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Note

Methods of identifying organic compounds using high-performance liquid chromatography with ultraviolet detection

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In 1963, in order to increase reliability of the identification of organic compounds, Talrose[1] suggested determining the ratios of the peak heights of two mass numbers obtained from mass spectra. The development of spectrophotometric single- and dual-beam detection (UV–VIS) (with possible variation of the wavelengths) in high-performance liquid chromatography (HPLC) has opened up new possibilities for characterizing the peaks of HPLC eluates. This made it possible to perform highly reliable identifications of organic compounds by finding peak-height ratios (absorption ratios) at two different wavelengths[2–12], or to determine the purity of poorly resolved components[13–16].

This method has been used for the identification of nucleosides, nucleotides and some other serum constituents[3,4], drugs of different classes[5], barbiturates[6], alkaloids[7], furan derivatives[8,9], nitrosamines[10], carotenoids[11], aromatic hydrocarbons[12] and phenols[17]. Positive results were obtained in all instances, but unfortunately there was no unity on some important points: (a) the wavelengths chosen by different workers for the ratio determination were not the same; (b) the number of ratios determined varied from one to nine; and (c) several different methods of ratio determination were used. Hence it is impossible to compare the data and results obtained by different workers or to establish the optimum procedure for a particular determination. Also, it is not clear whether the ratios found by HPLC can be determined by other methods. These problems have been investigated in this work.

EXPERIMENTAL

Liquid chromatography was performed with a Perkin-Elmer Model 601 liquid chromatograph equipped with LC-55 (Coleman Instruments Division) and Acta V (Beckman) ultraviolet detectors and a Spectra-Physics Model SP 8000B chromatograph equipped with an SP 8440 detector and SP 8010 autosampler; Model 36 and DU-8B spectrophotometers were used to obtain absorption spectra.

Acetonitrile for chromatography was supplied by Merck (Darmstadt, F.R.G.). Distilled water was redistilled over potassium permanganate. Furfural, 5-methylfurfural and *m*-cresol were distilled under reduced pressure. Other chemicals were supplied by Sigma (St. Louis, MO, U.S.A.) and by Applied Science Labs. (State College, PA, U.S.A.).

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To distinguish organic compounds of different classes we used several chromatographic systems, as follows:

(A) Chrompak 4.6 × 250 mm I.D. LiChrosorb RP-18 (10 μm) column with a mobile phase flow-rate of 2.0 ml/min; mobile phase, water–acetonitrile (75:25); column temperature, 50°C.

(B) Perkin-Elmer 2.6 × 250 mm I.D. HC-ODS Sil X (10 μm) column with a mobile phase flow-rate of 1.0 ml/min; mobile phase, water–acetonitrile (90:10); column temperature, 60°C.

(C) Column and flow-rate and column temperature as in (A); mobile phase, water–acetonitrile (20:80) with 0.1% of orthophosphoric acid.

Determination of a_{ij} ratios

Several methods were used, as follows.

(1) Two UV detectors LC-55 and Acta V spectrophorometers) were connected in series to the outlet of the column. Broadening coefficients for the peaks of each sample compound in the second flow cell were calculated using the equation

$$f_i = \frac{h_1^{254}}{h_2^{254}}$$

where f_i is the broadening coefficient for compound i and h_1^{254} and h_2^{254} are the peak heights at 254 nm for the first and the second detectors, respectively. Then,

$$a_{ij} = \frac{h_1^x}{h_2^y f_i}$$

where a_{ij} is the peak-height ratio for compound i and x and y are the wavelengths for first and second detectors, respectively.

(2) One Model SP 8440 variable-wavelength spectrophotometer was connected to the outlet of the column, then the first chromatographic run was registered at wavelength x and the other at wavelength y:

$$a_{ij} = \frac{h^x}{h^y}$$

where h^x and h^y are the peak heights at wavelengths x and y, respectively. Chromatographic runs were controlled by the microprocessor and samples were injected with the autosampler.

(3) Instrumentation as in (2); the stopped flow technique was used.

(4) The preparative method was performed with the help of a liquid chromatograph. For each component, absorption values were found at chosen wavelengths using the single-beam DU-8B spectrophotometer.

(5) Spectra of components collected as in (4) were registered with a Model 36 double-beam spectrophotometer, then the absorption value was found from spectra for all the chosen wavelengths.

TABLE I

RETENTION DATA AND ABSORPTION RATIOS a_{ij}

Compound	*Chromatographic conditions*	*k'*	a_{ij} *determination mode*	a_1	a_2	a_3	a_4	a_5	a_6	a_7
5-Hydroxy methyl-furfural	A	1.03	1	0.72 ± 0.02	0.60 ± 0.02	1.32 ± 0.02	1.40 ± 0.02	1.97 ± 0.05	0.71 ± 0.02	0.22 ± 0.01
	B	2.14	2	0.80 ± 0.10	0.63 ± 0.05	1.25 ± 0.15	1.50 ± 0.15	1.90 ± 0.07	0.74 ± 0.03	0.24 ± 0.02
			3	0.71 ± 0.03	0.58 ± 0.03	1.27 ± 0.05	1.38 ± 0.02	2.10 ± 0.10	0.74 ± 0.03	0.25 ± 0.02
			4	0.75 ± 0.05	0.57 ± 0.03	1.2 ± 0.07	1.42 ± 0.10	2.0 ± 0.10	0.73 ± 0.04	0.23 ± 0.02
			5	0.70 ± 0.03	0.56 ± 0.03	1.32 ± 0.02	1.50 ± 0.05	1.95 ± 0.05	0.71 ± 0.02	0.21 ± 0.02
Furfural	A	2.02	1	2.10 ± 0.05	0.57 ± 0.03	1.28 ± 0.04	1.45 ± 0.05	1.70 ± 0.05	0.93 ± 0.03	0.34 ± 0.02
	B	3.66	2	2.20 ± 0.20	0.60 ± 0.05	1.20 ± 0.20	1.40 ± 0.07	1.60 ± 0.10	0.95 ± 0.10	0.38 ± 0.08
			3	2.07 ± 0.04	0.57 ± 0.03	1.29 ± 0.07	1.44 ± 0.05	1.71 ± 0.05	0.90 ± 0.04	0.34 ± 0.02
			4	2.0 ± 0.10	0.57 ± 0.02	1.22 ± 0.04	1.40 ± 0.20	1.71 ± 0.05	0.90 ± 0.03	0.34 ± 0.02
			5	2.02 ± 0.05	0.54 ± 0.03	1.27 ± 0.03	1.35 ± 0.05	1.65 ± 0.05	0.94 ± 0.03	0.34 ± 0.02
Phenol	C	5.05	1	1.01 ± 0.04	3.37 ± 0.12	0.09 ± 0.01	4.20 ± 0.20	1.73 ± 0.05	3.00 ± 0.15	0.97 ± 0.10
			2	0.95 ± 0.05	3.50 ± 0.20	0.08 ± 0.02	3.00 ± 0.50	1.50 ± 0.10	2.95 ± 0.20	1.00 ± 0.10
			4	1.02 ± 0.02	2.90 ± 0.20	0.12 ± 0.02	4.00 ± 0.70	1.70 ± 0.20	2.50 ± 0.30	0.65 ± 0.10
			5	1.05 ± 0.05	2.70 ± 0.20	0.10 ± 0.02	4.00 ± 0.70	1.70 ± 0.10	2.50 ± 0.30	0.62 ± 0.10
o-Cresol	C	6.40	1	0.86 ± 0.02	2.90 ± 0.10	0.16 ± 0.01	5.10 ± 0.10	1.80 ± 0.05	1.95 ± 0.10	0.50 ± 0.05
			2	0.85 ± 0.02	2.90 ± 0.015	0.16 ± 0.01	4.80 ± 0.02	1.60 ± 0.15	1.94 ± 0.15	0.50 ± 0.10
			4	0.89 ± 0.04	2.50 ± 0.30	0.18 ± 0.03	5.50 ± 0.05	1.80 ± 0.15	1.70 ± 0.20	0.45 ± 0.10
			5	0.89 ± 0.04	2.65 ± 0.10	0.18 ± 0.03	4.50 ± 0.50	1.79 ± 0.15	1.72 ± 0.15	0.50 ± 0.15

RESULTS AND DISCUSSION

For the identification of organic compounds most authors calculate one ratio, *e.g.*, double-beam detection at 254 and 280 nm. However we[9,10] and others[6,16] have demonstrated that determination of several ratios at different wavelengths enables compounds to be discriminated better.

We concluded that if a compound is being analysed for the first time it is advisable to measure seven ratios. In further analyses, the number of determinations can be reduced to two or three. The choice of wavelengths the determination of ratios for a compound i depends on the wavelength of maximum absorption. We suggest finding the following ratios: $a_1 = h_{215}/h_{205}$; $a_2 = h_{215}/h_{225}$; $a_3 = h_{230}/h_{220}$; $a_4 = h_{230}/h_{240}$; $a_5 = h_{270}/h_{260}$; $a_6 = h_{270}/h_{280}$; and $a_7 = h_{254}/h_{280}$.

In this work we used five variants of the a_{ij} ratio method and the results are given in Table I. The data given were obtained in the course of 2–5 years; mean values of the ratios are given with their standard deviations. It can be clearly seen that the best reproducibility over a long period of time can be achieved if the first variant of ratio determination is used, *i.e.*, when two spectrophotometers are connected in series. This can probably be explained by the fact that chromatographic peaks at compared wavelengths are recorded under strictly the same conditions of chromatographic separation. Also, the solvent concentration remains constant, which eliminates possible deviations in the ratio values, which can be considerable[18]. When the stopped flow technique (mode 3) is used, the reproducibility achieved can also be satisfactory, although in further chromatographic steps false peaks appear and the separations deteriorate. This mode also requires more complex chromatographic and spectrophotometric equipment controlled by microprocessors.

The lowest reproducibility occurs when a_{ij} is determined from spectra recorded with the aid of double- or single-beam spectrophotometers (modes 4 and 5), owing to the dependence on the time of measurement.

If we now compare numerical values of the same parameter obtained by the different methods, in most instances these values coincide if we allow for the error of the method itself. Only the a_6 and a_7 ratios for phenol calculated from the spectra are significantly different from those calculated from the chromatograms. It can be assumed that the highest reproducibility is achieved by simultaneous recording at two wavelengths according to the method suggested by Webb *et al.*[19]. Unfortunately, they did not give any reproducibility data and we have no means of verifying their results.

It must be borne in mind that data in Table I were collected in the course of 2–5 years and so the difference between the time of measurements in some instances is about 1–2 years. Naturally, it is very difficult to ensure constancy of all the factors that influence the value of the measured parameters during such a long period. If all the a_{ij} values are determined within a period of not more than 1 week the reproducibility achieved will be much higher. Table II gives values obtained within 3 days, *i.e.*, each set of parameters was determined within 1 day only. It is obvious that the reproducibility here is much higher.

The results in Table I indicate that the highest reproducibility of a_{ij} for the same compound occurs when the wavelengths chosen for the ratio determination are close to the absorption maxima. For example, furfural and 5-hydroxymethylfurfural,

TABLE II

ABSORPTION RATIOS a_{ij} DETERMINED BY MODE 2 DURING 3 DAYS (CHROMATOGRAPHIC SYSTEM C)

Values represent the mean of three determinations.

Compound	a_1	a_2	a_3	a_4	a_5	a_6	a_7
Vanillin	0.56	0.55	1.41	3.04	1.83	0.89	0.30
	0.56	0.56	1.41	3.05	1.69	0.86	0.31
	0.56	0.55	1.39	3.14	1.73	0.89	0.30
Phenol	0.93	3.55	0.07	3.50	1.56	3.04	1.02
	0.94	3.58	0.09	3.39	1.61	2.80	1.01
	0.94	3.57	0.07	3.58	1.60	2.70	1.09
m-Cresol	0.88	2.20	0.18	8.80	1.81	1.64	0.50
	0.87	2.30	0.21	9.00	1.78	1.62	0.51
	0.88	2.22	0.19	8.60	1.75	1.70	0.48

the absorption maxima of which are at about 225 and 280 nm, have the highest reproducibility of a_2, a_5 and a_6. The reproducibility decreases abruptly when the chosen wavelength occurs close to the minimum on the absorption curve (*e.g.*, a_1 and a_4 for furfural and 5-hydroxymethylfurfural; a_2 and a_4 for *o*- and *m*-cresols).

Let us now consider the dependence of a_{ij} on the structure of the organic compound. The data in Tables I and II and in ref. 9 indicate that substitution of the organic compound molecule (*e.g.*, furfural, 5-methylfurfural and 5-hydroxymethylfurfural or phenol and vanillin) and alterations to the position of substitution (*e.g.*, *o*- and *m*-cresol) change the value of a_{ij}. This effect arises because these structural changes cause shifts of the absorption spectra. In such instances one or more a_{ij} ratios are calculated. Therefore, if all seven numerical values of the a_{ij} ratios coincide it confirms the identity of the sample compound with the assumed reference substance. Among several scores of inorganic compounds belonging to different classes for which all seven ratios were obtained, we have not found a single instance where the sets of values coincided (see Table I and refs. 9 and 10), although among these compounds there were homologues with analogous structures.

The situation is different when the structure of the sample compound contains a chromophore that determines the absorption of the molecule as a whole. Then the a_{ij} ratios can be expected to have very close or even identical numerical values. In order to verify this supposition, we found a_{ij} values for DNPH derivatives of carbonyl-containing compounds and for *p*-bromophenacyl esters of saturated and unsaturated fatty acids. It was found that, as the absorption of DNPH derivatives of aldehydes and ketones is completely determined by the dinitrophenyl radical, the values of all seven parameters for the respective derivatives of simple aldehydes and ketones were so close that there was no possibility of distinguishing them from these sets of values. Also, all seven values of the ratios were identical for the *p*-bromophenacyl derivatives of thirteen saturated fatty acids, which made identification from a_{ij} values impossible.

CONCLUSION

This work demonstrates that the major advantage of the method based on measuring the absorption value at two different wavelengths and calculation of the absorption intensity ratio at these wavelengths is that it permits the rapid and simple identification of organic compounds. If all measurements are made within a short period of time, high reproducibility of the results can be achieved by any of the five methods described.

The main disadvantage is that the absorption value at a definite wavelength depends considerably on various factors (instrumental operating conditions, composition of the solvents, contaminants of the solvents and sample compounds, etc.) that are difficult to maintain constant. Hence inter-laboratory reproducibility of absorption ratios is not possible at present.

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