ELSEVIER

PII: S0308-8146(96)00319-6

Food Chemistry, Vol. 60, No. 1, pp. 109–117, 1997
© 1997 Elsevier Science Ltd
All rights reserved. Printed in Great Britain
0308-8146/97 $17.00+0.00
Analytical, Nutritional and Clinical Methods Section

A modified method to minimise losses of carotenoids and tocopherols during HPLC analysis of red palm oil

G. Lietz & C. J. K. Henry

School of Biological and Molecular Sciences, Oxford Brookes University, Oxford OX3 0BP, UK

(Received 5 July 1996; accepted 7 October 1996)

A well-established saponification method for red palm oil was compared with a newly developed enzymatic hydrolysis method followed by analysis using high-performance liquid chromatography. Applying the saponification method to extract carotenoids from red palm oil resulted in soap precipitation and significant losses of α- and β-carotene ($P < 0.01$) and α-tocopherol ($P < 0.05$). The process also facilitated the isomerisation of α-carotene and β-carotene ($P < 0.05$). Using the non-specific *Candida cylindracea* lipase, complete hydrolysis of red palm oil was achieved after 4 h and no significant loss of carotenoids and tocopherols occurred during the extraction procedure. Mild hydrolysis with the use of *Candida cylindracea* lipase enables quantitative analysis of carotenoids and tocopherols in red palm oil without isomerisation or destruction of either carotenoids or tocopherols. The present method is comparable or shorter in time than the conventional saponification method. © 1997 Elsevier Science Ltd

INTRODUCTION

The edible grade of unrefined red palm oil is the richest and cheapest natural source of β-carotene. It contains about 15–300 times the retinol equivalent of carrots, leafy green vegetable and tomatoes (May, 1994). β-Carotene is the most important provitamin A source and there is evidence that this carotenoid may serve to protect against certain cancers (Olson, 1986; Temple & Basu, 1988). The major source of vitamin A in the diet of most communities in less industrialised countries is β-carotene from plant foods, since preformed vitamin A in meat, liver and eggs is out of reach of the economically deprived. To determine the provitamin A values of plant foods accurately, the following aspects of analysis need to be considered:

- Separation from biologically inactive carotenoids.
- Separation and quantification of each provitamin individually.
- Quantification of the *cis* and *trans* isomers of each provitamin.

At each stage of the analysis there is considerable scope for losses and isomerisation of carotenoids and fat-soluble vitamins. Thus, provitamin A determination has been accomplished with varying degrees of success (Rodriguez-Amaya & Tavares, 1992). In fruits and vegetables, β-carotene is demonstrated to undergo isomerisation during food processing such as cooking, drying and canning (Sweeney & March, 1970, 1971; Chandler & Schwartz, 1987). Similarly, pure β-carotene isomerises in response to light and heat and in the presence of iodine or acids (Zechmeister, 1944; Tsukida *et al.*, 1982). On the other hand, the vitamin A activity of *cis* isomers of β-carotene and other carotenoids is less than that of the all-*trans* carotenes (Sweeney & March, 1971; Gaziano *et al.*, 1995). The isomerisation of carotenes during analysis has to be restricted in order to maximise the success of obtaining a true value of *cis* and *trans* isomers in the sample.

High-performance liquid chromatography (HPLC) is an excellent method for the separation and quantification of provitamin A carotenoids and their isomers. Prior to the analysis of carotenoids and tocopherols, saponification has often been employed as an effective purification step for the removal of chorophylls and lipids and to release the esterified xanthophylls. However, several researchers have found significant losses of carotenoids after saponification (Khachik *et al.*, 1986; Kimura *et al.*, 1990; Craft & Granado-Lorencio, 1992). Furthermore, it has been shown that prolonged exposures to alkaline conditions resulted in significant losses of α-tocopherol (Craft & Granado-Lorencio, 1992).

Table 1. The effect of extraction method on carotenoid and tocopherol concentration

	Undigested standard	Standard digested by enzymatic hydrolysis[b]	Standard digested by saponification[b,c]
trans-α-Carotene (μg ml^{-1})[a]	0.54 ± 0.005	0.54 ± 0.009	0.44 ± 0.02**
cis-α-Carotene (%)	0.36 ± 0.1	0.43 ± 0.08	0.683 ± 0.08
trans-β-Carotene (μg ml^{-1})[a]	4.6 ± 0.04	4.6 ± 0.06	3.3 ± 0.2**
cis-β-Carotene (%)	4.0 ± 1.2	4.3 ± 0.2	8.0 ± 0.4*
δ-Tocopherol (μg ml^{-1})[a]	58.2 ± 1.0	60.5 ± 4.8	65.9 ± 1.3**
γ-Tocopherol (μg ml^{-1})[a]	60.6 ± 1.3	60.9 ± 2.5	64.1 ± 2.3
α-Tocopherol (μg ml^{-1})[a]	100.9 ± 3.2	106.5 ± 2.8	83.2 ± 6.8*

[a]Values are given as the mean ± SEM.
[b]Corrected for losses during extraction using the internal standard.
[c]According to Ng and Tan (1988).
*$p < 0.05$ significantly different from undigested standard.
**$p < 0.01$ significantly different from undigested standard.

In order to minimise these effects, an HPLC method developed by Hart and Scott (1995) has been modified using *Candida cylindracea* lipase for mild hydrolysis. The microbial lipase from *C. cylindracea* is a non-specific lipase (Macrae, 1983) and preferentially hydrolyses C_{14} to C_{18} saturated and mono-unsaturated fatty acids (Lie & Lambertsen, 1986). As red palm oil consists of 44% $C_{16:0}$ and 39% $C_{18:1}$ (May, 1994), *C. cylindracea* seems to be an optimal tool to hydrolyse red palm oil.

MATERIALS AND METHODS

Materials

Red palm oil was provided by the Palm Oil Research Institute of Malaysia (PORIM). *Candida cylindracea* lipase (943 units mg^{-1} solid), ascorbic acid and butylated hydroxytoluene (BHT) were purchased from Sigma Chemicals (Poole, UK). All other reagents and adsorbents were of analytical grade and were purchased from Merck (Lutterworth, UK).

Calibration solution

α- and β-carotene, α-, γ- and δ-tocopherol were purchased from Sigma; *trans*-β-apo-10′-carotenal for the synthesis of the internal standard *trans*-β-apo-10′-carotenal oxime according to Handelman *et al.* (1988) was a kind gift from Hoffman-La Roche (Basle, Switzerland). The stock and working solutions of the carotenoids were prepared according to Hart and Scott (1995); the tocopherols were dissolved in heptane to prepare stock solutions, and the concentration determined in ethanol using the specific extinction coefficients given by Franke *et al.* (1993).

Lipase-catalysed hydrolysis of the oil

Hydrolysis using separating funnels
The lipase-catalysed hydrolysis of the oil was carried out in a 50 ml flask at 35°C containing 20 mg of *C. cylindracea* lipase, 200 mg of red palm oil, sodium phosphate buffer (pH 7.0, 0.08 M, 35 ml) and ascorbic acid (50 ppm). The mixture was stirred under an atmo-

Table 2. Hydrolysis of red palm oil catalysed by *Candida cylindracea* lipase at 35°C in phosphate buffer (pH 7.0, 0.08 M)

Time (h)	Component	Amount in total products (wt%)	Fatty acid composition (%)				
			14:0	16:0	18:0	18:1	18:2
0	Acylglycerols	97.1	3.6	46.4	6.2	35.1	8.8
0	Fatty acids	2.9	35.3	43.2	11.2	7.1	3.2
0.5	Acylglycerols	77.3	0.0	36.8	11.3	36.3	15.7
0.5	Fatty acids	22.7	14.3	35.2	6.6	31.7	12.2
1	Acylglycerols	64.0	0.0	40.2	13.3	29.5	17.1
1	Fatty acids	36.0	15.5	40.4	5.9	27.7	10.4
2	Acylglycerols	29.5	0.0	44.4	20.2	31.0	4.4
2	Fatty acids	70.5	4.0	51.3	4.8	33.8	6.1
3	Acylglycerols	12.9	4.2	14.5	42.0	13.9	25.5
3	Fatty acids	87.1	1.3	41.5	4.0	41.0	12.1
4	Acylglycerols	3.8	0.0	70.9	0.0	0.0	29.0
4	Fatty acids	96.2	0.9	42.1	4.2	41.4	11.3
8	Acylglycerols	6.6	0.0	85.2	0.0	0.0	14.8
8	Fatty acids	93.4	3.9	43.5	4.8	38.8	9.0
16	Acylglycerols	2.8	0.0	86.3	0.0	0.0	13.7
16	Fatty acids	97.2	2.7	44.8	4.9	39.5	8.1

sphere of nitrogen. This method was a modification of the procedure reported by Rahmatullah *et al.* (1994).

The reaction mixture was extracted three times with 20 ml diethyl ether/petroleum ether (2:1, v/v) containing 0.1% BHT. The three extracts were combined and washed three times with 50 ml 10% sodium chloride solution, dried over anhydrous sodium sulphate and finally the organic solvent was evaporated by rotary evaporation under reduced pressure at 30°C under an atmosphere of nitrogen. The residue was redissolved by ultrasonic agitation to a final volume of 5 ml in acetone, filtered through a 0.45 μm PVDF syringe filter (Whatman), and 10 μl were injected directly after preparation for HPLC analysis according to Hart and Scott (1995).

Small-scale hydrolysis using universal containers

The lipase-catalysed hydrolysis of the oil was carried out in universal glass containers at 35°C containing 10 mg of *C. cylindracea* lipase, 100 mg of red palm oil, sodium phosphate buffer (pH 7.2, 0.08 M, 10 ml) and ascorbic acid (1 ml of a 1% solution). This was stirred under an atmosphere of nitrogen.

The reaction mixture was extracted four times with 3 ml diethyl ether/petroleum ether (2:1, v/v) containing 0.1% BHT and centrifuged at 2000 rpm for 5 min. The four extracts were combined and dried over anhydrous sodium sulphate, and finally the organic solvent was evaporated by rotary evaporation under reduced pressure at 30°C under an atmosphere of nitrogen. The residue was redissolved by ultrasonic agitation to a final volume of 5 ml in acetone, filtered through a 0.45 μm PVDF syringe fitter (Whatman), and 20 μl were injected directly after preparation for HPLC analysis according to Hart and Scott (1995).

High-performance liquid chromatography

The HPLC system comprised of a dual-piston solvent delivery pump (Polymer Laboratories), an AS3500 autosampler (Dionex), a LC1210 UV/VIS detector, a LC1250 fluorescence detector (Polymer Laboratories) and a LC/GC software system for data acquisition (Polymer Laboratories). The column system, column temperature and mobile phase were as described by Hart and Scott (1995), except for the omission of BHT from the moblie phase, which was done to increase the sensitivity of the fluorescence detection. Samples were injected via the AS3500 autosampler with a volume of

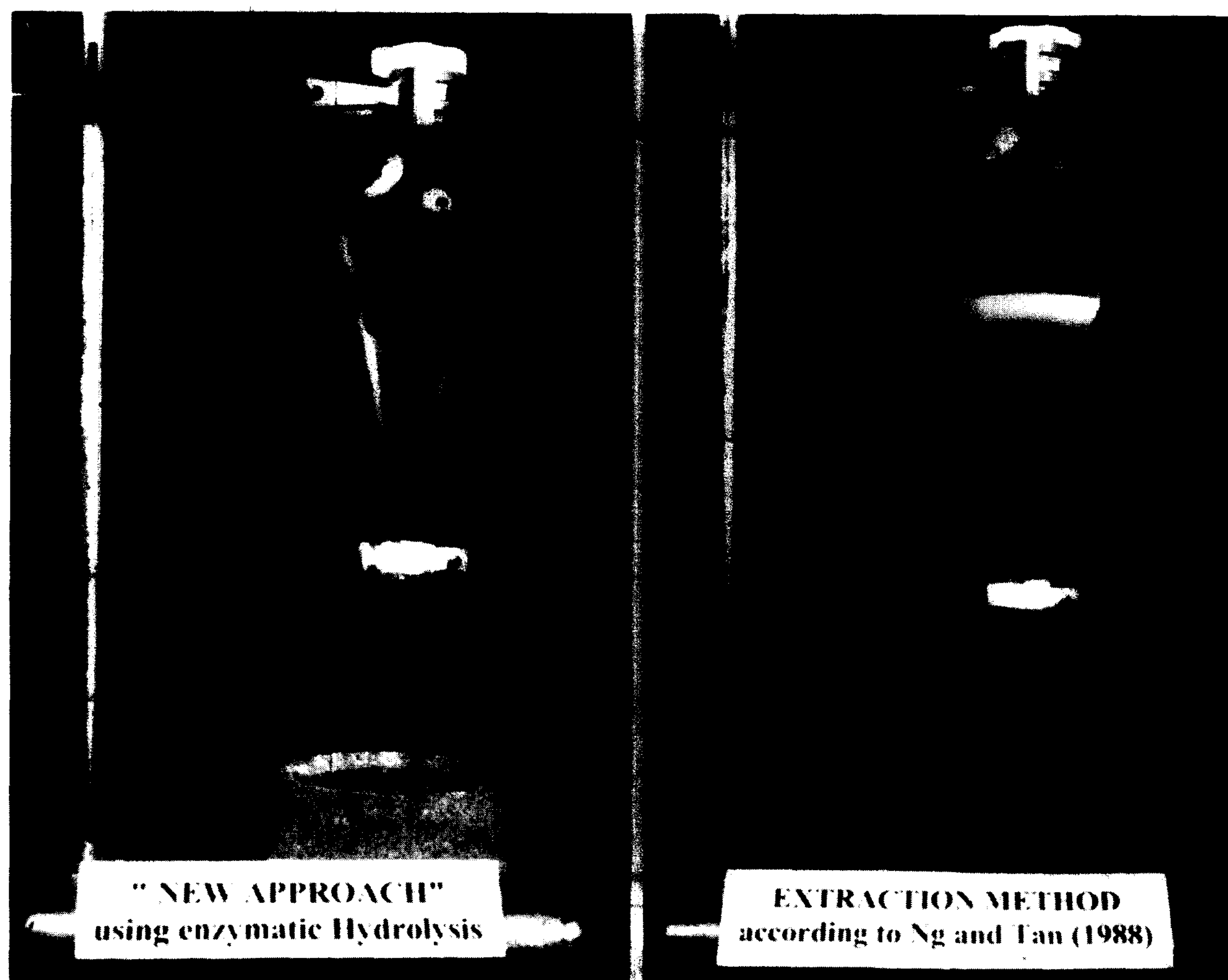

Fig. 1. Ether extraction of red palm oil samples digested by saponification and enzymatic hydrolysis.

10 or 20 μl per sample, and were held at 15°C in sealed amber vials to avoid evaporation and degradation. Peak responses of the carotenoids were measured at 450 nm using the UV/Vis detector, and the peak responses of the tocopherols were measured using the fluorescence detector with excitation and emission wavelength set at 298 nm and 328 nm, respectively. Columns were flushed first with chloroform/methanol (50:50, v/v) and than with methanol alone at the end of each day. This was done to ensure a reasonable column lifetime and to prevent precipitation problems.

Analysis of fatty acid and acylglycerol fractions after enzymatic hydrolysis

The fractions of fatty acids and acylglycerols recovered from lipase-catalysed hydrolysis of red palm oil were fractionated into fatty acids and acylglycerols (tri-

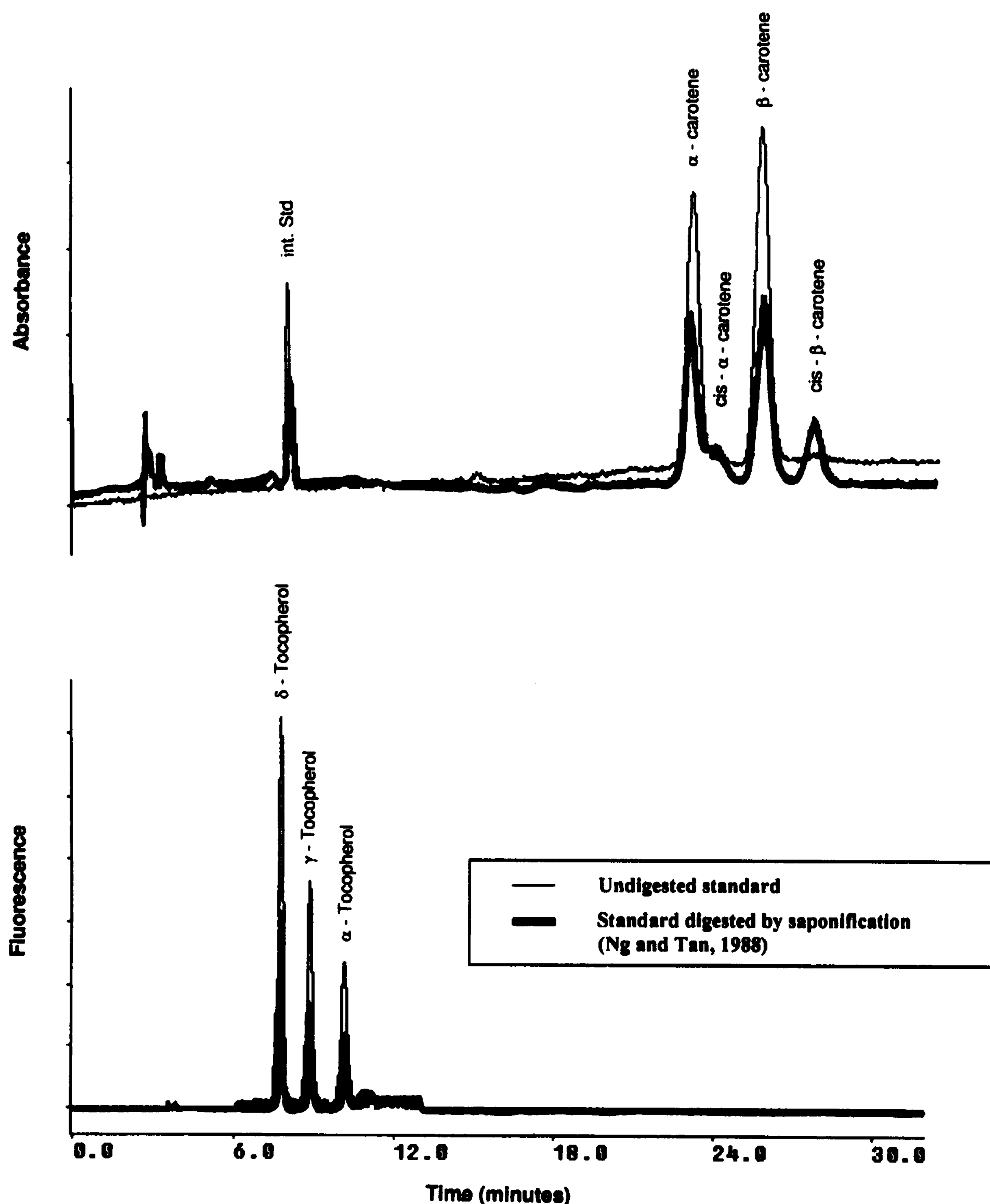

Fig. 2. HPLC chromatogram of a saponified and undigested standard. Internal standard: apo-10′-carotenal oxime.

+ di- + monoacylglycerols) by thin-layer chromatography (TLC) according to Rahmatullah *et al.* (1994). Both acylglycerols and fatty acids were then converted to methyl esters by adding 1 ml of sulphuric acid (2.5% in dry methanol) and heating for 2 h at 70°C. After cooling the samples, 5 ml of 5% sodium chloride solution were added and the methyl esters were extracted three times with redistilled petroleum ether (60–80°C b.p.). The combined ether phase was dried under nitrogen and resuspended in 30 μl redistilled petroleum ether prior to GC as described previously (Avery *et al.*, 1994).

Statistical analysis

Differences between methods were tested using Student's *t*-test. All values shown are mean ± standard error of the mean.

RESULTS AND DISCUSSION

To circumvent problems associated with the destruction and/or isomerisation of carotenoids and fat-soluble vitamins during sample preparation, a mild extraction

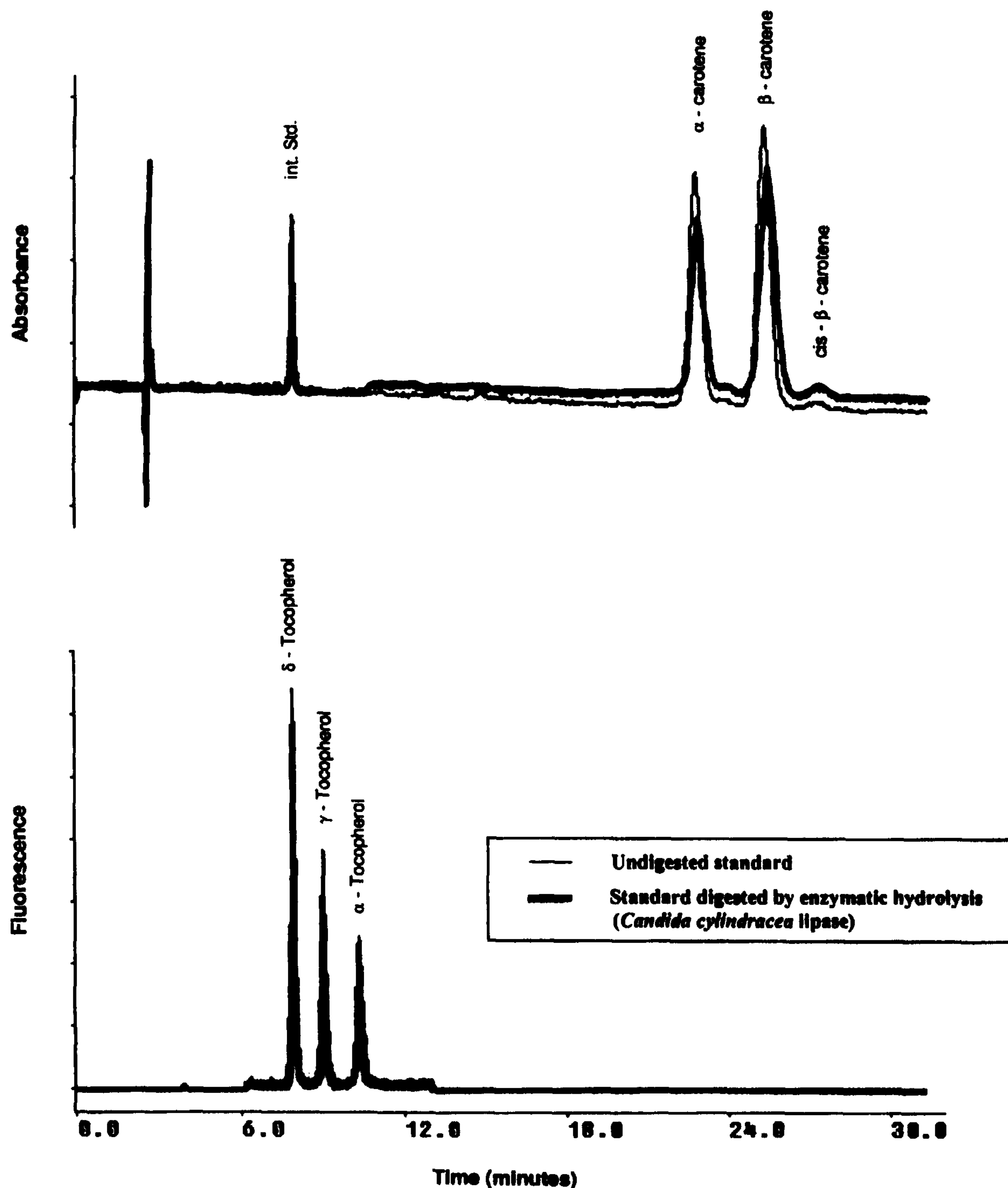

Fig. 3. HPLC chromatogram of an enzymatically hydrolysed and undigested standard. Internal standard: apo-10′-carotenal oxime.

procedure with the use of the non-specific *C. cylindracea* lipase was developed (Table 1). Complete hydrolysis of the red palm oil was achieved after 4 h of digestion under an atmosphere of nitrogen and in the presence of ascorbic acid (Table 2). Using this approach, no significant loss of either carotenoids and tocopherols or isomerisation of carotenoids occurred during the extraction procedure in a standard mixture (Table 1 and Fig. 3). After correcting for losses during the extraction, recovery of all-*trans* α- and β-carotene, δ-, γ- and α-tocopherol in the enzymatically digested standard was 100%, 100%, 104%, 101% and 106%, respectively. This calculation was done using the internal standard *trans*-β-apo-10′-carotenal oxime.

Carotenoid extracts from natural sources are usually saponified to remove chlorophyll, unwanted lipids and for hydrolysing carotenol esters (Kimura *et al.*, 1990). The saponification of carotenoid extracts can result in the destruction and structural transformation of some carotenoids. Ng and Tan (1988) stated that there was an inadvertent *cis*-isomerisation of carotenoids during extractions. Their method, which is the only comparable method to extract carotenoids from red palm oil, resulted in soap precipitation during saponification. These soaps could not be filtered sufficiently, and filtered soaps contained large amounts of carotenoids. In an attempt to re-extract these carotenoids using an ether mix, it was found that the soaps redissolved into the ether phase and precipitated in the separating funnel (Fig. 1). It was therefore not possible to achieve a clear separation between the water and ether epiphase and thus to determine the amount of carotenoids quantitatively. Using the method from Ng and Tan (1988), significant losses of α- and β-carotene ($P < 0.0\ 1$) and α-tocopherol ($P < 0.05$), as well as isomerisation of α-carotene and β-carotene ($P < 0.05$), occurred in a standard mixture (Table 1 and Fig. 2). After correcting for losses during the extraction, recovery of all-*trans* α- and β-carotene, δ-, γ- and α-tocopherol in the saponified standard was 81%, 72%, 113%, 106% and 82%, respectively. These percentages were calculated using the internal standard *trans*-β-apo-10′-carotenal oxime.

Khachik *et al.* (1986) investigated the effect of saponification on both qualitative and quantitative distribution of carotenoids in green vegetables by evaluating HPLC profiles before and after alkali treatment. Their quantitative evaluation showed that saponification was accompanied by significant loss of xanthophylls, particularly the epoxycarotenoids, whilst losses of carotenes were not significant. In addition, Kimura *et al.* (1990), looking at the effect of saponification on the carotenoids of tomato, kale and papaya, observed that saponifica-

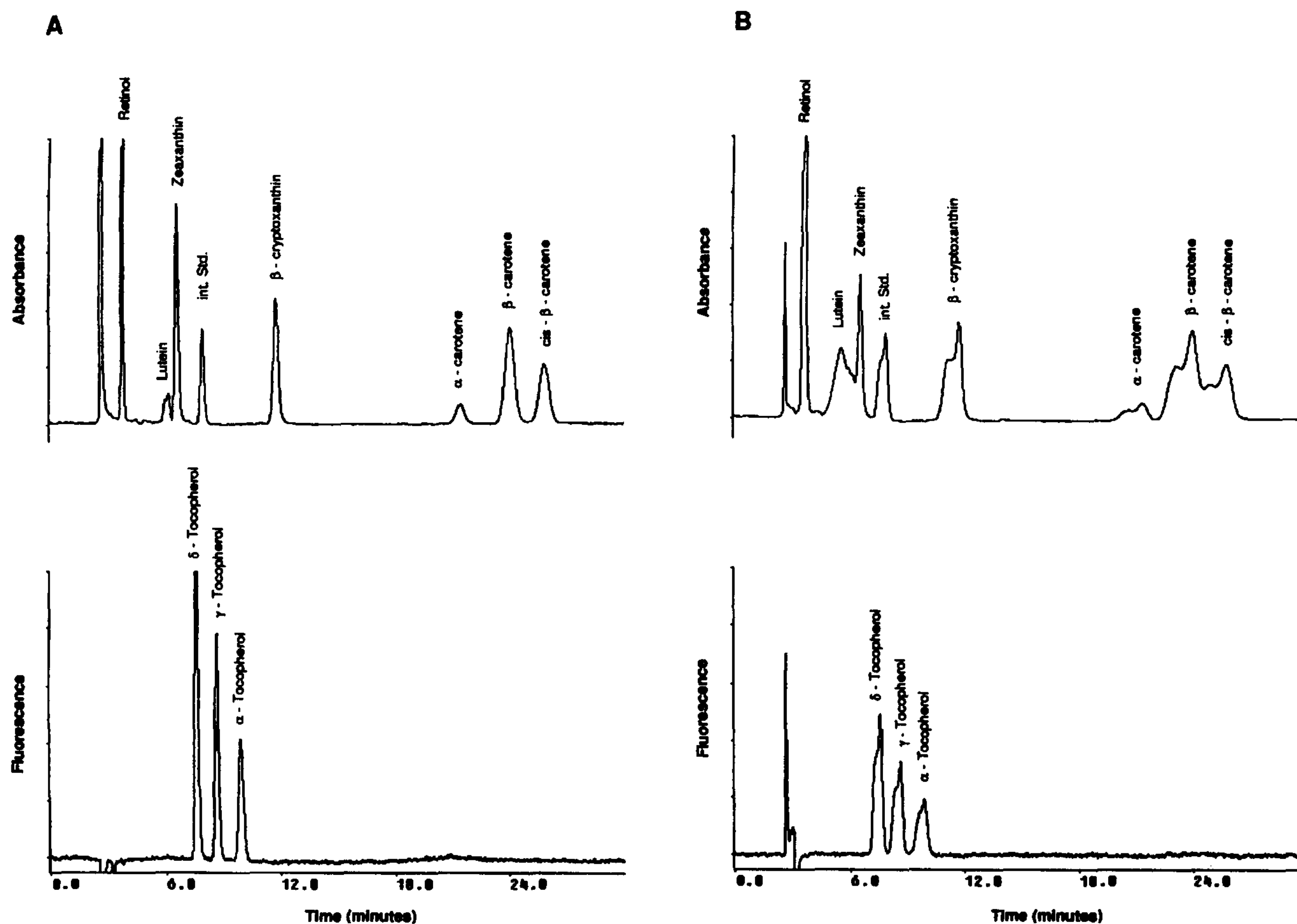

Fig. 4. Dependency of the separation of carotenoids and tocopherols on the sample solvent. A, injecting solvent acetone, injecting volume 20 μl. B, injecting solvent dichloromethane, injecting volume 20 μl. Internal standard: apo-10′-carotenal oxime.

tion retained β-, γ-carotene, β-apo-8′-carotenal and lycopene and completely hydrolysed the carotenol esters in papaya. However, even with this mild saponification, lutein, zeaxanthin and violaxanthin degraded significantly. In a study to analyse the effects of sample preparation on fat-soluble vitamin and carotenoid concentration (Craft & Granado-Lorencio, 1992), significant losses of α-tocopherol and isomerisation of retinol and β-carotene occurred. It is well known that losses of carotenoids can be reduced by using antioxidants during saponification. Butylated hydroxytoluene (BHT) is often used for this purpose. However, BHT

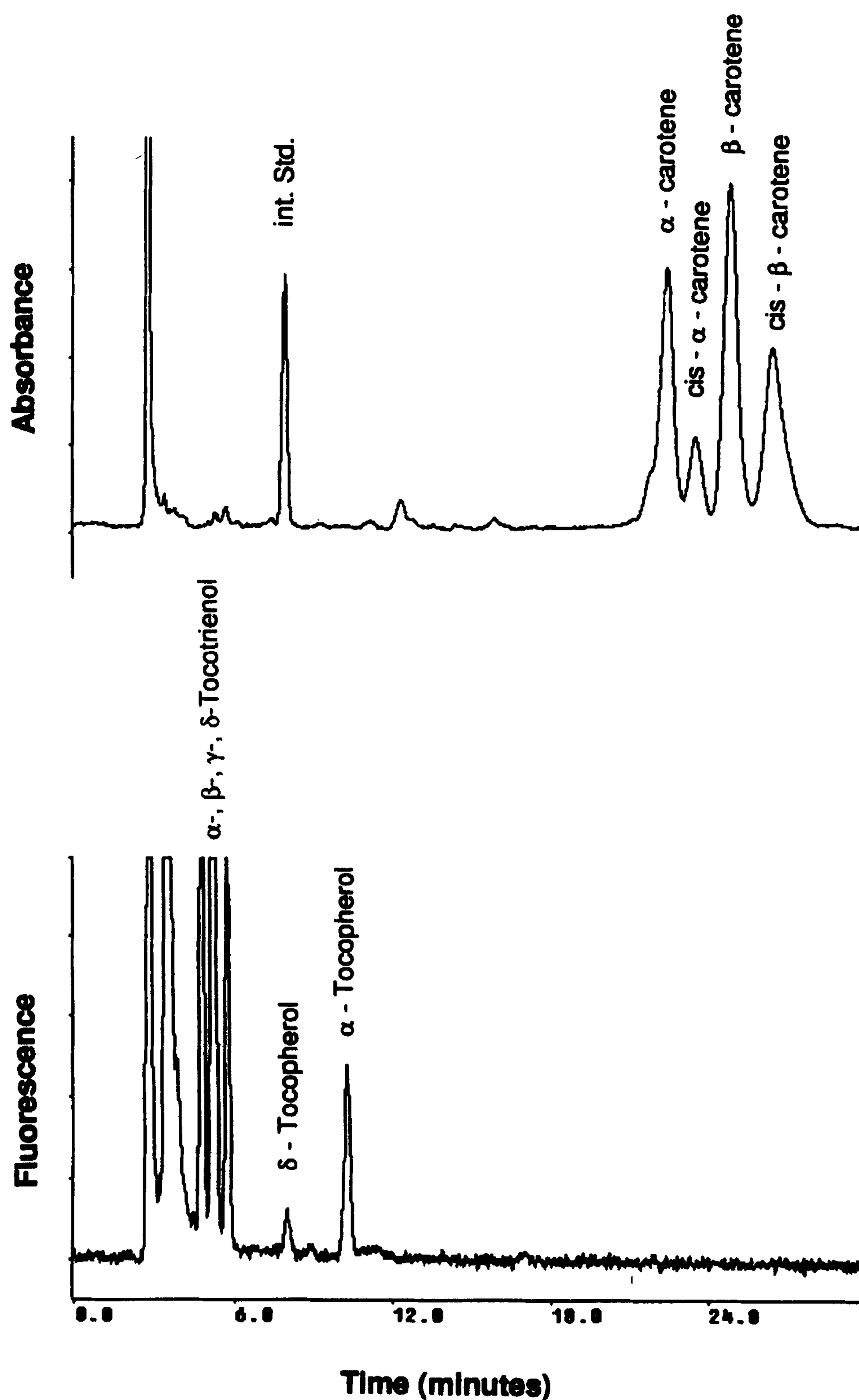

Fig. 5. HPLC chromatogram of an enzymatically hydrolysed red palm oil sample. Internal standard: apo-10′-carotenal oxime.

polymerises during saponification, producing a series of peaks reported to coelute and interfere with the measurement of retinol, tocopherols and xanthophylls (Craft & Granado-Lorencio, 1992).

The choice of extraction and injection solvent of carotenoids and fat-soluble vitamins plays a central role in the analysis. The extraction solvent used in this study was a mixture of diethyl ether/petroleum ether. This mixture was found to be a more effective extractant for carotenoids and fat-soluble vitamins than petroleum ether alone (Craft & Granado-Lorencio, 1992). The injection solvent for the analysis of carotenoids by HPLC was carefully selected so that chromatographic artefacts were not produced (Fig. 4). Khachik *et al.* (1988) showed that injection solvents such as methylene chloride, chloroform, tetrahydrofuran, benzene and toluene can result in HPLC peak distortion of carotenoids. This was thought to be due to the incompatible polarity and solubility properties of the injection solvent and the mobile phase. Khachik *et al.* (1988) thus concluded that the polarity and solubility properties of injection solvent and mobile phase should be compatible. The chosen injection solvent in this study was acetone because it has similar polarity and solubility properties as the mobile phase used. Acetone also solubilised the sterols of red palm oil after sample concentration and kept them in solution at 15°C in the autosampler. To circumvent the problem of peak distortion, only 10–20 μl of sample were injected. We decided to analyse the samples in acetone directly after preparation, in contrast to Ng and Tan (1988) who precipitated the sterols overnight in a freezer at −20°C and stored the extracts until analysis in dichloromethane. This was carried out since the isomerisation of carotenoid pigments begins as soon as they are solubilised and cannot be prevented by storing solutions in the cold (Lesellier *et al.*, 1993). Additionally, it was shown by Pesek *et al.* (1990) that the rate and/or extent of isomerisation of β-carotene was greatest in chlorinated solvents.

CONCLUSIONS

Applying a mild hydrolysis process using *C. cylindracea* lipase enables quantitative analysis of carotenoids and tocopherols in red palm oil without isomerisation or destruction of either carotenoids or tocopherols (Fig. 5). Complete hydrolysis of red palm oil is achieved only after 4 h and therefore compares favourably with the conventional saponification methods, which normally consumes 3–16 h. The sample preparation time can be significantly shortened when the analysis is carried out in universal containers. Four to eight samples may easily be prepared in the same time as it takes to prepare a single sample using the separating funnel. The new hydrolysis method may be used to analyse any oil sample, where saponification is required as a first step. More reliable data of stercoisomers of carotenoids as well as quantities of fat-soluble vitamins in various oil samples should be collated using this new analytical technique.

ACKNOWLEDGEMENTS

Our gratitude extends to Dr Kumar D. Mukherjee of the Bundesanstalt für Getreide-, Kartoffel- und Fettforschung in Münster, Germany, and to Dr Tony Bradshaw at Oxford Brookes University for their help and advice. We also acknowledge the funding from Oxford Brookes University, Dr W. Schüep from Hoffman–La Roche for trans-β-apo-10′-carotenal and PORIM for the red palm oil sample.

REFERENCES

Avery, S. V., Harwood, J. L. & Lloyd, D. (1994). Low-temperature-induced adaptations in fatty acid metabolism of *Acanthamoeba castellanii* cultures of different ages: relationship to changes in cell division, oxygen uptake and phagocytotic activity. *Microbiology*, **140**, 2423–2431.

Chandler, L. A. & Schwartz, S. J. (1987). HPLC separation of *cis–trans* carotene isomers in fresh and processed fruits and vegetables. *J. Food Sci.*, **52**, 669–672.

Craft, N. E. & Granado-Lorencio, F. (1992). Effect of sample preparation on fat-soluble vitamin and carotenoid concentrations. Paper presented at the Experimental Biology Meeting, 25 March to 2 April 1992, New Orleans, USA.

Franke, A. A., Custer, L. J. & Cooney, R. V. (1993). Synthetic carotenoids as internal standards for plasma micronutrient analyses by high-performance liquid chromatography. *J. Chromatogr.*, **614**, 43–57.

Gaziano, J. M., Johnson, E. J., Russel, R. M., Manson, J. E., Stampfer, M. J., Ridker, P. M., Frei, B., Hennekens, C. H. & Krinsky, N. E. (1995). Discrimination in adsorption or transport of β-carotene isomers after oral supplementation with either all-*trans*- or 9-*cis*-β-carotenc. *Am. J. Clin. Nutr.*, **61**, 1248–1252.

Handelman, G. J., Dratz, E. A., Reay, C. C. & Kuijk, F. J. G. M. (1988). Carotenoids in the human macula and whole retina. *Invest. Ophthalmol. Vis. Sci.*, **29**, 850–855.

Hart, D. J. & Scott, K. J. (1995). Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. *Food Chem.*, **54**, 101–111.

Khachik, F., Beecher, G. R. & Whitaker, N. F. (1986). Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. *J. Agric. Food Chem.*, **34**, 603–616.

Khachik, F., Beecher, G. R., Vanderlice, J. T. & Furrow, G. (1988). Liquid chromatographic artefacts and peak distortion: sample–solvent interactions in the separation of carotenoids. *Anal. Chem.*, **60**, 807–811.

Kimura, M., Rodriguez-Amaya, D. B. & Godoy, H. T. (1990). Assessment of the saponification step in the quantitative determination of carotenoids and provitamins A. *Food Chem.*, **35**, 187–195.

Lesellier, E., Tchapla, A., Marty, C. & Lebert, A. (1993). Analysis of carotenoids by high-performance liquid chro-

matography and supercritical fluid chromatography. *J. Chromatogr.*, **633**, 9–23.

Lie, Ø. & Lambertsen, G. (1986). Fatty acid specificity of *candida cylindracea* lipase. *Fette Seifen Anstrichm.*, **88**(9), 365–367.

Macrae, A. R. (1983). Lipase catalysed interesterification of oils and fats. *J. Am. Oil Chem. Soc.*, **60**, 291.

May, C. Y. (1994). Palm oil carotenoids. *Food Nutr. Bull.*, **15**(2), 130–137.

Ng, J. H. & Tan, B. (1988). Analysis of palm oil carotenoids by HPLC with diode-array detection. *J. Chromatogr. Sci.*, **26**, 463–469.

Olson, J. A. (1986). Carotenoids, vitamin A and cancer. *J. Nutr.*, **116**, 1127–1130.

Pesek, C. A., Warthesen, J. J. & Taoukis, P. S. (1990). A kinetic model for equilibration of isomeric carotenes. *J. Agric. Food Chem.*, **38**, 41–45.

Rahmatullah, M. S. K. S., Shukla, V. K. S. & Mukherjee, K. D. (1994). Enrichment of gamma linolenic acid from evening primrose oil and borage oil via lipase catalysed hydrolysis. *J. Am. Oil Chem. Soc.*, **71**(6), 569–573.

Rodriguez-Amaya, D. B. & Tavares, C. A. (1992). Importance of *cis*-isomer separation in determining provitamin A in tomato and tomato products. *Food Chem.*, **45**(4), 297–302.

Sweeney, J. P. & March, A. C. (1970). Separation of carotene stereoisomers in vegetables. *J. Assoc. Off. Anal. Chem.*, **53**, 937–940.

Sweeney, J. P. & March, A. C. (1971). Effect of processing on provitamin A in vegetables. *J. Am. Diet. Assoc.*, **59**, 238–243.

Temple, N. J. & Basu, T. K. (1988). Does β-carotene prevent cancer? A critical appraisal. *Nutr. Res.*, **8**, 685–701.

Tsukida, K., Saiki, K., Takii, T. & Koyama, Y. (1982). Separation and determination of *cis/trans* β-carotenes by high-performance liquid chromatography. *J. Chromatogr.*, **245**, 359–364.

Zechmeister, L. (1944). *Cis–trans* isomerization and stereochemistry of carotenoids and diphenylpolyenes. *Chem. Rev.*, **34**, 267–344.