CHROM. 3512

CORRELATIONAL ANALYSIS OF SEVEN PROCEDURES FOR DETERMINING AREAS OF CHROMATOGRAPHIC CURVES*

ROY B. MEFFERD, JR., RUBY M. SUMMERS AND JIM D. CLAYTON
Psychiatric and Psychosomatic Research Laboratory, Veterans Administration Hospital, Houston, Tex. (U.S.A.)

(Received March 18th, 1968)

SUMMARY

Seven widely different methods were used to measure 184 peaks from gas chromatograms. Nine ancillary measures known to influence area measurements were also determined. A correlational analysis of these was made to determine the exact influence of the ancillary variables on each of the area measurements, and to determine whether any one of the methods was superior to another. The ancillary variables had only minor influence on any of the procedures. Peak height was the primary determinant of precision with any method. All methods were essentially identical in precision justifying the use of a correction constant with any of the methods where it is desirable also to report an accurate result. Even a procedure as approximate as the rectangular one yields quite adequate results and at great economy in time and money, and is easily adaptable to automatic determination.

A number of procedures have been advocated for the determination of the areas of peaks on recorder output, ranging from crude approximations to extremely meticulous, time consuming procedures[1,2]. A theoretical discussion of error in the measurement of chromatographic peaks by a number of common manual methods has recently appeared[3]. In this investigation we experimentally examined the results of seven procedures, using the peaks obtained from esterified fatty acids of serum. These peaks were of a wide range of both peak sizes (from 0.1 to 246 cm^2) and of instrumental errors (*e.g.*, the amount of tailing and non-symmetry of the peaks and of baseline drift). Small laboratories and those in which gas chromatographs are used at irregular intervals seldom can justify the large investment required for an electronic integrator. Therefore, the comparisons were all made using methods that are available in the average small laboratory, and that can be performed rapidly by any careful person regardless of educational level.

This study was designed to determine how much precision varied between methods of area measurements. In the chromatographic literature the stress seems to be increasingly on accuracy as distinct from precision[4-6]. Such accuracy is usually

* The computations were made at the Common Research Computer Facility, Texas Medical Center, under USPHS Grant No. FR 00254, and by R. B. MEFFERD, JR. at Baylor University College of Medicine, Houston, Tex.

obtained at a considerable trade-off in time and cost, irrespective of the fact that two methods having equal precision may also have equal accuracy merely by applying suitable correction constants. In such cases, the consideration reduces to one of the relative cost of area measurements of the various methods.

METHODS

Samples

Although any chromatographic curves would have been suitable for this study, we desired to retain the error involved in the analysis of natural samples and of the variation due to individual differences. Large variation is desirable for correlational analyses. The samples were esterified free fatty acids isolated from the serum samples of 200 adult males. These were dissolved in *n*-hexane and injected into a 6 ft. gas chromatographic column on washed and silanized firebrick coated with 15 % diethyleneglycol succinate. The chromatograph used was a Warner-Chilcott (formerly Research Specialties) Series 600 instrument with a hydrogen flame detector. An E. H. Sargent and Co. SRL recorder was used.

Every tenth recording was taken from the pool of 200 samples for this area analysis. This insured that there would be a wide range of instrumental variation between the recordings (*e.g.*, seven different columns were involved, and the samples were injected at different stages during the life of these columns). Using the same eleven fatty acids from each sample, there were 184 peaks whose areas were at least 0.1 cm^2. These were used for the statistical analysis (*i.e.*, $N = 184$). Not only did the peak sizes for the different fatty acids vary greatly ($\bar{x}$ 0.7 to 85.5 cm^2), but, of course, the subjects also varied in their quantities of each fatty acid.

Area measurements

Seven methods were used to estimate the area of each of the 184 peaks. Each method was applied to all peaks by two technicians, who worked independently. Their measurements were averaged after all peaks were finished. A total of eight technicians were used, so that each applied from two to three of the methods. A technician completed one set of measurements before he started another, and he did not use two similar methods (*e.g.*, the two planimeter measurements). A baseline was drawn on each of the 20 charts and decisions that might influence area estimations (*e.g.*, the dividing lines between poorly resolved peaks) were made before the area measurements were started.

The methods were as follows:

(1) *Rectangular*: area of the rectangle formed by the product of the peak height and its true retention time (determined automatically with the OSCAR).

(2) *Template*: area obtained by fitting the peaks as nearly as possible to the size of one of a large set of calibrated clear plastic templates constructed from another group of similar chromatograms.

(3) *Triangular*: area obtained from the product of the peak height and the peak width at the midpoint of the altitude.

(4) *Integrator*: area obtained from a Disc integrator attached to the recorder.

(5) *Planimeter-rapid*: area obtained with a planimeter which was operated rapidly and with no effort to correct for minor departures in tracing the curve.

(6) *Planimeter-meticulous*: area obtained using the same planimeters but now different technicians traced slowly and meticulously until equal replicate measurements were obtained.

(7) *Automated ordinate summation*: area obtained using a Benson-Lehner Model F OSCAR with ordinate heights being summed for 1 cm intervals along the baseline and corrected by a precalculated constant.

Other variables

Variables that could influence areas of peaks were included in the analysis as follows: the quantity of sample injected into the chromatograph, true retention time, peak height, peak width (at both half-peak height and at the baseline), the number of readings required in the ordinate summation using the OSCAR for each peak, two objective tailing factors (the ratio of the horizontal distance from the altitude to the leading edge and this distance to the following edge at half-peak height, and the same ratio computed at the baseline) and a subjective judgment of tailing with a scale of 1 to 4 (1 = no tailing, 4 = severe tailing).

Statistical analysis

Linear components of variance[7,8] were extracted from the matrix of Pearson product moment correlation coefficients of the seven area measurements and of the nine ancillary variables listed above. The principal axis technique (PAX), was used for this with unities in the diagonals of the correlation matrix. Components were extracted until the associated eigen values fell below 1.0. The four components that resulted were rotated by the varimax (VAX) procedure, and orthogonal factor scores were computed for each of these so that the relative position of each peak on each component could be ascertained.

RESULTS

The means and standard deviations resulting from the use of the seven different methods for measuring the areas of the 184 peaks are shown in Table I. The intentionally wide range in size of the peaks is shown by the large standard deviations. The distribution of the areas of each peak was reasonably normal though slightly skewed

TABLE I

MEANS, STANDARD DEVIATIONS, AND CORRELATIONS (LEADING DECIMALS OMITTED) OF SEVEN AREA MEASUREMENTS

Method of measurement	*Mean*	*S.D.*	*Correlation variables*						
			1	*2*	*3*	*4*	*5*	*6*	*7*
1. Rectangular	248.0	371.7	X						
2. Template	20.2	32.4	97	X					
3. Triangular	17.9	28.7	98	98	X				
4. Integrator	19.2	31.7	97	98	99	X			
5. Fast planimeter	19.5	32.4	97	98	99	99	X		
6. Slow planimeter	19.5	32.9	97	98	99	99	99	X	
7. OSCAR	19.7	32.9	96	97	99	99	99	99	X

toward the small ends. The rectangular method, of course, yielded much larger areas than the others. Within the more similar procedures, the use of templates yielded high results and the triangular method low, as compared to those of the meticulous planimeter measurements. The other methods yielded results nearly identical both with respect to their means and standard deviations. The correlations between the seven areas are also shown in Table I. The extremely high precision of all seven methods is evident from the correlations that ranged from 0.96 to 0.99. Such high correlations indicate that for all practical purposes the area measurements are interchangeable. This was shown also by the fact that the correlations of the areas with the nine ancillary measures varied between methods at most by $r = 0.04$. The means of the z scores of the correlation coefficients for the seven methods are shown with those of the ancillary measures in Table II. Sample size was correlated only with the subjective judgment of tailing. This judgment was correlated only with sample size and the objective measure of tailing at the baseline. Retention time was, of course, positively correlated with measure of peak width (including the number of OSCAR readings per peak, since these were made every 1.0 cm) but it was uncorrelated with tailing. All the measures of peak height and width correlated with the area values. Both of the objective measures of tailing (but not the subjective) correlated moderately with the areas.

TABLE II

CORRELATIONS BETWEEN THE SEVEN AREA MEASUREMENTS AND NINE VARIABLES THAT INFLUENCE AREAS (DECIMALS OMITTED)

Variables	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
1. Sample size	X								
2. True retention time	10	X							
3. Peak height	—08	10	X						
4. Peak width, 1/2 alt.	—05	83	24	X					
5. Peak width, baseline	—09	73	37	90	X				
6. No. OSCAR readings	—07	70	42	89	90	X			
7. Tailing, 1/2 alt.	—05	13	18	27	—01	26	X		
8. Tailing, baseline	—19	23	33	48	45	50	55	X	
9. Tailing, judgment	—71	—10	09	10	11	06	14	36	X
10. Mean, areas	—09	50	76	66	66	75	47	55	11

Individual peaks varied greatly in areas, *e.g.*, peak 1 was about 10 × the area of peak 9 (85.8 *vs.* 8.1 cm^2 with standard deviations of 14.1 and 2.0 cm^2, respectively). The areas of very small peaks have a larger relative measurement error than do those of large peaks. The influence of this on the correlations is shown in Table III, where the intercorrelations of the seven area-measurement methods are compared for peak 1 (in the upper half of the matrix) and peak 9 (in the lower half of the matrix). It is evident that both the template and the rectangular methods introduced large errors into the area estimates of very small curves. Although it is not shown here, templates were also poor for estimating the areas of peaks when there was marked baseline drift.

The variance of the correlation matrix of the 16 variables was partialled into linear components (PAX) and rotated (VAX). The results are shown in Table IV. All

TABLE III

INTERCORRELATIONS (DECIMALS OMITTED) OF THE METHODS OF AREA MEASUREMENT FOR A LARGE PEAK (PEAK 1, UPPER HALF OF THE MATRIX) AND A VERY SMALL ONE (PEAK 9, LOWER HALF OF THE MATRIX)

Method	*1*	*2*	*3*	*4*	*5*	*6*	*7*
1. Rectangular	X	88	89	87	86	87	89
2. Template	73	X	99	99	99	99	99
3. Triangular	94	88	X	99	99	99	99
4. Integrator	81	87	92	X	99	99	99
5. Fast planimeter	78	92	90	89	X	99	99
6. Slow planimeter	88	88	94	96	90	X	99
7. OSCAR	87	89	94	95	93	96	X

TABLE IV

LOADINGS (*i.e.*, CORRELATIONS) OF THE VARIABLES ON THE PRINCIPAL AXIS (PAX) AND VARIMAX (VAX) COMPONENTS (DECIMALS OMITTED)

The percentage of total variance accounted for by each PAX and VAX component is shown in parentheses under the component's number.

Variable	*Components*							
	PAX				*VAX*			
	1 (63)	*2* (12)	*3* (10)	*4* (7)	*1* (43)	*2* (26)	*3* (11)	*4* (11)
1. Sample size	11	67	54	26				91
2. True retention time	−61	57	−36	11		88		
3. Peak height	−71	−24	41	−41	94			
4. Peak width, 1/2 alt.	−78	36	−45	13	−30	91	20	
5. Peak width, baseline	−76	36	−44	−17	40	87		
6. No. OSCAR readings	−85	28	−30	00	48	80		
7. Tailing, 1/2 alt.	−46	−35	18	74	26		92	
8. Tailing, baseline	−62	32	−19	43	32	30	65	−30
9. Tailing, judgment	−14	−72	−56	−09				−91
10. Area, rectangle	−95	01	19	−17	90	38		
11. Area, template	−97	−07	14	−09	88	38	23	
12. Area, triangle	−98	−07	15	−04	88	38	28	
13. Area, integrator	−98	−06	14	00	86	40	31	
14. Area, fast planimeter	−98	−08	15	00	87	38	31	
15. Area, slow planimeter	−98	−07	14	−02	87	39	29	
16. Area, OSCAR	−98	−10	16	−01	87	36	31	

PAX loadings are presented so that an oblique rotation can be made if the reader desires. Only loadings above 0.20 are shown for the VAX components.

Upon rotation three major orthogonal sources of variance in all the area measures were evident. There were essentially no differences between any of the methods with these. By far the most important source of variance for all measures was peak height (VAX 1, Table IV), explaining about 75 % of the variance of each of the areas (*i.e.*, the squares of the loadings). Next most important was peak width (VAX 2) explaining about 15 % of the areas' variance. Tailing tended to result in larger areas. Retention time was positively related to both width and the areas. A third component

of slight importance (*i.e.*, less than 10 % of the areas' variance) involved tailing (VAX 3). The fourth component did not involve the areas. Rather it concerned only judged tailing and sample size.

DISCUSSION AND CONCLUSION

For most peaks the methods of area measurement used here were equally precise. It was immaterial whether the measure was only an apparently crude approximation, as in the rectangular method, or an exceedingly accurate one, as with the meticulous planimeter measurement. The large amount of the total variance explained by VAX 1 shows that the precision of measurement is determined primarily by the proper location of the peak's apex, the location of the baseline under the apex, and the measurement of the altitude at this point. Quantitatively, of course, the measures differed considerably, but the precision was very high as evidenced by the intercorrelations of from 0.96 to 0.99. The use of correction constants to achieve accurate values is clearly justified. Very small peaks require special consideration since their relative error is large with any of the methods. The rectangular method is poor for small peaks that have long retention times and, as was pointed out above, the template method was poor when the baseline was shifting sharply. Both had higher relative errors than the other methods.

One of the reasons for the very high intercorrelations among the area measurements was the large range of values used. Had only one of the eleven peaks been used as well as a standard sample size and with as many of the other parameters held constant as possible, there would have been far less variance available for analysis. The correlations would have been reduced, accordingly—but correlations are only meaningful when there is adequate variance.

A judgmental artifact involving sample size became evident in the analysis. In the last VAX component, every peak that was judged to have no tailing (*i.e.*, given a rating of 1) was obtained from a small-sized sample (1.5 μl), whereas every peak that was judged to have severe tailing (*i.e.*, a rating of 4) was from a large sample (4.5 μl). Yet, this apparent tailing was uncorrelated with either the objective measures of tailing or the area. Thus, visual inspection of routine charts is an unreliable procedure for determining precision of a method.

REFERENCES

1 M. DIMBAT, P. E. PORTER AND F. H. STROSS, *Anal. Chem.*, 28 (1956) 290.
2 H. H. HARMAN, *Modern Factor Analysis*, University of Chicago Press, Chicago, 1960, pp. 154, 301, 337.
3 INTERNATIONAL CONFERENCE OF BENZOLE PRODUCERS, *J. Chromatog.*, 12 (1963) 293.
4 E. C. HORNING, E. H. AHRENS, JR., S. R. LIPSKY, F. H. MATTSON, J. F. MEAD, D. A. TURNER AND W. H. GOLDWATER, *J. Lipid Res.*, 5 (1964) 20.
5 D. L. BALL, W. E. HARRIS AND H. W. HABGOOD, *Separation Sci.*, 2 (1967) 81.
6 F. T. EGGERTSEN, H. S. KNIGHT AND S. GROENNINGS, *Anal. Chem.*, 28 (1956) 303.
7 J. JANÁK, *J. Chromatog.*, 3 (1960) 308.
8 J. E. OVERALL, *Psychol. Rept.*, 10 (1962) 651.