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Analytical Methods Section

Chromatographic investigation of carotenoids, sugars and organic acids from *Diospyros kaki* fruits

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A study was conducted to separate and determine carotenoid-type pigments, sugars and organic acids in persimmon fruits using high-performance liquid chromatography (HPLC) under isocratic conditions. The carotenoids were identified as *cis*-mutatoxanthin, antheroxanthin, zeaxanthin, neolutein, cryptoxanthins, α-carotene and β-carotene and fatty acid esters of cryptoxanthin and zeaxanthin. The fruits were found to be an excellent source of vitamin A: 1 g of edible portion provides about 54 IU of this vitamin. With respect to soluble sugars, the fruit contained mainly glucose, fructose and an unidentified oligosaccharide, but no sucrose could be detected in both unripe and ripe fruits. Ion-pair HPLC allowed the separation and quantification of malic, isocitric, citric, ascorbic, fumaric and gallic acids, with malic acid being predominant. When fully ripe, metabolic processes led to a considerable loss in soluble sugars and organic acids in the fruit.

INTRODUCTION

The attractive orange colour, sweet taste and fleshy texture of persimmon (date plum) fruits (*Diospyros kaki* L.) make them of special interest to food scientists. The ripe, sweet and non-astringent fruits can be used as a sweetening ingredient in baked products and fruity ice creams and as a material for other products such as jams, jellies, nectars, etc. Dried persimmon is a very common product in Japan and the US. Raw and processed persimmon appear to be quite important commercial products, since considerable quantities are marketed each year in EC markets.

Despite the high number of papers published on the general chemical composition, quality attributes and response to technological processes of this type of sub-tropical fruit more details are still needed on individual components such as carotenoids, sugars and organic acids, which are important quality indices. The analysis of these metabolites has been carried out either by unspecific titremetric and colorimetric methods or by time-consuming, unreliable chromatographic methods. Hirai and Yamazaki (1983) used thin-layer chromatography (TLC) and gas chromatography (GC) for the analysis of soluble sugars. Qualitative and quantitative TLC determination of free organic acids was achieved by Tsuyuki and Abe (1986). The first separation of the carotenoids of fruit pigment was performed on open columns (OC) following conventional procedures (Curl, 1960; Brossard & Mackinney, 1963).

Recently, due to such advantages as rapidity, reliability, sensitivity and cost-effectiveness, high-performance liquid chromatography (HPLC) has been of increasing interest in the analysis of food constituents (Macrae, 1982). Apart from the studies of Philip and Chen (1988*a*,*b*) and Homnava *et al.* (1990), in which fruit pigments were initially fractionated on alumina columns and then separated using gradient HPLC systems, this technique has not been used for the determination of soluble sugars and organic acids of persimmon fruit. In previous work, the present authors developed a simple and rapid HPLC method for the determination of fatty acid esters of carotenoids (Czinkotai *et al.*, 1989). The elution system of the latter method has also been modified to suit the analysis of unesterified xanthophylls and hydrocarbons (Daood *et al.*, 1989). The objective of the present study was the application of one-step rapid HPLC procedures for the determination of carotenoids, soluble sugars and organic acids from persimmon fruit.

MATERIALS AND METHODS

Materials

Round-shaped, orange-coloured *Diospyros kaki* fruits (unknown cultivar, imported from Albania) were purchased from a local market. The fruits were not fully ripe at the time of purchase. One-half of the material purchased was stored under ambient conditions in a sealed nylon bag for 1 week to permit ripening. The other half was packed in the same way and stored at −20°C until analyses were performed.

Methods of analysis

Carotenoid-type pigments were extracted from fruit samples with 2 : 1 (v/v) carbon-tetrachloride/methanol, as described previously (Daood *et al.*, 1987). The extracted pigments were saponified by refluxing with 10% methanolic potassium hydroxide for 30 min. Saponified carotenoids were then extracted using diethyl ether, washed three times with distilled water, dried over anhydrous sodium sulphate and evaporated under vacuum at 30°C. The organic acids and sugars were extracted following the method of Biacs *et al.* (1988) using 2% metaphosphoric acid solution.

HPLC separation of the different metabolites was carried out using a Beckman series liquid chromatograph equipped with a Model 114 pump, a Model 340 organizer fitted with a 20-μl sample loop, a Model 165 variable-wavelength detector (for carotenoids and organic acids) and an RI detector (for sugars). The detector signal was electronically integrated by a Shimadzu C-R3A integrator. Sugars were separated on Carbopack (Biorad) 300 × 7 mm i.d. column eluted with doubly distilled water at a flow rate of 1·5 ml min^{-1} (Valverde *et al.*, 1985).

Carotenoid pigments were chromatographed on a Chromasil C-18 column eluted with a mixture of acetonitrile/2-propanol/water (39 : 57 : 4, v/v/v) at a flow rate of 1 ml min^{-1}. The separated components were detected at 438 nm. The chromatographic separation of organic acids was carried out under ion-pair conditions using a Lichosorb C-18 column eluted with potassium-phosphate (1M)/methanol/tetrabutyl-ammonium-hydroxide (97 : 3 : 0·5, v/v/v). Detection of separated acids was carried out at 220 nm.

Identification of the individual components of the carotenoid fraction of the fruit was based on chromatographic behaviour on TLC plates using two different mobile phases: (1) petroleum-ether/acetone (95 : 5, v/v); (2) petroleum-ether/benzene/acetone (70 : 20 : 10, v/v/v) as described by Philip (1973). Visible absorption spectra were compared with authentic and pure standards (Buckle & Rahman, 1979), and specific chemical reactions such as acetylation with acetic anhydride and reaction with hydrochloric acid gas were carried out to confirm the presence of hydroxy and epoxy functions, respectively (Padula & Rodriguez-Amaya, 1986). Standard pigments (Sigma) were used for the quantification of γ-carotenes and β-carotenes, whereas β-cryptoxanthin was quantitatively estimated by using an authentic standard prepared from mandarin peel (Philip, 1973). The vitamin A value was calculated according to the NAS–NRC (1980) ratio of 0·6 μg of β-carotene or 1·2 μg of cryptoxanthin to 1 IU. Identification and quantification of sugars and organic acids were carried out by using standards (Fluka, Switzerland). The absorption spectra of the individual organic acids were also scanned and compared with those of the standards.

RESULTS AND DISCUSSION

Carotenoid composition and vitamin A value

TLC separation of carotenoids in the unsaponified extract indicated that most of them are of epiphasic nature. The major components travelling near the solvent front were tentatively identified as carotenes and fatty acid esters of β-cryptoxanthin and zeaxanthin. In addition to these pigments, four bands eluted with lower R_f values (near the origin). According to their position on the plate, spectral characteristics and response to different chemical reactions, they were identified as *cis*-mutatoxanthin, antheroxanthin, zeaxanthin and monoesters of some diol xanthophylls. The yellow–orange band having a R_f value of about 0·5 represented unesterified β-cryptoxanthin. Fatty acid monoesters and diesters of xanthophylls disappeared completely after saponification, which resulted in a marked accumulation of the free xanthophylls. Better separation of the saponified extract was achieved by using a solvent system of higher polarity petroleum-ether/benzene/acetone (70 : 20 : 10, v/v/v).

Figure 1 shows typical chromatograms of carotenoids in the unsaponified and saponified extract of fruit pigment. The order of elution of compounds was as expected from the general pattern of reversed-phase chromatographic separation, as described by Simpson (1976): the greater the compound polarity, the shorter its retention time. The first band on the TLC plate (mixed carotenes) could be fractionated on the HPLC column into two constituents. By their chromatographic behaviour and typical absorption spectra, compared with those of standard materials, they were identified as γ-carotene and β-carotene. When chromatographed on a C-18 column, fatty acid esters of cryptoxanthin and zeaxanthin eluted after β-carotene. Their sorption in the C-18 phase of the column and, consequently, their retention time seemed to be proportional to the fatty acid moiety of the molecules. Therefore, carotenoids esterified with long-chain fatty

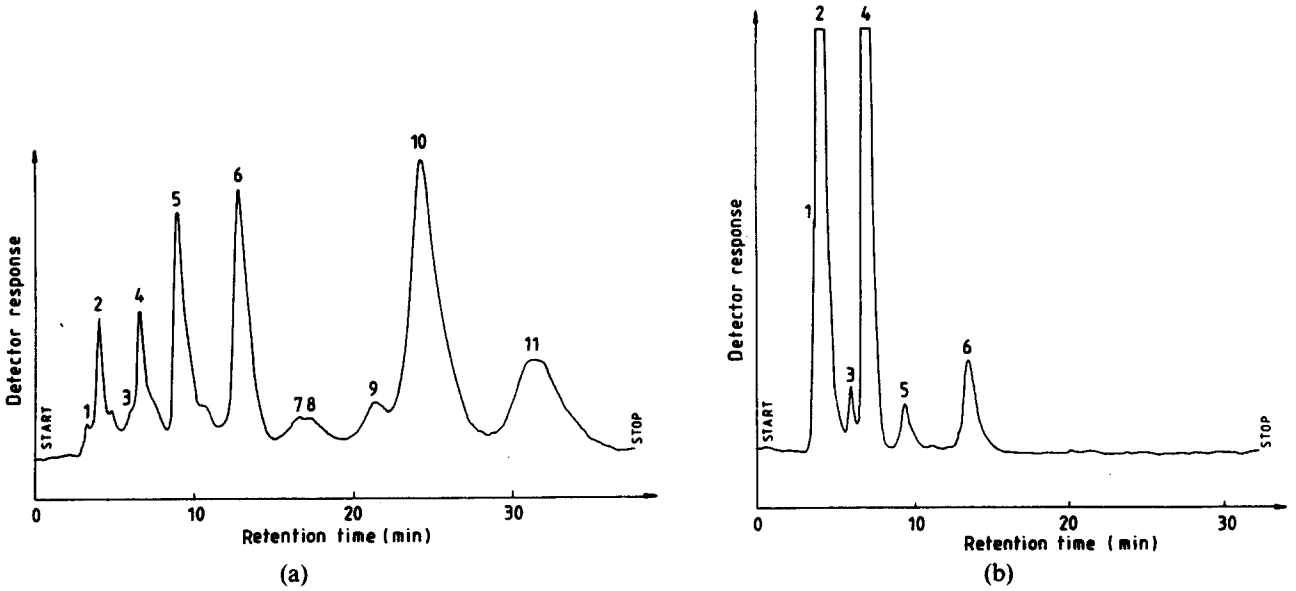

Fig. 1. HPLC profile of unsaponified (a) and saponified (b) extract of persimmon fruit on Chromsil C-18 column eluted with acetonitrile/2-isopropanol/water (39 : 57 : 4, v/v/v). Flow rate was 1 ml/min. Detection was at 438 nm. 1—Mutatoxanthin, 2—zeaxanthin, 3—α-cryptoxanthin, 4—β-cryptoxanthin, 5—γ-carotene, 6—β-carotene, 7,8—unidentified, 9—α-cryptoxanthin ester, 10—β-cryptoxanthin ester, 11—zeaxanthin diester.

acids eluted with long retention times and appeared as broad peaks. Furthermore, some of these compounds may associate strongly with the C-18 phase and need a mobile phase of decreasing polarity (gradient elution) for complete resolution (Philip & Chen 1988*a*,*b*). This held true for zeaxanthin diesters in the present study, as the total area of zeaxanthin diesters from the unsaponified extract was much smaller relative to that of the unesterified form present in the saponified extract (Figs 1(b) and 2). As a result of saponification, a substantial variation was also observed on the peak areas of both γ-carotene and β-carotene. This variation means that monoesters of some xanthophylls elute together with γ-carotene and β-carotene. Thus, carotenoids having vitamin A activity were quantified only in the saponified extracts. To obtain a conclusive separation and identification of the constituents of the saponified extract, a mobile phase consisting of acetonitrile/methanol/ethyl-acetate (53 : 40 : 7, v/v/v) was applied. A typical chromatogram of this separation is shown in Fig. 2. *cis*-Mutatoxanthin, antheroxanthin, zeaxanthin, neolutein, α-crypotxanthin and β-crypto-

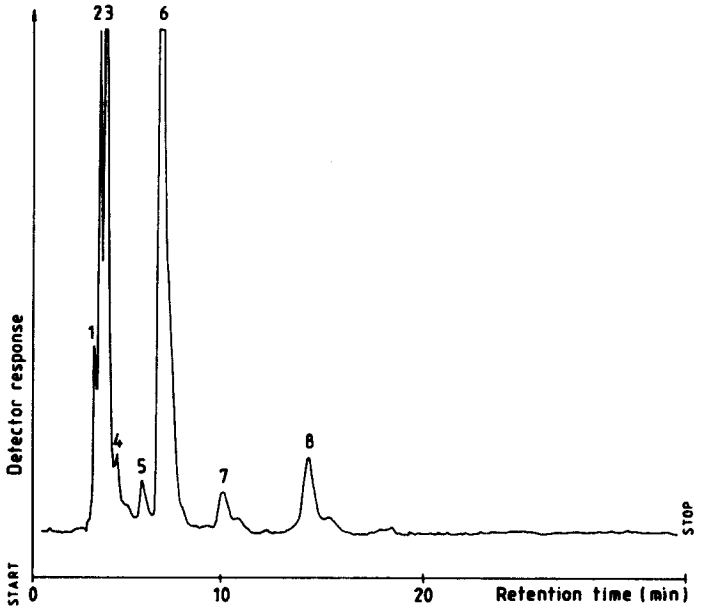

Fig. 2. HPLC profile of saponified extract of persimmon fruit of chromsil C-18 column eluted with acetonitrile/methanol/ethyl-acetate (50 : 45 : 5, v/v/v). Detection was at 438 nm. (1—*cis*-Mutatoxanthin, 2—antheroxanthin 3—zeaxanthin, 4—neolutein, 5—α-cryptoxanthin, 6—β-cryptoxanthin, 7—γ-carotene, 8—β-carotene.

xanthin could be identified as xanthophylls of persimmon fruit. The results are somewhat similar to those obtained by Philip and Chen (1988*a*).

From the data given in Table 1, it is evident that provitamin A carotenoids (β-cryptoxanthin, γ-carotene and β-carotene) are predominant among other constituents of fruit pigments. Thus, the fruit can be considered as a rich source of vitamin A. One edible gram of fruit can supply 54 IU of vitamin A. This value is much higher than those reported for other fruits, such as apple (Cecchi & Rodriguez-Amaya, 1981), grapefruit, orange, peach, strawberry (Bureau & Bushway, 1986) and guavas (Padula & Rodrigues-Amaya, 1986).

Soluble sugars

Persimmon fruit was found to contain mainly glucose, fructose and some unidentified oligosaccharides. As shown in Table 2 a ratio of 1 : 1 was determined for glucose : fructose, and this remained during the final stages of ripening. This ratio is evidence of the presence of very active invertase, which is thought to be in contact with its substrate even at the early stage of ripeness. In studies by Hirai *et al.* (1986) and Zheng and Sugiura (1990), the activity of invertase has been found to increase in accordance with progressive ripening. The changes in sugar composition of the fruits have also been related to invertase activity. Therefore, it is believed that the presence of such an active enzyme may explain the absence of sucrose in both the unripe and the ripe samples.

Once the fruit has ripened, it has exhausted considerable amounts of its easily assimilable carbohydrates. The rapid decrease in the sugar content of the fruit, as a result of increasing respiration rate, may explain why ripe persimmon should be served or processed as soon as possible. Nevertheless, the concentration of sugars retained by ripe fruit was high enough (210 mg g^{-1}) to make it very delicious and desirable when consumed fresh or processed.

Table 1. Carotenoid composition and content of date-plum fruit at the last stage of ripeness

Carotenoid pigments	Relative percentage[a]	Provitamin A activity[b]	
		(μg g^{-1})	(IU g^{-1})
cis-Mutatoxanthin	0·7	—	—
Zeaxanthin	3·0	—	—
α-Cryptoxanthin	trace	trace	trace
β-Cryptoxanthin	4·3	2·7±0·2	2·25±0·17
γ-Carotene	11·8	8·2±0·31	6·86±0·27
β-Carotene	18·7	11·6±0·70	19·33±1·16
Unidentified esters	3·8	—	—
α-Cryptoxanthin ester	4·6	3·2±0·28	2·67±0·23
β-Cryptoxanthin ester	35·8	27·8±1·42	23·17±1·18
Zeaxanthin diesters	16·1	—	—
Unidentified carotenoids	1·0	—	—
Total	—	53·5±2·91	54·3±3·01

[a] Calculated from the area of each peak to the total area.
[b] Each value represents the mean of three determinations ± standard deviation.

Organic acids

Figure 3 shows an HPLC profile of organic acids extracted from unripe fruit. Of the identified acids, malic, citric, isocitric and ascorbic acids were predominant. The maximum concentration estimated for ascorbic acid was 27 mg g^{-1} in the unripe fruit, but it rapidly declined to 12 mg g^{-1} towards the end of ripening. Relative to the values reported for commercially produced cultivars (Homnava *et al.*, 1990), the vitamin C content of the cultivar used in the present study is significantly lower. This may be due to the varietal variations and geographic and environmental effects. Phenol-carbonic acids are another interesting quality component in fruits and vegetables. They are related to the characteristic astringent taste of unripe fruits, as well as to the discolouration taking place during storage and processing. The only phenolic acid identified in this study was gallic acid. The largest peak that appeared with a long retention time represented a mixture of soluble phenolics. By using peak areas, a decrease of about 70% was recorded (from 792 to 240 mm^2) in the content of unidentified phenolics. As this dramatic change was in accordance with the disappearance of the astringency, it is believed that the unidentified phenolics belong to the soluble tannins that have been reported to be the major precursor of astringency in persimmons (Kato, 1990).

Table 2. Contents of reducing sugars and organic acids of date-plum fruit at two stages of ripeness

Constituents	Prior to ripeness			Ripe		
	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV
Reducing sugars (mg g^{-1})						
Glucose	149·8	7·01	4·6	110·3	8·1	7·3
Fructose	135·0	4·70	3·4	101·0	5·6	5·5
Organic acids (mg per 100 g)						
Dehydroascorbic acid	7·0	0·2	2·8	4·4	0·11	2·5
Ascorbic acid	27·2	1·0	3·7	12·2	0·5	4·2
Malic acid	1 560·0	110·0	7·1	354·5	1·8	5·1
Isocitric acid	119·30	3·00	2·5	109·10	1·70	1·6
Citric acid	135·90	2·80	2·1	65·60	1·50	2·3
Fumaric acid	5·50	0·22	4·0	5·10	0·17	3·3
Gallic acid	2·74	0·10	3·8	0·65	0·03	4·2

$\bar{x}$—Mean of three determinations.
SD—Standard deviation.
CV—Coefficient of variation (%).

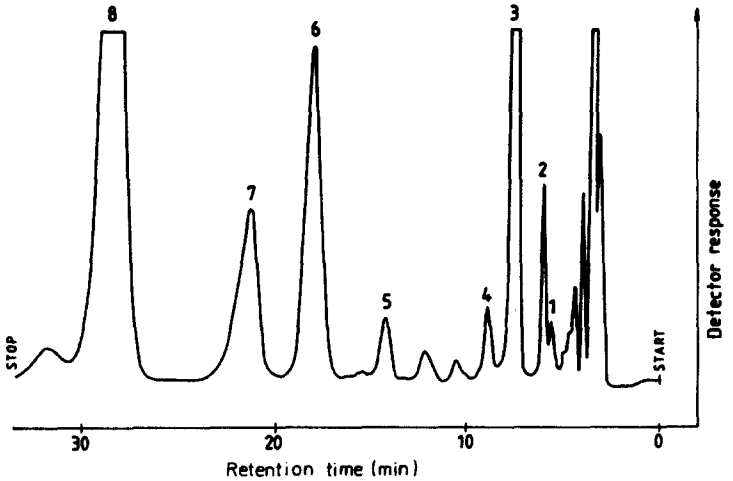

Fig. 3. HPLC separation of organic acid from persimmon fruit under ion-pair conditions using a Lichrosorb C-18 column eluted with potassium-phosphate (0·1 M)/methanol/tetrabutyl-ammonium-hydroxide (97 : 3 : 0·5, v/v/v, pH 2·75) at flow rate of 1 ml/min. Detection was at 225 nm. (1—Dehydroascorbic acid, 2—ascorbic acid, 3—malic acid, 4—isocitic acid, 5—citric acid, 6—fumaric acid, 7—gallic acid, 8—unidentified.)

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