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Development of a two-dimensional supercritical fluid chromatography system in multiple heart-cutting mode

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ABSTRACT: In this paper, we present the development of a loop-based two-dimensional supercritical fluid system in multiple heart-cutting mode (mSFC-SFC), with diode-array and mass spectrometric detection. The instrument design was developed to be as simple as possible, based on a single SFC instrument, with the sole addition of three external 2-port 6-position valves. The objective was to achieve the most complete transfer of a peak from the first to the second dimension, whatever the composition of the mobile phase, *i.e.* whatever the proportion of carbon dioxide and methanol co-solvent along a wide gradient elution. Thanks to fine adjustment of the valve switching times, the first-dimension peaks were parked in 50- or 100- μ L loops and later discharged to the second dimension. The interest of this instrument was then demonstrated with a sample application on a natural product: an extract of *Citrus aurantium* L. bark was analyzed, with a particular focus on chiral flavonoids, neohesperidin and naringin. In this system, the first dimension was an achiral separation of the flavonoids, based on a polar diethylamine-bonded silica stationary phase (ACQUITY Torus DEA), while the second dimension used a stereoselective polysaccharide stationary phase (CHIRALPAK IB-3) to resolve flavonoid diastereomers. Excellent repeatability was demonstrated, with relative standard deviation values on retention times and peak areas all below 2%, together with excellent peak capacity and peak shapes (no distortion observed), making it possible to quantify diastereomers in the second-dimension separation. This good repeatability was also shown for the transfer rate between the two dimensions, which reached a value of 83%. Finally, transferring of a compressible sample from the first to the second dimension is demonstrated to yield excellent performance, despite the large loop volume.

INTRODUCTION

Since its first applications in the 1970s¹, two-dimensional (2D) chromatography has evolved considerably¹. The rapid development of analytical technologies has opened up new perspectives in chromatography, with significant advances in multidimensional chromatography². By combining two chromatographic separation steps, this approach overcomes the limitations of one-dimensional (1D) chromatography. The advantages include superior peak capacity and the ability to separate closely related compounds by combining different selectivities in both dimensions.

From this perspective, 2D multiple heart-cut chromatography^{3,4} is characterized by the application of an initial separation, followed by the selection and selective reinjection of specific fractions containing the compounds of interest into a second chromatographic column. This approach enables a more advanced separation of compounds, providing a more detailed view of the analyzed mixture. Over the years, two-dimensional liquid chromatography has been widely developed. In particular, the applications of 2D liquid chromatography (LC) are vast and diverse. It is widely used in fields such

as pharmaceutical^{5,6} research, food analysis^{7,8}, natural products^{9,10}, and many others.

Compared to LC, supercritical fluid chromatography (SFC) offers a number of advantages, such as high efficiency at high flow rates, environmentally friendly and economically advantageous solvent (mainly CO₂)⁵. However, coupling LC with SFC can be challenging^{6,7,11-13} due to possible incompatibility of mobile phases and differences in pressure and flow rate between the two chromatographic systems. This typically causes broadening and distortion of the peaks.

Faced with these difficulties, the development of two-dimensional SFC (2D SFC) has emerged as a promising alternative. During the 1990s¹, capillary column systems developed and evolved over the years towards packed columns. The main difficulty in using SFC in two-dimensional systems was to successfully maintain back-pressure to keep CO₂ in its supercritical state. The first developments of these systems operated with neat CO₂, without any co-solvent^{8,14-16}. However, the applicability of SFC is significantly improved by the addition of a polar co-solvent to the non-polar CO₂, thereby increasing the polarity of the mobile phase and solvating power to a wider range of molecules. A first approach to such a

system was put forward in 2011 by Lu Zeng *et al*¹⁷, focused on selecting a single pair of enantiomers from the first dimension, to resolve them in the second dimension, particularly for transfer to preparative scale.

In this study, we present the development of a 2D SFC-SFC system in multiple heart-cutting mode. A major challenge in this development was to maintain the instrument as simple as possible to favor its applicability in different laboratories, without the need for complex equipment. The implementation of a dedicated valve system using multiple 6-port, 2-position switching valves allowed maintaining the system pressure with a single back-pressure regulator (BPR). However, optimization of the valve switching times was necessary to ensure good peak harvest. Combined with repeatability tests, it was demonstrated that the composition of the transferred fractions did not impact the proper functioning of the system, not even when transferring fractions including high co-solvent percentages. Finally, the usefulness of this system was demonstrated with an achiral-chiral method applied to major chiral flavonoids in a sample of bitter orange bark.

EXPERIMENTAL

1. Chemicals and reagents.

HPLC-grade methanol used as the co-solvent and sample diluent, ethanol HiPerSolv Chromanorm 99.7-100% and ammonium hydroxide were purchased from VWR (Fontenay-sous-Bois, France). CO₂ with a purity of 99.7% was delivered by Air Liquide (Paris, France). Methanesulfonic acid (MSA) was purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Ultra-pure water was supplied by a Milli-Q® IQ 7000 system from Merck (Darmstadt, Allemagne).

Reference compounds (naringin and neohesperidin) were purchased from Extrasynthese (Genay, France). Standards of epicatechin and caffeine were obtained from Sigma-Aldrich. *Citrus aurantium* L. peels were bought from Louis herboristerie (Charleville-Mézières, France).

2. Sample preparation.

Standard solutions were prepared at a concentration of 1 mg/mL in methanol (MeOH). Orange peels were finely ground, then 1 g of sample was extracted with 15 mL of ethanol (EtOH) for 30 min in an ultrasonic bath. The extract was then centrifuged (20 °C, 8000 rpm) for 5 min and filtered through 0.20 µm, 13-mm diameter PVDF syringe filters from Agilent Technologies (Les Ulis, France) and diluted by 4 in EtOH prior to analysis.

3. Chromatographic instrumentation.

The supercritical fluid chromatography system was a Waters Corporation (Millford, MA, USA) ACQUITY Ultra Performance Convergence Chromatography™ (UPC²®). It was equipped with an autosampler with a partial loop volume injection system, a back-pressure regulator, a column oven allowing the implementation of

two columns compatible with 150 mm length columns, via column switching valves. In the 2D mode, these column switching valves (V4, V5) were not used for column switching but for the implementation of multiple storage loops, as described in Section 3.1.1. The system also included a binary solvent delivery pump compatible with a maximum mobile phase flow-rate of 4 mL/min and a maximum pressure of 414 bar. Two detectors were available: a diode-array detector (ACQUITY PDA®) and a single-quadrupole mass spectrometer with an electrospray ionization source (ACQUITY QDa®). An isocratic solvent manager was used as a make-up pump and was positioned before the mass detector and with a split, where one portion was sent to the back-pressure regulator and the other to the MS. The use of MSA in the mobile phase involved adapting the exhaust valve of the QDa system as MSA may cause corrosion on certain parts.

Empower® 3 was used for chromatographic acquisition and for data treatment.

The system was extended with three 6-port, 2-position automatic valves (V1-V3) from Rheodyne (MXT715-000), supplied by CIL Cluzeau (Sainte-Foy-la-Grande, France). The loops to park the fractions were created in-house using tubing also provided by CIL Cluzeau. They were 1/16" stainless steel tubes with internal diameters of 0.020 and 0.030 inches. The tubing was cut to a length of 25 cm using a tube cutter, resulting in 50 µL and 114 µL loops. The tubes were then sanded to even out the cut. Each tubing was then rinsed with ethanol to remove any dust from the sanding to prevent system clogging.

4. Chromatographic conditions

Preliminary tests were carried out using identical stationary phases in both separation dimensions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm) from Waters. For applications on a real sample (bitter orange peel), two different columns were used. The first-dimension (dim1) column was again ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 µm). The second-dimension (dim2) column was CHIRALPAK IB-3 (150 × 4.6 mm, 3.0 µm) from ChiralTechnologies (Illkirch-Graffenstaden, France). Additionally, a Torus DEA pre-column (2.1 × 5.0 mm) from Waters was placed in front of the Torus DEA column.

Based on previous investigations¹⁸, the mobile phase used was CO₂ with MeOH containing 0.1% MSA.. The first-dimension method (Table 1a) was also adapted from J. Molineau *et al.* for flavonoid separation¹⁸. Only flow-rates were adapted due to the use of a pre-column causing additional pressure drop.

Table 1 - Gradient profiles in the a. first and b. second dimension

Time (min)	Co-solvent (%)	Back-pressure (bar)	Flow rate (mL/min)
a. First dimension method			
0.0	20	150	1.7
0.5	20	150	1.7
8.0	100	110	0.6
10.0	100	110	0.6
10.5	20	110	0.6
12.0	20	150	1.7
12.5	20	150	1.7
b. Second dimension method			
0.0	15	150	2.0
0.5	15		
17.0	50		
19.0	50		
19.5	15		
22.0	15		

The column oven was heated to 30°C and the sample compartment was kept at 10°C. All chromatograms were recorded in the 210–400 nm range, with 1.2 nm resolution. Scan rate was 20 Hz. Visualization and peak integration were done at 280 nm for standards and sample. The injection volume was 5 µL.

5. Mass spectrometry conditions

The optimal MS parameters were as follows: make-up fluid MeOH comprising 20 mM NH₄OH and 2% H₂O, pumped at 0.4 mL/min; probe temperature 600 °C; ion source temperature 120 °C; cone voltage 10 V; capillary voltage +/- 0.3 kV (both ionization modes examined in each run with voltage switching); scan rate 3.7 Hz. The mass range for total ion recording was set from *m/z* 100 to 1000, with SIM *m/z* values as indicated in Table 2.

Table 2 – Mass spectrometry identification of target compounds

	MW (g/mol)	[M + MSA] ⁺	[M - C ₁₂ H ₂₂ O ₉ + H] ⁺ (aglycone)
Neohesperidin	610.56	<i>m/z</i> 705	<i>m/z</i> 303
Naringin	580.54	<i>m/z</i> 675	<i>m/z</i> 273

RESULTS AND DISCUSSION

3.1. Characteristics of the 2D system

3.1.1. Description of operation

The 2D mSFC-SFC system was developed to allow for 4 possible configurations in a single instrument. It was based on a conventional one-dimensional SFC instrument. To add an extra dimension, three external 6-port, 2-position valves were added (Figure 1). Valves 1 and 3 were used to manage the flow directed to the columns: valve 1 directs the flow to the dim1 column while valve 3 was used to send the flow to the dim2 column. Valve 2 provides access to the selectors carrying

the storage loops. The selectors used for storage loops were already present on the column manager of the original system (valve 4 and valve 5). In its original configuration from the manufacturer, they were used to link the input (valve 4) and output (valve 5) of the column, and allow automated column switching. They were 9-port 8-position selectors. Thus, it was possible to install up to 8 storage loops. Because the minimum tubing length had to be 25 cm, the loops prepared had volumes of 50 or 114 µL. This minimum length was imposed by the system because the distance between the two selectors was fixed. The choice of loop volume depends on the quantity of sample to be recovered from the first dimension.

All valves were controlled automatically by Empower software, using contact closure located at the rear of the diode-array (valve 2) and MS (valve 1 and 3) detectors. Simultaneous control of valves 1 and 3 prevented the two columns from being in series which avoided causing excess pressure drop in the system. By applying the right valve positions, this configuration allowed different modes of analysis without any manual handling. These different modes included: 1) one-dimensional analysis on dim1 column (Figure 1-A) 2) one-dimensional analysis on the dim2 column (Figure 1-C), 3) two-dimensional analysis using a successive combination of all four settings (Figure 1-A,B,C,D). Whichever configuration was used, detection was carried out with a diode-array detector and MS placed in series at the outlet of valve 3. The first stage of the development of a 2D multiple heart-cut method was to examine the dim1 separation so that appropriate fractions can be selected. The settings of the dim1 analysis are shown in Figure 1-A. To do this, valve 1 was in position 2 (V1-2) to direct the flow from the pump and injector towards the dim1 column. Valve 2 was in position 1 (V2-1) to bypass the storage loops, and stoppers were placed in ports 2 and 3 to avoid any leaks when switching this valve. Valve 3 was in position 2 (V3-2) to bypass the dim2 column and transfer the effluent to the detectors. This first step allowed the identification of the target peaks as well as their elution window. The second step was to repeat the dim1 analysis but now including the loading of the fractions into the storage loops. Valves 1 and 3 remained in positions V1-2 and V3-2, respectively (Figure 1-A). Valve 2 would switch during analysis depending on the peaks to be recovered from the loops: it would be positioned at V2-1 when not loading, and would switch to V2-2 when the peaks of interest started eluting from the column (Figure 1-B).

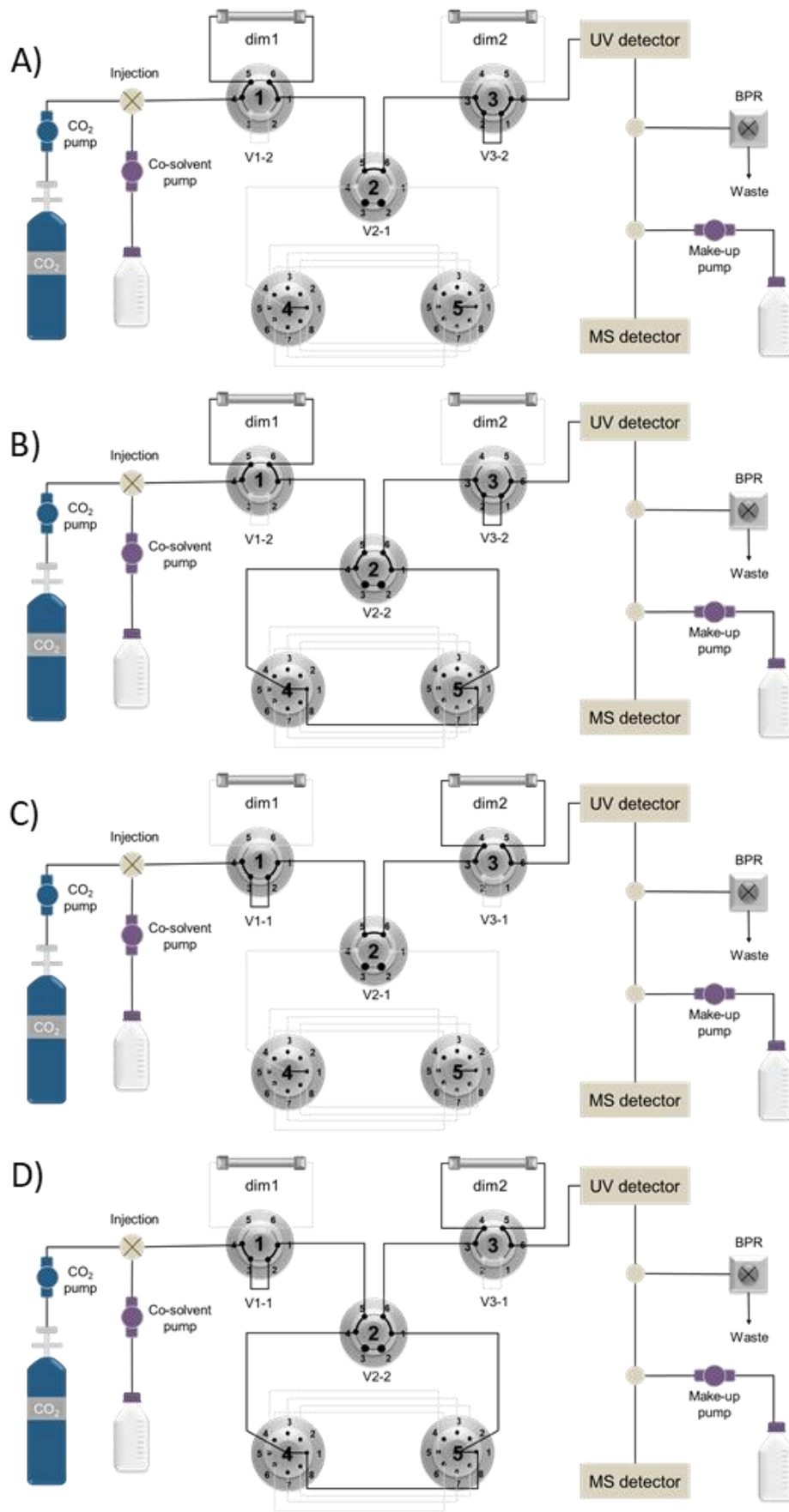


Figure 1 - 2D system configuration. A) first dimension only (dim1), B) first dimension with loading of the loops, C) second dimension only (dim2), D) second dimension with unloading of the loops.

In this case, the flow was directed through valve 4, a selected storing loop and valve 5 before going back to valve 2. Indeed, the initial presence of valves 4 and 5 in the instrument means that they could also be controlled automatically from the software, making switch programming easier and enabling the recovery of peaks that were very close to each other, which would otherwise not be feasible. In fact, when a peak was targeted to be retrieved and stored in a loop, valve 2 was set to V2-2, granting access to the selectors. Prior to this event, the loop selectors were already placed in the correct position towards the loop that was intended to be loaded, hence when valve 2 was still in position V2-1. As a result, when the selectors were turning, there was no flow in this part of the system. This absence of flow ensured that the fractions collected in the loops were maintained there, until their discharge to the second dimension was desired. Otherwise, the mobile phase flow would have started eluting the fractions as they passed in front of the loaded loops, during the rotation of the valve as observed during initial experiments.

Important to note was the fact that although the detectors remained in the flow path of the eluent, the dim1 chromatogram was impacted as the target compounds were stored in the loops, thus were not sent towards the detectors.

The final step in the 2D method was to unload the loops and send the fractions consecutively to the second dimension. This time, valve 1 switched to position V1-1 to bypass the dim1 column and valve 3 to position V3-1 to

unload the stored sample to the dim2 column. Valve 2 remained in position V2-2 to discharge the loop continuously (Figure 1-D), so the eluent was flowing through valve 4, the selected storing loop and valve 5, before going back to valve 2.

When several fractions had to be collected from dim1, the steps described in Figure 1A and 1B were repeated, storing the fractions in different selected loops with the simple control of valves 4 and 5. Then the step described in Figure 1D was repeated for as many times as the number of loops to obtain the dim2 chromatograms. Finally, by combining all these settings, it was possible to automatically perform several on-line separations with a single injection.

3.1.2. Preliminary tests

The main difficulty in developing this 2D system was to ensure good repeatability despite the modifications brought to the instrument. The addition of valves (1, 2 and 3) and numerous tubings can create significant variations in retention times. This phenomenon was observed during preliminary tests with a caffeine standard. In fact, significant variations in peak area were observed even for the analyses carried out in dim1 only. However, the retention times were highly repeatable. A leak test was then carried out at the level of each ferrule of each valve

which led to the observation of the presence of micro-leaks throughout the installation.

Following these leak tests and appropriate handling of the tubings and fittings, new repeatability analyses were carried out in isocratic mode with a caffeine standard and in gradient mode with an epicatechin standard (flavonoid compound selected to obtain sufficient retention, comparable to the target compounds). In order to check whether the entire system was operating correctly, the analyses were carried out in dim1 only (Figure 1A), in dim2 without passing through the loops (Figure 1C), and in dim2 passing through different loops (Figure 1D) of different volumes. Different features were measured (retention time, peak area, peak width, peak symmetry) and relative standard deviations (RSD) were computed from 20 and 10 replicates, for caffeine and epicatechin respectively. The full results are reported in Table S1 (supplementary information). Overall, they showed excellent RSD values, all below 2%. In conclusion, the modified instrument was repeatable, regardless of the mode and flow path used.

3.2. Sample application with achiral-chiral analysis

3.2.1. Preliminary study of the sample

Citrus aurantium L., also known as bitter orange, is a plant distinguished by its richness in various bioactive molecules, notably flavonoids. The latter are of particular interest for their potential medicinal properties¹⁹.

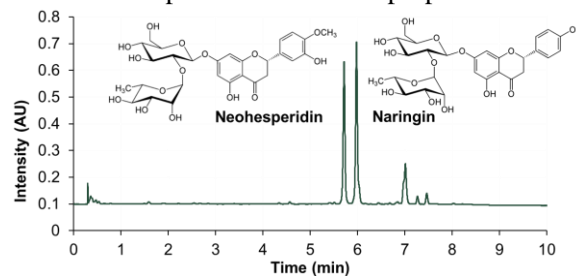


Figure 2 - dim1 chromatogram of a sample of sour orange peel. Analysis conditions: ACQUITY UPC² Torus DEA (100 × 3.0 mm, 1.7 μm), co-solvent: MeOH + 0.1% MSA, 150 to 110 bar, 1.7 to 0.6 mL/min (gradient conditions in Table 1.a).

When the extract of *Citrus aurantium* L. peels was analyzed in dim1, based on a previously developed method¹⁸, two major flavonoids were identified, neohesperidin and naringin. As shown in Figure 2, representing the analysis of the peel extract in dim1, they eluted between 5.5 and 6.0 min. These glycosylated flavanones have a chiral center on the aglycon part. The determination of diastereomeric excesses is of interest²⁰ thus the purpose of dim2 was to resolve the diastereomers, which could not be resolved with the first-dimension column but could be achieved with an enantioselective stationary phase.

For this purpose, the neohesperidin peak and the naringin peak were recovered in separate loops and re-analyzed in the second dimension. We aimed at collecting the full peak of each target compound in a single loop. Indeed,

because diastereomers were not resolved in the first dimension, a more accurate diastereomeric excess measurement should be obtained if the whole peak from the first dimension was retrieved and re-analyzed in a single second-dimension analysis, especially when the diastereomeric excess would be large. So the largest 114 μ L-loops were used to accommodate the peak width of both target compounds. (See Table S2 in supplementary information explaining fraction volume calculation.)

3.2.2. Calibration

When a compound is eluted from different columns in different operating conditions, the peak area measured with any detector will vary. In fact, it makes no sense to compare the intensity of peaks obtained on different columns. The column internal diameter, the flow rate and mobile phase composition will impact the detector response. Even under the same chromatographic conditions, but with a different column, the cumulative area of the peaks cannot be compared.

In order to be able to evaluate the quantity recovered from dim1 to be sent to dim2, it was necessary to quantify this difference in response. This was achieved by running a calibration curve for a neohesperidin standard on the two columns (Figure S1). This calibration was carried out with concentrations ranging from 0.1 to 0.7 mg/mL. Once the two linear regression equations were obtained, the response factor was obtained from the ratio of the two slopes. As a result, a factor of 2 was taken into account between the two dimensions.

3.2.3. Repeatability test

Assessing repeatability was important to ensure that the modified instrument was working properly. It was shown previously (section 3.1.2) that the system was repeatable in dim1 and dim2 when they were taken independently, but additional in-line tests with an automatic switch from dim1 to dim2 were necessary.

Sample analyses were made in triplicate with the extract of *Citrus aurantium* L. peels. Before each analysis, three blank analyses (injecting dilution solvent) were run to ensure that the column was conditioned correctly. A first analysis was carried out in dim1 to adjust the switching time as closely as possible to the retention time, as described above. A second dim1 analysis was then performed by loading two loops, one for the neohesperidin peak and one for the naringin peak. As the dim1 analysis was repeated 3 times, a total of six loops were loaded (3 for each target flavonoid). The dim2 configuration was then switched to unload the loops and analyze them in the second column. Prior to this, three blank runs were carried out using a bypass loop to condition the dim2 column. Six analyses were performed (one analysis per loop). In total, nine chromatograms were generated: three analyses in dim1 when the chromatogram was cut, three analyses in dim2 for the

neohesperidin peak and three in dim2 for the naringin peak.

RSD values from triplicate analyses are shown in Table 3 and include the values measured for retention time, peak area, width at half-height and asymmetry at 10% peak height. From the RSD values, it can be observed that the dim2 analyses were highly repeatable, especially as the most important RSD values for retention time and peak areas were both lower than 1.5%.

As described in Section 3.1.1, no data for areas or widths can be generated from this experiment for dim1, as the target compounds were removed after elution from dim1 and parked in the loop, thus not directed towards the detectors before elution from dim2. The data provided for retention time in dim1 correspond to two artefacts caused by the valves when they were switched, thus corresponding to the start of loop loading and end of loop loading. Indeed, turning the valves during analysis can create pressure gaps or slight overpressures that distort the baseline on the UV chromatogram. However, these baseline disturbances were also perfectly repeatable.

Table 3 - relative standard deviation in percentage of retention time, peak area, peak width at half height, asymmetry at 10% peak height, calculated after 3 replicates in dim1 and dim2 of a peel extract sample.

	Peak	Retention Time	Peak Area	Width 50%	Asymetry 10%
Dim 1 - cut	1	0.04	-	-	-
	1'	0.00	-	-	-
	2	0.22	-	-	-
	2'	1.0	-	-	-
Dim 2 -Neohesperidin	1	0.2	1.0	1.5	0.9
	2	0.2	1.1	3.4	-
Dim 2 - Naringin	1	0.2	1.3	3.7	20.1
	2	0.2	0.2	2.0	10.9

3.2.4. Optimization of the switching time

According to the repeatability test (Table 3), the dim1 analyses during loading, *i.e.*, when valve 2 was switched, appeared to be repeatable in terms of retention time. The RSD values were well below 2%. Prior to the optimization tests, the peak collection interval was determined by the delay between the column and the detector, the size of the tubing, the elution composition and the volume of the loop. Taking into account these factors and the exact retention times of the compounds, 5.71 min for neohesperidin and 5.98 min for naringin, the switch intervals for valve 2 could be determined. Initially, the valve was in position V2-1. To collect the neohesperidin peak at 5.71 min, valve 2 was switched to position V2-2 at 5.61 min and back to position V2-1 at 5.72 min. For the naringin peak at 5.98 min, the valve was switched in position V2-2 at 5.88 min and back to position V2-1 at 5.99 min.

At this stage, the fact that the system was known to be repeatable in 2D mode led to conclude that switch and collection times were also repeatable. However, despite this observed repeatability, optimum transfer was not guaranteed. Indeed, it was necessary to optimize the

switch time to maximize the quantity of peak transferred to the second dimension. Two tests were carried out, focusing particularly on the first peak of interest, neohesperidin. The first test involved extending the interval already tested by sampling before and after. A single injection was required but with six loop loadings at 5.47, 5.58, 5.69, 5.80, 5.91 and 6.02 min. Six loops of 100 μL were used and the interval duration remained constant, *i.e.*, 0.11 min. When the analysis in dim1 was complete, each loop was discharged to dim2 to be analyzed. It was then possible to calculate the transfer rate from the area of the peak in dim1 and the cumulative area of the peaks in dim2. As explained above, a response factor was observed between dim1 and dim2, which was important to take into account in this transfer calculation. Figure 3-A shows that, in the second fraction (cut2), which corresponds to the time interval from 5.58 to 5.69 min, more than 80% of the peak was transferred. Before that, neohesperidin was not detected. No overlap between the neohesperidin and naringin peaks was observed, as the MS trace showed no sign of the other compound.

Despite a good transfer rate in cut2, 18% of neohesperidin was still observed in cut3. Therefore, precise determination of the time when the maximum amount of target compound could be retrieved was necessary. To achieve this, a second test was conducted, still centered on the neohesperidin peak, but this time overlapping the collection times. One injection per loop loading was then required, *i.e.*, six injections to fill six loops. Loops were loaded at 5.50, 5.55, 5.61, 5.66, 5.72 and 5.77 min. The loop size and the intervals remained the same, *i.e.* 100 μL and 0.11 min.

When examining the results in Figure 3-B, representing the transfer rates per load, the maximum amount transferred was observed in cut3. This loading, done at 5.61 min (cut3), corresponds to the initial loading that was previously determined using calculations. Cut4 done at 5.66 min also had a high transfer rate because it overlapped the cut3 interval. By comparing cut3 and cut5, which were performed consecutively, the optimum of 83% was concluded to be in cut3, as cut5 had only a 2% transfer rate.

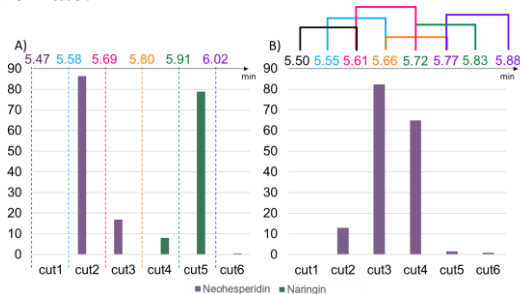


Figure 3 - Transfer rate calculated on peak areas in dim1 during loop loading. A) Consecutive cuts with 1 injection and 6 loads B) Overlapping cuts with 6 injections and 6 loads

To confirm this conclusion, the MS response was observed, in order to ensure that a maximum amount of the target compound had been loaded into the loop. In

Figure 4-A, the absence of UV peaks at the retention time of the compounds on the chromatogram of dim1 obtained when loading the loop confirms the previous results. The two spikes observed before and after the expected peak were the above-mentioned artefacts due to pressure changes. In addition, the study of the different single-ion monitoring (SIM) signals in positive and negative ionization mode confirms this observation (Figure 4-B). In dim1, the compounds were present, and their diagnostic ions were easily detectable. On the other hand, in dim1 with loading, none of the target m/z values were detected, indicating that the compounds had been correctly extracted.

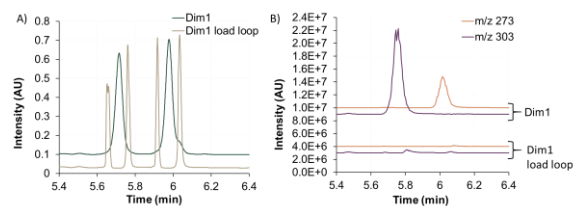


Figure 4 - A) Chromatogram of neohesperidin and naringin extracted from bitter orange peel, peaks analyzed in dim1 with and without loop loading. Artefacts due to pressure variations are visible when the valves rotate to fill the storing loops. B) Chromatograms obtained with single-ion monitoring (SIM) in dim1 with and without loop loading.

In conclusion, the major part of the peak of interest must have been recovered at the end of dim1, since it was no longer detectable thereafter. The loop unloading step in dim2 was very conclusive, transferring 83% of the peak. These various system and method optimizations for loading and storage in loops enabled reliable 2D analyses of the bitter orange peel sample. The dim2 chromatograms for neohesperidin and naringin both show 2 peaks (Figure 5). The diastereoisomers of neohesperidin and naringin were well resolved, with diastereoisomeric excesses of 97% and 35% respectively.

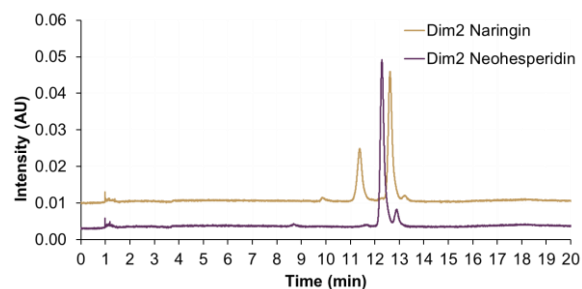


Figure 3 - Dim2 chromatograms of neohesperidin and naringin extracted from bitter orange peel. Analysis conditions: CHIRALPAK IB-3 (150 \times 4.6 mm, 3.0 μm), co-solvent: MeOH + 0.1% MSA (gradient conditions in Table 1.b), 150 bar, 2.0 mL/min.

Although the goal of this project was to demonstrate the feasibility and repeatability of a 2D mSFC-SFC set-up, another striking observation was worth noting. It is well known that the injection volumes in analytical 1D SFC

are limited to only a few μL 's, particularly when using injection solvents of higher polarity^{21,22}. When injecting larger volumes of “strong” solvent, peak distortion is usually observed. In the current research, however, storage loops of 50 and 100 μL were used. These storage loops were, in fact, the injection loops for the dim2 analyses in the 2D experiment. For both compounds, neohesperidin and naringin, which eluted from dim1 at co-solvent percentages of around 77%, excellent peak shapes were observed in dim2, despite the large injection volumes with large proportion of liquid co-solvent. Two factors can explain the good peak shapes: lower elution strength of the dim2 mobile phase (15% co-solvent at the gradient start), stacking the compounds at the column inlet, but also the higher pressure acting on the compressible fraction. Indeed, if the low elution strength was a sufficient explanation, injecting the same volume of a liquid sample would yield similar results. However, as can be seen in Figure S2, injecting the same volume (114 μL) of pure liquid in dim2 resulted in much lower chromatographic quality: pure methanol yielded strongly deteriorated peaks, while heptane-ethanol 75/25 (v/v), used to approximate the polarity of the CO_2 -methanol stored fraction, yielded slightly better peak shapes but still inferior quality to the injection of the compressible fluid sample. We must therefore conclude that the fact that the compounds were stored in supercritical/subcritical state prior to their injection onto the dim2 column clearly aided in ensuring good peak shapes. In addition, it is useful to examine the chromatographic features of “normal” and modified instrument (see Figure S3). In the first-dimension separation, adding valves and loops, thus increasing extra-column volume, caused a decrease in peak capacity of 11%. However, in the second-dimension separation, the decrease in peak capacity was only 5% when comparing a normal analysis on unmodified instrument to a dim2 analysis with loop-transfer injection. These observations clearly open perspectives for further research in this domain.

CONCLUSION

In this work the feasibility of a 2D mSFC-SFC system was demonstrated. This system only required the addition of three automatic valves to a conventional SFC system. This addition did not cause overpressure problems in the system, which was limited to a maximum pump head pressure of 414 bar, and a minimum back-pressure of 110 bar. Several configurations were then possible: a one-dimensional analysis with the first or the second column, and a two-dimensional analysis with loading of storage loops. The compliance of the repeatability in all these configurations proved the robustness of the set-up and the reliability of our results. In addition, the adjustment of valve switching times to load a peak into a storage loop was fully optimized to ensure total collection of the peak of interest from the first dimension. This resulted in 83%

of the peak being transferred to the second dimension. Sample application demonstrated the feasibility of determining stereoisomeric excesses of chiral flavonoids. This approach opens perspectives to any laboratory with an SFC instrument of which the users might be interested in occasionally and relatively easily implementing a second dimension to specific applications, without impacting the functionality of that system in routine 1D use.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website (PDF), including:

Figure S1 – Neohesperidin calibration curves obtained on dim1 and dim2

Figure S2 – Comparison between liquid and sub/supercritical samples injected in dim2

Figure S3 – Comparison between normal and modified instrument performance

Table S1 – Relative standard deviation on significant features calculated after analysis in different configurations (dim1, dim2 without loop, dim2 with loops).

Table S2 – Calculation of volume fraction retrieved from dim1 and cutting times depending on retention time (at peak apex)

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LR: Methodology, Investigation, Validation, Visualization, Writing – Original Draft; CDSJ: Methodology, Investigation, Writing – Review & Editing, Supervision; IF: Methodology, Resources, Writing – Review & Editing; CW: Conceptualization, Resources, Writing – Review & Editing, Supervision, Funding acquisition. The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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Supporting information to:

Development of a two-dimensional supercritical fluid chromatography system in multiple heart-cutting mode **Supplementary information**

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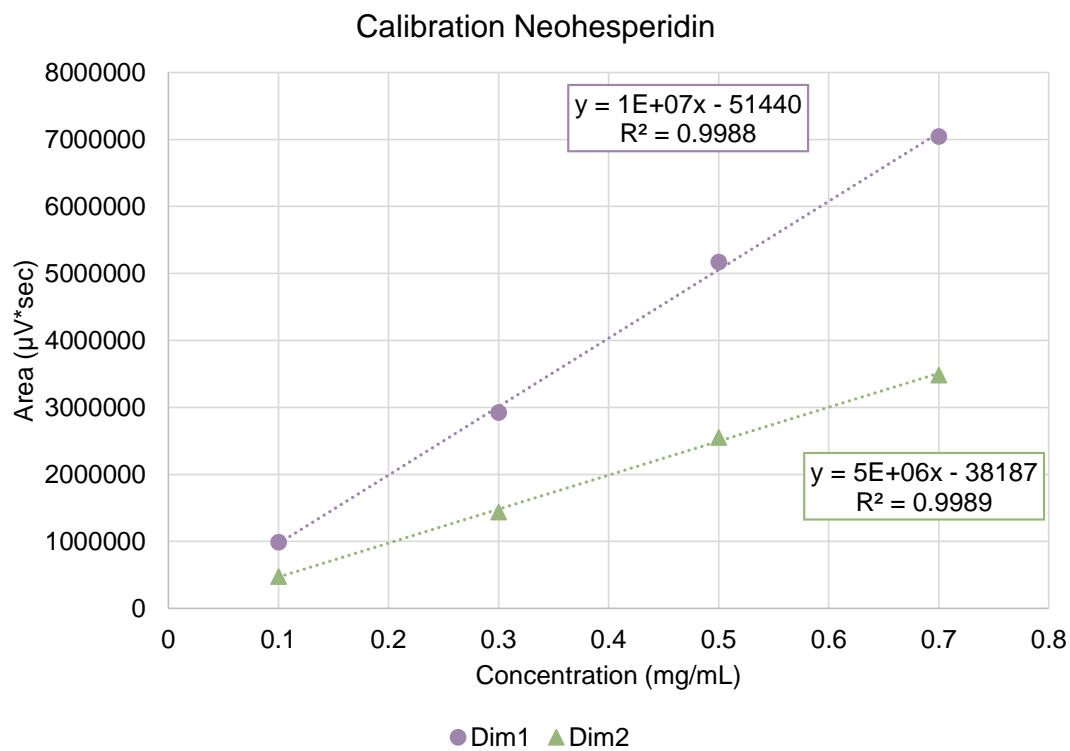
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Table of contents

Figure S1: Neohesperidin calibration curves obtained on dim1 and dim2	S11
Figure S2: Comparison between liquid and sub/supercritical samples injected in dim2	S12
Figure S3: Comparison between normal and modified instrument performance	S13
Table S1: Relative standard deviation on significant features calculated after analysis in different configurations (dim1, dim2 without loop, dim2 with loops)	S14
Table S2: Calculation of volume fraction retrieved from dim1 and cutting times depending on retention time (at peak apex)	S15

Figure S1 – Neohesperidin calibration curves obtained in dim1 and dim2. In dim1, the area of the whole neohesperidin peak comprising the two diastereomers was used. In dim2, the sum of areas of the two peaks (two diastereomers) was used.



Related data:

Dim1

Concentration (mg/mL)	Area (µV*sec)
0.1	988301
0.3	2924641
0.5	5172910
0.7	7046371

Dim2

Concentration (mg/mL)	Area Peak 1 (µV*sec)	Area Peak 2 (µV*sec)	Sum Area
0.1	83840	392513	476353
0.3	267107	1174470	1441577
0.5	469406	2082581	2551987
0.7	644158	2839832	3483990

Figure S2 – Comparison between (A) normal injection of 5 μL liquid sample in unmodified instrument, (B) sub/supercritical injection and (C, D) liquid samples injected in dim2. Naringin diastereomers analyzed on Chiralpak IB with dim2 method.

(A) Direct injection of 5 μL methanolic sample in the dim2 column (Chiralpak IB) but in normal operation of the instrument (without the added valves).

(B) Fraction stored from the first dimension (injection of 5 μL methanolic sample) prior to transferring to dim2.

(C) and (D) Liquid samples prepared in methanol and in heptane-ethanol 25-75, introduced through port 3 of valve 2 to fully load a 114 μL storage loop, thus with the same volume as a sub/supercritical fraction. The concentration of samples in liquid solvent was adjusted to retain identical quantity injected in the dim2 column in all cases.

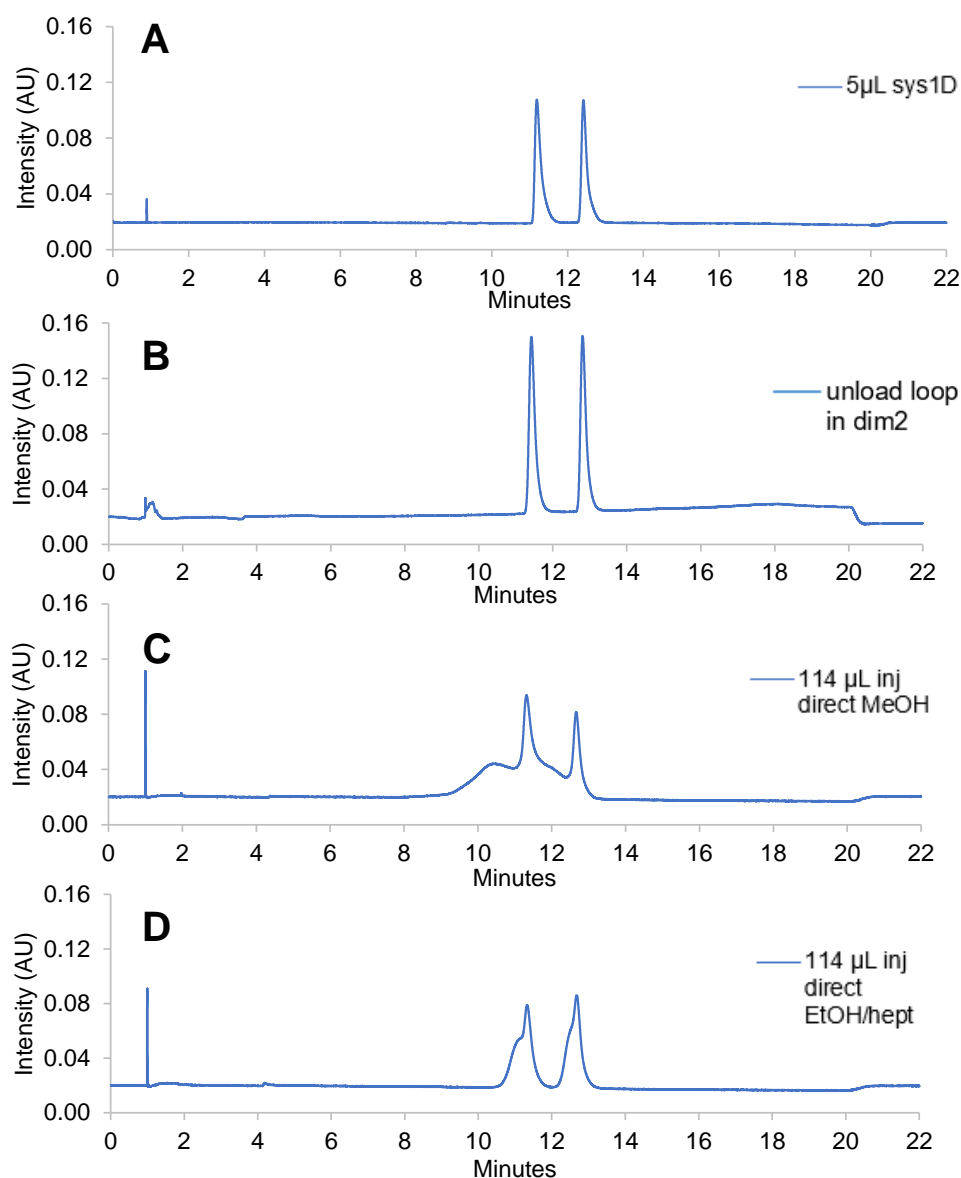


Figure S3 – Comparison between normal instrument and modified instrument.

(A, B) Analysis of naringin standard in the dim1 method (Torus DEA) with (A) the unmodified instrument and (B) modified instrument to see the impact of valves and tubings on efficiency.

(C, D) Analysis of naringin diastereomers in the dim2 method (Chiralpak IB) with (C) the unmodified instrument and (D) modified instrument operated in 2D, thus unloading the transfer loop to the second dimension.

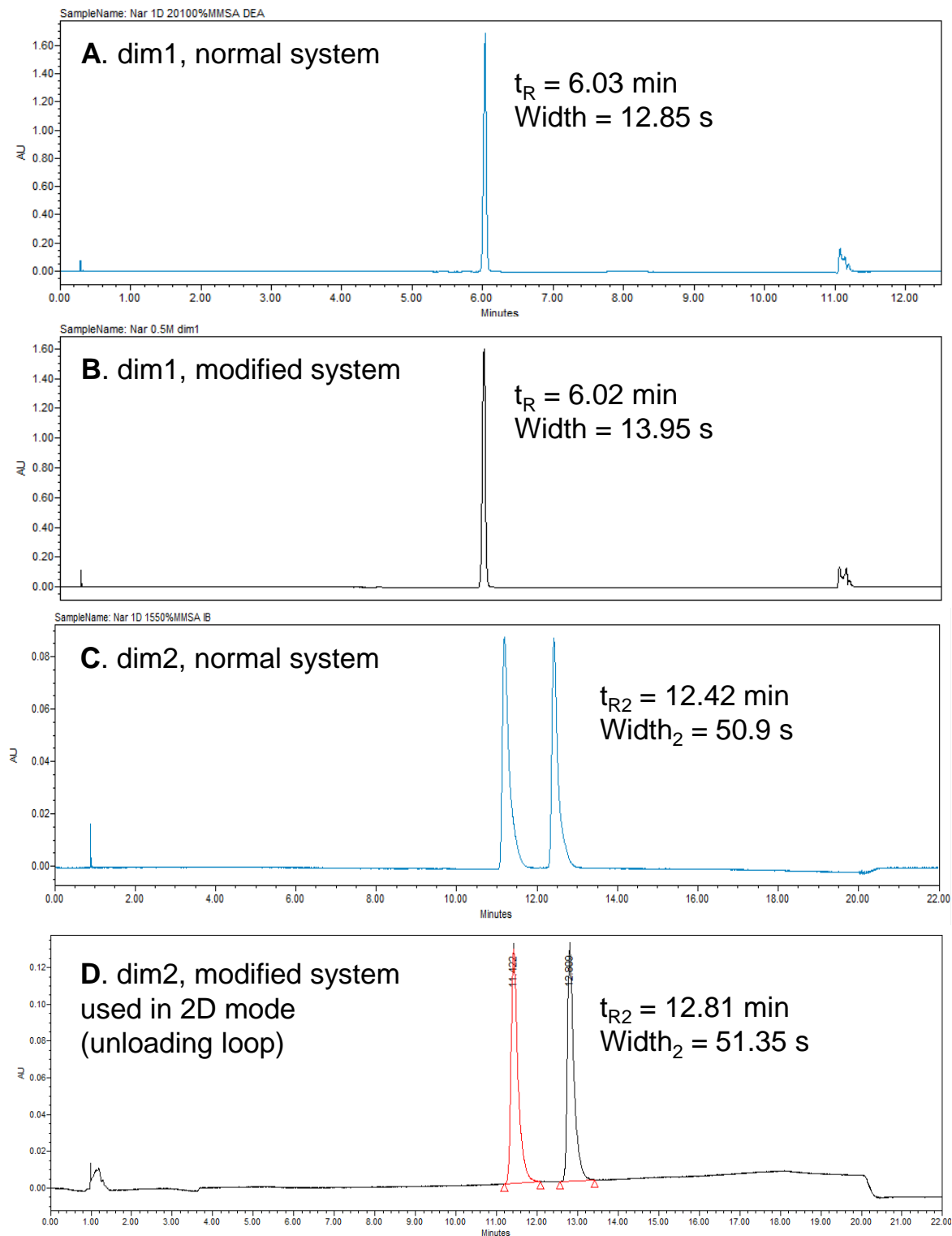


Table S1 – Relative standard deviation on significant features (retention time, peak area, width at 50% peak height, asymmetry at 10% peak height) calculated after analysis in different configurations (dim1, dim2 without loop, dim2 in 2 different loops of 100 μ L and dim2 in a loop of 50 μ L). A) 20 replicates of caffeine analysis in isocratic mode B) 10 replicates of epicatechin analysis in gradient mode. (*i.e.* P3 = position 3 of the selector and L3 = loop number 3)

	caffeine	retention time (min)	Area (μ V*sec)	Width 50%	Asym 10%
A)	Dim1	0.1	1.2	1.1	2.1
	Dim 2 – no loop	0.4	0.6	2.1	1.1
	Dim 2 – P3L3 100 μ L	0.3	0.2	0.9	0.7
	Dim 2 – P4L4 100 μ L	0.1	0.6	0.6	0.7
	Dim 2 – P5L5 50 μ L	0.2	0.6	0.5	0.6
	Epicatechin	retention time (min)	Area (μ V*sec)	Width 50%	Asym 10%
B)	Dim1	0.0	0.8	0.4	0.4
	Dim 2 – no loop	0.0	1.9	0.3	0.5
	Dim 2 – P3L3 100 μ L	0.0	0.9	0.1	0.5
	Dim 2 – P4L4 100 μ L	0.0	0.6	0.3	0.3
	Dim 2 – P5L5 50 μ L	0.0	0.4	0.1	0.4

Table S2 – Calculation of volume fraction retrieved from dim1 and cutting times depending on retention time (at peak apex)

Retention	Gradient	Dwell time	Co-solvent (%)		Flow rate (mL/min)	
t_R (min)	t_G (min)	t_D (min)	initial	final	initial	final
6	7.5	0.46	20	100	1.7	0.6
Elution composition						
1) Co-solvent (%)			2) Flow rate (mL/min)			
79.093			0.887			
Flow rate	Length	Radius	Volume			
(mL/min)	l (cm)	r (cm)	V (mL)			
0.887	169	0.009	0.043			
3) Delays Column 1/PDA (min)						
0.048						
V_{loop} (μ L)		100	50			
4) t_{loop} (min)		0.113	0.056			
Switch valve	5) ON	5.90	5.92			
PDA	6) OFF	6.01	5.98			

$$1) \%Cs_{elution} = \%Cs_{initial} + \frac{\%Cs_{final} - \%Cs_{initial}}{t_G} \times (t_R - t_D)$$

$$2) Fr_{elution} = \%Fr_{initial} + \frac{\%Fr_{final} - \%Fr_{initial}}{t_G} \times (t_R - t_D)$$

$$3) Delay_{column1/PDA} = \frac{V_{column}}{Fr_{elution}}$$

$$4) t_{loop} = \frac{V_{loop} \times 10^3}{Fr_{elution}}$$

$$5) Switch_{valve\ PDA\ (ON)} = t_R - delay_{column1/PDA} - \frac{t_{loop}}{2}$$

$$6) Switch_{valve\ PDA\ (OFF)} = t_R - delay_{column1/PDA} + \frac{t_{loop}}{2}$$