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Alignment of high resolution magic angle spinning magnetic resonance spectra using warping methods

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ABSTRACT

The peaks of magnetic resonance (MR) spectra can be shifted due to variations in physiological and experimental conditions, and correcting for misaligned peaks is an important part of data processing prior to multivariate analysis. In this paper, five warping algorithms (icoshift, COW, fastpa, VPdtw and PTW) are compared for their feasibility in aligning spectral peaks in three sets of high resolution magic angle spinning (HR-MAS) MR spectra with different degrees of misalignments, and their merits are discussed. In addition, extraction of information that might be present in the shifts is examined, both for simulated data and the real MR spectra. The generic evaluation methodology employs a number of frequently used quality criteria for evaluation of the alignments, together with PLS-DA to assess the influence of alignment on the classification outcome.

Peak alignment greatly improved the internal similarity of the data sets. Especially icoshift and COW seem suitable for aligning HR-MAS MR spectra, possibly because they perform alignment segment-wise. The choice of reference spectrum can influence the alignment result, and it is advisable to test several references. Information from the peak shifts was extracted, and in one case cancer samples were successfully discriminated from normal tissue based on shift information only. Based on these findings, general recommendations for alignment of HR-MAS MRS data are presented. Where possible, observations are generalized to other data types (e.g. chromatographic data).

1. Introduction

Nuclear magnetic resonance spectroscopy, or just magnetic resonance spectroscopy (MRS) in a medical context, is a highly reproducible and robust technique for examining the metabolic profiles of fluids or tissue specimens. By using high resolution magic angle spinning (HR-MAS) MRS, intact tissue samples can be analysed while peak broadening caused by anisotropic interactions is reduced [1]. The result is well-resolved spectra in which the metabolites are represented by sharp peaks. The peak positions in an MR spectrum may, however, be shifted, or misaligned, among spectra in a data set. In general, two types of misalignment are conceivable: non-systematic and systematic misalignments. Non-systematic misalignments can be caused by differences in temperature, intermolecular interactions and other variations due to imperfect control of experimental conditions [2–6], while systematic misalignments contain information about the biological origin of the sample. It has for instance been shown that tumour tissue has a lower pH than normal tissue, possibly due to the Warburg effect [7]. A different pH or ionic strength of samples influences the ionization state of basic or acidic groups and thus their associated chemical shifts [4,8,9]. Metabolite–protein interactions are another possible source of misalignment [10] which is especially important to consider when dealing with HR-MAS data of whole-tissue samples. In general, chemical interactions between substances and different background matrices might provide circumstantial evidence for differences between samples by systematic changes in chemical shifts [4,10,11].

Misalignments between corresponding peaks will affect multivariate analysis of the data. Therefore, it is generally recommended to correct for them [3,5,8,12]. Minor misalignment problems can be overcome by binning the data (typically using a bin width of 0.04 ppm), or by using more sophisticated peak alignment algorithms. An important disadvantage of binning is the loss of resolution and the resulting loss of interpretability [9]. For major misalignments, binning is not a feasible approach due to the resulting loss of resolution, and alignment would be preferable. Several different alignment methods exist. Amongst these, the so-called *warping* methods are most prominent [13–19], but other methods

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Table 1
Characteristics of alignment algorithms.

Alignment method	Optimization criterion	Optimization method	Alignment unit	Aligns by	Parameters to optimize
Icoshift	Correlation per segment	Cross-correlation by FFT	Segments	Shifting	2
COW	Total correlation	Dynamic programming	Segments	Stretching/shrinking	2
Fastpa	Correlation per segment	Beam search	Segments	Shifting and stretching/shrinking	3
VPdtw	L_1 norm	Dynamic Programming	Points	Shifting	2
PTW	Weighted cross-correlation	Nelder–Mead[a] simplex [46]	Complete spectrum	Polynomial model	2

[a] Default, different optimization algorithms are available.

have been described as well [6,20–22]. Most algorithms have their roots in chromatography, but some were specifically developed for MRS. It is not clear, however, which algorithm is the best choice for aligning HR-MAS MRS data and whether the methods originating in chromatography are indeed less suited for this type of data.

In this study, we investigated the suitability of five different warping algorithms—icoshift [13], COW [14,15], fastpa [16], VPdtw [17], and PTW [18]—for aligning HR-MAS MRS data. Of these, icoshift and fastpa were developed specifically for MRS data. The performances of COW and PTW have previously been compared for chromatographic data [23] and capillary electrophoresis (CE) data [24]. The performance of VPdtw for chromatographic data was briefly compared with that of PTW in the original VPdtw paper [17], and the original icoshift [13] paper discusses comparisons with COW and a number of fastpa-related methods for MRS data. We used three different cancer-related HR-MAS MRS data sets for evaluation, all containing samples from two distinct classes. All three data sets represent complex biological samples with varying degrees of misalignments. The various algorithms are compared, and their pros and cons will be discussed in this paper. Because there is no gold standard for assessing alignment quality, the alignments were evaluated with a number of commonly used criteria describing the similarity of the spectra and quantifying their change due to alignment. In addition, the data were classified using partial least squares discriminant analysis (PLS-DA) [25,26] to investigate the effect of alignment on the classification outcome. Although we limited our evaluation to MRS data, the presented evaluation methodology is generic and equally valid for other types of data.

Apart from the warping algorithm, the spectrum to use as the reference for aligning might influence the end result. Therefore, in all evaluations a number of different references were considered and their influence will be discussed.

A possible drawback of correcting for misalignments is that any information that might be present as systematic misalignments in the chemical shifts is lost from the spectra. In that case, correction via alignment or binning might be counterproductive. At the same time, it may be possible to align the spectra while extracting potential shift information from the warping path that describes the transformation from unaligned into aligned spectra. This possibility will be discussed in this article.

To evaluate the effect of alignment on data that display systematic shifts, a number of simple data sets were simulated in which class information was added as intensity differences, shift differences or a combination of the two. These data were aligned, and classification was performed on both the raw and aligned data, as well as on the shift information (i.e. the coefficients resulting from the alignment procedures) and to a combination of these with the aligned spectra. The insights from this procedure were subsequently used in an attempt to enhance the classification results for the real data.

Based on the results from this study, general recommendations for choosing an alignment method for HR-MAS MRS data and getting the optimal alignment are described. The validity of these results and recommendations for other types of data will be discussed.

2. Experimental

2.1. Description of the alignment algorithms

Five different alignment methods were used in this study, and will be elaborated here. Characteristics of the different alignment algorithms are summarized in Table 1. Fig. 1 shows typical warping paths, or warping *functions*, (the new *x*-axes as a function of the old *x*-axis) for all five methods. For clarity, the differences between the new *x*-axes and the old x-axis are drawn, rather than just the new *x*-axes.

2.1.1. Interval correlated shifting (icoshift)

Interval correlated shifting was developed specifically for MRS data [13]. It divides spectra into segments, and aligns these to the corresponding segments of a reference spectrum. The alignment is performed by shifting the segments sideways so as to maximize their correlation. In practice, this involves calculating the cross-correlation between the segments by a fast Fourier transform (FFT) engine that aligns all spectra of a data set simultaneously. The segments can be user-defined or of constant length. Missing parts on the segment edges are either filled with 'missing values', or by repeating the value of the boundary point. The maximum shift correction of the segments can either be equal to a constant defined by the user, or the algorithm can search for the best value for each segment [13]. Icoshift is available as a tool for Matlab from Ref. [27].

2.1.2. Correlation optimized warping (COW)

Correlation optimized warping [14,15] is another segmented warping method. It aims to optimize the overall correlation between two spectra. The spectra are aligned by shrinking or stretching the segments, rather than by shifting them as in icoshift.

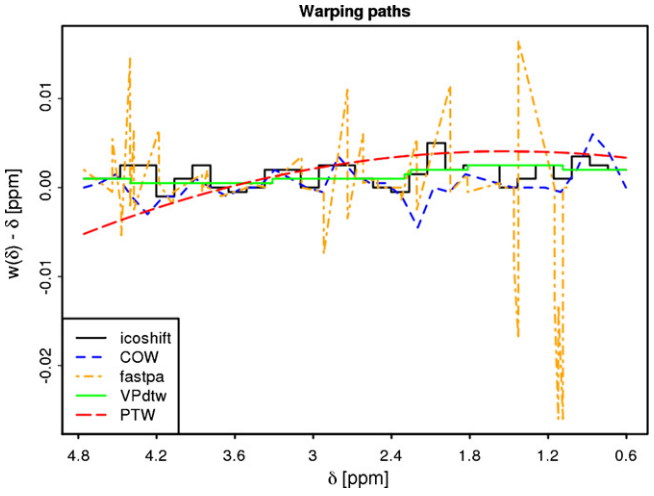

Fig. 1. A comparison of the warping paths of the different alignment methods. Warping paths depicting the fine structures of the different alignment methods are shown. The same query and reference spectra were used for all methods. For clarity, the *y*-scale is set to the difference between the warping paths proper and the original *x*-axis.

Another difference with icoshift is that the optimization takes all segments into account instead of aligning each segment separately, i.e. stretching a segment causes subsequent segments to shift. The maximum allowed change in segment length is determined by the so-called slack parameter defined by the user. In addition, the user must specify the segment length.

The alignment is performed using a dynamic programming algorithm [28]. The algorithm uses linear interpolation to create stretched (or shrunken) versions of the individual segments within the limits determined by the slack parameter. It calculates the sums of the individual correlation coefficients for all combinations of the stretched segments and picks the combination that leads to the largest sum (and hence the largest overall correlation) to construct the aligned spectrum. As all possible combinations are considered, dynamic programming will always yield the global optimum for the chosen parameters [14,15].

The slack and segment length parameters of COW can be optimized by a discrete simplex-like optimization routine described by Skov et al. [29]. An optimization space for both parameters must be specified, and the initial search is defined by a 5×5 grid in both parameter directions. Each of the 25 parameter combinations is evaluated by calculating the sum of the simplicity value and average peak factor (see Section 2.5) of the corresponding trial alignment of the data set. By default, the three best combinations are used as starting points for further simplex optimization. COW is available as a tool for Matlab from Ref. [27].

2.1.3. Peak alignment by beam search (fastpa)

Like icoshift, peak alignment by beam search was developed for MRS data [16]. It also divides the spectra into segments, but aligns these by both shifting and stretching/shrinking them to maximize their respective correlations. Fastpa is based on a routine by Forshed et al. [30] where the segments are chosen automatically to avoid cutting in a peak. However, instead of Forshed's genetic algorithm, fastpa uses a faster beam search [31,32] as the optimization routine for finding the optimal alignment. This change of optimization algorithm is possible because the segments are aligned independently, as opposed to COW.

Fastpa requires three input parameters to be specified: the maximum number of segments, the maximum range of shifting, and the maximum range of stretching or shrinking. In addition, the beam width k [31,32] has to be specified as either 1 or 2. From the viewpoint of optimization, a larger beam width is always preferable [32], and we considered k to be constant at a value of 2.

After choosing segments [30], the algorithm starts by adapting an initial trial solution of stretches and shifts for the individual segments. The 2 best adaptations are used as the next trial solutions in the algorithm. This is repeated until the optimal solution within the beam search space is found [16]. Fastpa is available as a Matlab tool upon request from the authors [16].

2.1.4. Variable penalty dynamic time warping (VPdtw)

Dynamic time warping (DTW) [33] is generally considered to be the first full-fledged warping method that has been developed. It works by shifting individual points of the query spectrum, rather than complete segments, as in icoshift. Many different sets of rules exist for allowed shifts [33,34]. Variable penalty DTW is a recent implementation of asymmetric DTW [17]. Instead of optimizing the correlation between the spectra, VPdtw tries to optimize the L_1 norm, i.e. the sum of the absolute differences between the variables in the spectra.

Regular DTW is notorious for causing artifacts in aligned data, by allowing too many shifts [17,35]. The variable penalty in VPdtw aims to prevent these from occurring by adding a penalty to the L_1 norm for each shift. Clifford and Stone [17] propose to use a morphological dilation (i.e. a running maximum) of the reference spectrum as a penalty. This results in a high penalty being added to the L_1 norm for a shift at or near the position of peaks, whereas in a baseline region, it would result in almost no extra increase. A maximum allowed shift and the penalty must be specified by the user [17]. VPdtw is available as a package in R from Ref. [36].

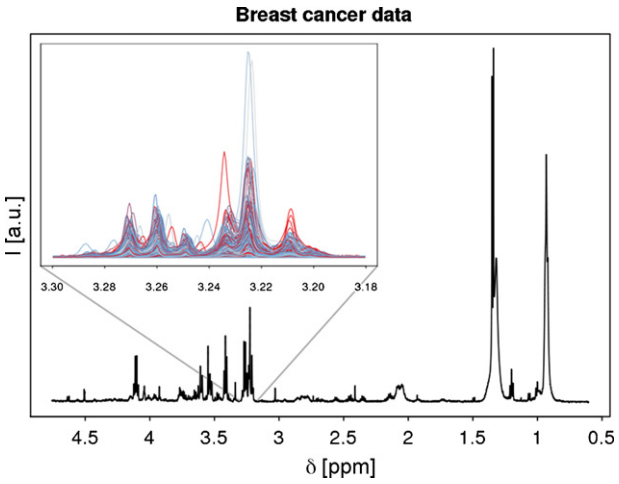

Fig. 2. Breast cancer data. A representative HR-MAS MR spectrum from the breast cancer data set. The inset shows the misalignment for the peaks between 3.18 and 3.30 ppm. a.u., arbitrary units.

2.1.5. Parametric time warping (PTW)

Rather than point-wise shifting, or dividing the spectra into segments that can subsequently be shifted and/or stretched, PTW [18] explicitly produces a global polynomial model (the warping function) of the misalignment:

$$w(t) = \sum_{k=0}^{K} a_k t^k$$

The first two coefficients, a_0 and a_1, in the warping function can readily be interpreted as an overall shift and stretch/shrinkage, respectively. Further coefficients correspond to higher order stretching or shrinking that are useful to model changes in the misalignment along the retention time axis. Bloemberg et al. recently proposed to use the weighted cross-correlation (WCC) [37] as the optimization criterion in PTW [19]. In their implementation, the user has to specify the order of the warping function and the width of the triangular weighting function for the WCC.

The continuity and smoothness of the PTW warping function imply that PTW does not lead to artifacts like 'decapitated' peaks. In the absence of many high-order terms, the polynomial model makes PTW a somewhat restrained method. This means that it may have difficulties in correcting strongly nonlinear misalignments, but also that overfitting is very unlikely to occur [18,19]. PTW is available as a package in R from Ref. [38].

2.2. Data

Three different cancer-related data sets were used in this study: data from cervix, breast, and colon tissue. All three data sets contain data from two biologically distinct classes of tissue. The data sets represent different degrees of misalignment: the cervical cancer data display minor misalignments, colon cancer has major misalignments and the breast cancer data show something in between. Fig. 2 shows the MR spectra from the breast cancer set as an example. In addition to these HR-MAS MRS data sets, simulated data sets with varying degrees of misalignment were generated.

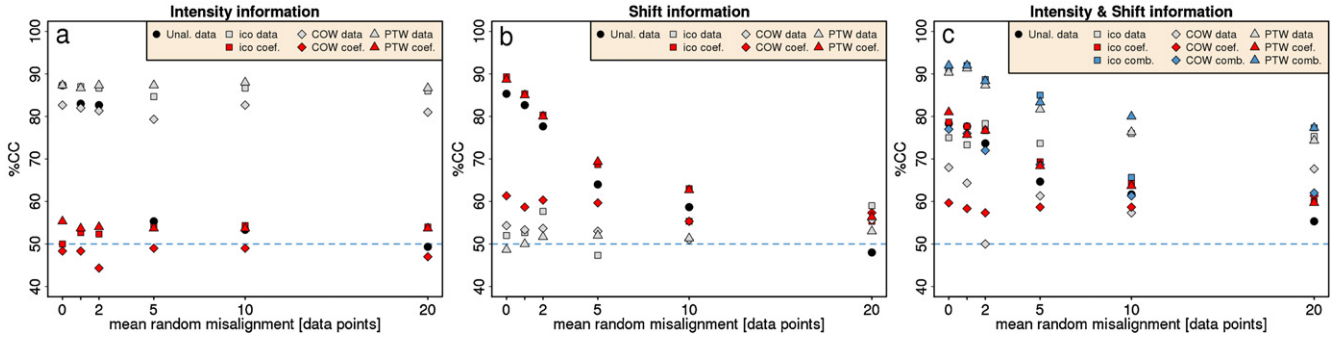

Fig. 3. Classification results of simulated data. The results are the averages of three replicates. (a) Class information is contained in the intensities; (b) class information is contained in the peak shifts; (c) class information is contained in both intensities and shifts. Abbreviations: Unal.: unaligned; coef.: coefficients; comb.: combined; %CC: percentage of correctly classified samples.

2.2.1. Simulated data

A large number of simple data sets were simulated. Each set consists of 100 spectra—50 of class 1 and 50 of class 2—with a spectral width of 1000 variables and two peaks only of width ~10 points. Bivariate class information was added as intensity differences (mean differences ranging from 1% to 10% of peak height), shift differences (means ranging from 1 to 25 points) or a combination of the two. For each set, six copies were produced with increasing non-systematic shifts (see Fig. 3), but exactly similar simulation parameters otherwise. Furthermore, all simulations were performed in triplicate; all reported results are the averages of the results obtained on three sets with similar settings for the randomly generated normal distributions that were used to generate realistic intensity and shift distributions.

2.2.2. Cervical cancer data

This data set is fully described in Ref. [39]. In short, cervical tissue samples ($n = 16$) were collected after hysterectomy of cervical cancer patients ($n = 8$) and patients with non-malignant disease ($n = 8$). The samples were analysed by HR-MAS MRS on a Bruker Avance DRX600, using a water and lipid suppressing spin-echo sequence (cpmgpr, Bruker BioSpin GmbH, Germany). All experiments were performed at room temperature and without buffering. The chemical shifts were referenced to the lactate doublet at 1.32 ppm, and the spectral region between 4.7 and 0.5 ppm was saved in a matrix of 16×3736 variables. The spectra were baseline corrected using asymmetric least squares [18] with parameters $\lambda = 1e5$ and $p = 0.0001$, and the minimum value of each spectrum was set to zero by subtracting the lowest value. The spectra were normalized to equal total area.

2.2.3. Breast cancer data

This data set is fully described in Ref. [40]. Breast cancer tissue samples ($n = 208$) were excised from estrogen receptor (ER) positive ($n = 161$) and negative ($n = 47$) patients. The samples were analysed by HR-MAS MRS using a cpmgpr sequence. All experiments were performed at 4 °C, and the samples were buffered with phosphate-buffered saline (PBS). Chemical shifts were referenced to the TSP peak at 0 ppm. The spectral region between 4.8 and 0.6 ppm, represented by 8251 variables, was extracted for further analyses. The spectra were baseline corrected by subtracting the lowest value of each spectrum, and normalized to equal total area.

2.2.4. Colon cancer data

Colon tissue samples ($n = 32$) were excised from the tumour area ($n = 17$) and normal mucosa ($n = 15$) of colon cancer patients, and the samples were analysed by HR-MAS MRS using a cpmgpr sequence. These samples are part of a larger patient cohort described in Ref. [41]. All experiments were performed at 4 °C, and the samples were buffered with phosphate-buffered saline. In order to induce random misalignments in the data, this data set was not chemical shift referenced. The spectral region between 4.8 and 0 ppm, represented by 9661 variables, was extracted for further analyses. The spectra were baseline corrected by subtracting the lowest value of each spectrum, and normalized to equal total area (excluding polyethylene glycol pollution at 3.71 ppm).

2.3. Alignment of simulated data

All simulated data sets were aligned using the five warping methods and alignment was performed using the first spectrum as the reference. Icoshift was set to align the data in two segments, whereas PTW was set to align using a linear warping function, corresponding to an overall shift and stretch. Unexpectedly, COW ran into memory problems when the segment length was chosen to be half the spectral width (500 points), and the segment length was set to one tenth of the spectral width (100 points). VPdtw was unable to produce well-aligned data consistently and the fastpa algorithm was too unstable in its current form to allow high-throughput analysis of a large number of data sets.

The obtained warping coefficients correspond to two (integer) shifts for icoshift (one shift coefficient per segment), a shift and a stretch coefficient for PTW and ten segment endpoints for COW. For classification purposes, the icoshift coefficients were used 'as is', whereas the stretch coefficient for PTW was multiplied by the number of data points (1000) after subtracting 1 (the default for 'no alignment') from it, as described in Ref. [19]. In this way, the shift and stretch coefficients are on comparable scales. For COW, the original segment endpoints were subtracted from the new endpoints, so as to provide the differences between them.

2.4. Alignment of real data

The cervix, colon, and breast cancer data sets were aligned using the five different warping methods, as described below. Ten different reference spectra were used subsequently for aligning the data; this in order to examine the influence of choosing different references and also to examine the robustness of the warping methods. Four spectra were chosen from each of the two classes in a data set: two randomly chosen ones and the two spectra having the highest average correlation with the other spectra in the data set. In addition, the mean and the median spectra were used as references.

2.4.1. Icoshift

The optimal number of segments for icoshift was determined by visual inspection of trial alignments and by the average overall correlation, and ranged from 20 to 150 for the different data sets and references. The maximum allowed shifts were determined by the

algorithm. For some matrices this led to artifacts, and the maximum allowed shift was manually determined instead. A full spectrum correction was performed prior to alignment of the segments. Missing parts on the segment edges were replaced by repeating the value of the boundary point. Both user-defined segments and segments of constant length were tested.

2.4.2. COW

Parameters for COW were determined using the optimization routine by Skov et al. [29]. The search interval for segment length was based on the average peak width, as suggested by the authors, and the slack size search space ranged from 1 to 15. The search space was increased if the limit values were chosen as the optimal parameter. Optimal segment length ranged from 30 to 300 variables.

2.4.3. Fastpa

Fastpa parameters were optimized by visual inspection of trial alignments, and the overall average correlation. The spectra were zero-padded prior to alignment to avoid cutting off peaks at the spectrum edges. This was also done because fastpa does not align the last segment of the spectra. The ranges of segment number, sideways movement and interpolation were 40–170, 10–150 and 10–100, respectively.

2.4.4. VPdtw

Alignments were performed on the normalized data, after additional square-root scaling, as this turned out to provide better alignment results than for unscaled data. The resulting warping paths were applied to the normalized data. The penalties that were used for the alignment were morphological dilations of the data, as described in Ref. [17]. Penalties were determined by trial and error until satisfactory alignment results were produced. The maximum allowed shift (the width of the Sakoe-Chiba band [33]) ranged from 100 to 300 variables.

2.4.5. PTW

Prior to alignment, the data were zero-padded, as described in Ref. [19]. The resulting warping coefficients were transformed accordingly and applied to the unpadded data. Triangle widths for the weighted cross-correlation measure were on the order of the largest misalignment in the data, as determined by visual inspection and ranged from 2 to 100 variables.

2.5. Evaluation criteria

The alignment results were assessed based on different measures:

2.5.1. Correlation

The spectra of a data set will be more uniform after successful alignment, and thereby have a higher correlation. The correlation between all the spectra of a data set was calculated before and after alignment.

2.5.2. Simplicity value

The simplicity value is related to principal component analysis (PCA) by singular value decomposition (SVD) of a matrix, where the singular values state how much variance is explained by each component. Aligned spectra will have more variance explained by the first components. The simplicity value of a matrix is defined as the sum of all singular values of the matrix—scaled to a total sum of squares of one—taken to the fourth power, and will be larger when more variation is explained by the first components [29].

$$\text{Simplicity} = \sum \left(\text{SVD} \left(\frac{X}{\sqrt{\sum_i \sum_j x_{ij}^2}} \right) \right)^4$$

2.5.3. Peak factor

The peak factor gives an estimate of how much the area and the shape of the peaks have changed in a spectrum after alignment. It compares the Euclidian length, or norm, of a spectrum before and after alignment. If the peak area and shape stay almost the same, the difference between the norms before and after alignment will be small [29]. The optimal value for the peak factor is 1, meaning that there is no change in peak shape.

$$\text{Peak factor} = \frac{\sum_{i=1}^{I} (1 - \min(c_i, 1)^2)}{I}$$

where

$$c_i = \left| \frac{\text{norm}(x_{i,\text{after}}) - \text{norm}(x_{i,\text{before}})}{\text{norm}(x_{i,\text{before}})} \right|$$

2.5.4. Classification

Correcting for misalignments should improve the classification results for data sets distorted by random shifts. However, it is also possible that information arising from biological differences between different classes may be removed when the spectra are aligned. In PLS, latent variables (LVs) are derived to maximize the covariance between the spectra and a quantity to be modelled. PLS-DA is a special case of PLS that attempts to discriminate between classes, represented by discrete numbers. Here, PLS-DA was used to evaluate the classifiability of aligned and unaligned data. In addition, the warping path or warping parameters were used as input to investigate possible shift information.

2.5.5. Visual inspection

Quantitative measures are valuable means for comparing specific characteristics of large sets of data at a glance, but they also have their limits. The human eye and brain are still unsurpassed as a pattern recognition tool. In the context of alignment, especially the assessment of alignment quality and detection of artifacts benefit from visual inspection.

2.6. Classification of simulated sets

Each data set was classified using PLS-DA. Classification was performed on the unaligned spectra, the aligned spectra, the warping coefficients and a combination of the aligned spectra and the coefficients. The latter was achieved by simply concatenating the spectra with the coefficients and multiplying the latter with a large number (on the scale of the average-scaled spectra, typically 100 was used) to make sure they would be contained in the first latent variables of the PLS model.

PLS-DA, including mean centering, was performed using full leave-one-out cross-validation (LOO-CV), and the number of LVs giving the first minimum in prediction error was chosen for the model. PLS-DA was performed in Matlab 7.7.0.471 (R2008b, The Mathworks, Inc., Natick, USA) using PLS_toolbox 5.5.1 (Eigenvector Research, Wenatchee, USA).

2.7. Classification of real data

The data sets were classified using PLS-DA, as described for the simulated data. Classification was performed on the unaligned spectra, the aligned spectra, the warping coefficients, and a combination of aligned spectra and coefficients (multiplied by 100).

Pollutions from ethanol and fatty residuals, and from polyethylene glycol for the colon cancer data, were removed from the spectra prior to classification. Single outlying spectra were removed from the colon cancer and breast cancer data sets. For the breast cancer data, the spectra were square-root scaled prior to analysis.

3. Results and discussion

3.1. Simulated data

Fig. 3a shows the average classification results for the sets in which classes are coded as intensity differences. Fastpa and VPdtw were not capable of correctly aligning the simulated data. As expected, classification rates for unaligned data decreased as random misalignment increased. Aligned data, on the other hand, delivered stable classification results. The warping coefficients did not contain class information, as expected, since the simulated shifts were completely random.

The average classification results for three data sets where class information is purely contained as peak shifts are depicted in Fig. 3b. As expected, the situation here was completely opposite to the previous one. The raw data gave better results than the aligned data; alignment removes the information of interest from the spectra. Now, the coefficients *do* contain information and in this case the coefficients give even better classification results than the raw data themselves. This is most likely due to less noise being present in the coefficients than in the data. Because classification is based on intensities, the intensity noise in the unaligned data will have a negative influence on the result. By extracting the positional information of the peaks into the warping coefficients, it effectively becomes available as information without the intensity noise that was present in the spectra.

When class information is present in both the shifts and the intensities, as might be expected with real data, the situation will be somewhere in between the two extremes discussed above. Where exactly depends on the data at hand. In that respect, any simulation is rather arbitrary and we should not over-interpret the results. It is clear from the example in Fig. 3c however, that—if discrimination is the main interest—there are situations in which aligned data on their own can be a sub-optimal choice as input for multivariate analyses when there is information present in the shifts. In these cases, for icoshift and PTW, the combination of aligned data with the warping coefficients delivered the best classification results. The COW results for the combination of data and coefficients were a lot worse than those of the other two methods; this may have to do with the sub-optimal parameter settings that were used to prevent the program from running into memory problems. Furthermore, it is likely that the good results for PTW are due to the simplicity of the data and the resulting suitability of a warping function of degree 1 for modelling the misalignments. Bearing in mind the conclusions from Refs. [23,24], it is to be expected that individual shifts in more complex MR data cannot be modelled very well with the global PTW model. Icoshift and COW are more likely to extract positional information in a way that is suited for multivariate analyses, although the cumulative character of the COW warping path might 'smear out' misalignment information over several segments, making it harder to interpret.

3.2. Real data

3.2.1. Correlation and simplicity value

A plethora of similarity and distance measures are used as optimization criteria in different alignment algorithms. Icoshift, COW and fastpa are all optimized using correlation as a criterion. DTW is available with various distance measures [34] and VPdtw employs the L_1 norm as a criterion for optimization. PTW optimization was originally based on the root mean square difference (RMS) between spectra [18], but the current implementation uses the weighted cross-correlation as a similarity measure [19]. There is still no generally accepted gold standard measure for assessing alignment quality. However, the combination of simplicity value and peak factor introduced by Skov et al. [29] is an interesting choice. Both measures, together with the correlation, were used to assess alignment quality in this study. In addition, the RMS and WCC criteria were examined, but these measures did not provide extra information. It should be kept in mind that methods optimizing the correlation will very likely be biased towards that measure and it is not certain that the results for the simplicity value will be completely independent.

Fig. 4 shows box plots of the mutual correlations between all samples in the three HR-MAS data sets, before alignment and after alignment with each of the five warping methods for ten different references. It is clear that for all methods, the correlation distributions of the aligned data are better than for the unaligned data. This is especially pronounced for the colon cancer data set which has the largest misalignments. Here, all warping methods greatly improved the correlations, with PTW scoring lower than the other methods. For the cervical cancer data, where the unaligned data displayed only minor misalignments, VPdtw resulted in the lowest correlation values, while the other methods performed comparable.

The simplicity values for the raw and aligned data in Fig. 4 convey the same general picture as the correlations. There are some differences, however. The most striking ones are the PTW and VPdtw results for the colon cancer data set. When looking at the correlations, the PTW correlations are clearly lower than the ones for icoshift, COW, and fastpa, and comparable to the VPdtw correlations. The PTW simplicity values, however, are comparable to those of icoshift, COW, and fastpa, whereas the VPdtw simplicity values are much lower. The simplicity value is influenced by the intensities of the peaks, and peaks with high intensities influence the value more than low intensity peaks. The colon cancer data displayed a high intensity peak at 3.71 ppm, resulting from polyethylene glycol. When the peak was removed from the data set, the simplicity values were more in accordance with the correlations. This example shows a weakness of the simplicity value, and it might be advisable to scale the data prior to simplicity calculations.

Fig. 4 also shows that the choice of reference can have a large influence on the alignment result for one-dimensional HR-MAS MR spectra, contrary to the observation made in Ref. [21] for LC–MS data. The breast cancer data generally provided stable results, but demonstrated that a bad choice of reference had a larger influence on the correlations than the particular warping method that is used. Icoshift, COW and fastpa, which are all segmented warping methods, appeared to be less influenced by the choice of reference, whereas PTW gave worse results than the unaligned data for some of the randomly chosen reference spectra. It is therefore advisable to try different references when aligning. Using the spectrum that has the highest average correlation to the other spectra does not seem to be a bad choice; however, it does not always give the optimal alignment. For data sets consisting of two or more classes, it is conceivable that the alignment will be affected by the class the reference belongs to. Trying references from both classes may therefore be advisable. Using the mean or median spectrum as a

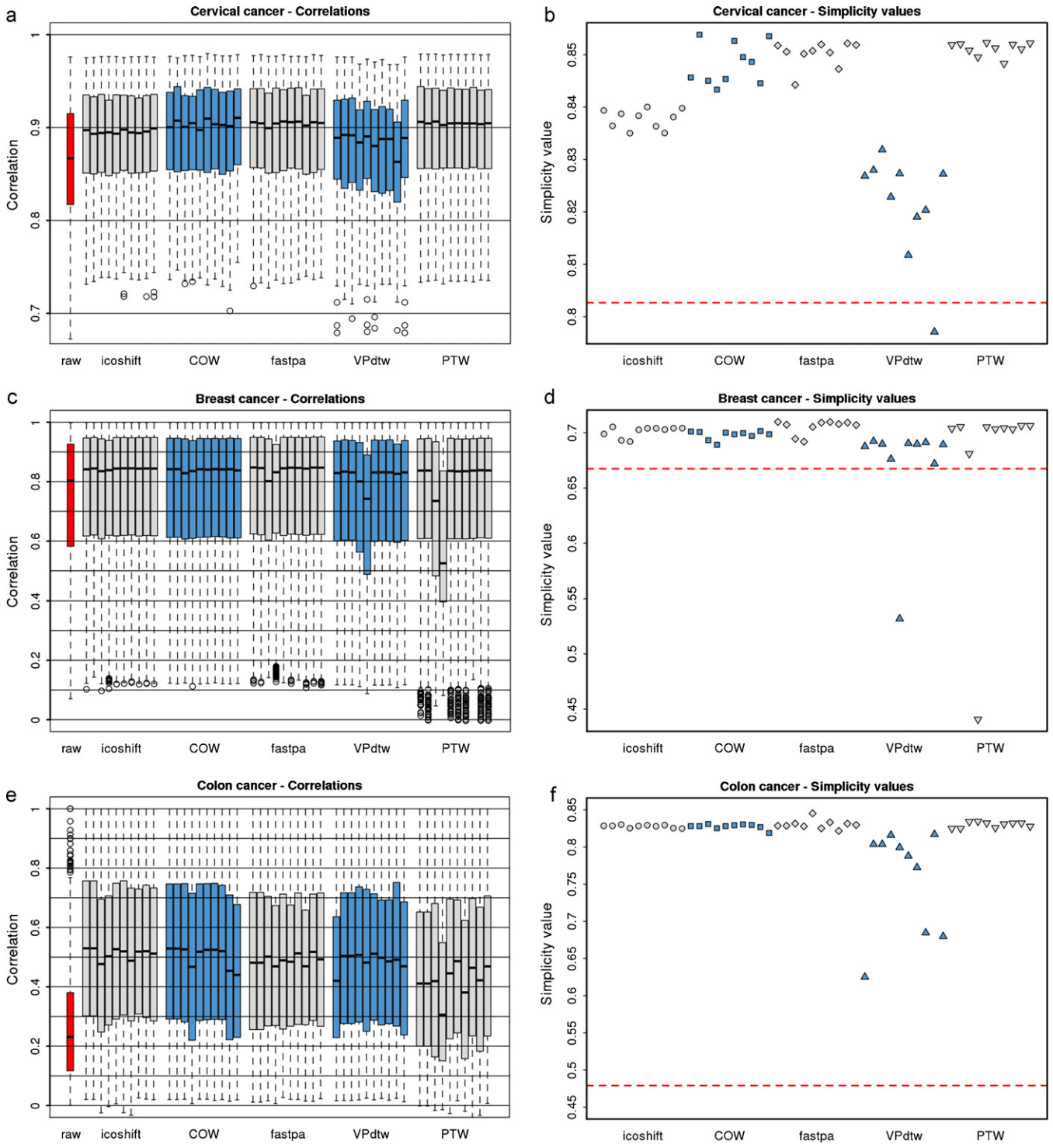

Fig. 4. Correlation and simplicity values for different alignment methods. In all plots, for each method, results from 10 different reference spectra are shown. From left to right: the spectrum having the highest average correlation with all other spectra, the (on average) second most highly correlated spectrum from the same class, and two random spectra from the same class; the most highly correlated spectrum, the second most highly correlated spectrum, and two random spectra from the other class, and the overall mean and median spectra. The box plots show the distributions of correlation values for the data sets; the red box stands for the unaligned data. The scatter plots show the simplicity values of the data sets; the red line depicts the simplicity value of the unaligned data. (a) Correlations of the cervical cancer data; (b) simplicity values of the cervical cancer data; (c) correlations of the breast cancer data; (d) simplicity values of the breast cancer data; (e) correlations of the colon cancer data; (f) simplicity values of the colon cancer data.

reference is also an option. This may not be a good choice for data sets with big misalignments though, as the mean/median spectrum will have broad peaks and may not resemble a real spectrum. This can be overcome by using an iterative procedure, i.e. by aligning the data and then recalculating the reference spectrum. This was tested for the colon cancer data in this study, and the resulting alignments then resembled those for the other references (results not shown).

In some cases, similarity measures can give the wrong impression of the alignment quality. An example of this is when peaks are badly deformed in order to give a high correlation, as in unpenal-

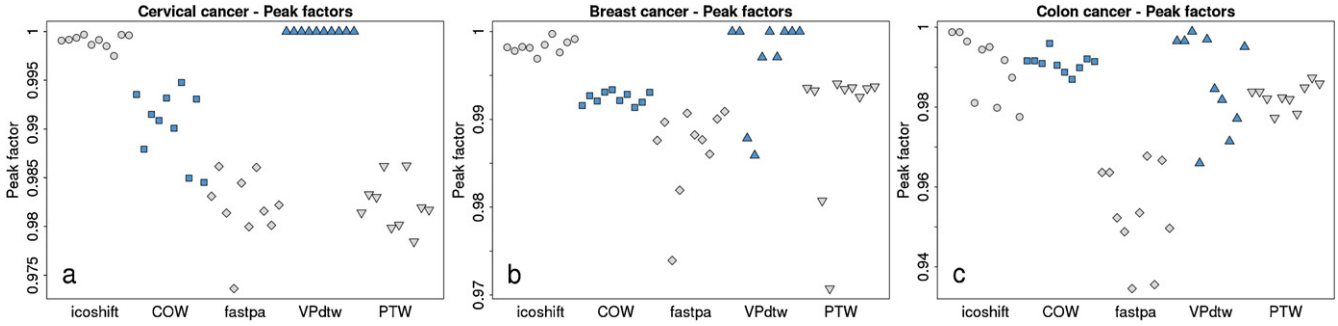

Fig. 5. Peak factors for different alignment methods. Results from the same 10 different references as in Fig. 4 are shown (a) peak factors for the cervical cancer data; (b) peak factors for the breast cancer data; (c) peak factors for the colon cancer data.

ized DTW [17,35]. Other examples were encountered when using icoshift: when using segments of constant length, the segment edges would sometimes be located in a peak, leading to major artifacts in the peak shapes, while the correlation of the spectra remained high. For some parameter choices for icoshift, the peaks were displaced to the wrong position, but again correlations remained high. Therefore, visual inspection of the data after alignment remains of the utmost importance. This will however put a limit to the complexity of the data of interest. While HR-MAS spectra of tissues have quite well-defined peaks, MR spectra from fluids may be more crowded, making visual inspection of the aligned data challenging. For these data, other methods may be more suitable, for instance the one described by Alm et al. [6].

3.2.2. Peak factor

Peak factor calculations for the different warping methods are shown in Fig. 5. The differences between the methods are small, and overall, all methods performed well. Icoshift and VPdtw gave the highest peak factors. This result was as expected for icoshift, as it only shifts segments of the spectra, as opposed to fastpa, COW and PTW that do shrinking and stretching. For VPdtw, the high peak factors are noteworthy, given that DTW has a history of strongly deforming peaks. Clearly, the variable penalty of VPdtw does what it is intended to do.

COW's parameters are optimized based on peak factor, and COW gave the best results of the warping methods that do shrinking and shifting. On average, fastpa had the lowest peak factor values, and was thus the method that changed the data most after alignment. This is also obvious from the warping functions in Fig. 1; fastpa typically had the most extreme warping path. As for correlation and simplicity value, icoshift and COW provided stable results for different reference spectra. The results for fastpa and PTW varied more, and it appears that the choice of reference is more critical for these methods.

3.2.3. Classification results

The classification results for unaligned and aligned data are shown in Fig. 6. For the cervical cancer data, the unaligned data already gave good results, with only one out of 16 samples misclassified. In general, the aligned cervical cancer data gave better classification results for all methods. For the breast cancer data, the average classification results, based on the 10 different references, improved for all methods except PTW. Here, the use of some reference spectra improved the classification results while others gave worse results. Despite the fact that both the correlation and the simplicity value of the colon cancer data greatly improved by aligning, the classification results did not improve. After alignment the average classification results decreased by one or two additional samples. It is not unlikely that the results for the unaligned data are better simply by chance. In theory, it is also possible that peaks have been aligned to the wrong peaks of the reference spectrum. This is unlikely however, as visual inspection of the alignment results gave no indication of wrong alignments.

Another possibility is that the shifts of the colon cancer data contained information, resulting from systematic misalignments of the spectra. Information present in the shifts would get lost after alignment. This was investigated for all the data sets by classification of the warping parameters and a combination of spectra and parameters. It should be noted that the prediction error from cross-validated PLS-DA was based on the same data set as the one used for choosing the optimal number of LVs. Thus, the prediction error will be slightly biased towards values lower than 0.5, and only results that differed strongly from 0.5 were considered important. Classification of the warping parameters alone did not give reliable predictions for the breast cancer and the colon cancer data. Furthermore, combining the warping parameters with the spectra did not improve classification. As previously described, cancer tissue can have a different pH than normal tissue, and the pH of a sample is an important source of shift variation. For the breast cancer samples, there are no established hypotheses for pH differences between ER positive and negative samples, and the results were as expected. For colon cancer, the samples in the data set were from either normal or cancer tissue. Therefore, differences in pH are more likely, even though the samples were buffered prior to HR-MAS analysis [42]. However, it is likely that shift information that might have been present in the data was masked by the major random shifts of the data set, similar to what is shown in Fig. 3b and c for the simulated data.

For the cervical cancer data, the results clearly indicate that the warping parameters of icoshift, COW and fastpa contain class information. Classification of the parameters gave an average correct classification of 87%, 84% and 76% for icoshift, COW and fastpa, respectively (Fig. 6d). Combining the spectra with the parameters was not beneficial for the overall classification. Thus, the information from the parameters was redundant. Nevertheless, the fact that shift information alone can discriminate between normal cervical tissue and cancerous tissue is very interesting. The cervical samples were analysed by HR-MAS without buffering, and therefore pH differences related to cancer-induced changes in tissue may be more pronounced here than for the colon cancer samples. So despite the redundancy of the shift information for the cervical data, this result indicates that measuring biological samples without buffering might reveal biologically relevant differences that would be obscured otherwise.

It is not hard to see why icoshift and fastpa provided warping coefficients that are suitable for subsequent multivariate analysis. These MRS-oriented methods align their segments independently; therefore corresponding shifts will always occur at the same position in the spectra. Opposed to that, for VPdtw, the effect of warping is cumulative, and the actual stretching occurs at slightly different places for different spectra even if they have similar misalignments (results not shown). The alignment of COW is also cumulative, but

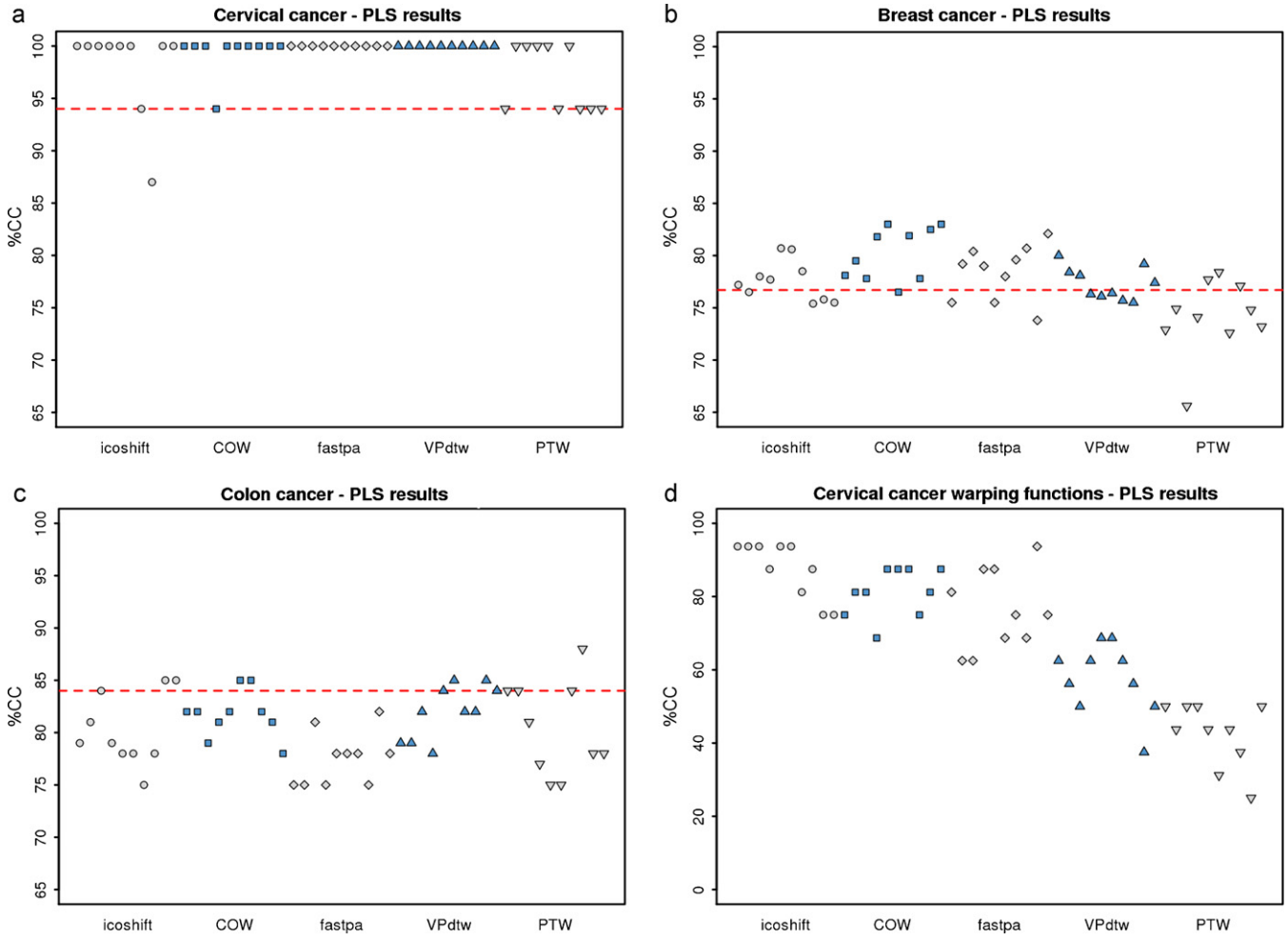

Fig. 6. PLS-DA classification results for different alignment methods. Results from the same 10 different references as in Fig. 4 are shown. The red line denotes classification results of the unaligned data (a) classification results of the cervical cancer data; (b) classification results of the breast cancer data; (c) classification results of the colon cancer data; (d) classification results of the cervical cancer warping coefficients.

its segmented nature largely prevents it from showing many local differences. Thus, it is not surprising that COW's warping coefficients for the cervical cancer data also led to good classification results. Both for VPdtw and COW, classification was also attempted using the cumulative sums of their warping functions instead, to (further) alleviate the local differences, but this did not improve the results. For PTW, alignment was performed using a quadratic function, and it can be assumed that the relevant shifts in the spectra were too complex to be modelled well by this function.

To summarize, the results presented here show that alignment of the data using the warping methods examined in this work not always improves the classification results compared to unaligned data. However, alignment improved the interpretability of the resulting model by providing less ambiguous loading profiles for PLS-DA, similar to the observations in Refs. [43–45]. This is especially important in situations where discriminating between two classes is not the only interest, but where one is also interested in looking at the differences in metabolic profiles to interpret biological incidences in the tissue. For that purpose, alignment will always be preferable.

3.2.4. Algorithms

Table 2 summarizes the evaluations of the different warping algorithms. Overall, icoshift and COW gave good alignment results and preserved the peak shapes. For COW, the optimization routine has a large part in this. Icoshift required quite some manual

Table 2
Evaluation of alignment methods[a].

Alignment method	Programming stability	Memory efficiency	Speed	Optimization of parameters	Peak conservation	Artifact- free	Alignment quality[c]
Icoshift	+	+	++	0	++	–	+
COW	+	–	–	+	+[b]	++	++
Fastpa	– –	+	0	–	0	0	+
VPdtw	+	+	+	0	+	0	0
PTW	+	+	0	0	0	++	0

[a] ++, very good; +, good; 0, moderate; –, improvement advisable; – –, improvement necessary.
[b] COW parameters were optimized to conserve peak shape prior to alignment.
[c] The alignment quality is assessed based on the end result after optimization of the parameters.

Table 3
Benchmarks. Time consumptions for alignment of the breast cancer data set using the same reference spectrum.

Alignment method	Time (s)
Icoshift	3.73
COW	292
Fastpa	87.0
VPdtw	42.8
PTW	149

optimization, but this was not considered a problem because of its speed, and the end results were satisfactory. For the parameter combinations allowed by the algorithm, fastpa also gave good alignment results, but at the expense of larger peak shape changes. This is not surprising when looking at Fig. 1: fastpa's warping paths were typically more extreme than those of the other algorithms. The segmentations of the spectra offered by fastpa and its predecessor [30] were typically good, however. A combination of this part of the algorithm with the alignment power of COW or icoshift may be worthwhile. Both VPdtw and PTW delivered variable results. The variable penalty in VPdtw clearly prevents the algorithm from deforming the spectra, but optimizing the resulting alignments is not trivial. The polynomial warping function of PTW is probably not flexible enough to model the local shifts occurring in NMR spectra very well.

The time consumption for alignment varies a lot among the methods. Table 3 shows the benchmarks for alignment of the breast cancer data set using the same reference spectrum. The benchmarks were obtained on a Dell Latitude E6400 laptop, equipped with an Intel Core 2 Duo P9500 processor running at 2.53 GHz and 3.48 GB of RAM. The operating system was Microsoft Windows XP SP3 (32 bit). Alignments with icoshift, COW and fastpa were performed in Matlab, version 7.7.0.471 (R2008b), while VPdtw and PTW alignments were performed in R, version 2.9.2.

Icoshift was the fastest warping method, and alignment of a data set of 209 spectra was done in a few seconds. COW, on the other hand, was the most time-consuming of the methods tested here, and used minutes to perform the same alignment. Also, COW sometimes runs into memory problems for large data sets. This can be overcome by choosing different parameters, or by dividing the data set in smaller subsets. Obviously, this may result in a final alignment that is not the optimal one for the data set.

The benchmarks shown here do not include optimization of the parameters, as that largely depends on the effort put into the procedures by the user. For fastpa, three parameters have to be optimized, as opposed to the other methods with only two parameters. This made fastpa more time-consuming to optimize. In addition, some combinations of fastpa parameters will give an error without any obvious reason. COW has an automatic optimization procedure that is time-consuming; however, it requires a minimum of effort from the user.

Although the evaluations in this paper were limited to MRS data, some of the observations above can safely be generalized to other types of data. Together with the conclusions in Refs. [23,24] it is clear that the rigidness of PTW's polynomial warping function limits its general applicability compared to COW. (At the same time, this is not to say that there are no situations in which such a rigid but also relatively simple warping function may be preferable; the data in the original PTW-paper were aligned in a satisfactory manner, for instance.) The 'wild' behaviour of fastpa is also something that seems to be inherent to that method and is expected to be independent of the exact type of data. Time consumption of alignments will mostly depend on data size and data complexity; the benchmarks in Table 3 can thus safely be used as an indication, regardless of the origins of the data.

3.2.5. General recommendations

Based on the observations in this study, we have the following recommendations for the alignment of HR-MAS MRS data from tissue samples:

- As a default method, icoshift is a good choice. It is fast, stable and gives good results. Its results should be thoroughly checked by visual inspection, though, and it may require some trial and error to prevent peaks from disappearing or artifacts to occur. However, its speed makes this feasible.
- When large local shifts occur in crowded data, or the results do not get satisfactory, COW is a good alternative. Although it is rather slow and memory intensive, this problem is alleviated somewhat by the computational power of current computers. COW robustly provides good alignment results and because it uses stretching instead of independent shifting for alignment, it is well suited to provide alignments exactly when icoshift runs into trouble. The pre-alignment optimization of the slack and segment length parameters ensures that peak shapes will be minimally affected.
- It is a good idea to try out a number of references for alignment. Although choosing the sample with the highest average correlation never seems to give bad results, it does not necessarily lead to the optimal result. Trying more references is a small effort and gives an idea of the variability of the results. Moreover, it is likely to provide a result close to the optimum that can be achieved.
- In general, a truly automatic warping procedure does not exist. The best alignment will not be achieved without putting some effort into optimizing alignment parameters, scaling, and finding a good reference.
- Visual inspection of the end result is an absolute necessity. Numerical measures can indicate a good result even if artifacts are present. At the same time, it should be kept in mind that visual inspection on its own is not infallible, since it is prone to subjective judgment.

4. Conclusion

In this paper, we investigated the suitability of five warping algorithms for aligning HR-MAS MR spectra to make them amenable to further multivariate analysis. Furthermore, we extracted shift information from the spectra and tested if it can be used in multivariate analysis.

Alignment of the data sets greatly improved their internal similarity compared to unaligned data. The differences in alignment quality between the algorithms examined in this study were not very large in general. Icoshift, COW and fastpa gave a good overall alignment result for HR-MAS data, but fastpa currently has too many drawbacks for general use. Both icoshift and COW also conserved the peak shapes of the spectra. Whether the algorithms were designed for chromatographic data or MR spectra did not seem to have an influence in general on their suitability for aligning MRS data. Comparison of our results with previous studies on chromatographical and CE data allowed generalization of some observations.

Both the choice of reference and the effort that is put into aligning are important factors in reaching the optimal alignment result. It is therefore advisable to try a number of different spectra as references and to optimize the parameter settings of the algorithms.

Based on the previous evaluations, general recommendations for aligning HR-MAS MRS data were proposed, including a suggestion for the algorithms to choose. The evaluation methodology discussed in this paper is generic and appropriate for assessing the suitability of warping methods for other types of data.

Finally, the extraction of shift information from spectra by means of the five warping algorithms has been demonstrated in

this paper. Cancer samples and samples from normal tissue in the cervical cancer data could successfully be discriminated based on the shift information, but this did not provide extra information next to the intensities in the aligned spectra.

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