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IDENTIFICATION OF CAROTENOIDS IN PARSLEY

(IDENTIFIKASI SENYAWA KAROTENOID DALAM PARSLEY)

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INTISARI

Telah dilakukan pemisahan karotenoid menurut golongan karoten, monol, diol dan polyol dari tanaman "parsley" dengan kromatografi kolom. Masing-masing golongan ini kemudian dipisahkan menjadi komponen individu dengan Kromatografi Cair Kinerja Tinggi (KCKT).

Masing-masing komponen individu telah diidentifikasi dengan dua metoda yaitu ko-kromatografi dengan senyawa standar dan metoda spektroskopi. Dari penelitian yang dilakukan telah berhasil diidentifikasi 12 senyawa karotenoid dari tanaman "parsley" yaitu beta-karoten, alfa-karoten, zeta-karoten, beta-cryptosantin, hidroksi-alfa-karoten, zea-santin, lutein, flavosantin, krisentemasantin, violasantin, luteosantin dan neosantin.

INTRODUCTION

Caratenoids are minor constituents in fruit and vegetables that are important as colouring agents enchancing the appearence of produce, and those with vitamin A activity provide a substantial source of vitamin A in diet. In recent years, interest in carotenoids has been heighteined by a possible role for certain compounds in prevention of human cancer.

Mathews-Roth reported that carotenoids such as beta-carotene, phytoene and canthaxanthin have been proved to retard the growth of tumor in mice[1]. Beta-carotene also has been proved to be anti-cancer in white rats[2].

The carotenoids are distributed in red or orange fruits and green leafy vegetables. Parsley (*Petroselinum crispum*) is a sort green leafy vegetables which popular in temperate countries, such as Australia. This vegetable belongs to family *Apiaceae*, the native plant of Mediterranean lands. The leaves were used by ancient Greeks and Romans as flavouring and garnish for foods. In Australia it is used as vegetables in soup and most of people just eat itself without cooking.

Parsley contains 0,5 percents essential oils, of which the main component is apiol, an oily, green liquid essential oil. This vegetable also contains carotenoids, the popular natural pigments[3]. However, there is no detailed report the composition of the individual components of such carotenoids.

This paper reports the method for separation of carotenoid components in parsley as well as the identification of the components.

MATERIAL AND METHODS

All chemicals were of analytical reagent grade, solvents of HLPC grade and water was double distilled. Chloroform was washed with double destilled water to remove trace acids and dried over anhydrous sodium sulphate. All solvents were distilled and purged with nitrogen for hydrogen and peroxyde axclusion.

Pigment standards were gifts from Roche Products (Sydney) and purchsed from Sigma Chemicals (St. Louis, Mo). Parsleys were obtained from local retair market in Sydney, Australia.

Analysis by liquid chromatography system were carried out on two column (Radialpak C18, 5 and 10μ, Water, Milford Mass.) connected in series.

The columns were attached to a water U6K injector and 41-mPa pump. The mobile phase contained water, methanol and acetontrile in various potions which filtered and degassed under vacuum before use and maintained at a flow rate of 3 mL min^{-1}. Solvent gradients were regulated with Waters Model 660 Solven Programmer, column effluents were monitored by a Waters Model 490 Programable Multivavelength Detector at 440 nm and recorded on a Waters Data Module.

Extraction and Saponification

Parsley fruit (1kg) extracted by a slight modification of the method described by Zakaria *et al.*,(1979) which involved cutting produce into small pieces and homogenising in aceton for 30 min in a blender with light being excluded by covering all vessel with aluminium foil. The homogenate was purged by nitrogen gas during the process to remove oxygen from the vessels. The homogenate was filtered and the residue re-extracted until all colours removed.

The combined acetone extracts were added to an equal volume of freshly distilled diethyl ether. Water was added and after shaking the layer was re-extracted with diethyl ether until the layer became colourless or clean green colour for green leavy vegetables. The combined organic extracts were washed with water to remove the acetone and died over anhydrous sodium sulphate.

The extracts were saponified to remove the chlorophylls and fats as well as hydrolysing carotenoid esters. A pleminary study on standards and fruit and vegetables extracts established the need for saponification and that the most effective method examined was that described by Curl[4] which was adopted routinely through the study. This involved addition of an equal volume of 20% KOH in methanol to diethyl ether extracts end leaving the mixture to stand at room tei ,perature overnight. The alkali and water soluble materials were removed by washing with water. The organic extracts were dried over sodium sulphate in chloroform and an alliquot (1-25 l) analysed by gradient elution HPLC.

Separation into carotene, monol, diol and polyol group

1. Mixture of carotenoids was chromatographed on column of MgO-hyflo-super-cel (1:1) mixture and eluted with 2-5% acetone in petroleum ether to remove carotene fraction. The polarity of solvent was increased into 10% acetone in petroleum ether to elute monol fraction and a combined diol-polyol fraction was eluted petroleum ether-ethanol (1:1). Elution with 2-5% acetone in petroleum ether to separate carotenes, poor separation was obtained, as a long band formed along the column and there was no clear gap between individual bands.
2. Mixture of carotenoids was chromatographed on column of MgO-hyflo-super-cel (1:1) and eluted with 10% acetone in petroleum to remove fraction of mixture of carotenes and monols. The carotenes and monols were then separated on t.l.c. of silica gel eluted with 10% acetone in petroleum ether. Two clear bands were formed, with the first band was carotene and the second band was monols.
   A combined diol-polyol fraction was washed from the column with petroleum ether-ethanol (1:1). This fraction was re-chromatographed on the same absorbent and eluted with 10% aceton in petroleum ether to give two sub-fractions, with diols of sub fraction 1 polyols of sub-fraction 2.

Separation of individual carotenoids

1. Carotene and monol group
   Carotenes and monols were separated into their individual components with HPLC. Each peak was collected for identification.

2. Diol group

2.1. Chromatographed on t.l.c. silica gel
   Solution of diol group in chloroform was chromatographed on t.l.c. of silica gel and eluted with petroleum ether-ethyl acetate-isopropanol (95:10:5). During developmen, green to blue colours were formed one fraction was still red and easy to separate from the green to blue components. This fraction was scraped off and dissolved in chloroform. When injected in HPLC system gave two peaks with retention times of 173 and 174 mins.

2.2. Chromatographed on alumina column.
   Solution of diol in chloroform was chromatographed on column of alumina and eluted with 35% ethyl acetate in petroleum ether to give to fractions. Fraction 1 gave four peaks and fraction 2 gave 2 peaks when analysed by HPLC. Fraction 1 was then re-chromatographed on alumina and eluted with 10% acetone in petroleum ether to give to sub-fractions. Sub-fraction 1 gave two peaks and sub-fraction 2 gave two peaks too.

3. Polyol group.
   Solution of polyol group in chloroform was chromatographed on alumina and eluted with 20% acetone in petroleum ether to give two fractions. Fraction 1 was a dark, sharp band and gave one peak on HPLC. Fraction was a mixture and in a small amount and was not examined further.

Acetylation of carotenoids

Acetic anhydride (0.1ml) was added to a solution of carotenoids in pyridine (0.1ml). The solution was heated at 40°Cfor 45 min. The reaction mixture was taken up in diethyl ether (10ml). Pyridine and excess acetic anhydride were washed out by shaking with 5 portions of 10ml of water. The ethereal solution was dried over anhydrous sodium sulphate, filtered and the solvent removed under reduced pressure.

Epoxide test

The carotenoids were dissolved in 0.1M HCl in ethanol. The appearance of green or blue colour indicated the presen-ce of epoxy group (Davies, 1976) and hypsochromic shift was measured by spectrophotometer by running the wavelength from 600-350nm.

RESULT AND DISCUSSION

The carotenoids of parsley were separated into carotene, monol, diol and polyol groups. Each group was then separated into individual carotenoids which were examined for identification by chromatographic behaviour, chemical reaction and spectroscopic evidence.

Total Carotenoids

Figure 1 shows the resolution of total parsley carotenoids with gradient elution on two-reserved phase columns (5 and 10μ) with mobile phases 25% water:15% methanol:60% acetonitrile(initial solvent), where the initial solvent was held for 60 min and conversion time from initial to final solvent was 120 min.

It can be seen from the chromatogram that there are at least 34 carotenoid components in parsley with the major components being peaks 1, 9, 10, 20, 22, 23, and 26.

Separation into carotene, Monol, Diol and Polyol Groups

The separation of carotenoids into carotene, monol, diol and polyol groups was carried out using a method modified from those described by Gross *et al.*[5] and Gross (1980).

Gross *et al.*[5] separated the carotenoids of Shamouti orange by column chromatography using a mixture of MgO-hyflo-super-cel (1:1) with the carotene fraction eluted with 2-5% acetone in petroleum ether, monol fraction with 10% aceton in petroleum ether and acombine diol-polyol fraction with petroleum-ethanol (1:1). The diol-polyol fraction was then rechromatographed on the same adsorbent and eluted with 10% aceton in petroleum ether-ethanol (97:3) to give separate diol and polyol sub-fractions.

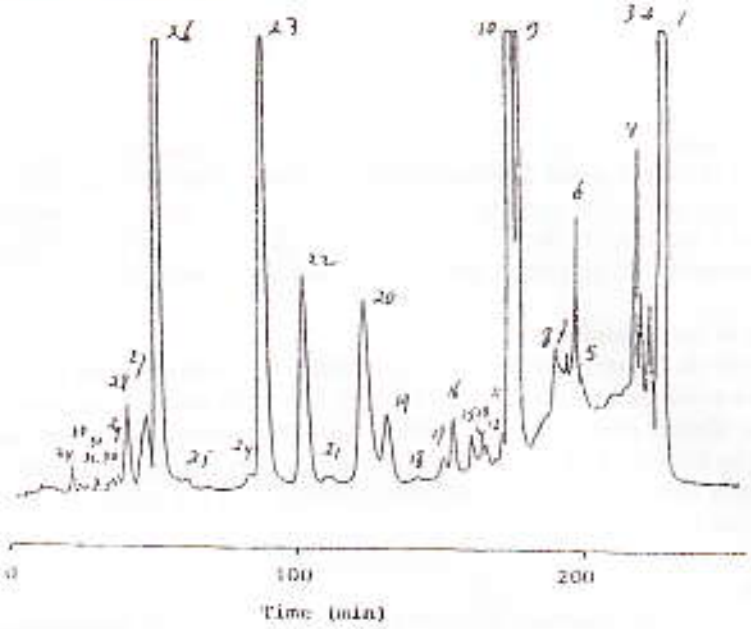

Figure 1. HPLC chromatogram of total carotenoids in parsley.

Gross (1980) separated the various carotenoid fractions of citrus by t.l.c. on silika gel⁵. The carotene fraction was developed with 10% acetone in petroleum ether and other fractions with 30% acetone in petroleum ether.

When parsley extract was chromatographed on a column of MgO-hyflo-super-cel as described by Gross *et al.* (1971) with 5-5% acetone in petroleum ether as eluent to separate caratonea from other groups, poor separation was obtained, as a long band formed along the column and there was no clear gap between individual bands. It was decided to elute the carotenes together with monols using 10% acetone in petroleum ether. This fraction was then separated by t.l.c. on silika gel with 10% aceton in petroleum ether as described by Gross (1980). Two clear bands were formed, with the first band having the same R_f as carotenes and the second band corresponding to monols; R_f values were determined by plate development with ß-carotene and ß-cryptoxantin, respectively.

The two bands were scraped off, extracted with chloroform and after evaporating the solvent, the residue was redissolved in chloroform and analysed on the HPLC gradient system. The chromatograms are shown in figures (2,I) and (2,II) respectively. Comparison of chromatograms with the total carotenoid profile in Figure 1 indicates that peaks 1-4 were carotenes and peaks 5-7 were monols.

For carotenes, examination of the chromatograms obtained before and after group separation shows that peaks 1 was present in both as the mayor peak. However, a slight relative decrease of peak 4 was observed compared to peaks 2 and 3. This indicates that peak 4 was either preferentially lost or decomposed

during the separation procedure. For monols, a better resolution of the peaks was obtained after separation, indicating that some impurities were probably removed during separation. However, one component, i.e.peak 8, disappeared.

A combined diol-polyol was washed from the column with petroleum ether-ethanol (1:1). This fraction was re-chromatographed on the same adsorbent and eluted with 10 % acetone in petroleum ether as described by Gross *et al.* (1991) to give two sub-fractions, containing diols (1) and polyols (2). Sub-fraction 1 was co-chromatographed with standard zeaxanthin on the some adsorbend and eluent. Only one band formed, indicating that sub-fraction 1 was diol conporents. No standart polyol was available to allow comparison of sub-fraction 2, but since carotenoids consist of carotene, monol, diol and polyol groups, sub-fraction 1 (diols) and sub-fraction 2 must be polyols. The chromatograms of sub-fraction 1 (diols) and sub-fraction 2 (polyols), when analysed by HPLC, are shown in Figures (2,III) and (2, IV), respectively. Comparison of the chromatograms with the total carotenoid profile in figure 1 indicates that peaks 9-23 were diols and peaks 26-34 were polyols. It can be seen in Figure (2, III) that a new peak (22a) appeared which overlapped with peak 23. A complex mixture of polyols with retention times at about the same to those of peaks 29-34 was also formed. The appereance of these peaks indicates that some carotenoids decompesed during the separation.

Thus, the separation of parsley carotenoids eith column and t.l.c. suggest that peaks 1-4 are carotenes, peaks 5-8 are monols, peaks 9-25 are diols and peaks 26-34 are polyols.

Separation and Identification of Individual Carotenoids

Carotenes

The carotene group was separated into individual components by gradient elution HPLC with each peak collected. After the solvent was removed, the residu was redissolved in a certain solvent and its absorption maxima measured.

Figure 2. HPLC chromatogram of carotenoid groups in parsley.
I = carotenes; II = monols; III = diols; IV = polyols.

Peak 1 had absorption maxima at 476, 449, 427 nm in petroleum ether, which is similar to Schwieter *et al.* (1965) reported that ß-carotene absorbed at 478, 451, 421 nm in petroleum ether. No colour change was observed when treated with solution of 0.1M HCl in ethanol indicating that it did not contain epoxy group. It's chemical ionisation mass spectrum showed a molecular ion (MH^+) at 537 indicating a molecular weight of 536. This corresponds to the molecular weight of ß-carotene. To confirm the identification, peak 1 was mixed with standard ß-catotenes and injected into the HPLC. The cromatogram gave a single peak with the retention time at 224 min.

Peak 2 had absorption maxima at 438, 457, 439 nm in chloroform, 473, 446, 428 nm in hexane and 473, 444, 434 in ethanol. Merlini and Cardilo reported that Ò-carotene absorbed at 485, 457, 432 nm in chloroform[7]. Valadon and Mummery (1967) found that Ó-carotene absorbed at 472, 442, 420 nm in hexane, and Hager and Stransky (1970a) reported Ó-caroten absorbed at 473, 444, 423 nm in ethanol. The absorption maxima of peak 2 in three solvents are thus similar to those reported in literature for Ó-carotene. No green or blue color was observed upon treatment with 0.1M hydrochloric acid in ethanol. This indicates that it is not an epoxide. Peak 2 was then co-chromatographed on HPLC with standard Ó-carotene, and a single peak was abtained.

Peak 3 had absorption maxima at 470, 440 nm in petroleum ether and 473, 446, 428 nm in hexane. The absorption maxima were similar to to those of prolycopene, a poly-cis-isomer of lycopene with unknown structure reported by Jungalwala and Cama (1962) which absorbed at 468, 440 nm in petroleum ether. The presence of prolycopene is usually associated with lycopene (Morgan, 1967; Johjima and Ogura, 1983). However, the presence of lycopene was not detected. It is not an epoxide, since it did not give green or blue colour upon treatment with 0.1M HCl in ethanol. Hence, no identity was assigned to it.

Peak 4 had absorption maxima at 425, 399, 379 nm in petroleum ether and at 425, 400, 379 nm in hexane. The absorption maxima were similar to those of ç-carotene; Jungalwala and Cama (1962) reported that ç-carotene absorbed at 425, 400, 380 nm in petroleum ether, Valadon and Mummery at 425, 400, 380 nm in hexane[8] and Davis *et al.* (1966) at 425, 400, 380 nm in hexane[9], whereas Liaaen-Jensen (1965) reported that ç-carotene absorbed at 418, 398, 376 in petroleum ether[10]. It did not give green or blue colour when treated with 0.1M HCl in ethanol indicating it is not an epoxide. Comparison of absorption maxima of peak 4 with those reported in literature, led to its identify being ç-carotene.

Monols

Monol were also separated into individual components by gradient elution HPLC and collected.

Peak 6 had absorption maxima at 478, 448, 425 nm in ethanol similar to those of ß-cryptoxanthin with reported absorption maxima at 473, 449, 428 nm (Hager and Stransky, 1970b) and 477, 449, 426 nm[11] in ethanol. When peak 6 was co-chromatographed with standard ß-cryptoxanthin, a single peak

appeared. The absorption maxima and cromatographic behaviour suggest that peak 6 was ß-cryptoxanthin.

Peak 5 had absorption maxima at 465, 439, 425 nm in ethanol. It did not give green or blue colour when treated with 0.1M HCl in ethanol indicating it did not contain epoxy group. No standard material was available to allow comparison of chromatographic behaviour and no comparable spectral data were found to enable identification of peak 5, which therefore remains unknown.

Peak 7 had absorption maxima at 470, 440, 424 nm in ethanol which was similar to that of OH-Ó-carotene which has been reported as 473, 444, 418 nm by Gross *et al.* (1971). No green or blue colour was observed upon treatment with 0.1M HCL in ethanol indicating it is not an epoxide. Figure (2, II) shows that peak 7 is more polar than peak (ß-cryptoxanthin) and figure (2, I) shows that Ó-carotene (peak 2) is more polar than ß-carotene (peak I). By analogy, it is suggested that peak 7 is OH-Ó-carotene.

Diols

The separation of diols into individual components by HPLC shows 6 main component, i.e. peaks 9, 10, 19, 20, 22 and 21. Each peak was collected from the HPLC and examined for identification. Visible spectra and epoxy group test results were able to be obtained but, because of small amounts of material available, it was not possible to obtain other spectra.

To obtain a larger amount of diols, compounds were separated by t.l.c. on silica gel G.The mixture was deve- loped by a mixture of petroleum ether-ethyl acetareisopropanol (95:10:5) as described by Gross *et al.*[5]. During development, most compounds decomposed into green to blue product. One fraction was stable and was easily separated from the decomposed fractions, which moved very slowly on the plate.

When this fraction was re-analysed by HLPC, two peaks were obtained with retention times at 173 and 174 min, which corresponded to peaks 9 and 10, respectively. The retention times were similar to those of lutein and zeaxanthin standards.

To try and better separate peaks 9 and 10, the mixture was chromarographed by t.l.c. on silica gel and developed with mixtures of petroleum ether-ethyl acetate and petroleum ether-acetone in most proportion, but none gave satisfactory separation. The best solvent used was 40% acetone in petroleum ether, which gave incomplete separation into a broad band which was dark on the top and bottom and light in the middle. Chromatograms on the top and bottom fractions gave single peaks corresponding to peaks 10 and 9, respectively.

Peaks 9 had absorption maxima at 486, 455, 438 nm in chloroform, 467, 441, 418 nm in petroleum ather and 478, 448, 425 nm in ethanol. The absorption maxima were similar to those at 488, 459, 434 nm reported for zeaxanthin by Stransky and Hager in chloroform, at 475, 448, 424 nm in petroleum ather as reported by Aasen and Liaaen-Jensen (1966) and at 478, 451, 423 in ethanol as reported by Gross[4]. No colour change was observed upon

treatment with 0.1 M HCL in ethanol indicating that it did not contain epoxy group.

Peaks 10 had absorption maxima at 480, 451, 431 nm in chloroform, 473, 442, 422 nm in petroleum ether and 469, 442, 420 nm in ethanol. The absorption maxima were similar to those reported by Stobart *et al*, (1967)[12] for lutein at 483, 454, 428 nm and 474, 444, 420 nm in chloroform and petroleum ether, respectively. The studies of Jungalwala and Cama (1962) showed that lutein absorbed at 475, 445, 420 nm in ethanol[13]. It gave negative reaction with 0.1 M HCL in ethanol indicating the absence of an epoxy group.

The chemical ionization mass spectra of component was similar. They both gave peaks at 569 (MH^+), 551 (MH^+-18), 533 (MH^+-36). The molecular ion at 569 indicates that the molecular weight of peak 9 and 10 is 568 which corresponds to the molecular weight of zeaxanthin and lutein. Fragmentation to give peaks at 551 and 533 indicates that both component has 2 hydroxyl group. However, neither component gave inter-pretable n.m.r. spectra.

The mixture of peaks 9 and 10 was esterified with acetic anhydride in pyridine. It was hoped that the ester mixture will be easily separated and gave better n.m.r. spectra. The chromatogram of esters (Figure 3, II) gave two peaks (9a and 10a). It can be seen from Figure (3,II) that the resolution of esters is better than that of the parent compounds.

Esters 9a and 10a were easily separated on silica gel column chromatography when eluted with 10% acetone in petroleum ether with the first fraction being 10%. Chemical ionization mass spectra of peak 9a gave molecular ion (MH^+) at 653 corresponding to the molecular weight of zeaxanthin diacetate of 652. Mass spectra of peak 10a gave a peak at 593, corresponding to (MH^+-60). It seems that peak 10a did not give a molecular ion, but the peak at 493 came from 653-60, i.e. loss of 1 acetate group.

On the basis of visible spectra, retention times and esterification, peaks 9 and 10 were identified as zeaxanthin and lutein, respectively. For structural confirmation, n.m.r. spectra of peaks 9a was compared to that of zeaxanthin diacetate which was synthesized from standard zeaxanthin. Figure 4 shows the n.m.r. spectra of peak 9b and zeaxanthin diacetate standard. Only peaks with chemical shift between 0.85-2.40 were compared, since these peaks are very clear and easy to compare. It can be seen that peak 9a and zeaxanthin also contained impurities. Peaks with chemical shift at 0.87, 0.88, 0.89, 1.11, 1.26, 1.56, 1.58, 1.01, 1.72, 1.96 and 1.97 are present in both peak 9a and zeaxanthin diacetate. However, peak 9a contaminated with impurities with chemical shitf at 1.00, 1.43, 1.90, 2.04, 2.17 and 2.35. Many attempts were made to remove the impurities, but were unsuccessful. The n.m.r. supported the view that peak 9a was zeaxanthin diacetate.

Since peak 9 was conssidered to be zeaxanthin, peak 10 should be lutein. For further confirmation, peak 10 was injected with standard lutein and both appeared as a single peak on the HPLC chromatogram. No n.m.r. comparison was made, as insufficient standard lutein was available for n.m.r. spectral analysis or ester synthesis.

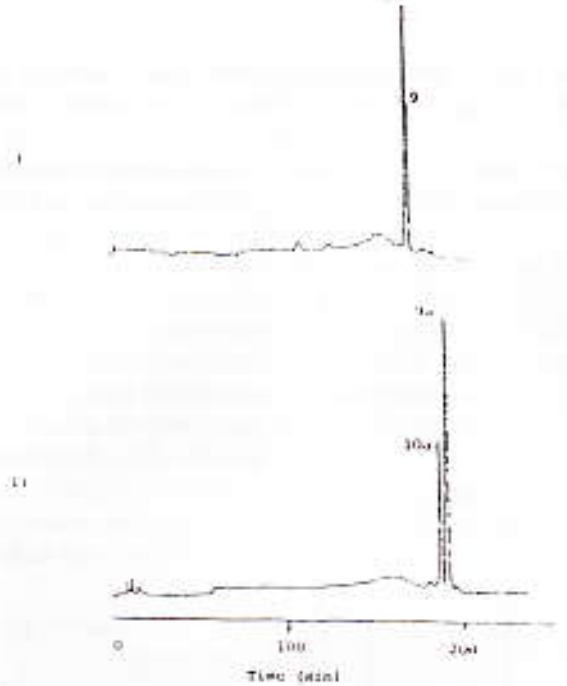

Figure 3. The chromatogram of a mixture of peaks 9 and 10 and their esters.

9 = zeaxanthin; 10 = lutein;
9a = zeaxanthin diacetate;
10a = lutein diacetate.

In an attempt to identify the remaining diol component which decomposed during t.l.c. development, the miture was re-applied on t.l.c. plates of silica gel G, but eluted with petroleum-ethyl acetate-isopropanol (95:10:5) which contained 1% butylated hydroxy toluene (B.H.T). It was hoped that B.H.T will protect the carotenoids from oxidation, but again the mixture decomposed to be green-blue product. The mixture was then developed

With the same solvent containing 1% dimenthylamine which may protect the carotenoids from trace acids thet could be present in the solvent, but no different results were obtained.

The mixture was further choramatographed on silica gel H Column and eluted with petroleum-ethyl acetate-isopropanol (95:10:5), however, the green-blue products were formed instantly at the top of the column. It seems that decomposition or oxidation of the mixture was not caused by the solvent but affected by silica gel. This was similar to studies of Strain et al. (1967) and Schaltegger (1965) which reported the conversion of epoxy substituents into furanoid groups when chromatographed on siliceous adsorbents such as silica gel, celite, kieselguhr and florisil.

Diols were then choramatographed on alumina and eluted with 35% ethyl acetate in petroleum ether to give two fractions which were analysed by HPLC. Fraction 1 consisted of peaks 9, 10, 19 and 20, where as fractions 2 was a mixture of peaks 22 and 23 (figure 5).

Fraction 1 was re-chomatographed on alumina and eluted with 10% acetone in petroleum ether to give two subfractions. Sub-fractions 1 consisted of peaks 9 and 20 and sub-fractions 2 was a mixture af peaks 19 and 20 when analyzed by HPLC. Sub-fractions 2 could not be better separated on alumina column. Solvents of petroleum ether-acetone and petroleum ether ethyl acetate in most proportion were used, but none gave improved separation.

Peak 19 or peak 20 separated by HPLC produced blue colour upon treatment with 0.1 M HCL in ethanol indicating that both were epoxides. Davies (1976)[9] demonstrated that 5,6-monoepoxides are characterised by a hypsochromic shift some 40 nm. Absorption maxima of peaks 19 and 20 in ethanol and 0.1 M HCL in ethanol did not exhibit such shifts. This indicates that both peaks did not contain 5,6-monoepoxides or 5,6,5',6'-diepoxides. As a consequence,peaks 19 and 20 should be 5,8-monofuranoids or 5,8,5',8'-difuranoids.

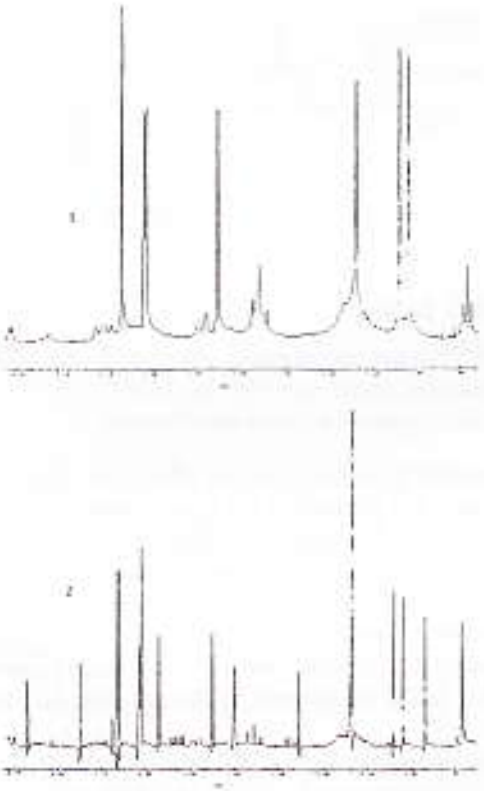

Figure 4. NMR spectra of standard zeaxanthin diacetate (1) and zeaxanthin diacetate synthesized from zeaxanthin isolated from parsley (peak 9a) (2). (Chemical shift between 0.85 - 2.40 ppm).

Peaks 19 and 20 had absorption maxima at 458,432 nm and 461, 438 nm in chloroform, respectively. In petroleum ether, peak 19 absorbed at 453, 431 nm, while peak 20 at 449, 432 nm. In ethanol, peaks 19 and 20 had absorption maxima at 452, 425, 405 nm and 444, 426 408 nm. It can be seen that both peaks had similar absorption maxima in the various solvents.

Absorption maxima of flavoxanthin and chrysanthemaxanthin reported in many published papers were similar to those of peaks 19 and 20. Davies (1976) reported that chrysanthemaxanthin had absorption maxima at 459, 430 nm in chloroform, while goodwin (1955) found that flavoxanthin had absorption maxima at 459, 430 nm in chloroform. Studies of Karrer and Jucker (1945) showed that flavoxanthin had maxima at 450, 421 nm in petroleum ether[13]. Jungalwala and Cama (1962) reported that flavoxanthin and chrysanthemaxanthin both had absorption maxima at 448, 421, 400 nm in ethanol. While Valadon and Mummery (1967) reported absorption maxima at 447, 420, 400 and 446, 420, 400 nm in ethanol, respectively. Valadon and Mummery (1967) and Jungalwala and Cama (1962) reported the difficulty of separating the isomeric flavoxanthin and chrysanthemaxanthin.

Absorption maxima, chemical reaction and chromatograp- hic behaviour, suggest that peak 19 is flavoxanthin and peak 20 is chrysanthemaxanthin.

Fraction 2 was chromatographed on alumina and eluted with 20% acetone in petroleum ether to give 2 sub-fraction.The retention times on HPLC showed that sub-fractions 1 corresponded to peak 22 and sub-fration 2 to peak 23.

Peak 22 produced a greenish-blue colour upon treatment with 0.1 M HCL in ethanol and colour turned to blue after 1 hr and gave absorption maxima at 427, 407, 379 nm. In ethaol, it had absorption maxima at 468, 442, 420 nm. Absorption maxima in ethanol and 0.1 M HCL in ethanol, showed a hysochromic shift of 41 nm indicating the presence of 2-epoxy groups (Davies, 1976). In cloroform and petroleum ether, peak 22 had absorption maxima at 483, 455, 432 nm and 468, 446, 422 nm. The absorption maxima were similar to those of violaxanthin which were reported as 467, 438, 418, nm in ethanol (Gross, 1971), 466, 422, 418 nm in petroleum ether (Davies, 1976) and 482, 452, 423 nm in chloroform (Stobart *et al.*, 1967). Absorption maxima and the epoxide test showed that peak 22 correponds to violaxanthin.

The chemical ionization mass spectra of peak 22 showed the presence of peaks at 565 and 521. Violaxanthin should give molecular ion peak (MH+) at 601. However, peak 565 could come from (607-36), since violaxanthin posseses two hydroxil groups and fragment to lose 2 molecules of water.

Vetter *et al.* reported that carotenoid with hydroxil groups at C-3 and an epoxy group at C-5,6 will give peak at (M-80)[14]. Although the mass Spectra did not show a moleculer ion, the fragmentation suggest that peak 22 is violaxanthin. It was thought that the acetate of peak 22 may give a mole-culer ion. The chromatogram of acetylated peak 22 gave a single peak with retention time at 172 min. However, the mass spectrum showed peak at 654, instead of 685,the mole-culer ion of violaxanthin diacetate and fragmented to give peaks at 642, 630, 621 and 604 nm which are difficult to interpret.

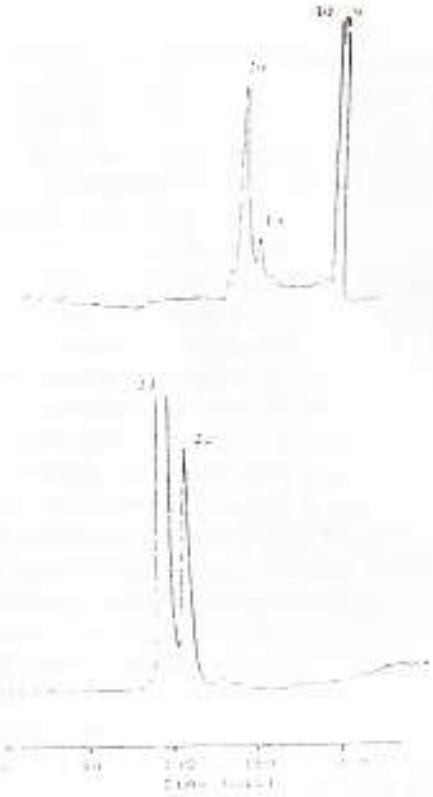

Figure 5. HPLC chromatograms of two fractions of diols separated on alumina column, eluted with 35% ethyl acetate in petroleum ether.

Sub-fraction 2 which corresponds to peak 23 had absorption maxima at 448, 423, 399 nm ethanol. It gave a blue colour with 0.1 M HCL in ethanol which had absorption maxima at 426, 407, 380 nm. A hyprochromic shift of 22 nm indicated the presence of a 5,6-monoepoxoxide. In chloroform and petroleum ether, peak 23 had absorption maxima at 453, 427, 406 nm and 443, 422, 402 nm, respectively. The absorption maxima were similar to those of luteoxanthin, reported as 458, 433, 410 nm in chloroform (Foppen, 1971), 446, 420, 396, nm in ethanol (Hager and Stansky, 1970a) and 448, 422, 401 nm in petroleum ether (Foppen, 1971). The chemical ionization mass spectra had peaks at 601 (MH^+), 583 ($MH\pm18$), 565 ($MH\pm36$). The peak at 601 indicated that the molecular weight was 600, corresponding to luteoxanthin. The fragmentation clearly gave peakss at 583, 565 which means loss of 2 molecules of water. This indicates that peak 23 has two hydroxyl groups. Based on the visible and mass spectra peak 23 was identified as luteoxanthin.

Polyhydroxy carotenes (Polyols)

Figure (2,IV) shows that polyols only gave three peaks. When chromatographed on alumina and eluted with 20% acetone in petroleum ether, 2 fraction were formed. The first fraction was a dark, sharp band which when chromatographed on HPLC corresponded to peak 26. The second fraction was

a light, broad band which when injected on HLPC gave many small peaks and was not examined further.

The visible spectra of peak 26 showed absorption maxima at 476, 446, 429 nm in chloroform, 464, 436, 424 nm in petroleum ether and 468, 442, 420 nm in ethanol. When treated with 0.1 M HCL in ethanol, a green colour was formed and had absorption maxima at 442, 422, 401 nm. A hypsochromic shift of 19-26 nm indicated the presence of a monopoxide. The absorption maxima were similar to those of neoxanthin reported as 467, 442, 418, nm (Davies, 1976) and 465, 435, 410 nm in petroleum ether (Donahue *et al.*, 1966), 477, 449, 422 nm in chloroform (Foppen, 1971) and 467, 438, 415 nm in ethanol[17] . Chemical ionization mass spectra gave peaks at 577, 559 (M-18), 541 (M-18-18), 523 (M-18-18-18). The peak at 577 was an unexpected peak, since it has a molecular weight of 600 and the parent ion peak would be 601. However, the fragmentation from peak 577 indicated the presence of three hydroxyl groups.

Mass spectral studies of cholnoky *et al.* (1969) showed that neoxanthin fragmented to give molecules of water indicating the presence of 3 hydroxyl groups. The appearance of peak 577 could be caused by the rupture of the molecule due to extreme lability of neoxanthin.

It was though that the ester of peak 26 may give more interpretable mass spectra. Therefore, it was then acetylated with acetic anhydride in pyridine. Acetylation for 30 min gave four peaks (26, 26a, 26b and 26c) on HPLC chromatogram with retention times of 49, 156, 167 and 178 min, respectively (Figure 7).

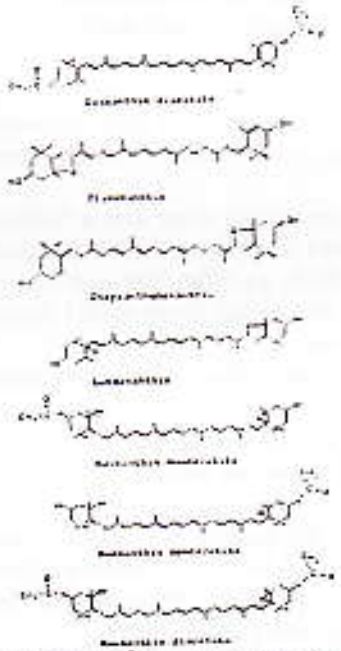

Figure 6. Structure of some carotenoids in parsley and their derivatives.

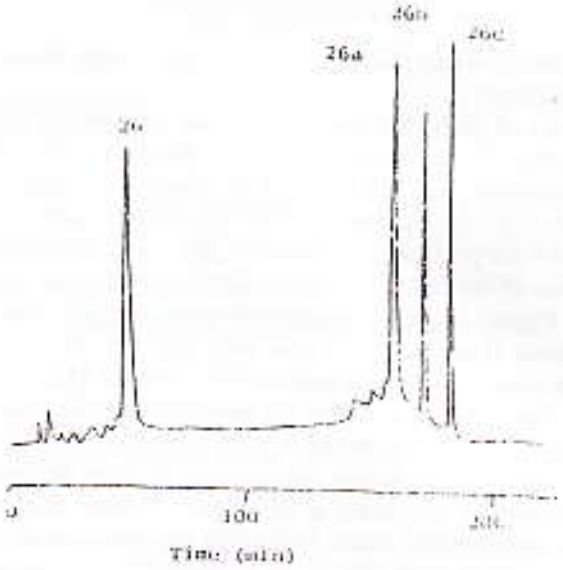

Figure 7. **HPLC Chromatogram of a miture of peak 26 and its acetates after acetylating for 30 min.**

When acetylated for 45 min, only peak 26c was observed. This indicated that only two hidroxyl groups were acetylated, i.e. hydroxyl groups at C-3 and C-3'. The apperance of peak 26a indicated the incompleteness of reaction. Peaks 26a and 26b were monoacetate. Peak 26a may be neoxanthin monoacetate and peak 26b, monoacetate or vice versa and peak 26c was neoanthin diacetate .Peak 26a and 26b cannot be precisely described, because they were not issolated and examined.

Hydroxyl group at C-5 was not acetylated, since it is a tertiary hydroxyl group. Aasen and Liaaen-Jensen reported that primary and secondary hydroxyl groups are acetylated by acetic anhydride pyridin, but not tertiary hydroyl group. If the group at C-5 had been acetylated, there would have been more than three ester peaks on chromatogram,

When acetylated for 30 min, since more than two mono- acetates would have been formed.

The mass spectral studies of peak 26c gave peaks at 577, 599, 541, 523 and 403. The unexpected peak at 577 still appeared instead of 785, the molecular ion (MH^+)
of neoxanthin diacetate. it seems that the peak at 577 was not the molecular ion peak, since it appeared in peak 26 and its acetate spectra. However, the fragmentation from peak at 577 to give peak at 559, indicated that diacetate of peak 26 contained 1 hydroxyl groups were acetylated.

The evidence of absorption maxima, epoxy group test, acetylation and mass spectral fragmentation supported that peak 26 was neoxanthin.

CONCLUSION

HPLC analysis of parsley carotenoids using the gradient system showed that at least 34 components were present. Separation with open column and t.l.c. indicated the pre sence of carotenes, monols, diols and polyols. Twelve components were positively identified -ß-, Ó-, Ç-carotenes, ß-cryptoxanthin, OH-Ó-carotene, zeaxanthin, lutein, flavoxanthin, chrysanthemaxanthin, violaxanthin, luteoxanthin, and neoxanthin based on chemical reaction, chromatographic behavior and spectroscopic evidence. The presence of ß-carotene in parsley was reported by Nutting *et al.* (1970), but other components have not been previously reported.

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