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Analytical Methods Section

An improved analytical method for the determination of carotenes and xanthophylls in dried plant materials and mixed feeds

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Various experiments were performed to evaluate the current Association of Official Analytical Chemists (AOAC) method used for the determination of carotenes and xanthophylls in dried plant materials and mixed feeds. Incorporation of nitrogen gas and antioxidants such as BHT during extraction and saponification increased carotene and xanthophyll contents from 2 to 4% and from 19 to 30%, respectively. Combination of extraction time and saponification time for 16 h increased xanthophyll content by approximately 23%. Cold saponification also resulted in higher xanthophyll contents than hot saponification. A 1:1 mixture of MgO and diatomaceous earth was found suitable to replace silica gel as the adsorbent to separate major carotenoids by open-column chromatography. A binary solvent system of hexane–acetone and a ternary solvent system of hexane–acetone–methanol in different ratios were used to separate carotenes, monohydroxy pigments, dihydroxy pigments and polyoxy pigments. The amounts of β-carotene and lutein were also substantially increased by using the modified AOAC method.

INTRODUCTION

Carotenoids are important biological compounds that are widely present in green plants. They include a class of hydrocarbons, called carotenes, and their oxygenated derivatives, xanthophylls. Of all the carotenes, β-carotene is the most important since it has been found effective in preventing photosensitization and formation of skin tumors in mice, as well as increasing immune response in rats (Bendich, 1989; Krinsky, 1989; Moon, 1989; Zieler, 1989). In addition, β-carotene plays an important role in nutrition because of its provitamin A activity (Olsen, 1989). Its concentration in ingredients of plant origin, particularly leafy plants, greatly exceeds that of other carotenes (Klaüi & Bauernfeind, 1981). Some other carotenes such as α-carotene and γ-carotene have only about one half the biological value of β-carotene (Bauernfeind *et al.*, 1981). Xanthophylls are of significant economic interest in the pigmentation of poultry. It has been well established that avian species preferentially deposit those orally ingested xanthophylls in the liver, egg yolks, body fat, skin, feather, beak and shanks (Marusich & Bauernfeind, 1970, 1981; Fletcher *et al.*, 1985; Tyczkowski & Hamilton, 1986; Bailey & Chen, 1989). Therefore, the degree of poultry pigmentation can be controlled by the dietary concentration of xanthophylls.

The current Association of Official Analytical Chemists (AOAC) method (1984) used for the determination of carotenes and xanthophylls in dried plant materials and mixed feeds may have the following drawbacks:

(1) Carotenoids are sensitive to oxygen, light, heat and acid. However, the AOAC method does not apply nitrogen gas or antioxidants during the analytical procedure.
(2) Saponification is necessary to remove unwanted lipids, chlorophylls and xanthophyll esters. Since the degree of xanthophyll esterification tends to increase during ripening of plants (Simpson *et*

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al., 1976), the amount of xanthophyll esters present in a plant should depend upon when the plant is harvested. Thus, the saponification time used in the AOAC method may not be enough for those plants containing high amounts of chlorophylls and xanthophyll esters. It has been reported that cold saponification could result in higher yields of carotenoids than those under hot saponification (Quackenbush, 1973). The author also concluded that hot saponification significantly lowered monohydroxy pigments and increased dihydroxy pigments in extracts from corn gluten. Since the epoxy-containing carotenoids were not taken into consideration in the experiment, the effect of hot and cold saponification on carotene and xanthophyll contents needs to be further investigated. Moreover, the extraction time and saponification time used by the AOAC method were 16 h and 1 h, respectively. This would make the extraction time too long and saponification time too short. Therefore, by combining extraction time and saponification time for 16 h it is possible to increase carotene and xanthophyll contents.

(3) The polarity of the solvent system of hexane–acetone–methanol (80:10:10, v/v/v) used to elute xanthophylls in the AOAC method may be too low to dissolve large amounts of xanthophylls present in other plants such as kale and yellow corn.

(4) The AOAC method uses a 1:1 mixture of activated magnesium oxide and diatomaceous earth as the adsorbent to separate carotenes and xanthophylls. The poultry industry also uses dietary concentration of xanthophylls to control the pigmentation of egg yolks. Since some major xanthophylls such as neoxanthin and violaxanthin are not effective yolk pigmentors (Marusich & Bauernfeind, 1981; Bailey & Chen, 1989), it is necessary to separate and quantify lutein so that the degree of yolk pigmentation can be properly controlled.

(5) The AOAC method also uses a 1:1 mixture of activated silica gel and diatomaceous earth as the adsorbent to separate carotenes, monohydroxy pigments, dihydroxy pigments and polyoxy pigments. It has been established that losses or degradation of carotenoids could occur on silica columns (Strain *et al.*, 1967). Moreover, the binary solvent system of hexane–acetone used in the AOAC method may not be able to resolve some polyoxy pigments such as neoxanthin and violaxanthin. Thus, it is necessary to use MgO instead of silica gel as the major adsorbent to separate carotenes, monohydroxy pigments, dihydroxy pigments and polyoxy pigments. The purpose of this study was to develop an improved AOAC method for the determination of carotenes and xanthophylls in dried plant materials and mixed feeds.

EXPERIMENTAL

Materials

Trans-β-carotene and lutein (75% purity) were purchased from Sigma (St. Louis, MO, USA). Lutein was further purified on a column (25 cm × 12·5 mm i.d.) containing a mixture (1:1) of MgO and diatomaceous earth with a solvent system of hexane–acetone–methanol (85:15:0:2, v/v/v). Cryptoxanthin was prepared from yellow corn according to Quackenbush *et al.* (1961). Violaxanthin and neoxanthin standards were prepared from spinach using a solvent system of hexane–acetone–methanol (90:29:1, v/v/v) by thin-layer chromatography (TLC). All solvents and adsorbents such as MgO, silica gel and diatomaceous earth were purchased from Merck (Taiwan) Ltd. (Taipei, Taiwan). The silica gel TLC plates were made with a CAMAG spreader (Germany). Several plants such as grass, water convolvulus, yellow corn, kale, garland chrysanthemum, amaranth, bell pepper and spinach were used as reference samples. All samples were purchased from a local supermarket except that grass was fresh-cut from a local farm.

Preparation and extraction of sample by AOAC method

A sample (1 kg) was freeze-dried to minimize oxidative loss before grinding into fine materials with a grinder. A freshly ground sample (1 g) was extracted with 30 ml extractant (hexane–acetone–ethanol–toluene, 10:7:6:7, v/v/v/v) in a 100 ml vol. flask. For cold saponification, the resulting slurry was saponified by adding 40% methanolic KOH (2 ml) after standing in the dark for 16 h. This saponification step was conducted in the dark for 1 h at 25°C. For hot saponification, the resulting slurry was saponified by adding 40% methanolic KOH (2 ml) and warmed at 56°C for 20 min. Hexane (30 ml) was then added to the flask and swirled gently for 1 min. After diluting to volume with 10% Na_2SO_4 and shaking vigorously for 1 min, the flask was allowed to stand in the dark for 1 h until two layers were formed.

Separation and quantification of carotenes and xanthophylls by AOAC method

A sample of the hexane layer (3 ml) was pipetted onto a column (25 cm × 12·5 mm i.d.) containing a mixture of adsorptive magnesia and diatomaceous earth (1:1). Carotenes and xanthophylls were eluted with hexane–acetone (90:10, v/v) and hexane–acetone–methanol (80:10:10, v/v/v), respectively, both of which were then poured into a 25 ml volumetric flask and diluted to volume with the same eluent. The absorbances of carotene and xanthophyll were measured at 436 and 474 nm, respectively, with a Hitachi 220S Double Beam Spectrophotometer (Tokyo, Japan). Both carotene and xanthophyll concentrations were determined using a

formula derived from Beer's Law as described by AOAC (1984).

Separation of carotenes, monohydroxy pigments, dihydroxy pigments and polyoxy pigments by AOAC method

A sample of the hexane layer (3 ml) was pipetted onto a column (25 cm × 12·5 mm i.d.) containing a 1:1 mixture of silica gel and diatomaceous earth. Carotenes such as β-carotene, monohydroxy pigments such as cryptoxanthin, and dihydroxy pigments such as lutein were eluted with a binary solvent system of hexane–acetone (96:4, 90:10, and 80:20, v/v, respectively). Polyoxy pigments such as violaxanthin and neoxanthin were eluted together with hexane–acetone–methanol (80:10:10, v/v/v).

Modification of AOAC extraction method

To determine the effect of antioxidants on carotene and xanthophyll concentrations, 0·0125% butylated hydroxyanisole (BHA) and 0·0125% butylated hydroxytoluene (BHT) were each added to the extractant. A combination of 0·00625% BHA with 0·00625% BHT (BHA–BHT) was also added to the extractant to determine the synergistic effect of these two antioxidants. To determine the effect of an inert atmosphere on carotene and xanthophyll concentrations, nitrogen gas was passed into the flask with a pipette for 25 s during extraction and saponification stages. Then the flask was sealed tightly with a rubber cap.

To determine the effect of extraction time and saponification time on carotene and xanthophyll concentrations, a combination of extraction time and saponification time for 16 h was used. Saponification was conducted in the dark at ambient temperature.

To determine the effect of hot saponification and cold saponification on carotene and xanthophyll concentrations, both methods were conducted according to the AOAC method as described previously.

To determine the effect of a ternary solvent system of hexane–acetone–methanol (80:10:10, v/v/v) on the elution ability of xanthophylls, several plants such as kale, bell pepper, yellow corn and water convolvulus were used as reference samples to determine if there is any opaque layer formed in the eluate by using this solvent system. Since the original solvent system was found inadequate to dissolve polar xanthophylls in these plants, a new solvent system of hexane–acetone–methanol (70:20:10, v/v/v) was developed.

Modification of AOAC method for the separation of carotenes, monohydroxy pigments, dihydroxy pigments and polyoxy pigments

A 1:1 mixture of activated MgO and diatomaceous earth was used as the major adsorbent to separate carotenes, monohydroxy pigments, dihydroxy pigments and polyoxy pigments, Each standard of β-carotene, cryptoxanthin, lutein, violaxanthin and neoxanthin, and a mixture of these standards were pipetted onto the column (25 cm × 20 mm i.d.) to determine the ability of various solvent systems at different ratios to elute these pigments. β-Carotene and cryptoxanthin standards were eluted with a binary solvent system of hexane–acetone at 96:4 and 90:10, (v/v) respectively. Lutein, violaxanthin and neoxanthin standards were eluted with a ternary solvent system of hexane–acetone–methanol at 85:15:1, 85:15:1 and 85:15:2, (v/v/v) respectively. Grass, spinach, amaranth and garland chrysanthemum were used as reference samples in order to confirm the separation efficiency of these solvent systems. An aliquot of crude extract (3 ml) prepared from each sample was pipetted onto the column containing the same adsorbent. β-Carotene, lutein, violaxanthin and neoxanthin were eluted with the same solvent systems and ratios as described above. Each separated band eluted from the column was evaporated to dryness and dissolved in ethanol to determine absorption spectra. The major carotenoids in each sample were identified by comparing absorption spectra of unknowns with reference standards. A hypsochromic shift upon acidification with HCl was used to identify epoxy-containing carotenoids such as violaxanthin and neoxanthin.

Quantification of major carotenoids in dried grass, spinach, amaranth and garland chrysanthemum

The eluate of each separated band obtained from the column was evaporated to dryness and dissolved in ethanol. Concentrations of β-carotene, lutein, violaxanthin and neoxanthin were determined using the following formula:

$$\text{concentration (g/ml)} = E/E^{1\%}_{1\,\text{cm}} \cdot 100$$

where E = extinction at given wavelength (β-carotene 453 nm, lutein 445 nm, violaxanthin 443 nm, neoxanthin 439 nm)
$E^{1\%}_{1\,\text{cm}}$ = extinction coefficient of 1% solution measured in cell with 1 cm light path (β-carotene 2620, lutein 2550, violaxanthin 2550, neoxanthin 2243) (Davies, 1976)

Statistical analysis

Data were analyzed using analysis of variance (PROC ANOVA) and Duncan's multiple range test procedures of the Statistical Analysis System (SAS, 1985).

RESULTS AND DISCUSSION

Table 1 shows the effect of antioxidants and nitrogen gas on carotene and xanthophyll contents in dried kale.

Table 1. Effects of antioxidants and nitrogen gas on carotene and xanthophyll contents in dried kale

Treatments	Carotene (μg/g)[a]	Xanthophyll (μg/g)[a]
Control	230·7[b]	490·3[b]
BHA (0·0125%)	232·8[bc]	486·0[b]
BHT (0·0125%)	234·7[bcd]	585·4[c]
BHA–BHT (0·00625% + 0·00625%)	237·7[cd]	595·3[c]
N_2	239·6[d]	637·4[d]

[a]Mean of triplicate sample analyses.
[b–d]Means within a column with different superscripts are significantly different ($p < 0·05$).

Samples containing nitrogen gas had significantly higher ($p < 0·05$) carotene concentration than control samples and samples containing BHA. However, there was no significant difference ($p < 0·05$) in carotene concentration among samples treated with BHT, combination of BHA and BHT (BHA–BHT), and nitrogen gas. Samples containing nitrogen gas also had significantly higher ($p < 0·05$) xanthophyll concentration than the other four treatments. However, the difference in xanthophyll concentration between control and BHA-treated samples was not significant ($p < 0·05$). The same results was also found on samples treated with BHT and BHA–BHT. Samples treated with BHA–BHT were also found to have higher carotene and xanthophyll concentration than when BHA or BHT was used alone, although the increase was not necessarily significant ($p < 0·05$). This result demonstrates the synergistic effect of both BHA and BHT. The increase in oxidation stability of xanthophylls by incorporation of nitrogen gas and antioxidants was greater than that of carotene. This result is expected since xanthophylls are more susceptible to oxidative loss than carotene (Livingston *et al.*, 1968; Davies, 1976; Moss & Weedon, 1976; Bailey & Chen, 1988).

Table 2 shows the effect of extraction time and saponification time on carotene and xanthophyll concentration in water convolvulus, kale, bell pepper and yellow corn. The modified AOAC method (extraction and saponification proceed simultaneously for 16 h) was found to have significantly higher ($p < 0·05$) xanthophyll concentration in all samples than in the AOAC method (extraction for 16 h plus saponification for 1 h). The average increase of xanthophyll concentration is about 23%. On the contrary, the difference in carotene concentration between AOAC method and modified AOAC method was not significant ($p < 0·05$). It has been well established (Simpson *et al.*, 1976) that during senescence there is a drop in the level of chlorophyll and an increase in esterified xanthophylls. Therefore the degree of xanthophyll esterification should depend on when plants are harvested. This result demonstrates that prolonged saponification time for 16 h is necessary for plants containing high amounts of xanthophyll esters and chlorophylls.

Table 2. Effects of extraction time and saponification time on carotene and xanthophyll contents in several dried plants

Sample (μg/g)[a]	AOAC method[b]		Modified AOAC method[c]	
	Carotene	Xanthophyll	Carotene	Xanthophyll
Kale	193·6[d]	328·8[e]	207·8[d]	509·6[f]
Bell pepper	18·1[d]	75·6[e]	20·4[d]	91·2[f]
Yellow corn	2·7[d]	15·2[e]	2·7[d]	19·9[f]
Water convolvulus	109·5[d]	240·5[e]	108·8[d]	262·8[f]

[a]Mean of triplicate sample analyses.
[b]Extraction for 16 h plus saponification for 1 h.
[c]Combination of extraction and saponification for 16 h.
[d–f]Means within a row with different superscripts are significantly different ($p < 0·05$).

Table 3. Effects of hot saponification and cold saponification on carotene and xanthophyll contents in several dried plants

Sample (μg/g)[a]	Hot saponification		Cold saponification	
	Carotene	Xanthophyll	Carotene	Xanthophyll
Kale	215·6[b]	367·5[c]	228·4[b]	423·6[d]
Bell pepper	14·8[b]	64·8[c]	16·5[b]	74·5[d]
Yellow corn	3·2[b]	14·6[c]	3·5[b]	18·2[d]
Water convolvulus	127·5[b]	275·8[c]	120·8[b]	298·5[d]

[a]Mean of duplicate sample analyses.
[b–d]Means within a row with different superscripts are significantly different ($p < 0·05$).

Table 3 shows the effect of hot saponification and cold saponification on carotene and xanthophyll contents in several dried plants. The difference in carotene content for hot saponification and cold saponification was not significant ($p < 0.05$). On the contrary, all samples under cold saponification were found to have significantly higher xanthophyll contents than those under hot saponification. This result should be due to the fact that the epoxy-containing carotenoids such as violaxanthin and neoxanthin are more susceptible to heat loss than other carotenoids (Davies, 1976). Since these polyoxy pigments are present in significant amounts in green plants, the application of heat during saponification for this kind of sample should be excluded. Additionally, the cold saponification is easier to operate than hot saponification. This is because the latter requires a condenser on the neck of flask to prevent solvent loss, which in turn can affect quantitation of both carotene and xanthophyll contents significantly.

The ternary solvent system of hexane–acetone–methanol (80:10:10, v/v/v) used in the AOAC method to elute xanthophylls was found inadequate to dissolve polar xanthophylls in several plants such as water convolvulus, kale, bell pepper and yellow corn. Thus, this solvent system was modified to hexane–acetone–methanol (70:20:10, v/v/v) to increase the solubility of xanthophylls without affecting the elution ability of the original solvent system. This difference in adsorption affinity of magnesium oxide is probably due to its nature, and how it is prepared. The ability of using this new solvent system to elute xanthophylls in several plants can be found in Tables 1, 2 and 3.

Table 4 shows the identification data of a mixture of carotenoid standards by open-column chromatography. β-Carotene was eluted with a binary solvent system of hexane–acetone (96:4, v/v), followed by cryptoxanthin with the same solvent system (90:10, v/v). However, this binary solvent system failed to resolve some polyoxy pigments such as neoxanthin and violaxanthin. Thus, by using methanol as a modifier and adding it to the binary solvent system it is possible to resolve some major xanthophylls such as lutein, violaxanthin and neoxanthin. Lutein and violaxanthin were eluted next with a ternary solvent system of hexane–acetone–methanol (85:15:1, v/v/v). Neoxanthin was the last to be eluted with the same ternary solvent system (85:15:2, v/v/v). Neoxanthin and violaxanthin were also confirmed by a hypsochromic shift on acidification with HCl.

Table 5 shows the quantitative data of major carotenoids in several dried plants by using silica gel (AOAC method) and MgO (modified AOAC method) as adsorbents. Cryptoxanthin was not found in all samples. Neoxanthin and violaxanthin were not quantified in samples using silica gel as the adsorbent because the binary solvent system used by the AOAC method failed to resolve these two pigments. On the contrary, by using MgO as the adsorbent both neoxanthin and violaxanthin could be adequately resolved. Violaxanthin

Table 4. Identification data of a mixture of carotenoid standards by open-column chromatography[a]

Pigment	Visible spectra (nm)[b]			Epoxide test	
	Max. observed	Solvent	Max. reported[c]	Hypsochromic shift	Color
β-Carotene	(428),450,476	ethanol	(427),449,475	—	—
Cryptoxanthin	(427),448,472	ethanol	(428),449,473	—	—
Lutein	421 ,445,474	ethanol	422 ,445,474	—	—
Violaxanthin	418 ,441,470	ethanol	417 ,440,469	381,402,432	blue
Neoxanthin	415 ,437,465	ethanol	415 ,438,467	398,420,448	green

[a] Each standard was eluted from the column with a solvent system as described in the text.
[b] Values in parentheses represent shoulders on spectral absorption curves.
[c] Reported values of visible spectra are from Davies (1976).

Table 5. Quantitative data of major carotenoids in several dried plants by using AOAC method and modified AOAC method[a]

Sample (μg/g)[b]	AOAC method[c]		Modified AOAC method[d]			
	β-carotene	lutein	β-carotene	lutein	violaxanthin	neoxanthin
Grass	203·8	461·2	228·3	537·3	115·6	98·5
Spinach	135·6	276·8	151·2	329·4	72·8	60·3
Amaranth	151·0	219·5	166·7	273·8	47·2	16·8
Garland chrysanthemum	33·2	89·6	37·8	108·4	21·5	14·7

[a] Cryptoxanthin was not found in all samples.
[b] Mean of duplicate sample analyses.
[c] Silica gel was used as the adsorbent. The current AOAC method failed to resolve violaxanthin and neoxanthin.
[d] MgO was used as the adsorbent.

was present at higher concentrations than neoxanthin in all samples. In addition, β-carotene and lutein could be eluted and quantified by both methods. The concentrations of β-carotene and lutein were found to be higher in samples when MgO was used as the adsorbent. The lower concentrations of β-carotene and lutein in silica gel-used samples are probably due to the irreversible adsorption which occurred between these pigments and hydroxy groups on the silica gel surface. These results demonstrate that with MgO as the adsorbent it is possible to increase β-carotene and lutein concentrations ranging from 10 to 15% and from 16 to 25%, respectively. Since β-carotene is the most important provitamin A compound, this method would make the quantification of vitamin A more accurate.

In conclusion, both carotene and xanthophyll contents can be increased by using the modified AOAC method. Magnesium oxide can replace silica gel as the adsorbent to separate major carotenoids in dried plant materials and mixed feeds. This would prevent degradation or irreversible adsorption of carotenoids on silica gel as used by AOAC method. Also some polyoxy pigments such as violaxanthin and neoxanthin can be separated by using this method. In addition, the poultry industry can use this method to isolate and quantify lutein in dried feeds so that the degree of yolk pigmentation can be properly controlled.

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