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POSITIVE PEAK IDENTIFICATION IN LIQUID CHROMATOGRAPHY USING ABSORBANCE RATIOING WITH A VARIABLE-WAVELENGTH SPECTROPHOTOMETRIC DETECTOR

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SUMMARY

It is frequently necessary to confirm the identification of component peaks isolated in high-performance liquid chromatograms. The UV spectrum of the pure compound is usually a very powerful signature for most compounds. For liquid chromatographic instruments that permit stop-flow operation, a simple approximation is obtained with a variable wavelength UV detector by determining the absorbance ratios at several specific wavelengths to characterize the compound. If the wavelengths are well chosen, these absorbance ratios provide a very specific and a very reliable technique for peak identification.

The absorbance ratios are independent of the concentration of the compounds in the detector flow cell. Identity or purity of individual peaks can be confirmed when absorbance ratios are taken from the peaks of individually injected pure standards. Flow can be stopped several times on the leading and trailing edges of broad peaks, and ratios calculated. If the ratios are identical, the peak can be considered to be pure; if ratios differ, the presence of unresolved components can be suspected.

Stop-flow ratioing can be performed during gradient elution: standards are injected under rapid elution conditions and the resulting ratios are compared with those of the peaks from the gradient run. Ratios are found to be identical for similar components, regardless of the differences in retention time, thus providing rapid identification of the compounds in the gradient chromatogram. Factors that influence the overall precision of the method are: ability to stop and re-start eluent flow without loss of chromatographic efficiency, ability to obtain numerical absorbance data from the detector, the wavelength repeatability of the detector, and the requirement that the detector cell design allows flow interruptions without baseline upset.

INTRODUCTION

In addition to its usefulness for quantitative analysis, high-performance liquid chromatography (HPLC) is a very important qualitative tool. In many applications it is critically important that tentative identification of a compound by its retention

volume be confirmed. In other cases it is important that the purity of a chromatographic peak be checked. In both of these cases the UV absorption spectrum of the chromatographic peak may provide the required information.

Typically this technique can be applied in several ways:

(1) A single-wavelength UV detector can be set to an appropriate wavelength in series with the variable-wavelength UV detector set to some other wavelength. This will provide two absorbance readings and a ratio for each peak for the two selected wavelengths. This is a very fast and simple procedure, but on the other hand, several absorbance ratios at different wavelengths provide a more positive compound identification than a single ratio.

(2) The chromatograph can be stopped at each peak and the variable-wavelength detector changed successively to the several selected wavelengths[1]. The chromatogram should also be stopped periodically at the baseline to determine the absorbance values for each selected wavelength. The peak ratios are then calculated as in the first case, thus characterizing the sample from a single chromatographic run, and a single-variable wavelength detector.

(3) A variation of the second technique can be used when there is doubt as to the identity or purity of a particular peak in the chromatogram. Having previously measured peak ratios for pure standard compounds, a suspect peak can be tested for identity by measuring peak ratios in the stop-flow mode described above. Variations of more than about 2% in peak ratios can be taken as a reasonable indication that the peaks are not identical.

(4) This technique can also be used to provide rapid identification of individual peaks in complex gradient runs. While a series of gradient runs made to determine retention time data for each standard typically consumes hours, rapid isocratic elution of pure standards can provide ratios for each peak in minutes. These ratios are characteristic for each peak and can be compared with ratios run on peaks eluted during the gradient run, to confirm identity.

(5) Even if spectral data on standards are not available, the purity of a chromatographic peak can be tested by measuring peak ratios at several points along the peak, *e.g.* the leading edge, the peak and the trailing edge. If the compound under the peak is pure, the ratios will agree. An example of this technique is presented in this paper.

Regardless of the approach selected, wavelengths are selected according to the following criteria: (a) absorption maxima of either the primary compound or the suspected impurities, (b) a general wavelength, such as 250 nm, where many materials have some absorption, and (c) low wavelengths (*e.g.*, 195–215 nm) where most compounds have strong absorption. It is important to determine the ratios between three or more wavelengths, because it is quite possible for the ratios to match between two wavelengths, but it becomes very unlikely for them to match fortuitously with three or more well-separated wavelengths.

Beer's law requires that there be a linear relationship between absorbance and concentration at every wavelength. Since the concentration of the compound does not change between the several absorbance measurements suggested in this procedure, the ratio of the absorbances becomes the ratio of the extinction coefficients, the constant of proportionality in Beer's law. The extinction coefficient at each wavelength, and therefore their ratio, is an intrinsic characteristic of a compound. This also ex-

plains why the technique is independent of the concentration of the compound in the photometer cell.

EXPERIMENTAL

The successful use of the technique is highly dependent upon the ability of the operator to re-set the wavelength when making repeated ratio readings and when returning to the operating wavelength.

Table I illustrates the changes in signal intensity, or absorbance, for the components of this five-compound mixture with the wavelength re-set within (a) 0.1, (b) 0.5, and (c) 1.0 nm. Peak areas were obtained for each of the separated compounds and are expressed as area ratios relative to acetophenone. Run (a) was repeated ten times and runs (b) and (c) were repeated six times, with the wavelength dial re-set within the limits listed for each run. The resulting relative standard deviations indicate that the precision of any method utilizing frequent re-setting of wavelengths requires that the instrument in use should preferably have $\pm$ 0.1 nm re-set capability. The pair of chromatograms shown in Fig. 1 illustrates the relative changes in signal intensity when the detector wavelength is set at (a) 253.5 and (b) 254.0.

TABLE I

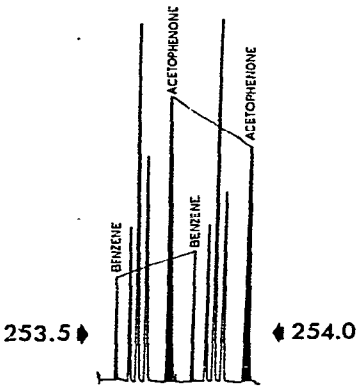
STANDARD DEVIATIONS AT VARIOUS WAVELENGTH REPEATABILITY LIMITS

Compounds are listed in order of elution.

Repeatability limit (nm): λ = 254 nm	*Compound*	*Mean area ratio relative to acetophenone*	*Standard deviation*	*Relative standard deviation (%)*	*n*
$\pm$ 0.1	Benzene	0.1723	0.0019	1.1	10
	Nitrobenzene	0.2899	0.0036	1.25	
	Methyl benzoate	0.8301	0.0070	0.86	
	Benzaldehyde	0.5104	0.0045	0.88	
$\pm$ 0.5	Benzene	0.184	0.075	41	6
	Nitrobenzene	0.290	0.047	16	
	Methyl benzoate	0.829	0.128	15	
	Benzaldehyde	0.507	0.006	1.27	
$\pm$ 1.0	Benzene	0.220	0.159	72	6
	Nitrobenzene	0.293	0.091	31	
	Methyl benzoate	0.843	0.263	31	
	Benzaldehyde	0.498	0.014	2.7	

A Perkin-Elmer Model LC-55 variable-wavelength UV detector has been used successfully for absorbance ratio identification. Its design makes it particularly insentitive to flow effects, refractive index gradients and temperature changes. It has a relatively narrow bandpass (2 nm) and is precisely re-settable ($\pm$ 0.1 nm) to wavelengths from 190 to 800 nm.

A Perkin-Elmer Model 601 liquid chromatograph was used. This instrument utilizes positive displacement syringe pumps driven by digital stepping motors. The flow was stopped by simultaneously shutting off pump power and closing the valve



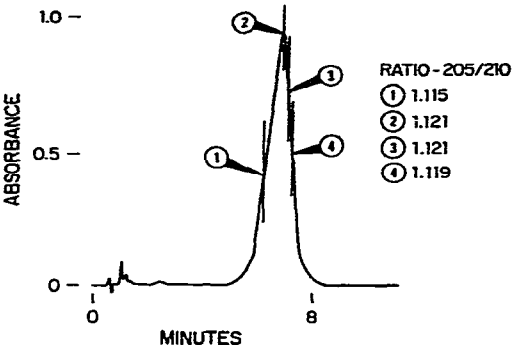


Fig. 1. Effect of 0.5-nm wavelength change on relative peak areas. Conditions: column, Silica A, 2.6 mm × 50 cm; eluent, chloroform–hexane (4:96); flow-rate, 2 ml/min; temperature, 35°; detection, 254.0 and 253.5 nm (0.4 a.u.f.s.).

Fig. 2. Absorbance ratio measurements to test the purity of the propyl paraben peak. See Table II for the measured ratios.

located at the injector inlet. This action holds the unit at operating pressure and immobilizes the material in the detector cell.

If solvent gradients are used, the blank values are usually obtained at the beginning, middle and end of the run and then interpolated to provide the proper values. These gradient values are alternatively obtained with a "blank" gradient or by obtaining them on each peak of interest.

RESULTS

Purity

During a chromatographic run of a propyl paraben standard (Fig. 2) the flow was stopped at four points and absorbance ratios were obtained between three different wavelengths. The resulting data are presented in Table II. Three successive sets of ratios were analyzed at three of the points and five sets at point 4. The absorbance statistical data are calculated for point 4. The absorbance statistical data on the ratios are calculated for all fourteen measurements. The ratios are precise to better than 1%, making them a practical analytical tool.

Impurities eluting with the peak of interest can be detected readily with this technique. In Fig. 3 a niacinamide standard produced a symmetrical peak. However, the absorbance ratios (Table III) calculated at four points produced very different values indicating that the peak was not homogeneous.

Confirmation of methyl paraben in mascara

A sample of mascara was separated on an ODS reversed-phase column, shown on the left in Fig. 4. The purpose of the analysis was to detect the presence of preservatives. Standards of potential preservatives are seen on the right of Fig. 4. Since the retention time of the methyl paraben standard agreed with that of peak 2 in the mascara chromatogram, methyl paraben appeared to be in the sample. To confirm this identification, the absorbance ratios were calculated for the peaks in both chro-

TABLE II

PROPYL PARABEN ABSORBANCE RATIOS (SEE FIG. 2)

S.D. = standard deviation.

Point	*Wavelength (nm)*			*Ratio*	
	205	*210*	*212*	*205/210*	*212/210*
1	0.494	0.443	0.406	1.115	0.916
	0.494	0.443	0.406	1.115	0.916
	0.493	0.442	0.404	1.115	0.914
2	0.932	0.829	0.763	1.124	0.920
	0.929	0.826	0.754	1.125	0.913
	0.926	0.826	0.755	1.121	0.914
3	0.890	0.794	0.725	1.121	0.913
	0.891	0.795	0.727	1.121	0.914
	0.894	0.797	0.728	1.122	0.913
4	0.581	0.519	0.473	1.119	0.911
	0.582	0.521	0.473	1.117	0.908
	0.587	0.519	0.472	1.131	0.909
	0.585	0.522	0.474	1.121	0.908
	0.586	0.520	0.473	1.127	0.910
Mean	0.584	0.520	0.473	1.121	0.913
S.D.	0.0025	0.0013	0.0007	0.047	0.0034
Relative S.D.	0.44	0.25	0.15	0.42	0.37

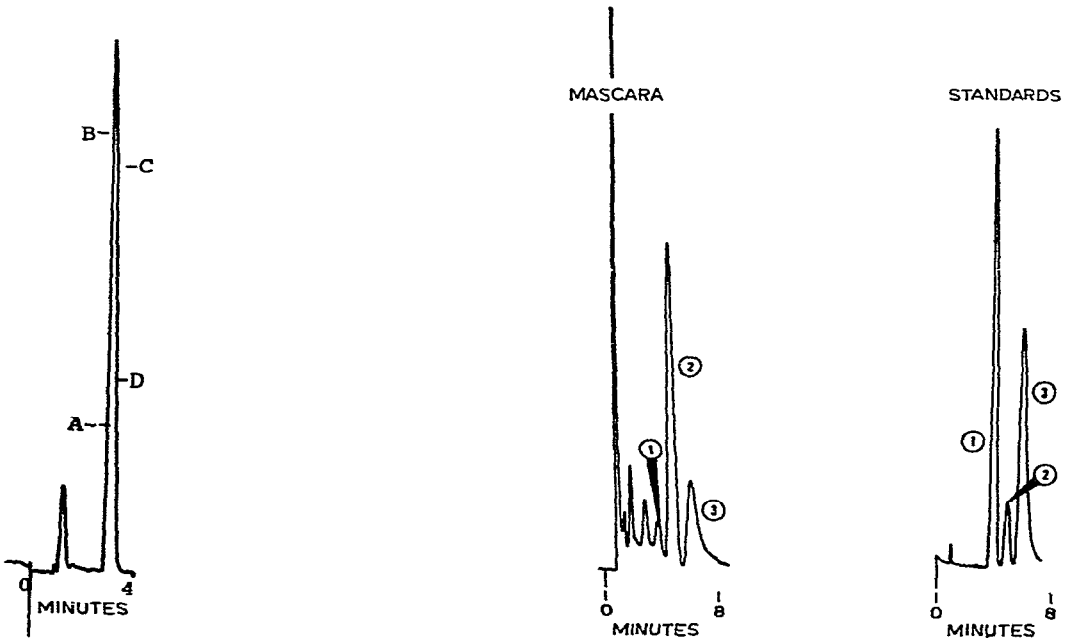


Fig. 3. Absorbance ratio measurements to determine an impurity eluting with a niacinamide standard. See Table III for the measured ratios. Conditions: column, ODS SIL-X-I, 2.6 mm × 50 cm; eluent, water; flow-rate, 1 ml/min; temperature, 40°; detection, 240 nm.

Fig. 4. Determination of preservatives in mascara. Conditions, column, ODS SIL-X-I, 2.6 mm × 50 cm; eluent, acetonitrile–water 7:93, flow-rate, 2 ml/min; temperature, 50°; detection, 230 nm. Peak identification: 1 = sodium benzoate; 2 = methyl paraben; 3 = sodium hydroacetate.

TABLE III

NIACINAMIDE STANDARD ABSORBANCE RATIOS (SEE FIG. 3)

Point	*Absorbance ratio*	
	210/240	*260/240*
A	2.84	0.90
B	3.11	1.10
C	2.58	0.93
D	1.83	0.70

matograms. The agreement shown in Table IV supports the identity of the two peaks Four chromatograms were run of both sample and standard. Note that the concentration (absorbance) of the methyl paraben differed considerably between the four chromatograms. Nevertheless, the ratios did not vary.

Identification of drugs in urine

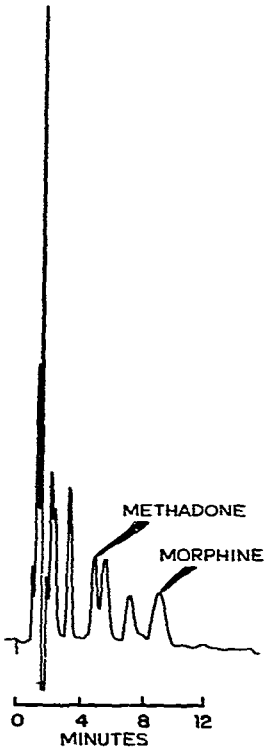
Liquid chromatography is very useful in the separation and isolation of drugs of abuse, *e.g.*, morphine standards can be detected at levels as low as 1 ng (ref. 2). It is important in the analysis of complex body fluids for these drugs that the peaks be identified accurately, both for medical and legal reasons. A commercially available sample of human urine* was analyzed, containing 1 μg/ml each of morphine and methadone (Fig. 5). The absorbance ratios were used to confirm the presence of methadone and morphine. The readings taken at the peak maxima of this chromatogram and of the chromatogram for standards are presented in Table V. The absorbance ratios confirm the identification and purity of the methadone, but indicate that the peak eluting with the proper retention time for morphine is not due entirely to that drug.

TABLE IV

METHYL PARABEN ABSORBANCE RATIOS (SEE FIG. 4)

Sample	*Run*	*Absorbance ratio*	
		230/220	*220/210*
Standard	1	0.076/0.095 (0.80)	0.095/0.279 (0.34)
	2	0.076/0.096 (0.79)	0.096/0.279 (0.34)
	3	0.057/0.073 (0.78)	0.073/0.214 (0.34)
	4	0.057/0.072 (0.79)	0.072/0.213 (0.34)
Mascara	1	0.120/0.169 (0.71)	0.169/0.497 (0.34)
	2	0.123/0.170 (0.72)	0.170/0.501 (0.34)
	3	0.242/0.327 (0.74)	0.327/1.012 (0.32)
	4	0.248/0.331 (0.75)	0.331/1.014 (0.33)

* Urine toxicology control, drugs 1, Lederle Diagnostics, American Cyanamid Company, Pearl River, N.Y., U.S.A.



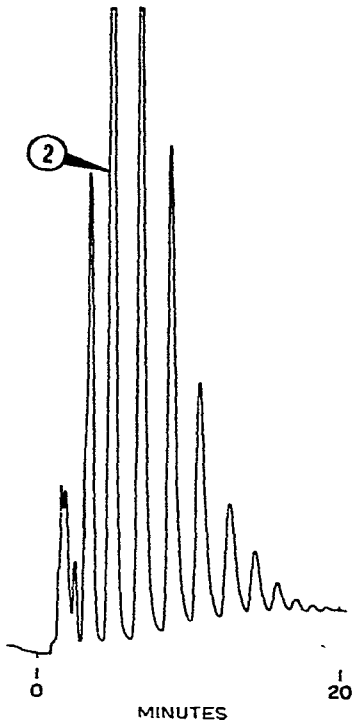


Fig. 5. Determination of morphine and methadone in urine. See Table V for the measured ratios. Conditions: column, fluoro ether SIL-X-I, 2.6 mm × 50 cm; eluent, water–acetonitrile containing 0.3% H_3PO_4 (8:92); flow-rate, 2 ml/min; inlet pressure, 600 p.s.i.g.; temperature, 70°; detection, 210 nm (0.1 a.u.f.s.).

Fig. 6. Analysis of polystyrene 600 oligomers. See Table VI for the measured ratios. Conditions: column, ODS SIL-X-I, 0.26 mm × 25 cm; eluent, gradient, (65:35) methanol–water to 100% water in 25 min (70% convex); flow-rate, 1 ml/min; temperature, 70°; detection, 260 nm (0.1 a.u.f.s.).

Purity determination of polystyrene

A polystyrene 600 sample was analyzed under conditions designed to separate the oligomeric series into separate peaks, as shown in Fig. 6. Since all of the major components seen here are chemically similar, they would be expected to have very similar UV spectra, thus very similar absorbance ratios. Stop-flow absorbance readings were taken on six major peaks. The resulting absorbance ratios are shown in Table VI.

The data suggest an impurity in peak 2. To confirm this, the gradient program was modified to begin at a lower methanol concentration (55%, previously 65%) and the gradient time was adjusted to provide a slower rate of change, as shown in Fig. 7. The resulting increase in separating power allowed resolution of the impurity that had been concealed by peak 2, which was responsible for the different UV response. A separate run with the detector wavelength set at 300 nm, where polystyrene should have no absorption, confirmed the presence of the impurity.

TABLE V

ABSORBANCE RATIOS FOR DRUGS IN URINE (SEE FIG. 5)

Drug	*Absorbance ratio*		
	205/210	*220/210*	*225/210*
Methadone standard	1.32	0.49	0.31
Methadone in urine	1.32	0.49	0.30
Morphine standard	0.90	0.75	0.42
Morphine in urine	0.94	0.67	0.53

Rapid identification of peaks in gradient run

In complex runs where gradient elution must be performed, the normal method of identifying peaks requires that individual pure standards be run under exactly the same conditions as those used during the sample analyses. It is common knowledge that to obtain precise repeatability of retention times, the column must be re-equilibrated between runs, preferably for exactly the same amount of time. Frequently, re-equilibration consumes as much time as the actual gradient. As a result, a series of gradient runs may require an entire day, simply to confirm peak identity. As an alternative, peak ratioing can be performed on each pure standard after the solvent concentration has been adjusted to obtain rapid elution (k' values of 1–2, if there are no solvent interferences). The resulting absorbance ratios are characteristic for the particular compounds at the wavelengths selected regardless of the differences in retention time. Fig. 8 shows a gradient separation of polynuclear aromatics performed on an ODS column. Stop-flow absorbance ratioing was performed on each of the peaks. Readings were made at 230, 250, 270 and 290 nm, ratios for each peak were calculated and were listed in the horizontal "Gradient" rows of Table VII. The standards were then chromatographed rapidly as described previously, stop-flow ratioing was performed, and the results listed in the "Standard" row for each. It then becomes a simple matter to compare the ratios listed for each component. Note that each compound has a distinctive profile, that standard and sample ratios generally agree very closely, and that peak identities can be easily confirmed on this basis. Even when all ratios do not match closely, the technique is still useful. Since readings were taken at four widely varying wavelengths it was possible to obtain several ratios which confirmed peak 2 to be naphthalene, even though, under the rapid elution conditions

TABLE VI

POLYSTYRENE ABSORBANCE RATIOS (SEE FIG. 6)

Peak no.	*Absorbance ratio*	
	240/260	*240/200*
1	0.24	18.6
2	0.32	2.82
3	0.24	18.2
4	0.25	18.3
5	0.26	18.0
6	0.26	17.9

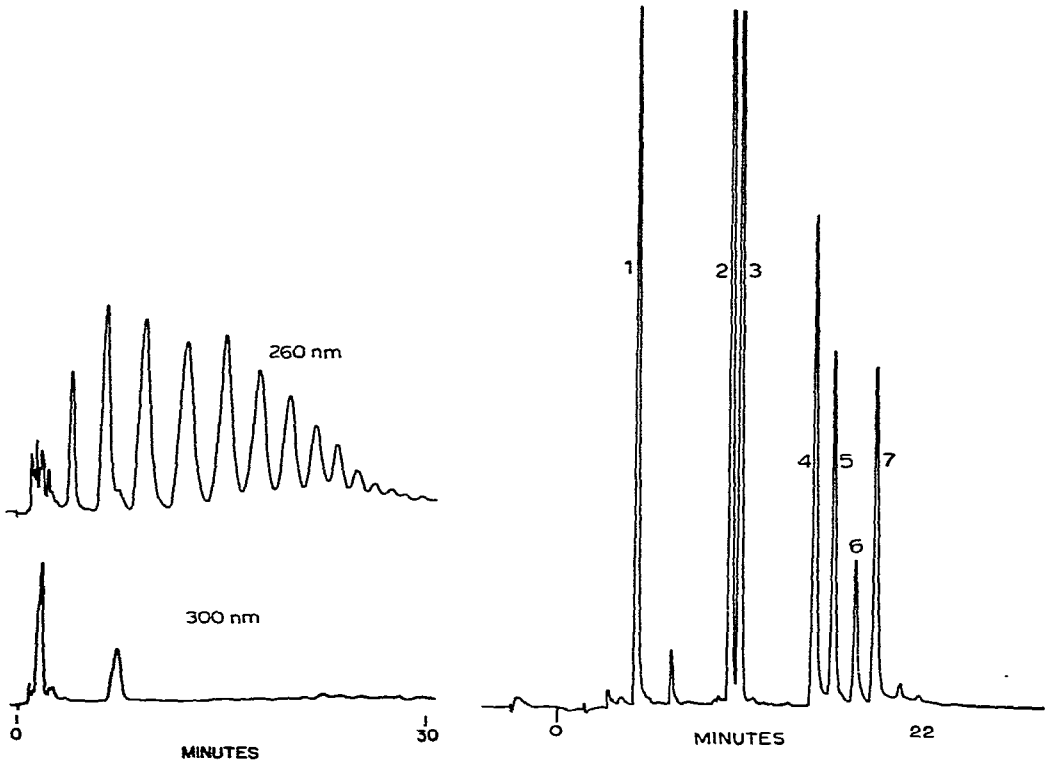


Fig. 7. Analysis of polystyrene 600 oligomers. Conditions as in Fig. 6 except that the gradient was from methanol–water (55:45) to 100% water in 50 min. Detection, 260 and 300 nm.

Fig. 8. Separation of polynuclear aromatics for absorbance ratio peak identification. Conditions: column, ODS SIL-X-I, 2.6 mm × 25 cm; eluent, gradient, acetonitrile–water (40:60) to 100% acetonitrile at 5%/min; flow-rate, 2 ml/min; temperature, 60°; detection, 250 nm. Peak identification: 1 = benzene; 2 = naphthalene; 3 = phenanthrene; 4 = perylene; 5 = benzo(*a*)pyrene; 6 = dibenzanthracene; 7 = dibenzphenanthrene.

TABLE VII

POLYNUCLEAR AROMATIC GRADIENT-RUN ABSORBANCE RATIOS

Compound	*Method**	*Absorbance ratio*				
		290/250	*290/270*	*290/230*	*250/230*	*270/250*
Naphthalene	Standard	0.46	0.22	0.67	1.44	2.96
	Gradient	0.49	0.24	0.19	0.38	2.06
Phenanthrene	Standard	0.17	0.76	0.66	3.9	0.22
	Gradient	0.17	0.82	0.62	3.7	0.20
Perylene	Standard	0.03	0.33	0.11	3.56	0.09
	Gradient	0.04	0.38	0.14	3.46	0.11
Benzo[*a*]pyrene	Standard	0.88	1.13	2.96	3.35	0.78
	Gradient	0.91	1.12	2.70	2.57	0.81
Dibenzanthracene	Standard	8.34	1.99	2.83	0.34	4.19
	Gradient	7.96	1.98	2.77	0.35	4.01
Dibenzphenanthrene	Standard	0.71	0.51	1.32	1.74	1.52
	Gradient	0.79	0.52	1.28	1.62	1.52

* See text.

used for the standards, an impurity was apparently not separated from the major peak, as is evidenced by the variance of some of the ratios. Note, also, that though the 250/230 and 270/250 ratios for benzo[*a*]pyrene do not match, the others do. More importantly, no other components of the mixture have a profile reasonably similar to that of the benzo[*a*]pyrene, therefore there is no danger of mis-labeling. The time savings can be tremendous since total analysis time for each standard, including the stop-flow ratioing process, is less than 5 min.

TABLE VIII

GRADIENT-RUN BASE-LINE ABSORBANCE READINGS

Solvent	*Wavelength (nm)*				
	210	*230*	*250*	*270*	*290*
Acetonitrile–water (40:60)	0.307	0.82	−0.66	0.05	0.238
Acetonitrile	0.303	0.80	−0.66	0.05	0.239

Table VIII illustrates a series of readings taken to ascertain the need for base-line correction during this run. Since all readings were taken at wavelengths where the water and acetonitrile have no UV absorption, there is no difference in the total absorbance readout at various solvent concentrations, thus it was not necessary to make base-line corrections.

CONCLUSIONS

It has been shown that absorbance ratios provide a very sure qualitative identification of HPLC peaks. The technique is easily applied to compound identification, detection of impurities chromatographically unresolved, and rapid identification of peaks eluting from gradient profiles.

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