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Colouring of Arctic charr

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Vinnsla, virðisaukning og eldi

Skýrsla Matís 11-11
Maí 2011

ISSN 1670-7192

Skýrsluágrip Matís ohf
Icelandic Food and Biotech R&D

Report summary

ISSN: 1670-7192

Titill / Title	**Colouring of Arctic charr /** Litun bleikjuholds		
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Skýrsla / Report no.	11-11	*Útgáfudagur / Date:*	Maí 2011
Verknr. / project no.	1887	Lokuð í 6 mánuði	
Styrktaraðilar / funding:	Tækniþróunarsjóður		
Ágrip á íslensku:	Tilraun var framkvæmd með það að markmiði að meta virkni lífræns litarefnis, Ecotone™, og ólífræns litarefnis, Lucantin® Pink, á litun bleikjuholds. Einnig voru áhrif 25% og 30% fitu í fóðri á virkni litarefnanna rannsökuð. Allir tilraunaliðir voru prófaðir í þrítekningu. Meðal þungi tilraunafiska var 564 g við upphaf tilraunar og 1381 g við lok tilraunar eftir 131 dag. Hitastig á tilraunatímanum var að meðaltali 8°C og selta eldisvökva 20 ‰ .Meltanleiki astaxanthins í Lucantin® Pink reyndist mun hærri en í Ecotone™. Munur á holdlit sem mældur var með mismunandi aðferðum reyndist mun minni og bendir það til betri nýtingar á litnum í Lucantin® Pink. Lítil áhrif á holdlitun fundust af mismikilli fitu í fóðri og gilti það um bæði litarefnin. Lífræna litarefnið er dýrara í innkaupi en það ólífræna og af því leiðir að u.þ.b. 5,5 % dýrara er að lita bleikju með Ecotone™ samanborið við Lucantin® Pink. Fram kom við greiningu á litarefni í fóðri í upphafi og við lok tilraunar 16 vikum seinna að verulegt tap var á litarefni úr fóðrinu og virtist það tap vera óháð tegund litarefnis.		
Lykilorð á íslensku:	*Bleikjueldi, fóður, litarefni, holdlitur*		
Summary in English:	A feeding trial was conducted to compare the pigmenting efficiency of the biological colorant Ecotone™ containg astaxanthin and prepared from the red yeast *Phaffia rhodozyma*, and the synthetic colorant Lucantin® Pink in Arctic charr. Both colorants were incorporated into diets containing either 25 or 30% lipid. All treatments were run in triplicate. The initial average weight of the fish was 564 g and the final weight 1381 g after a trial period of 131 days at 8°C and 20 ‰ salinity. The digestibility of astaxanthin seems to be very much dependent upon the astaxanthin source. Differences in flesh colour indicate a better utilization of astaxanthin from the synthetic source (Lucantin® Pink) as compared to the biological source (Ecotone™). There was only a minor effect of lipid content on utilisation of the astaxanthin. The biological astaxanthin source is more expensive than the synthetic source, resulting in about 5,5% higher production cost of fish produced with the "organic" colorant Ecotone™ as compared to fish produced with the synthetic source of astaxanthin (Lucantin® Pink). The astaxanthin content in all diets proved to be very unstable when the feed was stored under conditions that are common in production of Arctic charr (10 – 20˚C indoors). The loss of astaxanthin ranged from 21-40% and tended to be higher in diets containing Ecotone™. Thus, it is very important to avoid high temperatures, light and oxygen during storage of the feed.		
English keywords:	*Arctic charr, feed, colorants, flesh colour*		

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Introduction

The characteristic pink colour of the flesh is one of the most important quality criteria for salmonid fishes (Sigurgísladóttir et al., 1997). Animals, including salmonid fishes, are unable to biosynthesize carotenoids. In captivity such animals rely on diets supplemented with carotenoids to obtain a colour that is typical for the species and to meet other nutrient requirements. Different sources of astaxanthin and canthaxanthin, either alone or in combination, are the carotenoids most commonly used for pigmentation in farming of aquatic animals. Chemically synthesized and formulated astaxanthin (typically containing 10% astaxanthin) is currently the most widely used colorant in salmonid fish farming. In recent years, new astaxanthin containing products based on biological manufacture of astaxanthin have become available in the market. One such product is Ecotone™ (ADM, Decatur IL, USA) made from the red yeast *Phaffia rhodozyma* (or *Xanthophyllomyces dendrorhous*) and which is registered for use in EU. This product is also accepted as a colorant in organic production of farmed fish in some European countries. In a recent experiment, Bjerkeng et al. (2007) compared synthetic formulated astaxantin (Lucantin® Pink containing 10% astaxanthin; BASF, Germany) and Ecotone™ in a feeding trial with Atlantic salmon. Astaxanthin from *P. rhodozyma* was considerably more efficiently utilized for muscle pigmentation than Lucantin® Pink. This was reflected by the higher retention of consumed astaxanthin in the muscle by salmon fed the diet with Ecotone™ (astaxanthin from *P. rhodozyma* cells had 86% higher retention than the synthetic control astaxanthin). The effect can be explained by the higher apparent digestibility coefficient of the astaxanthin from the red yeast, or 64-68% in salmon fed the diet supplemented with *P. rhodozyma versus* 38-42% in the salmon fed the synthetic control (Bjerkeng et al., 2007).

Practical production scale trials with both Atlantic salmon and rainbow trout have shown a good effect of Ecotone™ on flesh colour (information from ADM). However, no direct comparison between different astaxanthin sources has to our knowledge been carried out with Arctic charr so far. Tabachek (1993) indicated that Arctic charr is not as efficient as other salmonid fishes in utilizing astaxanthin and later studies of Hatlen et al (1995), showed a very similar utilization. However, an early study with canthaxanthin indicated that Arctic charr was better able to deposit this pigment than either Atlantic salmon or rainbow trout (Christiansen and Wallace, 1988). The coloration of Arctic charr is, however, interesting because the deposition of carotenoids in the flesh of the charr is so different compared to what has been found in other salmonid fish species. Arctic charr is distinguished from other farmed salmonid species by the accumulation of high amounts of idoxanthin, a metabolite of astaxanthin, in the muscle. Depending on the age and the physiological status of the fish, idoxanthin may comprise as much as 70% of the total carotenoids in Arctic charr muscle (Aas et al., 1997; Hatlen et al., 1997), which is much higher than has been found in other salmonid fishes of similar size. Idoxanthin has less conjugated double bonds than astaxanthin and therefore has a more yellowish colour. Immature charr has less idoxanthin in the muscle than maturing fish (Bjerkeng et al., 2000) and this may influence the colour even though the

total amount of carotenoids in the muscle is similar. Furthermore, there may be differences between strains of Arctic charr, and the proportion of idoxanthin has only been reported for the Svalbard and Hammerfest strains. The Svalbard strain is more yellow than the Hammerfest strain, but this effect was apparently not caused by differences in carotenoid content (Hatlen et al., 1998).

Variability in flesh color has been recognized as a problem in the production of Arctic charr. A genetic variation in pigmentation has been demonstrated in salmonid fishes, including Arctic charr (Torrissen and Naevdal, 1984, 1988; Iwamoto et al., 1990; Elvingson and Nilsson, 1994). It is of considerable interest for -farmers of Arctic charr to determine to what extent this variation is due to total carotenoid content and carotenoid composition. The latter is related to metabolic turnover of carotenoids, and this turnover is very high where~70% of the absorbed carotenoid is transformed into colourless compounds (Bjerkeng 2008).

The utilization of carotenoids is found to be dependent on the feed composition. In particular, the lipid content in the diet has been shown to affect the utilization of the carotenoids. Thus, in Atlantic salmon a slightly higher astaxanthin concentration was found in fish fed a diet containing 39% compared to 31% lipid (Bjerkeng et al., 1997, and references therein). Efficient utilization of the pigment in the feed is important since the pigments comprise a significant part of the raw material cost of the feed.

The aim of this project is to compare different ways to pigment Arctic charr. Use of natural pigment sources is important in marketing the Arctic charr as a natural or organic product. A comparison will therefore be made between a synthetic formulated and a biologically manufactured astaxanthin product. In addition the effect of different lipid content in the diet on pigment utilization will be tested.

Materials and methods

Feed production

Four extruded diets based on fish meal, fish oil and wheat, were produced at Laxa Feedmill Ltd. (Akureyri, Iceland). Vitamins and minerals were added to all diets to cover the needs of Atlantic salmon as needs for Arctic charr have not been determined. Two of the diets were formulated with Lucantin® Pink containing 10% astaxanthin (BASF, Ludvigshafen, Germany) as a source of astaxanthin, while in the other two diets, the source was Ecotone™ (ADM, Decatur IL, USA). Both types of pigment were tested at two different lipid contents in the diet (25 and 30%). The feeds are denoted L25, L32, E25 and E32, reflecting pigment source and lipid content. The chemical composition of the diets is shown in table 1.

The astaxanthin concentration was 10% higher in the diets with 30% lipid, after production as compared to the diets with 25% lipid (independent of astaxanthin source). To have an equal astaxanthin concentration in all diets, 10% of the basal feed with no colorant was added to the diets with the higher astaxanthin content (table 1). The mixing of the coloured feed and the same diet without colorant was carried out before the onset of the feeding trial for all the feed used in the trial.

Yttrium oxide was used as an inert marker for the determination of apparent digestibility coefficient (ADC)

Growth trial

The growth trial was conducted in land based tanks in the research facility of The University of Hólar at Verið in Sauðárkrókur Iceland . The fish was of Holar strain from Holalax

Fish of average weight 564 g was randomly distributed in to tanks (1x1x0.8 m) supplied with brackish water (~ 20‰). The mean temperature during the trial was 8°C. Before the onset of the trial the fish was fed a commercial feed (LF 23 4mm) from Laxa Feedmill Ltd for three weeks during acclimatization to the tanks. All diets were fed in triplicate.

Some sexual maturation occurred in the trial but care was taken to avoid sampling of fish that had clear signs of maturation.

Sampling

Diets

The experimental diets were analysed for astaxanthin and canthaxanthin in freshly produced feed as well as in feed stored until the end of the experimental period. The feed was stored in a warehouse at ambient temperature ranging from 10-20°C for a period of 16.weeks between samplings for analyses.

Growth

Individual weighing of the fish was performed three weeks before the start feeding with the experimental diets (initial weight measured 12.02.08). One intermediate weighing was carried out 69 days after the initial weighing (22.04.09). At the end of trial, 131 days after the initial weighing (24.06.09) all fish was individually weighed and the length measured.

Samples for colorant analyses of fillets

A total of 15 fish were sacrificed at the start of feeding the experimental diets (04.03.09). The fish were sent to Islandsbleikja in Grindavík Iceland for filleting and evaluation of visible colour using Roche Salmofan® by the personnel at Islandsbleikja.
The meat from 3* 5 fillets from each replicate was minced together and all three samples immediately put on dry ice (-80 °C). The samples were kept as close to -80 °C as possible until analyses were performed at Nofima Marin, Sunndalsöra, Norway. The corresponding fillets were analyzed by Minolta colorimeter at Matis ohf Reykjavík Iceland at the sampling day.
In the intermediate and final samplings, 5 fish from each replicate were sacrificed and treated in the same way as described above.

Sampling of faces

Faeces was sampled by stripping as described by Austreng (1978) at the intermediate and final sampling.

Analyzes

Visual measurement of fillet colour.

The estimate of flesh colour was done on freshly filleted fish using Roche Salmofan® by the personnel at Islandsbleikja. The persons undertaking the evaluation are in charge for the production line in the company and used to evaluate colour of Arctic charr. The assessment was done in the production hall without using a special light box.

*Measurement of fillet colour by colorimetry (Minolta L*a*b*).*

Flesh colour was measured according to *International Commission on Illumination, CIE (1976)* using a Minolta light wave meter. L*-value from 0 – 100 represent lightness where 0 is black and 100 is white, giving an indication of the overall lightness and darkness of the filet. The a* value (a+ = red, a- = green) indicates how much red or green colour there is in the filet. The b* value (b+ = yellow, b- = blue) indicates the yellowness or blueness of the fillet. In the initial sampling, 15 fillets were measured at three different locations; close to the head, in the middle and at the tail of the fillet. In the intermediate and final samplings, 5

fillets from each replicate were measured in the same manner as described for the initial sampling.

Chemical assessment of colorants in diets, faces and fillet.

The determination of carotenoids was carried out using HPLC and performed by trained personnel in the laboratory of Nofima at Sundalsöra. The feed samples containing Lucantin® Pink were analysed according to standard procedures for pelleted fish feeds (FHF 2004). The faeces samples were analysed by the same method, with slight modifications to adjust for the lower dry matter content in the faeces samples. The feed and faeces samples containing Ecotone™ were analysed according to ADM's method for analysis of astaxanthin from pelleted fish feed containing *Phaffia* (ADM 2004).
The procedure for analysis of astaxanthin from fish flesh was developed at Nofima (former Akvaforsk) and is described in Bjerkeng et al., (1997). Briefly, the carotenoids are extracted by methanol and chloroform and the solvent is subsequently removed from an aliquot under reduced pressure, re-dissolved in mobile phase (acetone/*n*-hexane/methanol 20:80:0.1) and filtered through a 0.45 μm filter (Minisart SRP 15, Göttingen, Germany). An isocratic HPLC system was used to determine carotenoid concentrations. It consisted of an A LC-10 AS liquid chromatograph connected to a SPD-M10A VP photodiode array detector (detection wavelength at 470 nm), a SIL-10AD VP auto-injector and a SCL-10A VP system controller (Shimadzu, Kyoto, Japan). Chromatogram re-integrations were performed using the LC Workstation Class-LC10 software (Shimadzu, Kyoto, Japan). External standards of all-*E*-astaxanthin (Hoffmann-La Roche, Basel, Switzerland) with known concentrations were prepared to establish a response line and concentrations were calculated using peak areas for the chromatograms. Mobile phases were renewed daily. The concentrations were calculated using $E_{1\%,1cm}$-values of 2100 for all-*E*-astaxanthin (Britton, 1995), 1350 and 1750 for 13*Z* and 9*Z*- astaxanthin (Schüep and Schierle, 1995). The concentration of astaxanthin standards were determined spectrophotometrically in *n*-hexane containing 4.5% $CHCl_3$ (v/v). All analyses were performed in duplicate.

Analyses of Yttrium oxide in feed and faces

Yttrium oxide was determined by inductively coupled argon plasma spectrometry (ICP) at Jordforsk (Ås, Norway) as described by Refstie et al. (1997).

Calculations

Specific growth rate (SGR) = 100* LN(W_f) – LN(W_i)/ feeding days

Thermal growth coefficient (TGC) = 1000*($W_f^{1/3}$ – $W_i^{1/3}$)/ day degrees

Apparent digestibility (ADC) of astaxanthin = 100 – 100*(% marker in diet/ % marker in faeces)*(% astaxantin in faeces/ % astaxanthin in diet)
W_i and W_f are initial and final weight of the fish, respectively.

Results

Diet composition

The diets were very similar in protein content and the lipid content was very similar in the diets that were intended to be equal (Table 1).

There was a difference in the astaxanthin content in the diets produced. The content of astaxanthin was equalized by adding respective diets without added colorant.

Analyses of the diets at the end of the experimental period showed considerably lower amount of astaxanthin, compared to values found at the time of production (Table 1).

Table 1. Composition of the different diets

Feed type	L-32	L-25	E-25	E-32
Diet no	2938	2939	2940	2941
Dry matter (DM)%	93,5	92,7	92,7	93,3
In DM				
Crude protein %	44,0	44,8	44,8	45,5
Crude lipid %	30,6	25,7	25,0	30,1
Ash %	8,7	8,4	8,2	8,8
Analysed astaxanthin start mg/kg	48	42	40	46
Corrected astaxanthin start mg/kg*	44	42	40	42
Analysed astaxanthin end mg/kg	26	28	32	29
Average astaxanthin mg/kg	35	35	36	36
ADC of average astaxanthin %	53,9	59,5	18,8	14,1

**) non colour feed used for adjustment*

Growth results

The fish grew from on average 564 g to 1381 g during the 131 days of the experiment (Table 2). The growth in the trial was good and well in line with what can be expected for fish at this size (Sigurgeirsson et al., unpublished results). The average terminal growth coefficient (TGC) in the trial as whole is 2.74, supporting the high values of the specific growth rate (SGR) shown in Table 2.

Table 2. Mean weight development and SGR in the first half of the growth trial

Feed type	L32	L25	E25	E32
Diet no	2938	2939	2940	2941
Growth:				
Mean weight 1	578	568	507	603
Mean weight 2 day 69	1002	979	1024	1049
Mean weight 3 day 131	1393	1333	1359	1440
SGR 1 % per day (69d)	0,80	0,78	1,07	0,80
SGR 2 % per day (62d)	0,53	0,49	0,45	0,51
SGR Tot % per day (131d)	0,67	0,65	0,78	0,66

Pigmentation

a. Visual colour

There was a somewhat unexpected development in the values for visual estimation of the filet colour, with lower overall values observed at the intermediate sampling as compared to the initial values (Table 3). The values obtained at the final sampling were however better in line with what could be expected for fish of this size.

Table 3. Visual colour estimated by Roche fan® by workers at Islandsbleikja

Feed type	L32	L25	E25	E32
Diet no	2938	2939	2940	2941
Roche value initial	23,7	23,7	23,7	23,7
Roche value 29.4.09	22,9	23,2	22,7	23,2
Roche value 24.6.09	26,7	27,8	24,9	26,2

b. Minolta measurements

The L* values declined with time in the experiment (Table 4). The b* values were also reduced whereas no particular trend could be seen in the a* values. The overall results indicate an increased intensity in colour (L* values) and a reduction in the yellowish colour (b* values) while the intensity of the red colour does not show as consistent trend (a* values). There is no consistent effect of neither type of colorant or lipid content on the measured values.

Table 4. Results from colour measurements by the Minolta method

Feed type	L32	L25	E25	E32
Diet no	2938	2939	2940	2941
L* Mean initial	42,9	42,9	42,9	42,9
L* Mean 29.4.09	37,5	37,4	37,5	37,5
L* Mean 24.6.09	35,4	35,2	36,7	36,9

a* Mean initial	**5,3**	**5,3**	**5,3**	**5,3**
a* Mean 29.4.09	**5,2**	**5,7**	**5,6**	**5,8**
a* Mean 24.6.09	**4,8**	**7,0**	**4,4**	**5,5**
b* Mean initial	**14,9**	**14,9**	**14,9**	**14,9**
b* Mean 29.4.09	**10,8**	**11,7**	**11,4**	**11,1**
b* Mean 24.6.09	**10,6**	**12,0**	**10,2**	**11,4**

c. Chemical measurements

The amount of the astaxanthin metabolite idoxanthin increases during the experiment in all treatments (Table 5). The concentration of idoxanthin was 43% of total carotenoids in the initial samples and ranged between 48.7-55.7 at the end of the experiment. There was no clear effect of pigment source and lipid content on the concentration of idoxanthin. The concentration of astaxanthin increased in all treatments from the start of the experiment until the intermediate sampling, and there was a higher carotenoid concentration in the flesh of charr that had been fed with Lucantin Pink as compared to Ecotone (5.2 and 4.2 mg/kg, respectively). From the intermediate sampling until the end of the experiment the carotenoid concentration only increased in one treatment (L25), in the other treatments the concentration decreased or remained stable.

Table 5. Chemical analyses of colorants in fillets

Feed type	**L32**	**L25**	**E25**	**E32**
Diet no	**2938**	**2939**	**2940**	**2941**
Initial values:				
Asta mg/kg	**1,8**	**1,8**	**1,8**	**1,8**
Idoxanthin mg/kg	**1,4**	**1,4**	**1,4**	**1,4**
Asta +Ido mg/kg	**3,1**	**3,1**	**3,1**	**3,1**
Ido/ (Asta + Ido) %	**43,1**	**43,1**	**43,1**	**43,1**
29.4.09:				
Asta mg/kg	**2,8**	**2,8**	**2,1**	**2,4**
Ido mg/kg	**2,4**	**2,5**	**2,0**	**1,9**
Asta + Ido mg/kg	**5,2**	**5,2**	**4,1**	**4,2**
Ido/ (Asta + Ido) %	**47,2**	**47,3**	**49,4**	**44,0**
24.6.09:				
Asta mg/kg	**2,1**	**3,4**	**1,6**	**2,1**
Ido mg/kg	**2,6**	**3,2**	**2,0**	**2,2**
Asta + Ido mg/kg	**4,6**	**6,6**	**3,5**	**4,3**
Ido/ (Asta + Ido) %	**55,7**	**48,7**	**55,7**	**51,7**

Ido: Idoxanthin, Asta: Astaxanthin

Discussion

Diet composition

As seen from Table 1 the content of protein is very similar in the different diets. As intended, the lipid content in the diets was different and the difference between the diets with different colorants was very similar to the intended difference between the diets. At the production of the diets there was, however, an unintended difference in the content of astaxanthin in the different diets. This was partly adjusted by adding 10% of the same diets without astaxanthin to the diets with the highest colorant content in order to reduce the content of astaxanthin. This resulted in acceptable variation in the concentration of astaxanthin in the different diets. A second analysis of astaxanthin in the different experimental diets was performed after 16 weeks of storage. A considerable reduction in the concentration of astaxanthin in the experimental diets was observed at this time point. However, the reduction neither correlated to the initial analysed values nor the calculated adjusted content of colorants. This indicates that both sources of astaxantin do have limited stability at the conditions used for storing the diets. In comparison, Bjerkeng et al (2007) found minimal losses of pigment in diets with different sources of astaxanthin after storage in darkness at 10-20°C. The storage of the feed in the present trial would, however, be similar to practical storage of feed at commercial production sites, i.e. in an unheated room, avoiding direct light. This fact calls for further investigations of the stability of colorants in fish feed under practical conditions.

Digestibility

The present results show astaxanthin source effect on apparent digestibility in Arctic charr, with Lucantin Pink (synthetic astaxanthin, BASF, Ludvigshafen) showing higher digestibility than the biological source Ecotone™ (ADM, Decatur IL, USA). This is an opposite result compared to the observations of Bjerkeng et al. (2007) in a similar trial with Atlantic salmon. The numeric values for the ADC of astaxanthin are also different from what was found in the study by Bjerkeng and co-workers (2007), indicating that the Arctic charr digests Lucantin Pink more effectively than does Atlantic salmon. There is also a less pronounced effect of lipid content in the diet on ADC with higher ADC in the low fat diets. Bjerkeng et al. (1997) found a tendency to higher astaxanthin content in Atlantic salmon fed a diet with 39% lipid compared to a diet containing 31% lipid. Torrisen et al. (1990) found a positive effect, of increasing lipid from 4.1 – 23%, on ADC of canthaxanthin in rainbow trout. These results are not in agreement with the findings in the present study.

Growth

The growth in the trial was good compared to other trials with Arctic charr of similar size (Arnason and Sigurgeirsson, unpublished results). Thus the trial should be representative for well growing Arctic charr. There were minimal effects of diets on the weight development and growth measured as SGR.

Flesh colour

The flesh colour was evaluated by visual assessment using Roche Salmofan® , instrumental color analyses (L*, a*, b*) Minolta chroma meter CR-300 (Minolta, Osaka, Japan) and by chemical analyzes using HPLC. The different methods show the same effect of pigment source on the colouring of the flesh, with slightly better results obtained when using Lucantin Pink. This is in contrast with the results found by Bjerkeng et al (2007) who found a significant increase in pigmentation in Atlantic salmon when using Ecotone™ as colorant as compared with Lucantin Pink. Much of this difference can probably be explained by the differences in ADC of astaxanthin found in the different studies. The difference between the Arctic charr and the Atlantic salmon raises questions whether the difference in the metabolism of the two species can be the reason or if there can be a variable availability of astaxanthin between production batches of Ecotone™. These questions have to be addressed in future trials.
The results of the present study show no pronounced effect of lipid content in the diet on the efficiency of flesh colouring. This is partly in line with the findings of Bjerkeng et al (1997), working with Atlantic salmon, even though the authors found a tendency to increased flesh colour by increasing the lipid content from 31% to 39% in the diet.

Conclusion

The astaxanthin content in the diets seems to be unstable if the feed is kept under “normal” storage conditions. It is therefore of high importance to avoid oxygen, high temperatures and light during storage.
The digestibility of astaxanthin seems to be very much dependent upon the type of astaxanthin source and very different from what was found for Atlantic salmon. The measured values for digestibility are however uncertain due to the reduction of the astaxanthin content of the feed, and conclusions based on these results should be made with caution.
In the flesh, however, the differences were much less, indicating better utilization of the astaxanthin from the source less digestible. The results furthermore showed minimal effect of lipid content on utilisation of the astaxanthin.
The biological astaxanthin is a more expensive source than the synthetic one and therefore the production cost of fish produced with the “organic” colorant Ecotone™ will be ~ 5.5% higher than for fish produced with synthetic source of astaxanthin.

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