THIN LAYER CHROMATOGRAPHY OF FREE PORPHYRINS

NILS ELLFOLK AND GUNNEL SIEVERS
Department of Biochemistry, University of Helsinki, Helsinki (Finland)
(Received March 28th, 1966)

Chromatographic methods have been successfully used for the analysis and preparation of porphyrins. The different techniques employed by column and paper chromatography have been reviewed by FALK[1-3].

A general feature in the investigation of unknown porphyrin material seems to be in performing the first chromatographic analysis of the neutralized porphyrin acids in a water–lutidine (WL) system[4,5]. This system fractionates the porphyrins approximately according to the number of free carboxyl groups present. Different dicarboxylic porphyrins have been separated as esters by paper chromatography according to a method of CHU AND CHU[6,7]. The use of porphyrin derivatives for chromatographic identification has, however, a practical disadvantage, particularly in cases when only a limited amount of material is available. A technique in which the porphyrins could be analyzed without a *prior* conversion to a derivative would therefore be of analytical value.

Recently, thin layer chromatography (TLC) has been used to identify the isomers I and III of coproporphyrin[8] and of some porphyrin esters as well[9]. Since TLC provides several advantages over other procedures, its use in the analysis of porphyrin free acids was studied. This communication describes the application of TLC to the separation of a variety of porphyrin free acids, in particular to that of the dicarboxylic type. The use of this technique in the purification of several porphyrins has been previously reported by ELLFOLK AND SIEVERS[10].

EXPERIMENTAL

Materials

Protoporphyrin IX. This was prepared from commercial crystalline protohemin (Hoffman-LaRoche) by a method of MORELL AND STEWART[11]. The purity of the preparation was checked spectrophotometrically using chloroform and 5 % HCl as solvents.

Mesoporphyrin IX. This was prepared according to MUIR AND NEUBERGER[12] from protoporphyrin IX by catalytic reduction. The final purification was performed in semimicro scale by TLC as described below. The purity of the preparation was checked spectrophotometrically as above.

Deuteroporphyrin IX. This was obtained by heating protohemin IX (Hoffman-La Roche) with resorcinol at 170–180°[13,14] and finally purified by TLC as described below. The purity of the preparation was checked as above.

Hematoporphyrin IX. A commercial preparation of hematoporphyrin dihydrogen chloride (Fluka) was used and was purified according to PORRA AND JONES[15], the

final purification being performed by TLC as described below. The purity of the preparation was checked as above.

2(4)-Vinyl-4(2)-hydroxyethyldeuteroporphyrin IX. Isolation was according to PORRA AND JONES[15] from the commercial hematoporphyrin IX (Fluka) preparation. The final purification was performed by TLC. The purity of the preparation was checked spectrophotometrically in ether and 5 % HCl.

Etioporphyrin III. This was prepared from protoporphyrin IX as described by SCHUMM[16] and finally purified by TLC. The purity of the preparation was checked spectrophotometrically in dioxane and 5 % HCl.

Pyrroporphyrin XV. This was prepared by heating an alkaline solution of pheophorbide a_5[17,18], which was prepared from spinach leaves[19,20]. The purity was checked spectrophotometrically in acetone and ether.

Coproporphyrin I. A commercial preparation (Sigma) was used.

Uroporphyrin I. A commercial preparation of the octamethylester (Sigma) was hydrolyzed for 48 h at room temperature in 25 % (w/v) HCl. The free porphyrin was taken up in ethyl acetate at pH 3.2.

All solvents were prepared from analytical reagent grade materials. Chloroform was washed just before use with 1 % (w/v) K_2CO_3 and with water.

Spectrophotometric measurements

A Beckman Recording Spectrophotometer model DK-1 was used in most experiments.

Preparation of chromatographic plates

Approximately 0.250 mm thick layers were prepared on degreased glass plates (10 × 20 or 20 × 20 cm) using a Desaga applicator. The plates were coated with a slurry prepared by mixing 30 g of Silica Gel G (Camag) and 60 ml of water. Acidic plates were prepared by mixing Silica Gel G (Merck) in 60 ml of 0.3 *M* oxalic acid solution. The plates were allowed to dry overnight on a horizontal support at room temperature.

Chromatographic procedure

To ensure equilibrium conditions inside the chromatography chamber, the walls were lined with strips of filter paper dipped in the solvent system.

The starting line was at a distance of 2.5 cm from the lower edge of the plate. The porphyrins were dissolved in chloroform, methanol or a mixture of these (50 μg per 1 ml) and 2 μl of each solution were applied with a micropipette (Camag) in several portions in order to obtain a spot as small as possible.

The chromatoplates were pre-equilibrated in the saturated atmosphere in the tanks for 30 min before development of the chromatograms. The plates were developed by the ascending technique. The experiments were performed at room temperature (21–23°). 30–45 min were usually required for the solvent front to reach a distance of 10 cm from the starting line. The plates were taken out to dry in the air at room temperature.

Detection

The porphyrins on the developed chromatoplates were located by inspection in ultraviolet light (3500 Å) in which the porphyrins show a strong red fluorescence. In

order to increase the intensity of the fluorescence the plates were sprayed with isooctan[21] or with a solution of oxalic acid in methanol. In order to obtain permanent records the chromatograms were occasionally photographed in incident ultraviolet light (3500 Å). The camera was loaded with a panchromatic film (ADOX, KB 14), a red filter being placed in front of the lens. The exposure time was 5 min. The conditions will of course depend on the properties of the U.V.-lamp used.

RESULTS

Neutral plates

The WL system, which has been found to fractionate porphyrins according to the number of free carboxyl groups present, was studied as a developing solvent for different dicarboxylic porphyrins on thin layers. However, it gave a poor fractionation of the different porphyrins and it is evident that the solvent cannot be used for the analysis of dicarboxylic porphyrins.

After a systematic study of different developing solvents one was finally worked out consisting of a combination of a nonpolar solvent, an alcohol and an organic acid. A solvent containing a nonpolar solvent and an alcohol only was not satisfactory because of the immobility of the material at the origin. By addition of an organic acid, however, the solvent became an effective developing system. Formic, acetic and propionic acids were tested in a chloroform–methanol (9:1) system, the acid concentration being equal to 0.30 *M*. Solvents consisting of several components may become only partially miscible while penetrating a dry adsorbent. This was not observed with the solvent containing formic acid as indicated by spraying the plates with an indicator solution. No front formation was observed by applying the test mixture of the porphyrins to several starting points located on a straight line from the lower left to the upper right corner of the chromatoplate. However, acetic and propionic acids both showed front formation with a γ-front corresponding to R_F values of 0.85 and 0.77, respectively.

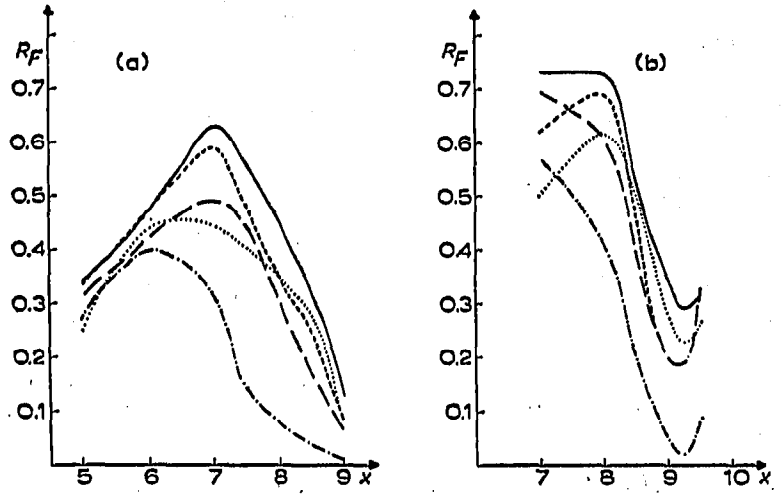

Fig. 1. R_F values of proto- (————), meso- (– – –), hemato- (. — . — . —), deutero- (. . .) and 2(4)-hydroxyethyl-4(2)-vinyl-deuteroporphyrin (— — —) as a function of the ratio benzene: methanol (x/(10–x), v/v) (a) and that of chloroform:methanol (x/(10–x), v/v) (b), both solvent systems containing a constant portion of formic acid equal to 0.3 *M*. The R_F values are mean values from several plates with an individual variation within a range of ± 0.05.

The specific effect of methanol, ethanol and propanol was tested in a benzene–alcohol/formic acid system (7:3/0.3 *M*). Methanol formed an excellent developing solvent, whereas ethanol and propanol both formed fronts, the γ-front corresponding to R_F values of 0.41 and 0.39, respectively.

A solvent system of benzene–dimethylsulfoxide (DMSO) was also tested. With lower concentrations of DMSO the solvent was not completely miscible. Only at a ratio of about 1:1 was the solvent system completely miscible. However, this solvent was not found to be very satisfactory for the chromatography of the porphyrins because of very strong tailing effects.

Fig. 1a gives the R_F values for five dicarboxylic porphyrins in a benzene–methanol–formic acid (BMF) system of a constant formic acid content (0.30 *M*), and Fig. 1b gives the R_F values of the same porphyrins in a chloroform–methanol–formic acid (CMF) system with a formic acid content equal to 0.3 *M*.

Typical chromatograms of different porphyrins in BMF (8.5:1.5/0.3 *M*) and in CMF (9:1/0.3 *M*) are reproduced in Figs. 2 and 3. Even though these solvent systems do not give the highest R_F values, they are usually employed because with higher alcohol contents and higher R_F, the spot areas increase noticeably. Fig. 4 shows the separation pattern of porphyrins with an increasing number of carboxyl groups in a BMF (8.5:1.5/0.3 *M*) system. The coproporphyrin I preparation seems to contain a component with three carboxyl groups as decided on the basis of the chromatogram.

The possibility of using the BMF-system for the chromatography of porphyrins with a higher carboxylic content was also studied. In a benzene–methanol solvent

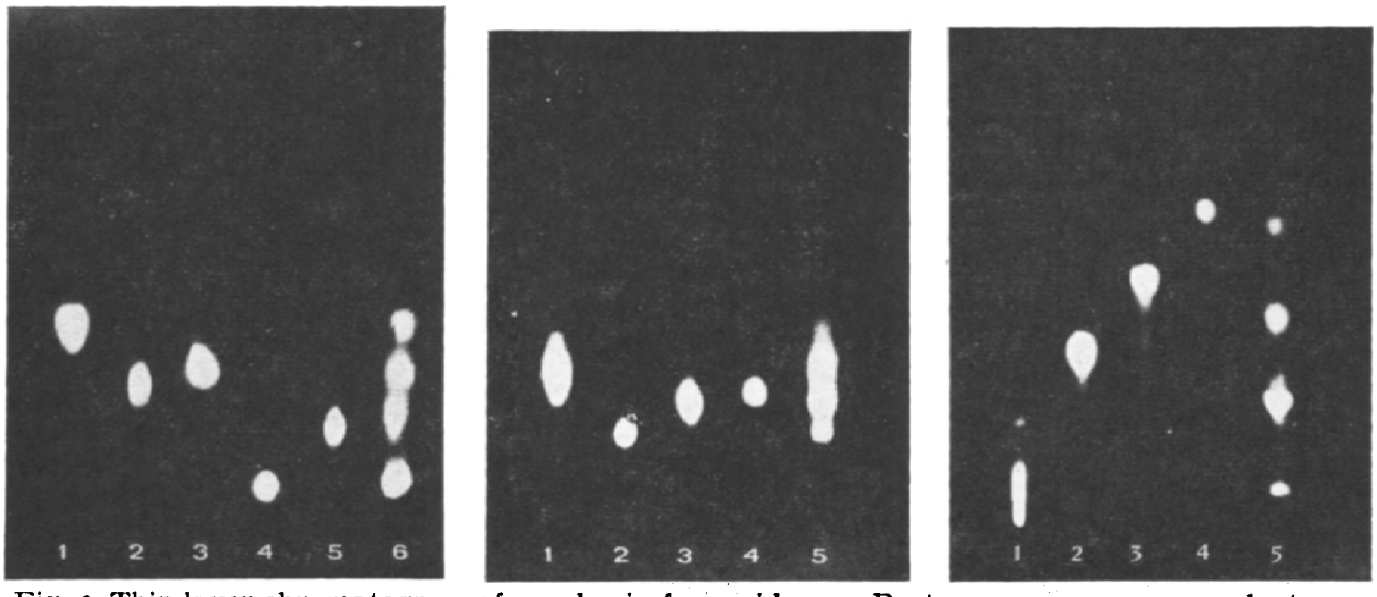

Fig. 2. Thin layer chromatogram of porphyrin free acids. 1 = Proto-; 2 = meso-; 3 = deutero-; 4 = hemato-; 5 = 2(4)-hydroxyethyl-4(2)-vinyl-deutero-porphyrin; 6 = a mixture of 1–5. Adsorbent: neutral Silica Gel G. Solvent: benzene–methanol/formic acid (8.5:1.5/0.3 *M*). Time 45 min, U.V.-photograph, red filter. Amount 0.1 μg.

Fig. 3. Thin layer chromatogram of porphyrin free acids. 1 = Proto-; 2 = meso-; 3 = deutero-; 4 = hemato-porphyrin; 5 = a mixture of 1–4. The experimental conditions are identical to those of Fig. 2 with the exception of the solvent, which was chloroform–methanol/formic acid (9:1/0.3 *M*).

Fig. 4. Thin layer chromatogram of 1 = copro-; 2 = meso-; 3 = pyrro-; 4 = etio-porphyrin; 5 = a mixture of 1–4. The experimental conditions are identical to those of Fig. 2.

(1:1), coproporphyrin was found to move with an R_F value of 0.66 and uroporphyrin with an R_F value of about 0.05.

Acidic plates

Formic acid could be excluded from the solvent systems by preparing acidic plates containing oxalic acid. Different alcohols in a benzene–alcohol (6:4) system were tested as developing solvents on these plates. Methanol, ethanol, *n*-propanol, isopropanol and *n*-butanol did not form fronts under these conditions, contrary to the case with solvents containing formic acid. Fig. 5 illustrates the specific chromato-

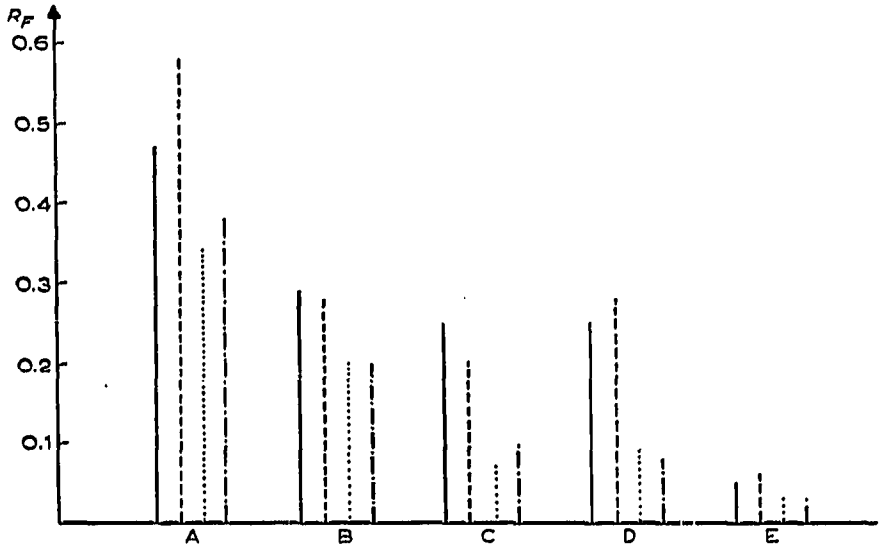

Fig. 5. The specific effect of different alcohols on R_F values of proto- (————), meso- (– – –), deutero- (...) and hematoporphyrin (. — . — . —) in a benzene–alcohol mixture (6:4) on acidic plates. A = Methanol; B = ethanol; C = *n*-propanol; D = isopropanol; E = *n*-butanol.

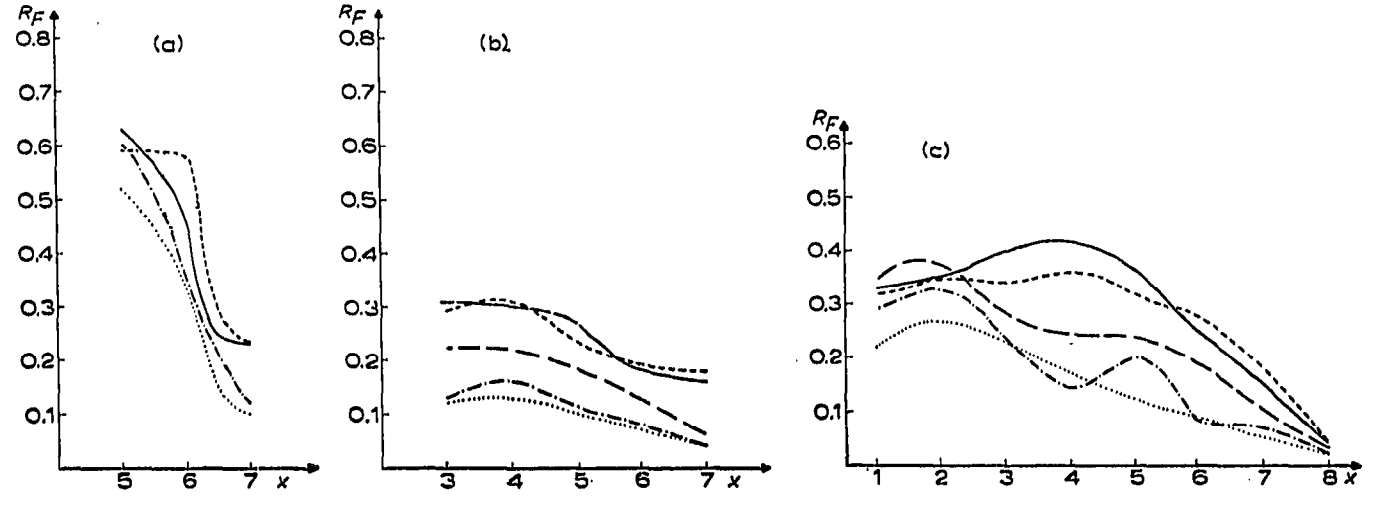

Fig. 6. R_F values of proto- (————), meso- (– – –), deutero- (...), hemato- (. — . — . —), and 2(4)-hydroxyethyl-4(2) vinyl-deutero-porphyrin (— — —) as a function of the ratio of benzene: methanol (x:(10–x), v/v) (a), that of toluene:isopropanol (x:(10–x), v/v) (b), and that of benzene: isopropanol (x:(10–x), v/v) (c) all on acidic plates. The R_F values represent mean values of several plate with a variation of ± 0.05.

graphic properties of the individual alcohols in the benzene–alcohol system on acidic plates. Methanol, *n*-propanol and isopropanol all showed exellcent fractionating properties. Fig. 6a gives the R_F values for five dicarboxylic porphyrins on acidic plates in a benzene–methanol solvent; in Fig. 6b, toluene–isopropanol solvent was used and in Fig. 6c, a benzene–isopropanol solvent. A typical chromatogram of different porphyrins on an acidic plate in a toluene–isopropanol (5:5) solvent is shown in Fig. 7.

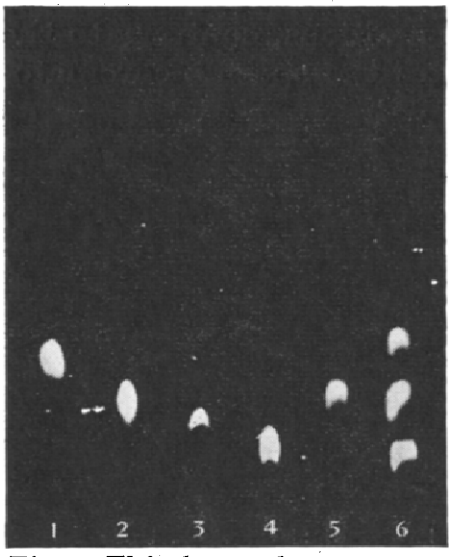

Fig. 7. Thin layer chromatogram of porphyrin free acids. 1 = Proto-; 2 = meso-; 3 = deutero-; 4 = hemato-; 5 = 2(4)-hydroxyethyl-4(2)-vinyl-deutero-porphyrin; 6 = a mixture of 1–5. Adsorbent: acidic Silica Gel G. Solvent: toluene–isopropanol (5:5). Time 45 min, U.V.-photograph. The experimental conditions are identical to those of Fig. 2.

The two procedures on neutral and acidic plates were satisfactory over the rather wide range of relative humidity prevailing in the laboratory, although there were limits beyond which it was less satisfactory. The upper limit was often exceeded when the relative humidity in the laboratory was in excess of 50 %. The use of plates of controlled humidity according to a technique of DALLAS[22] was not satisfactory.

Semimicro preparative separation

The porphyrin preparations to be purified were dissolved in a small amount of methanol of chloroform and the solution was pipetted on to the plates in individual spots close to each other. The plates were usually developed with BMF (8.5:1.5/0.3 *M*). The individual bands were scratched off and the porphyrins eluted with a dry mixture of chloroform and methanol, the composition of which depended on the solubility of the porphyrin. The silica was separated by filtration. The eluates from the individual zones were evaporated to dryness *in vacuo* by rotation. The individual fractions were dissolved in a suitable solvent (chloroform, dioxan etc.) and the identity of the component determined spectrophotometrically.

DISCUSSION

The results of this investigation demonstrate that thin layer chromatography on neutral and acidic plates is a suitable technique for the separation of porphyrin free acids.

The mobility of the porphyrins in chloroform–methanol–formic acid was found to be correlated to the number of carboxyl groups, so that etioporphyrin which has no carboxyl groups showed the highest mobility and the mobility of other porphyrins with one, two, three or four carboxyl groups decreased linearly.

The different solvent systems seem to differ only slightly in their chromatographic properties. Benzene–methanol–formic acid and chloroform–methanol–formic acid gave rather similar chromatograms on neutral plates, chloroform–methanol–formic acid, however, seemed to give better separation of meso- and deuteroporphyrins. The slight difference in the dielectric properties of the two solvent systems is evidently a reason for this difference. On acidic plates mesoporphyrin moves faster than deuteroporphyrin, in contrast to its behaviour on neutral plates. It is evident that the polarity of the compound strongly influences the R_F values, which tend to decrease with increasing polarity.

SUMMARY

Thin layer chromatography procedures are described for the separation and identification of a number of porphyrin free acids. Dicarboxylic porphyrins have been separated on neutral plates in benzene–methanol–formic acid and chloroform–methanol–formic acid, and on acidic plates in toluene–isopropanol, benzene–isopropanol and benzene–methanol.

A straight-line relationship for the mobility of porphyrins containing from nought to four carboxyl groups in the porphyrin ring was obtained on neutral plates in benzene–methanol–formic acid.

REFERENCES

1 J. E. FALK, *J. Chromatog.*, 5 (1961) 277.
2 J. E. FALK, in M. FLORKIN AND E. H. STOTZ (Editors), *Comprehensive Biochemistry*, Vol 9, Elsevier, Amsterdam, London, New York, 1963, p. 3.
3 J. E. FALK, *Porphyrins and Metalloporphyrins* (*B.B.A. Library*, Vol. 2), Elsevier, Amsterdam, London, New York, 1964, p. 189.
4 R. KEHL AND W. STICH, *Z. Physiol. Chem.*, 289 (1951) 6.
5 J. E. FALK, E. I. B. DRESEL, A. BENSON AND B. C. KNIGHT, *Biochem. J.*, 63 (1956) 87.
6 T. C. CHU, A. A. GREEN AND E. J. -H. CHU, *J. Biol. Chem.*, 190 (1951) 643.
7 T. C. CHU AND E. J. -H. CHU, *J. Biol. Chem.*, 208 (1954) 537.
8 J. JENSEN, *J. Chromatog.*, 10 (1963) 236.
9 T. C. CHU AND E. J. -H. CHU, *J. Chromatog.*, 21 (1966) 46.
10 N. ELLFOLK AND G. SIEVERS, *Acta Chem. Scand.*, 19 (1965) 2409.
11 D. B. MORELL AND M. STEWART, *Australian J. Exptl. Biol. Med. Sci.*, 34 (1956) 211.
12 H. M. MUIR AND A. NEUBERGER, *Biochem. J.*, 45 (1949) 163.
13 O. SCHUMM, *Z. Physiol. Chem.*, 178 (1928) 1.
14 T. C. CHU AND E. J. -H. CHU, *J. Am. Chem. Soc.*, 74 (1952) 6276.
15 R. J. PORRA AND T. G. JONES, *Biochem. J.*, 87 (1963) 186.
16 O. SCHUMM, *Z. Physiol. Chem.*, 181 (1929) 141.
17 H. FISCHER AND H. ORTH, *Die Chemie des Pyrrols. Pyrrolfarbstoffe*, Vol II, 1, Akademische Verlagsgesellschaft, Leipzig, 1937, p. 342.
18 A. TREIBS AND E. WIEDEMANN, *Ann.*, 466 (1928) 264.
19 H. FISCHER AND A. STERN, *Die Chemie des Pyrrols. Pyrrolfarbstoffe*, Vol. II, 2, Akademische Verlagsgesellschaft, Leipzig, 1940, p. 59.
20 A. STOLL AND E. WIEDEMANN, *Helv. Chim. Acta*, 16 (1933) 183.
21 M. BLUMER, *Anal. Chem.*, 28 (1956) 1640.
22 M. S. J. DALLAS, *J. Chromatog.*, 17 (1965) 267.