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Articles

Concept for Facilitating Analyst-Mediated Interpretation of Qualitative Chromatographic−Mass Spectral Data: An Alternative to Manual Examination of Extracted Ion Chromatograms

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A chemometrics-based data analysis concept has been developed as a substitute for manual inspection of extracted ion chromatograms (XICs), which facilitates rapid, analyst-mediated interpretation of GC- and LC/MS$^{(n)}$ data sets from samples undergoing qualitative batchwise screening for prespecified sets of analytes. Automatic preparation of data into two-dimensional row space-derived scatter plots (row space plots) eliminates the need to manually interpret hundreds to thousands of XICs per batch of samples while keeping all interpretation of raw data directly in the hands of the analyst—saving great quantities of human time without loss of integrity in the data analysis process. For a given analyte, two analyte-specific variables are automatically collected by a computer algorithm and placed into a data matrix (i.e., placed into row space): the first variable is the ion abundance corresponding to scan number *x* and analyte-specific *m/z* value *y*, and the second variable is the ion abundance corresponding to scan number *x* and analyte-specific *m/z* value *z* (a second ion). These two variables serve as the two axes of the aforementioned row space plots. In order to collect appropriate scan number (retention time) information, it is necessary to analyze, as part of every batch, a sample containing a mixture of all analytes to be tested. When pure standard materials of tested analytes are unavailable, but representative ion *m/z* values are known and retention time can be approximated, data are evaluated based on two-dimensional scores plots from principal component analysis of small time range(s) of mass spectral data. The time-saving efficiency of this concept is directly proportional to the percentage of negative samples and to the total number of samples processed simultaneously.

Targeted analyses of samples for specific analytes by GC/MS and LC/MS$^{(n)}$ remains, to date, the workhorse of several applied branches of analytical chemistry. In particular, when mass spectrometry is employed, forensic-related fields employ targeted analyses almost exclusively.[1−7] Within the forensic-related disciplines, the goal of hundreds of thousands of annual tests is the qualitative assessment of samples for substances that are expected to be absent from most samples. In order to give the most accurate qualitative (present or absent) answer, regulating bodies and laboratory clients will often require qualitative test results that are based on reporting concentration limits that are near or at the scientifically valid limits of detection for the assay. For example, the World Anti-Doping Agency (WADA) requires positive reports for steroid concentrations that are at or very near screening-assay limits of detection (to which WADA generally tunes its "minimum required performance limits", MRPLs): 10 ng/mL urine for most anabolic agents and metabolites and 2 ng/mL urine or less for a select subset of anabolic agents and metabolites.[8] (WADA has also established ion ratio criteria for qualitative chromatographic-mass spectral assays, which can be found at http://www.wada- ama.org/rtecontent/document/criteria_1_2.pdf) This proximity of reporting threshold to limits of detection often presents difficulties for software designed to automate the qualitative decision making process. In other words, chromatographic integrators, as good as they are and as good as they may get, cannot be considered flawless enough for laboratory

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directors to blindly rely upon them to provide zero false positive and zero false negative reports. Thus, many analytical laboratories are all but forced to manually examine at least one extracted ion chromatogram (XIC) for each analyte from every sample. This task requires many hours of tedious human effort and up until now has been the only option available for scientists who feel that they simply cannot rely upon chromatographic integrators for interpretation of qualitative results. The approach to qualitative analysis of chromatographic−mass spectral data described here is a rapid, efficient alternative to manual interpretation that keeps the interpretation of raw, uncompromised data in the hands of the analyst rather than turning interpretation over to computer software.

This article reports a data analysis concept based on the chemometric principle of row space (defined under Theory below) that dramatically simplifies and quickens the above-described data interpretation process by making it unnecessary to examine individual analyte XICs (Figures 1 and 2), while constantly keeping the interpretation of raw data in the hands of the analyst. Instead of having to examine thousands of XICs per batch of data, the analyst need only examine as many easy-to-interpret, two-dimensional row space-derived scatter plots (Figure 3, heretofore referred to as "row space plots") as there are analytes in the method.

METHODS

Theory. The data analysis concept presented here relies on the chemometric concept of row space. Row space, as used here, is a two-dimensional numerical data matrix containing information describing multiple samples where all data for a given sample are represented in a single row and data for each sample variable (e.g., abundance of a specific ion at a particular retention time) are represented in a single column (see Table 1). Graphically, row space is plotted with a single data point for each sample on an n-dimensional plot where the number of dimensions equals the number of sample variables used to describe at least one aspect (component) of a sample. In the case presented here, two variables are used to describe each analyte, resulting in a single two-dimensional row space plot per analyte. The intensity of each variable defines the data point's location along that variable's axis. For example, in the application described here, ion abundance serves as the quantitative measure, or intensity, of the sample variables. Samples that are similar in terms of the quantitative values for the sample variables plotted will cluster together on row space plots, distinguishing, for example, negative samples, which give a low number of counts for given variable(s), from positive samples, which give a higher number of counts for the same variable(s). Obviously, row space plots must contain three or fewer dimensions if they are to be visually useful. (This is one of the reasons why they are simplified into scores plots following principal components analysis of samples containing large numbers of variables.)

In practice, not all variables that describe an object (component) need be included in a row space matrix describing that object in order to distinguish it from other, similar objects. In many cases, only the two or three most distinguishing features (variables) may be necessary. As demonstrated below, this is generally the case for XICs from chromatographic−mass spectral data when the distinguishing features are background-subtracted ion abundances for analyte-specific ions at precise retention times. For example, two variables describing a single analyte might be (1) background-subtracted m/z 432 ion abundance at 15.75 min, and (2) background-subtracted m/z 417 ion abundance at 15.75 min. Batchwise, plotting the ion abundances for these two variables as a single data point per sample on a two-dimensional scatter plot creates a row space plot of a batch of samples for the analyte in question in which samples containing signal corresponding to

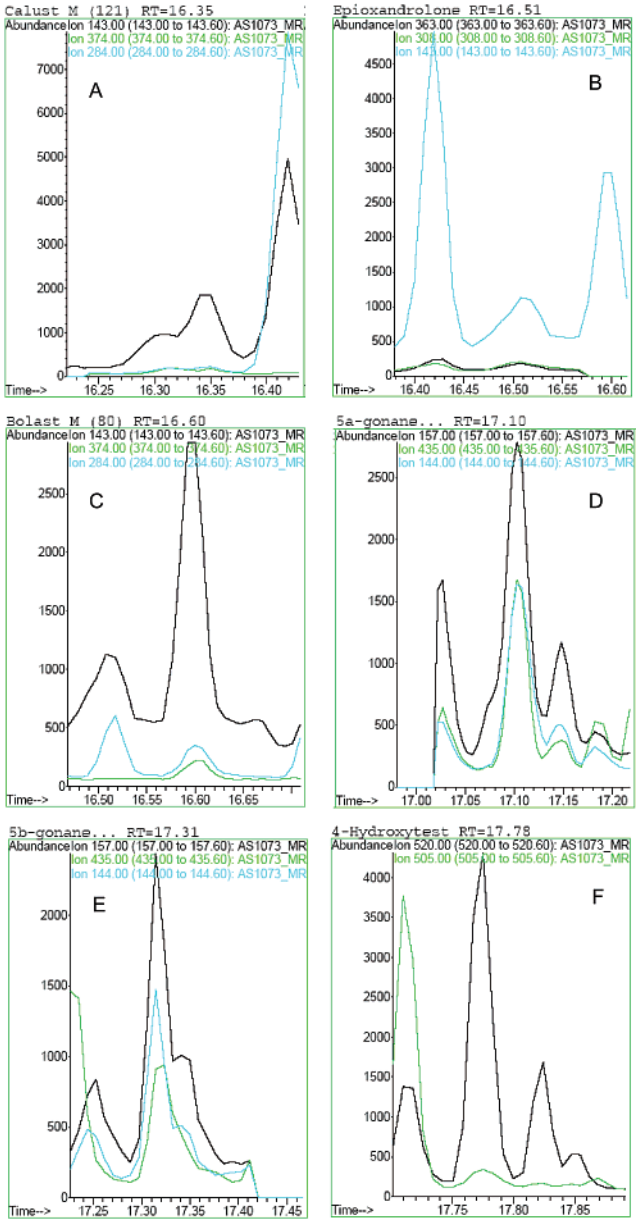


Figure 1. XICs for six analytes in a GC/MS-based urine steroid screen analysis. Analytes were spiked into authentic drug-free urine at the WADA's MRPL of 10 ng/mL each. Individual XIC windows correspond to (A) calusterone metabolite, 7β, 17α-dimethyl-5β-androstane-3α, 17β-diol; (B) epioxandrolone, a metabolite of oxandrolone; (C) Bolasterone metabolite: 7α, 17α-dimethyl-5β- androstane-3α, 17β-diol; (D) norbolethone metabolite, 13β, 17α-diethyl-3α, 17β-dihyroxy-5α-gonane; (E) norbolethone metabolite 13β, 17α-diethyl-3α, 17β-dihyroxy-5β-gonane; and (F) 4-hydroxytestosterone. To assess signal intensity and actual retention time, each panel in this figure should be compared to its corresponding negative control panel in Figure 2.

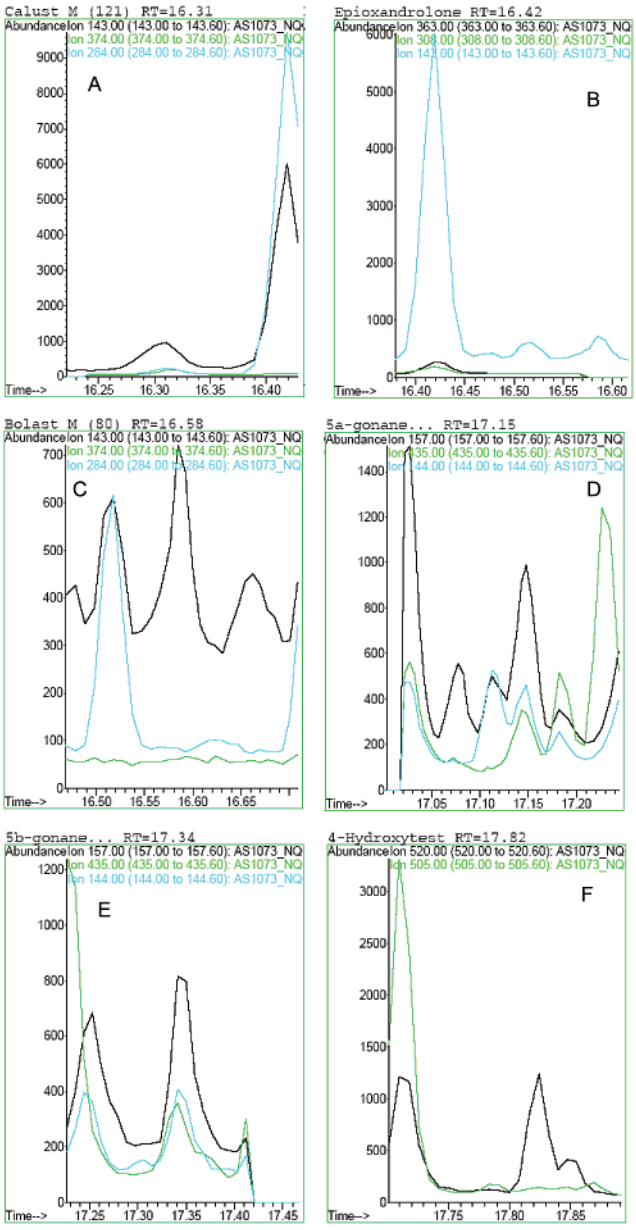


Figure 2. XIC windows corresponding to the expected retention times of the analytes described in Figure 1. This sample did not contain any of the analytes described in panels A−F of Figure 1; all peaks are those of endogenous chemical "noise". Retention times reported above XICs indicate observed retention times, which, for these negative data, indicate that incorrect peaks were integrated.

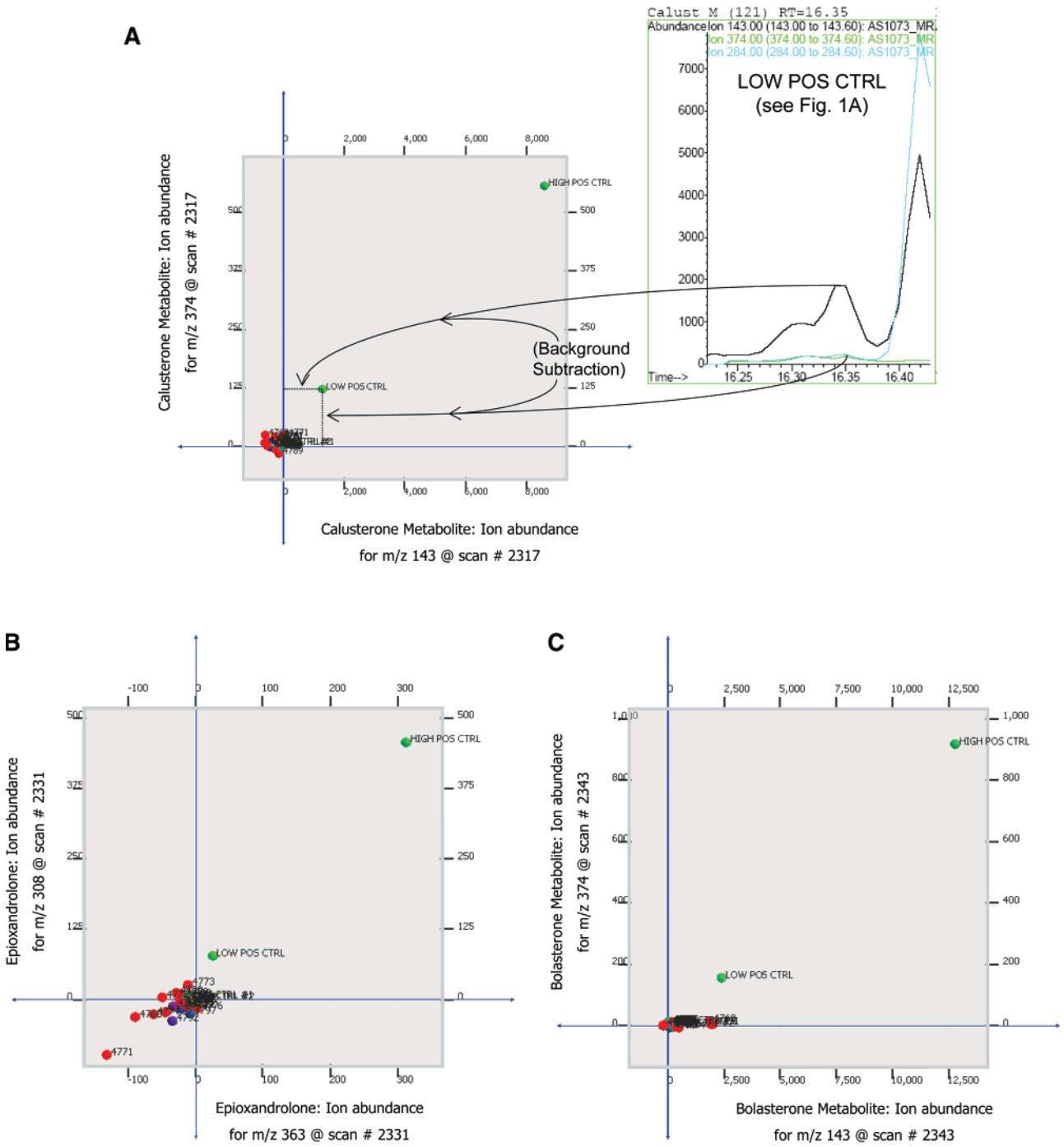
the presence of the analyte separate spatially from those that do not (Figure 3).

All chromatograph−mass spectral software packages are equipped with the ability to automatically display XICs of interest. However, in order to create row space plots of XIC objects, the appropriate data must be selectively extracted and tabulated into a row space matrix. This task is only efficient when performed in an automated fashion. Unfortunately, this particular data extraction feature is not available in any commercial GC- or LC/MS software packages. Thus, in order to implement the above-described data analysis concept, custom code was written for Agilent's GC/MS Chemstation software that automatically extracts data of interest and places it in a comma separated values (.csv) file that can be opened by most common spreadsheet applications.

Implementation. Row space data are extracted as illustrated in Table 1. Each analyte is assigned two analyte-specific variables (each variable represented by a single column in Table 1), which will serve as the two axes of the aforementioned row space plots: the first variable is the ion abundance corresponding to scan number x and analyte-specific m/z value y, and the second variable is the ion abundance corresponding to scan number x and analyte-specific m/z value z (a second ion, see Table 1). Thus, sufficient data are extracted from every sample in a batch to facilitate creation of a separate two-dimensional row space plot for every analyte of interest—in which a data point is included for every sample in the batch. In the current implementation, background subtraction is carried out by first subtracting the ion abundance of a scan that is four scans prior to the scan number of interest, then subtracting the ion abundance of a scan taken four scans after the scan of interest, and then comparing the two differences and reporting the larger value to the row space data matrix. Such background subtraction eliminates ambiguities caused by high baselines and tends to tighten and focus data in row space plots.

In chromatographic−mass spectral data sets (which, for non-MS/MS data, can be envisioned as continuous three-dimensional surface plots), scan number (retention time) and m/z value serve as the independent coordinates from which the corresponding value of ion abundance may be retrieved. Thus, in order to implement extraction of row space data in an automated fashion, a data-retrieval algorithm must be told what ion abundance values to glean and place in a table (in this implementation, a .csv file): Analyte-specific m/z values are readily gleaned from a data analysis method file, but to ensure that the correct data are extracted from batch to batch, analyte-specific scan numbers must be gleaned from a template data file produced by a sample spiked with all the analytes of interest. For the two variables corresponding to a single analyte, the data extraction algorithm gleans a single scan number from the template data file by finding the scan number for the target (most diagnostic) ion that corresponds to its XIC peak apex within the expected retention time range. Once all analyte variables are defined, a header file is automatically written (see second row in Table 1), and the data (background-subtracted ion abundances) specified by the independent coordinates in the second row of Table 1 are automatically extracted for each sample in turn.

Two potential difficulties must be addressed at this point: First, within-batch retention time consistency is important. If retention times shift by more than three or four scan numbers (~2 s) within a batch of samples, then execution of the data-retrieval algorithm without a chromatographic peak alignment algorithm (which is planned for a future version of the data extraction software) may cause incorrect data to be extracted for row space plots. To provide a consistent check on retention time reproducibility for the data presented here, batches were designed such that each sample was spiked with internal standard (in addition to naturally containing several endogenous steroids and steroid metabolites) and each batch was run with interspersed negative controls and positive controls spiked with all analytes. These sample parameters provided a check on retention time reproducibility in every sample: If the internal standard or endogenous analytes did not appear as expected in row space plots or if any analyte in any one of the positive control samples did not appear as expected in its


A
Calust M (121) RT=16.35
Abundance
Ion 143.00 (143.00 to 143.60): AS1073_MR
Ion 374.00 (374.00 to 374.60): AS1073_MR
Ion 284.00 (284.00 to 284.60): AS1073_MR
LOW POS CTRL
(see Fig. 1A)
Time-->
(Background
Subtraction)
HIGH POS CTRL
LOW POS CTRL
Calusterone Metabolite: Ion abundance
for m/z 374 @ scan # 2317
Calusterone Metabolite: Ion abundance
for m/z 143 @ scan # 2317
B
Epioxandrolone: Ion abundance
for m/z 308 @ scan # 2331
Epioxandrolone: Ion abundance
for m/z 363 @ scan # 2331
C
Bolasterone Metabolite: Ion abundance
for m/z 374 @ scan # 2343
Bolasterone Metabolite: Ion abundance
for m/z 143 @ scan # 2343

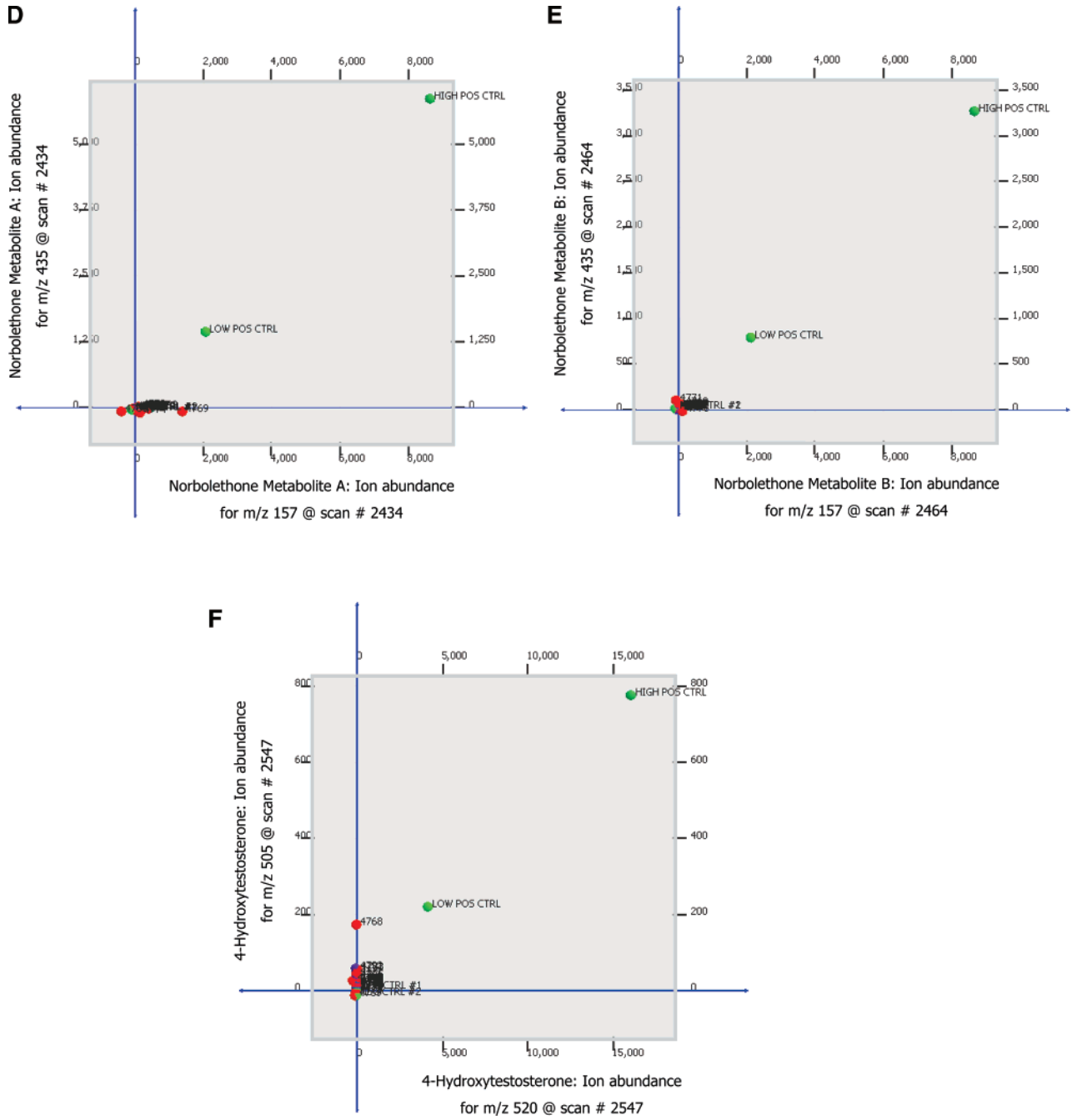


Figure 3. Two-dimensional row space plots corresponding to the six analytes described in Figures 1 and 2, panels A−F. (Panel A provides an illustration of how the row space plots work.) Numbers adjacent to data points are sample names. "LOW POS CTRL" indicates a low-concentration (10 ng/mL of urine) positive control and "HIGH POS CTRL" indicates a high-concentration (50 ng/mL of urine) positive control. Each plot contains a single data point for every sample in an entire 33-sample batch. Note that the time it takes to assess one XIC (in either Figure 1 or Figure 2) for the presence of the analyte of interest is at least as long as it takes to determine the absence or presence of the analyte in every sample from an entire batch using the row space plots shown in this Figure.

row space plot, this would have been an immediate red flag that something likely went awry during acquisition. Finally, since retention times are reset with a "model" positive control sample (spiked with all analytes) in each batch, retention times are not required to be extremely consistent between different batches with the implementation described here.

The second difficulty that requires addressing lies in the fact that it may not be possible to include all analytes in the standard reference material mix that is used to fortify the template or "model" sample (the data file from which batch-specific scan numbers are extracted as described above). One means of dealing with this problem might be to return to manual examination of XICs relevant to these particular analytes. Fortunately, however, this is not necessary: In such cases of scarce reference material, if diagnostic ions and an approximate retention time for a missing analyte can be determined once (e.g., from an excretion study urine sample such as is occasionally distributed by WADA to accredited laboratories), it is possible to implement principal components analysis (PCA) and construction of scores plots instead of row space plots to avoid manual examination of XICs

Table 1. Row Space Data Matrix from which Row Space Plots can be Constructed for the Two Analytes Indicated[a]

sample names	background-subtracted ion abundance data for analyte 1 (mesterolone metabolite)		background-subtracted ion abundance data for analyte 2 (methyltestosterone metabolite)	
	X-axis of row space plot of mesterolone met: scan no. 2317; *m/z* 448	*Y*-axis of row space plot of mesterolone met: scan no. 2317; *m/z* 433	*X*-axis of row space plot of methyltestosterone met: scan no. 2333; *m/z* 270	*Y*-axis of row space plot of methyltestosterone met: scan no. 2333; *m/z* 143
solvent	0	1	−1	8
negative QC	2	12	10	720
MRPL (10 ng/mL QC)	258	358	40	1029
DSTND (50 ng/mL QC)	1939	2616	252	3025
special QC 1	0	−1	401	4298
special QC 2	4	2	363	3753
unknown 31	-2	2	9	71
unknown 32	-1	8	12	72
unknown 33	0	0	14	62
unknown 34	3	2	10	48
unknown 35	4	10	7	26
unknown 1815	4	4	13	78
unknown 2564	−3	3	42	104
unknown 2683	−7	14	14	81
unknown 2871	305	356	10	70
unknown 2965	−3	19	130	27

[a] These data, including column headers and sample names, were automatically extracted as described in the text. The left-most column consists of sample labels. Remaining columns correspond to individual analyte variables, two of which are used per row space plot (as *x*- and y-axes). Numbers in the matrix correspond to background-subtracted ion abundances for the ion and scan numbers indicated. Only "MRPL" and "DSTND" samples were positive for both analytes. Unknown 2871 was found to be positive for only the mesterolone metabolite through visual inspection of the corresponding row space plot (not shown).

for these analytes. The principle behind row space plots and PCA scores plots is the same: PCA simply takes into account more than two or three variables (in this case, for each analyte) of a row space data matrix and then extracts the most distinguishing (covarying) dimensionally amalgamated features from *n*-dimensional row space and plots their intensity (as a scores plot) in two or three reconstructed dimensions. In essence, scores plots from PCA are dimensionally simplified row space plots where the least interesting (least covarying) dimensions are dropped. In effect, as used here, one is looking for outlying data points that do not group with other data points a given scores plot. See Beebe at al.[9] for a more detailed description of PCA and how it works.

To create the data (row space) matrices intended for PCA, ion abundance data on all scan numbers for all diagnostic *m/z* values within a prespecified retention time range must be extracted. That is, instead of extracting only two variables that are known to be highly diagnostic for an analyte, we generally include 100 or more (potentially) analyte-specific variables per analyte for PCA in order to ensure that the correct retention time is covered. To implement automatic extraction of these row space matrices for PCA, missing analytes must be flagged (e.g., by a custom field) in the data analysis method such that a separate row space matrix (one intended for PCA) is automatically extracted (and two variables for the analyte are not included in the matrix for constructing row space plots). PCA is then performed on each PCA-specific row space data matrix (i.e., for each analyte missing from the standard reference material mix), followed by construction of analyte-specific scores plots (instead of row space plots) to be visually inspected for outliers.

To date, we have implemented automatic extraction of row space data matrices for both row space plots and PCA, but not automatic construction of the actual row space plots and PCA scores plots themselves—features that, when implemented in the future, will save even more time. Row space data matrices (.csv files) are simply opened in Microsoft Excel and plotted (one after another with the aid of a simple Excel macro for selecting new data) with 3D-Plot, a feature of the Excel add-in XLSTAT (Addinsoft, New York, NY). For each analyte, PCA is performed and a scores plot is constructed using the PCA data analysis algorithm of XLSTAT.

GC/MS. As stated previously, the data analysis concept presented here is applicable to any chromatographic (and/or spectral) data. As illustrated below, the concept has been applied to gas chromatographic−mass spectral (GC/MS) data. GC/MS data were acquired from urine samples extracted for the purpose of analyzing for anabolic/androgenic steroids: Urine samples (3 mL, from a variety of unknown, mostly male donors) were buffered, treated with β-glucuronidase, mildly basified with a 20% (w/v) solution of potassium carbonate/potassium bicarbonate (1:1 w/w), and extracted with 6 mL of methyl *tert*-butyl ether, 2 mL of which was dried under air. Samples were reconstituted with 50 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide/ammonium iodide/ethanethiol (1000/2/10 v/w/v) and heated at 75 °C for 25 min to effect trimethylsilyl derivatization of steroidal alcohol and ketone groups. This method is described in greater detail by Geyer et al.[10] Analysis of extracted urine samples by GC/MS was

(9) Beebe, K. R.; Pell, R. J.; Seasholtz, M. B. *Chemometrics: A Practical Guide;* Wiley: New York, 1998.

(10) Geyer, H.; Schanzer, W.; Mareck-Engelke, U.; Nolteernsting, E.; Opfermann, G. *Recent Advances in Doping Analysis*; 14th Cologne Workshop on Dope Analysis, Cologne, Germany, 1998, 1997; Sport & Buch Strauss; Köln, Germany, 1997; pp 99−119.

done on an Agilent GC-MSD instrument consisting of an Agilent 6890 GC coupled to an Agilent 5973 inert MSD (G2579A Performance turbo EI MSD model). One-microliter samples were injected in split mode (10:1) onto an injector kept at 280 °C. The GC was operated in constant flow mode with a carrier gas (He) linear velocity of 35 cm/s through a DB-1MS, 30 m × 0.25 mm i.d., 0.1-μm film capillary column. The initial oven temperature was set to 180 °C and without any initial hold time was ramped at 3.3 °C/min. to 231 °C, followed by an immediate ramp to 310 °C and hold for 2 min, at which time the oven was then heated to 325 °C and held for 1 min. The GC/MS transfer line was kept at 280 °C. The ion source was operated in EI mode at 230 °C, with a 70-eV filament. The quadrupole was operated at 150 °C in SIM mode to obtain data on analyte ions of interest. The spectra from each scan were recorded individually, i.e., without averaging.

RESULTS/DISCUSSION

Methodological Comparison. Figure 1 displays six XICs from a single GC/MS run designed to detect over 80 total analytes. Each commercially available analyte was spiked into authentic drug-free urine at WADA's MRPL, which corresponds to 10 ng/mL of urine for each analyte shown. (Analyte name abbreviations are explained in the figure caption.) By carefully examining each XIC and comparing it to analogous data from an unspiked urine sample from the same urine pool (negative control, Figure 2), it is possible to determine that each analyte is present in the sample for which example XIC data are shown in Figure 1. (Readers should note that these data were obtained from a "screening" method from which any positive findings must be confirmed by a more sensitive and selective analytical method prior to reporting results.) Under routine conditions an analyst must not only verify that control samples in a batch are positive for each spiked analyte (and negative for unspiked analytes) but also check every XIC from every unknown sample to determine if any analyte(s) are present in any sample. As can be seen by simply assessing each panel in Figures 1 and 2 for peaks that indicate the presence of the analyte of interest, the process of reviewing a single batch (30−50 samples consisting of over 80 analytes each) of such data is highly consumptive of human time.

In contrast, Figure 3 shows six two-dimensional row space plots—one for each of the six analytes shown in Figure 1—with each row space plot containing data for the single analyte from every sample from an entire batch of 33 samples. The two positive control samples in the batch are indicated as "LOW POS CTRL" (which indicates an authentic urine sample spiked with the analyte at 10 ng/mL of urine) and "HIGH POS CTRL" (which indicates an authentic urine sample spiked with the analyte at 50 ng/mL of urine). Practical interpretation of such row space plots suggests that samples with data points falling outside (i.e., to the left and lower than) an "L"-shaped bracket positioned with its vertex at the "LOW POS CTRL" sample can be considered negative for the analyte in question (i.e., if they contain the analyte, it is at a concentration lower than the predefined testing limit and thus can be considered negative). Notice the distinct spatial separation of the two positive control data points in each row space plot from the cluster of data points describing negative control and unknown samples that were found to be negative. Using row space plots, it is arguably faster to assess an entire batch for the presence of a single analyte than to assess one sample for one analyte using the traditional approach of examining individual XICs. Within a matter of seconds, it is possible to determine that all samples in the batch (except for the two positive control samples) are negative for each of the six analytes described by a row space plot in Figure 3. Inclusion of negative controls and low-concentration and high-concentration positive control samples in each batch provides an excellent check on the validity of row space plots generated for a given batch: if something goes wrong with either data acquisition or analysis, these controls will not display as expected in row space plots. That is, the negative, low-concentration and high-concentration controls will not vary linearly with a positive slope on row space plots.

In theory, if the vast majority of samples are negative for most analytes (as they are in the steroid assay discussed here), using the row space data analysis concept to process multiple batches of data together ought to multiply the human time savings capacity of the concept. For example, if three batches are analyzed together, the total number of row space plots that must be visually inspected for the same number of samples is decreased to one-third without significantly complicating the plot inspection process. Therefore, time savings should be roughly tripled by analyzing three batches together. Figure 4 demonstrates the potential of this row space data analysis technique to aid review of multiple batches simultaneously. Three batches of data that were acquired on separate days were extracted for row space data one at a time. Ion abundance data from the second and third batches (which were based on scan numbers from positive control samples run within those individual batches) were simply appended to the .csv file in which the data from the first batch was written.

Each batch contained two negative control samples, one low-concentration positive control, one high-concentration positive control, and one high-concentration positive quality control (with "positive control" differing from "positive quality control" in that separate stock solutions of pure standard materials were used to make the solutions from which these samples were fortified). After a quick check of Figure 4 to ensure that each quality control sample from among the three batches was represented as expected (i.e., in the negative range, low positive range, or high positive range) in the row space plot, an analyst quickly realized that the plot shows the presence of an unknown sample (4830) with signal well above the MRPL concentration. Visual inspection of the relevant XICs (data not shown) unambiguously demonstrated the presence of dehydrochlormethyltestosterone (DHCMT), as expected from the row space plot. A closer examination of the data near the origin (inset) reveals that two additional samples (4649 and 4656) ought to be examined by their XICs as a precaution to avoid false negative reports. Upon examination of the relevant XICs, both of these samples were found to be negative for DHCMT (data not shown), as suspected, based on the fact that the ion abundance for at least one of the axes is less than that of the lowest MRPL positive control. Since these two samples (which were the unknowns with the greatest outlying magnitude in the "*x*" and "*y*" directions, respectively) proved negative upon additional investigation, no further investigation of other graphically close unknowns was deemed necessary. This logic was applied during the validation described below and has proven its merit. In summary, the plot shown in Figure 4 allows for the assessment of a single analyte across 3 batches (112 samples

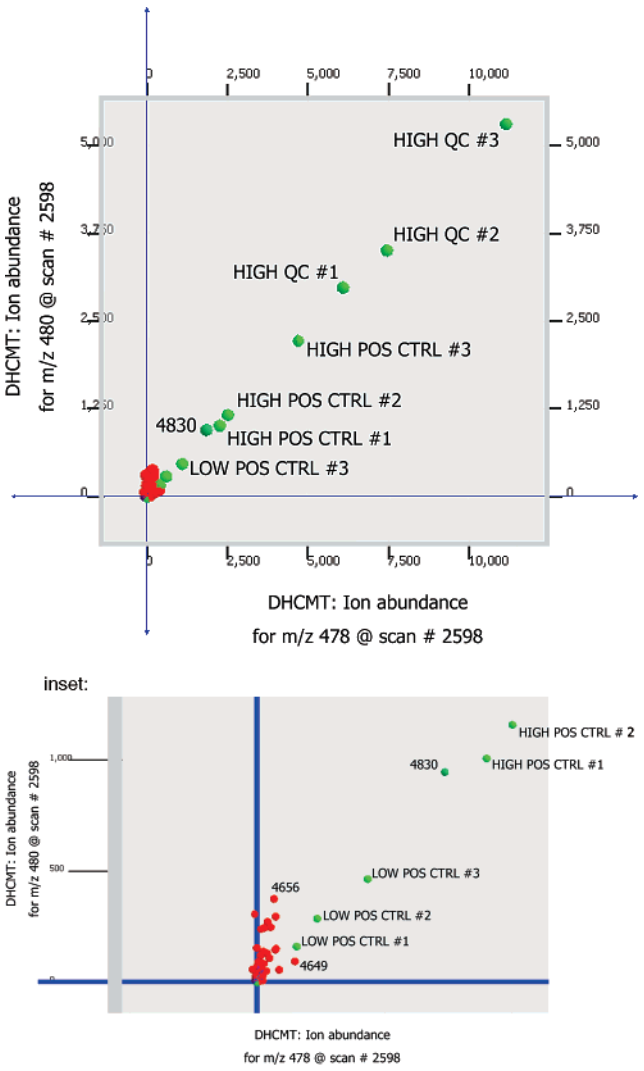


Figure 4. Row space plot containing ion abundance data corresponding to the anabolic steroid DHCMT. These data are from three separate batches and represent 112 samples (including 15 control samples: Two negative controls (not labeled), one low-concentration positive control, one high-concentration positive control, and one high-concentration positive quality control sample per batch). The relevant XICs for sample 4830 were visually inspected and found to be positive for DHCMT. Samples 4656 and 4649 (inset) were also examined by visual inspection of XICs for DHCMT and found to be negative.

including 15 controls) in a matter of seconds. But one note of caution must be mentioned: As the data are based on raw ion abundance, it is theoretically possible that a change in an instrument setting (such as an electron multiplier voltage or other tune settings) could preclude the effective analysis of multiple batches simultaneously. Functionally, however, issues with raw instrument signal only become a problem if the low-concentration positive control samples from one batch drift in among the noisier unknown samples from a different batch.

Practical Features. Three practical features of the row space plots shown in Figures 3 and 4 merit mention:

First, following visual inspection of row space plots, it is always possible to manually evaluate XICs for samples with data points near the MRPL (as described for samples 4649 and 4656 in Figure 4). Because of this, there is no greater danger of reporting false positives than in the traditional "manual" individual XIC examining method of looking at such data. In a fully developed software algorithm, it will be ideal to hyperlink each data point to a display of relevant XICs from the raw chromatographic−mass spectral data.

Second, the ratios of ion abundances between ions generated by the same analyte are generally consistent in most forms of mass spectrometry (including MS/MS) and therefore can serve as rough indicators of chromatographic peak identity. In fact, WADA[11] and other analytical governing bodies (such as the U.S. Food and Drug Administration) have established mandatory guidelines for ion abundance ratios for confirmatory analyses. Row space plots such as the ones shown in Figures 3 and 4 inherently provide graphical information on ion abundance ratios. In the future, enhanced row space plotting implementations will provide ion ratio trajectory lines (based on governing body guidelines) between the low-concentration positive control and high-concentration positive control (i.e., in the shape of a tilted "V" between the two positive control samples). This feature will allow rapid ion abundance ratio assessment during the screening process for samples that provide a signal that is stronger than the low-concentration positive control.

Third, row space plots can be used to assess chromatographic peak purity when data from more than a few samples are plotted simultaneously: In theory, ion abundance ratios between samples for a particular analyte should remain the same regardless of analyte concentration, suggesting that a row space plot representing many samples at a variety of concentrations (for any single analyte) will produce a linear trendline—provided that no underlying sample impurities affect the chromatographic peaks (XICs) in question. In the steroid assay discussed above, minor chromatographic impurities in the XICs for the endogenous steroid dihydrotestosterone (DHT) can usually be noted in at least several samples within a batch. This results in row space plots for DHT that are not perfectly linear (i.e., R^2 values are typically in the range of 0.95 from batch to batch). On the other hand, chromatographic XICs representing the endogenous steroid androsterone almost never contain evidence of interference. Row space plots for androsterone generally give linear distributions with R^2 values of 0.99 or higher. (Data not shown.)

Concept Validation. The data analysis concept presented here does not alter traditional analytical method validation criteria: Namely (and as with all analytical methods), analytes in sample runs within a batch must not undergo retention time shifts, relevant XICs must not contain excessive background noise that prohibits identification of an analyte, and pertinent ion ratios must fall within a predefined range relative to those in a positive control sample. These criteria should be validated as part of the development of any analytical method. Nevertheless, it remains conceivable that retention times or sample background noise may change during a batch-wise analysis and that this may not necessarily be as readily recognized by changes in row space plots as it may be via visual inspection of individual XICs. (Ion ratios, however, are readily observable in row space plots.) Granted, evaluation of these parameters belongs more in the realm of instrument and method validation, but it may be of interest to those who prefer not to rely on theory alone to see a practical evaluation of the data

(11) The World Anti-Doping Agency. WADA Technical Document, TD2003IDCR, 2003.

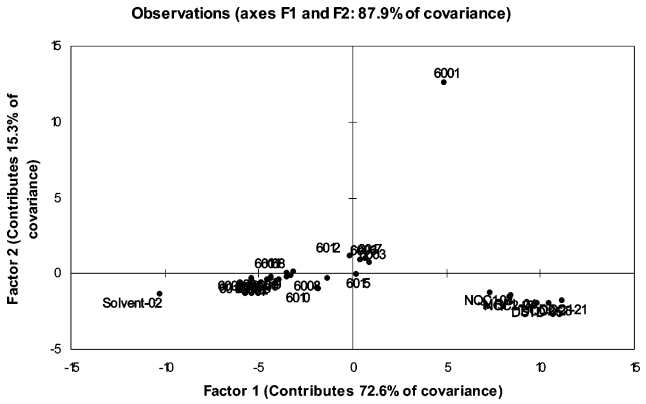


Figure 5. Two-dimensional scores plot from PCA analysis of a set of data variables gleaned from ~25 samples corresponding to a retention time window for 2-hydroxymethylethisterone, a metabolite of the anabolic steroid danazol. A sample containing this metabolite at about one-tenth its MRPL concentration (sample "6001") readily separates from the rest of the data points.

analysis concept presented here using blind controls and genuine unknown samples. (In examining the data presented below, it may be important to realize that, in general, retention times for the GC/MS runs corresponding to the data discussed in this paper do not vary by more than one to two scan numbers (less than 1 s) within a batch.)

During the process of laboratory accreditation by WADA, we were provided with four sets of five unknown urine samples each and a separate final set of 20 unknown urine samples. Data from a GC/MS-based anabolics screen was evaluated by inspection of individual XICs and by the batchwise data evaluation concept described here in a head-to-head comparison. In all cases, data analysis via row space plots and PCA agreed with a careful inspection of individual XICs. Interestingly, two positive samples were initially missed by visual inspection of XICs but were instantly discovered by examination of the relevant row space plot (data not shown).

To illustrate the utility of including PCA to expedite the analysis of those analytes for which no pure standard material is available, Figure 5 shows a scores plot from PCA of a tertiary danazol metabolite that was present at ~1 ng/mL of urine (which is ~10-fold less than the WADA's MRPL).

The sample was not reported positive for this metabolite because the signal of the qualifier ion was too weak, but the example serves to illustrate that even when analytes are below reporting threshold, this form of PCA and subsequent evaluation of scores plots can readily extract differences between negative samples and those which generate any signal—even if that signal is not robust enough to warrant identifying a sample as positive. In summary, after receiving sample content reports on each sample provided by WADA, no false negatives and no false positive results were generated by the row space and PCA data analysis processes, and all controls samples behaved as expected. Of course, as mentioned above, it is not possible for this data analysis technique to lead to the generation of false positive reports relative to traditional XIC inspection since any samples that appear positive in the batchwise row space or scores plots are always visually inspected at the XIC level. Even so, row space plots did not prompt many unnecessary investigations of XIC data. (Figures 1–3 illustrate the clarity of row space plots even when the corresponding XIC data may be difficult to interpret.)

To date, we have pitted the row space data analysis concept against manual inspection of over 80 analyte XICs across a total of over 1000 genuinely unknown samples. Zero false negative (and zero false positive) indications were given by row space plots relative to visual inspection of XICs—and all control samples behaved as expected. One must always remember, however, that the utility and reliability of row space plots are directly proportional to the quality and reproducibility of the raw chromatographic–mass spectral data.

In conclusion, it should be pointed out that the use of this data analysis technique in no way prohibits the use of additional, already-available chromatographic analysis tools such as integrators for cases of analytes where quantitation is required. For example, the bulk of the information gleaned from the steroid assay used as an example in this article is qualitative, but there are a few quantitative aspects of the method that are processed using traditional quantitative software tools (along with a custom macro to summarize any quantitative data on endogenous steroids that exceeds reporting thresholds).

Finally, it is important to understand that an element of subjectivity exists any time interpretation of raw data is left in human hands (as was intended with the data analysis concept presented in this article). Though it is not ideal, some degree of subjectivity will remain in the interpretation of assays—such as the GC/MS method used as an illustration here—until it becomes a perfected technological possibility to remove all human interplay with data interpretation. Until then, the concept presented here will hopefully provide a time-efficient, effective alternative to manual examination of individual XICs.

CONCLUSIONS

A new analyst-mediated data analysis concept pertaining to the qualitative analysis of targeted analytes by chromatographic–mass spectral techniques is described that substantially simplifies data interpretation and dramatically decreases the quantity of human time required to review such data relative to manual inspection of individual extracted ion chromatograms. The concept requires a computer algorithm to automatically extract data and construct row space and PCA scores plots, which are then visually inspected by an analyst. (The data analysis concept described here is intellectual property of the University of Utah and will soon be under development for commercial application across most (if not all) proprietary chromatographic-$MS^{(n)}$ platforms by a third party software company specializing in analytical informatics.) The technique does not generate more false negatives and cannot generate false positives relative to visual inspection of analyte-specific XICs—and is more robust with regard to accurately identifying positive samples than one-time visual inspection of relevant XICs. The time-saving efficiency of this concept is directly proportional to the percentage of negative samples and to the total number of samples processed simultaneously.

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