Vol. 2, No. 2, Th. 1993 ISSN : 0853 - 0033

THE NEEDS OF SAPONIFICATION FOR ANALYSIS OF CAROTENOIDS (PERANAN SAPONIFIKASI DALAM ANALISIS KAROTENOID)

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ABSTRACT

Telah dilakukan penelitian tentang peranan saponifikasi dan analisis karotenoid. Untuk saponifikasi ini digunakan larutan KOH 20% dalam metanol. Reaksi saponifikasi ini telah dicoba terhadap beberapa jenis karotenoid standar seperti beta-karoten, alfa- karoten, ekinenon dan apokarotenal serta beberapa jenis sayuran dan buah-buahan yang mengandung karotenoid seperti "parsley" (*Petroselinum crispum*), English spinach (*Spinacia oleracea*), tomat, wortel dan jeruk "Sunkist".

Hasil penelitian memperlihatkan bahwa beta-karoten, alfa-karoten, ekinenon dan apokarotenal stabil terhadap reaksi saponifikasi. Namun karotenoid yang terdapat dalam parsley, English spinach, tomat dan jeruk Sunkist ada yang mengalami perubahan.

INTRODUCTION

Saponification is generally carried out in carotenoid analysis to remove unwanted esterified lipids, acidic materials and chlorophylls which interfere with adsorption chromatography (Gross, 1987). However, saponification is often avoided where examinations of material for carotenol esters is desired and for analysis of extracs containing alkali labile carotenoids, such as astaxanthin, fucoxanthin, peridinin and related compounds. For carotenoids which are labile to saponification, special care must be taken such as the maintenance of aerobic conditions during saponification to proceed efficiently without degradation (Ruddat and Will, 1985).

Potassium-methanol solution is the common reagent used for saponification. Two main methods are used for saponification. The alkaline mixture is either: (i) heated for up tu 10 min in the dark on a boiling water-bath and

under stream of nitrogen, and then cooled; or (ii) left in the dark at room temperature under nitrogen for 12-16 hours (Davies, 1976; Gross, 1987).

Quackenbush (1973) reported that there was no statistical difference for the quantitative analysis of carotenoids between the "cold" overnigth procedure and the "hot" rapid method. While the "hot" method is time saving, the "cold" method has the advantage that the carotenoids, such as xanthophylls, which are particularly thermobile are not subjected to heat.

Many researchers have used different modifications of these methods. Some examples are : Curl (1953), by dissolving Valencia orange in diethyl ether and equal volume of 20% potassium hydroxide in methanol was added and allowed to stand at room temperature overnight. Johjima and Ogura (1983) saponified tomato carotenoids by dissolving the sample in hexane and the same volume of 20% potassium hydroxide in 85% methanol was then added and the mixture allowed to stand for 60 min at room temperature.

These methods were used throughout the investigation and this paper reports the results of the experiment.

MATERIALS AND METHODS

All chemicals were of analytical reagent grade, solvents of HPLC grade and water was double distilled. Chloroform was washed with double distilled water to remove trace acids and dried over anhydrous sodium sulphate. All solvents were distilled and purged with nitrogen for oxygen exclusion.

Pigment standard were gifts from Roche Products (Sydney). English spinach, parsley, tomato ans Sunkist orange were obtained from local retail marked in Sydney, Australia.

Analysis by liquid chromatography system were carried out on Novapak C18 column (Waters, Milford, Mass.). The column was attached to a Waters U6K injector and 41-mPa pump. The mobile phase contained 22.5% water: 2,5% chloroform: 75% acetonitrile which filtered and degassed under vacuum before use and maintained at a flow rate of 3 ml^{-1}. Column

effluents were monitored by a Waters Model 490 Programmable multi-wavelength detector at 440 nm and recorded on a Waters Data Module.

Fruit and vegetables (1000g) were extracted by a slight modification of the method described by Zakaria *et al* (1979) which involved cutting produce into small pieces and homogenising in acetone for 30 min in a blender with light being excluded by covering all vessel with aluminum 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 mixture was allowed to settle and the organic layer removed. The aqueous layer was re-extracted with diethyl ether until the layer became colourless. The combined extracts were washed with water to remove the acetone and dried over anhydrous sodium sulphate.

The extract was saponified to remove the chlorophylls and fats as well as hydrolysing carotenoid esters according to the methods of Curl (1953) and Johjima and Ogura (1983). Johjima and Ogura method involved the addition of an equal volume of 20% potassium hyroxyde in 85% methanol to solution of produce extract in hexane and the mixture was allowed to stand at room temperature for 60 min. The alkali and water soluble materials were removed by washing with water. The organic layer was dried over sodium sulphate and the solvent removed under vacuum. The Curl's method involved the addition of an equal volume of 20% KOH in methanol to diethyl extracts leaving the mixture to stand at room temperature overnight. The alkali and water soluble materials were removed by washing with water. The ether layer was dried over anhydrous sodium sulphate and the solvent removed under vacuum. Both residues were dissolved in chloroform and an aliquot (1-25 ul) analysed by HPLC.

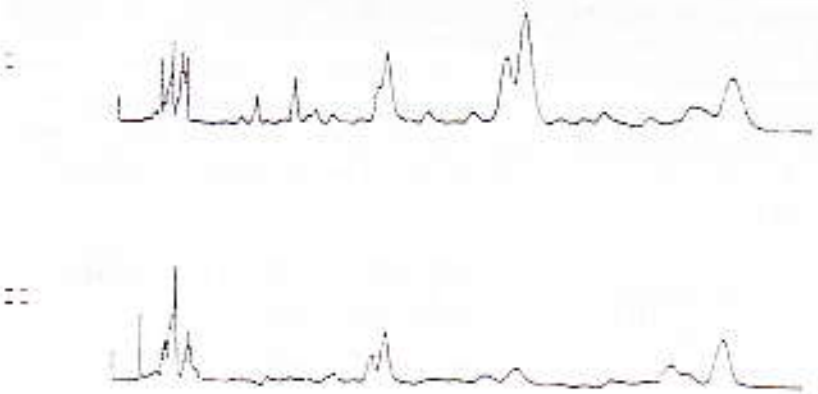
The standard carotinoids were also saponified by the method of Curl and injected to HPLC system with the same volume.

RESULTS AND DISCUSSION

The saponification methods used by Curl (1953) and Johjima and Ogura (1983) were compared. The Curl method was more satisfactory, since all esters were saponified. The method, however, was time-consuming, since saponification was carried out overnight. The method of Johjima and Ogura (1983) had a shorter saponification time, but an experiment with orange showed that some esters were not fully saponified as indicated by the presence of a few peaks with retention times longer than beta-carotine.

Figure 1 shows the chromatograms of orange carotenoids before saponification and after saponification according to Johjima and Ogura (1983) and Curl (1953).

It was noted in both methods that after saponification, a white residue always formed and difficult to remove from carotenoids. The methods used by Curl (1953) and Gross *et al* (1971) to remove the white residue (identified as sterols) by precpitating with methanol at -20^{0}C, were unsuccessful, since some carotenoids were co-precipitated. The residue was not very soluble in hexane, petroleum ether, acetone, methanol and ethanol and present in colloidal form. However, the residue was soluble in chloroform which was, therefore, used during the studies to dissolve the samples. The residue did not interfere with the analysis for carotenoids since it did not absorb at 440 nm.



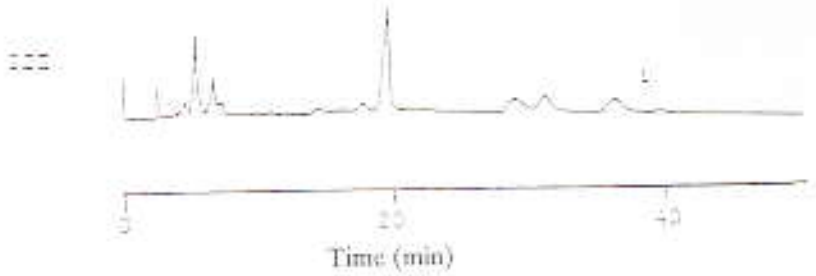


Figure 1. Chromatograms of orange carotenoids eluted with 22.5% methanol : 2.5% chloroform : 75% acetonitrile ; I = chromatogram of unsaponified carotenoids; II = chromatogram of carotenoids saponified by the method of Johjima (1983); III = chromatogram of carotenoids saponified by the method of Curl (1953); 1 = α -carotene.

The use of chloroform was some concern, since the presence of trace acids in the chloroform could transform epoxy groups into furanoid groups (Davies, 1976). The solvent was, therefore, washed with water, dried over anhydrous sodium sulphate and destilled prior to use to remove any trace of hydrochloric acid.

Saponification of standards, beta and alpha-carotenes, echinenone and apocarotenal by the method of Curl (1953), gave no significant loss of carotenoids (Figure 2). The peak areas before saponification were 5.2 $\pm$ 0.7, 20.7 $\pm$ 0.4, 23.2 $\pm$ 0,9 and 53,4 $\pm$ 0,5, respectively, and 6.1 $\pm$ 1.6, 23 $\pm$ 1.7, 23.3 $\pm$ 1.4 and 52.1 $\pm$ 2.3 integration area units after saponification respectively. Each carotenoid was therefore stable during saponification.

Studies on carrot, parsley and English spinach indicated that the carotenoids in these vegetables were stable during saponification. Figure 3 show the chromatograms of carrot carotenoids. Before saponification the area of peaks corresponding to beta and alpha-carotenes were 3.4 $\pm$ 0.2 and 2.7 $\pm$ 0.2 integration units, respectively, and 3.3 $\pm$ 0.1 and 2.5 $\pm$ 0.1, respectively, after saponification.

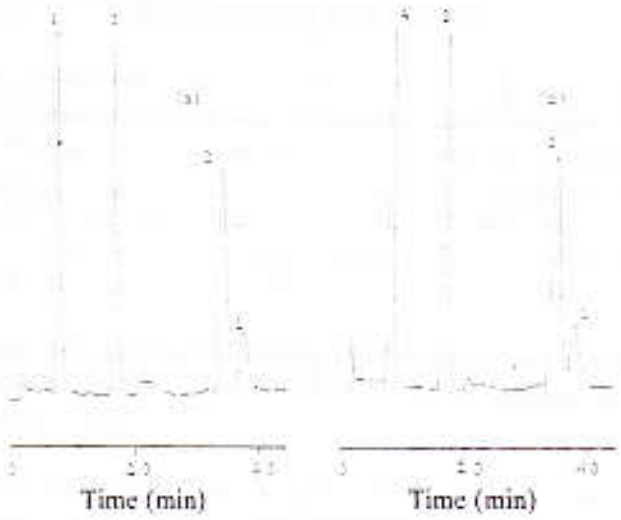


FIGURE 2: Chromatograms of standard carotenoids before saponification (a) and after saponification (b). 1 = β-carotene; 2 = α-carotene; 3 = echinenone; 4 = apocarotenal. Conditions : solvent : 22.5 % methanol : 2.5 % chloroform : 75 % acetonitrile, 5 and 10 u radialpak columns connected in series, flowrate 3 mL min^{-1}, detection at 440 nm.

The chromatograms of carotenoids in parsley (Figure 3, chromatogram II) and English spinach (Figure 4, chromatogram I) show that chlorophylls (which are esters) were are completely removed by saponification (peaks 3, 4, 5 in Figure 3, chromatogram IIa and peaks 3, 4, 5, and 6 in Figure 4, chromatogram Ia). The peak area of beta-carotene remain stable being 41.0 and 41.5, respectively and in English spinach 58.1 and 58.4 respectively.

Studies of carotinoids in tomato showed that saponificatio reduced the area of some peaks associated with carotenoids (Figure 4, chromatogram II). The area of the peaks corresponding to beta- carotene (peak 1) was 41.5 $\pm$ 1.1 and 31.0 $\pm$ 1.2 before and after saponification and of lycopene (peak 2) was 76.1 and 65.6, respectively. Since the investigation with standards, carrot, parsley and English spinach demonstrated that beta-carotene was

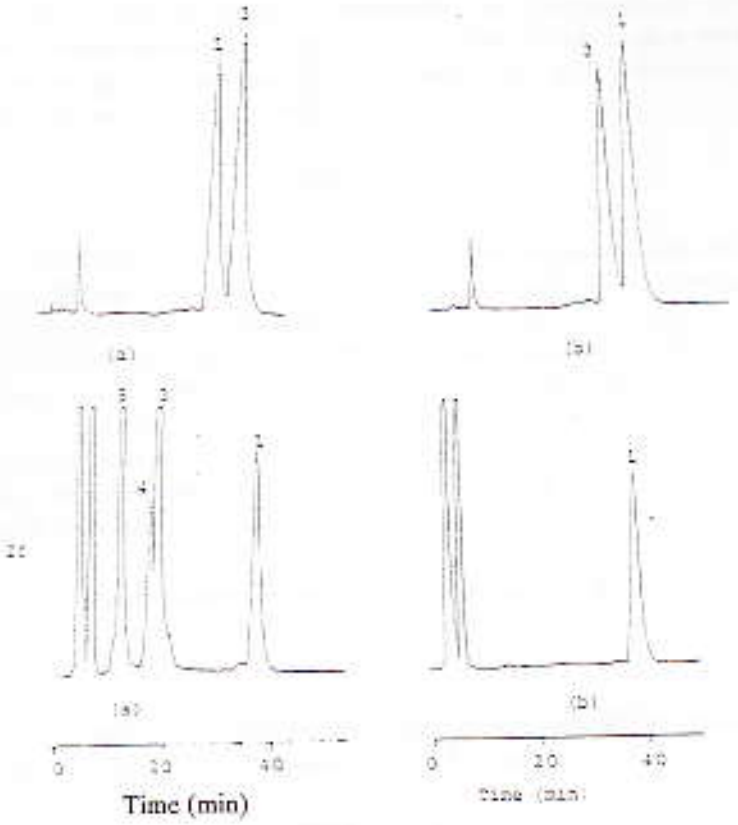


Figure 3 : Chromatograms of carrot (I) and parsley (II) carotenoids before (a) and after (b) saponification. 1 = β-carotene; 2 = α-carotene; 3, 4, 5 = chlorophyll. Solvent and operating conditions were similar to those in Figure 2.

stable during saponification, it is suggested that peak 1 (beta- carotene) and peak 2 (lycopene) in tomato were overlapped by other peaks, presumed to be esters which were removed by saponification.

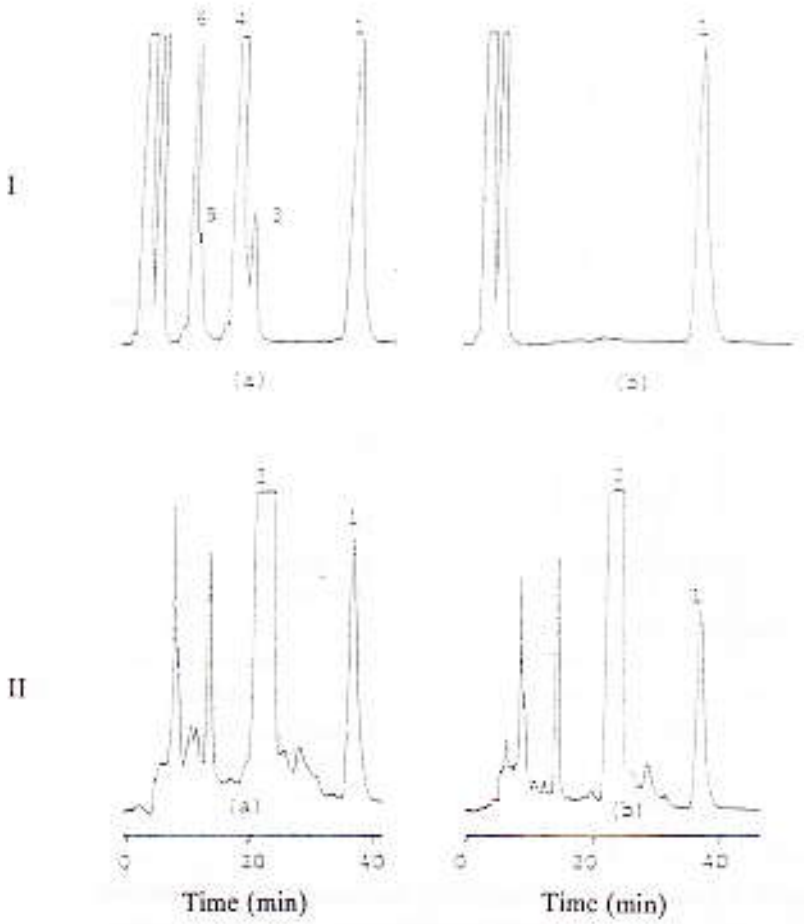


Figure 4 : Chromatograms of English spinach (I) and tomato (II) before (a) and After (b) saponification. 1 = β -carotene; 2 = licopene; 3, 4, 5, 6 = chlorophyll. Solvent and operating systems were similar to those in Figure 2.

The studies of standard carotenoids and carotenoids in carrot, parsley, English spinach and tomato indicate that saponification is needed for the analysis of carotenoids, since some peaks may overlapped by carotenoids and other ester peaks.

CONCLUSION

The experiment showed that saponification was needed for analysis carotenoids, since chlorophylls, fats and unwanted materials may give peaks which overlap with the peaks of carotenoids. The studies with orange, English spinach, parsley and tomato showed the different chromatograms before and after saponification. For English spinach and parsley all peaks with retention times 15-22 min disappeared after saponification. The diappearance of these peaks corresponded to the disappearance of chlorophylls. For orange and tomato the peaks which did not belong to carotenoids were removed after saponification and clean peaks will be obtained after saponification.

For saponification Curl method was more preferable to be used than Johjima and Ogura method, since complete saponification obtained with Curl method.

The experiment with standard carotenoids showed that the compounds examined were stable during saponification.

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