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Efficient enantioresolution of aromatic α -hydroxy acids with Cinchona alkaloid-based zwitterionic stationary phases and volatile polar-ionic eluents

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(Article begins on next page)

1 **Efficient enantioresolution of aromatic α -hydroxy acids with**
2 ***Cinchona* alkaloid-based zwitterionic stationary phases and**
3 **volatile polar-ionic eluents**

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26

27 **Highlights**

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29 • ZWIX(-) allows the enantioresolution of the three aromatic α -hydroxy acids.

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31 • The optimized polar-ionic conditions are fully compatible with MS detectors.

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33 • The developed LC method can be used to study biological matrices with MS detectors.

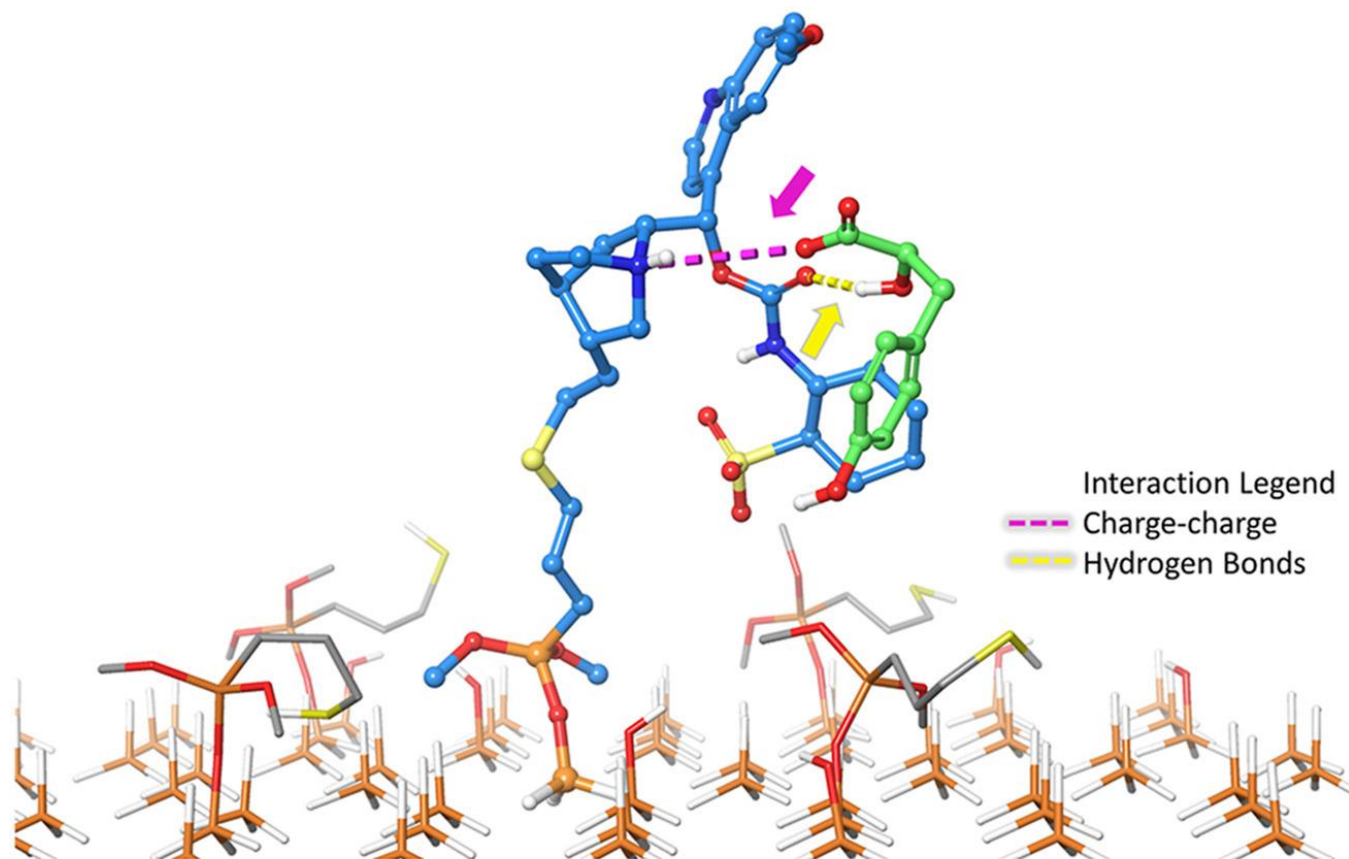
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35 • Combining in silico simulations and ECD analyses is useful to derive the EEO.

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37 • The retention mechanism can be explained with molecular dynamic simulations.

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Interactions engaged by the CSP 1 (cyan sticks) and (S)-3 (green sticks)

41 **ABSTRACT**

42 Single enantiomers of mandelic acid (1), 3-phenyllactic acid (2), and 3-(4-hydroxyphenyl)lactic acid (3) are the
43 subject of many fields of investigation, spanning from the pharmaceutical synthesis to that of biocompatible
44 and biodegradable polymers, while passing from the interest towards their antimicrobial activity to their role
45 as biomarkers of particular pathological conditions or occupational exposures to specific xenobiotics. All
46 above mentioned issues justify the need for accurate analytical methods enabling the correct determination
47 of the individual enantiomers. So far, all the developed liquid chromatography (LC) methods were not or
48 hardly compatible with mass spectrometry (MS) detection. In this paper, a commercially available Cinchona-
49 alkaloid derivative zwitterionic chiral stationary phase [that is, the CHIRALPAK® ZWIX(-)] was successfully used
50 to optimize the enantioresolution of compounds 1–3 under polar-ionic (PI) conditions with a mobile phase
51 consisting of an acetonitrile/methanol 95/5 (v/v) mixture with 80 mM formic acid. With the optimized
52 conditions, enantioseparation and enantioresolution values up to 1.46 and 4.41, respectively, were obtained.
53 In order to assess the applicability of the optimized enantioselective chromatography conditions in real-life
54 scenarios and on MS-based systems, a proof-of-concept application was efficiently carried out by analysing
55 dry urine spot samples spiked with 1 by means of a LC-MS system. The (S)<(R) enantiomer elution order (EEO)
56 was established for compounds 1 and 2 by analysing a pure enantiomeric standard of known configuration.
57 This was not possible for 3 because not commercially available. For this compound, the same EEO was
58 identified applying a procedure based on ab initio time-dependent density-functional theory simulations
59 coupled to electronic circular dichroism analyses. Moreover, a molecular dynamics simulation unveiled the
60 role of the phenolic OH in compound 3 in the retention mechanism.

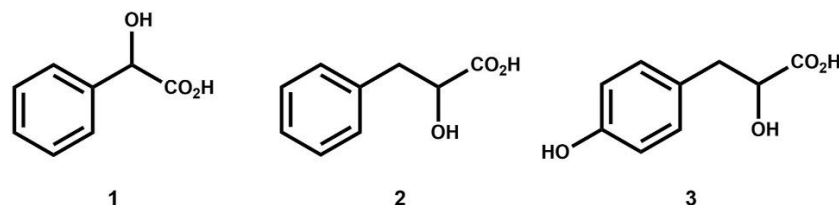
61
62 **KEYWORDS:**

63 Ab-initio simulations; Aromatic α -hydroxy acids; Dry urine spots; Electronic circular dichroism;
64 Enantioresolution; MS-Compatible conditions.

65 **INTRODUCTION**

66
67 Mandelic acid (1), 3-phenyllactic acid (2), and 3-(4-hydroxyphenyl)lactic acid (3) (Fig. 1) are aromatic α -
68 hydroxy acids, with compounds 2 and 3 belonging to the class of phenyl propionic acids.

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71 **Fig. 1.** Structure of the compounds investigated in the study: (1) mandelic acid; (2) 3-phenyllactic acid;
72 (3) 3-(4-hydroxyphenyl)lactic acid.

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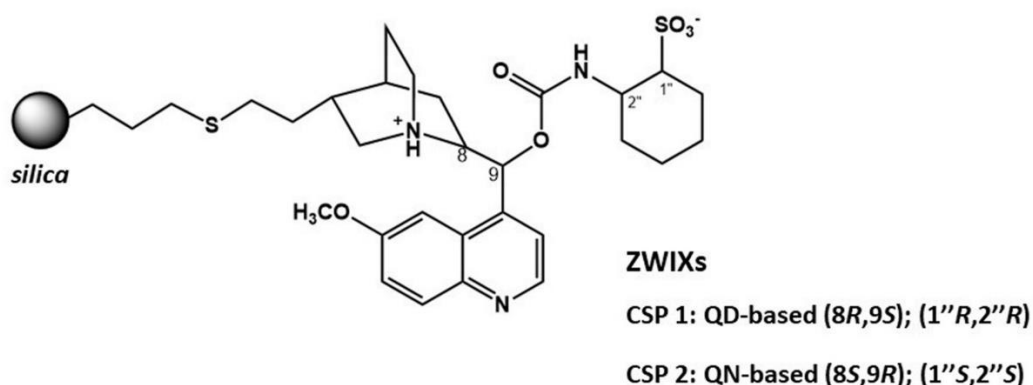
74 Single enantiomers of compounds 1–3 are versatile chiral building blocks for the large-scale production of
75 numerous active pharmaceuticals ingredients including semi-synthetic penicillins, cephalosporins and other
76 antibiotics [[1], [2], [3], [4]], antitumor [5,6] and antiobesity agents [7], just to cite some. The three
77 molecules are also very well-known (in an enantiomerically specific manner) for their antimicrobial activity
78 [8,9], as well as for their use in the synthesis of biocompatible and biodegradable polymers also for
79 promising biomedical applications [10,11].

80 Various approaches have been developed so far for the preparation of the enantiopure form of compounds
81 1–3. These approaches include traditional organic synthesis methods, as well as enzymatic and biological
82 routes [9,12,13]. In this scenario, over the last two decades, both biocatalytic asymmetric synthesis and
83 fermentation processes have gained great interest both in industry and academia owing to the high
84 efficiency, simple operation, environment-friendly nature and, in particular, excellent stereoselectivities
85 [9,12,13]. Also importantly, the enantiomers of compounds 1–3 are often measured in biological matrices
86 such as plasma and urine as biomarkers of particular pathological conditions (including phenylketonuria,
87 hyperphenylalaninemia, tyrosinemia, gut microbial alterations, etc.) [14,15] as well as of environmental and
88 occupational exposures to specific xenobiotics (such as styrene and ethylbenzene) [[16], [17], [18]].

89 All above mentioned issues justify the need for accurate analytical methods enabling the correct
90 determination of the individual enantiomers. So far, various direct enantioselective chromatography
91 methods with commercially available chiral stationary phases (CSPs) have been developed for this aim.
92 Accordingly, efficient enantioselective liquid chromatography (LC) analyses were performed with
93 polysaccharide- [9,[19], [20], [21], [22]], glycopeptide- [23,24], protein- [25] and ligand-exchange-based [26]
94 CSPs. However, all these methods were not or hardly compatible with mass spectrometry (MS) detection.

95 In order to fulfil this gap, we have developed the first LC method fully compatible with MS detectors. This

96 efficient enantioselective LC method relies upon the use of a polar-ionic (PI) mobile phase in combination
 97 with a zwitterionic CSP operating as an anion-exchanger. This zwitterionic CSP (CSP 1, Fig. 2) consists of a
 98 Cinchona-alkaloid derivative type chiral selector (SO), which has proven to produce enantioselectivity for
 99 many ionic and ionizable analytes under the most relevant elution regimes for LC applications [[27], [28],
 100 [29]]. Since commercial enantiopure standards are unavailable for compound 3, its enantiomeric elution
 101 order (EEO) in the optimized mobile phase conditions has been obtained with the application of a well-
 102 established method based on *ab initio* time-dependent density-functional theory (TD-DFT) simulations
 103 coupled to electronic circular dichroism (ECD) analyses [30,31]. Finally, in order to prove the compatibility
 104 of our method with MS detectors, dried urine spot (DUS) microsamples [32,33] spiked with mandelic acid
 105 (1) have been extracted and efficiently analysed by means of an ultra-high performance liquid
 106 chromatography (UHPLC)-MS system exploiting the optimized enantioselective chromatography conditions.
 107



108 **Fig. 2.** Structure of zwitterionic CSPs operating according an ion-exchange mechanism. The two CSPs belong
 109 to the family of Cinchona alkaloid-based enantiorecognition materials and are commercialized by Chiral
 110 Technology Europe (Strasbourg, France) under the trade names CHIRALPAK® ZWIX(-) (CSP 1) and
 111 CHIRALPAK® ZWIX(+) (CSP 2).
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115 2. MATERIAL AND METHODS

116 2.1. Chemicals

117 All the reagents and solvents used in the study were of analytical grade. Acetonitrile (ACN), methanol
 118 (MeOH), formic acid (FA), acetic acid (AcOH), as well racemic mandelic acid (1), 3-phenyllactic acid (2), 3-(4-
 119 hydroxyphenyl)lactic acid (3) and 3-phenylpropionic acid - used as internal standard (IS) - were purchased
 120 from Merck Life Science (Merck KGaA, Darmstadt, Germany). The pure enantiomer of 1 and 2 were also
 121 from Merck Life Science. Water for HPLC analysis was purified with a New Human Power I Scholar water

122 purification system (Human Corporation, Seoul, Korea). For UHPLC-MS analysis, MS-grade ACN, MeOH and
123 FA were employed and were purchased from Merck Life Science. Whatman FTA™ DMPK-B IND cards for
124 DUS sampling were purchased from Merck Life Science, while a Whatman (Maidstone, MA, USA) Harris Uni-
125 Core Punch, 6.0 mm was used for punching the DUS discs out of the spotting cards.

127 2.2. Instrumentations

128 The HPLC-UV analyses were performed on a Shimadzu HPLC equipped with a SCL-10Avp system controller,
129 two LC-10AD high pressure binary gradient delivery systems, a SPD-10A variable-wavelength UV-Vis
130 detector, and a Rheodyne 7725i injector with a 20 µL stainless steel loop. The enantioselective analyses
131 were carried out with the CHIRALPAK® ZWIX(-) (CSP 1, Fig. 2) and CHIRALPAK® ZWIX(+) (CSP 2, Fig. 2) (150
132 mm × 4.0 mm I.D, 120 Å pore size, 3 µm particle diameter) columns kindly provided by Chiral Technologies
133 Europe (Strasbourg, France). All the analyses were performed at a 0.2 mL min⁻¹ eluent flow rate. The
134 column temperature was fixed at 25 °C with a Grace (Sedriano, Italy) heater/chiller (Model 7956R)
135 thermostat. A 254 nm detection wavelength was selected for the analyses. Acetone was selected as the
136 unretained marker to evaluate the chromatographic parameters.

137 An ultra-high performance liquid chromatography coupled to single-quadrupole mass spectrometry
138 (UHPLC-MS) system was exploited in order to assess the applicability of the optimized enantioselective
139 chromatography conditions on MS-based systems and to obtain a proof-of-concept application by analysing
140 spiked DUS samples. The system was composed of a UHPLC Vanquish pump, Vanquish Autosampler,
141 Vanquish column compartment, and ISQ EC single-quadrupole mass spectrometer (Thermo Fisher Sci.,
142 Waltham, MA, US), while data elaboration was carried out by means of Thermo Fisher Scientific Dionex
143 Chromeleon 7.3 Chromatography Data System software. Enantioselective separations were obtained on the
144 same CHIRALPAK® ZWIX(-) (CSP 1) column previously described, a 0.2 mL min⁻¹ flow rate and 20 µL
145 injection volume; the autosampler needle was washed with 10% (v/v) MeOH (10 s) after each sample
146 injection. Isocratic mobile phase consisted of a mixture of ACN/MeOH 95/5 (v/v) with 80 mM FA. Optimized
147 mass spectrometric parameters were the following: vaporizer temperature (VT), 117 °C; ion transfer tube
148 temperature (ITT), 300 °C; sheath gas pressure (SGP), 30 psig; auxiliary gas pressure (AGP), 3 psig; sweep
149 gas pressure (SWGp), 0.5 psig. Negative ionisation mode was exploited, which resulted in extracted
150 mandelic acid (1) chromatograms at m/z 151.1. Column and autosampler temperatures were maintained at
151 25 °C and 10 °C, respectively.

152 The ECD spectra were recorded by a Jasco J-810 Spectropolarimeter (Jasco Corporation, Tokyo, Japan) at 25
153 °C, using 10 mm quartz cell. The samples were solubilized in MeOH and analysed in the global spectral
154 range of 210–400 nm.

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2.3. Computational studies

2.3.1. Theoretical ECD spectra simulations

Compounds 1–3 were built by the Maestro interface (Schrödinger, LLC, New York, NY, 2020) present in the Schrödinger Suite 2020-2; Ligprep Protocol set at the default options was adopted to assign the correct protonation state. MMFF94s force field was used to perform a conformational search with the MacroModel module (Schrödinger, LLC, New York, NY, 2020) executing 200 steps with the “Mixed torsional/Low-mode sampling” using water as solvent and retaining at most 50 conformers, lying in a window of 5.0 kcal mol⁻¹ from the global minimum energy. The torsional sampling involved either multiple Monte Carlo minimum searches for global exploration, and the low mode conformational search allowed for automatic local exploration. The conformers that exceeded the threshold of 0.5 Å of the maximum atom deviation for any pair of corresponding atoms were considered to be different. Using the same settings applied in a recently published paper to simulate the ECD spectra of compound 1 [34], the resulting conformers were all submitted to a quantum mechanical energy optimization using the wB97X-D as DFT and the 6–311++G**. Finally, the level of accuracy was set to Ultrafine (iacc = 1) in the Jaguar module (Schrödinger, LLC, New York, NY, 2020) [[35], [36], [37], [38]]. A RMSD threshold value of 0.01 Å for heavy atoms was applied to eliminate the high-lying or redundant conformers. The theoretical chiroptical properties of all the compounds were determined using a standard protocol for stereochemical characterization using TD-DFT calculations [30]. The sTD-DFT [39] calculations were carried out using the ORCA 4.1.2 software [40] using the wB97X-D3 as DFT and the 6–311++G** basis set. The 50 lowest energy electronic transitions of each optimized conformer were used to calculate the theoretical values of oscillator strength (f_j), rotational strength in dipole velocity formalism (R_j), and excitation energy (expressed as wavelength, λ_j). By approximation of f_j and R_j values to Gaussian bands with a σ value of 0.3 eV [41] the theoretical spectra of the optimized conformers were then derived. The weighted average of the contribution of all conformers according to their Boltzmann equilibrium populations at 298.15 K and 1 atm, based on free energy values (G), was used to derive the theoretical ECD spectra of the compound and compared to the experimental spectra. This last step was performed using the SpecDis software [42]. The in silico spectra obtained by the different basis set used were compared with the experimental outcome, and the one showing the most similar profile was then selected for the EEO determination.

2.3.2. Molecular dynamics simulations

The Maestro interface (Schrödinger, LLC, New York, NY, 2020) present in the Schrödinger Suite 2020-2 was used to reproduce the chromatographic environment. As reported in the previous work [43], a cubic box

188 was built with a 30 Å side length. For a realistic reproduction of the stationary phase environment, four 3-
189 mercaptopropyl-functionalized silanols (~1.97 mol m⁻²), eight free silanols (~8.0 mol m⁻²) and forty-five
190 silicon atoms were considered for each grafted selector (SO) unit (~0.5 mol m⁻²), at the base of the box. All
191 the silicon atoms in the base layer were set frozen during the molecular dynamics. The box was solvated
192 with ACN/MeOH 95/5 (v/v). The simulations were performed in the canonical ensemble at 298 K. The
193 temperature in the simulation cell was maintained constant through use of a Nosé-Hoover thermostat
194 [44,45]. All the other parameters in the simulation study were left to default values in the Desmond
195 Molecular Dynamics System present in the Schrödinger Suite 2020-2 [46]. The production run produced
196 1000 frames during the 300 ns dynamics, with an integration time of 2 fs.

197 198 **2.4. Preparation of dry urine spots (DUSs) and UHPLC-MS analysis**

199 Urine samples, used as blank matrix, were obtained from healthy volunteers. Aliquots of 200 µL were
200 spiked with 10 µL of standard mixtures containing 1 and IS to obtain urine concentrations of 100 ng mL⁻¹, 1
201 µg mL⁻¹ and 100 µg mL⁻¹ of 1 and a fixed IS concentration of 100 ng mL⁻¹. Then, the obtained fortified
202 urine was subjected to DUS sampling and pretreatment before UHPLC-MS analysis. Aliquots of 10 µL of
203 urine were transferred onto a Whatman FTA™ DMPK-B IND card by means of micropipetting; the obtained
204 spots were left to dry for 1 h at room temperature, then stored in the dark with suitable desiccant until
205 pretreatment and analysis. At the time of analysis, a 10-mm diameter circle was punched out from the card
206 with a puncher and placed into a vial with 250 µL of 80 mM FA in MeOH, subjected to ultrasound-assisted
207 extraction (UAE) at room temperature for 10 min and centrifuged at 4000 rpm for 5 min at 4 °C. The
208 supernatant was brought to dryness under a gentle N₂ stream, re-dissolved with 100 µL of an ACN/MeOH
209 95/5 (v/v) mixture with 80 mM FA and analysed by UHPLC-MS without the need for further treatment.
210 Resulting chromatograms were checked and compared with chromatograms from 1 standard solutions,
211 then the overlapping of retention times and the correspondence of chromatographic parameters were
212 verified.

213 214 **3. RESULTS AND DISCUSSION**

215 **3.1. Enantioseparation study of compounds 1–3**

216 One of the main advantages of using CSP 1 and CSP 2 (Fig. 2) is their well-documented flexibility to behave
217 as cation-exchangers (CXs) for the enantioseparation of chiral bases [47,48], as anion-exchanger (AXs) for
218 the enantioseparation of chiral acids [27,49,50], and as zwitterionic ion-exchangers for the
219 enantioseparation of ampholytic compounds [28,51,52].

220 CSP 1 and CSP 2 are commercially available as CHIRALPAK® ZWIX(-) and CHIRALPAK® ZWIX(+) (Chiral

221 Technology Europe, Strasbourg – France), and present the following structural characteristics: in CSP 1, QD
222 is fused via a carbamoyl group with (R,R)-trans-2-aminocyclohexanesulfonic acid (R,R-ACHSA), whereas in
223 CSP 2 QN is identically fused with (S,S-ACHSA); for the chiral selectors (SOs) incorporated in CSP 1 and CSP
224 2, the absolute configuration of N1, C3 and C4 is the same (that is, 1S,3R,4S), while an opposite
225 configuration is present at C8 and C9 of the Cinchona scaffold (that is, 8R, 9S in CSP 1, and 8S, 9R in CSP 2).
226 Moreover, CSPs 1 and 2 feature opposite configuration also at C1" and C2" in the ACHSA ring, which is the
227 reason why these two CSPs very often display a so-called "pseudo-enantiomeric" behaviour in terms of
228 their chiral recognition ability [49,[53], [54], [55]].

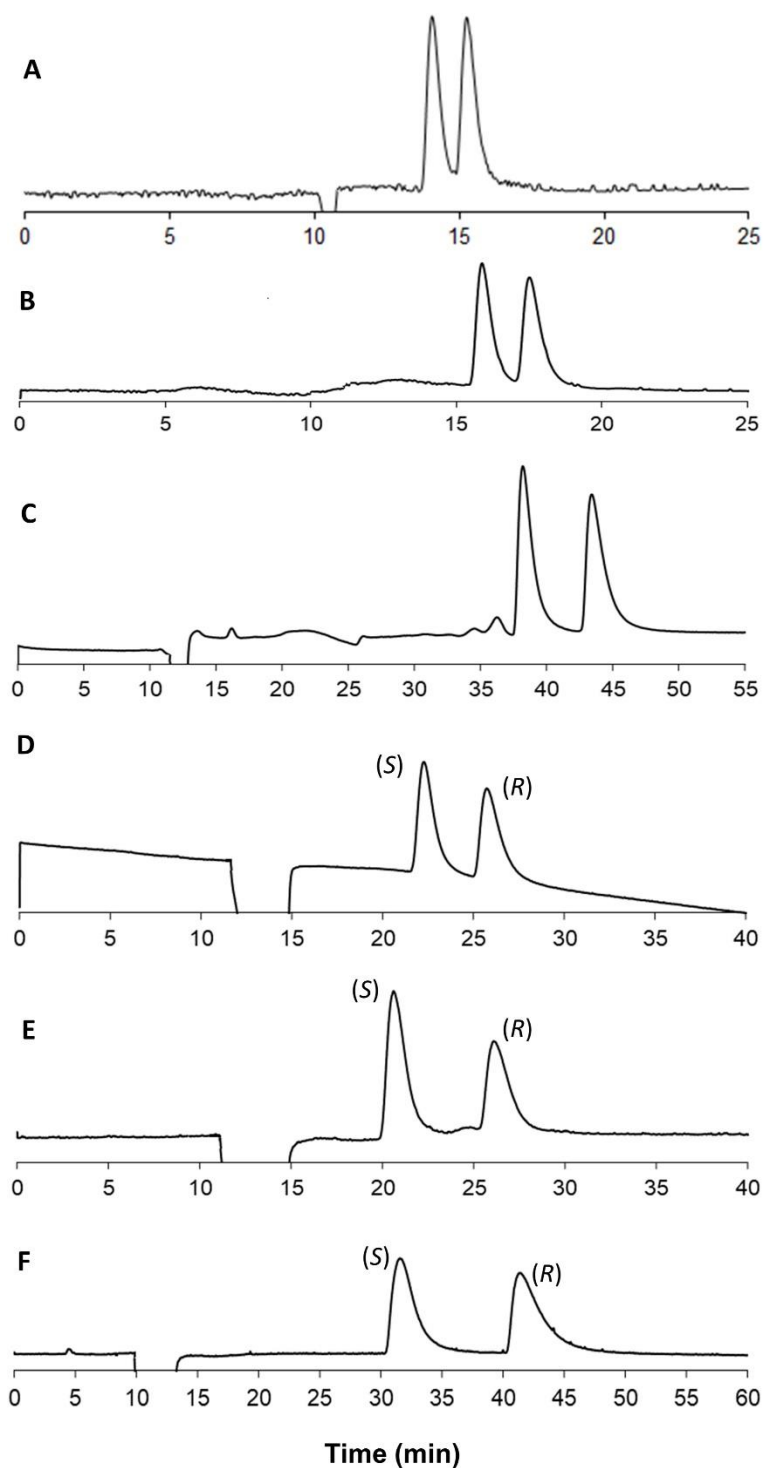
229 The use of polar-ionic (PI) eluents was here preferred over reversed-phase (RP) ones since the former often
230 produce higher enantioselectivities as a result of the following mechanisms. PI mobile phases are capable
231 to attenuate or even inactivate non-enantioselective interactions (both hydrophobic and other types of
232 interactions) with the stationary phase functionalities [52,54,56]. Moreover, a strong solvation of ionic
233 selectands (SAs) and zwitterionic SOs takes place under RP conditions, which in turn reduces the strength of
234 the ion pairing process with a detrimental effect on enantioselectivity. Furthermore, the solvation by
235 aqueous eluents might mitigate or even abolish non-ionic but stereo discriminating interactions, with a
236 negative impact on the overall enantioselectivity extent.

237 Due to the IMCI effect, the elution of anionic analytes might in principle occur even without any mobile
238 phase counterion [28]. However, this possibility is disadvantageous in practice since it may lead to
239 extremely long retention times [28]. Therefore, acidic additives (often used in combination with basic
240 additives or replaced by buffers) are added to the eluent as counter-ions to trigger the enantioseparation
241 process. The incorporation of acids in the eluent ensures, inter alia, the protonated state of the
242 quinuclidine nitrogen, which is a fundamental pre-requisite for the ion-pair and ion-exchange processes.

243 A solution consisting of MeOH with 160 mM AcOH was used as initial mobile phase. The use of the highly
244 volatile and more MS compatible FA was avoided for its well-known relatively fast reaction with MeOH
245 yielding FA methyl ester [52,56]. With that mobile phase and CSP 1, only compound 1 was partially
246 separated (Fig. 3A), while base-line resolution was obtained for compounds 2 and 3 (Table 1, entries 1, 8
247 and 10). CSP 2 did not distinguish the enantiomers of 1, while exhibited good enantioseparation capability
248 for the other two analytes (data not shown). The two zwitterionic SOs are diastereomeric in nature and
249 therefore characterized by geometrically and conformationally oriented interaction sites, such as the
250 carbamate group (mostly involved in H-bonding- and dipole-dipole-interactions) and the quinoline moiety
251 (mostly participating in π - π -interactions). These structural elements are together or separately involved in
252 the overall enantiorecognition process. The different chromatographic behaviour exhibited by CSP 1 and
253 CSP 2 can be rationally explained on this basis [47]. CSP 1 was therefore selected for the following

254 optimization steps aimed at improving the performance on compound 1.

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Fig. 3. Chromatograms of compounds 1–3 obtained under PI conditions with CSP 1. Mobile phase conditions can be retrieved from Table 1 (A) cpd 1, entry 1; (B) cpd 1, entry 2; (C) cpd 1, entry 6; (D) cpd 1, entry 8; (E) cpd 2, entry 10; (F) cpd 3, entry 12. The EEO has been reported in the best chromatographic conditions.

Table 1. Chromatographic performances obtained for compounds 1–3 with CSP 1.

Compound	Entry	Mobile phase ^a	Chromatographic parameters					
			k_1	k_2	N_1	N_2	α	R_s
1	1	A	0.74	0.89	4568	4422	1.20	1.37
	2	B	0.96	1.17	4466	4059	1.21	1.59
	3	C	0.83	1.01	3354	2655	1.23	1.32
	4	D	0.91	1.13	4314	4024	1.24	1.74
	5	E	1.45	1.72	4514	4280	1.19	1.73
	6	F	3.73	4.37	6354	5398	1.17	2.44
	7	G	2.95	3.68	873	454	1.25	1.04
	8	H	1.76 (S)	2.18 (R)	3092	2862	1.24	1.97
2	9	A	0.37	0.77	2438	2793	2.06	3.23
	10	H	1.55 (S)	2.23 (R)	1982	2283	1.44	2.72
3	11	A	0.27	0.65	3159	3179	2.39	3.64
	12	H	2.90 (S)	4.12 (R)	1447	1688	1.42	2.69

^a Composition: (a) MeOH with 160 mM AcOH; (b) MeOH with 80 mM AcOH; (c) MeOH/ACN 98/2 (v/v) with 80 mM AcOH; (d) MeOH/ACN 95/5 (v/v) with 80 mM AcOH; (e) MeOH/ACN 90/10 (v/v) with 80 mM AcOH; (f) ACN with 80 mM AcOH; (g) ACN/MeOH 95/5 (v/v) with 80 mM AcOH; (h) ACN/MeOH 95/5 (v/v) with 80 mM FA. The EEO has been reported in the best chromatographic conditions.

Testing different additive concentrations can be a useful tool to adjust column performance, e.g., to slow analysis with improved enantioresolution. When AcOH concentration was reduced from 160 mM (Table 1, entry 1) down to 80 mM (Table 1, entry 2), retention factors significantly increased in accordance to a typical AX process [27,49] and the stoichiometric displacement model [28,51,[56], [57], [58]]. At the same time, the α value remained nearly unchanged, suggesting a negligible effect of counterion on other tentative binding increments mainly responsible for enantioselectivity (namely H-bond and van der Waals/steric interactions). Instead, resolution factors (R_s) increased with the reduction of the eluent ionic strength, as a result of the increased retention of both enantiomers. With the lower AcOH concentration, base-line separation (R_s of 1.59; Table 1, entry 2) (Fig. 3B) of **1** was obtained.

In order to further improve the chromatographic performance, we scrutinized the impact of the combination of a protic and an aprotic eluent component. Accordingly, we continued to use MeOH as a protic solvent (which can suppress H-bonding interactions), this time in combination with a low amount of

281 ACN, which is an aprotic solvent known to support ionic interactions but to interfere with aromatic (π - π -
282 stacking) interactions [59]. In order to optimize the ratio of these two solvents, three different binary
283 eluents were evaluated (Table 1, entries 3–5), while keeping the AcOH concentration fixed at 80 mM. As
284 expected, a clear increase of the retention was observed with increasing concentrations of ACN from 2 up
285 to 10% (v). This behaviour can be explained by considering the reduced solvation power of ACN leading to
286 the enforcement of the electrostatic interactions between the anionic group of the analyte and the
287 protonated quinuclidine moiety of the SO. However, the presence of an aprotic component in the eluent
288 did not significantly enhance the separation properties of the system (Table 1, entries 3–5), thus indicating
289 that H-bonding interactions are important for analyte retention, whilst these seem not to support the chiral
290 recognition process. Conversely, increasing the ACN concentration in the eluent led to an improvement of
291 RS values as a result of the higher retention and enhanced efficiency.

292 Replacing MeOH with ACN, while keeping constant the acid concentration in the eluent, led to the
293 formation of a thinner solvation shell around the charged moieties of both SO and SA, thus amplifying the
294 strength of the electrostatic attractions, with a consequent elongation of the retention time (Table 1,
295 entries 2 and 6) [28,47,49,55,60]. The longer retention times recorded with the aprotic mobile phase can be
296 plausibly attributed to a less effective contribution of the IMCI effect. This outcome is in strict accordance
297 with the recognized ability by ACN to promote stronger intermolecular SO-SA interactions disfavouring
298 intramolecular interactions. A mobile phase consisting of only ACN (plus ionic additives) is usually not
299 recommended as it reduces the solubility of many ionizable solutes. As a consequence, these compounds
300 could partially precipitate on the top of the column and a slow dissolution of this portion of solute takes
301 place only with the passage of large volumes of mobile phase. Severe peak tailing is often promoted in such
302 circumstances. This might explain the stronger peak asymmetry observed with the ACN- (tailing factor
303 measured at 10% of peak height equal to 2.2 and 2.4 for the first and the second eluted enantiomer,
304 respectively; Fig. 3C) over the MeOH-based (tailing factor measured at 10% of peak height equal to 1.7 for
305 both enantiomers; Fig. 3B) eluent. The stronger tailing observed with ACN was accompanied by the
306 remarkable gain in resolution (Table 1, entries 2 and 6), ascribable to the higher retention and improved
307 efficiency. Based on this result, and aimed at reducing the elution time while keeping safe the quality of the
308 enantioseparation process, also in terms of peak shape, 5% (v) of MeOH was added to ACN with AcOH (80
309 mM). With this eluent system an expected reduction of the retention time turned out along with a sensitive
310 deterioration of the kinetic properties of the system (Table 1, entry 7). Following this observation, and
311 aware that the type and concentration of acidic additive can have a profound impact on the retention
312 profile and the quality of the enantioseparation process [27,50,55], AcOH was replaced with FA at the same
313 concentration (80 mM). With this eluent, no problems with mobile phase stability actually exist due to the

314 high content of ACN, making the mobile phase more suited to MS detection systems. In the last eluent,
315 excellent chromatographic performances were obtained for compound 1 (Table 1, entry 8; Fig. 3D), which
316 stimulated us to test these conditions also for the analysis of 2 and 3. Also for these two compounds, very
317 successful results were obtained under the last PI eluent tested (Table 1, entries 10, and 12; Fig. 3E and 3F,
318 respectively for compound 2, and 3).

319 It has been recently documented [59] that FA and AcOH can differently contribute to the overall
320 enantiorecognition event with the CSPs employed in this study. Indeed, these two acids have been
321 demonstrated to participate differently in the formation and composition of the solvation shells of the CSPs
322 and SAs. In spite of their non-chiral nature, the acidic additives display an intimate participation to the
323 chromatographic process as a whole. Indeed, these additives are capable to affect the conformation of the
324 SO and SA, in turn modifying the orientation of the complementary interacting functional groups on SO and
325 SA. The higher retention in the presence of AcOH (Table 1, entry 7) over FA (Table 1, entry 8) can be
326 explained with the ability by the former to produce solvation shells of smaller dimension on CSP 1,
327 ultimately leading to a stronger Coulomb attraction and higher enantiomer retention [59]. On the other
328 side, the highly polar FA is better adsorbed onto the CSP surface, thereby implying a remarkable change of
329 its retention properties.

330 The poor kinetic properties produced by eluent "g" (Table 1, entry 7) can be tentatively explained by a
331 competition between AcOH and MeOH in the solvation layer of the CSP, which can impair the
332 conformational flexibility of the SO unit [59].

333 Very intriguingly, a rather different chromatographic behaviour was found for all of the three compounds
334 when solubilized in ACN and analysed with the optimal [ACN/MeOH 95/5 (v/v) with 80 mM FA] mobile
335 phase. A sensitive reduction of the retention time was always observed, accompanied by very appreciable
336 enantioseparation and enantioresolution factors (Fig. 4). In a previous paper [61], some of us already
337 highlighted that the sample diluent may have a deep effect on the enantioseparation profile with ZWIX-
338 type CSPs under PI elution conditions. Ideally, for Chiralpak ZWIXs used under PI condition, the sample
339 diluent should have low elution strength, and therefore plain ACN should theoretically represent one of the
340 best options. However, further investigations are required to rationalize the effect produced by ACN as
341 sample diluent, which is out of the scope of the present paper.

342

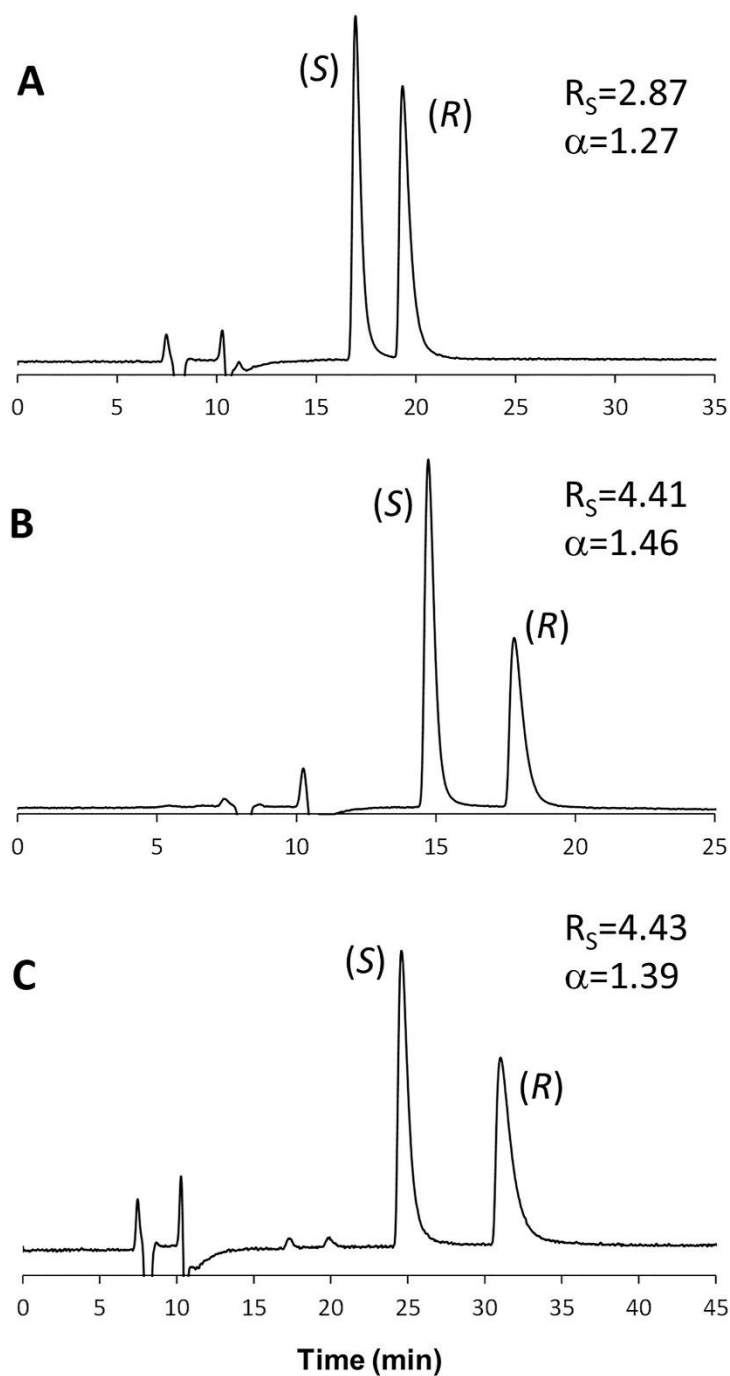


Fig. 4. Chromatograms of compounds (A) 1, (B) 2, (C) 3, obtained with the optimal [ACN/MeOH 95/5 (v/v) with 80 mM FA] mobile phase after the solubilization in plain ACN.

The higher retention of compound 3 under the applied eluent conditions with CSP 1 can be tentatively explained with a stabilizing H-bonding interaction by the phenolic OH group (see section 3.2. for further details).

351 The chromatogram of 2 depicted in Fig. 3E reveals the non-racemic nature of this analyte, although it was
352 purchased as such. The analysis of this compound was performed under the same eluent conditions with
353 CSP 2 in order to verify this issue. Thanks to the pseudo-enantiomeric behaviour often exhibited by the two
354 zwitterion-type CSPs, a reversal of the elution order was observed for compound 2 when analysed with the
355 CSP carrying the quinine scaffold (Fig. S1, Supplementary Material). The scalemic nature of sample 2 was
356 tentatively ascribed to an incorrect storage of the sample powder in our laboratory.

357 Mobile phase "h" was also used in combination with CSP 2. As a result, only compound 2 was almost base-
358 line separated, while a hint of separation turned out for 1 and co-elution for the enantiomers of 3.

359 The (S)<(R) enantiomer elution order (EEO) was established for compounds 1 and 2 injecting a pure
360 enantiomeric standard of known configuration. This was not possible for 3 because not commercially
361 available. For this compound, the same EEO was identified applying a well-established molecular modelling
362 procedure described in detail in sections 2.3 Computational studies, 3.2 Assignment of the absolute
363 configuration through ECD studies and molecular dynamics simulations.

364 **3.2. Assignment of the absolute configuration through ECD studies and molecular dynamics simulations**

365 Due to the lack of pure enantiomeric standards of known stereochemistry for compound 3, an indirect
366 protocol was applied to establish the enantiomer elution order (EEO) under the optimized eluent
367 conditions. Accordingly, based on a well-established protocol [30,31], ECD studies were coupled to ab initio
368 TD-DFT simulations. Experimental ECD spectra of the two enantiomers of compound 3 after their off-line
369 collection with the CSP 1 have been retrieved.

370
371 As readily evident from the ECD spectra displayed in Fig. 5, the two isolated peaks exhibit specular Cotton
372 effects typical of enantiomeric compounds, with the main band centred at around 230 nm. The ECD
373 spectrum of the first eluted peak is well mimicked by the TD-DFT theoretical spectrum of (S)-3 obtained by
374 the use of the wB97X-D functional with the 6-311++G** basis set.

375

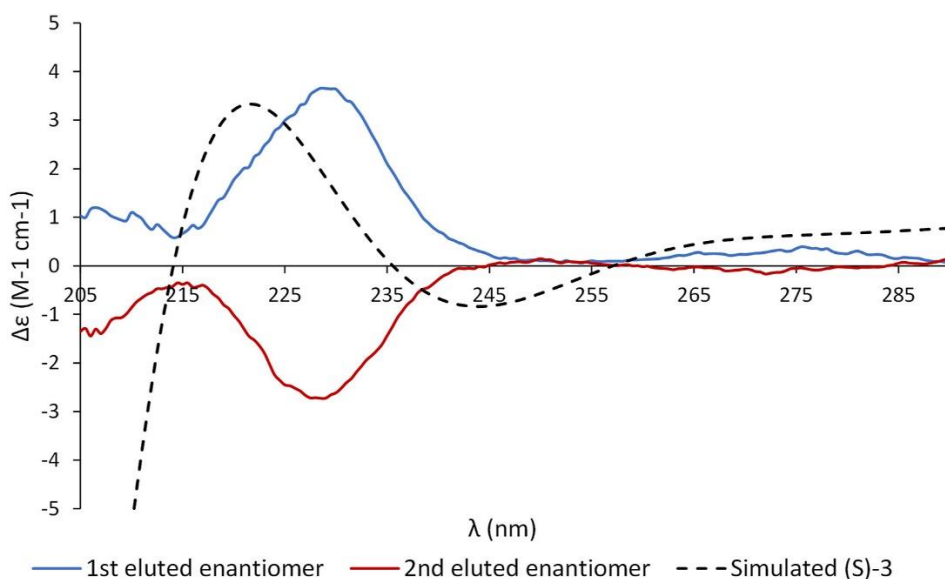


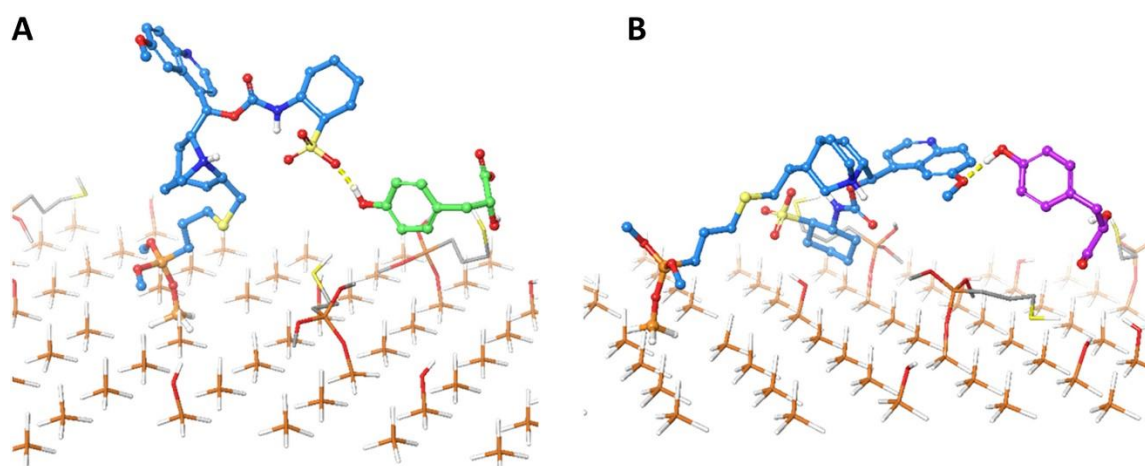
Fig. 5. Experimental ECD spectra of the first- (blue line) and second-eluted (red line) enantiomeric fraction of 3. The theoretical spectrum of the (S)-enantiomer is reported as black dashed-line. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In order to confirm the correctness of the sTD-DFT settings used for the assignment of the EEO of compound 3, the same protocol was also applied to calculate the theoretical ECD spectrum for the enantiomers of compounds 1 and 2 (Fig. S2, Supplementary Material). The ECD spectra of compounds 1 and 2 confirmed that the employed computational method is capable to successfully reproduce the Cotton effects for these two compounds. Moreover, the results obtained for the enantiomers of 1 and 2 are in strict accordance with those produced by other authors [34,62], in turn confirming the quality of our approach any longer.

The main positive Cotton effect at around 230 nm, that is a shared signature of the ECD spectra of both compounds 1 and 2, is also evident in the theoretical spectrum of (S)-3, supporting a safe conclusion about the EEO. Based on this result, the (S)-3 < (R)-3 can be deduced.

With respect to compounds 1 and 2, analyte 3 bears a unique feature that is a para-hydroxy moiety that was found to play a role in the retention mechanism. In order to investigate this aspect and with the additional aim to further support the indirect EEO assignment, a molecular dynamic run has been performed on both (S)- and (R)-3 enantiomers using a simulation box representing the chromatographic environment, as described in details in section 2.3.2. The monitoring of the H-bond established by the hydroxy group during the whole trajectory revealed that (R)-3 enantiomer interacts 48 times with the sulfonic acid moiety of the ZWIX(-) while the (S)-3 molecule only three times engages a hydrogen bond

399 with the methoxy group of the quinoline moiety of the selector (Fig. 6A and B, respectively). The obtained
400 results are in line with the retention behaviour of this compound over the other two examined.
401



402
403
404 **Fig. 6.** Exemplary frames of the H-bond interactions (yellow dashed lines) engaged by the CSP 1 (cyan sticks)
405 and (A) the (S)-3 (green sticks) and (B) (R)-3 (magenta sticks). (For interpretation of the references to colour
406 in this figure legend, the reader is referred to the Web version of this article.)
407

408 3.3. Dried urine spot (DUS) analysis

409 In order to assess the applicability of the optimized enantioselective chromatography conditions in real-life
410 scenarios and on MS-based systems, a proof-of-concept application was carried out by analysing DUS
411 samples spiked with mandelic acid (1) by means of a UHPLC-MS system. Details on this subject are reported
412 in section 2.4.

413 For this proof-of-concept application, it should be considered that, for the occupational exposure to
414 styrene, the Biological Exposure Index (BEI) for mandelic acid is 800 mg g⁻¹ creatinine (corresponding to a
415 concentration of mandelic acid of 8 µg mL⁻¹ urine, considering an average urinary creatinine concentration
416 of 1 mg dL⁻¹), according to the American Congress of Governmental Industrial Hygienists (ACGIH) [63]. On
417 the other hand, physiological concentrations of mandelic acid in urine samples from workers not exposed
418 to styrene were estimated to be in the order of magnitude of 25–250 ng mL⁻¹ [64].

419 In the present study, method sensitivity in terms of lower limit of quantitation (LLOQ) was evaluated based
420 on international bioanalytical method validation guidelines [65] as the lowest concentration of 1 in DUS
421 samples which can be quantified reliably, with an acceptable accuracy and precision, and corresponding to
422 5 times the signal of a blank sample. The LLOQ value estimated for the methodology proposed herein was 5
423 ng racemic 1 per mL urine, thus being well below the BEI limit value but also lower than physiological 1

424 urinary levels in non-exposed workers.

425 In Fig. 3S (Supplementary Materials), the ESI-UHPLC-MS chromatogram obtained from the analysis of a DUS
426 sample spiked with rac-1 is shown.

427 **4. CONCLUSIONS**

428
429 In this paper we have demonstrated that the Cinchona-alkaloid derived zwitterionic CSPs are suitable
430 materials for the enantioresolution of the three aromatic α -hydroxy acids under investigation. The
431 optimized chromatographic method based on the use of a volatile PI mobile phase makes it compatible
432 with MS detection systems. The possibility to perform analysis by means of mass spectrometry opens the
433 way to carry out important in-depth investigations in crucial fields such as biomarker and environmental
434 monitoring and food safety assessment, just to cite some. In this framework, the possibility to combine the
435 developed advanced chromatographic system to automated microsampling protocols and platforms for
436 ultra-high performance liquid chromatography coupled to mass spectrometry analysis can make it easier to
437 set-up high-throughput screening campaigns. This last point can be concretely pursued thanks to the
438 possibility to efficiently scale the developed chromatographic method down to the same commercial
439 columns of smaller dimensions.

440 Based on the encouraging results obtained in the present study, we intend to apply the developed method
441 for the following purposes. The optimized chromatographic method coupled to the described DUS
442 microsampling approach will be implemented (i) for workplace exposure controls aimed at feasibly and
443 reliably monitoring the incidence of styrene and ethylbenzene vaporous in workroom environments
444 (through the analysis of 1); (ii) to further investigate the possible use of compound 2 as a biomarker of both
445 ovarian and cervical cancers; (iii) for in-depth investigations of specific tyrosinemic disorders (through the
446 analysis of 3). Moreover, the developed chromatographic method will be applied to identify alimentary
447 sources rich in (R)-2 being used as food and feed additives to limit microbial contaminations.

448 449 **CRedit authorship contribution statement**

450 Ina Varfaj: Methodology, Investigation, Writing – original draft. Michele Protti: Methodology, Investigation,
451 Writing – original draft. Alessandro Di Michele: Investigation. Alceo Macchioni: Writing – review & editing.
452 Wolfgang Lindner: Writing – review & editing, Supervision. Andrea Carotti: Conceptualization,
453 Methodology, Validation, Formal analysis, Writing – original draft, Data curation. Roccaldo Sardella:
454 Conceptualization, Methodology, Validation, Writing – original draft, Supervision, Writing – review &
455 editing, Project administration. Laura Mercolini: Conceptualization, Methodology, Validation, Writing –
456 original draft, Supervision, Writing – review & editing, Project administration.

457

458 **Declaration of competing interest**

459 The authors declare that they have no known competing financial interests or personal relationships that
460 could have appeared to influence the work reported in this paper.

461

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Supplementary Material

Efficient enantioresolution of aromatic α -hydroxy acids with *Cinchona* alkaloid-based zwitterionic stationary phases and volatile polar-ionic eluents

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Figure 1S. Chromatograms of compound 2 obtained with CSP 2 [CHIRALPAK® ZWIX(+), from Chiral Technology Europe - Strasbourg, France) under the optimal [ACN/MeOH 95/5 (v/v) with 80 mM FA] mobile phase composition and experimental conditions: eluent flow rate, 0.2 mL/min; column temperature, 25 °C; detection wavelength, 254 nm.

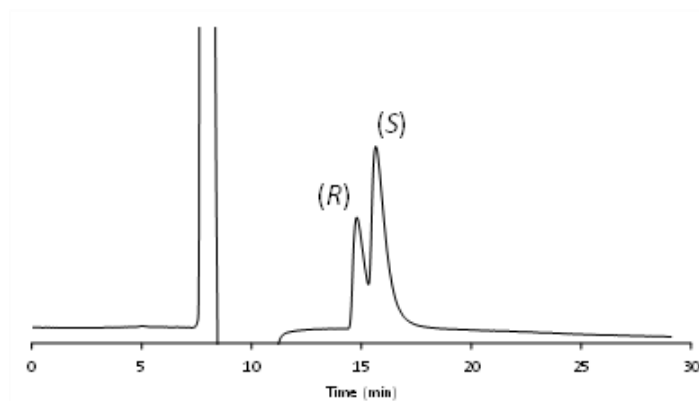


Figure 2S. Experimental ECD spectra of the first- (blue line) and second-eluted (red line) enantiomeric fraction of (A) compound 1 and (B) compound 2. The theoretical spectrum of the (S)-enantiomer is reported as black dashed-line.

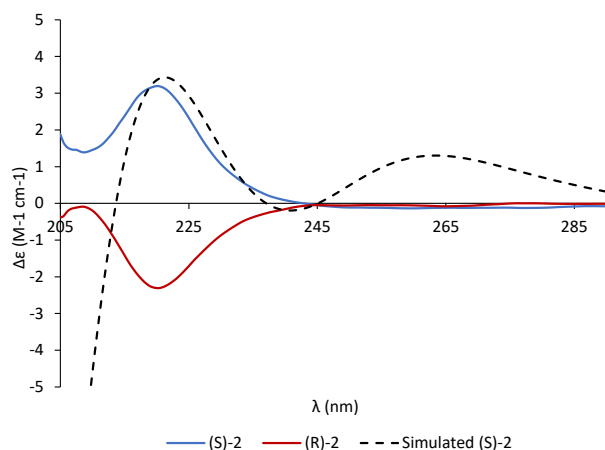
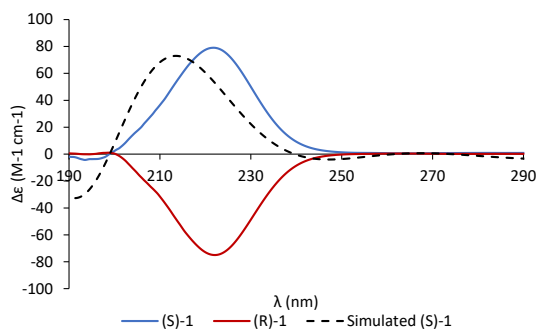


Figure 3S. ESI-UHPLC-MS chromatogram obtained from the analysis of a DUS sample spiked with *rac-1* (on-spot concentration: 100 ng/mL). Extracted ion: m/z 151.17.

