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Comprehensive impurity profiling of nutritional infusion solutions by multidimensional off-line reversed-phase liquid chromatography × hydrophilic interaction chromatography–ion trap mass-spectrometry 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 multi-constituent 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 reversed-phase liquid chromatography (RPLC) × hydrophilic interaction chromatography (HILIC) separation and 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. Impurities detected comprised di-, tri- and tetrapeptides, cyclic dipeptides (diketopiperazines), pyroglutamic acid derivatives and their condensation products. Cross-validation with HPLC–MS/MS methods using synthesized authentic standards confirmed the results obtained by the presented multidimensional analysis assay.

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 [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) and relevant degradation products are quantified by so-called stability indicating analysis methods [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 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.

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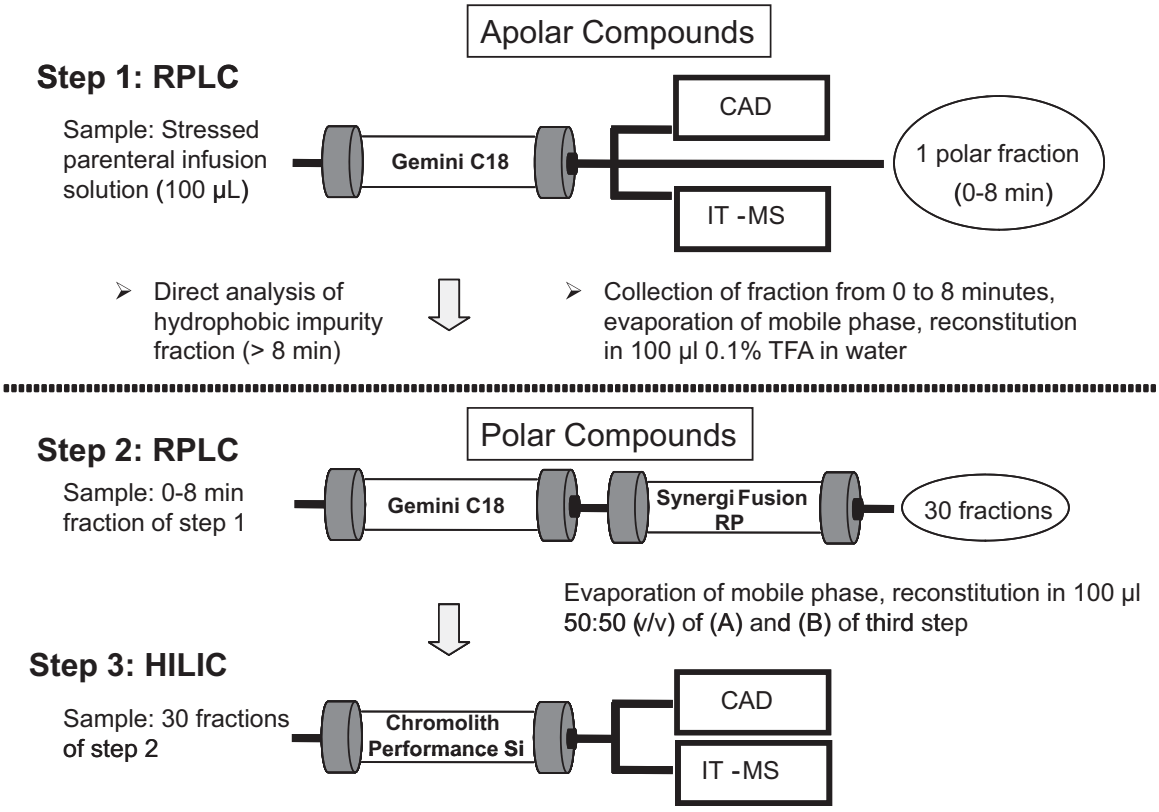

Fig. 1. Scheme of the workflow of the multidimensional analysis assay for establishing the impurity profile of a stressed nutritional infusion solution.

First, when the selectivity and peak capacity of a one-dimensional (1D) LC assay is insufficient to resolve all sample compounds low abundance impurities may easily be masked by high quantity ingredients due to ion-suppression and thus remain undetected, even if highly specific and sensitive mass spectrometric detection is employed. Second, considering the separation of isomeric or more generally isobaric compounds, MS selectivity is insufficient and appropriate chromatographic separation is required. 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 detected 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].

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 with orthogonal retention mechanisms selectivity and peak capacity can be enhanced [14–22]. We used herein a combination of RP and hydrophilic interaction 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 MS^2 mode.

The problem of quantification of impurities with unknown structure was solved by use of a charged aerosol detector (CAD) which is considered a universal detector for non-volatile compounds with relatively consistent detector response [24–26]. A unified calibration function was utilized for this purpose. Comparison between 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 [29–35]. The generated quantitative results allowed classification of the compounds as proposed by ICH guidelines and a ranking of impurities according to their relevance based on concentrations. 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 RPLC × HILIC-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

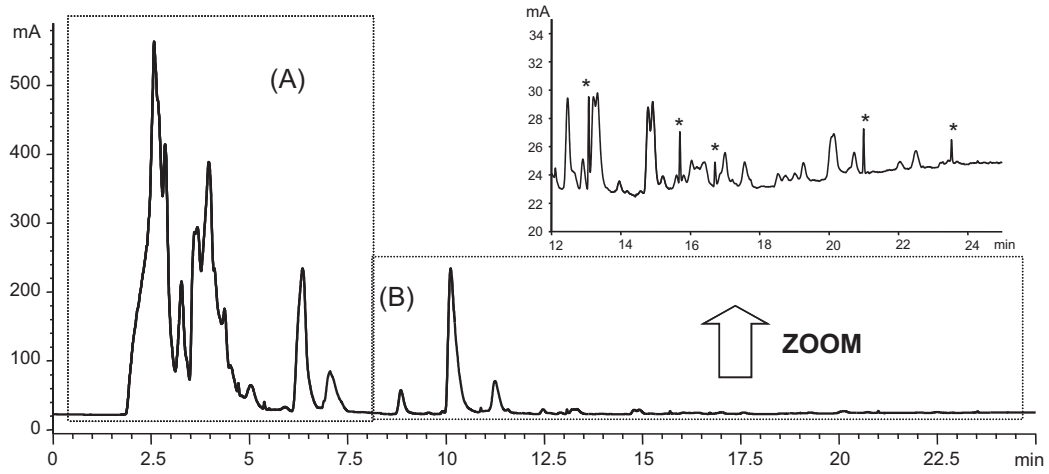

Fig. 2. RPLC chromatogram of the stressed formulation recorded with the CAD detector A Gemini C18 column was employed (step 1). Part (A) contains polar hydrophilic compounds, which were investigated in detail using the multidimensional analysis assay. Part (B) of the chromatogram was directly analyzed using the RPLC method with CAD detection and unified calibration. Peaks denoted with an asterisk are spikes.

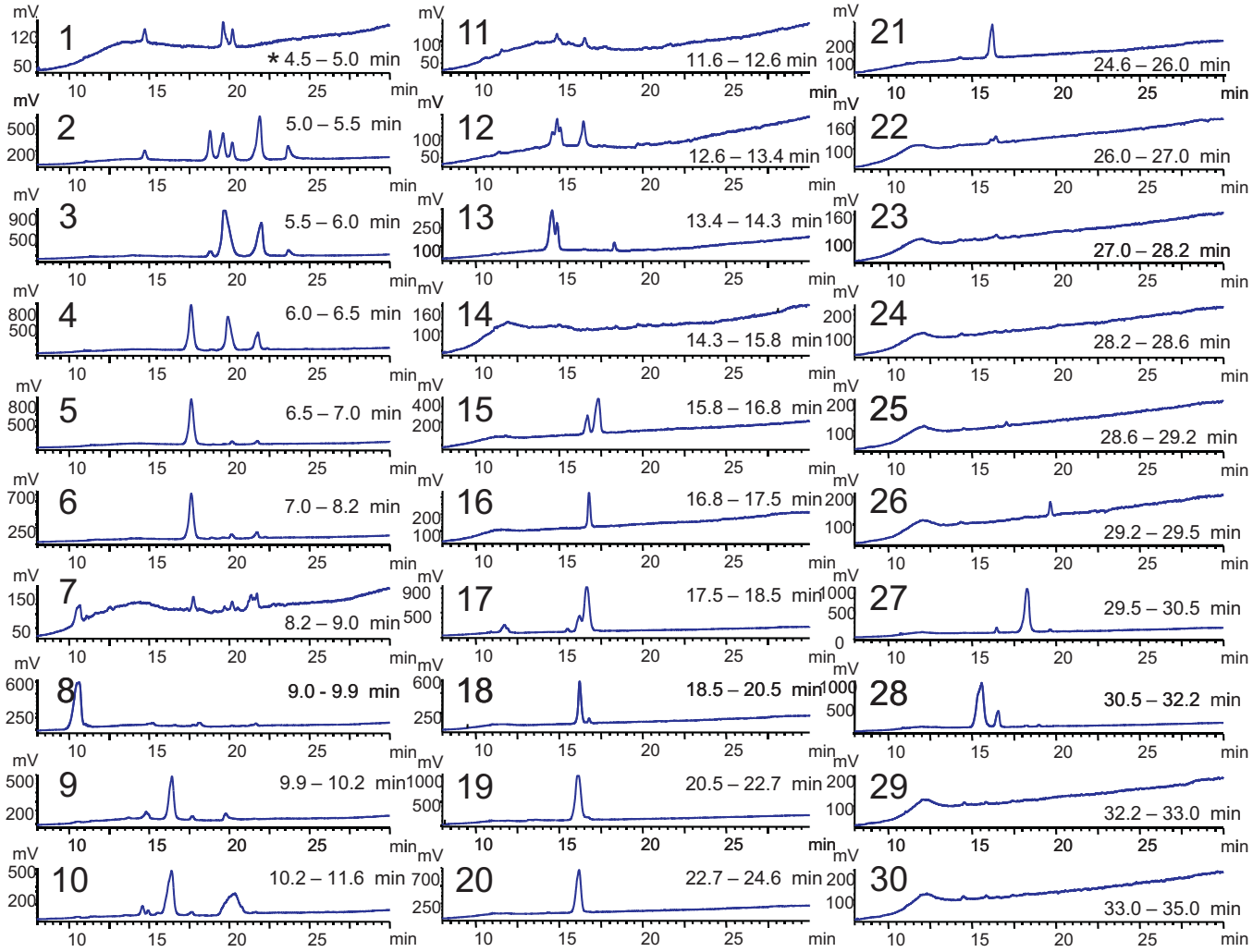

Fig. 3. Representative 2nd dimension HILIC chromatograms (step 3) of the 30 collected fractions from the 1st dimension tandem RP column). The chromatograms were recorded with the CAD. The injection volume was 2 μl for fractions 1, 2, 16, 18 and 20 μl for the remaining fractions. * Fraction collection time intervals.

ingredients 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, GlyTyr, cyclo(AlaGlu), LeuTrpMetArg (LWMR), N,N′-diacetylcystin ($(AcCys)_2$) and cyclo(AlaGln) were from Bachem (Bubendorf, Switzerland). L-Glu and L-Phe were purchased from Sigma–Aldrich (Vienna, Austria). L-Trp, L-Leu and L-pyroglutamic acid (pyroGlu) 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, glacial acetic acid (AcOH) and trifluoroacetic acid (TFA) were obtained from Fluka and formic acid (FA) 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 103–600.

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 Fig. 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 to 8 min was collected into one fraction which was subjected to off-line 2D RPLC × HILIC separation, whereby the second dimension separations on the HILIC columns were performed once by coupling to a CAD and once by hyphenation to IT-MS.

2.4. RPLC separation of the hydrophobic compounds (step 1)

In the first step 100 μl of the stressed infusion solution were injected onto a Gemini C18 column (150 mm × 3.0 mm; 3 μm) equipped with a guard column (4.0 mm × 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 gradient profile was as follows: from 5 to 52.5%B in 30 min, then re-equilibration for 15 min. The flow rate was set to 0.3 ml/min. The column effluent was collected into a single fraction (polar fraction) between 0 and 8 min. 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). Compounds eluting after 8 min were directly analyzed.

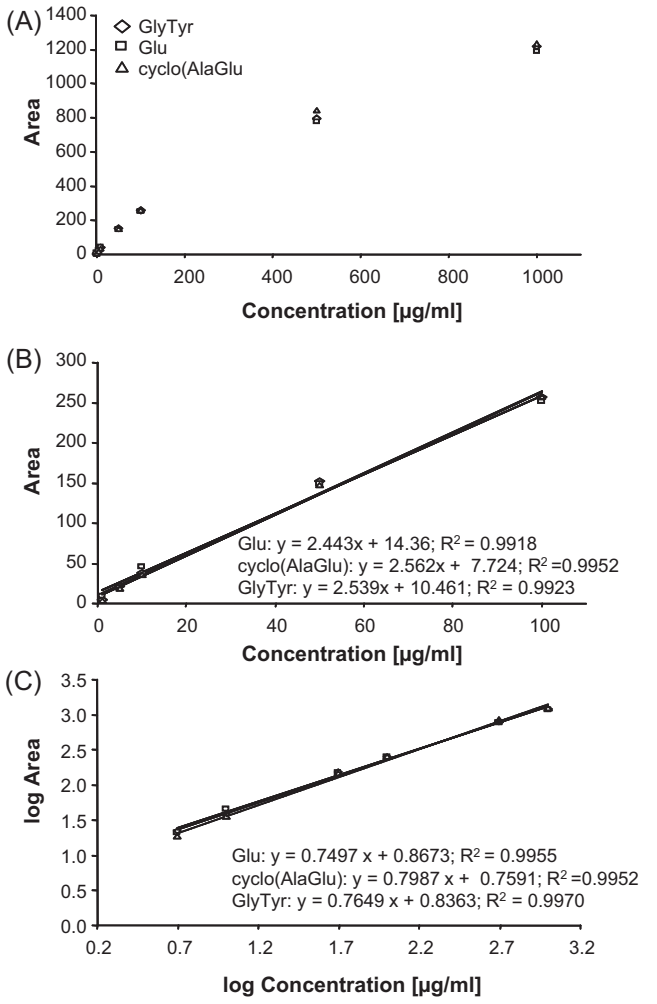

Fig. 4. Calibration curves: entire range (A), linear calibration function (B), double logarithmic over full concentration range (C) of the three compounds Glu, cyclo(AlaGlu) and GlyTyr using the RP method of the first separation step (Gemini C18).

2.5. RPLC prefractionation of the polar fraction (step 2)

A tandem column consisting of Gemini C18 (150 mm × 3.0 mm; 3 μm) equipped with a guard column (4.0 mm × 3.0 mm) coupled in series with a polar embedded Synergi Fusion-RP (150 mm × 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 channel (B) 0.1% TFA in ACN. The following gradient profile was employed: 2% B from 0 to 15 min, then increase to 51% B from 15 to 35 min, then reequilibration for 10 min. The flow rate was 0.3 ml/min. 30 fractions were collected into Eppendorf vials with sampling intervals as indicated in Fig. 3. The same run was repeated. The corresponding fractions from the two injections were combined and evaporated to dryness.

2.6. 2nd dimension HILIC separation (step 3)

The fractions collected from step 2 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) consisted 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 mm × 4.6 mm) from Merck (Darmstadt, Germany) was employed as column. Two different gradients were used. The low elution strength gradient was as follows: from 100% to 52.5% B in 30 min at a flow rate of 0.5 ml/min, then reequilibration for 10 min at a flow rate of 1 ml/min. The high elution strength gradient was as follows: from 100% to 52.5% B in 30 min at a flow rate of 0.5 ml/min, then steep decrease of %B to 7.5% within 1 min, hold of 7.5% B from 31 to 50 min at 1 ml/min, then reequilibration for 10 min at a flow rate of 1 ml/min.

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 HILIC system connected to the CAD. Furthermore, a third run was conducted with an injection volume of 10 μl on the HILIC 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 high elution strength gradient as well.

2.7. Calibration

Calibration of the RP method, which was used for the separation in step 1, 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 in step 3 used as second separation dimension for the polar fractions collected in step 2, calibration functions were constructed for cyclo(AlaGln), GlyTyr, AlaGlu, Glu, Gln, Trp, Leu and pyroGlu at concentrations of 1, 5, 10, 50, 100, 500 and 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

Initial RPLC runs of the stressed pharmaceutical formulation composed of amino acids and two dipeptides (stored at 40 °C for 12 months) revealed the majority of compounds eluting unresolved close to the front. 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 2D separation using a combination of RP and HILIC was envisioned.

The late eluting compounds from the RPLC column were directly analyzed (Fig. 2, part B). Early eluting substances of the RPLC chromatogram (<8 min) were collected into a single fraction (Fig. 2, part A). All active ingredients were eluted before 11 min and no impurities were eluting after 24 min. The polar fraction from step 1 was re-injected into a Gemini C18 column coupled in series with a more polar Synergi Fusion-RP column (step 2). On this tandem column, the hydrophilic compounds were spread over a wider retention time window facilitating separation in the HILIC separation dimension. 30 fractions were collected (sampling times are indicated in Fig. 3). In the early eluting part a sample was taken every half minute, while this sampling period was 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 increased undersampling.

All 30 fractions were finally analyzed three times by HILIC (step 3): 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 Fig. 3. 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 high background, ions in the mass spectra stemming from the column bleed would complicate the interpretation of the MS spectra in the course of structure elucidation of unknown impurities. For this reason, a bare silica monolith column Chromolith Performance Si was selected.

Blank injections confirmed absence of strong background signal and compatibility of the silica monolith with both the CAD and the IT-MS Moreover, the selected HILIC method showed sufficient complementarity [41] to the first dimension RPLC separation (see Fig. S1 of Supplementary Material). Further, column and separation conditions were selected which minimized the risk that impurities are not eluted from the columns, as they would be not detected and not show up on the impurity list. The RP method in the first separation step had weak retentivity for the polar compounds so that one can be sure everything was eluted. Likewise, in HILIC separation more strongly retentive ion-exchangers were avoided for this reason. Since basic amino acids (e.g. Arg and Lys) and corresponding peptides might give strong adsorption on the weakly acidic silanol surface of the Chromolith, a second run for each of the early very polar fractions 1–10 from the tandem RP column of step 2 was performed with a stronger eluting gradient (see Section 2.6).

An off-line multidimensional LC 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, 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.

3.2. Calibration

The suitability of the CAD as universal detector for preliminary quantification by using universal calibration functions 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 step 1. 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 Fig. 4A, 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 (nearly) a linear trend (see Fig. 4B). Alternatively, double logarithmic calibration functions can be used over the full concentration range (Fig. 4C).

It becomes also evident from Fig. 4 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 1. 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, a closer look reveals that individual slopes vary systematically. Slopes of calibration functions for later, i.e. with higher organic content, eluting compounds are significantly larger (see Table 1 and Fig. S2 of Supplementary information). 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 technically simpler and more straightforward strategy is to use a correction function to calculate calibration slopes specific for a certain retention time. If individual slopes of calibration functions from Table 1 (RP-method) are plotted versus % of organic modifier at the elution time a linear relationship ($y = 0.0894x + 1.5641$, wherein y = corrected slope and x is %B at elution time) with coefficient of determination $R^2 = 0.9034$ can be obtained (see Fig. S2A of Supplementary Information). This way it is possible to calculate corrected slopes specific for each compound in dependency of their retention times. The same trend was also noticed for the HILIC method (Table 1 and Fig. S2B of Supplementary Material). The slopes deviated within the set of distinct compounds by 14% RSD in the range of 65-85% ACN with larger slopes for compounds that elute at higher percentage of ACN. Corrected slopes in dependence of elution times can be calculated from the linear trend line (see Fig. S2B of Supplementary Information).

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 subjected to preliminary validation 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 distinct ACN content of eluates in 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 to 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 2). 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 (Fig. S2 of Supplementary Information). 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 75–130% (acceptable for a preliminary rough quantification). 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 1. 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.

The components that eluted in the RP method (Fig. 2) after 8 min were directly analyzed by this method (one injection of 20 μl 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 full list of detected impurities and results of the quantitative analysis by the RPLC-CAD method are summarized in Table S1 (see Supplementary Information) along with respective retention times and m/z of the respective components. Several of the impurity peaks were coeluting or partially overlapping (see Fig. 2, 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

Table 1
Linear calibration functions for individual standards.

Compound	%ACN[a]	Slope	Intercept	R^2	Corrected slopes[c]
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[b]		3.12	10.51		
Standard 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[b]		14.92	−4.81		
Standard dev.		2.1	37.6		
%RSD		14.3	−782.1		

[a] Content of ACN in the mobile phase at individual elution times.
[b] unified calibration function.
[c] Calculated using linear functions established by plotting slopes versus % ACN as shown in Fig. S2 of Supplementary Material.

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 S1 (Supplementary Information), 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. A number of relevant impurities above reporting, identification, but also qualification threshold remained (see Table S1, Supplementary Material). 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 (data not shown).

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 (Fig. 3). The complete list of components detected in the 30 chromatograms is presented in Table S2 (see Supplementary Material). 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

Table 2
Validation results of the RP method ($n = 6$) and the HILIC method ($n = 5$) at a concentration level of 10 μg/ml and 5 μg/ml, respectively.[a]

Compound	Intraday precision	Accuracy compound-specific calibration function	Accuracy unified calibration function	Accuracy slope-corrected calibration function[b]
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

[a] 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).
[b] Slopes of calibration functions were calculated for each compound using the linear relationship established in Fig. S2. The same intercept as for unified calibration functions were used (see Table 1).

Table 3
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, 261, 477, 498	89, 130, 147, 173, 184, 201, 136, 182, 193, 221
GlyTyr	239		
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

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. 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. Table 3 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 (i) concentrations as determined by RPLC-CAD and HILIC-CAD, respectively, (ii) MS(MS) data of specific peaks in native form or after derivatization of respective fractions with Sanger's reagent, as well as (iii) retention times of standards of known ingredients (see Table 3 and Fig. S3 of Supplementary Material).

More challenging was the identification of unknown impurities and those with higher molecular weight and peptidic nature, respectively, were of particular interest. For example, in fraction 6 at 22.3 min of the HILIC chromatogram (step 3) a peak was detected by the CAD (see Fig. 5A). In the corresponding spectrum, *m*/*z* 418 was found to be the dominant mass besides several others (Fig. 5B). The extracted ion chromatogram (Fig. 5C) revealed clearly two peaks between 20 and 25 min with same *m*/*z* supposed to be isobaric components. Examination of the fragmentation spectra provided structural information, which allowed to identify the compounds as AlaGlu(AlaGln) (Fig. 5D) and AlaGluAlaGln (Fig. 5E), respectively. Structure identification was confirmed with the help of standard compounds.

Similarly, the CAD chromatogram in Fig. 6B shows three low abundance impurities which can be also found in the TIC of the IT-MS run. 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 allowed to conclude 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 (Fig. 6). Unfortunately, it was not possible to differentiate whether the basic amino acid was attached at the C-terminal or side chain carboxylic group. Further elucidation of these structures with synthesized standards of these isomeric forms could clarify this matter [36].

Several other impurities were found, quantified and identified including cyclo(AlaGln), cyclo(AlaGlu), AlaAlaGln, amongst others. Extracted ion chromatograms (EIC) and fragmentation spectra are shown in Fig. S4 of Supplementary Material. 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]. Similarly, in the RP method of step 1 (13.2 min) two ions with *m*/*z* 332

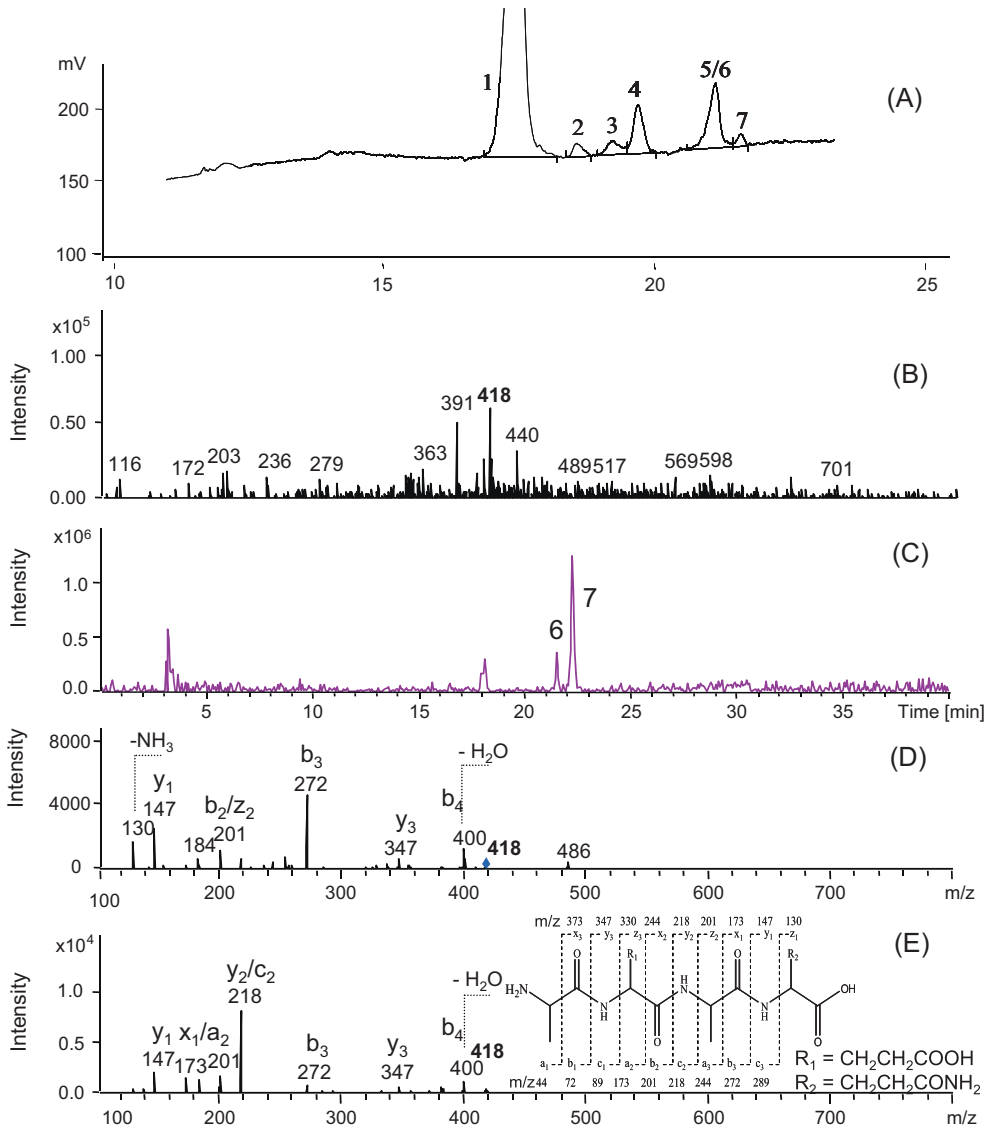

Fig. 5. Evaluation of fraction 6 of tandem Gemini C18/Synergi Fusion-RP column: (A) HILIC-CAD chromatogram of fraction 6, (B) scan spectrum of peak 6, (C) extracted ion chromatogram of *m/z* 418: (6) AlaGlu(AlaGln); (7) AlaGluAlaGln, (D) fragmentation spectrum of AlaGlu(AlaGln), (E) fragmentation spectrum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

Table 4
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.[b]
14.8	201	Cyclo(AlaGlu)/pyroGluAla	332.5[a]	275[a]	386.4[a]
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 pyroGluAlaGly-Tyr/pyroGluAlaMet	30.8[a]	n.a.	4.5[a]

[a] Determined as the sum of the indicated compounds.
[b] Not available.

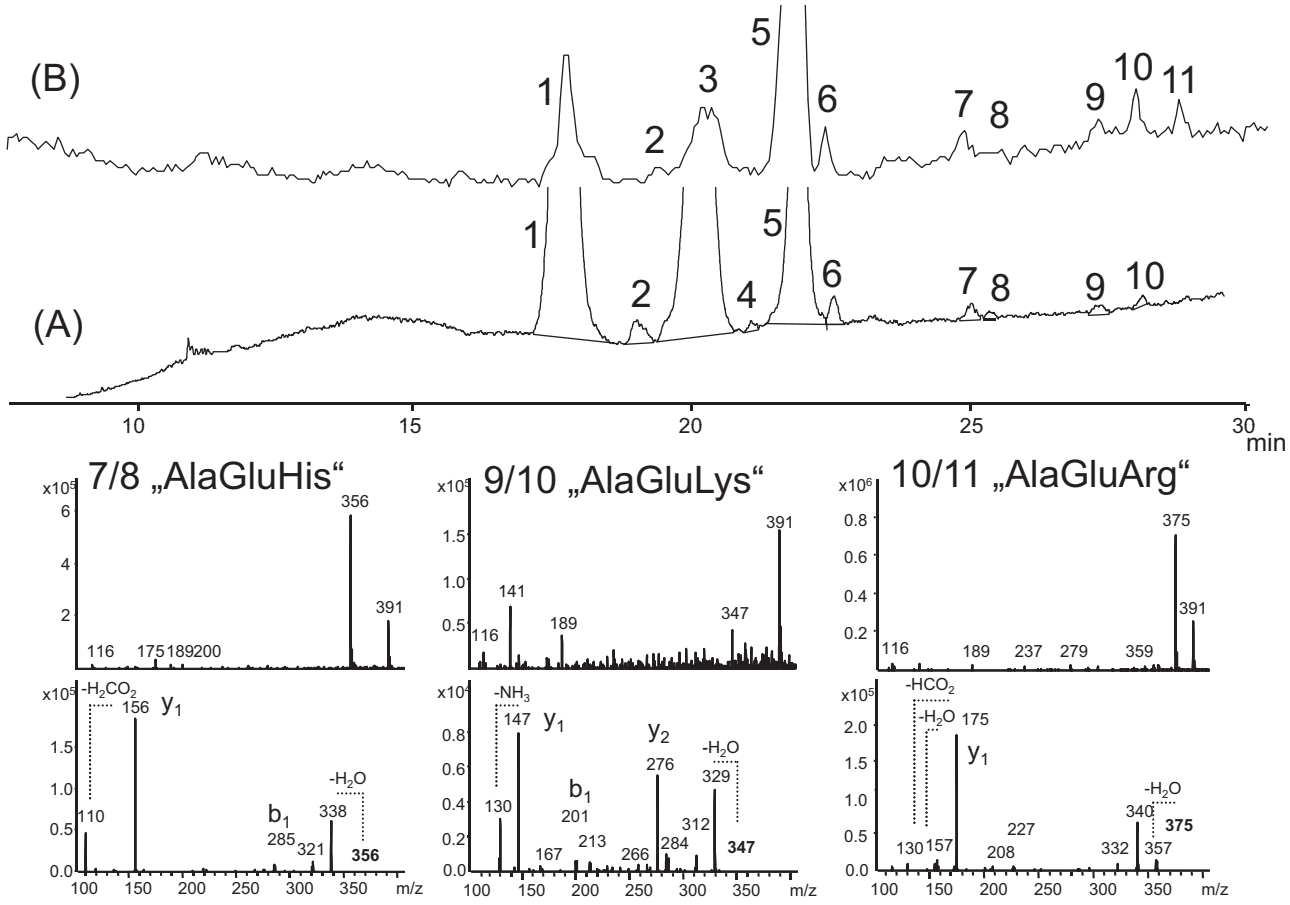

Fig. 6. Evaluation of fraction 4 of tandem Gemini C18/Synergi Fusion-RP column: top: (A) CAD chromatogram and (B) TIC trace of the ion trap of fraction 4; bottom: spectra of peaks 7–11 and corresponding fragmentation spectra (below). Peak annotation: 1 Val; 2 *m*/*z* 369; 4 no specific *m*/*z* found;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

and 421 were detected, and for each two isobaric structures were found to match the fragmentation spectra. For *m*/*z* 421 the structures pyroGluAlaGlyTyr or cyclo(AlaGlu)GlyTyr were suggested and for *m*/*z* 332 pyroGluAlaMet and cyclo(AlaGlu)Met, respectively (Fig. S4 of Supplementary Information). Also these compounds could be identified after standards have been supplied as described in detail elsewhere [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 derivatization with Sanger's reagent and complementary chromatographic conditions. Unfortunately, several of these unknown peaks in the CAD chromatograms remained unidentified.

Yet only a few impurities above the identification/qualification threshold could not be identified. 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; calculations according to ICH guideline Q3B, R2) and thus, need to be examined with regard to potential bioactivity.

3.4.3. Cross-validation

Quantitative results of compounds 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 4). However, it was striking that quantitative results obtained with 2D 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 complementary detectors. 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.01.009.

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