CHROMATOGRAPHIC AND SPECTRAL ANALYSIS OF PHENOLIC ACIDS AND RELATED COMPOUNDS

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(Received January 17th, 1967)

INTRODUCTION

Recognition of the presence of phenolic acids and related compounds in many biological systems has stimulated the development of numerous chromatographic methods of separation and identification. Nearly all texts on chromatographic procedures devote a section to these compounds, *e.g.*, SMITH[1] on paper chromatographic separations, and RANDERATH[2] and STAHL[3] on thin layer separations. Gas chromatography of some mono- and dihydroxy aromatic acids, as the methyl esters or as the methoxy substituted methyl esters, was described by WILLIAMS[4] and WILLIAMS AND SWEELEY[5]. These investigators conceded certain limitations for this procedure, the most important being that reactions of hydroxy aromatic acids with diazomethane to form methyl esters frequently result in the formation of variable amounts of the methoxy derivative or mixtures of derivatives, particularly if the reaction time is prolonged. However, the methylation procedure is simple and generally yields a major product with an unique retention time. As an alternative to methylation, BLAKLEY[6] recently reported the preparation of trimethylsilyl derivatives of a number of phenolic acids which he then successfully gas chromatographed. The hydrolysis of trimethylsilyl derivatives in 50% methanol[7] offers a simple and convenient method for the recovery of the original compound for further characterization studies.

Our interest in the characterization of phenolic compounds derives from their presence and that of the flavonoids in the cotton bud. After the sugars are removed from the flavonoid glycoside by refluxing in dilute acid, subsequent alkaline hydrolysis yields a phenol from the "A" ring and a phenolic acid from the "B" ring. In addition, the glycoside is often acylated with a substituted cinnamic acid which can be removed by mild alkaline hydrolysis[8]. Methylation of the glycoside before this hydrolysis permits deduction of the pattern of glycosidation. Although a number of investigators have published chromatographic data on these flavonoid degradation products, we were able to assemble literature values for only a fraction of the compounds that we expected to find in our study of plant constituents. Consequently, we chose 41 compounds for which we obtained chromatographic values with two gas chromatographic phases, and two paper chromatographic and two thin layer chromatographic systems. Also we obtained spectral maxima in the ultraviolet region for these compounds and observed characteristic bathochromic shifts when aluminum chloride was added. Our rationale for selection of the various chromatographic systems and comparisons of our results with the literature are included.

EXPERIMENTAL

Apparatus

Gas chromatographic determinations were conducted with an Aerograph Model A-95-P3 hydrogen flame unit*. The columns were of stainless steel, 0.0032 × 1.828 m. The first contained 20 % Apiezon L on 60/80 mesh Chromosorb W treated with hexamethyldisilazane, and the second contained 3 % SE-52 on 60/80 Chromosorb P treated with hexamethyldisilazane. Gas chromatographic operating conditions are listed in Table I.

Thin layer chromatography (TLC) plates were prepared with Brinkmann apparatus on 20 × 20 cm glass, 250 μ bed depth, and developed in the ascending fashion in solvent vapor saturated chambers to a height of 15 cm from the start. Paper chromatograms (PC) were achieved with Whatman No. 1 chromatography paper in the ascending fashion in solvent vapor saturated cylindrical jars to a height of 25 cm.

Spectra were obtained with a Beckman DK-2A ratio recording spectrophotometer in 1.0 cm matched silica cells.

TABLE I

CHROMATOGRAPHIC AND SPECTRAL ANALYSIS OF SELECTED PHENOLIC ACIDS AND PHENOLS; KOVÁTS' (I_K) INDICES, R_F VALUES, AND U.V. MAXIMA (mμ), RESPECTIVELY

Compound[a]	*GLC*[b]		*TLC–SGG*		*PC*		*U.V.*	
	APL[c]	*SE-52*[d]	*BMA*[e]	*PEAc*[f]	*BuAc*[g]	*BzAcW*[h]	*MeOH*	*$AlCl_3$*[i]
Bz	710[j]	732[j]					255	255
HBz	956[j]	994[j]	0.78	0.99	0.95	0.90	271, 277(s)[m]	271, 277(s)
1,2-DHBz	1215	1250	0.62	0.44	0.88	0.23	277	230(s), 284
1,3-DHBz	1292	1304	0.57	0.16	0.85	0.07	217(s), 274, 280	217(s), 274, 280
1,2,3-THBz	1469	1450	0.34	0.13	0.72	0.02	267, 276(s)	268, 277(s)
1,3,5-THBz	[k]	[k]	0.27	0.00	0.71	0.00	267, 270(s)	267, 271
MBz	956	986						
1,2-DMBz	1109	1257						
1,3-DMBz	1220	1317						
1,2,3-TMBz	1270	1548						
1,3,5-TMBz	1469	1691						
BA	1109	1160	0.80	0.90	0.85	0.90	230, 271, 277(s)	275, 282(s)
2-HBA	1220	1295	0.69	0.59	0.95	0.21	235, 302	241, 309
3-HBA	1417	1542	0.62	0.18	0.88	0.21	234, 295	242, 302
4-HBA	1449	1572	0.65	0.18	0.85	0.21	253, 280(s)	262
2-MBA	1320	1336	0.65	0.20	0.87	0.90	232, 291	240, 299
3-MBA	1331	1458	0.58	0.47	0.87	0.95	232, 292	242, 299
4-MBA	1375	1498	0.69	0.42	0.87	0.88	251, 270(s)	260, 272(s)
2,3-DHBA	1372	1364	0.34	0.28	0.88	0.28	256, 317	252, 321
2,4-DHBA	1532	1636	0.67	0.24	0.94	0.31	256, 293	266, 299
2,5-DHBA	1510	1595	0.55	0.16	0.88	0.11	237, 332	242, 342
2,6-DHBA	1397	1440	0.42	0.03	0.66	0.15	249, 314	266, 342
3,4-DHBA	1557	1666	0.58	0.05	0.85	0.03	258, 293	283, 318
3,5-DHBA	1617	1745	0.50	0.02	0.80	0.01	248, 306	257, 316
2,3-DMBA	1414	1518	0.69	0.22	0.90	0.89	292	242, 300
2,4-DMBA	1567	1675	0.72	0.16	0.90	0.87	253, 288	261, 294
2,5-DMBA	1431	1479						

(*continued on p. 45*)

* Mention of a proprietary product does not necessarily imply endorsement of this product by the U.S.D.A.

TABLE I (*continued*)

Compound[a]	*GLC*[b]		*TLC–SGG*		*PC*		*U.V.*	
	APL[c]	*SE-52*[d]	*BMA*[e]	*PEAc*[f]	*BuAc*[g]	*BzAcW*[h]	*MeOH*	*$AlCl_3$*[i]
2,6-DMBA	1460	1590	0.71	0.11	0.90	0.91	239(s), 277	246(s), 284
3,4-DMBA	1561	1652	0.73	0.23	0.90	0.92	257, 288	267, 294
3,5-DMBA	1559	1697	0.77	0.44	0.92	0.95	249, 303	257, 312
2,3,4-THBA	1529	1575	0.34	0.12	0.72	0.03	266, 295(s)	241(s), 294
2,4,6-THBA	1728	1807	0.18	0.03	0.67	0.02	262, 298	233, 275, 321
3,4,5-THBA	1754	1872	0.21	0.00	0.64	0.00	272	226, 307
2,3,4-TMBA	1627	1721						
2,4,6-TMBA	1642	1701						
3,4,5-TMBA	1657	1780	0.65	0.20	0.91	0.92	262	272
3-H-4-MBA	1560	1661	0.73	0.10	0.84	0.34	257, 293	264, 301
4-H-3-MBA	1498	1562	0.76	0.19	0.88	0.61	259, 289	268, 295
4-H-3,5-DMBA	1738	1867	0.59	0.11	0.82	0.59	272	292
trans-CA	1393	1434	0.75	0.60	0.93	0.95	213, 219(s), 272	217, 223(s), 282
2-HCA	1630	1718	0.76	0.24	0.89	0.38	272, 322	279, 331
3-HCA	1621	1772	0.70	0.19	0.87	0.26	233, 274, 311(s)	237, 283, 320(s)
4-HCA	1635	1807	0.68	0.18	0.87	0.24	288(s), 308	233, 297(s), 316
2-MCA	1625	1661	0.78	0.49	0.94	0.93	222, 272, 317	224, 278, 326
3-MCA	1631	1640	0.77	0.42	0.94	0.91	232, 274, 307(s)	236, 281, 313(s)
4-MCA	1672	1675	0.76	0.43	0.94	0.91	223(s), 296(s), 304	232, 303
3-M-4-HCA	1752	1899	0.78	0.18	0.89	0.66	237, 288(s), 318	241, 300(s), 331
Butter Yellow			0.99	0.89	0.99	0.98		

[a] Bz = Benzene, HBz = hydroxybenzene (phenol), BA = benzoic acid, 2-HBA = 2-hydroxybenzoic acid (salicylic acid), 2-MBA = 2-methoxybenzoic acid, 2-HCA = 2-hydroxycinnamic acid (*o*-coumaric acid), *etc.*

[b] KOVÁTS' indices (I_k) for the carboxylic acid methyl esters, free phenols, and methoxybenzenes.

[c] 0.0032 × 1.828 m stainless steel column packed with 20% Apiezon L on 60/80 mesh Chromosorb W treated with HMDS. Column temperature 140°, 68 ml/min N_2, FID, injector 240°, detector 260°.

[d] 0.0032 × 1.828 m stainless steel column packed with 3% SE-52 on 60/80 mesh Chromosorb p treated with HMDS. Column temperature 140°, carrier gas flow 32 ml/min N_2, FID, injector 240°, detector 260°.

[e] Benzene–methanol–acetic acid (45:8:4, v/v/v).

[f] Pentane–ethyl ether–acetic acid (75:25:1, v/v/v).

[g] *n*-Butanol–acetic acid–water (60:15:25, v/v/v).

[h] Benzene–acetic acid–water (2:2:1, v/v/v), upper layer.

[i] Three drops 10% $AlCl_3$ in methanol.

[j] Determined at 100°.

[k] Not successfully eluted under these or several other operating conditions. Degradation products observed.

[m] s = Shoulder.

Materials

The phenols and phenolic acids, with eight exceptions to be discussed later, were obtained from one of the following commercial sources: K & K Laboratories, Eastman Organic Chemicals, J. T. Baker Chemical Company, Chemical Procurement Laboratories, or Matheson, Coleman and Bell Organic Chemicals. The isovanillic acid (3-hydroxy-4-methoxybenzoic acid) was obtained by oxidation of isovanillin with neutral permanganate; it exhibited the expected melting point, ultraviolet spectra, and chromatographic behavior. Methyl esters were prepared by reaction of approximately 2 mg of the acid with diazomethane in ethyl ether at room temperature according to the procedure of WILLIAMS[4]. The reaction time for the hydroxy acids was limited to about 3 min by evaporating the ethereal solution *in vacuo*. Methoxy-

benzoic acids were allowed to react with the ethereal diazomethane overnight though shorter times would presumably suffice. Since 2,5-dimethoxybenzoic acid, 2,3,4-trimethoxybenzoic acid, 2,4,6-trimethoxybenzoic acid, 1,2-dimethoxybenzene, 1,3-dimethoxybenzene, 1,2,3-trimethoxybenzene, and 1,3,5-trimethoxybenzene were not commercially available, 2 mg quantities of the hydroxy analogues were allowed to react overnight with an excess of the ethereal diazomethane to form the corresponding methoxylated esters[5]. A series of standard normal paraffins obtained from Microtek Instrument Company were gas chromatographed as a mixture on both columns to provide reference retention times for conversion of the data to the KOVÁTS' indices (I_k) system[9].

TLC and PC chromatograms were visualized in a 2537 Å light and by spraying with a freshly prepared mixture of 3 volumes of (a) 2.5 g benzidine in 7 ml concentrated HCl and 500 ml H_2O, and 2 volumes of (b) 10 % $NaNO_2$ in water. Solvent systems are listed in Table I. Spectral shifts were produced by adding about 3 drops of 10 % $AlCl_3$ in absolute ethanol or methanol directly to the silica cell.

RESULTS AND DISCUSSION

Gas chromatographic analysis

The KOVÁTS' indices (I_k) for the carboxylic acid methyl esters and phenols are listed in Table I. One major component was obtained in each case though the methoxy analog occasionally was seen. The chromatography of these compounds was first investigated on the 3 % SE-52 column which was being used concurrently for separation of TMS-sugars as described by SWEELEY *et al.*[10] and KAGAN AND MABRY[11]. Relative retention times were generally comparably to the methyl esters of WILLIAMS[4] and WILLIAMS AND SWEELEY[5], who employed 15 % SE-30 on 100/200 mesh Chromosorb W treated with HMDS, and also to the trimethylsilyl derivatives of BLAKLEY[6] who employed 10 % SE-52 on Chromosorb W, 60/80 mesh, treated with HMDS. Since positional *e.g.*, *o*-, *m*-, *p*-isomers were usually separated satisfactorily, this column has the considerable advantage of providing dual service for studies of flavonoid glycoside degradation.

The Apiezon L column was chosen to study elution behavior of these compounds under conditions essentially free of polar effects. To achieve reasonable elution times while maintaining the same temperature and dimensions as with the SE-52 column, the flow rate was increased. Separations of positional isomers were again usually adequate. For faster analysis, decreased column length and/or increased temperature may be desirable.

The identity of the compounds in Table I is coded for more convenient comparison of positional effects: Bz = benzene, HBz = hydroxybenzene (phenol), BA = benzoic acid, 2-HCA = 2-hydroxycinnamic acid (*o*-coumaric acid), etc. A number of effects are apparent. Two that will be discussed more fully later are that (1) the retention time increases with molecular weight as expected, and (2) it varies as a function of the site of substitution, with *ortho* substituted acids possessing lowest elution times. The I_k values for the polar SE-52 column, as expected, were greater than for the Apiezon L column. However, the magnitude of the difference permits some deductions about the polarity and intra- and inter-molecular hydrogen bonding of the compound. Very small to moderate differences are observed for mono-, di- and

tri-hydroxybenzoic acids, when there is *ortho* substitution, and for the methoxy-cinnamic acids and the unsubstituted organic acids. The ΔI_k values for hydroxylated and methoxylated acids where the substitution is *meta* or *para* to the carboxy or vinyl-carboxy groups are normally 100 units or greater. Differences approaching 200 units were observed for the di- and tri-methyl ethers of benzene.

KOVÁTS[12] and EVANS AND HIGGINS[13] showed that nearly linear I_k (apolar) *versus* boiling point curves are obtained for members of homologous series of compounds and also that deviations exist where polar and steric considerations are important. LITTLEWOOD[14,15] discussed some of the forces involved in solution of various types of solutes in both apolar and polar solvents and showed that in apolar solvents the major effect involves solute–solvent dispersion force interactions. He also showed the direct proportionality between the logarithm of the specific retention volume (V_g) and the molecular weight of the solute, which is obvious for the relationships between log V_g, I_k, and the free energy of solution, ΔG_s, of the solute in solvent[12,16]. These considerations place the retention index among other free energy relationship functions[17] and justify its subdivision into additive partial free energy contributions by solute–solute and solute–solvent interactions, or in other ways, as long as all interactions are considered. KOVÁTS assigned ΔI_k values to individual polar groups as a measure of their effect on I_k in going from apolar to polar GLC solvents, and found that these frequently are additive. Thus, if one knows the apolar index value ($I_k{}^a$), one might predict the polar value ($I_k{}^p$) if suitable polar increments are known.

In like manner, we assigned a δI_k value to each functional group of these compounds chromatographed on the apolar phase, Apiezon L. The ponderal, or pure mass contribution (δI_{kM}), and the polar part (δI_{kP}) of these partial index values were separated to afford insight into some of the structure–vapor pressure relationships existing among members of this group of related compounds. We also chose to express the polar substituent group effects as *polar equivalent masses* (M_p) by the conversion:

$$M_p = \frac{14(\delta I_{kP})}{100} \tag{1}$$

When the M_p values for each substituent group are added to the total molecular weight, an *effective molecular weight*, M_e, is obtained which then gives an apolar index value:

$$I_k{}^a = \frac{100\, M_e}{14} \tag{2}$$

This effective molecular weight is related to the true, or ponderal value, M, by

$$M_e = M(1 + \alpha) \tag{3}$$

and

$$M_e = M + M_p \tag{4}$$

where α is the fraction associated because of polar interactions. This treatment is similar to that of EVANS AND SMITH[18], who also recommended the use of such an effective molecular weight as a parameter for correlating retention data.

Ponderal effects of 414, 114, 214, and 196 index units are expected for attachment of carbomethoxy, hydroxy, methoxy, and *trans*-vinyl groups, respectively, to an aromatic nucleus, but larger partial index substituent values were usually observed. Complex solute and solvent interactions on a GLC column, as well as polar group interactions within the solute molecule, occasionally led to negative M_p values. This was particularly true when the polar effects of a third and fourth polar substituent on the ring were calculated, since the method employed tended to put all substituent interactions in the M_p value of the "new" substituent. Some negative M_p values could be rationalized on the basis of steric crowding factors, however.

The calculations leading to the values given in Table II were made in the following manner:

(a) Ring, carbomethoxy, hydroxy, methoxy, and vinyl group M, M_p, and M_e values were computed.

Example: $\delta I_k{}^a$ (BA–Bz) = $\delta I_{k(\mathrm{COOMe})}$ = 399; M_e = 56, M = 58, M_p = —2. Since all δI_{kM} values were calculated for the group substituted on benzene, the M values were taken one mass unit lower (one proton) than the group mass to reflect the actual ponderal increase in the system.

(b) First substituent values were calculated from monosubstituted benzoates as illustrated in the following example:

$I_k{}^a$ compound pairs: 2-HBA–BA; 2-MBA–BA; 3-HBA–BA; 3-MBA–BA; 4-HBA–BA; 4-MBA–BA. (See Table III).

(c) Second substituent values were obtained by a similar process in which di- and mono-substituted benzoates were compared. The order of substituents was always taken in order of ring positions.

(d) Third substituent values were calculated similarly from tri- and di-substituted benzoates.

(e) Phenols, benzene methyl ethers, and cinnamates were treated similarly.

(f) Differing values obtained for the same substituent and position by different methods of calculation were averaged or adjusted to give a best overall fit of all predicted I_k values to the experimental data. In polysubstituted rings, values obtained for a function in a given position were different, depending on whether or not adjacent positions were occupied. Two values were tabulated for the second and third groups, depending on whether they were adjacent (A) or nonadjacent (NA) to another hydroxy or methoxy group. Positions *ortho* to the carbomethoxy or vinylcarbomethoxy groups were considered nonadjacent unless positions 3 or 5 were occupied.

The M_p values in Table II were combined with molecular weights to reconstruct I_k values for all the compounds except benzene. The calculated values agreed extremely well in most cases, as would be expected. The average deviation from experimental value was 0.9 % relative, with a maximum of 5.3 %. Presumably, I_k values for compounds of types other than those listed could be predicted within the same range of error. Further, by application of I_k values for the individual polar groups as suggested by KOVÁTS[12], $I_k{}^p$ values for the unknown compounds might also be predicted.

Examination of the polar effects operative among the various compounds investigated here suggested interesting structure–vapor pressure correlations. Use of equation (3) with the monosubstituted benzoates, for example, gives the alpha values in Table IV.

TABLE II

GROUP M_p CONTRIBUTIONS TO APOLAR GLC RETENTION INDICES OF PHENOLIC ACIDS AND RELATED COMPOUNDS[a, b]

A. Skeletal groups

C_6H_6	21
-COOMe	−2
-CH=CH-	14

B. Phenols and methoxybenzenes, OH or OMe substituents

1st group		*2nd and 3rd groups*			
OH	*OMe*	*OH*		*OMe*	
		A[c]	*NA*[c]	*A*[c]	*NA*[c]
19	5	20	31	−8	6

C. Methyl benzoates, OH or OMe substituents

1st group			*2nd group*					*3rd group*				
Position	*OH*	*OMe*	*Position*	*OH*		*OMe*		*Position*	*OH*		*OMe*	
				A[c]	*NA*[c]	*A*[c]	*NA*[c]		*A*[c]	*NA*[c]	*A*[c]	*NA*[c]
2	0	0	3	2	19	−3	2	3	11	—	−20	—
3	27	1	4	4	28	2	5	4	6	30	−13	−4
4	32	7	5	2	19	—	−6	5	11	—	−5	−15
—	—	—	6	—	9	—	−10	6	—	12	—	−19

D. Cinnamates, OH or OMe substituents

1st group			*2nd group*				
Position	*OH*	*OMe*	*Position*	*OH*		*OMe*	
				A[c]	*NA*[c]	*A*[c]	*NA*[c]
2,3	16	3	3,5	−2[d]	11[d]	−19	—
4	18	10	4	1	16[d]	−14[d]	—

[a] M_p = polar equivalent mass; acids as methyl esters; on Apiezon L.

[b] Sample calculation:

2,4-DHBA M = 168 Eqn. (2): $\frac{(215)(100)}{14}$ = 1536 I_k calc.

M_p:	
C_6H_6	21
-COOMe	−2
1st OH (2)	0
2nd OH(4NA)	28
M_e	215

I_k exp. = 1532

[c] A = Adjacent, NA = nonadjacent to OH or OMe. See text.

[d] Estimated from trends in substituted methyl benzoates.

These values represent associative effects which might reasonably be attributed to dipole–dipole and hydrogen bonding interactions.

In the three monomethoxybenzoates, the effect of necessity must be of a dipole–dipole nature. Contribution of resonance extremes such as (I) in the *para* isomer would

TABLE III

SAMPLE CALCULATIONS OF GROUP M_p CONTRIBUTIONS TO I_k^a OF PHENOLIC ACID METHYL ESTERS

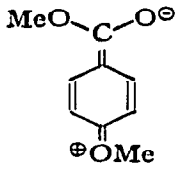
Function	*First substituent*					
	2-OH	*3-OH*	*4-OH*	*2-OMe*	*3-OMe*	*4-OMe*
δI_k	111	308	340	211	222	266
Less δI_{kM}	*114*	*114*	*114*	*214*	*214*	*214*
δI_{kP}	0	194	226	0	8	52
M_p	0	27	32	0	1	7

TABLE IV

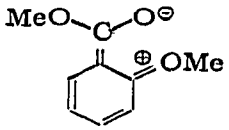
FRACTION OF SOLUTE ASSOCIATION OF HYDROXY AND METHOXY METHYL BENZOATES ON APIEZON L

Position	*Substituent*	
	OH	*OMe*
Ortho	0	0
Meta	0.18	0.006
Para	0.21	0.042

account for the increased association over that in the *meta* isomer, for which no such structure can be formulated. A similar resonance extreme (II) can be drawn for the *ortho* isomer, but the resultant dipole would be expected to produce much less intermolecular association than in the *para* isomer. The observed δI_k in the *o*-methoxybenzoate is consistent with the simple ponderal effect.



I



II

In the three hydroxybenzoates, the *o*-hydroxy group similarly appeared to contribute only a ponderal effect. This might be expected in analogy to the *o*-methoxy case if the hydroxy is prevented from intermolecular hydrogen bonding by effective intramolecular hydroxy-carbomethoxy hydrogen bonds. The *meta* and *para* isomers, on the other hand, are sterically unable to hydrogen bond intramolecularly and would be expected to associate to a greater extent intermolecularly, as observed. The dipole interaction presumed operative in the methoxy derivatives, if it is present in the hydroxy series, is apparently swamped by the H-bonding, which seems to account for about 17 % of the association in methyl *m*- and *p*-hydroxybenzoates.

Well known precedents for this *ortho* (or adjacent) effect are the isomeric hydroxybenzaldehydes, in which the *ortho* isomer has a markedly lower boiling point than the *meta* or *para* isomers. The *para* effect, *i.e.*, marked increase in polarity by electron donating groups *para* to the carbomethoxy function, is similarly well documented. Significantly, the *ortho* effect disappears among the monosubstituted cinnamates, in which the *trans*-vinyl extending group places the carbomethoxy

function out at a distance unfavorable for intramolecular hydrogen bonding. The *para* effect is still present, however, and could be rationalized by the method used previously.

The negative M_p values found for *ortho*-dimethoxy groups may be associated with steric crowding. Decreased vapor pressure expected from the mass increase may be offset partially by steric hindrance to approach of bulkier molecules in which the methyl groups may tend to lie in opposite directions perpendicular to the ring. Alternative explanations are also possible. As LITTLEWOOD[15] has pointed out, polar effects are difficult to assess in interacting systems.

Thin-layer chromatographic analysis

The R_F values for the free phenolic acids and phenol chromatographed on Silica Gel G are listed in Table I. The values are averages of at least three determinations and were normalized internally by including selected compounds with each series of plates. The benzene–methanol–acetic acid system (BMA) was employed by PASTUSKA AND PETROWITZ[19] for separation of a series of phenolic compounds. For the compounds which were common to their series and ours, the values generally compared within 10% or less. The pentane–ethyl ether–acetic acid system (PEAc), which was developed by us, gave considerably lower values. In addition, selection of different ratios of pentane to ether permitted us to manipulate the R_F values over a wide range so that complete separation of critical pairs was often achieved. Varying the acetic acid concentration was occasionally helpful. Visualization of the plates with the diazotizing spray produced a variety of colors and hues which aided in identification.

The following effects were observed: The attachment of hydroxy groups on the benzene or benzoic acid nucleus decreased the R_F observed with both systems. Successive hydroxy groups caused further depressions of the R_F values. Methoxylation of the benzoic acid nucleus slightly depressed the R_F for compounds chromatographed in the BMA system. This decrease was not particularly related to the number of methoxy groups, but positional effects were often significant. With the PEAc system, methoxylation depressed the R_F appreciably as a function of numbers of groups and sites of attachments. The addition of hydroxy and methoxy groups to the cinnamic acid nucleus did not appreciably depress the R_F values in the BMA system, but *o*- and *p*-coumaric acid could be nicely separated. With the PEAc system, R_F values were decreased appreciably.

Paper chromatographic analysis

The R_F values for the free phenolic acids and phenols chromatographed on Whatman No. 1 paper are listed in Table I. The values are averages of at least three determinations and were normalized by including selected compounds with each series of papers. The upper phase of the *n*-butanol–acetic acid–water (4:1:5) system (BuAc) has been utilized by numerous investigators, among them BATE-SMITH AND WESTALL[20] and LYNN AND LUH[21]. Chromatography in the upper phase of the benzene–acetic acid–water (2:2:1) system (BzAcW) has been described by BRAY *et al.*[22] and employed by LYNN AND LUH[21]. For those compounds which were common to our series, the values generally compared within 10% or less.

The following effects were observed: Hydroxylation of the benzene nucleus caused a moderate decrease in R_F values for the BuAc system and an appreciable

decrease with the BzAcW system. The R_F decreased with the number of hydroxy groups; positional (*o*, *m*, *p*) effects were observed. Hydroxylation of the benzoic acid nucleus moderately depressed R_F values in the BuAc system and caused large depressions in the BzAcW system. Positional effects were observed in both cases. Methoxylation of benzoic acid failed to depress R_F values appreciably for either system, and very few positional effects were noted. Hydroxylation of cinnamic acid caused only a slight decrease in the R_F for the BuAc system but contributed to a large decrease in the BzAcW system as well as to positional effects. Methoxylation of cinnamic acid did not affect the R_F in either system. An empirical treatment of these observed changes and the TLC observations would in many ways be similar to that found in the GLC section.

Analysis of U.V. maxima

The U.V. maxima in absolute methanol before and after treatment of the compounds with aluminum chloride are listed in Table I. No efforts were made to observe maxima below 215 mμ.

SCOTT[23] assigned base values for parent compounds and wave length increments for substituents attached to aromatic carbonyl compounds; when the parent is benzoic acid or a benzoic acid ester, the base value is 230 mμ. For each hydroxy or alkoxy substitution, 7 mμ are added for *ortho* and *meta* groups, and 25 mμ are added for *para* groups to give the theoretical maxima. When we used this treatment, our observed values could be reconciled. They also compared favorably with those listed by SCOTT[24], who determined maxima in ethanol.

Phenol exhibits a long wave length band at 270 mμ (our value 271 mμ) which is considered to be the displaced and intensified local excitation band of benzene (254 mμ)[24]. Maxima for the other phenols also compared closely with those listed by SCOTT[24]. *Trans*-cinnamic acid and *ortho* and *meta* substituted hydroxy and methoxy derivatives exhibited the electron transfer (E.T.) band at 273 mμ and a local excitation band above 300 mμ. *Para*-OH or -OR substituted cinnamic acids exhibited an electron transfer band at about 305 mμ which compared closely with that of *p*-methoxycinnamic acid methyl ester (308 mμ)[24]. The E.T. band of ferulic acid, which possesses both a 3-OMe and a 4-OH group, underwent a bathochromic shift to 318 mμ.

When aluminum chloride was added, the following bathochromic shifts were observed: phenol, but no other monohydroxybenzene, 7 mμ; all hydroxy, methoxy, hydroxy-methoxybenzoic, and cinnamic acids, at least 5 mμ and more if chelation occurred. Characteristic shifts of 13 to 35 mμ in both E.T. bands were observed for 3,4-DHBA, 3,6-DHBA, 2,3,4-THBA, 2,4,5-THBA, 3,4,5-THBA, and 4-H-3,5-DMBA. This feature was of practical significance to our flavonoid research since it distinguished between protocatechuic acid and the vanillic acids which have very similar absorption patterns. We are not aware of previous use of this technique though characteristic spectral shifts have been observed in phenolic compounds when alkali is added[24].

SUMMARY

Chromatographic data were obtained for 41 phenolic acids and related compounds by using two gas chromatographic supports and two paper chromatographic and two thin layer chromatographic systems. Spectral maxima in the ultraviolet

region and observed characteristic bathochromic shifts were also obtained when aluminum chloride was added. Conversion of the gas chromatographic relative retention values to the KOVÁTS' indices system permitted an analysis of the effects of substituents and sites of attachment on retention time. Relative contributions of molecular weight and intra- and intermolecular hydrogen bonding to I_k values are discussed. Hydroxy and methoxy substitution of benzene and benzoic and cinnamic acids decreased the R_F values in both TLC and PC, with some exceptions. Positional effects were often observed. Aluminum chloride elicited a small bathochromic shift for all acids; larger shifts were observed with dihydroxy and trihydroxybenzoic acids when the substitution pattern permitted chelation.

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