

# A method for assessing and maintaining the reproducibility of mass spectrometric analyses of complex samples

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**Direct injection mass spectrometric analysis of biological samples is potentially an attractive approach to the discovery of diagnostic patterns for specific pathophysiological conditions because of its speed and simplicity. Despite the possible benefits offered by such a method, its extensive application has been limited so far by several factors, including the inadequate reproducibility of the analytical results. We describe a method for monitoring and optimizing the performance of mass spectrometers used for biomarker discovery studies, based on the analysis of patterns of standardized spectral features. The method was successfully applied to maintaining spectral reproducibility during a multi-day analysis of hundreds of serum samples despite an ion source failure, which necessitated minor maintenance. The monitoring method allowed the early detection of that failure and the restoration of the spectral profiles after the system was restarted. Copyright © 2009 John Wiley & Sons, Ltd.**

The development of biomarkers for the early detection of disease, monitoring of drug therapy responses and the personalization of medicine is an area of intense interest. Traditionally, single markers have been utilized with relatively simple absolute cut-offs, but the attempts to develop markers for complex multi-factorial diseases have led to the recognition that panels of markers are potentially more useful and appropriate than individual markers;<sup>1</sup> one gene expression-based assay has already been clinically validated.<sup>2</sup> Such multi-variate assays can, in principle, employ a series of individual absolute cut-offs for the multiple molecular markers. However, the natural biological variation between individual people means that the 'normal' value for an individual marker may depend on the levels of other markers; thus a more useful approach may be to look at the relative expression levels between a series of informative markers.<sup>3–6</sup>

Regardless of the nature of the biomarker panel and the thresholds (relative or absolute) that are used to define a particular biological state, the relevant biomarkers must first be found, and then independently validated for their informational value. Mass spectrometry (MS) is an attractive method for discovering biomarkers because of its potential to rapidly identify and quantify large numbers of potential markers in complex biological samples. However, to date, the biomarkers discovered through MS have yielded a high false discovery rate, often failing to validate in independent clinical studies<sup>7,8</sup>. This may be, in part, because the signals

from diseases are subtle and immersed in a background of significant normal biological variability. In order to confidently discover putative biomarkers, a sufficiently large number of biological samples (many tens to hundreds) must be analyzed to provide a robust statistical basis for the analysis. Current methods often employ a discovery MS platform and then follow that with an orthogonal, higher precision quantitative MS/MS method to 'improve' confidence in the validity of the proposed biomarker.<sup>9</sup> Such discovery methods can require many hours to analyze even a single fractionated serum sample. Consequently, the analysis of sufficient samples to achieve statistical robustness can take weeks to months. That is a time scale over which changing instrumental, environmental, and sample factors can exacerbate the system analytical variability to a point where the improved statistical robustness cannot be achieved. Additionally, if limited numbers of samples are used in the second stage of analysis as well, then the many false positive markers may not be excluded until a much more expensive trial is performed down stream in the validation process.

An approach to addressing some of the challenges described above is to use a higher throughput method for biomarker discovery such as direct injection mass spectrometry (DIMS).<sup>10</sup> This reduces the burden of maintaining platform reproducibility by reducing the time required to analyze a given set of samples by as much as an order of magnitude and reduces the number of factors that need to be controlled (e.g., no retention time variability).<sup>11</sup> DIMS has been widely used for the evaluation of complex samples such as biodiesel,<sup>12</sup> natural compounds,<sup>13</sup> and beverages.<sup>14</sup> However, clinical applications of DIMS have been limited

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to the assessment of well-defined components in biological mixtures, such as hemoglobins,<sup>15</sup> lysophospholipids,<sup>16</sup> and purines.<sup>17</sup> In the vast majority of DIMS analyses, the selectivity to monitor specific compounds within complex mixtures is obtained by tandem mass spectrometry (MS/MS). The recent availability of mass spectrometers capable of yielding spectra containing thousands of well-resolved features in a dynamic range spanning over four orders of magnitude<sup>18,19</sup> now makes DIMS much more appealing for high-throughput biomarker discovery, especially in metabolomics applications. Additionally, since DIMS essentially analyzes many thousands of molecular components in parallel, it is ideally suited to be used in combination with pattern recognition algorithms to find those markers which, when taken together, form a pattern of relative relationships that is informative.

In DIMS analyses, matrix effects can mask the majority of the molecular entities present in a biological mixture and variation among individual samples makes achieving reproducible spectral profiles particularly challenging. Nevertheless, even a simple clean-up procedure, such as removing the most abundant proteins and reducing the concentration of inorganic salts by precipitation with organic solvents, appears sufficient to provide samples that are well suited for DIMS analysis, and yield content-rich spectra featuring hundreds if not thousands of well-defined peaks.<sup>18,20</sup> Such solvent-based serum-fractionation methods are particularly useful in metabolomics,<sup>21</sup> especially when DIMS is combined with the latest generation MS instruments that offer unprecedented sensitivity, resolution and dynamic range. In that context DIMS, can be very informative and a useful adjunct to hyphenated MS methods for serum analysis. Thus DIMS has the potential to become a valuable method for biomarker discovery, if reproducibility issues can be addressed.

In support of that goal we describe a method of monitoring and improving analytical reproducibility using a specific pattern of well-defined spectral features generated by spiking a mixture of standard compounds into serum samples. Assessing similarity among spectra, and hence variability in the analysis, is done by evaluating the Euclidean distances between centroids derived from patterns created by spiking serum with a selected set of compounds. Such a Euclidean distance-based strategy has been used previously to compare spectra from unknown compounds to spectra in databases.<sup>22,23</sup> Additionally, we demonstrate how, within limits, it is possible to compensate for some of the instrument-induced changes in the spectral profiles obtained by DIMS analyses of complex samples such as serum.

## EXPERIMENTAL

### Materials

Standard compounds, reserpine (RES), 9-aminoacridine-HCl (AMI) and *O*-acetyl-L-carnitine-HCl (ALC) were purchased from Sigma (St. Louis, MO, USA) while 1,2-diundecanoyl-sn-glycero-3-phosphocholine (PC-11) and 1,2-dilauroyl-sn-glycero-3-phosphocholine (PC-12) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). HPLC grade acetonitrile

and water were purchased from EM Science (Gibbstown, NJ, USA). Formic acid was obtained from Pierce (Rockford, IL, USA) and ammonium formate from Sigma. Standards and chemicals utilized for these experiments were used without further purification.

Normal human sera were acquired from ProMedDx (Norton, MA, USA) and stored at  $-80^{\circ}\text{C}$  until use. The sera were all collected under an IRB approved protocol by ProMedDx. A pool of sera, used to monitor instrument performance (use of pooled sera separates instrument effects from person-to-person variation), was generated by combining equal volumes of serum from ten individuals in a large tube. Following brief mixing using a vortex mixer, to ensure homogeneity, the pooled serum was aliquoted ( $100\ \mu\text{L}$ ) and stored at  $-80^{\circ}\text{C}$ .

### Preparation of the standard reference solution (BioMix)

Each standard compound (RES, AMI, ALC, PC-11 and PC-12) was individually dissolved in 90% aqueous acetonitrile (v/v) to give initial solutions with a nominal concentration of  $250\ \mu\text{M}$ . The standards were then mixed and diluted with 90% aqueous acetonitrile (v/v) to give a concentrated stock solution in which each standard, except AMI ( $12.5\ \mu\text{M}$ ), was present at approximately  $50\ \mu\text{M}$ . Aliquots of  $100\ \mu\text{L}$  of this standard solution were dried in  $1.5\ \text{mL}$  Eppendorf tubes via centrifugal evaporation at ambient temperature and then stored at  $-20^{\circ}\text{C}$  for later use.

### Spiking of pooled serum extracts with BioMix

Extracts of pooled sera were prepared by adding  $5\ \mu\text{L}$  of serum to a  $1.5\ \text{mL}$  Eppendorf tube, and then adding  $1245\ \mu\text{L}$  of aqueous acetonitrile/formic acid buffer (90% AcN, 10%  $\text{H}_2\text{O}$ , 0.2% FA v/v/v) and gently mixing. Samples were incubated for 20 min at  $4^{\circ}\text{C}$  and then spun at  $2010\ \text{g}$  for 15 min at  $4^{\circ}\text{C}$ . Aliquots of the resultant supernatant were mixed with the BioMix solution as described below.

Prior to each experiment a tube of dried BioMix was reconstituted in  $1\ \text{mL}$  of 90% aqueous acetonitrile (v/v) by mixing thoroughly on a vortex mixer for 1 min at full speed and then spinning in a centrifuge for 10 min at  $15000\ \text{g}$ . A  $100\ \mu\text{L}$  aliquot of the supernatant was diluted 50-fold using a buffer comprised of 90% aqueous acetonitrile (v/v) containing 0.2% formic acid (v/v) and  $10\ \text{mM}$  ammonium formate. The resultant solution was then mixed 1:1 (v/v) with an aliquot of the extracted pooled sera to yield a final concentration of approximately  $50\ \text{nM}$  for each standard compound, other than AMI, whose estimated final concentration was  $12.5\ \text{nM}$ . The resultant spiked serum samples were placed in 96-well microtiter plates for analysis.

### Preparation of samples for the multi-day reproducibility study

Individual sera for the multi-day reproducibility experiment were prepared as follows:  $5\ \mu\text{L}$  of serum were added in each well of a 96-well plate, followed by the addition of  $1245\ \mu\text{L}$  of aqueous acetonitrile/formic acid buffer (90% AcN, 10%  $\text{H}_2\text{O}$ , 0.2% FA v/v/v) and subsequent gentle mixing. Samples were incubated for 20 min at  $4^{\circ}\text{C}$  and then spun at  $2010\ \text{g}$  for 15 min at  $4^{\circ}\text{C}$ . Aliquots of the supernatant ( $200\ \mu\text{L}$ )

were withdrawn and mixed with an equal volume of a buffer comprised of 90% aqueous acetonitrile (v/v) containing 0.2% formic acid (v/v) and 10 mM ammonium formate.

Samples were freshly prepared for each day of the 4-day long experiment. The individual sera were placed in columns 3 to 12 of each of two 96-well plates. Column 1 on each plate was used as a solvent blank. Column 2 of each plate contained the spiked pooled serum samples which were used to assess analytical variability independent of matrix effects. With this arrangement, 192 total analyses were performed on each day, with the individual sera serving to place a realistic stress on the analytical platform. Samples were analyzed by row, resulting in approximately one reference BioMix measurement every hour.

### Instrumentation and data acquisition

Mass spectrometric analyses were performed, in positive ion mode, on an AccuTOF LC-plus, model JMS-T100LP instrument equipped with an electrospray ionization (ESI) source (JEOL, Peabody, MA, USA).

To improve the analytical reproducibility the flow rate of the nebulizing gas was verified before each experiment with a mass flow controller (model MC-5SLPM-D, Alicat Scientific, Tucson, AZ, USA). Default instrument settings were as follows: spray needle voltage (NV), 1900 V; orifice 1 voltage (Ori1), 40 V; Orifice 2 voltage (Ori2), 5 V; ring lens voltage (RL), 10 V; ion guide peaks voltage (PV), 1000 V.

Prior to the first day of each set of experiments the mass scale was calibrated with a mixture of polypropylene glycol and polyethylene glycol (1:1; v/v), diluted to a concentration of approximately 200 ng/mL with a solution of 90% aqueous acetonitrile containing 0.2% formic acid and 10 mM ammonium formate. An additional calibration was performed after the system maintenance necessitated by the blocked spray tip.

The AccuTOF was interfaced to an Agilent 1100 liquid chromatography (LC) system configured with a binary pump and a well-plate autosampler equipped with a Peltier cooler set at 4°C. For the analysis, samples were directly introduced into the mass spectrometer using a partial loop injection of 40 µL. The LC flow rate was set at 50 µL/min with a mobile phase consisting of 90% aqueous acetonitrile (v/v) containing 0.2% formic acid (v/v).

For each sample, data were acquired for 3 min from  $m/z$  100 to 1000 at a rate of 1 spectrum every 2 s. The injection yielded a broad elution profile, with a time segment of ~40 s during which the total ion current was stable and equivalent to infusion-like conditions.

### Data processing and analysis

Thirty seconds of data acquired during the stable segment of the sample elution profile (20–50 s post-injection) were summed into a single spectrum for each sample. The summed spectra were then recalibrated using a ubiquitous endogenous serum feature at  $m/z$  758.569 (diacyl glycerophosphocholine) as an internal reference. Finally, the mass and intensity values of the spectra were exported as comma-separated text files to facilitate further analysis with software tools developed in-house.

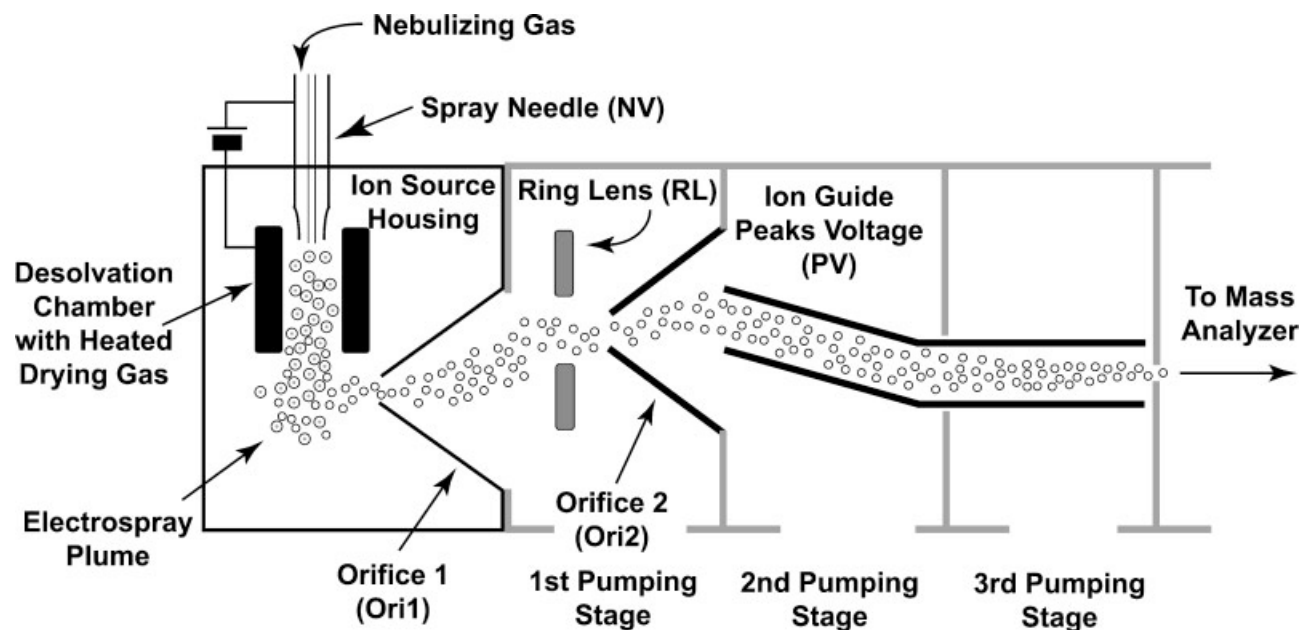
Centroid analysis was performed using SpectraViewer™ (Correlogic Systems, Inc., Rockville, MD, USA). In the case of the reference standards, the intensity of the peaks corresponding to the five compounds: AMI, ALC, PC-11, RES, PC-12; at  $m/z$  195.091, 204.123, 594.412, 609.281 and 622.444, respectively, were extracted, and normalized for each spectrum by dividing the intensity of each feature by the intensity of the most intense feature. System stability for the multi-day serum analysis was determined by reference to the system performance at the start of the experiment. As such, the eight spiked serum samples analyzed on the first 96-well plate were used to define a reference centroid for the experiment. To do this, the five BioMix peak intensities for a given sample were normalized and plotted as a single point in five-dimensional space; the eight spiked samples yielded eight points. The reference centroid was defined as the center-of-mass of these eight points. System drift was then determined using the other spiked samples analyzed throughout the 4-day analytical run. For each spiked sample a similar five-dimensional point was established and the drift expressed as the Euclidean distance between the reference centroid and the five-dimensional point. Because the spectra are internally normalized, resulting in the maximum distance in any dimension being equal to one, the maximum Euclidean distance that can separate two points if they are at 'opposite ends' of the normalized  $n$ -dimensional space is the square root of  $n$ . For a five-dimensional normalized space this maximum distance is the square root of 5 or approximately 2.24. Similarly, centroid and drift calculations for individual non-pooled sera were made using endogenous peaks in the sera; each centroid was calculated using five endogenous features. For simplicity, each Euclidean distance ( $y$ ) is expressed as a percentage of the maximum distance, in this case ( $100 \cdot y / 2.24$ ). For experiments in which we characterized the effect of the various tuning parameters, the reference centroids were determined from samples acquired using the default instrument conditions ( $n = 11$ ).

## RESULTS AND DISCUSSION

### Instrumentation

A simplified schematic of the source region of the AccuTOF mass spectrometer is shown in Fig. 1. The high-pressure region of the source has a series of ion optical elements that are exposed to varying degrees of contamination as samples are analyzed. A significant portion of the variability in instrument performance over time is a consequence of this contamination. Therefore, we chose to investigate the effects of intentional variation of the voltage on the spray needle (electrospray voltage), orifice 1, the ring lens, orifice 2 and the quadrupole ion guide radio-frequency (RF) voltage (i.e., peaks voltage in the AccuTOF software) on the artificial pattern defined by the five standard reference compounds (BioMix). The voltages in question refer to the potential difference between the adjacent ion optical elements, with the elements on the left in Fig. 1 having the higher voltage. Table 1 lists the specific conditions investigated in this study.

Figure 2 shows a representative spectrum of a pooled serum sample spiked with the BioMix; the spectrum displays many metabolite-derived signals. The concentration of the



**Figure 1.** Schematic of the ESI source of the JEOL AccuTOF.

BioMix has been carefully optimized relative to the serum preparation to yield peaks that are readily detectable in the background, but not so strong that they create their own 'matrix effect' that changes the spectral profile significantly. To ensure reproducibility of both this artificial pattern and the pattern of endogenous serum peaks it is of course important to exercise careful control of the BioMix preparation, since any sample-dependent changes in the BioMix profile will affect the data interpretation.

Figure 3(a) shows the calculated Euclidean distance for 11 individual replicate analyses of the BioMix relative to their combined center-of-mass centroid – the reference centroid. Injections which yield BioMix spectra that have identical relative intensity ratios to the reference centroid will yield a Euclidean distance calculation that falls on the axis at  $y = 0$ . For any given injection, the larger the  $y$ -value, the more discrepancy there is between the relative intensity ratios of the reference centroid and the sample. A consistent set of replicate injections should yield a minimal variation in  $y$ -values, preferably close to zero. As shown in Fig. 3(a), analyses performed using the default instrument parameters have consistent patterns of the BioMix, justifying their use in establishing a reference centroid. The BioMix pattern remains stable when there is no variation in system voltages or external factors, such as sample contamination.

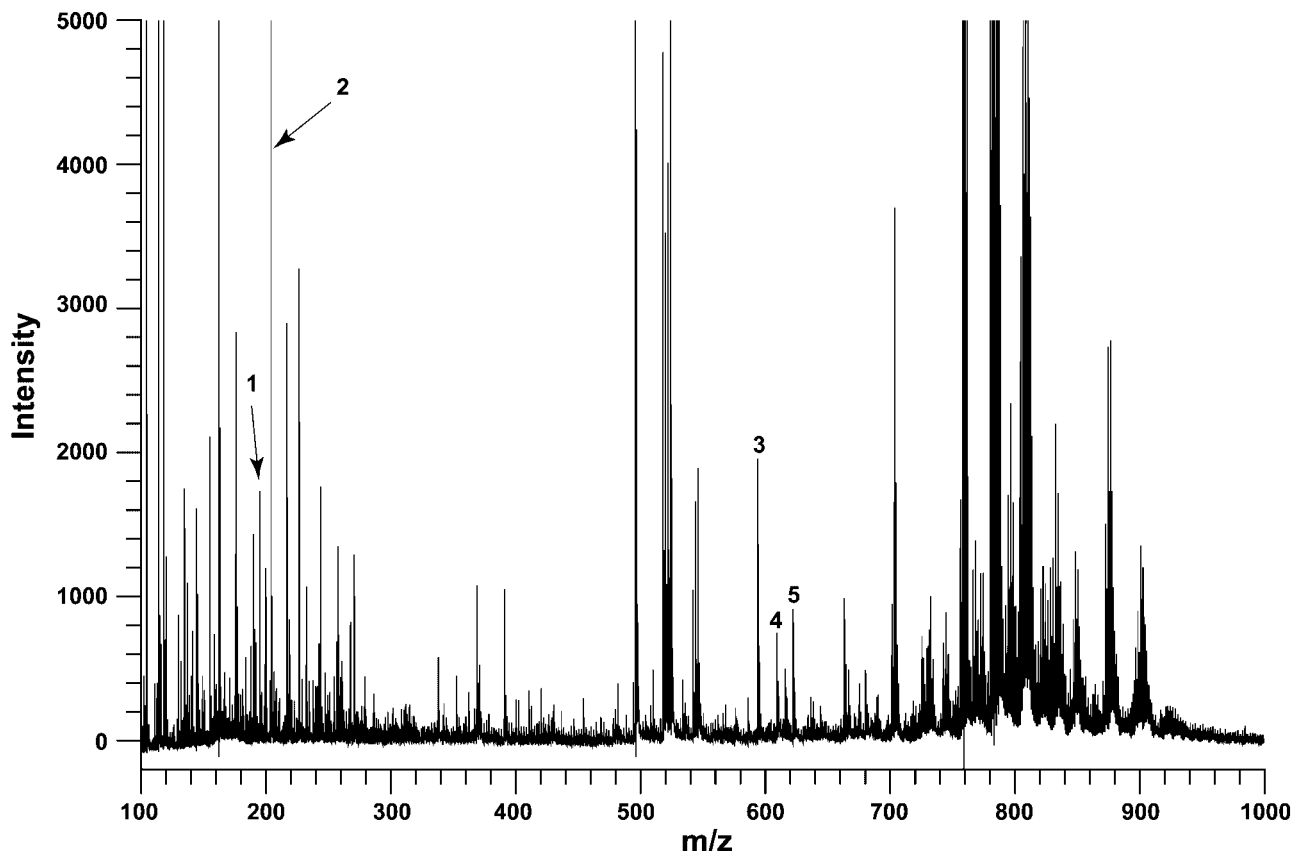
**Table 1.** Ion source voltage adjustments made to probe source tuning space

| Parameter adjusted <sup>a</sup> | Voltage range monitored | Step increments | Default voltage settings |
|---------------------------------|-------------------------|-----------------|--------------------------|
| Needle voltage                  | 1700–2300               | 100             | 1900                     |
| Orifice 1 voltage               | 34–44                   | 1               | 40                       |
| Ring Lens voltage               | 6–14                    | 2               | 10                       |
| Orifice 2 voltage               | 5–15                    | 2               | 5                        |
| Peaks voltage                   | 850–1100                | 50              | 1000                     |

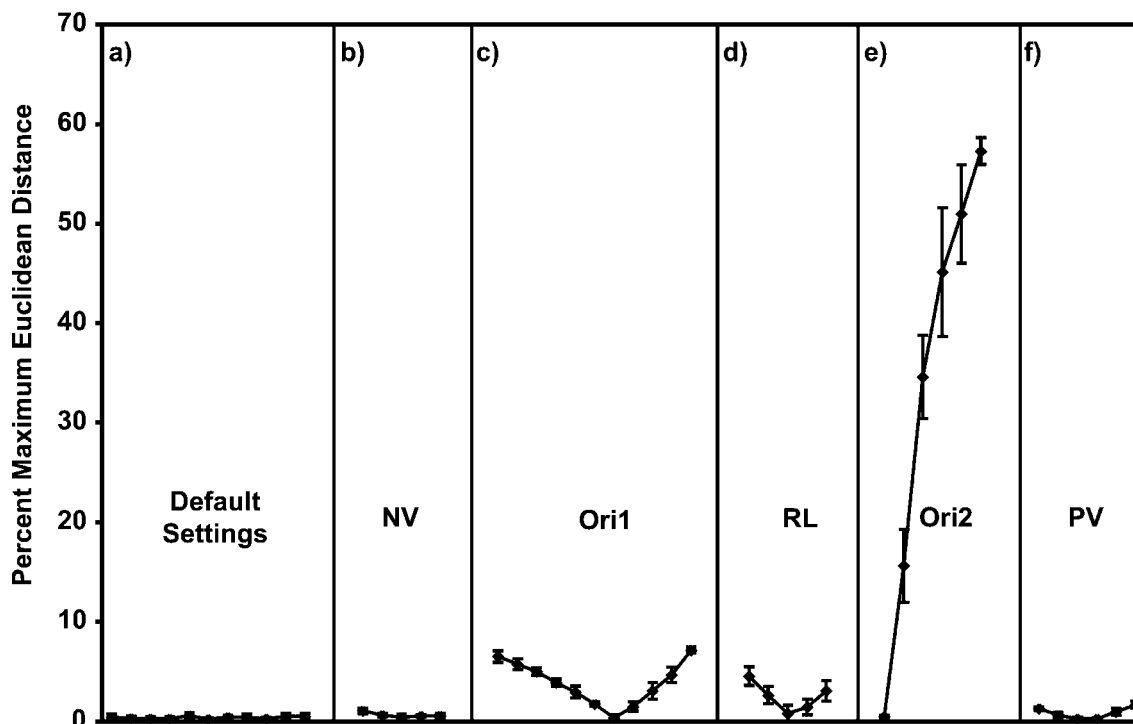
<sup>a</sup>Source parameters were adjusted in the order listed in the table.

Figures 3(b)–3(f) demonstrate the impact on the BioMix centroid of the variation of the voltage for each ion optical element. Some elements clearly have a larger impact than others. For example, while changes in the needle voltage (NV) had minimal impact, changes in the orifice 2 voltage resulted in large changes in the centroid position. Not evident from these figures is the fact that some of the optical elements have a differential impact at different  $m/z$  values. Specifically, the ring lens and the RF ion guide exhibit  $m/z$ -dependent tuning characteristics: an effect that has been reported for other instruments.<sup>24–26</sup> Additionally, the spray needle voltage has a differential effect depending on the physicochemical properties of the reference compounds. The lipids, probably because of their surface-active properties, tended to behave differently than AMI, RES and ALC as the spray voltage varied. In general, when a shift in a centroid was observed, it was the result of a change in more than one component in the BioMix, but which component(s) changed depended on the circumstances. However, changes in the observed centroid were not simply the result of an overall change in sensitivity. Since the centroids are derived from normalized feature intensity values, a proportional change in all them yields no net change in the centroid.

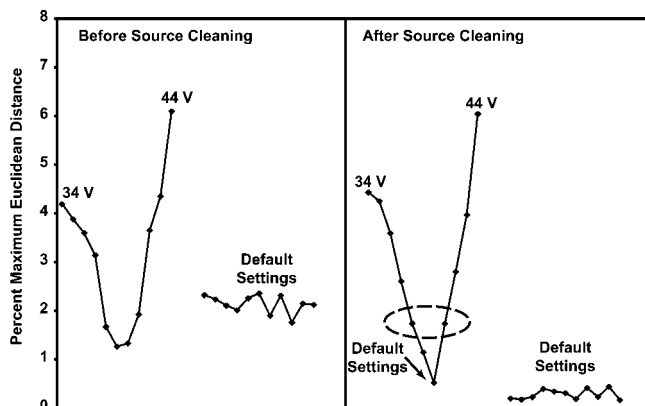
Having established that the BioMix centroid is sensitive to intentional variation of system parameters, we investigated if this tuning profile could be used to identify conditions that yield a similar profile after the system performance has changed. In particular, it had been noted in previous studies that instrument maintenance altered the relative intensity ratios of the BioMix.<sup>27</sup> Figure 4 shows an experiment in which the change in the BioMix centroid was monitored as a function of Ori1 voltage before and after the ion source was cleaned (cleaning included elements from Ori1 leftward in Fig. 1). Whether the change was purely a function of cleaning the source, or if venting the instrument and removing and replacing parts also had an impact, could not be determined from this experiment. Regardless, the BioMix data clearly



**Figure 2.** An expanded view of a DIMS spectrum of a typical extracted serum sample spiked with the BioMix. The BioMix features are indicated by the numbers in the spectrum: 1 = AMI, 2 = ALC, 3 = PC-11, 4 = RES, 5 = PC-12. The features are clearly visible, but do not overwhelm the endogenous serum signals.



**Figure 3.** The effect of various tuning parameters on the BioMix-derived Euclidean distance plot ( $n = 4$ ; error bars show 1 standard deviation). The distances are calculated relative to the average centroid derived from the BioMix-spiked serum under the default operating conditions: (a) overall mean and standard deviation for the default conditions is  $0.4 \pm 0.1$ . The values for adjustment of: the spray needle (NV) in 100 V increments (b); Ori1 in 1 V increments (c); the rings lens (RL) in 2 V increments (d); Ori2 voltage in 2 V increments (e); and the ion guide peaks voltage (PV) in 50 V increments (f) are also shown. For simplicity all of the distance plots are presented as a percentage of the maximum possible distance of 2.24 in the normalized five-dimensional centroid space.

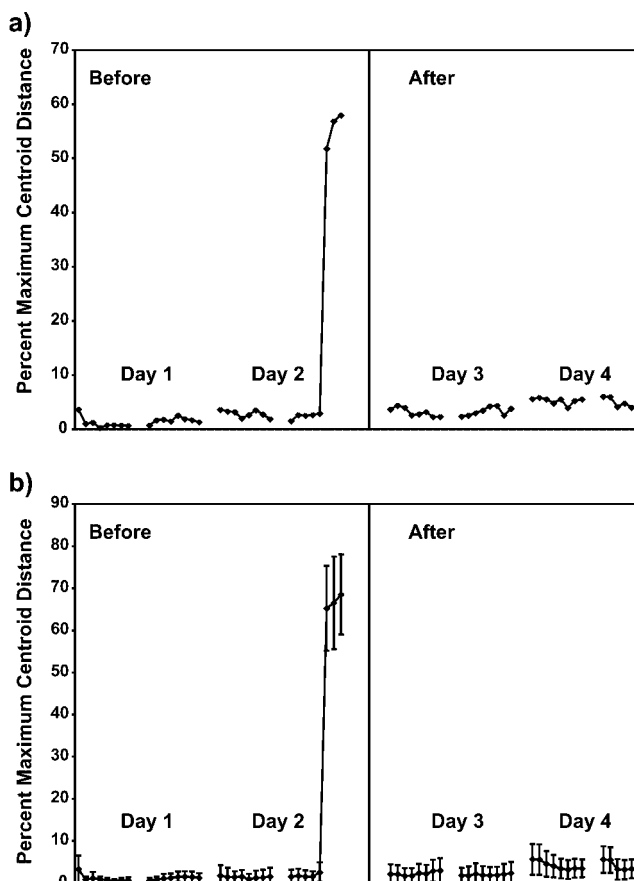


**Figure 4.** Ion source cleaning induced a shift in the Ori1-dependent BioMix profile. Changing the Ori1 voltage from the default 40 V to either 38 or 41 V (values inside the dashed circle) after the cleaning shifted the profile back to near the before cleaning default conditions. All distance values in this plot are presented as a percentage of the maximum possible distance of 2.24 in the normalized five-dimensional centroid space.

shows that the instrument performance has changed following the cleaning. However, the shift in the BioMix profile could be corrected by a small change in the Ori1 voltage (as indicated by the dashed circle) to yield a similar BioMix pattern to that obtained previously. In this case there appear to be two possible values that would restore the tuning; however, in cases requiring the adjustment of more than one parameter, it may be that only one value is truly optimal. While this is a simplistic case, it suggests that some changes in instrument performance resulting from environmental or sample factors can be compensated for by a corresponding change in instrument tuning.

To determine the practical value of this approach, we investigated if the BioMix could be used to monitor and adjust for changes in instrument performance during a multi-day analytical run of human serum extracts designed to study the reproducibility of DIMS data. The BioMix is composed of lipids, heterocyclics and a polar aliphatic compound. These components were chosen, in part, to have some similarity to the metabolic fraction of serum that was the focus of the analytical run. The microplates were laid out in such a way that a pooled serum sample, containing the spiked BioMix, was analyzed after every 10 serum samples. Thus a periodic assessment of the system stability provided an indication of whether serum profiles were being affected by instrument performance drift. The intervening individual serum samples, with their variable matrix effects, provided a realistic stress on the analytical system. This was evident when in the course of the multi-day experiment the spray tip became blocked. This caused the run to be halted after which the spray tip was replaced and the ion source was cleaned.

Figure 5(a)-Before shows the variation in the BioMix centroid distance across the multi-day study. During day 2 there is a shift in the BioMix centroid that occurred several hours before the spray tip became completely blocked. After replacing the spray tip and cleaning the source, but prior to restarting the experiment, the then current BioMix centroid was compared to the pre-blockage centroid; the BioMix



**Figure 5.** A comparison of the Euclidean distance plots for the BioMix in the spiked serum (a) and endogenous serum centroids (b) across a 4-day serum analysis study. Only the data for the BioMix-spiked pooled sera are shown in these plots, but they act as a control for the unspiked individual sera. The data show that near the end of the day 2 analyses a significant deviation appeared in the distance plots for both the BioMix- and serum-derived plots (error bars in (b) represent 1 standard deviation). The mean centroid distance for the serum patterns before the blockage was  $1.3$  with a standard deviation of  $0.54$ . As a consequence of the blockage, and after the spray tip replacement and retuning of the instrument, the centroid distance values were  $66 \pm 1.7$  and  $3.1 \pm 1.3$ , respectively. The blockage clearly caused a significant shift in the measured centroid distance. After replacing the spray needle, the retuning procedure was performed with the objective of obtaining the smallest Euclidean distance from the initial reference conditions. The result was a negligible change in the post-blockage BioMix profile relative to the pre-blockage data.

pattern was significantly different. Iterative adjustments were made starting near the front of the ion source, by first minimizing the difference in the BioMix centroid with one voltage, then adjusting an adjacent voltage in the source to further minimize the centroid difference, and then re-optimizing the previously adjusted voltage. In this fashion it was found that adjustments of the needle voltage and the Ori1 voltage proved sufficient to restore the original BioMix centroid (within error limits). Once the BioMix centroid was determined to have been returned to near its previous

position, the experiment was restarted. Figure 5(a)-After shows that the BioMix centroid was indeed similar to the pre-blockage data.

Notwithstanding the BioMix results, none of these data speak to the effects, if any, that the spray tip blockage or the instrument re-tuning had on the serum data. Figure 5(b) shows a Euclidean distance plot built on sets of five endogenous serum features present in the pooled sera. The spray tip blockage induced a similar shift in the Euclidean distance plot derived from serum features, as was observed in Fig. 5(a) for the BioMix-derived plot. Similarly, after retuning the instrument, the serum-derived spectral profile (as suggested by the distance plot) was restored close to the initial position. The serum centroid-derived plot in Fig. 5(b) was based on arbitrarily selected peaks in the spectra of the sera. To minimize the possibility that only a well-behaved set of features was inadvertently selected for the centroid analysis, 24 separate sets of five-feature serum centroids were examined (Table 2). These features were selected to broadly cover the mass range analyzed and to cover a range of molecular species, based on several that could be identified based on their mass alone. The error bars shown in Fig. 5(b) represent the standard deviation of the Euclidean distance calculation for the average of the 24 individual serum centroids. Generally the 24 sets of serum centroids mirrored what was found for the BioMix pattern.

Based on the results shown in Fig. 5 it appears that adjusting instrument tuning, using a relatively simple set of proxy molecules, can have a beneficial effect on the reproducibility of serum profiles. However, there are several considerations that must be noted. It is not clear, for example, if the specific serum centroids selected in this study would be representative of the physicochemical properties of real serum biomarkers. Nevertheless, in any biomarker discovery

study any potential biomarkers will be unknown; therefore, at this stage of a study some compromise will need to be made. In this work we made a compromise between choosing a simplified set of 'useful' proxy molecules vs. a larger set of molecules with a more comprehensive representation of the complexity of a serum sample to increase the practicality of the method. Our data suggest that the compromise may be justified, although further testing is needed.

Another consideration is that the serum results presented here are for a single experiment on a single instrument. While this approach may be customized for a given instrument, it is not clear how well a given centroid may be generalized to multiple instruments; especially since there are many reports in the literature of the difficulties in obtaining similar spectra from the same samples across multiple MS instruments.<sup>27-31</sup> Therefore, calibration of each individual instrument's characteristics is probably necessary in this approach.<sup>27</sup> Additionally, the artificial pattern used to make instrument adjustments is wholly dependent on the nature of the underlying reference compound mixtures used. Since the molecules in question span a variety of chemical classes it is reasonable to expect them to exhibit differential chemical stability and absorptive losses, which may change the reference pattern over an extended period of time. Therefore, it is prudent to select a set of reference compounds and/or use a strategy in which these differential effects are mitigated.

In future work we intend to examine this approach across multiple platforms. We have recently published a strategy using thermometer ions to address the spectral equivalency across multiple instruments,<sup>31</sup> and are now interested to understand if centroids must be established for individual instruments or if they might be more universal. This strategy also deals with many of the potential differential effects of the

**Table 2.** Set of serum features used to construct monitored serum centroids

|                   | Feature #1   | Feature #2   | Feature #3     | Feature #4   | Feature #5     |
|-------------------|--------------|--------------|----------------|--------------|----------------|
| BioMix Pattern    | 195.09 (AMI) | 204.12 (ALC) | 594.41 (PC-11) | 609.28 (RES) | 622.44 (PC-12) |
| Serum Pattern #1  | 232.07       | 496.32       | 703.58         | 758.56       | 782.56         |
| Serum Pattern #2  | 288.29       | 524.38       | 680.49         | 701.58       | 782.56         |
| Serum Pattern #3  | 232.07       | 270.98       | 542.32         | 760.58       | 848.75         |
| Serum Pattern #4  | 244.07       | 524.38       | 663.47         | 786.57       | 834.61         |
| Serum Pattern #5  | 257.94       | 524.38       | 616.39         | 704.58       | 832.59         |
| Serum Pattern #6  | 267.97       | 546.36       | 782.56         | 806.55       | 898.77         |
| Serum Pattern #7  | 232.07       | 518.33       | 680.49         | 898.77       | 902.81         |
| Serum Pattern #8  | 288.29       | 544.35       | 680.49         | 744.58       | 758.56         |
| Serum Pattern #9  | 257.94       | 369.36       | 544.35         | 810.58       | 900.78         |
| Serum Pattern #10 | 267.97       | 520.34       | 704.58         | 784.56       | 834.61         |
| Serum Pattern #11 | 288.29       | 546.36       | 760.58         | 806.55       | 900.78         |
| Serum Pattern #12 | 162.11       | 496.32       | 524.38         | 784.56       | 902.81         |
| Serum Pattern #13 | 184.07       | 369.36       | 518.33         | 544.35       | 762.58         |
| Serum Pattern #14 | 191.04       | 288.29       | 522.36         | 784.56       | 848.75         |
| Serum Pattern #15 | 244.07       | 496.32       | 522.36         | 784.56       | 902.81         |
| Serum Pattern #16 | 267.97       | 544.35       | 704.58         | 758.56       | 762.58         |
| Serum Pattern #17 | 162.11       | 369.36       | 524.38         | 759.56       | 810.58         |
| Serum Pattern #18 | 288.29       | 369.36       | 704.58         | 760.58       | 810.58         |
| Serum Pattern #20 | 257.94       | 544.35       | 758.56         | 812.59       | 902.81         |
| Serum Pattern #21 | 162.11       | 496.32       | 524.38         | 782.56       | 898.77         |
| Serum Pattern #22 | 267.97       | 518.33       | 616.38         | 663.47       | 902.81         |
| Serum Pattern #23 | 184.07       | 496.32       | 758.56         | 812.59       | 898.77         |
| Serum Pattern #24 | 184.07       | 518.33       | 544.35         | 760.58       | 808.57         |
| Serum Pattern #24 | 162.11       | 496.32       | 525.39         | 785.57       | 902.81         |

reference compounds. We believe that once informative markers have been identified, careful selection of combinations reference mixture components will allow this approach to be used to monitor and control instruments in a clinical diagnostic setting.

## CONCLUSIONS

The use of artificial patterns in serum, generated by spiking mixtures of selected compounds into serum, appears to provide a useful way of tracking the performance and reproducibility of the analytical method. Furthermore, it appears that some level of control of instrumental drift may be achieved by compensating for that drift with adjustments of several source voltages. The artificial patterns, represented as centroids in multidimensional space, provide a useful tool for making such instrumental adjustment and appear to be a useful tool to facilitate the initial discovery of robust and reproducible biomarker features.

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