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Assessment of the Saponification Step in the Quantitative Determination of Carotenoids and Provitamins A

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ABSTRACT

The saponification step in the determination of carotenoids was reassessed. Synthetic carotenoids (β-apo-8'-carotenal, β-carotene) and food samples (tomato, kale, papaya) were used and different procedures were evaluated. Hot saponification resulted in greater losses and cis- *and epoxycarotenoids were formed. The degradation was aggravated by a more direct contact between the carotenoids and the alkali. The AOAC procedure, performed in the presence of acetone, led to complete transformation of β-apo-8'-carotenal to citranaxanthin. Saponification was unnecessary for kale and tomato but was needed for good separation of papaya carotenoids which included carotenol esters. Saponification of the carotenoids dissolved overnight at room temperature in petroleum ether, with equal volume of 10% methanolic KOH, retained β-, γ-carotene, β-apo-8'-carotenal and lycopene and completely hydrolysed the carotenol esters. However, even with this mild saponification, lutein, zeaxanthin and violaxanthin degraded significantly. These losses could be reduced to insignificant levels by using an atmosphere of nitrogen or antioxidant.*

INTRODUCTION

The persistence of the vitamin A deficiency problem in developing countries and the recognition of a possibly wider physiological role for carotenoids beyond the provitamin A activity has drawn a lot of attention to the analytical procedure for these compounds. Nevertheless, the state of development of the rather complicated methodology involved gives 'conflicting' results (Beecher & Vanderslice, 1984). Part of the problem is that

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there are many naturally occurring carotenoids, and food samples vary markedly not only in quantitative but also in qualitative composition. Another major problem is the possibility of quantitative losses and artifact formation during the analysis, because of the instability of the highly unsaturated carotenoid molecule.

Saponification with potassium hydroxide has long been an integral part of both vitamin A and carotenoid or provitamin A analyses. For carotenoids, it is considered the most efficient procedure for removing chlorophyll and unwanted lipids and for hydrolysing carotenol esters (Liaaen-Jensen, 1971; Davies, 1976). For vitamin A, it serves to free the vitamin from the food matrix, convert esters to free alcohol and remove bulk components such as triglycerides (Van Niekerk, 1982). Due to the slightly differing purposes, saponification in vitamin A analysis is accomplished directly with the sample prior to extraction, while in carotenoid determination extraction generally precedes saponification.

There have been attempts to eliminate this inconvenient and time-consuming step in vitamin A analysis (Van Niekerk, 1982; Parrish, 1984). However, Thompson (1986) affirmed that vitamin A is not destroyed during alkali hydrolysis with pyrogallol and organic solvents do not extract vitamin A quantitatively from protected preparations, such as those coated by gelatin, without saponification. In terms of carotenoids, Khacik *et al.* (1986) reported a 6% loss in the fraction consisting of β-carotene and 15,15′-*cis*-β-carotene and 17–84% losses in the other carotenoids of raw broccoli as a result of saponification with 30% methanolic KOH at room temperature for 3 h under an atmosphere of nitrogen. On the other hand, Bushway and Wilson (1982) did not observe any difference in the concentration of α- and β-carotene in saponified and unsaponified samples (conditions unspecified). Baranyai *et al.* (1982) claimed no loss of carotenoids on saponification of an extract of native paprika with 30% methanolic KOH under nitrogen at room temperature for 16 h. In a previous study (Rodriguez-Amaya *et al.*, 1988), we have shown that loss of provitamins α-, β- and γ-carotene of squash, kale and tomato is not a problem with the saponification procedure used in our laboratory, i.e. saponification of the carotenoids dissolved overnight at room temperature in petroleum with equal volume of 10% methanolic KOH. Due to the discrepancy in the results reported, the saponification step was investigated in detail in this study.

MATERIALS AND METHODS

Materials

Two synthetic carotenoids, β-carotene and β-apo-8′-carotenal (Hoffman–La Roche, Basel, Switzerland), and three food samples, tomato, kale and

papaya, were used as test materials. Considering their instability, the synthetic carotenoids were purified immediately preceding each experiment. Kale was cut finely, mixed thoroughly and 5-g samples were taken for analysis. Tomato and papaya were homogenised in a Waring blender (Thomas Scientific, Swedesboro, NJ, USA) and 10- and 20-g samples, respectively, were taken for analysis.

Methods

Except for the saponification step, for which different conditions were tested, carotenoid determinations were accomplished according to standard procedures in the laboratory (Rodriguez *et al.*, 1976).

The following widely used saponification procedures were evaluated:

Procedure I: To the petroleum ether solution of the carotenoids an equal volume of 10% methanolic KOH was added. The mixture was left overnight (about 16 h) in the dark at room temperature.

Procedure II: The same as procedure I except that instead of leaving the mixture overnight it was heated in a steam bath for 10 min. The temperature reached 70–75°C.

Procedure III: To the carotenoids dissolved in ethanol a 60% aqueous solution of KOH (1 ml per 10 ml of the pigment solution) was added. The mixture was left in the dark at room temperature for 12–16 h.

Procedure IV: The same as procedure III except that instead of leaving the mixture overnight it was heated for 5–10 min in the dark in a boiling water bath. The temperature reached 80–85°C.

Procedure V: To the carotenoid or sample in a 100-ml volumetric flask 30 ml of the extracting solvent (hexane:acetone:ethanol:toluene—10:7:6:7) was added and the mixture agitated for 1 min. After standing for 16 h in the dark, 2 ml methanolic solution of 40% KOH was added, the mixture agitated for 1 min and left for 1 h in the dark; 30 ml hexane was added and, after agitation for 1 min, the volume was completed with aqueous Na_2SO_4. After vigorous agitation for 1 min, the mixture was again left in the dark for 1 h.

Procedure VI: After addition of the extracting solvent as described in procedure V, 2 ml methanolic KOH was immediately added. After 1 min agitation, the flask was placed in a 56°C water bath for 20 min, cooled and left for 1 h in the dark at room temperature.

Procedures V and VI were taken from AOAC (1984). Some results were submitted to analysis of variance and Tukey's test.

RESULTS AND DISCUSSION

Effect of saponification on β-apo-8′-carotenal and β-carotene

From Table 1 it can be seen that loss of carotenoids during saponification depends on the conditions under which it is accomplished. While procedure I led to losses of up to 3% of β-apo-8′-carotenal, procedures V and VI resulted in complete loss. Comparing procedures I and III, both conducted at room temperature, to the equivalent procedures II and IV, accomplished at elevated temperature, saponification at ambient temperature favoured retention of the carotenoid. On the other hand, samples dissolved in ethanol (procedures III and IV) degraded more than those dissolved in petroleum ether (procedures I and II), apparently due to greater contact between the alkali and the carotenoids. In procedure I, two phases can be discerned throughout saponification. Procedure II started with two phases, but with the application of heat petroleum ether evaporated towards the end. Petroleum ether is believed to make the saponification less harsh (due to temperature reduction and a protective effect on the released carotenoids) (Brubacher *et al.*, 1985).

With β-carotene, procedure I also showed best retention (losses of up to 1%) and procedure IV the poorest (21–56% losses). Similar results were obtained in relation to temperature and contact between the carotenoid and

TABLE 1
Loss of β-Apo-8′-Carotenal and β-Carotene on Saponification

Trial	*Initial amount* (μg)	*Per cent loss on saponification*					
		Procedures	*I*	*II*	*III*	*IV*	*V*	*VI*
β-Apo-8′-carotenal								
1	354		3	18	12	60	100	100
2	443		1	19	1	46	100	100
3	538		0	29	4	49	100	100
β-Carotene								
1	513		0	14	6	21	20	16
2	521		1	4	11	56	12	16
3	548		0	16	17	31	17	25

TABLE 2
Carotenoids Formed on Saponification of β-Apo-8′-Carotenal[a]

Carotenoid	*Amount (μg)*[b]			
Procedures	*II*	*IV*	*V*	*VI*
Cis-β-apo-8′-carotenal	11–82	44–66	—	—
5,8-Monoepoxy-β-apo-8′-carotenal	—	0–18	—	8–30
Citranaxanthin	—	—	429–688	206–219
Cis-citranaxanthin	—	—	—	58–205

[a] These carotenoids were not detected in procedures I and III.
[b] Ranges obtained from three trials, starting from 354–538 μg β-apo-8′-carotenal.

the alkali, although β-carotene appeared to resist saponification better than β-apo-8′-carotenal.

In an attempt to better understand what was happening during saponification, the products formed were isolated and identified (Tables 2 and 3). *Cis*-β-apo-8′-carotenal was detected in procedures II and IV; in the latter procedure, 5,8-monoepoxy-β-apo-8′-carotenal was also encountered. In procedure V, β-apo-8′-carotenal was completely transformed to citranaxanthin, apparently by aldol condensation with acetone. This condensation reaction has already been reported to occur during saponification, in the presence of acetone, of carotenoid extracts from photosynthetic bacteria (Schmidt *et al.*, 1971) and from citrus (Stewart & Wheaton, 1973). In procedure VI, aside from citranaxanthin, 5,8-monoepoxy-β-apo-8′-carotenal and *cis*-citranaxanthin were also detected.

Formation of *cis*-β-carotene was observed in all procedures except procedure I. On hot saponification, 5,6-epoxy- (procedures II and IV) and 5,8-epoxy-β-carotene (procedures IV and VI) were also detected. This result

TABLE 3
Carotenoids Formed on Saponification of β-Carotene[a]

Carotenoid	*Amount (μg)*[b]					
Procedures	*II*	*III*	*IV*	*V*	*VI*
Cis-β-carotene	0–6	3–32	39–49	5–13	20–37
5,6-Monoepoxy-β-carotene	4–16	—	0–45	—	—
5,8-Monoepoxy-β-carotene	—	—	0–4	—	4–9

[a] These carotenoids were not detected in procedure I.
[b] Ranges obtained from three trials, starting from 513–548 μg β-carotene.

TABLE 4
Effect of Ambient Temperature, Overnight Saponification on the Carotenoid Composition (μg/g) of Tomato[a]

Carotenoid	*Unsaponified*	*Saponified*
β-Carotene	4·5 ± 0·2*	4·5 ± 0·3*
γ-Carotene	0·8 ± 0·1*	0·7 ± 0·1*
Lycopene	20·8 ± 1·3*	19·8 ± 1·0*
Total	26·1 ± 0·8*	25·0 ± 0·7*
Vitamin A value (retinol equivalent/100 g)	821 ± 1*	800 ± 1*

[a] Means and standard deviations of duplicate determinations.
Values in the same horizontal line sharing the same superscript are not significantly different ($P \leq 0{\cdot}05$).

is surprising, considering Parrish's (1977) affirmation that use of an atmosphere of nitrogen gas is unnecessary during hot saponification because the area above the liquid is filled with solvent vapours, displacing most of the oxygen. Since β-carotene is not an aldehyde, aldol condensation was not observed in procedures V and VI.

The fluctuating amounts of the carotenoids formed during saponification were to be expected, considering that these compounds are known initial intermediate products of isomerisation or oxidation.

An added inconvenience of hot saponification is the necessity of controlling the conditions rigorously. Any slight change, especially in the temperature and time, altered the results.

Effect of saponification on the carotenoids of tomato and kale

Table 4 presents a comparison of the carotenoid composition of unsaponified and saponified subsamples of tomato using the saponification procedure least likely to cause transformation or degradation (procedure I). No significant difference was observed, although the carotenoid concentrations of the saponified subsamples were slightly lower.

In kale, the β-carotene content was also not affected by overnight cold saponification. On the other hand, the xanthophylls, lutein, zeaxanthin and neoxanthin, were degraded significantly. However, these losses could be reduced to insignificant levels by the use of an atmosphere of nitrogen or the antioxidant pyrogallol.

Tables 4 and 5 also show that saponification is not necessary for tomato and kale and probably other leafy vegetables, and should be omitted from

TABLE 5
Effect of Cold Overnight Saponification on the Carotenoid Composition (μg/g) of Kale[a]

Carotenoid	*Unsaponified*	*Saponified*	*Saponified under nitrogen*	*Saponified with pyrogallol*
β-Carotene	30·4 ± 0·4*	28·1 ± 0·7*	28·1 ± 0·7*	28·8 ± 0·1*
Lutein + violaxanthin	53·5 ± 2·8*	43·4 ± 1·1**	51·5 ± 1·1*	53·8 ± 3·5*
Zeaxanthin	4·4 ± 0·7*	1·8 ± 0·1**	2·2 ± 0·3*	1·9 ± 0·0*
Neoxanthin	8·5 ± 0·1*	5·2 ± 0·4**	6·9 ± 1·3*	7·3 ± 0·5*
Total	96·8 ± 4·0*	80·5 ± 2·7**	88·5 ± 3·0*	91·9 ± 5·0*
Vitamin A value (retinol equivalent/100 g)	506 ± 0*	468 ± 2*	468 ± 1*	481 ± 0*

[a] Means and standard deviations of duplicate determinations.
Values in the same horizontal line sharing the same superscript are not significantly different ($P \leq 0{\cdot}05$).

the analytical procedure. This would eliminate two time-consuming steps (saponification and subsequent washing) during which significant errors could be introduced, if not properly accomplished.

Effect of saponification on the carotenoids of papaya

Procedures I and IV, both widely used, were compared using a food sample (papaya) (Table 6). No significant loss of β-carotene was seen in either procedure. However, while saponification at ambient temperature also retained the other carotenoids, procedure IV led to significant losses of 5,6-monoepoxy-β-cryptoxanthin, β-cryptoxanthin, anteraxanthin and lycopene, and mutachrome and *cis*-lycopene were formed.

Table 6 also shows that good separation of papaya carotenoids was not possible without saponification because of the presence of carotenol esters. Overnight room temperature saponification was as efficient as hot saponification in completely hydrolysing the carotenol esters, contrary to what has been repeatedly stated in review articles and chapters. A more careful perusal of the literature reveals, however, that the collaborative study (Quackenbush, 1973) cited to prove the inefficiency of cold overnight saponification actually involved procedure V, i.e. standing of the sample overnight with the extracting solvent and saponification for only 1 h at room temperature. This misconception in the literature should be immediately corrected.

It could therefore be concluded that quantitative losses, *tran–cis*

TABLE 6
Effect of Two Saponification Procedures on the Carotenoid Composition (μg/g) of Papaya Cultivar Tailândia[a]

Carotenoid	*Unsaponified*	*Saponified procedure I*	*Saponified procedure IV*
β-Carotene	1·7 ± 0·0*	1·7 ± 0·1*	1·5 ± 0·1*
ζ-Carotene	1·7 ± 0·2*	1·6 ± 0·0*	1·4 ± 0·4*
Mutatochrome	ND	ND	0·2 ± 0·4
5,6-Monoepoxy-β-cryptoxanthin ester	1·0 ± 0·0	ND	ND
β-Cryptoxanthin ester	9·1 ± 0·4[b]	ND	ND
5,6-Monoepoxy-β-cryptoxanthin	ND	2·2 ± 0·4*	1·1 ± 0·3**
β-Cryptoxanthin	3·1 ± 0·4[c]	10·3 ± 0·1*	9·6 ± 0·3**
Antheraxanthin	3·5 ± 1·0	6·1 ± 1·3*	4·1 ± 0·7**
Cis-lycopene	ND	ND	3·1[d]
Lycopene	47·5 ± 0·5*	45·5 ± 0·8*	41·7 ± 1·6**
Total	67·5 ± 0·2*	67·4 ± 2·4*	62·8 ± 3·4**
Vitamin A value (retinol equivalent/100 g)	139 ± 7*	133 ± 2*	117 ± 5**

[a] Means and standard deviations of duplicate determinations.
[b] Also contains some 5,6-monoepoxy-β-cryptoxanthin ester.
[c] Also contains some antheraxanthin ester.
[d] Separated in only one of the duplicate samples.
Values in the same horizontal line not showing the same superscript are significantly different ($P \leq 0{\cdot}05$).

isomerisation and epoxidation can occur during saponification, depending on the operating conditions. Thus the saponification procedure in any laboratory should be evaluated to guarantee that such transformations do not happen. The degradation process is aggravated by higher temperature and greater contact between the alkali and the pigments. Per cent losses varied with different carotenoids, the provitamins being much more resistant than the notoriously unstable lutein and violaxanthin. This step is not necessary for all samples; when needed, saponification of the extract overnight at room temperature in petroleum ether with an equal volume of 10% methanolic KOH, under an atmosphere of nitrogen or in the presence of an antioxidant, is the recommended procedure. Not only are the carotenoids better retained, but the hydrolysis of carotenol esters is also complete.

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REFERENCES

AOAC (1984). *Official Methods of Analysis* (14th edn). Association of Official Analytical Chemists, Arlington, USA, pp. 834–5.

Baranyai, M., Matus, Z. & Szabolcs, J. (1982). Determination, by HPLC, of carotenoids in paprika products. *Acta Alimentaria*, **11**, 309–23.

Beecher, G. R. & Vanderslice, J. T. (1984). Determination of nutrients in foods: Factors that must be considered. In *Modern Methods of Food Analysis*, ed. K. K. Stewart & J. R. Whitaker. AVI, Westport, USA, pp. 29–54.

Brubacher, G., Muller-Mulot, W. & Southgate, D. A. T. (1985). *Methods for the Determination of Vitamins in Food.* Elsevier Applied Science Publishers, London and New York, pp. 33–50.

Bushway, R. G. & Wilson, A. M. (1982). Determination of α- and β-carotene in fruit and vegetable by high performance liquid chromatography. *Can. Inst. Food Sci. Technol. J.*, **15**, 165–9.

Davies, B. H. (1976). Carotenoids. In *Chemistry and Biochemistry of Plant Pigments, Vol. 2* (2nd edn), ed. T. W. Goodwin. Academic Press, London, pp. 38–165.

Khacik, F., Beecher, G. R. & Witaker, 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**, 603–16.

Liaaen-Jensen, S. (1971). Isolation, reactions. In *Carotenoids*, ed. O. Isler. Berkauser Verlag, Basel, Switzerland, pp. 61–188.

Parrish, D. B. (1977). Determination of vitamin A in foods—A review. *CRC Crit. Revs Food Sci. Nutr.*, **9**, 375–94.

Parrish, D. B. (1984). Recent developments in chromatography of vitamins in foods and feeds. In *Food Constituents and Food Residues: Their Chromatographic Determination*, ed. J. F. Lawrence. Marcel Dekker, New York and Basel, pp. 125–58.

Quackenbush, F. W. (1973). Use of heat to saponify xanthophyll esters and speed analysis for carotenoids in feed materials: Collaborative study. *J. Assoc. Off. Anal. Chem.*, **56**, 748–53.

Rodriguez, D. B., Raymundo, L. C., Lee, T. C., Simpson, K. L. & Chichester, C. O. (1976). Carotenoid pigment changes in ripening *Momordica charantia* fruits. *Ann. Bot.*, **40**, 615–24.

Rodriguez-Amaya, D. B., Kimura, M., Godoy, H. T. & Arima, H. K. (1988). Assessment of provitamin A determination by open column chromatography–visible absorption spectrophotometry. *J. Chromatogr. Sci.*, **26**, 624–9.

Schmidt, K., Francis, G. W. & Liaaen-Jensen, S. (1971). Bacterial carotenoids. XXXVI: Remarkable C_{43}-carotenoids artefacts of crossconjugated carotenals and new carotenoid glucosides from *Athiorhodaceae* spp. *Acta Chem. Scand.*, **25**, 2476–86.

Stewart, I. & Wheaton, T. A. (1973). Conversion of β-citraurin to reticulaxanthin and β-apo-8′-carotenal to citraxanthin during the isolation of carotenoids from citrus. *Phytochem.*, **12**, 2947–51.

Thompson, S. Y. (1986). Problem of official methods and new techniques for analysis of foods and feeds for vitamin A. *J. Assoc. Off. Anal. Chem.*, **69**, 727–38.

Van Niekerk, P. J. (1982). Determination of vitamins. In *HPLC in Food Analysis*, ed. R. Macrae. Academic Press, London, pp. 187–225.