Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 830–836
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

A Study of spectral integration and normalization in NMR-based metabonomic analyses

Bobbie-Jo M. Webb-Robertson [a,*], David F. Lowry [a], Kristin H. Jarman [a], Sam J. Harbo [b], Q. Ryan Meng [b], Alfred F. Fuciarelli [c], Joel G. Pounds [a], K. Monica Lee [b]

[a] *Pacific Northwest National Laboratory, P.O. Box 999, K7-90, Richland, WA 99352, USA*
[b] *Toxicology Northwest of Battelle Memorial Institute, Richland, WA 99352, USA*
[c] *Colorado State University, Fort Collins, CO 80523, USA*

Received 1 March 2005; received in revised form 9 May 2005; accepted 11 May 2005
Available online 28 June 2005

Abstract

Metabonomics involves the quantitation of the dynamic multivariate metabolic response of an organism to a pathological event or genetic modification [J.K. Nicholson, J.C. Lindon, E. Holmes, Xenobiotica 29 (1999) 1181–1189]. The analysis of these data involves the use of appropriate multivariate statistical methods; Principal Component Analysis (PCA) has been documented as a valuable pattern recognition technique for ^{1}H NMR spectral data [J.T. Brindle, H. Antti, E. Holmes, G. Tranter, J.K. Nicholson, H.W. Bethell, S. Clarke, P.M. Schofield, E. McKilligin, D.E. Mosedale, D.J. Grainger, Nat. Med. 8 (2002) 1439–1444; B.C. Potts, A.J. Deese, G.J. Stevens, M.D. Reily, D.G. Robertson, J. Theiss, J. Pharm. Biomed. Anal. 26 (2001) 463–476; D.G. Robertson, M.D. Reily, R.E. Sigler, D.F. Wells, D.A. Paterson, T.K. Braden, Toxicol. Sci. 57 (2000) 326–337; L.C. Robosky, D.G. Robertson, J.D. Baker, S. Rane, M.D. Reily, Comb. Chem. High Throughput Screen. 5 (2002) 651–662]. Prior to PCA the raw data is typically processed through four steps; (1) baseline correction, (2) endogenous peak removal, (3) integration over spectral regions to reduce the number of variables, and (4) normalization. The effect of the size of spectral integration regions and normalization has not been well studied. The variability structure and classification accuracy on two distinctly different datasets are assessed via PCA and a leave-one-out cross-validation approach under two normalization approaches and an array of spectral integration regions. The first dataset consists of urine from 15 male Wistar–Hannover rats dosed with ANIT measured at five time points, mimicking drug-induced cholangiolitic hepatitis [D.G. Robertson, M.D. Reily, R.E. Sigler, D.F. Wells, D.A. Paterson, T.K. Braden, Toxicol. Sci. 57 (2000) 326–337; J.P. Shockcor, E. Holmes, Curr. Top. Med. Chem. 2 (2002) 35–51; N.J. Waters, E. Holmes, A. Williams, C.J. Waterfield, R.D. Farrant, J.K. Nicholson, Chem. Res. Toxicol. 14 (2001) 1401–1412]. The second data is serum samples from young male C57BL/6 mice subjected to instillation of pancreatic elastase producing emphysema type symptoms [C. Kuhn, S.Y. Yu, M. Chraplyvy, H.E. Linder, R.M. Senior, Lab. Invest. 34 (1976) 372–380; C. Kuhn, R.M. Senior, Lung 155 (1978) 185–197]. This study indicates that independent of the normalization method the classification accuracy achieved from metabonomic studies is not highly sensitive to the size of the spectral integration region. Additionally, both datasets scaled to mean zero and unity variance (auto-scaled) have higher variability within classification accuracy over spectral integration window widths than data scaled to the total intensity of the spectrum. Of the top 10 latent variables for the ANIT dataset the auto-scale normalization has standard deviations larger than the total-scale in seven cases. In the case of the elastase all standard deviations are larger for the auto-scaling.

Keywords: Metabonomics; Nuclear magnetic resonance (NMR); Principal component analysis (PCA); Normalization; Integration; Cross-validation

1. Introduction

Metabonomic analyses involve the interpretation of dynamic metabolic responses of an organism to a pathological event or genetic modification [1]. The metabolic profile of

* Corresponding author. Tel.: +1 509 375 2292; fax: +1 509 375 2604.
E-mail address: bobbie-jo.webb-robertson@pnl.gov (B.-J.M. Webb-Robertson).

0731-7085/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.jpba.2005.05.012

the low molecular weight components in biofluids (e.g., urine, serum) reflects the concentration and fluxes of endogenous metabolites involved in key intermediary cellular pathways that are involved in the processes used to acquire and use energy, biosynthesize cellular components, and catabolize wastes. Although metabonomics is one of the most recently named "omics" technologies, it is based on decades of biochemistry with an emphasis on metabolism [10]. Historical approaches used for measuring changes in the metabolite composition of biofluids relied on the ability to monitor one (or at best a very limited number) of metabolic products and/or byproducts. These approaches were limited by the number of variables that affect metabolite concentrations in situ and by the commonality of biochemical processes disrupted by any change in metabolism. Metabonomics is a superior approach because the analytical measurements describe an overall pattern, or "fingerprint", of biochemical change that is more consistent and predictive of metabolic changes.

Metabonomics involves the use of advanced analytical methods and the application of appropriate multivariate statistical techniques to search for patterns in the data. The premise is that changes in this profile of endogenous molecules can be used as a rapid screen for human risk assessment or as a tool to diagnose disease and monitor treatment outcomes. Metabonomic analyses have revealed patterns of metabolic markers associated with various toxins and pathophysiologic changes [2,6]. The basic process from raw spectra to pattern recognition usually involves four steps: (1) align the spectra to an internal reference standard, (2) removal of regions devoid of endogenous peaks or associated with resonances not of interest, (3) an integration step to reduce the size of the observation vectors and minimize the effects of resonance shifts due to pH and temperature variations, (4) normalization, and (5) exploratory data analysis (EDA), most commonly principal component analysis (PCA). The third step is usually achieved by summing the intensity of all data points over a chemical shift region of width 0.04 ppm [2,4–7,11–13]. As ^{1}H NMR spectral size can vary this typically reduces the observations to between 100 and 300 intensities. However, to date there has not been a comprehensive analysis of spectral integration regions to determine if a width of 0.04 ppm achieves maximum separation of the patterns in the data. In addition, the fourth step typically normalizes the data to the total intensity of the spectrum (total-scale) or to mean zero with unity variance (auto-scale). Again, there has been no investigation as to the most appropriate normalization technique for ^{1}H NMR spectral evaluation.

The variability structure and classification accuracy via PCA is assessed for two distinctly different ^{1}H NMR spectra datasets under an array of spectral integration regions and both normalization techniques. The first processed dataset prior to spectral integration is represented as 75 observations over 47317 variables; five time points for 15 male rats exposed to α-naphthylisothiocyanate (ANIT). These numbers are smaller for the second dataset, 12 observations over 12720 variables; two dosages at two time points. Of specific interest is to determine if a specific window width integration region or normalization approach results in improved classification accuracy for both datasets.

2. Experimental description of data

The data motivating the evaluation of metabonomic data processing, specifically the spectral integration window width and normalization, were from two in-house studies at Pacific Northwest National Laboratory. The first dataset is of the urine of 15 male Wistar–Hannover rats dosed with ANIT and measured at five time points to create a state similar to drug-induced cholangiolitic hepatitis in humans [4,6,7]. The second data set is of the serum of 12 pooled samples of young male C57BL/6 mice subjected to instillation of pancreatic elastase [8,9] to produce structural damages in the lung that mimic emphysema in humans [14–17]. The experimental descriptions of each of these two datasets are given as reference.

2.1. Administration, sample collection and sample preparation

2.1.1. ANIT

Young male Wistar–Hannover rats (∼12 weeks) were dosed with a single gavage administration of ANIT in corn oil at a dosage of 20 mg/kg of body weight (BW) in a dosing volume of 5 mL/kg of BW. Following the dose administration the urine was collected continuously in the following time intervals: 0–23, 24–48, 48–72, and 72–96 h. Urine was collected in polypropylene tubes containing 1 mL of 1% sodium azide in water (Aldrich Chemical Co.) and maintained on ice. Both cold collection and sodium azide treatment are essential to prevent bacterial contamination from interfering with NMR analysis and minimizes metabolic degradation. Frozen urine samples (−70 °C) were thawed and diluted approximately 60:40 biofluid:buffer (0.2 M sodium phosphate, pH 7.2) to minimize pH variations. D_2O and 2,2′,3,3′-deuterotrimethylsilylproprionic acid (TSP) were added to a final concentration of 0.1 mM to provide an internal frequency lock and chemical shift reference, respectively.

2.1.2. Elastase

Young male C57BL/6 mice (∼14 weeks) were subjected to intra-tracheal instillation to porcine pancreatic elastase at a dosage of 37.5 U/kg of BW or saline; dosing volume: ≤ ∼0.07 mL. On weeks one or four post-dosing, the mice were euthanized by IP injection of pentobarbital (IP; ∼150 mg/kg) and bled via vena cava. Blood was drawn into glass tubes without coagulation activators. Samples were pooled in cases of low yield. Sample volumes ranging from 0.2 to 0.3 mL of serum from each mouse was diluted ∼60:40 serum:buffer [0.133 to 0.200 mL of 0.2 M sodium phosphate (pH 7.2),

Aldrich Chemical Co.] to minimize pH variations. An aliquot (27 to 41 μL) of 2,2′,3,3′-deuterotrimethylsilylproprionic acid (TSP; Aldrich Chemical Co.) prepared in deuterium oxide (D2O; Aldrich Chemical Co.) was added to the diluted serum which resulted in a final concentration of 0.1 mM TSP in 7.5% (by volume) D2O. The D2O and TSP were added to provide an internal frequency lock and chemical shift reference, respectively. Volumes ranging from 360 to 541 μL of each sample were transferred to 178 mm glass ultra precision NMR tubes (Norell, Inc.).

2.2. NMR methods

NMR free-induction decays (FIDs) were acquired on a Varian Unity 600 NMR spectrometer (Varian Inc., Palo Alto, CA) in a manual sampling mode. Final spectra were accumulations of 16 individual FIDs. Each FID was induced using a nonselective, 90° excitation pulse (6 μs at 63 dB) following a selective soft pulse (1.5 s at 3 dB) set on the water resonance and digitized into 32 K complex data points. A total inter-excitation pulse delay of 3.0 s, initiated by a gradient homogeneity spoil, was used to destroy residual transverse magnetization. A spectral width of 6983.24 Hz resulted in an FID acquisition time of 4.679 s for a total recycle time of 7.7 s [4].

2.3. Electronic data collection, archival and transmittal

Datasets consisting of NMR FIDs taken over time were written to data files and transferred to a Silicon Graphics Indigo workstation (Silicon Graphics, Inc., Mountain View, CA) where they were processed with Felix 97.0 (Accelrys, San Diego, CA). Run identification numbers were associated with each file that identified animal subjects and experimental protocols (i.e., treatment group, NMR scan date and time). The Varian data FID files were converted to Felix format. The FIDs were multiplied by a squared sinebell decay function, and converted to frequency domain using fast Fourier transformation (FFT). After FFT, the data were individually phased and subjected to convolution baseline correction using the "abl" routine in Felix.

3. Basic Statistical methods

The raw spectral files were imported into a common statistical analysis package; MATLAB® 6.50 Release 13 (Mathworks, Inc., Natick, MA). Raw data pre-processing, i.e., baseline correction and endogenous region removal, was performed with in-house MATLAB® code. These endogenous peak removals minimize the effects of properties such as imperfect water saturation and peaks of non-interest, such as urine. To assure as little information loss as possible, the regions excluded from analysis were kept as conservative as possible; the regions less than 0.5 ppm and regions containing urea (5.5 to 6.0 ppm) and water (4.5 to 5.2 ppm) resonances. This full reduced dataset prior to the integration, normalization and exploratory data analysis (EDA) were 47317 and 12720 measurements for ANIT and elastase, respectively. Fig. 1 gives a representative ^{1}H spectrum for treated ANT and elastase samples.

3.1. Normalization

The purpose of normalization is to remove systematic variation which affects the measured spectra. The multivariate NMR spectral dataset can be described as p random variables on n observations (or spectrums), X_1, X_2, ... X_n; x_{ij}

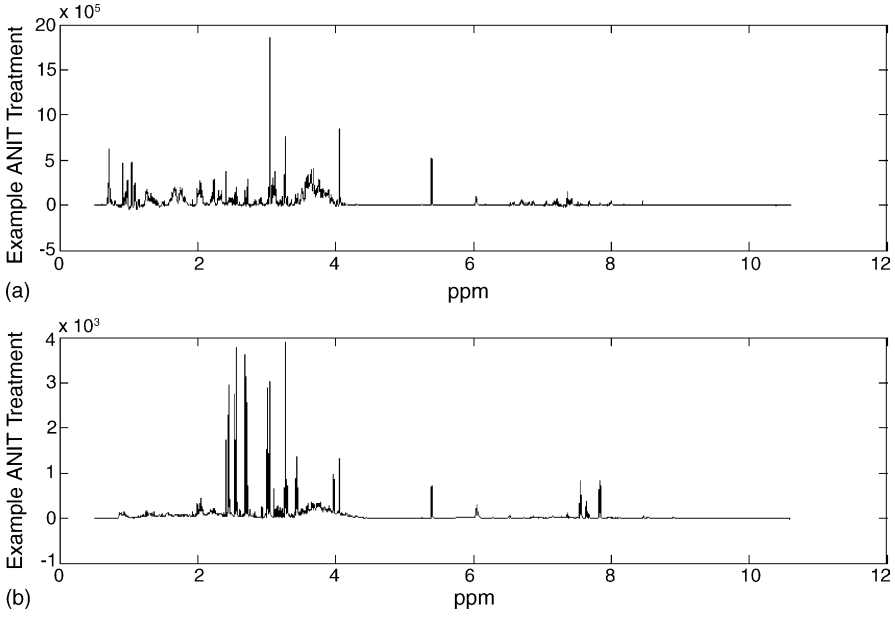

Fig. 1. Representative ^{1}H NMR spectra of a dosed sample from (a) ANIT and (b) elastase.

is the *j*th intensity measure of spectrum *i*. The most common approach to normalization in metabonomics has been to divide each element in each spectrum by the total intensity of the spectrum, forcing each spectrum to sum to one [3,7,13]

$$Z_i = \frac{X_i}{\sum_j x_{ij}}$$

referred to here as total-scale. Alternatively, other analyses have auto-scaled the data to mean zero and unity variance [4,12]

$$Z_i = \frac{X_i - \mu_i}{\sqrt{\sigma_i^x}}$$

where μ_i and σ_i^2 are the mean and variance of the *i*th spectrum, respectively. Both normalization approaches are applied post spectral integration.

3.2. Principal component analysis

PCA is a statistical approach that yields patterns and relationships in multivariate datasets, facilitating an understanding into the causes and effects behind the relationships. At the heart of this method is the generation of a new coordinate system where the new variables are independent linear combinations of the original variables and simultaneously capture some features in the original data. A feature depicts some aspect of the data, described as numerical values for each object. The fundamental tenet behind PCA is the existence of relationships between these numerical values for each object. Although all *p* variables are required to reproduce all information in the data, often most of the structural information in the original variables can be accounted for by a smaller number of factors, $k < p$, often referred to as loading vectors or latent variables. Possessing the ability to take large multivariate datasets from high- to low-dimensional space with little information loss, redundancy in the data is often reduced, leading to a set of features easy to visually explore and more computationally attractive to classification [18].

In PCA, *p* gives the formal dimension of the problem, the underlying eigenvalue problem of PCA is generally solved using the covariance matrix (*pxp*). Due to the size of *p* in this case (47317 or 12720), this is a computational challenge and is one reason for the spectral integration step accompanying metabonomics analyses. But the effective dimension of the data is actually just one less than the number of samples

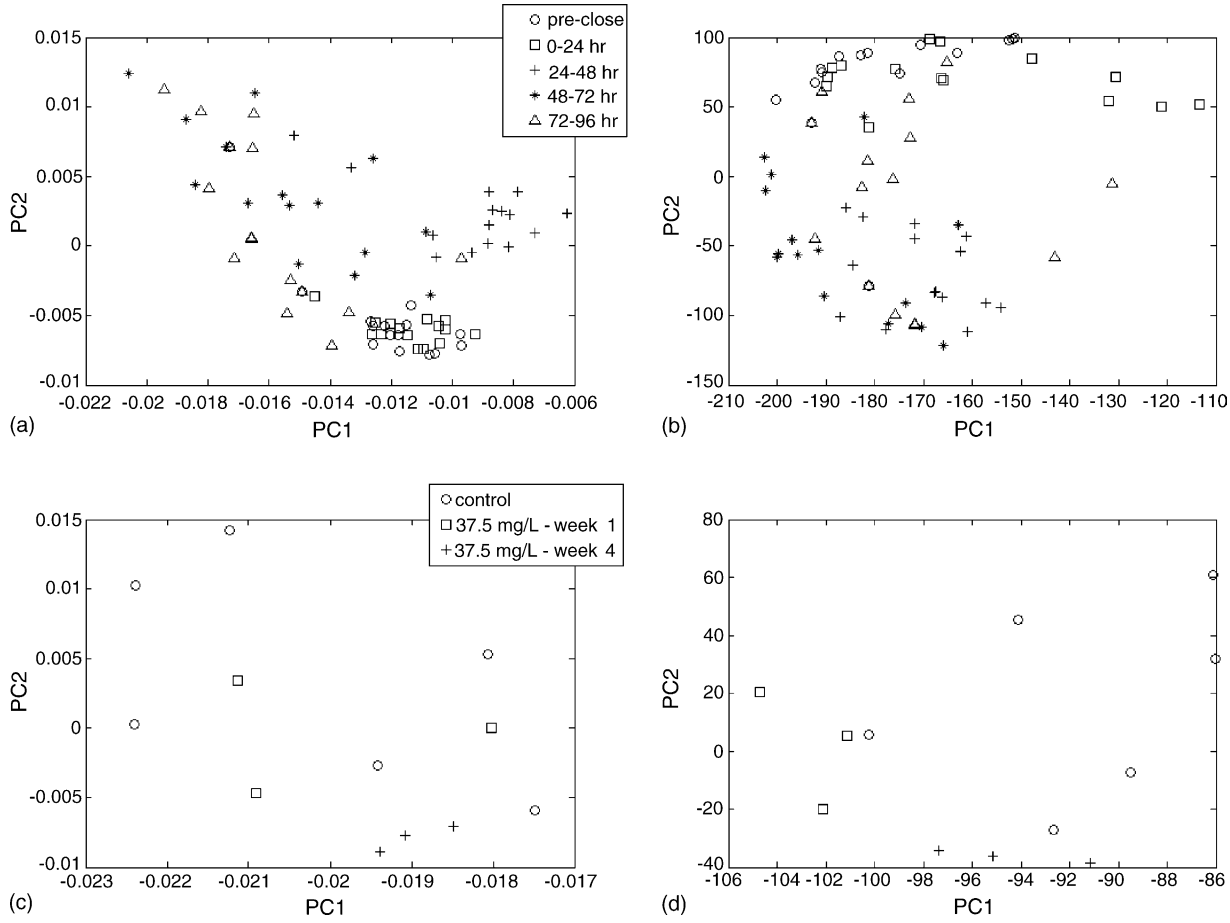

Fig. 2. Plot of PCI vs. PC2 for the un-integrated datasets under total-scale ((a) and (c)) and auto-scale ((b) and (d)) normalization for ANIT and elastase, respectively.

$(n-1)$; hence the PCA problem can be solved using a singular value decomposition (SVD) approach. Both datasets were subjected to PCA analysis using the "pca.m" function that performs PCA using SVD in the PLS_TOOLBOX (Eigenvector Research Inc., Manson, WA) of MATLAB®.

4. Results and discussion

An evaluation of an array of spectral integration window widths, ranging from 0 to 0.1 ppm in increments of 0.001 ppm, using PCA is undertaken. This approach resulted in datasets ranging from 84 variables to the size of the full spectrum. The change in information content for each principal component (PC) in respect to variance is first evaluated. Subsequently, a leave-one-out cross-validation scheme to evaluate the classification accuracy obtained from a given number of PC's and scores is used.

4.1. Percent variance explained

PCA transforms the large ^{1}H NMR multivariate dataset into a low-dimensional space that is conducive to visualization. Each principal component (PC) is a linear combination of the original variables where the first PC represents the largest portion of the variance in the data and subsequent PCs contain incrementally less of the variance. Thus, the majority of the structure in the data can be represented in a small number of PCs. The patterns in the data are represented by the scores associated with these PCs for each observation. Scores for individual observations with similar spectra, will have similar scores; $s_{ij} = X_i \mathrm{PC}_j^T$, where s_{ij} is the score of observation i with PC_j. Fig. 2 gives the basic clustering patterns on the first two PCs under the total-scale and auto-scale normalization schemes for the un-integrated ANIT and elastase datasets, respectively. There are apparent clustering patterns in the first two PCs for both datasets. The number of variables that are retained in the new low-dimensional dataset is usually determined by the percentage of the variance explained; a cut-off of 90 to 95% is typical. For ANIT, the first two PCs describe ~77.0% for the total-scale normalization (Fig. 2a) and ~76.1% of the variance for the auto-scaled normalization (Fig. 2b). The total-scale and auto-scaled normalization schemes return ~85.6% (Fig. 2c) and ~85.4% (Fig. 2d), respectively, for the elastase data.

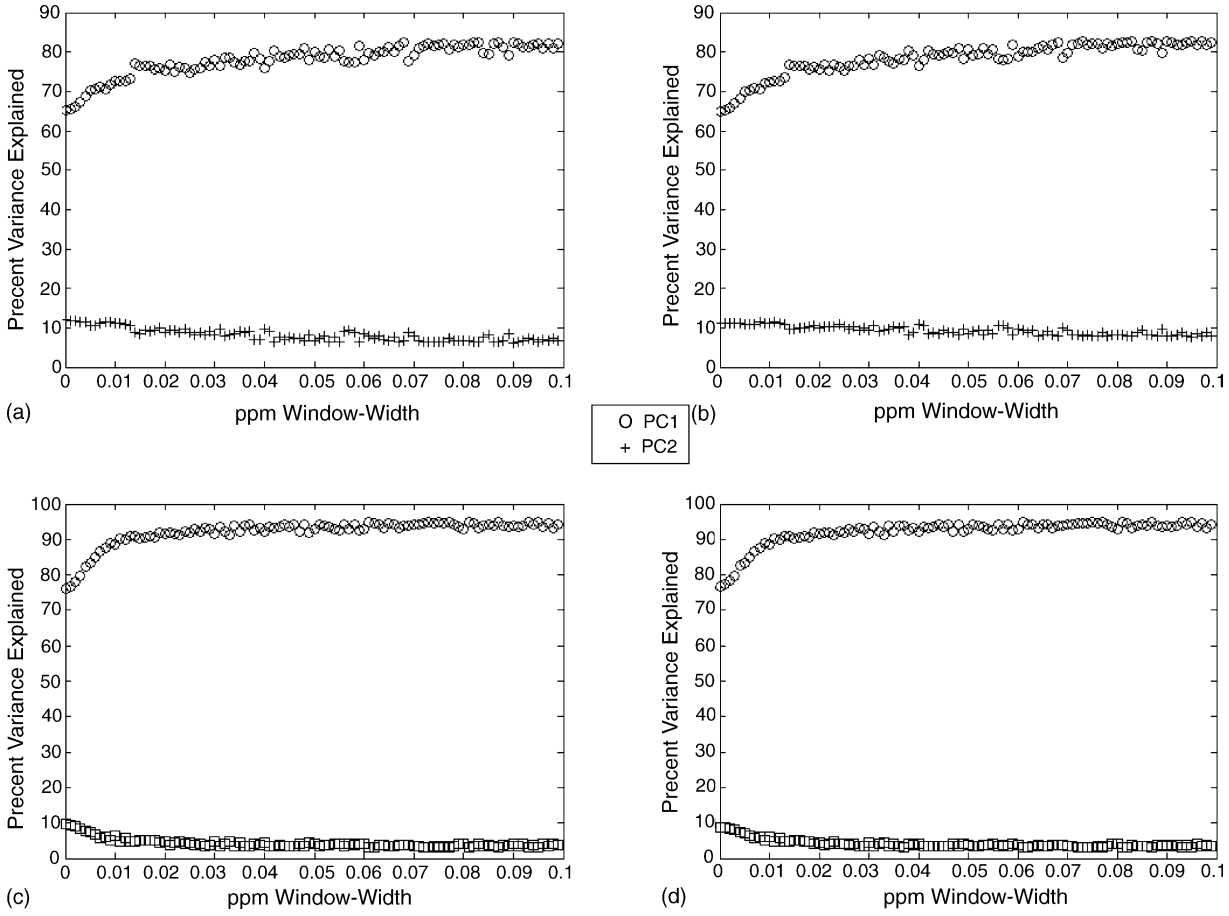

Fig. 3. Plot of ppm window-width vs. the percentage of variance explained by each of the top 2 PCs for the total-scale ((a) and (c)) and auto-scale ((b) and (d)) normalization for ANIT and elastase, respectively.

The ANIT and elastase datasets require 6 and 3 PCs, respectively, to be retained in order to explain over 90% of the variability. PCA was performed on each of 101 datasets resulting from spectral integration over regions ranging from 0 (un-integrated) to 0.1 ppm. To visually analyze the percentage of the variance explained for each of these datasets the top 2 PCs were evaluated for ANIT (Fig. 3a–b) and elastase (Fig. 3c–d). It appears that there is a slight improvement in the amount of variance explained by the first PC as the size of the spectral integration region increases for both datasets and both normalization approaches.

4.2. Classification accuracy

PCA is an unsupervised pattern recognition approach, however classification into specific groups is usually the end goal. Previously, a slight trend of increased variance explained by the first PC is observed in Fig. 3. PCA is used as a precursor to a leave-one-out cross-validation to determine if this observation is translated into an improved ability to classify observations into dose and time groups based on their PC scores. This approach removes one observation from the dataset and runs PCA on the remaining observations. Class centroids are calculated from the PC score vectors based on time and dosing information. The score for the left out observation is obtained by projecting it on the PC latent variables retained. The left out observation is then classified based on the Euclidean distance of this score from the class centroids. This operation is repeated for all observations and the classification accuracy is computed as the proportion of correctly classified observations to the total number of observations. This classification accuracy is dependent upon the number of PC latent variables and score vectors used to build the classifier. Fig. 4 gives the classification accuracy for 1–5 PCs for the ANIT and elastase datasets.

The most apparent observation in Fig. 4 is that the variability of the classification accuracy achieved at each spectral integration window width becomes less as more PCs are retained. Additionally, there is not a specific window width that stands out as achieving significantly better classification accuracy. Also, for ANIT the classification accuracy is relatively uniform, while it is much more erratic for elastase. The conclusion reflected in an analysis of the variance of the classification accuracy obtained over the 100 integrated datasets. Table 1 gives the standard deviation for the top 10 PCs.

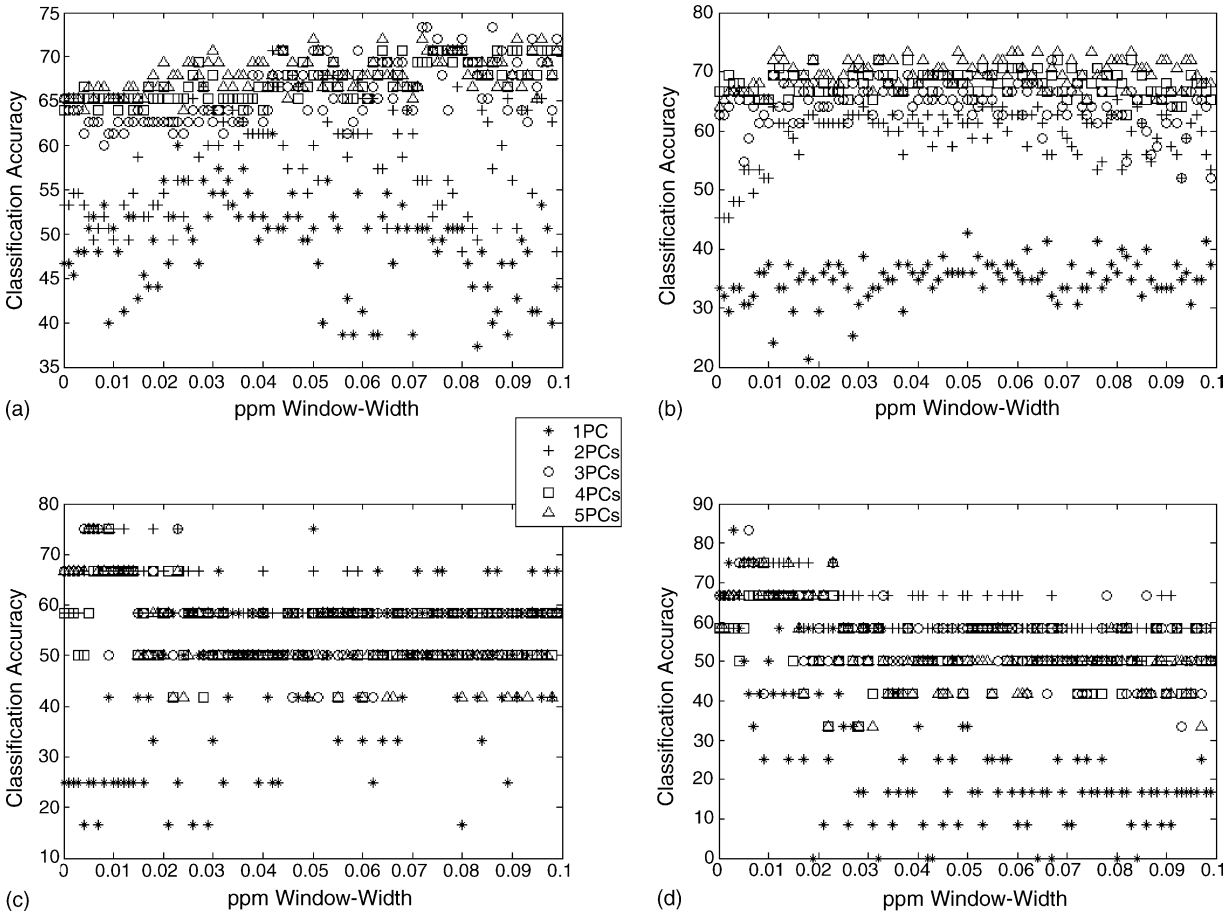

Fig. 4. Plot of the ppm window-width vs. the classification for 1 to 5 PCs for the total-scale ((a) and (c)) and auto-scale ((b) and (d)) normalization for ANIT and elastase, respectively.

Table 1
Standard deviation over classification accuracies obtained for each normalization scheme

PCs	ANIT		Elastase	
	Total-scale	Auto-scaled	Total-scale	Auto-scaled
1	5.32	3.41	15.53	18.11
2	5.73	4.51	5.79	6.50
3	3.44	3.55	7.04	9.30
4	2.19	1.99	6.52	8.43
5	1.95	2.34	8.09	10.06
6	1.54	2.02	8.51	10.75
7	1.48	2.06	8.48	10.21
8	1.60	1.84	7.71	10.07
9	1.56	1.74	7.99	10.73
10	1.57	1.73	7.90	10.65

In general it appears that the total-scale normalization scheme is slightly more robust to the window width than the auto-scale normalization. Of the 10 PCs for the ANIT dataset the auto-scale normalization has standard deviations larger than the total-scale in seven cases. In the case of the elastase all standard deviations are larger for the auto-scaling. Additionally, the auto-scale datasets (Fig. 3b and d) generally have lower classification accuracy than the total-scale datasets (Fig. 4a and c). Lastly, it is apparent from both Fig. 4c–d and Table 1 that the elastase data is much more variable in terms of classification accuracy. This result is not surprising due to the small sample size associated with this dataset; the leave-one-out cross-validation strategy only had 2 or 3 scores on which to calculate class centroids whereas the ANIT dataset has 14 or 15.

5. Conclusions

Spectral integration and normalization are basic steps associated with NMR-based metabonomic analyses. Using PCA and a leave-one-out cross-validation classification approach the sensitivity of pattern recognition to spectral integration regions and normalization schemes is evaluated on two distinctly different datasets. The first dataset of 75 rat urine samples is focused on simulating drug-induced cholangiolitic hepatitis in humans; 15 male Wistar–Hannover rats dosed with ANIT with urine collection over 5 time periods. The second dataset of 12 pooled mouse serum samples is focused on mimicking structural damages in the lung similar to emphysema in humans; 24 male C57BL/6 mice subjected to instillation of pancreatic elastase. The classification accuracy is evaluated using both the auto-scaled and total-scaled normalization approaches for the 101 datasets resulting from spectral integration over regions ranging from 0 to 0.1 in increments of 0.001. Despite the difference in the sizes and variability within the two datasets, clear patterns emerge from the study. First, not surprisingly as the number of PCs retained is increased, the variability within the classification accuracy over all integration regions decreases. In addition, this variability is generally larger for the auto-scaled data than the total-scaled data, Table 1. Finally, it appears that the classification accuracy is not highly sensitive to the size of the spectral integration region chosen, but that less variability is observed with data that are scaled to the total intensity of the spectrum. The discoveries associated with spectral integration and normalization are from a limited study, however PCA is a common approach shown to have applicability to many target samples. Thus, it is believed that the findings can be used as general guidance in most metabonomics analyses.

Acknowledgements

The Research Support was provided by Battelle BSTI IR&D (Drs. Mark Gritz, Bruce Westerberg, and John LaFemina) and the U.S. Department of Energy (DOE) through the Pacific Northwest National Laboratory (PNNL). PNNL is a multiprogram national laboratory operated by Battelle for the U.S. DOE under contract DE-AC06-76RL01830. In addition, the authors thank Ms. Judy Pierce, Lynne Wisse, and Lori Reed for the dosing, sample collection and preparation.

References

[1] J.K. Nicholson, J.C. Lindon, E. Holmes, Xenobiotica 29 (1999) 1181–1189.
[2] J.T. Brindle, H. Antti, E. Holmes, G. Tranter, J.K. Nicholson, H.W. Bethell, S. Clarke, P.M. Schofield, E. McKilligin, D.E. Mosedale, D.J. Grainger, Nat. Med. 8 (2002) 1439–1444.
[3] B.C. Potts, A.J. Deese, G.J. Stevens, M.D. Reily, D.G. Robertson, J. Theiss, J. Pharm. Biomed. Anal. 26 (2001) 463–476.
[4] D.G. Robertson, M.D. Reily, R.E. Sigler, D.F. Wells, D.A. Paterson, T.K. Braden, Toxicol. Sci. 57 (2000) 326–337.
[5] L.C. Robosky, D.G. Robertson, J.D. Baker, S. Rane, M.D. Reily, Comb. Chem. High Throughput Screen. 5 (2002) 651–662.
[6] J.P. Shockcor, E. Holmes, Curr. Top. Med. Chem. 2 (2002) 35–51.
[7] N.J. Waters, E. Holmes, A. Williams, C.J. Waterfield, R.D. Farrant, J.K. Nicholson, Chem. Res. Toxicol. 14 (2001) 1401–1412.
[8] C. Kuhn, S.Y. Yu, M. Chraplyvy, H.E. Linder, R.M. Senior, Lab. Invest. 34 (1976) 372–380.
[9] C. Kuhn, R.M. Senior, Lung 155 (1978) 185–197.
[10] C.M. Henry, Chem. Eng. News 80 (2002) 66–70.
[11] B.M. Beckwith-Hall, J.K. Nicholson, A.W. Nicholls, P.J. Foxall, J.C. Lindon, S.C. Connor, M. Abdi, J. Connelly, E. Holmes, Chem. Res. Toxicol. 11 (1998) 260–272.
[12] B.M. Beckwith-Hall, E. Holmes, J.C. Lindon, J. Gounarides, A. Vickers, M. Shapiro, J.K. Nicholson, Chem. Res. Toxicol. 15 (2002) 1136–1141.
[13] Y. Wang, E. Holmes, J.K. Nicholson, O. Cloarec, J. Chollet, M. Tanner, B.H. Singer, J. Utzinger, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 12676–12681.
[14] P.J. Barnes, N. Engl. J. Med. 343 (2000) 269–280.
[15] E.C. Lucey, J. Keane, P.P. Kuang, G.L. Snider, R.H. Goldstein, Lab. Invest. 82 (2002) 79–85.
[16] C.B. Rich, I. Carreras, E.C. Lucey, J.A. Jaworski, J.A. Buczek-Thomas, M.A. Nugent, P. Stone, J.A. Foster, Am. J. Physiol. Lung Cell. Mol. Physiol. 285 (2003) 354–L362.
[17] S. Kononov, K. Brewer, H. Sakai, F.S. Cavalcante, C.R. Sabayanagam, E.P. Ingenito, B. Suki, Am. J. Respir. Crit. Care. Med. 164 (2001) 1920–1926.
[18] R.A. Johnson, D.W. Wichern, Applied Multivariate Statistical Analaysis, 3rd ed., Prentice Hall, Upper Saddle River, 1992.