Journal of Chromatography A, 1596 (2019) 217–225

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# The use of alteration analysis in supercritical fluid chromatography to monitor changes in a series of chromatograms

József Simon [a], Csanád Rédei [b], Attila Felinger [a,b,c,*]

[a] MTA-PTE Molecular Interactions in Separation Science Research Group, Ifjúság útja 6, H-7624 Pécs, Hungary
[b] Department of Analytical and Environmental Chemistry and Szentágothai Research Center, University of Pécs, Ifjúság útja 6, H-7624 Pécs, Hungary
[c] Institute of Bioanalysis, Medical School, University of Pécs, Szigeti út 12, H-7624 Pécs, Hungary

ARTICLE INFO

*Article history:*
Received 21 November 2018
Received in revised form 13 March 2019
Accepted 17 March 2019
Available online 18 March 2019

*Keywords:*
Two-dimensional correlation
Chromatography
Alteration analysis
R-project

ABSTRACT

Two-dimensional correlation analysis (2DCOR) is a unique chemometric method introduced in spectroscopy which became successful in a wide range of analytical fields. It was applied in chromatography as well but has not gained wide-spread popularity. In our previous work, we introduced an alternative method, Alteration Analysis (ALA), which is built upon the basic properties of 2DCOR, but it is fine-tuned for chromatographic applications and can be used on higher dimensional data sets as well. We explored its merits through computer generated examples. In this study, we present the application of ALA to two various data-sets in chromatography. First, we used a series of samples where the concentrations of the compounds were adjusted according to the changes we studied in our previous in-silico experiments.

We compared the alteration maps from the computer generated and measured sources. The results demonstrated that ALA can provide the same properties from measured data as laid down in theory.

The second one is a test concerning the effect of sample solvent composition in supercritical fluid chromatography (SFC). ALA maps show the influence of increasing methanol concentration on the peak location and shape of compounds in the chromatogram.

With these two examples, we demonstrate that ALA can be used not only in theory, but it has also practical potentials and importance.



## 1. Introduction

More than thirty years ago Noda introduced a novel method in infrared spectroscopy [1]. It was aimed to create a two-dimensional measurement system, similar to what is used in nuclear magnetic resonance spectroscopy (NMR). It was named two-dimensional correlation analysis (2DCOR). In its existence, the method has gained a remarkable popularity and spread to numerous applications. However, there are only a few instances where 2DCOR stepped out of the boundaries of spectroscopy despite its simple applicability, versatility and promising potentials. It has a simple mathematical background and the improved generalized method [2] – which is used thoroughly – in theory has no limitation regarding the employed data. Furthermore, its goal is simple yet intriguing: to represent the changes in a chemical system triggered by an external perturbation on easily understandable graphs and through it to get a glimpse of the qualities of that system.

Our interest was, however, to use this method in chromatography. Understandably that is quite different from 2DCOR's original field and it shows by the lack of publications in this matter, despite the various advantages of the method. It certainly has a few applications, but has not gained a wide-spread acceptance [3–16]. We also tested 2DCOR in chromatography for a comparison of high-performance liquid chromatography (HPLC) columns [17], but quickly realized the disadvantage. In our opinion the reason why 2DCOR cannot be popular among chromatographers lies in a single reason: the peaks in chromatograms correlate much less than their spectroscopic counterparts. In spectroscopy it is highly probable that one peak will not change without another, but in chromatography independent changes are more likely to occur and 2DCOR relies heavily on correlation as its name suggests, thus it is less effective in a field where unrelated changes are the norm.

We still believe, however, that the benefits of this method can be advantageous in chromatography and thus we introduced ALA, which includes the basic properties of 2DCOR, but has more emphasis on individual changes. It was first introduced to extend the

* Corresponding author at: Department of Analytical and Environmental Chemistry and Szentágothai Research Center, University of Pécs, Ifjúság útja 6, H-7624 Pécs, Hungary.
*E-mail address:* felinger@ttk.pte.hu (A. Felinger).

https://doi.org/10.1016/j.chroma.2019.03.033
0021-9673/

boundaries of 2DCOR to three-dimensional data sets compiled from a series of two-dimensional measurements [18]. 2DCOR can already deal with 3D data, but what we propose is somewhat different, because the third dimension is another perturbation [19], so the allocation of dimensions is as follows: 1st – 1D measurement, 2nd – perturbation, 3rd – another perturbation. Two-dimensional measurements where the dimensions would be: 1st – first dimension of a 2D measurement, 2nd – second dimension of a 2D measurement, 3rd – perturbation have not been introduced yet.

Furthermore, the formulas allow ALA to be used in conventional two-dimensional data-sets obtained from one-dimensional measurements and in that case it can be compared to 2DCOR. The results of this comparison are summarized in our previous paper [20].

Our previous work was based on computer generated data, and in this study we present two examples using ALA on measured chromatograms. The first one serves as a transition between simulated and measured experiments, because we change the concentrations of the individual solutes in the samples separately and intentionally and with that we make sure that the theoretical changes we studied so far can be processed with measured chromatograms as well. The second example is a practical problem in SFC, the influence of methanol content in the sample solvent on the separation, where ALA can offer a new point of view.

During the experiments it has become clear that the minor reproducibility issues of the chromatographic system play a significant part in disturbing the signals on correlation and alteration maps. Time warping techniques are available, which provide potential solution to the problem [21–23]. Based upon those methods, we build our own simple algorithm and tested its effect on the maps.

## 2. Experiments and methods

### 2.1. Two-dimensional correlation

The calculation for 2DCOR begins with arranging the measured chromatograms to the rows of a $n \times m$ matrix, where $n$ is the number of chromatograms and $m$ is the number of points in the chromatogram:

$$\mathbf{X} = \left(x_{i,j}\right) \qquad \begin{matrix} i = 1, 2, \ldots, n \\ j = 1, 2, \ldots, m \end{matrix} \tag{1}$$

Before the actual correlation matrices are calculated, pretreatment is applied to the data. Usually the average chromatogram ($\bar{\mathrm{x}}$) is subtracted from each row.

$$\begin{matrix} \bar{\mathrm{x}} = \left(\bar{x}_j\right) \\ \mathbf{Y} = \left(y_{i,j}\right) \end{matrix} \qquad \begin{matrix} \bar{x}_j = \dfrac{1}{n}\displaystyle\sum_{i=1}^{n} x_{i,j} \\ y_{i,j} = x_{i,j} - \bar{x}_j \end{matrix} \qquad \begin{matrix} i = 1, 2, \ldots, n \\ j = 1, 2, \ldots, m \end{matrix} \tag{2}$$

The calculation of synchronous correlation map (SCM) ($\boldsymbol{\Phi}$) consists in a simple matrix multiplication. We calculate the covariance for retention times.

$$\boldsymbol{\Phi} = \frac{1}{n-1}\mathbf{Y}^T\mathbf{Y} \tag{3}$$

The calculation of asynchronous correlation map (ACM) ($\boldsymbol{\Psi}$) is only a little more complicated, as it includes a multiplication with the Hilbert–Noda transform matrix ($\mathbf{N}$).

$$\boldsymbol{\Psi} = \frac{1}{n-1}\mathbf{Y}^T\mathbf{N}\mathbf{Y} \tag{4}$$

$$\mathbf{N} = \frac{1}{\pi}\begin{bmatrix} 0 & 1 & 1/2 & 1/3 & \ldots \\ -1 & 0 & 1 & 1/2 & \ldots \\ -1/2 & -1 & 0 & 1 & \ldots \\ -1/3 & -1/2 & -1 & 0 & \ldots \\ \ldots & \ldots & \ldots & \ldots & \ldots \end{bmatrix} \tag{5}$$

The elements of this matrix are generated as follows:

$$N_{i,j} = \begin{cases} 0, & \text{if } i = j \\ (j-i)/\pi, & \text{otherwise} \end{cases} \tag{6}$$

### 2.2. Alteration analysis

ALA takes a rather different approach. Instead of enlarging the data, the goal is to minimize it. From the same $n \times m$ data matrix ($\mathbf{X}$) it creates $1 \times m$ vectors as opposed to bigger $m \times m$ matrices. The first step is the calculation of basic alteration map (BAM) ($\mathbf{b}$). It gives simply the scale of changes. It functions to give perspective to the scaled synchronous alteration map (SAM) and asynchronous alteration map (AAM).

$$\mathbf{b} = \left(b_j\right) \qquad b_j = \max(x_j) - \min(x_j) \qquad j = 1, 2, \ldots, m \tag{7}$$

The building of the last two alteration maps begins with the difference matrix ($\mathbf{D}$).

$$\mathbf{D} = \left(d_{i,j}\right) \qquad d_{i,j} = x_{i+1,j} - x_{i,j} \qquad \begin{matrix} i = 1, 2, \ldots, n-1 \\ j = 1, 2, \ldots, m \end{matrix} \tag{8}$$

SAM uses the BAM, difference matrix and its properties – average ($\bar{d}_j$) and standard deviation ($\sigma_{d_j}$) – to show only monotonous behaviours.

$$s' = \left(s'_j\right) \qquad s'_j = \frac{b_j \bar{d}_j}{\sigma_{d_j} + 1} \qquad j = 1, 2, \ldots, m \tag{9}$$

SAM is finally maximum scaled. That step is necessary because AAM utilizes a fundamentally different calculation, but we still have to be able to compare the various methods.

$$\mathbf{s} = \left(s_j\right) \qquad s_j = \frac{s'_j}{\max(|\mathrm{s}'|)} \qquad j = 1, 2, \ldots, m \tag{10}$$

AAM also uses BAM and the difference matrix, but its task is to quench every monotonous changes and focus on less systematic behaviours.

$$\begin{aligned} \mathrm{a}' &= \left(a'_j\right) \qquad j = 1, 2, \ldots, m \\ a'_j &= \left(b_j - |\sum_{i=1}^{n-1} d_{i,j}|\right) \sigma_{d_j} \left(\max(x_j) + \min(x_j) - 2\bar{x}_j\right) \end{aligned} \tag{11}$$

And again, it is scaled just like its counterpart.

$$\mathbf{a} = \left(a_j\right) \qquad a_j = \frac{a'_j}{\max(|\mathrm{a}'|)} \qquad j = 1, 2, \ldots, m \tag{12}$$

The mathematical background for 2DCOR and ALA is described in more details in our previous study [20].

### 2.3. Chromatographic peak simulation

The numerical simulations of the chromatograms were carried out with exponentially modified Gaussian peaks (EMGs), its peak shape model is expressed in Eq. (13) [24]. The model has five parameters: $t$ is the time, $t_R$ the retention time, $A$ the peak area, $\sigma$ the

**Table 1**
The formulas of the changes (perturbations).

| # | Type | Formula |
|---|---|---|
| 1 | Linear | $a\,x+b$ |
| 2 | Quadratic | $ax^2+b$ |
| 3 | EMG | Eq. (13) |
| 4 | sine | $a\sin x+b$ |
| 5 | cosine | $a\cos x+b$ |
| 6 | Single | $\begin{cases} ax+b, & \text{if } x=c \\ 0, & \text{otherwise} \end{cases}$ |

standard deviation, defining the width and $\tau$ the time constant, specifying the asymmetry.

$$y(t)=\begin{cases} \frac{A}{2\tau}\alpha(\beta+1) & \text{if } \tau>0 \\ \frac{A}{2\tau}\alpha(\beta-1) & \text{if } \tau<0 \end{cases} \quad \text{where} \quad \begin{aligned} \alpha &= \exp\left(\frac{\sigma^2}{2\tau^2}-\frac{t-t_R}{\tau}\right) \\ \beta &= \operatorname{erf}\left(\frac{t-t_R}{\sqrt{2}\sigma}-\frac{\sigma}{\sqrt{2}\tau}\right) \end{aligned} \tag{13}$$

### 2.4. Software

All calculations and plots were executed in the programming language R [25] with the help of the RStudio software [26].

### 2.5. Experimental parameters

For all experiments, we used a Waters ACQUITY UPC$^2$ System and a Supelcosil ABZ+Plus column (4.6 mm × 150 mm column packed with $d_p$ = 3 μm particles). The experimental parameters were: column temperature 60 °C; mobile phase 100% $CO_2$; flow rate 1.0 mL/min; sample temperature 25 °C; injection volume 2 μL; detection at 192, 200 and 260 nm; back pressure 150 bar.

Two series of experiments are presented with experimental data aimed to highlight the advantages of ALA. The first one is constructed by changing the individual concentration of seven compounds of a mixture through a series of chromatograms and then comparing the results to computer generated data. The concentrations were changed by a few functions summarized in Table 1. Most functions have two parameters, only a single function has three, where the third parameter is the point in the series of chromatograms where the perturbation occurs. The EMG parameters are the same as in Eq. (13). The parameters of the generated data are presented in Table 2 where $A$ is equivalent to the concentrations of the measured chromatograms. The experimental conditions are listed below. The second set of experiments has the same conditions, except the sample concentrations were always 0.4 mg/mL and the sample solvent composition was changed. This perturbation was started at 0:100% methanol:acetonitrile ratio and ended at 100:0% with 10% steps.

### 2.6. Retention time alignment

A simple algorithm is build where the maximum points of the first and last peaks in the series of chromatograms are aligned. First the chromatograms were shifted so the first peaks are aligned. Then considering the last peaks, evenly spaced points were added to all but one chromatogram in order to align the last peaks. These points were the mean of their neighbours.

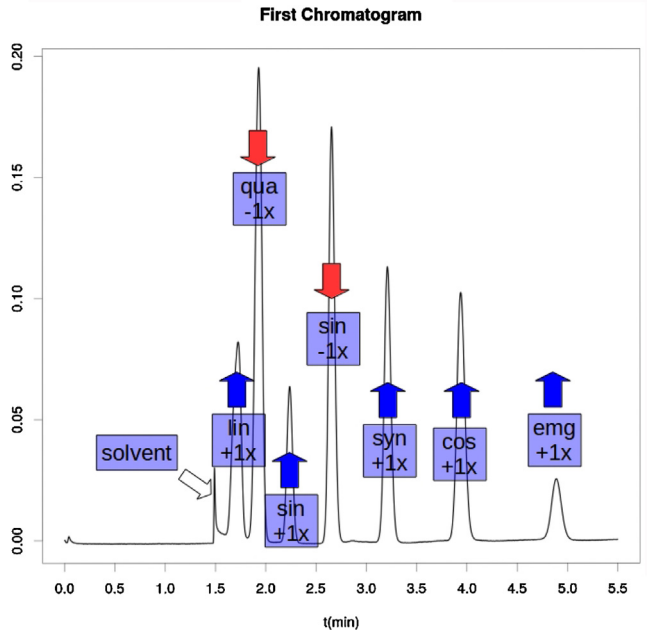


**Fig. 1.** Visual representation of the given changes in measured chromatograms, projected on the first point in the series: one linear, one quadratic and two single changes with opposite directions, sine and cosine and an EMG change.

## 3. Results and discussion

### 3.1. Peak height changes in a series of chromatograms

This experiment was designed simply to employ the theoretical properties of ALA – described in our previous studies [18,20] – in practice. A series of SFC measurements were executed with seven compounds present in the sample and their concentration was changed according to different functions from sample to sample (Fig. 1).

Computer simulation was also made with the peak parameters of the measured chromatograms in order to check the exact information we should get in the alteration maps. Thus, Fig. 2a shows the generated data and Fig. 2b shows the measured counterpart. At a first glance, the two images are different, but closer examination reveals that those differences can be explained and the overall information is untouched as explicated below. The major difference is in the BAM peak heights and their ratios. This issue comes from the fact that simulation does not take into account the differences in the absolute value of UV absorption of the individual compounds. The simulation does not consider the calibration between actual concentration and measured absorption.

**Table 2**
The parameters of chromatograms for Fig. 2a and Fig. 3a.

| # | $t_R$ (min) | Compound | $A$ | | | | | $\sigma$ | $\tau$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | Type | $a$ | $b$ | $c$ | $d$ | | |
| 1 | 1.68 | Ethylbenzene | Linear | 0.005 | 0.025 | – | – | 0.03 | 0.03 |
| 2 | 1.91 | Butylbenzene | Quadratic | −0.0005 | 0.07 | – | – | 0.025 | 0.025 |
| 3 | 2.22 | Hexylbenzene | Single | 0.045 | 0.025 | 5 | – | 0.022 | 0.022 |
| 4 | 2.64 | Octylbenzene | Single | −0.045 | 0.07 | 5 | – | 0.022 | 0.022 |
| 5 | 3.2 | Decylbenzene | Sine | 0.0231 | 0.0471 | – | – | 0.022 | 0.022 |
| 6 | 3.92 | Dodecylbenzene | Cosine | 0.0231 | 0.0471 | – | – | 0.022 | 0.022 |
| 7 | 4.88 | Tetradecylbenzene | EMG | $A$ = 0.086 | $t_R$ = 5 | $s$ = 0.5 | $\tau$ = 0.5 | 0.025 | 0.025 |

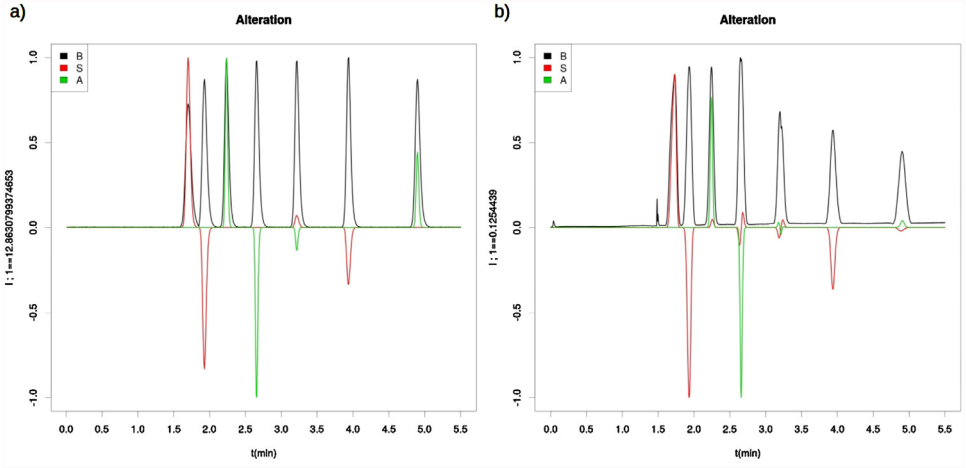


**Fig. 2.** Alteration maps for peak height changes in measured chromatograms. (a) Computer simulated alternative and (b) measured data. B: basic alteration map; S: synchronous alteration map; A: asynchronous alteration map.

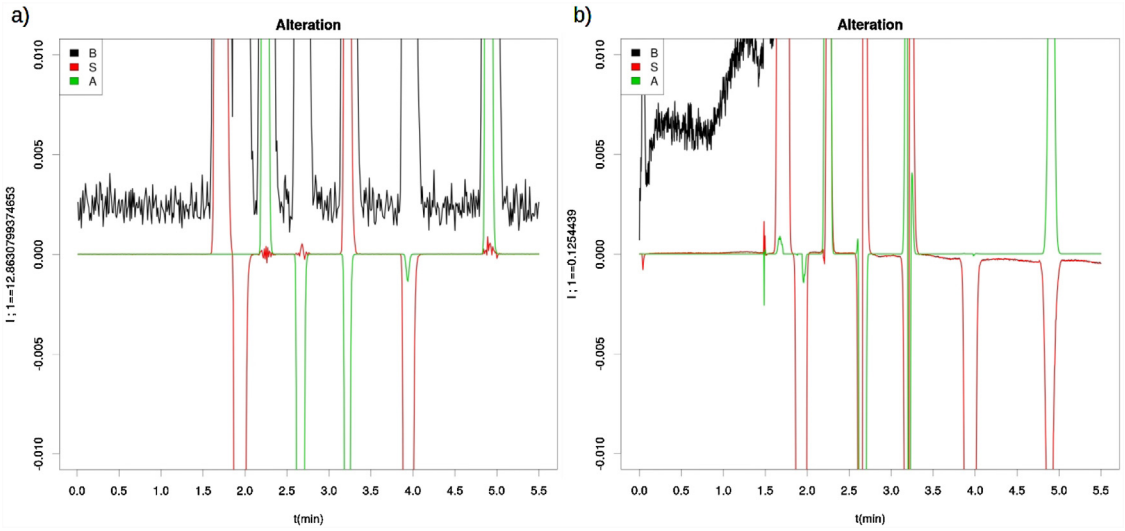


**Fig. 3.** Magnified alteration maps for peak height changes in measured chromatograms. (a) Computer simulated alternative and (b) measured data. B: basic alteration map; S: synchronous alteration map; A: asynchronous alteration map.

One can see that the patterns of three peaks are identical, the first two and the sixth (in the order of the generated maps). On the other peaks, the difference comes from appearing SAM values. It is because there is a small retention time shift and peak shape issues which is triggered by the imperfect reproducibility of the chromatographic system. Unfortunately, in real life of chromatographic measurements, we cannot expect perfect alignment in all cases. Retention times are naturally not as stable as the locations of spectroscopic peaks, and these relatively small shifts are affecting the alteration and correlation maps. However, there is still plenty of information which is delivered by ALA.

The monotonous changes (first two peaks) are easily separable from the rest, because they have no visible asynchronous value, only the magnified maps of measured chromatograms (Fig. 3b) show small peaks, but they are noisy and rather different from the shape of the original peak. This indicates that these are only caused by the imperfections of measurement system.

Also the quadratic change (second) has BAM and SAM rates different from linear change (first), not as much as in the generated data, but still noticeable. This means that ALA can separate different monotonous changes as well. Among non-monotonous changes, single changes (third, fourth) have the highest AAM peaks, demonstrated in the generated example (Fig. 2a). The measured map (Fig. 2b) is showing the same, so this feature is also reliable.

Some changes, such as monotonous, single and also EMG have a concrete direction, which is featured in ALA maps. The directions of peaks are directly representative to the trends of changes, SAM peaks for monotonous and AAM peaks for non-monotonous changes.

Wave-like changes (fifth – sine, sixth – cosine) are a little different. They have both SAM and AAM peaks in generated data, although cosine has only a small asynchronous peak. We can distinguish them further, because sine's two peaks have opposite directions while cosine peaks have the same directions. The sine change has the expected pattern in measured data with the added peak shift: the SAM and AAM peaks are the opposites, only the AAM peak is split into three because of the movement of the peak tailing. Unfortunately cosine's asynchronous peak cannot be found, which leaves it with similar pattern as the monotonous changes. However, in a case where we are looking for wave-like changes, sine and cosine can still be separable.

The biggest differences are at the two ends of the chromatogram. One small peak emerged before the first expected one. It is due to the solvent and because of that it is highly unlikely that it will dis-

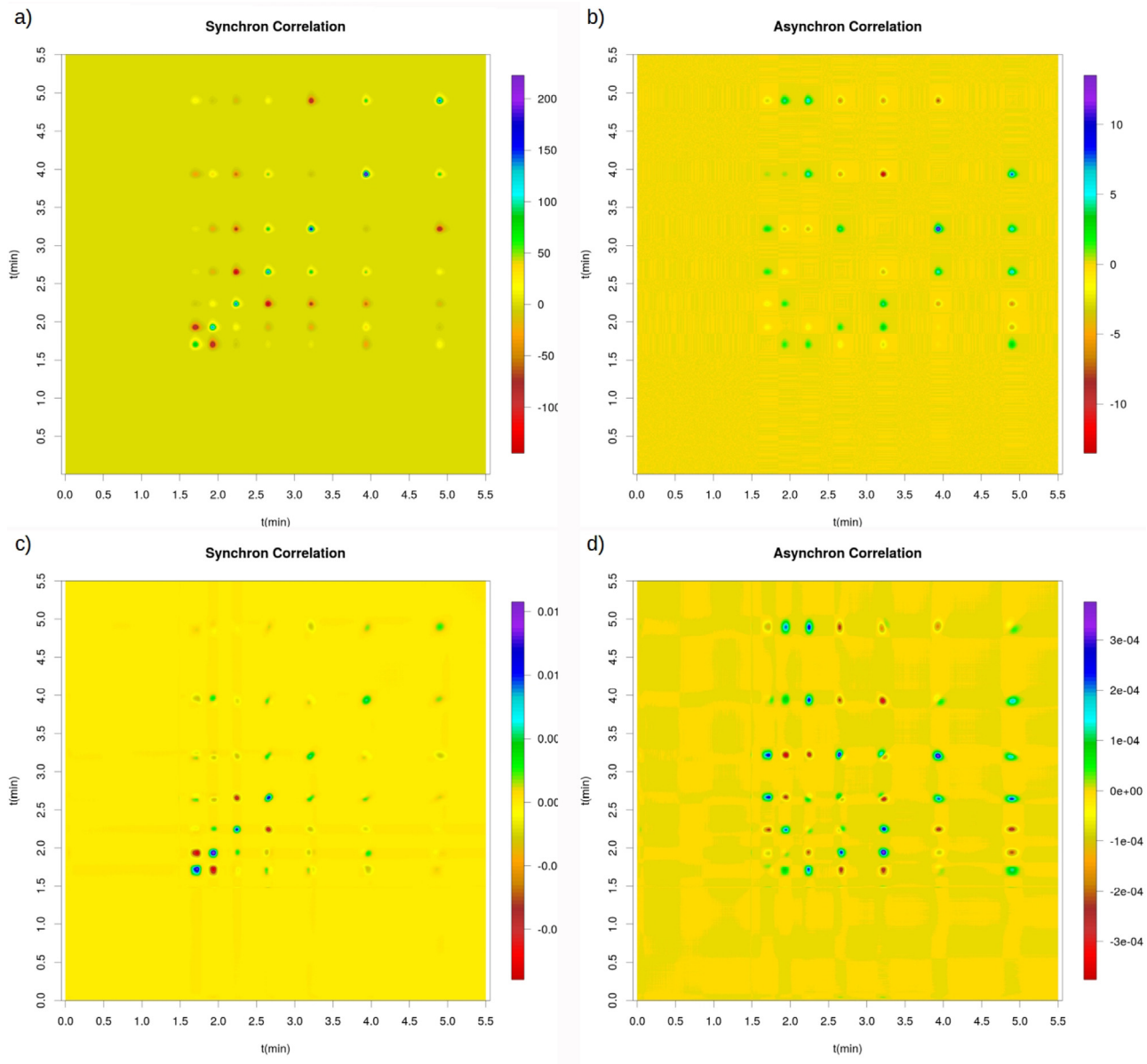


**Fig. 4.** Correlation maps for peak height changes in measured chromatograms. (a) Synchronous map of computer simulated alternative, (b) asynchronous map of computer simulated alternative, (c) synchronous map of measured data, and (d) asynchronous map of measured data.

turb the evaluation. On the one hand it is not overlapping with any of the peaks, on the other hand usually we have some knowledge about the chromatographic system, especially when we were the ones developing the method. The other difference is the big existing SAM value, relative to AAM, on the last peak.

The corresponding correlation maps presented in Fig. 4a and b are the SCM and ACM of the computer generated version and Fig. 4c and d are the measured ones. The evaluation process of these maps is very different from the previous paragraphs and arguably far more cumbersome. For the seven different changes we have to consider a total of 91 peaks, 49 in SCM and 42 in ACM, instead of 21, 7 for all 3 alteration maps. In this case not every peak is apparent, but in the process we have to look for all of them, because the lack of cross-peak (off-diagonal peak) is informative too. Similar information can be gathered at the end for sure, but there are scaling issues, which are of course apparent in ALA as well, but they can be more easily overcome. The main issue of scale is that in visual representation the smaller peaks can be overshadowed by the larger ones, therefore only the largest peaks are apparent in the maps, although, small peaks can hold meaningful information as well. It is necessary to harvest all information the maps contain, not just the most apparent one. There are several scaling techniques used in 2DCOR as well, they have both benefits and flaws [27], but undeniably they put an additional calculation step into the method, which has to be treated with caution. And most importantly ALA does not necessarily need that, because with a simple adjustment of the *y* axis the smaller features can be explored, as it is demonstrated in Fig. 3.

When evaluating correlation maps, we first look at the auto-peaks (diagonal peaks) of SCM. These show all the changes apparent in the measurements, in some way similarly to BAM. The cross-peaks of SCM and ACM form a delicate system. The fundamental rule is that peaks only appear where there is correlation between changes. SCM shows peaks between similar type of changes, while

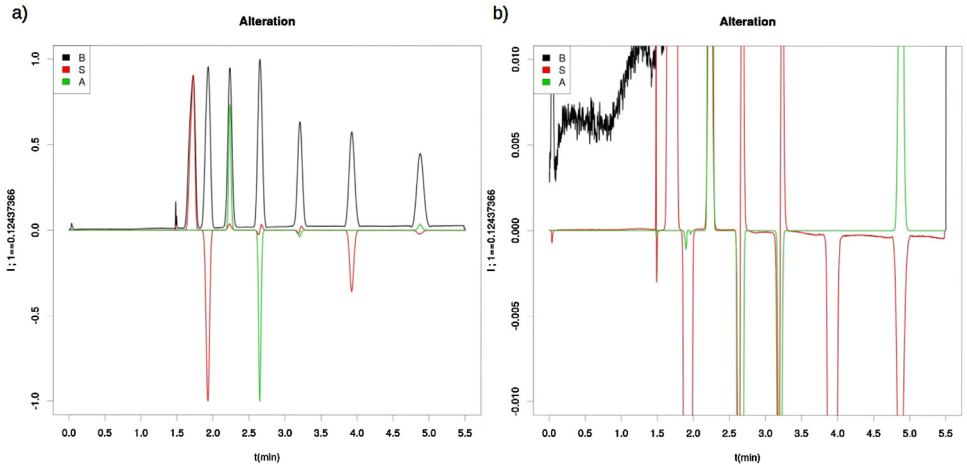


**Fig. 5.** Alteration maps for peak height changes in measured chromatograms with retention time alignment. (a) Normal and (b) magnified. B: basic alteration map; S: synchronous alteration map; A: asynchronous alteration map.

ACM shows the differences. However this rule is not completely strict. Usually SCM peaks are present no matter the changes, like in this case (Fig. 4a). Only the ratios tell the differences, for example first two peaks – which are both monotonous changes – have great cross-peaks and the second and third have significantly smaller peaks, because one is a monotonous change and the other is not. Positive and negative cross-peaks tell the relative directions of the changes. The first monotonous change is positive and the second is negative, so accordingly their cross-peaks are negative. Among similar changes the rule is the same, like the #3, #4 peaks are also positive and negative only they are single changes, and their cross-peak is negative as well. With different types of changes, this rule, however, is flexible. Between monotonous and single changes, the directions of the cross-peaks are the opposite. The EMG changes follow the pattern of the monotonous changes. The wavelike changes are hard to fit into this rule, because they have no directions in the original sense. However, they also have an order corresponding to other changes and the interesting thing is sine and cosine changes differ in their orders but not the opposite of each other, because with single changes they have the same pattern.

The ACM has only cross-peaks (see Fig. 4b). These are more exclusive for different types of changes. This case has only two examples from the same group, the two single changes, which aptly have no asynchronous peaks. Every other pair has cross-peaks. The main function of those peaks is to tell the sequential order of changes, which of them occurred sooner with the perturbation. This case does not contain suitable examples for proper presentment, because it is most useful observing similar changes. Combined with the synchronous cross-peaks, they can be used to separate the different types of changes. Arguments can be made how linear change precedes quadratic one, as suggested by ACM, but from it we can see that they are separate changes.

The connection between the 2DCOR maps of generated (a and b) and measured (c and d) data is similar to ALA. They are overall alike with a few differences only caused by peak shifts.

In conclusion 2DCOR can show less information about the changes with more complex graphs than ALA, because it emphasizes the connections between changes and not the individual parameters. For example the exact directions of the changes are only shown on ALA maps, correlation maps only give us the relative directions compared to other changes. Despite all of this, we do not want to diminish the value of 2DCOR. We only took an approach very different from what is used in correlation, because we tried to tune the method especially to chromatography and we are quite certain that simpler graphs and emphasis on separate changes can be beneficial.

### 3.2. The effect of retention time alignment on alteration maps

In the previous section we did not apply any pretreatment on the data. We saw that the imperfections of reproducibility in the chromatographic system have effect on the alteration and correlation maps compared to the computer generated data. In this chapter we added retention time alignment before the maps have been calculated and we will see if it will diminish the disturbance.

Fig. 5 shows the results. Compared to their previous state, Fig. 2b and Fig. 3b, there are a few changes, although not that significant. On the positive side the asynchronous signal on the first peak is disappeared so the alignment works on it just fine. The signals on the fifth peak are also improved, because now only the synchronous signal is split into two, the asynchronous one is as intended (Fig. 2a). However, on the other peaks the disturbed signals are arguably smaller but still present. We anticipated that the middle peaks are still affected, because the first and last peaks are aligned, but the last peak also stayed in a similar way as before.

To get to the bottom of this problem we also executed alignment on only one peak. We chose the fifth peak because the original alignment is partially worked on it, so there is evidence for its effectiveness but there is still room for improvement. We can see in Fig. 6 that the signals are still not the same as in the generated data (Fig. 2a) and seemingly they have not been improved from the previous setup. The asynchronous peak is still fine, the synchronous peak is the problem, because it is still split into two. Additionally it has changed direction from negative-positive to positive-negative and also it is more noisy, it is not a smoothly shaped curve, but fluctuations interfere with it.

The problem with this situation is the alteration maps and their graphs are scaled. The appearances of the peaks are relative to other changes in the maps. Thus, in order to see the actual size of peaks we have to look it up in the unscaled maps (Eq. (9)). In this case the maximum points of the first of two synchronous peaks in absolute values are:

- without alignment: 0.0632
- with first and last peak aligned: 0.0219
- with this peak aligned: 0.0074

It is clear that these values decrease step by step. We conclude that the distortions are the results of slight imperfections in the

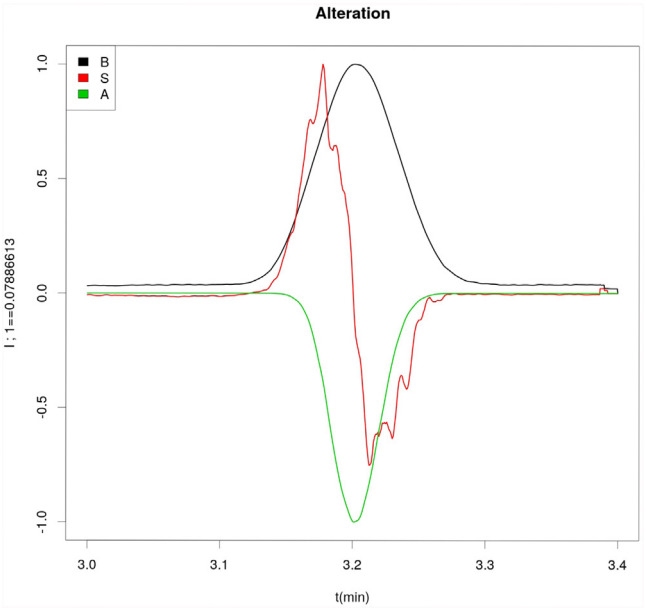


**Fig. 6.** Alteration maps for peak height changes on the fifth peak in measured chromatograms with retention time alignment. B: basic alteration map; S: synchronous alteration map; A: asynchronous alteration map.

shapes of peaks due to the nature of measured conditions and they will be apparent unless the system has perfect reproducibility.

In conclusion, retention time alignment does seem to lower the distortion of signals in the maps. Its impact is, however, far from perfect and it cannot get rid of the effect of reproducibility issues entirely. We suggest that it can be left out from pretreatment because the alteration maps are not improved much by it and in that case it gives unnecessary complications to the process. The maps can be still evaluated regardless of the alignment and in our opinion one of the main virtues of ALA is its simplicity. It is a method to give quick information about changes in the system without any pretreatment.

### *3.3. The influence of methanol as sample solvent in an SFC separation*

The next example comes from a series of experiments where the effect of methanol was studied in SFC. Eleven alkylbenzene samples were prepared in various sample solvent compositions: acetonitrile and methanol solvents were mixed by varying the percentage of the latter from 0 to 100%. UV detection was carried out at different wavelengths: (a) 192 nm and (b) 260 nm. The reason behind this is that methanol has negligible absorption at 260 nm so the compounds can be studied here without the disturbance of the methanol peak (see Fig. 7). Fig. 8 shows the resulting alteration maps.

The adsorption of the methanol solvent on the stationary phase affects the retention and the peak shape of the alkylbenzene analytes in various extent via competition. The higher the methanol content of the sample solvent, the more it disturbs the elution of alkylbenzenes. The peaks of the analytes eluting before methanol are compressed due to a displacement effect, whereas the analytes eluting after the front of the methanol peak are broader due to the tag-along effect.

First of all, there are far more signals in Fig. 8a and b than one would expect. Every peak has comparable changes not just the ones which are affected directly by competing adsorption caused by methanol. In theory, the last peak (at about 7.5 min) cannot be influenced by the perturbation, but alteration patterns can still be seen. We assume that it is due to the poor repeatability of the SFC measurements, because its pattern is different than those of any other peak. It features high asynchronous value concentrated at the middle of the peak and the maximum is split into two in AAM and bam. This leads us to the assumption that it is only due to a relatively small unintentional peak shift.

The main feature is the big basic peak of methanol in Fig. 8a ranging from about 2.5 min to 6.0 min. The direction of change is not clear at the first glance, because the linked synchronous peak is positive everywhere, but other peaks along the way show – with positive-negative synchronous peak combinations – that its migration started in the column together with earlier elution times. Also, asynchronous peaks are getting bigger in this direction in Fig. 8b, which is the result of the lack of methanol present for a longer time during migration in the series of chromatograms for those solutes. The peak at 4.7 min is mostly synchronous because methanol is a constant factor at that retention time, but it is arriving late in the series at 2.5 min. The peak eluted at 2.2 min is also influenced by methanol but not as strongly as the previously mentioned ones.

The signals obtained due to the first peak (at around 1.6 min) have the exact opposite directions than the peaks between 2.2 and 5.0 min and there are some irregular shapes before that. This means that a different effect causes that change, since the disturbing effect

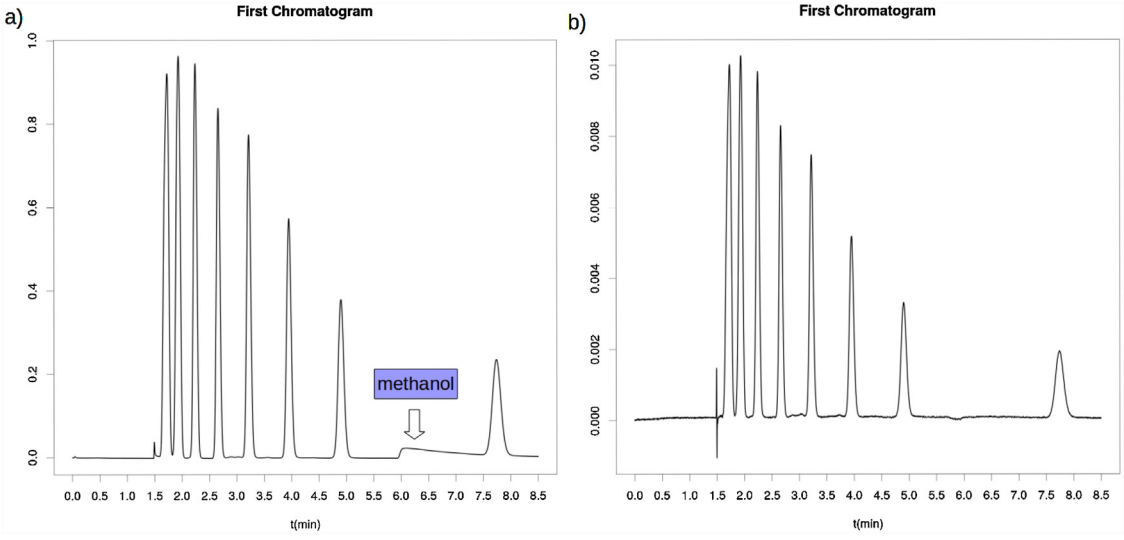


**Fig. 7.** First chromatograms for the effects of methanol in a SFC separation. (a) Detection at 192 nm and (b) detection at 260 nm.

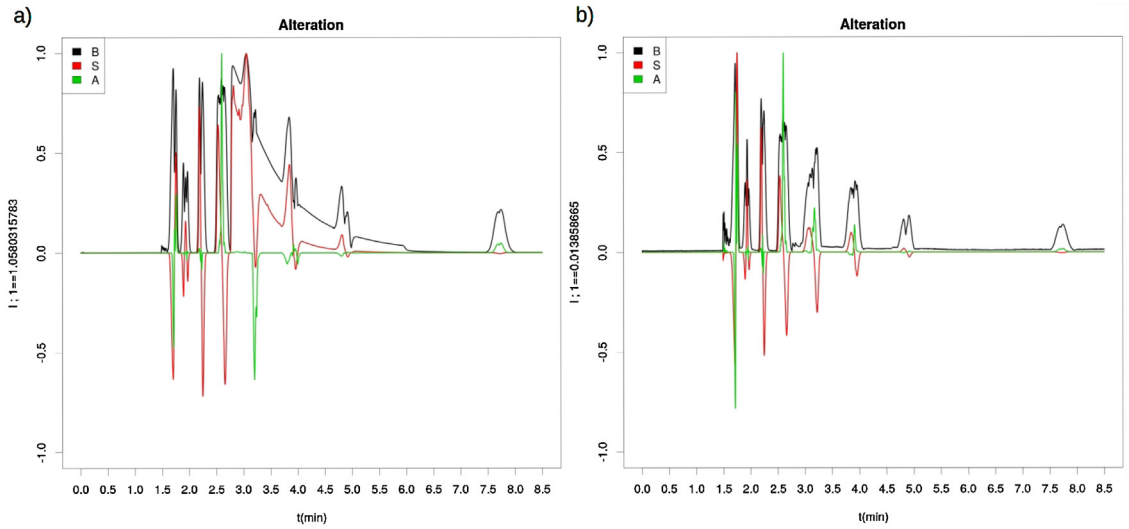


**Fig. 8.** Alteration maps for the effects of methanol in a SFC separation. (a) Detection at 192 nm and (b) detection at 260 nm. B: basic alteration map; S: synchronous alteration map; A: asynchronous alteration map.

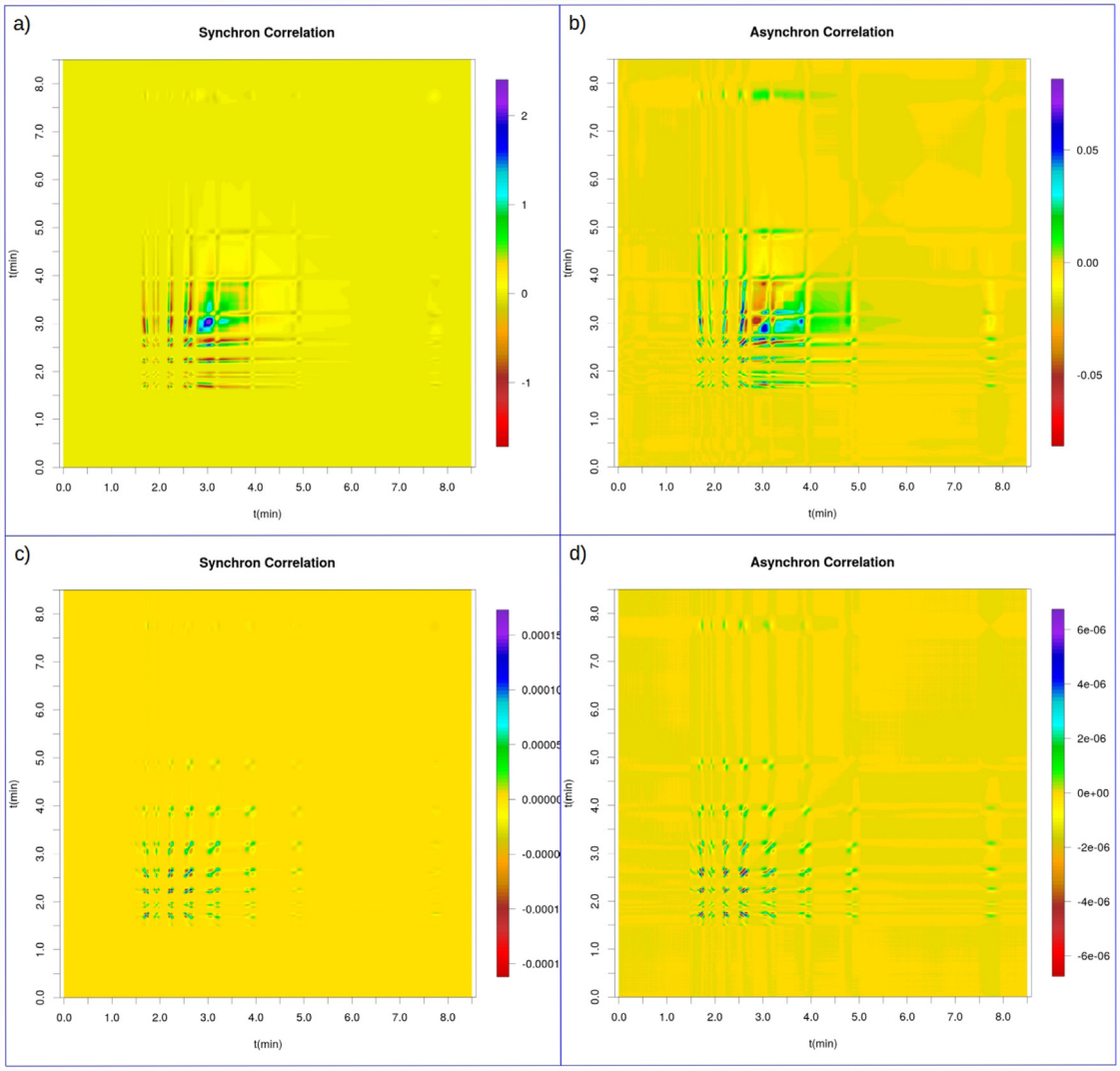


**Fig. 9.** Correlation maps for the effects of methanol in a SFC separation. (a) Synchronous map of detection at 192 nm, (b) asynchronous map of detection at 192 nm, (c) synchronous map of detection at 260 nm, and (d) asynchronous map of detection at 260 nm.

of methanol cannot reach that far. Further investigation showed that the irregular shapes are due to acetonitrile that influenced the behavior of the first compounds and thus, it was the source of this aberration. The most interesting part is the second peak because seemingly no mentioned effect has contact with it and clearly it has a unique alteration pattern which shows peak shape changes in contrary to the shifting tendency of any other peak.

Fig. 9 shows the correlation maps of the same experiments. The first impression is, especially on the maps (a) and b), recorded at 192 nm, that they are overcrowded and with this set up it is hard to evaluate the minor details. It needs scaling and/or magnifications and focusing on several parts separately to obtain the right maps. Thus, we do not aim to further discuss them, they already made their purpose, which is to show how much easier it is to evaluate ALA maps.

## 4. Conclusions

ALA is a chemometric method, which provides a unique perspective on an experimental problem. If there is a series of chromatograms and the changes between them are the subjects of interest, our method can provide an easy solution to examine them. ALA can tell where the changes are in the chromatogram, which directions they take and most importantly it can separate different types of changes with synchronous and asynchronous alteration maps. It can differentiate between monotonous and non-monotonous changes and many other among them.

ALA's strengths were proven in previous studies through computer generated data. In this study, two practical examples were presented with experimental SFC chromatograms. In the first case, the concentrations of the compounds were perturbed in a similar manner as we discussed in theoretical experiments and the results were also compared against the computer-generated counterpart. Our method delivered the same information in both cases, only the slight retention time shifts made a difference in the experimental series, which means the theoretical properties can be transferred to practice with ease. The second case is about the effect of methanol concentration in the sample solvent. The methanol/acetonitrile ratio was changed step by step from one extreme point to the other and the behavior of the chromatographic peaks were monitored and analyzed by ALA. The shift of the location of the methanol peak and its effects on the analyte peaks could be clearly seen on the alteration maps, which provided a unique look on this problem. Correlation maps are also given to compare the novel method to its predecessor and highlight its upgrades.

## Acknowledgement

The work was supported by the NKFIH OTKA grants K125312 and FK 128350.

The study was partly financed by the Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary, within the framework of the 20765-3/2018/FEKUTSTRAT Innovation for sustainable and healthy living and environment thematic programme of the University of Pécs.

The project was supported by the European Union, co-financed by the European Social Fund Grant no.: EFOP-3.6.1.-16-2016-00004 entitled by Comprehensive Development for Implementing Smart Specialization Strategies at the University of Pécs.

The work was also supported by the ÚNKP-18-3-III New National Excellence Program of the Ministry of Human Capacities.

We thank Dr. Abhijit Tarafder and Waters Corporation (Milford, MA, USA) for the long-term generous free loan of the Acquity UPC$^2$ equipment.

## References

[1] I. Noda, Two-dimensional infrared (2D-IR) spectroscopy, Bull. Am. Phys. Soc. 31 (1986) 520.
[2] I. Noda, Generalized two-dimensional correlation method applicable to infrared, Raman, and other types of spectroscopy, Appl. Spectrosc. 47 (1993) 1329–1336.
[3] K. Izawa, T. Ogasawara, H. Masuda, H. Okabayashi, I. Noda, Application of generalized two-dimensional correlation theory to gel permeation chromatographic analysis, PhysChemComm 4 (2001) 57–59.
[4] K. Izawa, T. Ogasawara, H. Masuda, H. Okabayashi, C.J. O'Connor, I. Noda, 2D gel permeation chromatography (2D GPC) correlation studies of the growth process for perfluoro-octyltriethoxysilane polymer aggregates, Phys. Chem. Chem. Phys. 4 (2002) 1053–1061.
[5] K. Izawa, T. Ogasawara, H. Masuda, H. Okabayashi, C.J. O'Connor, I. Noda, Two-dimensional correlation gel permeation chromatography (2D GPC) study of 1H,1H,2H,2H-perfluorooctyltriethoxysilane sol-gel polymerization process, J. Phys. Chem. B 106 (2002) 2867–2874.
[6] K. Izawa, T. Ogasawara, H. Masuda, H. Okabayashi, I. Noda, Two-dimensional correlation gel permeation chromatography study of octyltriethoxysilane sol-gel polymerization process, Macromolecules 35 (2002) 92–96.
[7] J.R. Hyde, R.A. Bourne, I. Noda, P. Stephenson, M. Poliakoff, 2DCOR-GC: an application of the generalized two-dimensional correlation analysis as a route to optimization of continuous flow supercritical fluid reactions, Anal. Chem. 76 (2004) 6197–6206.
[8] K. Suzuki, J.-i. Oku, K. Izawa, H.-F. Okabayashi, I. Noda, C.J. O'Connor, Two-dimensional correlation gel permeation chromatography (2D GPC) study of the $CH_3SO_3H$-catalyzed polymerization of triethoxysilyl-terminated polystyrene. Molecular weight effect on the aggregate-aggregate interactions, Colloid Polym. Sci. 283 (2004) 306–316.
[9] K. Suzuki, J.-i. Oku, K. Izawa, H.-F. Okabayashi, I. Noda, C.J. O'Connor, Two-dimensional correlation gel permeation chromatography (2D GPC) study of aggregate-aggregate interactions during polymerization of 3-(triethoxysilyl)propyl-terminated polystyrene, Polym. J. 36 (2004) 959–970.
[10] K. Suzuki, J.-I. Oku, K. Izawa, H.-F. Okabayashi, I. Noda, C.J. O'Connor, Two-dimensional correlation gel permeation chromatography study of aggregate-aggregate interactions during acid-catalyzed polymerization of triethoxysilyl-terminated polystyrene, J. Polym. Sci. Part B 42 (2004) 3447–3460.
[11] R. Amandi, J.R. Hyde, S.K. Ross, T.J. Lotz, M. Poliakoff, Continuous reactions in supercritical fluids; a cleaner, more selective synthesis of thymol in supercritical $CO_2$, Green Chem. 7 (2005) 288–293.
[12] J.R. Hyde, B. Walsh, M. Poliakoff, "Hydrogen-catalyzed" dehydrogenation: a supercritical conundrum, Angew. Chem. 117 (2005) 7760–7763.
[13] K. Suzuki, J.-i. Oku, K. Izawa, H.-F. Okabayashi, I. Noda, C.J. O'Connor, Two-dimensional gel permeation chromatography (2D GPC) correlation studies of the aggregate-aggregate interactions in acid-catalyzed triethoxysilyl-terminated polystyrene systems. The effect of specific catalysts on growth process, Colloid Polym. Sci. 283 (2005) 551–558.
[14] T.J. Kamerzell, M. Li, S. Arora, J.A. Ji, Y.J. Wang, The relative rate of immunoglobulin gamma 1 fragmentation, J. Pharmaceut. Sci. 100 (2011) 1341–1349.
[15] J. Andary, J. Maalouly, R. Ouaini, H. Chebib, D.N. Rutledge, N. Ouaini, Application of 2D correlation spectroscopy on olive stones acid hydrolysates: effect of overliming, Chemometr. Intell. Lab. Syst. 113 (2012) 58–67.
[16] Y.-K. Lee, J. Hur, Using two-dimensional correlation size exclusion chromatography (2D-CoSEC) to explore the size-dependent heterogeneity of humic substances for copper binding, Environ. Pollut. 227 (2017) 490–497.
[17] J. Simon, A. Felinger, Two-dimensional correlation analysis of the reproducibility of high-performance liquid chromatography columns, J. Chromatogr. A 1384 (2015) 115–123.
[18] J. Simon, A. Felinger, Correlation analysis on 3D data – introducing the alteration analysis, Chemometr. Intell. Lab. Syst. 158 (2016) 54–60.
[19] H. Shinzawa, S.-I. Morita, K. Awa, M. Okada, I. Noda, Y. Ozaki, H. Sato, Multiple perturbation two-dimensional correlation analysis of cellulose by attenuated total reflection infrared spectroscopy, Appl. Spectrosc. 63 (2009) 501–506.
[20] J. Simon, A. Felinger, Exploring the changes in a series of measurements – the comparison of the two-dimensional correlation analysis and the alteration analysis, Chemometr. Intell. Lab. Syst. 168 (2017) 28–37.
[21] C.P. Wang, T.L. Isenhour, Time-warping algorithm applied to chromatographic peak matching gas chromatography/Fourier transform infrared/mass spectrometry, Anal. Chem. 59 (1987) 649–654.
[22] N.-P.V. Nielsen, J.M. Carstensen, J. Smedsgaard, Aligning of single and multiple wavelength chromatographic profiles for chemometric data analysis using correlation optimised warping, J. Chromatogr. A 805 (1998) 17–35.
[23] P.H.C. Eilers, Parametric time warping, Anal. Chem. 76 (2004) 404–411.
[24] A. Felinger, Data Analysis and Signal Processing in Chromatography, Elsevier, 1998.
[25] R Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2013 http://www.R-project.org/.
[26] RStudio Team, RStudio: Integrated Development Environment for R, RStudio, Inc., Boston, MA, 2016 http://www.rstudio.com/.
[27] I. Noda, Scaling techniques to enhance two-dimensional correlation spectra, J. Mol. Struct. 883-884 (2008) 216–227.