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VARIABLES AFFECTING PRECISION AND ACCURACY IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Quantitative analysis by peak height or peak area measurement using a UV photometric detector can be performed with a high degree of precision, by both isocratic and gradient methods, if variables such as mobile phase composition and flow-rate are carefully controlled.

If flow control is poor but composition can be maintained precisely, peak height measurement yields better quantitative results because height is relatively independent of flow-rate. This may be a common condition with older liquid chromatography pumping systems. If flow control is good but solvent composition cannot be maintained precisely, peak area measurement is better because area is relatively independent of composition. This may be a common condition in adsorption chromatography where trace water and polar contaminants in the mobile phase are difficult to control, and in any type of affinity chromatography where mobile phases comprise volatile solvents.

Retention time is strongly affected by both composition and flow-rate, but retention time precision can be inherently better in gradient elution than in isocratic analysis.

INTRODUCTION

Analytical precision and accuracy in high-performance liquid chromatography (HPLC) depend on the reproducibility of peak retention time, peak height, and peak area. This peak behavior is a function of many chromatographic variables, including sample size, mobile phase composition, mobile phase flow-rate, and column temperature.

In this study, mobile phase composition and flow-rate were systematically varied in both isocratic and gradient analysis, while sample size and column temperature were held constant. The resulting peak behavior was observed and compared with theory. General guidelines were developed for quantitation techniques, depending on the equipment available.

Theory suggests that the composition and flow dependence of peak behavior will be different for peaks with different capacity factors (k') and for packings with

different chemical functionality. Therefore, the experiments attempted to define relationships for peaks of widely varying k' values on the most commonly used new packing, hydrocarbon reversed phase.

EXPERIMENTAL

Columns, solvents, and samples

250 × 3.1 mm I.D. Type 316 stainless-steel columns (Li-Chroma I.D.™ tubing; Handy and Harman, Norristown, Pa., U.S.A.) were packed by a partial balanced density slurry technique[1]. The packing used was 10-μm Spherisorb™ ODS, a totally porous spherical material with covalently bonded octadecyl functionality (Spectra-Physics, Santa Clara, Calif., U.S.A.).

Mobile phases were prepared from distilled-in-glass solvents (Burdick and Jackson Labs., Muskegon, Mich., U.S.A.). No drying or adjustment of water content was undertaken. Water was glass distilled. Trace organics in the water were not removed. All solvents were degassed prior to use.

Multicomponent samples were prepared by adding enough of each pure component to a 1:1 mixture of water and methanol to produce approximately equal peak areas. The approximate concentration was 0.1 mg/ml for each component.

Control of flow, composition, temperature, and sample volume

A Spectra-Physics Model 3500B gradient liquid chromatograph was used. It employs a continuous-flow, reciprocating-piston pump for Solvent A and an identical but independent one for Solvent B. Each pump has its own electronic flow feedback system for precise flow control. The two pump outlets are mixed at high pressure in a dynamically stirred chamber. The mobile phase composition and the flow-rate through the column are determined, respectively, by the ratio and the sum of the A and B pump flow-rates. This two-pump architecture has become the most common type in HPLC.

The flow feedback restrictors were placed in a 3-l insulated container of water to eliminate minor flow changes caused by any large ambient temperature fluctuations in the laboratory. Under these conditions, the flow-rate can be maintained to ± 0.2% or better. The temperature dependence of flow-rate produced by the pump due to the feedback system is typically 1% change in flow per °C change due to a combination of viscosity, pressure, and compressibility effects in the flow restrictor and the chromatographic column. The flow-rate was volumetrically measured at each setting to confirm accuracy.

The Model 3500B forced-air oven controlled column temperature at 40.0 ± 0.1°. No attempt was made to compensate for changes in the internal temperature of the column due to mobile phase composition and flow-rate changes[2].

The sample injector employed was of the 10-μl external loop type. The loop was filled slowly, taking care not to form microbubbles inside the loop. Volumetric precision was determined independently to be about 0.05% relative standard deviation.

The detector was a Spectra-Physics Model SP 8200 multiple-wavelength UV photometer operated at 254 nm.

Measurement of peak retention time, height, and area

Peak behavior was measured by computing integrators—the Minigrator® and the SP 4000™ Chromatography Data System (Spectra-Physics)—which are capable of retention time, peak height, and peak area determination with a precision of better than 0.1 %[3]. The SP 4000 built-in program for calculating relative standard deviations of peak areas from several runs was also used.

Selection of chromatographic conditions

Fig. 1 shows the chromatogram and operating conditions of the isocratic analysis on the Spherisorb ODS column. The conditions were chosen to be representative of current practice. For example, although the 7,000 p.s.i. (478 bar) capability of the chromatograph would permit a faster flow-rate and faster analysis, most workers continue to operate in the 1–2 ml/min range. Sample components and solvent composition were chosen to provide a range of k' from 0–8 in order to provide data on the peak behavior for this variable.

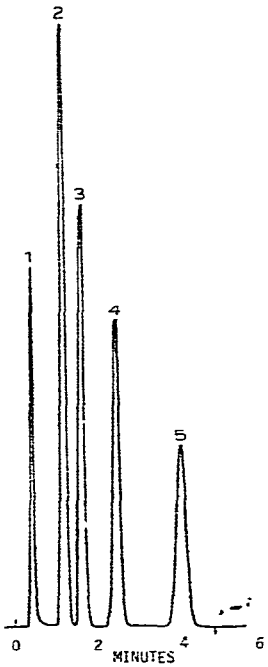

Fig. 1. Isocratic analysis. Column, 250 × 3.1 mm I.D.; packing, 10-μm Spherisorb ODS; solvent, water–methanol (1:1); flow-rate, 2.00 ml/min; pressure, 1840 p.s.i.; temperature, 40°; detector, Model SP 8200 multiple-wavelength UV photometer operated at 254 nm; sample size, 10 μl. 1 = Tartrazine; 2 = methyl *p*-hydroxybenzoate; 3 = ethyl *p*-hydroxybenzoate; 4 = propyl *p*-hydroxybenzoate; 5 = butyl *p*-hydroxybenzoate.

Fig. 2 shows the chromatogram of the gradient analysis on Spherisorb ODS. Again, conditions are representative of current practice. The speed of the analysis can be improved by starting the run at 30%B. However, the intention here was to provide large k' values. An extra component, benzyl alcohol, was added to the test

TABLE I

EFFECT OF FLOW-RATE VARIATIONS ON RETENTION TIME, PEAK AREA AND PEAK HEIGHT IN ISOCRATIC ANALYSIS

Compound	*k'*	*Retention time (sec)*				*Peak area (integrator counts*)*				*Peak height (scale divisions)*			
		Flow-rate (ml/min)				*Flow-rate (ml/min)*				*Flow-rate (ml/min)*			
		2.00	*1.92*	*1.84*	*1.76*	*2.00*	*1.92*	*1.84*	*1.76*	*2.00*	*1.92*	*1.84*	*1.76*
Tartrazine	0	26	27	28	30	782	815	864	903	1245	1260	1299	1323
Methyl *p*-nydroxybenzoate	1.8	73	76	80	85	1800	1865	1974	2061	2057	2058	2097	2127
Ethyl *p*-hydroxybenzoate	2.8	98	103	109	115	1686	1749	1845	1932	1553	1549	1572	1588
Propyl *p*-hydroxybenzoate	4.7	147	154	164	174	1655	1714	1811	1901	1090	1080	1093	1099
Butyl *p*-hydroxybenzoate	8.3	242	254	271	289	1491	1546	1632	1710	620	615	620	620

* 1 integrator count = 0.2 mV sec.

TABLE II

EFFECT OF COMPOSITION VARIATIONS ON RETENTION TIME, PEAK AREA AND PEAK HEIGHT IN ISOCRATIC ANALYSIS

Compound	*k'*	*Retention time (sec)*					*Peak area (integrator counts)*					*Peak height (scale divisions)*				
		Composition (%B)					*Composition (%B)*					*Composition (%B)*				
		50	*49*	*48*	*47*	*46*	*50*	*49*	*48*	*47*	*46*	*50*	*49*	*48*	*47*	*46*
Tartrazine	0	26	26	26	26	26	799	779	789	782	793	1259	1255	1277	1279	1250
Methyl *p*-hydroxybenzoate	1.8	74	76	79	82	86	1788	1795	1796	1810	1820	2028	2000	1978	1935	1857
Ethyl *p*-hydroxybenzoate	2.8	100	103	110	117	124	1675	1680	1682	1687	1698	1520	1480	1438	1372	1303
Propyl *p*-hydroxybenzoate	4.8	151	159	171	186	203	1644	1648	1649	1654	1665	1055	1012	959	890	825
Butyl *p*-hydroxybenzoate	8.6	250	268	294	327	363	1479	1482	1485	1489	1494	602	557	515	468	420

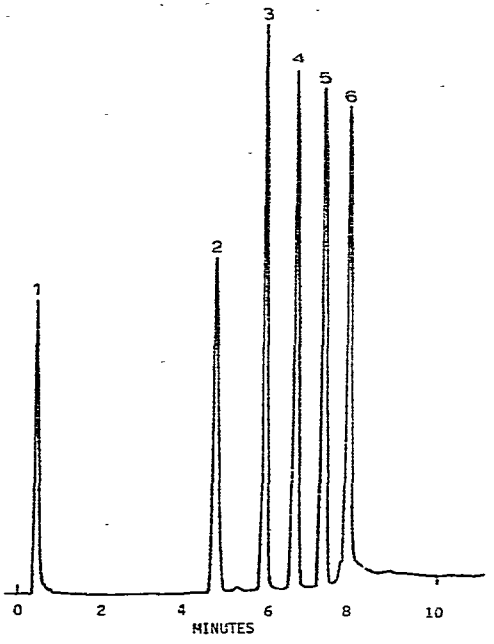

Fig. 2. Gradient analysis. Column conditions, same as for Fig. 1, except: Solvent A, water; Solvent B, methanol; gradient profile, 0 → 99%B in 10 min. 1 = Tartrazine; 2 = benzyl alcohol; 3 = methyl *p*-hydroxybenzoate; 4 = ethyl *p*-hydroxybenzoate; 5 = propyl *p*-hydroxybenzoate; 6 = butyl *p*-hydroxybenzoate.

sample for this analysis. The small peak at 5.3 min and the small peaks which interfere with the last major peak are impurities in the water which are concentrated at the top of the column and subsequently eluted by the gradient. These impurities can be removed, but were intentionally allowed in order to study their effect on the precision of observed peak behavior.

RESULTS

Isocratic analysis: Effects of flow-rate variations

Table I shows the effect of flow-rate variations on retention time, peak area, and peak height, respectively. The 2.00 ml/min is the reference flow-rate, the other three representing successively 4, 8, and 12% reductions from the reference.

Fig. 3 presents the following data for one compound, propyl *p*-hydroxybenzoate: per cent change in retention time, peak area, and peak height *versus* per cent change in flow-rate. The other compounds with different k' values exhibited similar behavior.

Results show that retention time and peak area are inversely proportional to flow-rate, while peak height is relatively independent of flow-rate.

Isocratic analysis: Effects of composition variations

Table II shows the effect of composition variations on retention time, peak area, and peak height, respectively. The 50.0%B is reference composition, the other

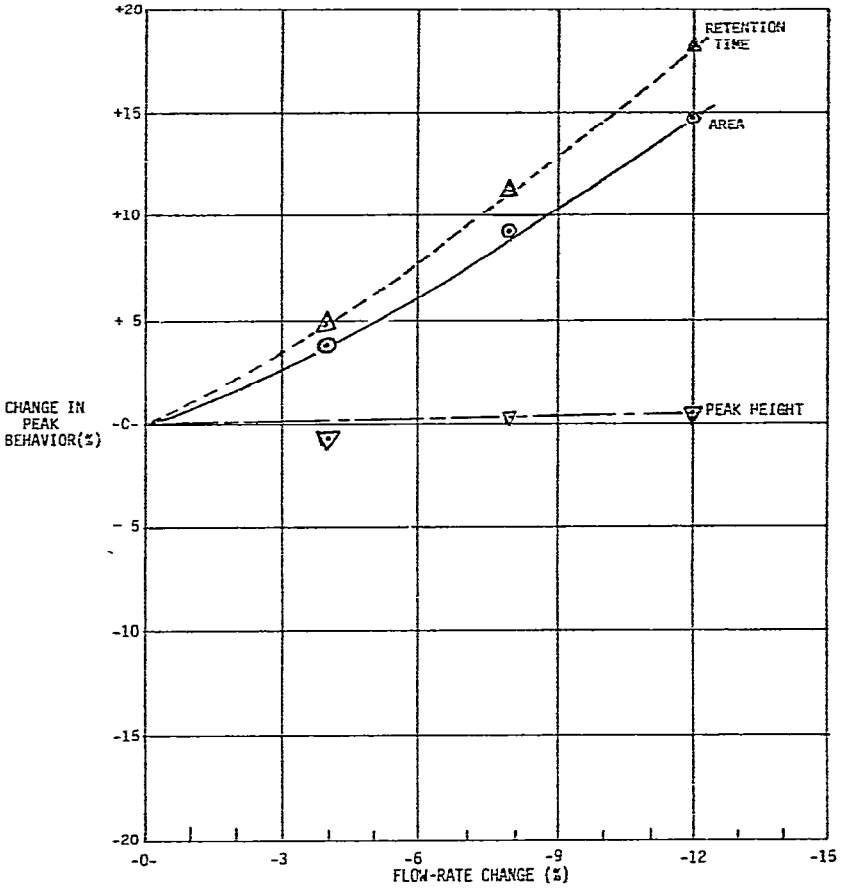

Fig. 3. Retention time, peak area, and peak height *versus* flow-rate in isocratic analysis.

four representing successively 2, 4, 6, and 8 rel. % reductions from the reference. Since the absolute value of composition itself is expressed in per cent, the following nomenclature clarification is made: 2% relative reduction from 50%B means 2% of 50% = 1% absolute reduction, or a drop from 50 to 49%B.

Fig. 4 presents peak area data for propyl *p*-hydroxybenzoate as a graph of per cent change in composition; other compounds with different k' values behaved the same. Fig. 4 also presents the retention time and peak height data for the other four compounds.

Results show that retention time is inversely proportional to composition. However, the dependence increases with increasing k'. Peak height is directly proportional to composition. However, the dependence increases with increasing k'. Peak area is independent of composition for all k' values.

Gradient analysis: Effects of flow-rate variations

Table III shows the effect of flow-rate variations on retention time, peak area, and peak height, respectively. The 2.00 ml/min is the reference flow-rate, the other four representing successively 2, 4, 6, and 8% reductions from the reference.

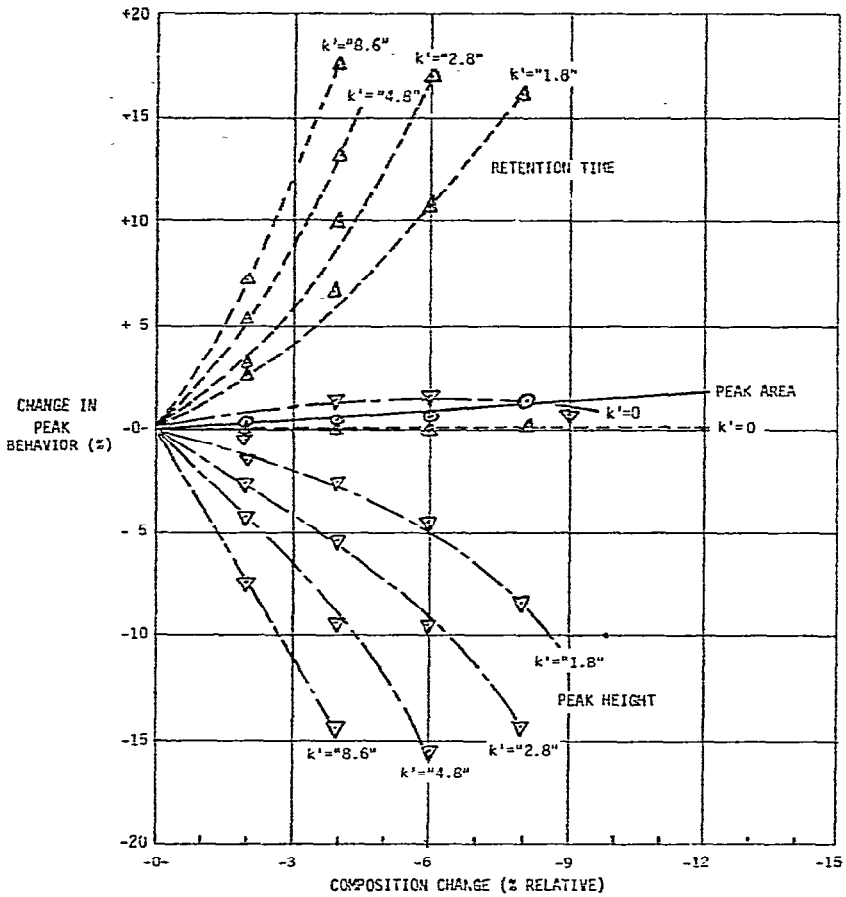

Fig. 4. Retention time, peak area, and peak height *versus* composition in isocratic analysis.

Fig. 5 presents peak area and peak height data for propyl *p*-hydroxybenzoate as a graph of per cent change in retention time, peak area, and peak height *versus* per cent change in flow-rate; it also presents retention time data for several other components. The peak height data are only for the adjusted 1.84 ml/min conditions as noted in Table III.

Results show that retention time is inversely proportional to flow-rate. However, the dependence decreases with increasing k'. Peak area is inversely proportional to flow-rate and peak height is relatively independent of flow-rate, for all k' values.

Gradient analysis: Effects of composition variations

Table IV shows the effect of composition variations on retention time, peak area, and peak height, respectively. The %B composition of 49.5, 5 min after the start of the gradient run from 0–99%B in 10 min, is the reference composition, the other three representing successively 3, 6, and 9 rel. % reductions from the reference. All compositions are expressed as %B 5 min after the start of the run.

TABLE III

EFFECT OF FLOW-RATE VARIATIONS ON RETENTION TIME, PEAK AREA AND PEAK HEIGHT IN GRADIENT ANALYSIS

Compound	*k'*	*Retention time (sec)*						*Peak area (integrator counts)*						*Peak height (scale divisions)*					
		Flow-rate (ml/min)						*Flow-rate (ml/min)*						*Flow-rate (ml/min)*					
		2.00	*1.96*	*1.92*	*1.88*	*1.84*	*1.84**	*2.00*	*1.96*	*1.92*	*1.88*	*1.84*	*1.84**	*2.00*	*1.96*	*1.92*	*1.88*	*1.84*	*1.84**
Tartrazine	0	27	28	29	29	30	30	755	770	789	810	830	820	999	990	989	993	997	985
Benzyl alcohol	9.5	283	287	293	297	302	314	1252	1275	1301	1330	1356	1356	1150	1167	1190	1213	1239	1163
Methyl *p*-hydroxybenzoate	12.2	355	359	365	368	373	393	1690?	1717	1746	1786	1824	1835	1935	1979	2019	2059	2095	1995
Ethyl *p*-hydroxybenzoate	13.8	400	404	409	412	417	442	1514	1535	1565	1601	1633	1642	1770	1813	1850	1885	1915	1827
Propyl *p*-hydroxybenzoate	15.3	440	[illegible]	448	452	456	486	1581	1594	1606	1641	1695	1690	1705	1747	1785	1817	1840	1759
Butyl *p*-hydroxybenzoate	16.6	475	478	483	486	490	524	1811	1841	1875	1915	1955	1998	1630	1669	1700	1730	1753	1673

* Conditions adjusted to provide a gradient program, in volumetric terms, equal to the reference run at 2.00 ml/min. The adjusted conditions are 0–99%B in 11 min, compared to the reference conditions of 0–99%B in 10 min.

TABLE IV

EFFECT OF COMPOSITION VARIATIONS ON RETENTION TIME, PEAK AREA AND PEAK HEIGHT IN GRADIENT ANALYSIS

Compound	*k'*	*Retention time (sec)*				*Peak area (integrator counts)*				*Peak height (scale divisions)*			
		Composition (%B) at 5 min				*Composition (%B) at 5 min*				*Composition (%B) at 5 min*			
		49.5	*48.0*	*46.5*	*45.0*	*49.5*	*48.0*	*46.5*	*45.0*	*49.5*	*48.0*	*46.5*	*45.0*
Tartrazine	0	27	28	27	27	756	757	748	754	993	1008	1008	1010
Benzyl alcohol	9.3	279	286	287	293	1247	1252	1244	1255	1163	1150	1108	1105
Methyl *p*-hydroxybenzoate	12.0	350	359	363	371	1673	1680	1672	1694	1951	1989	1887	1873
Ethyl *p*-hydroxybenzoate	13.6	395	405	411	420	1495	1504	1500	1514	1780	1765	1723	1707
Propyl *p*-hydroxybenzoate	15.2	436	446	454	465	1550	1562	1562	1574	1713	1707	1667	1640
Butyl *p*-hydroxybenzoate	16.4	471	482	492	503	1789	1805	1733	1760	1633	1623	1577	1558

Fig. 6 presents data for propyl *p*-hydroxybenzoate as a graph of the per cent change in retention time, peak area, and peak height *versus* the relative per cent change in composition. The other compounds with different k' values exhibit similar behavior.

Results show that retention time is inversely proportional to composition, peak height is directly proportional to composition, and peak area is independent of composition.

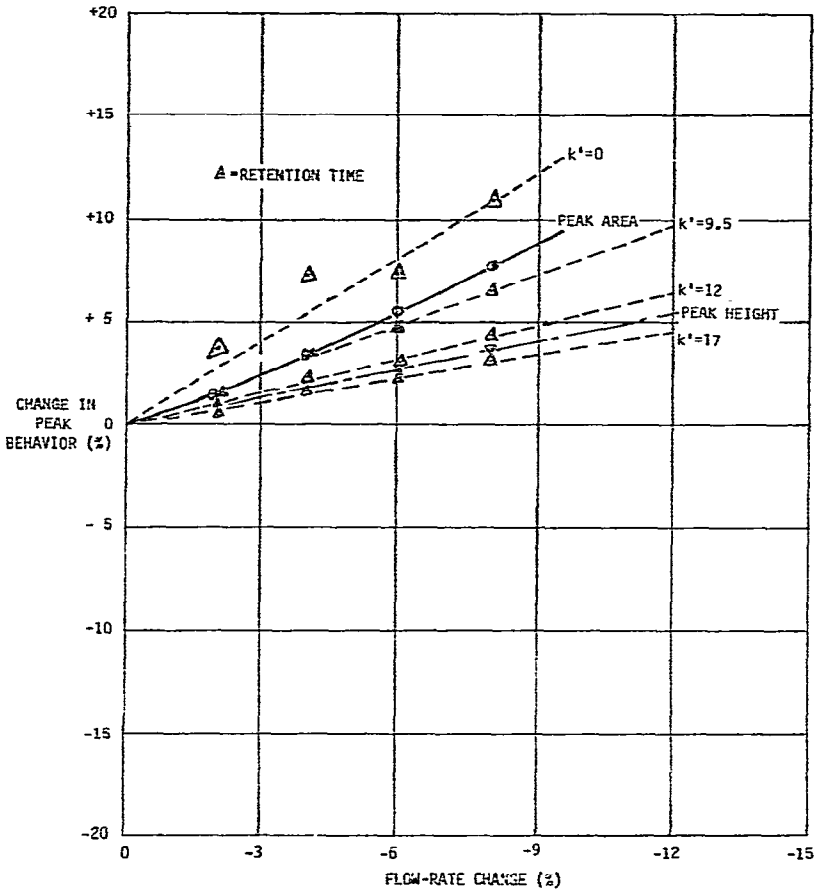

Fig. 5. Retention time, peak area, and peak height *versus* flow-rate in gradient analysis.

Precision of gradient analyses

The inherent precision of the flow-rate and the composition of the experimental system was measured by a series of gradient runs, as shown in Table V. Data show that the precision of the experiments was sufficient to validate the data obtained in the previous tables.

TABLE V

PRECISION OF RETENTION TIME AND PEAK AREA IN GRADIENT ANALYSIS

Compound	*Retention time (sec)*						*% RSD*	*Peak area (integrator counts)*						*% RSD*
	Run No.							*Run No.*						
	1	*2*	*3*	*4*	*5*	*6*		*1*	*2*	*3*	*4*	*5*	*6*	
Tartrazine	28	27	27	27	27	27	1.50	761	756	756	754	753	756	0.36
Benzyl alcohol	285	279	279	281	282	283	0.83	1249	1247	1247	1248	1251	1252	0.17
Methyl *p*-hydroxybenzoate	354	350	350	352	354	355	0.62	1677	1673	1673	1680	1684	1690	0.40
Ethyl *p*-hydroxybenzoate	398	395	395	397	399	400	0.52	1502	1495	1497	1503	1509	1514	0.48
Propyl *p*-hydroxybenzoate	438	436	435	437	439	440	0.43	1554	1550	1556	1568	1555	1581	0.75
Butyl *p*-hydroxybenzoate	472	471	470	472	474	475	0.39	1792	1789	1780	1793	1814	1811	0.74

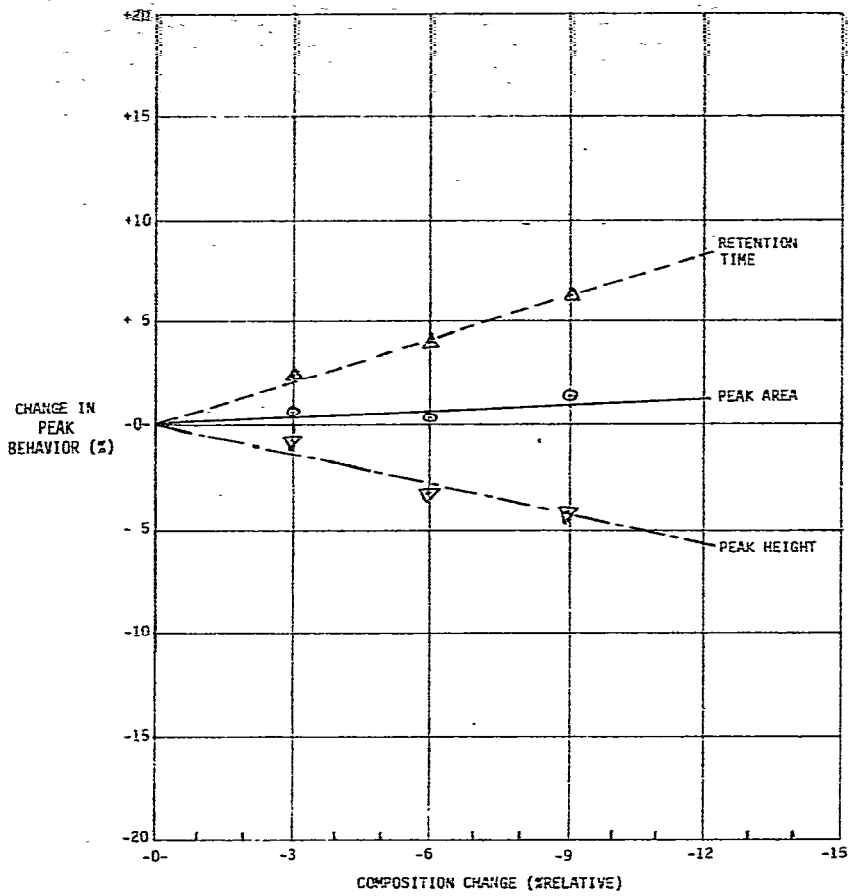

Fig. 6. Retention time, peak area, and peak height *versus* composition in gradient analysis.

DISCUSSION

Isocratic analysis

It is possible to explain the results observed in terms and equations that are familiar to the chromatographer. In the case of flow-rate dependence, peak behavior can be defined generally as follows

$$\text{Peak behavior} = f(F)$$

where F is the mobile phase flow-rate in ml/min. In the case of composition dependence, peak behavior can be defined as follows

$$\text{Peak behavior} = f(S)$$

where S is a mobile phase composition parameter intended to be directly related to

the %B of a binary solvent mixture. Since here the B solvent is always considered the stronger of the two, S may be thought of as a solvent strength parameter.

Of the different types of peak behavior studied, retention time is the easiest to understand, and is explained below.

Retention time versus flow-rate. Retention time can be expressed by the fundamental chromatography equation[4]

$$t_R = \frac{L}{u}(1 + k') \tag{1}$$

where L is column length in cm, u is mobile phase linear velocity in cm/sec, and k' is the capacity factor. u depends on the flow-rate F in ml or cm^3/sec and on the average cross-sectional area of voids between packing particles A_{xs} in cm^2

$$u = \frac{F}{A_{xs}} \tag{2}$$

A_{xs} and L are constants for a given column and k' is constant for a given sample component and mobile phase composition. Substituting eqn. 2 into eqn. 1 and grouping constants into a new constant K

$$t_R = \frac{K}{F} \tag{3}$$

For small changes, a per cent change in flow-rate causes a per cent change in retention time of the same numerical value but of opposite sign. This is consistent with the data in Fig. 3.

Retention time versus composition. Referring to eqn. 1, retention time is seen to depend on k', which is a function of mobile phase composition. The relationship of k' to composition is complex and not well understood; therefore, a nonrigorous statement will suffice as follows

$$k' = \frac{K}{S} \tag{4}$$

Substituting eqn. 4 into eqn. 1, considering L and u constant for a given column and flow-rate, and grouping constants

$$t_R = K_1 + \frac{K_2}{S} \approx \frac{K}{S} \tag{5}$$

A per cent change in composition (%B) causes a change in retention time of opposite sign. The magnitude of the change can be small or large, depending on the nature of the mobile and stationary phases, and depending on the k' of the peak. The experimental results of Fig. 4 agree with the description. The reason for the k' dependence is related to the dependence of resolution on k'[5].

Peak area versus flow-rate. In order to understand the effect of variables on peak area, it is useful to refer to the concentration profile of a component zone, independent of detector considerations, as illustrated in Fig. 7. In this concentration-

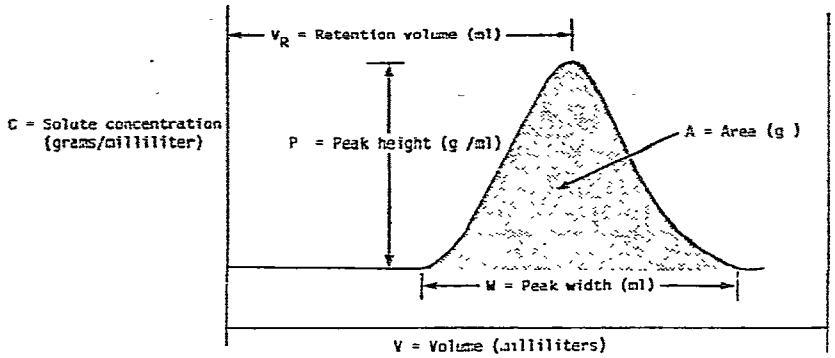

Fig. 7. Volumetric peak behavior.

volumetric type of description, the area of the peak can be expressed as the integral of concentration C with respect to volume, from the volume V_1 at peak onset to the volume V_2 at peak end

$$A_V = \int_{V_1}^{V_2} C \, dV \tag{6}$$

The concentration profile may change shape due to any number of causes, but the total weight of sample component remains constant, and thus the peak area remains constant. For example, the peak may become taller and narrower due to a decreased k' caused by a stronger mobile phase. Or it may become shorter and wider due to a decrease in efficiency caused by increased flow-rate. But the area, expressed in terms of (g/ml) (ml) = g, remains unchanged for a given column and is independent of both flow-rate and composition

$$A_V = K \tag{7}$$

Because we must depend upon a detector to monitor the compounds as they emerge from the column, Fig. 8 defines a peak in terms of detector signal and time as opposed to the terms of concentration and volume in Fig. 7. The detected area of the peak is

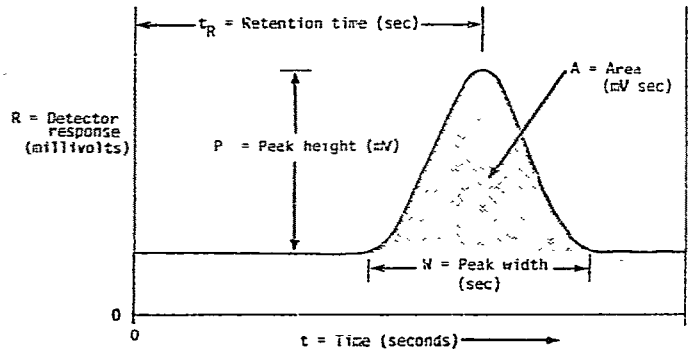

Fig. 8. Detected peak behavior.

expressed as the integral of detector signal R with respect to time, from the time t_1 at peak onset to the time t_2 at peak end

$$A = \int_{t_1}^{t_2} R \, dt \tag{8}$$

The photometric detector instantaneous signal R in mV is directly proportional to the instantaneous solute concentration C. Since there is a one-to-one correspondence between R and C, detected peak area will behave the same as actual peak area insofar as detector response is concerned; however, eqn. 3 shows that retention time is inversely proportional to flow-rate. This applies to all retention times, including peak onset and end. Thus, the time $t_2 - t_1$ over which area integration takes place expands or contracts inversely with flow-rate changes. As a consequence, eqn. 7, expressing the volumetric area dependence on flow-rate, must be modified to convert it to an expression for detected area dependence on flow-rate. The modification simply takes into account the inverse dependence of retention time on flow-rate

$$A = \frac{K}{F} \tag{9}$$

For small changes, a per cent change in flow-rate causes a per cent change in peak area of the same numerical value but of opposite sign. The experimental results of Fig. 3 agree. It is obvious that this conclusion would be different for a detector whose signal is proportional to mass rate instead of concentration.

Peak area versus composition. When mobile phase composition is changed to a weaker eluting strength, peaks elute slower and occupy more volume. At constant flow-rate, a direct translation can be made from C to R and from dV to dt in eqns. 6 and 8. As a consequence, eqn. 7, which expresses the dependence of volumetric area on composition, also applies to detected area

$$A = K \tag{10}$$

Peak area is independent of mobile phase composition. This was experimentally observed in Fig. 4.

Peak height versus flow-rate. Fig. 7 shows that the peak height is the maximum concentration of the peak, and Fig. 8 shows it as the maximum instantaneous detector response

$$P_V = C_{max} \tag{11}$$

$$P = R_{max} \tag{12}$$

R_{max} is independent of how fast the peak goes through the detector, except for time constant considerations discussed later. However, C_{max} has a small flow dependency related to column efficiency factors. By approximating the peak as a triangle of height P, width W, and area A

$$P_V = \frac{2A}{W} \tag{13}$$

Per eqn. 7 the volumetric area is constant, so we can express eqn. 13 as

$$P_v = \frac{K}{W} \tag{14}$$

Peak width W is related to efficiency by the definition of plate height H, the degree of zone spreading per unit length of column[6]

$$H = \frac{\sigma^2}{L}, \quad \sigma = (LH)^{0.5} \tag{15}$$

Since one σ is approximately $W/4$, we can substitute into eqn. 15 and group constants

$$W = KH^{0.5} \tag{16}$$

Most columns have a plate height dependence on flow-rate which is approximated by the empirical expression

$$H = Du^n \tag{17}$$

where D and n are constants for a given column and mobile phase[7]. Normally $0.3 < n < 0.6$. Substituting eqn. 17 into eqn. 16, grouping constants, and using the 0.6 value for n since it represents the worst case of flow dependence

$$W = Ku^{0.3} \tag{18}$$

Substituting eqn. 18 into eqn. 14, using the relationship between linear velocity and flow-rate expressed in eqn. 2, and grouping constants

$$P_V = \frac{K}{F^{0.3}} \tag{19}$$

This expresses the relationship between flow-rate and peak height when the latter is in concentration terms as in eqn. 11. Since there is a one-to-one correspondence between R and C, the relationship also applies in detector response terms

$$P = \frac{K}{F^{0.3}} \tag{20}$$

A per cent change in flow-rate causes a much smaller change in observed peak height of opposite sign. For example, a 10% reduction in flow-rate causes a 3% increase in peak height. This is consistent with the experimental results in Fig. 3, which shows a 1% peak height increase for a 10% flow reduction.

Peak height versus composition. It is well known that peak height, peak width, and retention time change dramatically with composition. Eqn. 14 states that the height in concentration is inversely proportional to the width in volume. This relationship is also true for detector signal and time, at constant flow-rate

$$P = \frac{K}{W} \tag{21}$$

The well known zone-spreading equation implies a direct relationship between width and retention time[8], since N is constant for all retention times.

$$N = 16\left(\frac{t_R}{W}\right)^2 \tag{22}$$

$$W = Kt_R \tag{23}$$

Substituting eqn. 23 into eqn. 21

$$P = \frac{K}{t_R} \tag{24}$$

Eqn. 5 expressed t_R in terms of S

$$t_R = \frac{K}{S} \tag{5}$$

Substituting eqn. 5 into eqn. 24

$$P = KS \tag{25}$$

A per cent change in composition causes a corresponding change in peak height. This was experimentally observed in Fig. 4. As with retention time as a function of composition, a family of curves is produced.

Gradient analysis

The previously derived isocratic relationships can be easily modified to describe peak behavior in gradient analysis.

Retention time versus flow-rate. As in isocratic analysis, a reduction in flow-rate during gradient elution causes an increase in retention time. The isocratic expression

$$t_R = \frac{K}{F} \tag{3}$$

must be modified, however, for gradient analysis because there are some important differences. In gradient analysis, composition is not constant and the instantaneous k' for each peak decreases during the run. As an oversimplified but instructive model, consider that a peak eluting with $t_R = 10$ min is not migrating at all during the first 9 min of the run. During this time, flow changes have no effect on t_R. Only during the last minute, when the peak is migrating, do flow changes affect t_R. A 10% flow-rate change causes only a 1% retention time change. This behavior is roughly approximated by the empirical expression

$$t_R = \frac{K}{F/(1 + k')} \tag{26}$$

At $k' = 0$ the numerical value of the retention time change equals that of the flow-rate change, and eqn. 26 reduces to eqn. 3. At large k' values there is less flow de-

pendence of retention time. This relationship was experimentally observed in Fig. 5 and has often been seen in published data[9] without explanation.

Retention time versus composition. The purpose of gradient elution is to reduce retention time by increasing solvent strength during the run. Eqn. 5, which expressed the inverse dependence of retention time on composition for isocratic analysis, also applies to gradient operation

$$t_R = \frac{K}{S} \tag{5}$$

A percent change in composition causes a change in retention time of opposite sign. The magnitude of the change can be small or large, but for a given column and set of conditions it is relatively constant for any compound. This was observed in Fig. 6. This k' independence is in contrast to the relationships in Fig. 4. Gradient operation has a leveling effect, which makes all compounds behave as though they had the same k' value.

Peak area versus flow-rate. Eqn. 9 expressed the inverse dependence of peak area on flow-rate in isocratic analysis. Eqn. 10 showed that area is independent of composition. The fact that composition changes throughout the run does not change the above relationships, so eqn. 9 is valid

$$A = \frac{K}{F} \tag{9}$$

For small changes, a per cent change in flow-rate causes a per cent change in peak area of the same numerical value but of opposite sign. This was experimentally observed in Fig. 5.

Peak area versus composition. Eqn. 10 expressed the peak area independence of mobile phase composition in isocratic operation. All of the arguments used to deduce this relationship also apply to the gradient mode.

$$A = K \tag{10}$$

Peak area is independent of mobile phase composition. This was experimentally observed in Fig. 6.

Peak height versus flow-rate. Eqn. 20 describes for isocratic analysis the small dependence of peak height on flow-rate which is due to efficiency phenomena. The fact that the composition is changing during the run should not influence efficiency significantly, so this same expression applies

$$P = \frac{K}{F^{0.3}} \tag{20}$$

A per cent change in flow-rate causes a much smaller change in peak height of opposite sign. This was experimentally observed in Fig. 5. We should note, however, that peak height data for Fig. 5 were taken only from the adjusted values in Table III. The adjusted %B *versus* time gradient program was changed in proportion to flow-rate in order to hold the %B *versus* volume gradient program equal to the reference program. Without this adjustment, reducing the flow-rate causes an increase in the

slope of the composition *versus* volume program, consequently, causing peaks to elute earlier, narrower, and taller. Since, in practice, chromatographs do not make such adjustments when flow-rate changes, there is usually a higher peak height dependence on flow-rate than is indicated in Fig. 5 and by eqn. 20.

Peak height versus composition. The reasoning employed in developing the isocratic relationship also applies to gradient conditions

$$P = KS \tag{25}$$

A per cent change in composition causes a corresponding change in peak height. This is consistent with the experimental results of Fig. 6. Again, as with gradient retention time behavior, there is a leveling effect. All sample components exhibit the same dependence.

CONCLUSIONS

We have demonstrated that high-precision quantitative analysis is achievable with HPLC using a photometric detector for both isocratic and gradient operation. During gradient analysis, the flow precision of the system is indicated by the early eluting peaks, while the composition precision is indicated by the late eluting peaks.

With photometric detectors, as with all detectors responding to concentration, it is important to control flow-rate when area is used for quantitation, and to control composition when height is used for quantitation. Stated differently, peak height should be used for quantitation if flow-rate cannot be carefully controlled, and peak area should be used if composition cannot be carefully controlled.

For detectors responding to mass rate, such as the flame ionization detector, other relationships apply. However, the peak behavior dependence described herein should apply to gas chromatography as well as liquid chromatography, when the differences between detectors are taken into account.

Preliminary experimental work, not reported here, has indicated that the retention time, peak area, and peak height dependence on flow-rate can be significantly affected by the detector time constant. The semiquantitative expressions for flow dependence presented here apply only when the rate of change of peak profile is small compared to the detector time constant.

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