High-throughput enantioseparation of Nα-fluorenylmethoxycarbonyl proteinogenic amino acids through fast chiral chromatography on zwitterionic-teicoplanin stationary phases

High–throughput enantioseparation of *N*α–fluorenylmethoxycarbonyl proteinogenic amino acids through fast chiral chromatography on zwitterionic-teicoplanin stationary phases

In this study, 31 racemates of Nα-FMOC (fluorenylmethoxycarbonyl) amino acids (AAs) with different chemico-physical characteristics (neutral nonpolar, neutral polar, acidic and basic) have been success- fully resolved in fast enantioselective chromatography on recently-developed zwitterionic-teicoplanin chi- ral stationary phases (CSPs). The CSPs were prepared by covalently bonding the teicoplanin selector on fully-porous particles of narrow dispersion particle-size distribution (particle diameter 1.9 μm) and superficially-porous particles (2.0 μm). Both the zwitterionic-teicoplanin CSPs have proved to be ideal media for the separation of this important class of compounds. In particular, the zwitterionic CSP pre- pared on superficially-porous particles exhibited superior enantioselectivity and resolution, compared to that made of fully porous particles, in virtue of more favorable thermodynamics. The zwitterionic na- ture of these CSPs allowed avoiding the annoying effect of Donnan's exclusion of enantiomers from the stationary phase. This effect, on the opposite, was frequently observed on a commercial teicoplanin CSP (Teicoshell) employed for comparative purposes. Noticeably, on the zwitterionic-teicoplanin CSPs, by us- ing either acetonitrile- or methanol-rich mobile phases (MPs), it was possible to favor speed over enan- tioresolution and vice versa. This work gives further replies to the request for rapid determination of enantiomeric excess of Nα-FMOC proteinogenic (and non–proteinogenic) AAs, typically used as preferred chiral synthons in the solid-phase synthesis of therapeutic peptides.

1. Introduction

Since the pioneering work by Fisher [1], the chemical synthesis of peptides has intrigued and challenged the curiosity of chemists [2,3]. In this field, 1963 marked a turning point, when Merrifield introduced the concept of solid-phase synthesis as the tool for the preparation of synthetic peptides [4]. Moreover, the increasing success of peptide drugs in therapy — notably glucagon-like peptide-1 receptor agonists — and the promising pipeline of peptide drugs, has stimulated the interest in therapeutic peptides by pharmaceutical industries [5-7]. As a matter of fact, nowadays, many peptide drugs are prepared via solid-phase synthesis by employing either Nα-FMOC (fluorenylmethoxycarbonyl) proteinogenic and non-proteinogenic amino acids (AAs) as preferred chiral synthons [5,8].

From a stereochemical point of view, native peptides are mainly made up of *L*-AAs [9]. Therefore, since the activity of peptides strongly depends on the stereochemical purity of their chiral synthons, the determination of the enantiomeric excess of *N*-protected AAs becomes pivotal. Otherwise, diastereoisomeric peptide impurities could be originated during the manufacturing process, with additional costs in the purification of finished products following their removal [10]. One of the preferred methods for assessing stereochemical purity is the direct chromatographic separation of enantiomers [11-13]. When it comes to the separation of

*N*α–FMOC AAs, the chiral stationary phase (CSP) must be chosen by considering that *N*α-derivatization leads to the formation of predominantly acidic species, whose chromatographic behavior is distinctly different from that of the starting AAs. Several CSPs have been used for the enantioseparation of *N*α-derivatized AAs [14], including polysaccharide-type [15,16], ion-exchange quinine-based [17-20] and macrocyclic glycopeptides (or antibiotics) [21-23]. A great structural variety characterizes macrocyclic antibiotics. Indeed, not only they have a wide range of molecular weights (roughly from 600 to 2000 Da), but also they may have acidic, basic, or neutral character. All of these features make this class of CSPs suitable for the separation of a wide range of chiral molecules under a variety of chromatographic modes (reversed- and normal-phase, polar organic mode, hydrophilic interaction chromatography HILIC, supercritical fluid chromatography SFC). It is worth mentioning that, since their introduction by Armstrong in 1994 [24], teicoplanin, teicoplanin aglycon, vancomycin, vancomycin aglycon, and ristocetin-A CSPs have been made commercially available (under different commercial names). Among them, teicoplanin is the most popular one. Thanks to the numerous interactions that can be established between selectands and chiral selector, commercial teicoplanin CSPs have been successfully employed for the resolution of many sorts of racemates [25,26].

Recently, a new kind of teicoplanin CSP has been prepared by some of the authors of this paper [22, 23]. Differently from the commercially available [24,26,27] — where the teicoplanin selector, bonded to silica via ureidic-bond, globally imparts a negative charge to the CSP — an innovative bonding protocol has been used for its preparation. This has allowed preserving on the CSP not only the carboxylic- but also the amino-group. Thus, depending on the pH of the surrounding mobile phase (MP), these groups can be respectively deprotonated (to give a carboxylate) or protonated (quaternary ammonium). At neutral pH, the carboxylate and the quaternary ammonium groups are both presents. For this reason, the CSP was named zwitterionic-teicoplanin [22,23,28]. The zwitterionic character of the selector has been proven to further extend the applicability of teicoplanin CSPs to very challenging separations, thanks to mixed ion-exchange mechanisms not achievable on the commercial version of the CSP [29-31].

The advantage of using chiral superficially porous particles (SPPs) over fully porous (FPP) ones to perform high performance fast or ultra-fast enantioseparations (the expression enantioselective ultra high performance liquid chromatography, eUHPLC, will be used in this paper to define these modes of operation) has already been thoroughly investigated [23,32-36]. Thus, the kinetic superiority of CSPs based on SPPs makes them ideal supports for high-throughput eUHPLC more and more required at the industrial level for the control of chiral active pharmaceutical ingredients (APIs), reaction intermediates, chiral synthons, etc. [37-40].

In this paper, the two columns, which were used, were in-house packed with zwitterionic-teicoplanin CSPs prepared on both SPPs of 2.0 μm particle diameter (90 Å nominal pore size) and FPPs of 1.9 μm particle diameter and narrow particle-size distribution (120 Å pore size). Both columns were employed to resolve 31 racemates of *N*α-FMOC AAs, by using either acetonitrile (ACN)- or methanol (MeOH)-rich MPs. For the sake of comparison, the separations of *N*α-FMOC AAs were also performed, under the same experimental conditions, on a commercially available teicoplanin CSP (Teicoshell column, packed with 2.7 μm particle diameter SPPs).

2. Materials and methods

2.1. Materials and chemicals

Water, MeOH, ACN, and ammonium formate ($HCOONH_4$) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. HPLC gradient grade solvents were filtered on 0.2 μm Omnipore filters (Merck Millipore, Darmstadt, Germany). Naphthalene was purchased from Sigma-Aldrich (St. Louis, MO, USA). The 32 *N*α-FMOC AAs (including the nonchiral FMOC-Gly) were available from previous studies or kindly provided by Fresenius-Kabi IPSUM (Villadose, RO Italia).

2.2. Instrumentation

An UltiMate 3000 RSLC LC (Thermo Fisher Dionex, Sunnyvale, CA, USA) equipped with dual gradient pumps, an in-line split-loop well-plate sampler, a thermostated-column ventilated compartment (temperature range: 5- 110°C), and a diode array detector (Vanquish) with a 2.0 μL low-dispersion flow-cell was employed. The UV detector was set at 0.10 s time-constant and 100 Hz data collection rate. UV detection was performed at 214 nm. Data acquisition and processing were performed with Chromeleon 7.2 (Thermo Fisher Dionex, Sunnyvale, CA, USA).

2.3. Chiral stationary phases

Two 50 × 4.6 mm (L. x I.D.) stainless steel columns were in house packed with zwitterionic CSPs prepared in our laboratories [41] by using the slurry packing method: slurry solvent $CHCl_3$ /IPA, 1/1 (*v*/*v*); conc. CSP 3% (*w*/*v*). Pushing solvent acetone, packing pressure until 1000 Bar by a constant pressure Haskel Pump.

Two particle formats were employed: (i) FPPs of narrow dispersion particle-size distribution (Titan®), 1.9 μm particle diameter, 120Å nominal pore size (surface area 282 m^2/g); (ii) SPPs (Halo®), 2.0 μm particle diameter, 90 Å pore size (surface area 125 m^2/g). These columns/CSPs will be in the following referred to as UHPC-FPP-Titan-Tzwitt-1.9μm-120Å and UHPC-SPP-Halo-Tzwitt-2.0μm-90Å, respectively [22,23].

Through CHN analysis of UHPC-FPP-Titan-Tzwitt-1.9μm-120Å and UHPC-SPP-Halo-Tzwitt-2.0μm-90Å [23], the values of absolute loading (both as μmol selector/g CSP and μmol selector/g silica) and specific loading (μmol selector/m^2) were obtained (Table S1 of Supplementary Information SI). Moreover, data concerning average distance (Å) between molecules of selector, μmol selector/column (taking into account that about 35-40% more material is needed in columns packed with SPPs than those packed with FPPs) and porosity (ε_{tot}) are reported.

Moreover, a commercially available teicoplanin-based column (Teicoshell, 100 4.6 mm L.xI.D., AZYP, Arlington, TX, USA) packed with SPPs of 2.7 μm particle diameter and 120 Å pore size was also employed.

2.4. Mobile Phases and Methodology

Two different MPs were used: (i) ACN/H_2O 85:15 *v*/*v* + 15 mM $HCOONH_4$ (^{MP}pH = 7.5), and (ii) MeOH/H_2O 85:15 *v*/*v* + 15 mM $HCOONH_4$ (^{MP}pH = 7.0). All measurements were performed in isocratic elution mode using a single solvent line. The injection volume was 0.1 μL. The flow-rate was 1.0 mL/min. Measurements were replicated twice. Average values were used for the calculation (according to the European Pharmacopeia through peak-width at half height) of retention factor (*k*), enantioselectivity (α), enantioresolution (*Rs*) and efficiency (N/m). The hold-up time was estimated by using naphthalene as the marker.

3. Results and Discussion

Proteinogenic AAs can be classified, consistently with their side-chain, in (i) neutral-nonpolar (NN-); (ii) neutral-polar (NP-); (iii) acidic (A-); and (iv) basic (B-). For reasons of clarity, we have maintained this classification for the *N*α-FMOC AAs considered in this

Table 1

Retention factor (*k*), enantioselectivity (**α**), enantioresolution (*Rs*) and efficiency (N/m) for all the 32 protected amino acids (AAs) considered in this work on the three columns employed and at the two MP compositions (ACN-rich MP: ACN/H_2O 85:15 *v/v* + 15 mM $HCOONH_4$; MeOH-rich MP: MeOH/H_2O 85:15 *v/v* + 15 mM $HCOONH_4$) Column-1: UHPC-FPP-Titan-Tzwitt-1 9μm-120Å (50 × 4 6mm); column-2: UHPC- SPP-Halo-Tzwitt-2 0μm-90Å (50 × 4 6mm); column-3: Teicoshell (100 × 4 6 mm, 2 7μm SPP diameter, 120 Å pore size) Side chain: NN-(Neutral-Nonpolar); NP- (Neutral Polar); A- (Acidic); B- (Basic) See text for details

Entry	Side chain	AAs	Column	ACN-rich MP				MeOH-rich MP			
				k	α	Rs	N/m	k	α	Rs	N/m
1	NN-	FMOC-Gly	1	2 75	–	–	227 100	7 26	–	–	177 560
			2	3 77	–	–	225 300	11 8	–	–	150 300
			3	0 96	–	–	166 220	0 08	–	–	93 370
2	NN-	FMOC-(*D,L*)-Ala	1	2 11			232 000	5 00			192 820
				2 51	1 19	2 88	138 000	8 44	1 69	8 06	76 560
			2	2 77			224 780	7 35			170 220
				3 87	1 43	4 96	68 900	14 9	2 02	9 35	51 540
			3	0 75			177 540	-0 031 05[t]			132 220
				0 88	1 16	2 16	143 330	0 221 32[t]	–	4 44	35 760
3	NN-	FMOC-(D,L)-Ile	1	1 16			235 920	3 16			201 240
				1 34	1 16	2 07	188 560	4 53	1 43	5 33	79 880
			2	1 25			225 740	4 05			182 180
				1 71	1 37	4 26	131 520	6 89	1 70	6 43	43 820
			3	0 42			194 910	-0 110 97[t]			147 900
				0 52	1 22	1 98	136 910	-0 011 08[t]	–	2 18	39 970
4	NN-	FMOC-(*D,L*)-Phe	1	1 31			219 000	4 96			173 580
				1 41	1 07	1 03	197 060	6 47	1 3	4 56	108 160
			2	1 56			217 420	7 34			151 440
				1 80	1 15	2 18	170 040	10 5	1 43	5 45	71 780
			3	0 44			190720	-0 051 03[t]			129 300
				0 50	1 13	1 32	164060	0 041 13[t]	–	2 23	81 720
5	NN-	FMOC-(*D,L*)-Leu	1	1 18			233 800	3 35			193 200
				1 34	1 14	1 90	208 540	4 95	1 48	6 37	107 180
			2	1 25			224 460	4 33			175 900
				1 67	1 33	4 08	158 860	7 44	1 72	7 83	70 140
			3	0 43			177 990	-0 100 98[t]			123 880
				0 51	1 19	1 83	158 520	0 041 13[t]	–	2 78	45 160
6	NN-	FMOC-(*D,L*)-Met	1	1 44			231 040	4 60			186 760
				1 69	1 18	2 53	194 520	7 10	1 54	7 33	101 820
			2	1 70			225 780	6 47			165 520
				2 41	1 41	5 41	146 760	11 8	1 82	8 94	71 340
			3	0 50			181 870	-0 110 97[t]			119 900
				0 62	1 23	2 30	155 870	0 091 18[t]	–	4 15	47 970
7	NN-	FMOC-(*D,L*)-Trp	1	1 77			157 680	8 63			145 520
				1 85	1 05	–	–	10 3	1 19	3 07	101 660
			2	2 25			200 620	15 1			124 520
				2 50	1 11	1 75	154 040	19 5	1 29	3 91	65 600
			3	0 48			148300	-0 090 99[t]			105 920
				0 53	1 10	0 97	129330	-0 011 08[t]	–	1 97	66 890
8	NN-	FMOC-(*D,L*)-Trp-(Boc)	1	0 87			–	4 43			–
					–	–	–		–	–	–
			2	0 98			–	6 51			–
					–	–	–		–	–	–
			3	0 26			–	-0 081 00[t]			102 540
					–	–	–	0 071 16[t]	–	2 93	38 690
9	NN-	FMOC-(*D,L*)-Pro	1	2 60			–	5 04			–
					–	–	–		–	–	–
			2	2 67			–	6 28			–
					–	–	–		–	–	–
			3	1 06			–	-0 081 00[t]			–
					–	–	–		–	–	–
10	NN-	FMOC-(*D,L*)-Val	1	1 42			233 180	3 52			201 500
				1 53	1 08	1 19	198 720	4 68	1 33	4 89	121 280
			2	1 54			228 880	4 59			179 300
				1 83	1 19	2 67	161 480	6 98	1 52	6 38	76 540
			3	0 51			185 730	-0 140 93[t]			140 580
				0 56	1 10	1 13	156 780	-0 051 03[t]	–	2 36	71 260
11	NP-	FMOC-(*D,L*)-Tyr	1	2 20			195 620	6 08			153 240
				2 30	1 05	–	–	7 66	1 26	3 86	97 100
			2	2 97			186 040	9 90			129 020
				3 35	1 13	1 87	97 220	13 6	1 38	4 63	59 840
			3	0 64			171 580	-0 110 97[t]			118 380
				0 70	1 09	1 05	139 830	-0 011 08[t]	–	2 37	64 410
12	NP-	FMOC-(*D,L*)-Tyr-(tBu)	1	1 10			217 420	4 04			159 200
				1 24	1 13	1 61	190 100	6 47	1 60	6 78	74 100
			2	1 25			201 300	5 56			132 180
				1 58	1 26	3 10	149 780	10 1	1 82	7 56	51 300
			3	0 36			189 870	-0 081 00[t]			94 920

(*continued on next page*)

Table 1 (*continued*)

				0 43	1 21	1 74	156 310	0 151 25[a]	–	4 16	39 390
13	NP-	FMOC-(*D,L*)-Ser	1	2 68			237 480	5 60			205 240
				2 82	1 05	0 98	211 360	6 46	1 15	2 92	166 240
			2	3 23			197 620	8 23			184 680
				3 65	1 13	2 37	201 440	10 0	1 21	3 92	132 940
			3	0 93			169 430	-0 090 99[a]			123 770
				0 99	1 06	0 96	164 950	-0 011 08[a]	–	2 21	78 020
14	NP-	FMOC-(*D,L*)-Ser-(tBu)	1	1 15			232 240	3 25			199 320
				1 25	1 09	1 23	212 760	4 14	1 27	4 30	137 620
			2	1 18			229 220	4 08			180 140
				1 43	1 21	2 71	186 340	5 77	1 41	5 54	92 700
			3	0 42			191 250	-0 110 97[a]			131 280
				0 48	1 13	1 27	172 230	-0 021 06[a]	–	2 35	76 050
15	NP-	FMOC-(*D,L*)-Thr	1	1 78			–	8 62			149 520
				1 86	1 05	–	–	10 2	1 18	2 92	105 000
			2	2 23			191 760	14 9			129 680
				2 48	1 11	1 67	137 400	18 9	1 27	3 74	68 280
			3	0 48			175 710	-0 120 96[a]			145 350
				0 52	1 10	1 00	162 120	-0 071 01[a]	–	1 65	98 880
16	NP-	FMOC-(*D,L*)-Thr-(tBu)	1	1 40			–	2 63			188 240
					–	–	–	2 81	1 07	1 16	171 180
			2	1 00			–	2 99			169 800
				1 04	–	–	–	3 32	1 11	1 75	149 900
			3	–			–	-0 130 94[a]			–
				–	–	–	–		–	–	–
17	NP-	FMOC-(*D,L*)-Asn	1	3 96			–	8 06			171 380
					–	–	–	9 10	1 13	2 41	142 980
			2	4 90			–	12 8			155 360
					–	–	–	15 0	1 17	2 93	118 820
			3	1 02			–	0 031 12[a]			51 340
					–	–	–	0 171 27[a]	–	–	–
18	NP-	FMOC-(*D,L*)-Asn-(Trt)	1	0 87			–	5 23			–
					–	–	–		–	–	–
			2	0 91			–	7 6			135 100
					–	–	–	8 14	1 07	1 15	99 200
			3	0 28			–	-0 061 02[a]			–
					–	–	–		–	–	–
19	NP-	FMOC-(*D,L*)-Gln	1	4 15			214 020	6 42			166 320
				4 42	1 07	1 17	126 240	8 33	1 30	7 99	117 480
			2	4 94			236 600	9 75			144 900
				5 87	1 19	3 81	201 620	13 6	1 39	4 71	90 600
			3	1 37			155 170	-0 051 03[a]			99 120
				1 54	1 12	2 05	126 420	0 021 11[a]	–	1 58	84 040
20	NP-	FMOC-(*D,L*)-Gln-(Trt)	1	0 84			–	3 90			146 840
				0 89	1 06	–	–	4 76	1 22	3 23	114 500
			2	0 86			186 800	5 52			134 780
				0 99	1 16	1 64	168 620	7 31	1 32	4 46	93 920
			3	0 28			–	-0 100 98[a]			122 150
					–	–	–	-0 021 06[a]	–	1 96	57 040
21	NP-	FMOC-(*D,L*)-Cys	1	1 70			–	6 10			128 020
					–	–	–	6 97	1 14	1 14	90 180
			2	2 41			–	11 0			62 380
					–	–	–	12 8	1 16	1 68	37 620
			3	0 65			–	-0 061 02[a]			100 460
					–	–	–	0 041 13[a]	–	1 82	40 110
22	NP-	FMOC-(*D,L*)-Cys-(Trt)	1	0 58			–	4 19			152 900
					–	–	–	4 59	1 10	1 59	137 520
			2	0 61			184 640	6 26			140 740
				0 69	1 12	1 05	159 360	7 24	1 16	2 54	120 020
			3	0 13			–	-0 110 97[a]			–
					–	–	–	-0 090 99[a]	–	–	–
23	A-	FMOC-(*D,L*)-Asp	1	23 1			–	186			–
					–	–	–		–	–	–
			2	34.3			–	459			–
					–	–	–	467	1.02	–	–
			3	3 35			–	-0 021 06[a]			–
					–	––	–		–	–	–
24	A-	FMOC-(*D,L*)-Asp-(OtBu)	1	23 1			–	4 40			179 220
					–	–	–	4 88	1 11	1 95	150 760
			2	34 7			–	6 68			165 840
					–	–	–	7 78	1 16	2 09	122 800
			3	0 35			–	-0 090 99[a]			–
					–	–	–	-0 100 98[a]	–	–	–
25	A-	FMOC-(*D,L*)-Glu	1	35 0			–	160			108 598

(*continued on next page*)

Table 1 (*continued*)

					–	–	–	170	1 06	1 42	106 8 82
			2	51 0			–	364			110 728
					–	–	–	405	1 11	2 76	103 374
			3	4 88			–	-0 081 00[a]			98 380
					–	–	–	0 021 11[a]	–	2 21	51 980
26	A-	FMOC-(*D,L*)-Glu-(OtBu)	1	1 10			229 200	3 50			168 800
				1 21	1 10	1 35	201 580	5 01	1 49	4 28	80 840
			2	1 26			204 460	5 06			139 780
				1 55	1 23	2 72	133 980	8 32	1 64	5 99	48 880
			3	0 27			160 050	-0 090 99[a]			121 850
				0 32	1 16	1 11	194 440	0 091 18[a]	–	3 34	31 680
27	B-	FMOC-(*D,L*)-Lys	1	7 11			224 180	0 77			97 400
				7 63	1 07	1 58	184 140	1 28	1 66	3 49	45 400
			2	4 64			226 960	0 68			81 440
				5 54	1 19	3 50	147 440	1 41	2 06	4 26	33 580
			3	6 74			152 680	0 99			60 500
				7 56	1 12	2 85	111 490	1 30	1 31	2 98	5 630
28	B-	FMOC-(*D,L*)-Lys-(Boc)	1	1 63			224 640	2 82			181 660
				1 93	1 18	2 65	180 560	5 12	1 82	8 93	95 420
			2	1 28			205 540	3 65			162 220
				1 91	1 49	5 12	113 300	8 23	2 25	10 9	66 800
			3	0 33			202 580	-0 120 96[a]			135 120
				0 43	1 30	2 41	146 150	0 101 19[a]	–	4 55	44 570
29	B-	FMOC-(*D,L*)-Arg	1	5 65			207 420	0 66			103 080
				6 37	1 13	2 42	153 160	1 38	2 09	4 74	40 040
			2	4 10			150 600	0 69			82 580
				5 29	1 29	4 71	123 400	1 68	2 44	5 26	30 960
			3	5 72			150 170	0 97			58 390
				6 87	1 20	4 24	95 450	1 28	1 32	2 01	19 210
30	B-	FMOC-(*D,L*)-Arg-(Pbf)	1	1 46			183 020	5 62			82 760
				1 65	1 13	1 71	145 120	9 64	1 72	6 03	42 300
			2	1 78			161 000	8 63			78 480
				2 38	1 34	3 75	93 040	17 5	2 03	7 47	35 280
			3	0 54			157 620	0 001 09[a]			44 320
				0 66	1 22	2 19	120 540	0 351 47[a]	–	3 35	15 940
31	B-	FMOC-(*D,L*)-His	1	5 78			177040	5 64			86 560
				6 11	1 06	–	–	7 33	1 30	3 21	52 120
			2	6 09			162 480	8 16			56 760
				6 89	1 13	2 25	126 740	11 1	1 36	3 14	34 340
			3	3 44			115 100	1 19			25 070
				4 00	1 16	2 75	66 810	1 74	1 46	1 44	3 540
32	B-	FMOC-(*D,L*)-His-(trt)	1	1 35			–	4 18			133 480
					–	–	–	5 65	1 35	4 45	85 080
			2	1 25			190 160	5 35			126 960
				1 36	1 09	1 13	155 500	8 20	1 53	5 61	55 280
			3	0 57			–	0 001 09[a]			24 660
					–	–	–	1 382 58[a]	–	3 05	1 660

[*] Retention time (minutes) for analytes with negative k (column 3- t_0 =1 086 minutes**).**

work (see Table 1). Since these compounds are used for polypeptide production through solid-phase synthesis, many of them are not only protected in the **α**-amino function but also in other reactive sites. The structural variability of *N***α**-FMOC AAs thus represents an important "testbed" to evaluate the chromatographic behavior of the different CSPs used in this work. Two different aqueous-organic MPs have been employed on the three columns. In both cases, the MP composition was organic/water 85:15 % v/v + 15 mM $HCOONH_4$. Organic can be either ACN or MeOH. In the following, the expression ACN- and MeOH-rich will be used to refer to these MPs.

The *N***α**-FMOC AAs were injected as single racemates, and all data regarding the 32 analytes (31 racemates plus the achiral FMOC-Gly) collected in this work have been summarized in Table 1. We anticipate that to facilitate the comparison between columns (and since this was not the scope of this work), chromatographic conditions have not been optimized on each column to favor the separation of a specific compound. On the opposite, the columns were all operated under the same experimental conditions.

3.1. *N***α**-FMOC neutral-nonpolar (NN-) amino acids

Let us start by considering NN-AAs (entries 1-10 Table 1) with the ACN-rich MP. A first general observation is that, under these conditions, AAs were little retained on all columns, even if the zwitterionic-teicoplanin columns were significantly more retentive than the Teicoshell one. Indeed, only in two cases (Table 1, entries 1 and 2), the retention factor of the more retained enantiomer exceeded 3 on the zwitterionic-teicoplanin columns. In both cases, this was observed on the UHPC-SPP-Halo-Tzwitt-2.0µm-90Å column. On the opposite, on the Teicoshell column, none of FMOC AAs exhibited $k > 1$ with the only exception of FMOC-*(D,L)*-Pro (Table 1 entry 9), whose enantiomers, however, were not separated. In addition to FMOC-(*D, L*)-Pro, also the enantiomers of FMOC-(*D, L*)-Trp (Boc) (Table 1 entry 8) could not be resolved on any column.

As an example of chromatographic separations of NN-AAs with the ACN-rich MP, Fig. 1A compares the chromatograms for the separation of FMOC-(*D,L)*-Phe (Table 1 entry 4) on the three columns. It is worth noticing not only the shorter retention but also the significantly less efficiency achieved on the commercial column.

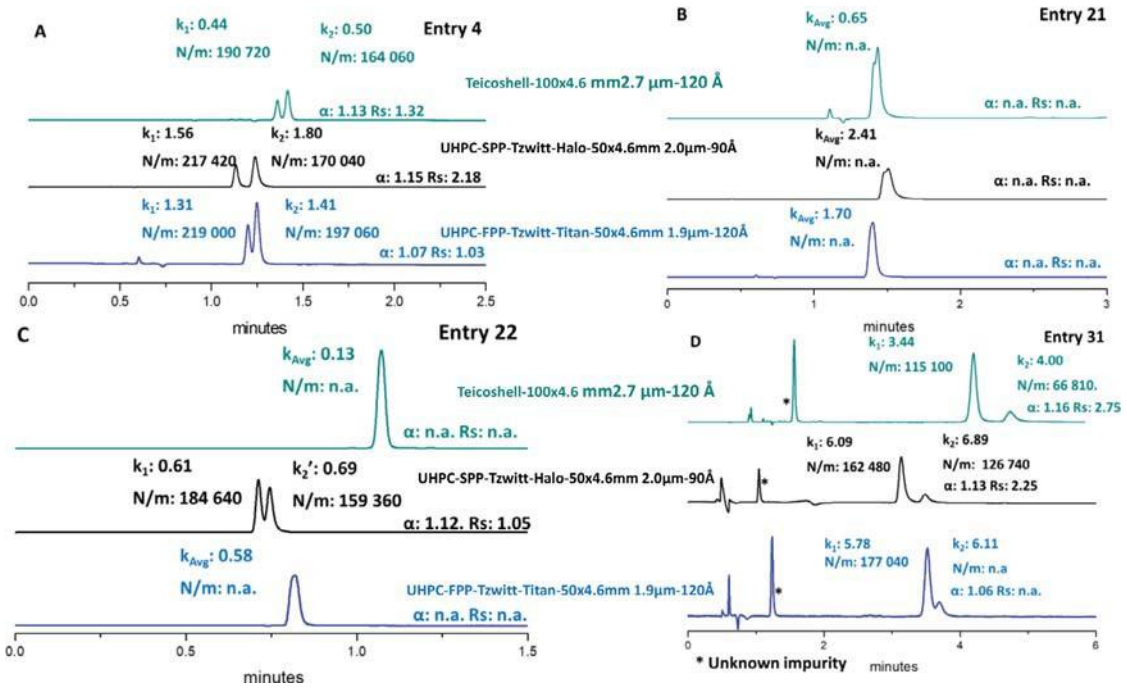

Fig. 1. Enantioseparation of A) FMOC-(*D,L*)-Phe (Table 1 entry 4), B) FMOC-(*D,L*)-Cys (Table 1 entry 21), C) FMOC-(*D,L*)-Cys-(Trt) (Table 1 entry 22), and FMOC-(D,L)-His (Table 1 entry 31) Comparison on the three columns employed in this work From top to bottom: commercial Teicoshell, UHPC-SPP-Halo-Tzwitt-2 0µm-90Å, UHPC-FPP- Titan-Tzwitt-1 9-120Å MP: ACN/H_2O 85/15% *v/v* + 15 mM $HCOONH_4$. Flow rate: 1 0 mL/min UV detection at 214 nm

By comparing the zwitterionic-teicoplanin CSPs, it is evident that chiral SPPs have allowed to improve both the thermodynamics of the separation (through larger enantioselectivity) and, as a consequence of that, the enantioresolution.

By considering, now, the chromatographic behavior of NN-AAs with the MeOH-rich MP, some very interesting things can be noticed. Firstly, on both zwitterionic-teicoplanin columns, retention, enantioselectivity, and resolution were found to be dramatically larger compared to the ACN-rich conditions. Secondly, this increase was significantly more pronounced on the UHPC-SPP-Halo-Tzwitt-2.0µm-90Å column than on the UHPC-FPP-Titan-Tzwitt-1.9-120Å one. Thirdly, when the Teicoshell column is operated under this condition, the retention is practically lost for all considered AAs. The maximum k observed with this column was 0.2 (Table 1 entry 2), but often $k < 0.1$ was found. There are several cases where enantiomers are eluted before the hold-up time of the Teicoshell column. These cases are evidenced in Table 1 with negative retention factors (however, retention times are reported). This phenomenon can be explained by considering the electrostatic negative repulsion (Donnan's effect) between the negatively charged CSP and analytes. In fact, considering the mobile phase ^{MP}pH =7-7.5, both the silica bonded teicoplanin (see introduction section), and the *Nα*-FMOC protected amino acids are expected to be deprotonated [42]. As it was pointed out before, the ureidic linkage — through which the chiral selector was bound to the silica surface — imparts a globally-negative charge to the surface of the commercial teicoplanin. On the other hand, FMOC AAs are also negatively charged in these conditions. Thus, the Teicoshell column suffers from a strong Donnan's effect in this MeOH-rich condition.

As examples of separations with the MeOH-rich MP, Fig. 2A shows what happens by moving from the ACN- to the MeOH-rich MP for FMOC-(*D,L*)-Phe (Table 1 entry 4) on the UHPC-SPP-Halo-Tzwitt-2.0µm-90Å On the other hand, Fig S1 of SI reports the chromatograms for FMOC-*(D,L)*-Ala (Table 1 entry 2) on the Teicoshell column.

Thus, to summarize the information regarding the zwitterionic CSPs, it may be said that both enantioselectivity and resolution are always significantly larger on the column made of SPPs than on that packed with fully porous ones. This happens with both ACN- and MeOH-rich MPs, being more evident with the latter. On the opposite, the efficiency decreases (especially for secondly eluted enantiomers) if the MeOH-rich MP is employed in place of the ACN-rich one.

32. *Nα-FMOC neutral-polar (NP-) amino acids analysis*

FMOC NP-AAs (entries 11-22 Table 1) are characterized by the presence of polar groups (e.g., OH, SH) in their side-chains. Due to the reactivity of these groups, and to avoid undesired reactions, they are often derivatized before solid-phase synthesis. Chemical protecting groups typically employed for this purpose are, e.g., Trt: trityl; Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Tbu: *tert*-butyl.

Entries 12, 14, 16, 18, 20, and 22 in Table 1 are examples of FMOC NP-AAs protected on the side-chain. As before, the study on the chromatographic behavior of FMOC NP-AAs on the three columns starts by considering the ACN-rich MP. A first observation is that on both the zwitterionic teicoplanin-based CSPs, retention of FMOC NP-AAs was always slightly larger than that of FMOC NN-AAs (Table 1 entries 2-10). Moreover, side-chain protected AAs are less retained than their analogous unprotected ones. In Figs. 1B, 1C, and 2B, 2C, the interesting example of separations of FMOC-(*D, L*)-Cys (Table 1 entry 21) and FMOC-(*D, L*)-Cys(Trt) (Table 1 entry 22) are reported. From these chromatograms (Fig. 1B and 1C) it is evident that separation of enantiomers of FMOC-(*D, L*)-Cys and FMOC-(*D, L*)-Cys(Trt) was not possible when the ACN-rich MP was employed (apart for those of FMOC-(*D, L*)-Cys(Trt) on the UHPC-SPP-Halo-Tzwitt-2.0µm-90Å, Fig 1C) This result can be, in part, generalized to the category of NP-AAs. Indeed, in half of the cases, FMOC NP-AAs could not be separated with this MP composition. As it happened before for NN-AAs, noticeably larger retention on

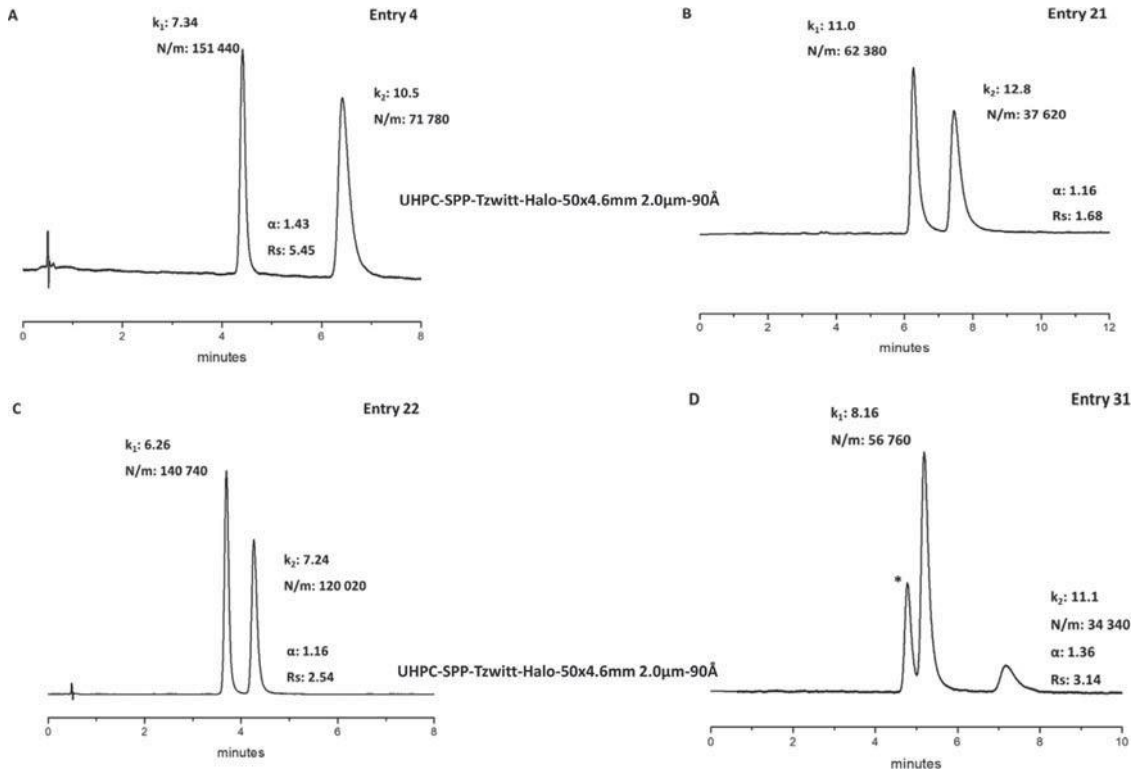

Fig. 2. Enantioseparation of A) FMOC-(D,L)-Phe (Table 1 entry 4), B) FMOC-(D,L)-Cys (Table 1 entry 21), C) FMOC-(D,L)-Cys-(Trt) (Table 1 entry 22), and FMOC-(D,L)-His (Table 1 entry 31), on the UHPC-SPP-Halo-Tzwitt-2 0µm-90Å column with the MeOH-rich MP (MeOH/H_2O 85/15% *v/v* + 15 mM $HCOONH_4$) Flow rate: 10 mL/min UV detection at 214 nm

the zwitterionic-teicoplanin CSPs compared to the Teicoshell one was observed.

The effect of the use of the MeOH-rich MP on the behavior of FMOC NP-AAs can be introduced by referring again to the chromatograms of Fig. 2. Figs. 2B and 2C show that a quasi-baseline separation of the two couples of enantiomers previously unresolved (Table 1 entries 21 and 22) was achieved on the UHPC-SPP-Halo-Tzwitt-2.0µm-90Å column. Only slightly worse results were obtained with the UHPC-FPP-Titan-Tzwitt-1.9-120Å (see Table 1), while on the commercial Teicoshell teicoplanin column, both FMOC-(*D, L*)-Cys and FMOC-(*D, L*)-Cys(Trt) were excluded from the stationary phase, such as the other FMOC NP-AAs considered in this work (see Fig S1 under SI for an example).

In conclusion, for the separation of NP-AAs on zwitterionic teicoplanin-based CSPs, very similar considerations to those drawn for NN-AAs can be done. Shortly, the ACN-rich MP allowed faster and more efficient separations, but the MeOH-rich one permitted to increase the enantioselectivity and the resolution. Moreover, comparing the zwitterionic CSPs, the UHPC-SPP-Halo-Tzwitt-2.0µm-90Å has proven to the best choice to maximize the thermodynamics of separation (enantioresolution).

3.3. *Nα-FMOC acidic (A-) and basic (B-) amino acids analysis*

It is remarkable to observe that with the ACN-rich MP, all *Nα*-FMOC acidic (A-) AAs considered in this work (Table 1 entries 23-26) could never be separated on any of the columns but in one case (FMOC-(*D,L*)-Glu OtBu, Table 1 entry 26). Admittedly, this result should not be taken as the final one, since no attempt to improve the separation through changes in MP composition was done. As was pointed out before, indeed, this was not the purpose of this paper.

On both the zwitterionic-teicoplanin CSPs, in three cases (Table 1 entries 23-25), the retention of A-AAs was extremely large, also with the ACN-rich MP, even if the separation of enantiomers did not occur. As an example, *k* as large as 51 was observed for FMOC-*(D,L)*-Glu (Table 1 entry 25). In general, the zwitterionic-teicoplanin CSPs were significantly more retentive than the commercial phase. Separation of *Nα*-FMOC A-AAs can be improved by using MeOH-rich conditions (as it happened before for the other classes of AAs) but at the cost of a dramatic retention increase. As an example, $k > 400$ (!) was obtained for the second eluted enantiomer of FMOC-(*D,L*)-Glu (Table 1 entry 25) that makes, in any case, this separation quite unpractical. On the opposite on the commercial Teicoshell column, FMOC A-AAs were excluded (see Fig S1 under SI for an example) with MeOH-rich MP.

Contrary to acidic ones, *Nα*-FMOC basic (B-) AAs (entries 27-32 in Table 1) were always separated with the ACN-rich MP on all the three columns (the only exception is FMOC *(D,L)*-His-(trt), Table 1 entry 32), that was separated only on the UHPC-SPP-Halo-Tzwitt-2.0µm-90Å). With the MeOH-rich MP, on the other hand, only one of the six FMOC B-AAs (Table 1 entry 28) was excluded by the stationary phase of the Teicoshell column. This is not surprising since *Nα*-FMOC B-AAs, for their chemical characteristics, do not suffer (or only in minimal part) of Donnan's repulsion effect [42]. As an example, Fig. S1 of SI shows the chromatogram for the separation of FMOC-(D,L)-Arg (Table 1 entry 29) on the Teicoshell column.

As for the enantioselectivity with ACN, it is worth noticing that the UHPC-SPP-Halo-Tzwitt-2.0µm-90Å column always outperforms the other two. Interestingly, in many cases, α on the commercial Teicoshell CSP was larger than that on the UHPC-FPP-Titan-Tzwitt-1.9µm-120Å CSP. On the other hand, with the MeOH-rich MP, the enantioselectivity increased compared to ACN-rich conditions no matter what happens to retention (that in some cases

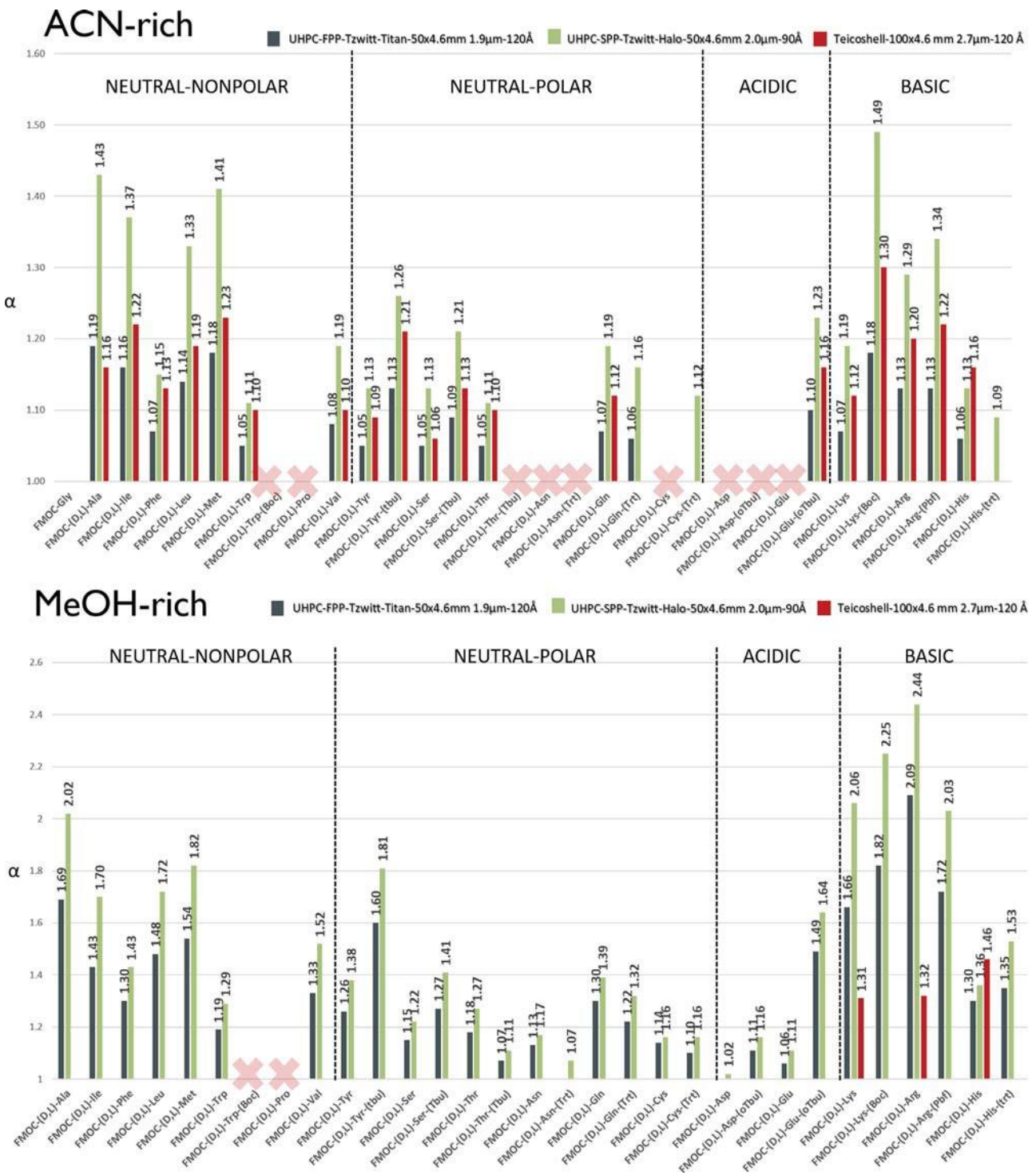

Fig. 3. Bar chart representing the enantioresolution (α) for the FMOC AAs considered in this work For the sake of comparison, AAs have been divided based on their chemical characteristics (see text for details) Top: α with the ACN-rich MP (ACN/H_2 O 85/15% v/v + 15 mM $HCOONH_4$) Bottom: α with the MeOH-rich MP (MeOH/H_2 O 85/15% v/v + 15 mM $HCOONH_4$) Symbol × means no data available (see Table 1 and text for details)

increases while in other decreases). Figs. 1D and 2D compare the chromatograms for FMOC-(*D,L*)-His (Table 1 entry 31) on different columns and experimental conditions (see figure caption for details).

4. Conclusive remarks

This work aimed at evaluating the separation capability of two zwitterionic-teicoplanin CSPs prepared on superficially- and fully-porous particles towards the critical class of *N*α-FMOC AAs. To the best of our knowledge, this is the most extensive work dedicated to this topic. Indeed, 31 chiral analytes were evaluated. For the sake of comparison, also a commercially available teicoplanin CSP (Teicoshell) has been employed.

Even if no optimization of the MP composition was performed (in the attempt to improve the separation when enantiomeric mixtures could not be resolved), the results of this work

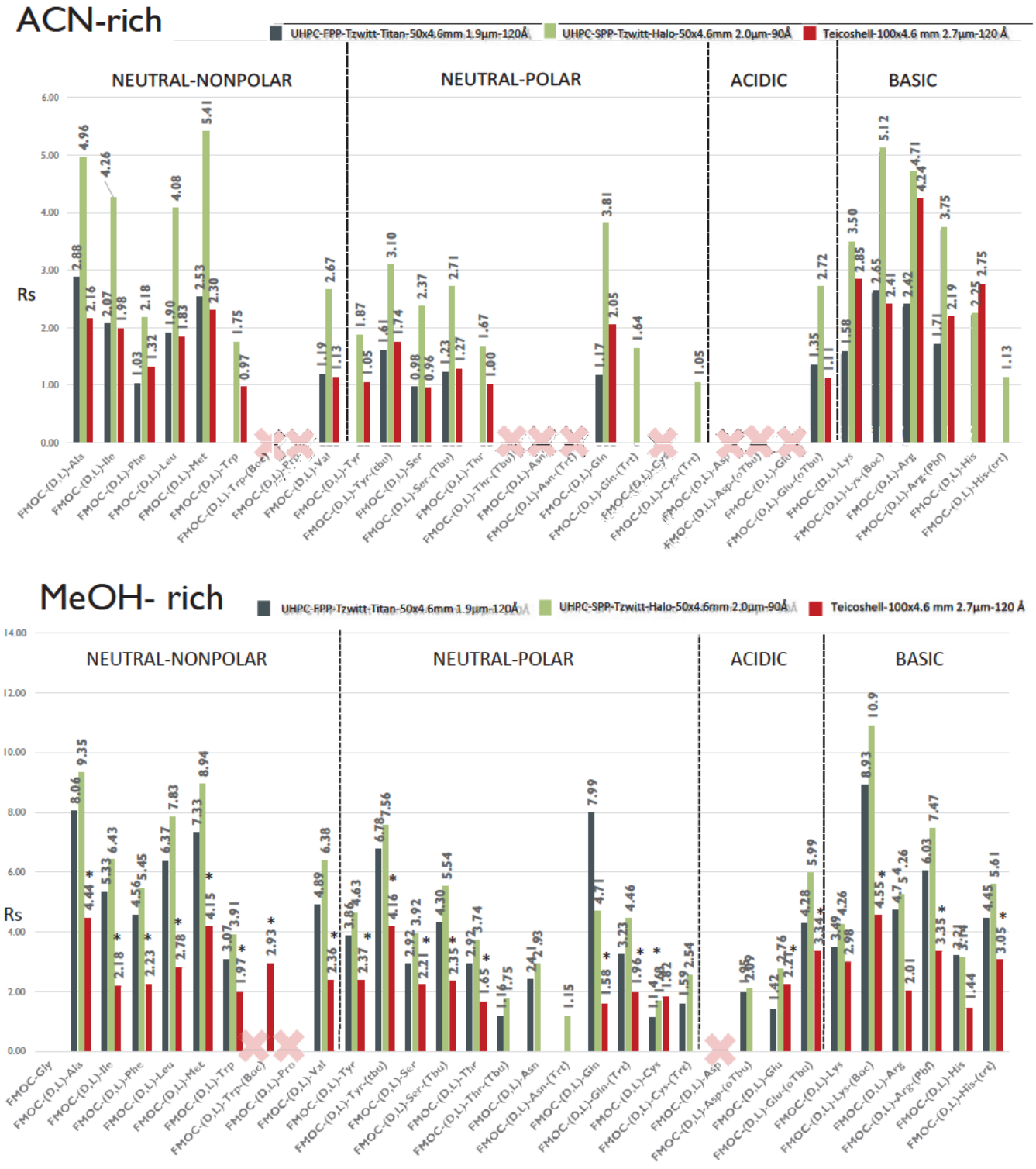

Fig. 4. Bar chart representing the resolution (*Rs*) for the FMOC AAs considered in this work For the sake of comparison, AAs have been divided based on their chemical characteristics (see text for details) Top: *Rs* with the ACN-rich MP (ACN/H_2 O 85/15% *v/v* + 15 mM $HCOONH_4$) Bottom: *Rs* with the MeOH-rich MP (MeOH/H_2 O 85/15% *v/v* + 15 mM $HCOONH_4$), the asterisk (*) denotes the *Rs* for those analytes with *k* negative Symbol × means no data available (see Table 1 and text for details)

are striking. They clearly evidence the capability of zwitterionic-teicoplanin CSPs to separate *NΩ*-FMOC AAs, no matter the class to which they belong (neutral nonpolar, neutral polar, acidic, basic). They also show that the zwitterionic-teicoplanin columns has a broader application field than the commercial teicoplanin one . Thanks to their zwitterionic character, indeed, the annoying phenomenon of Donnan's repulsion was never observed on both the UHPC-SPP-Tzwitt-Halo-2.0µm-90Å and UHPC-FPP-Titan-Tzwitt-1.9µm-120Å columns. This was, instead, a limiting factor with the commercial Teicoshell column, at the point that it could not be used to analyze *NΩ*-FMOC AAs with the MeOH-rich MP, but in a few cases. Indeed, in these conditions, the exclusion from the Teicoshell stationary phase of *NΩ*-FMOC AAs was always observed, except for basic AAs. Instead, under MeOH-rich conditions, on both

the zwitterionic CSPs, the enantioselectivity is dramatically enhanced compared to ACN-rich conditions, at the cost of longer retention.

Moreover, when it comes to the comparison of the two zwitterionic CSPs, it was clearly evident that the UHPC-SPP-Tzwitt-Halo-2.0µm-90Å allowed to achieve better results than the UHPC-FPP-Titan-Tzwitt-1.9µm-120Å The different morphology of SPP and FPP and, primarily, the higher density of chiral selector on SPP (see ta- ble S1) affect thermodynamics of the enantiorecognition process, reflected by larger enantioselectivity and resolution.

To visually summarize these concepts, Figs. 3 and 4 show, as bar-charts, the enantioselectivity (α), and enantioresolution (Rs) measured on the three columns under the different experimental conditions. From these representations, clearly emerges the outstanding performance of the UHPC-SPP-Tzwitt-Halo-2.0µm-90Å in terms of both enantioselectivity and resolution, not only with the MeOH- but also with the ACN-rich MP. In particular, with this CSP, the success rate in the separation of Nα-FMOC AAs enantiomers was as large as 74% by using the ACN-rich MP and even more than 90% with the MeOH-rich MP. Finally, Fig. S2 reports a series of chromatograms, obtained on the UHPC-SPP-Tzwitt-Halo-2.0µm- 90Å column for the separation of several Nα-FMOC AAs, excluding the acidic ones.