Verbindungen zu detektieren und mit einer universellen Kalibrationsfunktion zu quantifizieren. Dadurch war eine Differenzierung zwischen relevanten und nicht-relevanten Verunreinigungen und eine Klassifizierung der Verunreinigung in solche, die berichtet, identifiziert und qualifiziert (d.h. toxikologisch getestet) werden müssen, möglich. Richtlinien für eine derartige Klassifizierung wurden von der ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) verfasst. Die Kombination mit IT-MS Detektion ermöglichte es, die mit dem CAD gefundenen Verbindungen an Hand ihres Masse-

zu-Ladungsverhältnisses bzw. ihrer Fragmentierungsspektren größtenteils zu identifizieren. Mit Hilfe des multidimensionalen Analysenansatzes konnte erfolgreich ein vorläufiges Verunreinigungsprofil erstellt werden. Hauptsächlich wurden Verunreinigungen gefunden, welche durch 1) Peptidkondensation entstanden sind (Di-, Tri-, Tetrapeptide), 2) Hydrolyse der Glutamin Seitenkette bzw. der Peptidbindung erhalten wurden, 3) durch Cyclisierung von Dipeptiden (Diketopiperazin-Derivate) entstanden sind, sowie 4) durch Cyclisierung von Glutaminsäure zur Pyroglutaminsäure und anschließender Kondensation mit Aminogruppen oder Peptiden gebildet wurden. Zur genaueren Quantifizierung identifizierter relevanter Verunreinigungen wurden drei LC-MS/MS Methoden im Multiple Reaction Monitoring (MRM) Modus entwickelt, validiert und für die Untersuchung von unterschiedlich gestressten Infusionslösungen verwendet.

Im zweiten Projekt mit der Firma Sandoz (Wörgl, Österreich) wurden LC-MS/MS Methoden (MRM Modus) für die Prozessoptimierung und -kontrolle der biotechnologischen Herstellung von β-Lactam Antibiotika erstellt und validiert. β-Lactam Antibiotika werden großtechnisch in Fermentoren mit über 200 m^3 Fassungsvermögen hergestellt. Kontinuierliche Kontrolle des Prozesses ist daher von höchster Wichtigkeit.

Die Gruppe der Zielanalyte umfasste ca. 70 Metabolite (Aminosäuren, organische Säuren, Vitamine, Fettsäuren, biogene Amine bzw. Penicillin und Cephalosporin als auch die Zwischenprodukte ihrer Biosynthese). Die Fragestellung erforderte daher die Entwicklung eines gezielten Analysenansatzes.

Die Zielverbindungen waren sehr divers und wiesen zum Teil beträchtliche Unterschiede in ihren Polaritäten auf. Der überwiegende Anteil der Verbindungen war als eher polar und hydrophil einzustufen. Aus diesem Grund wurde zuerst ein Säulenscreening diverser HILIC Phasen (ZIC-HILIC von Merck Sequant AB, Luna Amino und Luna HILIC von Phenomenex, Biobasic AX von Thermo Scientific, Obelisk N und Obelisk R von Sielc Technologies, TSK-Gel Amide 80 von Tosoh, Acclaim Mixed-mode WAX von Dionex sowie Chromolith Si Performance von Merck), die für die Chromatographie polarer Verbindungen bevorzugt eingesetzt werden, durchgeführt. Auf Grund der Labilität der β-Lactam Antibiotika und ihrer Zwischenstufen im basischen Milieu wurden im Zuge des Säulenscreenings nur saure (pH 3-5) mobile Phase Buffer getestet. Bei einer derartigen Anzahl an Analyten ist eine vollständige Auftrennung mit ein-dimensionalen (1-D) chromatographischen Methoden nahezu unmöglich. Deshalb stand bei der Wahl der endgültigen Analysensäule die Auftrennung kritischer Paare (Isobare, Verbindungen ähnlicher Struktur) im Vordergrund. Für die Unterscheidung nicht-isobarer Verbindungen, wurde die Selektivität der

massenspektrometrischen Detektion ausgenutzt. Eine ZIC-HILIC Säule von Merck Sequant AB wurde schlussendlich für die Methodenfeinoptimierung gewählt. Fettsäuren und einige andere unpolare Verbindungen konnten unter HILIC Bedingungen nicht ausreichend retardiert werden. Für die Analyse dieser eher unpolaren Verbindungen wurde eine zweite Methode unter RP Bedingungen auf einer X-Bridge C18 Phase von Waters erstellt.

Ein häufig auftretendes Problem bei der quantitativen Analyse in biologischen Matrices sind Matrixeffekte. Sie werden durch koeluierende nicht-detektierte Substanzen aus der Matrix verursacht und äußern sich in Signalschwankungen, die quantitative Ergebnisse verzerren können. Aus diesem Grund wurden im Zuge der Methodenentwicklung verschiedene Ansätze wie Matrix-angepasste Kalibrierung bzw. die Verwendung von isotopenmarkierten internen Standards und struktur-analogen internen Standards zur Kompensation von Matrixeffekten getestet und nach ihrem Aufwand/Nutzen Verhältnis bewertet.

Die Präzision der HILIC Methode für 57 Analyte war für drei über den linearen Bereich verteilte Konzentrationen zu 90% unter 10% RSD, wenn keine internen Standards verwendet wurden und zu 70% unter 10% RSD für den Fall, dass interne Standards verwendet wurden. Die Richtigkeit für externe Kalibrierung mit reinen Standardlösungen (ohne interne Standards) war für eine mittlere Konzentration mit nur wenigen Ausreißern innerhalb eines akzeptablen Intervalls von 80-120%. Bei Verwendung von Matrix-angepasster Kalibrierung war die Accuracy noch enger verteilt und lag hauptsächlich zwischen 95 und 105%. Grundsätzlich waren die Validierungsergebnisse für die verschiedenen Kalibrationsansätze zufriedenstellend. Wie erwartet konnte mit Matrix-angepasster Kalibrierung eine bessere Accuracy erzielt werden als mit externer Kalibrierung mit reinen Standardlösungen.

Die Verwendung von internen Standards (isotopenmarkierte und struktur-analoge) hat keine wesentliche Verbesserung der Validierungsergebnisse erbracht. Allerdings haben die Ergebnisse der interday Präzision und Accuracy gezeigt, dass interne Standards zum Teil Geräteschwankungen ausgleichen können.

Bei der Validierung der RP-Methode traten Komplikationen auf. Die Auftrennung von Fettsäuren unter RPLC Bedingungen funktionierte zwar sehr gut, aber starke Memory Effekte, besonders von Ölsäure, Palmitinsäure und Stearinsäure, beeinträchtigen die quantitative Analyse. Diverse Untersuchungen dieses Effekts haben gezeigt, dass dieser chromatographischen Ursprungs ist und nicht durch Verschleppungen bei der Probeninjektion bedingt ist. Da die Richtigkeit bei mittleren und höheren Konzentrationslevels akzeptabel war, sollten bei der Quantifizierung der relativ hohen Konzentrationen in den Extrakten keine ernsthaften Probleme auftreten.

Der lineare Bereich umfasste für die meisten Verbindungen über zwei bis drei Größenordnungen. Durch den Umstand, dass die zu analysierenden Verbindungen in den Fermentationsextrakten in sehr unterschiedlichen Konzentrationen vorliegen, stellen verbindungsspezifische Sensitivitätsunterschiede und der gerätetechnisch limitierte lineare Kalibrationsbereich ein Problem dar. Eine Analyse von mehreren Verdünnungen der Proben ist möglicherweise nötig, damit die Konzentrationen aller Verbindungen über der Bestimmungsgrenze liegen und in den linearen Bereich fallen. In der Prozesskontrolle wird allerdings ein hoher Probendurchsatz gefordert und Mehrfachanalysen sind daher unerwünscht. Trotz allem sind die Methoden für das Monitoring bestimmter Analytgruppen wie z.B. Aminosäuren, die in ähnlichen Konzentrationen vorliegen, durchaus geeignet.

Die Bewerkstelligung von Multikomponentenanalysen ist heutzutage in vielen wissenschaftlichen Bereichen wie z.B. in Metabolomics und Proteomics ein sehr aktuelles Thema. Weiterentwicklungen in der Massenspektrometrie leisten einen wesentlichen Beitrag zum Fortschritt und ermöglichen die Analyse von immer größeren Analytzahlen. Trotzdem ist nach wie vor die Kopplung mit chromatographischen Trenntechniken unerlässlich. Die Stärke von LC-MS/MS Techniken ergibt sich durch die Kombination der chromatographischen Trennselektivität mit der massenspektometrischen Selektivität Verbindungen an Hand ihrer Masse zu unterscheiden, woraus sich ein leistungsfähiges multidimensionales Analysensystem ergibt, welches zum Standard in vielen Fragestellungen moderner Anwendungen geworden ist.

Abstract

Mass Spectrometry-based Multicomponent Analysis in Impurity Profiling and Metabolic Approaches

The main subject of the PhD thesis was the development of multicomponent analysis assays for pharmaceutical applications. The work was carried out in the course of two industry projects.

The objective of the first project with Fresenius Kabi was to establish a comprehensive qualitative and quantitative impurity profile of a recently developed infusion solution intended for parenteral supplementation of amino acids and dipeptides.

First, a qualitative impurity profile of stressed infusion solutions was established. Thereby, a challenge was to achieve separation of low abundant impurities from highly concentrated ingredients. Since main constituents in the infusion solution were highly polar conventional reversed phase liquid chromatography (RPLC) was not suitable for separation. Furthermore, quantification of impurities had to be accomplished without authentic standards. Thus, a multidimensional analysis assay was developed. The infusion solution was submitted to multidimensional liquid chromatography employing a combination of RPLC and Hydrophilic Interaction Liquid Chromatography (HILIC) and two complementary detectors. A charged aerosol detector (CAD) exhibiting consistent detector responses was used for quantification and an ion trap mass spectrometer (IT-MS) for identification. For accurate quantification of identified relevant impurities three LC-MS/MS methods were developed and validated.

The goal of the second project with Sandoz was to develop LC-MS/MS methods intended for process control and optimization of the biotechnological production of β-lactam antibiotics. A RPLC and a HILIC method were developed for the quantification of about 70 metabolites (amino acids, organic acids, vitamins, fatty acids, biogenic amines as well as penicillin and cephalosporin and their corresponding biosynthesis intermediates). Matrix effects constitute a special issue for quantification of compounds in biological samples. They emerge due to coeluting non-detected compounds of the matrix and cause signal fluctuations which may severely distort quantitative analysis results. Thus, in the course of method development several approaches i.e. matrix matched calibration and application of

stable isotope labeled internal standards were investigated with regard to their capability to compensate for matrix effects.

Abstract

Massenspektrometrie-basierende Multikomponentenanalysen zur Erstellung von Verunreinigungsprofilen und für Metabolomics Anwendungen

Der Fokus der Doktorarbeit lag auf der Erstellung von Methoden zur Multikomponentenanalyse für pharmazeutische Fragestellungen im Zuge zweier Industrieprojekte.

Das Ziel des ersten Projekts mit der Firma Fresenius Kabi war die Erstellung eines umfassenden Verunreinigungsprofils einer neu entwickelten Infusionslösung zur parenteralen Zufuhr von Aminosäuren und Dipeptiden.

Zunächst wurde ein qualitatives Verunreinigungsprofil einer gestressten Infusionslösung erstellt. Die Schwierigkeit bestand darin, eine unbekannte Zahl an niedrig konzentrierten Verunreinigungen möglichst gut von den Inhaltsstoffen, die in der Lösung in vergleichsweise hoher Konzentration vorlagen, aufzutrennen und zu detektieren. Die Inhaltsstoffe der Infusionslösung sind sehr polar und können daher mit konventionellen Reversed Phase (RP) Methoden kaum retardiert werden. Eine weitere Herausforderung bestand darin, die gefundenen Verbindungen, für die größtenteils keine Standards verfügbar waren, zu quantifizieren. Aus diesen Gründen wurde ein multidimensionaler Analysenansatz entwickelt. Die Infusionslösung wurde mittels multidimensionaler Chromatographie unter Verwendung einer Kombination aus RP Flüssigchromatographie (Liquid Chromatography, LC) und Hydrophiler Interaktionschromatographie (Hydrophilic Interaction Liquid Chromatography, HILIC) aufgetrennt, und mit zwei komplementären Detektoren, einem Charged Aerosol Detector (CAD) mit substanzunabhängiger Signalempfindlichkeit zur Quantifizierung und einem Ionenfallen Massenspektrometer (IT-MS) zur Identifizierung, detektiert. Zur genaueren Quantifizierung identifizierter relevanter Verunreinigungen in gestressten Infusionslösungen wurden drei LC-MS/MS Methoden entwickelt und validiert.

Im zweiten Projekt mit der Firma Sandoz wurden LC-MS/MS Methoden für die Prozessoptimierung und -kontrolle der biotechnologischen Herstellung von β-Lactam Antibiotika erstellt und validiert. Zur Analyse von ca. 70 Metaboliten (Aminosäuren, organische Säuren, Vitamine, Fettsäuren, biogene Amine bzw. Penicillin und Cephalosporin sowie Zwischenprodukte ihrer Biosynthese) wurden eine RP und eine

HILIC Methode entwickelt. Eine Problem bei der quantitativen Analyse in biologischen Matrices sind Matrixeffekte. Sie werden durch koeluierende nicht detektierte Substanzen in der Matrix verursacht und äußern sich in Signalschwankungen, die quantitative Ergebnisse verfälschen können. Aus diesem Grund wurden im Zuge der Methodenentwicklung verschiedene Ansätze wie Matrix-angepasste Kalibrierung bzw. die Verwendung von isotopenmarkierten internen Standards zur Kompensation von Matrixeffekten getestet, und nach ihrem Aufwand/Nutzen Verhältnis bewertet.

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Sprachkenntnisse

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Französisch Grundkenntnisse

Wissenschaftliche Beiträge

Publikationen

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