Pairwise alignment of chromatograms using an extended Fisher–Rao metric

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HIGHLIGHTS

- A new approach to the alignment of chromatograms is presented.
- The entire chromatogram is aligned without priority given to any user-selected features.
- This eliminates operator bias and allows for unattended alignment of chromatograms.

GRAPHICAL ABSTRACT
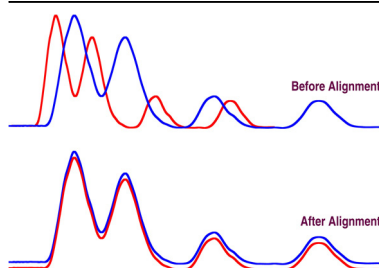
ABSTRACT

A conceptually new approach for aligning chromatograms is introduced and applied to examples of metabolite identification in human blood plasma by liquid chromatography–mass spectrometry (LC–MS). A square-root representation of the chromatogram's derivative coupled with an extended Fisher–Rao metric enables the computation of relative differences between chromatograms. Minimization of these differences using a common dynamic programming algorithm brings the chromatograms into alignment. Application to a complex sample, National Institute of Standards and Technology (NIST) Standard Reference Material 1950, Metabolites in Human Plasma, analyzed by two different LC–MS methods having significantly different ranges of elution time is described.

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1. Introduction

In most types of chromatographic separation [1], irreproducibility in run-to-run retention time arising from instrument instability is a common occurrence. In liquid chromatography, changes in separation column temperature, mobile phase composition, mobile phase flow rate, stationary phase age, and instrument usage history are each sources of unintentional measurement variability. Retention time variability may also arise

intentionally as when instrument parameters are varied systematically to find the conditions under which the best separation occurs [2]. Additionally, variability can occur when samples are analyzed on different instruments to compare separation performance or to find as many components in a mixture as may be identified by a suite of methods [3]. In any circumstance chromatograms must be retention-time aligned in order to allow run-to-run comparisons to be made [4,5].

Broadly considered, there are two approaches to chromatogram alignment [6]. One approach identifies common features in the chromatograms to be aligned, forces alignment of these features, and then interpolates an alignment function between these fiducial features. The popular XCMS program [7] uses this method.

These features can be individual peaks, clusters of peaks, or entire segments of the chromatogram. The difficulty in this approach lies in defining and detecting features with consistency in real data. For instance, a single missed peak or a false positive identification can alter the alignment of the remaining spectrum. A second approach seeks to align all points in the chromatogram without any added importance given to chromatographic features by finding a warping function that minimizes the discrepancy between two chromatograms across the entire range of retention time. An example of this is parametric time warping [8]. The algorithm introduced here uses this second full-chromatogram approach but relies on the derivative of the chromatogram to create a warping function. Use of the derivative enhances the sensitivity of the alignment to subtle features in the chromatograms.

In this work a new approach for alignment is presented for consideration by the chromatography community. A purely geometric framework for separating the phase and the amplitude variability [9] based on an extension of the Fisher–Rao metric is described and applied to the problem of chromatogram alignment. The chromatograms are treated as mathematical functions f_i without regard to any specific features or details as to how the data was taken. The user must select the beginning and ending points of each chromatogram that will remain fixed during the alignment procedure. For highly misaligned chromatogram pairs it has been observed that the method functions best when the chromatograms to be aligned have the same, or similar, number of points. This may entail pruning or re-sampling of the chromatograms as will be discussed below. Alignment examples are given on liquid chromatography–mass spectrometry data (LC–MS) on National Institute of Standards and Technology (NIST) Standard Reference Material 1950, Metabolites in Human Plasma [3,10], a sample that is chromatographically complex.

2. Mathematical framework

We treat individual chromatograms as functions on an interval $[a, b]$ and consider the issues and challenges that arise in aligning such functions. Given two functions f_1 and f_2, our goal is to find a warping function $\gamma:[a, b] \to [a, b]$ such that f_1 is optimally aligned to $f_2 \circ \gamma$. The most basic idea in alignment is to solve a problem of the type:

$$\inf_\gamma \|f_1 - (f_2 \circ \gamma)\| \tag{1}$$

where inf means the infimum (the greatest lower bound), $\|\cdot\|$ denotes the standard Euclidean norm and quantifies the difference between f_1 and the warped f_2. However, there are some known issues with this formulation, including the possibility of a degenerate solution known as the pinching effect. This occurs when one can pinch the whole of f_2 into a single point, via warping, and consequently reduce the alignment cost to zero despite f_1 and f_2 being very different. A common solution that prevents pinching is to regularize γ by including its roughness in the optimization, according to:

$$\inf_\gamma (\|f_1 - (f_2 \circ \gamma)\| + \lambda \mathcal{R}(\gamma)) \tag{2}$$

here $\mathcal{R}(\gamma)$ is a measure of roughness associated with the warping function γ, and λ is a positive constant. While this is a commonly-used solution, it suffers from the problem of asymmetry. That is, the optimal registration of f_1 to f_2 can be different from that of f_2 to f_1. This asymmetry makes it difficult to provide a meaningful interpretation to the alignment. An additional issue is the choice of λ that provides a balance between the matching term and the roughness term. Different values of λ can lead to very different solutions.

More recently there has been a large interest in developing alignment criteria that result in proper distances between aligned functions. The advantages of this approach are: (1) the solutions are symmetric, i.e. the optimal alignment of f_1 to f_2 is the same as that of f_2 to f_1, resulting in a better interpretability of alignments, (2) the regularization term is already included in these distances, i.e. one does not need any explicit roughness penalty term and, thus, avoiding the tricky issue of selecting λ, and (3) the distance can be used for ensuing statistical analysis such as PCA or classification. The last item is important because the same distance can be used for both the alignment and the accompanying analysis, rather than using two different distances for these steps.

In this paper a specific distance formulation, termed elastic functional data analysis [11] is used. It is based on extending the traditional Fisher–Rao metric used in statistics [13–17] to include more general functions such as the chromatograms. The details of this construction are provided in papers [9,11,12,18,19] and, therefore, here we simply state the alignment solution and apply it to chromatography data.

This new approach is based on a mapping that takes the original functions and transforms them into new functions. This new function, $q : [a, b] \to \mathbb{R}$, is called the square-root slope function (SRSF) of the original function f, and is defined as follows:

$$q(t) = \text{sign}(\dot{f}(t)) \sqrt{|\dot{f}(t)|}. \tag{3}$$

As described in earlier papers [18,19], there are several reasons for choosing SRSFs for the alignment problem. The main reason is that at the Fisher–Rao metric, which has the requisite mathematical properties to facilitate alignment, but is difficult to work with, becomes a standard Euclidean norm of the difference, when we use SRSFs instead of the original functions. The use of Euclidean norm naturally simplifies the alignment solution as it is a familiar quantity. While the Fisher–Rao metric has been used for studying probability density functions and cumulative density functions in the past, the SRSFs allow an extension to more general functions. Notice that we do not require the chromatograms to be positive or have positive derivatives; we allow general functions. For every SRSF $q(t)$, the original function f can be obtained precisely using the equation $f(t) = f(0) + \int_0^t q(s)|q(s)|\mathrm{d}s$, since $q(s)|q(s)| = \dot{f}(s)$. If we warp the function f by γ, the SRSF of the warped function $f_1 \circ \gamma$ is given by $\tilde{q}(t) = (q, \gamma)(t) = q(\gamma(t))\sqrt{\dot{\gamma}(t)}$. With this expression it can be shown that for any f_1, f_2 and a warping γ, we have:

$$\|q_1 - q_2\| = \|(q_1, \gamma) - (q_2, \gamma)\|, \tag{4}$$

where q_1, q_2 are SRSFs of f_1, f_2, respectively. This is called the isometry property, and implies preservation of distances under identical warping. Readers may be familiar with more common isometries such as the preservation of Euclidean distance between vectors under identical rotation and/or translation. SRSF allows a similar behavior for warping. This property is central in suggesting a new cost term for pairwise registration of functions: $\inf_{\gamma \in \Gamma} \|q_1 - (q_2, \gamma)\|$. Therefore, in this framework, one aligns the SRSFs of any two functions first and then maps them back to the original function space to obtain the registered functions. We point out that due to the presence of the square-root of the derivative of γ inside the norm, this cost function has a built in regularization term and does not require any additional penalty term. In the case one wants to further control the amount of warping this can be done by using an additional penalty term.

The pairwise alignment problem can now be solved using the optimization:

$$\gamma^* = \inf_{\gamma \in \Gamma} \left\| q_1 - (q_2 \circ \gamma)\sqrt{\dot{\gamma}} \right\| = \inf_{\gamma \in \Gamma} \left\| q_2 - (q_1 \circ \gamma)\sqrt{\dot{\gamma}} \right\|. \tag{5}$$

The minimization over γ can be performed in many ways. In the case where γ is represented by a parametric family then one can use the parameter space to perform the estimation as in [20]. In this paper we have used the dynamic programming algorithm in Ref. [21] to solve for the optimal alignment. This algorithm uses the discrete time nature of chromatograms to form a finite grid over the square $[0,1]^2$ and seeks the optimal piecewise linear warping function passing through the grid points. It should be noted that for any fixed partition on the interval $[0,1]$ this algorithm provides the exact optimal γ^* that is restricted to the graph on this partition.

While in this paper we are focused on pairwise alignment of chromatograms, this framework is naturally extendible to study groupwise alignment of several chromatograms. The basic idea is to compute a mean or average chromatogram and then align the given functions to that mean. As the objective function used in Eq. (5) is a proper metric, it can be used to define a mean in a consistent manner. For details, we refer the reader to Ref. [18] or [20].

3. Computational implementation

The alignment code was implemented in MATLAB (MathWorks Inc., Natick, MA, USA) [22]. First, the differences in the number of points between the two chromatograms must be reconciled. The chromatogram with the larger number of points is reduced in size. This is accomplished by either selecting every nth point, or by sampling uniformly with a fixed number of points equal to the number in the smaller chromatogram. Next the SRSFs for each of the input chromatograms are computed using Eq. (3) and passed to the dynamic programming algorithm to solve for an optimal γ according to Eq. (5). The optimal γ is applied to the first function, f_1, to align it with f_2. The interpretation is that a height $f_2(t)$ is matched to the height $f_1(\gamma(t))$, for all t in the common range. In practice, some numerical issues may be a small source of asymmetry in warping even though the theory provides a symmetric solution. The computational cost of finding optimal matching depends on the size of chromatograms — on a desktop computer, the matching takes approximately 10 s when the chromatograms have about 1000 sample points. The code can be downloaded from the web address given in Ref. [23].

4. Experimental methods

4.1. Reagents

All the reagents were HPLC (high performance liquid chromatography) grade. Water and acetonitrile were manufactured by J.T. Baker and obtained from VWR (Radnor, PA, USA). All the remaining reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA).

4.2. Sample preparation

SRM 1950 was handled at biosafety level (BSL) 2 as recommended in the NIST Certificate of Analysis. The material was treated with ethanol which also resulted in protein precipitation. Ethanol is a potent bactericidal and virucidal agent at concentrations of 60–80% [24]. An ethanol concentration of 70% for 30 min [25] with the sample held at 0 °C was used. Following treatment, the material was considered safe for handling at BSL 1. Briefly, the SRM 1950 samples were allowed to thaw at room temperature. Ice-cold ethanol (200 proof) was added to a final concentration of 70%. The samples were vortexed for 15 s then allowed to sit on ice for 30 min. The percent of ethanol was then adjusted to 80% and samples were vortexed and stored at −20 °C overnight. The next day, the samples were centrifuged at 4 °C for 15 min at 14,000 × g and the supernatant was evaporated to dryness in a CentriVap

(Labconco, Kansas City, MO, USA). The metabolites were reconstituted in 1% acetonitrile with 0.1% formic acid on the day of analysis and centrifuged at 4 °C for 15 min at 14,000 × g to remove any insoluble material prior to analysis. The supernatant, excluding a yellow hydrophobic material on the top, was stored at −20 °C until analysis.

4.3. Measurement

Approximately 550 μg of the prepared plasma sample was analyzed by conventional HPLC-MS/MS using a 1290 Infinity HPLC coupled to a 6530 Accurate-Mass Q-TOF mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA) with an electrospray ionization (ESI) interface. The column was a SunFire C18 column (1.0 mm × 150 mm, 100 Å, 3.5 μm; Waters Corporation, Milford, MA). HPLC mobile phases A and B consisted of 0.1% formic acid in water and in acetonitrile, respectively. The flow rate was 75 μL min^{-1} and the gradient started at 1% B, increased linearly to 20% B in 30 min, to 90% B in 10 min, was maintained at 90% B for 5 min, returned to 1% B in 2 min and then was maintained at 1% B for 20 min. Two blanks using the same method were run between each sample injection. The mass spectral data was collected in positive ion mode.

For the UHPLC analysis, approximately 110 μg of the prepared plasma sample was analyzed using a ZORBAX Eclipse Plus C18 RRHT column (2.1 mm × 50 mm, 95 Å, 1.8 μm, Agilent Technologies). The flow rate and gradient used for conventional HPLC were scaled using the HPLC calculator version 3.0 [26] to maintain a similar separation. The flow rate was 500 μL min^{-1}. The gradient started at 1% B, increased linearly to 20% B in 6.73 min, to 90% B in 2.21 min, was maintained at 90% B for 1.1 min, returned to 1% B in 0.44 min, and then was maintained at 1% B for 4.41 min. The data was collected on the same mass spectrometer with the same instrument parameters as for the conventional LC.

4.4. Data processing

The data was displayed as base peak chromatograms (BPCs) with a full MS scan filter. The chromatograms were exported into Excel (Microsoft, Redmond, WA) and saved as two-column ASCII files for alignment. The chromatograms are created using only the base peak intensity of the underlying mass spectrum at each point. By examining the entire mass spectrum at each point a verification that the alignment has been done correctly can be made.

5. Results and discussion

Fig. 1 shows a typical chromatogram of SRM 1950 as well as q_1, its corresponding square-root slope function (SQSF) as defined by Eq. (3). Alignment is achieved by calculating γ^* at each point in q_1 according to Eq. (5). Loosely speaking this is equivalent to matching the zero crossings of q_1 to those of q_2. Fig. 2 shows two chromatograms, f_1 and f_2, taken in succession under identical instrument conditions. The bottom panel of the figure shows the optimal γ^* for alignment. The values of γ^* have been normalized to $\gamma^*(t) - t$ by subtracting the expected values for a perfectly aligned chromatogram, that is, a straight line on the t vs. γ^* plot from [0 min, 0] to [50 min, 1]. At early elution times chromatogram 1 needs to be shifted to shorter times as indicated by the positive values of γ^*. These shifts end at about the 20 min mark where chromatogram 1 is brought back gradually toward its original time base as γ^* goes negative. Shifts to shorter times for chromatogram 1 are once again required at the end of the run to bring the two chromatograms into alignment. Note that the value of $\gamma^*(t) - t$ is zero both at the beginning and at the end of the
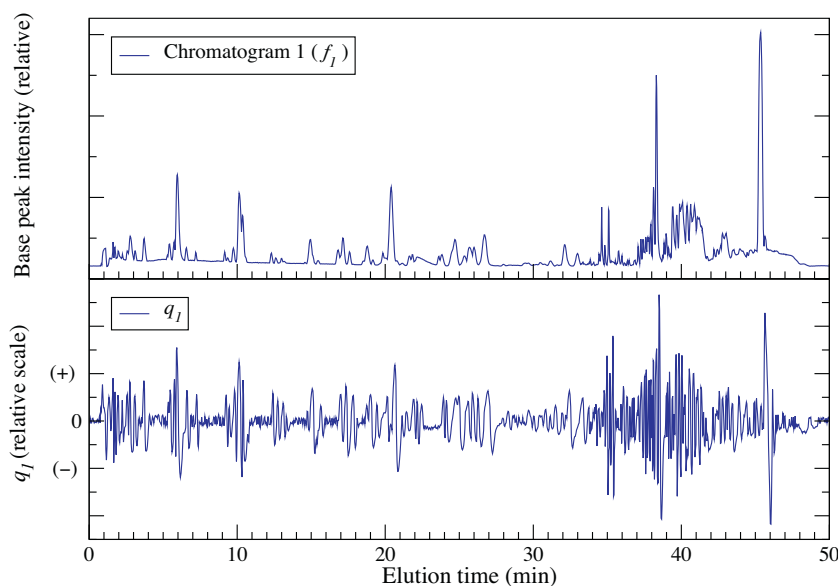
Fig. 1. Top: a typical chromatogram of SRM 1950 taken using the conventional HPLC protocol. Bottom: the square-root slope function (SQRF) of the chromatogram used in the alignment method. Positive and negative values of the SQSF are indicated by (+) and (−), respectively.

chromatogram run times because by construction these points are not allowed to vary during the warping procedure.

Figs. 3 and 4 show details of the chromatographic alignment. Shown are original chromatograms 1 and 2 as well as chromatogram 1 shifted in time to overlay as best as possible chromatogram 2. Circled points in the chromatograms correspond to mass spectra taken at those elution times and are shown in the bottom panels of each figure. In each case chromatogram 1 is brought into close alignment with chromatogram 2. The effect of elution time drift is particularly dramatic in Fig. 4 where the alignment method shifts chromatogram 1 to shorter times by only one MS scan (equivalent to approximately 3 s in real time) yet this results in a significant improvement in the match between mass spectra. Recall that the

alignment method employs only the shape of the chromatograms, the mass spectra are shown simply to verify the accuracy of the alignment.

Fig. 5 shows γ^* for three replicate chromatographic runs aligned to the initial run: the first as shown in Fig. 1 ($\gamma^*_{1\to2}$) and two subsequent runs. The overall shapes are similar; however, small differences can be observed. The sensitivity to these small differences is a consequence of using the derivative of the chromatogram for alignment. At approximately 23 min each run has the same negative excursion in γ^* suggesting that runs 2, 3 and 4 are more closely matched in the position of this peak than they are to run 1. On the other hand, at approximately 44 min runs 2 and 4 behave similarly while in run 3 the peaks in this region shift in
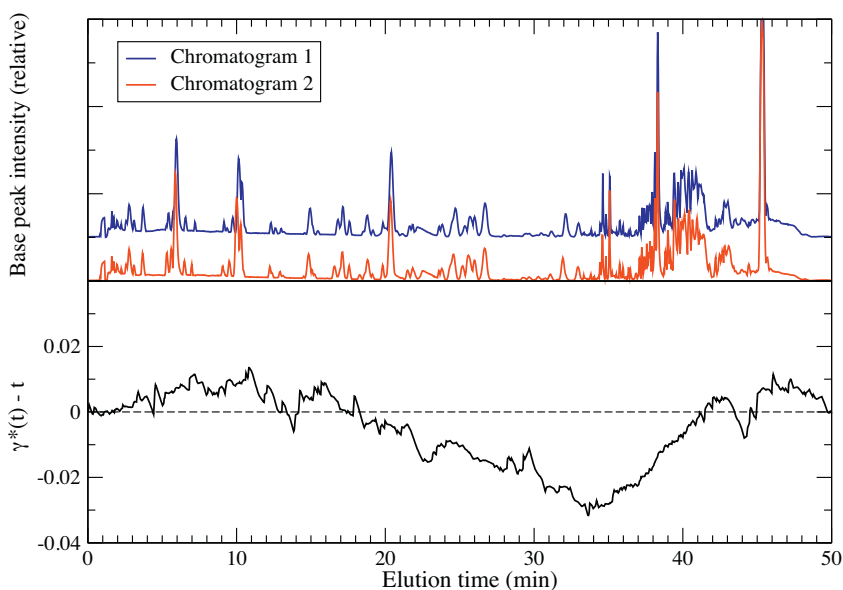

Fig. 2. Top: two chromatograms taken in immediate succession under the conventional HPLC method described in Section 4. Chromatogram 1 is the same as in Fig. 1. Observation shows slight shifts at various elution times. Bottom: the normalized warping function calculated from Eq. (5) that when applied to chromatogram 1 brings it into alignment with chromatogram 2.
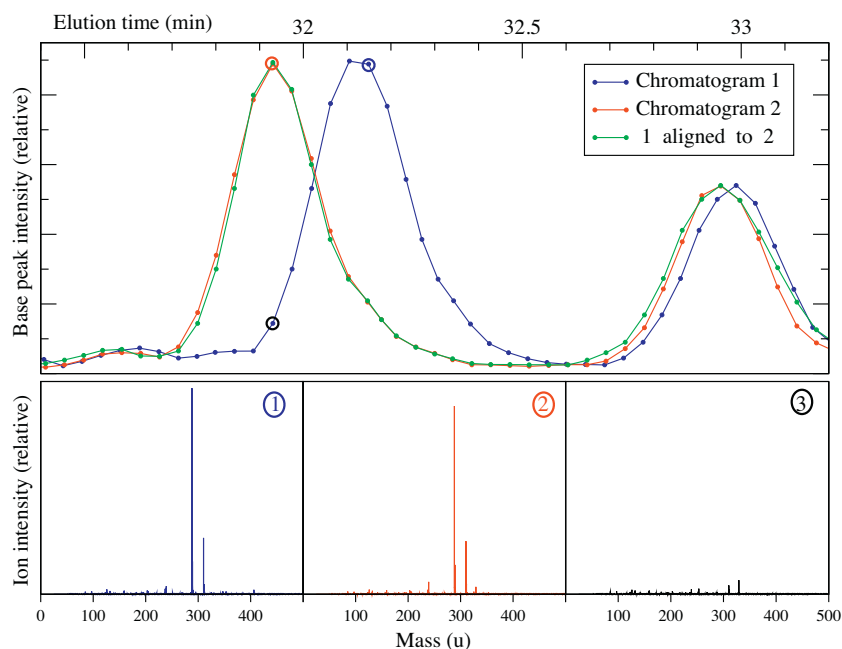
Fig. 3. Top: detail from Fig. 2 in the range of 32 min elution time showing chromatograms 1 and 2 as well as chromatogram 1 aligned to chromatogram 2. Circles represent the locations of the mass spectra in the bottom panel. Bottom: mass spectra 1 and 2 are very similar showing the proper alignment of the chromatograms. Mass spectrum 3 occurs at the same elution time as mass spectrum 1 but is clearly not equivalent. Mass spectra are plotted on the same intensity scale.

the opposite direction. It is conceivable that such information could be used to, for example, optimize measurement conditions or diagnose the condition of the column.

In Fig. 6 a more difficult alignment problem is presented. An aliquot of SRM 1950 from the sample preparation used to perform a conventional HPLC measurement (as shown in Fig. 1) was used to perform an ultrahigh-performance liquid chromatography (UHPLC) measurement as described in the Section 4.2. The different flow volumes of the two columns (due to differences in internal diameter and length) create a dramatic time shift

between the two chromatograms. In addition, the changes in selectivity between the packing materials from different manufacturers create differences in the chromatograms. In conventional chromatography the total elution time was about 50 min while for the UHPLC it was about 11.5 min. To perform the alignment the number of points in the conventional chromatogram was reduced to be the same number as in the shorter UHPLC chromatogram ($n = 336$). This was done by taking every fourth point of the conventional chromatogram. After warping the two chromatograms align to within one MS scan. For example, phenylalanine
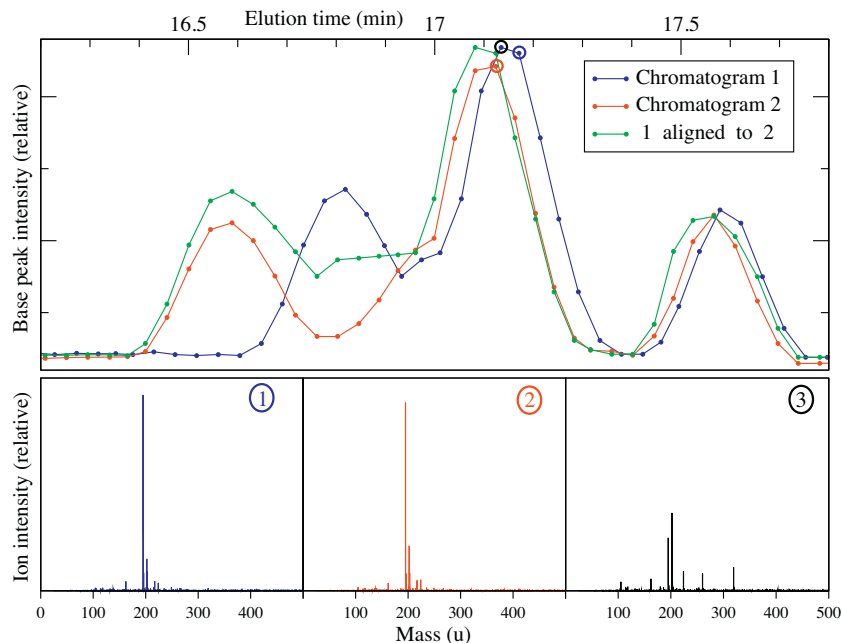

Fig. 4. The same notation as Fig. 3 but showing a more demanding alignment requirement. The difference in mass spectra indicate a degree of alignment to within one MS time step in the chromatogram.
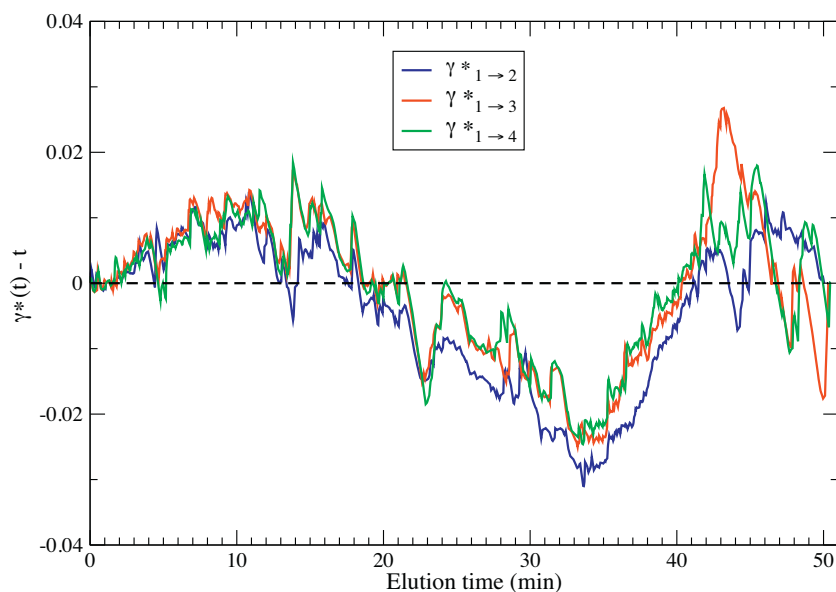
Fig. 5. The warping function calculated from Eq. (5) that, when applied to chromatogram 1, brings it into alignment with chromatograms 2, 3 and 4. The broad sigmoidal misalignment of chromatogram 1 to the others remains virtually the same but small local peak shifts can be observed.
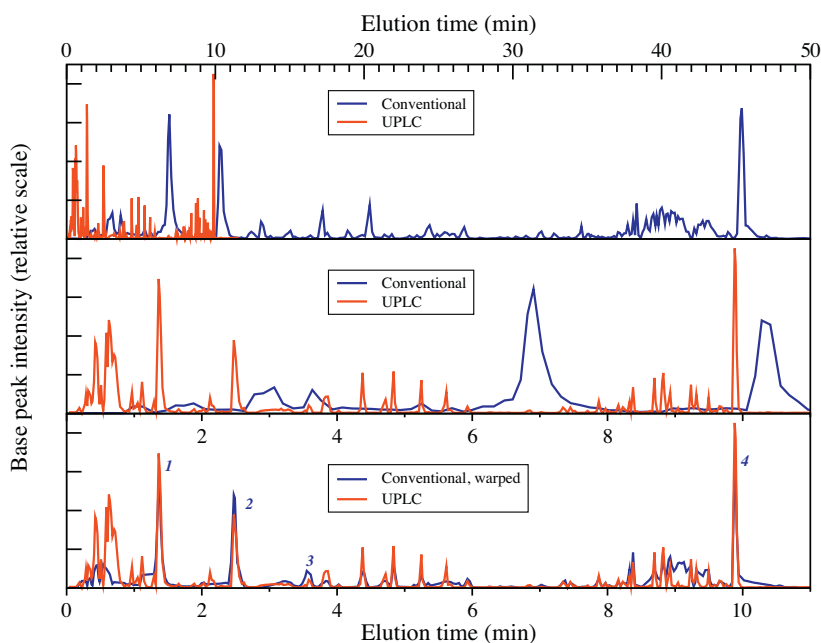

Fig. 6. A more difficult alignment problem comparing a chromatogram taken by the conventional HPLC method (same conditions as chromatogram 1 above) and the same sample analyzed using UHPLC chromatography. Top: two chromatograms as taken plotted in a elution time range to show the entire conventional HPLC chromatogram. Middle: two chromatograms as taken plotted in a elution time range to show the UPLC chromatogram in detail. Bottom: conventional HPLC chromatogram aligned to the UHPLC chromatogram. The blue numerals in the bottom panel refer to the following compounds: (1) phenylalanine, (2) caffeine, (3) tryptophan, (4) cholanoic acid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

eluted at 6.906 min by conventional chromatography and 1.361 min by UHPLC. After warping the phenylalanine peak in the conventional separation moved to 1.362 min which places it in time virtually on top of the UHPLC scan and well within the 2 s window on which the UHPLC data was taken. Other examples of conventional peaks shifted shown in Fig. 6 include caffeine (17.176 min shifts to 3.580 min (3.581 min in UHPLC)), tryptophan (10.344 min shifts to 2.472 min (2.473 min in UHPLC)) and 2b,3a,7a,12a-tetrahydroxy-5b-cholanoic acid (45.371 min shifts to 9.896 min (9.881 min in UHPLC)). In each case the aligned point is the nearest neighbor of the corresponding point in the UHPLC

chromatogram. These values indicate a strong agreement between the aligned conventional chromatogram and the UHPLC chromatogram even though these data were collected under significantly different conditions.

6. Conclusions

An extended Fisher–Rao Riemannian metric based on the square-root slope function has been introduced as a means of aligning chromatograms. A key aspect of this work is the use of the derivative of the chromatogram as the function to be aligned.

Alignment is demonstrated on LC–MS data for a metabolomic reference sample. Underlying mass spectra were used to confirm the accuracy of the alignment which was found to be within one MS scan. Future work will focus on relaxing the criterion of requiring fixed end-points during alignment.

Acknowledgement

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