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Analytical Methods Section

Observations on some of the problems associated with the analysis of carotenoids in foods by HPLC

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There is considerable current interest in the influence of certain foods on human health. In the study of the relationship between nutrient intake, nutritional status and morbidity, the need for accurate qualitative and quantitative data on the nutrients in these foods has become increasingly important. β-Carotene has attracted particular attention not only because of its nutritional role as a vitamin A precursor but also because of its antioxidant potential. More recently interest has been extended to the possible importance of other naturally occurring carotenoids. Their measurement in natural products involves extraction and chromatography with organic solvents. The potential for loss during sampling, extraction and saponification has been recognised, although definitive methods have yet to be described satisfactorily. It is however often overlooked that the response of carotenoids may be affected by artefacts produced during chromatography. Two areas in particular are (i) the reaction between carotenoids and metal surfaces with particular reference to stainless steel frits and (ii) the reaction between carotenoids, injection solvents and the mobile phase. These may result in peak distortion and the production of artifacts which can lead to misinterpretation of the chromatogram and the production of erroneous and unreliable data. Studies to identify the problems, and preliminary conclusions on the way they may be overcome are reported.

INTRODUCTION

Comparatively recently interest has intensified on the possible effect of subclinical or marginal nutrition on morbidity. The relationship between dietary intake and nutritional status is poorly defined and that between status and morbidity remains to be fully explored. A number of studies have concentrated on the role of individual dietary components or nutrients in health and disease, however it is becoming clear that although single nutrients may play a predominant role the picture is usually far more complex.

Together with the antioxidant vitamins C and E, the intake of β-carotene has been linked to cancer prevention (see Antioxidant vitamins and β-carotene in Disease Prevention (1991)). β-Carotene may be protective against lung cancer and possibly stomach cancer (Stahelin *et al.*, 1984; Menkes *et al.*, 1986; Wald *et al.*, 1988). Free radical mediated events have been proposed to be critically involved in several disease states (see Antioxidant vitamins and β-carotene in Disease Prevention (1991)). Human plasma is normally endowed with an array of antioxidant radical scavengers. Low blood β-carotene levels have been associated with a high risk of cancer in a number of prospective case studies (Stahelin *et al.*, 1984; Nomura *et al.*, 1985; Menkes *et al.*, 1986). However, it has been demonstrated that β-carotene itself is not a particularly effective antioxidant in high oxygen tension (i.e. blood or lung) (Burton & Ingold, 1984). Therefore β-carotene from foods may only be acting as a proxy for a more relevant carotenoid(s).

Di Mascio *et al.* (1991) reported that lycopene was found to be the most efficient carotenoid regarding singlet oxygen quenching. Terao (1989) in a comparison of the effect of zeaxanthin, canthaxathin, astaxanthin and β-carotene on peroxyl radical mediated lipid peroxidation demonstrated that canthaxathin and astaxanthin retarded the hydroperoxide formation more efficiently than β-carotene and zeaxanthin. Thurnham *et al.* (1988) indicated that lutein together with β-carotene occurred most consistently in all blood samples studied. Little is known of the action or biological turnover of lutein, however, it might be of physiological importance and is found specifically in the rods of the eye. Information from our own preliminary studies and other data (Heinonen *et al.*, 1989; E-Siong Tee, 1991)

suggests that lutein is also one of the major carotenoids in fruit and vegetables.

Carotenoids are present in all fruit and vegetables except some root vegetables. Four classes of compounds have been shown to be present, xanthophylls (the oxygenated carotenoids), hydrocarbon carotenoids, carotenol mono fatty acid esters and carotenol bis fatty acid esters.

Fruit and vegetables are complex foods containing a wide variety of substances and several specific compounds which are widely believed to confer protective properties for human health. Since carotenoids are amongst the most abundant micronutrients in fruits and vegetables, the determination of accurate qualitative and quantitative data on these compounds has become increasingly important.

The analysis of carotenoids is complicated by the fact that there are several hundred different naturally occurring carotenoids, however, there are far fewer, estimated between 7 and 20, in human blood (Bieri *et al.*, 1985; Thompson *et al.*, 1985).

Traditional separation methods employ open column and thin layer chromatography. The development of HPLC has allowed for advances in the analysis of carotenoids. In addition the elucidation of structures and stereochemistry has been made possible by the development of powerful spectroscopic techniques.

However, despite the introduction of advanced technology for analysis and data handling which allows the separation, identification and quantification of complex mixtures of carotenoids from natural sources, it is important that the analyst is aware of the inherent practical difficulties and problems associated with carotenoid analysis. It is far too easy to obtain the 'wrong' answer with a high degree of precision.

Historically much of the 'carotene' data in tables of food composition have been obtained by measuring total absorption at a specified wavelength and quantified against β-carotene, or more usually by open column chromatography (e.g. AOAC method) which has been widely used to separate carotenoid pigments which are then quantified spectrophotometrically. The AOAC method is a relatively 'crude' procedure which can give reasonably good estimates of 'carotene' where β-carotene predominàtes although the presence of other 'carotenes' and their isomers can result in the over estimation of vitamin A activity. Modification of the chromatographic conditions allows the elution of total xanthophylls which are quantified against lutein.

The development of more sensitive methodology such as HPLC means that artefacts produced during extraction, purification and chromatography will be more readily detected. The isolation of carotenoids from natural products involves extraction and chromatography with organic solvents. Carotenoids are highly sensitive to light, heat, air and active surfaces and their isolation and analysis may be accompanied by degradation, formation of stereoisomers, structural rearrangements and other physicochemical reactions. Although steps may be taken to avoid or lessen the effects of these problems during handling of samples and sample extraction, it is often overlooked that artefacts affecting the response of carotenoids may be readily produced during chromatography. Therefore, once a potential separation system has been developed it is necessary to carry out an in depth study to assess the validity of the 'peak response' obtained and various factors which may affect it.

Important factors to be considered in developing a routine HLPC method for carotenoids are, selectivity, sample solubility, column stability, chromatographic convenience and its application to materials (i.e. fruit and vegetables) of interest. One of the priorities was to develop a system which took into account all the factors mentioned above, with the maximum chromatographic convenience. This involved, if possible, the use of an isocratic system which would allow separation of the carotenoids of interest in a time suitable for routine analysis.

It was considered reasonable in attempting to assess the 'potential' intake of carotenoids from foods to concentrate on those carotenoids predominating in blood, i.e. lutein, β-cryptoxanthin, lycopene and α- and β-carotene.

It is not intended to review or discuss all the developments in the HPLC methods for carotenoids which have taken place mainly during the past 10 years, but to consider some of the problems which have arisen in the author's laboratory and which may be applicable to other analysts attempting to develop chromatographic techniques for the determination of carotenoids, and report on studies to identify the problems and preliminary conclusions on the way they may be overcome.

RESULTS AND DISCUSSION

Chromatography: column and solvent systems

In 1983 Nelis and De Leenheer investigated the use of an isocratic non-aqueous reversed phase system, on Zorbax ODS. Nine standard carotenoids, lutein, zeaxanthin, canthaxthin, β-cryptoxanthin, echinenone, lycopene, torulene and α- and β-carotene were 'separated' using a mobile phase consisting of acetonitrile (ACN) dichloromethane (DCM) methanol (METH) (70 : 20 : 10, v/v). The use of eluents not containing methanol (80ACN : 20DCM) was reported to yield better resolution of zeaxanthin and lutein, but at the expense of incomplete separation of lycopene and echinenone. In our experience, even when methanol was omitted, base line separation of zeaxanthin and lutein was not achieved. In a study of carotenoids in Finnish foods Heinonen *et al.* (1989) reported that zeaxanthin and lutein were not separated on a Zorbax ODS with 70 ACN : 20 DCM : 10 METH and results were reported as lutein + zeaxanthin. Additionally in our experience the use of 80 ACN : 20 DCM, although initially giving some but not complete separation, was found on continued use to produce a considerable

lowering of lutein response accompanied by peak distortion. This was suggestive of non-specific absorption or deterioration of lutein on the column. Peak sensitivity and shape were only temporarily restored by washing the column with 100% methanol and re-equilibrating with mobile phase. Earlier tests had suggested this deterioration in response did not occur when ~5% methanol was included in the solvent mix (e.g. 75 ACN:20 DCM:5 METH).

Trials in our laboratory using a 5 μm Vydac 201TP 54 column (Separations Group) coupled to an ODS2 guard column (Pharmacia LKB) with various mobile phase combinations showed that effective separation of lutein, zeaxanthin, β-cryptoxanthin, lycopene and α- and β-carotene was obtained with a mobile phase 75 ACN:20 METH:5 DCM (1·5 ml min^{-1}). An even better separation, particularly of β-cryptoxanthin, lycopene and α-carotene, was obtained by coupling a 100 mm ODS 2 spherisorb cartridge column (Pharmacia LKB) before the Vydac; total elution time was ~25 min. Surprisingly these early tests showed that the responses were considerably diminished when this guard column was used. Later tests also showed that when individual standard carotenoid solutions were run singly, responses were variable and lower than their response in a mixture. The response of lutein for instance could be up to 40% higher in the mixture. This increase in response (in a mix) could not be adequately accounted for by 'contaminants' in other carotenoids. Sequential addition of other carotenoids (zeaxanthin, β-cryptoxanthin, lycopene, α- and β-carotene) to lutein showed that the presence of zeaxanthin was, for the most part, responsible for the increase in lutein response, although these peaks were well separated. Furthermore although the addition of graded concentrations of zeaxanthin showed a linear response of zeaxanthin, the increase/decrease in lutein response was not linear. Bearing in mind the apparent effect of the guard column on responses found earlier two steps were taken, i.e. the replacement of the guard column with a new one and the removal of the guard column completely. The former did not produce any significant effect, however the removal of the guard column not only resulted in increased responses but the responses of carotenoids run singly were more similar to those in a mixture taking into account any 'contaminants'. The individual responses were lutein 102%, zeaxanthin 98%, β-cryptoxanthin 96%, α-carotene 100% and β-carotene 98% respectively of their responses in a mix.

Protein-biochemists have long been aware that standard stainless steel HPLC fittings, filters and frits may be damaging to the integrity of the analytes. It has been suggested that column frit materials may be partially responsible for low recoveries of carotenoids (Nierenberg & Lester, 1986; MacCrehan & Schönberger, 1987). Currently available Pharmacia LKB guard columns (10 cm) and cartridge columns have large surface area sintered frits. These replaced the frit system, in guard columns (30 mm) and cartridge columns, composed of a fibre glass filter pad between two stainless steel sieves. The current guard column fits into the cartridge column, effectively producing one column. There is obviously a large 'frit' area, especially where the two 'mate' together. Peak response problems with the Pharmacia LKB guard column, when used in conjunction with the Pharmacia LKB cartridge column, have also been noted by Thurnham (personal communication).

In order to test the possibility that stainless steel frits were mainly or partially responsible for the irregularities in peak response, i.e. the differences between peak response of individual and 'mix' carotenoids and the occurrence of 'artefacts', the Pharmacia LKB ODS guard column was replaced with a 5 μm ODS 2 metal free guard column (Alltech Associates). The use of this guard column with the column system showed a much closer relationship between the individually run carotenoids and carotenoids in a 'mix'.

Replacement of the Pharmacia LKB ODS2 cartridge column (100 mm) with a similar but metal free column (Alltech) gave response values for individual carotenoids which were essentially the same as those in a mix, when the mix values were corrected for 'contaminant' peaks eluting at the same time. Individual responses were lutein 99%, zeaxanthin 101%, β-cryptoxanthin 93%, α-carotene 99% and β-carotene 99% respectively of their response in a mixture. However, this column system still resulted in the appearance of possible artefacts associated with certain carotenoids. The metal frits on two VYDAC columns were replaced with metal free biocompatible frits (Alltech) and the exercise repeated. This resulted in the apparent reduction or elimination of certain minor peaks associated particularly with β-cryptoxanthin and α- and β-carotene. There was, however, a difference between the two columns (Fig. 1). The response of all carotenoids tested were linear over the range 0–0·02 AU. Replacement of the (50 μl) injection loop with a non-metal loop ('Flexon' - Alltech) did not affect responses.

Stacewicz-Sapuntzakis (1991) has reported that C_{18} Novapak column, earlier reported to be useful for the separation of serum carotenoids (Stacewicz-Sapuntzakis *et al.*, 1987), proved to give progressively worse results with newly purchased columns. It was found that all peaks were greatly diminished in size, especially β-carotene and lycopene, and accompanied by the appearance of artefacts which seemed to arise from the destruction of β-carotene or lycopene. Some columns did not release any carotenoids injected onto them, absorption or destruction being complete. The problem was traced to a change in the stainless steel frits in Novapak columns to 'sintered frits' with irregular pores and larger surface area. When replaced with custom-made teflon frits the problem disappeared.

The carotenoid profile of a standard carotenoid 'mix' and an unsaponified 'food' mix, using the metal free Alltech columns and metal free frits (Vydac column) is shown in Fig. 2.

Further to the question of the suitability of different columns Epler *et al.* (in press) studied 60 commercial

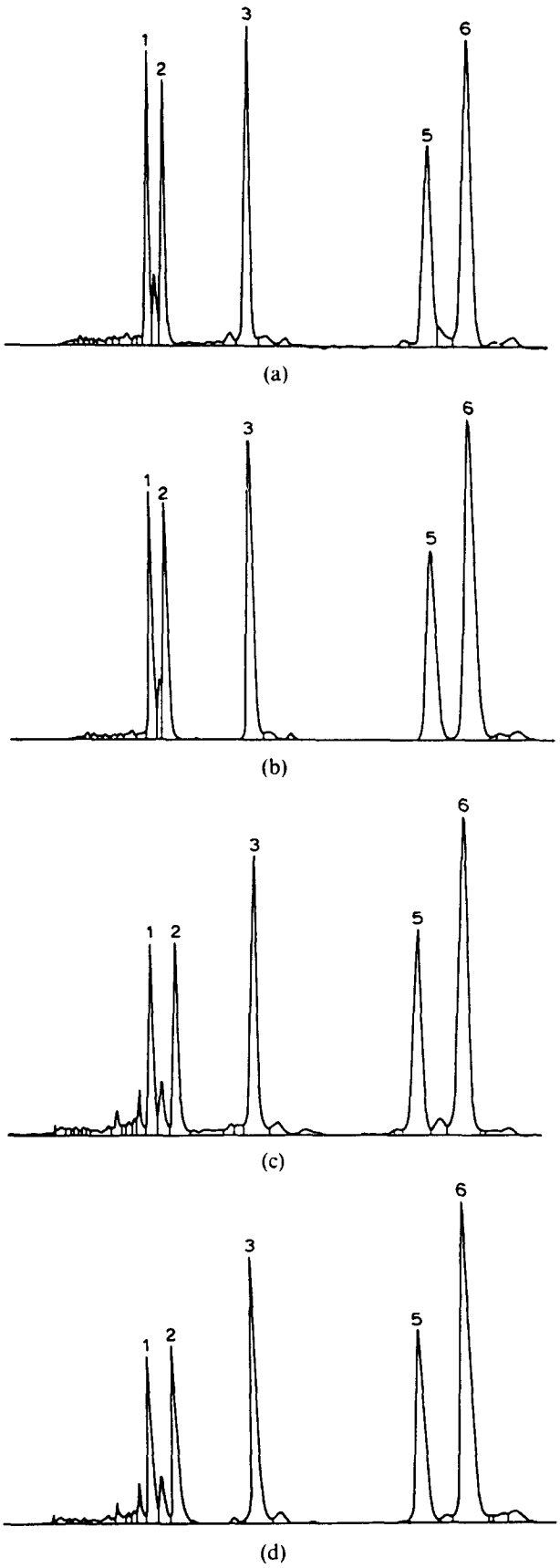

Fig. 1. Comparison of metal and metal 'free' frits in VYDAC TP201-54 columns; (a) column 2, metal frits; (b) column 2, metal free frits; (c) column 3, metal frits; (d) column 3, metal free frits. 1, lutein; 2, zeaxanthin; 3, β-cryptoxanthin; 5, α-carotene; 6, β-carotene. Vydac 201 TP 54 (250 mm) (Separations group); ODS 2, 5 μm (100 mm) ODS 2, 5 μm guard column (Alltech); mobile phase: acetonitrile 75: methanol 20: dichloromethane 5; flow rate 1·5 ml min^{-1}; range 0·02 AU monitored at 450 nm.

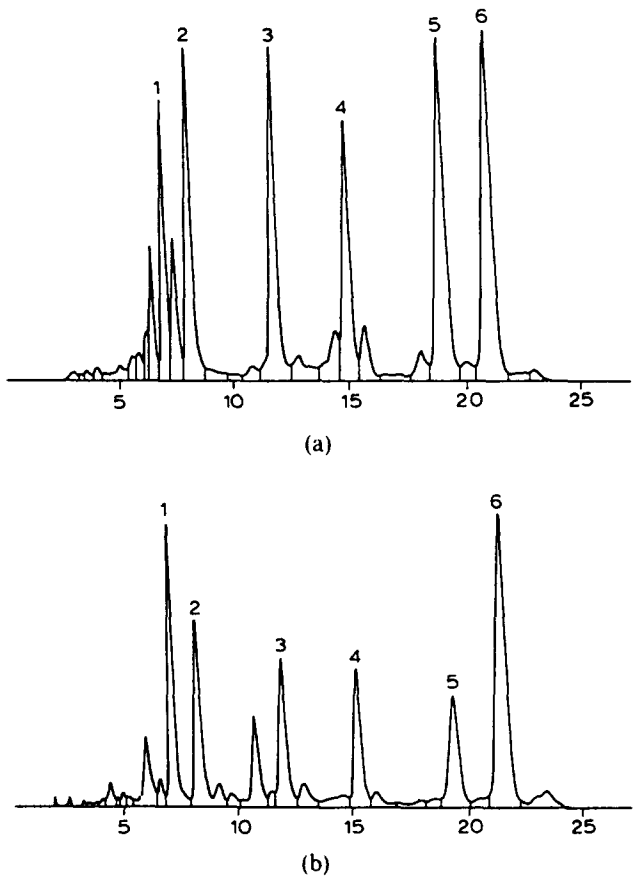

Fig. 2. Carotenoid profile of (a) standard carotenoid 'mix' and (b) unsaponified 'food mix' containing sweet corn, mandarin orange, tomato and carrot (conditions as in Fig. 1). 1, lutein; 2, zeaxanthin; 3, β-cryptoxanthin 4, lycopene; 5, α-carotene; 6, β-carotene.

and five experimental columns to determine the recovery of carotenoids injected on to a column, the ability of a column to separate the carotenoids present in human serum, and the reproducibility of columns. This study found that in general separations using acetonitrile-based solvent resulted in lower recoveries than separations using methanol or methanol-based solvents. Monomeric C_{18} columns, e.g. Zorbax ODS, often provided high recoveries, but did not resolve lutein and zeaxanthin using methanol or methanol-based eluents. Polymeric C_{18} columns, e.g. Vydac, were usually able to resolve lutein and zeaxanthin in methanol based solvents. Recovery might be improved by using a non-stainless steel frit. Columns with the 'same' stationary phase do not necessarily retain compounds for the same length of time, nor do they necessarily yield the same percentage recovery, several runs on a new column may be necessary before the separation becomes reproducible.

The results of a trial in our laboratory on between column variation using three Vydac TP20154 columns containing different batches of packing material are shown in Fig. 3. Note the peak responses, elution times, differences in profiles of minor peaks associated with main peaks and in particular peaks X and Y on column 3 which are 'contaminants' of zeaxanthin, on column 2 peak X co-elutes with lutein, on column 1,

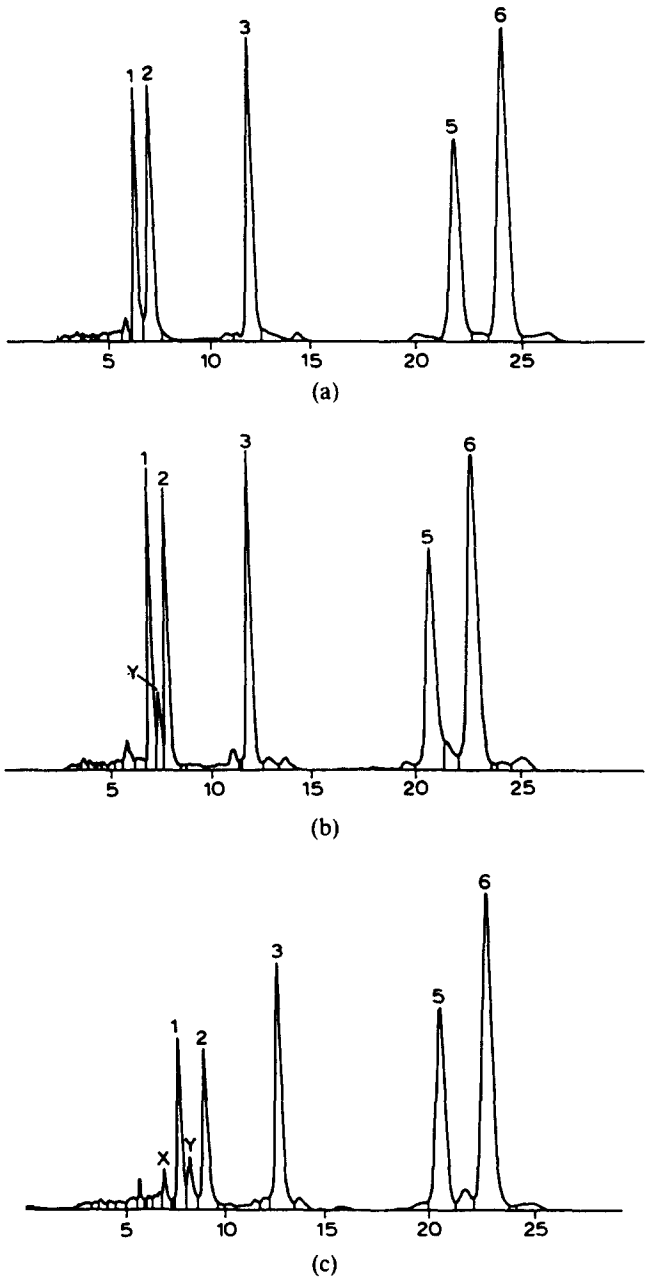

Fig. 3. Comparison of carotenoid responses on three VYDAC TP 201-54 columns containing different batches of packing materials: (a) VYDAC 1; (b) VYDAC 2; (c) VYDAC 3. 1, lutein; 2, zeaxanthin; 3, β-cryptoxanthin; 5, α-carotene; 6, β-carotene. Other conditions were as in Fig. 1. The conventional frits in VYDAC columns had not been replaced with metal free frits.

neither X or Y are eluted as individual peaks. A recently obtained batch of zeaxanthin did not contain these 'contaminants'.

Interactions between carotenoids, injection solvents and mobile phase can result in peak distortion and the production of artefacts which can lead to misinterpretation of the chromatogram. To ensure compatibility of the injection solvent with the mobile phase the carotenoids should preferably be injected in the mobile phase. However, owing to the wide range of solubilities of carotenoids extracted from natural sources, they can be more conveniently solubilised and injected in solvent(s) other than the mobile phase. Khachik *et al.* (1988) studied the effect of sample–solvent interactions on the resolution of HPLC peaks for α and β-carotene, lycopene, β-apo-8'-carotenal, canthaxanthin and zeaxanthin. They were injected in various solvents and volumes and chromatographed under various isocratic and gradient HPLC conditions and mobile phase composition. Depending on the solubility of the carotenoids in the HPLC mobile phase and the nature of the injecting solvent, single, double, and in some cases multiple HPLC peaks were produced. Isolation and structural elucidation of the individual carotenoid components indicated the additional peaks were artefacts of chromatography. The multiple peak formation was shown to be dependent on the relative solubility of the carotenoids in the injection and eluting solvents and the interaction of the solvents as the sample bolus first interacts with the column.

In our laboratory, it is the practice to evaporate stock carotenoid solutions under N_2 and dissolve in the mobile phase, containing 0·1% BHT. All the carotenoids were soluble, however some were more readily soluble than others. Our studies using lycopene confirmed Khachik's findings that the nature of the injection solvent effected peak response and elution time.

Lycopene in serum is reported to exist as several different isomers. Some columns and solvent systems resolve or partially resolve these isomers. In our studies a standard solution of predominantly *trans*-lycopene in chloroform degraded on standing to form several isomers. Over a 20 day period (stored at *c.* −20°C) the concentration of a stock lycopene solution in chloroform, calculated from absorption at 472 nm in hexane fell by 48%. There was also an apparent slight shift in the absorption maximum. In chloroform containing 0·1% BHT the loss was 11%. The chromatographic response of *trans*-lycopene from stock solution in chloroform injected in the mobile phase decreased by 56% (total lycopene by 46%). The corresponding decrease in stock solution containing BHT was *c.* 7% (total lycopene *c.* 3%). In stock solutions with or without BHT which had been stored evaporated the decrease was *c.* 7% (total lycopene *c.* 4%). However, in stored working solutions prepared on day zero in the mobile phase + BHT there was no apparent decrease over the 20 day period.

Figure 4 shows the effects of storage in chloroform with and without BHT and Fig. 5 in mobile phase containing BHT.

Extraction and saponification

Extraction and saponification of test materials is another very uncertain area. There are no universally accepted or standard methods. For extraction, the literature contains many different recommendations regarding choice of extracting solvent, e.g. acetone, methanol and ethanol are often used singly or in combination, others include THF, THF/petroleum ether, petroleum ether/acetone and diethylether/methanol.

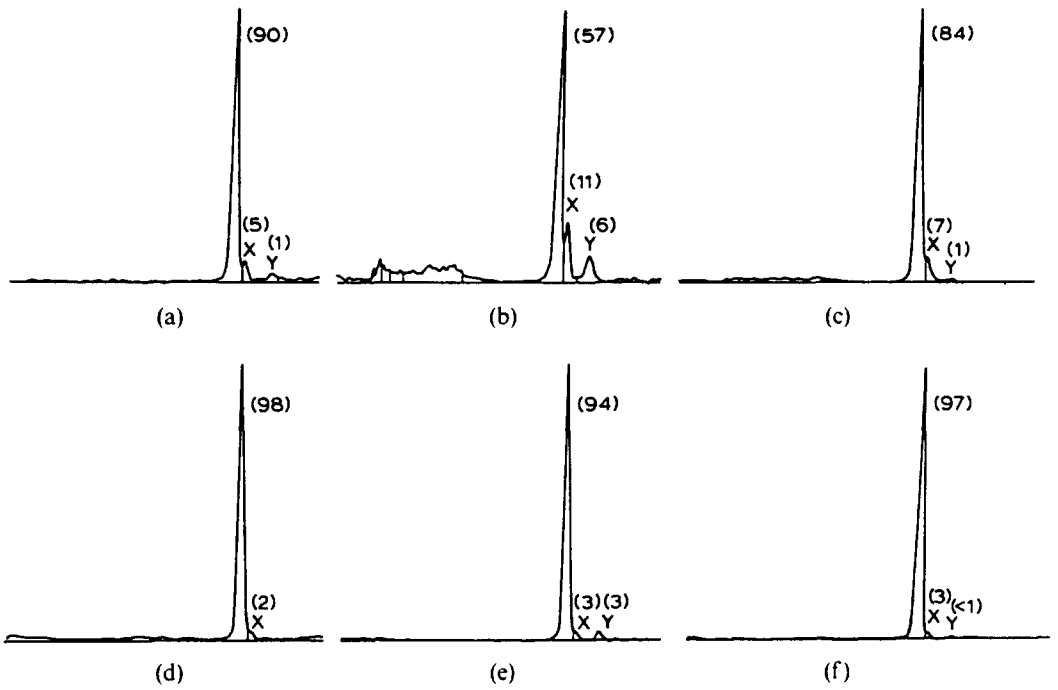

Fig. 4. Effect of storage on lycopene stock solution in chloroform with and without BHT: (a) from chloroform stock solution, day 0; (b) from chloroform stock solution stored, day 20; (c) from chloroform stock solution stored evaporated, day 20; (d) as (a) but containing 0·1% BHT; (e) as (b) but containing 0·1% BHT; (f) as (c) but containing 0·1% BHT. For analysis all stock solutions were evaporated as necessary and injected in mobile phase + 0·1% BHT. Figure in parentheses shows the areas of peaks expressed as % of total area. Other conditions as Fig. 1.

The use of THF stabilised with BHT has been employed by Khachik *et al.* (1986) for the extraction of fruit and vegetables. It not only solubilises carotenoids and chlorophylls but denatures protein-complexed carotenoids preventing the formation of emulsions. Concern has been expressed that this solvent is known to promote peroxide formation that may contribute to the production of artefacts. However in comparison with petroleum ether/acetone and diethyl ether/methanol no significant changes in qualitative or quantitative distribution of carotenoids and chlorophylls was observed (Khachik *et al.*, 1986).

In our laboratory the effectiveness of various solvents in the extraction of carotenoids was tested on dry and liquid materials (i.e. dried tomato soup and dried tomato soup suspended in water and carrot juice). As

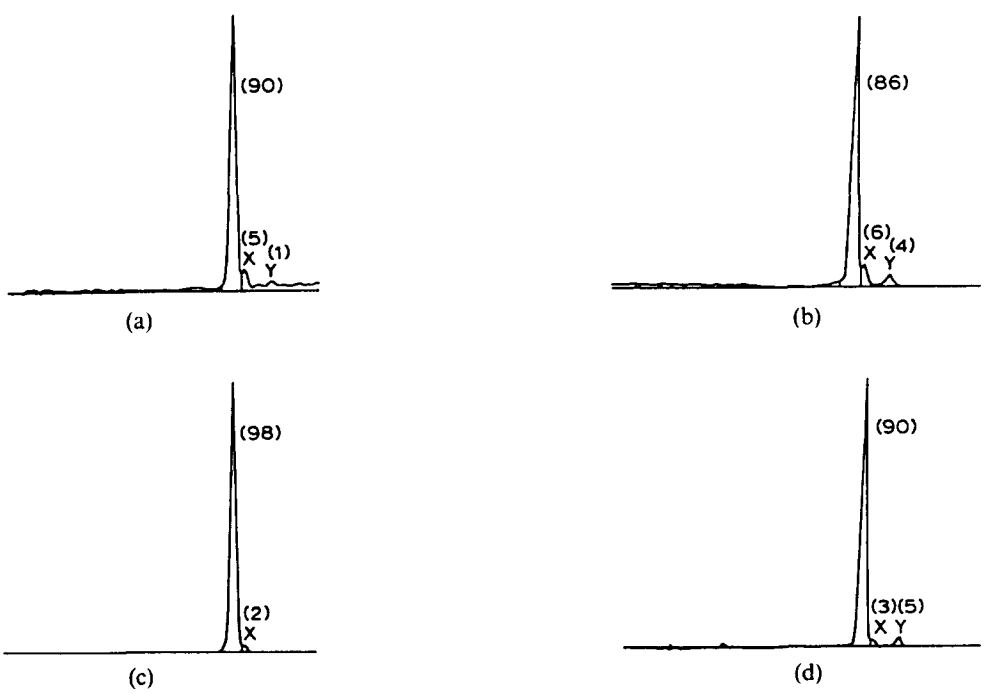

Fig. 5. Effect of storage of lycopene in mobile phase + BHT: (a) working solution (from chloroform stock solution) in mobile phase + 0·1% BHT, day 0; (b) as (a), day 20; (c) working solution (from chloroform stock solution + 0·1% BHT, day 0; (d) as (c), day 20. Figure in parentheses shows the areas of peaks expressed as a percentage of total area. Other conditions as in Fig. 1.

judged by the visual disappearance of colour from sampling to extracting fluid, for the dried material methanol were the most effective followed by ethanol; THF, acetone and hexane was less so. With liquid materials THF was the best, with carrot juice it also appeared to solubilise precipitable material, although with the dried tomato soup suspended in water not all material was solubilized. The use of methanol followed by THF, or THF followed by methanol, or a combination of THF and methanol appeared to result in efficient extraction and solubilisation. Subsequent tests with carrots and peas confirmed this finding.

Saponification of plant extracts combined with solvent partitioning prior to analysis is carried out as a 'purification' step to remove chlorophylls and hydrolyse carotenol esters. Various saponification conditions are suggested in the literature ranging from 5–60 min at 80–100°C, to 3–16 h at RT both in the presence and absence of antioxidants and nitrogen. Depending on the nature of the carotenoid, saponification may result in destruction or structural transformation. Khachik *et al.* (1986) noted that using 30% methanolic KOH for 3 h at RT under N_2 resulted in significant losses of xanthophylls but not carotenes. Similar findings have been noted in the author's laboratory. Miki *et al.* (1990) using a microwave oven and 2% methanolic KOH for 3 min to saponify an extract of marine fish obtained a yield of over 95% without *cis*-isomerisation compared to 6–10% methanolic KOH for 1 h at 80–90°C or 5–10 h at RT where the yield was < 85% and isomerisation frequently occurred. It may seem obvious to state that the mildest conditions that will do the job are best, but these are yet to be described satisfactorily. Only where problems related to the chromatography have been overcome is it possible to address those associated with extraction and saponification. Currently studies are underway in our laboratory to assess optimum conditions both for extraction and saponification on a wide range of materials, and will form the basis of a subsequent publication.

Quantification

Whilst most publications will give details relating to the concentration of stock standard solution calculated from extinction coefficients at absorption maximum, e.g.

$$\text{concentration (mmol l}^{-1}) = \frac{\text{Absorbance} \times \text{dilution factor}}{\text{mM ext. coefficient}}$$

invariably no information is given relating to 'purity', i.e. the peak area response as a percentage of the total area when a working solution is run through the column under assay conditions. Unless purities of carotenoids are taken into consideration large inaccuracies may occur.

$$\therefore \text{concentration} = \frac{\text{Absorbance} \times \text{dilution factor} \times \text{purity}}{\text{mM ext. coefficient}}$$

Bioavailability

There are over 500 naturally occurring carotenoids. Man, like other animals, cannot synthesise carotenoids and they must be obtained from the diet. An important nutritional function of certain carotenoids is their ability to act as vitamin A precursors. The structure of a carotenoid provitamin A compound must include at least one unsubstituted β-ione ring and a polyene side chain. The other end of the molecule may have a cyclic or an acyclic structure and be lengthened to not less than an 11 carbon polyene chain. β-carotene possesses two β-ione rings, one at either end of a long polyene chain. α-Carotene has approximately half of the vitamin A activity of β-carotene. Several factors may influence the biological activity (absorption and utilisation) of carotenoid pro-vitamin A precursors—the physical form of the carotenoid, the state of isomerisation, the dietary level administered, the dietary fat level and its state of saturation and oxidation, the dietary protein level, the presence of dietary antioxidants and vitamin A status. The availability of carotenoids from foods has been noted to be considerably less than that from purified sources. The bioavailability of β-carotene from carrots for example can also be affected by grating, homogenisation and cooking. Brown *et al.* (1989) reported the maximum plasma response to 29 mg β-carotene contained in cooked carrots was 21% of the response to 30 mg β-carotene in capsule form.

Although blood levels of carotenoids will reflect their consumption from foods, the amount which is bioavailable cannot be assumed to be the same as that calculated from analysed levels in those foods.

CONCLUSIONS

Our studies have demonstrated that there are a number of potential pitfalls associated with the analysis of carotenoids by HPLC.

1. Adequate separation of carotenoids and their isomers is complicated by the difference in responses between columns, not only on columns with different stationary phases, but even on columns with the same stationary phase.
2. Metal surfaces and in particular stainless steel frits may be damaging to the analytes. We suggest this problem may be overcome by the use of metal free columns or the replacement of stainless steel frits with biocompatible, e.g. teflon, frits.
3. Reactions between carotenoids, injection solvents and the mobile phase can cause chromatographic artefacts.
4. Suitable precautions, such as the addition of an antioxidant (BHT) or evaporation, should be taken to avoid losses in stock solutions of the more labile carotenoids like lycopene.
5. The potential for losses occurring during extraction and saponification requires further investigation.

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