DAEHAN HWAHAK HWOEJEE
(Journal of the Korean Chemical Society)
Vol. 18, No. 4, 1974
Printed in Republic of Korea

무당개구리의 복피 Carotenoid 색소에 관한 연구(제 1 보). β-Carotene 과 4-Hydroxy-echinenone 의 분리 및 확인

張 世 熙·鄭 義 埈

서울대학교 문리과대학 화학과

(1974. 5. 27 접수)

Studies on the Carotenoid Pigments in the Abdominal Skin of Bombina Orientalis(I). Occurrence of β-Crotene and 4-Hydroxy-echinenone in the Abdominal Skin of Bombina Orienialis

Sae Hee Chang and Ui Chun Chong

Department of Chemistry, College of Liberal Arts and Sciences, Seoul National University, Seoul, Korea

(Received May 27, 1974)

요 약. Bombina Orientalis (무당개구리)의 복피로부터 암적색의 색소를 추출하여 TLC 및 column chromatography 로 10여종의 carotenoid를 분리하여 visible 및 IR-spectrum을 조사하였다. 물리, 화학적 성질, chromatomatographic 성질 visible 및 IR-spectral 특성을 이용하여 주색소는 β-carotene 과 4-hydroxy-echinenone 임을 확인하였다.

Abstract. More than ten cartenoid pigments were separated from the dark red extracts of abdominal skin of Bombina Orientalis by TLC, and column chromatography, and the visible and infrared spectrum of purified carotenoids were determined.

Two major pigments were identified as β-carotene and 4-hydroxy-echinenone respectively through physical and chemical properties, chromatographic, visible and infrared spectral charateristics.

Introduction

The chemical studies of animal carotenoid pigments have been laged for behind that of the plant pigments because of difficulty of sample collection and the separation of the pigments from accompanying lipids. Recently several studies[1~9] of animal carotenoids containing carbonyl and hydroxy group, mostly from marine invertebrate, were isolated and exa mined.

A study of carotenoid pigments of the Amphibian had been initiated from the retina of frog by St. Capranica[10] in 1877. He reported the presence of carotenoids of unknown constitution. Krukenberg[11] reported the occurrence of xanthophyll, unidentified, in the Bombinator igneus in 1882. Zechmeister and Tuzson[12] had

been recently reported the presence of carotene, xanthophyll, and zeaxanthin in the skin of tissues of Runa esculenta in 1936. The other studies of carotenoid pigments of the Amphibian had been made[13~17], but reported almost unknown constitution of carotenoids.

Bombina Orientalis is a rather rare kind of frog which is distributed only in Korea, Manchuria and the northern part of China. It lives in cold and clean water of mountain streams or nearby rice pads. They have a reddish orange abdoman with scattered black spots. The frog is believed as poisonous toward the common enamies of the frogs like snakes. The color is suggested as a warning color.

We have separated two major pigments from the abdominal skin of Bombina Orientalis and identified them as β-carotene and 4-hydroxy-echinenone repectively.

The abdominal skins of about twenty thousand of Bombina Orientalis were peeled off and extracted with acetone and petroleum ether. The combined extract was washed, dried, and evaporated in vacuo. The residue was dissolved in a small portion of acetone and allowed to stand overnight in a refrigerator(−15 °C). The steroids, separated out as white solid, were filtered off and the filterate was evaporated to a very viscous liquid. This procedure was repeated four or five times in order to remove steroids and other lipids from the pigment.

More than ten carotenoid pigments were detected and separated by PLC, TLC, and column chromatography. The absorption spectra in the visible range of all separated carotenoids were determined in the various solvents.

β-Carotene which is the most common carotenoid pigment in the nature was separated from the least polar portion of the pigments on PLC. 4-Hydroxy-echinenone, which was isolated from the several marine invertebrate[18], was isolated from the moderatly polar portion of the pigments.

Experimental

Materials and Methods. As far as possible, all operations were carried out in an inert atmosphere, usually of nitrogen. The adsorbant used for chromatographies were neutral aluminum oxide by E. Merck and silica gel for chromatography(100 mesh) by Kanto Chemical Co. Unless otherwise indicated, petroleum ether referes to the fraction boiling 30~70°C. For PLC and TLC, precoated plates(in case of A. 1203, type T) by E. Merck Co. were used. Melting points were determined in evacuated capillary tubes without correction. IR spectra were determined within the range of wave length 4000 cm^{-1} to 400 cm^{-1} with IR spectrophotometer. Model IR—G by Japan Spectroscopic Co. Ltd. The visible absorption spectra determined within the rang of wave length through 360 nm to 560 nm with Hitach—124 Spectrophotometer. Solutions of carotenoids were concentrated in a rotary evaporator operated under reduced pressure at 35~40°C.

Extractions of Pigments. About twenty thousand heads of Bombina Orientalis were captured during May and early June through out the Kyong-Ki province, Korea. The frogs were washed and frozen in the refrigerator(−15 °C) for more than twelve horus. The abdominal skin was carefully peeled off from the frozen body, and extracted with acetone in the refrigerator three times and then with petroleum ether until all pigments were removed (usually 4~5 times). The extracts were combined, washed with water, and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure in a rotary evaporator at room temperature. The dark viscous residue was dissolved in a small portione

and put to stand overnight in the refrigerator

The considerable amounts of precipitate, mainly sterols, were separated by decanation, and the remaining solution was concentrated further under reduced pressure. The same procedure was repeated four or five times to remove sterols and lipids from the pigments. The dark red residue was dissolved in hexane and subjected to chromatographic separation.

Separation of Pigments. The dark red hexane solution, without saponification alumina plate by Merck appeared quite satisfactory. The subsquent separation were carried out on the precoated alumina plate for PLC by Merck with hexane—benzene(1 : 1) solution as the developing solvent. Six main zones were separated in the following order of increasing adsorptive power as shown in *Fig.* 1: (*A*) a narrow deep orange and a broad yellow bands, (*B*) a narrow deep yellow band, (*C*) a broad pink band, (*D*) a narrow red band, (*E*) a narrow deep red band, and (*F*) a narrow purple. Each zone was scraped off from the plate, and the pigment were extracted from the adsorbant with benzene for *A* and *B* and acetone for other zones. Each extract was concentrated and examined by TLC.

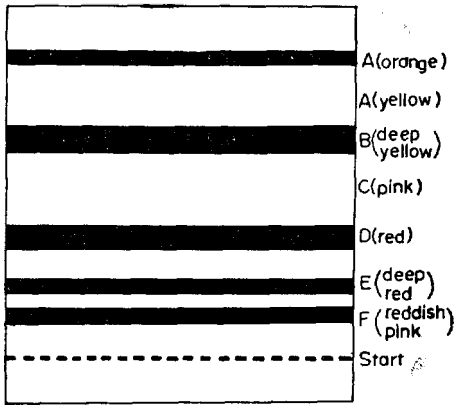


Fig. 1. Al_2O_3 PLC chromatogram solvent, hexane-benzene (1 : 1)

(*A*). This was further separated on Al_2O_3 PLC plate with petroleum ether. Three zones were separated in the following erder of increasing adsortive power: (A_1) a narrow deep orange band, (A_2) a broad yellow band, and (A_3) a broad pink band. The pigments in each zone were extracted with benzene. The extracts were evaporated and purified as follows:

(A_1), Chromatographed on an active Al_2O_3 column with petroleum ether. The broad yellow major band was extracted with petroleum ether. The extract was dried and evaporated. The residue was again examined by TLC on silica gel with hexane—benzene(1 : 1). No further separation was observed. The melting point of dark reddish violet prismshaped crystal was 181 ~182 °C after recrystallization from chloroform-benzene. The VIS—UV absorption maxima in nm: (425), 450, 477, in hexane; (442), 466, 496 in benzene; 466, 497 in $CHCl_3$; (450), 485, 520 in CS_2; (425), 451, 482 in petroleum ether(*Table* 1). IR(KBr pellet): 3.47 μ(C—H), 6.87 μ(C=C), 7.26 μ(CH_3), and 10.4μ(C—H in *trans* olefin). Upon the mixed TLC with A_1 and β-carotene from carrots, no separation was achieved. The R_f-value of pigment A_1 on SiO_2 with hexane—benzene(10 : 1) was 0.56, identical with that of a authentic β-carotene from carrots.

(A_2), Further purification was carried out on an active alumina column with petroleum ether containing 20 % benzene. After final purification the visible specrum was determined in the various solvents(*Table* 1).

(A_3), This was further purified on active alumn a column adsorption followed by elution with 40% benzen in petroleum ether. The spectrum of A_3 was determined after final purification.

(*B*). This was further separated on silica gel PLC plate with petroleum ether—benzene

(1 : 1). Two zones were separated, (B_1) a narrow pink band and (B_2) a broad red band. The pigments in each zone were extracted with benzene. The solvent was evaporated and the residue was purified further.

(B_1), Further purification was carried out on SiO_2column with benzene containing 30 % petroleum ether as eluent.

(B_2), This was further purified on SiO_2 column with benzene-hex-ane(1 : 1).

(C). This was further separated on SiO_2 PLC-plate with benzene containing 3 % acetone. There zones, (C_1) a narrow yellow band, (C_2) a narrow deep red band, and (C_3) a narrow pink band, were separated. The pigments in each zone were extracted with acetone, transfered to petroleum ether layer by adding water. The petroleum ether layer was washed with saturated NaCl solution, dried over anhydrous sodium sulfate, and concentrated. The residue were purified further as follows.

(C_1), Chromatographed on SiO_2 column 3 % acetone as eluent.

(C_2), This was further purified as (C_1).

(C_3), Chromatographed on SiO_2 column with benzene containing 3 % acetone, The major red zone was extracted with benzene. The extract was washed, dried and evaporated to dryness.

(D). This was further separated on SiO_2 PLC-plate with benzene containing 5 % acetone. Three zones, (D_1) a narrow pink band; (D_2) a broad red band; and (D_3) a narrow red, were obtained. Each zone was extracted with benzene containing 5 % acetone and purified further as follows.

(D_1), Chromatographed on SiO_2 column with benzene containing 5 % acetone. The major red zone was eluted out. The eluted solution was washed with water, dried and evaporated. The residue was examined by TLC on SiO_2 with benzene—acetone(20 : 1). No further separation was observed.

(D_2), The same procedure was used as (D_1).

(D_3), Chromatographed on SiO_2 column with benzene containing 5 % acetone. The major broad red zone was extracted with benzene. The extract was washed with water, dried and concentrated. The residue was crystallized from hexane, as the dark red diamond-shaped plates, m. p 165~167 °C. The VIS—UV absortpion maxima in nm:458 in petroleum ether; 465 in hexane 475 in benzene; 473 in $CHCl_3$ and 495 in CS_2 as a broad round-shaped curve. IR (KBr pellet): 2.90 μ(—OH), 6.04 μ(conjugated carbonyl), and 10.4 μ(C—H in *trans* olefin). The partition ratio, determined by the method of Zechmeister[22], was 33 : 67.

(E). This was further separated on SiO_2 PLC-plate with benzene containing 10 % acetone. Three major zones were separated in the following order of increasing adsorptive power: (E_1) a narrow red band, (E_2) a narrow red band, and (E_3) a narrow purple band. The pigments in each zone were extracted with acetone and transfered to petroleum ether layer. The petroleum ether layer was washed with water, dried, and evaporated to dryness. Each residues were further purified on SiO_2 column with benzene-acetone(10 : 1). The purified pigments were examined by TLC on SiO_2 with benzene—acetone(10 : 1). No further separations were observed.

(F). To the original acetone extract of zone (F), was added a twice the volume of petroleum ether. The resulted solution were washed with saturated NaCl solution, finally with water, dried, and evaporated. The residue was issolved in a small volume of hexane, and stood overnight in the refrigerator. The sterols, separated out as white solid, were filtered off. The filterate was futher chromatographed on SiO_2 PLC-plate with benzene containig

10 % acetone. Three obscure zones and a major zone were obtained. The red colored major zone was extracted with acetone, transfered to petroleum ether layer. Petroleum ether layer was washed with water, dried, and evaporated. The residue was further purified by PLC on SiO_2-plate with benzene containing 10 % acetone.

Reduction of Pigment D_3. To a solution of 0.5 mg of D_3 in 20 m*l* was added dropwise at 10 °C, with stirring under nitrogen atmosphere. The color of the solution turned to deep yellow within 5 minutes. The resulting solution kept for ten more minutes at this tempearture and the excess $NaBH_4$ was decomposed by dropwies addition of water at 5° C. The solution was washed with saturated NaCl solution, finally with water, dried and evaporated under reduced pressure. The residue was dissolved in 3 m*l* of hexane and poured on the top of the $Ca(OH)_2$-celite ($Ca(OH)_2$, made by Kanot Chem. Co., celite, No. 545, Hayashi Chem. Co.) column (1×10 cm) and developed with hexane containing 30 % $CHCl_3$. From the yellow major zone the solid was obtained as reddish orange needles m.p 143~144 °C from hexane $CHCl_3$. The visible spectrum of D_3 was determined in hexane, (428), 451, 478 nm.

Results and Discussion

The present investigation has demonstrated the presence of β-carotene and 4-hydroxy-echinenone in the abdominal skin of Bombina Odientalis as two major pigments. The visible absorption spectra of all the purified pigments were determined in the various solvents, as shown in *Table* 1.

The absorption specrum of pigment Al in the visible region, as shown in *Fig.* 2, were almost superimposable to that of β-carotene, previously reported; (425), 451, 482 nm in petroleum ether; (450), 485, 520 nm in CS_2; 466, 497 nm in $CHCl_3$; 425, 450, 477 nm in *n*-hexane by Goodwin[19].

The melting point of pigment A_1, the dark reddish violet prismshaped plates, was 181~182 °C (uncorr. evacuated capllary), and in good consistence with that of β-carotene mea-

Table 1. Visible absorption maxima of all separated pigments in various solvents(in nm)

Pigment	Benzene	Chloroform	Carbon disulfide	*n*-Hexane	Pet. ether
A_1	(442)466, 494	466, 497	(450)485, 520	(425)450, 477	(425)451, 482
A_2	466, 494	(440)465, 495	(449)483, 517	(425)451, 476	(427)450, 477
A_3	(447)477, 510	(446)475, 508	(463)496, 533	437, 461, 492	437, 462, 493
B_1	466, 494	466, 495	483, 515	(426)450, 477	(427)450, 477
B_2	433, 457, 488	433, 457, 489	458, 477, 505	421, 446, 475	420, 446, 476
C_1	479	480	489	464	463
C_2	480	43 , 456, 487	(450)475, 506	465	416, 443, 471
C_3	460	472	493	457	454
D_1	485	483	500	467	466
D_2	473	475	492	457	456
D_3	475	473	495	465	458
E_1	(440)462, 487	(440)498, 476	(455)482	(430)450(468)	450, (468)
E_2	433, 457, 487	(435)456, 487	(453)475, 505	421, 444, 472	416, 444, 471
E_3	462, 484	460, 486	480, 503	450, 472	450, 470
F_1	485	482	502	466	465

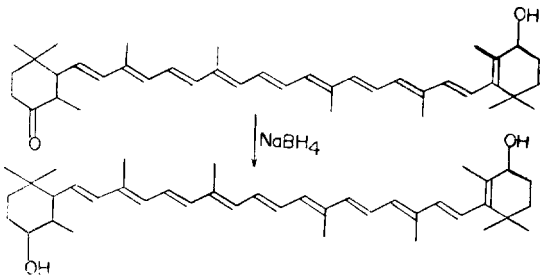
sured by Karrer[20] and his coworkers. The R_f-value of pigment A_1 on SiO_2 with *n*-hexane-benzene(10:1) was 0.56, identical with that of β-carotene isolated from the carrots. The IR-spectra of pigment A_1 shows a distinct absorption band at 3.47 μ, 6.87 μ, 7.26 μ and 10.4 μ (*Fig.* 3), which are in excellent agreement with that of β-carotene measured by Zechmeister[21]. On the mixed thin layer chromatography of pigment A_1 and an authentic sample of β-carotene from carrots, only one colored spot was observed. All these data strongly supported that pigment A_1 was undoubteply β-carotene itself.

The IR spectrum of pigment D_3 showed the presence of conjugated carbonyl group(6.04 μ), hydroxyl group(2.90 μ) and out-of-plane vibration of two hydrogen atoms in a *trans* olefin group(10.4 μ), as shown in *Fig.* 4

The spectrum was exactly superimposable to that of 4-hydroxy-echinenone by Zechmeister[21] and Jensen[22]. The crystalline form was the dark red diamond-shaped plate from $CHCl_3$—hexane, with m.p 165~167 °C, and the patition ratio, determined by the method of Zechmeister[23], was 33 : 67. The absorption maximum as a round broad curve at 458 nm in hexane(*Fig.* 2) shows the presence of keto group cojugated with the rest of the double bonds of the molecules.

(*Fig.* 2) in hexane, are in good agreement with the data reported for 4, 4-dihydroxy-β-carotene(isozeaxanthin) by Grob, *et al*[24].

All these data were in well agreement with 4-hydroxy-echinenone as previously reported[21, 22]. To confirm the assignment, D_3 was reduced with $NaBH_4$. The resulted deep yellow com-



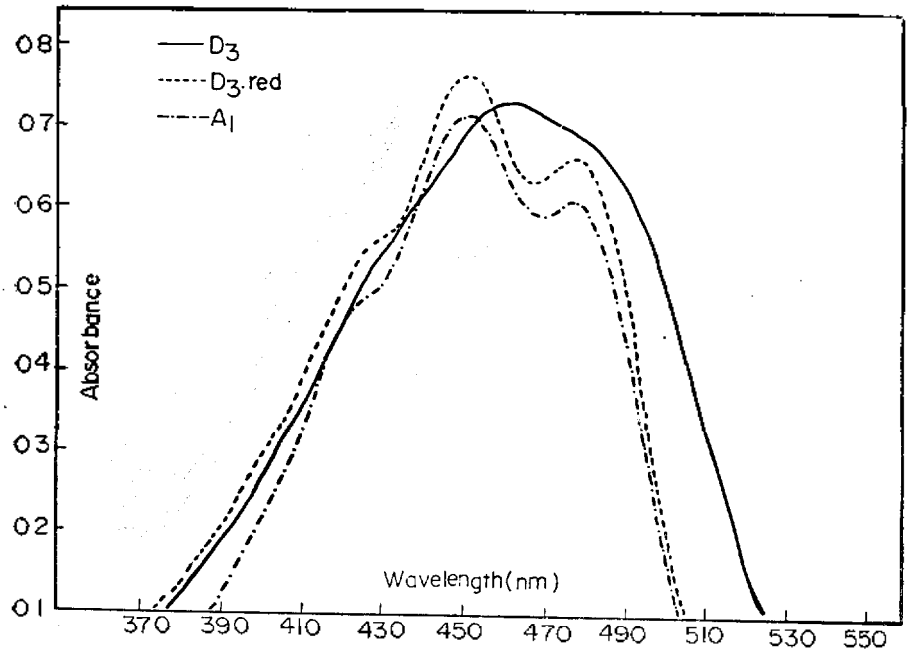


Fig. 2. Visible absorption curver of pigment A_1, D_3 and D_3 reduction product; Solvent, in *n*-hexane

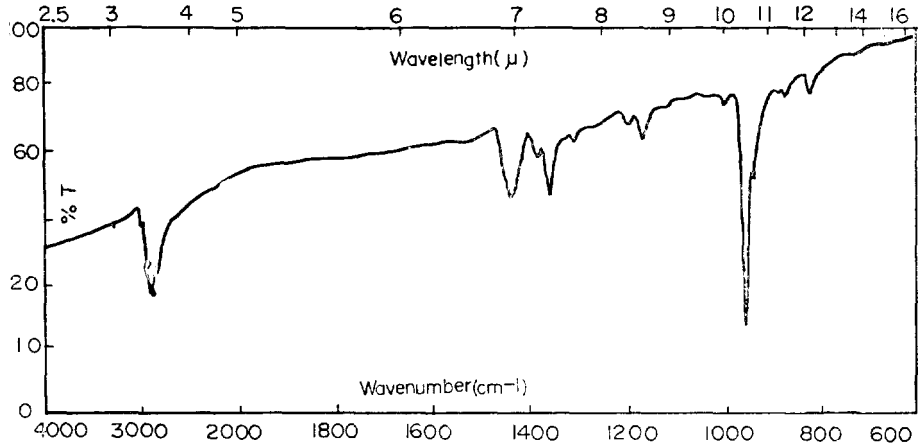


Fig. 3. IR Spectrum of pigment A_1 (KBr pellet)

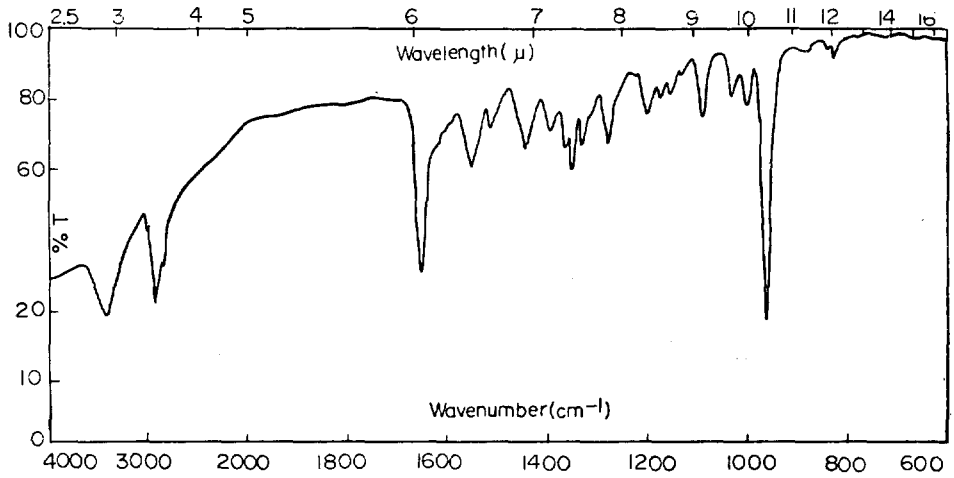


Fig. 4. IR Spectrum of pigment D_3 (KBr Pellet)

pound showed abs. max. at (428), 451, 478 nm

From these data D_3 was proved unequivocally as 4-hydroxy-echinenone. As far as literatures were searched by the authors, this is the first example that 4-hydroxy-echinenone has been found in vertebrate.

Acknowledgement

The authers wish to express their gratitute to the Dong-Ah Natural Science Foundation for a generous grant in partial support of this research. We are also indebted to the kind help of all the members of our organic chemitry labratory.

References

1. Marina de Nicola, *Biochem. J.*, **56**, 555(1953).
2. T. W. Goodwin and S. Srisukh, *ibid.*, **45**, 263 (1949).
3. T. W. Goodwin and S. Srisukh, *ibid*, **45**, 293 (1949).
4. R. Fabre and E. Lederer, *Bull. Soc. Chim.*

Biol., **16**, 105(1934).
5. T.W. Goodwin and M.M. Maha, *Biochem. J.*, **47**, 244(1950).
6. R. Kuhn and E. Lederer, *Ber. Dtsch. Chem. Ges.*, **66**, 488(1933).
7. P. Karrer and F. Benz, *Helv. Chim. Acta*, **17**, 412(1934).
8. R. Kuhn and N.A. Sorensen, *Z. Angew. Chem.*, **51**, 465(1938).
9. C.R Lederer, *Acad. Sci.*, **201**, 300(1935).
10. St. Capranica, *Arch. Physiol.* 287(1877).
11. C.F.W. Krukenberg, Cf. Physiol. Stud, Part 2, 50 Ser. **11**, (1882).
12. L. Zechmeister and P. Tuzson, *Z. Physiol. Chem.*, **238**, 197(1938).
13. C.F.W. Krukenberg, Cf. Physiol. Ser. 11, Part 2, 43, (1882).
14. F.G. Dietel, *Klin. Wochschr*, **12**, 601(1933).
15. C. Rand, *Biochem. Z.* **281**, 200 (1935).
16. O. Brunner and R. Stein, *Biochem. Z.* **282**, 47 (1935).
17. A. Magnan, *Compt. rend.*, **144**, 1130, 1068 (1907).
18. W.L. Lee, B.M. Gilchrist, and R.P. Dales, *J. Mar. Biol. Ass. U.K.*, 47 (1), 33~7(1967).
19. T. W. Goodwin, "Modern Methods of Plant Ana lysis" Vol. 111, P. 272, 1955.
20. P. Karrer and Coworkers, *Helv. Chim. Acta*, **14**, 615(1931).
21. K. Lunde and L. Zechmeister, *J. Amer. Chem. Soc.*, **77**, 1647(1955).
22. Synnove Liaaen Jensen, *Acta. Chim. Scad.* **19**, 1166(1965).
23. L. Zechmeister and F. J. Petracek, *Anal. Chem.*, **28**, 1484(1956).
24. E. C. Grob and R. P. Pflugshaupt, *Helv. Chim. Acta*, **48**, 490(1965).