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Enantioselective multiple heartcut two-dimensional ultra-high-performance liquid chromatography method with a Coreshell chiral stationary phase in the second dimension for analysis of all proteinogenic amino acids in a single run☆

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ABSTRACT

A multiple heartcut (MHC) 2D-UHPLC method with UV detection has been developed for the enantioselective analysis of complex amino acid mixtures in a single run. The MHC method is based on an achiral gradient RPLC separation with 1.8 μm C18 phase (100 × 2.1 mm ID column) in the first dimension (^{1}D) and enantioselective isocratic separation on a *tert*-butylcarbamoylquinine-based 2.7 μm Coreshell particle column (50 × 3 mm ID) in the second dimension (^{2}D). Pre-column derivatization has been performed with Sanger's reagent (2,4-dinitrofluorobenzene) yielding chromogenic 2,4-dinitrophenylated amino acids (DNP-AAs). Heartcuts of 40 μL fractions of the ^{1}D peaks were sampled into the ^{2}D system via a two-position four-port dual valve connected to two loop decks each equipped with six 40 μL parking loops. Using this setup, 25 amino acids (20 proteinogenic plus allo-Thr, allo-Ile, homoserine (Hse), Orn, β-Ala) have been analyzed enantioselectively in a fully automated manner with a single chiral column within 130 min total run time (^{1}D and ^{2}D). All ^{2}D separations together took 101.5 min (29 cuts with 3.5 min run time each) and thus the total analysis time was quite efficiently utilized. Faster separations were restricted by some software constraints which did not allow to adjust run times in ^{2}D individually. The practical utility of this enantioselective MHC method is documented by application for the absolute configuration determination of the amino acids in gramicidin and bacitracin. Further optimizations should lead to a generic enantioselective amino acid analyzer for the quality control of synthetic peptides and the structural characterization of non-ribosomal peptides.

1. Introduction

Synthetic and non-ribosomal peptides are gaining interest as novel therapeutic agents, antibiotics, and diagnostics. For their full structural characterization and quality control, the analysis of the amino acid enantiomer composition and stereochemical integrity is part of common protocols in QC/QA [1]. In practice, this is accomplished by enantioselective amino acid analysis after chemical digestion.

The benchmark method for the simultaneous analysis of complex amino acid enantiomer mixtures is enantioselective GC using Chirasil-Val and double derivatization (e.g. trifluoroacetylation at *N*-terminus and esterification at *C*-terminus) of resultant free amino acids of peptide hydrolysate [2,3]. A strength of this method is its consistent enantiomer elution order and the good chemoselectivity between isobaric amino acids such as Thr and allo-Thr as well as Leu, Ile, and allo-Ile. Difficulties, on the other hand, arise for polar amino acids such as Arg, His and Cys (unless an additional side chain derivatization concept is introduced). Other enantioselective GC methods for amino acid analysis have been reported as well [4]. Electrokinetic methods (CE and MEKC) have

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also been quite powerful methods for resolving complex amino acid mixtures [5–8]. On the LC side, the most prominent methods are those based on indirect approach [9], using Marfey's reagent [10], 1-(9-fluorenyl)ethyl chloroformate (FLEC) [7] or the OPA method (*ortho*-phthaldialdehyde/*N*-acetyl-L-cysteine derivatization) [11] followed by reversed-phase separation of the resultant diastereomeric derivatives after pre-column derivatization. The advantage of these indirect approaches is that one can take benefit of the high efficiencies and high peak capacities of RP columns, but the approach may suffer from problems such as enantiomeric impurities in chiral derivatizing agent, kinetic resolution and racemization during derivatization (which all may lead to false quantitative results). Direct LC enantiomer separation of a complex amino acid mixture typically requires MS detection [3,12–14] in order to overcome the problem of poor chemoselectivity of chiral stationary phases and overlaps of distinct amino acids through the assay specificity of MS detection.

On the other hand, online 2D-LC approaches [15] have also been proposed to resolve complex amino acid mixtures, often in the context of their analysis in complex matrices [16]. Multiple heartcut (MHC) methods have been reported for this purpose [17–19]. Such column coupling technologies are not new in the field of enantioselective LC [20–24]. It has been a common strategy in enantioselective bioanalysis before tandem mass spectrometry has been broadly available in (bio)analytical labs and before triple quadrupole instruments have become the prime detection principle in quantitative bioanalysis. In the majority of cases of enantioselective MHC 2D-LC analysis of amino acids, a limited number or a subset of amino acids of particular interest have been analyzed by MHC technology [25–28]. The major challenge is to cover all 20 proteinogenic amino acids plus some practically relevant biogenic congeners such as allo-Thr and allo-Ile within one analytical run in acceptable analysis time i.e. comparable to one-dimensional enantioselective GC without MS detector. Multiple chiral columns were often necessary because it is still challenging to get a full separation of all amino acids by a single CSP. Hamase and coworkers have proposed 2D and 3D-LC workflows involving 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD)-tagging by precolumn derivatization, RP achiral separation on a monolithic C18 silica microcolumn and chiral separation on narrowbore columns packed with Pirkle-type CSPs in the second dimension [29]. The fraction transfer involved a multiloop valve, e.g. with 9 loops in which the fractions from the first dimension were stored until analysis in the second dimension [17]. With such a setup, D-amino acids in complex biomatrices have been analyzed as disease biomarkers [29].

The aim of the present work was to develop a fully automated 2D-LC workflow for the enantioselective separation of all proteinogenic amino acids (plus allo-Thr, allo-Ile, Hse, Orn, β-Ala) in a single run with pre-column derivatization by Sanger's reagent, fast separations in the second dimension using Coreshell particle CSPs based on *tert*-butylcarbamoylquinine selector [12] (Fig. 1) and UV detection. The targeted field of application is the determination of the absolute configurations in non-ribosomal peptides such as gramicidin and bacitracin.

2. Materials and methods

2.1. Materials

Superficially porous particles (SPP) covalently modified with *tert*-butylcarbamoylquinine (tBuCQN) were used in the second dimension and prepared using established methods [12,30]. In short, Halo Sil 2.7 μm (1.7 μm solid core), 90 Å (from Advanced Materials Technologies, Wilmington, DE USA) was modified with (3-mercaptopropyl)trimethoxysilane (1.5 mmol/g silica,

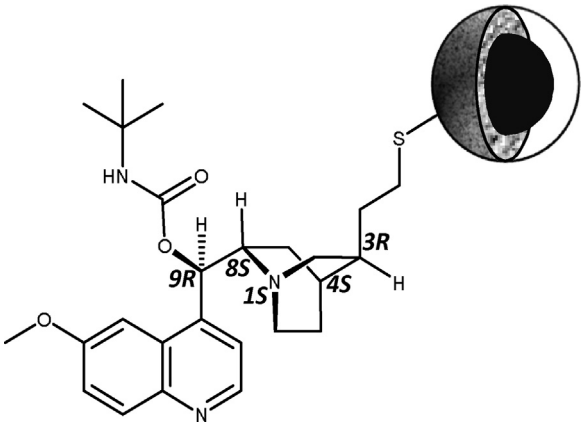

Fig 1. Structure of Coreshell tBuCQN-based chiral stationary phase.

from ABCR, Karlsruhe, Germany) by refluxing it in dry toluene in presence of small quantities of 4-dimethylaminopyridine (0.04 mmol/g silica). Subsequently, in-house synthesized tBuCQN was bonded to 3-mercaptopropyl-modified SPP by thiol-click reaction (0.6 mmol/g silica) using azobisisobutyronitrile (0.07 mmol/g silica) as radical initiator and methanol as solvent. These particles were slurry packed into a stainless steel column of 50 × 3 mm ID. A Zorbax SB-C18 (100 × 2.1 mm ID, 1.8 μm) column from Agilent Technologies (Waldbronn, Germany) was used for the first dimension separation.

Gramicidin with a specified composition of 73.2% Gramicidin A1 and a total content of 98.2% Gramicidin A1, A2, B1, C1 and C2 was from Alfa Aesar (Karlsruhe, Germany). Bacitracin was provided by Sigma Aldrich (Munich, Germany). Reference amino acids were obtained from various vendors (as specified in Suppl. Table S6). Peptide hydrolysis and preparation of DNP-amino acid samples by precolumn derivatization is described in supplementary data (Section 2).

HPLC grade methanol and acetonitrile were supplied by VWR (Fontenay-sous-Bois, France). Ammonium acetate, acetic acid, formic acid, ammonium formate and ammonia were purchased from Sigma Aldrich. Highly purified water for chromatography was obtained by an Elga PureLab Ultra purification system (Celle, Germany).

Sodium hydrogen carbonate, sodium carbonate, and 1-fluoro-2,4-dinitrobenzene (DNFB, Sanger's reagent) were supplied by Sigma Aldrich. Dichloromethane (HPLC-grade) was from Fisher Scientific (Loughborough, UK).

2.2. Instrumentation

Multiple heart cutting online-2D-LC experiments were performed using an Agilent 1290 Infinity II 2D-LC Solution from Agilent Technologies (Waldbronn, Germany) with components as follows: ^{1}D-Quaternary low-pressure gradient UHPLC pump (G7104A), ^{1}D-autosampler (G7167B), ^{1}D-column compartment (G7116B), ^{1}D UV-detector (G7114B) with 14 μL flow cell (G1314-60186) and a pressure relief device (G4236-60010) between ^{1}D UV-detector and ^{1}D/^{2}D-interface, ^{2}D-binary high-pressure gradient UHPLC pump (G7120A), valve drive (G1170A) with two-position four port dual valve and connected with two multiple heart-cutting valves (G4236A) carrying six 40 μL parking loops each as ^{1}D/^{2}D-interface, ^{2}D-column compartment (G7116B) and ^{2}D DAD (G7117B) with 1 μL flow cell (G4212-60008). The instrument was controlled with Open Lab CDS Rev. C.01.07SR3.

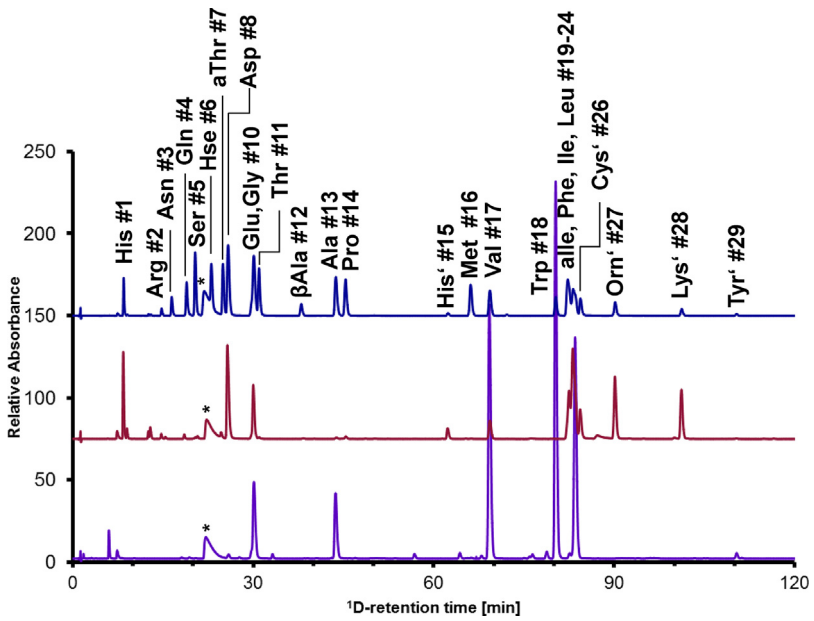

Fig. 2. ^{1}D RP separations of DL-mix (blue), Bacitracin (red) and Gramicidin (purple). *Reagent peak; for zoomed region of isobaric leucines see Supplementary Fig. S1. (Corresponding ^{2}D separations of sampled fractions are depicted in Fig. 5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Online MHC 2D-LC method for complete amino acid mix

In the first dimension, an achiral reversed-phase separation of the DNP-amino acid mixtures was carried out. Thus, a Zorbax SB-C18 (100 × 2.1 mm ID, 1.8 μm) column was operated with the following conditions: Mobile phase, (^{1}A) water + 0.05% formic acid (v/v), (^{1}B) acetonitrile + 0.05% formic acid (v/v); flow rate, 0.25 mL/min; gradient, 10%-50% ^{1}B in 120 min; column temperature, 25 °C; detection, UV at 360 nm (40 Hz sampling frequency).

Enantiomers were then isolated from the achiral column in online-multiple heartcutting mode and subjected to enantioselective 2D-analysis on a home-made tBuCQN-Coreshell CSP (50 × 3 mm ID, 2.7 μm diameter, 1.7 μm solid-core diameter, 90 Å pore size). Fractions were isolated into MHC-loops (40 μL) according to a previously established timetable (see Suppl. Table S1). For ^{2}D-chiral analysis the following conditions were applied: mobile phase, (^{2}A) methanol/water (98/2, v/v) with 100 mM formic acid and 100 mM ammonium formate, (^{2}B) methanol/water (98/2, v/v) without additive; flow rate, 2 mL/min; mobile phase composition, isocratic separations in each ^{2}D-analysis with 25% ^{2}A/75% ^{2}B (25 mM formic acid and 25 mM ammonium formate, for His, His' and Arg), 50% ^{2}A/50% ^{2}B (50 mM formic acid and 50 mM ammonium formate, for Asn, Gln, Ser, Hse, allo-Thr, Gly/Glu, Thr, Ala, β-Ala, Pro, Met, Val), 75% ^{2}A/25% ^{2}B (75 mM formic acid and 75 mM ammonium formate, for Trp, allo-Ile, Ile, Leu, Phe, Cys', Orn', Lys', Tyr') and 100% ^{2}A (100 mM formic acid and 100 mM ammonium formate for Asp) (note, dashed means double DNP-labeled); column temperature, 45 °C; detection, DAD set to 360 nm at 40 Hz sampling frequency. The second dimension cycle time was 3.5 min.

3. Results and discussion

3.1. Optimization of the ^{1}D gradient RPLC method

The ^{2}D chiral separation on the weak anion-exchange Coreshell tBuCQN-CSP requires tagging of the amino acids to convert them into acidic amino acid derivatives (DNP-AAs). This provides the amino acids with suitable chromophoric groups for sensitive detection by UV, suitable for the intended application of enantioselective amino acids analysis in hydrolyzed peptides. Acidic analytes are typically separated in RPLC by use of 0.1% trifluoroacetic acid (TFA) as additive to the eluent (water in channel A and acetonitrile in channel B). This secures sufficient retention due to suppression of dissociation of the carboxylic acid group and provides excellent peak shapes in RPLC. In the current approach, TFA was replaced by formic acid (FA). TFA is a strong counterion in weak anion-exchange chromatography. Since the eluent of ^{1}D is the sample matrix in ^{2}D, its elution strength must match with the ^{2}D separation. It must have weaker elution strength than the ^{2}D eluent in order to avoid peak broadening and peak distortions. FA is a weaker counterion than TFA and thus better compatible with the ^{2}D separation. Furthermore, its concentration has been decreased to 0.05%. The peak shape in ^{1}D is not compromised by this reduction of the FA concentration, yet the elution strength in ^{2}D is further significantly reduced, thus better compatible.

The gradient time in the first dimension is dictated by the speed of the ^{2}D chiral separations on the one hand and the number of available parking loops on the other. The adjusted run time in the ^{2}D separations is 3.5 min × 29 cuts (Suppl. Table S1) which makes a total ^{2}D run time of 101.5 min (*vide infra*). This would be the minimum total run time. Furthermore, the elution times in the ^{1}D chromatogram need to be adjusted so that at least one free loop is available for fraction storage. Under this umbrella, the ^{1}D RPLC gradient separation has been established by DRYLAB based on two linear gradients with same start and end%B differing by factor of 3 in gradient time. A straightforward linear gradient with a maximum of 120 min gradient time was considered acceptable for the present purpose. A simple optimization on basis of 2 experimental runs with gradients of 5–95%B in 60 min and 180 min was done. A linear gradient, from 10% to 50% B in 120 min was finally selected. This maximum gradient time was required due to the critical set of isobaric amino acids allo-Ile, Ile, and Leu. Other components coeluted in the ^{1}D RPLC separation such as Hse/reagent peak, Glu/Gly and Phe/Ile were not critical because of sufficient chemoselectivity in the ^{2}D separation.

The chromatogram of a mixture of all examined amino acids (20 proteinogenic plus allo-Thr, allo-Ile, Hse, Orn, β-Ala) is depicted in Fig. 2 (top trace). It can be seen that each amino acid elutes as a

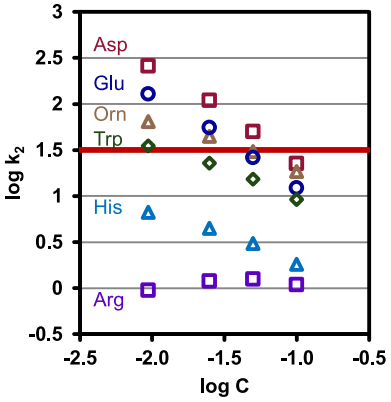

Fig. 3. Control of retention factors of various DNP-amino acids by counterion-concentration (stronger retained enantiomers shown).

single peak except for His which appears as mono- and bis-DNP-labeled amino acid. The majority of amino acids are separated from each other, except for a few uncritical ones (*vide supra*). The isobaric amino acids Thr and allo-Thr, the enantiomers of which coelute in the ^{2}D chromatogram if not separated in ^{1}D, as well as Hse are well resolved. On the other hand, the RPLC separation of the isobaric amino acids allo-Ile, Ile, and Leu is not fully optimal. The partial separation, however, allows a distinction of these amino acids by smart cutting strategy (rising and tailing edges) (*vide infra*) (Supplementary Fig. S1). Overall, a sufficient ^{1}D separation of the complete mixture was obtained in less than 120 min.

3.2. Optimization of the ^{2}D isocratic chiral separation method

The ^{2}D chiral separations must be performed as fast as possible in order to keep the total run times acceptable. Coreshell particle columns are certainly supportive in this regard [12]. However, it is still difficult to achieve fast separations with the same method for all proteinogenic amino acids on a Coreshell tBuCQN-CSP column. Some acidic amino acids (Asp, Glu) as well as double-labeled amino acids (Orn, Lys) require long run times, when using conditions that are proper for non-polar (single labeled, such as Val, Ile, Phe) and polar neutral AAs (such as Asn, Gln, Ser). On the other hand, basic (single labeled, such as Arg) may not be sufficiently retained with mobile phases having higher elution strength. Therefore, a MHC-method was developed by using the gradient shift module. It allows to program isocratic ^{2}D-separations with ionic strengths individually optimized for each DNP-AA.

The rationale for the optimization of the ^{2}D chiral separation is illustrated in Fig. 3. It shows the influence of the ionic strength on retention factors for the most (Asp, red square; Glu, blue circle; Orn, gold triangle) and least retained DNP-AAs (Arg, purple square; mono-DNP-His, blue triangle). These amino acids bracket all the others and are therefore most critical to the separation and analysis time, respectively. Retention factors typically decrease with increase of ionic strength on this WAX-type CSP. Arg with its guanidinium side chain exhibits a peculiar behavior. Retention first increases with increase of ionic strength due to shielding of repulsive electrostatic interaction at high ionic strength. At higher ionic strength attractive electrostatic interactions seem to dominate and thus a further increase of ionic strength reduces retention. It is important to point out that a lower ionic strength produces much better selectivities between different amino acids because they spread over a wider elution window, however, at expense of run time (Fig. 3). Squeezing the 24 heartcuts or more into one 2D-LC run, requires log *k* values smaller than 1.5 (which corresponds to 3.5 min in a MHC method) to keep the overall run time reasonable (within 2.5 h). Finally, ionic strengths (i.e. equal molarity of formic acid and ammonium formate) of 25 mM for His, His' (double DNP labeled) and Arg, 50 mM for Asn, Gln, Ser, Hse, allo-Thr, Gly, Glu, Thr, βAla, Ala, Pro, Met, Val, 75 mM for Trp, allo-Ile, Ile, Leu, Phe, Cys', Orn', Lys', Tyr' (dashed means double DNP-labeled) and 100 mM for Asp have been adjusted to elute all amino acids within a ^{2}D run time of 3.5 min. Unfortunately, the software does not allow to adjust distinct flow rates or distinct analysis times in the different ^{2}D separations. A lower flow rate for mono-DNP-His and DNP-Arg could improve their enantiomer separation to a full baseline separation. On the other hand, total analysis time could have been reduced if the run time in the individual ^{2}D separations can be adjusted specifically. Currently, the longest retained enantiomer pair has dictated the ^{2}D run time.

An important optimization parameter of the ^{2}D chiral separation was the temperature. By increase of the temperature from 15 °C to 45 °C the retention times could be significantly reduced (ca. factor 1.5) (Supplementary Fig. S2), at expense of separation factors which dropped a little bit (Supplementary Table S2). However, the slight loss of selectivity was more than compensated by higher efficiencies. The chromatographic results of the finally resulting ^{2}D chiral separations are summarized in Table 1. As can be seen the elution order is always L- before D-enantiomer except for Glu and Orn which show reversed elution order.

3.3. Establishing the MHC 2D-UHPLC method

A multiple heartcutting interface consisting of a two-position four-port duo valve equipped with two parking loop decks each with six 40 μL loops was used to hyphenate the two dimensions (Fig. 4). In Fig. 4a the valve position at the beginning of the ^{1}D separation is shown. The 2D-LC separation was performed in the time-based mode, i.e. the sampling from the ^{1}D separation was carried out automatically by a time program with defined fixed times for start of sampling (Supplementary Table S1). The effluent from the ^{1}D column enters the ^{1}D detector and then into the central switching valve. The first peak (mono-DNP-His) is collected into loop 1 of the lower loop deck (deck A). Immediately after collection, the valve is switched to the position shown in Fig. 4b and cut #1 is immediately analyzed in concurrent mode (i.e. loop is emptied in the same direction as it was filled) by the ^{2}D chiral separation method. While cut #1 is analyzed in ^{2}D, the next fraction is collected into loop 1 of the upper parking deck (deck B). While this sample is analyzed in ^{2}D, the next fractions are collected into loops 1,2 and 3. A smart MHC algorithm is working in the background and reduces valve switches to a minimum but attempts to inject heart-cuts that are sampled as soon as possible. In each loop deck only 5 loops can be utilized simultaneously for sample parking while the 6th loop is used as flow path for the mobile phase. In order to ensure uncompromised analysis of a more complex sample, the ^{2}D separation must be fast enough so that always at least one loop in either of the two decks is available for sampling of a fraction from the ^{1}D chromatogram. Typically parking of heart-cuts begins in loop-1 of a deck. Subsequently, parking of additional cuts proceeds in loops with increasing number. On contrary, injection of these cuts into ^{2}D occurs in the reversed order. Supplementary Table S1 summarizes further details about sampling in ^{1}D and provides information in which loop the respective sample was stored. The resultant corresponding ^{1}D and ^{2}D chromatograms are shown in Fig. 2 and Fig. 5, respectively.

For the majority of DNP-amino acid peaks sampling in ^{1}D occurred without problems. For peaks which were well resolved, a 40 μL heart cut around the peak maximum (sampling time 0.16 min) ensured maximal sensitivity in ^{2}D. In case of Hse, a tailing reagent peak is partly overlapping. It is, however, well resolved in the ^{2}D and no interference can be observed from this reagent peak in the ^{2}D chromatogram (cut #6) (Fig. 5). Glu and Gly coelute

Table 1
Chromatographic parameters obtained by achiral-chiral MHC 2DLC of dinitrophenylated amino acids.

DNP-Amino acid[a]	Symbol[a]	Cut #	^{1}D-Cut start [min][b]	^{1}D-RT [min]	^{2}D-RT 1 [min]	^{2}D-RT 2 [min]	Enantioselectivity $^2\alpha_{DL}$	Elution order	Resolution $^2R_{DL}$
Histidine	His	1	8.34	8.45	0.607	0.685	1.16	L < D	0.69
Arginine	Arg	2	14.58	14.76	0.241	0.284	1.32	L < D	0.97
Asparagine	Asn	3	16.35	16.45	0.802	1.142	1.49	L < D	3.61
Glutamine	Gln	4	18.82	18.92	0.771	0.984	1.32	L < D	2.46
Serine	Ser	5	20.28	20.35	0.876	1.196	1.42	L < D	3.33
Homo-Serine	Hse	6	22.94	23.04	0.693	0.915	1.38	L < D	3.01
allo-Threonine	aThr	7	24.86	24.96	0.684	0.927	1.42	L < D	3.26
Aspartic acid	Asp	8	25.75	25.82	1.969	2.466	1.27	L < D	1.99
Glutamic acid	Glu	10	29.99	30.09	2.861	3.165	1.11	**D < L**	0.91
Glycine	Gly	10	29.99	30.09	1.217	–	–	–	–
Threonine	Thr	11	30.87	30.96	0.674	0.984	1.55	L < D	4.08
β-Alanine	βAla	12	37.87	37.99	0.51	–	–	–	–
Alanine	Ala	13	43.61	43.73	0.859	1.012	1.20	L < D	1.83
Proline	Pro	14	45.24	45.34	0.767	1.009	1.37	L < D	2.83
Histidine'	His'	15	62.33	62.37	2.019	2.172	1.08	L < D	0.65
Methionine	Met	16	66.06	66.14	0.928	1.136	1.25	L < D	2.24
Valine	Val	17	69.23	69.34	0.596	0.823	1.47	L < D	3.53
Tryptophan	Trp	18	80.21	80.29	1.053	1.444	1.41	L < D	2.99
allo-Isoleucine	alle	19	81.96	82.29	0.446	0.668	1.66	L < D	4.22
Phenylalanine	Phe	21	82.82	n.d.	0.727	0.931	1.33	L < D	2.46
Isoleucine	Ile	21	82.82	83.22	0.473	0.648	1.48	L < D	2.86
Leucine	Leu	24	83.82	n.d.	0.489	0.633	1.38	L < D	2.73
Cysteine'	Cys'	26	84.44	84.39	1.899	2.304	1.23	L < D	1.95
Ornithine'	Orn'	27	90.11	90.17	2.461	2.645	1.08	**D < L**	0.63
Lysine'	Lys'	28	101.18	101.23	1.956	2.343	1.21	L < D	1.72
Tyrosine'	Tyr'	29	110.27	110.35	1.193	1.385	1.18	L < D	1.34

[a] Dash indicates bis-labeled amino acid derivatives.
[b] Sampling time, 0.16 min.

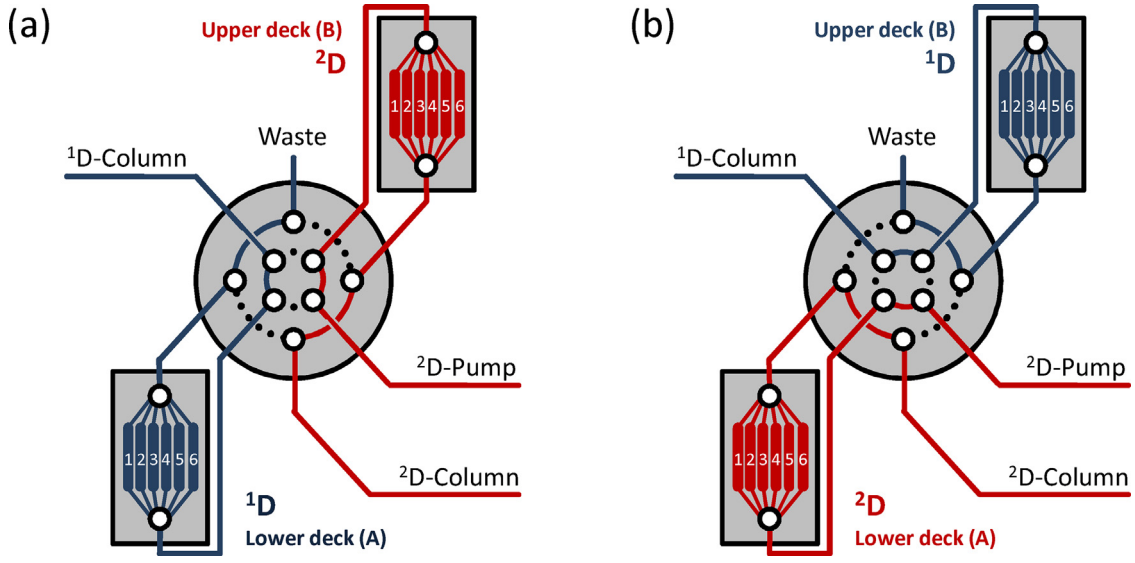

Fig. 4. Valve design for multiple heartcutting 2D-LC.

in the ^{1}D RPLC separation, however, they are well resolved in the ^{2}D chiral separation (cut #10). Glu as an acidic amino acid with a second carboxylic group in the side chain is significantly stronger retained on the WAX-type tBuCQN CSP and elutes between 160 and 210 s, while Gly already elutes at ca. 73 s. While the isobaric allo-Thr/Thr/Hse, the enantiomers of which would coelute in ^{2}D chiral separation, are well resolved in ^{1}D, a challenge remains for the isobaric Leu/Ile/allo-Ile which further coelute with Phe. The isobaric allo-Ile peak is relatively well resolved from Ile and Leu. Cut #19 which is taken on the rising edge of the allo-Ile RPLC-peak does contain only allo-Ile. Ile is also cut at the rising edge of the peak (cut #21). It is superimposed by Phe which is, however, well resolved in the ^{2}D chiral separation. Leu is cut on the tailing edge (cut #24) and is well separated from Phe, Ile, and allo-Ile. The disadvantage of cutting fractions at rising or tailing edges is that the sensitivity is somewhat lower. However, sensitivity is not an issue in the current application because the analyzed peptide drugs are available in sufficient quantities. The full set of chromatographic data obtained by this enantioselective MHC 2D-UHPLC method is given in Table 1. It can be seen that there is sufficient enantiomer resolution for all chiral amino acids. Although no full baseline separation was obtained for some amino acids (His, Arg, Glu, Orn), the degree of resolution was good enough for determination of their stereoconfiguration. Overall the separations look promising and are useful for a complete amino acid analysis. Further optimizations will focus on better ^{1}D separation of isobaric leucines and a reduction of total run time (possible through further acceleration of ^{2}D chiral separations).

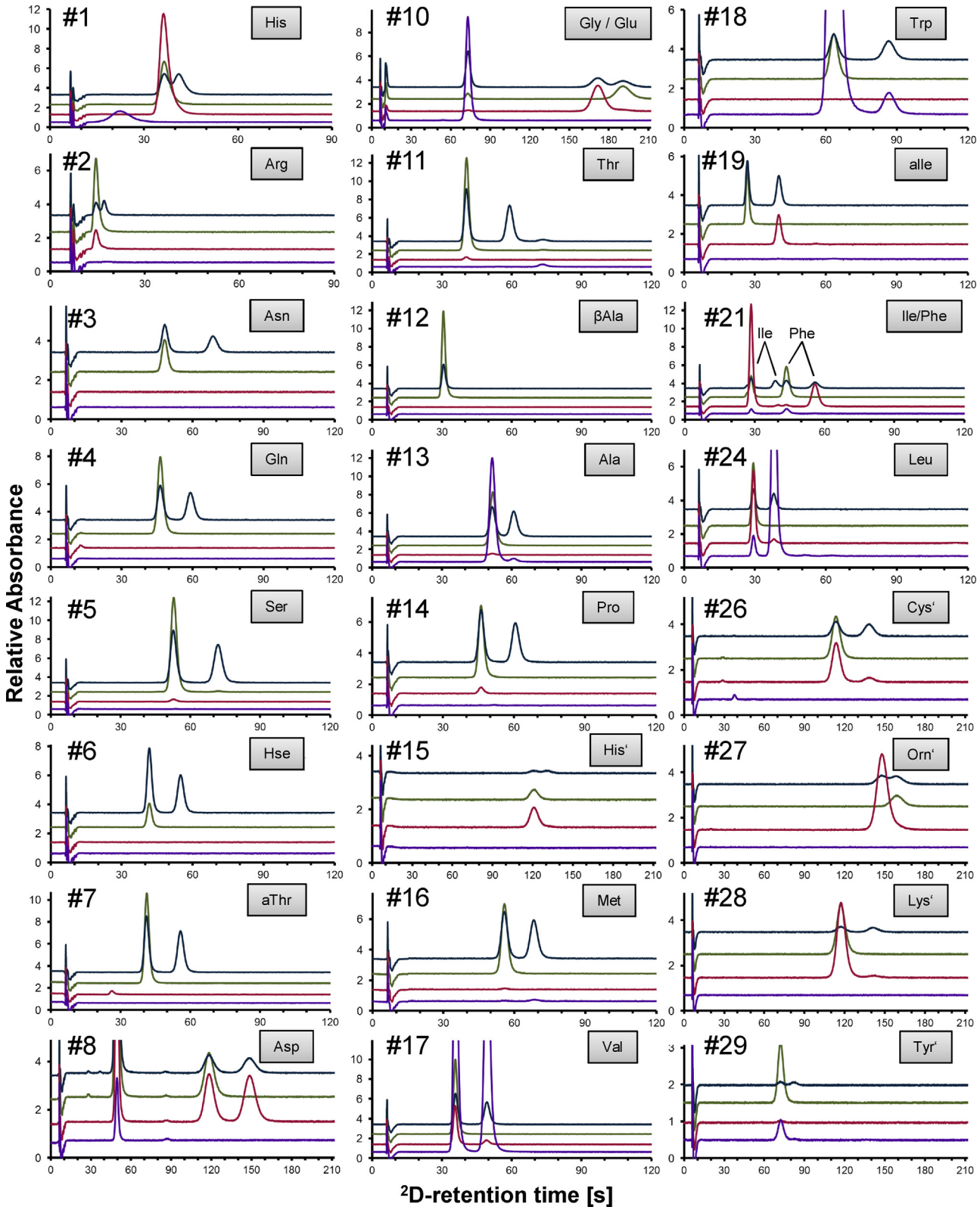

Fig. 5. [2]D-chiral separations of DL-mix (blue), L-mix (green), Bacitracin (red) and Gramicidin (purple). (Corresponding [1]D chromatograms are shown in Fig. 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Application for the qualitative analysis of nonribosomal peptides

The applicability and practical utility of the established automated enantioselective MHC 2D-UHPLC assay was evaluated by its application for the qualitative analysis (of identity) of the amino acids, including their stereochemistry, in bacitracin and gramicidin A (Fig. 6). The chromatograms of their [1]D separation are shown in Fig. 2 and the corresponding chromatograms of the cuts in Fig. 5.

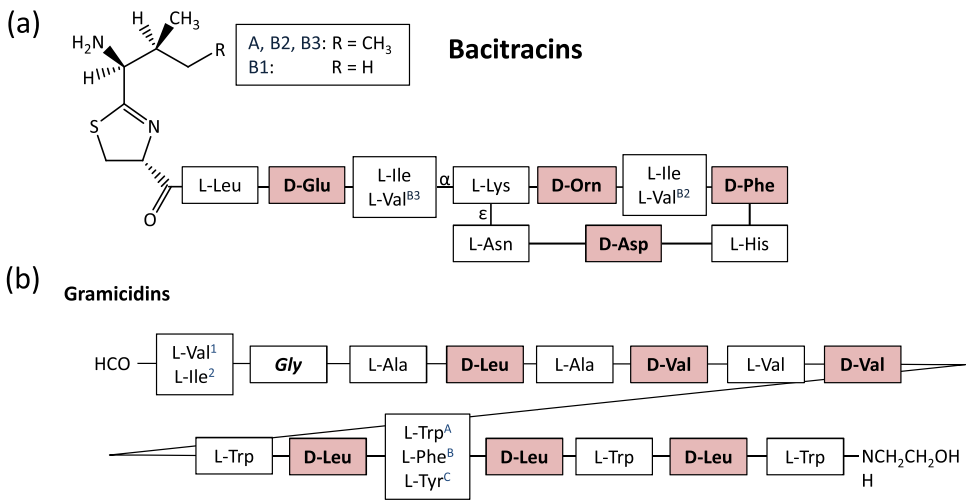

Fig. 6. Schematic structures of Bacitracin (a) and Gramicidin (b). Superscripts refer to structure variants (see also Supplementary Tables S3 and S4).

For both peptides the same cuts were made in the ^{1}D separation as outlined above for the 25 amino acid standard mixture, although not all amino acids were present in bacitracin and gramicidin. This, however, allows to test whether impurities or minor component peptides, such as observed in case of gramicidin A, are present. According to ICH guidelines, the analysis of the identity requires the validation of the assay specificity [32]. The fact that separations are performed by two distinct complementary assays in ^{1}D and ^{2}D provides enhanced assay specificity because each of the two dimensions can resolve some impurities. This becomes clearly evident in the chromatograms of bacitracin and gramicidin in Figs. 2 and 5. Several impurities have been well resolved in ^{1}D. In some rare cases an additional impurity peak can be found in the ^{2}D chromatogram, e.g. in cut #1 of the chiral separation of mono-DNP-His. This impurity, however, is well resolved in ^{2}D and does not influence the specificity for the determination of the His enantiomers. Of importance for the method validity is also the retention time stability over several sample injections. This has been tested by replicate injections of amino acid mixtures. It turned out that the run-to-run repeatability in this MHC 2D-UHPLC system is excellent. Mean RSD values of 0.09% for ^{1}D retention times (<0.2% RSD, except for His 0.42%, Arg 0.22%, and Asp 0.23% RSD) and 0.17% for ^{2}D retention times (<0.3% RSD, except for His' 0.40%, Tyr' 0.34%, and Cys' 0.31% RSD) have been observed (for detailed data see Suppl. Table S3). This clearly assures the validity of this method for qualitative analysis of the peptide hydrolysates.

The amino acids found for bacitracin (Fig. 6a) hydrolysate are summarized in Table 2. The ^{1}D separation for bacitracin (red trace in Fig. 2) indicates the presence of the amino acids His, Arg, Asp, Gly or Glu, Val, isobaric leucines (allo-Ile/Ile/Leu or Phe), Cys, Orn and Lys. Cys originated from the hydrolysis of the thiazoline ring. Val was not expected for bacitracin but may stem from bacitracin variants e.g. bacitracin B1 (see Eur. Pharmacopoeia [33]). The origin of Arg is unclear as no variant with this amino acid is listed in the Eur. Pharmacopoeia. For His, Arg, Val, Cys, and Lys the L-form (cuts #1,2,17, 26, 28) was found in the ^{2}D-chromatograms and for Orn the D-form (cut #27) (Fig. 5). Cut #8 shows that both L and D-form of Asp are contained in the amino acid hydrolysate sample at a ratio of 50:50. This can be explained by the hydrolysis of the Asn side chain and L-configuration of this amino acid in bacitracin. Since Asp exists in D-form, the resulting chromatogram shows a 1:1 mixture for this amino acid. Cut #10 clearly indicates that no Gly is present and that Glu is present in the D-form. A specific challenge is the correct identification of the configuration of

Table 2
Amino acids found in hydrolysate of Bacitracin.

^{1}D – RP[a]	^{2}D – cWAX	Cut #	Comment
His	**L**	1,15	
Arg	*L*	2	*impurity*
Ser	*L*	5	*traces*
Asp	**DL**	8	*ratio L:D = 1:1.18*
Glu	**D**	10	
Thr	*L*	11	*traces*
Ala	*L*	13	*traces*
Pro	*L*	14	*impurity*
Val	*DL*	17	*Bacitracin B1 and B2*
alle	*D*	19	*epimerization of Ile-1*
Phe	**D *(L in traces)***	21	
Ile	**L**	21	
Leu	**L (D in traces)**	24	
Cys	L (D in traces)	26	
Orn	**D**	27	
Lys	**L**	28	

[a] Bold, expected amino acids according to Eur. Pharmacopoeia.

the fractions containing the isobaric leucines in presence of Phe. Cut #19 indicates the presence of D-allo-Ile. Since there is no peak at the retention time of L-Ile, it can be concluded that there is no contamination from Ile in cut #19. Likewise, absence of a peak in the gramicidin trace in cut #19 proves that this heartcut is not contaminated with Leu. Cut #21 shows a clear peak for D-Phe. It can also be seen that there is no peak at the retention time of D-allo-Ile and D-Leu. Hence, it can be concluded that this cut is essentially free of these two amino acids which are isobaric to Ile. In cut #24 we clearly can see that the configuration of Leu is L. To some extent unexpectedly, cut #26 reveals the presence of an L-Cys residue. It is the result of hydrolysis of the thiazoline ring which represents a masked Cys residue. Except for the unexpected amino acid Arg, all other findings were in agreement with the reported bacitracin structure (Fig. 6a and Supplementary Table S5).

The results for gramicidin (Fig. 6b) hydrolysate are summarized in Table 3. The analyzed gramicidin sample was a mixture of gramicidin A1 (ca. 73%) and some minor structural variants (gramicidin A2, B1, C1 and C2) (altogether with a specified purity of 98.2%) (see also Supplementary Table S5). The ^{1}D chromatogram (Fig. 2, bottom trace) reveals peaks for Gly/Glu, Ala, Val, Trp, allo-Ile/Ile/Leu/Phe, and Tyr. The ^{2}D chromatogram of cut #10 confirms Gly and absence of Glu in the sample (Fig. 5). Cuts #13 and 18 reveal L-forms for Ala and Trp. For Val (cut #17) both forms are detected in a ratio of about 1:1. Tyr is detected as L-form and is present in minor quantities. It

Table 3
Amino acids found in hydrolysate of Gramicidin.

^{1}D – RP[a]	^{2}D – cWAX	Cut #	Comment
Gly	**-**	10	
Ala	**L (D in traces)**	13	
Val	**DL**	17	*ratio L:D = 1: 1.07*
Trp	**L (D in traces)**	18	
Phe	L (D in traces)	21	*Gramicidin B1*
Ile	L (D in traces)	21	*Gramicidin A2, C2*
Leu	**D *(L in traces)***	*24*	
Tyr	L	29	*Gramicidin C1, C2*

[a] Bold, expected amino acids according to Eur. Pharmacopoeia.

may stem from gramicidin C1 and/or C2. Likewise, a minor peak for L-Phe is detected in cut #21 which may originate from gramicidin B1 and/or B2. Cut #21 also shows a minor peak for L-Ile which is due to gramicidins A2, B2 and C2. No allo-Ile is detected in cut #19 and the major peak in cut #24 shows D-configuration for Leu. Once more, the results are in agreement with the reported structures and document the practical usefulness and applicability of the present enantioselective MHC 2D-UHPLC method.

4. Conclusions

Combining the chromatographic selectivity of two orthogonal chromatographic modes such as achiral separation by reversed-phase LC and enantioselective separation by a chiral stationary phase in a multiple heart cut mode can be a powerful methodology for the amino acid composition analysis combined with absolute configuration determination of peptide hydrolysate samples. Robust commercial solutions for such a MHC-2D-UHPLC are nowadays available which give well repeatable separations with excellent run-to-run precision of retention times in spite of two dimensions and multiple loop interface for fraction transfer by column switching technologies. The utility of this 2D-LC approach has been documented for 25 amino acids (20 proteinogenic amino acids plus allo-Thr, allo-Ile, Hse, Orn, β-Ala) using Sanger's reagent as a tagging strategy for introduction of favorable interaction sites supporting chiral separation on the 2D tBuCQN–CSP as well as introducing a chromophoric group for UV detection. The achiral separation of the isobaric leucines can be further improved which will be subject of future studies. This may be of importance for bioanalytical investigations of minor quantities of D-amino acids in presence of large amounts of L-amino acids in biological samples where minor traces of contamination into other cuts may be more critical. For such bioanalytical studies a different tagging strategy should be used such as 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) reagent. Amino acid derivatives of this derivatization reagent can be well resolved on quinine and quinidine carbamate CSPs [34] and allow sensitive fluorescence detection or highly sensitive and selective mass spectrometric detection. For the latter method, much faster separations should be feasible because not all components must be separated due to the selectivity of the MS detector.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2018.05.062.

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