Mass Spectrometry Proteomic Diagnosis: Enacting the Validation Paradigm. *

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Abstract

This paper presents an approach to the evaluation and validation of mass spectrometry data for construction of an ‘early warning’ diagnostic procedure. We describe implementation of a designed experiment and place emphasis on the consistent and correct use of validation based evaluation - which is a key requirement to achieve unbiased assessment of the ability of mass spectrometry data for diagnosis in this setting. Strict adherence to validation as a scientific principle will however typically imply that the analyst must make choices. Like all choices in statistical analysis, validation comes at a cost! We present a detailed and extensive discussion of the issues involved and propose that much greater emphasis and requirement for validation would enter clinical proteomic science.

Keywords. Clinical proteomics, mass spectrometry, spectroscopy, classification, double cross-validation, statistical design, diagnosis, pattern recognition.

1 Introduction

There currently is much interest in application of mass spectrometry for the construction of new diagnostic proteomic approaches for the early detection of disease. This is particularly the case in situations where no reliable diagnostic tools have yet been developed.

Generally, in diagnostic research, we may be interested in two key problems or objectives. The first and foremost of these is to ascertain whether there is any information in the data (in our case mass spectra) to allow future cases to be identified with a high degree of reliability. The second is the identification of the separating information (in this case proteomic markers) itself. At this stage, some of our readers may be puzzled, as naturally, the ability to do the first would imply the presence of prognostic (marker) information. From a purely statistical methodological point of view however, the two problems are subtly different and pose different demands on the analysis. This is because a proper answer to the first research question requires rigorous emphasis on fully validated estimation of diagnostic performance. The second objective on the other hand poses a feature extraction or definition problem and hence, by its very nature, may easily cause the analyst to introduce so-called data ‘tuning’
and optimization steps. This can arise through the need or desire to employ some form of data cleaning, data preprocessing steps, use of algorithmic optimization techniques based on the complete observed data and other similar interventions with the data. Hence, a conflict may arise and the analyst should carefully weigh the options and consider the objectives to ensure that the primary research question can be answered. In particular, if the first objective is of interest, then special care must be taken to strictly avoid introducing any optimization steps which could result in optimistically biased assessment of the diagnostic ability of the technology, unless some form of validation can be introduced to ‘counteract’ and assess the effects. Irrespective of these considerations, both research questions require carefully designed experimentation to ensure validity of any study results.

In this paper, we discuss the problem of ascertaining the viability of using mass spectroscopic analysis of serum samples for the construction of a diagnostic test for colorectal cancer at an early stage of the research effort. In other words, we describe - in essence - a feasibility study. A crucial objective of such studies is to provide information which allows us to make decisions as to the continuation of the research effort (which may involve experiments of much greater cost and complexity in comparison to the first-stage evaluation). Hence, it is essential to get a fully validated and unbiased assessment of predictive error rates and thus the primary research objective is clearly concerned with the first research question discussed above.

We will now first discuss some additional complicating special issues which are a concern with mass spectrometry proteomics as well as a brief discussion of the data, prior to a summary of our objectives and outline description of the remainder of the paper.

1.1 Mass spectrometry proteomics, sample size and clinical science

A key problem in many proteomic studies - but similar problems arise in many other similar settings (e.g.: microarray diagnostics, chemometric discriminant studies) - is the difficulty to collect a sufficient number of samples. In oncology applications this will tend to happen, simply because the cancer of interest may be (relatively) rare. Our example is again a typical one, as although colorectal cancer is one of the most common human malignancies, the number of patients any hospital may expect to encounter on a yearly basis will be limited. On the other hand, clinicians and biomedical researchers who wish to explore application of proteomic mass spectrometry technology for the construction of new diagnostic procedures, will be interested first to get an indication of whether there is information in the spectra to allow groups to be separated and what the likely error rates of misclassification will be. Both these reasons conspire to cause many proteomic studies to be of small sample size initially.

While small sample size causes problems of precision of model calibration in the first instance, it causes special problems in the proteomics setting because of two complicating reasons. First is the complexity of the mass spectrometry signal which can consist of hundreds of possibly overlayed peaks, which are typically stored on a discretised predefined fine grid of bins. As a consequence and in combination with small sample size, this provides the ideal setting for so-called ‘data-dredging’ exercises which easily generate unsubstantiated claims. The second problem is that across institutions and research groups we can expect many such attempts being undertaken to explore application of proteomic mass spectrometry for diagnosis. For all these reasons, there is an urgent need for fully validated methods of discriminatory assessment of proteomic patterns which are sufficiently critical and conservative and give unbiased estimates of error rate in small sample size situations.

1.2 Mass spectrometry data

The experiment and data discussed and analyses in this paper are derived from a MALDI-TOF (Matrix Assisted Laser Desorption Ionisation Time-Of-Flight) mass spectrometer (specifically a Ultraflex TOF/TOF instrument, Bruker Daltonics, equipped with a SCOUT ion source which was operated in linear mode). The spectrometer produces a sequence of intensity readings for each sample on an ordered
set of contiguous bins in the m/z range from UUU to VVV Dalton. Bin sizes (length) of the unprocessed spectra gradually increase with increasing m/z values, ranging from 0.07 Dalton at the lower end of the mass/charge scale up to 0.24 Dalton at the upper end of the scale.

We now summarize our objectives with this paper, which are to...

- explain and propose full double cross-validation based methodology for assessment of diagnostic potential.
- explain the limitations which an analyst and researcher must endure and impose on themselves in order to maintain the benefits from a fully validatory approach.
- propose design suitable for first-stage evaluation of diagnostic potential and which can also support double cross-validation.
- to describe application to the colon cancer mass spectral data.
- to propose that validation should be given much greater prominence in wider scientific diagnostic research and that double cross-validation can play an important role to achieve this in the first stages of evaluating a new methodology for diagnostic purposes.

We will discuss design first, followed by a description of the discriminant method and a double cross-validatory approach to joint estimation and validation of the allocation rule, which allows for validated error rate evaluation. We pay special attention to the implied consequences for both the design and analysis strategy if we are to avoid bias as a consequence of optimization. As we have explained, this will apply particularly to preprocessing steps which should be carefully considered to prevent such problems from affecting the credibility of the first evaluation of the discriminatory potential of the data. Put simply: any assessment of such potential must reflect the data, not the abilities of the analyst to - perhaps inadvertently - artificially induce it. In practice, this implies that an analyst must make choices - perhaps even sacrifices - and we describe the issues involved. Subsequent to description of the methodological approach, we discuss application to the colon cancer data and present a post hoc exploratory data analysis to interpretation of the results. While we will focus on our example to structure the discussion, the issues apply quite generally to similar problems in proteomics and many other related problems in bioinformatics, chemometrics, statistical prediction and beyond. We will assume that the reader has some knowledge of standard leave-one-out cross-validation.

2 Design and validation

2.1 Design

A key and characteristic problem of proteomic mass spectrometry design is the need to cope with the presence of what we may loosely refer to as so-called ‘batch effects’. Examples are plate-to-plate variability, day-to-day variation and so on. The presence of such effects is in reality unavoidable. A naive response to the problem could be to construct designs and experiments, which avoid or eliminate the presence of such effects (assuming that we can anticipate them properly). However, this will typically imply sample size restrictions on the designs which are undesirable. A better approach is - rather than try to eliminate them from the design - to account and accommodate for these effects in such a way that they do not lead to erroneous or artificially induced between-group separation. In addition, this makes experimentation more realistic also as any ‘real-life’ application of the methodology would also have to cope with the presence of such effects.

The problem is a standard example on application of principles of statistical design which have been well described in many classical textbooks and have now been known for almost a century. The typical
The first step in applying this knowledge is to first try and identify - or at least think about - what the potentially important batch effects might be in advance. In our case plate variation, but at least also day-to-day variation - such that we may think of each plate-by-day combination as a batch - commonly referred to as a ‘block’ in standard statistical terminology. We may then randomly distribute the available samples from each group (colon cancer and controls) across the blocks such that proportions are (as near as) equal within and across blocks for each group. For colon cancer, we randomized samples to plates in such a manner that the distribution of disease stadia was in approximately equal proportions across plates as well. The position on the plates of samples allocated to each plate was also randomized. Each plate was then assigned to a distinct day, which completes the design. Table 1 summarizes the design as executed on the first week, which provides us with mass spectra on 63 colon cancer patients and 50 healthy controls.

Table 1: Design as executed on the first week. A replicate of the entire experiment was run on the subsequent week using plate duplicates. ‘Stage’ refers to the distribution of cases across the four respective disease stages.

| Group       | Plates | Total |
|-------------|--------|-------|
|             | 1      | 2     | 3     |
| Controls    | 17     | 17    | 16    | 50    |
| Cases       | 22     | 22    | 19    | 63    |
| Stage       | 1  | 2  | 3  | 4  | 1  | 2  | 3  | 4  |
| Cases       | 4  | 10 | 4  | 4  | 4  | 10 | 4  | 4  |

In our case, it was decided to carry out the experiment in a single week using three plates only, each of which was assigned to a consecutive day in the middle of the week - Tuesday to Thursday. Generally speaking however, it is often wise to try and increase the number of blocks rather than seek to reduce it when batch effects are at issue - which is the typical, but actually rather naive first response of the ‘uninitiated’ in these circumstances. For example, we could have spread the experiment across several weeks and use several plates per day with the same design configuration across weeks, which would allow for a separate analysis to study and disentangle batch-to-batch variability (besides the analysis of the main research question). More importantly, this tends to make the design more robust as the sample material gets spread across a larger number of blocks which implies reduced loss of sample material in case problems arise during the experiment with any specific block (see further on for an example). An advantage of running the experiment in a single week is that it may be more easy to maintain the study protocol across the study, which can be a particular issue if the research facility carrying out the measurement has to provide for several customers using the same facilities. Good laboratory management makes the latter argument less credible however.

The above described design is also referred to as randomized block design in the statistical literature and will ensure that the batch effects - if they materialize - will not induce an artificial between-group effect or separation. We refer the reader to the statistical literature on design of experiments for further discussion and details of the issues involved, as well as many other examples of these basic design
2.2 Validation

We can exploit design to augment cross-validatory analysis. This is because while sample sizes may be small (i.e. it is difficult to get new independent samples), the amount of sample material available for each sample may be more abundant. This allows the introduction of so-called replicate samples into the design.

As the samples are pre-arranged on rectangular plates, a second ‘copy’ of any plate can be made provided sufficient sample material is available from each sample. (In our case, sufficient sample material was available for a second copy only). Thus, we can duplicate the entire design from the first week and re-measure the replicate plates through the same design on the second subsequent week, using new sample material from each sample (but of course not new samples themselves). With this approach, we thus generally have available from each $i^{th}$ sample an observation $x^1_i = (x^1_{i1}, \ldots, x^1_{ip})$ of the associated recorded mass spectrum in the first week, where the vector elements refer to the measured mass/charge intensities on a predefined and ordered grid of mass/charges of dimensionality $p$. In addition, we have for each sample a duplicate measurement $x^2_i = (x^2_{i1}, \ldots, x^2_{ip})$ obtained from the corresponding replicate on the corresponding plate measured on the same day one week later. We may denote the associated class label from each $i^{th}$ observation as $c(i)$ which takes value in the set of group indicators $\{1, \ldots, G\}$, where $G$ is the number of groups. [Note we will drop use of the suffixes 1,2 when the context makes clear to which week the data relates.]

Unfortunately, the replicate measurements from the third plate are unavailable due to a technical malfunction which occurred on the last day of the second week. As a consequence we only have available the 78 replicates from the first 2 plates in week 2 for further analysis.

3 Integrated calibration and validation for classification by double cross-validation

Given the need to provide fully validated error rates and to keep the problem of overfitting under control, we will restrict attention to double cross-validated linear discrimination for joint calibration and validation (Stone 1974). We explain shrinkage-based estimation and the need for it in linear discrimination first. Then we explain the double cross-validatory implementation.

3.1 Linear classification and shrinkage estimation methodology

We base classification on Fisher linear discrimination (Fisher 1936), which is one of the oldest statistical allocation methods and certainly the most widely used and successful approach to statistical classification and pattern recognition to this day. It has been derived and may be justified based on a variety of principles of inference, such as maximization of the between-group separation relative to within-group error in the two-group case (Fisher’s original argument) or the likelihood principle for normally distributed within-group populations, among others. The methodology has been amply studied and has been established as an extremely reliable and robust form of classification and discriminant analysis - for example, even when assumptions to pool covariance matrices are not fully met. It is also important to note that Fisher discrimination does not require an assumption of within-group normal dispersion. We must refer to the voluminous literature for further details on the method (Hastie 2001), (Seber 1984), (McLachlan 1992), (Ripley 1996) which is well established in the applied sciences, such as biology and medicine. Hastie et al. (2001) contains an up-to-date account of many new applications which demonstrate the continuing success of the approach.
Fisher linear discriminant allocation may be defined as assigning a new observation with feature vector \( \mathbf{x} \) to the group for which the distance measure
\[
D_g(\mathbf{x}) = (\mathbf{x} - \mu_g)\Sigma^{-1}(\mathbf{x} - \mu_g)^T
\]
is minimal, where \( g \) denotes the group indicator with \( g \in \{1, \ldots, G\} \), \( \mu_g \) the population means and \( \Sigma \) the population within-group dispersion matrix which is assumed equal across groups. In practice, the population means and dispersion matrix will be unknown and hence must be estimated from the data. In a high-dimensional problem such as in mass spectrometry proteomics, this leaves us with a difficulty in estimating the dispersion matrix as we will typically not be able to achieve a full rank estimate.

At the risk of some oversimplification of the discussion, there are basically two ways in which we may remedy the problem so that the above methodology may again be applied. The first is through the selection or construction of a set of features which is reduced in dimensionality, while capturing most of the variability in the data. In essence, this is the approach which is currently applied in most of the mass spectrometry proteomics literature (at the time of writing) through so-called ‘peak’ selection and construction algorithms, spectral alignment algorithms and other similar preprocessing procedures, which are applied prior to subsequent further analysis to construct diagnostic rules, or other prognostic models. Typical examples are found in papers by Baggerly, Yasui, Sauve, Morris ((Baggerly 2003),(Morris and Coombes et al. 2005),(Sauve 2004),(Yasui et al. 2003)), among others. It is important that readers understand that we do not consider this approach to be fundamentally flawed for mass spectrometry proteomic data. On the contrary, it is self-evident that mass spectra consist of mixtures of possibly overlaid intensity peaks corresponding to substances present in the analyte. Thus, to try to elucidate this structure (first) is in principle of interest. Problems will however arise when validation and unbiased assessment of error rates or any other measure of predictive performance are crucial. This is because preprocessing may involve a high degree of optimization which is itself data-driven. This applies particularly to problems of peak selection and definition, and certainly to alignment.

For these reasons and because unbiased error rate estimation is crucial to our goals, we will pursue the second option, which is to reduce the preprocessing steps to such an extent that they no longer introduce any optimization which can no longer be validated. We then leave the dimensionality of the data intact but introduce a regularized estimation of the dispersion matrix to cope with the singularity of the sample dispersion matrix. This approach can be conservative and may well not provide the most optimal classifier and error rate, but will at least not be optimistically biased.

We will explore two distinct forms of regularization, both of which may be expressed in terms of the canonical decomposition of the ‘observed’ (or sample) pooled dispersion matrix \( \mathbf{S} = \mathbf{Q}\Lambda\mathbf{Q}^T \) where \( \mathbf{Q} \) and \( \Lambda = \text{diag}(\lambda_1, \ldots, \lambda_r) \) are the matrices of principal component weights (or loadings) and variances respectively, with \( \lambda_1 > \ldots > \lambda_r > 0 \) respectively (\( r \) is the rank of the pooled covariance matrix). We may now re-estimate the within-group covariance matrix by only retaining the first \( 1 \leq k \leq r \) components only, which gives an estimate
\[
\mathbf{S}_{(k)} = \mathbf{Q}_{(k)}\Lambda_{(k)}\mathbf{Q}_{(k)}^T,
\]
where \( \Lambda_{(k)} = \text{diag}(\lambda_1, \ldots, \lambda_k) \) and \( \mathbf{Q}_{(k)} \) denotes the corresponding reduced matrix of component loadings. The associated linear discriminant allocation rule hence assigns observations to the group for which the smallest sample-based distance estimates
\[
\hat{D}_g(\mathbf{x}) = (\mathbf{x} - \mathbf{\bar{x}}_g)\mathbf{S}_{(k)}^{-1}(\mathbf{x} - \mathbf{\bar{x}}_g)^T
\]
are observed, with \( \mathbf{\bar{x}}_g \) the sample group means for \( g \in \{1, \ldots, G\} \). In the two-group case, this is also equivalent to least-squares regression analysis using the Moore-Penrose inverse of the pooled covariance matrix when \( k = r \) (all components kept, also known as shortest least squares regression), or else
is equivalent to so-called shrunken least-squares regression (Ripley 1996) or (Hand 1997) for more details. An alternative is to employ a ridge regularization

\[ S(\gamma) = Q[(1 - \gamma) \Lambda + \gamma I]Q^T, \]

where \( 0 < \gamma \leq 1 \) is the ridge regularization or ‘tuning’ parameter, in which case the sample distance measures \((x - \bar{x}_g)S^{-1}(\lambda)(x - \bar{x}_g)^T\).

### 3.2 Double cross-validatory estimation and validation

Application of the above described classification approaches still requires choice of the tuning parameters \( k \) or \( \gamma \) involved. As we are specifically interested in an evaluation of predictive performance of any diagnostic allocation rule, it becomes crucial that any optimization - such as the choice of the tuning parameters - does not take place on the same data used for validation. On the other hand, predictive tuning is clearly highly desirable if diagnosis is of interest, so we would not wish to base the choice of tuning parameters on the full calibration data itself (and thus effectively drop predictive tuning from the analysis), but use a truly validatory choice instead. This implies we either set aside a so-called separate ‘tuning set’ from the available calibration data prior to validation of predictive performance itself or appeal to some form of cross-validation. Good predictive optimization or tuning becomes particulary important in a high-dimensional setting, such as proteomics, as it provides a crucial opportunity to safeguard model choice against overfitting (in other words: over-interpreting the data). Meanwhile, even if we were able to effectively choose good tuning parameters, the predictive performance (in our case essentially the error rates) of any implied allocation rule should again be validated, which again introduces a need for yet another set-aside validation set or cross-validation.

We may solve both problems by carrying out a so-called double-cross-validatory approach, which avoids the need to introduce separate test (tuning) and validation sets. The method has been first proposed and investigated by Stone (Stone 1974) and integrates predictive optimization and unbiased validated error rate estimation in a single validatory procedure. While the principle of the methodology is sound and well described, this procedure has until recently not been applied in practice due to the considerable computational cost and (algebraic) complexity of the method. (See (Mertens 2003) for a first full implementation in the related setting of discriminant allocation on microarray data.)

As with ordinary leave-one-out cross-validation, double cross-validation removes each individual (sample) in turn from the data, after which the discriminant rule is fully recalibrated (and optimized for prediction) on the leftover data and using the same procedure in each case. The resulting classification rule is then applied to the left-out datum to obtain an unbiased allocation for this sample. This procedure is then repeated across all individuals and for each person separately, after which misclassification rates are calculated on the basis of the thus validated classifications. The double-validatory aspect results from the fact that the discriminant rule constructed to classify each left-out datum is optimized through a secondary cross-validatory evaluation within the first cross-validatory layer (i.e. full cross-validation again on each ‘leftover’ set after removal of an observation). In this manner, we are able to integrate predictive optimization and predictive unbiased validation in the same procedure, without loss of data - which is a crucial requirement to get realistic estimates of error rate with high-dimensional data.

### 4 Application and evaluation

#### 4.1 Preprocessing

Preprocessing is potentially hazardous as it may induce optimistic bias into the error-rate evaluation. On the other hand, pre-processing can be beneficial and justified if it removes variation from the data which does not relate to the group separation and might obscure an existing group separation. Put simply,
preprocessing is allowed as long as the preprocessing steps undertaken for any sample are not based on any borrowing of information or ‘learning’ on the basis of the other samples. In other words: it must be ‘within-sample’ preprocessing. Note that this conflicts with some of the preprocessing procedures which have been applied by other authors in the proteomics literature (e.g. Coombes, but also Baggerly and Yasui). At this point, we will describe the preprocessing steps that were employed in our analysis. We defer a detailed discussion of pre-processing and the specific potential problems and differences with other authors to the discussion.

First, we calculated for each sample the average intensity within each bin across the four mass spectra from the associated spots on the plate. Then, we aggregated contiguous bins on the m/z scale, such that the new aggregated bin size spans approximately one Dalton at the left side of the spectrum and gradually increases to a width of approximately 3 Dalton at the right hand side. For each of these new aggregated bins, we calculated for each spectrum the associated aggregate intensity by summing the intensities across the bins being aggregated. Subsequently, spectral baseline was then removed from each of the thus aggregated spectra separately using an asymmetric least squares algorithm.

Suppose \( \mathbf{x}_{bi} = (x_{b1i}, \ldots, x_{bp_i}) \) denotes the ordered sequence of baseline corrected m/z intensity values for the \( i \)th sample at this stage of preprocessing. We then correct the spectrum for the typical intensity and variability across the spectrum by calculating the standardized values

\[
x_{sbij} = \frac{x_{bij} - \text{median}(\mathbf{x}_{bi})}{(q_{0.75}(\mathbf{x}_{bi}) - q_{0.25}(\mathbf{x}_{bi}))},
\]

where \( q_{0.25}(\mathbf{x}_{bi}) \) and \( q_{0.75}(\mathbf{x}_{bi}) \) denote the 25th and 75th percentiles of the baseline corrected intensity values for the \( i \)th sample. Some readers will note that these steps bear close resemblance to the pre-processing procedure proposed by (Satten et al. 2004), although ours is a cruder version which does not employ local estimates. The final preprocessing step is a log-transformation

\[
x_{ij} = \log(x_{sbij} + \alpha) - \beta
\]

of each spectrum, where \( \alpha \) and \( \beta \) are two real constants. We chose \( \alpha = 100 \) and \( \beta = 4 \). The main purpose of the log-transform is to ensure numerical stability of calculations.

The above preprocessing steps were applied for each sample and within each week separately, which thus gives us the observations \( \mathbf{x}_1^i \) and \( \mathbf{x}_2^i \) from the first and second weeks. It is important to stress that the preprocessing of the data of any \( i \)th sample does not involve use of any information based on the remaining samples \( \{k | k \neq i\} \), nor of the duplicate replicate measured spectrum of the same sample on another week. This is a vital requirement to ensure the validity of any cross-validatory evaluation. In addition, there are more general reasons to avoid any such processing in the classification/diagnosis context at least, for reasons we will go in to more detail in the general discussion.

### 4.2 Double cross-validatory error rates

Using the above described preprocessed data, we may carry out a double- cross-validatory evaluation of predictive performance of the classifiers we discussed. In the first instance, we restrict ourselves to the data from the first week. Table 2 displays the estimated recognition rates and performance measures from an analysis of the first week data (leftmost 3 columns). All of the estimates are based on double cross-validation. We used the average of sensitivity (Se) and specificity (Sp) as our estimate of the total recognition rate (T), which implies we assume prior class probabilities to equal 0.5. A threshold of 0.5 was also used to assign observations on the basis of the a-posteriori class probabilities within the cross-validatory calculations. B denotes the Brier distance which we define as

\[
B = \sqrt{\frac{1}{n} \sum [1 - p(c(i) | \mathbf{x}_i)]^2},
\]
Figure 1: Mean spectra for each group separately, after preprocessing. We plot negative intensity value for the control group (bottom mean spectrum).

where \( p(c(i) | x_i) \) is the double cross-validated predicted a-posteriori class probability for the correct class \( c(i) \) for each \( i^{th} \) sample and \( n \) is the total sample size. Likewise, AUC is a double cross-validation estimate of the area under the empirical ROC curve defined as

\[
AUC = \frac{1}{n_1 n_2} \sum_{i \in G_1} \sum_{j \in G_2} [I(p(1 | x_i) > p(1 | x_j)) + 0.5 * I(p(1 | x_i) = p(1 | x_j))]
\]

where \( G_1 \) and \( G_2 \) refer to the sample index labels for samples from the first and second group respectively.

The rightmost three columns of the table refers to a repetition of this entire double cross-validatory exercise, which replaces each sample feature vector \( x_i^1 \) with the corresponding replicate measurement \( x_i^2 \) immediately prior to classification of that \( i^{th} \) sample (i.e. replacing the feature vectors with the data from week 2 in the outermost layer (only!) of the double cross-validatory calculation). Crucially and importantly, construction of the corresponding discriminant rule for the classification of each such \( i^{th} \) sample in the internal ‘calibration’ layer of the double cross-validatory procedure does of course remain based on the data from week 1. Note that as the replicate data from the third plate are not available, these results are based on the double cross-validated predictions for the remaining 78 replicate samples from week 2 only.

At first sight, the Moore Penrose implementation (top line of the table, both weeks one and two) would seem to be the best performing and most consistent method. In week 1, Moore-Penrose, PCA-selection (both using the Mahalanobis distance) and ridge estimation perform equally well, but there seems to be an increase in error rate for week 2 for both the PCA-selection and ridge implementation. The Euclidean distance based implementations are worse in the evaluation on the first week, but recognition rates are consistent across both weeks when compared to the other methods.

These results should be interpreted with some caution and require some explanation. First of all, the ‘plain’ Moore-Penrose is leave-one-out only as it does not involve choice of shrinkage or data
Table 2: Double cross-validated classification results for the colon cancer data. T is the total recognition rate. Se and Sp are sensitivity and specificity respectively. B is the Brier distance and AUC is the estimated area under the ROC curve.

| Method                  | First week                      | Second week                   |
|-------------------------|---------------------------------|-------------------------------|
|                         | T (Se,Sp) B AUC                 | T (Se,Sp) B AUC               |
| Moore-Penrose $S_{(r)}$ | 92.6 (95.2,90.0) 0.0905 97.6    | 94.4 (91.7,97.1) 0.0885 97.4  |
| PCA-selection $S_{(k)}$ | 92.6 (95.2,90.0) 0.0786 97.3    | 88.8 (80.6,97.1) 0.0935 96.8  |
| Moore-Penrose Euclidean $S_{(r)}$ $A_{(r)} = I_{(r)}$ | 89.4 (88.9,90.0) 0.179 96.0 | 87.2 (86.1,88.2) 0.190 97.0 |
| PCA-selection Euclidian $S_{(k)}$ $A_{(k)} = I_{(k)}$ | 88.7 (87.3,90.0) 0.184 96.0 | 90.0 (88.9,91.2) 0.192 97.0 |
| Ridge $S_{(\gamma)}$   | 92.0 (95.2,88.0) 0.0909 98.4    | 95.8 (91.7,100.0) 0.0918 97.9 |

reduction parameter ($k$ or $\lambda$). The deterioration of the PCA-selection implementations is partly due to the uncertainty in estimating the shrinkage terms or choice which is introduced by the double-cross-validatory estimation. For the ridge implementation, performance is comparable to that from Moore-Penrose in week 1, which is not surprising since the chosen ridge shrinkage parameter $\lambda < 0.0001$ for most observations. The effects of uncertainty in the determination of the shrinkage term becomes particularly apparent for PCA-selection using Mahalanobis distance (second line in the table) in week 2.

The two Euclidean distance based implementations on the other hand seem more consistent across both weeks. The reason is that component selection is much more stringent for these two implementations, which selects only the first 2 components for nearly all observations (with exception of two observations out of 113 for which only the first principal component is retained). This explains the reduced performance but also the greater consistency of the classification results. It is precisely because of this reason that these results (from the Euclidean based implementations) are more credible and may well turn out to be more repeatable if the classifier were applied in the future to data from a new repeat experiment. For comparison, component selection in the Mahalanobis distance based PCA implementation is much less stringent and selects ($k = 23$ for 53 observations, $k = 28$ for 28 observations and the remainder of the samples uses even more components). There is thus some evidence of insufficient shrinkage for this method, and similarly for the ridge implementation.

4.3 Investigating bias: a permutation exercise

We have proposed double cross-validatory integrated estimation and assessment of statistical diagnostic rules on the basis of the argument that it should protect against optimistically biased evaluations. We may check this property by ‘removing’ the class labels $c(i)$ from the samples $i \in \{1,\ldots,n\}$, randomly permute and then reassign them to the samples. We then carry out the double cross-validatory procedure again for any of our classification methods. Repeating this procedure several times will give an indication of the biases involved, as the typical recognition rate - for example - should equal 50% across a large number of permutations for an unbiased method.

Table 3 shows results from such an exercise for the pca-selection based algorithm across more than 600 such permutations. The results, both for misclassification rate and area under the (ROC) curve clearly demonstrate the method to be free from bias as we find median rates and areas of 50% exactly.
Table 3: Permutation-based evaluation of double cross-validatory calculations for linear discrimination using principal component selection. DBCV refers to the actual double cross-validatory results (see table 2). \( q_{2.5} \) and \( q_{97.5} \) are the 2.5 and 97.5 percentiles. B is the Brier distance and AUC is the estimated area under the ROC curve.

| measure           | DBCV | median | \( q_{2.5} \) | \( q_{97.5} \) |
|-------------------|------|--------|--------------|--------------|
| misclassification rate | 7.4  | 50.0   | 36.3         | 72.7         |
| AUC               | 97.3 | 49.4   | 24.8         | 64.2         |
| B                 | 0.0786 | 0.324 | 0.200       | 0.446       |

On the other hand, table 3 also includes 95% confidence intervals for the permutation-based performance measures. These give an indication of the variability which can be expected with purely random data and can be compared with the actually observed double-cross-validation results in our study (second column of the table). Clearly, the distance between the validated measures actually observed and even the extreme bounds of the random permutation confidence intervals is considerable, which demonstrates the presence of discriminating information in the mass spectra.

4.4 Data reduction and post-hoc exploratory analysis

It is a key feature of our analysis that it places strong emphasis on estimating fully validated error rates and all steps in the analysis are geared towards that end. This choice forces a discipline upon us not to (re-) induce bias via preprocessing, which specifically precludes any form of either peak ‘selection’ or ‘definition’. Therefore, while the analysis is strong on establishing such unbiased error rates (and other such measures of predictive performance) and the presence of ‘predicting’ or ‘discriminating’ information, the analysis is necessarily not equally outspoken in telling us where this information is to be found in the spectra. Thus, at this point, we may have convinced clinicians of the wisdom of randomized block design and (double cross-) validatory analysis, but still leave an uneasy feeling as the approach would appear not to be very transparent as to ‘how’ the classifier assigns observations. We should wish to get an indication of what the markers are which drive the classification - or at least an assurance that the classifier is not classifying in the noise region of the spectra. Of course, it would be wrong to label this issue as a deficiency of the methodological approach. Rather, it is a necessary consequence of strict adherence to proper protocols for evaluating predictive ability in a high dimension/small sample size situation.

The dilemma is however not as acute or serious as would appear at first sight. Indeed, there is nothing stopping us from carrying out a post-hoc exploratory analysis of both the results and data (after the validatory calculation). This may include repeating the analysis after (possibly) some more elaborate preprocessing which does not have to abide by the limitations of full validation. We describe two post-hoc exploratory analyses. The first is based on a very ad hoc algorithmic approach through pre-selection of a small set of adjacent bins which together account for most of the variation in the spectra. The second explores the linear discriminant weights from a post-hoc fit on the full data.
4.4.1 Data reduction

Initialize $I = \{1, \ldots, p\}$ as the ordered set of bin indices and $V = \{v_1, \ldots, v_p\}$ the associated set of variances for all $p$ bins in the preprocessed spectra and across all $n$ samples, such that $v_j = \sum_i [(x_{ij} - \bar{x}_j)^2]/(n-1)$, where $\bar{x}_j = \sum x_{ij}/n$ is the sample mean and $j$ is the bin index number. Calculate the constant $v_{ref} = q_{0.95}(V)$ as the 95% percentile of all $p$ bin variances. Now initialize the bin selection set $B$ as the set containing the bin indicator $j$ for which the maximum variance $v_j$ is observed in the set $V$. Initialize the set of intensity readings $X_s = \{x_{[j]} \mid j \in B\}$ corresponding to the set $B$, where $x_{[j]} = (x_{1j}, \ldots, x_{nj})^T$. We write $m = (m_1, \ldots, m_n)^T$ as the set of means $m_i = \text{mean}(\{x_{ij} \mid j \in B\})$, $i : 1, \ldots, n$. Define $\text{cor}(a, b)$ to be the coefficient of correlation between two vectors $a$ and $b$.

Now run the following algorithm.

\{Start of outer loop\}

\{Start of inner loop\}

1. Set $k=1$, $I = I - \{j\}$ and $V = V - \{v_j\}$
2. Now iterate the following procedure until termination.
3. Calculate $\rho_{lower} = \text{cor}(m_i, x_{[j-k]})$ and $\rho_{upper} = \text{cor}(m_i, x_{[j+k]})$.
4. If $\rho_{lower} > 0.9$ and $\rho_{upper} > 0.9$ then
   \begin{enumerate}
   \item Add $j-k$ and $j+k$ to the bin selection set: $B = \{j-k\} \cup B \cup \{j+k\}$.
   \item Update the means $m_i: i : 1, \ldots, n$.
   \item Remove indices $j-k$ and $j+k$ from the index set $I$, such that $I = I - \{j-k, j+k\}$.
   \end{enumerate}
5. Similarly update $V = V - \{v_{j-k}, v_{j+k}\}$
6. Set $k = k+1$
7. Else
   \begin{enumerate}
   \item $k = k-1$
   \end{enumerate}
8. End iteration.

Now select the bin index $j$ for which $v_j = \text{max}(V)$.

\{Start of inner loop\}

1. If $v_j > v_{ref}$ then
   \begin{enumerate}
   \item Update the index set $B = B + \{j\}$ and likewise $X_s$ and $m$.
   \end{enumerate}
2. Go to \{Start of inner loop\}
3. Else
   \begin{enumerate}
   \end{enumerate}

End algorithm.

The algorithm identifies a set of ‘clusters’ of bins. It is important to note there is no assumption on either shape of the signal or on monotonicity involved (a single cluster may span mixture of underlying peaks). Running this algorithm on the data from the first week finds the set of indices $B$ that corresponds to the bins which account for most of the variation in the data. Applying this to our data results in a subset of 330 bins (in 32 bin clusters - but it is possible that we visit the same contiguous region of bins several times). Repeating the entire double cross-validatory procedure using the principal component selection shrinkage procedure on this reduced set yields recognition rates as described in table 4 which are not inconsistent with those from the full double cross-validatory evaluation shown in table 2. Hence, we may conclude that classification is not in the noise region of the spectra.

4.4.2 Post-hoc data exploration

The second aspect which is of interest is a post-hoc exploration of the (linear) discriminant coefficients $\beta = (\beta_1, \beta_2, \ldots, \beta_p)^T = S_{(k)}^{-1}(\overline{x_1} - \overline{x_2})^T$ [see (Seber 1984) or (Hand 1997)], where $\overline{x_1}$ and $\overline{x_2}$ are the two sample group means (for cases and controls). An appropriate and convenient way to summarize and present the information contained in these coefficients is via the associated correlations of the measured intensities for each $j^{th}$ bin with the class indicator, which are easily calculated as $\rho_j = s_{xj}/s_j$, for $j = 1, \ldots, p$ where $s_{xj} = \sqrt{\sum}$ is the standard deviation at the $j^{th}$ bin and $s_j$ the standard deviation of class
Table 4: Results from re-running double cross-validatory calculations after bin-selection for the colon cancer data (week 1 data only). T is the total recognition rate. Se and Sp are sensitivity and specificity respectively. B is the Brier distance and AUC is the estimated area under the ROC curve.

| Method                                | T (Se,Sp)       | B    | AUC  |
|---------------------------------------|-----------------|------|------|
| PCA-selection $S_{(k)}$               | 90.0 (92.1,88.0)| 0.115| 96.4 |
| PCA-selection Euclidisch $S_{(k)} \Lambda_{(k)} = I_{(k)}$ | 89.0 (92.1,86.0)| 0.173| 95.4 |

indicators. We will base this investigation on the linear discriminant fit using the Euclidean distance on the first two principal components (use $S_{(k)}$, with $k = 2$ and $\Lambda_{(k)} = I_{(k)}$), as the double validatory assessment of this classifier clearly identifies the first 2 components as containing the discriminatory information.

At this point, we can carry out the analysis starting from a linear discriminant fit based on the full data. Alternatively, we may equally well base the evaluation on a recomputation of the linear discriminant fit on the reduced data described in previous subsection (in both cases we use the data from the first week). Figure 2 (middle section) shows a plot of the correlation coefficients, subsequent to data reduction (previously described selection of 330 bins, but of course now using all 113 samples from the first week). We only show results within the m/z region between AAA and BBB Dalton, as the correlations are effectively zero in the remainder of the m/z range. Evidently, this immediately implies that the separating information is to be found within the AAA to BBB m/z range. We note that the picture shown is virtually indistinguishable by eye from that which results from an analysis of the full data (not shown to save space). The reason for this is that the data reduction restricts attention to the dominant sources of variation, which is not very different from what is achieved through principal component reduction. Immediately above the correlation coefficients graph, figure 2 displays the first two principal components (vertically offset and rescaled to aid visual interpretation) and again based on the reduced data. In this case, the distinct bin subsets selected by the previous data reduction step are clearly visible in the two components, and display the characteristic ‘peaks’ we would expect to identify. Disjoint neighboring bin sets are connected with straight lines. The thus calculated components are a close approximation to those which would result from an analysis of the full data, as we should expect (results not shown). As for the correlation coefficients, any conclusions are therefore identical whether we use the reduced data or not, although the data reduction step perhaps makes the component plot easier to ‘read’. At the bottom of the graph we give the mean spectrum again for each group separately and from the original data within the m/z range of interest, as shown in figure 1 also, along the complete m/z range.

From this graphical analysis, it is immediately obvious how the linear discriminant correlation coefficients identify two major discriminating contributions, the first of which is centered at XXX Dalton and the second at YYY Dalton. Furthermore, the correlations have opposite signs at these locations, which would indicate that the discriminating information can be summarized through a contrast effect between corresponding measured intensities in the spectra. An investigation of the principal components plots above, learns that the contribution at XXX Dalton is primarily accounted for by the first component, which also already contains the contrast with intensities recorded at YYY Dalton. This contrast is then further amplified by the second component which identifies a second orthogonal source.
Figure 2: Discriminant correlation coefficients $\rho_j = s_{x_j} \beta_j / s_g$ of observed intensity values with the class indicators in the m/z range from AAA up to BBB Dalton. We have plotted the first two principal components above these correlations for visual comparison and interpretation. Below the correlations, we plot mean spectra per group (i.e., the vectors $\mathbf{x}_1$ and $\mathbf{x}_2$, as in figure 1). The y-axis is only relevant to the correlation coefficient, while we have vertically offset and rescaled both components and mean spectra to aid visual comparison across the m/z range.
of variation relative to the first component, centered predominately at the already identified peak at YYY Dalton. Note how each component identifies several other smaller contributions, which could also be of interest for further investigation. Comparing these graphs with the within-group mean spectra, the resemblance with the principal components plots at the top of the figure are striking and would suggest that the first component may be primarily explained through variation within the control group at XXX Dalton. Likewise, the second component accounts for a substantial intensity peak at Dalton within the colon cancer group.

Figure 3: Scatter plots distinguishing cases (o) from controls (+). On the left we plot the second versus the first principal component. The right plot shows intensity values at YYY m/z versus those at XXX m/z.

Figure 4: Plot of the contrasts (differences) between intensities at XXX m/z and m/z across all observations, using distinct plotting symbols for each group: cases (o) and controls (+).

To investigate this further, figure 3 provides scatter plots of cases and controls versus the first 2 components (left plot) and between intensities at XXX and YYY Dalton respectively (right plot). The resemblance between both graphs is striking as the right plot can be obtained (virtually) after clockwise rotation of the left plot. As we can see, increases in intensity at XXX Dalton separates controls from cases. Similarly, an increase in intensity at YYY Dalton separates cases from controls. The same interpretation applies to the principal components scatter plot, which confirms our interpretation of the data in figure 2. Figure 4 provides a concise summary graphical illustration of the results. We calculate the contrast (difference) for all 113 individuals participating in the study between the measured
intensities at XXX and YYY Dalton and display the differences in a dotplot using distinct plotting symbols for cases and controls respectively, which demonstrates the separation between both groups.

For further discussion of the clinical background, study rationale, setup, execution and interpretation of results from a substantive clinical perspective, we refer to (de Noo et al. 2005) and subsequent papers from these authors.

5 Discussion

5.1 Current statistical ‘proteomic’ practice

Most analyses and papers on use of mass spectrometry proteomics for diagnosis, prognosis and prediction of treatment response are currently employing a so-called two-stage approach, of which the first step consists of (extensive) pre-processing procedures and the second application of some classification or more general ‘prediction’ algorithm (e.g. (Baggerly 2003) (Yasui 2003) among others). While this approach may at times appear fast on the way to establishing a ‘proteomic dogma’, there is actually no agreement as to precisely what pre-processing steps should be applied and how these may find their place in a coherent analysis strategy. Thus, while there is much enthusiasm in ad hoc algorithm-writing, the methods proposed (and perhaps also results) may be conflicting. Meanwhile, the dangers of such pre-processing are little recognized, particularly with respect to the potential effect on error rate calculation. This may not matter too much if the objective of the analysis is purely data-exploration. However, if the objective is to calculate an estimate of prediction error or the identification of markers that allow us to discriminate between or prognosticate for future patients, it may be a recipe for trouble.

Compare these developments with the analysis presented in Krzanowski’s paper (Krzanowski et al. 1995) (for example ) on classification in the related setting of discrimination with near infrared spectroscopic data. The analysis carried out there is actually rather cautious and conservative in comparison and does not require the amount of pre-processing typically presented in proteomics papers. Nevertheless, Krzanowski is able to demonstrate presence of an optimistic bias of 10% in the calculated recognition rates for one of his classifiers - even though leave-one-out cross-validation is applied, as a result of the tuning of only two (!) model parameters based on the full data (and with a sample size considerably larger than for many current proteomics projects). In comparison, preprocessing efforts in proteomics are typically near reckless with extensive preprocessing steps employed on the full data first, following which ordinary leave-one-out calculations are frequently presented as if the first optimization step had never taken place. To make matters worse, the classifiers employed are often highly complex and new procedures which must yet pass the test of time and easily lead to overfitting (e.g. genetic algorithms (Baggerly 2003)(Petricoin, et al. 2002) ). Good (and standard) statistical advice however, will be to employ a conservative and inflexible class of predictors instead (e.g. Hand, page 153, or McLachlan among others), based for example on first and second moments only.

The extent to which all this will induce optimistically biased assessments is hard to prejudge, but there can be no doubt that the current state of affairs will sooner or later induce false-positive claims on the abilities of mass spectrometry in specific research projects - if this has not yet materialized. Long term, the impact on proteomic science itself may be disillusionment with proteomics and an averse reaction, as overly optimistic claims due to improper analysis and study design do not materialize. A more conservative approach and more stringent emphasis on full validation in the first stages of evaluation can help to counteract this, as well as free valuable time and resources to those projects that are truly worthy of further attention. In other words, the whole argument for proper validation and cautious calibration does not restrict itself to what is ‘proper’ from a purely statistical point of view - but also to the economics, viability and credibility of (proteomic) research and thus to research planning. In this respect, good statistics leads to good scientific practice.
5.2 Preprocessing, in particular alignment and feature ‘definition’

We want to discuss spectral ‘alignment’ and peak selection in more detail, as they pose special problems when applied in any pre-processing of mass spectra that the analyst should be aware of, irrespective of the precise methods employed.

Peak ‘selection’ (or discovery as some authors would phrase it) is really as much peak ‘definition’ as anything else. From a practical point of view, the separation of the construction of the classifier into two phases, the first of which identifies and aligns the ‘peaks’ may be useful. However, statistical science has always regarded the distinction as artificial and more in tune with the pattern recognition literature (Hand 1997)(page 51). Feature extraction and the construction of the classifier both form part of the optimization of a diagnostic allocation rule. For that reason, proposals such as by Morris et al. to base peak identification on the full data (by means of the average spectrum) make sense. However as we have already pointed out, it implies use of the full data and therefore contravenes (cross-)validatory logic. Another potential problem is that the approach is dependent on the within-group sample sizes, which may cause difficulties with unbalanced designs. In principle, cross-validation can be applied to account for peak-selection as well, but this would likely be a very costly procedure from a computational point of view. The other option is to develop new classification methods which have some form of peak discovery built into the classifier in such a way that it allows for efficient (cross-)validation (and for both choice and assessment). If all else fails, use of separate tuning and validation sets is our only alternative, but as we have discussed this will often be undesirable in the first instance due to the sampling costs (see also further on for further comments on this issue). Even if we were to attempt to bypass the whole issue by insisting that our only purpose is discovery of new ‘markers’ (proteins or peptides), some form of validation will be of the essence in the construction of the discriminant models employed as a means to protect against overfitting.

Alignment is a more tricky problem than peak discovery as it involves, by definition, reference to other (calibration) samples. Hence, if cross-validatory analysis is employed within the same data to either calibrate or validate the classification rule, bias will be implied by the referencing which has been induced through aligning previously. At first sight, this might of course be taken as an argument to avoid cross-validatory evaluation all together and insist on collecting additional sample material for separate tuning and test sets. However, (besides the increased costs this will imply) we can not really escape the problems posed by alignment within classification studies as the issue will return with a vengeance as soon as we must allocate a new sample: with respect to which group do we align the new sample for which diagnosis is required? Perhaps, this could be taken as an argument for employing so-called ‘nearest-neighbour type’ classifiers (try alignment of the new sample with - possibly a few samples from - each group separately). However, these rules are notoriously unstable in high-dimensional problems. Furthermore, the alignment problem exists already within the calibration set itself and leads to strange dilemma’s: aligning spectra from one group with those from another reduces between-group separation (makes them more alike) and does not seem the right course of action in the discriminant setting. The opposite choice is equally unappealing as to align spectra within-group only is sure to induce an artificial between-group separation.

From a statistical inferential point of view, the whole issue is therefore more difficult than appears at first sight. While a proper treatment of the problem on statistical grounds is lacking (assuming this is possible at all), it might be advisable not to align and - in the meantime - place some emphasis on maintaining good laboratory practice and protocols instead. The only ‘error’ such choice implies is to make our analyses more conservative only.

5.3 Double validatory analysis

As in our experiment, use of a separate validation set is often precluded in high dimensional problems, due to sample size restrictions. Likewise, the need for predictive optimization and protection against
overfitting may be addressed through use of an additional tuning set, but this greatly increases the burden of collecting sufficient sample material in practice - often to the point of rendering the whole endeavour impossible.

For such reasons, predictive optimization is usually carried out on the full data instead, which results in optimistically biased error rate evaluations, particularly with high-dimensional data such as in mass spectrometry proteomics. The other option is to reduce the available calibration data prior to optimization so as to set aside data (perhaps for both a ‘predictive tuning’ as well as ‘validation’ set) but this approach is not as innocuous as appears at first sight as it will often reduce the calibration set beyond what is needed for reasonable calibration. More generally still, reducing the size of the calibration data changes the condition of the estimation itself. To put this simply: we are not only reducing the data by setting-aside aside data from the calibration set, but also changing the discriminant problem itself. This is again particularly the case in high-dimensional cases such as in proteomics where the problem will typically be ill-conditioned.

The approach we have described in this paper avoids these above discussed difficulties and dilemmas by reducing the pre-processing to such an extent that it no longer induces the biases discussed and then focuses on application of validation for both model calibration and evaluation through double cross-validation. Subsequently, a more exploratory analysis can be carried out, provided we are carefully to interpret results cautiously without contradicting the primary validated evaluation. We discuss a number of issues related to application of (double) cross-validation.

5.3.1 Full validation

One potential cause for concern is whether double cross-validation precludes the need for a completely separate validation set entirely. Is ‘double-cross’ also ‘full’ validation?

The answer to this question will of course depend on what we mean by ‘full validation’. Double cross-validation should give reasonable protection against overfitting and unbiased estimates of error rate at the time of study. Typically however, the performance of any decision rule or classifier has a tendency to ‘decay’ over time. To assess this, subsequent experiments are needed to verify the estimated error rates. More generally, good scientific practice requires that we replicate results in a separate repeat study. This is because cross-validation must ultimately always remain ‘within-study’ validation and there can be factors beyond our knowledge which have influenced the study results. Note however that this applies particularly to the definition of the case and control group, as the impact of systematic effects due to measurement can be minimized through use of randomized block design. Repeat studies may help to detect such problems. In this sense, double-cross represents the maximum usage we can make of the data for joint calibration and validation within a single experiment in order to facilitate decision making for further follow-up confirmatory experimentation. It is desirable to have such information available as early as possible, as subsequent experimentation may be expensive and time consuming. See also (Ransohoff 2004) for more discussion on these aspects.

5.3.2 What classifier are we evaluating?

Two related questions to the previous discussion are ‘What classifier does double cross-validation evaluate?’ and ‘How to assign a new observation?’ Indeed, each observation has its own classifier in the double cross-validatory evaluation. This seems to run counter to the intuition that we calibrate a discriminant rule first and only then evaluate. In that case, the estimated error rate is taken as a reflection of the diagnostic abilities of that particular classifier and the allocation of a new sample is immediate. There is however no logical inconsistency here. Double cross-validation estimates the error rate we would get ‘if we were to apply leave-one-out’ on the whole data. Once we know what the error rate is, we may choose the specific classifier (choice of $k$ or $\lambda$ in our case) for allocation of future samples.
(if required) through application of ordinary leave-one-out on the whole data (this is in line with the discussion presented by Mervin Stone (Stone 1974)).

With double cross-validation, there are however other options to allow allocation of new samples which have not yet been discussed in the literature. In our case for example, we may use the mode of the number of components selected \((k)\) across all samples and then re-estimate the discriminant model with this choice from the full data. More adventurous still, we could retain each of the \(n\) classification rules which are calibrated within the double-cross procedure and use this ensemble (of classifiers) for allocation of any future new observation \(x\). This could be done by calculating the associated a-posteriori class probabilities \(p_i(g | x)\), for each \(i \in \{1, ..., n\}\) and \(g \in \{1, ..., G\}\), where \(p_i\) is obtained from the discriminant model calibrated in the double-cross procedure when the \(i^{th}\) datum has been removed from the data (in the outer shell of the double-cross procedure). Classification may then be based on the mean across these \(n\) a-posteriori class probabilities for any \(g^{th}\) class. We will not pursue these options further in this paper.

### 5.4 Validation and the future of (statistical) proteomics

Rigorous emphasis on validation and proper design can help to establish long-term credibility for proteomic research and more general bioinformatics applications. The double-cross approach with randomized block design described in this paper represents one contribution towards this goal. Many other steps may however be taken to enhance the quality of such research studies. One example is to promote use of ‘truly’ separate validation sets, as obtained from subsequent separate and additional sampling from the population of interest and measurement through identical protocols as applied in the first study. Of course, in practice, this will only be relevant for those studies which indicate potential from the first within-study verification of diagnostic ability.

It would be desirable to stimulate greater awareness within the discipline on the requirements and restrictions which validation imposes on any analysis. However, validation and the need for it, does not only pose a test on the data or any model constructed from or for it. It does (or should) as much lead to verification and restriction on the methodological soundness of any proposed data analysis procedure itself. In this respect we have made reference to the considerable problems caused by pre-selection and similarly, so-called alignment methods. The difficulty to gracefully combine such ad hoc approaches with proper validation may well indicate a more fundamental problem with the data-analytic procedures which are used. As a rule of thumb we should promote forms of analysis which may be validated. This also implies feasibility of the validation schemes proposed. Of course, validation is in principle still possible for pre-selection or construction of markers in principle at least through use of a separate testing set, but this is not so evident for alignment procedures. In this paper, we have taken the view that any methodology should be chosen to err on the conservative side, whenever such problems arise. Editors of scientific journals can also contribute much to inspire such conservative attitude by careful scrutiny of the papers presented for publication. Perhaps simple check lists could be developed to prevent the major mistakes from slipping through the net. This may lead to considerable annoyance in some cases when we face the difficulties of establishing results in the short term, but may enhance scientific credibility of the science as a whole in the long run. Results from the present study show that, with good designed experimentation, these precautions need not form unsurmountable obstacles.
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References

Baggerly, K. A., Morris, J. S., Wang, J., Gold, D., Xiao, L.-C. and Coombes, K. R. (2003) A comprehensive approach to the analysis of matrix-assisted laser desorption/ionization-time of flight proteomics spectra from serum samples. Proteomics, 3, 1667-1672.

Box, G.E.P., Hunter, W.G. and Hunter, J.S. (1978) Statistics for experimenters. Wiley: New York.

Coombes, K. R. (2005) Analysis of mass spectrometry profiles of the serum proteome. Clinical Chemistry, 51, 1-2.

Cox, D.R. and Reid, N. (2000) The theory of the design of experiments. Chapman and Hall: New York.

Hand, D. J. (1997) Construction and Assessment of Classification Rules. Chichester: Wiley.

Hastie, T., Tibshirani, R. and Friedman, J. (2001) The Elements of Statistical learning. Springer-Verlag: New York.

McLachlan, G. J. (1992) Discriminant analysis and statistical pattern recognition. Wiley: Chichester.

Morris, J. S., Coombes, K. R., Koomen, J., Baggerly, K. A. and Kobayashi, R. (2005) Feature extraction and quantification for mass spectrometry in biomedical applications using mean spectrum. Bioinformatics, 21, 1764-1775.
de Noo, M.E., Deelder, A.M., Mertens, B.J., Ozalp, A., Bladergroen, M.R., van der Werff, M.P.J., Tollenaar, R.A.E.M. (2005) Detection of colorectal cancer using maldi-tof serum protein profiling. Submitted.

Petricoin, E. F. III, Ardekani, A. M., Hitt, B. A., Levine, P. J., Fusaro, V. A., Steinberg, S. M., Mills, G. B., Simone, C., Fishman, D. A., Kohn, E. C. and Liotta, L. A. (2002) Use of proteomic patterns in serum to identify ovarian cancer Lancet, 359, 572-577.

Sauve, A. C. and Speed, T. P. (2004) Normalization, baseline correction and alignment of high-throughput mass spectrometry data. Proceedings Gensips 2004, http://stat-www.berkeley.edu/users/terry/group.

Seber, G.A.F. (1984) Multivariate Observations. Wiley:Chichester.

Ransohoff, D. F. (2004) Rules of evidence for cancer molecular-marker discovery and validation. Nature Reviews, 4, 309-314.

Ripley, B. D. (1996) Pattern Recognition and Neural Networks. Cambridge: Cambridge University Press

Satten, G. A., Datta, S., Moura, H., Woolfitt, A. R., Carvalho, M. da G., Carlone, G. M., De, B.K., Pavlopoulos, A. and Barr, J. R. (2004) Standardization and denoising algorithms for mass spectra to classify whole-organism bacterial specimens. Bioinformatics, 20, 3128-3136.

Stone, M. (1974) Cross-validatory choice and assessment of statistical predictions (with discussion). J. Roy. Statist. Soc. 36—, 111-147.

Stone, M. and Brooks, R. J. (1990) Continuum regression: cross-validated sequentially constructed prediction embracing ordinary least squares, partial least squares and principal components regression. (with discussion) J. Roy. Statist. Soc.,52, 237-269.

Stone, M. and Jonathan, P. (1993) Statistical thinking and technique for QSAR and related studies. Part 1: General theory. Journal of Chemometrics, 7, 455-475.

Stone, M. and Jonathan, P. (1994) Statistical thinking and technique for QSAR and related studies. Part 2: Specific methods. Journal of Chemometrics, 8, 1-20.

Yasui, Y., McLerran, D., Adam, B.-L., Winget, M., Thornquist, M. and Feng, Z. (2003) An automated peak identification/calibration procedure for high-dimensional protein measures from mass spectrometers. Journal of Biomedicine and Biotechnology, 4, 242-248.

Yasui, Y., Pepe, M., Thompson, M. L., Adam, B.-L., Wright, J. L. Jr., Qu, Y., Potter, J. D., Winget, M., Thornquist, M. and Feng, Z. (2003) A data-analytic strategy for protein biomarker discovery: profiling of high-dimensional proteomic data for cancer detection. Biostatistics, 4, 449-463.

Yasui, Y. (2005) Analysis of high-throughput proteomic data for diagnostic cancer-biomarker discovery. Presentation to the International Statistical Institute, 55th Session.