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DETERMINATION OF COMPOUND IDENTITY BY GROUP TYPE AND CLASS USING DIODE-ARRAY DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A format for spectral data interpretation employing an absorbance-weighted mean wavelength of a spectrum, labeled the purity parameter™ (Varian), was applied to spectra acquired with high-performance liquid chromatography and a UV photodiode-array detector. The purity parameter format was used to categorize solutes into compound group types or classes sharing similar spectral characteristics. Multiple purity parameters (each covering a different wavelength range) were utilized to span distinctive spectral features of a class. The data format was applied to class determination of petroleum hydrocarbons and drugs.

INTRODUCTION

UV spectral characteristics of a compound have been exploited by high-performance liquid chromatography (HPLC) photodiode-array detectors to confirm peak identification and determine peak homogeneity. The high spectral acquisition rates of photodiode array-detectors (up to *ca.* 25 Hz) provide a large data base from which to extract information on solutes. Methodologies for interpretation of spectral data include both graphical techniques for spectra manipulation and presentation as well as numerical techniques for data reduction.

Commonly employed graphical interpretation techniques include spectral overlays[1,2], isoabsorbance plots[3,4], three-dimensional presentation of wavelength–time–absorbance data[5], and higher-derivative transformation of spectra[6,7]. In general, these methodologies yield a characteristic picture or data profile for qualitative comparison of two or more spectra.

Numerical techniques for spectral data interpretation, such as the absorbance ratio[8,9], yield a singular value which is objectively compared to a standard reference or characteristic value. Information regarding peak identification and homogeneity can be obtained in this manner. One limitation in the use of absorbance ratios for peak homogeneity determination is the proper selection of wavelengths when impurities are unknown. However, such numerical methodologies for data interpretation offer advantages of enhanced objectivity and applicability of statistical treat-

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ments for verification that are lacking in most graphical techniques for spectral data interpretation. Deconvolution techniques such as principal component analysis[10] and factor analysis[11] have also been applied to spectral data in order to determine the number of components in a chromatographic band.

Another numerical approach to spectral data interpretation, the purity parameter™ (Varian) data format, has been recently developed which utilizes an absorbance-weighted mean wavelength of a spectrum to reduce spectral data to a single value[12]. Absorbance weighting minimizes the effect of noise in the calculation. This format yields a single value ($\bar{\lambda}_w$) in units of nanometers which represents a characteristic average wavelength of a spectrum. The calculation of $\bar{\lambda}_w$ is analogous to statistical calculation of the moment of a distribution. The wavelength range over which $\bar{\lambda}_w$ is calculated can be selected to focus on a characteristic absorbance band of a compound spectrum or to enhance discrimination between the spectrum of the compound of interest and that of an impurity.

Any conditions which affect the spectrum acquired on a chromatographic peak will affect the $\bar{\lambda}_w$ value calculated from the spectral data. Changes in chromatographic conditions such as mobile phase buffers, pH or organic modifier content can induce spectral shifts. Similarly, changes in chemical structure of a compound such as functional group changes or branching can cause shifts in spectral data. As with other spectral data formats, these variations will affect $\bar{\lambda}_w$ values calculated using the purity parameter format. However, under identical chromatographic conditions the same compound will yield the same $\bar{\lambda}_w$ value as long as the spectral data is unchanged.

The purity parameter data format can be applied to spectra to confirm chromatographic peak identity and homogeneity as well as to determine compound class or group type. Peak identity can be confirmed by comparison of $\bar{\lambda}_w$ of a peak spectrum with that of a spectrum from a reference compound or standard acquired under the same chromatographic conditions. Peak homogeneity can be ascertained from comparison of the $\bar{\lambda}_w$ values for spectra collected at several points across the elution volume of the peak of interest. The $\bar{\lambda}_w$ values can then be compared with each other to determine peak homogeneity as long as the peak of interest and suspected impurities differ in retention time. For the special case of exact coelution of the peak of interest and an impurity, comparison of $\bar{\lambda}_w$ values of the compound under analysis with that of a standard compound or reference material analyzed under identical chromatographic conditions is required due to the additivity of spectra.

Multiple $\bar{\lambda}_w$ values, each calculated for a different wavelength region of a spectrum, can focus on characteristic absorbance bands or spectral fragments specific to a wavelength region of the UV spectrum. By selecting a series of overlapping wavelength ranges, the spectrum can be mathematically defined in greater detail. $\bar{\lambda}_w$ values, calculated over the following wavelength ranges, have been found useful in focusing on spectral fragments of a group or class of compounds with a common chromophore: 210–249 nm ($\bar{\lambda}_w$-low); 239–278 nm ($\bar{\lambda}_w$-mid); 273–367 nm ($\bar{\lambda}_w$-high). $\bar{\lambda}_w$-mid and $\bar{\lambda}_w$-high values support confirmation of identity and purity even if a low-wavelength UV interference destroys the information content of other calculated $\bar{\lambda}_w$ values. In addition, a 210–367 nm wavelength range ($\bar{\lambda}_w$-broad) was calculated that in many cases is adequate by itself to confirm peak identity and purity. These values with their corresponding wavelength ranges are illustrated in Fig. 1 for the spectrum of theophylline.

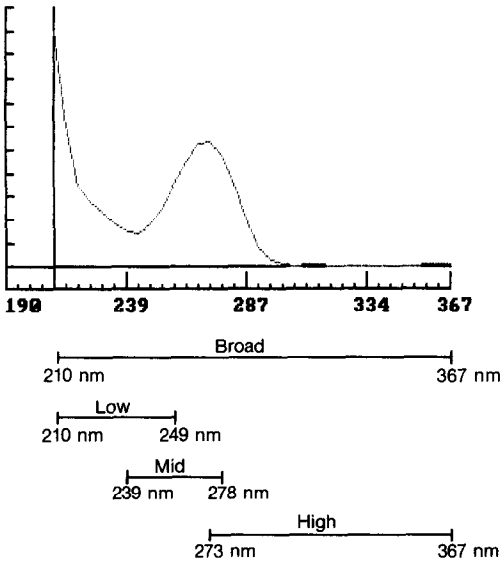


Fig. 1. Spectrum of theophylline standard, acquired at peak apex with multiple purity parameter format $\bar{\lambda}_w$ values. (See Fig. 9 for chromatography conditions under which spectrum was obtained). $\bar{\lambda}_w$-broad = 239.19 nm, $\bar{\lambda}_w$-low = 214.60 nm, $\bar{\lambda}_w$-mid = 267.18 nm, $\bar{\lambda}_w$-high = 277.34 nm.

The purpose of this report is to evaluate the application of the purity parameter data format to determination of compound identity in HPLC separations. Use of multiple $\bar{\lambda}_w$ values focusing on different wavelength ranges allowed accentuation of spectral fragment characteristics for identification of a group or class of compounds. Applications are demonstrated for drug classes and hydrocarbon group types.

EXPERIMENTAL

Instrumentation

A Varian Series 5560 liquid chromatograph with a DS600 chromatography data system (Varian Assoc., Walnut Creek, CA, U.S.A.) was employed for all chromatographic separations and data collection and a Rheodyne (Cotati, CA, U.S.A.) 7125 manual loop valve injector for sample injection. A Varian Polychrom 9060 photodiode array detector coupled to an IBM personal computer with Polysoc (Varian) applications software was used to acquire, manipulate, and store spectral data. A Varian RI-3 refractive-index detector was also employed.

Solvents and chemicals

Acetonitrile, *n*-hexane, *n*-pentane, and methanol were distilled-in-glass solvents obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Buffers were prepared from HPLC-grade potassium dihydrogen phosphate (Fisher, Pittsburgh, PA, U.S.A.). HPLC-grade water was generated with a HydroService water purification system (Varian, Sunnyvale, CA, U.S.A.).

Hydrocarbon standards were obtained from Varian, Aldrich (Milwaukee, WI, U.S.A.), and Fluka (Hauppauge, NY, U.S.A.). Linear isomers of nonane, decane,

undecane, dodecane, tridecane, and tetradecane were employed as alkane standards. Olefin standards consisted of hexene-1, heptene-1, octene-2, and nonene-1. Cyclohexane, methylcyclohexane, and cyclopentane were used as naphthene standards. One-ring aromatics, such as benzene, toluene, *m*-xylene, *p*-cymene, *n*-butylbenzene, hexylbenzene, and ethylbenzene, and two-ring aromatic standards (naphthalene, acenapthene, and acenaphthylene) were also utilized. Samples of gasoline and jet fuel were obtained from round-robin tests conducted by the American Society for Testing and Materials (ASTM). Additional gasoline samples were purchased locally.

Xanthine drug standards (Sigma, St. Louis, MO, U.S.A.) used in this report consisted of theobromine, theophylline and caffeine. Barbiturate drugs employed were aprobarbitol, butalbital, pentobarbital, amobarbital, butabarbital, secobarbital, phenobarbital, mephobarbital, and hexobarbital (Sigma).

Chromatographic procedures

Hydrocarbon samples and standards were diluted, filtered and injected manually onto two MicroPak PONA Analysis (Varian) columns (30 cm × 8 mm each) connected in series. A mobile phase of *n*-pentane or *n*-hexane was employed. Detection was performed initially at a wavelength of 205 nm to allow detection of olefins, followed by a time-programmed wavelength change to 254 nm to coincide with elution of the aromatic hydrocarbons (24 min). Peak apex spectra were acquired automatically with the Polychrom 9060 photodiode-array detector. A refractive-index detector was also used in series with the 9060 photodiode-array detector.

Drug samples were filtered and injected manually into a 15 cm × 4 mm MicroPak PTHAA-5 (Varian) column. A 0.05 *M* phosphate buffer (pH 3.0)–acetonitrile–methanol ternary gradient as described in Fig. 9 was used to achieve separation.

RESULTS AND DISCUSSION

Petroleum hydrocarbon separations

One advantage of HPLC analyses for petroleum hydrocarbons is the ability

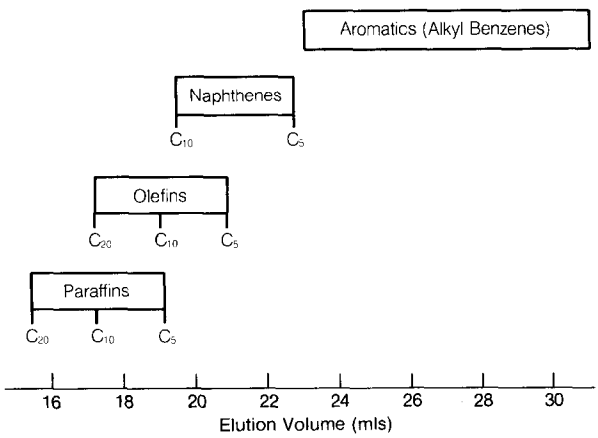


Fig. 2. Selectivity of MicroPak PONA column for various hydrocarbon classes. Columns, 30 cm × 7.5 mm (two columns in series); mobile phase, *n*-hexane; flow-rate, 1.0 ml/min; detection, RI-3 refractive-index detector; sample, hydrocarbon standards.

to achieve group-type separations. Normal-phase packings can be employed to achieve resolution of saturate, olefin, and aromatic group types[13]. For this work, a resin column (MicroPak PONA Analysis column) was utilized that displayed unusual selectivity for saturated hydrocarbons, allowing resolution of paraffin and naphthene (cycloalkane) classes for gasoline boiling-point-range samples. Olefins can also be partially resolved from saturated hydrocarbons and aromatics are strongly retained with resolution of one- and two-ring aromatics[14]. The packing was used in an earlier report from this laboratory on column switching schemes for rapid hydrocarbon group-type separations[15]. Fig. 2 displays the selectivity of the column for various hydrocarbon classes when *n*-hexane is used as mobile phase. Overlap of paraffins, olefins, and naphthenes can occur depending upon the carbon number distribution of each class. The predominant mode of separation is believed to be adsorption while a secondary exclusion mechanism is also in operation among each class of hydrocarbons.

Fig. 3 shows the separation of a synthetic mixture of hydrocarbon standards. Apex spectra (designated by the S number above each peak) were acquired to aid in class determination. The purity parameter data format was employed to calculate $\bar{\lambda}_w$ values for each spectrum over the wavelength regions 190–239 nm (elution volume interval for the olefin group) and 239–316 nm (elution volume intervals for the one- and two-ring aromatics). Figs. 4 and 5 show the separations of supreme unleaded gasoline and jet fuel respectively, under identical conditions as the hydrocarbon standards. Spectra were again collected at the apex of each peak. Note that for the higher boiling range jet fuel the resolution between paraffin, olefin, and napthene classes is diminished. Selected olefin spectra, one-ring aromatic spectra, and two-ring aromatic

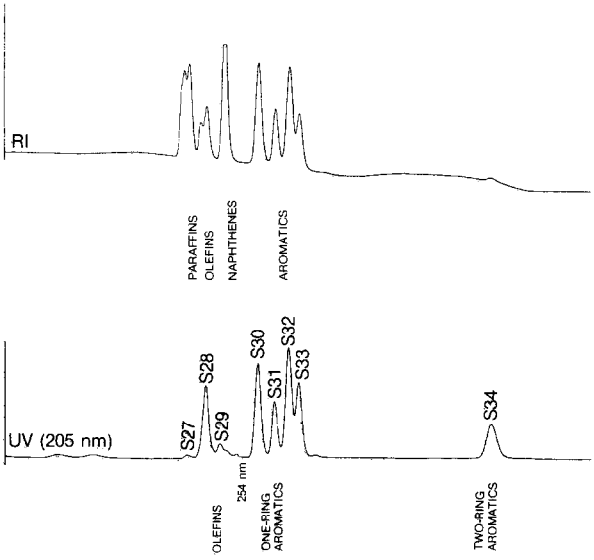


Fig. 3. Analysis of hydrocarbon standards with spectra acquired at peak apices (identified by S number) for olefin and aromatic group types. Columns, MicroPak PONA Analysis (30 cm × 8 mm) (two in series); mobile phase, *n*-hexane; flow-rate, 1.0 ml/min; detection, RI-3 refractive-index detector and Polychrom 9060 diode-array detector in series.

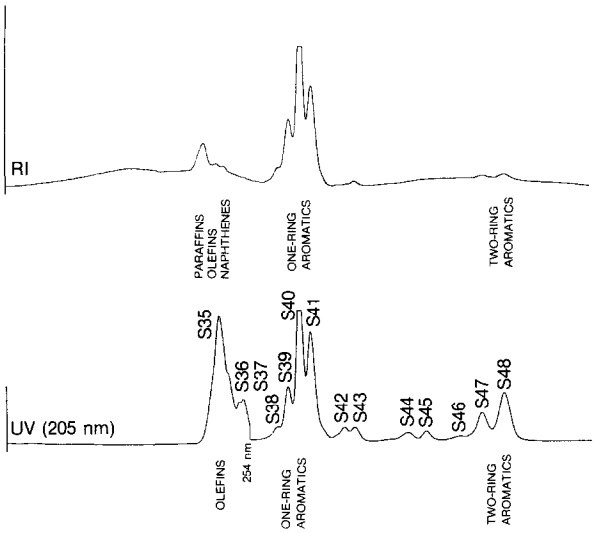


Fig. 4. Analysis of supreme unleaded gasoline sample with spectra acquired at peak apices (identified by S number) for olefin and aromatic group types. Conditions same as Fig. 3.

spectra from separation of the hydrocarbon standards, gasoline, and jet fuel are displayed as spectral overlays in Figs. 6–8 along with the $\bar{\lambda}_w$ values from application of the purity parameter format to all acquired spectra for these separations. Results are summarized in Table I. Olefin spectra yielded a $\bar{\lambda}_w$ value range of 192–195 nm with an average value of 194.0 nm. The one-ring aromatic spectra resulted in $\bar{\lambda}_w$ values ranging from 256 to 266 nm with an average of 261.6 nm. The two-ring aromatic spectra resulted in $\bar{\lambda}_w$ values of 267–271 nm with an average of 269.0 nm. Variation among each set of $\bar{\lambda}_w$ values is the result of bathochromic shifts induced

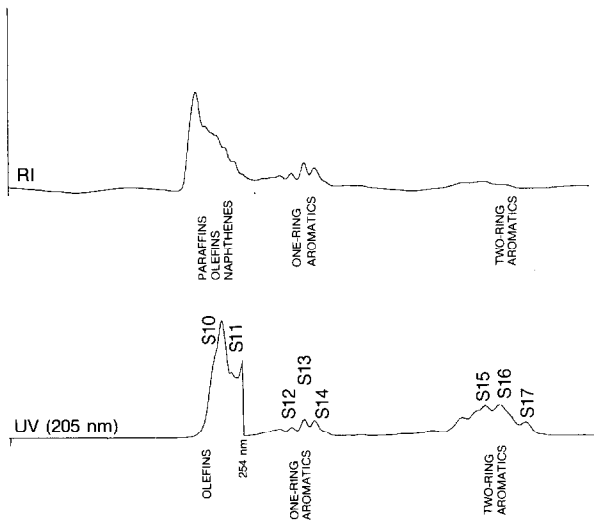
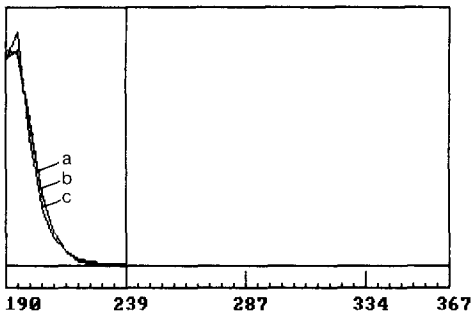


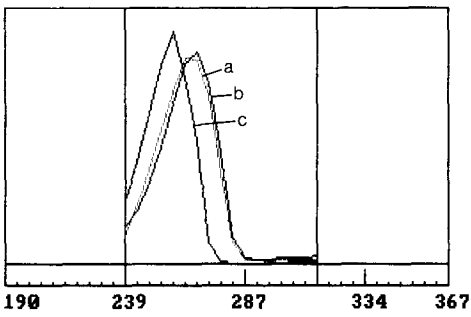
Fig. 5. Analysis of jet fuel sample with spectra acquired at peak apices (identified by S number) for olefin aromatic group types. Conditions same as Fig. 3.



Purity Parameter $\bar{\lambda}_w$ Values
Wavelength Range: 190-239 nm

Jet Fuels	Gasolines	Hydrocarbon Standards
S0: 194.466 nm	S35: 194.723 nm	S27: 192.560 nm
S1: 195.970 nm	S36: 194.121 nm	S28: 193.397 nm
S10: 193.517 nm	S54: 194.027 nm	S29: 194.334 nm
S11: 193.810 nm		

Fig. 6. Comparison of olefin spectra for petroleum samples. Overlay of spectra: a = jet fuel spectrum S10, b = gasoline spectrum S35, c = hydrocarbon standard spectrum S28. Purity parameter data format $\bar{\lambda}_w$ values listed for all peaks in elution volume interval for olefins. Jet fuel samples: spectra S0, S1, S10, S11; gasoline samples: spectra S35, S36, S54; hydrocarbon standards: spectra S27, S28, S29.



Purity Parameter $\bar{\lambda}_w$ Values
Wavelength Range: 239-316 nm

Jet Fuels	Gasolines	Hydrocarbon Standards
S12: 264.995 nm	S39: 263.084 nm	S30: 261.338 nm
S13: 265.138 nm	S40: 263.497 nm	S31: 256.746 nm
S14: 266.659 nm	S41: 258.418 nm	S32: 259.241 nm
S4: 263.705 nm	S56: 264.332 nm	S33: 257.634 nm
S5: 265.208 nm	S57: 264.779 nm	
S6: 264.546 nm	S58: 258.760 nm	
S7: 257.866 nm		

Fig. 7. Comparison of one-ring aromatics spectra for petroleum samples. Overlay of spectra: a, b = jet fuel spectrum S4, S12; c = hydrocarbon standard spectrum S31. Purity parameter data format $\bar{\lambda}_w$ values listed for all peaks in elution volume interval for one-ring aromatics. Jet fuel samples: spectra S12–S14 and S4–S7; gasoline samples: spectra S39–S41 and S56–S58; hydrocarbon standards: spectra S30–S33.

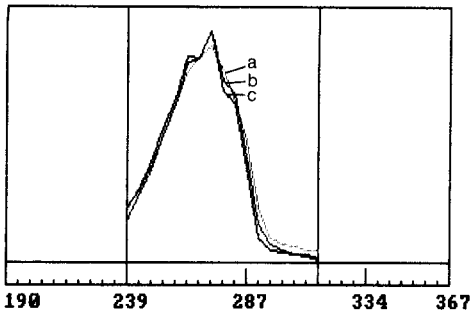


Fig. 8. Comparison of two-ring aromatics spectra for petroleum samples. Overlay of spectra: a, b = gasoline spectra S47 and S48; c = hydrocarbon standard spectrum S34. Purity parameter data format $\bar{\lambda}_w$ values listed for all peaks in elution volume interval for two-ring aromatics. Jet fuel samples: spectra S15–S17; gasoline samples: spectra S47, S48 and S8, S9; hydrocarbon standards: spectrum S34.

by alkyl substitutions on the aromatic rings. Two-ring aromatics give higher $\bar{\lambda}_w$ values than one-ring aromatics due to the additional condensed ring. For the olefins, alkyl substitution also induces a bathochromic shift to absorption at longer wavelengths[16].

Drug analysis

UV spectral acquisition has been found to be an invaluable tool for confirmation of compound identification in toxicological separations[17]. Additionally, determination of peak homogeneity through spectral acquisition can confirm accurate quantitation of drug levels.

Fig. 9 displays a gradient elution separation of several xanthine and barbiturate drugs. The apex spectrum of each peak was automatically acquired and stored during the run. Figs. 10 and 11 show the overlay of selected xanthine spectra and selected barbiturate spectra, respectively, for this separation. The purity parameter data for-

TABLE I

SUMMARY OF PURITY PARAMETER DATA FORMAT $\bar{\lambda}_w$ VALUES FOR CLASS DETERMINATION OF PETROLEUM HYDROCARBON SAMPLES

	Purity parameters (nm)				*Retention time range (min)*
	Low $\bar{\lambda}_w$-value	*High $\bar{\lambda}_w$-value*	*Average $\bar{\lambda}_w$-value*	*Wavelength range*	
Olefins	192	195	194.0	190–239	18–21
One-ring aromatics	256	266	261.6	239–316	26–33
Two-ring aromatics	267	271	269.0	239–316	47–53

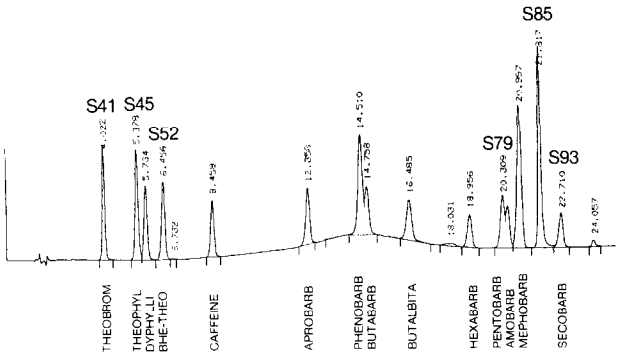


Fig. 9. Analysis of drug standards with spectra acquired at peak apices (identified by S number). Column, MicroPak PTHAA-5 (15 cm × 4.0 mm); mobile phase gradient: A–B (95:5) for 3 min, A–B–C (85:5:10) at 8 min, A–B–C (79:5:16) at 12 min, A–B (82:18) at 16 min, A–B (70:30) at 25 min. A = 0.05 *M* phosphate buffer (pH 3.0), B = acetonitrile, C = methanol. Detector, Polychrom 9060 diode-array at 210 nm. Samples: xanthine and barbiturate standards.

mat was applied to the spectra and $\bar{\lambda}_w$-low, $\bar{\lambda}_w$-mid, $\bar{\lambda}_w$-high, and $\bar{\lambda}_w$-broad values were calculated (each value covering a different wavelength range). Results are summarized in Table II for xanthine drugs. Structural variations in the xanthines involve both the position of the alkyl groups on ring nitrogens as well as the character of the alkyl groups themselves. Spectral overlays (Fig. 10) show that these variations create shifts in spectral maxima without large changes in the band-width or proportionality of the absorbance bands. This is reflected in the multiple $\bar{\lambda}_w$ values calculated for the xanthines. The spectral shifts cause large differences in the $\bar{\lambda}_w$-broad values. Similarity in the band-widths and contours generated excellent agreement among the $\bar{\lambda}_w$-low values (214.21–214.74 nm), $\bar{\lambda}_w$-mid values (267.18–267.77 nm), and the $\bar{\lambda}_w$-high values (277.34–278.31 nm) for this class of compounds.

One characteristic of a numeric interpretation scheme such as the purity parameter format is the ability to statistically evaluate the significance of deviation in the data. In a similar study of xanthine peak purity[18], the $\bar{\lambda}_w$ value for caffeine was statistically evaluated for a wavelength range of 220 nm to 367 nm. A $\bar{\lambda}_w$ value of 258.92 ± 0.10 nm (±2 standard deviations: 95% confidence limit) was obtained for ten repetitive chromatographic runs of a caffeine standard at a 10 μg/ml concen-

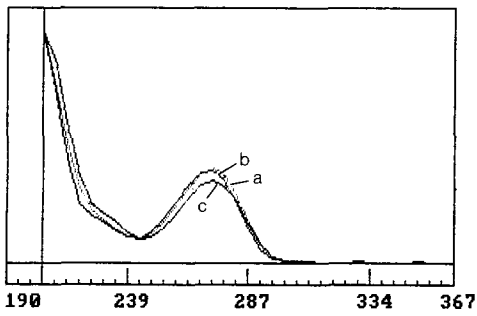


Fig. 10. Comparison of xanthine spectra for drug separation. Overlay of spectra: a = theobromine spectrum S41, b = theophylline spectrum S45, c = β-hydroxyethyltheophylline spectrum S52.

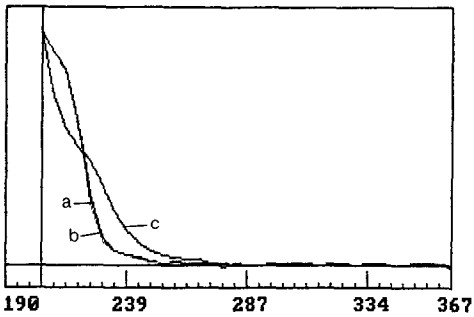


Fig. 11. Comparison of several barbiturate spectra for drug separation. Overlay of spectra: a = pentobarbital spectrum S79, b = secobarbital spectrum S93, c = mephobarbital spectrum S85.

tration. The establishment of this type of statistical confidence interval allows unambiguous determination of peak purity and identification.

Table III summarizes the results of the purity parameter format for interpretation of the barbiturate spectra. The similarity between the $\bar{\lambda}_w$-broad and $\bar{\lambda}_w$-low values for each barbiturate drug confirms the lack of significant absorptivity above 240 nm for this class of compounds (see Fig. 11). A value of "baseline" was recorded for the $\bar{\lambda}_w$-mid and $\bar{\lambda}_w$-high values since all diodes showed absorbances less than 1 ma.u. The N-methyl substitution in hexo- and mephobarbital can be identified by the increased $\bar{\lambda}_w$-broad value for these two drugs (all other barbiturates yielded $\bar{\lambda}_w$-

TABLE II

SUMMARY OF XANTHINE SPECTRAL EVALUATION BY MULTIPLE PURITY PARAMETER FORMAT $\bar{\lambda}_w$ VALUES

	$\bar{\lambda}_w$-*broad (nm)*	$\bar{\lambda}_w$-*low (nm)*	$\bar{\lambda}_w$-*mid (nm)*	$\bar{\lambda}_w$-*high (nm)*
Theobromine	238.45	214.74	267.77	278.00
Theophylline	239.19	214.60	267.18	277.34
Diphylline	230.81	214.63	267.46	278.31
β-Hydroxyethyltheophylline	231.65	214.58	267.45	278.19
Caffeine	232.28	214.21	267.60	277.89

TABLE III

SUMMARY OF BARBITURATE SPECTRAL EVALUATION BY MULTIPLE PURITY PARAMETER FORMAT $\bar{\lambda}_w$ VALUES

	$\bar{\lambda}_w$-*broad (nm)*	$\bar{\lambda}_w$-*low (nm)*	$\bar{\lambda}_w$-*mid (nm)*	$\bar{\lambda}_w$-*high (nm)*	*Alternate* $\bar{\lambda}_w$-*mid (nm)*
Aprobarbital	214.21	214.19	Baseline	Baseline	225.88
Butabarbital	214.14	214.09	Baseline	Baseline	226.20
Pentobarbital	213.96	213.95	Baseline	Baseline	225.92
Phenobarbital	214.26	214.11	Baseline	Baseline	229.88
Hexobarbital	218.70	218.59	Baseline	Baseline	227.81
Mephobarbital	217.58	217.45	Baseline	Baseline	228.88

broad values from 213.96 to 214.26 nm). An alternative $\bar{\lambda}_w$-mid value, calculated over a wavelength range of 224–278 nm, identified the less pronounced contributions of the phenyl side chain in phenobarbital.

It should be noted that if functional group substitution causes large spectral shifts, identification of drug metabolites by compound class will be extremely difficult.

CONCLUSIONS

The purity parameter spectral interpretation format was successfully employed for peak confirmation and identification. Use of multiple $\bar{\lambda}_w$ values provided unambiguous determination of compound class. The calculation of $\bar{\lambda}_w$-broad, $\bar{\lambda}_w$-low, $\bar{\lambda}_w$-mid, and $\bar{\lambda}_w$-high values (each calculated over a different wavelength region) allowed accentuation of characteristic spectral fragments for both xanthine and barbiturate drug classes. Alkyl substitution on the xanthine nitrogen ring affected $\bar{\lambda}_w$-broad values that were useful for individual compound identification. Class determination was achieved from examination of $\bar{\lambda}_w$-low, $\bar{\lambda}_w$-mid, and $\bar{\lambda}_w$-high value patterns which were very similar for each xanthine compound.

Multiple $\bar{\lambda}_w$ values allowed confirmation of olefin, one-ring and two-ring aromatic hydrocarbon classes in gasolines and jet fuels.

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