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Evaluation of Principal Components Analysis with High-Performance Liquid Chromatography and Photodiode Array Detection for the Forensic Differentiation of Ballpoint Pen Inks

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ABSTRACT: Inks from seven black and eight blue ballpoint pens were separated by a high-performance liquid chromatography (HPLC) method utilizing a photodiode array detection (PDA). A classifier flowchart was designed for the chromatographic data based on the presence or absence of certain peaks at different wavelengths to qualitatively discriminate between the inks. The same data were quantitatively classified by principal components analysis (PCA) to estimate the separation between a pair of classes of ink samples. It was found that the black ballpoint pen inks were discriminated satisfactorily utilizing two-dimensional data of the peak areas and retention times at the optimum wavelengths. The blue pens were discriminated by analyzing the chromatographic data at four different wavelengths simultaneously with a cross-validated PCA. The results of this study indicated that HPLC-PDA coupled with chemometrics could make a powerful discriminating tool for the forensic chemist, especially when analyzing extensive and/or complex data.

KEYWORDS: forensic science, chemometrics, principal components analysis, ballpoint pen inks, classification, high-performance liquid chromatography.

The authenticity of a questioned document may be examined through the analysis of the inks used to prepare the document (1). Inks are considered as evidence in forensic cases, as the manufacturing involves a large-scale blend of chemicals (2). Although it is very difficult to determine whether an individual pen was used to write a document, it is feasible to identify the ink manufacturer. This requires some kind of classification or discrimination between the different ink formulations. In this work, the classification of ballpoint pen inks with a traditional approach using flow charts has been compared with multivariate chemometric strategies using PCA.

Ballpoint pen inks are a complex mixture of about 45% dyes with the remaining 55% being contributed by solvents and additives such as resins and organic acids (3). Lyter (4) found that though the resins and organic acids of the ballpoint pen inks were not always readily detectable, the dyes were sufficiently detectable to provide a satisfactory discrimination between the different ballpoint pen ink formulations.

A number of analytical techniques have been applied to the analysis of inks. Thin layer chromatography (TLC) is the simplest of those methods and is effective for separating dyestuff components (1,5). Fourier transform infra red (FTIR) (5) spectroscopy, microspectrophotometry (6) and X-ray emission analysis (7) of printing inks has also been reported. Capillary electrophoresis has been successfully employed by Vogt and Rodhe et al. for the separation of fountain pen inks (2,8). The analysis of inks using HPLC has been attempted by many (4,9,10). The HPLC method used in this work to separate the ballpoint pen ink components has been modified on the basis of Lyter's work (4) with the added advantage of using a diode array detection, as writing inks contain compounds detectable at different wavelengths.

As forensic chemists have access to increasingly complex methods of analysis capable of generating multidimensional data, it is becoming more important to examine methods of efficiently extracting useful forensic conclusions from the data. One area of data analysis is classification, which can be realized using induction, pattern recognition, or statistical methods of comparison. Each of the above methods aims at building a predictive model for a class of objects. The aim of this project was to build and test classification models for ballpoint pen inks.

Pattern recognition methods have been applied to address classification problems in chemical analysis, environmental chemistry, food science, pharmaceuticals, and biological sciences. Their areas of application continue to grow. These methods include PCA (11,12), principal components regression (PCR) (13,14), discriminant analysis (DA) (14–16), partial least squares regression (PLS) (13,17), and K-nearest neighbour (KNN) (18). PCA has been used to analyze instrumental data gathered from a variety of techniques like liquid chromatography (18), near infrared spectroscopy (NIR) (11,12,16,19), gas chromatography (15), and instrumental neutron activation analysis (17). Data analysis techniques such as PCA, PCR, and DA are beginning to find applications in the forensic science area. Synthetic fibers have been classified using Raman microprobe spectroscopy and PCA (20). PCA together with PLS have been used to discriminate between hard ivories, soft ivories, and mammoth tusks by nondestructive techniques, for instance, NIR Fourier transform Raman spectroscopy (21). The forensic determination of carboxyhemoglobin in blood was performed using PCR applied to UV-visible spectra (22). DA was used in the analysis of sexual dimorphism in the Thai femur (23) and for the sex determination of the petrous portion of the temporal bone (24).

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Materials and Methods

Reagents

HPLC grade acetonitrile and methanol was purchased from Selby-Biolabs, Victoria, Australia. Milli-Q Water from Millipore filtration system was used.

Pen Samples and Extraction of Inks from Documents

Eight blue ballpoint pens and seven black ballpoint pen varieties were chosen for analysis and labeled as S1-S15. Six pens of each variety were purchased and two injections of each ink were assayed, resulting in a total of 12 chromatograms for each pen variety, which are as listed in Table 1.

The ink from these 90 pens was extracted and applied to several sheets of blank Victoria copy paper from the same ream to reduce the interference from the paper support. Each blank sheet of paper was cut into nine equal portions on which continuous lines of words were written by one ballpoint pen sample. Fifty millilitres of 80% acetonitrile were used as an extracting solvent. The sample size was chosen on the basis of previous work by Lyter. Lyter used a micro-hole punch with an internal diameter of 1.8 mm. Utilizing ten of these plugs with 50 mL of the extracting solvent results in a sample size of 18 mm/50 mL. To maintain the same sample size in this work, since a hole punch of internal diameter of 6 mm was used, only three plugs were utilized with 50 mL of the solvent. Each ink sample was sonicated with the extraction solvent for 2 min to maximize the amount of ink extracted.

HPLC Equipment and Conditions

All HPLC work was carried out on a Watersð 2690 separations module with a Watersð 996 PDA detector. The software management system used to operate, collect, and manipulate data was Waters MassLynx version 3.3. The column used for this work was a Watersð Nova-Pak C18 150Ç 3.9 mm. The sample injection volume was 20 mL. A gradient of 0 to 100% acetonitrile was run for 15 min at a flow rate of 1.0 mL/min to separate the ink components. The Alliance HPLC machine was primed (drawing solvent through the solvent line) before each run to remove any air bubbles. Fresh solvents were collected and filtered through 0.45 μm each day using a Duopore filtration system.

TABLE 1—*Sample identification.*

Sample ID	Description	Color
S1	UniBall Laknock	Blue
S2	Zebra Rubber 1.0	Blue
S3	Pilot BP-S	Blue
S4	Pilot BPS-GP	Blue
S5	Bic Soft-feel Rubber	Blue
S6	Bic Crystal	Blue
S7	Bic Cliptop Retractable	Blue
S8	UniBall SA-S	Blue
S9	UniBall Laknock	Black
S10	Pilot BP-S	Black
S11	Pilot BPS-BP	Black
S12	Bic Soft-feel Rubber	Black
S13	Bic Crystal	Black
S14	Bic Cliptop Retractable	Black
S15	UniBall SA-S	Black

Statistics

The chromatographic data were saved in Excel prior to importing to Sirius version 6.52. The chromatographic information was condensed to a matrix ($n \times p$). The n rows represented each peak area for the corresponding p retention times for the black inks and the wavelength retention time for blue inks.

A max plot is a chromatogram based on the optimum wavelength for each component. Although it represents a sum of all the peaks, it is unable to correlate the occurrence of a peak at a particular wavelength, which limits the information from it to only two dimensions, the peak area and retention time. The max plot chromatogram of black inks succeeded in classifying them, but it failed to provide a satisfactory classification for the blue inks. This necessitated the extraction of the chromatogram of blue inks at four different wavelengths—254, 279, 370, and 400 nm. A three-dimensional data of blue pens including wavelength, retention time, and peak areas provided a better classification. The same chromatographic method was used for the analysis, which meant that the retention time specified the approximate identity of a particular compound in a given ballpoint pen.

Data Pretreatment and Analysis

The max plot data of black pens was normalized to equalize the amounts of sample analyzed at different wavelengths. Each subset in the data was mean centered and autoscaled. Since the blue pen data was three dimensional, it was not needed to equalize the variance between the different wavelengths; hence, these data were not normalized. However, it was mean centered and standardized to make it comparable within every individual wavelength.

The data were stratified into subsets depending on the 15 pen types originally purchased. Each subset in every data set was then tested for the presence of outliers. No outlying observations were detected in this work. PCA was performed on each subset using the cross validation option. The cross validation algorithm remodels the same subset a number of times, each time leaving out one observation and using the same to test how well it fits in the predicted model. This leave-one-out validation procedure iterates until every object in each subset is used for validation.

PCA is a common method of classification that can place similar objects into clusters that can be visualized with the score plots. PCA reduces the large number of data points in the original series of chromatograms to fewer variables that contain all of the information in the original data. These variables are known as latent variables or principal components. A score plot is a visualization of the individual samples (in this case ballpoint pen inks) as they are represented for two of these principal components. If they are well separated on a score plot, a satisfactory ink classification has been achieved.

The class distances provided by the PCA were used to evaluate the discriminating power of the pens. The class distance is a measure of the discriminating power between each pair of inks using a single numerical value. It is a numerical estimate of the distance between the inks in terms of the principal components. A value of less than 1 implies that there was insufficient information to discriminate between a pair of inks. A value of 1 to 3 implies some degree of discrimination, and a value of greater than 3 represents a satisfactory discrimination between the different classes.

Results and Discussion

The max plot chromatograms for the black pens—Bic Cliptop Retractable and Pilot BP-S—are as shown in Fig. 1.

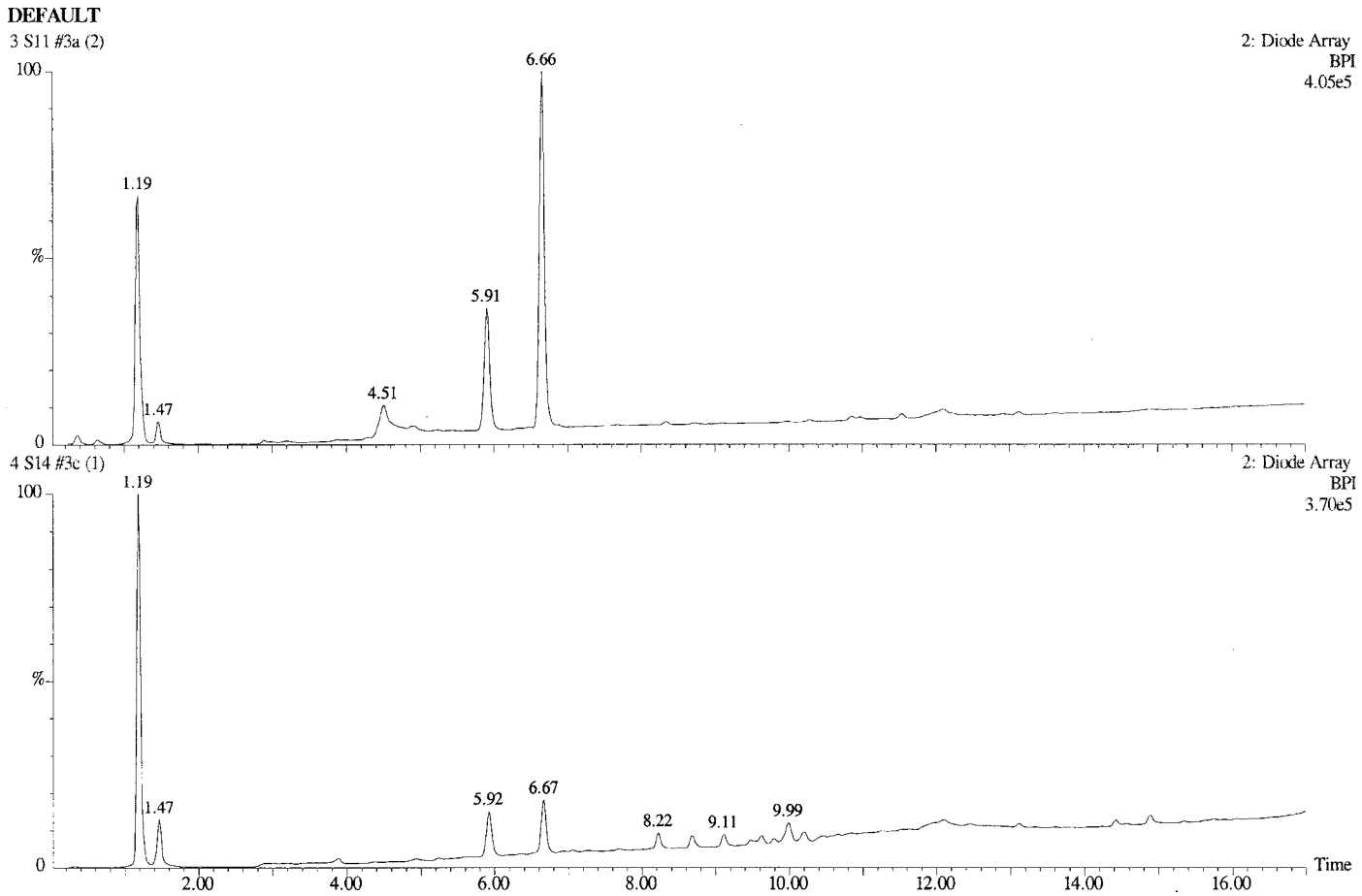

FIG. 1—*Chromatogram of different black pens.*

In this work the flow chart classification of ballpoint pen inks was compared with the classification produced by the application of the PCA algorithm to the data.

Classification Flow Chart

Flow charts were constructed on the basis of the visual comparison of peaks in the ink chromatograms. The classification was achieved by considering the presence or absence of a chromatographic peak at a particular retention time or wavelength-retention time combination, for instance, is there a peak at 3.48 min in the 400 nm chromatogram? Yes or No. The max plot data in case of black ballpoint pens were used for the construction of flow charts as shown in Fig. 2.

The flow charts were validated by subjecting the 84 chromatograms of the seven different black pens to the chart. Table 2 summarizes the validation results of the black ballpoint pen types; it can be seen that the poorest classification was for the Pilot BPS-BP, where only 77% were successfully predicted. All other pens were classified within 80 to 100% success rates. These results indicate that the majority of black ballpoint pens were classified correctly with a high degree of certainty, indicating a high degree of probability that an unknown pen ink would also classify correctly.

The flowcharts classified black ballpoint pen inks into five distinct groups:

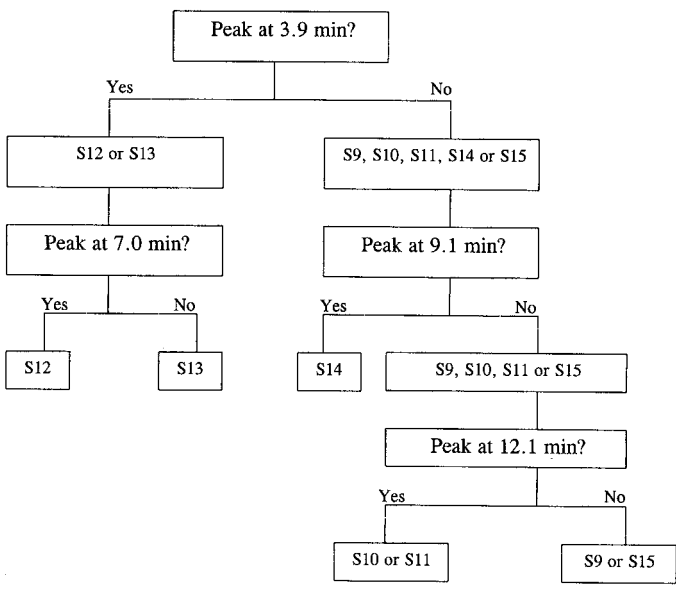

FIG. 2—*Classification flowchart for black ballpoint pens.*

ñ Pilot BP-S and Pilot BPS-BP
ñ UniBall Laknock and UniBall SA-S
ñ Bic Soft-feel Rubber
ñ Bic Crystal
ñ Bic Cliptop Retractable

TABLE 2—*Summary of the prediction for the black inks.*

Sample	210 to 799 nm
UniBall Laknock	83%
Pilot BP-S	83%
Pilot BPS-BP	77%
Bic Soft-feel Rubber	100%
Bic Crystal	100%
Bic Cliptop Retractable	100%
UniBall SA-S	100%

The flowcharts were successful in classifying the three Bic black ballpoint pen types individually, whereas they could not provide discrimination between the Pilot and the UniBall pens, indicating that the flowcharts could distinguish between the black pens of different manufacturers but not essentially the same manufacturer.

The chromatograms at four different wavelengths 254, 279, 370, and 400 nm were compared for the blue ballpoint pen inks and flow charts were designed both on the basis of single wavelength analysis as well as with a combination of all the four wavelengths simultaneously. The flow chart for the blue pens at a combination of all the four wavelengths is shown in Fig. 3.

The validation results of blue ballpoint pens are tabulated in Table 3. It can be seen from Table 3 that, apart from the pen Bic Crystal at 370 nm, the majority of the pen chromatograms were correctly classified by the flow chart. From the high level of certainty seen, it would be expected that unknown pen ink could be correctly identified using this flow chart classification method.

Flowcharts designed for the blue ballpoint pens at 254, 279, and 400 nm produced the same classification. At these wavelengths, the eight blue pens were discriminated into five groups as:

ñ UniBall Laknock and UniBall SA-S
ñ Pilot BP-S and Pilot BPS-GP
ñ Bic Crystal and Bic Cliptop Retractable
ñ Zebra Rubber 1.0
ñ Bic Soft-feel Rubber

Again here it was demonstrated that except for the three Bic blue ballpoint pens, which were shown to be partially discriminated into pen types, at a majority of the wavelengths, none of the other Pilot and UniBall pens could be discriminated, limiting the characterization of the blue pens only up to a manufacturer level at the above three wavelengths. Flowcharts produced a significant differentiation of blue ballpoint pens at 370 nm where, except for the two Pilot pens, the Pilot BP-S and the Pilot BPS-GP, all the pens were shown to be discriminated individually.

The classification produced at the combination of all four wavelengths for the blue ballpoint pens enabled the differentiation of all the pen types except the two Bic pens, the Bic Crystal, and the Bic Cliptop Retractable. It was found that these two pen types were separable only by the flowchart at 370 nm with a distinguishing peak at 10.3 min. Analyzing the flowcharts at both 370 nm and at a combination of all the four wavelengths the blue ballpoint pens could be successfully discriminated.

PCA Classification of Ballpoint Pens

The classification of black ballpoint pens produced with the PCA algorithm identified four groups:

ñ UniBall Laknock, Pilot BP-S and Bic Crystal
ñ Pilot BPS-BP and Bic Soft-feel Rubber

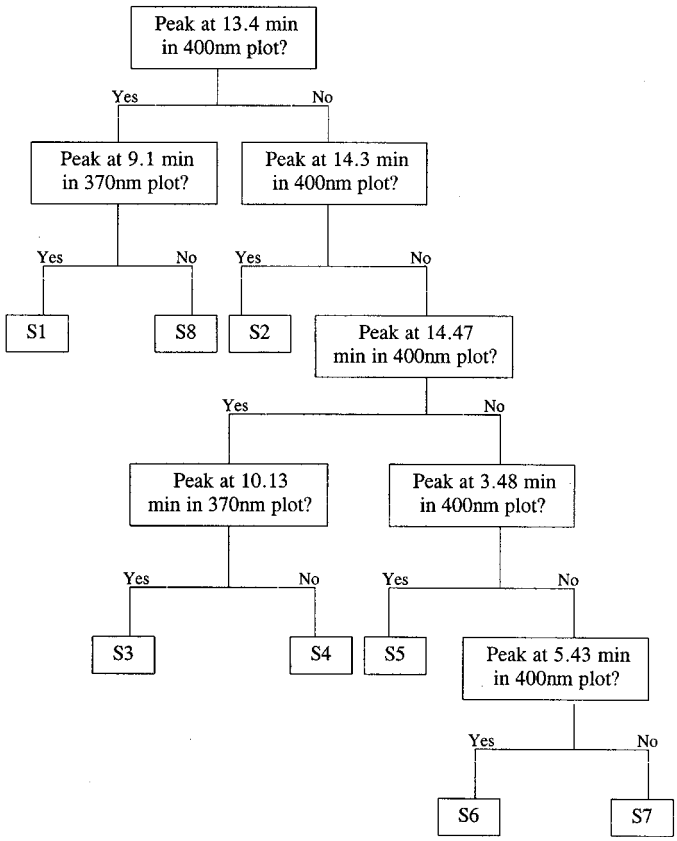

FIG. 3—*Classification flowchart for blue ballpoint pens at a combination of all the four wavelengths—254, 279, 370 and 400 nm.*

TABLE 3—*Summary of the predictions for the blue inks at 254, 279, 370, and 400 nm.*

Sample	254 nm	279 nm	370 nm	400 nm	Total
UniBall Laknock	92%	100%	67%	83%	100%
Zebra Rubber 1.0	83%	100%	100%	75%	100%
Pilot BP-S	92%	100%	100%	100%	100%
Pilot BPS-GP	100%	100%	100%	100%	100%
Bic Soft-feel Rubber	83%	83%	83%	83%	83%
Bic Crystal	100%	100%	50%	92%	100%
Bic Cliptop Retractable	100%	100%	100%	100%	100%
UniBall SA-S	83%	100%	100%	100%	100%

ñ UniBall SA-S
ñ Bic Cliptop Retractable

These results indicate that PCA could differentiate between pens of the same manufacturer but not necessarily between those of different manufacturers. This could be due to different manufacturers using similar or the same ink components.

The class distances for black pens are as listed in Table 4; it can be seen that the distance is less than 3 for the inks UniBall Laknock with Pilot BP-S and Bic Crystal and for the pair Pilot BPS-BP and Bic Soft-feel Rubber. All other class distances are greater than 3, indicating a satisfactory discrimination between the pens.

The wavelength of 254 nm differentiates the eight blue pens into six groups. The two pairs of pens, which do not get separated at this wavelength, are: the two Pilot pens; Pilot BP-S and Pilot BPS-GP and the two Bic pens; Bic Crystal and the Bic Cliptop Retractable.

TABLE 4—*Class distances for the max plot data of black ballpoint pen inks.*

	S9	S10	S11	S12	S13	S14	S15
S9	. . .	2.619	7.794	4.533	1.988	6.244	7.576
S10	2.619	. . .	4.127	4.064	4.302	8.204	5.656
S11	7.794	4.127	. . .	2.694	5.888	5.005	5.578
S12	4.533	4.064	2.694	. . .	6.435	6.589	5.725
S13	1.988	4.302	5.888	6.435	. . .	4.922	10.685
S14	6.244	8.204	5.005	6.589	4.922	. . .	11.074
S15	7.576	5.656	5.578	5.725	10.685	11.074	. . .

TABLE 5—*Class distances for the blue ballpoint pen inks at a combination of all the four wavelengths.*

	S1	S2	S3	S4	S5	S6	S7	S8
S1	. . .	3.232	8.999	11.080	14.702	16.497	9.381	9.489
S2	3.232	. . .	9.385	8.344	9.854	12.890	13.831	10.102
S3	8.999	9.385	. . .	7.930	9.228	9.141	7.223	5.897
S4	11.080	8.344	7.930	. . .	8.740	14.588	9.293	13.975
S5	14.702	9.854	9.228	8.740	. . .	15.373	15.115	15.930
S6	16.497	12.890	9.141	14.588	15.373	. . .	1.981	18.134
S7	9.381	13.831	7.223	9.293	15.115	1.981	. . .	15.839
S8	9.489	10.102	5.897	13.975	15.930	18.134	15.839	. . .

The classification produced at 279 nm is similar to that with the flowcharts, except that PCA classifies the Zebra pen with the above two Pilot pens. In the flowchart, however, the Zebra pen is shown to be separated from the two Pilot pens by the presence of a distinguishing peak at a retention time of 14.4 mins. At 370 nm and at a combination of all the four wavelengths PCA can identify all the blue ballpoint pens individually except the two Bic pens, the Bic Crystal, and the Bic Cliptop Retractable, which remain inseparable with the PCA classification.

The class distance values for blue pens at the four wavelengths simultaneously are tabulated in Table 5. It was clear that the only class distance less than 3 was observed between the Bic Crystal and the Bic Cliptop Retractable.

An interesting situation was found to develop at a wavelength of 400 nm for the discrimination of blue pens. The flow charts at a combination of all the four wavelengths feature two wavelengths predominantly, the 370 nm and the 400 nm. The class distance readings for 370 nm were accordingly higher and, except for the two Bic pens, all the pens were identified at this wavelength with the PCA classification. At a wavelength of 400 nm, in the case of certain pen pairs as listed below, the software was unable to compute a value for the class distances. This was noticed because Sirius 6.52 clusters similar objects on the basis of any discrepancies detected in the variables. Hence, to achieve a PCA classification, it is an essential prerequisite to have at least two or more common variables with differing values. It was noteworthy that no class distance reading was recorded between the classes exemplified by UniBall Laknock and Bic Crystal, Zebra rubber 1.0 and Bic Crystal, Pilot BP-S and Bic Soft-feel Rubber, Pilot BP-S and Bic Crystal, Pilot BP-S and Bic Cliptop Retractable, and Pilot BP-S and UniBall SA-S. These pens did not have any common variable/s in them at a wavelength of 400 nm to enable them to produce discrimination. This was noticed because the chromatographic peaks eluted at different retention times at 400 nm that indicated the possibility of different components in them, implying that these pen samples were completely discriminated at a wavelength of 400 nm.

Flowcharts Versus PCA Classification for the Ballpoint Pen Inks

The flowcharts identified black ballpoint pens up to a manufacturer level. PCA was able to differentiate between the same brands, but not necessarily different brands, indicating the possibility of different manufacturers using similar ink composition. Using the same data, conflicting results were produced by the flowcharts and the PCA. This is probably because with the max plot data there was a loss of one dimension of information from the wavelength.

PCA succeeded in achieving a better classification at a wavelength of 254 nm as compared with the flowcharts. It is probable that the components eluted from the different pen inks were the same at this wavelength except that they differed in their concentrations across the various brands of inks, which was reflected in a difference in their peak areas. Since similar or the same components were eluted at this wavelength, less difference was justifiably seen in the flowcharts, resulting in a relatively poorer classification at 254 nm with the flowchart. The situation at 400 nm, however, is completely contrasting, where different ink components seem to be separated, resulting in a better classification with the flowcharts than the PCA.

Flowcharts at 370 nm failed to separate the two Pilot pens. These two pens were separated with the PCA. However, PCA failed to discriminate between the two Bic pens, the Bic Crystal, and the Bic Cliptop Retractable at this wavelength. A combination of flowchart classification and the PCA, however, has succeeded in separating all the blue pens at a wavelength of 370 nm. The wavelength combination failed to differentiate between the above two Bic pens with the PCA classification. The separation chemistry of these two pen types is noteworthy because there was a peak at 400 nm at 5.43 min, which could separate the two pens. However, the integration performed indicated that this peak was small and not always detectable. Further experimental work could confirm the presence of this peak.

This shows that though PCA is a good tool for exploring/classifying the data, the visual comparison of the data with the chromatograms provided improved results. This is especially true for the smaller chromatographic peaks. However, if a large number of samples were to be classified, the PCA can automate this process with a minimum loss of information.

Another interesting feature of this classification was that both the black and the blue Bic pens were easily discriminated, at least partially, into their individual pen types, indicating a possibility of a greater variation in their ink composition as compared to the other manufacturers.

Conclusions

The results of this study indicate that it is feasible to use the PCA for the forensic characterization of ballpoint pen inks. This research has demonstrated the usefulness of extracting the chromatographic data at various wavelengths prior to classification. The blue ballpoint pens showed better discrimination compared to the black ones, with the correct identification possible with seven out of eight or nearly 87.5% of blue ballpoint pens. The work done on this project could lead to the construction of a database of pen inks that could be used for the forensic classification of pen inks.

It was possible to design classification flowcharts for this project because only 12 samples of 15 different ink types were analyzed. It would be very confusing and time consuming to do the same for classifying huge sets of chromatographic data, in which case HPLC-PDA coupled with chemometric multivariate strategies would provide a powerful discriminating tool for the forensic chemist.

The application of mass spectrometry (MS) would probably allow the development of a unique fragmentation pattern for every ink sample, which would help in the identification of its components. The introduction of an additional dimension of fragmentation along with the retention time, peak height/area, and wavelength would result in an enhanced discriminating power and hence provide an exclusive classification of pen inks. Thus it is possible that the additional application of MS with the HPLC and the evaluation of other chemometric packages and algorithms could eventually lead to a unique identification of pen inks.

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