RESEARCH PAPERS

THE IMPORTANCE OF EXTINCTION RATIOS IN THE SPECTROPHOTOMETRIC ANALYSIS OF MIXTURES OF TWO KNOWN ABSORBING SUBSTANCES

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The accuracy obtainable from Vierordt's method largely depends upon the establishment of numerical coefficients, which apply to the analyst's own instrument at the time of analysis. For the special case of a two component mixture, the method has been formulated in terms of extinction ratios, which can be determined from solutions of unknown concentration—so facilitating the task of obtaining up-to-date numerical coefficients. A further development of the same formulation has led to a simple theoretical criterion, which enables the analyst to avoid pairs of wavelengths and concentration ratios that are unsatisfactory for precision. This criterion has been tested in an unfavourable application of the method. A procedure for choosing the best pair of wavelengths is described in detail.

The value of this new formulation and criterion of precision is discussed with special reference to a general procedure for the assay of injections which contain absorbing bacteriostatics. Any assay for a two component mixture depends upon some difference between the two components and in this respect Vierordt's method appears to be less exacting than the average quantitative separation process. For this reason, it seems to have greater scope than the latter.

THE method whereby a mixture of two known absorbing substances, A and B, may be determined spectrophotometrically is an old one. It was first applied by Vierordt[1] almost 90 years ago and involves extinction measurements at a pair of suitable wavelengths. The concentrations of A and B are then evaluated from a pair of simultaneous equations of the following form:—

$$E_1 = \alpha_1 c_A + \beta_1 c_B \quad .. \quad .. \quad .. \quad .. \quad (1)$$

$$E_2 = \alpha_2 c_A + \beta_2 c_B \quad .. \quad .. \quad .. \quad .. \quad (2)$$

The subscripts, "1" and "2" refer to wavelengths; E denotes an extinction of a 1 cm. layer of the solution of a mixture of A and B. c_A and c_B are the concentrations of A and B, whilst α and β are their respective extinction coefficients.

Despite its simplicity, this method seems to lack popularity among analysts, possibly for the reasons that follow. Thus, the method is far more sensitive to wavelength errors than is the spectrophotometric determination of a single absorbing substance, in which it is easy to choose a wavelength, λ, of maximum absorption (where, of course, $dE/d\lambda = O$). In Vierordt's method, on the other hand, it is almost certain that some of the extinction measurements will have to be made on the slopes of absorption curves. Here there is not only an increased source of error at the

time of measurement, but also a greater tendency for the extinction coefficients calculated from such measurements to go out of date—on account of changes in the instrument parameters (especially wavelength calibration) of a spectrophotometer, which inevitably occur with time. In this connection, the publication of equations involving numerical coefficients, which only apply to individual instruments at a particular time, suggests a lack of appreciation that such coefficients are impermanent. However, with regard to this important matter of calibration, the analyst is discouraged from setting up fresh coefficients by the "usual expressions" for the solution of equations (1) and (2). Thus, the expressions, which the author has seen, are all written in a form, which suggests that accurately prepared solutions of A and B are essential for the setting up of the numerical coefficients. One of the main purposes of this paper therefore is to point out that by the use of extinction ratios, the necessary coefficients can be obtained from solutions of A and B, of unknown concentration. It is hoped that this suggestion will encourage analysts to set up their own up-to-date coefficients and so obtain rather greater accuracy from Vierordt's method than hitherto. Quite apart from this however, the use of extinction ratios leads to expressions which give a much clearer idea of the conditions for precision. In particular, it is possible to set up a numerical criterion for satisfactory precision, which enables the analyst to avoid application of the method, to such examples as are doomed from the outset.

Formulation of Vierordt's method in Terms of Extinction Ratios

The formulation of equations (1) and (2) in terms of extinction ratios is readily achieved by substituting the following expressions into equation (2):

$$m = E_2/E_1 \qquad a = \alpha_2/\alpha_1 \qquad b = \beta_2/\beta_1$$

Note that m refers to the mixture, a to substance A and b to substance B. This leads to the following:

$$E_1 = \alpha_1 c_A + \beta_1 c_B \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

$$mE_1 = a\alpha_1 c_A + b\beta_1 c_B \quad .. \quad .. \quad .. \quad .. \quad (3)$$

which can be solved in the usual way to give

$$c_A = \frac{E_1}{\alpha_1}\left[\frac{b-m}{b-a}\right] \quad .. \quad .. \quad .. \quad .. \quad (4)$$

$$c_B = \frac{E_2}{\beta_2}\left[\frac{b(m-a)}{m(b-a)}\right] \quad .. \quad .. \quad .. \quad .. \quad (5)$$

The last two expressions can each be regarded as an apparent concentration multiplied by a correction term, T. Thus, equation (4) can be written, $c_A = c_A' T_A$, where $c_A' = E_1/\alpha_1$ which is the usual Beer's Law expression for the determination of substance A in the absence of any other absorbing substance. Just as in the determination of a single component, it is of course necessary that E_1 should be obtained from an accurately prepared solution of the analytical sample and that α_1 should

be an accurately known extinction coefficient. However, the correction term, T_A, depends upon the ratios *a*, *b* and *m* and these involve no knowledge of the concentration of absorbing material. Although the ratio, *m*, must be measured in each determination, this involves no more than the second reading, E_2, in addition to E_1—that is, without removing the cells from the instrument. The ratios, *a* and *b*, are simply and rapidly determined from solutions of A and B of unknown concentration, with the sole proviso that all extinctions lie within a reasonable range (e.g., 0·2–1·0). Once having determined *a* and *b*, there is no need to re-measure them until the analyst judges that instrument parameters have changed sufficiently for *a* and *b* to need revision.

Conditions for Precision

Inspection of the correction term, T, of both (4) and (5) shows that as *a* approaches *b*, the denominator tends toward zero. Furthermore, since *m* always lies between *a* and *b*, the numerator of T will also tend toward zero. This means that T will become very sensitive to small errors in *a*, *b* and *m* and the precision of the final result will suffer accordingly. We therefore see that if the absorption curves of A and B are sufficiently similar, it may be impossible to choose any pair of wavelengths, for which *a* and *b* are sufficiently different to obtain a reasonable measure of precision. It follows that such a pair of substances cannot be determined by Vierordt's method. This situation is not always easy to appreciate from graphs of extinction coefficient against wavelength, for although the curve of A may be substantially displaced on the ordinate scale relative to that of B, the ratios associated with various pairs of wavelengths may be insufficiently different for a successful application of the method. As will be shown later, the best test involves graphs of log E against wavelength. Then, if the curves for A and B are nearly superimposable, it is useless to apply Vierordt's method.

A Numerical Criterion for the Successful Application of Vierordt's Method to a Two Component Mixture

Assuming that for a given set of instrument parameters, the coefficient of variation, *u*, of an extinction measurement is constant between the limits, 0·2–1·0, and that all relative extinction errors are less than 20 per cent, it has been shown theoretically[2] that in a two component analysis, the coefficient of variation of the measurement of c_A, c.v.(c_A), is $u\sqrt{H_A}$. In a similar way, c.v.(c_B) is $u\sqrt{H_B}$.

H_A and H_B are different functions of the same extinction ratios, *a*, *b* and *m*. Having started with so simple an assumption about *u*, these relationships are but crude representations of practical observation. Nevertheless, there is experimental evidence[2] that large values of H are associated with high c.v.(c), so that the relationships provide a rational basis for the setting up of a practical criterion. The equation for H_A in terms of *a*, *b* and *m* shows that provided the ratio, *b*/*m*, lies outside the limits, 0·1–2·0, $\sqrt{H_A}$ will not exceed $\sqrt{7}$. Under these conditions,

c.v. (c_A) should not exceed $u\sqrt{7}$. This means that provided b/m is outside the above limits, c.v.(c_A) in the presence of B will not be more than $2\frac{1}{2}$ times c.v.(c_A) in the absence of B. A similar criterion exists for B. That is, c.v.(c_B) will not exceed $u\sqrt{7}$ provided the ratio, m/a, is outside the limits, 0·1–2·0.

The range of exclusion, 0·1–2·0, implies that for each ratio, b/m or m/a, there are two satisfactory ranges, that is, 0–0·1 and 2·0–∞. Inspection of the explicit forms[2] of H_A and H_B shows that of these two ranges, the range, 2·0–∞, is decidedly the better one to use. Sometimes, however, there is no option but to use the lower range.

This criterion limits not only the analyst's choice of wavelengths in any given case, but also the concentration ratio, c_A/c_B. Thus, in the determination of A, there is a limit for the ratio, c_A/c_B, below which c.v.(c_A) exceeds $u\sqrt{7}$.

The Choice of Wavelengths

Although quite general, the present section is really intended for those cases where the best choice of wavelengths is not immediately obvious and the author hopes that its inclusion will not confuse what is usually a very simple operation. Thus, in many applications of Vierordt's method, A and B have absorption peaks that are well separated in terms of wavelength. The choice is then very simple; that is, λ_{max} of A is chosen as λ_1, whilst λ_{max} of B is chosen as λ_2.

When, however, A and B do not possess such well separated peaks, the following discussion should help toward the best choice of wavelengths. Nevertheless, *the over-riding consideration is always that the appropriate ratio* (b/m for A; m/a for B) *should lie outside the limits*, 0·1–2·0, *and preferably be in excess of* 2·0. Thus, if the analyst chooses wavelengths that lead to unacceptable precision, then the mistake is readily shown up by the fact that the ratio for at least one of the components will lie between 0·1 and 2·0. Since a, b and m are all concerned in the final calculation, it is just a matter of inspection to check that the ratio is satisfactory for the particular component that is being calculated.

The ratios, b/m and m/a, are overall criteria, which limit both the choice of wavelengths and the concentration ratio. Although the latter may profoundly affect the precision of the result, it has no direct relevance to the best choice of wavelengths, which depends entirely upon the shapes of the absorption curves of A and B. Inspection of the explicit forms[2] of H_A and H_B, shows wavelength choice to be governed by the ratio, b/a, in the case of *both* A and B. If either b/m or m/a are to lie outside the limits, 0·1–2·0, then so also must b/a. The latter ratio is, in fact, always optimistic. Thus, since m always lies between a and b, the ratios b/m and m/a are always nearer to 0·1 or 2·0 than is the corresponding value of b/a (provided that the latter lies outside the limits).

It follows that when choosing wavelengths, b/a must certainly not lie within the range, 0·1–2·0. If b/a has to be less than 0·1, then it should be as small as possible. If, on the other hand, the *choice of wavelengths* can

achieve the more favourable condition, then in theory, *b/a should be as large as possible.* However, in practice* there is little point in making *b/a* much larger than about 25. It is therefore possible to pay some attention to general considerations of the kind mentioned on page 606.

The choice of wavelengths in accordance with the above requirements becomes simple when the absorption curves are plotted in the form of log E vs. λ, as in Figure 1 (*a*) and (*b*). It is then only necessary to superimpose the "B" graph upon the "A" graph, as in Figure 1 (*c*), and to slide the "B" graph along the ordinate axis, until the curves intersect at or near a λ_{max} of A. The wavelength of intersection must be designated λ_1, if the result is to be calculated by means of the expressions on p. 596. Then, to get a satisfactory value of *b/a*, choose λ_2 so that the distance between the "A" and "B" curves is fairly large, as in Figure 1 (*c*). It is usually necessary to try several values of λ_1 (intersection points) by sliding the "B" graph to various positions and noting the possibilities for λ_2. It is also desirable that at λ_2, the point on the "B" curve should lie above the point on the "A" curve, so that *b/a* comes within the range, 2·0–∞. Once the choice has been made, the value of log (*b/a*) can be read directly from the superimposed graphs as shown in Figure 1 (*c*). During this process, it is essential to keep the wavelength scales in register and the same ordinate and abscissa scales must be used for both graphs.

The above procedure is justified as follows. Thus,

$$b/a = \frac{\beta_2.\alpha_1}{\beta_1.\alpha_2}$$

$$\therefore \log (b/a) = \log \beta_2 - \log \alpha_2 + \log \alpha_1 - \log \beta_1$$
$$= (\log \beta_2 + K) - \log \alpha_2 + [\log \alpha_1 - (\log \beta_1 + K)]$$

When the "A" and "B" curves intersect, the value of K is such that $\log \alpha_1 - (\log \beta_1 + K) = O$, so that,

$$\log (b/a) = (\log \beta_2 + K) - \log \alpha_2$$

* The theoretical gain in precision to be expected from large values of *b/a* is partially offset by the large relative errors which are usually associated with the measurement of extreme values of *a*, *b* and *m*. Thus, the present treatment is based upon the assumption that all extinction measurements lie within the range, 0·2–1·0. Then, if path length and solution concentration are to be kept the same during the determination of one of the ratios, *a*, *b* or *m*, the ratio cannot fall outside the range, 0·2–5·0, unless one of the extinctions also falls outside the assumed extinction range. This obviously restricts the minimum and maximum values of *b/a* that are associated with precisely measurable ratios of *a*, *b* or *m*. The use of different (accurately known) path lengths or dilutions would, of course, allow one to observe ratios outside the range, 0·2–5·0, whilst keeping within the assumed limits of extinction. However, such steps are not practical in ordinary analysis.

In practice, one may have to measure quite large ratios and these will usually imply a very small value for the lower extinction. Nevertheless, although the resultant ratio will be correspondingly imprecise, the final answer for the concentration will not be greatly affected. Thus, suppose that in equation (4), *a* is very small and *b* very large, and therefore subject to considerable error. Now, since $b \gg a$, *a* can be neglected from the denominator, so that (4) becomes:—

$$c_A = \frac{E_1}{\alpha_1}(1 - m/b)$$

Unless the ratio c_A/c_B is very small, *m* will be small, so that errors in *b* will have little effect on c_A.

Finally, it must be mentioned that although in the light of the simple criterion for b/a, the best choice of wavelengths for A will also be the best for B, practical considerations may favour different pairs of wavelengths for A and B. This is because the criterion for b/a is based upon a purely photometric argument, which can accommodate wavelength setting errors only on an average basis. That is, it cannot take account of the fact that wavelength setting errors depend upon the actual slopes of the absorption

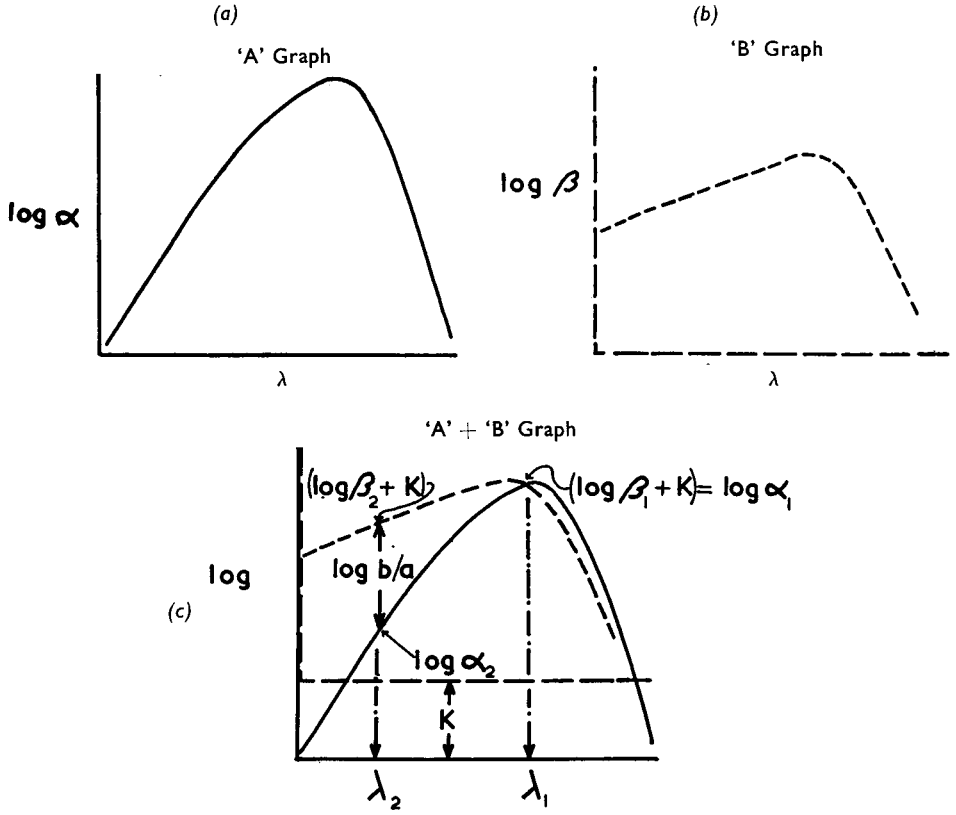


FIG. 1. Procedure for choosing wavelengths in difficult cases. In (*c*), the 'B' graph has been superimposed upon the 'A' graph and then displaced through the distance, K, along the ordinate.

curves of A and B at λ_1 and λ_2 as well as upon the ratio, c_A/c_B. When choosing wavelengths, therefore, it is undesirable to be too rigid in applying the criterion for b/a. However, this statement does not invalidate the importance of the ratios, b/m and m/a, as indications of the applicability of Vierordt's method.

Possible Interaction Between A and B

The validity or otherwise of Lambert's and Beer's Laws is important in any spectrophotometric method. These considerations apply to

Vierordt's method with greater force than they do in the determination of a single substance. Thus, the latter can be determined in conditions where both laws are disobeyed, provided that the analyst is prepared to accept a non-linear calibration curve (E *vs.* c). With Vierordt's method on the other hand, such deviations require the final result for c_A and c_B to be calculated by a series of successive approximations[3], which is tedious to the point of being impractical.

Nevertheless, Vierordt's method contains an additional potential hazard in that A and B may form complexes (e.g., AB)[4] the extinction coefficients of which may not be simple sums (e.g., $\alpha_1 + \beta_1$) of those of

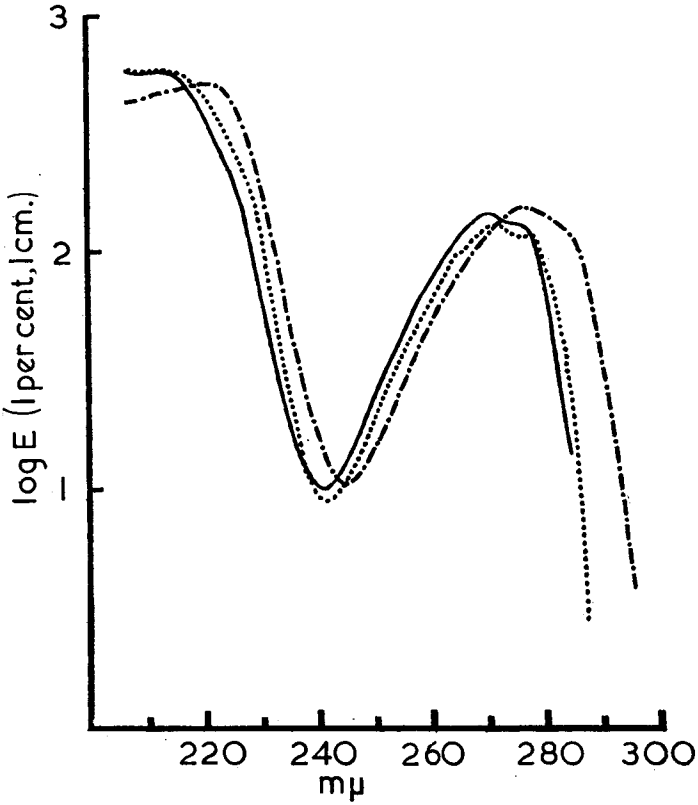


FIG. 2. The absorption curve of *o*-, *m*-, *p*-cresols in 0·1N aqueous H_2SO_4.
—— = *o*-cresol; · · · · = *m*-cresol; — · — · = *p*-cresol.

the uncomplexed substances. This means that although A may obey Beer's law on its own, it may not do so in the presence of B. Such interactions are governed by chemical equilibria, which means that in a given case their effects become more pronounced as c_A and c_B increase. Large flat aromatic systems such as dyes are especially likely to form complexes, even at the rather low concentrations used in spectrophotometry. Vierordt's method then becomes very tedious and some other method of analysis should be invoked.

The author believes that the above interactions are unlikely to be significant, where neither A nor B is a large molecule. It is, however, desirable to formulate a simple test for the absence of experimentally significant interactions. Thus, if at a particular wavelength, the E(1 cm.) of a mixture of A and B at concentrations, c_A and c_B, is the sum of (i) E(1 cm.) of A alone at concentration, c_A, and (ii) E(1 cm.) of B alone at concentration, c_B, then such interactions are absent. This is the basis of the test given in the experimental section.

An Unfavourable Application of Vierordt's Method

Mixtures of *o* and *p*-cresols in 0·1N H_2SO_4 were analysed for *o*-cresol in an attempt to evaluate the method under the conditions of an unfavourable application. Thus, it will be noted from Figure 2 that the

TABLE I

COEFFICIENTS OF VARIATION OF GROUPS OF EIGHT RESULTS OBTAINED IN AN UNFAVOURABLE APPLICATION OF VIERORDT'S METHOD: THE DETERMINATION OF *o*-CRESOL (SUBSTANCE A) IN THE PRESENCE OF *p*-CRESOL (SUBSTANCE B)

No.	$\frac{c_A}{c_B}$	λ_1*	*a*	*b*	*m*	*b*/*a*	*b*/*m*	c.v. (c_A)	Percentage error of mean value of c_A
1	3·85	272·5	0·96	1·14	0·99	1·19	1·15	0·89	−1·2
2	3·85	275	0·91	1·26	0·97	1·39	1·30	0·42	−0·4
3	3·85	277·5	0·83	1·30	0·92	1·56	1·42	1·01	−0·9
4	3·85	280	0·55	1·20	0·67	2·17	1·79	0·60	−1·2
5	3·85	282·5	0·26	1·10	0·41	4·28	2·70†	0·40	−1·0
6	3·85	285	0·10	0·96	0·25	9·76	3·81†	0·37	−0·6
7	1·44	272·5	0·96	1·14	1·03	1·19	1·11	0·57	−2·2
8	1·44	275	0·91	1·26	1·03	1·39	1·22	0·64	−1·3
9	1·44	277·5	0·83	1·30	1·00	1·56	1·29	0·74	−1·9
10	1·44	280	0·55	1·20	0·80	2·17	1·51	1·18	−3·5
11	1·44	282·5	0·26	1·10	0·57	4·28	1·93	0·48	−3·1
12	1·44	285	0·10	0·96	0·42	9·76	2·30†	0·34	−2·7
13	0·64	285	0·10	0·96	0·58	9·76	1·65	0·72	−2·3
14	0·24	285	0·10	0·96	0·77	9·76	1·25	1·25	−7·0

* λ_1 = 270 mμ throughout.

† These sets of results are the only ones that would be allowed by the rule that *b*/*m* should lie outside the limits, 0·1–2·0.

The values of *a* and *b* in rows 1–6, were used to calculate results throughout the whole table; hence, the repetitions in the columns for *a*, *b* and *b*/*a* (from row 7 onward).

absorption curves of the three cresols are similar. With *o* and *p*-cresols, it was therefore easy to select a range of wavelengths and ratios, c_A/c_B, that enabled one to explore the performance of the method in the region of the greater of the proposed limits. The results of the survey are given in Table I.

All but three of the results (that is, Nos. 5, 6 and 12) are disallowed by the criterion that *b*/*m* should lie outside the limits, 0·1–2·0. Nevertheless, the coefficient of variation of a number of these disallowed results is not large and on the whole the precision is surprisingly good.

The correlation of c.v.(c_A) and *b*/*m* was rather poor, probably because each set of results was associated with its own particular set of wavelength setting errors. As already mentioned, such errors can only be accommodated within this simple theory on an average basis. Nevertheless, the criterion for *b*/*m* was effective in disallowing all instances in which c.v.(c_A) exceeded 0·5. Note that *b*/*m* was not only effective in controlling the choice of wavelengths, but also the minimum ratio of c_A/c_B, that could be tolerated in the determination of c_A. Thus, for λ_1 = 270 mμ

and $\lambda_2 = 285$ mμ, b/m was satisfactory for Nos. 6 and 12, but not for Nos. 13 and 14, which were associated with the lowest ratios of c_A/c_B.

Since an analytical method must usually stand or fall by its accuracy rather than its precision, the matter of bias is important. The results in Table I show a consistent bias toward low values of c_A, which might be expected to arise from an interaction between A and B. Nevertheless, no significant interaction was detectable and this bias remains unexplained. On the whole, the bias became worse as c_A/c_B moved to lower values. For $c_A/c_B = 3{\cdot}85$, the bias was, however, small and the ratio, b/m, did have some effect in disallowing those results, which had a large bias.

A Generalised Design for Two Component Spectrophotometric Assays

The present theory offers a generalised specification for two component spectrophotometric assays in that having decided upon limits for b/m, the conditions for satisfactory wavelengths and concentration ratios follow with substantial certainty. The particular choice of limits used in this paper is only tentative; they could be more or less stringent than the range, 0·1–2·0. The general idea might be helpful in the rational design of official assays for particular combinations of substances. Such an assay would specify equations (4) and (5), as well as λ_1, λ_2, α_1, β_2, together with the allowable limiting concentration ratios. The analyst would, however, be expected to measure the ratios, a and b, on his own instrument, using pure samples of A and B. There would be no need to mention the limits of b/m, upon which the specification was based.

By comparison with existing specifications, the main advantage of the above would reside in the improved accuracy to be gained from the use of individually determined ratios, a and b. Nevertheless, the theory offers much more scope in dealing with a situation in which a particular absorbing substance, A, has to be determined in the presence of an "absorbing nuisance", B, which, although known to the analyst, could be one of a large range of possibilities. Hitherto, this situation has posed an apparently insoluble problem to the designers of official assay processes. Thus, if substance B is quite unspecified, the official assay process must be a completely general one and it is difficult to design a process of this kind, which is neither vague nor cumbersome. However, a general specification that uses equation (4) in conjunction with suitable limits for b/m seems to provide a satisfactory answer.

A small problem arises in that any such official process would need to quote an extinction coefficient, α_0, for a certain wavelength, λ_0. However, when assaying A in the presence of a particular substance, B, it might be impossible to choose λ_1 equal to λ_0. The analyst would then need to set up a value of α_1 based on the official value, α_0, but this would only necessitate an additional extinction measurement at λ_0 when measuring a.

A Solution to the Problem of Absorbing Bacteriostatics in the Spectrophotometric Assay of Pharmacopoeial Injection Solutions

The fact that on occasions any suitable bacteriostatic can be added to a pharmacopoeial injection has undoubtedly retarded the inclusion of

spectrophotometric assays in a number of monographs that would otherwise have constituted ideal spectrophotometric applications. Thus, a simple spectrophotometric determination breaks down in the presence of an appreciably absorbing bacteriostatic. However, although the range of bacteriostatics that might be found in an injection solution is rather a wide one[5], the answers[6,7] that have been offered so far have been restricted in that they have referred to the particular absorbing bacteriostatics, phenol and chlorocresol.

In an attempt to produce "an ideal method . . . by which any injection solution containing an interfering bacteriostatic agent could be determined (spectrophotometrically)", Elvidge, Proctor and Baines[7] have successfully used oxidised cellulose for the quantitative separation of active constituents from either phenol or chlorocresol in the case of some 13 injection solutions. This method has given good results and appears to be of wide application. Nevertheless, it seems doubtful whether so general an aim can be achieved by a particular separation process. The process, which will quantitatively separate any two substances in a reliable and predictable manner surely does not exist? If the analyst applies a standard separation process to a bacteriostatic, which he has not previously met, he must presumably run a quantitative recovery experiment. This is apt to be time consuming and may merely lead to the conclusion that the standard separation process is inapplicable. On the other hand, the decision whether a particular combination of substances will be feasible by Vierordt's method is a matter of simple arithmetic and it is often possible to assess the feasibility of the method by inspecting the molecular formulae of A and B and noting their approximate relative concentrations, where such information is available.

It is fundamental to most quantitative separations that A should belong to one class of substance and B to another. In merely requiring a sufficient difference between the absorption curves of A and B, Vierordt's method is much less stringent and therefore of correspondingly wider application than any one separation process. The mathematical character of Vierordt's method also leads to a precise but simple general specification, which would be difficult to equal in the case of the separation process. Nevertheless, both general approaches are valuable and it is to the analyst's advantage to have two strings to his bow. Furthermore, where the two components cannot be completely separated from one another, and where the concentration ratio is unfavourable to Vierordt's method, a combination of the two general approaches may succeed in circumstances which defeated either one alone.

Collections of Spectra of Active Constituents and Bacteriostatics

When assaying injections that contain absorbing bacteriostatics, it is useful to build up a collection of absorption curves for all substances encountered. The same graph scales should be used throughout and all curves should be determined in the same solvent. In this connection, the author has found 0·1N aqueous H_2SO_4 to be the solvent of choice, for when A and B contain acid-base auxochromes, its high buffering power favours

the obedience to Beer's law. The concentration of H_2SO_4 should be controlled to within about ± 10 per cent, since variations of say ± 100 per cent may produce significant spectral changes, especially when the absorbing compound contains a very weakly basic auxochrome[8,9].

By way of example, a small collection is shown in Figure 3, in which log E(1 cm.) refers to the pharmacopoeial concentrations of the substances in question. Whilst facilitating the choice of wavelengths, this particular ordinate scale also gives an immediate impression of the relative absorptions of active constituent and bacteriostatic that arise from given combinations. A diagram of this kind is easy to construct by first plotting extinctions directly onto logarithmic graph paper and then tracing off the curve at the correct height on the diagram.

Unlike the bacteriostatic concentration, the concentration of a particular active constituent is variable. In this connection, Figure 3 has

TABLE II

WAVELENGTHS CHOSEN FROM FIGURE 3

Injection	Bacteriostatic	λ_1 (mμ)	λ_2 (mμ)	b/a
Aminophylline 2·5 per cent w/v	Chlorocresol	245	—	—
	Phenol	240	271	5
	Phenylmercuric Nitrate	271	—	—
Aneurine Hydrochloride 0·5 per cent w/v	Chlorocresol	249	—	—
	Phenol	246	270	12
	Phenylmercuric Nitrate	246	—	—
Atropine Sulphate 0·03 per cent w/v ..	Chlorocresol*	247	280	210
	Phenol*	247	270	31
	Phenylmercuric Nitrate	257	220	2·7
Morphine Sulphate 1·0 per cent w/v ..	Chlorocresol	285	249	0·07
	Phenol	282	265	12
	Phenylmercuric Nitrate	285	—	—
Nicotinamide 5·0 per cent w/v	Chlorocresol	261	—	—
	Phenol	238	—	—
	Phenylmercuric Nitrate	261	—	—

The absence of a figure for λ_2 means that at λ_1, the extinction due to the bacteriostatic is less than 0·5 per cent of that due to the active constituent. In such a case, the bacteriostatic's absorption can be ignored, so that a normal single component determination can be applied at λ_1. This approximation was referred to by Brealey and Proctor[8] as the "Direct Method".

* Assuming that the bacteriostatic concentration is first reduced by partial extraction.

been constructed pessimistically by using the lowest concentrations of active constituent that are likely to be met in practice. It is, of course, easy to adjust any curve in the Figure to a different concentration by simply sliding it bodily up or down the ordinate scale. For example, curve No. 7 refers to Atropine Sulphate 0·03 per cent w/v and the curve for twice this concentration could be obtained by a vertical displacement of curve No. 7 through a distance equal to log 2.

Figure 3 contains a good deal of information that is available at a glance: (i) the extent to which an injection must be diluted to bring E(1 cm.) into a measurable range at a particular wavelength, (ii) wavelengths at which the bacteriostatic absorption contributes less than 0·5 per cent to the total extinction of the mixture[6], (iii) of the three bacteriostatics shown, Phenol provides the worst problem, (iv) Phenylmercuric Nitrate only makes a significant contribution in the case of Atropine Sulphate Injection, (v) Vierordt's method cannot be applied to Atropine Sulphate Injection containing phenol or chlorocresol, unless the bacteriostatic concentration has been reduced to about 1 per cent of its nominal value by partial extraction.

The list of wavelengths given in Table II has been compiled from Figure 3 according to the considerations; (i) b/a to be as large as possible, (ii) avoidance of absorption curve slopes, particularly for E_1 (for example,

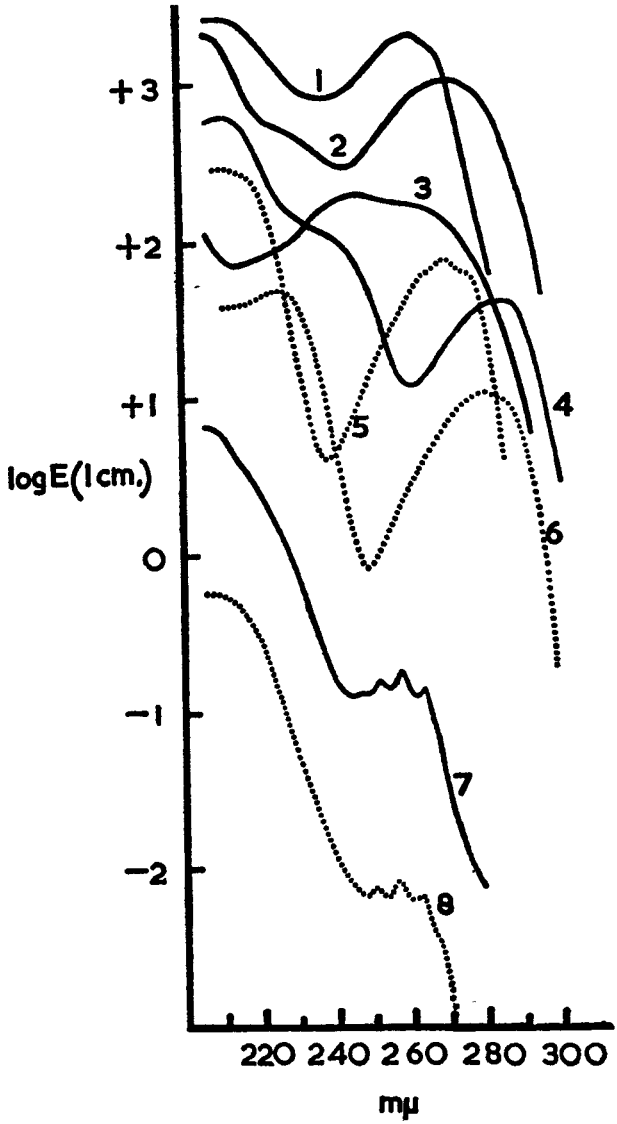


FIG. 3. Ultra-violet absorption curves of constituents of injection solutions (in 0·1N aqueous H_2SO_4).

1. Nicotinamide (5 per cent w/v); 2. Theophylline (2 per cent w/v) equivalent to Aminophylline (2·5 per cent w/v); 3. Aneurine Hydrochloride (0·5 per cent w/v); 4. Morphine Sulphate (1 per cent w/v); 5. Phenol (0·5 per cent w/v); 6. Chlorocresol (0·1 per cent w/v); 7. Atropine Sulphate (0·03 per cent w/v); 8. Phenylmercuric Nitrate (0·001 per cent w/v).

—— Active constituent; ···· Bacteriostatic.

regions of vibrational structure), (iii) preference for $\lambda_1 = \lambda_{max}$ of A, (iv) avoidance of measurements below 220 mμ, (v) avoidance of Vierordt's method where possible[6].

EXPERIMENTAL

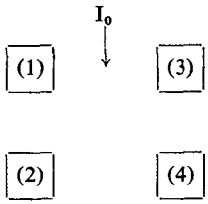
All extinctions were measured on a Uvispek Spectrophotometer (Mark III). 0·1N aqueous H_2SO_4 was used as solvent throughout.

Coefficients of Variation of the Determination of o-Cresol in the Presence of p-Cresol (Table I)

Since pure materials were not essential to this work, the *o*- and *p*-cresols were used without further purification. Solutions containing both *o*- and *p*-cresols were prepared by mixing known weights of Solution "A" (*o*-cresol) and Solution "B" (*p*-cresol). The results, "percentage error of mean value of c_A" in Table I were calculated with reference to the observed E(1 cm.) of comparison solutions. Each comparison solution was identical to the solution of mixed isomers to which it referred except that *p*-cresol was omitted.

The object of the experiment was to include photometric and wavelength setting errors, but to eliminate other sources of variance including cell filling. Each solution was therefore placed in a stoppered 10 mm. cell and subjected to a series of measurements, which continued until all necessary readings had been obtained from the solution in question. The differences in radiant power that occurred over the small wavelength interval, 270 mμ–285 mμ, were insignificant and deflection sensitivity was kept constant throughout the whole experiment. Throughout each series, measurements alternated between λ_1 and λ_2, each extinction being the mean of two readings for one particular setting of the wavelength scale. With the exception of a few measurements at 282·5 mμ and 285 mμ, all extinctions were within the range, 0·2–0·6.

Test for Interaction Between o-Cresol and p-Cresol (*see p.* 600)



Throughout the test, four 10 mm. cells were used as shown herewith. Solutions "A" and "B" (as above) were accurately diluted or mixed and placed in the cells according to the following arrangements. ("S" = 0·1N aqueous H_2SO_4)

Series	Cell (1)	Cell (2)	Cell (3)	Cell (4)
I	"A" 50 ml. "S" 50 ml.	"B" 50 ml. "S" 50 ml.	"A" 50 ml. "B" 50 ml.	"S"
II	"A" 50 ml. "B" 50 ml.	"S"	"A" 50 ml. "B" 50 ml.	"S"
III	"S"	"A" 50 ml. "B" 50 ml.	"A" 50 ml. "B" 50 ml.	"S"

For each series, the extinction of the four cell arrangement was measured at 270, 275, 280 and 285 mμ. Series III was included to detect any path length mis-match between cells (1) and (2), which would show itself as a difference between Series II and Series III. In all three series and at all four wavelengths, the readings differed insignificantly from 0·005, which value evidently arose from absorption mis-match between the cell pair, (1) + (2), and the pair, (3) + (4). Interaction between the *o*- and *p*-cresols was therefore experimentally insignificant under the conditions, which applied to the results in Table I.

Absorption Curves (*see Figures* 2 *and* 3)

With the exception of *o*-, *m*-, and *p*-cresols, all substances were of British Pharmacopoeial standard and dried according to B.P. requirements. Primary solutions were prepared from quantities of the order of 0·5 g. and then diluted to produce solutions for measurement. Extinctions were plotted directly onto logarithmic graph paper (Wightman & Mountain No. 11L).

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