Isolation and Identification of Carotenoids from the Petals of Rape (*Brassica napus*)

Daniel Kull and Hanspeter Pfander*

Institute of Organic Chemistry, University of Berne, Freiestrasse 3, CH-3012 Berne, Switzerland

From the yellow petals of rape flowers (*Brassica napus*), the carotenoids have been extracted and some compounds were identified. A HPLC analysis of the whole extract showed at least 80 different carotenoids, ranging from hydrocarbons to highly oxygenated species, including naturally occurring *E*/*Z*-isomers. The most polar part of the extract was further investigated by HPLC and UV–vis photodiodearray detection. The major carotenoids were isolated and identified, from UV–vis, CD, 400 MHz ^{1}H-NMR, and mass spectra, as (8′*R*)- and (8′*S*)-luteoxanthin, (13*Z*,8′*R*)-luteoxanthin, flavoxanthin/chrysanthemaxanthin, taraxanthin, and (*all-E*)-, (9*Z*)-, and (13*Z*)-violaxanthin. This shows the existence of different *E*/*Z*-isomers in nature. It was shown that luteoxanthin is found as an 8′-epimeric mixture, whereas there were no epimers found for violaxanthin.

Keywords: *Carotenoids; isolation; structure elucidation; Brassica napus*

INTRODUCTION

Rape, which belongs to the family of the Brassicaceae (cabbage plants), is in the temperate regions of the northern hemisphere one of the most important plants for oil and albumen. From the fruit is produced vegetable oil which is used for lubricating and for food purposes. Recently the production of rape has increased, mainly due to the fact that varieties with a small amount of erucic acid have been cultivated (Shahidi, 1990).

In general, petals owe their brilliant colors to flavones, anthocyanins, or carotenoids. In previous investigations the carotenoid composition of the petals of roses (Eugster and Märki-Fischer, 1991) and daffodils (Berset and Pfander, 1985) has been investigated, whereby large amounts of epoxy- and polyhydroxycarotenoids have been identified.

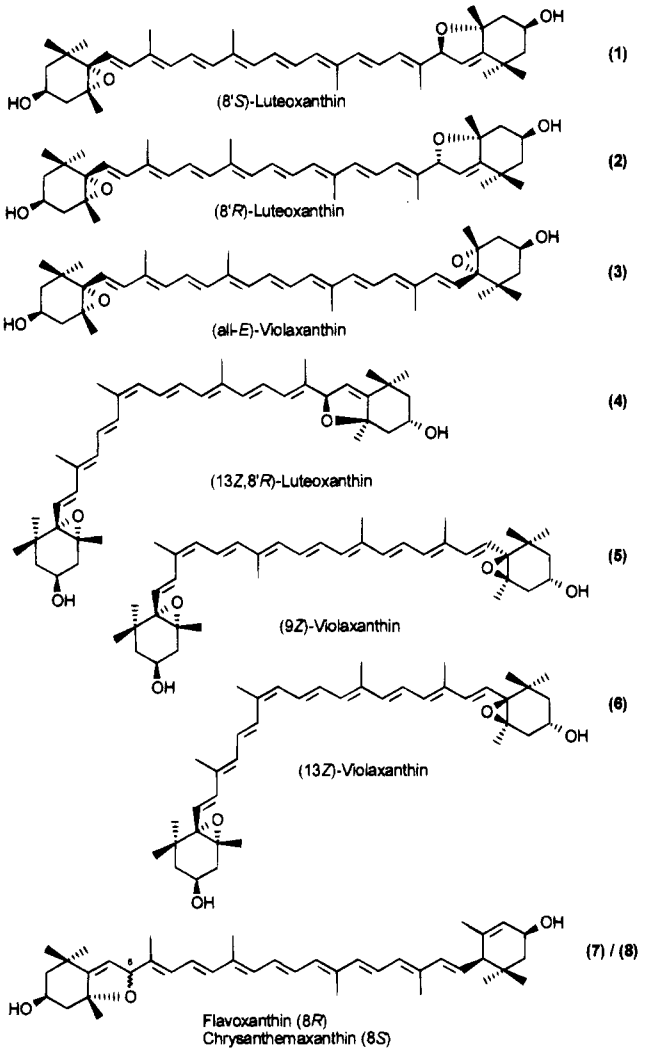
Recently we reported the isolation of (9′*Z*)-neoxanthin, (8′*S*)-luteoxanthin (**1**), (8′*R*)-luteoxanthin (**2**), (*all-E*)-violaxanthin (**3**), and (9*Z*)-violaxanthin (**5**) as the major carotenoids from the petals of rape (Debrunner and Pfander, 1991). In continuation of this work we report in the present work the isolation of four additional polar carotenoids.

MATERIALS AND METHODS

Plant Material. After harvesting, the fresh petals were frozen in liquid nitrogen and crushed immediately to a fine powder. This yellow powder was lyophilized for 5 days. The plant material could then be stored under argon at −20 °C for months, or even years, without loss of carotenoids or change in the carotenoid pattern.

Special Precautions. All work was carried out under an argon atmosphere and in dim light. All possible contaminations with acids were avoided to prevent degradation of the carotenoids. The solvents for extraction were of p.a. quality or freshly distilled. The solvents for chromatography were all of HPLC grade and purchased from Romil Chemicals. During workup the temperature was kept below 25 °C.

Chromatography. For flash chromatography Al_2O_3 III neutral, obtained from Al_2O_3 I neutral by addition of 6% water and stirring for 12 h, was used (Müller et al., 1985).

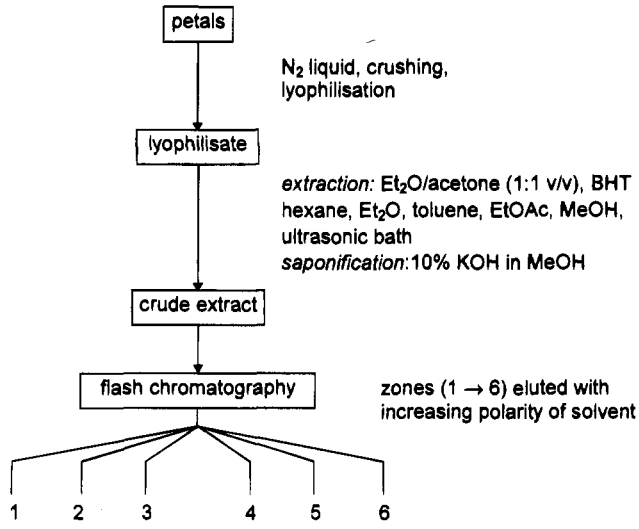


Apparatus. High-performance liquid chromatography (HPLC) was performed with a Waters PDA 991 photodiode array system, two Kontron T414 LC-pumps, and a Rheodyne 7125 injection valve. UV–vis spectra were measured with a Perkin-Elmer 554 spectrophotometer or with the PDA detector 991 of the HPLC apparatus. Electron impact mass spectrometry (EI-MS) was performed with an MS 9 (AE, Manchester,

* Author to whom correspondence should be addressed (fax ++ 41 31 631 34 25; e-mail pfander@ioc.unibe.ch).

0021-8561/95/1443-2854$09.00/0

Scheme 1. Extraction and Preseparation



U.K.) with a ZAB console (VG, Altrincham, U.K.) and a datasystem SS 300 (Finnigan MAT, Bremen, Germany). The samples were introduced by direct probe insertion and ionized with a 70 eV electron beam. The circular dichroism (CD) spectra were measured with a modified Dichrograph II (Jobin-Yvon) with MeOH as solvent. The ^1H nuclear magnetic resonance (NMR) spectra were recorded with a 400 MHz NMR instrument from Bruker Spektrospin, Model AM 400 WB, with dual head; $CDCl_3$ was used as solvent with trimethylsilane as internal standard.

Extraction. To 100 g of lyophilized petals was added 600 mL of acetone/diethyl ether (1:1 v/v), and the slurry was mixed for 15 min with a high-performance mixer. To prevent oxidation during this process, 0.1 g of 2,6-di-*tert*-butyl-4-methylphenol (BHT) was added. Afterward, the solution was put for 15 min in a cooled ultrasonic bath, and the green-yellowish pulp was filtered over Celite. The filter cake was transferred into a 2 L flask and extracted several times with different mixtures of solvents, consisting of acetone, diethyl ether, ethyl acetate, methanol, toluene, and hexane until the residue was almost colorless (3 h each time, dark, under N_2). After each extraction, the extract was filtered over Celite and immediately evaporated in vacuo to dryness. The combined extract was dried for 8 h under high vacuum and saponified with 700 mL of 10% methanolic KOH solution at room temperature. Afterward, the solution was concentrated to about 100 mL, and 300 mL of H_2O and 300 mL of Et_2O, were added. The H_2O phase was extracted six times with Et_2O and twice with EtOAc. For better phase separation NH_4Cl was added. The combined organic phase was washed neutral three times with phosphate buffer, dried over $MgSO_4$, and concentrated in vacuo to dryness. The dark red extract was dissolved in a small amount of methanol, and 100 mL diethyl ether was added. At −20 °C colorless sterols were precipitated and removed by filtration over Celite. This procedure was repeated until no more precipitates were obtained. After concentration and drying for 8 h at high vacuum, 2.61 g of saponified, dark red honey-like crude extract was obtained.

Flash Chromatography. The extract was dissolved in 20 mL of toluene/petroleum ether (1:1 v/v) and submitted to flash chromatography on a column (5 cm o.d.) which was packed with Al_2O_3 III neutral. With a slight nitrogen pressure the different fractions were eluted. The elution was carried out with increasing polarity: toluene/petroleum ether (1:1 v/v) → toluene/diethyl ether (1:1 v/v) → diethyl ether → ethyl acetate → methanol. The change to the next polar solvent was performed when the eluate was colorless. The fractions were immediately concentrated, dried under high vacuum, and stored at −20 °C. With methanol, a sharp red zone was eluted, which contained the most polar carotenoids (310 mg) and which was further investigated by HPLC.

HPLC Separation. For the HPLC separation a reversed-phase column (250 × 10 mm Nucleosil 7 C_{18}; Macherey-Nagel, Switzerland), developed with A/B [85:15 v/v; A, water/methanol/triethylamine (10:90:1 v/v); B, ethyl acetate/methanol/triethylamine (20:80:1 v/v), at a flow rate of 4.0 mL/min], was used. A separation of seven main peaks was observed with retention times from 12 to 26 min. For the further purification of the sample from peak 1 a nitrile-phase column (250 × 10 mm Nucleosil 5 CN; Macherey-Nagel) developed with hexane (1% TEA)/methylene chloride (2% MeOH) (7:3 v/v) at a flow rate of 3 mL/min was used. By this procedure three carotenoids were identified: (8′*S*)-luteoxanthin (**1**), t_R = 15.5 min; (8′*R*)-luteoxanthin (**2**), t_R = 16.0 min; and (*all-E*)-violaxanthin (**3**), t_R = 16.8 min.

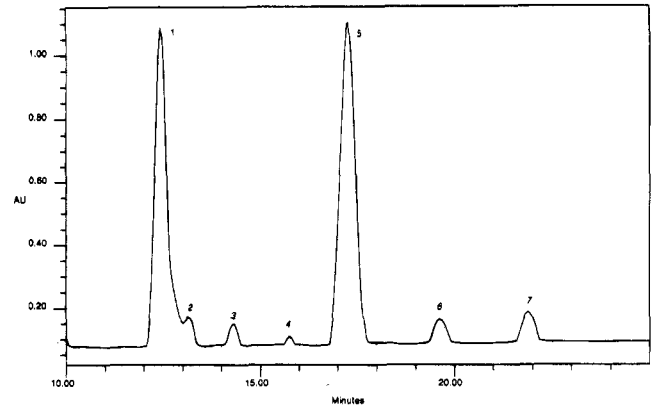


Figure 1. Chromatogram of the crude extract of *B. napus*.

For the separation of peak 2, the same separation as described above, with a flow rate of 1.5 mL/min, was used to give (13*Z*,8′*R*)-luteoxanthin (**4**), t_R = 14.0 min, and (9*Z*)-violaxanthin (**5**), t_R = 15.0 min.

For the separation of peak 3 the solvent ratio was 1:1 (v/v) with a flow rate of 1.5 mL/min to give as major compound (13*Z*)-violaxanthin (**6**), t_R = 16.5 min.

For the separation of the sample of peak 5 a reversed-phase column (250 × 10 mm Nucleosil 7 C_{18}; Macherey-Nagel) developed with 2-propanol (1% TEA)/H_2O (9:1 v/v) at a flow rate of 3 mL/min was used to give a mixture of flavoxanthin (**7**) and chrysanthemaxanthin (**8**), t_R = 6.2 min.

RESULTS

After extraction and saponification of 100 g of lyophilized petals of *Brassica*, 2.61 g of crude extract was obtained. Afterward, the extract was separated by flash chromatography on Al_2O_3 III neutral by a stepwise gradient elution to give six fractions of carotenoids with different polarities (Scheme 1).

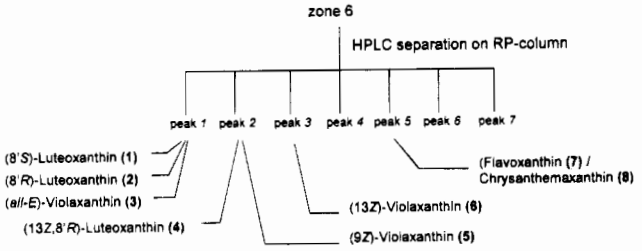
For an overview all of the fractions were investigated by HPLC, and in total more than 80 different carotenoids were observed. The most polar fraction, which contained 310 mg of carotenoids, was subject to further HPLC separations.

The separation of this fraction on a reversed-phase column gave seven main peaks (Figure 1).

Four peaks were further separated with different HPLC systems using either reversed-phase or nitrile columns. The identification was based on UV−vis, CD, 400 MHz ^1H-NMR, and mass spectra as well as cochromatography with authentic reference samples (Scheme 2).

From peak 1 three carotenoids have been isolated as major compounds. According to their UV−vis, CD, 400 MHz ^1H-NMR, and mass spectra as well as their cochromatography with authentic reference samples they were identified as (8′*S*)- (**1**) and (8′*R*)-luteoxanthin (**2**) as well as (*all-E*)-violaxanthin (**3**), which have been isolated earlier from *Brassica napus* (Debrunner and Pfander, 1991). The distinction between the 8′*S*- and 8′*R*-isomers **1** and **2** was based on their ^1H-NMR

Scheme 2. Isolation of the Carotenoids



spectra, where a shift difference of 0.1 ppm for H-C(8′) can be observed. The signals can be observed at δ 5.17 for the 8′*R* form and δ 5.07 for the 8′*S* form, respectively. The luteoxanthins as 5,8-epoxides are formed by an acid-catalyzed reaction of violaxanthin. This reaction may also occur in nature in the living plant during the aging process (Eugster and Märki-Fischer, 1991).

From peak 2 (13*Z*,8′*R*)-luteoxantin (**4**) and (9*Z*)-violaxanthin (**5**), which has been isolated earlier from the petals of rape (Debrunner and Pfander, 1991), were isolated. The identification of **4** is based on data published earlier (Märki-Fischer and Eugster, 1984; Englert, 1982). Compound **4** displayed UV−vis maxima at λ 298, 310, 395, 416, and 442 nm in MeOH with an α/β ratio of 0.95; CD (EtOH) 205 (0), 225 (−), 243 (0), 256 (+), 274 (0), 310 (−), 348 (0); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.98 [s, 3H, CH_3(16)], 1.14 [s, 3H, CH_3(17)], 1.18 [s, 3H, CH_3(16′)], 1.21 [s, 3H, CH_3(18)], 1.32 [s, 3H, CH_3(17′)], 1.68 [s, 3H, CH_3(18′)], 1.71 [s, 3H, CH_3(19′)], 1.91 [s, 3H, CH_3(19)], 1.94 [s, 3H, CH_3(20′)], 1.95 [s, 3H, CH_3(20)], 2.12 [m, 1H, H_{ax}-C(4)], 2.37 [m, 1H, H_{eq}-C(4)], 3.91 [m, br, 1H, H-C(3)], 4.24 [m, br, 1H, H-C(3′)], 5.17 [d, 1H, H-C(8′)], 5.25 [s, 1H, H-C(7′)], 5.91 [d, 1H, H-C(7)], 6.08 [m, 1H, H-C(14)], 6.19 [m, 2H, H-C(10/10′)], 6.22 [m, 1H, H-C(14′)], 6.29 [d, 1H, H-C(8)], 6.32 [d, 1H, H-C(12′)], 6.49 [m, 1H, H-C(11′)], 6.61 [m, 1H, H-C(15′)], 6.74 [dd, 1H, H-C(15)], 6.82 [d, 1H, H-C(12)], MS 600 (M^+, 8%), 520 ($M^+ - 80$, 6), 440 ($M^+ - 80 - 80$, 22), 396 (30), 352 (6), 313 (8), 287 (10), 234(8), 221 (40), 181 (21), 165 (18), 120 (25), 105 (27), 91 (37), 69 (30), 55 (43), 43 (100).

From peak 3 (13*Z*)-violaxanthin (**6**) was isolated as major compound. Compound **6** displayed UV−vis maxima at λ 312, 327, 417, 440, and 468 nm in EtOH; CD (EtOH) 195 (−), 199 (0), 209 (+), 219 (0), 234 (−), 247 (0), 269 (+), 267 (0), 316 (−), 329 (−), 350 (0); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.98 [2s, 6H, CH_3(16/16′)], 1.15 [2s, 6H, CH_3(17/17′)], 1.18 [2s, 6H, CH_3(18/18′)], 1.24 [m, 2H, H_{ax}-C(2/2′)], ~1.62 [m, 2H, H_{ax}-C(4/4′)], ~1.64 [m, 2H, H_{eq}-C(2/2′)], 1.92 [2s, 6H, CH_3(19/19′)], 1.94 [s, 3H, CH_3(20)], 1.96 [s, 3H, CH_3(20′)], 2.34 [dq, 2H, H_{eq}-C(4/4′)], 3.88 [m, 2H, H-C(3/3′)], 5.81 [d, 1H, H-C(7′)], 5.85 [d, 1H, H-C(7)], 6.05 [d, 1H, H-C(14)], 6.14 [d, 1H, H-C(10′)], 6.17 [d, 1H, H-C(10)], 6.19 [d, 1H, H-C(14′)], 6.24 [d, 1H, H-C(8′)], 6.25 [d, 1H, H-C(8′)], 6.31 [d, 1H, H-C(12′)], 6.48 [dd, 1H, H-C(15′)], 6.53 [dd, 1H, H-C(11′)], 6.54 [dd, 1H, H-C(11)], 6.73 [dd, 2H, H-C(15)], 6.84 [d, 1H, H-C(12)], MS 600 (M^+, 10%), 520 ($M^+ - 80$, 6), 440 ($M^+ - 80 - 80$, 8), 427 (4), 352 (8), 340 (8), 299 (8), 287 (20), 274 (14), 234 (12), 221 (57), 181 (38), 165 (23), 122 (28), 105 (30), 91 (48), 69 (19), 57 (41), 43 (100).

The spectral data were in agreement with the literature (Märki-Fischer and Eugster, 1985; Acemoglu et al., 1988).

The isolation of (*all-E*)-violaxanthin (**4**) and its *Z*-isomers **5** and **6** confirms the importance of these compounds for the formation of large amounts of carotenoids in petals. The violaxanthin cycle is responsible for the introduction of oxygen in the epoxides (Eugster and Märki-Fischer, 1991). The 5,6-epoxides exhibit always 5*R*,6*S* chirality, which means that the introduction of the oxygen occurs from the opposite side of the hydroxy group. The order of elution of the *E*/*Z*-isomers of violaxanthin on a reversed-phase column is in agreement with data obtained earlier (Riesen, 1991). Comparison of the UV−vis spectra of **3**, **5**, and **6** exhibits hypsochromic shifts of 5 nm of **5** and of 7 nm of **6**, respectively, compared to the *all-E*-isomer **3**. Furthermore, the intensity of the "*cis* peak" at ca. 327 nm was significantly higher in **6** compared to **5**. As violaxanthin exhibits strong conservative CD spectra, the spectra of **5** and **6** are opposite in sign to the spectrum of **3**. In the NMR spectra of **5** and **6** most of the signals for the olefinic protons are doubled due to the *Z* bond.

From peak 5 a mixture of flavoxanthin (**7**) with the 8*R* stereochemistry and chrysanthemaxanthin (**8**) with the 8*S* stereochemistry was isolated. The mixture of compounds **7** and **8** displayed UV−vis maxima at λ 447, 422, and 398 nm in MeOH; CD (MeOH) 208 (+), 227 (+), 250 (+), 322 (0), 335 (−), 345 (0); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.83 [s, 3H, CH_3(16′)], 0.98 [s, 3H, CH_3(17′)], 1.16 [s, 3H, CH_3(16)], 1.28 [s, 3H, CH_3(17′)], 1.62 [s, 6H, CH_3(18/18′)], 1.70 [s, 3H, CH_3(19)], 1.89 [s, 3H, CH_3(19′)], 1.93 [s, 3H, CH_3(20′)], 1.94 [s, 3H, CH_3-(20)], 2.39 [s, 1H, H-C(6′)], 4.23 [m, br, 2H, H-C(3/3′)], 5.12 [s, 1H, H-C(8)$_{chrys}$], 5.22 [s, 1H, H-C(8)$_{flavo}$], 5.30 [s, br, 1H, H-C(7)], 5.41 [dd, 1H, H-C(7′)], 5.55 [m, 1H, H-C(4′)], 6.10 [s, 1H, H-C(8′)], 6.14 [d, 1H, H-C(10′)], 6.176 [m, 1H, H-C(10)], 6.21 (m, 2 H), 6.30 (d, 1H), 6.32 (d, 1H), 6.49 [dd, 1H, H-C(11)], 6.59 (m, 2H); MS 584 (M^+, 4%), 566 ($M^+ - 18$, 7), 551 ($M^+ - 18 - CH_3$, 2), 504 ($M^+ - 80$, 8, typ Epoxid), 486 (5), 412 (4), 365 (8), 352 (12), 287 (9), 247 (8), 221 (60), 165 (31), 145 (41), 119 (38), 105 (53), 91 (51), 69 (51), 55 (60), 43 (100).

Experiments toward the preparative separation of **7** and **8** have been unsuccessful. However, on the basis of high-resolution ^{1}H-NMR spectra, the structures were elucidated univocally (Märki-Fischer et al., 1984; Englert, 1982). The two compounds **7** and **8** are 5,8-epoxides which are formed in a nonenzymatic manner in nature (Märki-Fischer and Eugster, 1985). Therefore, the 8*R*- and 8*S*-isomers are formed in a ratio of 1:1, which is in accordance with our ^{1}H-NMR data. Taraxanthin could not be isolated from the extract, but UV−vis spectra gave strong indications of its presence.

ACKNOWLEDGMENT

We express our sincere gratitude to F. Hoffmann-La Roche Ltd., Basel, for material and for measuring spectral data.

LITERATURE CITED

Acemoglu, M.; Uebelhart, P.; Rey, M.; Eugster, C. H. Syntheses of enantiomerically pure violaxanthins and related compounds. *Helv. Chim. Acta* **1988**, *71*, 931.

Berset, D.; Pfander, H. Investigation of the carotenoid composition in the petals of a garden hybrid narcissus cvv Golden Harvest. *Helv. Chim. Acta* **1985**, *68*, 1149.

Debrunner, B.; Pfander, H. Isolation and characterisation of carotenoids from *Brassica napus*. *Chimia* **1991**, *45*, 27.

Englert, G. In *Chemistry and Biochemistry of Plant Pigments*; Pergamon Press: Oxford, U.K., 1982.

Eugster, C. H.; Märki-Fischer, E. The chemistry of the colorants in roses. *Angew. Chem.* **1991**, *103*, 671.

Märki-Fischer, E.; Eugster, C. H. Carotenoids from the anthers and petals of *Lilium tigrinum* cv. Red Light. *Helv. Chim. Acta* **1985**, *68*, 1708.

Märki-Fischer, E.; Buchecker, R.; Eugster, C. H. Reinvestigation of the carotenoids from *Rosa foetida*, structures of 12 novel carotenoids; stereoisomeric luteoxanthins, auroxanthins, latoxanthins and latochromes. *Helv. Chim. Acta* **1984**, *67*, 2143.

Müller, R.; Keese, R.; Toube, T. P. *Fundamentals of Preparative Organic Chemistry*; Juris Verlag: Zürich, 1985.

Riesen, R. *(E/Z)* isomerisation of bacterioruberin. Ph.D. Dissertation, University of Berne, Switzerland, 1991.

Shahidi, F. *Canola and Rapeseed*; Van Nostrand Reinhold: New York, 1990.

Received for review May 3, 1995. Accepted August 23, 1995.[⊗] We are grateful to the Swiss National Foundation for financial support of this project.

JF950267V

[⊗] Abstract published in *Advance ACS Abstracts,* October 1, 1995.