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Cover letter

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31 July 2018

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Dear dr. FANALI,

please find enclosed our revised version of the manuscript "*Direct analysis of chiral active pharmaceutical ingredients and their counterions by ultra high performance liquid chromatography with macrocyclic glycopeptide-based chiral stationary phases".*

The paper has been enriched comparing the zwitterionic CSPs with the traditional and commercially available ones. We believe that all suggestions have been satisfied in order to improve the quality of work. In addition, same parts were restyled to increase readability.
Together with the revised paper, we attached a file with specific answers to each observation/comment of Reviewers.

With best Regards,

Francesco

Reply to Reviewers

Reviewer #2 The manuscript entitled "Direct analysis of chiral active pharmaceutical ingredients and their counterions by ultra high performance liquid chromatography with macrocyclic glycopeptide-based chiral stationary phases" describe study on separation of chiral API and their counterions using the zwitterionic macrocyclic glycolpeptide chiral selectors bonded to sub 2 micron silica particles. This study may be useful to the audience in the pharmaceutical field; unfortunately this article is poorly written and referenced. Please find comments below.

R: We thank the Reviewer for his/her comments and suggestions that improve the quality of work. We modified point-by-point as requested mainly focusing on references.

Highlights:
Remove below mentioned highlight: this has been developed and published in literature (see list of citations need to be added in the end) A family of macrocyclic glycopeptide-based CSPs has been developed onto sub-2µm fully porous silica particles for ultra-high performance applications.

In second highlight author mentioned "a brand new" stationary phase, please change this one and make it more scientific.

R: As suggested, the macrocyclic glycopeptide-based (namely teicoplanin and vancomycin) have already been developed on sub-2 micron particles, but with the sentence "a family of.." we wanted refer to all type of CSPs presented in the manuscript: the already known and the zwitterionic versions (Vzwit are introduced here for the first time). Maybe it was not well explained so, in line with suggestions, we have modified the Highlights.

Line 27 ---Authors mention that the macrocylic chiral selectors are tethered (bonded would be correct word) to sub 2 um monodisperse fully porous particles. Authors should provide the particle size distribution data to prove these stationary phases are monodisperse. If the particle size distribution is more than the unity then these phases cannot be considered as a monodisperse.

R. "Bonded" has been introduced in the main text. And additionally we replaced "monodisperse" with "narrow particle size distribution".

Line 102 glycopeptide is spelled wrong. *R. corrected.*

Line 106 here authors correctly mention terminology "narrow particle size distribution" but fail to cite the first paper mentioned this correct terminology for this particles the detailed list of citations authors need to add mentioned at the end. *R. We have introduced the appropriate references [now 36-39].*

Line 107 mention Very fast, high efficiency separations performed using UHPLC though there is no mention of separation efficiencies using these phases. Authors should mention the separation efficiency achieved on all the phases.

R. As suggested we have introduced in almost all separations the efficiency values (see figures 2, 3, 6, 7, 8). Notably, the values obtained from UV chromatographic traces correspond to the true kinetic performances of columns (up to 200 000 N/m). When efficiencies are calculated from CAD traces, the values suffer of the high dispersion of detector (high variance) that reduces the values up to 50%, as clearly visible in figure 6 where UV and CAD traces of the same mixture are reported.

3.1 chiral separations: Author mentions that newly synthesized zwitterionic phases capable of separating the N-derivatized amino acids, this has been very well known and studied in great details with the non-zwitterionic teicoplanin, TAG and profens with vancomycin stationary phase. Authors need to mention how these phases are different/better for this separations showing comparison at same chromatographic conditions on same analytes on non zwitterionic teicoplanin and vancomycin stationary phase this would be ideal experiment.

R. As already reported in some papers (i.e ref 35), the performances on separation of N-derivatized amino acids and profens by using conventional teico e vanco CSPs are known and they do not represent the focus of our paper. However, we introduced some comparison between zwitterionic and non zwitterionic CSPs. New figures were added (figure 2 and figures S1, S5, S7 and S8) in order to show the different performances of our CSPs compared to the conventional ones. We would underline that, to make an appropriate comparison and being the TeicoShell/VancoShell the only commercially available CSPs for UHPLC applications (developed on SPP), we employed the zwitterionic CSPs developed as well on SPP (2.7 µm and 2.0 µm).

Section 3.2 similarly as mentioned above authors should mention about the example shown in fig 3 have different selectivity than non zwitterioninc teicoplanin and vancomycin?

R. Figure S5 is now added to the manuscript showing the difference between zwitterionic and non zwitterionic teicoplanin CSPs in the analysis of acidic samples (salicylic and acetyl salicylic acid) in HILIC conditions.

Section 3.3 shows interesting separation of chiral APIs and their counterions in pharmaceutical salts again here comparison with non zwitterioninc vancomycin and teicoplanin stationary phase with same example shown in figure 6 and 7 is must in this case.

R. Figure 7 and Figure S7 in the revised manuscript show the separations of chiral APIs and their counterions in both versions of CSPs (zwitterionic and non-zwitterionic), emphasizing the usability of zwitterionic CSPs in this kind of analysis.

Unfortunately, authors missed many relevant references, please find the references below

1) F. Gritti, D.S. Bell, G. Guiochon, Particle size distribution and column efficiency. An ongoing debate revived with 1.9 um Titan-C18 particles, J. Chromatogr. A 14) 179e192.

2) Barhate, C.L., Wahab, M.F., Breitbach, Z.S., Bell, D.S. and Armstrong, D.W., 2015. High efficiency, narrow particle size distribution, sub-2 <mu>m based macrocyclic glycopeptide chiral stationary phases in HPLC and SFC. Analytica chimica acta, 898, pp.128-137.
3) F. Gritti, G. Guiochon, The quantitative impact of the mesopore size on the mass transfer mechanism of the new 1.9 um fully porous Titan-C18 particles. I: analysis of small molecules, J. Chromatogr. A 1384 (2015) 76e87.
4) Barhate, C.L., Joyce, L.A., Makarov, A.A., Zawatzky, K., Bernardoni, F., Schafer, W.A., Armstrong, D.W., Welch, C.J. and Regalado, E.L., 2017. Ultrafast chiral separations for high throughput enantiopurity analysis. Chemical Communications, 53(3), pp.509-512.
5) Guillarme, D., Bonvin, G., Badoud, F., Schappler, J., Rudaz, S. and Veuthey, J.L., 2010. Fast chiral separation of drugs using columns packed with sub-2 <mu>m particles and ultra-high pressure. Chirality: The Pharmacological, Biological, and Chemical Consequences of Molecular Asymmetry, 22(3), pp.320-330.
6) Ai, F., Li, L., Ng, S.C. and Tan, T.T.Y., 2010. Sub-1-micron mesoporous silica particles functionalized with cyclodextrin derivative for rapid enantioseparations on ultra-high pressure liquid chromatography. Journal of Chromatography A, 1217(48), pp.7502-7506.
R. References suggested by the reviewer have been added in the manuscript.

Reviewer #3: Authors have shown an application of simultaneous separation of small inorganic ions as well as enantiomers in the same chromatographic run. The only novel part is the separation of the ions. but it isn't compared to other ion separations. Before the article can be accepted for publications, the authors should major concerns.

R. We thank the Reviewer for his/her comments and suggestions that improve the quality of work. We modified point-by-point as requested.

Major comments:

1. line 24: Please remove the word "novel macrocyclic glycopeptide" from the abstract. A quick search in Google Scholar shows that both vancomycin and teicoplanin were initially employed in 1994 for chiral separations. It is somewhat surprising that the authors missed this original publication with more than 750 citations. Similarly, both vancomycin and teicoplanin have already been bonded to Titan particles in 2015. Please see the link to this paper here: https://www.sciencedirect.com/science/article/pii/S0003267015012064.
The authors should consult these suggested papers and many others of this genre and revise the novelty aspects of their writing in the light of standard literature survey principles.

R: We deleted "novel" from the abstract and for sake of completeness we added the references related to the introduction of teicoplanin as chiral selector for chromatography (HPLC and UHPLC).

2. line 26: The ureidic linkage chemistry is rather traditional. Unfortunately, the experimental section is not clear enough as to what is the novel aspect regarding stationary phase synthesis. Please improve the quality of Figure 1 as the text size extremely small.

R: Concerning the details of synthetic procedure, this does not represent the novelty nor the purpose of the work. So, we have referred to our first paper where this kind of stationary phase has been presented for the first time. We improved the resolution of figure 1, it should be clearer now.

3. line 57: Better rephrase "ion-exchange chromatography with conductivity detection" with simply "ion chromatography." *R: Corrected*

4. line 73 The authors remark that CAD allows obtaining higher sensitivity than ELSD. Could they comment on the reason behind this observation?

R. As reported from specific technical note and some papers, both CAD and ELSD are non-linear to the injected amount of sample, but CAD performs better for the measurement of low levels of analytes (more sensitivity), and it has a wider dynamic range up to four orders of magnitude. To support the sentence in line 73, we added two references without some explanation of this concept that we don't believe essential in this context.

References added are:

19- David Thomas, Bruce Bailey, Marc Plante, Ian Acworth. Charged Aerosol Detection and Evaporative Light Scattering Detection – Fundamental Differences Affecting Analytical Performance, technical note from Thermo Fisher Scientific.

20- R. Godoy Ramos, D. Libong, M. Rakotomanga, K. Gaudin, P.M. Loiseau, P. Chaminade, Comparison between charged aerosol detection and light scattering detection for the analysis of Leishmania membrane phospholipids, Journal of Chromatography A, 1209 (2008) 88–94.

5. line 84: "The concept of mixed-mode mechanism in chiral separations is not recent. The terminology may have been coined in 2008 by Lindner and co-workers", but most chiral separations have always been mixed mode. This statement is quite problematic both factually and semantically. First of all, what is the accepted definition of mixed mode? Just like HILIC, there is none. Secondly, the macrocyclic glycopeptides are extremely complex molecules offering several interactions. The standard 3- point interaction model for chiral retention is potentially "mixed mode." Interactions can be attractive, or repulsive. Please revise or clarify this statement. The mechanism of retention of teicoplanin has already been explained in very detailed studies more than a decade ago.
See Anal. Chem. 2001, 73, 22, 5499-5508 (for teicoplanin) and https://onlinelibrary.wiley.com/doi/abs/10.1002/(SICI)1520-636X(1996)8:8%3C590::AID-CHIR9%3E3.0.CO;2-D (for vancomycin)

R: We agree with the comment and almost all chiral separations are mixed mode. So we modified the sentence in: "The concept of mixed ion-exchanger mechanism (due to the same occurrence of cation and anion exchangers, line 84) in chiral separations is relatively recent. It was introduced in 2008 by Lindner and co-workers." In this way we would stress the concept of cation and anion exchange at the same time due to at least two differently charged sites in the structure of selector.

6. Line 114: The term "monodispersed" is misleading from a colloidal science perspective. In colloidal terminology, "monodisperse" silica is impossible. Secondly, the particle size distribution of Titan is narrow but nowhere monodisperse. According to the IUPAC "The adjectives 'monodisperse' and 'polydisperse' are deeply rooted in the literature, despite the former being non-descriptive and self-contradictory. They are in common usage, and it is recognized that they will continue to be used for some time; nevertheless, more satisfactory terms are desirable. After an extensive search for possible replacements, the terms 'uniform' and 'non-uniform' have been selected, and they are now the preferred adjectives." To support the statements, D90/D10 of this particular batch of Titan should be quoted. Titan is nowhere near monodisperse.

R: In agreement with the comment and in line with a similar observation of the first Reviewer, we modified "monodisperse" with "narrow particle size distribution".

7.line 232: It is suggested that these CSPs should not be used below pH 4. The upper range of silica is around 7. This is a very narrow working range. Could the authors add a few comments as to why one should not try using a pH of 4 and below?

R. As reported in the manuscript (lines 234-236), the evidence by long use of these columns a pH below pH 4 is remarkable by a strong peak distortion. We believe the loss of efficiency and separation power is due to some phenomenon of CSP degradation.

8. Please recheck your figures. There is a spelling mistake of minute on one of the axes. Secondly please use the decimal dot instead of the comma to indicate a decimal place. Mixed symbolism is seen in many figures. Also, number the peaks for labels, and state the column dimensions in the captions.

R: We have rechecked our figures. The spelling minute has been modified, dot was used for decimal place and symbolism was homogenized (mainly Arial). Column dimensions were inserted in the captions.

Minor comments:
1. Line 112: Correct the sentence "chiral samples were…. studied". It does not make sense as written. *R: Corrected*

Reviewer #4: This work reports a comparison of chiral stationary phases based on Teicoplanin and Vancomycin as chiral selectors which have been obtained by two distinct bonding chemistries. These two distinct bonding chemistries give the surface of the resultant CSPs a different character, namely either resultant an acidic or a zwitterionic chiral selector moiety. As a consequence, selectivity profiles are altered and this is documented in this work. The authors make a point on the fact that on the zwitterionic CSPs chiral compounds can be separated into enantiomers besides simultaneously analyzing their counterions. The work is of interest, sufficiently novel and innovative and the paper is mostly well written with a clear message. It can be accepted with minor revisions.

R. We thank the reviewer for appreciating the novelty of the work. We introduced all his/her suggestions that improve quality and style of the manuscript.

l. 49: Instead of "clinically drugs" I recommend "approved drugs". This applies not only to the drugs used in the clinics but also OTCs etc. *R: Corrected*

l. 112: previous studies (instead of previously studied) …. *R: Corrected*

l. 119: "proprietary bonding protocols" It is a weak point of this paper that the bonding chemistry is not described. Since the columns are not commercially available the work cannot be reproduced without procedure for the synthesis of the CSPs. Scientifically it is not correct because it is the essence of science that work can be reproduced.

R: see answer to Reviewer n 3.

l. 224: Probably better: (a) derivatized amino acids Boc-Met and Fmoc-Ala l. 256: chlorine must be replaced by chloride (anion!) (also elsewhere in the text). *R: Corrected*

l. 269: bromide *R: Corrected*

Table 2: Please insert resolution values and elution orders!

R. We introduced Rs values for all entries in table 2. In addition, for N-derivative and free amino acids we reported the elution order. Unfortunately, similar data are missed for separation of profens due to the lack of single enantiomer or alternately the CD detector for UHPLC equipment.

Table S1: Column 20mM top: header should be alpha instead of A! *R: Corrected*

Highlights

A zwitterionic vancomycin CSP has been introduced into the family of sub-2µm macrocyclic glycopeptide-based CSPs.

The zwitterionic CSPs are able to well resolve inorganic anions in less than 2 min by using HILIC conditions.

The simultaneous separation of chiral active pharmaceutical ingredients (API) in salt form from their counterions has been performed.

Direct analysis of chiral active pharmaceutical ingredients and their counterions by ultra high performance liquid chromatography with macrocyclic glycopeptide-based chiral stationary phases.

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Keywords

Direct chiral API/counterion separation, UHPC macrocyclic glycopeptide-based CSP, inorganic anion/cation separation, high-efficiency FPP/SPP CSPs.

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Abstract

In this work the simultaneous separation of chiral active pharmaceutical ingredients (API) in salt form from their counterions has been performed by using different high-efficiency macrocyclic glycopeptide-based chiral stationary phases (CSPs). Not only a new zwitterionic vancomycin-based CSP has been prepared (similarly to what was done for teicoplanin) but macrocyclic selectors have also been bonded to sub-2µm fully porous silica particles through traditional ureidic linkage to obtain versions of CSPs suitable for ultra-high performance applications. The direct separation of chiral APIs and counterions is particularly attracting since it simplifies the workflow traditionally used with reduction of analysis time and costs. The wide selection of macrocyclic antibiotics CSPs now available has allowed to manage different cases that can happen in the simultaneous separation of APIs and their counterions (either cations or anions). Indeed, while inorganic cations are retained on traditional vancomycin- and teicoplanin-based CSPs, inorganic anions are almost unretained (due to Donnan's effect). On the other hand, cations and anions can be both retained on the zwitterionic versions of these CSPs. Afterwards, zwitterionic CSPs allowed to the separation of other compounds including N-derivative amino-acids, profens, polyols, sugar anomers, oligosaccharides and inorganic anions/cations opening new perspectives in the use of this family of CSPs.

1. Introduction

Pharmaceutical drugs are often prepared in salt forms. The conversion of a drug into a salt has been demonstrated to improve not only some properties of drugs, such as their solubility and dissolution rate but also their chemical stability [1-9]. These properties directly affect other important characteristics of drugs, including pharmacokinetics,

pharmacodynamics, bioavailability and toxicity profile. As a consequence, the number of drugs produced in salt form has rapidly increased over the last years to the point that, according to the Orange Book database by the U.S. Drug and Food Administration [10], almost half of the approved drugs currently used are salts. Approximately 80% of these is formed by basic molecules and the remaining 20% by acidic ones [8,11,12]. Counterions can be both inorganic and organic acids and bases. Examples of commonly employed inorganic ions include chloride, sulfate, bromide, nitrate, sodium, calcium, etc. Among the most employed organic acids and bases there are lactate, succinate, malate, gluconate, etc.

In pharmaceutical analysis, APIs and their counterions are usually evaluated by using different methods, separation columns and instrumentation. A common approach, for instance, is to employ ion chromatography for the assay of counterions and ion-pairing liquid chromatography with ultraviolet diode array (UV-DAD) or mass spectrometry (MS) detection for that of APIs.

The need to determine together both APIs and the corresponding counterions in a single chromatographic analysis [1] has led to the development of separation systems based on mixed-mode or multimodal stationary phases (SPs) where both reversed phase (RP) and IEX retention mechanisms are combined. In this work, the term mixed-mode or multimodal will be used to refer to SPs operating with simultaneous IEX and RP/HILIC retention mechanisms, even though in literature the expression has been used in a broader sense to include any kinds of SPs where different modes of interaction contribute to analyte retention [13]. Through the fine control of experimental variables (basically, pH, ionic strength, organic modifier kind and amount [14-17]), the selectivity of these phases can be tuned so to simultaneously separate analytes with very different hydrophobicity/hydrophilicity and charge state [1,18]. On the other hand, to avoid the use

of multiple detection systems for APIs and counterions (that often lack UV chromophores), the use of refractive index (RI) and evaporative light scattering detection (ELSD) has been proposed [1]. Zhang et al. have recently employed charged aerosol detection (CAD), which allows to obtain larger sensitivity and enhanced signal stability under gradient elution compared to RI and ELSD [1, 19-20].

An even more challenging issue is the simultaneous separation of chiral APIs and their counterions. About more than a half of the drugs currently in use are indeed chiral (be they racemic, single-enantiomeric, or some other mixture of chiral stereoisomers) [21-23]. Even if the situation has partially changed today, since new regulations have imposed a strict control of stereochemical properties of new-released drugs, the need of evaluating the enantiomeric composition of pharmaceutical products is pressing [24-28]. Actually, most isomers of chiral drugs have very different toxicology profiles, metabolism, pharmacokinetics and biological activity.

The concept of mixed ion-exchanger mechanism (due to the same occurrence of cation and anion moieties) in chiral separations is relatively recent. It was introduced in 2008 by Lindner and co-workers [29] who developed a zwitterionic chiral selector, where a weak anion exchanger (WAX) based on cinchona alkaloid was combined with a strong cationic exchanger (SCX) based on trans-2-aminocyclohexanesulfonic acid. This combination was proved to be particularly effective in the separation of zwitterionic analytes (including α-, β-amino acids and peptides), usually unresolved by cinchona-based or other types of single anionic or cationic selectors [30, 31]. Other zwitterionic systems containing chiral moieties have been previously reported but not for separation of enantiomers [32-34]. In 2016, a new-concept zwitterionic teicoplanin-based CSP was prepared by some of the authors of this work [35]. Zwitterionic behavior of this CSP comes from the presence of the amino group that can be protonated and the acidic moiety of teicoplanin (**Figure 1a**). This

characteristic is lost if the traditional ureidic-linker based-protocol is employed for the preparation of teicoplanin-based CSPs (**Figure 1b**) [35]. It has been demonstrated that the zwitterionic teicoplanin CSP is able to effectively separate different classes of compounds, including N-protected amino acids, α-aryloxy acids and anti-inflammatory drugs under various types of elution conditions, such as RP, normal phase (NP), hydrophilic interaction (HILIC), polar organic mode (POM), both WAX and weak cation exchanger (WCX) and supercritical fluid (SFC). [35].

In this work macrocyclic glycopeptide chiral stationary phases (CSPs), including zwitterionic teicoplanin and a new zwitterionic vancomycin (**Figure 1c**), have been employed for the direct determination of chiral APIs and, at the same time, their counterions from chiral drugs prepared in salt forms. All the four CSPs (**Figure 1a-d**) were prepared on high-efficiency sub-2μm particles of narrow particle size distribution [35-39] to be suitable for very fast, high efficient separations in ultra high performance liquid chromatography (UHPLC) [40-42].

2. Experimental

2.1 Materials and chemicals

Reagents and solvents were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Chiral samples were from Sigma-Aldrich or already in labs from previous studies. HPLC gradient grade solvents were filtered before use on 0.2 μm Omnipore filters (Merck Millipore, Darmstadt, Germany). Titan monodispersed silica particles (pore size 120 Å, particle size 1.9 μm, specific surface area 282 m^2 g^{-1}) were a gift from Supelco (Sigma-Aldrich (St. Louis, Mo, USA). Empty stainless steel columns, 50×4.6 mm or 100x4.6 mm (L×I.D.), were from IsoBar Systems by Idex (Wertheim-Mondfeld, Germany). For comparison, additional columns were employed: TeicoShell and VancoShell (both 100*4.6 mm L x ID) from AZYP

LLC Arlington, TX, USA), and two zwitterionic CSPs *"ad hoc"* developed on superficially porous particles (SPP): UHPC-SPP-Halo90-T_{ZWIT}-2.7 (100*4.6 mm L x ID) and UHPC-SPP-Halo90-T_{ZWIT}-2.0 (100*4.6 mm L x ID) [35-40].

2.2 Preparation of chiral stationary phases

Two different proprietary bonding protocols were used to immobilize both teicoplanin and vancomycin selectors onto 1.9 μm silica particles to obtain respectively the zwitterionic macrocyliclic glycopeptide teicoplanin and vancomycin CSPs (**Figures 1a** and **1c**) and the traditionally ureidic-linked ones (**Figures 1b** and **1d**). Results of elemental analysis (C, H, N) of all CSPs are listed in **Table 1**, where also chiral selector loading (μmol/g base silica) and surface coverage (μmol/m^2) are reported. All CSPs were slurry packed with a pneumatically driven Haskel pump (operated at the maximum pressure of 1000 bar) into 50×4.6 mm stainless steel columns. **Figure 1** also reports the acronyms employed for theses CSPs.

2.3 Equipment

An UPLC Acquity Waters (Milford, MA, USA) made of a binary solvent system (maximum flow rate: 2 mL/min), an auto-sampler, PDA detector (500 nL flow cell, 80 Hz acquisition rate) was used for RP experiments (*vide infra*). The maximal back-pressure allowed by the system is 1000 bar at a flow rate of 1 mL/min. This value linearly decreases, in the range 1-2 mL/min, up to 600 bar at 2 mL/min. A standard UPLC Acquity Waters column heater (maximum temperature: 65°C; still air conditions) was used. To minimize the extra-column volume, standard inlet and outlet detector connections were replaced by two Viper capillaries (respectively, 250 and 350×0.100 mm L×I.D.). In this configuration, the extra-column volume was 7.33 μL and the extra-column variance (measured with uracil by employing a zero dead-volume connector between Vipers) was 1.39 μL^2 at 1.0 mL/min

144 (calculated via second central moment) [43]. Data acquisition, data handling and
145 instrument control were performed by Empower 3 (Waters).

146 The UHPLC chromatographic system used for HILIC experiments (vide infra) was an
147 UltiMate 3000 RS system (Thermo Fisher Dionex Sunnyvale, California), equipped with a
148 dual gradient pump, an in-line split-loop well plate sampler, a thermostated column
149 ventilated compartment (temperature range: 5-110 °C) and a diode array detector
150 (Vanquish diode array detector, 100 Hz acquisition rate) with a low dispersion 2.5 µL flow
151 cell. The extra-column variance of system (measured with uracil by employing a zero
152 dead-volume connector between Vipers) was 3.94 μL^2 at 1.0 mL/min (calculated via
153 second central moment) [43]. In addition, a CAD detector (Charged Aerosol Detector Ultra
154 by Thermo Fisher Dionex Sunnyvale, California) was used to detect samples lacking UV
155 chromophores. It was interfaced to the chromatograph through Viper capillaries
156 (350×0.10 mm L×I.D.). Data acquisition and processing were performed with Chromeleon
157 6.8 (Thermo Fisher).

158 **2.4 Chromatographic conditions**

159 All injections were performed by using mixtures of MeOH/H_2O or ACN/H_2O with 15%, 30%
160 and 40% variable water percentage. Ammonium acetate and ammonium formate were
161 added as additives at different concentrations (20 mM, 15 mM, 10 mM and 5 mM). In the
162 evaluation of pH effect, the ^{w}pH (pH measured in aqueous content of mobile phase before
163 mixing with organic solvent) was changed in the range 4.0 – 6.5. In HILIC conditions, a
164 mobile phase of ACN/H_2O, 85:15 v/v + 15mM ammonium acetate was mainly employed at
165 ^{w}pH= 7.0. Injection volume was 0.5 µl. Resolution (R_s) and efficiency (N/m) values were
166 calculated, according to the European Pharmacopeia, using peak width at half height
167 ($W_{0.5}$). Hold-up time was estimated by the elution time of an unretained marker

168 (naphthalene in HILIC and uracil in RP, respectively). Retention factor, *k*, has been
169 calculated as:

170 $$k = \frac{t_R - t_0}{t_0} \qquad (1)$$

171 where t_r and t_0 are the retention and hold-up time respectively. Enantioselectivity (α) was
172 calculated as the ratio between the retention factors of the second and the first eluted
173 enantiomer, respectively:

174 $$\alpha = \frac{k_2}{k_1} \qquad (2)$$

175 being k_1 and k_2 the retention factors of the first and second eluted enantiomer.

176 **3. Results and Discussion**

177 **Figure 1** shows the structure of chiral selectors and how they were bonded to the surface
178 of silica particles in the four CSPs employed in this work. On the left side of the figure
179 (squares **a** and **c**), the zwitterionic versions of teicoplanin (top) and vancomycin (bottom)
180 CSPs are reported. The selectors have at least two opposite ionizable groups (highlighted
181 in red and blue in **Figure 1**) responsible for the zwitterionic properties of the CSPs when
182 pH conditions are such to ensure, on the one hand, the deprotonation of the carboxylic
183 group and, on the other hand, the protonation of amino group. Moreover, vancomycin has
184 an additional free amino group, which could undergo protonation. On the right side of
185 **Figure 1** (squares **b** and **d**), the teicoplanin and vancomycin CSPs, via traditional ureido-
186 bonding chemistry [44, 45], are schematically represented. The combined effect of the
187 carboxylic group (in the form of carboxylate, under the typical working conditions used with
188 these CSPs) and the basket-like structure of chiral selectors has been demonstrated to be
189 pivotal in the enantiorecognition process on these CSPs [45-47]. Moreover, it was shown
190 that, on teicoplanin CSPs obtained via ureido-bonding, the negatively charged carboxylate

191 moiety can produce the exclusion of negatively charged analytes through Donnan effect
192 [45]. All CSPs of this work have been developed on sub-2µm fully porous particles (FPP,
193 Titan-120-1.9 µm). Taking to account that the only commercially available glycopeptide-
194 based CSPs for UHPLC applications are designed on superficially porous particles
195 (namely TeicoShell and VancoShell on 2.7µm SPP silica particles), some comparisons in
196 this work required the use of additional zwitterionic SPP CSPs. Specifically, T_{ZWIT} CSPs
197 based on SPP-2.7µm and SPP-2.0µm were included to properly compare different
198 selector capabilities (zwitterionic and non zwitterionic ones) [35, 40]. In fact, in the
199 evaluation of the overall performance of different CSPs (retention, selectivity, resolution),
200 the maximum degree of similarity should be maintained (same type of silica particles,
201 similar particle size and column geometry).

202 The focus of this work is the characterization of the zwitterionic macrocyclic-based CSPs
203 towards the separation of different classes of molecules, not only chiral but also achiral
204 ones, be they ionic, polar or neutral, organic or inorganic. Based on this information, the
205 CSPs have been employed for cutting edge applications for the direct separation of APIs
206 and their counterions.

207 **3.1 Chiral separations**

208 It is well known that chiral recognition on macrocyclic glycopeptide-based CSPs involves a
209 complex interplay of different mechanisms where experimental variables such as pH, ionic
210 strength, amount and kind of mobile phase (MP) modifier, buffer type, etc. may have a
211 dramatic effect on the outcome of separation both in terms of retention time and selectivity
212 of separation. MPs typically used with traditional, commercially available teicoplanin and
213 vancomycin CSPs (**Figure 1** squares **b** and **d**) are made of aqueous buffers and an
214 organic modifier (usually acetonitrile or methanol). MP pH is kept around 7 as to maintain

deprotonated the carboxylic unit, which strongly improves the chiral recognition ability of the CSP.

pH control is still more demanding with zwitterionic teicoplanin and vancomycin CSPs, in order to simultaneously guarantee the presence of both a negatively charged carboxylate unit and a positively charged amino group (pK_a of carboxylic groups of teicoplanin and vancomycin are, respectively, approximately 2.5 and 2.2 while the pK_a of NH_2 group is roughly 7.8).

The behavior of the new zwitterionic CSPs has been firstly evaluated in the classical field of enantiomeric separations by considering the effect of different experimental variables (e.g., organic modifier amount, buffer, ionic strength, pH etc.) on the separation of several classes of chiral compounds, including weak acids, basically profens and N-derivative amino acids (Boc-, Dansyl-, Fmoc-, Z-). The latter are particularly interesting since on non-zwitterionic macrocyclic CSPs they are excluded from the stationary phase due to Donnan repulsion [45]. **Table 2** reports the list of enantiomers resolved on the two new CSPs. In particular, by looking at these data, some important differences in terms of selectivity between the two CSPs can be noticed. Indeed, the teicoplanin-based CSP shows great selectivity for amino acid derivatives. On the other hand, the vancomycin-based CSP showed better selectivity for nonsteroidal anti-inflammatory profens. Moreover, the amino acid derivatives were better resolved on zwitterionic teicoplanin respect to traditional version of CSPs. As an example, **Figure 2** and **Figure S1** report the separation of Fmoc-*D,L*-Ser and Fmoc-*D,L*-Met in HILIC conditions. Moving to the zwitterionic version, a remarkable gain in retention of both enantiomers was obtained due to a reduced Donnan effect (k_1 = 1.02 on TeicoShell-2.7 respect to k_1 = 2.31 on UHPC-SPP-Halo90-T_{ZWIT}-2.7 for Fmoc-*D,L*-Ser). Looking to the kinetic performances, the efficiencies of UHPC-SPP-Halo90-T_{ZWIT}-2.0 and UHPC-FPP-Titan120-T_{ZWIT}-1.9 packed columns (green and blue traces in **Figure 2**) are comparable reaching in both cases 220 000 N/m. In addition, the

241 effect of amount of organic modifier (methanol) on chromatographic selectivity and
242 retention was preliminary investigated. The results showed that analyte retention
243 decreases by increasing the amount of organic modifier in the MP, thus following a typical
244 RP behavior (**Figure S2** of Supplementary Information). **Figure S3** shows the effect of
245 buffer (formate vs. acetate) on the separation of (a) derivatized amino acids Boc-*D,L*-Met
246 and Fmoc-*D,L*-Ala on the zwitterionic teicoplanin CSP and (b) suprofen enantiomers on
247 the zwitterionic vancomycin CSP. In both cases, methanol was the organic MP modifier.
248 As it can be seen, formate buffer always leads to significantly better selectivity and
249 resolution than acetate one. For the sake of completeness, the effect of ionic strength on
250 retention and enantioselectivity (**Table S1**) and the minimum MP pH at which these CSPs
251 can be used (**Figure S4**) were also investigated. Conclusions of these studies are that (i)
252 by increasing the ionic strength, retention decreases (in agreement with results reported
253 with traditional macrocyclic glycopeptide based CSPs [35,47]); and (ii) below pH 4, these
254 CSPs should not be used (in some cases severe peak distortion occurs after long use).
255 The loss of efficiency and separation power is probably due to some phenomenon of
256 selector degradation.
257
258 **3.2 Achiral separations**

259 The characterization of zwitterionic macrocyclic glycopeptide CSPs has been extended to
260 achiral compounds, including neutral and polar samples and inorganic ions. Polar samples
261 are often separated under HILIC conditions. **Figure 3** reports the chromatograms for the
262 separation of a mixture of salicylic acid and acetyl salicylic (square **a**) and some polyols (**b**)
263 on a zwitterionic Teicoplanin column with an acetonitrile-rich MP (typical HILIC mode). The
264 different performances of zwitterionic and non-zwitterionic phases are highlighted in
265 **Figure S5**, where salicylic and acetyl salicylic acid are not retained on TeicoShell-2.7 µm.

Once again, the zwitterionic behavior of this CSP reduces the Donnan effect. **Figure 3c**, on the other hand, shows the HILIC separation of uracil, adenosine and cytosine on the zwitterionic vancomycin column. The mixture represents the classical test to evaluate HILIC performance, where naphthalene has been added to determine the void volume. For fast analysis (traces **3b** and **3c**), the efficiency values, as plate count per column, are additionally reported. Notably, the values recorded by using UV detector (trace **3c**) show the true efficiency expressed by the sub-2 micron packed columns. Samples 8-10 are eluted in short times with efficiencies of approximately 230 000 N/m. When CAD detector was employed the plate column values dramatically decrease due to the high dispersion volume of detector. The loss of efficiency is clearly visible when the same separation was recorded by in series UV/CAD detectors (see following data). In **Figure 4**, finally, the separation of sugars is presented. Very interestingly, zwitterionic macrocyclic glycopeptide CSPs were found not only able to separate α- and β-anomers of monosaccharaides at sub-ambient temperature (e.g., *D*-(+)-glucose and *D*-(-)-fucose, see **Figure 4a**) but also mixture of oligosaccharides in gradient elution. As an example, in F**igure 4b** the chromatograms of the separation of six oligomers of maltose and that corresponding to the analysis of a commercial beer sample are reported (upper and lower traces, respectively).

The separation of inorganic anions is presented through **Figure 5**, where chromatograms for the elution of a mixture of iodide, nitrate, bromide and chloride (as sodium and potassium salts) on the zwitterionic vancomycin column are reported. The top of the figure refers to a MP made of ammonium acetate aqueous buffer and acetonitrile (40/60 v/v). The chromatograms recorded with CAD and UV detection are compared not only to show that chloride (as well as sodium and potassium) are not UV detected but also to highlight the band broadening due to CAD (UV peaks look significantly narrower than peaks recorded with CAD). On the bottom of this figure, the separation of the same anions mixture has been performed on the same column but by employing ammonium acetate

aqueous buffer and methanol as MP modifier (40/60 v/v). Interestingly, the order of elution is inverted in comparison to the separation with acetonitrile. Actually, the effect of elution inversion is under investigation in our laboratories. The selectivity of these CSPs towards anions can be significantly improved by moving to HILIC-like conditions. This has been shown in **Figure 6** for the separation of the mixture on the zwitterionic teicoplanin (left) and zwitterionic vancomycin (right) columns. Baseline separation of all anions has been reached in less than two minutes (at flow rate of 1.0 ml/min). Under these conditions, we observed on the zwitterionic vancomycin CSP coelution of sodium and potassium cations (always unresolved on zwitterionic macrocyclic glycopetide CSPs) together with bromide anion.

The impossibility to separate cations on zwitterionic stationary phases has been also previously reported in literature [48]. On the other hand, they can be easily separated on a non-zwitterionic teicoplanin column. Chromatograms showing the separation of monovalent cations (Li^+, Na^+, K^+, Cs^+) as bromide and chloride salts are reported in **Figure S6**. It can be noticed that retention increases with the size of cations ($Li^+ < Na^+ < K^+ < Cs^+$). On the opposite, Br^- and Cl^- anions were almost unretained. In addition, this study shows that an increase of water in MP reduces retention, most likely due to medium effects in charge-charge interaction.

3.3 Simultaneous separation of chiral APIs and their counterions in pharmaceutical salts

In virtue of above described characteristics, macrocyclic glycopeptide zwitterionic CSPs seem particularly suitable for applications of great practical relevance such as the separation of chiral APIs and their counterions in drugs used as salts by performing a single chromatographic run [10,49]. Indeed, these CSPs are able to retain both inorganic anions or cations (**Figure 6**), the retention of which can be easily modulated by changing

water content and/or pH of mobile phase. The proof of concept of this idea is demonstrated in **Figure 7** and **Figure S7** where the separations of propionyl-D,L-carnitine hydrochloride and potassium suprofen salt have been performed on zwitterionic teicoplanin (**Figure 7**) and on zwitterionic vancomycin (**Figure S7**) columns. As it can be observed, zwitterionic columns allow to baseline resolve the enantiomers of chiral active ingredients, which are also very well separated from their counterions. On the other hand, in the same figures, the performances of traditional columns were reported attesting how the TeicoShell and VancoShell columns are not good candidates for the simultaneous resolution of chiral drugs and their counter-anions. In different API systems, where the counterion is positively charged, the weak cation exchanger teicoplanin CSP can be employed. This phase indeed exhibits great selectivity for inorganic cations as demonstrated in **Figure S6**, where the separation of inorganic cations (Li+, Na+, K+, Cs+) from anions (Br- and Cl-) was performed. As an example of the application of this concept to chiral APIs in salt form, the enantiomers of carglumic acid have been successfully separated from sodium cation, which under these experimental conditions is more retained than the chiral molecules (**Figure 8**).

4. Conclusions

In this proof-of-concept study, macrocyclic glycopeptide (vancomycin and teicoplanin) CSPs were demonstrated to be suitable for the single run direct resolution of chiral APIs in salt form and the simultaneous separation from their counterions. New CSPs were prepared on sub-2µm fully porous silica particles with a narrow particle size distribution by employing either the traditional protocol (to get the typical version of teicoplanin and vancomycin-based CSPs but suitable for UHPLC applications) or a proprietary one that allows to obtain zwitterionic forms of these CSPs. Thanks to the different selectivity of these phases, it was possible to separate chiral APIs not only from inorganic cations

(where traditional vancomycin- and teicoplanin-based CSPs can be employed) but also from inorganic anions (by using the zwitterionic form of these CSPs). Anions are typically unretained (or even excluded by the stationary phase) on traditional teicoplanin or vancomycin CSPs due to electrostatic repulsion.

Zwitterionic CSPs were efficiently operated under RP, HILIC and weak ion exchange conditions to perform separations of great practical relevance, such as those of N-derivative amino acids and mixtures of inorganic anions and cations.

Supplementary data

Supplementary data associated with this article can be found in the online version of manuscript.

Conflict of interest

Authors declare no conflict of interest.

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Figure captions.

Figure 1. Schematic representation (and acronyms) of teicoplanin- (top) and vancomycin-based (bottom) CSPs. (a) and (c): zwitterionic versions; (b) and (d): traditional (commercially available) versions of CSPs. Red (amino groups or modified amino groups) and blue (carboxylic groups) colors were used to emphasize differences between CSPs. See text for details.

Figure 2. Comparison between commercially available column TeicoShell-2.7 (100 x 4.6 mm L. x I.D.) and T_{ZWIT} based columns (same geometry, packed with SPP 2.7 µm, 2.0 µm and FPP 1.9 µm). Sample: Fmoc-*D,L*-Ser, MP: ACN/H_2O 85/15 + 15 mM ammonium acetate. T: 30°C. Flow rate: 1.0 mL/min. Detection: UV 254 nm.

Figure 3. Examples of achiral separations of polar samples in HILIC mode. (a) salicylic (1) and acetyl salicylic (2) acid (Column UHPC-Titan120-T_{ZWIT}-1.9; MP: ACN/H_2O 85/15 + 10 mM ammonium formate: detector: UV 254 nm). (b) cis,cis-1,3,5-cyclohexanetriol (3), xylitol (4), alloinositol (5) and myoinositol (6) (Column UHPC-Titan120-T_{ZWIT}-1.9; MP: ACN/H_2O 85/15 + 15 mM ammonium acetate; detector: CAD). (c) naphthalene (7) (hold-up time marker), uracil (8), adenosine (9) and cytosine (10) (Column UHPC-Titan120-V_{ZWIT}-1.9, MP: ACN/H_2O 85/15 + 15 mM ammonium acetate, UV 254 nm. Flow rate: 1.5 ml/min, T: 30°C. Column dimensions: 50×4.6 mm.

Figure 4. Separation of carbohydrates in HILIC mode. (Cryo-)separation of anomers of *D*-(+)-Glucose on column UHPC-Titan120-T_{ZWIT}-1.9 (a-left) and of anomers of *D*-(+)-Fucose on column UHPC-Titan120-V_{ZWIT}-1.9 (a-right). MP: ACN/H_2O 85/15 + 15 mM ammonium acetate, T = 10°C, CAD detector, flow rate 1.5 ml/min. (b) Gradient separation of oligosaccharides of maltose (upper chromatogram) and sugar profile in a national beer sample (bottom chromatogram). Column: UHPC-Titan120-T_{ZWIT}-1.9: MP: ternary mixture:

i) ACN/H_2O 95/5, ii) ACN/H_2O 5/95, iii) 100 mM ammonium acetate. Gradient program: from (i) 100% to (i) 79%, (ii) 20% and (iii) 1% in 10 min. Flow rate 1.5 mL/min. T = 30°C, CAD detector. Column dimensions: 50×4.6 mm.

Figure 5. Separation of anions and cations by changing organic modifier (column UHPC-Titan120-V_{ZWIT}-1.9). MP: ACN (or MeOH)/H_2O 60/40 + 15 mM ammonium acetate, ^{w}pH: 5.5. T: 30°C. Flow rate: 1.0 mL/min. Detection: CAD and UV 214 nm. Column dimensions: 50×4.6 mm.

Figure 6. Separation of anions on UHPC-Titan120-T_{ZWIT}-1.9 (left side) and UHPC-Titan120-V_{ZWIT}-1.9 (right side) columns. MP: ACN/H_2O 85/15 + 15 mM ammonium acetate. T: 30°C. Flow rate: 1.0 mL/min. Detection: CAD and UV 214 nm. Column dimensions: 50×4.6 mm.

Figure 7. Separarion of Propionyl-*D,L*-carnitine hydrochloride on UHPC-Titan120-T_{ZWIT}-1.9 column. Comparison with commercially available column TeicoShell-2.7 and T_{ZWIT} SPP columns (packed with SPP 2.7 µm, 2.0 µm). Column dimensions: 100 x 4.6 mm L. x I.D.. MP: MeOH/H_2O 85/15 + 10 mM ammonium formate. Flow rate: 1.0 mL/min. Detector: CAD.

Figure 8. Direct gradient separation of *D,L*-Carglumic acid sodium salt on UHPC-Titan120-T_{COOH}-1.9 column. Binary gradient: (i) ACN/H_2O 90/10 + 10 mM ammonium acetate, (ii) ACN/H_2O 30/70 + 10 mM ammonium acetate. Gradient program: from (i)/(ii) 80/20 to (i)/(ii) 60/40 in 5 min. Flow rate: 1.5 ml/min, T: 40°C. Detection: CAD. Column dimensions: 50×4.6 mm.

Bibliography

[1] K. Zhang, L. Dai, N. P. Chetwyn, Simultaneous determination of positive and negative pharmaceuticalcounterions using mixed-mode chromatography coupled with charged aerosol detector, J. Chromatogr. A, 1217 (2010) 5776-5784.

[2] A.T.M. Serajuddin, Salt formation to improve drug solubility, Adv. Drug Deliv. Rev. 59 (2007) 603-616.

[3] D.J. Snodin, Residues of genotoxic alkyl mesylates in mesylate salt drug substances: real or imaginary problems?, Regul. Toxicol. Pharmacol. 45 (2006) 79-90.

[4] P.H. Stahl, C.G. Wermuth, Handbook of Pharmaceutical Salts; Properties, Selection, and Use, VHCA and Wiley–VCH, Zurich, Switzerland, and Weinheim, Germany, 2008.

[5] P.L. Gould, Salt Selection for Basic Drugs, Int. J. Pharm. 33 (1986) 201-217.

[6] I. Haumann, Simultaneous determination of inorganic anions and cations by capillary electrophoresis with indirect UV detection, J. Boden, A. Mainka, U. Jegle, J. Chromatogr. A 895 (2000) 269-277.

[7] H.S. Gwak, J.S. Choi, H.K. Choi, Enhanced bioavailability of piroxicam via salt formation with ethanolamines, Int. J. Pharm. 297 (2005) 156-161.

[8] M.B. Stephen, D.B. Lyle, C.M. Donald, J. Pharm. Sci. 66 (1977) 1-19.

[9] M. Catani, R. Guzzinati, N. Marchetti, L. Pasti, A. Cavazzini, Exploring Fluorous Affinity by Liquid Chromatography, Anal. Chem., 87 (2015), 6854-6860.

[10] U.S. Food and Drug Administration Orange Book, http://www.accessdata.fda.gov/scripts/cder/ob/.

[11] G.S. Paulekuhn, J.B. Dressman, C. Saal, Trends in active pharmaceutical ingredient salt selection based on analysis of the Orange Book database, J. Med. Chem. 50 (2007) 6665-6672.

[12] A. Patel, S. A. Jones, A. Ferro, N. Patel, Pharmaceutical salts: a formulation trick or a clinical conundrum?, Br J Cardiol 16 (2009) 281-286.

[13] A. Cavazzini, N. Marchetti, R. Guzzinati, L. Pasti, A. Ciogli, F. Gasparrini, A. Laganà, Understanding Mixed-Mode Retention Mechanisms in Liquid Chromatography with Hydrophobic Stationary Phases, Anal. Chem., 86 (2017) 4919–4926.

[14] M. Lämmerhofer, M. Richter, J. Wu, R. Nogueira, W. Bicker, W. Lindner, Mixed-mode ion-exchangers and their comparative chromatographic characterization in reversed-phase and hydrophilic interaction chromatography elution modes, J. Sep. Sci. 31 (2008) 2572-2578.

[15] E. Apfelthaler, W. Bicker, M. Lämmerhofer, M. Sulyok, R. Krska, W. Lindner, R. Schuhmacher, Retention pattern profiling of fungal metabolites on mixed-mode reversed-phase/weak anion exchange stationary phases in comparison to reversed-phase and weak anion exchange separation materials by liquid chromatography-electrospray ionisation-tandem mass spectrometry, J. Chromatogr. A 1191 (2008) 171-181.

[16] X. Liu, C. Pohl, A. Woodruff, J. Chen, Chromatographic evaluation of reversed-phase/anion-exchange/cation-exchange trimodal stationary phases prepared by electrostatically driven self-assembly process, J. Chromatogr. A 1218 (2011) 3407-3412.

[17] X. Liu, C. Pohl, HILIC behavior of a reversed-phase/cation-exchange/anion-exchange trimode column, J. Sep. Sci. 33 (2010) 779-786.

[18] X. Liu, C. Pohl, Comparison of reversed-phase/cation-exchange/anion-exchange trimodal stationary phases and their use in active pharmaceutical ingredient and counterion determinations, J. of Chromatogr. A, 1232 (2012) 190-195.

[19] David Thomas, Bruce Bailey, Marc Plante, Ian Acworth. Charged Aerosol Detection and Evaporative Light Scattering Detection – Fundamental Differences Affecting Analytical Performance, technical note from Thermo Fisher Scientific.

[20] R. Godoy Ramos, D. Libong, M. Rakotomanga, K. Gaudin, P.M. Loiseau, P. Chaminade, Comparison between charged aerosol detection and light scattering detection for the analysis of Leishmania membrane phospholipids, Journal of Chromatography A, 1209 (2008) 88–94.

[21] L. A. Nguyen, H. He, C. Pham-Huy, Chiral Drugs: An Overview, Int J Biomed Sci., 2 (2006) 85-100.

[22] E. J. Ariens, E. W. Wuis, Bias in pharmacokinetics and clinical pharmacology, Clinical Pharmacology and Therapeutics, 42 (1987) 361-363.

[23] S. Magni, M. Parolini, C. Della Torre, L. F. de Oliveira, M. Catani, R. Guzzinati, A. Cavazzini, A. Binelli, Multi-biomarker investigation to assess toxicity induced by two antidepressants on Dreissena polymorpha, Sci. Tot. Env., 578 (2017), 452-459.

[24] A. Cavazzini, N. Marchetti, R. Guzzinati, M. Pierini, A. Ciogli, D. Kotoni, I. D'Acquarica, C. Villani, F. Gasparrini, Enantioseparation by ultra-high-performance liquid chromatography, TrAC, 63 (2014) 95-103.

[25] A. Cavazzini, A. Felinger, K. Kaczmarski, P. Szabelski, G. Guiochon, Study of the adsorption equilibria of the enantiomers of 1-phenyl-1-propanol on cellulose tribenzoate using a microbore column, Journal of Chromatography A, 953 (2002) 55-66.

[26] K. Kaczmarski, A. Cavazzini, P. Szabelski, D. Zhou, X. Liu, G. Guiochon, Application of the general rate model and the generalized Maxwell-Stefan equation to the study of the mass transfer kinetics of a pair of enantiomers, Journal of Chromatography A, 962 (2002) 57-67.

[27] P. Vajda, A. Felinger, A. Cavazzini, Adsorption equilibria of proline in hydrophilic interaction chromatography, Journal of Chromatography A,1217 (2010) 5965-5970.

[28] O.H. Ismail, L. Pasti, A. Ciogli, C. Villani, J. Kocergin, S. Anderson, F. Gasparrini, A. Cavazzini, M. Catani, Pirkle-type chiral stationary phase on core–shell and fully porous

particles: Are superficially porous particles always the better choice toward ultrafast high-performance enantioseparations?, Journal of Chromatography A, 1466, (2016) 96-104

[29] C.V. Hoffmann, R. Pell, R.M. Lämmerhofer, W. Lindner, Synergistic effects on enantioselectivity of zwitterionic chiral stationary phases for separations of chiral acids, bases, and amino acids by HPLC, Anal. Chem. 80 (2008) 8780-8789.

[30] C.V. Hoffmann, R. Reischl, N.M. Maier, M. Lämmerhofer, W. Lindner, Stationary phase-related investigations of quinine-based zwitterionic chiral stationary phases operated in anion-, cation-, and zwitterion-exchange modes, J. Chromatogr. A, 1216 (2009) 1147–1156.

[31] I. Ilisz, N. Grecsó, A. Misicka, D. Tymecka, L. Lázár, W. Lindner, A. Péter, Comparison of the Separation Performances of Cinchona Alkaloid-Based Zwitterionic Stationary Phases in the Enantioseparation of β2 - and β3 -Amino Acids, Molecules, 20 (2015) 70-87.

[32] Yu, L. W.; Hartwick, R. A. Zwitterionic Stationary Phases in HPLC, J. Chromatogr. Sci. 1989, 27, 176–185.

[33] H. Arasawa, C. Odawara, R. Yokoyama, H. Saitoh, T. Yamauchi, N. Tsubokawa, Grafting of zwitterion-type polymers onto silica gel surface and their properties, React. Funct. Polym., 61 (2004) 153–161.

[34] W. Jiang, G. Fischer, Y. Girmay, K. Irgum, Zwitterionic stationary phase with covalently bonded phosphorylcholine type polymer grafts and its applicability to separation of peptides in the hydrophilic interaction liquid chromatography mode, J. Chromatogr., A 2006, 1127, 82–91.

[35] O.H. Ismail, A. Ciogli, C. Villani, M. De Martino, M. Pierini, A. Cavazzini, D.S. Bell, F. Gasparrini, Ultra-fast high-efficiency enantioseparations by means of a teicoplanin-based chiral stationary phase made on sub-2 μm totally porous silica particles of narrow size distribution, J. Chromatogr. A, 1427 (2016) 55–68.

510 [36] F. Gritti, D.S. Bell, G. Guiochon, Particle size distribution and column efficiency. An
511 ongoing debate revived with 1.9 um Titan-C18 particles, J. Chromatogr. A 1335 (2014)
512 179-192.
513 [37] M. Catani, O.H. Ismail, A. Cavazzini, A. Ciogli, C. Villani, L. Pasti, C. Bergantin, D.
514 Cabooter, G. Desmet, F. Gasparrini, D. S. Bell. Rationale behind the optimum efficiency of
515 columns packed with new1.9 µm fully porous particles of narrow particle size distribution,
516 Journal of Chromatography A, 1454 (2016) 78–85.
517 [38] C.L. Barhate, M.F. Wahab, Z.S Breitbach, D.S. Bell, D.W. Armstrong. High efficiency,
518 narrow particle size distribution, sub-2 µm based macrocyclic glycopeptide chiral
519 stationary phases in HPLC and SFC. Analytica chimica acta 898 (2015) 128-137.
520 [39] F. Gritti, G. Guiochon, The quantitative impact of the mesopore size on the mass
521 transfer mechanism of the new 1.9 µm fully porous Titan-C18 particles. I: analysis of small
522 molecules, J. Chromatogr. A 1384 (2015) 76e87.
523 [40] O.H Ismail, M. Antonelli, A. Ciogli, C. Villani, A. Cavazzini, M. Catani, S. Felletti, D.S.
524 Bell, F. Gasparrini, Future perspectives in high efficient and ultrafast chiral liquid
525 chromatography through zwitterionic teicoplanin-based 2-µm superficially porous particles,
526 J. Chromatogr. A, 1520 (2017) 91-102.
527 [41] C.L. Barhate, L.A. Joyce, A.A. Makarov, K. Zawatzky, F. Bernardoni, W.A. Schafer,
528 D.W. Armstrong, C.J. Welch, E.L. Regalado, 2017. Ultrafast chiral separations for high
529 throughput enantiopurity analysis. Chemical Communications, 53 (2017) 509-512.
530 [42] D. Guillarme, G. Bonvin, F. Badoud, J. Schappler, S. Rudaz, J.L. Veuthey, Fast chiral
531 separation of drugs using columns packed with sub-2 µm particles and ultra-high pressure.
532 Chirality: The Pharmacological, Biological, and Chemical Consequences of Molecular
533 Asymmetry, 22 (2010) 320-330.
534 [43] A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, Comparison of ultra-high
535 performance supercritical fluid chromatography and ultra-high performance liquid

536 chromatography for the analysis of pharmaceutical compounds, J. Chromatogr. A 1266
537 (2012) 158–167.
538 [44] D.W. Armstrong, Y.B. Tang, S.S. Chen, Y.W. Zhou, C. Bagwill, J.R. Chen.
539 Macrocyclic Antibiotics as a New Class of Chiral Selectors for Liquid Chromatography
540 Anal. Chem. 66 (1994) 1473-1484.
541 [45] A. Berthod, X.H. Chen, J.P. Kullman, D.W. Armstrong, F. Gasparrini, I. D'Acquarica,
542 C. Villani, A. Carotti, Role of the Carbohydrate Moieties in Chiral Recognition on
543 Teicoplanin-Based LC Stationary Phases Anal. Chem. 72 (2000) 1767-1780.
544 [46] I. D'Acquarica, F. Gasparrini, D. Misiti, M. Pierini, C. Villani, HPLC chiral stationary
545 phases containing macrocyclic antibiotics: practical aspects and recognition mechanism,
546 Adv. Chromatogr. 46 (2008) 109-173.
547 [47] A. Cavazzini, G. Nadalini, F. Dondi, F. Gasparrini, Ciogli A., Villani C. Study of
548 mechanisms of chiral discrimination of amino acids and their derivatives on a teicoplanin-
549 based chiral stationary phase. J. Chromatogr. A 1031(2004) 143-158.
550 [48] W. Hu, P.R. Haddad, K. Hasebe, K. Tanaka, P. Tong, C. Khoo, Direct determination
551 of bromide, nitrate, and iodide in saline matrixes using electrostatic ion chromatography
552 with an electrolyte as eluent, Anal. Chem., 71 (1999) 1617–1620.
553 [49] X. Liu, C.A. Pohl, Comparison of reversed-phase/cation-exchange/anion-exchange
554 trimodal stationary phases and their use in active pharmaceutical ingredient and
555 counterion determinations J. Chromatogr. A 1232 (2012) 190–195.
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Figure 1

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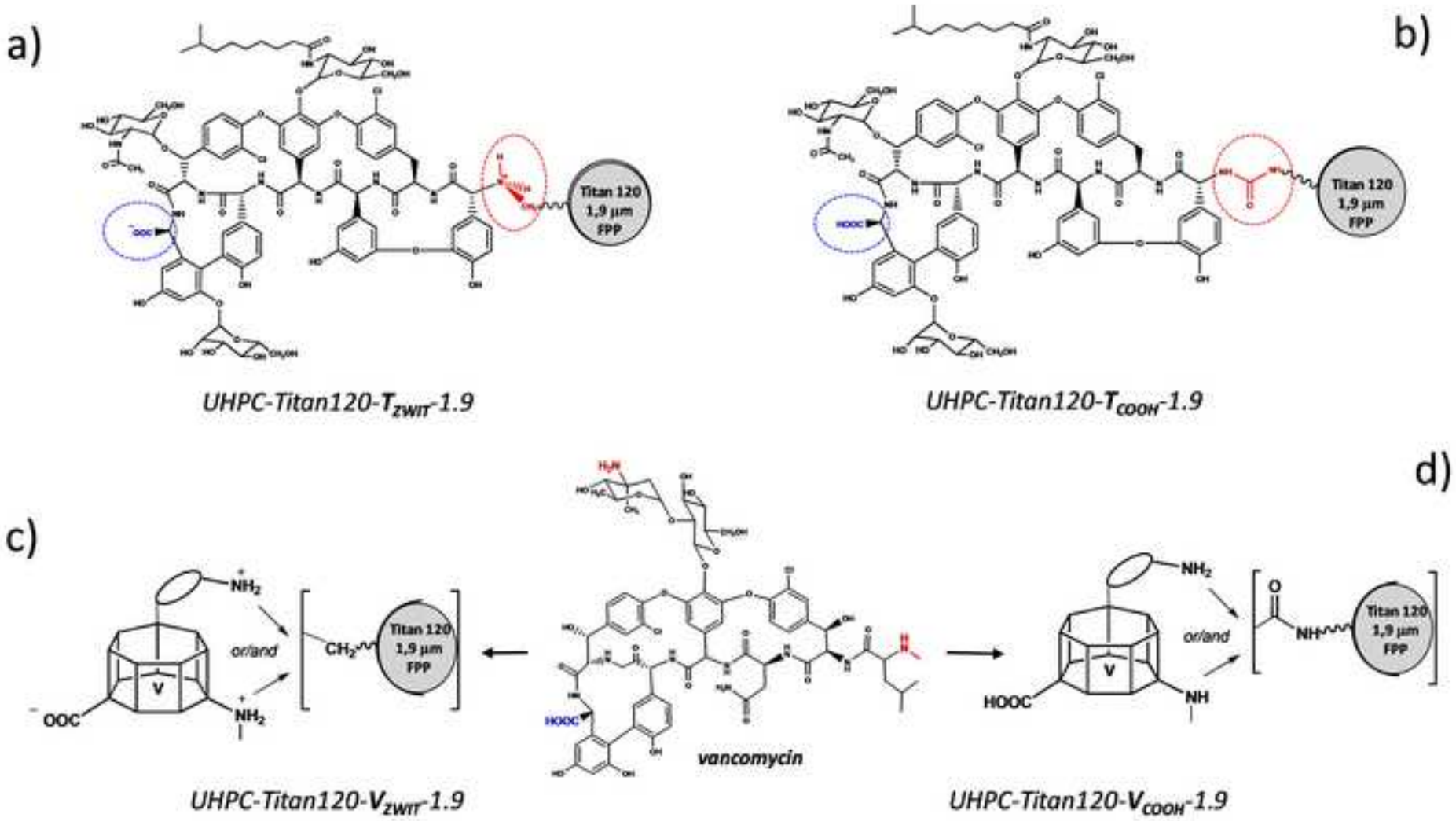

Figure 2

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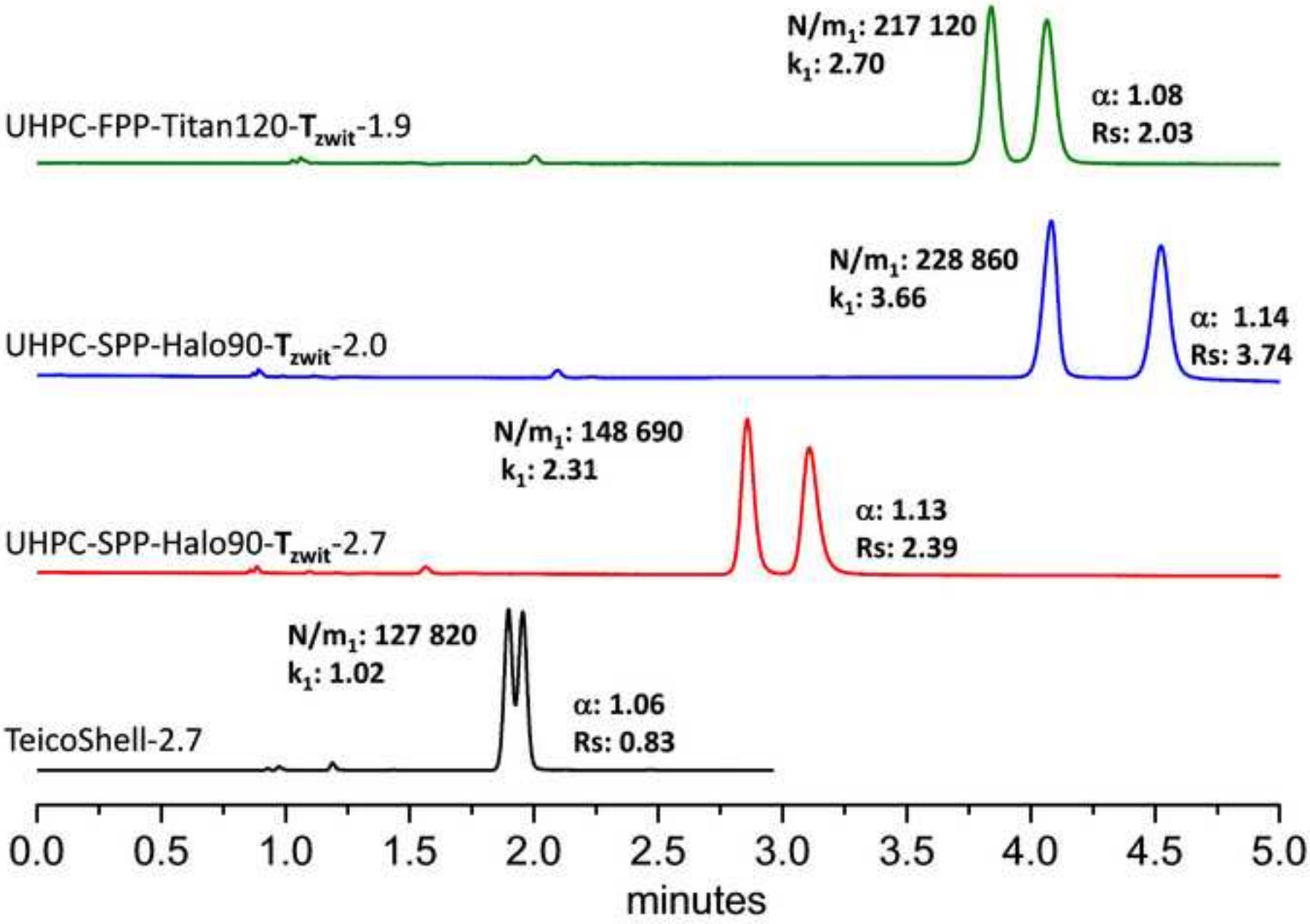

Figure 3

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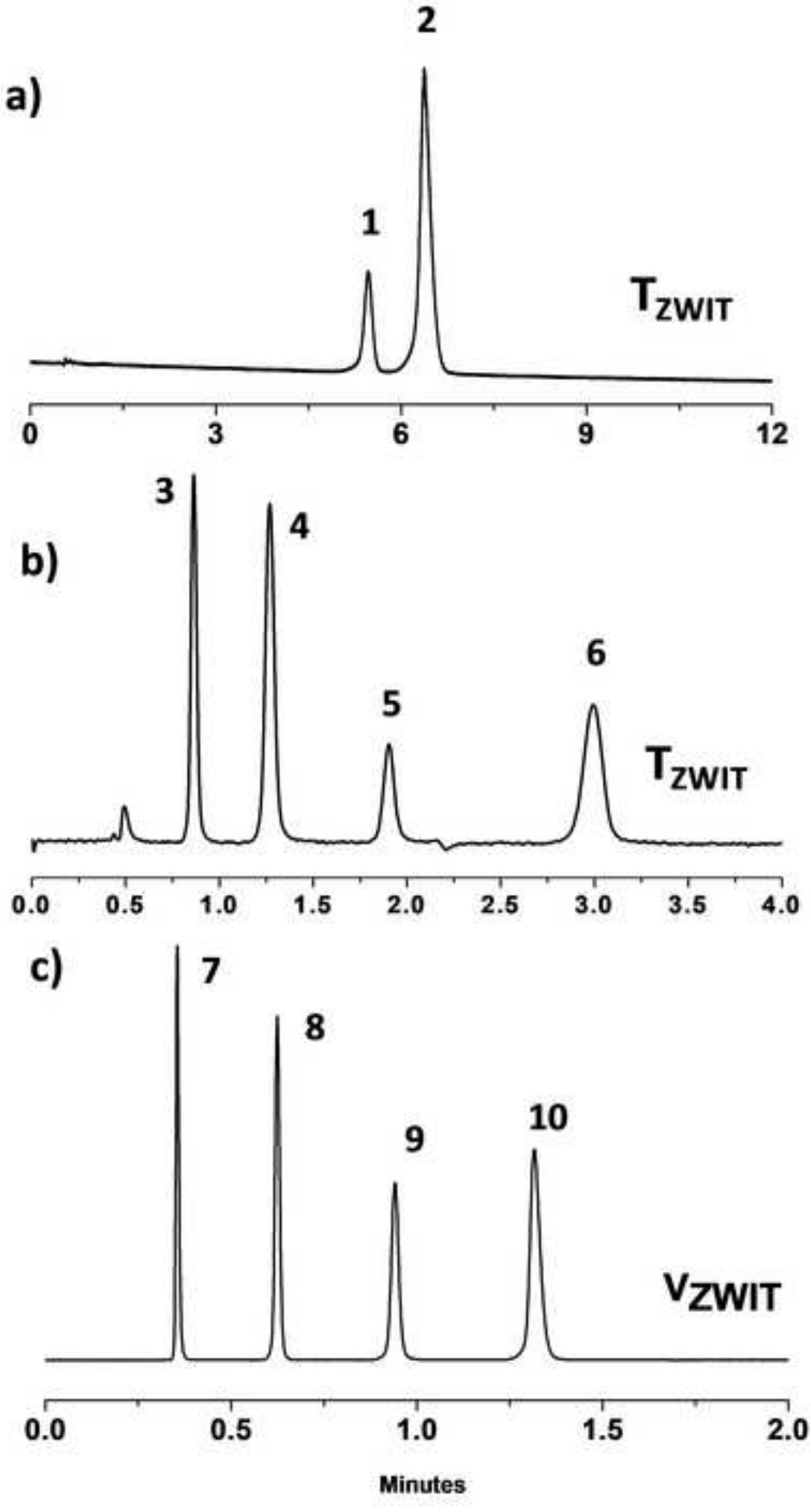

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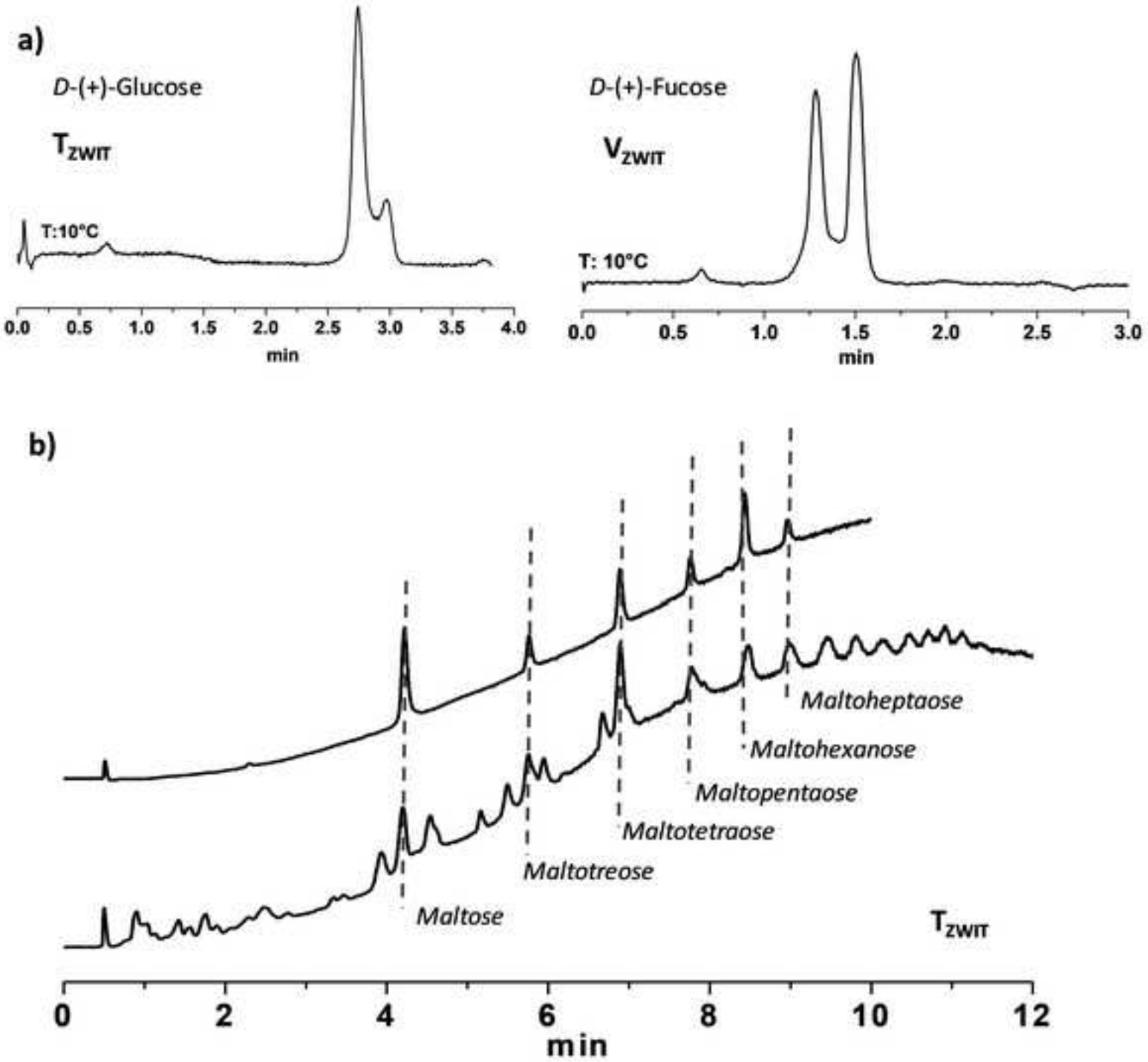

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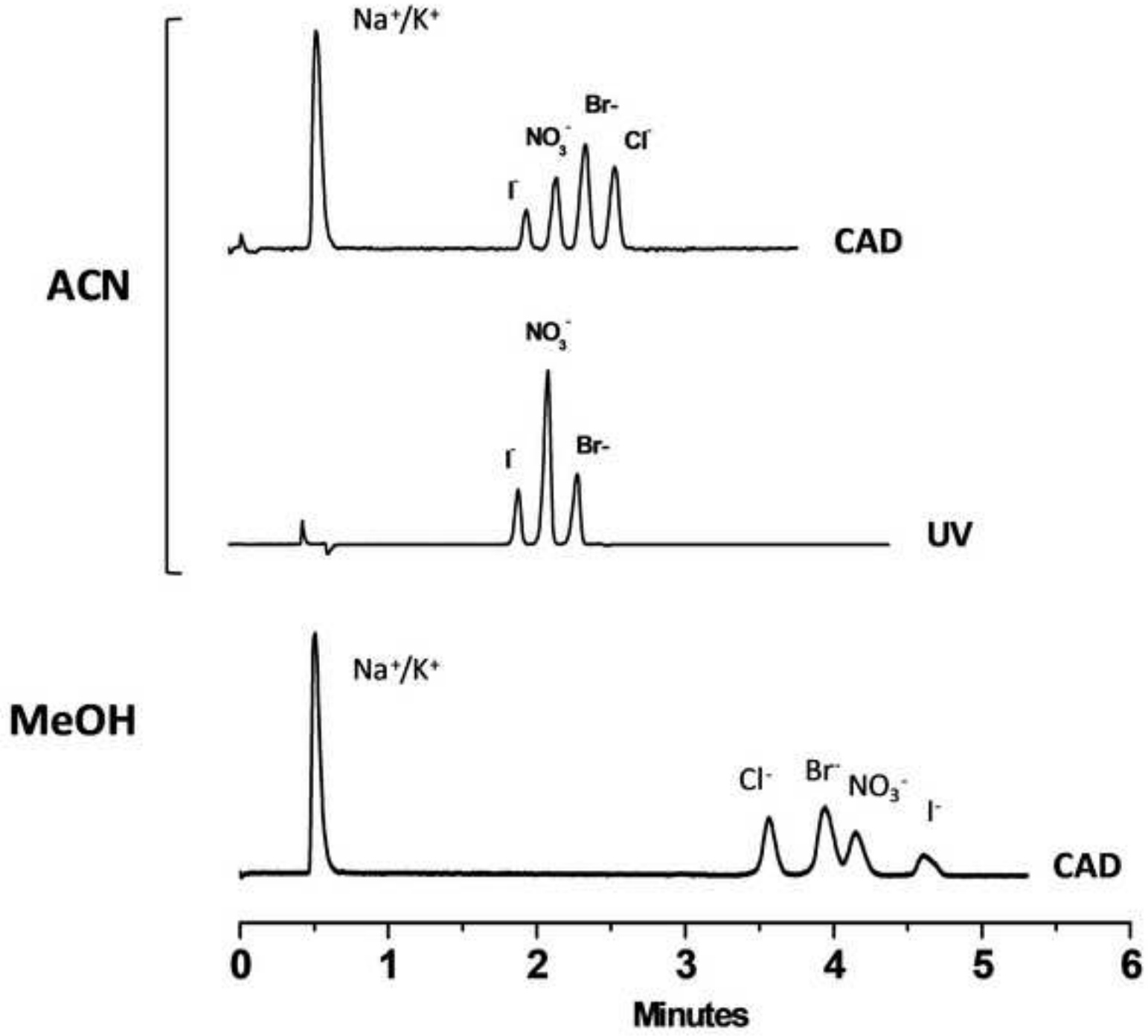

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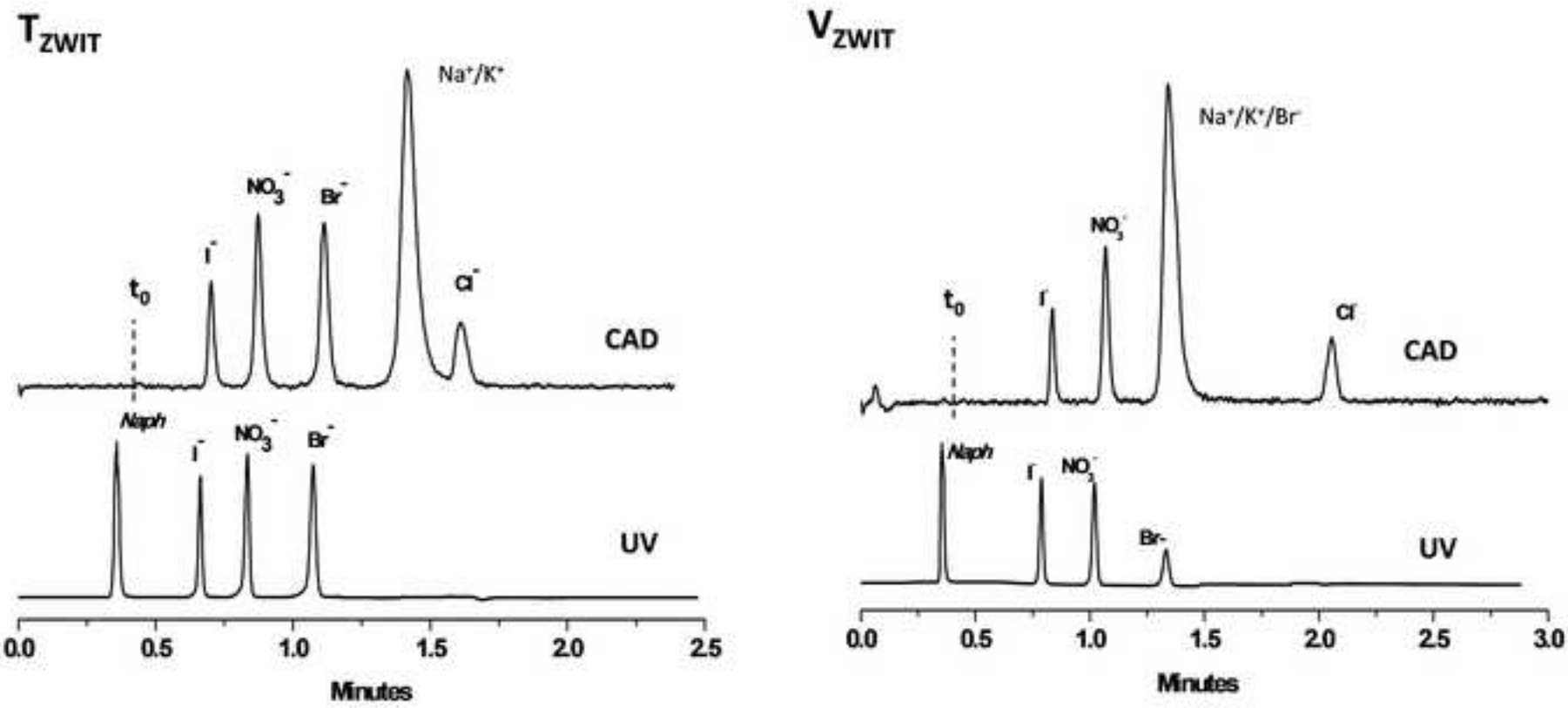

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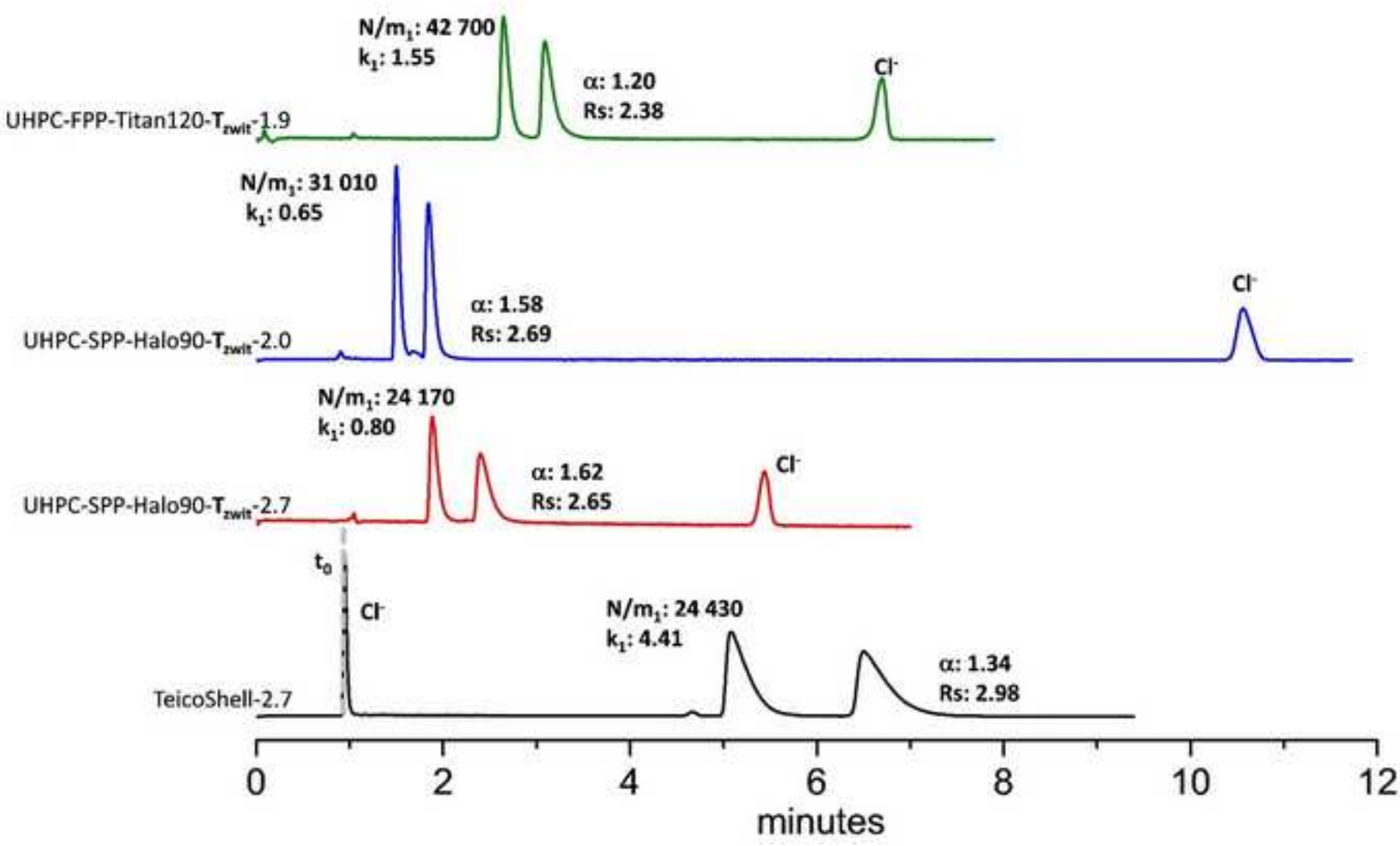

Figure 8

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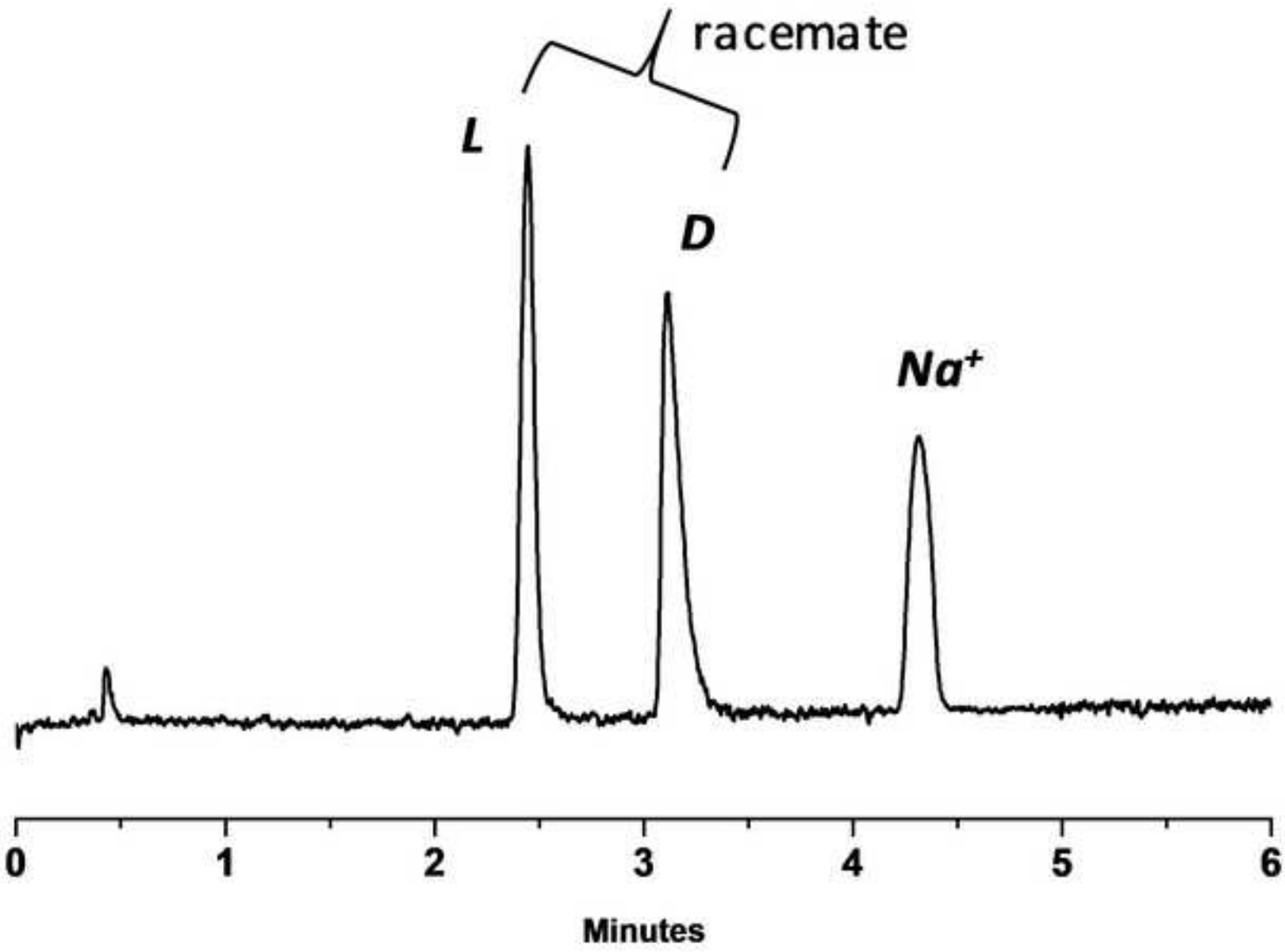

Tables

Table 1- Elemental analysis of chiral stationary phases and calculated bonding densities (both as μmol per gram of base silica and as specific density).

CSP acronym	%C	%H	%N	μmol/g	μmol/m^2
UHPC-Titan120-**T**$_{ZWIT}$-1.9	12.13	1.60	1.35	138	0.49
UHPC-Titan120-**V**$_{ZWIT}$-1.9	8.18	1.21	1.01	91	0.32
UHPC-Titan120-**T**$_{COOH}$-1.9	6.72	1.11	0.84	68	0.24
UHPC-Titan120-**V**$_{COOH}$-1.9	4.62	0.98	0.73	55	0.20

Tables

Table 2 – Retention factor (first eluted enantiomer) and enantioselectivity of enantiomers considered in this work on the two CSPs (UHPC-Titan120-T_{ZWIT}-1.9 and UHPC-Titan120-V_{ZWIT}-1.9). Eluent: MeOH/H_2O 85/15 + 10 mM formate ammonium (^{w}pH =6.5); flow rate: 1.0 ml/min; T: 30°C.

Sample	UHPC-Titan120-T_{ZWIT}-1.9			UHPC-Titan120-V_{ZWIT}-1.9		
	k_1	α	R_s	k_1	α	R_s
Haloxyfop	10.7	1.91	10.5	10.3	1.06	1.23
Ketorolac	19.2	1.99	20	18.4	1.04	0.96
Ketoprofen	17.2	1.10	1.86	12.9	1.07	1.80
Indoprofen	29.4	1.27	1.54	22.3	1.08	1.20
Flunoxaprofen	17.2	/	/	11.4	1.11	1.76
Naproxen	18.7	/	/	17.7	1.06	1.17
Suprofen	23.4	1.06	0.90	15.4	1.09	1.72
Ibuprofen	9.5	/	/	7.65	1.06	1.00
Fmoc-*D,L*-Ala	18.8 (*L*)	2.52	12.6	17.6	1.14	3.21
Boc-*D,L*-Met	6.0 (*L*)	1.71	7.62	9.46	/	/
Fmoc-*D,L*-Glu	17.8 (*L*)	1.92	10.2	19.8	1.05	1.22
Dansyl-*D,L*-Met	14.5 (*L*)	2.50	11.9	20.3	1.15	2.86
Dansyl-*D,L*-Phe	18.1 (*L*)	1.20	3.54	24.8	1.12	2.30
D,L-Ala	1.06 (*L*)	2.42	8.11	*not retained*		
D,L-Pro	2.33 (*L*)	4.15	14.0	*not retained*		
D,L-Val	0.62 (*L*)	2.38	5.96	*not retained*		
Z-D,L-Ala*	8.66 (*L*)	2.62	13.2	7.04	1.41	7.24
Z-D,L-Leu*	5.51 (*L*)	2.60	14.7	6.03	1.08	1.72
Z-D,L-Met*	8.47 (*L*)	2.74	13.9	7.05	1.16	3.23

* Eluent for Z-AA was MeOH/H_2O 70/30 + 20 mM formate ammonium (^{w}pH =6.5)

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