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1 **Application of tandem coupling of columns in supercritical fluid chromatography for**
2 **stereoisomeric separation: optimization and simulation.**

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25 **model;** Semi-preparative chromatography.

26

27 ABSTRACT

28

29 Chromatographic separation of compounds with more than one chiral center is challenging,
30 requiring high resolution methods. Owing to the low viscosity of the mobile phase,
31 Supercritical Fluid Chromatography (SFC) enables the tandem coupling of columns which
32 increases resolution compared over a single column and can be effective in resolving
33 stereoisomers. Enantioseparation of a dihydropyridone derivative with two chiral centers,
34 synthetic API, was here studied using SFC. Six polysaccharide-based, chiral stationary phases
35 with a mobile phase consisting of a carbon dioxide/methanol mixture (80:20 v:v) were
36 investigated at 40°C and a flow-rate of 3 mL/min, but only incomplete separation of the four
37 expected stereoisomers was observed. We then examined different combinations of columns
38 in tandem. It was found that, among the thirteen successful tandems, the OJ-H//AD-H system
39 gave complete baseline resolution of the four stereoisomers with 4.98, 5.63, 6.06 and 6.89 as
40 retention times and 2.97, 1.83 and 3.54 as resolution values. The conditions were further
41 optimized to obtain the best resolution in the shortest elution time. The best conditions were
42 transposed to semi-preparative scale to obtain the pure isomers, with yield increased by using
43 stacked injections. The four fractions allowed the attribution of elution order on all tandem
44 performed previously. The column order itself had no impact on the stereoisomeric elution
45 order but the type of stationary phase and column order strongly influenced the resolution. In
46 parallel, a previously reported mathematical model was used to predict the retention times of
47 the four stereoisomers on each of the six polysaccharide-based, chiral stationary phase column
48 tandems. This mathematical model was successfully applied to predict separation the
49 dihydropyridone derivative's isomers on two columns with chlorinated stationary phases.

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52 **1. Introduction**

53 Owing to the impact of chirality on both the physical and biological properties of molecules,
54 the separation of enantiomers (and stereoisomers in general) has been extensively studied.
55 Among the 30-top selling drugs on the market in September 2014, ten were pure
56 stereoisomers showing clearly that separating racemates continues to be a key step in the
57 development of new drugs [1]. In this field, thanks to continued improvement in
58 instrumentation and chiral column design, Supercritical Fluid Chromatography (SFC) is now
59 outperforming traditional High Performance Liquid Chromatography (HPLC). Since
60 polysaccharide-based, chiral stationary phases (CSP) can be used in both techniques, SFC has,
61 in a relatively short frame, benefited from 40 years of stationary phase development in HPLC
62 applications [2-5]. Polysaccharides are currently very popular for many reasons: i) their broad
63 range of applications, ii) the availability of numerous different chemistries and, iii) their high
64 loading capacity which is useful at the preparative scale [6; 7]. Okamoto *et al.* developed the
65 tris(3, 5-dimethylphenylcarbamate) of cellulose and amylose stationary phases, together with
66 the cellulose tris(4-methylbenzoate) and the amylose tris((S)- α -methylbenzylcarbamate)
67 phases that make up the so-called “golden four”. Chlorinated polysaccharides have also come
68 to market [8]. Immobilized versions of these coated phases were also patented by Daicel
69 Industries and developed in collaboration with E.R. Francotte. Later, Phenomenex patented its
70 two first immobilized columns in collaboration with B. Chankvetadze.

71 In SFC, the mobile phase consists of at least 60% supercritical carbon dioxide ($T = 31^{\circ}\text{C}$ and
72 $P = 74$ bar). The low viscosity and high diffusivity of the mobile phase, together with a lower
73 pressure drop, permits the use of a high flow-rate with a reduced influence on efficiency [9].
74 Carbon dioxide has an elution strength close to that of hexane: hence, a polar modifier such as
75 an alcohol or acetonitrile must be added to elute chiral compounds from the column. It is

worth noting that while the addition of a liquid modifier moves conditions to subcritical, the previously stated advantages are conserved.

The intrinsic properties of supercritical CO₂ are particularly suitable for use with multiple columns in series. In addition, there is no distinction in SFC between normal- and reverse-phase separations. This feature enables the coupling of two (or more) columns in series, as well as combinations of columns with different polarities, in order to achieve high separation efficiency with greater column length and targeted selectivity. Berger and Wilson, for example, coupled 11 columns in series (4.6 mm × 200 mm, 5 μm, resulting in 2.2 m long column) and demonstrated only a 160 bar pressure drop [10]. This concept was more recently illustrated by West [11] with an achiral-achiral tandem of columns for impurity profiling. The number of potential combinations, comprising matched chiral-chiral phases [12], different chiral-chiral phases [13-15] and achiral-chiral combinations [16-17] is large, allowing tailored, high efficiency and giving rise to many novel and unique chiral selectivity properties. However it must be kept in mind that the two implemented stationary phases should provide orthogonal behaviors, *i.e* complementary selectivity, and not opposing properties resulting in a loss of resolution. It is not until recently that Poole [18] and Welch [19] have highlighted that tandem columns separation may depend on the order in which the columns are coupled. To date, excepting the work by Wang [14], no experimental data illustrating the effect of column order on tandem, chiral column SFC separations has been published.

Therefore, continuing our previous work in which preparative liquid separations were run with hydrocarbon mixtures [20], column coupling SFC was tested to perform cleaner enantioseparation of the dihydropyridone derivative **1** which bears two asymmetric carbons and was synthesized as a drug-candidate for the treatment of type-2 diabetes (Figure 1a). Compound **1** is representative of a large, patented series of molecules entrusted to our laboratory. In the early-stage of the drug discovery process, the pure stereoisomers of final

compound **1** are required. Thus, in this work we compared the ability of single column and tandems of columns to separate the four stereoisomers of the dihydropyridone derivative.

The first aim of this work was to identify and optimize a suitable tandem-column system in order to generate individual isomers of this derivative using small-scale, preparative chromatography. The resolving power and elution order of the four isomers was tested for thirteen column tandems. The impact of column order on selectivity and retention times was carefully explored. The second goal was to apply Wang's mathematical model [14] to predict elution times of the dihydropyridone derivative's stereoisomers on the various tandems. Lastly, mathematical parameters for two chlorinated chiral stationary phases, not included in the Wang's model, were determined and used to simulate the retention times of this synthetic API on these phases.

2. Material and methods

2.1 Chemicals

Compound **1** (2C*) was synthesized according to procedures previously described [21]. The four fractions containing individual diastereoisomers (FR1, FR2, FR3 and FR4) were obtained after a small-scale, preparative separation on a tandem column system (OJ-H//AD-H). The methanol (MeOH), ethanol (EtOH), propan-2-ol (IPA) and acetonitrile (ACN) were HPLC grade and were purchased from Carlo-Erba Reagents (Val-de-Reuil, France). Carbon dioxide (CO₂, 99.995% purity) was purchased from Linde (Saint Priest, France). Probe molecules (2 achiral and 9 chiral) were used for determination of the simulation parameters: achiral sulfamoylbenzoic acid and acetazolamide were purchased from Alfa-Aesar (Karlsruhe, Germany) and chiral warfarin, ketoconazole, propranolol, ibuprofen, bupivacaine, 3-methyl-1-indanone, paliperidone from Sigma-Aldrich (Saint-Quentin Fallavier, France), while compound **1** (proprietary structure with two asymmetric carbons) was prepared by our team. All observed peaks were used for assessment of the retention factors.

2.2 Sample solutions

For analytical screening, methanolic solutions were prepared at 0.8 mM (0.31 mg.mL⁻¹). For small-scale preparative separation, solutions of samples were prepared in methanol at 19 mM (7.29 mg.mL⁻¹). Solutions of probe molecules were prepared in methanol at 1.0 mg.mL⁻¹. The solutions were always degassed in an ultrasonic bath and filtered with a 0.45 µm PTFE syringe-filter (15 mm diameter) prior to use.

2.3 Supercritical Fluid Chromatography Apparatus

2.3.1 Stationary phases

Six chiral analytical columns were used for this study. Chiralpak AS-H amylose tris((S)-α-phenylethylcarbamate), Chiralcel OJ-H cellulose tris(methylbenzoate), Chiralcel OD-H cellulose tris(3,5-dimethylphenylcarbamate) and Chiralpak AD-H amylose tris(3,5-dimethylphenylcarbamate) were purchased from Chiral Technologies Europe (Illkirch, France) and Lux Cellulose-2 cellulose tris(3-chloro-4-methylphenylcarbamate) and Lux Amylose-2 amylose tris(3-chloro-4-methylphenylcarbamate) from Phenomenex® (Le Pecq, France). All columns had dimensions of 250 mm x 4.6 mm i.d. with 5 µm particle size and were coated, silica-gel phases. The tubing used to couple columns was a 1/32" Inox flexible tube of 0.170 x 400 mm purchased from PIC Solution (Avignon, France) with a negligible influence on pressure.

2.3.2. Chromatographic system

The chromatographic system used was an SFC-PICLAB hybrid 10-20 apparatus (PIC Solution, Avignon, France) equipped with an autosampler comprising a 48-vial plate (model Alias, Emmen, Netherlands), three model 40 P pumps (two for CO₂ and a third for the modifier (Knauer, Berlin, Germany)), a column oven with a Valco ten-position column selection valve, and a Valco six-position solvent switching valve. The system was also composed of a Smartline 2600 diode array detector (DAD) (Knauer, Berlin, Germany).

151 Detection wavelength was set at 210 nm. The system was controlled and the data was
 152 acquired using the SFC PicLab Analytic Online v.3.1.2 software (PIC Solution, Avignon,
 153 France).

154 2.3.3 Chromatographic conditions

155 During the separation optimization, the mobile phase was always a CO₂-modifier mixture
 156 with 20% of methanol as a modifier at 3 mL/min flow rate. All analyses were run in isocratic
 157 mode. The column oven temperature was 40°C and the outlet pressure was maintained at 120
 158 bar for all experiments. The volume injected was 20 µL.

159 2.4 Calculations

160 The resolution factor from our study was calculated using the following equation:

$$161 \quad R_s = 2 \frac{(tr_2 - tr_1)}{(\omega_2 + \omega_1)} \quad \text{Eq. 1}$$

162 where tr₁ and tr₂ are the retention times of the peaks of interest and ω₁ and ω₂ are the peak
 163 widths measured at the baseline of the peak.

164 The retention factor (k) is a mean of measuring the retention of an analyte on the
 165 chromatographic column for each isomer, k₁ to k₄ were calculated using the equation:

$$166 \quad k = \frac{(tr - t_0)}{(t_0)} \quad \text{Eq. 2}$$

167 where t₀ represents the first disturbance of the baseline after injection, caused by the solvent
 168 peak. Retention times were average values of two replicate determinations. The peak variance
 169 σ² was back calculated as σ² = tr²/N, where N is the efficiency processed by SFC New Data
 170 Manager V.1.8.0 software (Pic Solution, Avignon, France).

171 2.5 Data processing for simulation parameters determination

172 Two columns were chosen: Lux Amylose-2 and Lux cellulose-2. Column outlet pressure was
 173 set at five different values, i.e., 100, 125, 150, 175 and 200 bar. For retention factor
 174 measurements, triplicate injections were made for each probe at each condition. For dead time

measurements, twelve replicate methanol injections were performed for each set of conditions.

3. Results and discussion

In order to separate the four stereoisomers of the dihydropyridone derivative **1**, six chiral phases differing in their polysaccharide backbone (cellulose or amylose), the derivative type (carbamate or ester) and nature of the substituents (namely Chiralpak AD-H, AS-H, Lux Amylose 2 and Chiralcel OD-H, OJ-H and Lux Cellulose-2) were chosen (Figure 1b). Most of the chiral analyses performed by HPLC and SFC have been performed with these stationary phases [22; 23].

3.1 Single-column screening

The analyses were run at 3 mL/min flow-rate, with a mobile phase constituted of either MeOH or EtOH or IPA or ACN/CO₂ 20:80 (v/v), at 40°C, under 120 bar outlet pressure. The best results observed with MeOH as a modifier provided three peaks (two peaks on LA-2 column) instead of the four expected. The enantioseparations are represented in Figure 2 and chromatographic parameters are summarized in Table 1. We thus proceeded to the testing of tandem pairs of chiral columns [24].

3.2 Tandem coupled-columns screening and stereoisomeric elution order study

Serially coupling two columns increases both the separation efficiency and the working pressure by a factor of two, thus allowing an improved resolution through: i) the complementarity of two different stationary phases, ii) increased separation efficiency and, iii) a change in the selectivity as a result of the pressure increase. It should be noted, however, that a total loss of enantioseparation may also result when coupling columns with competing separation mechanisms. In the case of n available columns, $n \times (n - 1)$ tandem combinations are possible. In our case the six chiral stationary phases (CSPs) were different, thus the 30 possible combinations corresponded to 15 possible tandems able to adopt two configurations

each (CSP_A upstream//CSP_B downstream) and (CSP_B upstream//CSP_A downstream). The screening was undertaken on the 30 possible tandems by injection of compound **1**. The results are summarized in the Table 2. Several systems proved to be effective in obtaining the four expected peaks (Fig.1 in Supplementary Information). To determine the best column tandem in order to implement a small-scale preparative separation, baseline resolution and analysis time were chosen as the criteria to be optimized.

Based on these criteria, the best tandem consisted of the OJ-H CSP as the upstream column and the AD-H CSP as the downstream column (hence OJ-H//AD-H – Figure 3a, top). It is noteworthy that a 50 cm long column has double the efficiency of a 25 cm length column, leading to narrower peaks and better resolution. The chromatogram obtained using the AD-H//OJ-H tandem is given in Figure 3a (bottom).

The four fractions isolated in the preparative stage (FR 1 to FR 4) were subsequently injected on all the “successful” tandems allowing us to study the stereoisomers’ elution order. The data in Table 2, Figure 3 and Figure 1 in the Supplementary Information show that the tandem column order influenced the retention of the isomers but does not influence their elution order. This is due to the low back pressure induced by the 5 µm particle size columns [14].

Wang *et al.* showed that if a CSP_A is placed to the front of CSP_B, the upstream column CSP_A experiences higher back pressure (P_u) than the downstream column CSP_B (P_d). The pressure difference between (P_u) and (P_d) is the pressure drop over the downstream column CSP_B. The overall retention time, t_{AB} is the addition of the retention times on both columns at corresponding outlet pressure: $t_{AB} = t_{A,P_u} + t_{B,P_d}$, where t_{A,P_u} is the retention time on the upstream column CSP_A at higher back pressure (P_u), and t_{B,P_d} is the retention time on the downstream column CSP_B at lower outlet pressure (P_d). When columns CSP_A and CSP_B are swapped, the overall retention time t_{BA} changes to $t_{BA} = t_{B,P_u} + t_{A,P_d}$. When moving the column CSP_A from upstream to downstream of the CSP_B column, the pressure induced a

retention gain on column CSP_A, ($t_{A,P_d} - t_{A,P_u}$), which rarely compensates exactly the retention loss on column CSP_B, ($t_{B,P_u} - t_{B,P_d}$). Consequently, tandem column retention times of a compound usually change when column coupling order is reversed, leading to very different separations. For example separations obtained on tandems consisting of OD-H and OJ-H CSPs in different orders are depicted in Figure 3b. It can be seen that FR 3 and FR 1 co-elute when the OD-H CSP is upstream of the OJ-H CSP (Fig. 3b, top), while FR 3 and FR 1 are well resolved when the OJ-H CSP is upstream of the OD-H CSP (Fig. 3b, bottom). When the OD-H is upstream to experience higher pressure (P_u) its selectivity contribution is reduced to a point that resolution between FR 3 and FR 1 is canceled by the opposing selectivity of the downstream OJ-H. Conversely, when the OJ-H is moved upstream, its contribution to the overall selectivity is lower and the selectivity between FR 3 and FR 1 from the OJ-H can only partially be opposed by that of the downstream OD-H CSP. Thus, the 1 and 3 isomers are resolved and conserve the elution order. A similar phenomenon can be observed with several different tandems (Fig. 3a for instance). Some combinations of stationary phases with opposing selectivity resulted, when coupled in tandem, in two peaks instead of the four expected (AS-H//LA-2; AD-H//LA-2 and LA-2//AD-H tandems, for instance).

3.3 Optimization of the conditions on OJ-H//AD-H tandem and small-scale, preparative separation

In order to improve the baseline resolution between the four peaks on the chosen tandem, different parameters were optimized (*i.e* type and percentage of modifier, outlet-pressure and flow-rate).

3.3.1 Influence of the type of co-solvent on enantioseparation

The analyses were initially run at 3 mL/min flow-rate, with a mobile phase of CO₂/MeOH 80:20 (v/v), at 40°C and 120 bar outlet pressure. Thus IPA, EtOH and ACN in 80% CO₂ were tested as modifiers. ACN resulted in two tailing peaks (probably because ACN is less capable

for hydrogen bonding), and EtOH and IPA resulted in three peaks instead of the four obtained with MeOH, even when tested at lower percentages (*i.e* 15 and 10%, data not shown). Thus MeOH was retained as a modifier.

3.3.2 Influence on enantioseparation of the percentage of MeOH as a modifier

Percentages under 20 were tested: 15 and 10%. An increase of the organic modifier percentage, led to a decrease in retention and selectivity. This phenomenon is mainly caused by an increase in the polarity of the mobile phase but also in the fluid density. Indeed, with the increase in the mobile phase density, its elution strength increases and the retention time of the analyte decreases. As a consequence, enantioselectivity is slightly reduced [25]. This has been reported by others in SFC with these proportions of modifier (under 20%) [26]. Thus, bearing in mind that the analysis time should be as short as possible, 15% was retained as a good compromise between resolution (with values equal to 2.30, 2.70, 4.40, between the four stereoisomers) and run time (under 10 minutes).

3.3.3 Influence of the outlet pressure and flow-rate on enantioseparation.

The outlet pressure was evaluated at 100, 120, 150 and 180 bar (data not shown). For liquid-like fluids (above 10% of co-solvent), the pressure effect is less important. However it is observed that an increase in outlet pressure leads to a decrease in both retention times and resolution, thus 120 bar was retained. The flow-rate was briefly explored: 2 mL/min was tested to improve separation and 4 mL/min to shorten the analysis time, but the lower flow-rate resulted in a longer analysis time (around 25 minutes) with similar resolution values compared to 3 mL/min, while the higher flow-rate led to a loss of resolution between the middle peaks (*i.e* FR 2 and FR3). Thus, small-scale preparative separation was run at 3 mL/min flow-rate, with a mobile phase made of CO₂/MeOH 85:15 (v/v), at 40°C and 120 bar outlet pressure, injecting 20 µL of 0.8 mM of compound 1.

When injecting a concentrated solution (*i.e* 19 mM) a slight loss of baseline resolution between Peaks 2 and 3 was observed. Therefore the percentage of modifier was decreased to 10% in CO₂. The chromatogram obtained under these conditions is depicted in Figure 4a.

3.3.4 Small-scale preparative separation

Boxcar (or overlapped or stacked) automated injections are used to increase the productivity of preparative separation. With this stacked injection method, a second injection is made during the first one, before the eluted enantiomers have completely left the column (Figure 4b). The cycle time was then reduced from 14 to 7 minutes, and the productivity was increased by 50%. The preparative separation was performed on a total volume of 1 mL, so an amount of 7.29 mg of compound **1** was resolved. Around 1 mg (55% yield) of each stereoisomer was collected after evaporation. Unfortunately, the isomeric purity was not sufficient to perform the pharmacological tests. This can be seen at a glance by injecting each fraction on tandem coupling of columns (Fig. 2 in Supplementary Information), but nevertheless allowed us to study the stereoisomeric elution order (see Section 3.2 and Tables 1 and 2).

3.4 Chromatographic simulation: application of the mathematical model

If the coupling of columns provides good stereoisomeric separations, the exhaustive screening approach (followed by an optimization step) is neither time nor cost-efficient, particularly if many different columns are available. Thus, the possibility of mathematically predicting the retention times at any pressure is a potentially useful tool. Based on a study of pressure effect on chiral separation using a binary mobile phase [27], Wang *et al.* [14] determined two empirical quantitative pressure-retention relationships, which can be used to predict retention times on a column tandem:

$$t_0 = aP^2 + bP + c \quad \text{Eq. 3}$$

where the fitted coefficients a , b , and c depend on each column and are summarized in Table 3; and:

$$k_{P_i} = k_{P_0} e^{d(P_i - P_0)} \quad \text{Eq. 4}$$

where k_{P_i} is the retention factor at any pressure P_i , k_{P_0} is the experimental retention factor at the fixed experimental outlet pressure P_0 , and d is a column constant (Table 3). Equation 3 provides a way to calculate retention time at different pressures based on a single measurement at P_0 , obviating the need to perform the injection itself. To illustrate the accuracy of the model, the previously described tandem of columns OJ-H//AD-H was chosen. Compound **1** was injected the OJ-H and AD-H columns individually at P_0 equal to 120 bar outlet pressure, and the inlet pressure P_0 was then measured to calculate the individual pressure drop of each column (Figure 5a). The pressure drops over the OJ-H and the AD-H were determined to be 30 and 27 bar, respectively. When the OJ-H column was upstream of the AD-H column, the P_i was 147 bar (120 bar system back pressure plus 27 bar pressure drop over the AD-H) (Figure 5b). Using the column parameter obtained in the Wang's studies [14; 27], retention times of the four fractions of compound **1** on the OJ-H CSP at 147 bar, were calculated (Table 4). The overall retention times for OJ-H//AD-H (OJ-H in the upstream of AD-H) tandem column separations were obtained by adding experimental retention times on the AD-H at 120 bar and calculated retention times on the OJ-H at 147 bar ($t_{r_{\text{OJ-H//AD-H}}} = t_{r_{\text{OJ-H } 147 \text{ bar}}} + t_{r_{\text{AD-H } 120 \text{ bar}}}$). The results are summarized in Table 4. The accuracy between the observed and predicted retention times were less than 3.30% (Table 4). The variances of chromatographic peaks from single OJ-H and AD-H columns at 120 bar outlet pressure were summed to give the peak variance in tandem columns separations. Thus, based on the calculated retention times and peak variance, tandem coupling of columns chromatograms were simulated and are shown in Figure 6. Similarly, the overall retention times for AD-H//OJ-H (AD-H in the upstream of OJ-H) tandem column separations were determined by

summing the experimental retention times on the OJ-H at 120 bar and the calculated retention times on the AD-H at 150 bar (Figure 5b). For FR1, FR2, FR3 and FR4 the simulated retention times were 4.78, 5.24, 6.16 and 6.74 min respectively while experimental retention times were 4.97, 5.41, 6.22 and 6.80 min. The accuracy between the experimental and the predicted retention time was evaluated as less than 3.82%. Using this approach, we accurately predicted many of the other column tandems studied here. This mathematical model is of great help to speed up method development. However in our case mathematical parameters were missing for two columns involved in the screening. Thus we determined these parameters (a , b , c and d) required for the simulation accordingly to the two Wang's studies [14; 27] previously described.

3.5 Chromatographic simulation: determination of new parameters of mathematical model on chlorinated columns LA-2 and LC-2

3.5.1 Column dead time function

In spite of the wider adoption of SFC, and an increasing number of published articles using this technique, studies of the relationship between pressure and retention using binary solvents are rather scarce [28]. Rajendran *et al.* described a decrease in retention times while increasing the outlet pressure. Wang *et al.* also explored the trend of dead time with outlet pressure, and the result was that t_0 increased with rising column outlet pressure [27]. They also demonstrated that t_0 became less sensitive to pressure change as methanol content in carbon dioxide increased in the 5 to 40% range, resulting in a less compressible mobile phase. The relationship between dead time and outlet pressure was studied on the two chlorinated CSPs, namely LA-2 and LC-2. Figure 3 in Supplementary Information depicts their interdependence at 3 mL/min with 20% of MeOH in CO₂ as the mobile phase. The observed data were well predicted by(?) the quadratic equation 3 (presented above). The determination coefficient (R^2) was 0.9943 for LA-2 and 0.9989 for LC-2 columns. The fitted parameters (*i.e*

a , b and c from Eq. 3) defining the relationship between dead time and outlet pressure are summarized in Table 3.

3.5.2 Retention factor function

Outlet pressure influences retention time in different ways, notably the mobile phase solvation power through changes to mobile phase density. When the pressure increases, the density of the fluid increases (and hence the elution strength) causing a decrease in retention times. The extent of retention variation depends on mobile phase composition. To illustrate the effect of outlet pressure on retention 11 compounds were chosen as probe molecules covering a wide range of retention factors (for instance ketoconazole was highly retained). The retention factors, k , of each probe compound were plotted over the five outlet pressures. Good linearity was found for all compounds on each column. Then the slope of a fitted line of k over P (i.e. dk/dP) was plotted against k (Figure 3b in Supplementary Information). The representation of dk/dP against k , for the 11 probe molecules, was linear with a R^2 equal to 0.9774 and 0.9336 for the LA-2 and LC-2 columns, respectively, for a least square regression through the origin. This observation that the retention factor rate changes with pressure in a packed column with a binary mobile phase consisted of MeOH/CO₂ was also reported by Wang and Zhang [27]. The regression results of dk/dP against k (namely parameter d) are summarized in Table 2 for the two columns. Finally, the four parameters (a , b , c and d) determined on the chlorinated columns permitted us to predict overall retention times on column tandems including the LA-2 and LC-2 columns.

3.5.3 Application of the model to the LC-2//LA-2 and LA-2//LC2 tandems

The validity of the new parameters in the mathematical model developed by Wang *et al.* was then undertaken with the LC-2//LA-2 and LA-2//LC2 column tandems, following the calculations in section 3.4. The pressure drops over the LC-2 and the LA-2 were determined to be 21 and 20 bar, respectively. When the LA-2 column was placed upstream, the predicted

retention times for FR1, FR2, FR3 and FR4 were 13.54, 8.01, 8.50 and 9.18 min, respectively, while observed retention times were 13.38, 7.91, 8.41 and 9.08 min. The accuracy between the predicted and observed retention times was thus evaluated as less than 1.26%. When the LC-2 was placed upstream, the accuracy was less satisfactory with values between 4.25 and 6.88%. All the results are summarized in Table 5. Based on the calculated retention times, a simulation of the chromatograms with the LA-2//LC-2 column tandem and the LC-2//LA-2 tandem are shown in Figure 7 (top and bottom, respectively) and are superimposed on corresponding experimental chromatograms.

4. Conclusion

To perform the pharmacological assay of stereoisomers from a potential anti-diabetic compound, their enantioseparation(?) is necessary. In this particular case, when separation on a single-column was insufficient, coupling columns of different selectivity in series, thus doubling the separation efficiency, is a potentially powerful technique to resolve complex mixtures. Its utility in resolving stereoisomers of a dihydropyridone derivative with multiple chiral centers is illustrated in this work. It can be seen that even if the column length is doubled, allowing improved resolution through an improved efficiency, analysis times increase by a factor of less than two. The upstream column is operated at elevated outlet pressure which attenuates its retention of compounds. In consequence, we here illustrated that the order of columns (and hence the different back pressures the individual columns experience), is particularly important in tandem column method development. Very different separations of compound **1** were observed by simply swapping the upstream and downstream columns, as shown in Fig. 3b or in Fig.2 in Supplementary Information. For the first time, the mathematical model developed by Wang was accurately applied to another probe (namely our compound **1**). In addition, this study has characterized the pressure effect on two new chlorinated, chiral stationary phases, Lux Amylose-2 and Lux Cellulose-2, using the most

widely-used binary solvents in SFC (MeOH/CO₂). We have here shown that compound retention at multiple outlet pressures can be calculated from a single retention time at a single working pressure. The model can further predict, with good accuracy, the molecules' retention time on tandems of coupled columns, including chlorinated CSPs, thus enabling faster and cheaper method development.

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Figure captions

Figure 1a Structure of the dihydropyridone derivative **1**.

Figure 1b Structure of the polysaccharide-based stationary phases.

Figure 2 Chromatograms obtained for compound **1** stereoisomers on AD-H, AS-H, OD-H, OJ-H, LA-2 and LC-2 CSPs with 20% methanol and a flow-rate of 3 mL/min, column temperature of 40°C and 120 bar outlet pressure, $\lambda = 210$ nm.

Figure 3a Chromatograms obtained for compound **1** stereoisomers on OJ-H//AD-H and AD-H//OJ-H tandem of coupling columns and stereoisomeric elution order with 20% methanol and a flow-rate of 3 mL/min, column temperature of 40°C and 120 bar outlet pressure, $\lambda = 210$ nm.

Figure 3b Chromatograms obtained for compound **1** stereoisomers on OD-H//OJ-H and OJ-H//OD-H tandem of coupling columns and stereoisomeric elution order with 20% methanol and a flow-rate of 3 mL/min, column temperature of 40°C and 120 bar outlet pressure, $\lambda = 210$ nm.

Figure 4a Small-scale preparative separation chromatogram obtained for compound **1** stereoisomers (at 19 mM), on OJ-H//AD-H tandem columns and points of collection of the fractions 1 to 4, with 10% methanol and a flow-rate of 3 mL/min, column temperature of 40°C and 120 bar outlet pressure, $\lambda = 210$ nm.

Figure 4b Small-scale preparative separation chromatogram obtained for compound **1** stereoisomers (at 19 mM), on OJ-H//AD-H tandem of coupling columns in stacked-mode of injection, with 10% methanol and a flow-rate of 3 mL/min, column temperature of 40°C and 120 bar outlet pressure, $\lambda = 210$ nm.

Figure 5a Pressure drop determination for single columns OJ-H and AD-H with 20% methanol, a flow-rate of 3 mL/min, column temperature of 40°C and 120 bar outlet pressure, $\lambda = 210$ nm.

Figure 5a Intermediate pressure determination for single column OJ-H placed upstream and AD-H placed downstream (upper part) and intermediate pressure determination for single column AD-H placed upstream and OJ-H placed downstream (lower part), with 20% methanol and a flow-rate of 3 mL/min, column temperature of 40°C and 120 bar outlet pressure, $\lambda = 210$ nm.

Figure 6 Superposition of predicted and experimental chromatograms obtained for compound **1** on OJ-H//AD-H column tandems with 20% methanol and a flow-rate of 3 mL/min, column temperature of 40°C and 120 bar outlet pressure, $\lambda = 210$ nm.

Figure 7 Superposition of predicted and experimental chromatograms obtained for compound **1** on LC-2//LA-2 and LA-2//LC-2 column tandems with 20% methanol and a flow-rate of 3 mL/min, column temperature of 40°C and 120 bar outlet pressure, $\lambda = 210$ nm.

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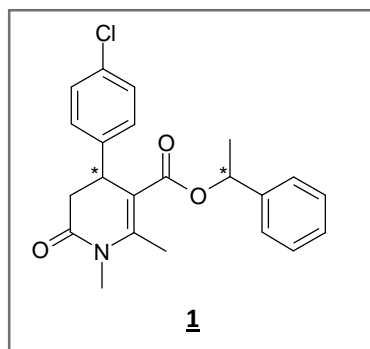


Figure 1a

Amylose-based			Cellulose-based		
Name	Nature	Substituent	Name	Nature	Substituent
Chiralpak® AD	Coated		Chiralcel® OD	Coated	
Chiralpak® AS	Coated		Chiralcel® OJ	Coated	
Lux™ Amylose-2	Coated		Lux™ Cellulose-2	Coated	

Figure 1b

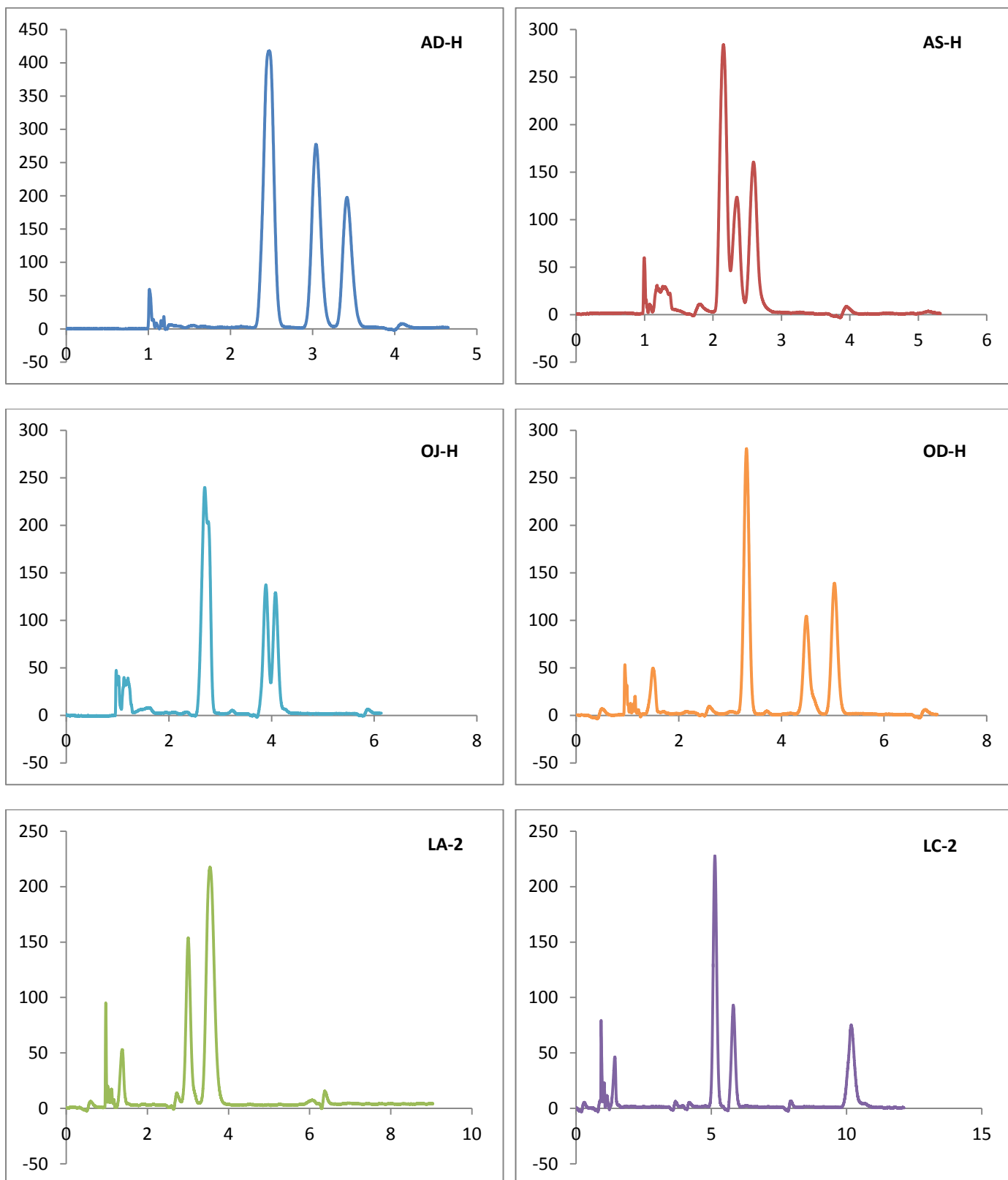


Figure 2

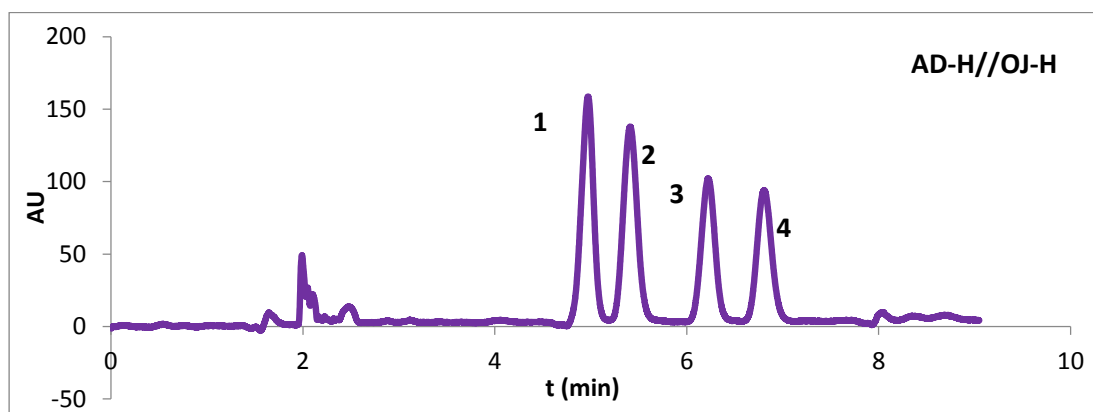
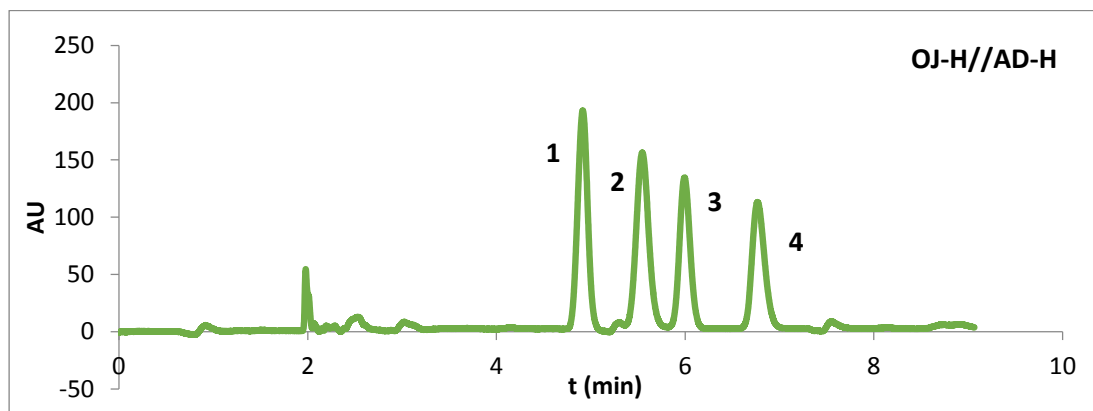


Figure 3a

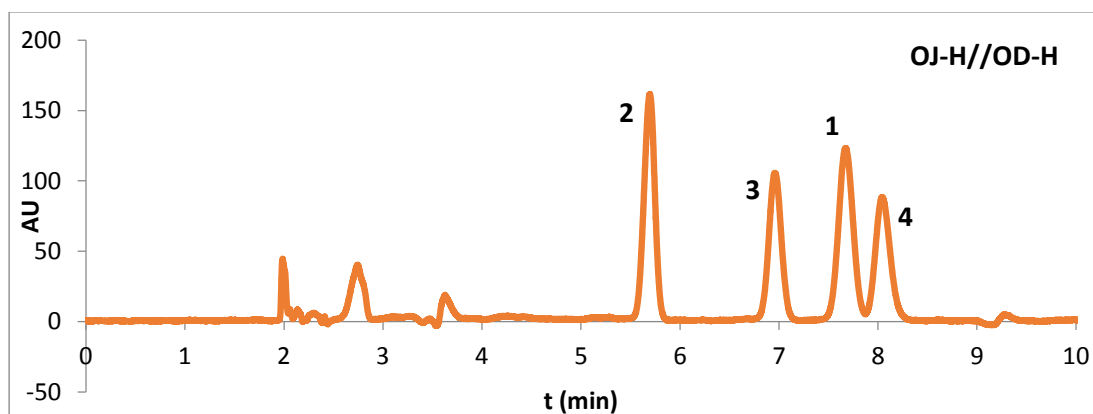
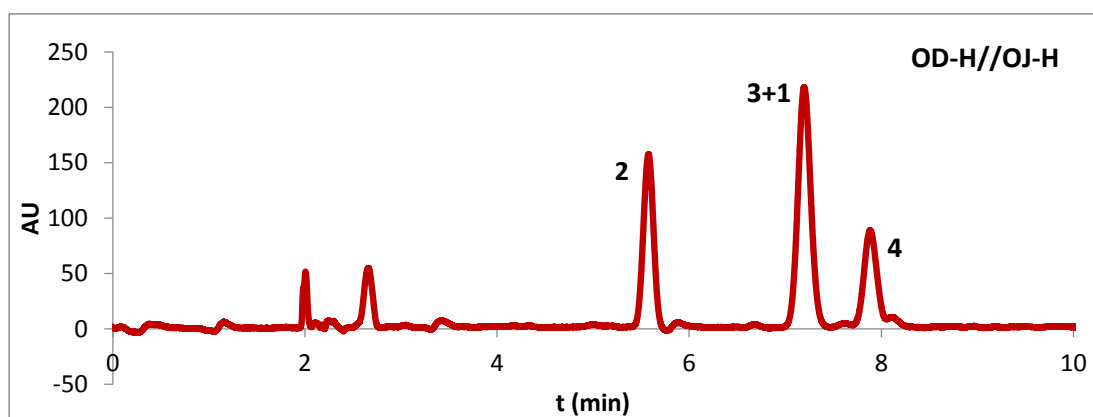


Figure 3b

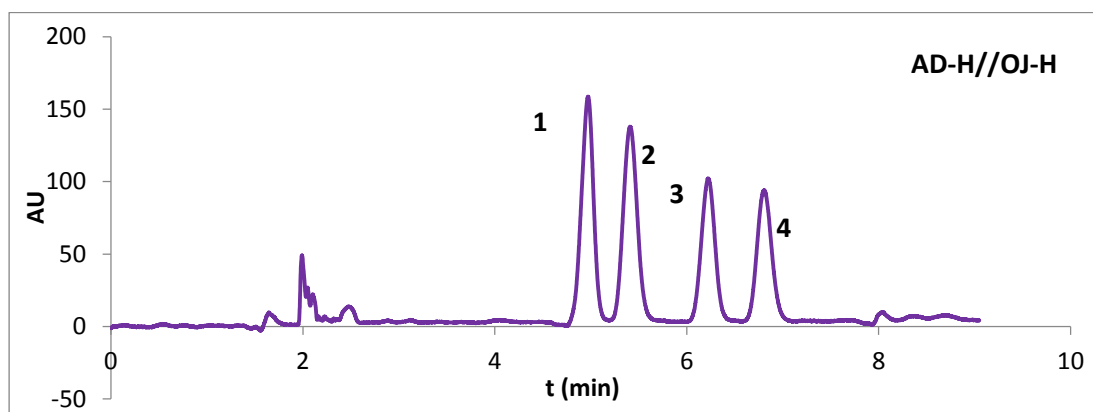
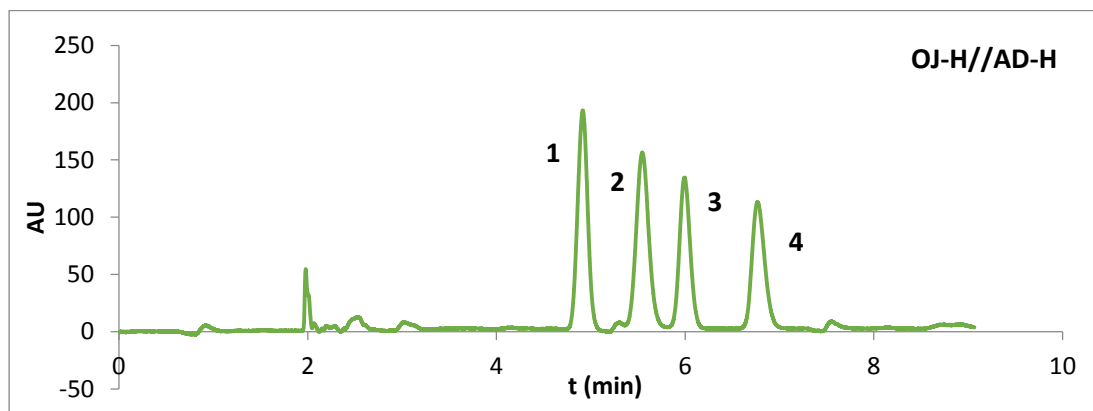


Figure 3a

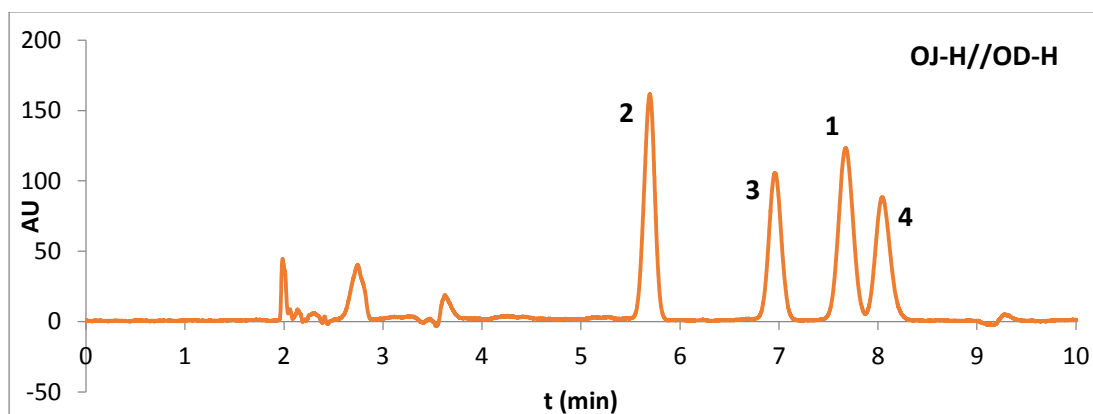
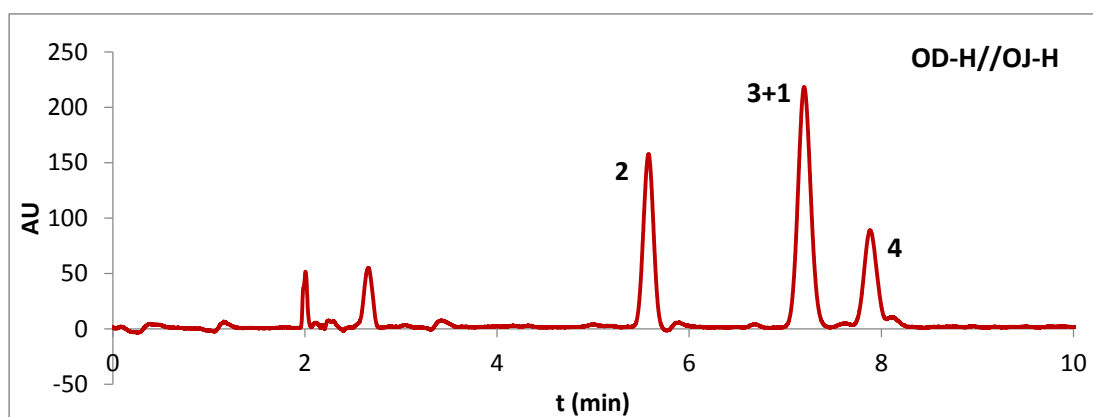


Figure 3b

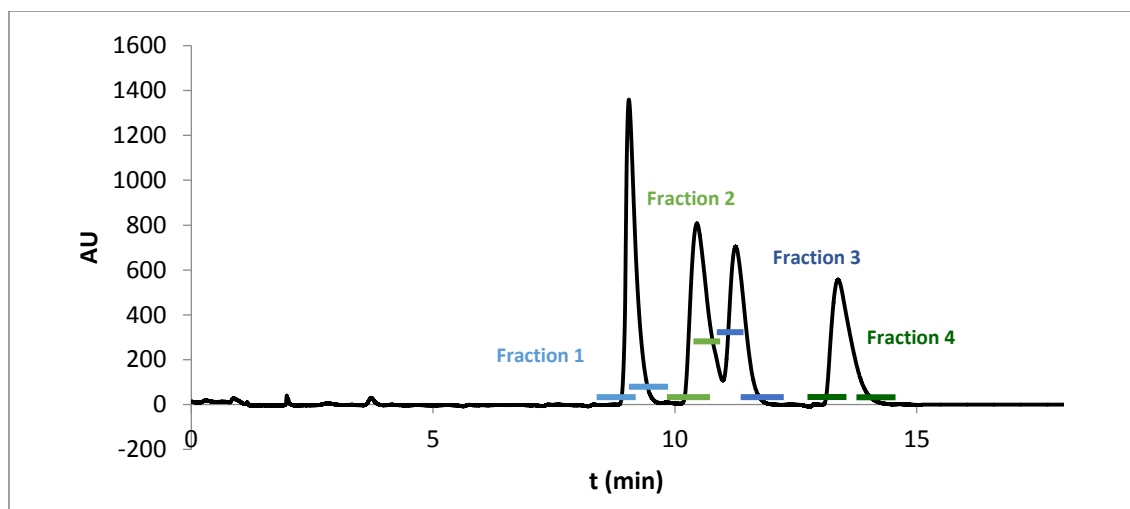


Figure 4a

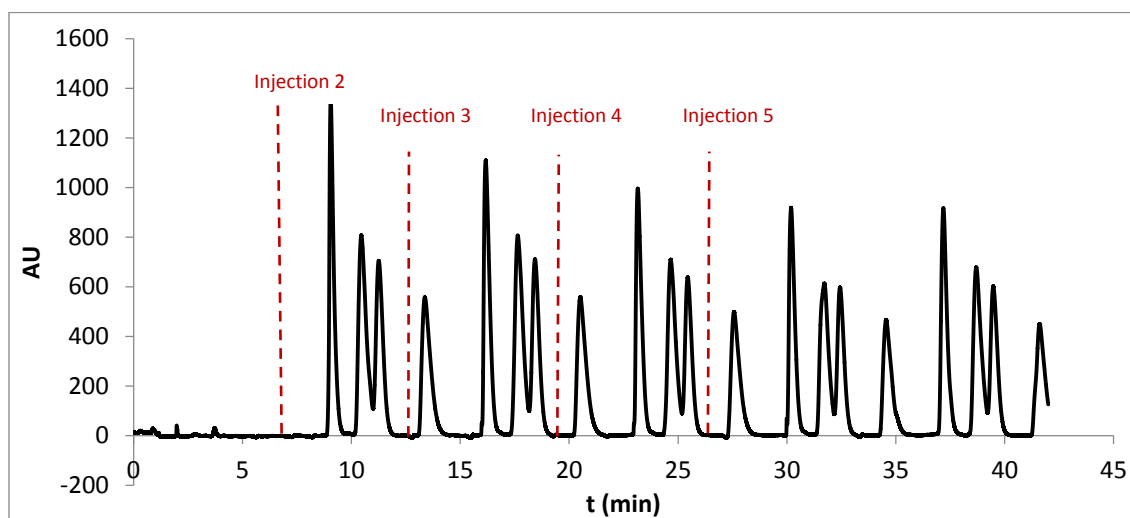


Figure 4b

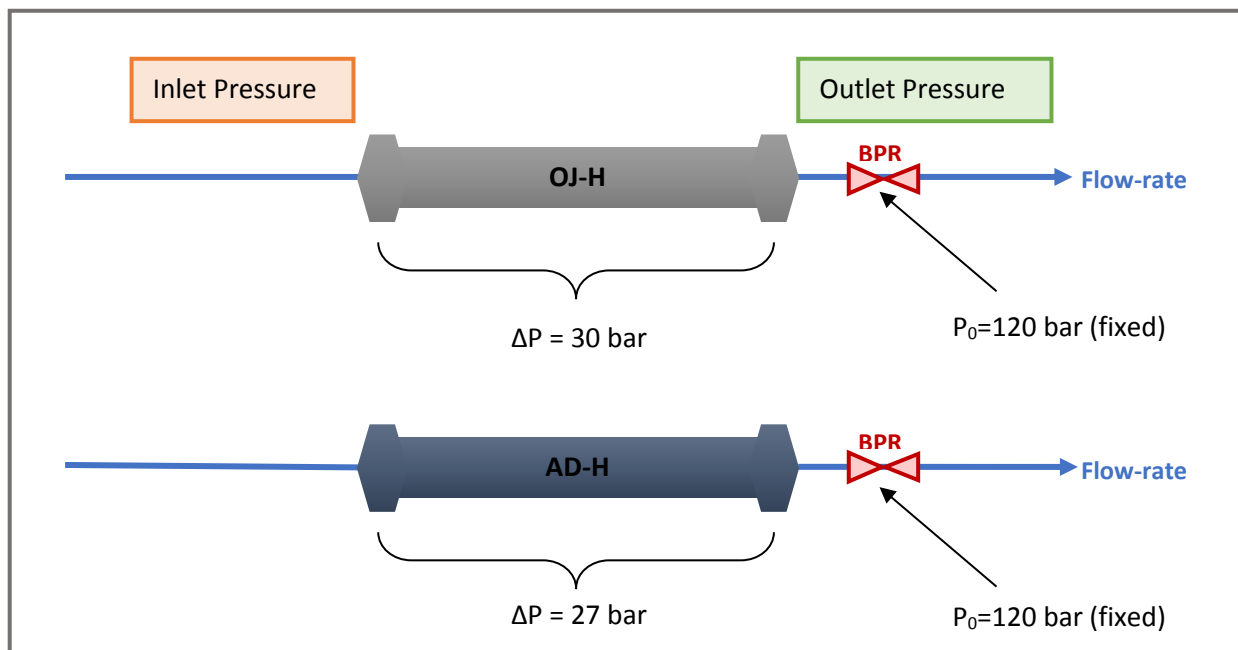


Figure 5a

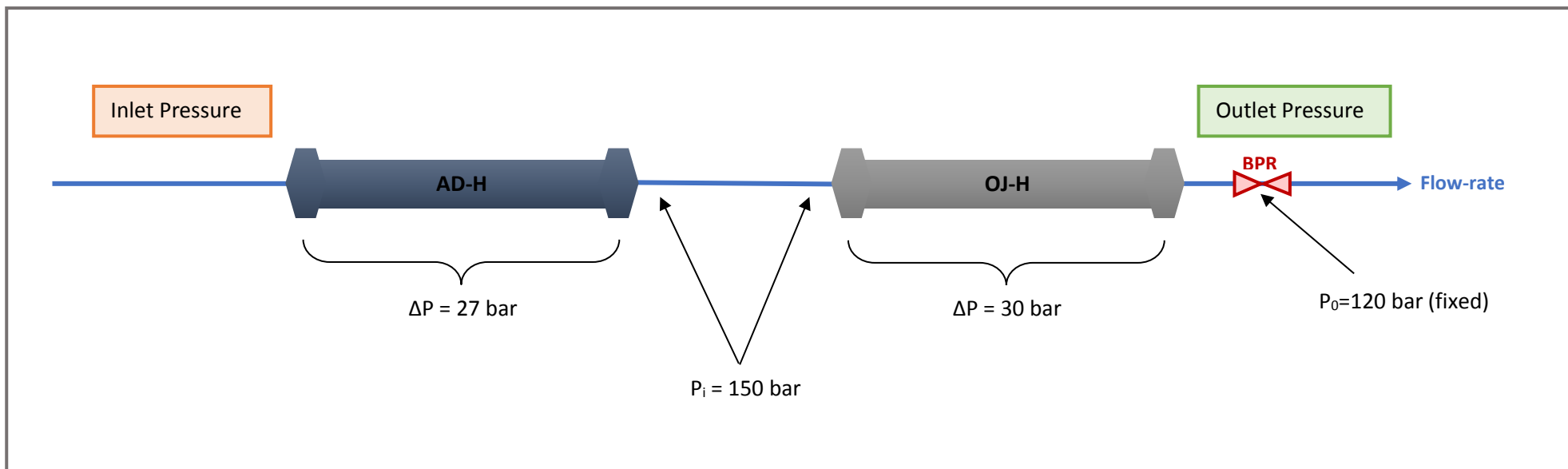
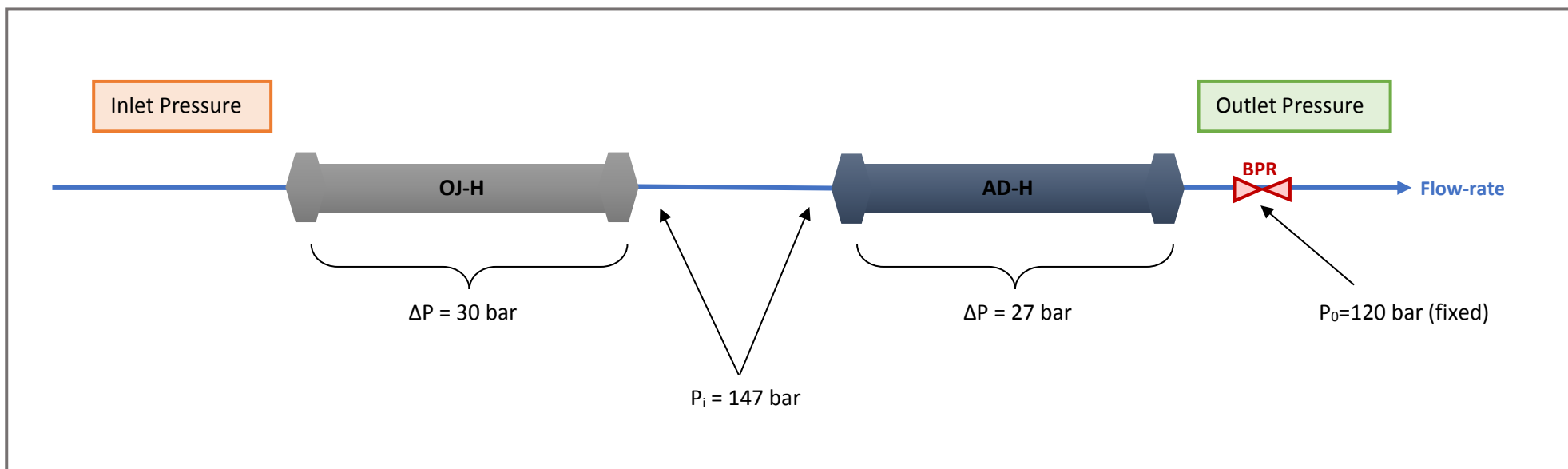


Figure 5b

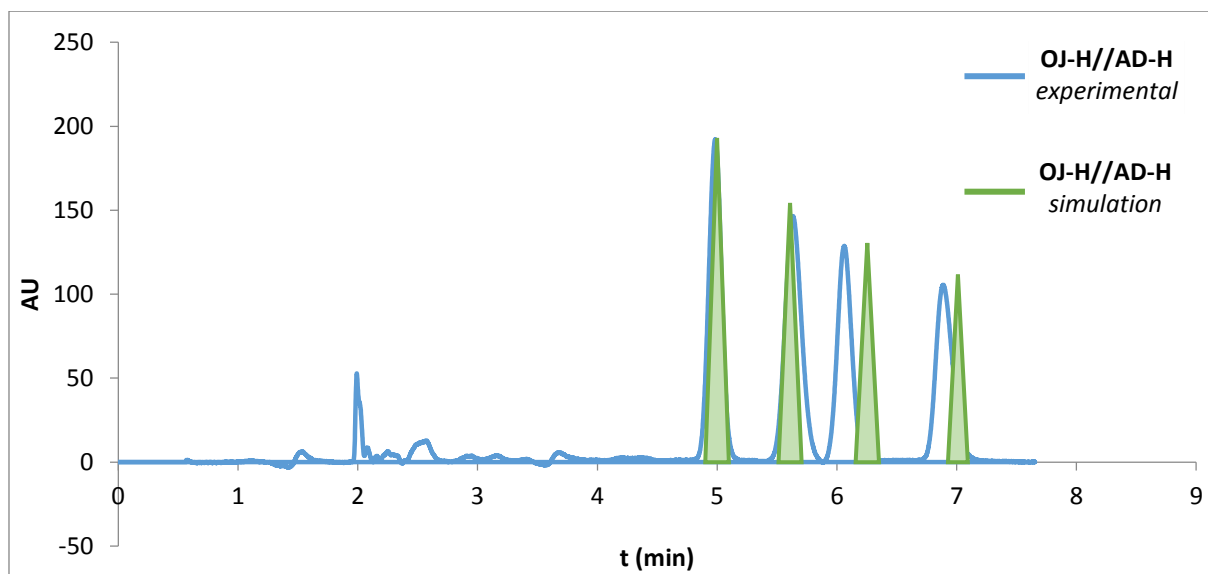


Figure 6

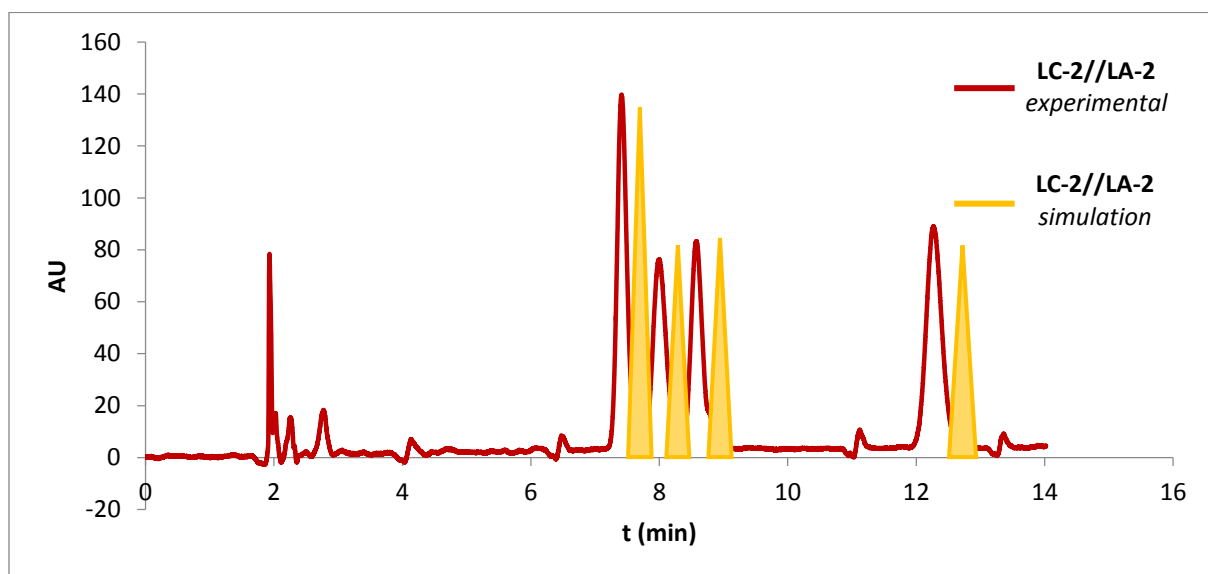
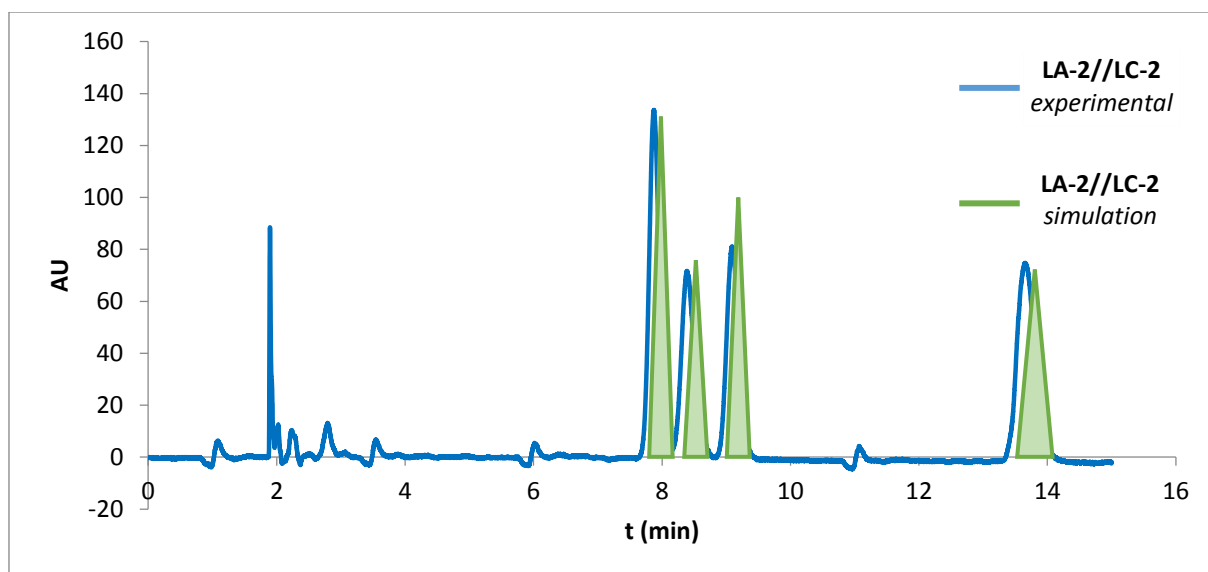


Figure 7

Table 1. Chromatographic parameters obtained for **1** on each single column and isomeric elution order, under 3 mL/min 20% MeOH, 40°C, 120 bar, $\lambda = 210$ nm.

Column	tr ₁ (min)	tr ₂ (min)	tr ₃ (min)	tr ₄ (min)	k ₁	k ₂	k ₃	k ₄	α_{1-2}	α_{2-3}	α_{3-4}	Rs ₁₋₂	Rs ₂₋₃	Rs ₃₋₄
	FR2 + FR3	FR4	FR1											
LC-2	5.13	5.81	10.17	-	4.52	5.25	9.94	-	1.16	1.89	-	2.67	12.82	-
	FR1 + FR3	FR2	FR4											
AD-H	2.59	3.02	3.47	-	1.65	2.55	-	-	1.39	1.20	-	2.48	1.77	-
	FR2 + FR 3	FR4	FR1											
OD-H	3.29	4.50	5.03	-	2.45	3.72	4.27	-	1.52	1.15	-	5.69	2.36	-
	FR2	FR1+FR3+FR4												
LA-2	2.99	3.52	-	-	2.05	2.59	-	-	1.26	-	-	1.89		-
	FR 1 + FR 2	FR4	FR3											
OJ-H	2.72	3.89	4.01	-	1.62	2.74	2.95	-	1.70	1.07	-	5.26	1.11	-
AS-H*	2.15	2.35	2.59	-	1.16	1.36	1.60	-	1.17	1.18	-	1.02	1.28	-

* FR1 to FR4 not injected

Table 2. Chromatographic parameters obtained for **1** on each tandem and isomeric elution order, under 3 mL/min 20% MeOH, 40°C, 120 bar, $\lambda = 210$ nm.

Tandem	tr ₁ (min)	tr ₂ (min)	tr ₃ (min)	tr ₄ (min)	k ₁	k ₂	k ₃	k ₄	α_{1-2}	α_{2-3}	α_{3-4}	Rs ₁₋₂	Rs ₂₋₃	Rs ₃₋₄
	FR 1	FR 2	FR 3	FR 4										
OJ-H//AD-H	4.98	5.63	6.06	6.89	1.50	1.83	2.04	2.46	1.22	1.12	1.20	2.97	1.83	3.54
	FR 1	FR 2	FR 3	FR 4										
AD-H//OJ-H	4.97	5.41	6.22	6.80	1.50	1.72	2.12	2.42	1.15	1.24	1.14	1.66	3.06	2.19
	FR 2	FR 3	FR 1	FR 4										
OJ-H//OD-H	5.69	6.96	7.67	8.04	1.86	2.50	2.86	3.05	1.34	1.14	1.07	7.47	3.84	1.85
OD-H//OJ-H	5.58	7.20	7.90	-	1.79	2.60	2.95	-	1.45	1.13	-	7.53	2.92	-
OJ-H//AS-H	4.45	4.99	5.58	-	1.24	1.51	1.80	-	1.22	1.20	-	2.57	2.57	-
AS-H//OJ-H	4.58	5.03	5.96	-	1.31	1.53	2.00	-	1.17	1.31	-	2.14	3.80	-
	FR 2	FR 3	FR 4	FR 1										
OJ-H//LC-2	7.50	8.68	9.17	12.48	2.97	3.60	3.86	5.62	1.21	1.07	1.45	4.55	2.14	10.51
	FR 2	FR 3	FR 4	FR 1										
LC-2//OJ-H	7.01	8.40	8.75	11.11	2.69	3.42	3.61	4.85	1.27	1.06	1.34	5.90	1.28	7.26
OJ-H//LA-2	5.33	5.79	6.74	-	1.72	1.96	2.44	-	1.14	1.25	-	1.74	2.68	-
LA-2//OJ-H	5.35	5.84	7.08	-	1.74	1.99	2.62	-	1.14	1.32	-	2.04	3.14	-
	FR 2	FR 3	FR 4	FR 1										
OD-H/LA-2	5.95	6.53	7.60	7.92	2.07	2.37	2.92	3.08	1.14	1.23	1.06	2.11	3.65	1.16
	FR 2	FR 3	FR 4	FR 1										
LA-2//OD-H	5.97	6.47	7.68	8.11	2.11	2.37	3.00	3.22	1.12	1.27	1.07	1.62	3.58	1.33
	FR 3	FR 2	FR 1	FR 4										
OD-H//AD-H	5.30	5.86	6.85	7.35	1.69	1.97	2.48	2.73	1.17	1.25	1.10	2.43	3.69	1.87
	FR 3	FR 2	FR 1	FR 4										
AD-H//OD-H	5.49	5.93	7.23	7.47	1.82	2.04	2.71	2.83	1.12	1.33	1.05	1.81	4.73	0.79
OD-H//AS-H	4.96	6.09	6.76	-	1.53	2.11	2.45	-	1.38	1.16	-	4.81	2.53	-
AS-H//OD-H	5.26	6.70	7.44	-	1.66	2.38	2.76	-	1.44	1.16	-	5.33	2.60	-
OD-H//LC-2	8.01	9.70	14.55	-	3.24	4.13	6.70	-	1.28	1.62	-	5.56	13.47	-
LC-2//OD-H	7.71	9.46	13.57	-	3.12	4.06	6.26	-	1.30	1.54	-	5.63	10.69	-

Tandem	tr_1	tr_2	tr_3	tr_4	k_1	k_2	k_3	k_4	α_{1-2}	α_{2-3}	α_{3-4}	Rs_{1-2}	Rs_{2-3}	Rs_{3-4}
	FR 2	FR 3	FR 4	FR 1										
LC-2//LA-2	7.41	8.00	8.58	12.69	2.84	3.14	3.44	5.58	1.11	1.10	1.62	1.67	1.54	9.68
	FR 2	FR 3	FR 4	FR 1										
LA-2//LC-2	7.91	8.41	9.08	13.38	3.14	3.40	3.75	6.01	1.08	1.10	1.60	1.54	1.91	10.12
	FR 3	FR 2	FR 4	FR 1										
LC-2//AD-H	6.71	7.25	8.25	10.94	2.49	2.78	3.30	4.70	1.11	1.19	1.42	2.12	3.45	7.58
	FR 3	FR 2	FR 4	FR 1										
AD-H//LC-2	7.26	7.70	8.73	12.24	2.83	3.07	3.61	5.46	1.08	1.18	1.51	1.51	3.51	9.00
LC-2//AS-H	6.38	7.09	10.82	-	2.36	2.73	4.69	-	1.16	1.72	-	2.84	11.13	-
AS-H//LC-2	7.02	7.85	12.57	-	2.68	3.11	5.58	-	1.16	1.79	-	2.96	12.10	-
AS-H//LA-2	4.97	5.84	-	-	1.49	1.92	-	-	1.29		-	1.89	-	-
LA-2//AS-H	4.74	5.17	5.40	5.57	1.38	1.60	1.71	1.80	1.16	1.07	1.05	-	-	-
AS-H//AD-H	4.27	4.73	5.43	-	1.16	1.39	1.74	-	1.20	1.25	-	1.52	1.94	-
AD-H//AS-H	4.21	4.69	5.19	-	1.13	1.37	1.62	-	1.22	1.18	-	1.98	1.92	-
AD-H//LA-2	5.66	6.60	-	-	1.90	2.38	-	-	1.25	-	-	2.91	-	-
LA-2//AD-H	5.60	6.61	-	-	1.81	2.32	-	-	1.28	-	-	2.56	-	-

Table 3. Fitted parameters for the relationship between dead time and column outlet pressure (a , b and c) and linear regression results of the $dk/dP = f(k)$ relationship for test compounds [14].

	a	b	c	r^2	d	r^2
Chiralpak AD-H	-2.00 ^{E-06}	0.0001	0.8868	0.9956	-0.0015	0.9123
Chiralpak AS-H	-2.00 ^{E-06}	0.0001	0.8738	0.9986	-0.0016	0.9348
Chiralcel OD-H	-2.00 ^{E-06}	0.0009	0.9172	0.9960	-0.0019	0.9516
Chiralcel OJ-H	-4.00 ^{E-06}	0.0016	0.8626	0.9940	-0.0024	0.9729
Chiralpak IA	-3.00 ^{E-06}	0.0011	0.8781	0.9889	-0.0024	0.9805
Chiralpak IB	-3.00 ^{E-06}	0.0012	0.8715	0.9948	-0.0020	0.9750
Whelk-O1 (R, R)	-2.00 ^{E-06}	0.0013	0.7222	0.9972	-0.0026	0.9942
Chirobiotic T	-4.00 ^{E-06}	0.0018	0.7680	0.9988	-0.0019	0.9939
Cyclobond I2000	-3.00 ^{E-06}	0.0016	0.8262	0.9859	-0.0021	0.9646
Larihc CF6RN	-2.00 ^{E-06}	0.0013	0.8035	0.9990	-0.0021	0.9922
Lux Amylose-2	-2.00 ^{E-06}	0.0023	0.7354	0.9943	-0.0054	0.9774
Lux Cellulose-2	-4.00 ^{E-06}	0.0027	0.6542	0.9989	-0.0037	0.9336

Table 4. Predicted retention times calculations of compound **1** on OJ-H//AD-H tandem and comparison to experimental retention times on OJ-H//AD-H tandem

	$t_{r_{OJ-H\ 120\ bar}}^*$	$k_{OJ-H\ 120\ bar}^\dagger$	$t_{r_{OJ-H147\ bar}}^\pounds$	$k_{OJ-H\ 147\ bar}^\S$	$t_{r_{OJ-H\ 147\ bar}}^\ddagger$	$t_{r_{AD-H\ 120\ bars}}^*$	$t_{r_{OJ-H147\ bar//AD-H\ 120\ bar}}^\ddagger$	$t_{r_{OJ-H//AD-H}}^*$	Accuracy
Iso 1 /FR 1	2.72	1.62	1.01	1.52	2.55	2.45	5.00	4.98	0.40%
Iso 2 /FR 2	2.72	1.62	1.01	1.52	2.55	3.02	5.57	5.63	1.07%
Iso 3 /FR 3	4.10	2.95	1.01	2.77	3.81	2.45	6.26	6.06	3.30%
Iso 4 /FR 4	3.89	2.74	1.01	2.57	3.60	3.41	7.01	6.89	1.74%

* *Experimental data*

† *Calculated data through Eq.2*

‡ *Calculated data through Eq.3*

§ *Calculated data through Eq.4*

$$\ddagger t_{r_{OJ-H//AD-H}} = t_{r_{OJ-H\ 147\ bar}} + t_{r_{AD-H\ 120\ bar}}$$

Table 5. Predicted retention times calculations of compound **1** on different tandems and comparison to experimental retention times

	$t_{r_{LA2\ 120\ bar}}^*$	$k_{LA2\ 120\ bar}^{\dagger}$	$t_{r_0\ LA2\ 141\ bar}^{\text{¥}}$	$k_{LA2\ 141\ bar}^{\text{§}}$	$t_{r_{LA2\ 141\ bar}}^{\dagger}$	$t_{r_{LC2\ 120\ bars}}^*$	$t_{r_{LA2\ 141\ bar//LC2\ 120\ bar}}^{\ddagger}$	$t_{r_{LA2//LC2}}^*$	Accuracy
Iso 1 /FR 1	3.52	2.59	1.02	2.31	3.37	10.17	13.54	13.38	-1.26%
Iso 2 /FR 2	2.99	2.05	1.02	1.83	2.88	5.13	8.01	7.91	-1.07%
Iso 3 /FR 3	3.52	2.59	1.02	2.31	3.37	5.13	8.50	8.41	-1.19%
Iso 4 /FR 4	3.52	2.59	1.02	2.31	3.37	5.81	9.18	9.08	-1.10%
	$t_{r_{LC2\ 120\ bar}}^*$	$k_{LC2\ 120\ bar}^{\dagger}$	$t_{r_0\ LC2\ 140\ bar}^{\text{¥}}$	$k_{LC2\ 140\ bar}^{\text{§}}$	$t_{r_{LC2\ 140\ bar}}^{\dagger}$	$t_{r_{LA2\ 120\ bars}}^*$	$t_{r_{LC2\ 140\ bar//LA2\ 120\ bar}}^{\ddagger}$	$t_{r_{LC2//LA2}}^*$	Accuracy
Iso 1 /FR 1	10.17	9.94	9.23	0.95	9.71	3.52	13.23	12.69	-4.25%
Iso 2 /FR 2	5.13	4.52	4.19	0.95	4.93	2.99	7.92	7.41	-6.88%
Iso 3 /FR 3	5.13	4.52	4.19	0.95	4.93	3.52	8.45	8.00	-5.60%
Iso 4 /FR 4	5.81	5.25	4.87	0.95	5.52	3.52	9.04	8.58	-5.36%

* Experimental data

† Calculated data through Eq.2

¥ Calculated data through Eq.3

§ Calculated data through Eq.4

$$\ddagger t_{r_{LA-2//LC-2}} = t_{r_{LA2\ 141\ bar}} + t_{r_{LC2\ 120\ bar}}$$

$$\ddagger t_{r_{LC-2//LA-2}} = t_{r_{LC2\ 140\ bar}} + t_{r_{LA2\ 120\ bar}}$$