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THIN-LAYER CHROMATOGRAPHIC BEHAVIOUR AND CHEMICAL STRUCTURE OF 6- AND 8-METHOXY-5-HYDROXYFLAVONES

F. A. T. BARBERÁN*, F. TOMÁS and F. FERRERES
Laboratorio de Flavonoides, Centro de Edafología y Biología Aplicada del Segura, C.S.I.C., Apdo. 195, Murcia (Spain)
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SUMMARY

Relationships have been found between the structure and the chromatographic behaviour of 5-hydroxyflavones with methoxy substituents at the 6- and/or 8-positions. Correlations involving ΔR_M increments have been attempted. The important role of permethylated derivatives as a tool in flavone structure determinations is discussed.

INTRODUCTION

Work on the chromatographic behaviour of flavonoids in relation to their structure, based on theoretical considerations put forward by Martin[1] and developed by Bate-Smith and Westall[2], is important in flavonoid identification[3–7]. In the last few years, much work on the paper and thin-layer chromatographic (TLC) isolation and purification of polyphenols has been published[8–14]. However, little work has been carried out on the chromatographic behaviour of 6- and 8-methoxyflavones[15] and the relationship between their TLC behaviour and their structure.

An opportunity to study the latter behaviour arose in an investigation of the flavones occurring in several species of *Sideritis* (Labiatae)[16–18], during which a large number of aglycones with substituents at the 6- and 8-positions were isolated. As expected, a close relationship between TLC R_F values and the structures of 6- and 8-methoxyflavones was found. In this paper, a number of considerations are put forward on the structural analysis of flavonoid aglycones by means of TLC as a complementary technique to instrumental assays.

EXPERIMENTAL

Flavones

The flavones used were tectochrysin (Fluka), chrysin and acacetin (Roth), apigenin from *Petroselinum sativum*[19], luteolin from *Capsicum annuum*[20], hispidulin and nepetin from *Centaurea aspera*[21], 5,3′,4′-trihydroxy-6,7,8-trimethoxyflavone, 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone, cirsimaritin, cirsiliol, cirsilineol and

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xanthomicrol from *Sideritis leucantha*[17–18], 5,6,3′,4′-tetrahydroxy-7-methoxyflavone supplied by Prof. Dr. B. Voirin (Université C. Bernard, Lyon, France), 5-hydroxy-6,7,3′,4′-tetramethoxyflavone and 5-desmethylnobiletin from *Sideritis mugronensis*[16] supplied by Prof. Dr. B. Rodriguez (Instituto Química Orgánica, C.S.I.C., Madrid, Spain), 5,7-dimethoxyflavone, 5,7,4′-trimethoxyflavone and 5,7,3′4′-tetramethoxyflavone obtained by diazomethane methylation of chrysin, apigenin and luteolin, respectively, and 5,6,7,4′-tetramethoxyflavone, sinensetin, isosinensetin, tangeretin and nobiletin from *Citrus unshiu*[22].

Chromatographic systems

The naturally occurring (partially methylated) flavones that were available were chromatographed in parallel on silica gel plates (20 × 20 × 00.2 cm; Carlo Erba) with benzene–methanol–acetic acid (45:3:2) and chloroform–*n*-hexane–methanol (40:40:3) as recommended by Randerath[23], slightly modified for our purpose. These solvents have proved useful for two reasons: first, they are specially suitable for the classes of substances being studied, and second, the difference in pH between the solvents makes it possible to calculate ΔR_M values[24] and apply them in structural studies.

All solvents were recently prepared, and the plates were heated at 110°C for 30 min. The chromatograms were developed at 22°C. Under these conditions almost no variation occurred from run to run.

The permethylated flavones were developed in parallel by TLC on silica gel plates (20 × 20 × 0.02 cm; Carlo Erba) with chloroform–ethyl acetate–dimethyl ketone (5:4:1 and 5:1:4)[25], *n*-butanol–*n*-hexane (15:85)[26], benzene–ethyl acetate (6:4)[26] and ethyl acetate[27].

Detection of developed chromatograms

Flavone spots were rendered visible under UV light (360 nm). The 5-hydroxyflavones with methoxy groups at the 6- or 8-positions showed dark purple colours, and the permethylated flavones exhibited typical bright fluorescences (blue, pink, cream).

Diazomethane methylation

The permethylation with diazomethane of flavones was carried out by adding diazomethane, prepared according to Vogel[28], to a methanol–diethyl ether (1:1) solution of the flavone and leaving it in a stoppered tube overnight at room temperature. The 5-hydroxy group is difficult to methylate, but this reaction allways yields the permethylated derivative in an appreciable amount, being easily distinguished by means of its characteristic bright colours under a UV lamp (360 nm), which are very different from those of the 5-hydroxyflavones (dark purple).

RESULTS AND DISCUSSION

Calculation of free hydroxyl number by means of a chromatographic chart

It is possible to establish the number of carboxylic groups in organic acids by means of chromatographic developments with two different systems, one acidic and the other alkaline[29]. We applied this technique to 5-hydroxyflavone, on the basis of

TABLE I

TLC OF 5-HYDROXYFLAVONES ON SILICA GEL

The available 5-hydroxyflavones were chromatographed on silica gel with benzene–methanol–acetic acid (45:3:2) (solvent 1) and with chloroform *n*-hexane–methanol (40:40:3) (solvent 2). Values (R_p) relative to 5,3′.4′-trihydroxy-6,7,8-trimethoxyflavone were also calculated.

5-Hydroxyflavone	*Trivial name*	R_F		R_F	
		Solvent 1	*Solvent 2*	*Solvent 1*	*Solvent 2*
5,7-Dihydroxyflavone	Chrysin	0.60	0.47	200	188
5-Hydroxy-7-methoxyflavone	Tectochrysin	0.84	0.85	280	340
5,7.4′-Trihydroxyflavone	Apigenin	0.20	0.07	67	28
5,7-Dihydroxy-4′-methoxyflavone	Acacetin	0.57	0.44	190	176
5,7,3′.4′-Tetrahydroxyflavone	Luteolin	0.05	0.00	17	0
5,7,4′-Trihydroxy-6-methoxyflavone	Hispidulin	0.24	0.11	80	44
5,4′-Dihydroxy-6,7-dimethoxyflavone	Cirsimaritin	0.38	0.32	127	128
5,6,3′,4′-Tetrahydroxy-7-methoxyflavone	Pedalitin	0.04	0.02	13	8
5,7,3′,4′-Tetrahydroxy-6-methoxyflavone	Nepetin	0.12	0.04	40	16
5,3′,4′-Trihydroxy-6,7-dimethoxyflavone	Cirsiliol	0.23	0.14	77	56
5,4′-Dihydroxy-6,7,3′-trimethoxyflavone	Cirsilineol	0.44	0.53	147	212
5-Hydroxy-6,7,3′,4′-tetramethoxyflavone		0.58	0.83	193	332
5,4′-Dihydroxy-6,7,8-trimethoxyflavone	Xanthomicrol	0.44	0.47	147	188
5,3′,4′-Trihydroxy-6,7,8-trimethoxyflavone		0.30	0.25	100	100
5,4′-Dihydroxy-6,7,8,3′-tetramethoxyflavone		0.49	0.62	163	248
5-Hydroxy-6,7,8,3′,4′-pentamethoxyflavone	5-Desmethylnobiletin	0.63	0.88	210	352

the acidic character of these polyphenols. The R_F and R_p values (R_F values relative to that obtained for 5,3′,4′-trihydroxy-6,7,8-trimethoxyflavone) obtained in acidic and neutral solvents (Table I) were plotted (Figs. 1 and 2) and from the chromatographic charts obtained it is possible to calculate the free hydroxyl number.

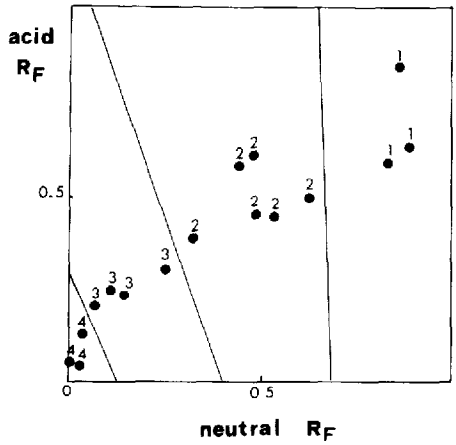

Fig. 1. Chromatographic chart of 5-hydroxyflavones. Acidic solvent, benzene–methanol–acetic acid (45:3:2). Neutral solvent, chloroform–*n*-hexane methanol (40:40:3). Numbers are free hydroxy groups on the flavone nucleus.

TABLE II

ΔR_M VALUES OF 6- AND 8-METHOXY-5-HYDROXYFLAVONES

The available flavones bearing methoxy groups at C-6 and/or C-8 were chromatographed on silica gel with benzene methanol–acetic acid (45:3:2) (solvent 1) and chloroform–*n*-hexane–methanol (40:40:3) (solvent 2). ΔR_M values were calculated as R_M (solvent 1) − R_M (solvent 2).

Flavone	*Solvent 1*		*Solvent 2*		ΔR_M	*Hydroxy/methoxy*	*Log(hydroxy/methoxy)*	*No. of substituents*
	R_F	R_M	R_F	R_M				
5,7,4′-Trihydroxy-6-methoxy-flavone	0.24	0.5006	0.11	0.9080	−0.4074	3.00	0.4771	4
5,4′-Dihydroxy-6,7-methoxy-flavone	0.38	0.2126	0.32	0.3272	−0.1147	1.00	0.0000	4
5,7,3′,4′-Tetrahydroxy-6-methoxy-flavone	0.12	0.8653	0.04	1.3802	−0.5149	4.00	0.6020	5
5,3′,4′-Trihydroxy-6,7-methoxy-flavone	0.23	0.5248	0.14	0.7883	−0.2636	1.50	0.1761	5
5,4′-Dihydroxy-6,7,3-methoxy-flavone	0.44	0.1047	0.53	−0.0522	−0.1569	0.66	−0.1804	5
5-Hydroxy-6,7,3′,4′-methoxy-flavone	0.58	−0.1402	0.83	−0.6886	0.5484	0.25	−0.6020	5
5,4′-Dihydroxy-6,7,8-methoxy-flavone	0.44	0.1047	0.47	0.0522	0.0525	0.66	−0.1804	5
5,3′,4′-Trihydroxy-6,7,8-methoxy-flavone	0.30	0.3680	0.25	0.4771	0.1091	1.00	0.0000	6
5,4′-Dihydroxy-6,7,8,3′-methoxy-flavone	0.49	0.0174	0.62	−0.2126	0.2299	0.50	−0.3010	6
5-Hydroxy-6,7,8,3′,4′-methoxy-flavone	0.63	−0.2311	0.88	−0.8653	0.6342	0.20	−0.6990	6

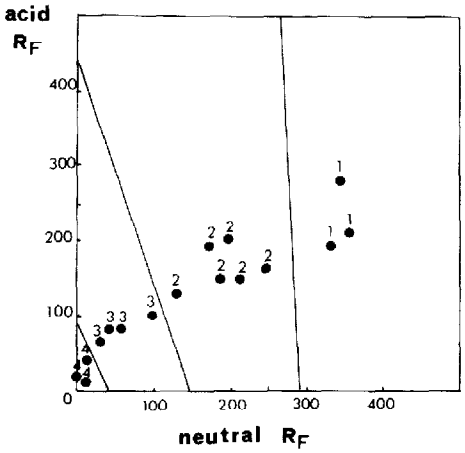

Fig. 2. Relative chromatographic chart of 5-hydroxyflavones. Acidic solvent, benzene–methanol–acetic acid (45:3:2). Neutral solvent, chloroform–*n*-hexane–methanol (40:40:3). Numbers are free hydroxy groups. The R_p values were calculated by giving to the 5,3′,4′-trihydroxy-6,7,8-trimethoxylflavone R_F value an R_p value of 100.

Correlation between R_M values and the number of methoxy groups

The R_M value proposed by Bate-Smith and Westall[2] is

$$R_M = \log\left(\frac{1}{R_F} - 1\right)$$

It is proportional to the free energy moving a molecule from one phase to the other, and was described for partition chromatography.

The available 6- and 8-methoxy-5-hydroxyflavones were chromatographed and their R_F and R_M values were calculated (Table II).

If the R_M values are plotted, for example, against the number of hydroxy or glycosy groups or carbon atoms, straight lines are obtained for all homologous series[2,30–32], which makes the calculation of the R_F values of unknown members fairly accurate. We plotted the R_M values obtained with the chromatographic systems used against the number of methoxy groups in each flavone studied (Fig. 3). Straight lines were obtained for each homologous series, considering homologous series as groups of flavones with four, five or six (hydroxy and methoxy) substituents on the flavone nucleus. The straight lines were parallel to each other. These results are similar to those obtained by Bate-Smith and Westall[2] with partition systems. Nevertheless, our system is an adsorption one, althought partition must play some role.

Relationship between ΔR_M and hydroxyl number/methoxyl number

It has been reported[24,33] that substances such as acids and bases can be chromatographed using solvents in the ionized or non-ionized form in order to obtain structural information. If a solvent pair is chosen in which most other groups than the ionizable ones have low group constants[24,33], the difference in R_M values will be proportional to the number of the ionizable forms in the molecule. We have tried to

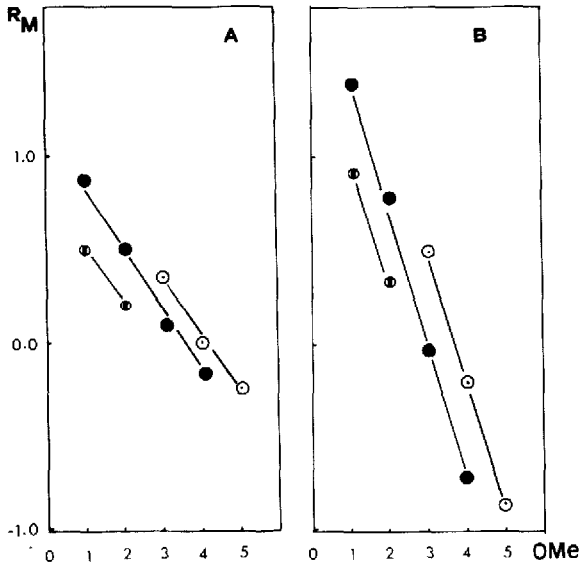

Fig. 3. Correlation between R_M values and methoxyl number. Chromatography on silica gel with benzene–methanol–acetic acid (45:3:2) (A) and chloroform–*n*-hexane–methanol (40:40:3) (B). Number of substituents: ⦶, 4; ●, 5; ⊙, 6.

apply this theory to flavones, which have an acidic character, developing in acidic and neutral media, by calculating ΔR_M values. In our experiment, it was not possible to establish a linear relationship between ΔR_M and the free phenolic hydroxyl number (Table II). However, a straight-line relationship between ΔR_M and the ratio of the hydroxyl number to the methoxyl number was found, by plotting ΔR_M values against log (hydroxyl number/methoxyl number) (Fig. 4). These results show that methoxy groups have some effect on ΔR_M, although they are non-ionizing groups. This can possibly be explained by the use of a neutral instead an alkaline solvent.

There are considerable restrictions on the use of benzene as a solvent because of its carcinogenity. We found that when it was replaced with toluene only slight differences in R_F values were observed and the general conclusions were unaltered.

Chromatographic behaviour of permethylated flavones

In the structural analysis of flavonoid aglycones, methylation with diazomethane yields permethylated derivatives. There are considerably fewer of these than the partially methylated flavones, and it is easy to obtain authentic permethylated samples. Chromatographic comparisons of these standards with the products obtained after permethylation with diazomethane of the sample compounds give important information about the substitution pattern of the flavone nucleus.

The available permethylated flavones were chromatographed using different systems (Table II) and similar results were obtained for all of them. From these data, it was possible to study the effect on the R_F values of introducing methoxy groups in several positions.

In general, 6-methoxyflavones show the highest R_F values and the only 8-methoxy compound available without 6-substitution exhibits the lowest R_F value; the

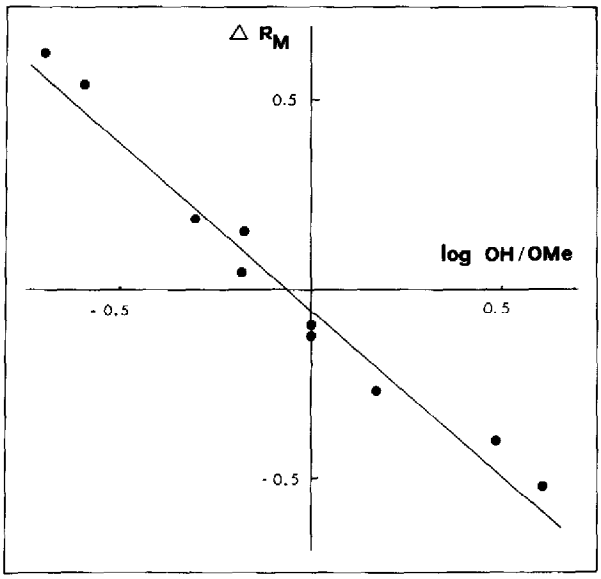

Fig. 4. Relationship between ΔR_M and log(hydroxyl number/methoxyl number). $\Delta R_M = R_M$ (acidic system) $- R_M$ (neutral system). Acidic system, benzene–methanol–acetic acid (45:3:2); neutral system, chloroform–*n*-hexane–methanol (40:40:3).

other compounds without methoxy groups in the 6- or 8-positions have intermediate R_F values. The introduction of a methoxy group at the 3'- or 4'-position leads to a decrease in R_F values, whereas its introduction at the 6- or 8-position when there is already a methoxy group in the 6-position increases the R_F value. Finally, the introduction of a methoxy group at the 8-position when there is no methoxy group in the 6-position leads to a decerase in the R_F value.

Elucidation of unknown structures

In the structural analysis of unknown flavonoids, it is possible to begin the elucidation by means of chromatographic assays. The substitution pattern is readily known by diazomethane methylation and chromatographic comparisons against permethylated controls. Additional information on this subject and on the number and localization of free hydroxy groups can be gained by means of UV–visible spectrophotometry[9].

The chromatographic charts (Figs. 1 and 2) reveal the total number of free hydroxy groups. By means of the R_M values it is possible to ascertain the number of methoxy groups on the flavone nucleus (Fig. 3), if the substitution pattern is already known. Calculation of the ΔR_M values allows the ratio of the hydroxyl number to the methoxyl number to be determined, which confirms the above results.

These TLC techniques are of interest in screening for flavonoid aglycones for chemotaxonomic and pharmacological purposes, considering the very small amounts (microgram scale) of substances required.

TABLE III

TLC OF PERMETHYLATED FLAVONES ON SILICA GEL

The available permethylated flavones were developed with the following systems on silica gel: benzene–methanol–acetic acid (45:3:2) (solvent 1), chloroform–*n*-hexane–methanol (40:40:3) (solvent 2), benzene–methanol–carbon tetrachloride (20:3:7) (solvent 3), benzene–ethyl acetate (6:4) (solvent 4), *n*-butanol–*n*-hexane (15:85) (solvent 5), ethyl acetate (solvent 6), chloroform–ethyl acetate–dimethyl ketone (5:4:1) (solvent 7) and chloroform–ethylacetate–dimethyl ketone (5:1:4) (solvent 8).

Flavone	*Trivial name*	R_F								*UV (360 nm) colour*
		Solvent 1	*Solvent 2*	*Solvent 3*	*Solvent 4*	*Solvent 5*	*Solvent 6*	*Solvent 7*	*Solvent 8*	
5,7-Dimethoxyflavone		0.67	0.69	0.59	0.13	0.21	0.33	0.43	0.61	Light blue
5,7,4′-Trimethoxyflavone		0.63	0.58	0.54	0.07	0.12	0.21	0.31	0.51	Blue
5,7,3′,4′-Tetramethoxyflavone		0.57	0.55	0.49	0.04	0.04	0.17	0.28	0.49	Blue
5,6,7,4′-Tetramethoxyflavone		0.67	0.72	0.65	0.25	0.22	0.60	0.59	0.73	Pink
5,6,7,3′,4′-Pentamethoxyflavone	Sinensetin	0.62	0.70	0.60	0.17	0.09	0.50	0.54	0.71	Blue
5,6,7,8,4′-Pentamethoxyflavone	Tangeretin	0.73	0.79	0.74	0.31	0.30	0.67	0.65	0.77	Dark orange
5,7,8,3′,4′-Pentamethoxyflavone	Isosinensetin	0.51	0.45	0.41	0.02	0.02	0.10	0.20	0.43	Dark red
5,6,7,8,3′,4′-Hexamethoxyflavone	Nobiletin	0.68	0.78	0.71	0.22	0.15	0.60	0.61	0.75	Light blue

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REFERENCES

1 A. P. J. Martin, *Biochem. Soc. Symp.*, No. 3 (1949) 4.
2 E. C. Bate-Smith and R. G. Westall, *Biochim. Biophys. Acta*, 4 (1950) 427.
3 E. A. H. Roberts, A. Cartwright and D. J. Wood, *J. Sci. Food Agr.*, 7 (1956) 637.
4 J. B. Harborne, *J. Chromatogr.*, 2 (1959) 581.
5 D. G. Roux and E. A. Maihs, *J. Chromatogr.*, 4 (1960) 65.
6 D. G. Roux, E. A. Maihs and E. Paulus, *J. Chromatogr.*, 5 (1961) 9.
7 D. G. Roux, *J. Chromatogr.*, 10 (1963) 473.
8 K. Egger, in E. Stahl (Editor), *Thin Layer Chromatography; A Laboratory Handbook*, Springer-Verlag, Berlin, 1969, p. 687.
9 T. J. Mabry, K. R. Markham and M. B. Thomas, *The Systematic Identification of Flavonoids*, Springer-Verlag, Berlin, 1970.
10 J. H. Tatun and R. E. Berry, *Phytochemistry*, 11 (1972) 2283.
11 B. Voirin, *Phytochemistry*, 11 (1972) 257.
12 K. R. Markham, in J. B. Harborne, T. J. Mabry and H. Mabry (Editors), *The Flavonoids*, Chapman and Hall, London, 1975, p. 21.
13 B. Voirin and M. Jay, *Phytochemistry*, 16 (1977) 2043.
14 K. Hostettmann and M. Hostettmann, in J. B. Harborne and T. J. Mabry (Editors), *The Flavonoids; Advances in Research*, Chapman and Hall, London, 1982, p. 1.
15 J. G. Nielsen, P. Norgaard and H. Hedjs, *Acta Chem. Scand.*, 24 (1970) 724.
16 B. Rodriguez, *Phytochemistry*, 16 (1977) 800.
17 F. Tomás, F. Ferreres and A. Guirado, *Phytochemistry*, 18 (1979) 185.
18 F. Tomás, F. Ferreres, A. Guirado and W. Trowitzsch, *An. Quím.*, 79-C (1983) 96.
19 F. Tomás, J. J. Mataix and O. Carpena, *Agroquím. Tecnol. Aliment.*, 12 (1972) 263.
20 F. Tomás and P. Salmerón, *Agroquim. Technol. Aliment.*, 16 (1976) 572.
21 F. Ferreres, F. Tomás, A. Guirado and F. A. Tomás, *Afinidad*, 37 (1980) 337.
22 F. Ferreres, F. Tomás and A. Guirado, *Agroquim. Technol. Aliment.*, 20 (1980) 285.
23 K. Randerath, *Dünnschicht-Chromatographie*, Verlag-Chemie, Berlin, 1962.
24 E. Lederer and M. Lederer, *Chromatography*, Elsevier, Amsterdam, London, New York, Princeton, 1957, p. 118.
25 M. L. Bouillant, J. Favre-Bonvin and J. Chopin, *Phytochemistry*, 14 (1975) 2267.
26 F. Serrano, *Tesis Doctoral*, Univ. Autónoma de Madrid, Fac. Ciencias (1975).
27 R. D. Schmid, *Tetrahedron*, 28 (1972) 3259.
28 A. I. Vogel, *A Textbook of Practical Organic Chemistry*, Longmans Green, London, 3rd ed., 1961, p. 969.
29 J. Layole, A. Lattes, B. Battie, H. Zamarlik and J. Carles, *J. Chromatogr.*, 76 (1973) 441.
30 H. Van Duin, *Biochim. Biophys. Acta*, 10 (1953) 198.
31 H. Van Duin, *Recl. Trav. Chim. Pays-Bas*, 73 (1954) 68.
32 G. Serchi, *Ann. Chim. (Rome)*, 43 (1953) 253.
33 E. R. Reichi, *Mikrochim. Acta*, (1956) 958.