**Vrije Universiteit Brussel**

VRIJE
UNIVERSITEIT
BRUSSEL

Stationary-phase optimized selectivity in supercritical fluid chromatography using a customized Phase OPtimized Liquid Chromatography kit

Grooten, Yasmine; Riasová, Petra; Salsinha, Inês; Mangelings, Debby; Vander Heyden, Yvan

1    **Stationary-Phase Optimized Selectivity in Supercritical Fluid Chromatography using a**

2    **customized Phase Optimized Liquid Chromatography kit: Comparison of different prediction**

3    **approaches**

4

5    Yasmine Grooten [a], Petra Riasová [a, b], Inês Salsinha [a], Debby Mangelings [a,*], Yvan Vander Heyden [a]

6

7

8    [a]   Department of Analytical Chemistry, Applied Chemometrics and Molecular Modelling,

9    Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, B-1090 Brussels, Belgium

10   [b]   Department of Analytical Chemistry, Faculty of Pharmacy, Charles University, Akademika Heyrovského 1203,

11   CZ-500 05 Hradec Králové, Czech Republic

12

13

14

15

16

17   *: Corresponding author. Tel: +32 2 477 43 29. E-mail address: Debby.Mangelings@vub.be

18   *ORCID numbers*: 0000-0003-1349-492X (Y. Grooten), 0000-0002-3015-8066 (P. Riasová), 0000-

19   0001-8773-6843 (D. Mangelings), 0000-0002-7788-9886 (Y. Vander Heyden)

20 **Abstract**

21 The use of Stationary-phase Optimized Selectivity in Liquid Chromatography (SOS-LC) was shown

22 successful for HPLC to analyze complex mixtures using a Phase OPtimized Liquid Chromatography

23 (POPLC) kit. This commercial kit contains five stationary-phase types of varying lengths, which can be

24 coupled to offer an improved separation of compounds. Recently, Stationary-Phase Optimized

25 Selectivity Supercritical Fluid Chromatography (SOS-SFC) has been introduced, transferring the

26 methodology to SFC. In this study, the applicability of a customized POPLC expert kit for isocratic SFC

27 runs was explored. Five stationary-phase chemistries were selected as potentially most suitable for

28 achiral separations of polar compounds: aminopropyl (amino), cyanopropyl (CN), diol, ethylpyridine

29 (EP) and silica. The retention factors (k) on the individual stationary phases were used for the prediction

30 of the best stationary-phase combination, based on the POPLC algorithm (via the included software).

31 As an alternative, the best column combination was predicted using multiple linear regression (MLR)

32 models on the results obtained from a simplex centroid mixture design with only three stationary-phase

33 types (Amino, Silica and EP). A third approach applied the isocratic POPLC algorithm on the same

34 three stationary-phase data. The proposed combinations were assembled and tested. The predicted and

35 experimental retention factors were compared. The predictions based on the POPLC algorithm provided

36 a stationary phase showing a complete separation of the mixture. The stationary phase suggested by the

37 MLR-models on the other hand, showed co-elution of two compounds, due to an unexpected

38 experimental retention shift. Overall, the customized POPLC kit showed good potential to be applied in

39 SFC.

## 1. Introduction

Supercritical Fluid Chromatography (SFC) has established itself as a preferred technique for the separation of chiral compounds, especially at the preparative level [1]. The major benefits of the technique are related to the supercritical-fluid properties of carbon dioxide ($CO_2$) in the mobile phase. $CO_2$ is by far the most used supercritical fluid in SFC since it has a low critical pressure and temperature of about 73 bar and 31°C, respectively, and is non-toxic, non-flammable and naturally produced [2].

Supercritical fluids possess a higher diffusivity and a lower viscosity than liquid mobile phases, which allow run times to be 3 to 5 times shorter than in High-Performance Liquid Chromatography (HPLC) [3]. Higher flow rates, which reduce the overall analysis time and increase the throughput capacity, do not compromise the efficiency of the technique. Moreover, the optimal Height Equivalent to a Theoretical Plate (HETP), which is directly related to the column efficiency, is similar for SFC and HPLC. In sub/supercritical conditions, the chromatographic column is equilibrated in a few minutes [4]. The low viscosity of the supercritical fluids is also the property responsible for the lower pressure drop across the column, allowing the coupling of several stationary phases in series [5].

Following the research of Berger and Wilson [6], showing the considerably high efficiency of coupling columns in SFC, several studies have been performed on column coupling [7–14]. Phinney et al. [15] showed the potential benefits of such couplings in SFC by separating groups of structurally related compounds (β-blockers and 1,4-benzodiazepines), through connection of different achiral and chiral stationary phases under subcritical experimental conditions. The results indicated that using $CO_2$ may solve mobile-phase incompatibility problems encountered in HPLC experiments. On the other hand, Wang et al. [5] changed backpressure to optimize the separation of stereoisomers on coupled systems, showing also that the coupling sequence can be an important parameter affecting selectivity changes. West et al. [16] compared the tandem coupling of two complementary achiral columns with the results obtained on the individual columns, for impurity profiling of synthetic drugs. The tandem-column method provided some advantages, such as an improved peak capacity, compared to the single-column analysis.

69 Stationary-phase selection is the most critical step for method development, with samples'

70 elution and retention depending mainly on the chemical properties of the selected phase [17]. However,

71 with an extensive number of stationary-phase types available on the market, the selection of an adequate

72 one may be less straightforward than hoped. The search for the proper stationary phase is challenging

73 and typically based on the analyst's experience, literature information and often on trial-and-error

74 approaches [18]. Additional steps in an optimization strategy include the selection of the mobile-phase

75 composition, temperature, backpressure, flow rate, gradient slope and, occasionally, the use of different

76 additives. All these factors can be varied to tune the selectivity depending on the properties of the

77 analytes. When analyzing complex mixtures, the difficulty in achieving maximal resolution is even

78 higher and often the use of one single column is not successful, not only in terms of resolution but also

79 of analysis time [18].

80 Column coupling seems to be a promising approach for solving selectivity problems when

81 analyzing complex mixtures. The term "serial tandem columns" is a synonym for coupled columns and

82 consists of basically connecting two (or more) columns in series. Zero Dead Volume (ZDV) couplers

83 are often used to assemble the systems because of the simplicity of connection [18]. The most significant

84 advance in this approach was noticed with the commercialization of the POPLC (Phase OPtimized

85 Liquid Chromatography) system, associated with the POPLC Optimizer Software [19]. The calculations

86 of the retention are based on the PRISMA model, initially applied for mobile-phase optimization, and

87 later adapted for stationary-phase optimization by Nyiredy et al [19,20]. In the latter approach, the

88 stationary phase is the optimization parameter, where connection of segments with variable lengths and

89 different chemistries may lead to an optimal separation. The commercialized POPLC kit includes five

90 types of column segments (ProntoSIL C18-SH2, C18-EPS, Cyano, Phenyl and C30), each with five

91 different lengths.

92 The first step is the screening of all compounds on a predefined length of each of the five types

93 of stationary phases. The retention factor of each component in the mixture is predicted for all possible

94 column combinations according to the following equation [19]:

95 $$k_{A,B,C,D,E} = \frac{\sum_{i=A}^{E} \Phi_i k_i}{\sum_{i=A}^{E} \Phi_i} \qquad \text{(Eq.1)}$$

96 where $k_{A,B,C,D,E}$ represents the retention factor predicted for a compound in a coupled system, and $k_i$ the

97 retention factor obtained for a compound on an individual stationary-phase segment (A-E). The symbol

98 $\Phi$ is the effective length of a stationary phase. Afterwards, the column combinations can be ranked

99 according to their ability to separate the critical peak pair and the combination providing the best

100 separation can be selected.

101 The POPLC kit was already successfully used in HPLC for the separation of mixtures containing

102 steroids [21], explosives [22], polymer electrolyte membrane degradation products [23], and synthetic

103 thyroid hormones and their impurities [24]. Deconinck et al. [25–28] have successfully applied the

104 POPLC method in the contexts of food-supplement analysis to screen several types of mixtures,

105 containing analgesics, adulterated dietary supplements, PDE-5 inhibitors in food supplements and to

106 develop herbal fingerprints of specific plants in plant food supplements. De Beer et al. [29,30] provided

107 a solution for the largest drawback of the POPLC method, namely that it is developed for application in

108 isocratic mode. This was based on applying a multiple step gradient for the separation of a mixture

109 composed of compounds with a broad range of polarities. Furthermore, the same research group

110 provided an algorithm allowing to use the POPLC kit in a linear gradient mode, accurately predicting

111 the optimal column combination after inserting a minimum of three preliminary isocratic measurements

112 on each individual stationary phase. This linear gradient approach was also applied to develop a green

113 method, in which a "green" mobile phase, composed of ethanol and formic acid, was defined and the

114 stationary phase was optimized using the POPLC kit [31]. The POPLC strategy was also used to predict

115 the optimal combination of chiral columns to acquire baseline separation of a mixture containing four

116 chiral pairs [32]. The experiments in isocratic mode did not provide a satisfying separation, in contrast

117 to the prediction and experimental results obtained by the algorithms for gradient analysis. In addition

118 to the implementation in HPLC, the application of the basic kit was tested in SFC by Delahaye and

119 Lynen [33], thereby introducing the term Stationary-Phase Optimized Selectivity Supercritical Fluid

120 Chromatography (SOS-SFC). With this approach, an acceptable separation of a mixture containing 15

121 achiral compounds was achieved. Subsequently, the same research group applied the SOS-SFC

122 methodology to chiral mixtures, optimizing the separation further via the mobile phase density and the

123 use of additives, such as isopropylamine and trifluoroacetic acid [34].

124     The aim of our actual study was to further investigate the suitability of the POPLC approach for

125 SFC experiments. Five column chemistries were selected based on previous research by Galea et al.

126 [17,35]. The selected chemistries are aminopropyl (amino), ethylpyridine (EP), cyanopropyl (CN), diol

127 and silica, since these were identified as being the best stationary-phase types for the analysis of polar

128 compounds in SFC. The intention was to adapt the kit and the methodology in the context of SOS-SFC.

129 In this way, the kit is composed of normal-phase stationary phases, which are more interesting for

130 implementation in SFC. Since these stationary-phase types are dissimilar, they are more likely to provide

131 complementary separations, which could be beneficial to separate mixtures of structural analogues, such

132 as impurities. An analgesics mixture was assembled in order to test the applicability of different

133 prediction approaches on the customized kit.

134

135 **2. Materials and methods**

136     *2.1. Instrumentation*

137 All experiments were performed on the SFC Acquity® Ultra Performance Convergence Chromatography

138 (UPC$^2$) System from Waters® (Milford, MA, USA). The system consists of a binary pump, an

139 autosampler with a fixed loop of 10 µL, a convergence manager, a photo diode array (PDA) detector, a

140 backpressure regulator and an external Acquity column oven without pre-heating system. During the

141 analysis, the sample manager compartment was kept at 10°C for sample stability.

142     *2.2. Chemicals and reagents*

143 The solvent used was HPLC grade methanol (MeOH) purchased from VWR Chemicals (Fontenay-sous-

144 Bois, France). $CO_2$ quality 4.5 (purity ≥ 99.995%) was from Messer (Sint-Pieters-Leeuw, Belgium).

145 Ammonium formate, used as mobile phase additive, was obtained from Sigma-Aldrich (Steinheim,

146 Germany). The mixture consisted of analgesic compounds. All samples were prepared using HPLC

147 grade MeOH. Compounds used for the mixture were acetaminophen, diclofenac, ibuprofen, ketoprofen,

148 naproxen (Sigma-Aldrich, Steinheim, Germany), caffeine (Fluka, Neu-Ulm, Switzerland), codeine

149 hydrochloride, morphine hydrochloride (Bios Coutelier, Brussels, Belgium) and indomethacin (Sigma,

150 St. Louis, MO, USA).

### 2.3. Test set

152 Since it was aimed to identify the benefits of using the kit for the separation of complex mixtures, a

153 number of analgesic compounds, composed of acetaminophen, caffeine, codeine, diclofenac, ibuprofen,

154 indomethacin, ketoprofen, morphine and naproxen, was mixed. These nine analgesics cover a variety of

155 molecular structures and were arbitrarily selected to assemble a mixture that was difficult to separate.

156 Some compounds are structural analogues, e.g. ibuprofen, ketoprofen and naproxen (arylpropionic acid

157 derivatives); morphine and codeine (opioids). Each individual compound solution contained 1 mg/mL

158 in methanol. To prepare the mixture, 1.0 mL of each individual solution and 1.0 ml MeOH were added

159 to a 10 mL tube, resulting in final concentrations of 0.1 mg/mL for each component. Both the individual

160 solutions and the mixture were injected, in order to determine the elution sequence.

### 2.4. Customized POPLC Expert kit

162 The SOS-SFC method was developed using a customized POPLC Expert kit 250-5-SFC,

163 commercialized by Bischoff Chromatography (Leonberg, Germany). The kit is composed of segments

164 with lengths of 10, 20, 60 and 80 mm, and of two segments of 40 mm for each of the five stationary

165 phases, which are ProntoSIL amino, cyano (CN), diol, ethylpyridine (EP) and silica. The columns have

166 an internal diameter of 3 mm and the stationary phases all have particle sizes of 5 µm. The segments

167 assembly is made via PEEK (PolyEther Ether Ketone) holders, with 10 mm and 20 mm cartridges, to

168 support pressure. The kit also includes the holder pieces and inlet and outlet filters, necessary for the

169 segments' assembly.

### 2.5. Mobile-phase composition

171 The fraction of MeOH necessary to elute the analgesic compounds was first evaluated in a segment with

172 100 mm length. A generic gradient was applied and the retention times ($t_R$) of the compounds recorded.

173 The generic gradient starts at 5% MeOH and increases up to 40% over 10 min, that fraction of modifier

174     is kept for 5 min and then returns to the initial value of 5% in 0.5 min, at which the column is re-

175     equilibrated for 2 more min. The overall run time for each sample is 17.5 min. Equation 2 is used to

176     calculate the modifier fractions $C_e$ at which the compounds are eluting [36].

177     $$C_e = C_i + \frac{C_f - C_i}{t_G} \; x \; (t_r - t_d - t_0)$$     (Eq. 2)

178     where $C_i$ and $C_f$ represent the compositions at the start and end of the gradient, respectively, $t_d$ is the

179     instrument dwell time, $t_r$ the residence time on the column, $t_G$ the gradient time and $t_0$ the column void

180     time. Then, the average modifier fraction was determined.


181     *2.6. Chromatographic conditions*

182     The runs were performed in isocratic mode with a mobile phase consisting of 15% MeOH with 10 mM

183     ammonium formate in $CO_2$. This additive was selected according to Muscat Galea et al. [37]. The flow

184     rate was 2 mL/min, the column oven was set at 25°C and the backpressure at 150 bar. The injection

185     volume was 2 µL and the detection wavelength 220 nm. The system void time ($t_d$) was determined by

186     coupling a ZDV union. The column void time ($t_0$) for each column was measured with a length of

187     100 mm and marked as the first baseline disturbance observed with the blank injection. In the first step

188     of the approach, the compounds are screened on the five separate stationary phases, using an arbitrary

189     chosen length of 100 mm. When making combinations of different stationary phases, the longest

190     stationary phase type was always coupled at the beginning of the column. The retention factors (k) were

191     calculated as $(t_r - t_0)/t_0$, in which $t_r$ represents the retention time of the compound.


192     *2.7. Approach 1*

193     The POPLC Optimization Software (version 1.04.03, Bischoff Chromatography, Leonberg, Germany)

194     is included in the kit and allows the prediction of the 'optimal' and 'best' combination of segments

195     (multiples of 10 mm segments) using equation 1. With the use of this equation, the selectivity α is

196     calculated for all consecutive peaks, after which a ranking can be made of the α values for the critical

197     peak pair of every column combination with the predefined total column length. The combined

198     stationary phase, leading to the highest selectivity for the critical peak pair is selected, considering also

199     the total analysis time. Furthermore, the algorithm of the software takes into account the efficiency of

8

200  the column, expressed as number of theoretical plates N. The efficiency of a combined column is

201  calculated using the efficiencies of the elementary stationary-phase segments. After entering the

202  retention times, column void time and plate counts on the pure stationary phases of 100 mm in the

203  POPLC Optimization Software, it provides the predicted column combinations. The predictions were

204  made, defining a maximum column length of 200 mm (to provide a wider variety of possible

205  combinations) and a minimal resolution of 1.5 (since a resolution higher than 1.5 represents baseline

206  separation). The maximal analysis time was set at 30 min. The 'optimal' prediction is the combination,

207  which meets the requirements for the resolution and has the shortest analysis time. The 'best'

208  combination is that with the best chromatogram (best resolution and selectivity).

209  *2.8. Approach 2*

210  The idea of approach 2 was to apply a mixture design to obtain the best result for the selectivity α. As

211  in the original SOS-LC idea of Nyiredy et al. [19] a triangular domain of possible combinations between

212  three stationary-phase types with a certain total column length was examined. In this mixture-design

213  approach, the variables are the lengths of the stationary-phase types, of which two are independent and

214  one is dependent at a selected total column length [38,39]. Instead of applying the classical equation

215  based on the addition of the retention factors, MLR models were built for the retention based on the

216  results from a 7-points simplex centroid mixture design. The obtained response surface models were

217  used to predict the retention of different points in the triangular domain, which in turn allowed the

218  prediction of the selectivity at these points. The goal of this approach is to predict the column-segments

219  combination with the best selectivity α of the worst separated peak pair. For the mixture design, a

220  simplex centroid design was chosen, which required seven measurement points for three segments: (1,

221  0, 0), (0, 1, 0), (0, 0, 1), (0.5, 0.5, 0), (0, 0.5, 0.5), (0.5, 0, 0.5) and (1/3, 1/3, 1/3). However, when

222  coupling segments with a minimal length of 10 mm, the centroid could not be assembled for a total

223  column length of 100 mm. Therefore, this point was shifted towards stationary phase composition (0.4,

224  0.3, 0.3) (see **Fig. 1**). In practice, this means that the three 100 mm individual stationary phases, three

225  50 mm - 50 mm combinations of two stationary-phase types and the 40 mm – 30 mm – 30 mm

226  combination of the three column types should be measured. With the results at these seven points,

9

227    multiple linear regression (MLR) models or response surfaces with the following type of equation were

228    built for each compound:

229           $\log k = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3$          (eq. 3)

230    where log k is the logarithm of the retention factor for a compound in the coupled system, $x_i$ the relative

231    lengths of stationary phases 1, 2 or 3, $b_0$ the intercept and $b_i$, the estimated coefficients.

232         The three selected stationary phase types were Amino, Silica and EP. When setting the total column

233    length to 100 mm, 66 possible combinations (multiples of 10 mm) can be assembled (see grid points in

234    **Fig. 1**). With the MLR-models for each compound, the log k and k values can be estimated for each

235    column combination. When sorting the k values, the selectivity (α) can be calculated for each

236    consecutive peak pair. The minimal α-value of every stationary phase combination can be visualized in

237    an $\alpha_{min}$ plot and then the maximal is selected. This value represents the column combination for which

238    the best separation of the critical peak pair is achieved.

239    *2.9. Approach 3*

240    A third approach was to perform predictions based on the POPLC algorithm with the three stationary-

241    phase types selected for approach 2, i.e. Amino, Silica and EP. Based on the retention factors obtained

242    from the measurements on 100 mm of each column, the corresponding k-values for all possible

243    combinations of these three stationary-phase types (with a total length of 100 mm) were calculated using

244    equation 1. For the 66 potential combinations (multiples of 10 mm), the retention factors were ranked

245    and the α-values calculated. The stationary phase with the highest predicted α-value for the critical peak

246    pair was selected and assembled to measure the mixture.

247    *2.10.        Procedure and software for data analysis*

248    Empower® 3 V7.10 software 2010 (Waters, Milford, MA, USA) was used for data acquisition and

249    processing, i.e. retention times ($t_r$), retention factors ($k$), USP resolutions (Rs) and  USP plate counts (N)

250    are determined for all injections. The resolutions were calculated as $2(t_{r2} - t_{r1})/(w_1 + w_2)$, in which w

251    represents the peak width at the baseline, applying the tangent method. The plate counts were calculated

252 as 16 $(t_r/w)^2$. The POPLC Optimizer Software was used to predict the 'optimal' and 'best' combinations

253 of approach 1 and to provide the predicted chromatograms at these combinations. Multiple linear

254 regression (MLR) models were calculated with m-files written in MATLAB® 2014a (The Mathworks,

255 Natick, MA, USA). The $\alpha_{min}$ plot for the mixture design was drawn with the ggtern package [40] in

256 RStudio version 1.2.5019 (RStudio Inc., Boston, MA, USA). Microsoft® Excel (Microsoft Office

257 Professional Plus 2016) was used to calculate relative deviations (|measured – predicted value|/average

258 value) and selectivities (α), and to create scatter plots. Furthermore, it was used to calculate the k-values

259 for approaches 2 and 3.

260 **3. Results and discussion**

261 *3.1. Basic measurements on 100 mm of each stationary phase type*

262 It was challenging to establish a chromatographic method that allowed a good separation of the selected

263 analgesics group, which presents heterogeneity in terms of molecular properties. A mobile phase with

264 10 mM ammonium formate and 15% MeOH as the average percentage necessary for the elution of all

265 compounds on the Amino column was selected using equation 2. Thereafter, the mixture was screened

266 on each stationary phase type with 100 mm column length. The most retained compound was morphine

267 (peak 8), eluting at 21.5 min from the silica stationary phase. All individual retention times and

268 corresponding retention factors for the nine compounds on the different segments are presented in **Table**

269 **S1 (**in the Electronic Supplementary Material (ESM)). The chromatograms obtained with the individual

270 stationary phase segments, in the screening step, are shown in **Fig. 2**. None of the stationary phases fully

271 separated the analgesics mixture. However, different elution sequences are observed, suggesting that

272 combining segments of the various stationary-phase types could possibly provide a baseline separation

273 of all compounds.

274 To assess the dissimilarities S between the different stationary phase types for this analgesics

275 mixture, the correlation r² between the retention factors was used in the following equation [41,42]:

276 $$S = 100 \text{ x } \sqrt{1 - r^2} \qquad \text{(Eq. 4)}$$

277  S values close to 100 show a high dissimilarity between columns, while a value close to 0 indicates a

278  similar separation on two columns. The results (see **Table 1**) show that the highest dissimilarity is found

279  between the column types Silica – Amino and CN – Amino. On the other hand, the retention on the CN

280  and Silica column shows high similarity (low S).

281  *3.2. Approach 1*

282  A next step consisted of entering the retention times, column void times and plate counts obtained by

283  the measurements on 100 mm of the individual stationary phases (**Table S1 in the ESM**) in the POPLC

284  Optimizer Software, applying equation 1 and determining that the critical peak pair consisted of

285  naproxen and ketoprofen for all advised predictions. The 'optimal' (meeting the requirements for the

286  resolution in the shortest analysis time) and 'best' combination (the best possible result for resolution

287  and selectivity) were both predicted via the POPLC Optimizer Software, setting the maximal analysis

288  time at 30 min. The proposed 'optimal' combination for a minimal resolution of 1.5 and a maximal

289  column length of 200 mm was 60 mm CN + 20 mm Amino + 10 mm EP. However, this combination

290  led to a chromatogram where only seven peaks were observed: a co-elution of naproxen (peak 5),

291  ketoprofen (peak 6) and codeine (peak 2) occurred (**Fig. 3 (a)**). This was caused by earlier elution of

292  ketoprofen (peak 6) and codeine (peak 2) than predicted (13.9% and 29.7% relative deviation for the

293  retention factors, respectively; see **Table 2**). Furthermore, a large deviation could also be noticed for the

294  retention factor of morphine (peak 8) (18.6%).

295  To improve the experimental separation of the predictions of the 'optimal' combination, a minimal

296  resolution of 2 was requested, leading to the predicted combination of 80 mm CN + 30 mm Amino + 10

297  mm Diol. The analysis of the analgesics mixture on this column composition led to an improved

298  separation (**Fig. 3 (b)**). However, four peak pairs showed a resolution below 1.5, with a minimal value

299  of 0.98 for indomethacin (peak 7) and codeine (peak 2) (**Table 2**). Once again, the largest relative

300  deviation in retention factors could be assigned to codeine (peak 2) (8.4%) and morphine (peak 8)

301  (8.9%). It may be noticed that both above suggested column combinations are shorter than 200 mm,

302  most likely to obtain the shortest analysis times, as requested for the 'optimal' combinations.

As mentioned above, this POPLC Optimizer software approach also provides the possibility to estimate the 'best' combination. In this case, less focus is put on the speed of the separation, while achieving the best resolution and selectivity is preferred. This 'best' combination, when a minimal resolution of 1.5 and a maximal length of 200 mm were entered as parameters, was 110 mm Silica + 50 mm Amino + 40 mm CN. This segment combination led to a complete baseline separated chromatogram (**Fig. 4**). The lowest resolution (2.00) was obtained between the peaks of naproxen (peak 5) and acetaminophen (peak 4) (**Table 2**). However, the use of this 200 mm long column implied a prolonged analysis time (25 min) compared to the shorter column combinations, where a total analysis time of approximately 3 and 4.5 min for 90 mm and 120 mm columns was achieved (in both cases without baseline separation!), respectively. For a shorter analysis, this run could possibly be further optimized by applying a gradient elution. This would allow for the same selectivity with a faster elution of the later eluting compounds.

*3.3. Approach 2*

The application of a simplex centroid mixture design was evaluated as an alternative to the SOS-LC approach. However, for this methodology, only three types of stationary phases were considered simultaneously. Amino, Silica and EP were the selected stationary phase types for the measurement of the mixture on seven column combinations. The selection was made based on the S values in **Table 1**: these three stationary phase types showed an overall high value in terms of dissimilarity. Furthermore, because the combinations CN – silica and diol – EP showed higher correlations, they were not considered together. Seven measurements (see **Fig. 1**) were required for this approach: the vertices, midpoints of the sides of the triangle and a point near the centroid (40 mm Amino + 30 mm Silica + 30 mm EP). The obtained retention factors (see **Table S1** and **Table S2** in the Electronic Supplementary Material (ESM)) were then used in the calculations to search for the best stationary-phase combination. Only combinations with a total column length of 100 mm were used in the predictions, leading to a total of 66 theoretical combinations (step size 10 mm, see **Fig. 1**). With multiple linear regression (MLR) models as in equation 3, the retention factors k on each column-fragment combination were modelled. From the predicted k values, the selectivities α for consecutive peaks were estimated and the minimal selectivity $\alpha_{min}$, i.e. α for the worst separated peak pair, indicated for each stationary-phase composition.

330 The column composition where $\alpha_{min}$ is maximal specifies the best predicted separation for the worst

331 separated peak pair. The $\alpha_{min}$ values were visualized by plotting them in a ternary phase diagram (**Fig.**

332 **5**).

333     It can be noticed, that the combinations between only EP and Amino, on the one hand, and the Silica

334 and EP combinations, on the other, seem to provide poor separations. An area of improved separations

335 is observed in the range, where the relative length of the EP segment is below 0.4, the Silica segment

336 between 0.3 and 0.7 and the length of the Amino segment between 0.3 and 0.6. The highest $\alpha_{min}$ value

337 could be found at a combination of 60 mm Silica + 40 mm Amino. The worst separated peak pair on

338 this stationary phase was predicted to be naproxen – ketoprofen. However, as seen in **Fig. 6 (a)**, a co-

339 elution of codeine (peak 2) and diclofenac (peak 9) is noticed when testing this column combination.

340 Large shifts in retention can again be seen for codeine (peak 2) and morphine (peak 8) (the last two

341 eluting compounds), resulting in an unforeseen overlap between codeine (peak 2) and diclofenac

342 (peak 9) (**Table 3**). For these compounds, a larger decrease than expected for the retention factors was

343 noticed. The other compounds were all baseline separated with the lowest resolution between naproxen

344 (peak 5) and ketoprofen (peak 6) (Rs 1.68). To verify whether a longer total column length could

345 promote the separation, the same fraction ratio was used in a 200 mm column, leading to a combination

346 of 120 mm Silica + 80 mm Amino (see **Fig. 6 (b)**). This stationary-phase combination indeed led to a

347 baseline separated chromatogram of the mixture. This observation could be attributed to a prolonged

348 retention of codeine (peak 2), which moreover resembles the predicted retention factor better than that

349 on the 100 mm column. However, notice that the total analysis time of the analgesics mixture almost

350 tripled compared to the measurements on the 100 mm column.

351     *3.4. Approach 3*

352 As a comparison to the predicted stationary-phase combination obtained from approach 2, predictions

353 were performed using equation 1 on the same three stationary phases: Amino, Silica and EP. The

354 retention factors of the nine compounds were predicted for the same 66 theoretical combinations and a

355 total column length of 100 mm, based on the data from the basic measurements on 100 mm columns.

356 Afterwards, identical steps were considered to predict the separation of a given stationary-phase

357 composition. A maximal $\alpha_{min}$ for the critical peak pair was now predicted for a stationary phase

358 combination composed of 70 mm Silica + 30 mm Amino. Although there was little difference compared

359 to the composition retrieved from approach 2, a full baseline separation was now achieved (**Fig. 6 (c)**).

360 The sequence of elution remained the same in both cases, as well as the overall analysis time (8.5 min).

361 Naproxen (peak 5) and ketoprofen (peak 6) proved to be the most difficult to separate, with a resolution

362 of 1.53 (**Table 4**). Once more, a large difference could be noticed in the predicted and measured retention

363 times of the last two eluting compounds: codeine (peak 2) (4.44 min instead of 8.73 min) and morphine

364 (peak 8) (7.95 min instead of 15.90 min). Overall, larger relative variations of retention factors could be

365 observed compared to the stationary-phase combinations tested with the other approaches, with relative

366 differences ranging from 11.4% to 69.0%.

367 *3.5. Comparison of the prediction approaches*

368 Three alternative approaches for the prediction of the best stationary-phase combination were

369 evaluated. The use of the POPLC Optimizer Software accompanying the kit, allowed the selection of

370 different responses, such as the desired minimal resolution or selectivity, maximal length of the column

371 and the maximal time of analysis. However, the two tested 'optimal' combinations, obtained for minimal

372 resolutions of 1.5 and 2, and a maximal column length of 200 mm, did not provide a baseline separation

373 of the mixture. The 'best' combination with approach 1, calculated for a minimal resolution of 1.5,

374 allowed a complete baseline-separated chromatogram.

375 Approach 2, using equation 3 models to predict the k and $\alpha$ values, which thereafter are ranked to

376 find the best predicted separation for the critical peak pair, seemed to be less successful, with a co-

377 elution of two compounds. Furthermore, to simplify the combination, only three of the five stationary-

378 phase types were selected for the measurements and calculations. The selection was based on

379 dissimilarities between the stationary-phase chemistries. A total column length of 100 mm was chosen,

380 the same length as the basic measurements, leading to 66 possible column combinations. In this way,

381 the same pressure and density will be obtained as with the basic measurements, together with overall

382 shorter analysis times in contrast to longer columns. To obtain enough data points for the calculations

383    of approach 2, seven column combinations were assembled. In contrast, only five and three basic

384    measurements (100 mm of each individual column type) are needed for approaches 1 and 3, respectively.

385    Approach 3, using the same three stationary-phase types as approach 2 and a column length of 100

386    mm, proposed a column combination capable of separating the mixture. With both approaches 2 and 3,

387    the largest deviations from predicted retention factors were found with codeine and morphine. Both

388    compounds are structural analogues and exhibit the strongest basic properties of the mixture.

389    Furthermore, these two compounds eluted last in most tested column combinations, which makes them

390    potentially more difficult to predict. Overall, larger deviations in predicted retention factors were noticed

391    with approaches 2 and 3, compared to approach 1. Doubling the length of the column combination

392    obtained with approach 2 did indeed give a completely separated mixture.

393    **4.  Conclusions**

394    The applicability of a customized POPLC kit in supercritical fluid chromatography was examined using

395    an analgesics mixture. The kit consisted of five stationary-phase types, specifically chosen for their

396    applicability in SFC, which could be easily combined to achieve a better separation of complex mixtures.

397    Two out of the three approaches (i.e. those using the POPLC algorithm) used to predict the best possible

398    combination indicated conditions that fully baseline separate the mixture. Approach 2 (using a different

399    equation than the POPLC algorithm to predict the best combination) did not predict conditions that gave

400    a complete separation. Large deviations between predicted and measured retention factors were noticed,

401    especially on the shorter columns. With the 90 and 100 mm columns, the retention times of especially

402    codeine and morphine seemed difficult to predict, leading to the highest relative deviations of their

403    measured retention factors. Both compounds elute last in the column combinations of approaches 2 and

404    3, which make their prediction even more difficult. As a general trend, the longer the total length of the

405    column, the better the overall predictions seem to be. However, this phenomenon should be confirmed

406    from additional separations considering different mixtures.

407    In conclusion, the customized POPLC kit can successfully be used for experiments in SFC.

408    Nevertheless, due to some experimental shifts in the predicted retention times, it may be necessary to

16

409 test several predicted combinations. This implies that the POPLC algorithm is possibly too simplified

410 for the application in SFC, where pressure and mobile-phase density are of a larger importance than in

411 HPLC. The experiments have only been conducted in isocratic mode in this study. The applicability of

412 the approach in gradient mode to possibly enhance the separation of the compounds has yet to be tested.

413 The complementarity of the isocratic POPLC and the gradient optimizations for compounds with similar

414 and different polarities, respectively, may also be another topic for future research. Furthermore, the use

415 of longer columns or changing the backpressure could possibly fine-tune the separation of the mixture

416 on failed stationary-phase combinations.

## 6. Conflict of Interest

421 The authors declare that they have no conflict of interest.

**Tables**

422    **Table 1** Dissimilarity S for the retention factors obtained on the five column types

| Column | Amino | Silica | EP | Diol | CN |
|--------|-------|--------|-------|-------|----|
| Amino | 0 | | | | |
| Silica | 99.67 | 0 | | | |
| EP | 68.70 | 85.94 | 0 | | |
| Diol | 89.27 | 61.16 | 38.08 | 0 | |
| CN | 99.80 | 16.91 | 82.90 | 57.72 | 0 |

423

424 **Table 2** Retention factors (k), selectivity (α) and resolutions (Rs) for the analgesics mixture measured

425 with the different combinations of approach 1: **A.** 60 mm CN + 20 mm Amino + 10 mm EP, **B.** 80 mm

426 CN + 30 mm Amino + 10 mm Diol, and **C.** 110 mm Silica + 50 mm Amino + 40 mm CN. Peak

427 numbering, see **Fig. 2**

| Combination | Peak | Retention factor (k) | | | Selectivity (α) | | | Measured resolution (Rs) |
|---|---|---|---|---|---|---|---|---|
| | | Measured | Predicted | Relative deviation (%) | Measured | Predicted | Relative deviation (%) | |
| A | 1 | 0.51 | 0.55 | 6.76 | - | - | - | - |
| | 3 | 1.28 | 1.33 | 4.18 | 2.48 | 2.42 | 2.59 | 3.86 |
| | 4 | 2.75 | 2.88 | 4.46 | 2.16 | 2.16 | 0.29 | 4.43 |
| | 5 | **3.13** | 3.24 | 3.63 | 1.14 | 1.13 | 0,84 | 0.82 |
| | 2 | **3.13** | 4.22 | 29.68 | 1.00 | 1.17 | 15.95 | 0.00 |
| | 6 | **3.13** | 3.59 | 13.90 | 1.00 | 1.11 | 10.28 | 0.00 |
| | 7 | 4.45 | 4.69 | 5.18 | 1.42 | 1.11 | 24.60 | 2.57 |
| | 8 | 7.07 | 8.52 | 18.58 | 1.59 | 1.82 | 13.43 | 3.96 |
| | 9 | 9.38 | 9.84 | 4.78 | 1.33 | 1.16 | 13.83 | 2.57 |
| B | 1 | 0.52 | 0.51 | 2.50 | - | - | - | - |
| | 3 | 1.46 | 1.40 | 4.45 | 2.80 | 2.75 | 1.95 | 6.15 |
| | 4 | 2.98 | 2.92 | 1.89 | 2.04 | 2.09 | 2.57 | 6.17 |
| | 5 | 3.59 | 3.42 | 4.71 | 1.21 | 1.17 | 2.82 | 1.86 |
| | 6 | 4.06 | 3.84 | 5.45 | 1.13 | 1.12 | 0.74 | 1.19 |
| | 2 | 4.73 | 4.35 | 8.38 | 1.17 | 1.13 | 2.94 | 1.44 |
| | 7 | 5.25 | 5.00 | 4.81 | 1.11 | 1.15 | 3.58 | 0.98 |
| | 8 | 9.90 | 9.06 | 8.91 | 1.89 | 1.81 | 4.11 | 6.50 |
| | 9 | 11.01 | 10.63 | 3.53 | 1.11 | 1.17 | 5.39 | 1.14 |
| C | 1 | 0.90 | 0.88 | 2.32 | - | - | - | - |
| | 3 | 1.61 | 1.62 | 0.28 | 1.78 | 1.83 | 2.60 | 6.26 |
| | 4 | 3.20 | 3.12 | 2.46 | 1.98 | 1.93 | 2.75 | 8.58 |
| | 5 | 3.70 | 3.77 | 1.92 | 1.16 | 1.21 | 4.38 | 2.00 |
| | 6 | 4.45 | 4.54 | 2.07 | 1.20 | 1.20 | 0.15 | 2.59 |
| | 7 | 5.87 | 6.06 | 3.32 | 1.32 | 1.34 | 1.25 | 4.05 |
| | 9 | 10.94 | 11.30 | 3.21 | 1.86 | 1.86 | 0.11 | 8.73 |
| | 2 | 22.92 | 23.51 | 2.53 | 2.10 | 2.08 | 0.68 | 12.36 |
| | 8 | 42.35 | 43.35 | 2.34 | 1.85 | 1.84 | 0.19 | 12.66 |

428 **Table 3** Retention factors (k), selectivity (α) and resolutions (Rs) for the analgesics mixture measured

429 on the best combination predicted with approach 2: 60 mm Silica + 40 mm Amino. Peak numbering:

430 see **Fig. 2**

| | Retention factor (k) | | | Selectivity (α) | | | Measured resolution (Rs) |
|---|---|---|---|---|---|---|---|
| Peak | Measured | Predicted | Relative deviation (%) | Measured | Predicted | Relative deviation (%) | |
| 1 | 0.78 | 0.89 | 13.21 | - | - | - | - |
| 3 | 1.99 | 2.07 | 3.93 | 2.54 | 2.32 | 9.29 | 6.94 |
| 4 | 3.40 | 3.54 | 4.00 | 1.71 | 1.71 | 0.07 | 5.20 |
| 5 | 4.76 | 4.82 | 1.18 | 1.40 | 1.36 | 2.81 | 3.53 |
| 6 | 5.57 | 5.83 | 4.63 | 1.17 | 1.21 | 3.44 | 1.68 |
| 7 | 7.07 | 7.65 | 7.93 | 1.27 | 1.31 | 3.31 | 2.63 |
| 9 | 14.82 | 14.48 | 2.34 | 2.10 | 1.89 | 10.27 | 7.21 |
| 2 | 14.82 | 25.86 | 54.24 | 1.00 | 1.79 | 56.41 | 0 |
| 8 | 27.72 | 46.47 | 50.54 | 1.87 | 1.80 | 3.98 | 7.76 |

431

432 **Table 4** Retention factors (k), selectivity (α) and resolution (Rs) for the analgesics mixture measured on

433 the best combination predicted with approach 3: 70 mm Silica + 30 mm Amino. Peak numbering: see

434 **Fig. 2**

| | Retention factor (k) | | | Selectivity (α) | | | Measured resolution (Rs) |
|---|---|---|---|---|---|---|---|
| Peak | Measured | Predicted | Relative deviation (%) | Measured | Predicted | Relative deviation (%) | |
| 1 | 0.81 | 0.97 | 17.80 | - | - | - | - |
| 3 | 1.60 | 1.89 | 16.67 | 1.97 | 1.95 | 1.14 | 4.75 |
| 4 | 3.04 | 3.40 | 11.35 | 1.90 | 1.80 | 5.35 | 5.46 |
| 5 | 3.78 | 4.41 | 15.23 | 1.25 | 1.30 | 3.90 | 2.09 |
| 6 | 4.44 | 5.36 | 18.87 | 1.17 | 1.22 | 3.66 | 1.53 |
| 7 | 5.62 | 7.15 | 24.00 | 1.27 | 1.33 | 5.19 | 2.37 |
| 9 | 11.71 | 13.38 | 13.34 | 2.08 | 1.87 | 10.75 | 7.41 |
| 2 | 14.00 | 28.63 | 68.60 | 1.20 | 2.14 | 56.55 | 2.17 |
| 8 | 25.85 | 53.09 | 69.02 | 1.85 | 1.85 | 0.47 | 8.50 |

435

437
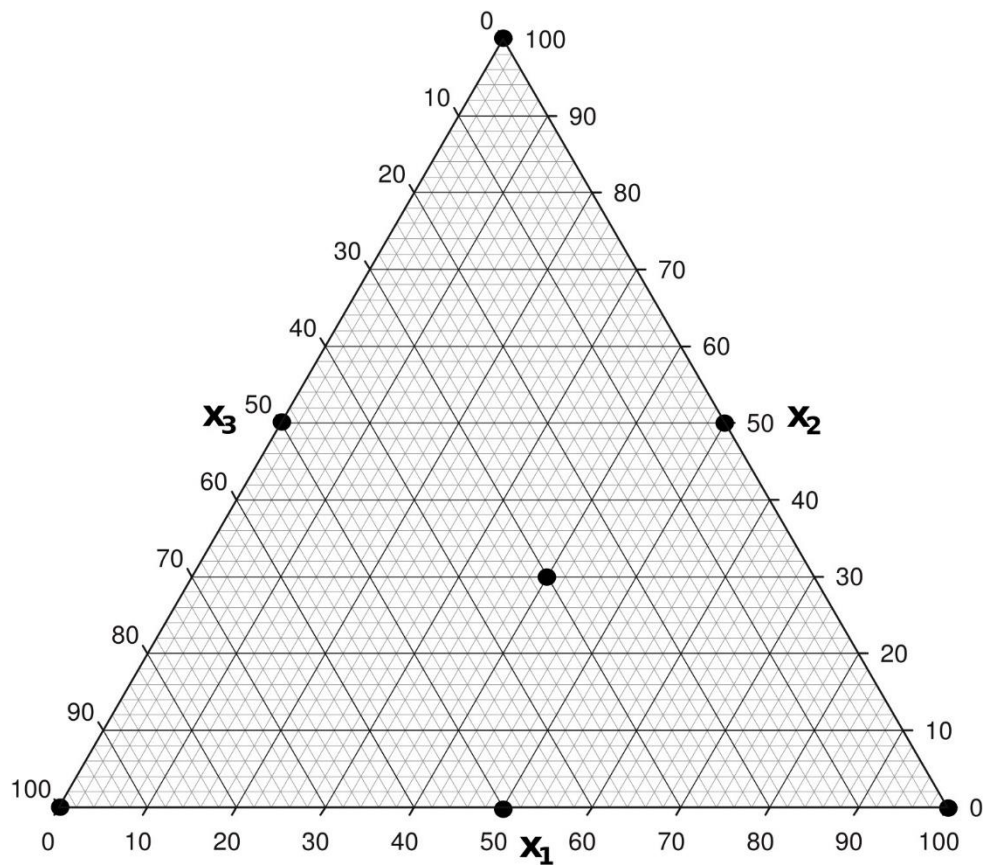
**Fig. 1** Simplex centroid design for a ternary mixture with an 'adjusted centroid', leading to seven measurement points: (1, 0, 0), (0, 1, 0), (0, 0, 1), (0.5, 0.5, 0), (0, 0.5, 0.5), (0.5, 0, 0.5) and (0.4, 0.3, 0.3)
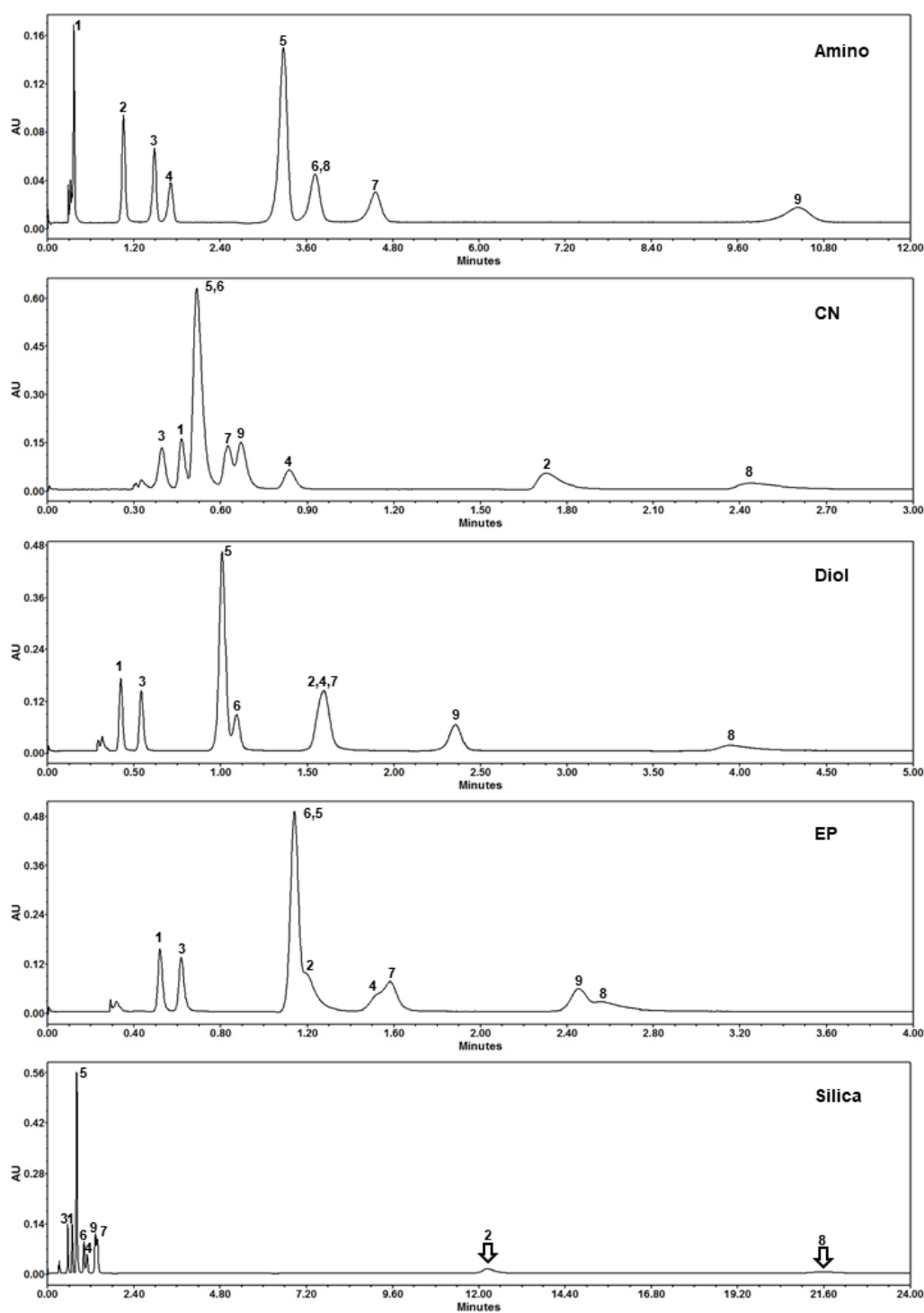
440

**Fig. 2** Chromatograms obtained for the separation of the analgesic components on the five different segments (Amino, Cyano (CN), Diol, Ethylpyridine (EP) and Silica) with 100 mm length. Mobile phase: see text. A flow rate of 2 mL/min and a backpressure of 150 bar were applied. **1.** caffeine, **2.** codeine, **3.** ibuprofen, **4.** acetaminophen, **5.** naproxen, **6.** ketoprofen, **7.** indomethacin, **8.** morphine, and **9.** diclofenac

446
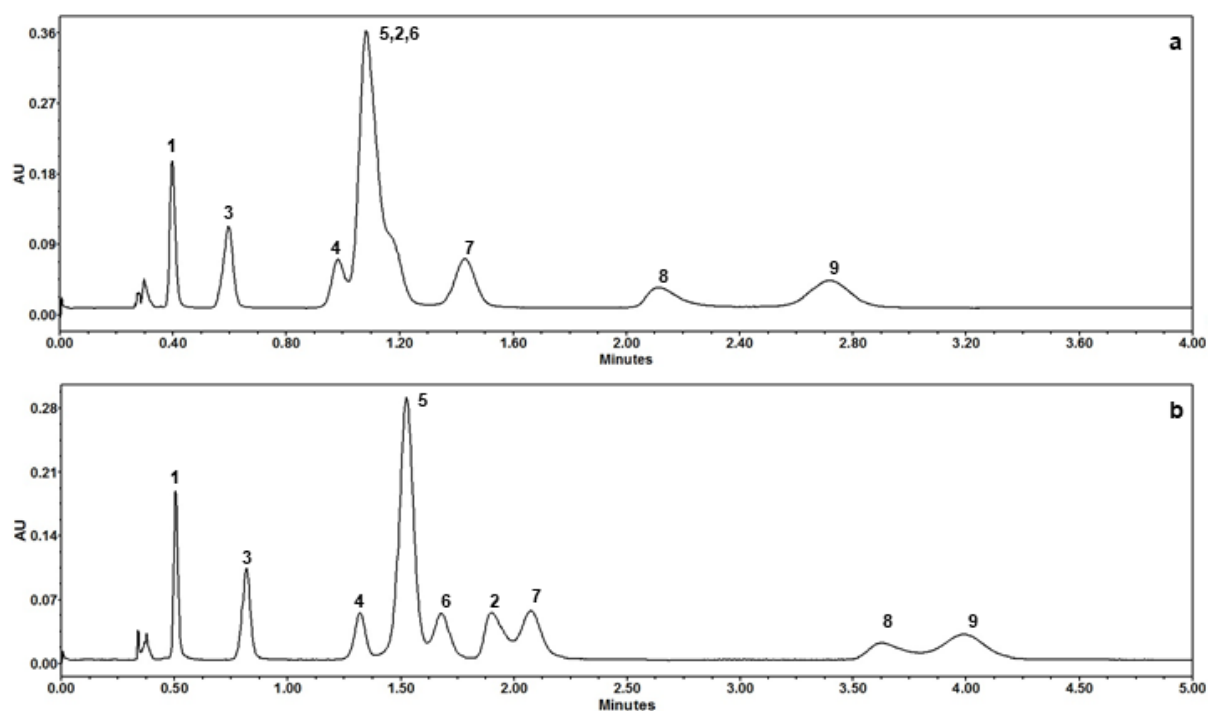
447 **Fig. 3 (a)** Experimental chromatogram for the 'optimal' combination, predicted minimal resolution of

448 1.5 and a maximal column length of 200 mm, for approach 1: 60 mm CN + 20 mm Amino + 10 mm EP.

449 **(b)** Experimental chromatogram for the 'optimal' combination, predicted minimal resolution of 2 and a

450 maximal column length of 200 mm, for approach 1: 80 mm CN + 30 mm Amino + 10 mm Diol.
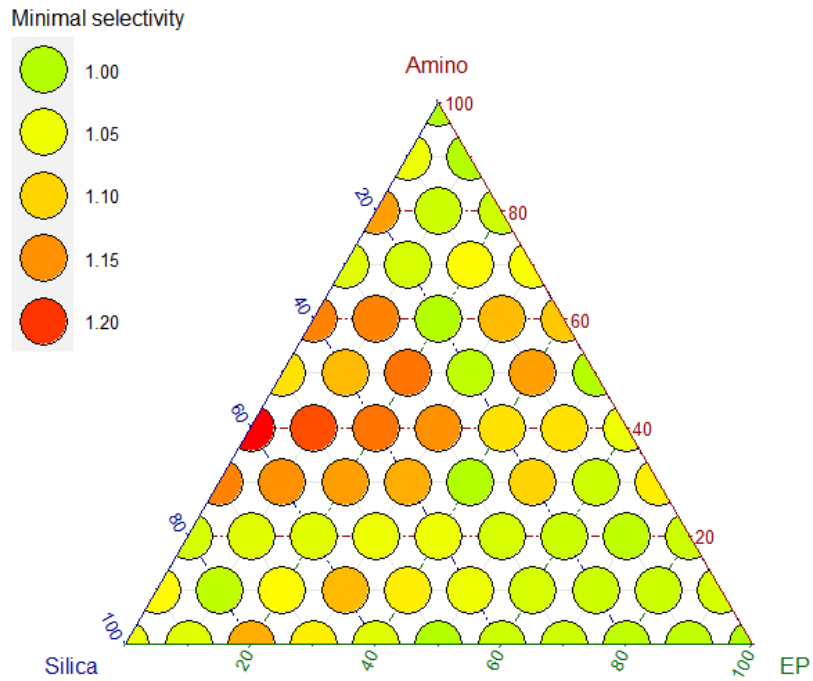
451 Flow rate: 2 mL/min. Backpressure: 150 bar. Mobile phase: see text. Numbering: see **Fig. 2**

452
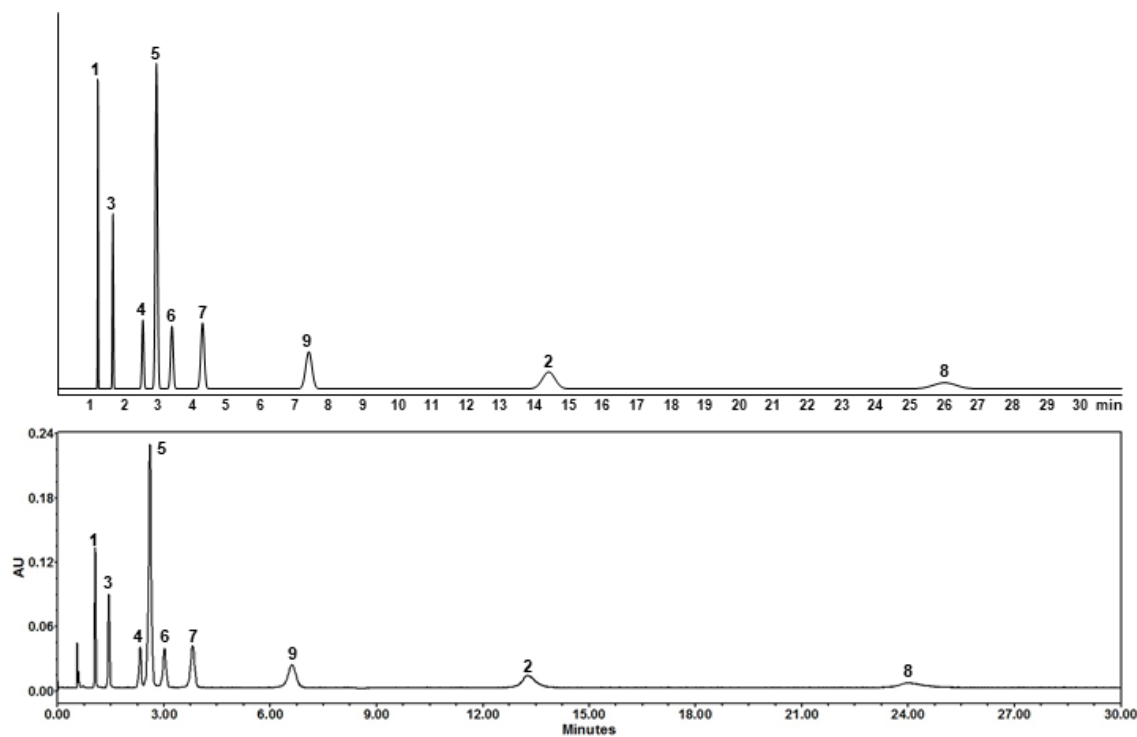


453

454 **Fig. 4** Predicted (top) and experimental (bottom) chromatogram of the 'best' combination for the

455 analgesics mixture, resulting from approach 1: 110 mm Silica + 50 mm Amino + 40 mm CN. Flow rate:

456 2 mL/min. Backpressure: 150 bar. Mobile phase: see text. Numbering: see **Fig. 2**

**Fig. 5** Ternary phase diagram, showing the minimal selectivity α of each combination of 100 mm between the Amino, Silica and EP stationary-phase segments

460
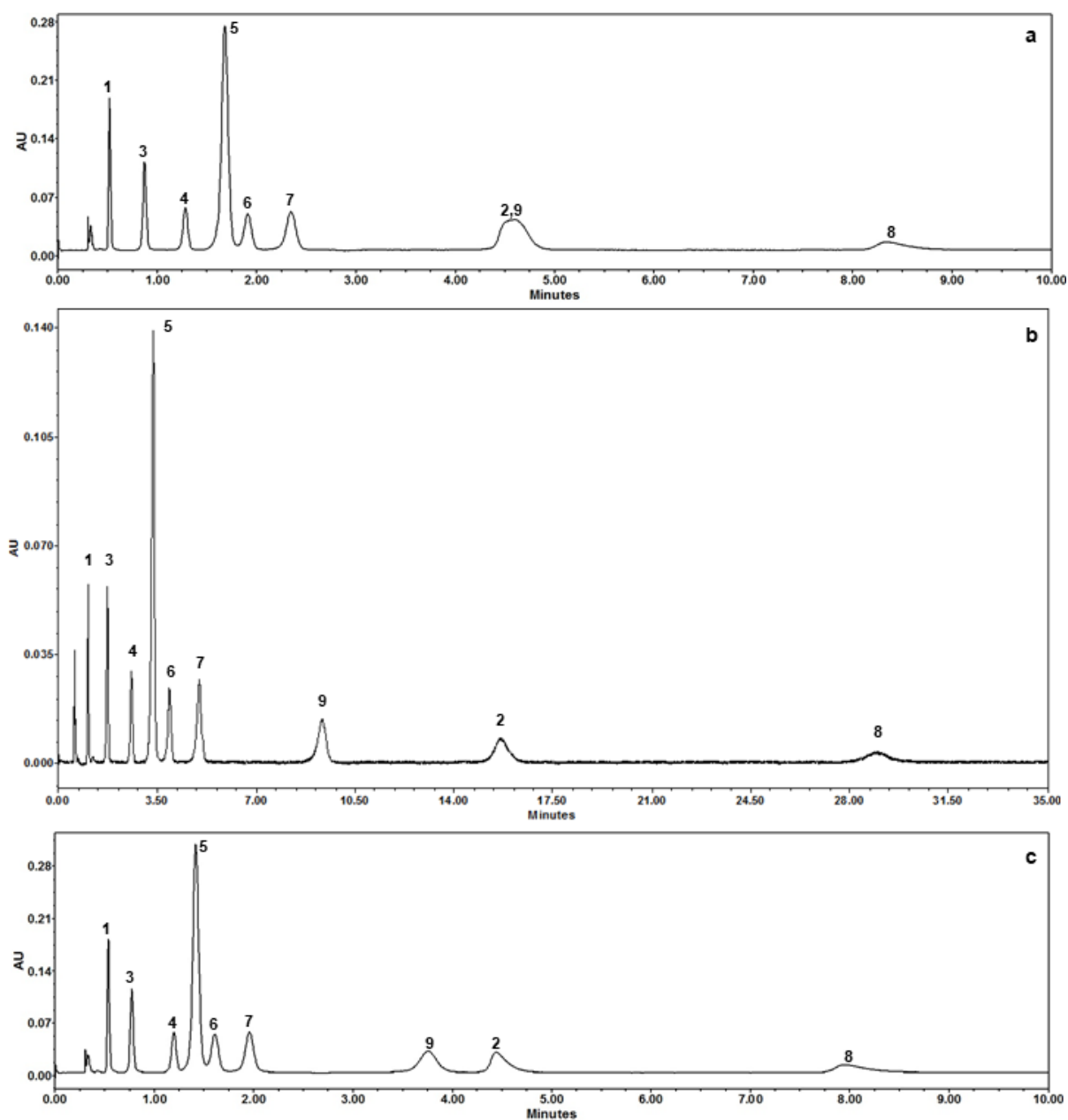
**Fig. 6** Chromatograms from the analgesics mixture **(a)** on 60 mm Silica + 40 mm Amino (approach 2), **(b)** on 120 mm Silica + 80 mm Amino (longer column with the same ratio of the stationary-phase combination with approach 2), and **(c)** on the best prediction from approach 3: 70 mm Silica + 30 mm Amino. Flow rate: 2 mL/min. Backpressure: 150 bar. Mobile phase: see text. See **Fig. 2** for the peak identification

466 **References**

467 1. Rajendran A. Design of preparative-supercritical fluid chromatography. J Chromatogr A.

468 2012;1250:227–49.

469 2. Taylor LT. Supercritical fluid chromatography for the 21st century. J Supercrit Fluids. 2009;47:566–

470 73.

471 3. Lesellier E, West C. The many faces of packed column supercritical fluid chromatography - A critical

472 review. J Chromatogr A. 2015;1382:2–46.

473 4. Majewski W, Valery E, Ludemann-Hombourger O. Principle and Applications of Supercritical Fluid

474 Chromatography. J Liq Chromatogr Relat Technol. 2005;28:1233–52.

475 5. Wang C, Tymiak AA, Zhang Y. Optimization and simulation of tandem column supercritical fluid

476 chromatography separations using column back pressure as a unique parameter. Anal Chem.

477 2014;86:4033–40.

478 6. Berger TA, Wilson WH. Packed Column Supercritical Fluid Chromatography with 220 000 Plates.

479 Anal Chem. 1993;65:1451–5.

480 7. Pirkle WH, Welch CJ. Some thoughts on the coupling of dissimilar chiral columns or the mixing of

481 chiral stationary phases for the separation of enantiomers. J Chromatogr A. 1996;731:322–6.

482 8. Gaudin K, Lesellier E, Chaminade P, Ferrier D, Baillet A, Tchapla A. Retention behaviour of

483 ceramides in sub-critical fluid chromatography in comparison with non-aqueous reversed-phase liquid

484 chromatography. J Chromatogr A. 2000;883:211–22.

485 9. Lesellier E, West C, Tchapla A. Advantages of the use of monolithic stationary phases for modelling

486 the retention in sub/supercritical chromatography: Application to cis/trans-β-carotene separation. J

487 Chromatogr A. 2003;1018:225–32.

488 10. Deschamps FS, Lesellier E, Bleton J, Baillet A, Tchapla A, Chaminade P. Glycolipid class profiling

489 by packed-column subcritical fluid chromatography. J Chromatogr A. 2004;1040:115–21.

490 11. Barnhart WW, Gahm KH, Thomas S, Notari S, Semin D, Cheetham J. Supercritical fluid

491  chromatography tandem-column method development in pharmaceutical sciences for a mixture of four

492  stereoisomers. J Sep Sci. 2005;28:619–26.

493  12. Welch CJ, Biba M, Gouker JR, Kath G, Augustine P, Hosek P. Solving multicomponent chiral

494  separation challenges using a new SFC tandem column screening tool. Chirality. 2007;19:184–9.

495  13. Abrahamsson V, Rodriguez-Meizoso I, Turner C. Determination of carotenoids in microalgae using

496  supercritical fluid extraction and chromatography. J Chromatogr A. 2012;1250:63–8.

497  14. Lesellier E, Latos A, de Oliveira AL. Ultra high efficiency/low pressure supercritical fluid

498  chromatography with superficially porous particles for triglyceride separation. J Chromatogr A.

499  2014;1327:141–8.

500  15. Phinney KW, Sander LC, Wise SA. Coupled achiral/chiral column techniques in subcritical fluid

501  chromatography for the separation of chiral and nonchiral compounds. Anal Chem. 1998;70:2331–5.

502  16. West C, Lemasson E, Bertin S, Hennig P, Lesellier E. Interest of achiral-achiral tandem columns for

503  impurity profiling of synthetic drugs with supercritical fluid chromatography. J Chromatogr A.

504  2018;1534:161–9.

505  17. Galea C, Mangelings D, Vander Heyden Y. Characterization and classification of stationary phases

506  in HPLC and SFC - a review. Anal Chim Acta. 2015;886:1–15.

507  18. Alvarez-Segura T, Torres-Lapasio JR, Ortiz-Bolsico C, Garcia-Alvarez-Coque MC. Stationary

508  phase modulation in liquid chromatography through the serial coupling of columns: A review. Anal

509  Chim Acta. 2016;923:1–23.

510  19. Nyiredy S, Szucs Z, Szepesy L. Stationary phase optimized selectivity liquid chromatography: Basic

511  possibilities of serially connected columns using the "PRISMA" principle. J Chromatogr A.

512  2007;1157:122–30.

513  20. Nyiredy S, Szűcs Z, Szepesy L. Stationary-Phase Optimized Selectivity LC (SOS-LC): Separation

514  Examples and Practical Aspects. Chromatographia. 2006;63:S3–S9.

515  21. Kuehnle M, Rehbein J, Holtin K, Dietrich B, Gradl M, Yeman H, et al. Phase optimized liquid

516 chromatography as an instrument for steroid analysis. J Sep Sci. 2008;31:1655–61.

517 22. Matysik F, Schumann U, Engewald W. Isocratic Liquid Chromatography with Segmented Columns

518 and Simultaneous UV and Dual Electrochemical Detection: Application to the Selectivity Enhancement

519 for the Determination of Explosives. Electroanalysis. 2008;20:98–101.

520 23. Zedda M, Tuerk J, Teutenberg T, Peil S, Schmidt TC. A strategy for the systematic development of

521 a liquid chromatographic mass spectrometric screening method for polymer electrolyte membrane

522 degradation products using isocratic and gradient phase optimized liquid chromatography. J Chromatogr

523 A. 2009;1216:8910–7.

524 24. Gostomski I, Braun R, Huber CG. Detection of low-abundance impurities in synthetic thyroid

525 hormones by stationary phase optimized liquid chromatography-mass spectrometry. Anal Bioanal

526 Chem. 2008;391:279–88.

527 25. Deconinck E, Kamugisha A, Van Campenhout P, Courselle P, De Beer JO. Development of a

528 Stationary Phase Optimised Selectivity Liquid Chromatography based screening method for

529 adulterations of food supplements for the treatment of pain. Talanta. 2015;138:240–6.

530 26. Deconinck E, Ghijs L, Kamugisha A, Courselle P. Comparison of three development approaches for

531 Stationary Phase Optimised Selectivity Liquid Chromatography based screening methods Part I: A

532 heterogeneous group of molecules (slimming agents in food supplements). Talanta. 2016;148:518–28.

533 27. Deconinck E, Ghijs L, Kamugisha A, Courselle P. Comparison of three development approaches for

534 Stationary Phase Optimised Selectivity Liquid Chromatography based screening methods Part II: A

535 group of structural analogues (PDE-5 inhibitors in food supplements). Talanta. 2016;148:346–55.

536 28. Deconinck E, Djiogo CAS, Kamugisha A, Courselle P. The use of Stationary Phase Optimized

537 Selectivity Liquid Chromatography for the development of herbal fingerprints to detect targeted plants

538 in plant food supplements. Talanta. 2017;170:441–50.

539 29. De Beer M, Lynen F, Chen K, Ferguson P, Hanna-Brown M, Sandra P. Stationary-Phase Optimized

540 Selectivity Liquid Chromatography: Development of a Linear Gradient Prediction Algorithm. Anal

541    Chem. 2010;85:1733–43.

542    30. De Beer M, Lynen F, Hanna-Brown M, Sandra P. Multiple Step Gradient Analysis in Stationary

543    Phase Optimised Selectivity LC for the Analysis of Complex Mixtures. Chromatographia. 2009;69:609–

544    14.

545    31. Chen K, Lynen F, De Beer M, Hitzel L, Ferguson P, Hanna-Brown M, et al. Selectivity optimization

546    in green chromatography by gradient stationary phase optimized selectivity liquid chromatography. J

547    Chromatogr A. 2010;1217:7222–30.

548    32. Hegade RS, De Beer M, Lynen F. Chiral stationary phase optimized selectivity liquid

549    chromatography: A strategy for the separation of chiral isomers. J Chromatogr A. 2017;1515:109–17.

550    33. Delahaye S, Lynen F. Implementing Stationary-Phase Optimized Selectivity in Supercritical Fluid

551    Chromatography. Anal Chem. 2014;86:12220–8.

552    34. Hegade RS, Lynen F. Chiral stationary phase optimized selectivity supercritical fluid

553    chromatography: A strategy for the separation of1. Hegade RS, Lynen F. Chiral stationary phase

554    optimized selectivity supercritical fluid chromatography: A strategy for the separation of mix. J

555    Chromatogr A. 2019;1586:116–27.

556    35. Galea C, Mangelings D, Vander Heyden Y. Method development for impurity profiling in SFC: The

557    selection of a dissimilar set of stationary phases. J Pharm Biomed Anal. 2015;111:333–43.

558    36. D'Attoma A, Grivel C, Heinisch S. On-line comprehensive two-dimensional separations of charged

559    compounds using reversed-phase high performance liquid chromatography and hydrophilic interaction

560    chromatography. Part I: Orthogonality and practical peak capacity considerations. J Chromatogr A.

561    2012;1262:148–59.

562    37. Muscat Galea C, Mangelings D, Vander Heyden Y. Investigation of the effect of mobile phase

563    composition on selectivity using a solvent-triangle based approach in achiral SFC. J Pharm Biomed

564    Anal. 2017;132:247–57.

565    38. Cornell JA. Experiments with Mixtures: Designs, Models, and the Analysis of Mixture Data, Third

566 Edition. New York: John Wiley & Sons; 2002.

567 39. Piepel GF, Cornell JA. Mixture experiment approaches: examples, discussion, and
568 recommendations. J Qual Technol. 1994;26:177–96.

569 40. Hamilton NE, Ferry M. ggtern: Ternary Diagrams Using ggplot2. J Stat Softw. 2018;87:1–17.

570 41. Neue UD, O'Gara JE, Méndez A. Selectivity in reversed-phase separations: Influence of the
571 stationary phase. J Chromatogr A. 2006;1127:161–74.

572 42. Romand S, Rudaz S, Guillarme D. Separation of substrates and closely related glucuronide
573 metabolites using various chromatographic modes. J Chromatogr A. 2016;1435:54–65.

574