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Method of Analysis of Astaxanthin and its Occurrence in some Marine Products

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A method for the determination of astaxanthin in free and esterified forms is based on silica gel chromatography of a lipid extract to obtain fractions of diester, monoester and free astaxanthin. The fractions are reduced with borohydride and the tetrahydroxy β -carotene is measured by ultra-violet spectrophotometry at 450 and 476 nm. The method has been applied to different crustacean samples and products, to fish oils and to organs from rainbow trout. Values down to 0.1 $\mu\text{g/g}$ sample could be measured. The fractions were identified by thin-layer chromatography.

Introduction

ASTAXANTHIN, 3,3'-dihydroxy-4,4'-diketo- β -carotene, is the most commonly occurring pigment in marine organisms. The pink to orange-pink colouring of many echinoderms and crustaceans, the skin of several fishes, and the meat of many salmonid fishes, consists partly or wholly of astaxanthin and its esters. The comparative occurrence of carotenoids, including astaxanthin, has been reviewed by Goodwin.¹ Modern methods of isolation and characterisation, and new and revised knowledge of the different forms of carotenoids have been reviewed by Liaaen-Jensen & Jensen.² The increase of fish farming, in particular of trout and salmon species, has led to an increased interest in the problems concerning pigments in fish feed, their uptake and possible *in vivo* conversions. In Norway, pigmentation of the flesh of trout and salmon is usually achieved by feeding offal from the shrimp industry but methods of catching smaller planktonic crustaceans (euphausiids and copepods) as a feed for this purpose are also being investigated.³ The introduction of canthaxanthin as a synthetic carotenoid pigment should also be considered in an analytical procedure.⁴

The absorption spectrum of astaxanthin in ultra-violet (u.v.) light is characterised by a broad, single peak with maximum at 480 nm. β -Carotene has a well-defined spectrum with two sharp peaks at 450 and 476 nm but these absorption details are lost with the introduction of two conjugated carbonyls, as in astaxanthin. The proposed method is, therefore, based on a borohydride reduction of the astaxanthin fractions followed by u.v. spectrophotometry. Astaxanthin is alkali-labile, so that saponification and chromatography on alumina must be avoided. The method involves direct separation of the astaxanthin and its mono- and di-esters on columns of silica gel. The astaxanthin fractions and their reduction products have been identified by thin-layer chromatography (t.l.c.).

Experimental

Extraction

Fresh material was frozen and ground in a meat grinder, and meals were ground in a small mill. The products were extracted with acetone followed by ethyl ether, until colourless. The extracts were evaporated in vacuum to dryness if little water was present, or, if necessary, were re-extracted with ethyl ether and again evaporated. The lipid residues were dissolved in hexane and stored in the freezer.

Column chromatography

0.5–5 g of lipids were separated on columns of 20 × 200 mm. The columns were filled with a slurry of 20 g silica gel (Woelm, 'for partition chromatography', containing ~20% water) in hexane and kept free of air during chromatography.

The main triglyceride fraction was eluted with pure hexane. The astaxanthin fractions were eluted in hexane containing increasing concentrations of ethyl ether. The astaxanthin diester was eluted by 10–15% ether in hexane, the monoesters by 20–30% and free astaxanthin by 50–60% ether. The columns had rather weak adsorption capacity, and in a few cases overloading occurred; re-chromatography of the three fractions was then carried out on new columns. The fractions were evaporated in vacuum, and the residues were taken up in a few ml of hexane.

Thin-layer chromatography of the astaxanthin fractions

The fractions from column chromatography were tested for purity and identity by t.l.c. Pre-coated aluminium plates of silica gel (Merck, 20 × 20 cm, 0.25 mm layer) were used straight from the box, and 10–50 μl of the fractions and of the original extract were applied to the plate. The elution solvent was 25% isopropyl acetate in benzene. Well-defined oblong spots were obtained with R_f values of 0.90–0.96 for the diesters, 0.75–0.80 for the monoesters and 0.35–0.45 for free astaxanthin. Known corresponding fractions from a *Calanus* lipid extract were usually run on each plate as standards. Canthaxanthin, if present, gave an R_f value between the mono- and di-esters of astaxanthin.

Reduction of the fractions

Aliquots of each fraction were transferred to ethanol and reduced with a few grains of potassium borohydride at room temperature. It was necessary to complete the reduction in less than 15 min, to avoid saponification of the astaxanthin esters. A pure yellow β -carotene colour showed that the keto groups were reduced to hydroxy groups. Visual observation was generally sufficient, as found subsequently by t.l.c. Surplus borohydride was filtered off if necessary.

Thin-layer chromatography of the reduced astaxanthin fractions

If necessary for further identification, aliquots of the reduced fractions were transferred to hexane and spotted on pre-coated silica gel plates as described previously. An elution solvent of dioxane-hexane (20 : 80 by vol.) gave the best separation of these fractions, with R_f values of ~0.90 for the dihydroxy di-fatty acid esters, 0.30–0.40 for the trihydroxy mono-fatty acid esters and 0.10 for tetrahydroxy- β -carotene. Dihydroxy- β -carotene (reduced canthaxanthin) gave an R_f value of 0.85.

Spectrophotometry of reduced astaxanthin

Absorption curves of original and reduced astaxanthin fractions in ethanol, shown in Fig. 1, were recorded on a Unicam SP700 spectrophotometer. Accurate measurements of absorption values, at wavelengths 450 and 476 nm,

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given by reduced fractions were taken on a Zeiss PMQII spectrophotometer, and were used for calculation. $E_{1\text{cm}}^{1\%}$ values of pure β -carotene were reduced by the molecular weight ratio between β -carotene and tetrahydroxy- β -carotene (0.8935), inverted and multiplied by 10^6 to give microgram per gram of sample. Pure β -carotene (Hoffman La Roche), 0.2 mg in 100 ml ethanol, was used as a standard. The calculation factors were:

$$\text{for } E_{1\text{cm}(450\text{nm})}^{1\%} = \frac{10^6}{2500 \times 0.8935} = 448$$

$$\text{for } E_{1\text{cm}(476\text{nm})}^{1\%} = \frac{10^6}{2140 \times 0.8935} = 520$$

The two calculated values were averaged to give astaxanthin (diester, monoester or free) in $\mu\text{g/g}$ of sample.

Results and Discussion

In Fig. 1, the recorded absorption curves for astaxanthin, reduced astaxanthin (tetrahydroxy- β -carotene) and β -carotene (Hoffman La Roche) in ethanol, are compared. The same β -carotene was used for the determination of the basic absorption values. Astaxanthin has a broad absorption curve with maximum at 480 nm on which most spectrophotometric methods have been based.⁵⁻⁷ Astacene, tetraketo- β -carotene, formed from astaxanthin in an alkaline medium, has a similar absorption curve, and this has also been used for astaxanthin determinations.

Reduction of keto-carotenoids has been used in structural investigations.² The reduction is usually carried out with sodium or potassium borohydride. Reduced astaxanthin has the same chromophore as β -carotene, and thus shows the same two peaks in the absorption curve (Fig. 1). The ratio

between the two peaks gives a measure of purity and identity; this absorption curve provides a better basis for a reproducible spectrophotometric method than the curve of astaxanthin or astacene. In the analyses, the ratio of $E_{1\text{cm}(476\text{nm})}^{1\%}$: $E_{1\text{cm}(450\text{nm})}^{1\%}$ was found to vary between the limits of 0.83 and 0.86 for acceptable determinations. For highly oxidised products, i.e. shrimp meals, the ratio may fall below this limit, giving erroneously high results. It is usually the non-esterified astaxanthin which is susceptible to oxidative damage. When the total quantity of astaxanthin falls below 1 $\mu\text{g/g}$ sample, the extinction ratio is greater than 0.86, indicating reduced precision at this low level.

As the amount of pigments present may vary considerably both in quantity and form, it was necessary to identify all samples both before and after reduction. The chromatographic methods reported by Egger,⁸ which were applied to keto-carotenoids of plant materials, were modified and found to give the best results. Solvent systems, isopropyl acetate/benzene and dioxane/hexane, used with pre-coated silica gel thin-layer plates, were most useful for keto and hydroxy forms, respectively.

Astaxanthin contents of different crustacean products are given in Table I. These species and products are recognised sources for the natural astaxanthin colouring of salmonid fishes. The use of shrimp-industry offal, consisting mainly of boiled prawns (*Pandalus borealis*) minus the tail meat, and of planktonic species of euphausiids and copepods, has gained wide application in the feeding programme in fish farms. Results show that the content varies considerably from one type of product to another, the values being in the range 10–100 $\mu\text{g/g}$ wet weight. Astaxanthin content in the fat is considerable; oils extracted from a planktonic copepod (*Calanus finmarchicus*), and from krill and prawns showed concentrations of 500–1100 $\mu\text{g/g}$. Analysis of the distribution between esters and the free form showed extensive

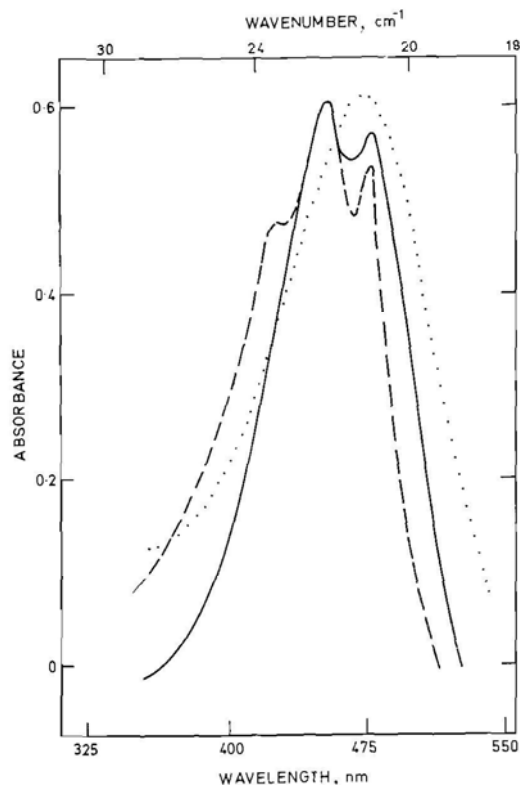


FIG. 1 Absorption curves for astaxanthin, tetrahydroxy- β -carotene and β -carotene in ethanol

— Tetrahydroxy- β -carotene; - - - β -carotene; ····· astaxanthin

TABLE I
Astaxanthin content of different crustacean products

| Sample | Astaxanthin, $\mu\text{g/g}$ | % Astaxanthin as | | |
|--|---------------------------------|------------------|-----------|------|
| | | Diester | Monoester | Free |
| Shrimp (<i>Pasiphaea</i> sp.) | 14.9 | 69 | 17 | 14 |
| Krill (euphausiids) mixed sample: A | 77.4 | 48 | 49 | 3 |
| | B | 33 | 36 | 31 |
| <i>Calanus finmarchicus</i> † | 46.5 | 37 | 38 | 25 |
| Stomach content* of herring, mainly <i>Calanus</i> | 90.8 | 55 | 26 | 19 |
| Lobster shells, boiled | 35.3 | 34 | 26 | 40 |
| Prawn offal, boiled | 66.0 | 67 | 28 | 5 |
| Prawn oil (<i>Pandalus borealis</i>) | 1095 | 79 | 18 | 3 |
| Krill oil (euphausiids) | 727 | 51 | 43 | 6 |
| <i>Calanus</i> oil (<i>C. finmarchicus</i>) | 520 | 27 | 33 | 40 |

† 3.5% Fat

* Represents 4% of whole herring

TABLE II
Astaxanthin content of some shrimp meals

| Sample | Astaxanthin, $\mu\text{g/g}$ | % as diester |
|-----------------------------|------------------------------|--------------|
| Industrial meals: | | |
| A | 8.9 | 82.0 |
| B | 3.9 | 82.8 |
| C | 0.0 | — |
| Unknown, of foreign origin: | | |
| A | 24.5 | 84.9 |
| B | 16.3 | 89.9 |
| C | 12.8 | 86.8 |
| Vacuum dried | 76.2 | 77.0 |

TABLE III
Astaxanthin content of different fish oils

| Oil sample | Astaxanthin, $\mu\text{g/g}$ | % Astaxanthin as | | |
|-------------|------------------------------|------------------|-----------|------|
| | | Diester | Monoester | Free |
| Capelin: | | | | |
| A | 94.3 | 59 | 26 | 15 |
| B | 39.5 | 53 | 33 | 14 |
| C | 6.6 | (100) | tr. | tr. |
| D | 5.7 | (100) | tr. | tr. |
| Mackerel: | | | | |
| A | 11.3 | 36 | 25 | 39 |
| B | 6.3 | 36 | 28 | 36 |
| Ocean perch | 0.8 | (100)* | — | — |
| Polar cod | 18.9 | 52 | 28 | 20 |

tr. = trace

* Concn. too small for separation

differences, and no particular trend. It appears that, in the live animal, the pigment is present as its fatty acid diester, and that active lipolytic enzymes cause hydrolysis to the monoester and to free astaxanthin after death of the animals. Thus, the prawn oil which was extracted from freshly caught samples, had 79% diester and only 18 and 3% monoester and free astaxanthin, respectively. It can be assumed that the free form is less stable towards oxidation than the esters and may therefore disappear more quickly.

Some analyses of shrimp meals are given in Table II. Shrimp meal is a normal industrial product of the shrimp industry. The values show that the astaxanthin content is generally low or even absent. A vacuum-dried meal shows a much higher value, indicating that loss is due to the processing method. Apart from the diester, only small amounts of monoester were found, and free astaxanthin seemed completely absent. Even the vacuum-dried shrimp meal showed a concentration of astaxanthin of the order of non-dried offal (Table I), indicating a very low stability of the pigment during drying.

Results of analysis of some red-coloured fish oils are given in Table III. All the species are known to feed on planktonic copepods. It may be noted that two of the capelin oils had fairly high contents of astaxanthin, making them feasible as feed supplements with regard to pigmentation. The skin, meat and liver of these species and of herring show little astaxanthin content, and the pigment of the oils is certainly derived from the stomach content (Table I).

TABLE IV
Astaxanthin content of fillet and roe of rainbow trout, and of cod roe

| Sample | Astaxanthin, $\mu\text{g/g}$ wet wt. | Form of astaxanthin |
|--|--------------------------------------|---|
| Commercial, adult fillet: A | 0.56 | Free |
| B | 0.19 | Free |
| Roe | 0.86 | Free |
| Fillet from feeding expt. (10 weeks) | | |
| A. Shrimpmeal | 0.0 | — |
| B. Shrimp offal, 2% | 0.12 | Free |
| C. Shrimp offal, 25% | 1.19 | Free |
| D. Herring with stomach filled with <i>Calanus</i> | 0.15 | Free |
| E. Synthetic canthaxanthin added | 0.38 0.06 | Canthaxanthin Free |
| Cod roe, unripe | 5.56 | Diester, 56% Monoester, 21% Free, 23% |
| Cod roe, ripe | 0.30 | Esters, 45% Free, 55% |

In Table IV analyses of fillet and roe from rainbow trout (*Salmo gairdneri*) and of cod roe are given. These samples were analysed prior to pigmentation studies at present in progress. In particular, it may be noted that only the samples of cod roe contained astaxanthin in esterified form, and that the unripe ovaries had eight times as much pigment (by wt.) as did the mature ovaries. This may be a result of the uptake of water during the final stages of maturation.⁹ Samples from rainbow trout showed no evidence of esters of astaxanthin; this was confirmed by t.l.c. This corresponds to the early observation of Steven,¹⁰ and is contrary to the findings of Thommen & Gloor.¹¹ The feeding experiments given in Table IV show that shrimp offal is a suitable source of pigment for the rainbow trout. Synthetic canthaxanthin also gave pigmentation, and the pigment was mainly deposited in the flesh as shown by t.l.c.

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