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Note

Reversed-phase high-performance liquid chromatographic separation of lutein and lutein fatty acid esters from marigold flower petal powder

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Lutein is a common carotenoid in nature, occurring in all green structures of plants and also in many flower petals[1]. It is frequently used in industry as a natural food colorant, for instance in poultry feeds, to enhance pigmentation of the skin and egg yolk[2]. Currently, the commonest commercial source of lutein is the flower of the marigold plant *Tagetes erecta*[3], where it is found esterified with one or two fatty acids, and constitutes about 90% (w/w) of the petals[4].

Free xanthophylls and their esters not only have different stabilities but also differ widely in their ability to act as coloring agents in food technology[5]. Hence it is of great interest to have methods to separate and determine lutein and lutein esters in every step of the processing of this coloring agent (*i.e.*, in raw materials, during saponification procedures, etc).

The aim of this work was to devise a fast, sensitive and quantitative method to investigate the pigment composition of lutein sources and the pigment composition of these materials during processing. In recent years high-performance liquid chromatography (HPLC) has been shown to be a useful and accurate technique for separating and identifying carotenoids[6]. We have developed a new reversed-phase HPLC method for separating in a single step lutein and the different lutein fatty acid esters in colour sources and in other coloured products. This method could also be of general interest in the study and control of xanthophyll saponification processes.

EXPERIMENTAL

Extraction and sample preparation

Carotenoids from marigold (*Tagetes erecta*) petal powder were extracted with acetone overnight in a tightly closed flask according to the general method of Britton[7]. Carotenoids were transferred to *n*-hexane and the *n*-hexane fractions were dried over anhydrous sodium sulphate and evaporated to dryness in a stream of nitrogen. Samples were dissolved in ethyl acetate and passed through a Sep-Pak C_{18} cartidge (Millipore) in order to remove any substance that may stick non-reversibly to the octadecylsilane. Samples were then filtered twice through a 0.45-μm HVLP Millipore filter to remove insoluble particles before analysis.

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Saponification

Saponification was performed in order to hydrolyse carotenoid esters. After evaporation of the *n*-hexane the carotenoid extracts were dissolved in ethanol and sufficient potassium hydroxide solution (60%, w/v) was added to bring the final overall potassium hydroxide concentration to 10%. The mixture was allowed to stand at room temperature under nitrogen and in the dark for different times to allow saponification to progress to different extents. The ethanolic phase was re-extracted with *n*-hexane, and the *n*-hexane layer washed with water until free from alkali. Then the saponified extracts were prepared as indicated above before performing the analysis.

HPLC procedure

The HPLC system consisted of a high-pressure pump (LKB 2150), a low-pressure mixer driver (LKB Ultrograd 11300), a mixing valve to form gradients (LKB 2040-203) and a Rheodyne injection valve (7125). The mobile phase consisted of a linear gradient of ethyl acetate from 0 to 100% in acetonitrile–methanol (9:1, v/v) over 30 min, performed with an LKB 2125 programmable solvent delivery controller. Acetonitrile, methanol and ethyl acetate (HPLC grade, Scharlau) were filtered (Ultipor NX 0.45-μm membrane filter) and degassed with a stream of helium prior to use. The stationary phases used were two different octadecylsilane reversed-phase columns: LiChrosorb RP-18 (LKB UltroPac) and Zorbax ODS (Thames Chromatography) (both 250 × 4.0 mm I.D., with 5-μm spherical particles).

Samples were loaded on to the column via a Rheodyne injection valve (20-μl sample loop). The flow-rate was 1 ml/min and the pressure ranged from 66 to 87 bar in the LiChrosorb column and from 22 to 29 bar in the Zorbax column. Carotenoids in the effluent were continuously monitored with a photodiode array detector (Waters 990) covering the range 300–600 nm and connected to an NEC APC III computer for storing and processing of chromatograms and spectra.

Identification and quantitative evaluation

Peak identification was based on retention times and comparison with a lutein standard, and also on visible spectra of the chromatographic peaks obtained with the photodiode array detector. All the peaks present in the chromatograms were identified as xanthophylls (lutein and lutein esters) by UV–VIS spectrophotometry, having maxima at 425, 446 and 475 nm.

To carry out quantitative analysis of the data, peaks were monitored at the λ_{max} of the carotenoid and simultaneous integration was achieved at this wavelength using the NEC APC III think-jet integration facility. Calibration was carried out with the lutein standard, plotting the peak-area ratio *versus* concentration. The response factor of lutein was obtained from separate injections of the standard solution at several concentrations. Quantitation was achieved using the extinction coefficient of the standard because all the fractions eluted were lutein and lutein esters.

The relative quantitation of the peaks of diesterified and monoesterified lutein was based on partial integration of each group of these peaks in the chromatograms with the NEC APC III think-jet.

RESULTS AND DISCUSSION

Serious difficulties have previously been reported when separating carotenoid mixtures in a single-step chromatographic procedure owing to the need to cover the whole range of polar and non-polar carotenoids[8,9]. This difficulty is increased when separations between xanthophylls and their fatty acid esters are to be achieved, owing to the large difference in polarity between these compounds. Gau *et al.*[10] used HPLC on C_{18} with eluent mixtures of medium polarity (dichloromethane–acetonitrile) in order to separate xanthophyll esters, but this isocratic method needed very high flow-rates (3 and 15 ml/min) and did not resolve simultaneously diesterified, monoesterified and free xanthophylls.

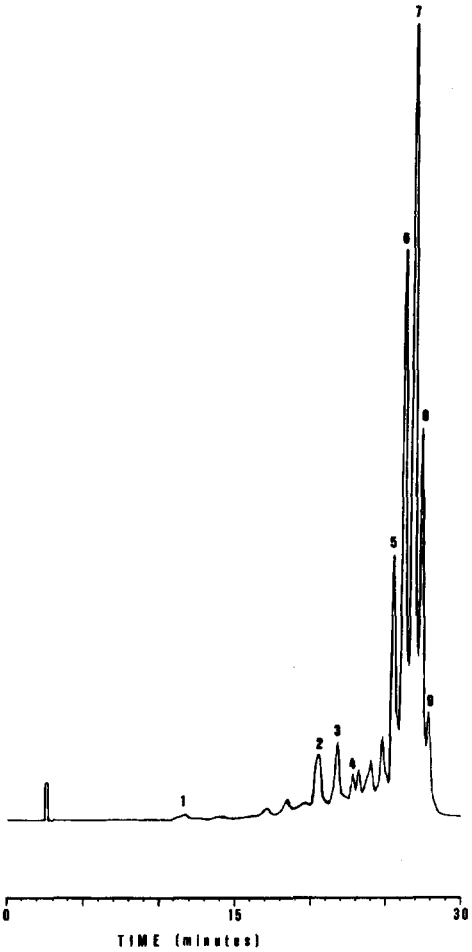

Fig. 1. HPLC of an unsaponified extract of marigold. Mobile phase, linear gradient of ethyl acetate from 0 to 100% in acetonitrile–methanol (9:1, v/v) over 30 min; flow-rate, 1.00 ml/min; column, Zorbax ODS, 5 μm (250 × 4.0 mm I.D.); detection, 450 nm; volume injected, 20 μl. Peaks: (1) lutein; (2) monomyristate of lutein; (3) monopalmitate; (4) monostearate; (5) dimyristate; (6) myristate–palmitate; (7) dipalmitate; (8) palmitate–stearate; (9) distearate.

The HPLC method proposed here separates in 30 min free, mono- and diesterified lutein with a simple linear gradient of ethyl acetate in acetonitrile–methanol. An example of the use of this method can be seen in Fig. 1, which shows a chromatogram run on a Zorbax column of the unsaponified extract from marigold petal powder. The chromatogram shows a lack of significant amounts of free lutein (peak 1). This confirms the notion that the xanthophyll esters constitute the majority of the carotenoid pool in marigold petal powder. In the chromatogram shown in Fig. 1, lutein esters eluted after 20 min, represented 95.5% of the total carotenoids in marigold petals, the last five peaks (5–9) being the major components (79.5%). Peaks not numbered in the figures are minor xanthophyll esters that were not identified.

The major peaks in the original unsaponified extract (Fig. 1) were identified as diesterified lutein (lutein esterified with two fatty acid chains). The two fatty acids can be the same (diesters) or different (mixed esters)[10–11]. These esters were associated with dimyristate (Fig. 2, peak 5), myristate–palmitate (peak 6), dipalmitate (peak 7), palmitate–stearate (peak 8) and distearate (peak 9) by comparing their relative retention times with those reported by Gau *et al.*[10] who separated xanthophyll fatty acid esters extracted from marigold flower petals by reversed-phase HPLC (LiChrosorb RP-18). These five fatty acid esters of lutein are the most abundant in marigold petals, and the quantitation (means for five samples) of the major ester peaks showed good agreement between the values found by Gau *et al.*[10] and our results. These data are presented in Table I.

Fig. 2 shows the carotenoid composition at three different times during the saponification of the lutein esters from marigold petal powder: immediately after the start of saponification (Fig. 2a), after 10 min of hydrolysis (Fig. 2b) and after 3 h of hydrolysis (Fig. 2c). This series of chromatograms shows the progressive hydrolysis of esters. Peaks corresponding to lutein esters decreased during the saponification process whereas the lutein peak exhibited a concomitant increase, until it became the major carotenoid in the final mixture (Fig. 2c, peak 1 = 91.3%).

LiChrosorb (Fig. 2) and Zorbax (Fig. 1) columns gave similar chromatograms, with lutein retention times of 9.33 ± 0.05 and 11.25 ± 0.16 min, respectively.

After partial saponification (Fig. 2a and b), two groups of peaks were present. One corresponds to diesterified lutein (peaks 6–9) and the other, with lower retention times, to monoesterified lutein (peaks 2–4). These monoesters originated from hydrolysis of one of the ester bonds.

TABLE I

PERCENT DETERMINATION OF DIESTERIFIED LUTEIN IN AN EXTRACT OF UNSAPONIFIED MARIGOLD PETALS

Peak No.	*Concentration (area-%)*		*Xanthophyll diesters and mixed esters*
	Gau et al.[10]	*This work (mean of 5 samples)*	
5	12.6	11.59 ± 0.39	Dimyristate
6	24.7	24.23 ± 0.87	Myristate–palmitate
7	35.5	37.57 ± 1.42	Dipalmitate
8	14.4	15.55 ± 0.47	Palmitate–stearate
9	2.4	3.63 ± 0.52	Distearate

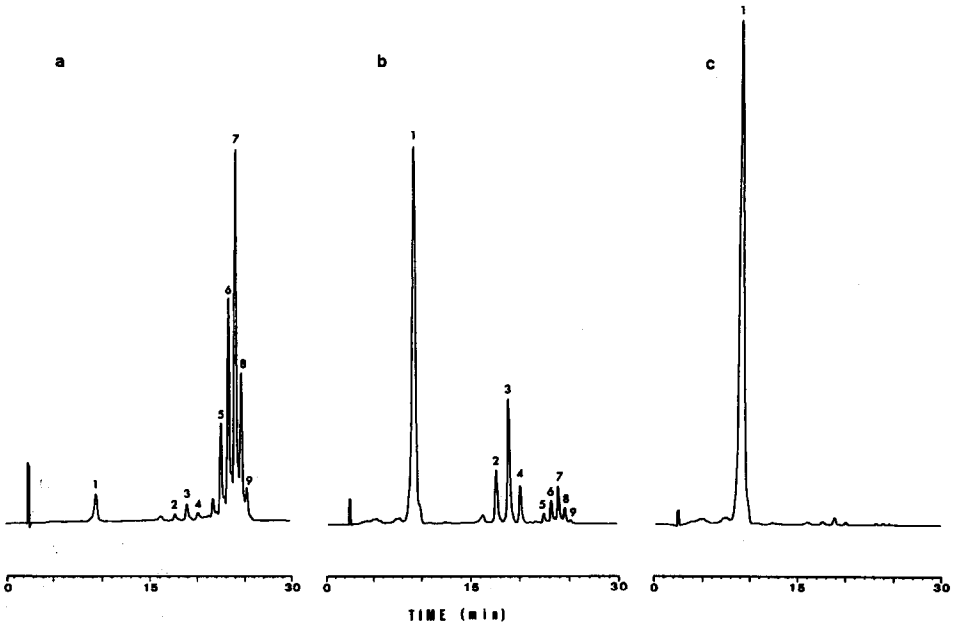

Fig. 2. HPLC absorbance (450 nm) chromatograms of marigold flower petal powder extracts saponified and semi-saponified: (a) immediately after the start of saponification; (b) after 10 min of hydrolysis; (c) after 3 h of hydrolysis. Chromatographic conditions as in Fig. 1, except the column was LiChrosorb RP-18,5 μm (250 × 4.0 mm I.D.).

The major fatty acid esters present in the hydrolysate of *Tagetes* are known to be myristate, palmitate and stearate[12]. In earlier work in our laboratory, the fatty acids isolated from marigold petals were identified by gas chromatography by comparison with pure standards; myristic, palmitic and stearic acids being the major components[13]. They correspond to the monoester peaks 2, 3 and 4, respectively. Moreover, the relative amounts of each of these three monoesters (*e.g.* monomyristate, peak 2: 24.0%) in the semi-saponified extract (Fig. 2b) was found to be equal to the sum of the corresponding diester (dimyristate, peak 5: 11.6%) plus half of the other mixed esters (myristate–palmitate, peak 6 24.2/2 = 12.1%) in the original unsaponified extract. This confirms the tentative identification, although it was not the original purpose of this work.

The HPLC method reported here is very suitable (simple, rapid and reproducible) for analysis of xanthophylls and their esters. This non-aqueous reversed-phase HPLC method surpasses thin-layer chromatography, as it allows the separation of mixed esters from the diesters of xanthophyll. Moreover, it is an improvement over other HPLC methods as it separates free xanthophylls from their fatty acid esters in a single step. Finally, this chromatographic system could be useful in the study and control of xanthophyll saponification processes.

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