Received: 3 June 2022 | Revised: 25 October 2022 | Accepted: 26 January 2023

DOI: 10.1002/chir.23543

RESEARCH ARTICLE

Chirality WILEY

Teicoplanin aglycone media and carboxypeptidase Y: Tools for finding low-abundance D-amino acids and epimeric peptides

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Funding information
This work was supported by the Robert A. Welch Foundation (Y0026).

Abstract

D-amino acids and epimeric peptides/proteins can play crucial biological roles and adversely affect protein folding and oligopeptide aggregation in age-related pathologies in humans. This has ignited interest in free D-amino acids as well as those incorporated in peptides/proteins and their effects in humans. However, such stereoisomeric analytes are often elusive and in low abundance with few existing methodologies capable of scouting for and identifying them. In this work, we examine the feasibility of using teicoplanin aglycone, a macrocyclic antibiotic, which has been reported to strongly retain D-amino acids and peptides with a D-amino acid on the C-terminus, for use as a solid phase extraction (SPE) medium. The HPLC retention factors of L-/D-amino acids and C-terminus modified D-amino acid-containing peptides and their L-amino acid exclusive counterparts on teicoplanin aglycone are presented. Retention curve differences between amino acids and peptides highlight regions of solvent composition that can be utilized for their separation. This approach is particularly useful when coupled with enzymatic hydrolysis via carboxypeptidase Y to eliminate all L-amino acid exclusive peptides. The remaining peptides with carboxy-terminal D-amino acids are then more easily concentrated and identified.

KEYWORDS

6-aminoquinoline-N-hydroxysuccinimidyl carbamate, carboxypeptidase, enkephalin, LC–MS, peptides

1 | INTRODUCTION

The study of biological sources of D-amino acids (D-AAs) and D-amino acid-containing peptides (DAACP) has intensified due to their occurrence in pathologies such as cancer,[1–4] age-related pathologies such as cataracts (α-crystalline)[4–7] and various neurological conditions including epilepsy, and Alzheimer's disease.[8–13] With the resurgence in interest involving biological roles and impacts of free D-AAs as well as those incorporated in peptides/proteins, there is an increasing demand for sequence-dependent stereospecific methodologies to aid in the search for these low-abundance analytes in complex biological matrices.[14–16] Traditional methods of isolating and characterizing D-AAs and DAACPs have analytical impediments including partial racemization during acid hydrolysis, and they are isobaric to dominant L-amino acids (L-AAs) and all L-AA peptides.[14–23] A

selective solid-phase extraction (SPE) type of format would be ideal if it could facilitate the extraction of D-AAs and D-AA-containing peptides from complex biological samples.

Teicoplanin is a macrocyclic glycopeptide antibiotic that was first isolated from the soil bacterium *Actinoplanes teichomyceticus*.[24,25] Aside from its use as an antibiotic, teicoplanin has also found success as a chiral stationary phase (CSP) for enantiomeric separations.[26–28] First utilized as a silica-based stationary phase in 1995, one of its first uses was for the enantioseparation of both unmodified and *N*-derivatized L- and D-AAs.[29] It was found that D-AAs were significantly retained on teicoplanin when compared to L-AAs.[30] Teicoplanin aglycone (TAG) is a product of synthetically modified teicoplanin in which all three carbohydrate groups of the precursor compound were removed.[31–33] The end product was found to have decreased solubility in water but increased specificity toward D-AAs.[34] Thus, the TAG CSP appeared to be an ideal choice when considered as a SPE approach targeting D-AAs and DAACPs.

TAG selectively interacts with the carboxylate functionality of amino acids and the carboxy-terminal D-AAs of peptides. Such a TAG stationary phase could selectively retain DAACPs that contain a carboxy-terminal amino acid. Presumably, this would be quite rare in biological matrices. Recently, we reported an enzymatic method using carboxypeptidase Y (CPY) that was capable of eliminating L-AA exclusive peptides and creating C-terminal DAACPs from embedded DAACPs.[35] This would allow the natural affinity of immobilized TAG to be used in either a chromatographic or SPE format. CPY is a serine carboxypeptidase that sequentially hydrolyzes amino acids at the peptide bond from the C-terminus of peptides.[36–39] This enzyme has been shown to have decreased enzymatic activity when cleaving D-AAs from peptide chains, sometimes by two or more orders of magnitude.[35] It has been previously shown that enzymatic screening with CPY is capable of eliminating L-AA exclusive peptides while possessing low activity toward DAACPs.[35,36,39]

Herein we provide screening results for amino acids, dipeptides, and longer peptides on TAG stationary phase under reversed phase conditions. The combination of a streamlined and targeted enzymatic methodology can potentially eliminate L-amino acid exclusive peptides while being largely inactive towards low-abundance DAACPs. This stereospecific enzymatic methodology when combined with the TAG stationary phase's natural affinity toward D-AAs/C-terminal DAACPs is potentially a powerful tool for selectively identifying such low-abundance species.

2 | METHODOLOGY

2.1 | Materials

Solvent sourcing for LC separations was as follows: methanol was purchased from Fischer Chemical (Fair Lawn, NJ, USA), anhydrous ethanol 200 proof from Decon Labs Inc. (King of Prussia, PA, USA), and DI water was obtained through a Barnstead GenPure Pro UV water purification system. The mobile phase additives ammonium formate and formic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Buffers such as 2-*N*-(morpholino)-ethanesulfonic acid hydrate (MES hydrate) and boric acid were also purchased from Sigma to make the enzyme buffer and 6-aminoquinoline-*N*-hydroxysuccinimidyl carbamate (AQC) derivatization buffer, respectively. Carboxypeptidase Y was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). All amino acid standards were sourced from Sigma Aldrich. All dipeptides were sourced from either Sigma-Aldrich or Tokyo Chemical Industry America (TCI) (Portland, OR, USA), whereas all tetra- and pentapeptides were purchased from Peptide 2.0 except for enkephalin analogues, which were obtained from Sigma Aldrich. AQC derivatization reagent was provided by AZYP LLC (Arlington, TX, USA).

2.2 | Methods

Initial screening of peptides was performed on a 1200 Agilent series HPLC and data analyzed with ChemStation. LC–MS analysis of enzymatic hydrolysis products was conducted on a Shimadzu 8040 LC–MS and the data analyzed using LabSolutions software. Screening separations were conducted using a 4.6 × 100 and 250 mm, 5 μm FPP Astec Chirobiotic TAG from Supelco, which utilizes a TAG stationary phase. The column used for LC–MS analysis of hydrolysis products was a 3 × 150 mm 2.7 μm SPP TeicoShell from AZYP LLC, which utilizes a teicoplanin stationary phase.

All HPLC separations were performed using a flow rate of 1 mL/min and LC–MS with 0.4 mL/min. Screening samples were prepared in 50:50 water methanol (v/v) at a concentration of 100 ppm. Retention factors (k) and optimal separation of amino acids were obtained using a mobile phase composition of 80:20 methanol: water with a sample injection volume of 1 μL on a 4.6 × 100 mm Chirobiotic TAG and detected at 220 nm. The retention factor curves were obtained by varying mobile phase percentages of water and methanol with identical conditions to the amino acid analysis. The optimal separation for glycyl- and alanyl-dipeptides was

obtained using a mobile phase composition of 80:20 methanol: water with a sample injection volume of 1 μL on a 4.6 × 250 mm Chirobiotic TAG and detected at 220 nm.

The AQC derivatization procedure was as follows. A 3 mM peptide sample was mixed with AQC reagent at a 10:20:70%(v/v) of sample: AQC reagent: borate buffer. Peptide samples were then heated in the oven for 25 min at 55°C. After heating, the 300 μM samples were left at room temperature for a minimum of 6 h before proceeding. In the CPY-catalyzed hydrolysis, the solution was prepared with 50 mM 2-(*N*-morpholino)ethanesulfonic acid hydrate (MES) and 0.1 M NaCl adjusted to pH 5.5 with 1 M HCl to make the MES solution. Enzyme solutions were prepared with 1.6 mg of lyophilized CPY in 800 μL of MES solution. 25 mM ammonium formate in CH_3OH was prepared as a post hydrolysis quenching solution to prevent microbial growth and halt enzymatic activity. When preparing the samples, 60 μL of the DAACP AQC-enkephalin was mixed with 140 μL of the L-amino acid exclusive enkephalin. 50 μL of 300 μM of the mixed enkephalin peptide solution was pipetted into 1400 μL of MES buffer for CPY (or tris • HCl buffer for CPA) and topped off with 50 μL of enzyme solution 3.3:92.4:3.3% (v/v) in 15 mL Eppendorf tubes and vortexed well. All samples had respective no-enzyme controls with 50 μL of 300 μM peptide sample added into 1450 μL of buffer. All tubes were placed in 37°C water bath and sampled at 5-, 15-, and 25-min intervals. 450 μL of the sampled solution was pipetted into Amicon centrifugal filters (3 kDa). Enzyme removal was achieved by spinning down the solution at 14,000 rpm for 12 min at 25°C. The filtrate was mixed with the 25 mM ammonium formate CH_3OH quenching solution at a 1:1 ratio in a sample vial and subsequently analyzed on LC–MS with a flow rate of 0.4 mL/min.

3 | RESULTS AND DISCUSSION

The difference in retention factor between representative D,L-amino acid enantiomeric pairs: alanine, serine, glutamine, and asparagine, is plotted in Figure 1 against varying concentrations of methanol in the mobile phase when screened on a TAG stationary phase support. An increase in $k_{D\text{-}AA}$-$k_{L\text{-}AA}$ (Δk) as well as enantiomeric selectivity (α) was observed with increasing methanol concentration in the mobile phase. As small zwitterionic analytes, amino acids are better solubilized in water while having limited solubility in higher % organic solutions under reversed phase conditions. Discrimination between D-AA and L-AA enantiomers is shown to be greatest at high methanol concentrations (Figure 1). This

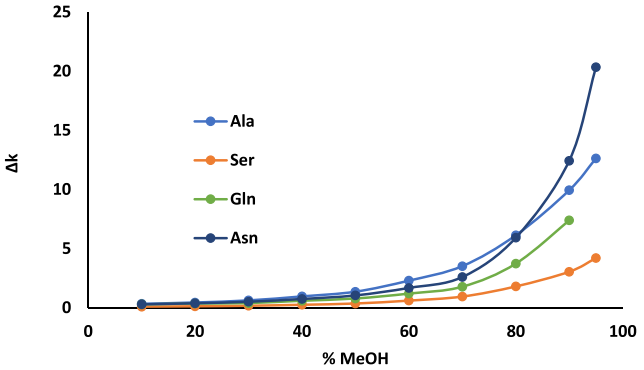

FIGURE 1 Difference in retention values ($k_2 - k_1$) for enantiomers of Ala, Ser, Gln, and Asn with varying ratios of methanol:water; 1 mL/min on Chirobiotic TAG, 5 μm particles and detected at 220 nm.

behavior can vary somewhat by changing the nature of the organic modifier, the concentration of buffer salts in the mobile phase, and the pH of the aqueous portion of the mobile phase. All proteinogenic amino acids were screened, and all their behaviors followed this trend (Table 1). However, the hydrophobic or charged nature of the amino acid side chains affected the shape and steepness (slope) of the curves. The effects of adding 50 mM ammonium formate buffered to pH 4 as the aqueous portion of the mobile phase under the optimized conditions of 90:10 methanol: water is detailed in Table 2. Consequently, some of the most retained L-enantiomer amino acids were retained longer than the D-enantiomer of the least retained amino acids under these particular conditions.

The overlaid chromatographic separations of eight glycyl-dipeptides is depicted in Figure 2 and is offset for clarity. As a result of glycine's achiral nature, the number of stereocenters in this group of peptides is limited to one. Thus, all variations in stereochemistry are situated at the C-terminus and retention differences due to side-chain characteristics were less apparent when screening glycyl-dipeptides. The selectivities of these enantioseparations are provided in the Supporting Information (Table S1). Under these separation conditions, all screened glycyl-peptides with a C-terminal D-amino acid were highly retained, with all of the L-enantiomer peptides eluting prior to the cutoff time of 33 min, or a k of 18.4. The dipeptides with C-terminal D-AAs eluted in the order as listed: Gly-D-Thr, Gly-D-Asp, Gly-D-Trp, Gly-D-Ser, Gly-D-Phe, Gly-D-Leu, Gly-D-Val, Gly-D-Met, and Gly-D-Ala. Peak identities were confirmed via pure standards.

In similar fashion, Figure 3 shows the overlaid chromatographic separations of alanyl-dipeptides offset for clarity. The additional chiral center results in four

TABLE 1 *k* values for different mobile phase concentrations of methanol:water

AAs	Ala		Phe		Ile		Leu		Met		Asn	
%MeOH	k_L	k_D	k_L	k_D	k_L	k_D	k_L	k_D	k_L	k_D	k_L	k_D
95	3.98	16.6	2.79	7.10	1.34	13.1	1.78	9.16	2.86	14.5	7.62	28.0
90	3.07	13.0	2.38	5.93	1.22	10.8	1.52	7.94	2.35	12.2	4.99	17.4
80	1.83	7.96	1.55	3.79	0.870	7.60	1.04	5.57	1.45	7.38	2.57	8.49
70	1.03	4.55	1.17	2.74	x	x	0.732	3.87	0.968	4.66	1.23	3.83
60	0.687	2.98	1.05	2.38	x	x	0.637	3.26	0.825	3.78	0.797	2.48
50	0.572	1.93	1.15	2.36	0.603	3.37	0.694	2.70	0.792	2.98	0.577	1.62
40	0.403	1.36	1.21	2.30	0.571	2.81	0.637	2.11	0.716	2.46	0.431	1.17
30	0.306	0.930	1.17	2.06	0.503	2.13	0.559	1.75	0.636	1.90	0.331	0.841
20	0.258	0.703	1.18	1.89	0.487	1.68	0.537	1.46	0.603	1.56	0.289	0.669
10	0.238	0.574	1.18	1.73	0.474	1.36	0.501	1.23	0.551	1.29	0.243	0.527
AAs	**Gln**		**Ser**		**Thr**		**Val**		**Trp**		**Tyr**	
%MeOH	k_L	k_D	k_L	k_D	k_L	k_D	k_L	k_D	k_L	k_D	k_L	k_D
95	x	x	3.08	7.28	1.75	5.03	1.71	9.02	3.27	10.1	2.50	6.53
90	4.38	11.8	2.18	5.22	1.34	3.64	1.41	7.71	2.75	8.01	2.02	5.16
80	2.39	6.12	1.22	3.02	0.726	2.16	0.957	5.00	1.93	5.34	1.30	3.29
70	1.24	3.02	0.634	1.58	0.387	1.18	0.630	3.27	1.58	4.12	0.928	2.34
60	0.850	2.05	0.405	1.01	0.271	0.800	0.535	2.48	1.64	3.90	0.861	2.07
50	0.649	1.43	0.316	0.676	0.253	0.590	0.554	1.90	1.88	3.78	0.871	1.85
40	0.507	1.10	0.215	0.468	0.176	0.424	0.424	1.44	2.07	4.00	0.851	1.75
30	0.445	0.843	0.165	0.336	0.145	0.322	0.367	1.07	2.32	4.06	0.857	1.61
20	0.374	0.728	0.146	0.271	0.134	0.267	0.338	0.837	2.61	4.15	0.903	1.56
10	0.330	0.618	0.110	0.205	0.105	0.210	0.297	0.658	2.76	4.11	0.891	1.43

Note: x denotes that analyte signal was below LOD.

possible stereoisomers in the form of LL, DL, LD, and DD that must be separated. The alanyl-dipeptides were separated under identical conditions as the glycyl-dipeptides. The αs of these epimeric/enantiomeric separations are given in the Supporting Information (Table S2). The solvent "cutoff" composition for glycyl-dipeptides was also acceptable for separating alanyl-dipeptides on TAG stationary phase with all C-terminal L-AA peptides eluting prior to all C-terminal D-AAs. The four possible stereoisomers of each peptide were classified into two groups, those with an L-AA on the C-terminus in contrast to those with a D-AA on the C-terminus. The majority of peptide stereoisomers followed the elution order of LL, DL, LD, and DD. All alanyl-dipeptides with a C-terminal L-amino acid were found to elute prior to the cutoff time of 33 min, or *k* of 18.4. The notable exception being the four stereoisomers of DL-Ala-DL-Met. These separation conditions were unable to provide chromatographic resolution between the pairs L-Ala-L-Met, D-Ala-L-Met and L-Ala-D-Met, D-Ala-D-Met. Hence, only two peaks were observed in this chromatogram. The general amino acid elution order of peptides was Ala-Ser, Ala-Asn, Ala-Val, Ala-Met, and Ala-Ala. Peak identity was confirmed with separate standards of the epimers. Notably D-Ala-D-Val was not detected within the 3-h method run time. This very highly retained dipeptide only eluted when the pH of the aqueous part of the mobile phase was lowered to pH 4. When the side chain of the N-terminal amino acid was kept constant, the elution order trend was dependent on the side chain characteristics of the C-terminus amino acid. The general amino acid elution order was acidic side chains<polar side chains<aromatic side chains<aliphatic side chains. The higher retention of peptides with aliphatic side chains compared to those with aromatic side chains, despite the presence of five aromatic rings on TAG, can be explained by the hydrophobic effect in reversed phase solvent systems as well as weakened pi–pi interactions in the same solvents. The aliphatic side chains of alanine, methionine, and valine interact more strongly with the

TABLE 2 Comparison of k for 90:10 methanol: Water with and without buffer

	90:10 without buffer		90:10 with buffer	
AA	k_L	k_D	k_L	k_D
Ala	3.07	13.0	2.50	10.4
Cys	3.02	14.1	2.62	8.50
Asp	1.21	2.71	2.22	5.35
Glu	1.24	4.04	1.77	6.85
Phe	2.38	5.93	1.96	5.13
His	x	x	x	x
Ile	1.22	10.8	0.937	8.87
Lys	x	x	21.1	x
Leu	1.52	7.94	1.17	6.39
Met	2.35	12.2	1.85	10.5
Asn	4.99	17.4	4.34	15.5
Pro	2.40	x	9.86	49.0
Gln	4.38	11.8	3.87	11.0
Arg	x	x	22.1	x
Ser	2.18	5.22	1.82	4.40
Thr	1.34	3.64	1.08	3.10
Val	1.41	7.71	1.14	6.38
Trp	2.75	8.01	2.20	6.46
Tyr	2.02	5.16	1.71	4.38

Note: x denotes that analyte signal was below LOD.

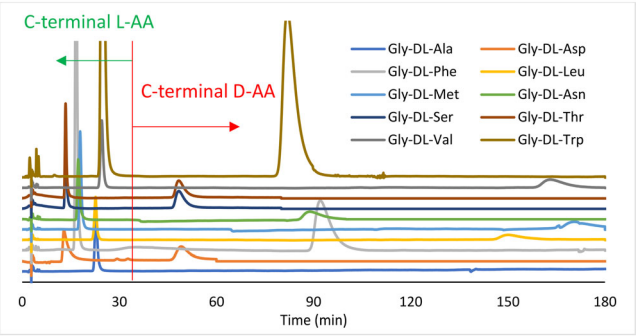

FIGURE 2 Enantiomeric separation of glycyl-dipeptides with all C-terminal L-amino acid peptides eluting prior to the 33 min cutoff; 1 mL/min 80:20 MeOH:water, 4.6 × 250 mm, Chirobiotic TAG 5 μm detected at 220 nm.

hydrophobic pocket of the aglycone, hence their increased retention. The selectivities of non-enantiomeric peptide pairs are listed in the Supporting Information (Table S3).

The chromatogram of an epimeric mixture of insulin B fragment 22–25 with the sequence L-Arg-Gly-L-Phe-L-Phe and a synthesized epimer with the sequence L-Arg-Gly-L-Phe-D-Phe is shown in Figure 4A. The terminal D-epimer was significantly more retained. In similar fashion, Figure 4B depicts the chromatogram of an epimeric mixture of bovine pineal antireproductive peptide with the sequence L-Thr-L-Ser-L-Lys-L-Tyr and its synthesized epimeric counterpart with the sequence L-Thr-L-Ser-L-Lys-D-Tyr. Higher retention was again observed for the terminal D-epimer. Both chromatograms shown in Figure 4 were optimized for maximum α within a reasonable time frame.

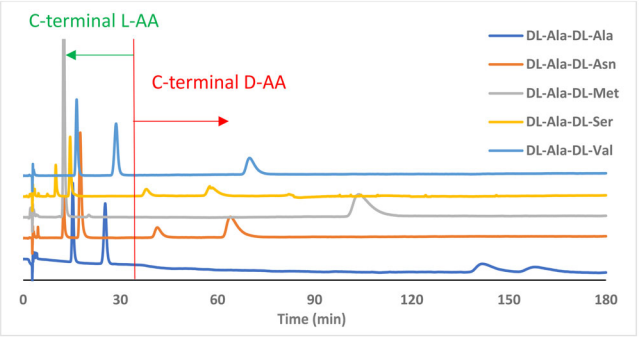

FIGURE 3 Separation of alanyl-dipeptides with all C-terminal L-amino acid peptides eluting prior to the 33 min cutoff. 1 mL/min 80:20 MeOH:water, 4.6 × 250 mm, Chirobiotic TAG, 5 μm, detected at 220 nm.

The retention behavior of selected peptides were investigated. Figure 5 is a plot of the retention factor of L-Arg-Gly-L-Phe-L-Phe and its C-terminus D-epimer at varying percentages of methanol: water. The C-terminal D-amino acid epimer is more retained than all the L-amino acid peptides at all ratios of methanol: water tested. The chromatograms are provided in the Supporting Information (Figure S1). Although both curves diverge (increase in α) when approaching solvent extremes, a larger Δk was observed at higher organic mobile phase compositions. These synthetic peptides with biological activity and their C-terminus D-epimers, which were screened at different methanol: water concentrations serve to demonstrate the preference of TAG toward C-terminal DAACPs at all solvent compositions.

Despite having screened many peptides with αs both large and small, differences in side-chain characteristics still play a significant role in their retention behavior. As a result, the data show that despite achieving high αs and resolutions, no all-inclusive cutoff could be determined for a wide array of peptide epimers. This, in part, was why a streamlined enzymatic method capable of eliminating most, if not all L-amino acid exclusive peptides while preserving the DAACPs, should be utilized. It should be noted that the AQC derivatization technique coupled with the enzymatic method aids in but is not required for detection. However, AQC derivatization is

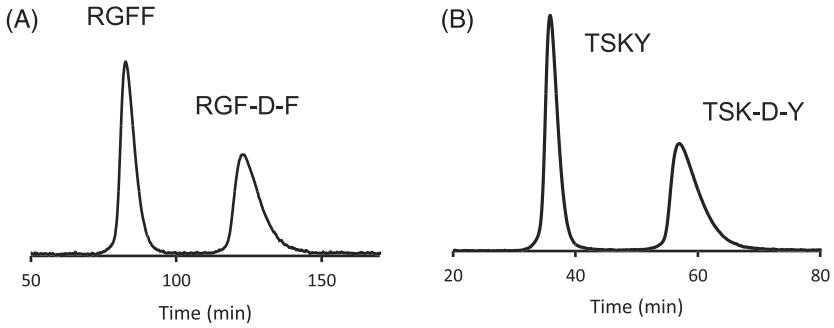

FIGURE 4 (A) Separation of Insulin B fragment (22-25) RGFF and synthesized RGF-D-F peptide on Chirobiotic TAG 4.6 × 100 mm, 5 µm. 1 mL/min, 80/20 MeOH/50 mM ammonium formate pH = 4.0. (B) Separation of bovine pineal antireproductive peptide TSKY and synthetic TSK-D-Y peptide on Chirobiotic TAG 4.6 × 100 mm, 5 µm. 1 mL/min, 80/20 MeOH/50 mM ammonium formate pH = 4.0. Both were detected at 220 nm.

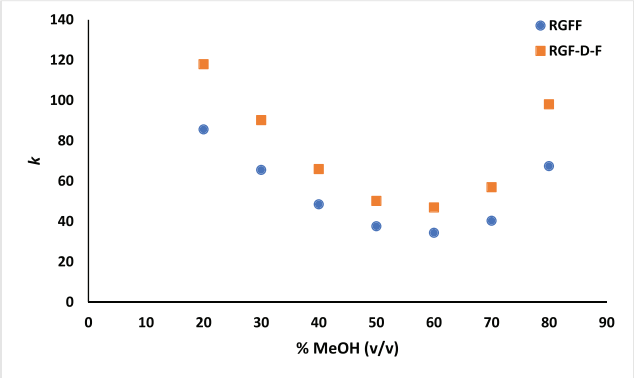

FIGURE 5 U-shaped retention curve of RGFF and synthetic RGF-D-F at varying ratios of methanol in 50 mM ammonium formate pH 4.0 mobile phases on Chirobiotic TAG 5 µm 4.6 × 100 mm at 1 mL/min and detected at 220 nm.

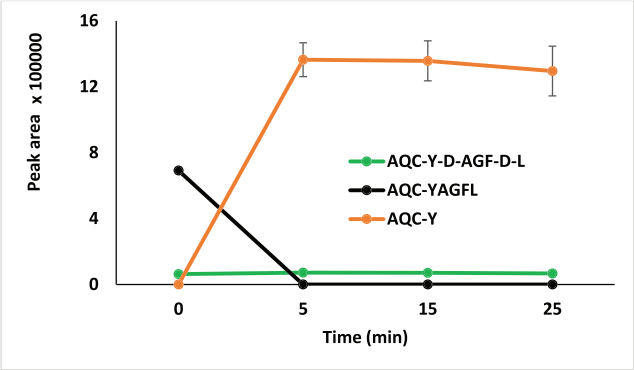

FIGURE 6 LC-MS peak area analysis of 70:30 (v/v) equimolar solutions of AQC-YAGFL:AQC-Y-D-A-GF-D-L hydrolyzed with CPY over time. Both starting materials were monitored at 5, 15, and 25 min as well as the final expected product. The separation was conducted on TeicoShell 2.7 µm SPP 3 × 150 mm column at 0.4 mL/min.

imperative if the second position from the N-terminus is to be interrogated for D-AAs as CPY has been largely found to be inactive toward native dipeptides.[34–38] The retention differences between peptides and amino acids become more apparent at low % organic mobile phases and may be exploited. This can be seen when comparing the *k* values in Table 1 with the U-shaped curves shown in Figures 5 under identical conditions. Additionally, AQC derivatization increases the hydrophobic character of the peptide. The hydrophobic AQC-peptide's preference for the hydrophobic pocket of the aglycone combined with the specificity of TAG toward C-terminal D-AAs is rare complimentary combination in stationary phases when targeting DAACPs. This streamlined methodology is capable of producing C-terminal DAACPs using enzymatic hydrolysis while fully hydrolyzing all L-amino acid exclusive peptides can now be combined with purification and preconcentration on a TAG stationary phase. Although a larger α was observed at higher % organic, a lower % organic would ensure the fast elution of endemic amino acids as well as hydrolyzed amino acids from the CPY enzymatic method. Under the same conditions that elute amino acids, intact AQC-peptides would be strongly retained on the stationary phase.

The CPY-catalyzed hydrolysis of a mixture of AQC-Tyr-Ala-Gly-Phe-Leu and AQC-Tyr-D-Ala-Gly-Phe-D-Leu in 70:30 and 90:10 molar ratios sampled at 5-, 10-, and 25 min over a period of 25 min of enzymatic hydrolysis is shown (Figure 6). The L-amino acid exclusive peptide shown in black was below the limit of detection after 5 min of the enzymatic hydrolysis. The D-amino acid terminal peptide peak area is shown in green did not change significantly over the course of the hydrolysis reaction, compared to the control. The levels of the expected hydrolysis end product of the all L-AA containing peptide (i.e., AQC-tyrosine) spiked sharply after 5 min and remained throughout the hydrolysis experiment. The appearance of AQC-tyrosine and the unchanged peak area of the DAACP suggest that the AQC-tyrosine observed was solely from the L-AA exclusive peptide proving that the all L-AA peptide was fully hydrolyzed by the 5-min sampling interval despite being present in 9 and 2.3 times excess in two separate experiments. This proof of concept could be translated to biological samples where CPY is capable of fully eliminating non-DAACPs while not degrading DAACPs,

which would remain intact for extraction and subsequent analysis.

4 | CONCLUSIONS

The TAG CSP was able to establish a L- and D-amino acid (C-terminus) cutoff for most amino acids and short peptides. This may be useful in a SPE format for the purpose of preconcentrating D-AAs and short C-terminal DAACPs while removing all L-AAs in a wash step. Amino acids with basic side chains were highly retained on TAG; amino acids with acidic side chains were much less retained. This resulted in some L-AAs eluting after D-AAs, only for these specific groups of amino acids. Addition of ammonium buffer hastened the elution of amino acids and decreased the selectivity between D- and L-AAs. In the case of peptides, the TAG media can be used to retain peptides with D-AAs on the C-terminus while eluting their all L-amino acid analogues. A usable solvent "cutoff" composition was established for both glycyl- and alanyl-dipeptides on the TAG stationary phase with all C-terminal L-AA peptides eluting prior to peptides with C-terminal D-AAs. TAG also excels at retaining peptides in general at high and low % organic mobile phase compositions as the U-shaped retention curves demonstrate. A CPY enzymatic hydrolysis was performed on a mixture of AQC-derivatized synthetic DAACP and its L-amino acid counterpart. It was demonstrated that the L-amino acid exclusive enkephalin peptide was eliminated, whereas the DAACP enkephalin epimer remained intact for subsequent extraction and analysis by LC–MS with a TeicoShell CSP.

ACKNOWLEDGMENTS

We thank AZYP LLC for providing the CSPs used in this project as well as their expertise in chiral separations when consulted.

DATA AVAILABILITY STATEMENT

All data is available upon request.

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How to cite this article: Sung Y-S, Khvalbota L, Dhaubhadel U, Špánik I, Armstrong DW. Teicoplanin aglycone media and carboxypeptidase Y: Tools for finding low-abundance D-amino acids and epimeric peptides. *Chirality*. 2023;1-8. doi:10.1002/chir.23543