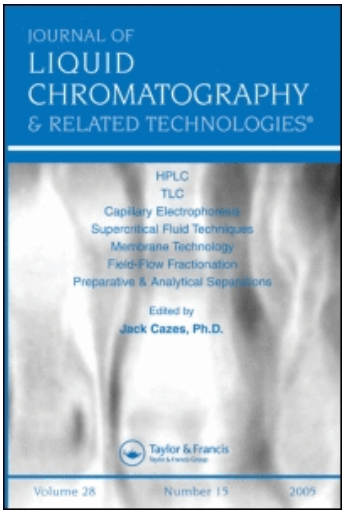


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

pH Gradient Layers for the TLC of Fluorescent Dyes

Egon Stahl[a]; Josef Müller[a]
[a] Pharmacognosy and Analytical Phytochemistry Universität des Saarlandes, Saarbrücken

To cite this Article Stahl, Egon and Müller, Josef(1980) 'pH Gradient Layers for the TLC of Fluorescent Dyes', Journal of Liquid Chromatography & Related Technologies, 3: 6, 775 — 787
To link to this Article: DOI: 10.1080/01483918008060190
URL: http://dx.doi.org/10.1080/01483918008060190

JOURNAL OF LIQUID CHROMATOGRAPHY, 3(6), 775-787 (1980)

pH GRADIENT LAYERS FOR THE TLC OF FLUORESCENT DYES

Egon Stahl and Josef Müller
Pharmacognosy and Analytical Phytochemistry
Universität des Saarlandes
D 6600 Saarbrücken

ABSTRACT

The advantages of pH gradient layers are presented with some fluorescent dyes as examples. In chromatography, transverse to the gradient (T-gradient) indications as to the chemical structure of the substance are obtained and contaminants are easily recognized. Chromatography in the direction of the gradient leads to a focusing, especially of the accompanying substances in the upper region of the chroma togram: These layers are suitable also for the chromatography of mixtures of substances of strongly differing polarities.

INTRODUCTION

Preparation of gradient layers in TLC with three different (1, 2, 3) directions of run was first successful 15 years ago; the usual neutral layers have only one available direction (Fig. 1). Chromatography can be carried out either transverse to the gradient or in two directions of the gradient. For the latter two cases, direction of chromatography should be selected in such a way that the substances to be examined are progressively more strongly braked during chromatography (4), thus yielding focusing. So far, not much use has been made of this phenomenon. The effect of

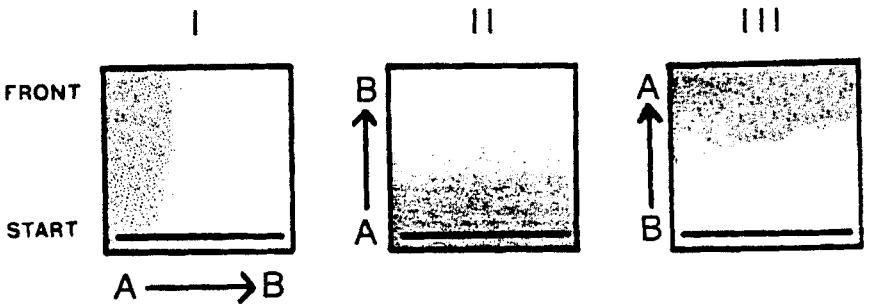


Fig. 1: A gradient layer yields three different areas for chromatography. The corresponding gradient and chromatography directions are marked by A⟶B.

focusing is to some extent contrary to the usual chromatography where, with increasing length of run, diffusion is so dominant as to cause formation of zones especially in the upper region of the chromatogram.

So far, of all the various possibilities of chromatography on a gradient layer, only the layers with increasing impregnation of silver nitrate (2, 3, 5-7) and pH-gradient layers (1-3, 8-14) have aroused interest. In spite of the numerous further possibilities of this method it was never widely employed; mostly chromatography transverse to the gradient (T-gradient-technique) was carried out. One of the reasons could have been the difficult preparation of the gradient layer with the former spreader which caused failures in the gradient. In the meantime, this spreader has been greatly improved (15). The advantages of this technique for a modern TLC are demonstrated below, using some commercial fluorescent dyes which were separated on pH gradient layers.

EXPERIMENTAL

Apparatus:

Gradient-TLC-Spreader 85 (New Model) with leveling table for 5 plates 20 x 20 cm. Manufacturer: DESAGA GmbH., D 6900 Heidelberg 1, West Germany

Preparation of pH-Gradient Layers:

14 g of silica gel HF_{254}, Type 60 (Merck, Darmstadt) are weighed into each of two 100 ml round flasks. To one is added 47.5 ml of 0.5 N sulphuric acid, to the other a solution of 0.8 g sodium sulfate in 47 ml 0.5 N sodium hydroxide, measured from burettes. After intensive shaking, Dexal 250 (a plastic dispersion of DESAGA, Heidelberg) is added into the flask which is hereby lightly shaken; the amounts are 0.3 ml to the acid suspension and 0.4 ml to the alkaline. The contents of the flask are mixed by careful shaking. Each suspension is then put at the same time in the corresponding chamber of the dividing trough and each surface is carefully leveled with a separate spatula. The separating wall is lifted and the mixing shaft put in with the help of the plastic holder. With its own motor drive, mixing is done for 15 sec in one direction. After disconnecting the motor, the lever of the spreader is turned 90° upwards (ventilation!) and the suspension is spread uniformly in one pull onto the 5 TLC plates. The layers are heated with an Infrared rod (length 90 cm) 35 cm above until the watery shine has disappeared; a warm air current from a hot air heater is then led over the surface until it is pure white. The thus predried plates are heated for 30 min at 110° to harden the plastic dispersion.

PREPARATION OF UNIFORM AND ABRASIVE-PROOF NEUTRAL LAYERS

Apparatus:

The same spreader but without diagonal separating trough and mixing shaft. 30 g silica gel HF_{254}, Type 60, and 100 ml demineralized water are mixed in a 250 ml round flask which is lightly shaken while 0.75 ml Dexal 250 is added. After the mixing, the spreading anddrying are done as usual.

TEST SOLUTIONS AND THEIR APPLICATION

The application is done with the DESAGA Autoliner 75, either transverse to the gradient or in the direction of the gradient (in the acid region). Solutions are made in methanol of fluorescein and of benzoflavine (both 0.4%) and of the other dyes (2%). The starting band is 18 cm long, 130 µl each are applied of fluorescein and benzoflavine and 25 µl each of the other dyes. The mixture of dyes for chromatography in the direction of the pH gradient and on neutral layers consisted of fluorescein (0.2%), Eosin "blue" (0.02%), rhodamine B (0.1%), benzoflavine (0.15%), and ß-naphthoquinoline (0.07%). 300 µl of this mixture were applied as a 18 cm long starting band.

CHROMATOGRAPHY CONDITIONS

Humidity: Humidity was not specially established, it amounted to 35% rel.

Solvent and Development:

For fluorescein: chloroform-methanol (95+5), 1 x 10 cm, CS

For eosin: chloroform-methanol (90+10), 1 x 10 cm, S-Chamber

For benzoflavine: chloroform-methanol (85+15), 1 x 10 cm, CS

For the mixture for chromatography in the gradient from acid to basic: chloroform-methanol (90+10), 2 x 18 cm, CS; the same for the neutral layer.

Detection and Photography:

Visualization was carried out by four high pressure lamps UV365 (DESAGA, Heidelberg).

Photography was done with a Leica, Film: ILFORD FP 4, 22 DIN, and a yellow and a UV-Filter.

TLC OF FLUORESCENT DYES IN THE pH GRADIENT

Fluorescein

Fluorescein shows amphoteric behaviour in the pH-T-gradient (Fig. 2); in the alkaline region the molecule exists as an anion (Formula) and the salt is so polar that no migration takes place. With descending pH, a point is reached (approx. at pH 6,5) where the molecule is uncharged and migrates in the solvent chloroform-methanol (95+5). A noncharged but colourless lactone form of fluorescein is also possible, however, it plays a minor role only as the complete set of curves is coloured intensively.

Approximately at pH 3 a recharge takes place. By addition of one proton each, charged particles are formed which migrate slowly. Care must be taken that this amphoteric behaviour does not show in any other too polar solvent. In ethyl acetate, for example, the fluorescein cation migrates in the acid region, too. This shows that one must work with solvents of various polarities if information about the chemical structure of an unknown substance is the aim of the tests (Fig. 2).

An interesting outcome of the pH-T-gradient chromatography of different commercially available products seemed the almost

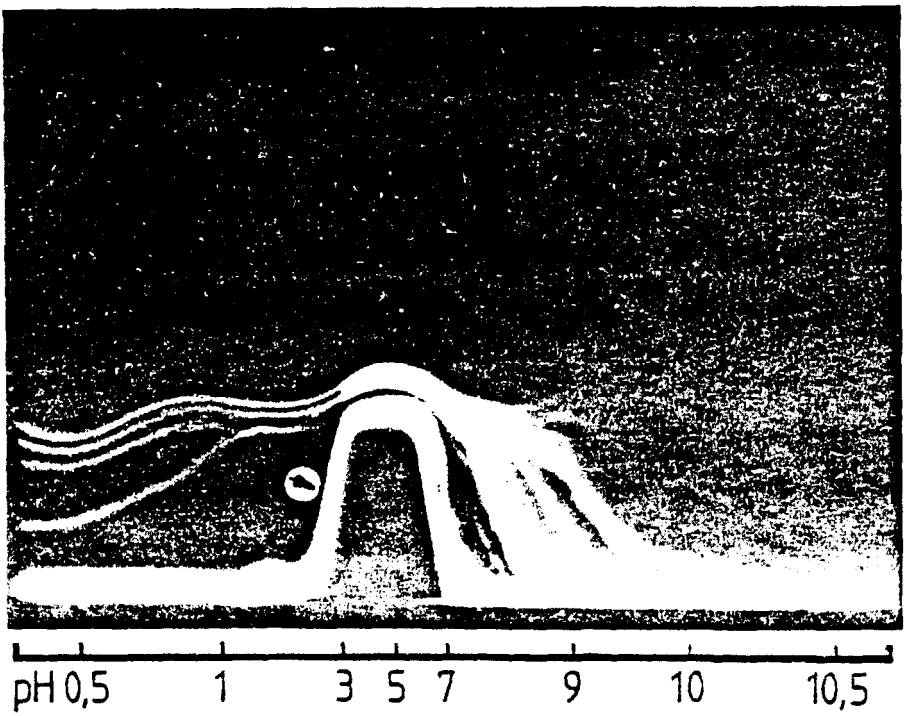
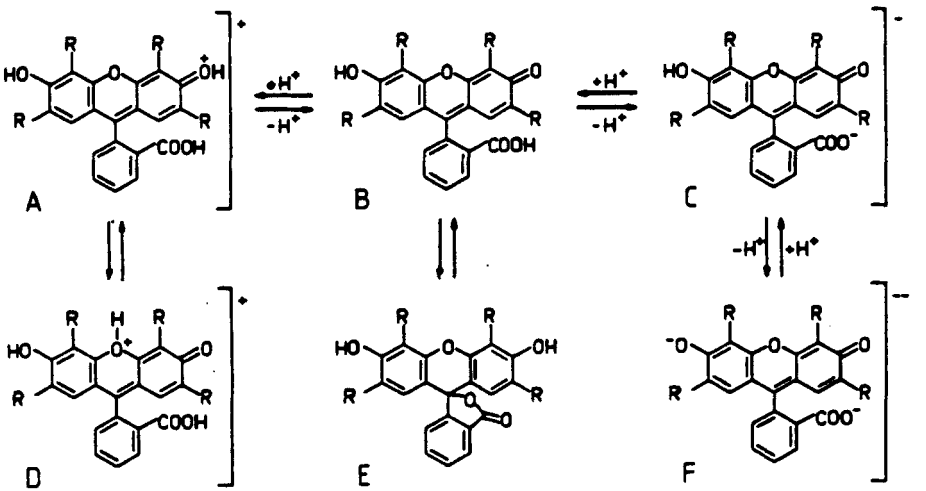


Fig. 2: Thin-layer chromatogram of commercially available fluorescein in the pH-T-gradient. Visualization and photography in long wave UV light 365 nm. The actual fluorescein zone is marked by an arrow. Note the by-products in the acid region and the splitting up in the pH-region between 8 and 9.

regular fact of a large number of by-products of the fluorescein. Most of the contaminants also show fluorescence in long wave UV light (365 nm) which makes them detectable in a concentration as low as approximately 10 ppm. Whereas in the acid pH region 4 to 5 substances of relatively weak fluorescence can be made out, a number of curves are detectable in the alkaline part of the plate (Fig. 2). All substances undergo salt formation in the alkaline medium. However, this occurs at different pH values which again

The various states of fluorescein and eosin in the acid (A and D), weakly acid (B and E), and basic (C and F) pH-regions.



R = H: Fluorescein
R = Br: Eosin

causes what can be called a fan of curves. To be on the safe side, we established by SRS technique (16) the fact that fluorescein decomposes neither on acid nor basic - impregnated silica gel layers. This, of course, would give the impression of contaminants which are contained in the original samples.

Eosin

Eosin, the 2.4.5.7-tetrabromoderivative of fluorescein, migrates in the pH-T-gradient (Fig. 3) in the acid region and remains on the starting line in the alkaline. The inflexion of the curve occurs at approx. pH 5. In contrast to fluorescein, there is no salt formation in the acid region since the halogen derivatives are more polarizable than fluorescein itself; the addition of protons is, therefore, more difficult. Leonhardt and Gordon (17) found this

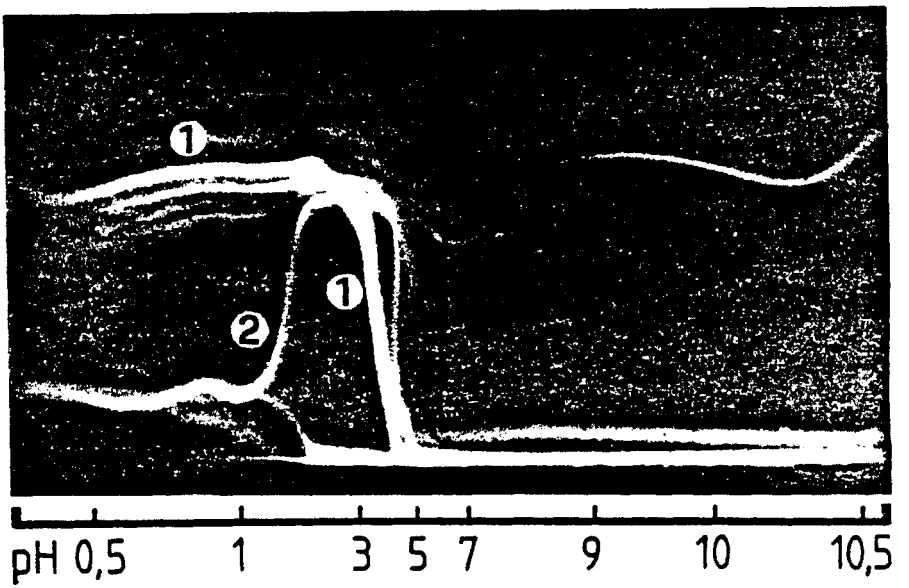


Fig. 3: Thin-layer chromatogram of eosin in the pH-T-gradient and visualization in long wave light, 365 nm. The thick curve (1) is attributed to the eosin, curve 2 is typical for fluorescein. Attention should be given to the numerous subsidiary curves which are due to the contaminants of the commercial product.

for dichlorofluorescein, too. The neutral form of Eosin thus exists up to the acid region of the layer.

The commercially available samples were all more or less contaminated with most of the by-substances forming the same curve course as eosin. This should support the idea of their being similar, e.g. partly halogenated fluorescein derivatives. According to Tsekos (18), tribromofluorescein represents the main contaminant. Eosin "blue" contains in addition dibromodinitrofluorescein. Fluorescein itself is still detectable in the product as starting product of the synthesis, as can be seen in Fig. 3.

A test shows that pure fluorescein is easily convertible on the TLC layer if applied as a band and if the layer is introduced into a chamber containing bromine. It can be seen that eosin is almost formed quantitatively in the neutral and in the acid medium. As shown in the pH-T-chromatogram, the thus formed dye is much purer than the commercially acquired products. Apart from eosin, only tribromofluorescein can be found. Through variation of time or concentration of the bromine vapours, the amount of the by-products formed can be influenced.

Benzoflavine

Commercially available benzoflavine has been chosen as another sample for the suitability of the pH-T-gradient technique for fast and simple detection of contaminants in substances. As seen in Fig. 4, they are detectable in the acid region as well as in the basic (Fig. 4).

TLC IN THE DIRECTION OF THE pH-T-GRADIENT

Chromatography is possible in two directions of the gradient. Basic considerations proved the method of chromatographic development with increasingly braked substances to be superior. We prefer to speak of this phenomenon as a focusing effect. Chromatography in reversed direction is possible, too, especially if one wishes to enlarge the distance between already separated zones, as could be the case in isolating experiments. In short, one chooses the direction according to the desired purpose. Fig. 5 shows the gradient chromatogram of a mixture of fluorescein, eosin "blue", rhodamine B, benzoflavine, and ß-naphthaquinoline. Development occurred in the gradient from acid to basic. In the upper region of the chromatogram, the contaminants

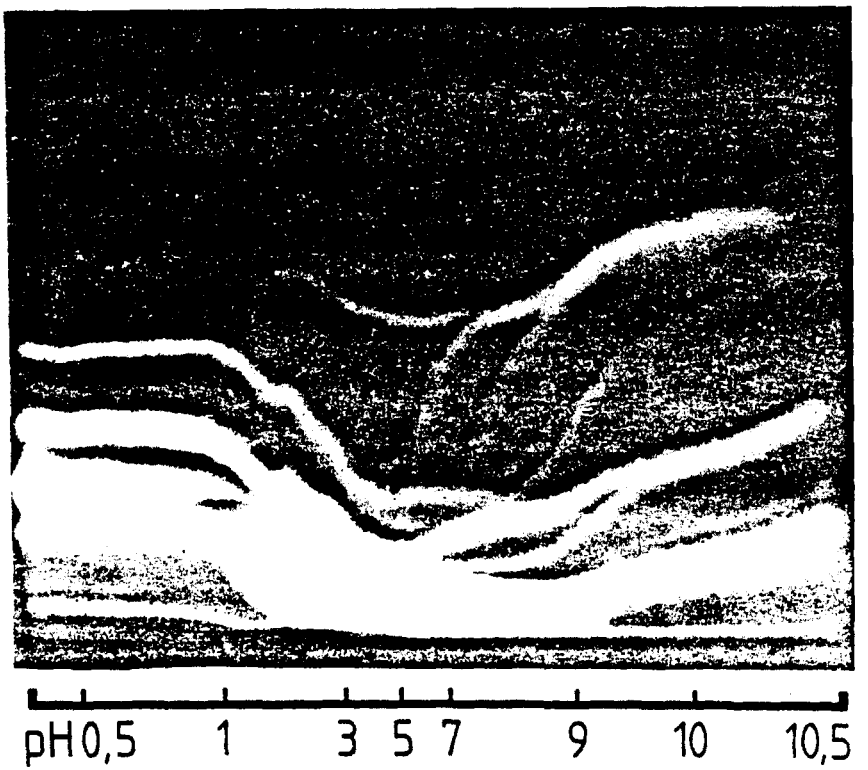


Fig. 4: Thin-layer chromatogram of benzoflavine in the pH-T-gradient. Visualization in long wave UV light, 365 nm. Besides the thick main zone numerous subsidiary zones are detected, especially in the pH-region between 7 and 9.

of the fluorescein can be detected as narrow and drawn together-that is: focused bands.

ADVANTAGES OF GRADIENT LAYERS

A comparison with the chromatography on neutral silica gel layers (Fig. 6) clearly shows the supremacy of the chromatography in the gradient compared to the hitherto used uniform layer of "neutral" silica gel.

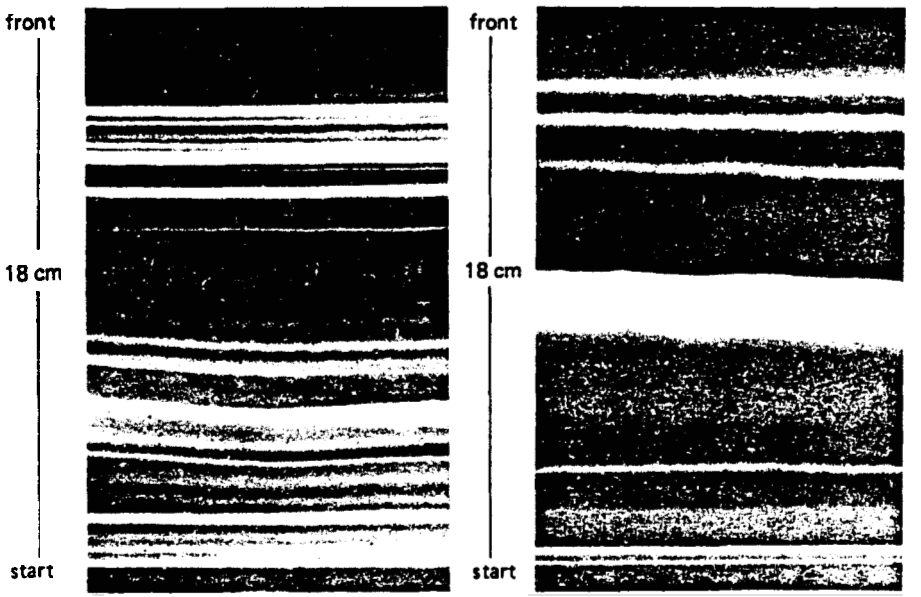


Fig. 5: (left) Thin-layer chromatogram of a mixture of fluorescein, eosin "blue", rhodamine B, benzoflavine, ß-naphthoquinoline. Chromatography was carried out from acid to basic in the pH-gradient. Visualization in long wave UV light 365 nm. Attention should be given to the focusing, especially in the upper region.

Fig. 6: (right) Thin-layer chromatogram of the mixture of Fig. 5 on a neutral silica gel layer. Some fluorescing substances are concentrated in the middle of the chromatogram in the strongly fluorescing main zone. A focusing effect as in Fig. 5 does not occur.

Chromatography transverse to the gradient (T-gradient technique) discloses rapidly whether the substances in a sample are acid, basic, neutral, and or amphoteric or even mixtures thereof.

Fluorescent dyes sometimes contain certain byproducts which differ only slightly from the main substance, yet they can be detected even in the ppm-region by their set of curves (Fig. 2-5).

The focusing effect which arises in chromatography in one direction of the gradient is especially interesting as it is possible with its help to move together substances with high Rf values into narrow bands and to increase greatly the number of detectable compounds per separation run.

Summarizing, gradient TLC yields the following advantages:

1. Details are obtained concerning the chemical structure of individual compounds in a mixture.
2. The purity of a sample can be ascertained with relatively simple means.
3. The stationary phase of TLC can be optimized in this way.
4. Mixtures of substances which differ strongly in their polarities can be separated on one single layer.
5. Concentration and detection by means of the focusing effect are possible in trace analysis.

In a further publication we shall report on the advantages of TLC in the gradient and on the resulting focusing effect.

REFERENCES

1. Stahl, E., Chem. Ing. Techn., 36, 941 (1964)
2. Stahl, E., Z. Anal. Chem., 221, 3 (1966)
3. Stahl, E., Proc. 6th Intern. Congr. Clin. Chem. Munich 3, 63(1966)
4. Snyder, L.R., and Saunders, D. L., J. Chromatog., 44, 1 (1969)
5. Stahl, E. and Vollmann, H., Talanta 12, 525 (1965)
6. Stahl, E. and Pfeifle, J., Naturwissenschaften 52, 620 (1965)
7. Rozumek, K.E., J. Chromatog. 40, 97 (1969)
8. Schorn, P.J., Vortragsref. III. Intern. Symp. Chromatog. Brussels, 1964
9. Shellard, E.J., Alam, M.C. and Armah, J., Sci. pharm. I, XXV. FIP-Congress, Prague, 1965

10. Poldermann, J., Pharm. Weekblad 101, 421 (1966)
11. Stahl, E. and Dumont,E., Talanta 16, 657 (1969)
12. Stahl, E. and Dumont, E., J. Chromatog. Sci. 7, 517 (1969)
13. Kraus, Lj. and Dumont, E., J. Chromatog. 48, 106 (1970)
14. Kraus, Lj., Stahl,Elis.and Thies,W., GIT 23, 645 (1979)
15. Stahl, E. and Müller, J., J. Chromatog., in press
16. Stahl, E. (Edit.), Thin-Layer Chromatography, A Laboratory Handbook, 2nd ed., p. 88, Springer-Verlag Berlin, Heidelberg, New York, 1969
17. Leonhardt, H. and Gordon, L., J. Phys. Chem., 75, 245 (1971)
18. Tsekos, I., Flora, Abt. A, 159, 519 (1969)