Transgenic Research
Revision

Engineered maize as a source of astaxanthin: processing and application as fish feed

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Abstract

Astaxanthin from a transgenic maize line was evaluated as feed supplement source conferring effective pigmentation of rainbow trout flesh. An extraction procedure using ethanol together with the addition of vegetal oil was established. This resulted in an oily astaxanthin preparation which was not sufficiently concentrated for direct application to the feed. Therefore, a concentration process involving multiple phase partitioning steps was implemented to remove 90% of the oil. The resulting astaxanthin raw material contained non-esterified astaxanthin with 12% 4-keto zeaxanthin and 2% zeaxanthin as additional carotenoids. Isomeric analysis confirmed the exclusive presence of the 3S,3'S astaxanthin enantiomer. The geometrical isomers were 89% all-E, 8% 13-Z and 3% 9-Z. The incorporation of the oily astaxanthin preparation into trout feed was performed to deliver 7 mg/kg astaxanthin in the final feed formulation for the first 3.5 weeks and 72 mg/kg for the final 3.5 weeks of the feeding trial. The resulting pigmentation of the trout fillets was determined by hue values with a colour meter and further confirmed by astaxanthin quantification. Pigmentation properties of the maize-produced natural astaxanthin incorporated to 3.5 µg/g dw in the trout fillet resembles that of chemically synthesized astaxanthin. By comparing the relative carotenoid compositions in feed, flesh and feces, a preferential uptake of zeaxanthin and 4-keto zeaxanthin over astaxanthin was observed.

Introduction

Carotenoids exert health promoting activities in humans and are important in nutrition. Their economic value is mainly based on their use as food colorants and as animal feed additives. For example, astaxanthin along with other ketocarotenoids are the most expensive ingredient used in salmon and trout feed. It is essential if the necessary quality in terms of aesthetic pink flesh coloration is to be achieved. This is an important criterion for consumer preference. In poultry feeding lutein and other carotenoids are used to provide pigmentation of meat and egg yolk. In chicken feeding, the carotenoids mostly originate from natural sources including marigold flowers rich in lutein (Tyczkowski and Hamilton 1986) and capsanthin from red pepper (Hamilton et al. 1990) both applied as powder. Most of the astaxanthin used in salmon and trout farming is of synthetic origin (Moretti et al. 2006). Only a small number of biological sources are available. This includes the bacterium *Paracoccus carotinifaciens*, the alga *Haematococus pluvialis* and the fungus *Xanthophyllomyces dendrorhous* (Ambati et al.

2014). At the moment, their potential is limited and is not sufficient to supply the global astaxanthin market with the needed 300 tons per year (Research and Markets 2015). Therefore, attempts have been made to discover or develop new biological sources. Alternatively, the enhancement of astaxanthin yield in already producing organisms will be assessed by genetic improvement (Gassel et al. 2014). Another approach is the extension of the carotenoid pathway in crop plants for the synthesis of astaxanthin through metabolic engineering. The latter approach was successful with carrot (Jayaraj et al. 2008) and tomato (Huang et al. 2013) in which astaxanthin biosynthesis was engineered.

Maize is a staple crop with a high potential for carotenoid pathway improvement. Maize has been successfully engineered for high β-carotene and zeaxanthin formation in seed endosperm (Zhu et al. 2008). This work laid the basis for the cloning of a hydroxylase and a ketolase gene into this maize line leading to the synthesis of astaxanthin. For enhanced yield of astaxanthin, lycopene ε-cyclase was knocked-down in addition to the over-expression of the phytoene synthase gene (Farré et al. 2016). The astaxanthin producing line was crossed into a high oil hybrid. The resulting maize seeds are a source of astaxanthin for direct feeding e.g. to chicken or can be used as a raw material for the recovery of astaxanthin. In thecurrent report, we demonstrated the usefulness of this transgenic maize line as an astaxanthin source for trout feeding. After its cultivation, a large-scale extraction process was established and the isomeric composition of the astaxanthin product determined. Application of a concentration step provided an oily astaxanthin preparation for use as fish feed ingredient. Current fish farming and production require the use of an oily astaxanthin preparation rather than incorporation of the raw maize product directly into the fish diet.

Materials and methods

Maize material

An oil producing maize transformant NSL76-bkt based on NSL 30876 genetically engineered for the biosynthesis of astaxanthin in the kernel endosperm (Farré et al. 2016) was used in this investigation. 700 NSL76-bkt plants were planted in 20 l pots, two times per year over a two year period. They were grown in a contained greenhouse at a day temperature of 30-32º C and a night temperature of 26 - 28º C with no supplementary lighting. Cobs were harvested at

maturity and dried. A total of 340 kg were recovered. Kernels were removed from the cobs manually.

Extraction process

A total of 294 kg of astaxanthin-producing maize was crushed in a cutting mill (Retsch, Haan, Germany) and the ground material (size around 1.5 mm) filled in 9 batches of 30 to 35 kg in polypropylene sacks. For improved extraction, 1 kg of maize oil was added to each batch. Extraction was carried out in two steps, first at 57°C with ethanol (1.6-fold the amount of seed powder each) for 6 and then again for 3h. The ethanol was pumped off and removed by vacuum distillation at 55-60°C. The resulting products were an oily fraction (10.8 kg) and a solid residue (7.8 kg).

Concentration of astaxanthin in the oily preparation

With the extracted oil-astaxanthin solution a concentration procedure by phase partitioning was adapted from one described earlier (Schiedt et al. 1995). This is illustrated in Fig. 1. Batches of 0.8 l of the astaxanthin solution were diluted with 0.5 l of hexane and partitioned in a first step against 0.5 l of dimethyl sulfoxide (DMSO). After the collection of the lower phase, it was partitioned twice against 300 ml of 10% ethanol in ethyl ether in a second step. Addition of ice water aided the separation of the two phases, minimizing loss of astaxanthin in the lower DMSO phase. Finally, the ether with the ethanol were removed under vacuum in a rotary evaporator at 40°C.

Carotenoid and pigmentation analysis

Astaxanthin at different stages of processing including the final oily concentrated preparation was analyzed for quantification, purity and isomeric composition. Carotenoids were extracted from maize seeds, freeze-dried fish, and freeze-dried feces, with tetrahydrofuran/methanol (50:50, v/v) by heating for 20 min at 60°C. After partitioning of each extract into 30% ether in petrol, the upper phase was collected, evaporated, and re-dissolved in acetone (Decourcelle et al. 2015). Three different HPLC systems were used. System I with a 15 cm Nucleosil C18, 3µ column and acetonitrile/2-propanol/methanol/water (85:5:10, v/v/v) as mobile phase; system II with a 25 cm C30 RP, 3µ column (YMC, Wilmington, NC, USA) (Sander et al. 1994) and

3% methyl tertiary-butyl ether (MTBE) in methanol as mobile phase for the separation of geometrical isomers; and with system III on a Chiralpak IC column and MTBE/acetonitrile (50:50, v/v) according to Wang et al. (2008) for enantiomer separation at 24°C and a flow of 1 ml/min, the others at 20°C and a flow of 0.8 ml/min. Standard carotenoids, zeaxanthin, 4-ketozeaxanthin, astaxanthin and β-carotene were generated by heterologous expression of appropriate genes in *Escherichia coli* (Sandmann 2002) and identified by their typical spectra. The astaxanthin enantiomer standard was purchased from Sigma Chemicals, Munich, Germany. Coloration of the fish fillets was measured as mean value at five positions of the fillet with equal distance to the neighboring point with a ColorLite sph 900 spectral color meter (ColorLite GmbH Katlenburg, Germany) calibrated with a white standard from the supplier.

Feed composition and fish treatment

In the feeding experiments, rainbow trout with a weight of about 100g were used. They were grown in a 400 l tank filled with 260 l of water. Three tanks with 10 fish were used per treatment over 7 weeks. Fish were fed for the first week with 2% equivalent of their weight and subsequently with 1.5%. Two different diets were applied differing only by the presence or absence of astaxanthin. The control feed contained (in g/kg) wheat gluten 200, fish meal 450, corn oil 150, fish oil 35, dextrin 100, cellulose 4.6, wheat starch 100, minerals 20, vitamins 10, TiO_2 10 (Gaye-Siessegger et al. 2011) as extruded diet. The astaxanthin feed initially supplied during the first half of the growth period contained 7 mg of astaxanthin per kg. After 3.5 weeks astaxanthin content of the feed was increased to 72 mg/kg. Fish were sampled after seven weeks when they had reached a weight around 230 g and their feces and fillets collected for analysis at the end of the experimental period

Results

Maize material

The germination, growth and development of the NSL76-bkt plants were normal and indistinguishable from those of the original Bkt transgenic line (Farré et al. 2016), the M37W near isogenic line and the NSL76 wild type parent. Even though it is not possible to estimate

yield parameters in the greenhouse, NSL76-bkt plants performed in a very similar manner in terms of fertility and seed set as the M37W, NSL76 and Bkt lines.

Extraction and concentration process

Several solvents were evaluated for astaxanthin extraction from ground maize material. The most effective solvent was ethanol in combination with added commercial maize oil which performed closest to the most efficient solvents tetrahydrofuran and chloroform, which were not preferred because of their toxicity. The efficiency of astaxanthin extraction from the maize material was over 80%. The resulting oily and solid fractions were analyzed for their astaxanthin content. The yield from 294 kg of maize was 10.8 kg of astaxanthin oil with a concentration of 120 mg/kg (Table 1). A solid residue (7.8 kg) was obtained after the ethanol was removed by distillation. From this solid deposit, astaxanthin was only poorly extractable even with strong solvents such as DMSO. Therefore, we focused only on the oily fraction and did not attempt to recover more astaxanthin from the solid phase.

The extraction process produced an oil-astaxanthin solution with a concentration not high enough to prepare a feed with an astaxanthin concentration equivalent to commercial trout feed (Choubert et al. 2006). In order to apply this oil as an astaxanthin source for the fish feed, we carried out a concentration procedure. Because it is difficult to separate the oil from the lipophilic astaxanthin, a two-step phase partitioning (Schiedt et al. 1995) modified as shown in Fig. 1 removed more than 90% of the oil. After transfer of astaxanthin into the DMSO phase, it was not possible to recover the astaxanthin by vacuum distillation since heat treatment degrades astaxanthin, which is evident by discoloration. Therefore, a second partitioning step into ether was necessary. From the final ether phase, a highly concentrated oily astaxanthin solution was obtained after evaporation, which now was 10-fold higher in concentration reaching a level of 1.2 g/kg of oil (Table 1). Astaxanthin loss during the whole concentration treatment was less than 15%.

Properties of astaxanthin extracted from transgenic maize

In order to quantify astaxanthin, to identify accompanying carotenoids and to determine the isomeric composition of astaxanthin the concentrated astaxanthin fraction was subjected to quantitative HPLC analysis in three different systems. Fig 2A shows an HPLC separation in system I of the oily astaxanthin extract after concentration. Three peaks were identified, (i)

one at 3.8 min with a diketo-type astaxanthin spectrum and an absorbance maximum at 470 nm, (ii) a peak of keto zeaxanthin at 5.9 min with an asymmetric mono keto spectrum and (iii) a small peak of zeaxanthin at 11.4 min with the typical absorbance shoulder and maxima (425, 450, 476 nm). All carotenoids were identified using reference compounds.

Geometrical isomers of astaxanthin enriched by an initial pre-separation in HPLC system I were identified in HPLC system II (Fig. 2B). In addition to the major peak of the all-E isomer at 16.8 min, smaller peaks were detected for the 13-Z isomer at 13.6 min and for the 9-Z isomer at 18.4 min. Identification was with all-E astaxanthin and according to Englert and Vecchi (1980) for the two Z-isomers via the relative position to all-E, the corresponding absorbance spectra and depending a cis peak at 370 nm (Visser et al. 2005).

An astaxanthin (Ax) standard with a mixture of all three enantiomers was separated with HPLC system III (Fig. 2C) and assigned according to Wang et al. (2008). The peak of 3S,3´S astaxanthin eluted first at 8.9 min, the 3S,3´R meso form at 10.2 min and the 3R,3´R enantiomer at 11.6 min. Separation of the astaxanthin HPLC fraction from the astaxanthin extract (peak Ax from Fig. 2A) resulted in a single isomer eluding at 9.0 min (Fig. 2D) identifying the astaxanthin from this transgenic maize as the 3S,3´S enantiomer.

Trout feeding trial

This feeding trial was carried out as a proof-of-concept experiment to demonstrate the potential of the astaxanthin from transgenic maize as a colorant for trout. All fish had a similar behavior and final weights 232±14 g (control) versus 230±6 g (astaxanthin supplemented) independent of the feed treatment. Since strong pigmentation of trout fillet occurs after 4 to 5 week of their growth phase (Choubert et al. 2006), the fish were fed r with astaxanthin supplement from the start of the trial with a lower dose of 7 mg/g feed which was increased 10-fold in the astaxanthin feed to 72 mg/g after 35 days to a concentration ensuring a maximum pigmentation effect. In contrast to the fillets from the astaxanthin-free feed, the application of astaxanthin resulted in a strong pink pigmentation (Fig. 3). Pigmentation of fillets from both groups was quantified by the hue values a and b. These color parameters are shown in (Fig. 4). The hue values of fillets from the control fish grouped in the region of negative a and b values in the lower left area of the diagram. This position reflects their extremely low pigmentation. In contrast, the color parameters of all fillets from astaxanthin-treated trout with one exception exhibited positive a values for redness and b values for yellowness around zero for most of the samples from individual fish. Their grouping in the

upper right part of the diagram corresponds to the reddish fillet colour of the astaxanthin-treated trout.

The only detectable carotenoid in the fillet of the control-diet fish was a small amount of zeaxanthin. Carotenoid quantification and distribution in the feed, trout fillet and feces are shown in Table 2. The comparison of the carotenoid distribution within the feed, fillet and feces highlights the preference of carotenoids uptake in the fish fillet. In the fillet, astaxanthin accounted for more than 60% of the incorporated carotenoids. The other carotenoids which were detected were 4-keto zeaxanthin and zeaxanthin, the same carotenoids present in the feed. However, the relative distribution of all three carotenoids changed to lower values for astaxanthin and higher values for the other carotenoids in the fillet compared to the feed. All three carotenoids were also found in the feces. Here, the amount of astaxanthin was relatively higher, whereas the other carotenoids were less abundant than in the trout fillet.

The relative carotenoid composition in feed, fillets and feces can be used to estimate the uptake of the individual carotenoids. We found that compared to the percentage of keto zeaxanthin and zeaxanthin in the feed, a higher relative accumulation of both carotenoids (especially of zeaxanthin) in the fish was evident in the final phase of the growth period. The relative distribution of both carotenoids in feces was also lower than in the feed. This indicates an uptake and incorporation preference for keto zeaxanthin and zeaxanthin over astaxanthin.

Discussion

The goal of this investigation was to assess the potential of astaxanthin from transgenic maize as fish colorant using simple processing steps to obtain an astaxanthin raw material compatible with the current requirements for commercial trout feeding. A previously generated transgenic astaxanthin-producing maize line (Farré et al. 2016) exhibited normal growth behavior and could be easily grown in a contained greenhouse to accumulate large amounts of maize kernels. The prevalent accumulated carotenoid in the kernels was astaxanthin reaching levels of 60% of total carotenoids. Astaxanthin in the transgenic maize was totally non-esterified (Fig. 2A). It has been shown that esterified astaxanthin has a lower pigmentation potential than free astaxanthin (Storebakken and No 1992). In contrast, astaxanthin fatty acid esters are the prevalent form of astaxanthin in *Haematococcus pluvialis*,

the alternative natural source of astaxanthin used for trout and salmon feeding (Lorenz and Cysewski 2000). The distribution of the optical isomers found in the astaxanthin preparations (Fig. 2B) where similar to the synthetic astaxanthin (Schüep and Schierle 1995). However, the composition of optical isomers in synthetic astaxanthin is different, i.e. the 3R,3'R, and the 3S,3'R enantiomers together with the meso form 3S,3'S are present in substantial amounts (Megdal et al. 2009). It has been shown that there is no preference for astaxanthin stereoisomers in pigmentation of trout fillets (Foss et al. 1984) but a preference for all-E astaxanthin over Z isomers has been reported (Ytrestøyl and Bjerkeng 2007; Zhao et al. 2016). Therefore, astaxanthin from transgenic maize in which the all-E isomer predominate (89%, Fig. 2) is suitable for intense fish flesh pigmentation. Since isomerization of carotenoids is caused by heat, light and solvents (Schiedt and Liaaen-Jensen 1995), the prevalence of the all-E isomer indicates that the processing of the transgenic maize was sufficiently mild.

Extraction of astaxanthin from the ground maize seeds with a combination of ethanol and 3% vegetable oil contributed to a good astaxanthin yield (Fig. 1, Table 1). This procedure provided enough astaxanthin for the fish feeding experiments. However, there was a substantial loss of astaxanthin during the extraction process caused by the solid residue which formed after evaporation of the solvent and which retained poorly extractable astaxanthin. An improvement of the extraction process as reported here needs to focus on the crystalline residue in order to increase astaxanthin recovery from it. Attempts were made to improve the extraction process by drying this residue in a freeze dryer, pulverization and extraction with organic solvents during sonication. Alternatively, other extraction procedures can be attempted such as super critical CO_2 extraction, which has been shown to be highly suitable for extraction of astaxanthin and other carotenoids (Nobre et al. 2006). An improved extraction procedure avoiding the formation of a solid residue has the potential to increase the available astaxanthin by up to 3-fold.

Due to the use of vegetable oil to aid extraction, the astaxanthin levels in the oil could not be achieved at a high enough concentration to be used for the preparation of the fish feed. The subsequent enrichment process was efficient in removing 90% of the oil matrix, corresponding to 10-fold concentration of astaxanthin in the oil with a loss of only 15% (Table 1). Avoiding this processing step would make the use of maize-derived astaxanthin more economical. With an optimized extraction using only one tenth of the applied oil, the enrichment step could be avoided since the resulting astaxanthin oily solution would be concentrated enough for direct application to the fish feed.

The most economical use of the ground astaxanthin maize is its direct application to the fish feed not only as an astaxanthin but also as a carbohydrate source. Calculations based on the replacement of the 40% carbohydrates in the feed by maize and a minimum astaxanthin concentration of 30 mg/kg feed sufficient for strong pigmentation (Kurnia et al. 2015) indicate that astaxanthin production in the transgenic maize needs to be increased by 5-fold. This can be attempted by further genetic improvement of the astaxanthin transformants by selective breeding, additional engineering of the early steps further directing metabolism into the terpenoid pathway, and by enhancing limiting reactions which finally provide larger amounts of precursors for specific carotenoid biosynthesis (Sandmann 2001).
Pigmentation of the trout fillets after the feeding regimen with transgenic maize-produced astaxanthin was as strong as in comparable experiments with similar doses of synthetic astaxanthin (Kurnia et al. 2015). In different feeding trials with synthetic astaxanthin, the resulting astaxanthin content in the trout muscle varied in a dose-dependent manner in the range from 1.4 to 8.2 μg/g (Torrissen et al. 1989). This corresponds to the astaxanthin concentration reached in the current experiments (Table 2). This result suggests a pigmentation potential of maize-produced astaxanthin equivalent to synthetic astaxanthin. A natural astaxanthin source for fish feeding alternative to maize-derived astaxanthin is krill meal which contains astaxanthin predominantly as fatty acid esters (Takaichi et al. 2003). It has been used in a trout feeding trial at high concentration of 90 mg/kg feed (Roncarati et al. 2011). Feeding this astaxanthin amount continuously over the same period as in our trial, the same amount of astaxanthin as in Table 2 was incorporated into the flesh although we applied substantially lower astaxanthin doses. This lower dose-dependent pigmentation potential of the krill meal compared to maize-derived astaxanthin may be due to the esterified form of krill astaxanthin (Storebakken and No 1992).

The pink trout fillets show a yellow hue (Fig. 4) which is visible in the fillets (Fig. 3) and reflected by the colour parameters determined in Fig. 4. This is due to the preferential uptake of zeaxanthin (Table 2). It has been shown earlier that lutein (differing in structure from zeaxanthin only by the position of one double-bond) from marigold exhibits a similar behavior causing a yellow pigmentation in trout (Büyükcapar et al. 2006). Since the zeaxanthin content in our astaxanthin preparation after astaxanthin enrichment is negligible (Fig. 2A), the yellowish contribution to the fillet color can easily be avoided in future experiments by omitting colorless vegetable oil instead of zeaxanthin-containing maize oil for dilution prior to and during feed preparation.

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Table 1 Astaxanthin in the individual processing steps

Starting material:	294 kg of transgenic maize containing a total of 4.9 g astaxanthin with a concentration of 16.7 mg/kg seeds
Extraction :	solvent 3% oil in ethanol; seed residual 0.8 g astaxanthin; pooled oil fractions with 1.3 g astaxanthin in 10.8 kg oil; concentration 120.4 mg/kg; solid residual fraction with calculated 2.8 g non-extractable astaxanthin;
Concentration:	recovery of 1.1 g astaxanthin in 0.9 kg oil; concentration 1,222 mg/kg oil, other carotenoids: 12% keto-zeaxanthin and 2% zeaxanthin
Fish feed:	dilutions with oil to 68 mg/kg and 680 mg/kg astaxanthin prior to feed application

Table 2 Final carotenoid concentration (µg/g dw) and distribution of carotenoids (%)

	Ax	K-Zx	Zx
Feed ()	71.6±10.4	11.4±0.5	8.6±1.5
(% distribution	77.7±1.2	12.4±0.6	9.9±+1.4)
Fillet	3.5±1.1	1.1±0.4	0.9±0.3
(% distribution	61.9+3.6	21.2±0.9	16.9±3.3)
Feces	354.5±39.5	43.3±12.0	17.0±8.6
(% distribution	86.2±2.2	9.0±1.9	3.9±1.6)

The only detectable carotenoid in the fillet of control fish was Zx 0.4±0.1

Ax astaxanthin; K-Zx 4-keto zeaxanthin; Zx zeaxanthin. n=5, ± standard deviation.

Figure 1 Concentration of the astaxanthin oil solution by a two-step phase partitioning procedure. Upper and lower phases as indicated in the illustration. Astax, astaxanthin.

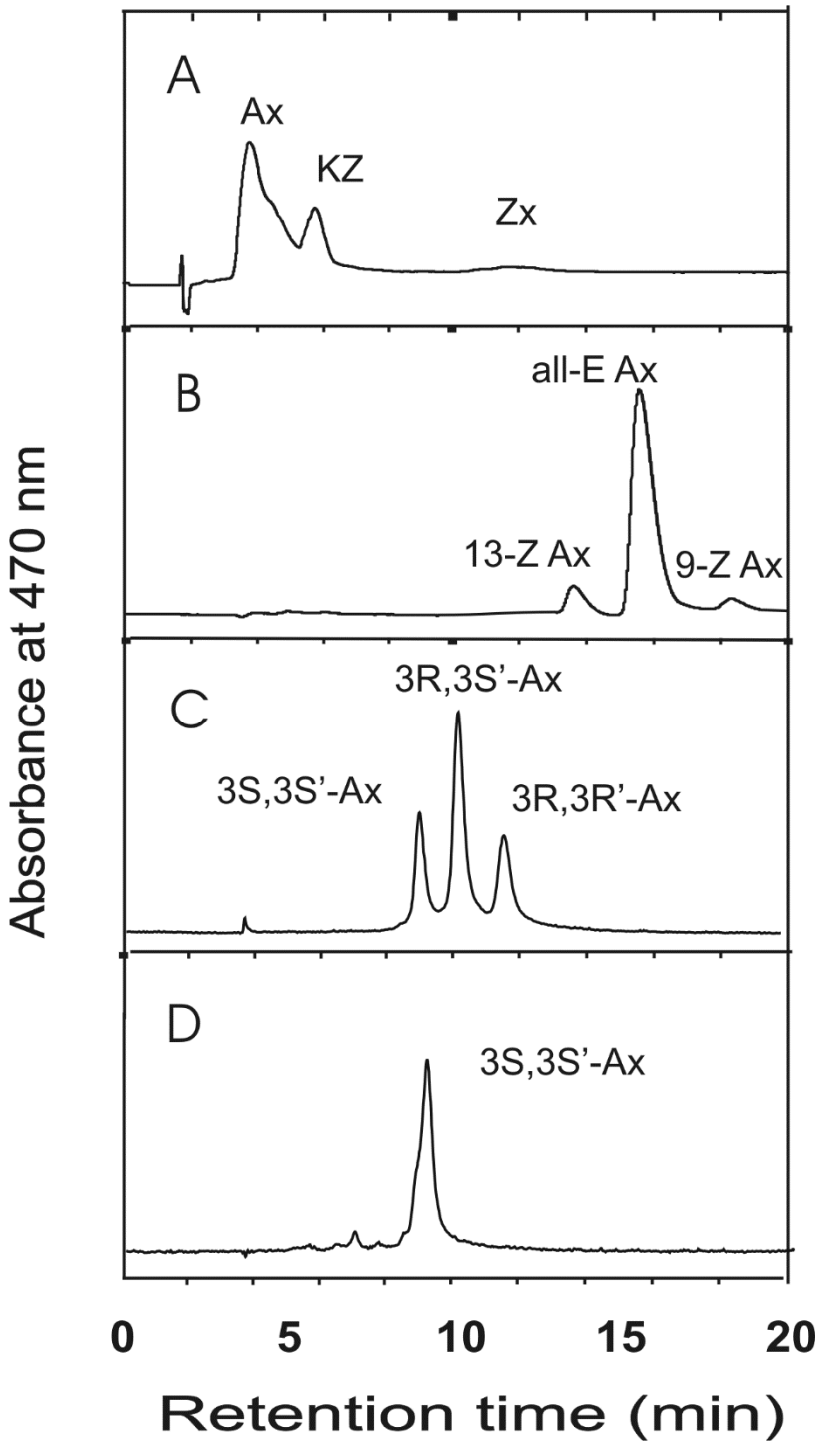

Figure 2 Analysis of extracted astaxanthin and determination of accompanying carotenoids in the astaxanthin oil. Trace A carotenoid composition in HPLC systems I, trace B composition of astaxanthin positional isomers in system II and determination of astaxanthin chirality in system III. Trace C, enantiomeric astaxanthin standard and trace D composition of geometrical astaxanthin isomers: all-E 89%, 9-Z 3%, 13-Z 8%. Ax astaxanthin, KZ 4-ketozeaxanthin, Zx zeaxanthin.

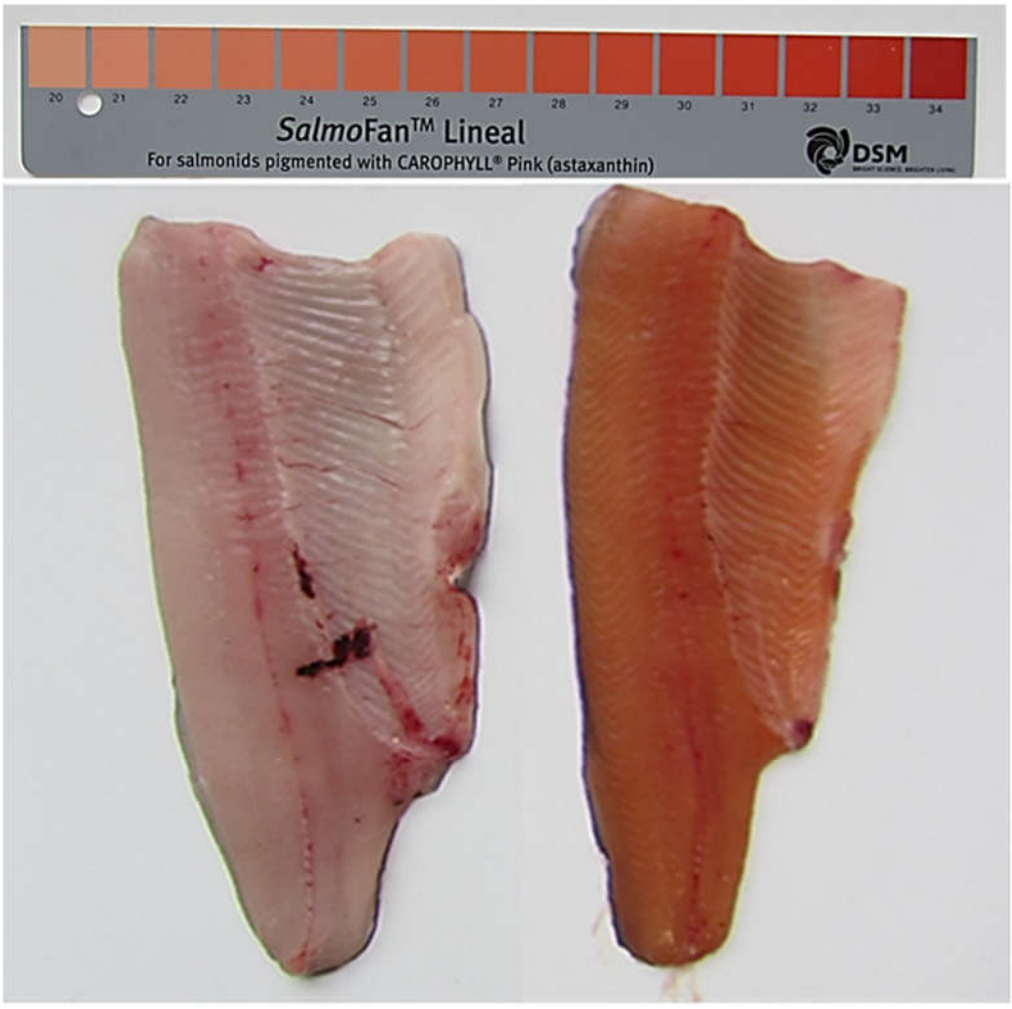

Figure 3 Pigmentation of trout fillet. On the right fillet from fish fed with feed supplemented with maize astaxanthin, on the left, fillet from fish fed with a basic feed (without astaxanthin).

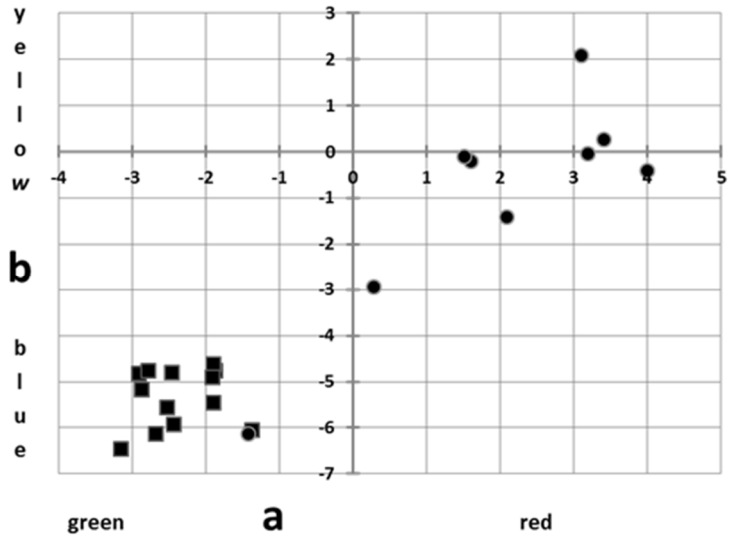

Figure 4 Colour parameters of fillets from trout fed without ■ or with ● a diet containing an oily astxanthin preparation from transgenic maize