temperature when obtained pure. It decomposes at 52° as compared with the crude product which explodes if heated rapidly to 50°. The pure azide

does not readily detonate when attacked with a hammer.

A Method for Standardization of Chromatographic Analysis[1]

BY ARTHUR L. LeROSEN

The chromatographic method of Tswett has been applied to many problems of the chemist with great success, making possible separations which could not be attained satisfactorily by any other means. As the use of this method increases it is desirable to obtain quantitative data for the comparison of adsorbents and the behavior of the adsorbed substances on these materials. A quantitative treatment of chromatography should be helpful in determining the best conditions for a given operation and in standardizing the properties of adsorbents.

Good books on chromatography are now available (Zechmeister,[2] Strain[3]). Theories of chromatography have been developed by Wilson[4] and by Martin and Synge,[5] and measurements of a quantitative nature have been made by Cassidy.[6,7] Brockmann and Schodder[8] have suggested a method of standardizing the adsorption affinity of certain alumina samples, using azo dye mixtures.

The investigations herein recorded were undertaken primarily with the purpose of ascertaining how far it is possible to determine the relative position of carotenoids on the adsorbent column by separate measurements of the rate of movement for each pigment.

It was first necessary to investigate the flow of the developing solvent through the adsorbent. The velocity of flow was found to vary directly with the pressure difference between the ends of

the column, inversely with the length of the column and to be essentially independent of the diameter of the tube (tubes of diameter 17, 43, and 70 mm. gave almost equal rates for columns of the same length). The exact nature of each of these dependencies has not been thoroughly studied.

The change of rate of flow with time was of more importance. Table I illustrates the behavior of calcium hydroxide columns. It is evident that the flow becomes constant after an initial decrease, a uniform rate being obtained soon after the solvent has reached the bottom of the column.

Three terms will now be introduced to simplify the following discussion: S = length of adsorbent column containing one unit volume of solvent/ length of tube required to contain the same volume of solvent; V_c = rate of flow of developing solvent through the column when a state of constant flow has been reached (mm./min.); R = rate of movement of adsorbate zone (mm./min.)/ rate of flow of developing solvent (V_c).

The ratio S may be of importance in characterizing the packing of the column, and, moreover, it gives the percentage of the tube volume occupied by the adsorbent (% volume adsorbent = $100(S-1)/S$). There is a variation in the degree of packing throughout the column; S was found to vary

(1) Presented before the Division of Analytical and Micro Chemistry of the American Chemical Society at the Memphis meeting, April, 1942.

(2) L. Zechmeister and L. Cholnoky, "Principles and Practice of Chromatography," John Wiley and Sons, Inc., New York, N. Y., 1941.

(3) H. H. Strain, "Chromatographic Adsorption Analysis," Interscience Publishers, Inc., New York, N. Y., 1941.

(4) J. N. Wilson, THIS JOURNAL, 62, 1583 (1940).

(5) A. J. P. Martin and R. L. M. Synge, Biochem. J., 35, 1358 (1941).

(6) H. G. Cassidy and S. E. Wood, THIS JOURNAL, 63, 2628 (1941).

(7) H. G. Cassidy, ibid., 63, 2735 (1941).

(8) H. Brockmann and H. Schodder, Ber., 74, 73 (1941).

TABLE I

RATE OF BENZENE FLOW THROUGH CALCIUM HYDROXIDE COLUMNS (17 MM. IN DIAMETER AND 150 ± 5 MM. LONG).

The data given indicate flow in mm. column length/min. Time was measured from the instant the solvent was poured on the column.[a]

Column no.	Time, min.								
	1	5	10	15	20	25	30	35	40
1	32.0	14.5	9.0	7.1	6.4	6.3	6.3	...	...
2	43.0	15.5	9.8	7.1	7.0	7.0	7.0	...	...
3	36.0	13.5	7.8	...	7.6	7.5	7.5	7.5	...
4	42.0	15.3	...	...	7.6	7.7	7.7	7.7	7.7
5	43.0	13.8	8.8	...	8.4	8.2	8.2	8.2	8.2
6	42.0	16.0	13.0	...	...	8.1	8.1	8.1	8.1

[a] The solvent reached the bottom of the column in about twelve minutes.

on the average from 1.97 for the top third to 1.79 for the bottom third of the calcium hydroxide columns used. No consistent relation has been observed between S and V_c, probably due to the greater importance of local variations in the column packing.

The rate of solvent flow, V_c, varies from column to column packed with the same adsorbent under similar conditions, but the variation is within reasonable limits; for example, in 14 of 15 cases considered, V_c was between 6.3 and 8.2, while in one instance it was much higher, 10.3.

V_c itself depends on such variables as particle size, shape, surface character, solvent, etc., but it is beyond the scope of this paper to discuss these factors.

The "strength" or adsorption affinity of a material may be best determined by measuring the adsorption isotherm, but this is not always prac-

TABLE II

RATES OF MOVEMENT OF CHROMATOGRAPHIC ZONES RELATIVE TO THAT OF THE DEVELOPING SOLVENT, ON CALCIUM HYDROXIDE AND DEVELOPED WITH BENZENE. MEASUREMENTS APPLY TO BOTTOM EDGE OF ZONE

Substance	R	Substance	R
Capsanthin	0.007	Lycopene	0.125
Celaxanthin[a]	.150	Kryptoxanthin	.340
β-Carotenone	.030	Physalien	.590
Zeaxanthin	.040	γ-Carotene	.790
Lutein	.070	Prolycopene[9]	.885
Hydroxy-γ-carotene[a]	.070	β-Carotene	1.000

[a]Obtained from the fruits of *Celastrus scandens* (unpublished).

TABLE III

CALCULATED AND OBSERVED RELATIVE POSITIONS OF THE COMPONENTS OF A MIXTURE SEPARATED ON CALCIUM HYDROXIDE, DEVELOPED WITH BENZENE. ZONES WERE IDENTIFIED SPECTROSCOPICALLY

Substance	Calculated		Observed[c] Position mm. from the top relative to lycopene	
	R	R/R_{lycopene}	I	II
Capsanthin	0.007	0.056	0.04	0.04
Celaxanthin[a]	.015	.12	.13	.11
β-Carotenone	.030	.24	.24	.19
Zeaxanthin	.040	.32	.35	.30
Lutein	.070	.56		
Hydroxy-γ-carotene[a]	.070	.56	.62[b]	.55
Lycopene	.125	1.00	1.00	1.00

[a] Obtained from the fruits of *Celastrus scandens*. [b] Not separated; rechromatographing on calcium carbonate showed the presence of both pigments in this zone. [c] Two readings were taken at different stages in the development of the chromatogram.

(9) L. Zechmeister, A. L. LeRosen, F. W. Went and L. Pauling, *Proc. Nat. Acad. Sci.*, **27**, 236 (1941).

tical, as Brockmann and Schodder have pointed out. The same end can be attained with somewhat less precision by measuring R, the relative rate of movement of an adsorbate zone with respect to the developing solvent. Table II gives the values of R for certain carotenoids. Table III shows the agreement of the relative positions of carotenoid zones, calculated from R, and positions observed when a mixture was separated chromatographically.

There should be a relation between the adsorption isotherm and R. Let us consider the element of volume of any adsorbent column which contains in its interstitial spaces one unit volume of solution of uniform concentration c/unit volume. The adsorbent in equilibrium with this unit volume contains $f(c) = A$ concentration units of the solute. The use of volume here rather than length or weight has an advantage in dealing with rates, because, after the relation is obtained for an adsorbent, it is independent of tube radius. In the case of lycopene, $C_{40}H_{56}$, the relation was approximately linear over the concentration range studied for the system lycopene–benzene–calcium hydroxide; the value $A = 8c$ was found for the calcium hydroxide columns studied.

Eight units of solution would have to pass through an element of column, as defined above, before it reached saturation; therefore, the front edge of the solvent moves through nine elements, while the pigment moves through one. R should, therefore, be equal to 0.111. The value observed for lycopene, 0.125, is in reasonably good agreement.

Martin and Synge,[5] in discussing the theory of a certain type of chromatogram, have used a term, R,[10] for the relative rate of movement of a chromatographic zone. Their definition differs slightly from that proposed above.

Dr. W. T. Stewart has suggested[11] an alternative procedure, namely, the measurement of rates of movement relative to some standard dye, for the determination of the relative positions of chromatographic zones.

From measurements of R certain inversions of the relative positions of pairs of carotenoids on different adsorbents were predicted. The first example was the case of kryptoxanthin–lycopene;

(10) The use of a somewhat different R in this paper is not only due to a delay in receiving the journal containing the work of Martin and Synge (received May 12, 1942), but also for convenience in measurement.

(11) W. T. Stewart, Thesis, California Institute of Technology, 1941.

kryptoxanthin is adsorbed above lycopene on calcium carbonate or alumina but below it on calcium hydroxide. Several other cases of this type have been observed. This phenomenon has already been noticed, e. g. by Duschinsky and Lederer.[12]

It has been found in this Laboratory that measurements of V_c of adsorbents led to the acquirement of better materials than before its use. For ordinary laboratory work a range of from about 5 to 15 mm./min. is desirable; this seems to be associated with an average particle size of 5 to 15 microns. If the adsorption affinity of a material shows much variation, as in the case of alumina, measurements of R for the substances to be separated should allow standardization. The most suitable value for R seems to be between 0.2 and 0.3. The values given for R in the tables are subject to correction as the measurement technique is improved.

Experimental

Determination of V_c.—For all the experimental work, except that concerning the influence of diameter, chromatographic tubes 250 by 17 mm. were used (obtainable from Scientific Glass Apparatus Co., Bloomfield, N. J.). The adsorbent was "Shell" brand lime, chemical hydrate, 325 mesh, obtained from the Braun Chemical Company in Los Angeles.

For the determination, the tubes were filled with the absorbent while suction was applied (about 25 mm. vacuum) and the absorbent slowly poured into the tube, the sides of which were then vigorously tapped in order to allow the absorbent to settle. The top of the column was pressed down firmly with a stamper, and the absorbent was removed from the walls above the column. Three successive 5-cc. portions of solvent were then pipetted onto the top of the column. Just as each portion disappeared into the absorbent the next one was introduced. At the same time, the position of the bottom edge of the solvent was noted. In this manner it was possible to determine the

(12) R. Duschinsky and E. Lederer, *Bull. soc. chim. biol.*, **17**, 1534 (1935); see also Strain. "Chromatographic Analysis" (ref. 3), page 6.

number of mm. column length equivalent to 1 cc. of solution. Next a buret was attached to the top of the absorption tube by a stopper provided with an outlet which could be closed when the air space above the column was filled with solvent. The buret stopcock was then opened, the tube filled with solvent and the outlet closed. It was then possible to determine the velocity of solvent in the column from the flow in cc. per minute shown by the buret.

Determination of R.—The procedure was the same as described above except that the initial portion of solvent (ligroin or ligroin–benzene) contained the carotenoid pigment, while all succeeding portions were pure benzene. The pigment was poured on the column in a solution containing ligroin, from which it is more strongly absorbed than benzene, in order to obtain a concentrated zone. The ligroin is immediately washed through the column by the benzene and, consequently, does not interfere with the determination. The buret reading and carotenoid position on the column were recorded at ten-minute intervals by a stop watch. The values, recorded in Table II, were obtained when V_c was reached.

All measurements were made with fresh solutions of pure crystals since the rates of movement may be profoundly influenced by impurities. It remains to be seen how far, even in very crude solutions, the relative rates remain proportional to those found with pure materials.

Summary

1. Some terms of importance to quantitative chromatography have been suggested; these are S, which indicates the average packing of the column, V_c, the rate of solvent flow when the velocity has become constant, and R, the rate of movement of an absorbate zone relative to that of the developing solvent.

2. Evidence has been given to show that the above mentioned quantities are of value in characterizing and standardizing absorbents, as well as predicting the relative positions of chromatographic zones.

3. Inversion of the sequence of some carotenoid pairs on different absorbents has been predicted and observed.