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Review

Analysis of organic volatile impurities as a forensic tool for the examination of bulk pharmaceuticals

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Abstract

This discussion offers an overview of some formally accepted methodology in the USA for the determination of organic volatile impurities in pharmaceuticals. Particular advantages of equilibrium headspace sampling with capillary gas chromatography for this task are outlined and some important considerations are expressed. Specific adaptations which we have made for forensic applications are described along with mention of select applications within the context of the detection of the counterfeiting of bulk pharmaceuticals. Finally, a brief description is provided of statistical techniques which can be used to effectively manipulate multivariate data sets for purposes of distinguishing between the manufacturers of a product based upon impurity profiles.

Keywords: Reviews; Headspace analysis; Forensic analysis; Statistical techniques; Residual solvents; Volatile organic compounds

Contents

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1. Introduction

It has been estimated that in excess of 70% of the bulk drugs that are used in the United States are imported. The Food and Drug Administration approves these products using the same guidelines that are followed in approving domestic manufacturers. Unfortunately, there have been incidents in which material manufactured by an unapproved source has been disguised as that of an approved source. This act of counterfeiting is of concern for reasons of health and safety as well as economic fraud.

As a forensic laboratory, we are interested in identifying and assessing trace impurities in bulk pharmaceuticals with a view towards using profiles of these contaminants as a "fingerprint" of the manufacturer. Once material from a variety of legitimate producers has been examined, this information often provides a means of ascertaining the probable source of a given material. In other circumstances, the comparison of samples can provide evidence that a pharmaceutical under investigation was not manufactured by the producer-of-record. In either case, impurity profiles are a valuable tool for detecting "counterfeit" drugs or illicit substitutions and tracking down their source.

There are a variety of publications in the open literature concerning the determination of impurities in bulk drugs for forensic purposes. Most often, these involve drugs-of-abuse where there is interest in determining the manufacturing process or in demonstrating that samples have a common origin. A few recent examples include the following. Capillary gas chromatography was used to develop impurity profiles of methamphetamine seized in Australia during a 2-year period. Sophisticated statistical analysis was used to draw conclusions concerning the mode of synthesis and/or the origin of these samples and this has been developed into a national drugs database [1]. A similar approach has been applied to heroin in Germany [2]. In addition to high-performance liquid chromatography and capillary gas chromatography, workers in our laboratory have employed more non-traditional techniques such as ion chromatography [3], capillary electrophoresis [4] and elemental analysis [5] to the same end.

The determination of residual solvents and other organic volatile impurities (OVIs) can make a significant contribution to this process. A recent article [6] has demonstrated the usefulness of equilibrium (or static) headspace analysis combined with gas chromatography–mass spectrometry (SHS-GC–MS) to detect and determine volatile impurities as a means of characterizing illicit heroin and cocaine samples.

2. Official analytical methods (USA) for organic volatile impurities

Although extensive work on the determination of residual solvents in pharmaceuticals and related substances (e.g. [7–10]) has been conducted in Europe and elsewhere, the focus of this section is on the genesis of officially accepted methods for the determination of OVIs in the USA.

After extensive discussions initiated in the mid-1980's, the evaluation of OVIs was formally recognized as an important element in the control of the quality of pharmaceuticals by the proposed introduction of a general chapter in the United States Pharmacopeia [11]. This chapter consisted of three analytical methods. Two of these (Method II and Method III) stipulated the analysis of 5 ml of a 1% (w/v) aqueous solution of the drug by dynamic headspace (i.e. purge and trap) procedures already in use by the USEPA: USEPA Test Method Purgeable Hydrocarbons - Method 601, and USEPA Test Method Purgeables - Method 624. In contrast, Method I proposed direct analysis of a 1% (w/v) solution of the drug in benzyl alcohol on a special column which consisted of serial sections of polydimethylsiloxane on acid-washed siliceous earth and polyethylene glycol on Carbopak B [12]. Subsequently, tolerances were recommended for six OVI residues: benzene (100 mg kg^{-1}); chloroform (50 mg kg^{-1}); 1,4-dioxane (100 mg kg^{-1}); ethylene oxide (10 mg kg^{-1}); methylene chloride (100 mg kg^{-1}) and trichloroethylene (100 mg kg^{-1}) based upon toxicological considerations concerning a projected dose in μg day^{-1} for 1 g ingested [13]. The potential applicability of these standards was to 490 drug substances and 267 excipients while the choice of which of the three methods to be used was deferred to the monograph on each substance [14].

In the light of work by Foust and Bergren [15], the special column was replaced by a combination of a

deactivated retention gap and a widebore capillary column (5% phenyl–95% dimethyl polysiloxane) for the final incorporation of these methods into the United States Pharmacopeia [16]. At the same time, a method for methylene chloride in coated tablets was also introduced which employed equilibrium headspace sampling above 5% (w/v) aqueous solutions of the tablet with subsequent analysis on a polyethylene glycol column. Almost immediately, a general method (Method IV) based upon SHS was added [17].

To address problems of identification which arise from the co-elution of a number of common solvents such as acetonitrile and acetone, hexane and methyl ethyl ketone, and cyclohexane and benzene on the 5% phenyl–95% dimethyl polysiloxane column specified for Method I, Cyr et al. [18] suggested that test results be confirmed on a poly(ethylene glycol) (DB-Wax, J&W Scientific, Folsom, CA, USA) column. Moreover, Foust and Bergren pointed out that the use of water or methanol (recommended in some monographs) in place of benzyl alcohol (which was used in their initial work [15]) created injection problems or interfered with the detection of some solvents and, consequently, invalidated Method I as a means of determining OVIs [16]. Subsequently, Chen et al. [19] pointed out that the resolution problems could also be remedied through the use of a 6% cyanopropylphenyl–94% dimethyl polysiloxane column (DB-624, J&W Scientific). They also recommended that test materials be dissolved in either dimethylsulfoxide or water for analysis. An additional method (Method V) was introduced to allow for the use of this column [20]. The use of dimethylsulfoxide was also recommended by workers at the USP Drug Research and Testing Laboratory [21]. Recently, Clark et al. proposed the use of dimethylacetamide in place of dimethylsulfoxide because of improvements in purity and a lower freezing point which was more compatible with refrigerated storage of standards [22]. The choice of columns and conditions has since been expanded further as Method VI [23] which is constrained only by the requirement for resolution in excess of 1 between components of the standard mixture and reproducibility no worse than 15% relative standard deviation (R.S.D.).

It was subsequently proposed that the dynamic headspace methods be dropped because they were seldom employed for OVI analysis [24] and as a consequence they do not appear in the most recent revision of the USP [25] although the other methods were not renumbered to reflect this change. Also, the static headspace procedure was modified to include the use of automated sampling systems.

3. Static headspace methods

The introduction of an automated equilibrium headspace method as an official USP method was championed by Dennis et al. [26] who separated 33 commonly used solvents on a DB-624 column (30 m×0.32 mm I.D., 1.8 μm film thickness). Drug samples were dissolved to the extent of 2% (w/v) in water [or 0.1 *M* HCl or 0.1 *M* $(NH_4)_2CO_3$], then 5 ml of the solution was placed in a 22-ml headspace vial which contained 1 g of Na_2SO_4 to enhance the partitioning of polar volatiles into the vapor. The vial was sealed and incubated at 85°C for 60 min after which a 2-ml aliquot of the headspace was injected at a 35:1 split ratio onto the column. Co-elution only occurred between the following pairs: benzene, isobutanol and cyclohexane, methylene chloride. They pointed out that at the 2% level of dissolved drug with the added salt, there was no significant matrix effect on the distribution ratio of the OVIs in keeping with a report by earlier investigators [27]. This permits the use of external standards. Headspace sampling is substantially more robust than direct injection since less of the dissolution medium is introduced onto the column which consequently can last for years rather than months as is usual when direct injection is employed. In order to readily achieve acceptable reproducibility with a headspace system, the use of automated sampling devices in contrast to manual injection is recommended. It is important to note that, unless the residual solvents are simply adsorbed to the surface of the drug, dissolution is necessary in order to access the OVIs which are occluded within the crystals.

Kidd [28] evaluated the method of Dennis et al. [26] and found that while it performed in a satisfactory manner for drugs dissolved in dilute acid or water, the recoveries of the test analytes from ammonium carbonate buffer (pH 9) were in the range of 66% to 75%. This failure was ascribed to the generation of carbon dioxide during the incuba-

tion interval and the author speculated that another buffer system might improve recoveries. He also pointed out that although the use of organic solvents to dissolve the drug for headspace analysis was not investigated, this approach might be useful if the purity of the solvent was adequate.

Kolb [29] has addressed the critical issue of calibration in the application of the equilibrium headspace technique to pharmaceuticals. Multiple headspace extraction consists of a series of extractions of the sample solution into a fixed headspace coupled with chromatographic analysis of each draw. Although this method accesses the total OVI content of the sample and is, therefore, immune to matrix effects, it requires multiple analyses and is impractical for routine use. Nevertheless, this method does serve as a benchmark against which other approaches can be evaluated during method development.

Internal standards can be successfully applied if they closely match the nature of analyte of interest but this can be complicated if multi-component mixtures are being analyzed. Standard additions is generally the most suitable quantitative approach. In special cases, levelling the matrix by the addition of high levels of salt [26,27] can allow for the use of external standards.

When the drug sample is poorly soluble and the OVIs are largely adsorbed to the surface rather than occluded in the crystalline matrix of the drug, OVIs can be displaced from the surface of the drug using an organic solvent. In this case, there is virtually no matrix effect and external standards can be used. This tactic can be employed by simply wetting the surface of the finely ground drug, which increases sensitivity.

Finally, a variety of relatively high boiling organic solvents are mentioned as candidates for the analysis of OVIs by equilibrium headspace analysis. These include: 2-ethoxyethanol (b.p. 135°C); glycols (b.p. about 200°C); dimethylacetamide (b.p. 166°C); dimethylformamide (b.p. 153°C); benzyl alcohol (b.p. 205°C); glycerol (b.p. 182°C at 3.3 Pa); propylene carbonate (b.p. 240°C) and acetic acid (b.p. 118°C). Propylene carbonate is recommended by virtue of its purity. We have used dimethylsulfoxide (b.p. 189°C) successfully although we observe significant impurity peaks due to dimethylsulfide and dimethyldisulfide. Dimethylacetamide can contain significant amounts of dimethylformamide as well.

Naughton [30] approached the problem of water-insoluble drugs by dissolving 500-mg samples in 2 ml of dimethylformamide and subsequently added 1 ml of saturated aqueous sodium sulfate prior to incubation at 85°C for 20 min with agitation. A variety of OVIs including ethanol and acetone were detected at the 1 mg kg^{-1} level. De Smet et al. [31] recently suggested the use of 1,3-dimethyl-2-imidazolidinone (b.p. 108°C at 2.9 Pa) and described a method for the determination of OVIs in hydrophobic drugs at the 50 mg/kg to 2500 mg/kg level in keeping with tolerances based upon toxicity. This solvent allows dimethylformamide and dimethylacetamide to be evaluated by the equilibrium headspace technique. Other examples of recent applications in this area include the determination of ethanol, acetone and diethyl ether in gonadotropin powders [32] and the evaluation of several solvents in the antibiotics, cephalosporin and tetracycline [33].

4. Performance characteristics and some recent applications of a SHS-GC–MS system

The particular system which we use for equilibrium headspace analysis is targeted at detection and identification of OVIs at trace levels [34]. An equilibrium headspace autosampler (Model 7000/7050, Tekmar, Cincinnati, OH, USA) is configured for use with 9-ml headspace vials and plumbed with electroform nickel tubing for increased inertness. Generally, a 0.25-ml sampling loop is employed. The gas flow-rate is set to about 0.9 ml min^{-1} (at 10°C) in keeping with the requirements of the mass spectrometer.

Aliquots of the headspace are transferred to a cryofocussing unit (Tekmar) which is maintained at −130°C. After enough time has passed so that the volume of the transfer line has been swept 2.5 times, the cold trap is heated to 200°C (at 600°C min^{-1}) to re-volatilize the sample.

Chromatography is conducted on a relatively thick film, cross-linked trifluoropropylmethyl polysiloxane capillary column (Rtx-200, 30 m×0.25 mm I.D., 1.0 μm film, Restek, Bellefonte, PA, USA). This column exhibits special selectivity for lone pair electrons which increases the relative retention of oxygenates, particularly ketones, with respect to the 6% cyanopropylphenyl–94% dimethyl polysiloxane col-

umn mentioned above. However, sub-ambient cooling is needed to obtain adequate retention of early eluting compounds such as methanol, diethyl ether and ethanol. In general, the temperature is programmed as follows: 10°C (2 min) to 200°C (at 10°C min^{-1}).

The column is directly connected to a benchtop mass spectrometer system (Model 5971A, Mass Selective Detector, Hewlett-Packard, Palo Alto, CA, USA). Typical scanning experiments for identification are conducted using the following operating parameters: range 20 amu to 250 amu at a repetition rate of 2.9 Hz; the threshold is set at 150. A total-ion chromatogram for a variety of solvents dissolved in dimethylacetamide and analyzed using the SHS-GC–MS system is presented as Fig. 1. While there are some significant peak overlaps, these can be deconvoluted using characteristics of the mass spectrum of each compound.

In a detailed study presented elsewhere [34], calibration data was generated for a variety of analytes dissolved in 0.5 ml of dimethylacetamide and incubated at 105°C. Detection limits were estimated by defining the noise as the measured width of the baseline divided by the square root of 2 and linearly extrapolating a low standard to a height equivalent to 3 times the noise of the baseline. Under these constraints, the limits of detection for selected compounds are as follows: acetone (0.4 mg l^{-1}); benzene (0.3 mg l^{-1}); chloroform (1.3 mg l^{-1}); chlorobenzene (1.4 mg l^{-1}); dioxane (1.2 mg l^{-1}); ethanol (2 mg l^{-1}); methylene chloride (0.5 mg l^{-1}); methyl ethyl ketone (0.5 mg l^{-1}); *n*-octane (0.2 mg l^{-1}) and trichloroethylene (0.5 mg l^{-1}). The important point is that at a factor of two times these levels, mass spectra were obtained that could be reliably matched to those in the Wiley Library of Mass Spectra.

In contrast, when water served as the dissolution medium with incubation at 85°C, non-polar analytes were enriched in the headspace by up to a factor of 50 while polar analytes were depleted by up to a factor of 4. When the analytes are known or for specific screening, the use of selective ion monitoring increases the sensitivity of the method by about two orders of magnitude.

Linearity was investigated using only selected ion monitoring at the most abundant ion of the analyte since it was anticipated that quantitative work would be conducted in this manner because of enhanced sensitivity and improved data sampling rates. The linearity exceeded four orders of magnitude for the analytes mentioned above.

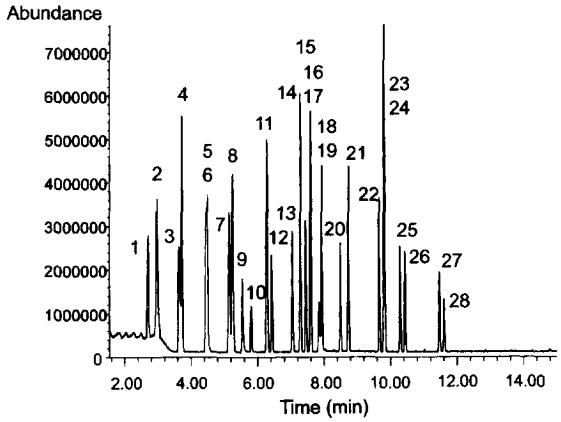

Fig. 1. Total ion current chromatogram of selected solvents sampled from 1 ml of dimethylacetamide in a 9-ml vial at 105°C using an equilibrium headspace autosampler with a 0.25-ml sampling loop. They are separated on an Rtx-200 column (30 m×0.25 mm I.D., 1.0 μm film) under a temperature program as follows: 10°C (2 min) to 130°C at 10°C min^{-1}, detector off, and to 200°C at 20°C min^{-1} to elute dimethylacetamide. Peaks identities and concentrations are: (1) methanol 100 mg l^{-1}; (2) pentane 11 mg l^{-1}; (3) ethanol 110 mg l^{-1}; (4) diethyl ether 14 mg l^{-1}; (5) 2–propanol 100 mg l^{-1}; (6) dichloromethane, 28 mg l^{-1}; (7) 2-methyl-2-propanol 89 mg l^{-1}; (8) 2-methoxy-2-methyl propane 14 mg l^{-1}; (9) 1-propanol 97 mg l^{-1}; (10) chloroform, 31 mg l^{-1}; (11) carbon tetrachloride 54 mg l^{-1}; (12) acetone 17 mg l^{-1}; (13) acetonitrile 54 mg l^{-1}; (14) benzene 35 mg l^{-1}; (15) tetrahydrofuran 19 mg l^{-1}; (16) 1,2-dichloroethane 41 mg l^{-1}; (17) trichloroethylene 55 mg l^{-1}; (18) 1-butanol 220 mg l^{-1}; (19) ethyl acetate 31 mg l^{-1}; (20) 2-butanone 24 mg l^{-1}; (21) octane 12 mg l^{-1}; (22) toluene 37 mg l^{-1}; (23) 1,4-dioxane 45 mg l^{-1}; (24) tetrachloroethylene 74 mg l^{-1}; (25) 3-pentanone 35 mg l^{-1}; (26) 2-pentanone 35 mg l^{-1}; (27) ethyl benzene 35 mg l^{-1} and (28) chlorobenzene 50 mg l^{-1}.

Raising the incubation temperature increases the concentration of analytes in the headspace at a rate of 3% to 4% $(°C)^{-1}$. However, higher temperatures also introduce more of the dissolution solvent into the column which can lead to pronounced distortion of early eluting peaks. Water significantly interferes with early-eluting hydrophilic analytes (e.g. methanol, ethanol) on the Rtx-200 column and at trace levels these compounds are determined more effectively when the bulk drug is dissolved in dimethylacetamide.

A 6% cyanopropylphenyl–94% dimethyl polysiloxane column can be operated successfully at a higher starting temperature (viz. 40°C) than the Rtx-200 column mentioned above and a megabore capillary column of this type (Rtx-1301, 30 m×0.53 mm I.D., 3.0 μm film, Restek) is installed in a second system which consists of an HS-100 headspace autosampler (Perkin Elmer, Norwalk, CT, USA) mated to a Sigma 2000 gas chromatograph (Perkin Elmer) with a flame ionization detector. Data acquisition is accomplished through an A/D convertor (Model 35900, Hewlett Packard) into ChemStation (HP 3365) software for subsequent reduction. This system is utilized for well characterized samples.

In a recent study, sulfamethazine was dissolved in dimethylacetamide at 40% (w/v) with 2-pentanone added as an internal standard to evaluate levels of acetone using SHS-GC–MS with the mass spectrometer operated in single-ion mode [27]. Acetone was detected at levels ranging from 0.2 mg kg^{-1} to 20 mg kg^{-1} in the pharmaceutical and this served as an effective means of distinguishing between material derived from different sources. Product which contained either 1.4 mg kg^{-1} or 13 mg kg^{-1} of acetone was evaluated in duplicate with each group of samples analyzed over a four month period on the same instrument by the same analyst. For 1.4 mg kg^{-1}, the within-day precision was 5% R.S.D. while the between-day precision was 13% R.S.D.. For 13 mg kg^{-1}, the within-day precision was 3% R.S.D. while the between-day precision was 6% R.S.D..

Ranitidine hydrochloride was dissolved in dimethylsulfoxide at the 20% (w/v) level and evaluated by equilibrium headspace sampling coupled to GC–MS for identification. Then, it was dissolved in water at the 50% (w/v) level for quantitative comparison using equilibrium headspace sampling coupled to GC-FID. Product from one source was distinguished from that obtained from another based upon the OVIs: benzene, chloroform, methanol, 4-methyl-2-propanone and 2-propanol. While 2-propanol and methanol were present at concentrations which exceeded 1000 mg kg^{-1}; the others, if present, were evident at levels as low as 1 mg kg^{-1}. Similarly, doxycycline hyclate from several different manufacturers was dissolved in dimethylacetamide at the 25% (w/v) level (or water at 37.5% (w/v)) and differences in the following OVIs served to specify the source: acetone; acetonitrile; ethanol; ethyl acetate; methanol and methylene chloride.

5. Statistical methods for data analysis

The evaluation of results from analysis can be relatively straightforward. If only one or two OVIs are involved, this can require little more than significance testing of the difference between the means of two groups. It is important to note that consideration also needs to be given to the lot-to-lot variability for material which is produced by a single manufacturer. The problem becomes more challenging when multiple variables are involved.

A number of different chemometric approaches have been used in this laboratory to interpret data obtained from the determination of OVIs in pharmaceuticals. The references cited below are meant to provide introductions to the various techniques. Multivariate OVI data can be processed with at least two goals in mind. The first is visualization and the second is classification.

5.1. Visualization

While a set of data can be described mathematically with regard to central tendency (mean, median, or mode), to dispersion (range or variance) and even to degree of skewness, these statistics may not prove as useful as a good picture [35]. An accurate graphical representation not only shows the size and shape of the data cluster for each class, but also the location of each class relative to the others. It can give the analyst an intuitive feel as to whether inter-group separation is adequate for accurate classification. Extracting this information from a table of sample statistics would be difficult and possibly misleading.

5.1.1. Scatterplots

Two- and three-dimensional graphs, or scatterplots, can be used to view OVI data. Each sample is treated as a point in the space defined by concentration axes of the analytes being considered. The use of separate plotting symbols or colors for each class of samples permits rapid examination of group

separation. When the important differences between classes can be described using only two or three analyte variables, the scatterplot is the visualization method of choice, owing to its simplicity.

5.1.2. Principal components analysis

In some circumstances more than three analyte variables or axes are required to effect the separation of all the classes in the data set. Unfortunately, it is not possible to directly view a space with a dimensionality greater than three. However, principal components analysis (PCA) can be employed to reduce the effective dimensionality of the data [36,37].

PCA capitalizes upon the fact that there are generally correlations between variables which introduce a certain amount of redundancy into a set of multivariate data. For example, data described by three variables may take the form of a 2-dimensional plane of points embedded in 3-dimensional space. Thus the points can be represented by two new factors which are linear combinations of the original three variables. The deviations (or residuals) of the points from this 2-dimensional plane are the errors incurred in this reduction of dimensionality. If the points lie very close to the plane, then little information is lost.

The chief benefit to be derived using PCA, which can justify some loss of information, is the ability to visualize a data set with many variables via a more compact representation. A parameter known as the eigenvalue is associated with each new axis or principal component extracted from the data set. It is a measure of the amount of variance in a data set explained by a given principal component. When there are more than two or three significant eigenvalues found, the data set is not completely amenable to visualization via PCA. Such a data structure is not readily reduced to two or even three dimensions without significant residuals or errors. Nevertheless, the 2-dimensional plot of principal component 2 vs. principal component 1 is the best possible representation of the data set using only two axes. It must be stressed that principal components are mathematical combinations of the original variables and not to be confused with the real chemical components of the system.

5.1.3. Spectral templates

The variables being used to visualize the data set can be arranged consecutively, generating for each sample a "spectrum" of concentration values. Unlike a traditional spectrum, however, the ordering of variables is arbitrary. The spectra for all members of a given class can be combined, producing a visualization of the entire class that shows not only the overall spectral pattern of the class, but also the distribution of values obtained for each analyte measured. This class spectrum can be treated as a template upon which the individual spectrum of an unknown can be placed. If the unknown is a member of the class in question, its value on each of the spectral variables should lie within, or close to, the ranges established for known members of the class. To accommodate variables in the spectrum having very different magnitudes, it may be necessary to scale each variable independently. This is done by dividing each sample's value for a given variable by a suitable constant, frequently the largest value of that variable for all the samples analyzed.

Fig. 2 Fig. 3 show data from the analysis of a bulk pharmaceutical powder from two different manufacturers. (The identity of the product and the manufacturers is confidential). The spectra are ex-

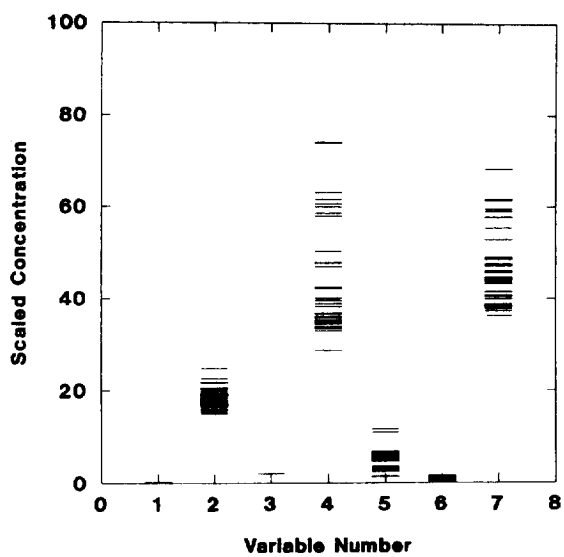

Fig. 2. Plot of scaled concentration values for seven different impurities in a bulk pharmaceutical from one manufacturer. Each short horizontal line corresponds to a scaled concentration value.

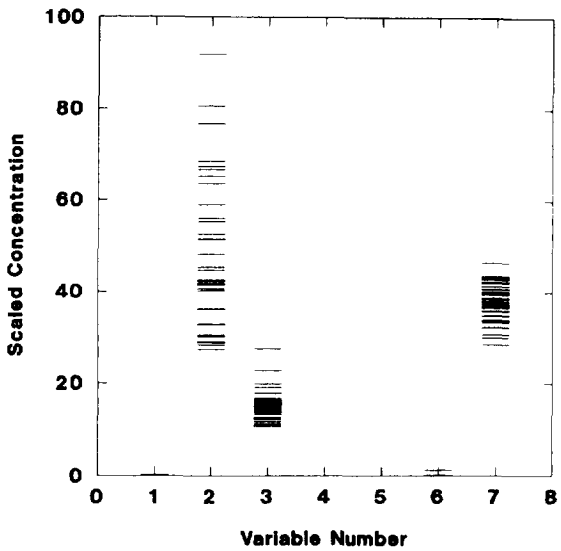

Fig. 3. Plot of scaled concentration values for seven different impurities in a bulk pharmaceutical from a manufacturer other than that in Fig. 2. Each short horizontal line corresponds to a scaled concentration value.

pressed in terms of 7 analyte concentrations, with approximately 40 samples (manufacturing lots) from each manufacturer. Each short horizontal line represents the scaled sample concentration value for a select analyte. Clearly there are significant differences between the two classes, particularly with respect to variables 3, 4 and 5 where the analyte is present in one manufacturer's product and essentially absent in the other's. Variables 1 and 6 show minimal response on either figure, but were significant for certain other manufacturers whose plots are not shown. The diagrams can be expanded further by connecting values in each category which are obtained from the same lot of material. This may be useful but it complicates the picture. The independence of the values in each analyte class can be assessed by correlation tests.

5.2. Classification

The second goal of data treatment is the assignment of unknown samples to correct classes. The class structure may be dichotomous (authentic vs. counterfeit) or polychotomous (manufacturer A, B, C, etc.). A number of classification approaches will be described below. Generally, the relationship between impurity profile and class membership is established using a training set consisting of samples with known identities. Regardless of the classification method employed, it is critical that results be subjected to validation with a suitable test set. The test set consists of samples with known class identities which are treated as though their class memberships were unknown. The accuracy of the chosen classification method can thus be assessed. It is important that members of the test set are distinct from the training set. The use of training set results to evaluate classification accuracy yields a false sense of confidence in the method being used [38].

5.2.1. Univariate statistics

Occasionally a single chemical component is adequate for differentiating between groups of samples, in which case a traditional statistical approach is called for [39]. Typically this involves parametric tests such as Student's *t*-test but non-parametric methods can be used also. A univariate classification system is desirable because of its simplicity; however, it is unlikely that a single variable will produce non-overlapped clusters for more than a few different classes of samples.

5.2.2. Linear discriminant analysis

PCA maximizes the amount of information retained while reducing dimensionality for easier visualization. It treats the data globally, without regard to class membership (such as the identity of the manufacturer). Discriminant analysis [40,41] also finds new axes for representing the multivariate data set. However, discriminant analysis serves not to maximize retained information, but to maximize instead the separation between the groups in the data. It therefore utilizes knowledge of group membership in finding new axes which are again linear combinations of the original variables. Discriminant analysis enhances group separation relative to PCA. Because plots can be generated showing the location of samples in a discriminant space defined by the new axes, discriminant analysis serves not only as a classification method but as a visualization tool as well. Like PCA, it sacrifices ease of interpretability because the axes are mathematical constructs and not

the actual analyte concentrations employed in simple scatterplots.

Unknowns are assigned to classes based upon their distance in discriminant space from the means (centroids) of each manufacturer's cluster. In order to evaluate the usefulness of such a technique in classifying new samples, it must be validated by withholding a certain percentage of the samples as a test set, treating them as unknowns, and noting whether they are assigned correctly.

5.2.3. Soft independent modeling of class analogy

Soft independent modeling of class analogy (SIMCA) is a more sophisticated classification approach which is based upon, but goes beyond, PCA [42,43]. In SIMCA, a separate principal components model is generated for each class of samples. Diagnostic tools are used to find the optimal number of factors to retain and to detect class members which may be outliers. Unknowns are classified with regard to how well they fit the model for each class and membership probabilities can be obtained. SIMCA does not force unknowns into a class: non-membership in all classes is a possibility.

5.2.4. k-nearest neighbors

A multivariate classification approach which is appealing because of both its simplicity and its effectiveness is the *k*-nearest neighbors method (*k*NN). The basic premise behind this technique is that the identity of an unknown sample can be obtained by examining the identities of its *k*-nearest neighbor samples [44].

The data set generally consists of a set of samples of known class membership, each measured with regard to n variables. These variables may be the concentrations of n volatile components determined in the set. Thus, an n-component vector of concentrations exists for each sample. The euclidean distance from an unknown sample's vector to that of each of the knowns is calculated in n-dimensional space. The k-closest known samples are examined, where k is typically a small integer such as 3. If the majority of these k neighbors belong to a particular class, the unknown is assigned to that class as well. In a sense, the algorithm is examining the point density for each known class at the site of the unknown sample.

Despite its simplicity *k*NN is a powerful classification tool, but its non-parametric nature does not allow assessment of the degree of confidence in a given classification. Generally, its overall performance is evaluated by cycling through the set of knowns, treating each consecutively as an unknown, and observing the percentage of correct class assignments.

5.2.5. Artificial neural networks

The artificial neural network (ANN) is a relative newcomer to multivariate classification. A large variety of networks have been developed to solve problems of classification, optimization, prediction, etc. [45,46]. The method is named because its assembly of interconnected processing units bears resemblance both in form and function to a simple biological nervous system. These processing units, or neurodes, which are mathematical rather than physical, treat incoming signals according to specified operations and thereby pass along an output signal. These neurodes are interconnected through weights which change their value as the system learns. The back propagation network has been the one most utilized in chemical pattern recognition.

Learning consists of providing the network with the input pattern (here, analyte concentrations) and corresponding class membership for known samples in the training set. The network adjusts its connection weights slightly after each iteration, learning gradually to produce the expected class membership when given the pattern of analyte concentrations. When training is complete, and this may entail thousands of passes through the training set, system performance is evaluated by consecutively inputting the known samples in the test set. The trained network produces a class membership for each input pattern and the accuracy of these assignments can be determined. If performance is satisfactory, the trained network is given unknowns to classify.

The chief advantage of the artificial neural network is its ability to successfully separate classes where linear classification methods fail. The effective boundary between classes need not be a line, plane, or hyperplane but may assume as complex a shape as necessary. However, training time can be lengthy and the network does not converge to exactly the same solution each time it is trained. The

interpretability issue which is a minor problem with linear discriminant analysis is more of a concern here: it is frequently difficult to interpret the role of each original analyte concentration in the separation scheme.

6. Conclusion

In conclusion, the object of the foregoing discussion has been to emphasize the important role that the analysis of organic volatile impurities can play in the detection of the unapproved manufacture and distribution of pharmaceuticals. Among a variety of formally endorsed methods, automated equilibrium headspace sampling with capillary gas chromatography offers a robust and reliable approach to the analysis of OVIs when adequate attention is paid to issues of calibration. Conjoining the technique with a mass spectrometer gives an added dimension of qualitative power which is important in a forensic setting. Finally, special statistical techniques such as those which are discussed above are often required for decision making when a variety of OVIs (or any impurities for that matter) are present.

References

[1] M. Perkal, Y.L. Ng and J.R. Pearson, Forensic Sci. Int., 69 (1994) 77.
[2] H. Neumann, Forensic Sci. Int., 69 (1994) 7.
[3] L.A. Kaine, D.T. Heitkemper, D.S. Jackson and K.A. Wolnik, J. Chromatogr. A., 671 (1994) 303.
[4] C.L. Flurer and K.A. Wolnik, J. Chromatogr. A., 674 (1994) 153.
[5] K.A. Wolnik, D.T. Heitkemper, J.B. Crowe, B.S. Barnes and T.W. Brueggemeyer, J. Anal. At. Spectrom., 10 (1995) 177.
[6] D.R. Morello and R.P. Meyers, J. Forensic Sci., JFSCA, 40(6) (1995) 957.
[7] G. Wynia, P. Post, J. Broersen and F.A. Maris, Chromatographia, 39 (1994) 355.
[8] "General Method for the Determination of Residual Solvents in Pharmaceutical Substances", Pharmeuropa, 5 (1993) 145.
[9] P. Billot and B. Pitard, J. Chromatogr., 623 (1992) 305.
[10] I.D. Smith and D.G. Waters, Analyst, 116 (1991) 1327.
[11] "Organic Volatile Impurities", Pharm. Forum, 14 (1988) 3601.
[12] J.E. Haky and T.M. Stickney, J. Chromatogr., 321 (1985) 137.
[13] "Organic Volatile Impurities", Pharm. Forum, 14 (1988) 4115.
[14] "Organic Volatile Impurities - Initial List of Monographs to Which OVI Requirements may be Applicable", Pharm. Forum, 15 (1989) 5256.
[15] D.W. Foust and M.S. Bergren, J. Chromatogr., 469 (1989) 161.
[16] "Organic Volatile Impurities", The United States Pharmacopeia XXII Revision, Supplement 3, The United States Pharmacopeial Convention, Rockville, MD, 1990, pp 2395–2397.
[17] "Organic Volatile Impurities", The United States Pharmacopeia XXII Revision, Supplement 4, The United States Pharmacopeial Convention, Rockville, MD, 1990, pp 2508–2510.
[18] T.D. Cyr, R.C. Lawrence and E.G. Lovering, Pharm. Forum, 16 (1990) 129.
[19] T.K. Chen, W. Moeckel and H.L. Suprenant, Pharm. Forum, 17 (1991) 1475.
[20] "Organic Volatile Impurities", The United States Pharmacopeia XXII Revision, Supplement 5, The United States Pharmacopeial Convention, Rockville, MD, 1991, pp 2707–2708.
[21] J.A. Krasowski, H. Dinh, T.J. O'Hanlon and R.F. Lindauer, Pharm. Forum, 17 (1991) 1969.
[22] L. Clark, S. Scypinski and A.-M. Smith, Pharm. Forum, 19 (1993) 5067.
[23] "Organic Volatile Impurities", The United States Pharmacopeia XXII Revision, Supplement 8, The United States Pharmacopeial Convention, Rockville, MD, 1993, pp 3352–3355.
[24] "Organic Volatile Impurities", Pharm. Forum, 19 (1993) 4917.
[25] "Organic Volatile Impurities", The United States Pharmacopeia XXIII Revision, The United States Pharmacopeial Convention, Rockville, MD, 1994, pp 1746–1748.
[26] K.J. Dennis, P.A. Josephs and J. Dokladalova, Pharm. Forum, 18 (1992) 2964.
[27] J.P. Guimbard, M. Person and J.P. Vergnaud, J. Chromatogr., 403 (1987) 109.
[28] W.C. Kidd III, Pharm. Forum, 19 (1993) 5063.
[29] B. Kolb, Pharm. Forum, 20 (1994) 6956.
[30] V.J. Naughton, Pharm. Forum, 20 (1994) 7223.
[31] M. De Smet, K. Roels, L. Vanhoof and W. Lauwers, Pharm. Forum, 21 (1995) 501.
[32] M.V. Russo, Chromatographia, 39 (1994) 645.
[33] Z. Penton, J. High Resolut. Chromatogr., 17 (1992) 329.
[34] K.J. Mulligan and H. McCauley, J. Chromatogr. Sci., 33 (1995) 49.
[35] W.S. Cleveland, The Elements of Graphing Data, Wadsworth and Brooks/Cole, Pacific Grove, CA, 1985.
[36] J.E. Jackson, A User's Guide to Principal Components, Wiley, New York, 1991.
[37] I.T. Joliffe, Principal Components Analysis, Springer Verlag, New York, 1986.
[38] S.M. Weiss and C.A. Kulikowski, Computer Systems That Learn, Morgan Kaufmann, San Mateo, CA, 1991, Chapter 2.

[39] G.J. Hahn and W.Q. Meeker, Statistical Intervals: A Guide for Practioners, Wiley, New York, 1991.
[40] B. Flury and H Riedwyl, Multivariate Statistics–a Practical Approach, Chapman and Hall, London, 1988, Chapter 8.
[41] W.R. Dillon and M. Goldstein, Multivariate Analysis–Methods and Applications, Wiley, New York, 1984, Chapters 10 and 11.
[42] M.A. Sharaf, D.L. Illman and B.R. Kowalski, Chemometrics, Wiley, New York, 1986, pp. 242–254.
[43] O.M. Kvalheim and T.V. Karstang, "SIMCA–Classification by Means of Disjoint Cross Validated Principal Components Models" in R.G. Brereton (Editor), Multivariate Pattern Recognition in Chemometrics, Illustrated by Case Studies, Elsevier, Amsterdam, 1992.
[44] M. Nadler and E.P. Smith, Pattern Recognition Engineering, Wiley, New York, 1993, pp. 372–375.
[45] M. Smith, Neural Networks for Statistical Modeling, Van Nostrand-Reinhold, New York, 1993.
[46] J. Zupan and J. Gasteiger, Neural Networks for Chemists–An Introduction, VCH, New York, 1993.