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Coupling of chiral and achiral stationary phases in supercritical fluid chromatography

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Link to publication

1 **Coupling of chiral and achiral stationary phases in supercritical fluid chromatography: evaluating**

2 **and improving retention prediction**

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22   **Abstract**

23   Isomers and stereoisomers are always challenging to separate. Column coupling may provide

24   improved chromatographic selectivity, necessary for the separation of the compounds with similar

25   chemical and structural properties. The relatively low viscosity of supercritical fluids, used as mobile

26   phases allows for the coupling of several columns in series in supercritical fluid chromatography

27   (SFC), without exceeding the pressure limits of the system. The aim of this study is to propose

28   reliable prediction of the retention behaviour of analytes on a coupled column system, based on a

29   limited number of initial analyses. The chiral compounds atenolol, ephedrine, propranolol,

30   mianserin, labetalol and nadolol, besides the diastereomers quinine and quinidine, and the

31   structural isomers of aminophenol and aminocresol were used as model analytes. The retention

32   behaviour of the analytes was determined on the individual chiral columns Lux Cellulose-1, Lux

33   Cellulose-2, Lux Cellulose-3, Lux Cellulose-4, Lux Amylose-2 and the achiral columns Luna NH_2, Luna

34   Silica, Synergi RP and FluoroSep RP. The mobile phase was composed of CO_2 mixed with 20% (v/v)

35   MeOH, which contained 0.1% (v/v) trifluoroacetic acid and 0.1% (v/v) isopropylamine. The retention

36   factors of the analytes on coupled stationary phases were predicted, and subsequently compared to

37   the experimentally obtained ones. Relative deviations of predicted and experimental retention

38   factors were in range from 0.00% to 51.91%. Flow rate and back pressure of the screening

39   conditions were adjusted to improve prediction precision on four column combinations, with varying

40   success rates. The average relative deviations of retention factors were reduced to 2.84% - 6.59% by

41   adjusting flow rate, and to 2.30% - 8.57% by adjusting back pressure. The most successful approach,

42   flow rate adjustment, was then applied to select a column combination providing improved

43   resolution of the structurally similar components of silymarin extract.

44   **Keywords:** supercritical fluid chromatography, column coupling, chiral and achiral separations,

45   retention prediction

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1. Introduction

Supercritical fluid chromatography (SFC) is a separation technique developed in the 1960s with an initial commercial growth in the 1980s [1,2]. The major benefits of the technique are attributed to the properties of supercritical fluids. SFC primarily uses CO_2 as mobile phase, which has a critical pressure and temperature of 73.8 bar and 31.1°C, respectively [3]. Thanks to the lower viscosity and better diffusion properties in comparison to LC, higher velocities in the column can be used [4]. This leads to an increased throughput as well as a reduced analysis time and organic solvent consumption, compared to conventional HPLC. These features of SFC are beneficial also in terms of organic solvent cost and waste reduction, being an important asset in the pharmaceutical industry, while they present a reduced impact on the environment as well [5].

The benefits for enantioseparation were recognised early on in SFC development, with the first separation of enantiomers by SFC reported in 1985 [6]. Nowadays, SFC is well established for the separation of chiral compounds [7]. Chiral method development is a labour intensive and time-consuming process, mainly based on a trial-and-error approach, given the unpredictability and complexity of enantioselectivity [8]. Because of the properties of the stationary phase, which are the main determinants of retention behaviour and enantioselectivity of the analytes, its choice is the most crucial step in method development [9].

The low viscosity of the mobile phase, responsible for the low pressure drop across the column, and the compatibility of the same mobile phase with a variety of stationary phases are the main features allowing column coupling in SFC. Several methods have been published taking advantage of the properties of SFC for column coupling [10–22]. Berger and Wilson [10] coupled several columns with the same stationary phase, achieving high efficiencies on a 2.2 m long column for the separation of polycyclic aromatic hydrocarbons. Phinney et al. [11] demonstrated the use of coupled chiral and achiral stationary phases for the separation of structurally related β-blockers and 1,4-benzodiazepines. In the work of Lesellier et al. [15,16] coupling of two to seven stationary phases

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72   was explored to separate the isomers of β-carotene. Serial coupling of silica and diol columns

73   improved the selectivity for glycolipids in the work of Deschamps et al. [17]. Barnhart et al. [18] used

74   the chiral columns Chiralpak AD-H and Chiralcel OD-H, coupled in tandem, to separate a mixture of

75   four in-house synthesized stereoisomers. Adequate separation of the four stereoisomers was not

76   achieved using a single chiral column, but the individual column results indicated the possibility of

77   the separation on a coupled column system [18]. Welch et al. [20] developed a column screening

78   tool for tandem column coupling to separate complex or multicomponent chiral mixtures. In other

79   studies, the advantages of column coupling were used for impurity profiling [12], for steroid profiling

80   [13], for the stereoisomeric separation of potential anti-diabetic compounds [14], to separate

81   spirostanol diastereomers [22], and when the separation on a single column was found insufficient

82   [19,21].

83   When coupling columns in series, the column positioned as first is operating under a higher

84   pressure. This causes an increase in density of the mobile phase, and consequently of its solvation

85   power, resulting in a decrease in retention compared to the column positioned downstream. The

86   importance of column back pressure as optimization parameter, and of column sequence in a

87   coupled column system was highlighted in the work of Wang et al. [23,24]. This, as well as the

88   advantage of coupling columns with different selectivities, was demonstrated on the separation of

89   stereoisomers of molecules with more than one chiral centre [23,24]. The importance of pressure in

90   a coupled-columns system was further illustrated in respective works of Lynen's group [25,26]. For

91   the prediction of an optimal column combination to separate an achiral and chiral compounds

92   mixture, they used the stationary-phase optimized selectivity supercritical fluid chromatography

93   methodology (SOS-SFC)[25,26], where a selection of stationary phases is made using retention

94   factors from a screening step. The columns are then connected with zero-dead-volume couplers.

95   Correspondence between predictions and experiments improved when the same average pressure

96   was used in all experiments by adapting the back pressure or the flow rate.

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97   In the actual study, five chiral and four achiral conventional columns are screened under

98   generic chromatographic conditions for their ability to separate enantiomers and/or structural

99   isomers. Combinations of coupled columns, a total of 60 systems coupled by PEAK couplers, were

100   screened under the same conditions. The aim of this work is to define a stationary-phase selection

101   approach in the method-optimization process in real lab conditions, maximizing the chance that the

102   most effective column combinations are chosen for the separation of given mixtures. The stationary-

103   phase selection is based on a prediction approach with the highest correspondence to the

104   experimental results. The effects of flow rate and back pressure changes are explored to further

105   improve the prediction ability of the employed model. The findings are then applied to select the

106   best column combination for the separation of silymarin, an extract from the *Silybum marianum*

107   plant. This mixture consists of six structurally similar flavonolignans, silybin A (SB A), silybin B (SB B),

108   isosilybin A (ISB A), isosilybin B (SB B), silychristin (SCH), silydianin (SD), and the flavonoid taxifolin

109   (TX).

110   **2. Material and methods**

111   **2.1 Chemicals and reagents**

112   The following compounds were used as a model sample set (Figure S-1): (R)-(+)-atenolol (99%), (S)-

113   (-)-atenolol (99%), (1R,2S)-(-)-ephedrine (99%), quinine (99%), labetalol, nadolol, *o*-aminophenol

114   (99%), *m*-aminophenol, *p*-aminophenol, 2-amino-*m*-cresol (96%), 2-amino-*p*-cresol (97%), and 5-

115   amino-*o*-cresol (97%), all purchased from Sigma-Aldrich (St. Louis, MO, USA), (1S,2R)-(+)-ephedrine

116   (99%), (R)-propranolol (99%), (S)-propranolol (99%), and quinidine (90%) obtained from Fluka

117   Chemie (Neu-Ulm, Switzerland), (S)-(+)-mianserin and (R)-(-)-mianserin were gifts. CO_2 quality

118   4.5 (purity ≥ 99.995%) was from Messer (Sint-Pieters-Leeuw, Belgium). HPLC grade methanol

119   was provided by VWR Chemicals (Fontenay-sous-Bois, France). Trifluoroacetic acid (99%) and

120   isopropylamine (≥ 99.5%) were purchased from Aldrich (Steinheim, Germany).

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121 The standards of SB B (≥95%), ISB A (≥95%), SCH (≥95%), SD (≥95%), and TX (≥85%) were

122 purchased from Sigma-Aldrich (Steinheim, Germany). SB (≥92.53%) was obtained from HWI

123 Analytik (Rülzheim, Germany). ISB B and SD were provided by PhytoLab (Vestenbergsgreuth,

124 Germany).

125 **2.2 Stationary phases**

126   Chiral stationary phases Lux Cellulose-1 (LC-1), Lux Cellulose-2 (LC-2), Lux Cellulose-3 (LC-3),

127 Lux Cellulose-4 (LC-4), and Lux Amylose-2 (LA-2) were from Phenomenex (Utrecht, The

128 Netherlands). The dimensions of all chiral columns were 250 mm x 4.6 mm i.d. with 3 μm

129 particle size. Achiral columns Luna NH2 (LNH2), Luna Silica (LS), both with dimensions 100 mm

130 x 4.6 mm and particle size 3μm, and Synergi Polar RP (SRP), 100 mm x 4.6 mm with 4 μm

131 particle size, were also obtained from Phenomenex. The achiral column FluoroSep-RP (FSRP)

132 Phenyl with dimensions 150 mm x 4.6 mm and 3 μm particle size was acquired from ES

133 Industries (West Berlin, Germany).

134 **2.3 Instruments**

135 All analyses were performed on an SFC Acquity Ultra Performance Convergence Chromatography

136 (UPC^2) system from Waters (Milford, MA, USA). The system is equipped with a binary pump, an

137 autosampler with a fixed loop of 10 μL thermostated at 10°C, a convergence manager, a photo diode

138 array (PDA) detector, a back pressure regulator and an external column oven. For data collection and

139 processing, the Empower software (3 V7.10, 2010, Waters) was used.

140 **2.4 Preparation of standard and test solutions**

141 Stock solutions for each individual compound were prepared by dissolving the appropriate amount

142 of test set standard in 10 mL MeOH to achieve a concentration of 0.5 mg/mL (or 1 mg/mL for

143 aminocresol). Standard solutions of each individual compound were prepared by diluting the stock

144 solutions with MeOH to achieve final concentrations of 0.25 mg/mL (0.20 mg/mL for aminocresol).

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145   Mixture solutions of structural of positional isomers of the same compound were prepared by

146   transferring appropriate amounts of stock solutions into a 10 mL test tube, leading to final

147   concentrations of 0.25 mg/mL for each analyte (concentrations of aminophenol and aminocresol

148   were 0.17 and 0.20 mg/mL, respectively).

149   Stock solutions of flavonolignans of the silymarin complex and of TX were prepared by dissolving the

150   appropriate amount of the standard in MeOH to achieve a concentration of 1 mg/mL (or 2 mg/mL

151   for SB). Test solutions were prepared by diluting 100 μL stock solution with MeOH to achieve 100

152   μg/mL (200 μg/mL for SB). A mixture solution was prepared by adding 100 μL of each standard

153   solution to 400 μL MeOH.

154   **2.5 Chromatographic screening conditions**

155   For the screening experiments, generic chromatographic conditions from an earlier defined chiral

156   separation strategy were used [27,28]. All screening experiments were performed in isocratic mode

157   at a constant flow rate of 3.0 mL/min, with a mobile phase consisting of 20% MeOH, containing 0.1%

158   (v/v) isopropylamine and 0.1% (v/v) trifluoroacetic acid, combined with 80% CO_2. The back pressure

159   was kept at 150 bar and the column temperature at 30°C, while the temperature of the sample

160   compartment was 10°C. The injection volume was 5 μL and the detection wavelength was 220 nm.

161   **2.6 Calculations**

162   The test set analytes were initially screened on the individual chiral and achiral columns using the

163   above screening conditions. To predict the retention factors of analytes in coupled column systems,

164   three equations were employed and compared. Eq. 1 was designed to calculate the retention factor

165   of a given compound on serially connected stationary phases in liquid chromatography [29]. Eq. 2

166   was an update for SFC to account for the void time [30], while Eq. 3 is based on a similar expression

167   used in gas chromatography [31].

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169 $$k_{A,B} = \frac{\Phi_A k_A + \Phi_B k_B}{\Phi_A + \Phi_B}$$ (Eq. 1) [29]

170 $$k_{A,B} = \frac{\Phi_A k_A t_{0A} + \Phi_B k_B t_{0B}}{\Phi_A t_{0A} + \Phi_B t_{0B}}$$ (Eq. 2) [30]

171 $$k_{A,B} = k_A t_{0A} + k_B t_{0B}$$ (Eq. 3) [31]

172 where $k_{A,B}$ is the predicted retention factor of an analyte in a coupled column system, k_A and k_B are

173 the experimental retention factors on the individual stationary phases, Φ_A and Φ_B represent the

174 column lengths of the stationary phases (see section 2.2), while t_{0A} and t_{0B} are the void times of the

175 individual stationary phases.

176 The experimental retention factors of the analytes on a given column are calculated using the

177 equation $k = (t_R - t_0)/t_0$, where t_R stands for the retention time of the analyte and t_0 the void time,

178 which was determined as the retention time of the first system peak on the chromatogram. The

179 equation $t_R = kt_0 + t_0$ was used to calculate retention times predicted on coupled columns from

180 predicted retention factors and predicted void times.

181 Correspondence between calculated (k_{cal}) and experimentally obtained retention factors (k_{exp}) values

182 was assessed by the relative deviation (RD) [32]:

183 $$RD(\%) = 100 \, \frac{|k_{cal} - k_{exp}|}{(k_{cal} + k_{exp})/2}$$ (Eq. 4)

184 **3. Results and discussion**

185 **3.1. Column screening and selection of prediction equations**

186 All model compounds were analysed on the chiral and achiral columns under the initial screening

187 conditions. The same chromatographic conditions (Section 2.5) were applied for screening of the

188 coupled column systems. All combinations coupling chiral - chiral (CH-CH), chiral - achiral (CH-ACH)

189 and achiral - chiral (ACH-CH) columns were tested. The combinations of achiral and achiral stationary

190 phases were not examined.

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191   The isomers were analysed first separately to determine the elution order of the isomers, and then

192   as a mixture. Values of k, t_R, and t_0 obtained from analysis of the mixtures were used in the

193   calculations.

194   To assess the best way to predict the retention factors of analytes on coupled columns, three

195   equations (Eqs. 1-3) were applied. The estimated retention factors were compared to the

196   experimental on the coupled columns. The best prediction model should provide the smallest

197   difference between predicted and experimental results. Averages and ranges of relative deviations

198   for categories of coupled combinations are listed in Table 1.

199   The least analogy between predicted and experimental retention factors of analytes on coupled

200   columns was achieved using Eq. 3. The equation predicts retention factors from the individual

201   retention factors and void times from single column screening, without considering the lengths of

202   the columns. The average relative deviations for ACH-CH and CH-ACH systems, which were 35 – 40

203   cm long, were 27.1% and 33.0%, respectively. The average relative deviation for 50 cm long CH-CH

204   systems was 59.2%. From the relative deviations obtained using Eq. 3 (Table 1A), it can be concluded

205   that the deviation increased with the length of the coupled columns.

206   Better but still poor prediction ability was obtained when using Eq. 2. This equation considers

207   retention factors, column lengths and void times. Opposed to the Eq.3, the best agreement between

208   predicted and experimental values was on the CH-CH coupled systems (average RD = 6.40%), which

209   are the longest coupled systems. However, this equation performed insufficiently well for the

210   systems where shorter achiral columns were connected to chiral columns, with average relative

211   deviations of 12.0% and 17.8% for the ACH-CH and CH-ACH systems, respectively.

212   Eq. 1 provided the best prediction ability, with average relative deviations of 7.3%, 9.8%, and 6.7%

213   for ACH-CH, CH-ACH, and CH-CH coupled systems, respectively, with 20 examined systems in each

214   category. This equation does not include the void time values of the individual columns for

215   prediction of retention factors on the coupled systems.

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216 The estimated void time of serially coupled columns was expressed as the sum of the single-column

217 void times. Using predicted retention factors and void times, the retention times of the analytes

218 were predicted and compared to the experimental values. The average relative deviations of

219 predicted and experimental retention times were 5.0%, 5.9% and 5.1%, respectively, for ACH-CH,

220 CH-ACH and CH-CH coupled systems (Table 1B). On average, the agreement between predicted and

221 experimental retention times is better than that of retention factors, where average relative

222 deviations are higher, providing promising results for retention time predictions in coupled systems.

223 Depending on its position in the coupled system, the column operates under different pressures.

224 Using the same column pair in different sequences may result in differences in the prediction quality

225 (Fig. 1). An example of such difference is seen for the combination of LA-2 and FSRP columns. When

226 the achiral column FSRP is first in the column sequence, the average relative deviation was 8.4%.

227 When FSRP is placed after the LA-2 column, the % RD increased to 39.2% (Fig. 1 A, B). The retention

228 of analytes in the first column decreases as a consequence of the higher pressure and mobile phase

229 density. Experimental retention factors were closer to the predicted when the FSRP column was

230 placed in first position, where the column had a less impact on the separation, compared to the LA-2

231 column connected as second in the sequence.

232 Another example is the combination of the LS and LC-3 columns (Fig. 1 A, B). Placing the chiral

233 column at the beginning of the system resulted in an average deviation of 3.2%, while having the

234 shorter achiral column first, leads to an increase in the deviation to 10.4%. An example of a

235 difference in a combination of two chiral columns with the same length is LC-1 and LC-3 (Fig. 1 C).

236 Upstream connection of LC-1 led to an %RD of 16.4%. The deviation decreased to 6.65% when LC-1

237 is coupled downstream in the system. In general, changing the sequence in CH-CH systems caused

238 smaller differences in relative deviations as opposed to when achiral and chiral columns were

239 coupled in series. This may be linked to the lower efficiencies of the chiral columns compared to the

240 achiral ones and different column lengths of achiral columns.

10

241   In four occasions, the effect of the pressure difference under which columns operate, resulted in

242   such significant shift between predicted and experimental results, that the elution sequence was not

243   predicted correctly anymore. This was the case for the closely eluting isomers of aminocresol on the

244   LC-3+LC-4 system and for aminophenol on the LC-2+LC-1, LC-1+SRP, and LS+LC-1 systems. In all

245   these examples, at least two of the three isomers co-eluted on one of the columns during single

246   column screening. Because the retention behaviour of co-eluting compounds was unclear in the

247   single column, it was more difficult to predict the retention of the compounds in the coupled

248   systems, even if relative deviations between predicted and experimental retention factors were

249   relatively small.

250   In the LS column, *o*- and *m*- aminophenol eluted as one peak, followed by *p*-aminophenol. The

251   elution sequence of the aminophenol isomers on the LC-1 column was *o*-, *p*-, and *m*-aminophenol.

252   Based on the results from the single column screening, the predicted elution sequence was the same

253   as the observed on the LC-1 chiral column. The experimental elution sequence, however, was as

254   follows: *o*-aminophenol, *m*-aminophenol, *p*-aminophenol, with deviations 1.1%, 33.6%, and 4.8%,

255   respectively. The difference in retention times between the first and last eluting analytes was again

256   less than one minute.

257   Overall, it was possible to predict the correct elution sequence of analytes in a majority of these

258   preliminary experiments (60 systems x 7 analytes). As the density of the mobile phase is of major

259   importance in SFC, the pressure differences complicate the retention behaviour prediction of the

260   analytes. Inconsistency in the mobile phase density as well as the pressure drops in single columns

261   and coupled column systems contributed to large deviations between predicted and measured

262   retention factors. This is especially important when analysing samples where analytes are eluting

263   closely to each other. Therefore, some adjustments of the chromatographic conditions were tested

264   in order to improve the prediction performance.

265   **3.2 Adjustment of chromatographic conditions**

11

266   So far, all the individual and coupled columns were screened under the same chromatographic

267   conditions. Back pressure and flow rate were kept constant, which led to differences in the

268   generated system pressure and therefore in mobile-phase properties. To minimize these differences,

269   flow rate and back pressure were adjusted to generate the same average pressure in the individual

270   column and the coupled system. The average pressure was calculated as the average of

271   backpressure and system pressure. The effectiveness of this approach was demonstrated on four

272   column combinations: LS+LA-2, LC-3+SRP, LA-2+FSRP, LC-2+LC-3, representing ACH-CH, CH-ACH and

273   CH-CH couplings and covering the range of screened columns. The selected column combinations

274   varied both in length of the system and in particle sizes, as well as in their averages and ranges of %

275   RD.

3.2.1 Flow rate adjustment

277   To improve the agreement between experimental and predicted values, the flow rate was adapted

278   to generate the same system pressure on the coupled columns as obtained during the screening of

279   single columns. The flow rate was also adjusted during the individual column screening step, when

280   columns of different lengths were coupled, so that the generated system pressure, and

281   consequently the average pressure, was the same throughout all experiments. For example, the

282   system pressures generated with 3 mL/min flow rate were 239, 256, and 311 bar for LS, LA-2, and

283   LS+LA-2, respectively. In the actual approach, the flow rate of LA-2 was adjusted to 2.66 mL/min to

284   generate 239 bar system pressure resulting in an average pressure of 194.5 bar. The retention

285   factors on the coupled system were predicted using the results from LS (3mL/min) and LA-2 (2.66

286   mL/min) individual column screenings. The predicted retention factors were then compared to the

287   experimental on LS+LA-2, analysed at a flow rate of 1.89 mL/min, which also generated 239 bar

288   system pressure. This approach of flow rate adjustment was applied on the four above-mentioned

289   coupled-column systems using equation 1 to calculate predicted retention factors (see Table 2).

290   For this approach, the void time was calculated using Eq. 5 to predict the retention time in a coupled

291   system:

292      $$t_{03} = \frac{t_{01}\ FR_1 + t_{02}\ FR_2}{FR_3} \qquad \text{(Eq. 5)}$$

293   where t_{01} and t_{02} are the void times of the individual stationary phases, FR_1 and FR_2 the flow rates on

294   the individual stationary phases, FR_3 the flow rate on the coupled-column system, and t_{03} the void

295   time of the coupled-column system.

296   The first examined combination was the ACH-CH system with LS and LA-2. The pressure generated

297   on LA-2 using the general screening conditions at 3 mL/min was 256 bar (average pressure 203 bar).

298   To reach the same system pressure, the LS column had to be operated at an increased flow rate of

299   3.40 mL/min. A flow rate of 2.20 mL/min generated the same pressure on the coupled column

300   system. The predicted retention factors were compared to the experimental as % RD obtained at

301   adjusted conditions (Table 2). The average deviation for retention factors (Table 2) and retention

302   times (Table 3) decreased from 10.61% and 8.2% for initial results, to 6.3% and 3.4%, respectively.

303   The same trend was seen on this system when flow rates were adjusted to generate the system

304   pressure of 239 bar (average pressure 194.5 bar) generated as described at the beginning of this

305   section. The average and maximal deviations of retention factors (Table 2) in the LS+LA-2 system

306   decreased from 10.6% and 21.1% to 6.6% and 16.4%, respectively. The retention times (Table 3) also

307   resembled the predicted values better when applying a flow rate change, as opposed to the initial

308   predictions, decreasing from 8.2% to 4.9% average RD.

309   The experimental retention factor of *o*-aminophenol correlated the worst for the system at 2.20

310   mL/min flow rate (average pressure 203 bar), with a deviation of 22.6%. Here the maximum RD was

311   higher than for the initial prediction, which was 21.1% and originated from 2-amino-*p*-cresol (Table

312   2). However, the maximal % RD for the retention times decreased from 17.1% to 11.5% and 10.7%,

313   depending on the applied flow rate (Table 3).

13

314   The flow rate was changed similarly for the three other column combinations to generate the same

315   system pressure both on the individual columns and on the coupled system. For the CH-ACH

316   combination with LC-3 and SRP, the flow rate was changed to 1.79 mL/min for the chiral column and

317   1.55 mL/min for coupled system to match the system pressure on the achiral column. On the other

318   hand, to reach the system pressure of the LC-3 column analysed at 3 mL/min, the SRP column

319   requires a flow rate higher than 4 mL/ml, which was above the operating limits of the equipment.

320   For this reason, the LC-3 and SRP combination was analysed with just one alternative flow rate, 1.55

321   mL/min. The highest deviations of retention factors in the original predictions were seen for *p*-

322   aminophenol and 5-amino-*o*-cresol with 41.8% and 44.3, respectively. By reducing the flow rate on

323   the LC-3+SRP system, the correspondence between predicted and experimental retention factors of

324   the analytes improved significantly to 9.8% for *p*-aminophenol and 0.8% for 5-amino-*o*-cresol. This

325   contributed substantially to the improvement in average and maximal deviations, which decreased

326   from 9.1% and 44.3% in the initial prediction, to 2.8% and 9.8%, respectively (Table 2). Retention

327   time predictions also improved considerably from average RD of 5.6% to 1.1% (Table 3).

328   The system pressures on LC-2 and LC-3 at general screening conditions were the same. Therefore,

329   the coupled system was also analysed only once, with the flow rate reduced to 1.93 mL/min, as

330   opposed to the initial flow rate of 3.0 mL/min. The relative deviations for the system LC-2+LC-3 were

331   not as high as in the previous example. The largest deviation in retention factor was 22.6%, caused

332   by *p*-aminophenol. By changing the flow rate of the coupled system, the retention of the analyte

333   increased. This resulted in a better correspondence between predicted and experimental retention

334   factors, where the maximal deviation improved to 18.4%. As in the previous system, the retention

335   behaviour of the analytes was better predicted, with average deviation of retention factors 5.3% for

336   and LC-2+LC-3, compared to the original average RD of 8.88% (Table 2). Also for retention times a

337   reduction in average RD was seen from 6.8% to 3.5% (Table 3).

338   Using the screening conditions with changed flow rate, the most substantial improvement in average

339   and maximal % RD values of retention factors and retention times was seen for the LA-2 and FSRP

340   coupled system. During the screening step, the LA-2 and FSRP columns generated system pressures

341   of 256 and 266 bar, respectively. To predict the retention factors, LA-2 was analysed with a flow rate

342   of 3.16 mL/min generating a system pressure of 266 bar. A system pressure of 256 bar was achieved

343   at 2.80 mL/min flow rate on the FSRP column. The LA-2+FSRP system was analysed with reduced

344   flow rates 1.88 and 2.04 mL/min. The most significant improvement seen was in maximal % RD for

345   the retention factor, which was reduced from 51.9% to 21.0% and 19.7%, respectively. The maximal

346   51.9% RD was in the initial prediction attributed to the ephedrine enantiomers. By using the

347   adjusted flow rate strategy, correlation with the predicted retention times improved drastically, with

348   RD 3.0% and 6.7% for these compounds at flow rates 1.88 and 2.04 mL/min, respectively. The

349   maximal deviation of retention factors under adjusted flow rates was caused by aminocresol

350   isomers. In general, the average retention factors deviations improved considerably from 39.2% to

351   6.6% and 4.4% for LA-2+FSRP system analysed with flow rates 1.88 and 2.04 mL/min, respectively.

352   For the retention times, average deviations decreased from 9.4% to 3.8% and 2.4%.

353   The strategy of changing the flow rates according to a generated system pressure provided better

354   results for all four column combinations relative to the initial predictions. The elution sequences of

355   the analytes were predicted correctly for each mixture, and the average % RD of the predicted

356   retention factors and retention times improved. By keeping the same back pressure of 150 bar for all

357   experiments and changing the flow rate depending on the generated system pressure, a smaller

358   pressure difference was created between the column inlet and outlet. The smaller pressure drop

359   allowed a more consistent mobile phase density than when a flow rate of 3 mL/min was used,

360   regardless of the length of a column and the stationary phase character.

361   The retention factors were also predicted using Equations 2 and 3, to determine whether also better

362   results from the flow rate change were reached. Using Eq. 3, there was still a considerable deviation

363 between predicted and experimental retention factors. The lowest average RD was 16.1% for the

364 LS+LA-2 system analysed at 2.20 mL/min. The average deviation between experimental and

365 predicted retention factors, calculated using Eq. 2, i.e. 4.8% for LA-2+FSRP at 2.04 mL/min flow rate

366 and 5.4% for LC-2+LC-3 at 1.93 mL/min flow rate (Table S-1), is comparable with the results obtained

367 using Eq. 1. However, when the predictions were made for shorter coupled systems, the deviations

368 became larger. The highest average deviation was 23.3%, obtained for the LS+LA-2 system, when

369 analysed at 2.20 mL/min flow rate (Table S-1). Because of this inconsistency in results related to the

370 different column lengths and the high deviations for shorter coupled systems, Eq. 1 provides the

371 most reliable predictions.

372 **3.2.2 Back pressure adjustment**

373 A second approach tested towards reducing the % RD for retention factors and retention times of

374 analytes was adjusting the back pressure in the coupled-column systems. The back pressure was

375 manipulated, to ensure that the average pressure inside the columns was the same during the

376 screening of the individual columns, and in the coupled systems to achieve consistency in the mobile

377 phase density during the experiments. The % RD values of retention factors and retention times

378 predicted with adapted back pressure were then compared with the predictions from the original

379 experiments (Tables 2 and 3).

380 For the ACH-CH combination, the LS+LA-2 system was again tested. During individual column

381 screening with flow rate 3 mL/min and back pressure 150 bar, the system pressure generated on the

382 LS column was 239 bar. The average of these pressures in the column was therefore 194.5 bar. To

383 simulate the same average pressure, the LA-2 column was screened at 141 bar back pressure, which

384 generated a system pressure of 248 bar (corresponding to an average pressure of 194.5 bar). To

385 achieve the same average column pressure as on the individual columns, the LS+LA-2 system was

386 tested at 118 bar back pressure, which generated a system pressure of 271 bar.

16

387   From the individual LS column and LA-2 column screening experiments with reduced back pressure,

388   the retention factors on the LS+LA-2 system were predicted using Eq. 1. The predicted values were

389   compared to the experimental retention factors obtained by analysing the coupled system with 118

390   bar back pressure. Improved correlation of predicted and experimental retention factors was

391   observed, reducing average and maximal deviations from 10.6% and 21.1%, to 6.33% and 18.1%,

392   respectively (Table 2).

393   The system pressure reached by screening the LA-2 column under back pressure 150 bar was 256

394   bar, making the average column pressure 203 bar. To accommodate the average pressure generated

395   by the LA-2 column during initial screening, LS was screened at 159 bar and the LS+LA-2 system at

396   125 bar. Overall, average retention factor deviation improved from 10.6% for the initial predictions,

397   to 6.8%. However, in this system with slightly increased average pressure, the maximal deviation

398   was larger (24.0%) than for the initial predictions (21.1%) (Table 2). The deviation of 24.0% was

399   caused by a prolonged retention of *o*-aminophenol, increasing the difference between predicted and

400   experimental retention factor values.

401   The three other coupled-systems, LC-3+SRP, LA-2+FSRP, and LC-2+LC-3, were tested by the same

402   approach. To simulate the average column pressure of 179 bar, generated in the SRP column with

403   150 bar back pressure, the LC-3 column and LC-3+SRP system were analysed with back pressures of

404   123 and 115 bar, respectively. In general, agreement between predicted and experimental values

405   improved, with average RD for retention factors 3.6% and for retention times 0.9%. However, only a

406   slight improvement was detected for the LC-2+LC-3 system. Both chiral columns generated the same

407   system pressure under initial screening conditions, i.e. 262 bar. To match the average pressure of

408   206 bar, the LC-2+LC-3 system was analysed with a back pressure of 113 bar, generating a system

409   pressure of 299 bar. In this system, the maximum relative deviation for the retention factors

410   increased from 22.6% to 29.7%. The worst agreement between predicted and experimental

411   retention factor values was found for labetalol. Moreover, the elution sequence of the aminocresol

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412   isomers was predicted incorrectly. The analytes eluted in the sequence: 5-amino-*o*-cresol, 2-amino-

413   *m*-cresol, and 2-amino-*p*-cresol, but the predicted order was 2-amino-*m*-cresol, 5-amino-*o*-cresol,

414   and 2-amino-*p*-cresol, with relative deviations 3.0%, 17.6%, and 11.8%, respectively.

415   The LA-2+FSRP system was analysed similarly, changing the back pressures to 145 and 154 bar to

416   screen the FSRP and LA-2 columns, respectively. To equal the average pressures on the columns

417   during the screening process, the LA-2+FSRP system was analysed with 112 bar and 117 bar back

418   pressures. In both cases, the back pressure adjustment led to significant improvements in the

419   agreement between predicted and experimental retention factors and retention times. The average

420   deviation of retention factors decreased from 39.2% for the initial predictions, to 5.3% and 2.3%

421   when analysed with 112 bar and 117 bar back pressures, respectively.

422   In general, this approach provided improved similarity between predicted and experimental results

423   of the analytes, comparable to the results obtained by the flow rate adapting strategy. An exception

424   is the LC-2+LC-3 system, where the average % RD of the retention factors and retention times

425   improved only slightly, and the maximum % RDs were higher than the initial predictions. Moreover,

426   the elution sequence of the aminocresol isomers, eluting within less than a minute of each other,

427   was incorrectly predicted on the LC-2+LC-3 system. This inconsistency in the results when applying

428   the back pressure change strategy may be caused by the various pressure drops generated in

429   columns of different lengths.

430   While system and back pressures on the columns were the same for the adjusted flow rate approach

431   (for instance LS, LA-2, LS+LA-2 all had a back pressure of 150 bar and a generated system pressure of

432   256 bar), this was not the case for the back pressure adjusting approach, where the aim was to reach

433   the same average pressure of 203 bar on the columns and system. For example, the LA-2 column

434   was analysed with a back pressure of 150 bar, generating a 256 bar system pressure, while the LS

435   column was analysed at 159 bar and a system pressure of 247 bar. The LS+LA-2 coupled system had

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436   a back pressure of 125 bar, generating a system pressure of 281 bar, leading to altered pressure

437   drops on the columns.

438   Because in all abovementioned cases the average column pressure was the same (203 bar), different

439   pressures were affecting the mobile phase properties in the columns. The differences between inlet

440   and outlet pressures of the columns may be the cause of inconsistencies in mobile phase densities,

441   and consequently the reason for the inferior accuracy (average RD = 6.8%) of retention behaviour

442   predictions than in the flow rate change approach (average RD = 6.3%).

443   The highest deviations were mostly observed for aminophenol or aminocresol analytes. These are

444   the only compounds from the sample set with one benzene ring and amino and hydroxyl groups. The

445   density of the mobile phase might affect the ionization of these analytes in a non-linear way, making

446   their behaviour even more difficult to predict.

447   **3.3 Application to silymarin**

448   The strategy of changing the operating flow rate to achieve consistency in average column pressure

449   was applied on the screening and selection of chiral and achiral columns for the separation of the

450   silymarin complex compounds. Silymarin compounds were strongly retained on the chiral stationary

451   phases, exceeding analysis time over 60 min when using the mobile phase with 20% (v/v) of

452   modifier. Therefore, the modifier content was increased to 40% (v/v) to keep the analysis time

453   below 30 min. The aim was to screen all columns in this study for silymarin separation and the

454   screening started with the SRP column. Based on the earlier experiments, this column was known to

455   generate the lowest system pressure due to the 4 μm particles and the 10 cm length. When the

456   screening was started with another column, the generated system pressure may be too high and

457   impossible to reach on the SRP column, because of the flow rate limit of the instrument. The system

458   pressure generated on the column at flow rate 3.5 mL/min was 237 bar. The flow rate of the method

459   was adjusted for each column depending on the system pressure generated on the SRP column (for

460   example the flow rate on LNH2 was 2.56 mL/min, and on LC-3 was 1.82 mL/min).

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461   None of the screened individual columns provided separation of all seven tested silymarin

462   compounds (data not shown). Analysis on achiral columns LS, LNH2, SPRP, and FSRP provided two,

463   four, two, and one peak, respectively. The chiral columns provided a more efficient separation, with

464   six peaks on the chromatograms of LC-1, LC-2, LC-3, and LC-4. SCH and SD co-eluted on LC-1, SB A

465   and ISB A on LC-2, SB A and ISB B on LC-3, and ISB A and ISB B on LC-4. Compounds SB A and SB B,

466   and ISB A and ISB B co-eluted on the LA-2 column, resulting in five observed peaks.

467   From the screening results, the retention factors and subsequently the selectivities on each column

468   combination were estimated using Eq. 1. Based on the predicted retention factors, the combination

469   of columns LC-2 and LC-3 was chosen as the most promising for the separation of the silymarin

470   compounds. Screening on the individual LC-2 column showed co-elution of SB A and ISB A, while on

471   the individual column LC-3 column compounds SB A and ISB B remained unseparated (Fig. 2A, B).

472   Two coupled-column systems, LC-2+LC-3 and LC-3+LC-2 (Fig. 2 C, D), were tested for separation of

473   silymarin. The predicted retention factors and retention times were compared to the experimentally

474   obtained ones (Table S-2). The relative deviations of retention factors ranged from 49.44% for SB B

475   to 67.14% for TX, both in LC-3+LC-2 system. Similarly, the predicted void times, 2.96 and 2.91 min for

476   LC-2+LC-3 and LC-3+LC-2 systems, respectively, were very different from the experimental values:

477   4.63 min for LC-2+LC-3 and 4.59 min for LC-3+LC-2 systems. This drastic increase in deviations,

478   compared to the previous experiments, can be explained by different compressibility of the mobile

479   phase with 40% modifier, resulting in different volumes of the mobile phase in the system. However,

480   the agreement between predicted retention times, calculated from predicted retention factors and

481   predicted void times, and experimental retention times was very good, with % RD not exceeding 7%

482   (Table S-2).

483   Even if the agreement between predicted and experimental retention factors was poor, the

484   combination of the two columns, LC-2 and LC-3, did show at least partial separation of all tested

485   compounds (Fig. 2 C, D). Baseline separation was not achieved on either system, but the best

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486   resolution for the critical peak pair, i.e. ISB B/SB A, was achieved when the LC-3 column coupled first

487   (Fig. 2D). Further optimisation of the separation was not examined since this was not the purpose of

488   the actual study.

489   **4. Conclusions**

490   Compounds with similar chemical structures and properties are always challenging to separate,

491   requiring a unique chromatographic selectivity, which may be created by column coupling. Chiral

492   and achiral columns were tested individually and serially coupled in SFC, to provide an insight into

493   the retention behaviour of the test analytes in such coupled-column systems. Three equations were

494   employed to predict the retention factors of analytes on the coupled systems, based on individual

495   column retentions. The highest similarity between the predicted and experimental retention factors

496   was seen for Eq. 1., with an average RD for all coupled systems (ACH-CH, CH-ACH, and CH-CH) of

497   7.96%, compared to 12.07% and 39.77% for Eq. 2 and Eq. 3, respectively (Table 1).

498   Instrumental conditions, i.e. flow rate and back pressure, were adjusted to achieve the same

499   average column pressure for single-column and coupled-system analysis, and consequently to

500   improve predictability. The results of the study show that while a change in back pressure may

501   provide faster analysis, the drawback of this approach is the higher inconsistency between the

502   experimental and predicted results when compared to the flow rate change approach. With back

503   pressure adjustment, an increased maximal % RD in coupled systems and an unreliable predictions

504   of the elution sequence was demonstrated on closely eluting compounds. An explanation is that the

505   tested Equation 1 was designed to be used primarily in LC where the properties of a mobile phase do

506   not change throughout the column. Hence, they do not account for changes in mobile-phase

507   properties caused by pressure variation.

508   The strategy of flow-rate change, generating a constant system pressure during screening as well as

509   after coupling, showed to be more reliable. The problems related to pressure drops were minimized

510   by keeping the back pressure constant, while generating the same system pressure. The

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511   improvement in % RD was demonstrated on the four examined coupled systems: LS+LA-2, LC-3+SRP,

512   LA-2+FSRP and LC-2+LC-3.

513   The applicability of this approach was demonstrated on the separation of seven structurally related

514   compounds of the silymarin complex. Based on the results from the screening, the most suitable

515   columns were selected. While baseline separation of all compounds was not achieved on single

516   columns, an improved resolution of the critical peak pairs was observed when a coupled column

517   system was used.

518   In conclusion, it is recommended to start the single column screening with the lowest system

519   pressure generating column, to ensure that the generated system pressure is achievable on the

520   other tested columns. The used flow rate should be selected with respect to the operating limits of

521   the instrument and the column. The generated system pressure should then be matched on each

522   single column by adjusting the flow rate. Finally, the flow rate should be adjusted on the coupled

523   systems to generate the same system pressure as achieved on the single columns.

524   **Conflict of Interest**

525   The authors declare no conflict of interest.

526   **Acknowledgements**

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528   **References**

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Figure captions

Figure 1: Relative deviations between experimental and predicted (using Eq.1) retention factors for

specific combinations of (A) ACH-CH, (B) CH-ACH and (C) CH-CH coupled systems, depicting average

(bars), minimum and maximum (dots) RD (%). Column abbreviations: see text.

Figure 2: Chromatograms of silymarin separation on (A) LC-2 and (B) LC-3 individual columns, and on

(C) LC-2+LC-3, and (D) LC-3+LC-2 coupled column systems, using the general screening conditions

with 40% MeOH and adjusted flow rate to generate a system pressure of 237 bar. TX - taxifolin, SCH

- silychristin, ISB B - isosilybin B, SB A - silybin A, ISB A - isosilybin A, SD – silydianin, SB B - silybin B.

628   **Table 1:** (A) Relative deviations (%) between predicted and experimental retention factors using the

629   three different equations, and (B) relative deviations (%) of retention times (in min) estimated from

630   retention factors and void time (see section 2.6).

631   A

	ACH-CH				CH-ACH				CH-CH			
	Avg. RD (%)	Med. RD (%)	Min. RD (%)	Max. RD (%)	Avg. RD (%)	Med. RD (%)	Min. RD (%)	Max. RD (%)	Avg. RD (%)	Med. RD (%)	Min. RD (%)	Max. RD (%)
Eq. 1	7.29	6.47	0.07	50.69	9.83	6.38	0.04	51.91	6.76	4.74	0.00	33.59
Eq. 2	12.03	8.98	0.01	50.19	17.79	14.55	0.01	56.34	6.40	4.51	0.01	29.57
Eq. 3	27.09	26.46	1.18	53.55	33.05	28.84	1.21	81.40	59.19	58.19	31.19	82.09

632

633   B

	ACH-CH				CH-ACH				CH-CH			
	Avg. RD (%)	Med. RD (%)	Min. RD (%)	Max. RD (%)	Avg. RD (%)	Med. RD (%)	Min. RD (%)	Max. RD (%)	Avg. RD (%)	Med. RD (%)	Min. RD (%)	Max. RD (%)
Eq. 1	4.99	3.91	0.02	24.91	5.88	4.51	0.07	22.24	5.05	3.32	0.03	27.92

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636 **Table 2:** Relative deviations (|% RD|) obtained when applying Eq. 1, for retention factors at the initial conditions (k_{ORG}), after the flow rate adjustment (k_{FR}), and

637 after the back pressure (k_{BP}) adjustment. AP – average pressure.

	LS+LA-2						LC-3+SRP			LC-2+LC-3			LA-2+FSRP					
AP (bar)		203			194.5			179			206			203			208	
|RD| (%)	k_{ORG}	k_{FR}	k_{BP}	k_{ORG}	k_{FR}	k_{BP}	k_{ORG}	k_{FR}	k_{BP}	k_{ORG}	k_{FR}	k_{BP}	k_{ORG}	k_{FR}	k_{BP}	k_{ORG}	k_{FR}	k_{BP}
(R)-(+)-atenolol	17.57	1.13	1.57	17.57	7.47	7.08	5.75	1.18	3.87	13.62	4.58	4.21	37.64	7.69	5.66	37.64	0.24	1.81
(S)-(-)-atenolol	17.57	1.13	1.57	17.57	7.47	7.08	5.75	1.18	0.56	13.50	4.46	4.73	37.64	7.69	5.66	37.64	0.24	1.81
(1R,2S)-(-)-ephedrine	12.10	2.65	2.70	12.10	6.07	5.92	6.65	1.74	0.57	6.31	1.23	5.22	51.91	3.02	0.45	51.91	6.72	0.18
(1S,2R)-(+)-ephedrine	12.10	2.65	2.70	12.10	6.07	5.92	6.65	1.74	0.57	5.40	3.22	5.27	51.91	3.02	0.45	51.91	6.72	0.18
(R)-propranolol	9.75	0.47	1.81	9.75	1.85	1.34	0.44	4.73	3.15	6.59	0.50	2.82	39.30	5.10	1.98	39.30	4.80	0.21
(S)-propranolol	9.75	0.47	1.81	9.75	1.85	1.34	3.66	4.73	1.00	6.67	1.01	3.05	39.30	5.10	1.98	39.30	4.80	0.21
(S)-(+)-mianserine	10.57	0.21	1.90	10.57	1.67	1.31	6.18	2.55	3.60	12.42	2.26	1.85	40.21	5.18	4.20	40.21	0.47	2.91
(R)-(-) mianserine	10.57	0.21	1.90	10.57	1.67	1.31	6.70	2.93	4.20	12.63	3.17	3.07	40.21	5.18	4.20	40.21	0.47	2.91
quinine	9.43	0.48	1.73	9.43	0.14	0.64	7.39	0.20	4.80	16.21	6.02	5.97	48.73	9.59	8.67	48.73	1.30	6.54
quinidine	9.49	12.41	0.48	9.49	0.81	0.82	4.88	3.37	7.98	13.78	3.58	3.76	45.56	10.50	9.34	45.56	0.98	5.91
labetalol (1)	1.09	10.36	12.77	1.09	11.62	10.28	0.20	4.60	5.95	3.58	16.85	29.74	38.20	1.93	2.37	38.20	3.90	0.23
labetalol (2)	1.09	10.36	12.77	1.09	11.62	10.28	0.20	4.60	5.95	4.80	8.49	21.49	38.20	1.93	2.37	38.20	3.90	0.23
labetalol (3)	1.09	10.36	12.77	1.09	11.62	10.28	0.20	4.60	5.95	4.80	8.49	21.49	38.20	1.93	2.37	38.20	3.90	0.23
labetalol (4)	1.09	10.36	12.77	1.09	11.62	10.28	0.20	4.60	5.95	4.80	8.49	21.49	38.20	1.93	2.37	38.20	3.90	0.23
nadolol (1)	11.61	5.41	6.29	11.61	2.29	2.87	3.66	2.26	3.82	9.14	0.97	0.50	39.06	3.14	0.97	39.06	1.26	0.08
nadolol (2)	11.61	5.41	6.29	11.61	2.29	2.87	3.66	2.26	3.82	7.54	1.40	0.35	39.06	3.14	0.97	39.06	1.26	0.08
nadolol (3)	14.20	3.22	4.05	14.20	4.89	5.45	3.66	2.13	3.82	13.07	1.68	0.09	40.03	3.14	0.97	40.03	2.34	0.08
nadolol (4)	14.20	3.22	4.05	14.20	4.89	5.45	3.66	2.13	3.82	11.37	2.13	0.47	40.03	3.14	0.97	40.03	2.34	0.08
o-aminophenol	13.42	22.58	23.95	13.42	16.41	18.14	10.49	0.58	2.47	6.27	9.84	19.79	26.17	9.54	8.45	26.17	11.33	7.65
m-aminophenol	5.24	3.17	4.58	5.24	1.25	0.01	16.35	0.58	2.47	2.52	4.01	5.05	40.65	1.70	1.94	40.65	2.82	2.07
p-aminophenol	19.84	6.44	4.97	19.84	16.20	14.48	41.76	9.75	2.03	22.58	18.36	12.87	49.48	1.70	2.59	49.48	13.10	3.48
2-amino-m-cresol	9.95	10.10	9.94	9.95	13.35	12.90	9.57	2.44	4.16	1.25	7.29	17.64	35.13	21.00	19.70	35.13	19.73	15.34
2-amino-p-cresol	21.14	10.10	9.94	21.14	12.02	12.58	26.21	2.51	4.02	4.44	5.43	11.83	23.29	21.00	19.70	23.29	4.24	1.36
5-amino-o-cresol	10.20	18.49	19.81	10.20	2.58	3.18	44.25	0.78	2.80	9.85	4.38	3.01	23.29	21.00	19.70	23.29	4.24	1.36
Average RD (%)	**10.61**	**6.31**	**6.80**	**10.61**	**6.57**	**6.33**	**9.09**	**2.84**	**3.64**	**8.88**	**5.33**	**8.57**	**39.22**	**6.59**	**5.33**	**39.22**	**4.37**	**2.30**
Min. RD (%)	1.09	0.21	0.48	1.09	0.14	0.01	0.20	0.20	0.56	1.25	0.50	0.09	23.29	1.70	0.45	23.29	0.24	0.08
Max. RD (%)	21.14	22.58	23.95	21.14	16.41	18.14	44.25	9.75	7.98	22.58	18.36	29.74	51.91	21.00	19.70	51.91	19.73	15.34

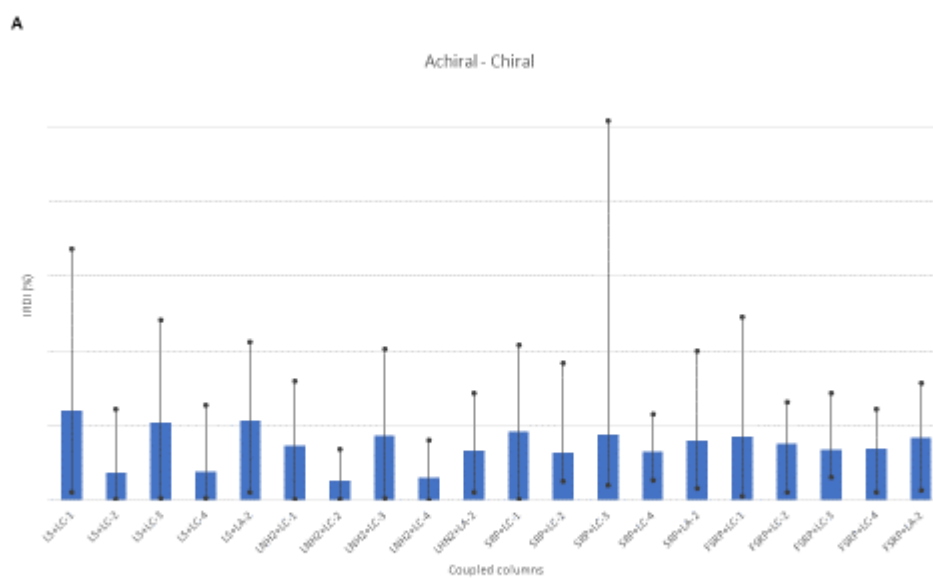638

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Table 3: Relative deviations (% RD) obtained when applying Eq. 1, for retention times (min) at the initial conditions ($t_{R\,ORG}$), after the flow rate adjustment ($t_{R\,FR}$), and after the back pressure adjustment ($t_{R\,BP}$). AP – average pressure.
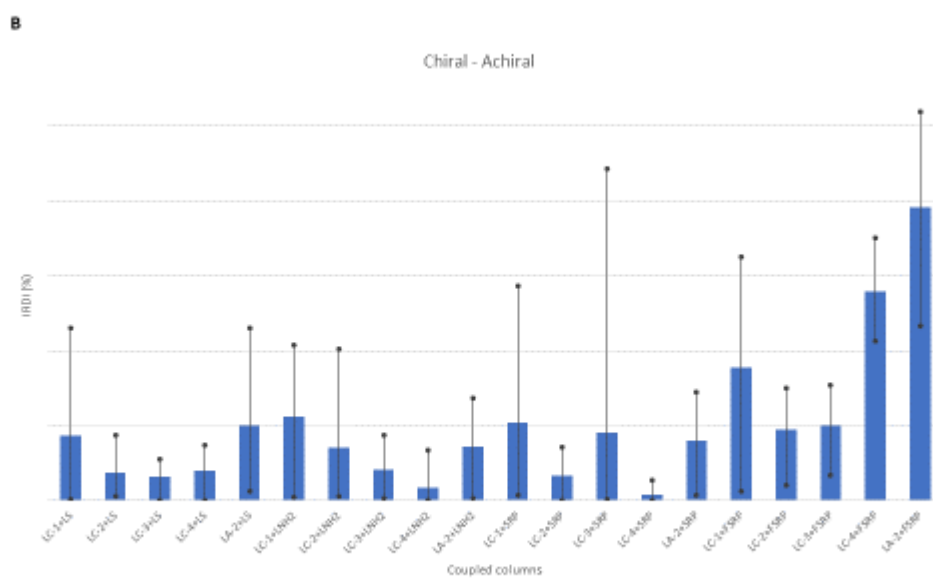
	LS+LA-2						LC-3+SRP			LC-2+LC-3			LA-2+FSRP					
AP (bar)	**203**			**194.5**			**179**			**206**			**203**			**208**		
|RD| (%)	$t_{R\,ORG}$	$t_{R\,FR}$	$t_{R\,BP}$	$t_{R\,ORG}$	$t_{R\,FR}$	$t_{R\,BP}$	$t_{R\,ORG}$	$t_{R\,FR}$	$t_{R\,BP}$	$t_{R\,ORG}$	$t_{R\,FR}$	$t_{R\,BP}$	$t_{R\,ORG}$	$t_{R\,FR}$	$t_{R\,BP}$	$t_{R\,ORG}$	$t_{R\,FR}$	$t_{R\,BP}$
(R)-(+)-atenolol	17.06	0.87	1.65	17.06	8.06	9.01	3.74	1.22	0.33	13.80	5.08	5.66	13.14	6.04	5.69	13.14	0.89	5.78
(S)-(-)-atenolol	17.06	0.87	1.65	17.06	8.06	9.01	3.74	1.22	1.78	14.14	5.11	6.28	13.14	6.04	5.69	13.14	0.89	5.78
(1R,2S)-(-)-ephedrine	8.76	0.39	1.52	8.76	5.04	6.24	2.94	0.30	1.89	3.62	0.45	0.10	7.86	2.11	2.07	7.86	3.45	4.66
(1S,2R)-(+)-ephedrine	8.76	0.39	1.52	8.76	5.04	0.80	2.94	0.30	1.89	3.66	0.50	0.47	7.86	2.11	2.07	7.86	3.45	4.66
(R)-propranolol	8.05	2.11	1.88	8.05	2.80	3.77	1.66	1.39	0.63	5.46	0.57	0.04	9.64	3.74	2.99	9.64	3.40	4.70
(S)-propranolol	8.05	2.11	1.88	8.05	2.80	3.77	3.02	1.39	2.48	5.61	0.24	0.15	9.64	3.74	2.99	9.64	3.40	4.70
(S)-(+)-mianserine	9.76	1.67	1.60	9.76	2.89	3.90	4.61	0.64	0.10	10.71	2.61	3.21	11.45	4.00	4.40	11.45	0.46	6.31
(R)-(-) mianserine	9.76	1.67	1.60	9.76	2.89	3.90	4.57	0.70	0.02	10.37	3.17	4.02	11.45	4.00	4.40	11.45	0.46	6.31
quinine	8.14	2.13	1.87	8.14	1.57	2.52	4.38	0.63	0.05	12.87	5.21	6.09	12.43	5.93	6.43	12.43	1.39	7.96
quinidine	8.43	9.98	2.66	8.43	1.12	2.39	3.51	0.74	1.46	10.82	3.37	4.42	12.86	6.81	7.15	12.86	0.20	7.86
labetalol (1)	1.34	5.46	6.05	1.34	6.56	4.33	1.38	1.61	1.05	0.97	10.49	18.75	10.02	0.25	0.47	10.02	1.56	4.72
labetalol (2)	1.34	5.46	6.05	1.34	6.56	4.33	1.38	1.61	1.05	4.61	4.92	13.21	10.02	0.25	0.47	10.02	1.56	4.72
labetalol (3)	1.34	5.46	6.05	1.34	6.56	4.33	1.38	1.61	1.05	4.61	4.92	13.21	10.02	0.25	0.47	10.02	1.56	4.72
labetalol (4)	1.34	5.46	6.05	1.34	6.56	4.33	1.38	1.61	1.05	4.61	4.92	13.21	10.02	0.25	0.47	10.02	1.56	4.72
nadolol (1)	11.10	2.35	1.88	11.10	3.45	5.19	2.75	0.16	0.50	8.08	1.61	1.44	12.39	2.89	2.50	12.39	1.53	4.64
nadolol (2)	11.10	2.35	1.88	11.10	3.45	5.19	2.75	0.16	0.50	7.04	1.94	1.54	12.39	2.89	2.50	12.39	1.53	4.64
nadolol (3)	13.13	0.67	0.15	13.13	5.50	7.22	2.75	1.55	0.50	11.25	2.17	1.74	13.04	2.89	2.50	13.04	2.21	4.64
nadolol (4)	13.13	0.67	0.15	13.13	5.50	7.22	2.75	1.55	0.50	10.21	2.56	2.18	13.04	2.89	2.50	13.04	2.21	4.64
o-aminophenol	5.35	10.74	10.40	5.35	7.61	7.35	7.54	0.35	0.49	2.52	5.30	10.89	0.13	3.58	2.11	0.13	4.54	0.99
m-aminophenol	5.13	0.01	0.33	5.13	2.39	2.94	11.05	0.35	0.49	0.34	1.89	1.69	6.91	1.72	2.83	6.91	2.09	5.58
p-aminophenol	13.93	5.62	5.92	13.93	11.46	11.72	16.73	5.40	1.11	11.05	9.12	5.11	11.58	1.72	3.14	11.58	7.24	6.27
2-amino-m-cresol	3.00	3.53	2.30	3.00	5.40	3.88	6.95	0.76	0.51	0.62	3.68	9.47	2.48	8.84	7.25	2.48	8.37	2.56
2-amino-p-cresol	9.31	3.53	2.30	9.31	5.15	4.21	17.04	0.89	0.56	1.52	2.68	6.06	1.55	8.84	7.25	1.55	1.30	3.93
5-amino-o-cresol	3.53	8.47	8.08	3.53	0.17	1.10	22.24	0.28	0.52	4.87	1.87	0.08	1.55	8.84	7.25	1.55	1.30	3.93
Average RD (%)	**8.25**	**3.42**	**3.14**	**8.25**	**4.86**	**4.94**	**5.55**	**1.10**	**0.85**	**6.81**	**3.52**	**5.38**	**9.36**	**3.78**	**3.57**	**9.36**	**2.36**	**4.98**
Min. RD (%)	1.34	0.01	0.15	1.34	0.17	0.80	1.38	0.16	0.02	0.34	0.24	0.04	0.13	0.25	0.47	0.13	0.20	0.99
Max. RD (%)	17.06	10.74	10.40	17.06	11.46	11.72	22.24	5.40	2.48	14.14	10.49	18.75	13.14	8.84	7.25	13.14	8.37	7.96

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642 Figure 1

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Achiral - Chiral

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Chiral - Chiral
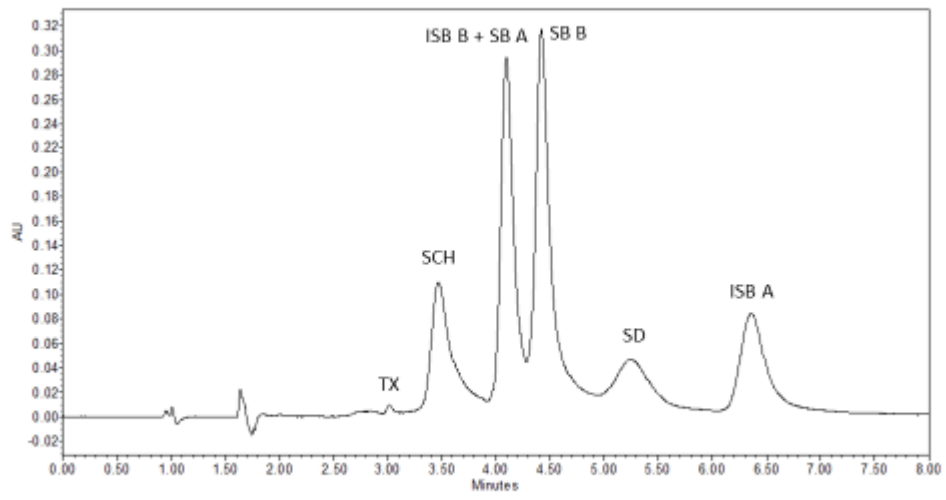
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646 Figure 2

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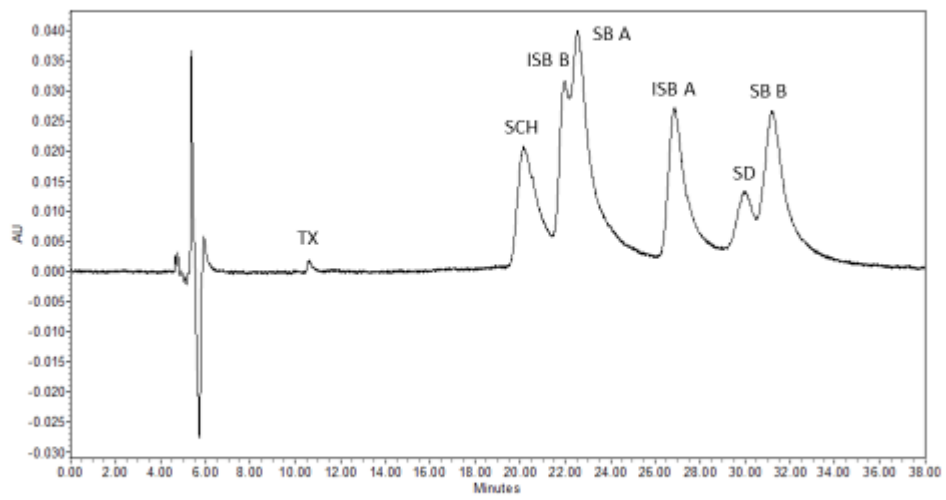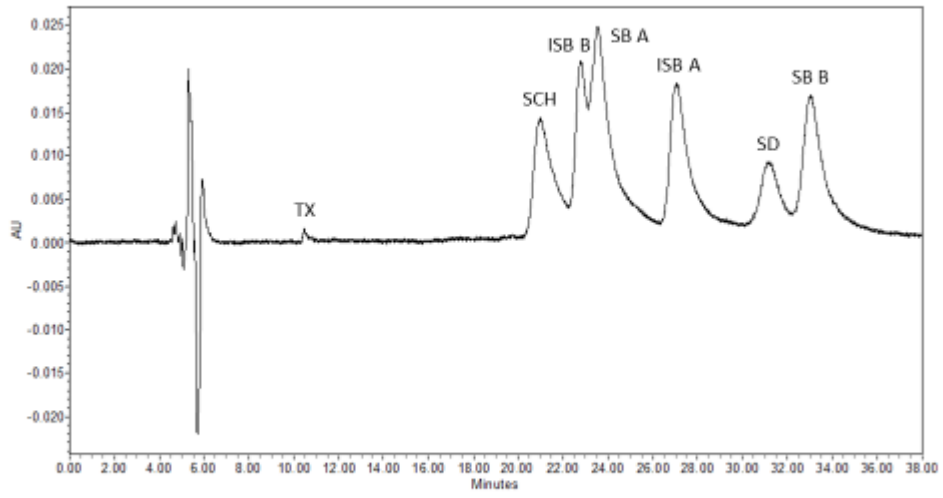
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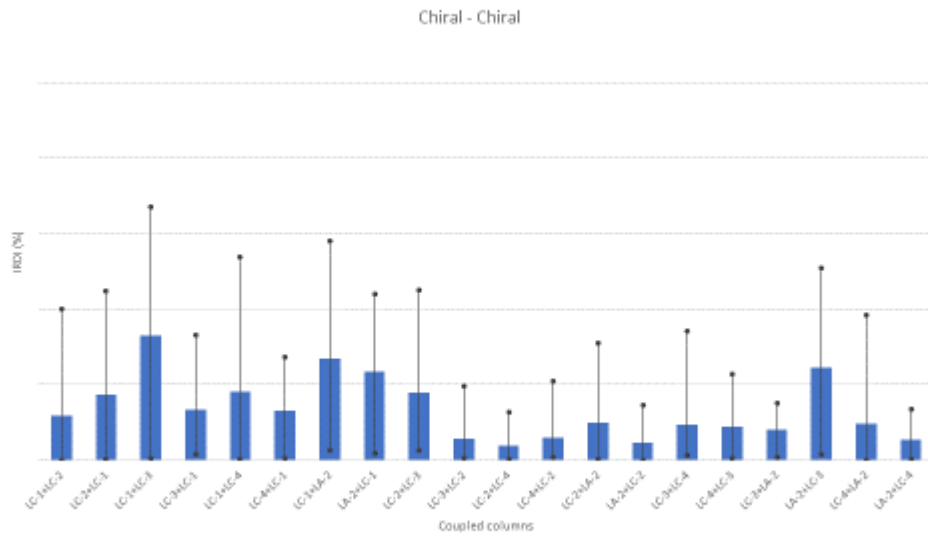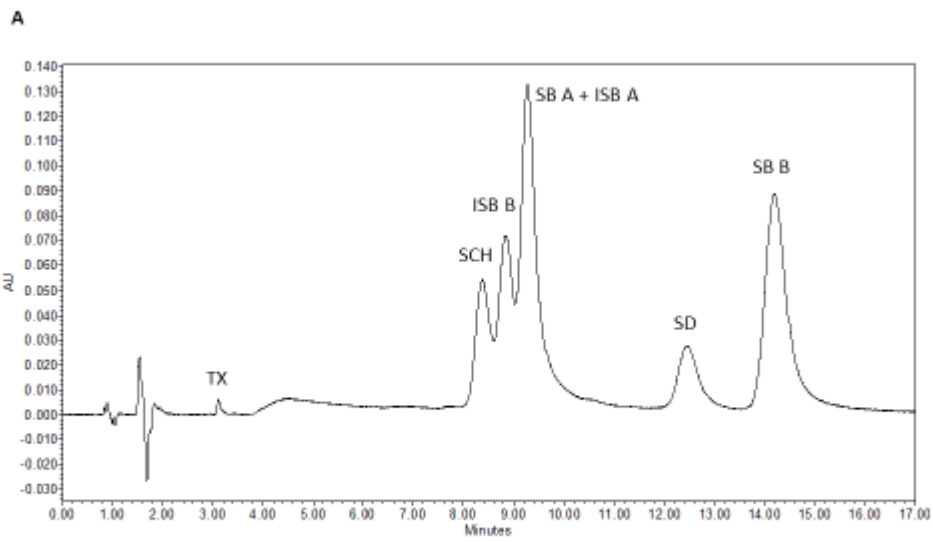
Supplementary material

Coupling of chiral and achiral stationary phases in supercritical fluid chromatography: evaluating

and improving retention prediction

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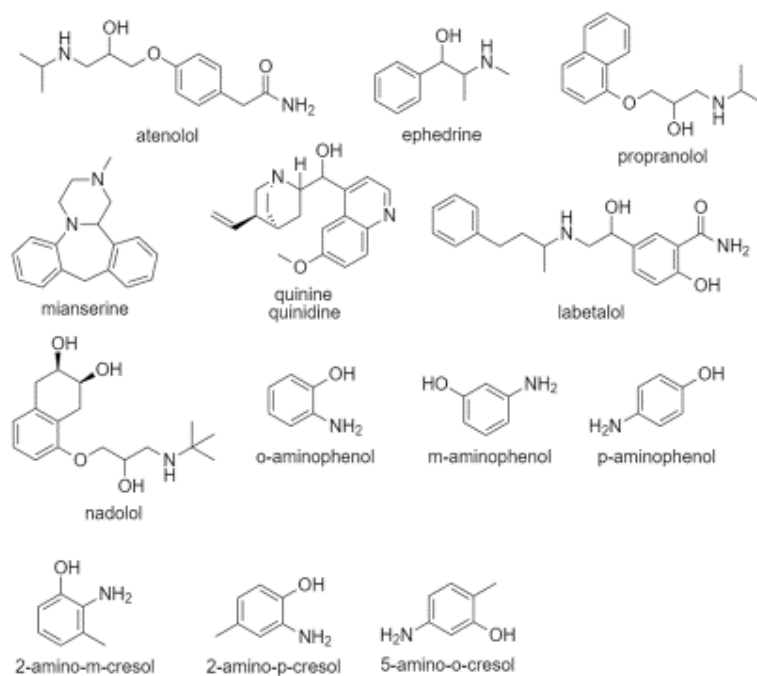**Email:** Debby.Mangelings@vub.be

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673   **Figure captions**

674   **Figure S-1:** Chemical structures of the sample set compounds.

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676 **Table S-1:** Relative deviations (% RD) obtained applying Eq. 2, for (A) retention factors and (B)

677 retention times (min), after the flow rate adjustment, and at the initial conditions.

	LS+LA-2		LC-3+SRP	LC-2+LC-3	LA-2+FSRP	
FR (mL/min)	**2.20**	**1.89**	**1.55**	**1.93**	**1.88**	**2.04**
SP (bar)	**256**	**239**	**208**	**262**	**256**	**266**
(A) Retention factors after flow rate adjustment						
Average RD (%)	**23.27**	**21.01**	**16.57**	**5.38**	**9.24**	**4.80**
Min. RD (%)	5.36	2.65	4.66	0.24	2.20	0.43
Max. RD (%)	43.20	38.32	58.81	19.44	20.00	13.29
Retention factors at initial conditions						
Average RD (%)	**12.73**		**16.47**	**7.16**	**35.21**	
Min. RD (%)	0.89		2.07	1.05	21.81	
Max. RD (%)	27.68		52.40	23.66	53.30	
(B) Retention time after flow rate adjustment						
Average RD (%)	**13.57**	**12.39**	**6.74**	**2.98**	**5.57**	**2.97**
Min. RD (%)	1.26	0.12	1.25	0.08	1.76	0.50
Max. RD (%)	26.95	24.92	21.25	11.85	12.41	6.90
Retention times at initial conditions						
Average RD (%)	**6.49**		**6.70**	**5.57**	**11.85**	
Min. RD (%)	0.63		0.10	0.66	0.64	
Max. RD (%)	12.64		26.76	11.62	85.38	

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679 FR – flow rate, SP – system pressure

680   **Table S-2:** Evaluation and overview of predicted and experimental retention factors (k) and retention

681   times (t_R) for silymarin compounds for (A) LC-2+LC-3 and (B) LC-3+LC-2 coupled systems.

(A)	Predicted		Experimental			
	k	t_R (min)	k	RD (%)	t_R (min)	RD (%)
Silybin A	7.01	23.72	3.86	57.88	22.54	5.12
Silybin B	10.26	33.36	5.72	56.79	31.16	6.80
Isosilybin A	8.23	27.35	4.80	52.79	26.86	1.80
Isosilybin B	6.74	22.93	3.75	57.15	21.99	4.17
Silychristin	6.11	21.04	3.35	58.31	20.16	4.31
Silydianin	9.62	31.46	5.48	54.89	30.02	4.67
Taxifolin	2.58	10.59	1.29	66.75	10.60	0.08

(B)	Predicted		Experimental			
	k	t_R (min)	k	RD (%)	t_R (min)	RD (%)
Silybin A	7.01	23.28	4.12	51.90	23.52	1.01
Silybin B	10.26	32.74	6.19	49.44	33.04	0.92
Isosilybin A	8.23	26.84	4.89	50.92	27.06	0.81
Isosilybin B	6.74	22.51	3.97	51.84	22.81	1.34
Silychristin	6.11	20.65	3.58	52.28	21.01	1.71
Silydianin	9.62	30.87	5.80	49.59	31.22	1.12
Taxifolin	2.58	10.39	1.28	67.14	10.48	0.79

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