

Simultaneous enantioseparation of nonsteroidal anti-inflammatory drugs by a one-dimensional liquid chromatography technique using a dynamically coated chiral porous silicon pillar array column

Naghdi, Elahe; Fakhari, Ali Reza; Baca, Martyna; De Malsche, Wim

Published in:
Journal of Chromatography A

DOI:
[10.1016/j.chroma.2019.460752](https://doi.org/10.1016/j.chroma.2019.460752)

Publication date:
2020

Document Version:
Accepted author manuscript

[Link to publication](#)

Citation for published version (APA):
Naghdi, E., Fakhari, A. R., Baca, M., & De Malsche, W. (2020). Simultaneous enantioseparation of nonsteroidal anti-inflammatory drugs by a one-dimensional liquid chromatography technique using a dynamically coated chiral porous silicon pillar array column. *Journal of Chromatography A*, 1615, [460752].
<https://doi.org/10.1016/j.chroma.2019.460752>

Copyright

No part of this publication may be reproduced or transmitted in any form, without the prior written permission of the author(s) or other rights holders to whom publication rights have been transferred, unless permitted by a license attached to the publication (a Creative Commons license or other), or unless exceptions to copyright law apply.

Take down policy

If you believe that this document infringes your copyright or other rights, please contact openaccess@vub.be, with details of the nature of the infringement. We will investigate the claim and if justified, we will take the appropriate steps.

1 **Simultaneous enantioseparation of nonsteroidal anti-inflammatory drugs by a one-**
2 **dimensional liquid chromatography technique using a dynamically coated chiral porous**
3 **silicon pillar array column**

4 **Elahe Naghdi^{1,2}, Ali Reza Fakhari¹, Martyna Baca², Wim De Malsche^{2*}**

- 5 1. Faculty of Chemistry, Shahid Beheshti University, G.C., Tehran, I.R. Iran.
6 2. μ Flow group, Department of Chemical Engineering, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels,
7 Belgium.

8 (*) corresponding author

9 Pleinlaan 2, B-1050, Brussels, Belgium

10 Tel.: +32 (0) 2 629 3781, Fax.; +32-2 629 3248, E-mail: Wim.De.Malsche@vub.be

11 **Abstract**

12 The preparation of a highly efficient chiral liquid chromatography (LC) column is explored by
13 dynamically coating a reversed-phase porous silicon pillar array column with hydroxypropyl- β -
14 cyclodextrin (Hp- β -CD) as the chiral selector. Analyte mixtures composed of non-steroidal anti-
15 inflammatory drugs were tested to reveal the enantioseparation potential of the column. The
16 mechanism of chiral discrimination was investigated. The adsorbed Hp- β -CDs on the column
17 surface experience different interaction with enantiomers. The chiral stationary phase showed
18 satisfying stability and could be easily restored by recovering the selector with sufficient flushing
19 and repeating the loading procedure. The peak capacity of the column was evaluated, and it was
20 found high enough to separate three enantiomer couples using a one-dimensional LC technique.

21 **Keywords:** Chiral; Dynamic coating; One-dimensional liquid chromatography; Non-steroidal
22 anti-inflammatory drugs; Porous silicon pillar array column.

23 1. Introduction

24 Chiral separation has become an important topic, not only for the analytical determination of
25 enantiomeric purity, but also for the isolation of enantiomers. As a result, the demand for
26 stereoselective separation techniques and analytical assays to evaluate the enantiomeric purity of
27 chiral compounds has increased. The relevance of enantioseparation has particularly increased in
28 the pharmaceutical industry and the development of various methods for analytical and
29 preparative chiral separations is therefore recognized as a critical point in pharmaceutical
30 research. This is related to the fact that the human body, with its numerous homochiral
31 compounds such as proteins and amino acids, operates as the chiral environment. It therefore
32 responds differently to each of the racemic drugs enantiomers and enantiomers display different
33 biological activities such as metabolism, toxicology, pharmacokinetics, and so on [1,2].
34 Nonsteroidal anti-inflammatory drugs (NSAIDs) play an important role in modern therapy. Since
35 most NSAIDs are chiral and each enantiomer shows different biological and therapeutic
36 activities, the enantioselective separation of this main drug family is important [3-5].

37 To achieve appropriate NSAIDs enantioresolution of individual chiral pairs, several liquid
38 chromatography (LC) methods have been proposed. While LC methods using polysaccharide-
39 based chiral stationary phase (CSP) are most popular [6-8], some methods using various chiral
40 selectors such as vancomycin [9] and hydroxypropyl- β -cyclodextrin (Hp- β -CD) [10, 11] as the
41 chiral mobile phase additive have been developed as well.

42 In general, the simultaneous chiral separation of analytes encounters several difficulties. The
43 achievement of a high peak capacity is a good measure of the general performance of the
44 column. While it is not common to require the separation of large amounts of enantiomers, the
45 need for high efficiency is very relevant. High efficiency is essential when e.g. aiming at the
46 (rapid) detection of low quantities of enantiomeric impurities. To the best of our knowledge there
47 are no successful one-dimensional (1D) approaches described in literature to separate multiple
48 pairs of NSAIDs.

49 However, chiral chemically bonded stationary phases may be perceived as the ideal stationary
50 phase format, since they give more stable retention times. Dynamic coating of non-chiral LC
51 columns with a chiral selector represents another viable approach. The latter approach is a
52 simple, reversible and inexpensive way to prepare CSPs. Change of the selector to another one is
53 straightforward if a suitable washing procedure is employed and followed by coating with a new
54 selector.

55 Advantages and drawbacks of chemical and dynamic coated CSPs are discussed in literature. For
56 instance, chemically and dynamically bonded CSPs were tested for enantioseparation of amino
57 acids, α -hydroxy acids, and dipeptides. The chemically bonded CSP was found to be superior to
58 the dynamically coated CSP in terms of enantioseparation of amino acids and dipeptides.
59 However, α -hydroxy acids were better resolved on the dynamically coated phase [12]. Several

60 authors have described the successful application of dynamically coated LC phase using different
61 types of chiral selectors on a monolithic support or on packed columns [13-15].

62 To further increase the attainable peak capacity and column efficiency, pillar array columns
63 (PACs) have been introduced as an alternative column format to packed bed and monoliths. The
64 order and design freedom of the pillar bed makes the PAC unique and offers several advantages
65 compared to conventional columns. The eddy diffusion (the A term in the van Deemter equation)
66 is negligible in this column due to homogeneous flow paths in the separation bed. In addition, the
67 much lower back pressure of PACs allows for the application of very long columns and
68 concomitantly very high plate counts [16, 17]. In terms of loadability, porous PACs are preferred
69 for most applications in LC [18], but also non-porous columns have been used successfully by
70 several groups to perform a wide range of applications [19-22] that can even benefit from the
71 non-porous nature of the column.

72 Ketoprofen, naproxen and ibuprofen are frequently used profens. While the (S)-naproxen
73 enantiomer binds to the cyclooxygenase enzyme to decrease prostaglandin production at the site
74 of injury for decreasing pain, (R)-naproxen isomer does not inhibit inflammation properties and
75 is furthermore toxic to the liver [23]. Also, (S)-ibuprofen is the only active form and (R)-
76 ibuprofen even causes side effects [24]. The (R) and (S)-ketoprofen enantiomers produce
77 different therapeutic actions. The (S)-enantiomer is used to relieve pain, while the(R)-enantiomer
78 is applied to prevent periodontal disease. In addition, the (R)-enantiomer of the ketoprofen has
79 been reported to convert into its antipode in human and animals' bodies [25]. Therefore, optical
80 purity of these drugs is a critical point and their enantioseparation is necessary.

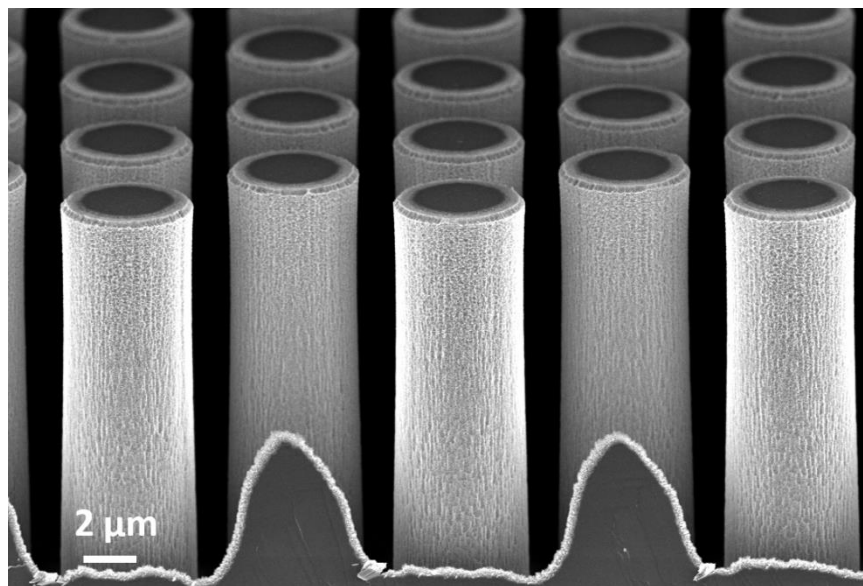
81 In the present study, we prepared a dynamically coated reversed-phase -porous silicon pillar
82 array column (RP-PAC) using Hp- β -CD as chiral selector and used it to simultaneously
83 enantioseparate selected NSAIDs (naproxen, ketoprofen, ibuprofen) and to get some insight in
84 the separation mechanism. The main goal of this work was to evaluate the chiral separation
85 potential of the pillar array column.

86 **2. Experimental**

87 **2.1. Apparatus**

88 A nano-LC (UltiMate 3000, Thermo Fischer Scientific, Massachusetts, USA) equipped with a
89 UV-detector system and column oven was used for (dynamic) chiral coating and
90 chromatography evaluation. The UV system was set at wavelength equal to 214, 254 and 230
91 nm. The sample injection was performed using an automated 4 nl valve system injections (C4N-
92 4004) obtained from Valco (Schenkon, Switzerland). RP-PACs were acquired from
93 PharmaFluidics (Zwijnaarde, Belgium). The chips contain channels with a length of 2 m and a
94 width of 315 μ m. The channels are filled with cylindrical pillars (5 μ m diameter, 18 μ m deep,
95 2.5 μ m interpillar distance, 59% bed channel porosity, 450 nm porous layer thickness) and were

96 fabricated in the same way as already described in detail by Callewaert et al. [26]. A typical
97 SEM image of the pillar array is depicted in Fig .1.



98

99

Fig. 1. SEM picture of the pillar array

100 A pH-meter (WTWinoLab, Weilheim, Germany) was used for pH adjustments of buffer
101 solutions.

102 2.2. Material

103 Ketoprofen, ketorolac, indoprofen, fenoprofen, suprofen, ibuprofen, S-ibuprofen, flurbiprofen,
104 benzoic acid, naphthalene, and uracil standard were purchased from Sigma-Aldrich (St. Louis,
105 MO, USA). Naproxen reference standard was kindly provided by Temad faculty (Tehran, Iran).
106 The chemical structures of solutes have been summarized in Fig. 1S. Some chemical and
107 physical properties of tested solutes are collected in table 1. Hp-β-CD with the substitution
108 degree of 3.5-5.5 was obtained from Cyclodextrin-Shop (Tilburg, Netherlands). Purified water
109 from a Milli-Q reagent water system (Millipore, Bedford, MA, USA) was used to prepare the
110 buffer and reagent solutions. The analytical grade H₃PO₄, NaH₂PO₄, NaOH were purchased from
111 Merck (Darmstadt, Germany). LC/MS grade acetonitrile, ethanol and methanol were also
112 purchased from Biosolve (Valkenswaard, Netherland).

113 2.3. Preparation of stock and standard Solution

114 The standard stock solution of the chemicals was prepared in organic solvent (methanol, ethanol,
115 acetonitrile) at a concentration of 1000 μg mL⁻¹. Standard working solutions were diluted with
116 water/organic solvent (50/50) to get adequate concentration.

117 2.4. Chromatographic conditions

118 All separations were carried out at 30 °C at a flow rate of 0.25 μL/min. LC/MS-grade methanol,
119 acetonitrile, ethanol, deionized water were used as components of the mobile phase.

120 For the chromatographic evaluation of chiral and non- chiral columns, peak capacity, retention
121 coefficient, and resolution were calculated using following equations:

122 Peak capacity as the number of peaks that can fit into a chromatogram between the first and the
123 'last peaks, can calculate based on retention time (n_r):

$$124 \quad (n_r) = (T_R - T_0) / W_b \quad (1)$$

125 Also, peak capacity based on gradient time (n_G) can calculate as:

$$126 \quad (n_G) = 1 + (T_G / W_b) \quad (2)$$

127 The retention coefficient and resolution were calculated by:

$$128 \quad \text{Retention coefficient} = (T_R - T_0) / T_0 \quad (3)$$

$$129 \quad \text{Resolution} = 1.18 (T_{R,2} - T_{R,1}) / (W_{2,50\%} - W_{1,50\%}) \quad (4)$$

130 with T_R , T_0 , T_G , $T_{R,2}$, $T_{R,1}$, $W_{2,50\%}$, $W_{1,50\%}$ and W_b the retention time of a retained compound, the
131 retention time of a non- retained compound, the gradient time, the retention time of more
132 retained compound, the retention time of less retained compound, the peak width of more
133 retained compound at 50% height, the peak width of less retained compound at 50% height and
134 the baseline peak width, respectively.

135 2.5. Chiral selector immobilization via physical absorption

136 The dynamic chiral coating process consists of the attachment of the Hp-β-CD chiral selector on
137 a PAC column coated with C_{18} groups through non-covalent coating. To immobilize the selector,
138 the 10mM Hp-β-CD was dissolved in phosphate buffer (25 mM, pH 4) and pumped through the
139 column [15,28] at a column temperature that was fixed at 30 °C. The chiral selector solution was
140 recycled through the column at a constant flow rate of 0.25 μl/min Changes in the absorbance of
141 the eluent were followed by UV detection. Equilibrium in the coating process was indicated by
142 an initial abrupt in the UV baseline and then stabilizes to the new baseline [29], which occurred
143 after 15 h. In practice the column was flushed for 28h with feed solution, which is equivalent to
144 50 column volumes.

145 Thereafter, the column was washed with water and organic solvent

146 **Table 1** Some properties of the tested solutes [27].

Analyte	Molar mass (g/mol)	Log D	pK _a	Van der Waals volume (Å ³)
Ketorolac	255.27	2.28	3.84	223.07

Indoprofen	281.31	2.86	3.74	250.7
Suprofen	260.31	3.53	4.01	225.21
Ketoprofen	254.28	3.61	3.88	233.86
Naproxen	230.23	2.99	4.19	213.06
Fenoprofen	242.27	3.65	3.96	223.4
Flurbiprofen	244.26	3.94	4.42	219.19
Ibuprofen	206.28	3.84	4.85	211.80
Naphthalene	128.27	2.96	-	125.17
Benzoic acid	122.12	1.63	4.08	109.73

147 **3. Results and discussion**

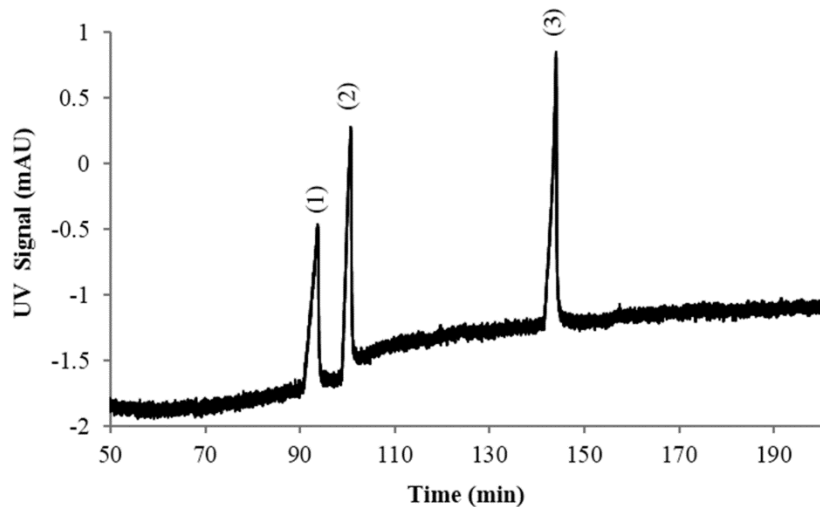
148 Before performing the dynamic coating, first two sample sets of the NSAID family were
149 separated on the RP-PAC . In a next step, a chiral column was simply prepared by pumping a
150 solution of Hp- β -CD through the column. This column was then used to simultaneously separate
151 ketoprofen, naproxen and ibuprofen enantiomers.

152 3.1. Separation of NSAIDs on the RP-PAC

153 Two different sample sets containing 1. naproxen, ibuprofen, ketoprofen and 2. ketorolac,
154 suprofen, indoprofen, ketoprofen, naproxen, ibuprofen, fenoprofen, flurbiprofen were injected
155 and separated using acetonitrile/water in the gradient elution as the mobile phase. The obtained
156 chromatograms are shown in Fig. 2 and Fig. 3. The chromatographic parameters are summarized
157 in table 2 and table 3.

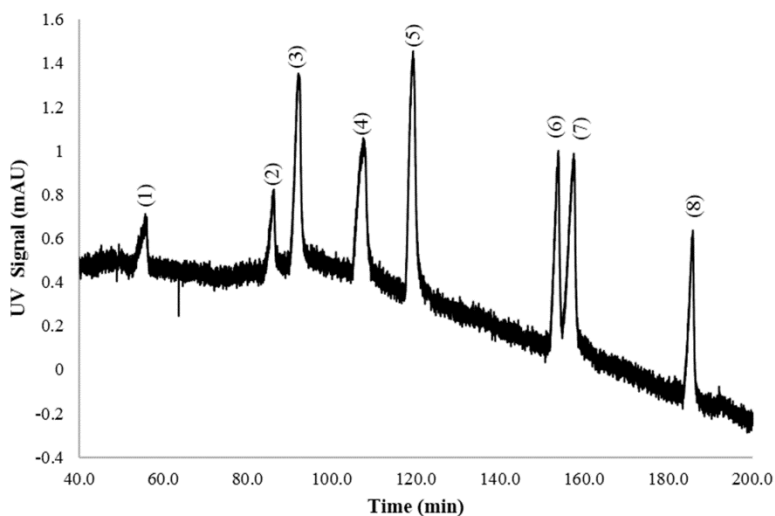
158

159



160

161 **Fig. 2.** Separation of some profens on the studied RP-PAC. Components: 1. ketoprofen 2. naproxen 3. Ibuprofen.
 162 Chromatographic conditions: column length 200cm. mobile phase: gradient elution of acetonitrile–water (15/85–
 163 80/20 v/v), flow rate: 0.25 μ l/min, injection volume: 4 nL, column temperature: 30°C and detection: UV at 214 nm.



164

165 **Fig. 3.** Separation of some profens. Components: 1. ketorolac, 2. indoprofen, 3. suprofen, 4. ketoprofen, 5.
 166 naproxen, 6. fenoprofen, 7. flurbiprofen, 8. ibuprofen. Chromatographic conditions: column: 200 cm RP-PAC.
 167 mobile phase: gradient elution of acetonitrile–water (15/85–40/60 v/v), flow rate: 0.25 μ l/min, injection volume: 4
 168 nL, column temperature: 30 °C and detection: UV at 214 nm.

169 **Table 2.** Chromatographic parameters for ketoprofen, naproxen and ibuprofen using the RP-PAC. Experimental
 170 conditions are the same as in Fig. 2.

Analyte	Retention time (min)	Retention coefficient	Width (50%)	Resolution
Ketoprofen	93.70	1.94	1.59	3.04
Naproxen	100.68	2.16	1.12	25.27
Ibuprofen	144.04	3.52	0.91	/

171 **Table 3.** Chromatographic parameters for eight profens on the RP-PAC. Experimental conditions are the same as in
172 Fig. 3.

Analyte	Retention time (min)	Retention coefficient	Width (50%)	Resolution
Ketorolac	55.68	0.68	1.11	16.98
Indoprofen	86.29	1.61	1.01	2.79
Suprofen	92.09	1.78	1.44	5.10
Ketoprofen	107.49	2.25	2.12	3.70
Naproxen	119.38	2.61	1.67	14.19
Fenoprofen	153.92	3.65	1.20	1.53
Flurbiprofen	157.59	3.76	1.62	12.03
Ibuprofen	185.90	4.62	1.16	/

173 3.1.1. Separation mechanism

174 To gain more understanding in the separation mechanism, first the elution order of eight
175 separated profens is investigated. The main interaction in reverse phase columns is hydrophobic
176 interaction. The octanol/water distribution coefficient (D), defined as the ratio of the molar
177 concentration of the solute in octanol or water at a specified temperature, is usually used to
178 represent molecular hydrophobicity. According to the chemicals' log D the analytes should elute
179 in this order: 1. ketorolac (2.28), 2. indoprofen (2.86) 3. naproxen (2.99) 4. suprofen (3.53) 5.
180 ketoprofen (3.61) 6. fenoprofen (3.65), 7. ibuprofen (3.84), 8. flurbiprofen (3.95). However, the
181 obtained results show another elution order: 1. ketorolac, 2. indoprofen, 3. suprofen, 4.
182 ketoprofen, 5. naproxen, 6. fenoprofen, 7. flurbiprofen, 8. ibuprofen.

183 This result indicates that hydrophobic partitioning interaction does not control the separation
184 process solely and that at least another retention mechanism plays a role. The steric hindrance of
185 solute molecules can be considered as an influencing factor in retention of the analytes. It is
186 suspected that steric hindrance made interactions less strong and that the order of elution was
187 therefore, to some extent, scrambled. For example, ibuprofen has a small volume and is expected
188 to experience less steric hindrance, resulting in high retention in comparison to e.g. flurbiprofen,
189 which is more hydrophobic but voluminous.

190 The elution order of the separated eight profens based on the size of compounds merely can be
191 estimated according to their volume (table 1): 1. indoprofen (250.7 Å³) 2. ketoprofen (233.86

192 A^{o3}) 3. suprofen (225.21 A^{o3}) 4. fenoprofen (223.4 A^{o3}) 5. ketorolac (223.07 A^{o3}) 6. flurbiprofen
193 (219.19 A^{o3}) 7. naproxen (213.06 A^{o3}) 8. ibuprofen (211.80 A^{o3}).

194 When the elution order is interpreted as a function of hydrophobicity and the volume of
195 chemicals it is observed that the separation remains mainly controlled by hydrophobic
196 interaction, however, the volume of molecules is clearly important too. A good example is
197 naproxen, its retention is the combination of both effects.

198 To get more insight in the separation order and mechanism, another sample set consisting of
199 benzoic acid, ketoprofen, naproxen, naphthalene and ibuprofen was injected.

200 By considering log D, the solutes should elute according to the following order: 1. benzoic acid,
201 2. ketoprofen 3. naproxen 4. naphthalene 5. ibuprofen. However, a different order was observed:
202 1. ketoprofen, 2. naproxen, 3. benzoic acid, 4. ibuprofen, 5. naphthalene.

203 To explain this order, we again considered the chemicals' volume. According to this parameter
204 and size-based separation assumption, the elution order should be: 1. ketoprofen, 2. naproxen, 3.
205 ibuprofen, 4. naphthalene, 5. benzoic acid. As can be seen, both factors (hydrophobicity and size)
206 are effective on the solutes retention and real elution order is a combination of the order
207 according size and hydrophobicity.

208 The separation mechanism on the RP-PAC for small molecules appears to be a combination of
209 partition and steric hindrance effects, with the hydrophobic interaction the most effective. A
210 similar conclusion has been reported for the separation of some NSAIDs on a C₁₈ packed column
211 [30].

212 3.2. Evaluation of the Separation Potential of a Dynamically Coated chiral RP-PAC

213 A simple and straightforward procedure was used to prepare the chiral stationary phase. A 25
214 mM phosphate buffer at pH 4.0 containing 10mM HP-β-CD was pumped through the column
215 for 28h.

216 Hp-β-CD is a suitable and commonly used chiral selector for enantioseparation of NSAIDs in
217 different analytical methods due to its ability to form inclusion complexes with analytes in its
218 hydrophobic cavity [10,11,31,32].

219 3.2.1. Effect of HP-β-CD concentration

220 The influence of the concentration of HP-β-CD on the resolution of the selected NSAIDs
221 enantiomers and performance of the chiral column was evaluated by adding different
222 concentrations of HP-β-CD (2, 5, 10 and 20 mM) to the coating solution. At concentrations
223 below 10 mM not enough chiral sites seemed to be available to achieve the enantioseparation of
224 selected profens. The coating solutions with higher concentrations (10 & 20 mM) modified the

225 surface column successfully as well as indicated by a successful chiral separation of analytes.
226 10mM was selected as the optimum concentration.

227 3.2.2. Suitable mobile phase

228 To obtain the best enantioselectivity the organic solvents methanol, acetonitrile and ethanol as
229 organic modifier in aqueous mobile phase were evaluated (water/methanol- water/ethanol-
230 water/acetonitrile). No considerable difference between selected organic solvents was observed.
231 The resolution was almost unaltered when shifting from methanol to ethanol, while applying
232 acetonitrile decreased the resolution slightly (around 0.05). A similar influence was observed for
233 the retention time. Methanol and ethanol caused an increase in retention times of around 0.5 min
234 in comparison to acetonitrile. In addition, the application of TFA (0.1%) in organic or/ and
235 aqueous solvents was investigated. As only a minor decrease in chromatographic parameters
236 were observed (differences in retention time and resolution below 1 min and 0.2, respectively),
237 all experiments were performed with the same mobile phase (water/acetonitrile)

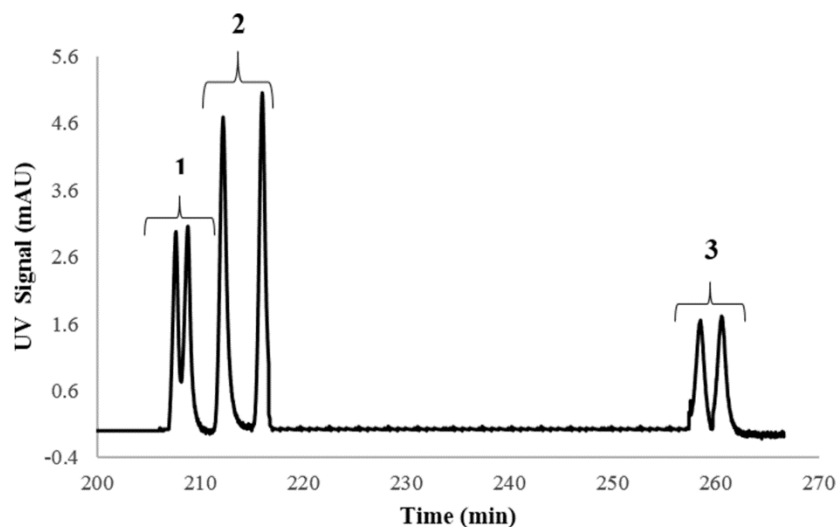
238 3.2.3. Chiral separation of some NSAIDs on a dynamically coated RP-PAC

239 After dynamic chiral coating of the column, the potential of the column was evaluated for
240 enantioseparation of the sample set containing ketoprofen, naproxen and ibuprofen, individually
241 or simultaneously.

242 Hence, determination and examination of chiral drugs s by single-run analysis needs sufficient
243 chiral and achiral sites on the stationary phase surface to enable adequate resolution between
244 enantiomers and other analytes. The simultaneous chiral separation of a sample set containing
245 ketoprofen, naproxen and ibuprofen was set as a key objective in this study.

246 The stability of column was evaluated. he racemic standard of naproxen was injected three times
247 during 6000 min running time and the relative standard deviation (RSD) of retention time of
248 each enantiomer data was less than 1.88 and 1.32%. This result showed that the dynamic coating
249 is adequately stable.

250 Fig. 4. shows the successful application of a dynamically coated column in enantioselectivity of
251 ketoprofen, naproxen and ibuprofen simultaneously. The accumulated chromatographic
252 parameters in table 4 indicate that retention of all enantiomers on the column is sufficient
253 (retention coefficient > 5 in all cases).



254

255 **Fig. 4.** Simultaneous enantioseparation of profens on a dynamical chiral coated RP-PAC with Hp- β -CD.

256 Components: 1. ketoprofen enantiomers (R & S) 2. naproxen enantiomers (R & S) 3. ibuprofen enantiomers (R &
 257 S). Chromatographic conditions: column: 200 cm dynamically chiral coated RP-PAC. mobile phase: gradient elution
 258 of acetonitrile–water (15/85–80/20 v/v), flow-rate: 0.25 μ l/min, injection volume: 4 μ L, column temperature: 30 $^{\circ}$ C
 259 and detection: UV at 214 nm

260 **Table 4.** Chromatographic parameters for ketoprofen, naproxen and ibuprofen enantiomers using the dynamically
 261 chiral coated RP-PAC. Experimental conditions are the same as in Fig. 4.

Analyte	Retention time (min)	Retention coefficient	Width (50%)	Resolution
Ketoprofen	207,63	5,27	0,54	1,27
Ketoprofen	208,79	5,31	0,54	3,28
Naproxen	212,21	5,41	0,68	3,37
Naproxen	216,01	5,52	0,65	34,01
Ibuprofen (R)	258,53	6,81	0,83	1,45
Ibuprofen (S)	260,57	6,87	0,83	/

262 Re-injection of the separated enantiomers of ibuprofen indicated that the second peak is related
 263 to (S)-enantiomer and that the (R)-enantiomer has less interaction with the chiral column. This
 264 order of elution has been observed in chiral separation of ibuprofen by β -cyclodextrin based
 265 [33,34] other chiral HPLC columns e.g. ((R)-1-naphthylglycine and 3,5-dinitrobenzoic acid
 266 amide) [35, 36].

267 The LOQs of each enantiomer (defined as the analyte concentration producing a signal that is ten
 268 times greater than the noise signal [37]) for naproxen, ketoprofen and ibuprofen are 0.18 ppm,
 269 0.55 ppm and 1.98 ppm, respectively. These different LOQs are related to the nature of the
 270 compound, e.g. ibuprofen is less efficiently detected with UV in comparison to naproxen. When
 271 injecting e.g. 50 ppm of sample, it can be extrapolated that the analysis of fraction values down

272 to 0.5, 1 and 4% enantiomeric impurity of naproxen, ketoprofen and ibuprofen would be possible
273 with the present setup

274 3.2.4. Chiral separation mechanism

275 After evaluation of chiral discrimination of some NSAIDs in the column, the separation
276 mechanism using chromatographic parameters was investigated. During flushing of the column
277 with buffer that contains chiral selector, Hp- β -CD, chiral selector can adsorb on the surface of
278 the stationary phase. The adsorbed chiral selector molecules on the surface make different
279 interaction with enantiomers and formed diastereomers. The host-guest inclusion complexation
280 mainly controls the chiral separation of analytes on the dynamic chiral coating column [38].

281 Adsorption of the chiral selector was suspected due to the following observation. In our
282 procedure, first the column was flushed with buffer phosphate containing Hp- β -CD for 28h and
283 then mobile phase was switched to water/organic solvent (without chiral selector). In this
284 situation enantioseparation of analytes was observed. If Hp- β -CD would not be adsorbed on the
285 surface of stationary phase, the chiral separation would not be possible. This separation indicates
286 that the chiral selector is most likely adsorbed on the stationary phase surface. Flushing the
287 column with the mobile phase for a long time (more than 100 h) leads to a decrease of the
288 resolution between two enantiomers of analytes (enantioselectivity), which is a result of removing
289 chiral selector from the surface. Also, the retention time of analytes started to decrease
290 substantially after 100h due to desorption of chiral selector from column surface. Flushing with
291 6 column volumes (after 100h) led to a decrease 2.6 % retention time of naproxen enantiomers.
292 Continued flush up to 18 column volumes further decreased retention times to 5.5 %.

293 Investigation of the analyte retention times provides valuable data and supports the proposed
294 mechanism. The retention time of the analytes at the dynamically chiral coated column is higher
295 than with the native reversed phase column (compare table 2 and 4), related to the fact that more
296 active sites in the column (adsorbed Hp- β -CD) interact with the analytes.

297 3.3. Comparison of RP-PAC with and without chiral coating

298 Comparison of chromatographic parameters of analytes using the same chromatography
299 conditions before and after application of the chiral coating shows the effect the chiral selector.
300 Four relevant factors were used to compare between two columns: 1. peak capacity 2. retention
301 time 3. retention coefficient 4. resolution between two analytes.

302 The peak capacities before and after chiral dynamic coating was calculated by eq. 1 & 2 and
303 collected in table 5. The peak capacity of the column decreases after dynamically chiral coating,
304 however, both columns provided high peak capacities, related to the inherent high plate count of
305 the column [17]. The peak capacity is affected by the packing structure and quality, which was
306 not altered during the present study. But it is also determined by the chemical nature of the
307 interaction between analytes and stationary phase, there with affecting dispersion and the elution

308 window. The latter parameter has changed when switching from (single mode) reversed phase
309 mode to multimode interaction in the dynamically chiral bonded column.

310 **Table 5.** Peak capacity for profens on RP-PAC before and after applying the chiral coating

Coating type in column	n _r	n _G
C ₁₈	345	582
C ₁₈ + Hp-β-CD	162	179

311 Inspection of table 2 and 4 parameters reveals that the column shows a higher retention time and
312 retention coefficient after application of the dynamic chiral coating, due to the adsorbed chiral
313 selector.

314 The observed resolutions reveal an interesting observation. The resolution between the second
315 enantiomer of ketoprofen and the first enantiomer of naproxen was 3.28, while the resolution
316 between ketoprofen and naproxen on the non-chiral column was 3.04. Also, the resolution
317 between the second enantiomer of naproxen and the first enantiomer of ibuprofen was 34.01,
318 while the resolution between ibuprofen and naproxen on non-chiral column was 25.27. These
319 results indicate that chiral interactions can improve the resolution of two different compounds.

320 3.4. Comparison of the developed method with other methods

321 The current method was compared with another method, which is one of the scarce methods
322 available in literature for the simultaneous chiral separation of profens. In these studies,
323 LC/MS/MS coupled instruments were used with (R)-1-naphthylglycine 3,5-dinitrobenzoic acid
324 [36] and amylose tris(3,5-dimethylphenylcarbamate) [39] as the packed separation column to
325 simultaneously achieve enantioselectivity of the selected NSAIDs. The dynamically coated RP-
326 PAC column approach from the present study provides sufficiently high peak capacities to allow
327 for enantioseparation of the selected profens using a single separation operation.

328 The prepared chiral column shows a similar, even in some case better, separation capacity and
329 enantioresolution as compared to the optimized commercial column. Appropriate
330 enantioresolution for naproxen and ibuprofen using polysaccharide- base column was achieved,
331 with a resolution of 1.0 and 1.1, respectively [39]. The obtained resolutions by another stationary
332 phase for naproxen and ibuprofen were 2.8 and 1.4 [36]. The dynamically coated RP-PAC
333 showed better resolutions (3.37 and 1.45 for naproxen and ibuprofen enantiomers, respectively).

334 The peak capacity of current chiral column was compared with a variety of commercial chiral
335 stationary phases used for enantioseparation of different chemicals (table 6).

336 The peak capacity of these cases was less than 25, while the new chiral column provides a value
337 of more than 160.

338

339 **Table 6.** Comparison of the peak capacity of commercial columns with proposed chiral column

Stationary phase	Peak capacity	Ref.
Amylose tris(3,5-dimethylphenylcarbamate)	8	39
(R)-1-Naphthylglycine 3,5-dinitrobenzoic acid	10	36
Amylose tris-((S)- α -methylbenzylcarbamate)	22	40
Ovomucoid	10	41

340 **4. Conclusion**

341 A temporary and stable chiral column was prepared via a dynamic coating approach on a
342 reverse- phase pillar array column. Some profens were separated, individually and
343 simultaneously. To better understand the chiral separation mechanism, the separation of some
344 profens before applying a chiral coating was investigated and possible mechanisms have been
345 discussed. Our data show that the developed method is flexible and simple over the use of chiral
346 stationary phases and shows good chromatography properties such as retention coefficient,
347 enantioresolution and peak capacity, which makes the simultaneous chiral separation of three
348 and more diastereomer compounds possible by a one-dimensional technique.

349 **Acknowledgments**

350 The authors gratefully acknowledge support from Vrije Universiteit Brussel and Shahid Beheshti
351 University.

352 **Conflicts of interest**

353 Wim De Malsche is co-founder of PharmaFluidics and has shares of the company.

354 **References**

- 355 [1] Y. Zhu, W. Liu, S. Qi, H. Wang, Y. Wang, G. Deng, Y. Zhang, S. Li, C. Ma, Y.
356 Wang, X. Cheng, C. Wang, Stereoselective glucuronidation metabolism,
357 pharmacokinetics, anti-amnesic pharmacodynamics, and toxic properties of vasicine
358 enantiomers in vitro and in vivo, *Eur. J. Pharm. Sci.* 123 (2018) 459-474.
359 <https://doi.org/10.1016/j.ejps.2018.07.058>
360
- 361 [2] Q. Shen, L. Wang, H. Zhou, H-D. Jiang, L-S Yu, S. Zeng, Stereoselective binding of
362 chiral drugs to plasma proteins, *Acta Pharmacol. Sin.* 34 (2013) 998–1006
- 363 [3] H. Ikuta, A. Kawase, M. Iwaki, Stereoselective Pharmacokinetics and Chiral
364 Inversion of Ibuprofen in Adjuvant-induced Arthritic Rats, *Drug Metab. Dispos.* 45
365 (2017) 316–324. <https://doi.org/10.1124/dmd.116.073239>
366
- 367 [4] J. Siodmiak, T. Siodmiak, A. Tarczykowska, K. Czirson, J. Dulęba, M.P. Marszall,
368 Metabolic chiral inversion of 2-arylpropionic acid derivatives (profens), *Med. Res. J.* 2
369 (2017) 1–5. <https://doi.org/10.5603/MRJ.2017.0001>
- 370 [5] E.J. Quann, F. Khwaja, K.H. Zavitz, D. Djakiew, The Aryl Propionic Acid R-
371 Flurbiprofen Selectively Induces p75^{NTR}-Dependent Decreased Survival of Prostate
372 Tumor Cells, *Cancer Res.* 67 (2007) 3254-3262. [https://doi.org/10.1158/0008-
373 5472.CAN-06-3657](https://doi.org/10.1158/0008-5472.CAN-06-3657)
- 374 [6] S. Magiera, A. Piwowarczyk, A. Węgrzyn, A study of the enantiospecific degradation
375 of ibuprofen in model aqueous samples using LLME-HPLC-DAD, *Anal. Methods* 8
376 (2016) 7789-7799. <https://doi.org/10.1039/C6AY02670B>
377
- 378
- 379 [7] G. D’Orazio, C. Fanali, S. Fanali, A. Gentili, B. Chankvetadze, Comparative study on
380 enantiomer resolving ability of amylose tris(3-chloro-5-methylphenylcarbamate)
381 covalently immobilized onto silica in nano-liquid chromatography and capillary
382 electrochromatography, *J. Chromatogr. A* (2019) in press.
- 383 [8] L. Zhang, W. Yu, Y. Rong, X. Guo, J. Ye, Z. Shen, S. Zeng, Enantiomeric separation
384 of 2-arylpropionic acid nonsteroidal anti-inflammatory drugs and β -blockers by RP-
385 HPLC using an amylose chiral stationary phase for the enantioselective skin permeation
386 study, *Anal. Methods* 6 (2014) 6058-6065. <https://doi.org/10.1039/C4AY00579A>
387
- 388 [9] D. Gherdaoui, H. Bekdouche, S. Zerkout, · R. Fegas, ·M. Righezza, Separation of
389 ketoprofen on an achiral NH₂ column by HPLC using vancomycin as chiral mobile phase
390 additive, *J. Iran. Chem. Soc.* 13 (2016) 2319-2323. [https://doi.org/ 10.1007/s13738-016-
391 0951-6](https://doi.org/10.1007/s13738-016-0951-6)
- 392 [10] A. Rocco, A. Maruska, S. Fanali, Cyclodextrins as a chiral mobile phase additive in
393 nano-liquid chromatography: comparison of reversed-phase silica monolithic and

394 particulate capillary columns, *Anal. Bioanal. Chem.* 402 (2012) 2935-2943.
395 <https://doi.org/10.1007/s00216-012-5764-6>
396 [11] J. Ye, W. Yu, G. Chen, Z. Shen, S. Zeng, Enantiomeric separation of 2-arylpropionic
397 acid nonsteroidal anti-inflammatory drugs by HPLC with hydroxypropyl- β -cyclodextrin
398 as chiral mobile phase additive, *Biomed. Chromatogr.* 24 (2010) 799-807.
399 <https://doi.org/10.1002/bmc.1365>
400 [11]
401 .
402 [12] E. Pittler, N. Grawatsch, D. Paul, G. Gubitz, M.G. Schmid, Enantioseparation of
403 amino acids, α -hydroxy acids, and dipeptides by ligand-exchange CEC using silica-based
404 chiral stationary phases, *Electrophoresis* 30 (2009) 2897–2904.
405 [13] G. Kucerova, H. Prochazkova, K. Kalíková, E. Tesarova, Sulfobutylether- β -
406 cyclodextrin as a chiral selector for separation of amino acids and dipeptides in
407 chromatography, *J. Chromatogr. A* 1467 (2016) 356-362. [https://doi.org/](https://doi.org/10.1016/j.chroma.2016.07.061)
408 [10.1016/j.chroma.2016.07.061](https://doi.org/10.1016/j.chroma.2016.07.061)
409 [14] E. Pittler, M.G. Schmid, Enantioseparation of dansyl amino acids by HPLC on a
410 monolithic column dynamically coated with a vancomycin derivative, *Biomed.*
411 *Chromatogr.* 24 (2010) 1213-1219. <https://doi.org/10.1002/bmc.1430>
412 [15] G. Kucerova, K. Kalíková, H. Prochazkova, M. Popr, J. Jindrich, P. Coufal, E.
413 Tesarova, Chromatographic Characterization of a New Cationic β -CD Based Stationary
414 Phase Prepared by Dynamic Coating, *Chromatographia* 79 (2016) 529-536.
415 <https://doi.org/10.1007/s10337-016-3050-z>
416 [16] W. De Malsche, J.O. De Beeck, S. De Bruyne, H. Gardeniers, G. Desmet,
417 Realization of 1×10^6 Theoretical Plates in Liquid Chromatography Using Very Long
418 Pillar Array Columns, *Anal. Chem.* 84 (2012) 1214-1219.
419 <https://doi.org/10.1021/ac203048n>
420 [17] M. Baca, G. Desmet, H. Ottevaere, W. De Malsche, Achieving a Peak Capacity of
421 1800 Using an 8 m Long Pillar Array Column, *Anal. Chem.* 91 (2019) 10932-
422 10936 <https://doi.org/10.1021/acs.analchem.9b02236>
423 [18] W. De Malsche, H. Gardeniers, G. Desmet, Experimental Study of Porous Silicon
424 Shell Pillars under Retentive Conditions, *Anal. Chem.* 80 (2008) 5391-5400.
425 <https://doi.org/10.1021/ac800424q>
426 [19] W. De Malsche, S. De Bruyne, J.O. De Beeck, P. Sandra, H. Gardeniers, G. Desmet,
427 F. Lynen, Capillary liquid chromatography separations using non-porous pillar array
428 columns, *J. Chromatogr. A* 1230 (2012) 41-47.
429 <https://doi.org/10.1016/j.chroma.2012.01.060>
430 [20] L. Zhang, B. Majeed, L. Lagae, P. Peumans, C. Van Hoof, W. De Malsche, Ion-pair
431 reversed-phase chromatography of short double-stranded deoxyribonucleic acid in silicon
432 micro-pillar array columns: Retention model and applications, *J. Chromatogr. A* 1294
433 (2013) 1-9. <https://doi.org/10.1016/j.chroma.2013.04.002>

434 [21] T. Nissilä, L. Sainiemi, T. Sikanen, T. Kotiaho, S. Franssila, R. Kostiainen, R.A.
435 Ketola, Silicon micropillar array electrospray chip for drug and biomolecule analysis,
436 Rapid Commun. Mass. Spectrom. 21 (2007) 3677-3682. [https://doi.org/](https://doi.org/10.1002/rcm.3266)
437 10.1002/rcm.3266. [22] Y. Song, K. Takatsuki, T. Sekiguchi, T. Funatsu, S. Shoji, M.
438 Tsunoda, Rapid quantitative method for the detection of phenylalanine and tyrosine in
439 human plasma using pillar array columns and gradient elution, Amino acids 48 (2016)
440 1731-1735. <https://doi.org/10.1007/s00726-016-2248-6>.
441 [23] P.A. Todd, S.P. Clissold, Naproxen, Drugs 40 (1990) 91-137.
442 <https://doi.org/10.2165/00003495-199040010-00006>
443 [24] A.R.M. De Oliveira, E.J. Cesarino, P.S. Bonato, Solid-phase microextraction and
444 chiral HPLC analysis of ibuprofen in urine, J. Chromatogr. B 818 (2005) 285-291.
445 <https://doi.org/10.1016/j.jchromb.2005.01.010>
446 [25] Z. Guo, H. Wang, Y. Zhang, Chiral separation of ketoprofen on an achiral C₈
447 column by HPLC using norvancomycin as chiral mobile phase additives, J. Pharm.
448 Biomed. Anal. 41 (2006) 310-314. <https://doi.org/10.1016/j.jpba.2005.10.045>
449
450 [26] M. Callewaert, J. O. De Beeck, K. Maeno, S. Sukas, H. Thienpont, H. Ottevaere, H.
451 Gardeniers, G. Desmet. W. De Malsche, Integration of uniform porous shell layers in
452 very long pillar array columns using electrochemical anodization for liquid
453 chromatography, Analyst 139 (2014) 618-625. <https://doi.org/10.1039/C3AN02023A>.
454 [27] <http://www.chemicalize.com/>
455 [28] Y-C. Guillaume, C. Andre, A novel chiral column for the HPLC separation of a
456 series of dansyl amino and arylalkanoic acids, Talanta 76 (2008) 1261-1264.
457 <https://doi.org/10.1016/j.talanta.2008.05.014>.
458 [29] B. Natalini, R. Sardella, A. Macchiarulo, R. Pellicciari, Dynamic Ligand-Exchange
459 Chiral Stationary Phase from S-Benzyl-(R)-cysteine, Chirality 18 (2006) 509-518.
460 <https://doi.org/10.1002/chir.20280>
461 [30] A. Hussain, M.F. Al-Ajmi, S. Amir. I. Ali, Development and validation of
462 SPMTE HPLC method for analysis of profens from human plasma, Biomed.
463 Chromatogr. 30 (2016) 1263-1269. <https://doi.org/10.1002/bmc.3676>
464 [31] B.K. Patel, M. Hanna-Brown, M.R. Hadley, A.J. Hutt1, Enantiomeric resolution of
465 2-arylpropionic acid nonsteroidal anti-inflammatory drugs by capillary electrophoresis:
466 Methods and applications, Electrophoresis 25 (2004) 2625-2656. [https://doi.org/](https://doi.org/10.1002/elps.200405928)
467 10.1002/elps.200405928
468 [32] S. Tong, Y.-X. Guan, J. Yan, B. Zheng, L. Zhao, Enantiomeric separation of (R, S)-
469 naproxen by recycling high speed counter-current chromatography with hydroxypropyl-
470 β-cyclodextrin as chiral selector, J. Chromatogr. A 1218 (2011) 5434- 5440.
471 <https://doi.org/10.1016/j.chroma.2011.06.015>

- 472 [33] Y. Lv, D. Mei, X. Pan, T. Tan, Preparation of novel β -cyclodextrin functionalized
473 monolith and its application in chiral separation, *J. Chromatogr. B* 878 (2010) 2461–
474 2464. <https://doi.org/10.1016/j.jchromb.2010.07.020>.
- 475 [34] G. Farkas, L.H. Irgens, G. Quintero, M.D. Beeson, A. Al-Saeed, G. Vigh,
476 Displacement chromatography on cyclodextrin silicas: IV. Separation of the enantiomers
477 of ibuprofen, *J. Chromatogr. A* 645 (1993) 67-74.
478 <https://doi.org/10.1002/jssc.201301184>
- 479 [35] J.L.C. Cardoso, V.L. Lanchote, M.P.M. Pereira, N.V. De Moraes, J.S. Lepera,
480 Analysis of ibuprofen enantiomers in rat plasma by liquid chromatography with tandem
481 mass spectrometry, *J. Sep. Sci.* 37 (2014) 944–949.
482 <https://doi.org/10.1002/jssc.201301184>
- 483
- 484 [36] C. Caballo, M.D. Sicilia, S. Rubio, Enantioselective analysis of non-steroidal anti-
485 inflammatory drugs in freshwater fish based on microextraction with a supramolecular
486 liquid and chiral liquid chromatography–tandem mass spectrometry, *Anal. Bioanal.*
487 *Chem.* 407 (2015) 4721-4731. <https://doi.org/10.1007/s00216-015-8675-5>.
- 488 [37] C.-F. Tsai, C.-H. Kuo, D.Y.-C. Shih, Determination of 20 synthetic dyes in chili
489 powders and syrup-preserved fruits by liquid chromatography/tandem mass spectrometry,
490 *J. Food. Drug. Anal.* 23 (2015) 453-462. <https://doi.org/10.1016/j.jfda.2014.09.003>.
- 491 [38] G.K.E. Scriba. *Chiral Separations, Methods and Protocols*, Springer. New York,
492 2019.
- 493 [39] R. Ma, H. Qu, B. Wang, F. Wang, Y. Yu, G. Yu, Simultaneous enantiomeric
494 analysis of non-steroidal anti-inflammatory drugs in environment by chiral LC-MS/MS:
495 A pilot study in Beijing, China, *Ecotoxicol. Environ. Saf.* 174 (2019) 83-91.
496 <https://doi.org/10.1016/j.ecoenv.2019.01.122>
- 497 [38]
- 498 [40] S. Mohr, M. Taschwer, M.G. Schmid, Chiral separation of cathinone derivatives
499 used as recreational drugs by HPLC-UV using a CHIRALPAK® AS-H column as
500 stationary phase, *Chirality* 24 (2012) 486-492. <https://doi.org/10.1002/chir.22048>
- 501 [41] S. Imre, A. Ormenișan, A. Tero-Vescan, D-L. Muntean, C-E. Vari, HPLC
502 Enantioseparation of β -Blockers on Ovomuroid Stationary Phase, *J. Chromatogr. Sci.* 54
503 (2016) 1578-1583. <https://doi.org/10.1093/chromsci/bmw107>.

