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Effect of Soybean Phospholipids on Canthaxanthin Lipoproteins Transport, Digestibility, and Deposition in Rainbow Trout (*Oncorhynchus mykiss*) Muscle

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This study was designed to assess the effect of dietary soybean phospholipids on canthaxanthin transport by serum lipoproteins and canthaxanthin muscle deposition in trout. Three groups of 12 immature trout in triplicate with a mean body weight of 130 g were fed with three experimental diets containing (1) canthaxanthin plus lecithin plus fish oil, (2) canthaxanthin plus lecithin, and (3) canthaxanthin alone, for 12 days. The two major lipoprotein classes in rainbow trout are high-density lipoproteins, which transport principally carotenoids present in the serum, and low-density lipoproteins, which are responsible for the transport of cholesterol, both independently of the administered diet. In addition, very low density lipoproteins are responsible for triglyceride transport in serum. Nevertheless, the amount of canthaxanthin in the serum increased when carotenoid was associated with phospholipids plus fish oil. When canthaxanthin is transported by lecithin plus fish oil, the amount of phospholipids, cantaxanthin, and cholesterol deposited in muscle increased but not significantly. The highest apparent canthaxanthin digestibility coefficient was obtained when canthaxanthin was carried by lecithin plus fish oil. The administration of canthaxanthin carried by phospholipids improved its accumulation in the muscle of rainbow trout. This accumulation could be enhanced if the time of administration of canthaxanthin is increased.

KEYWORDS: Canthaxanthin; transport; soybean lecithin; phospholipids; muscle; lipoproteins; digestibility; trout

INTRODUCTION

It is generally accepted that the color of salmonids is one of their most important quality parameters. This specific pink flesh color, only provided by carotenoids, has always been associated with salmonids and has distinguished salmonids from other fish species. Consumers perceive that redder salmon is equated to these characteristics: freshness, flavor, and high quality (*1*).

Color resulting from carotenoids deposition is considered to be significantly linked to the behavior of the animal. In addition to their color properties, carotenoids are suspected to have biological functions in salmonid such as growth and health (*2*). Carotenoids mobilization and their transport from flesh to skin and ovaries has led to the hypotheses that carotenoids have a function in reproduction. Possible carotenoid functions would be a fertilization hormone, a source of pigments for chromatophores, a function in cellular respiration, a protection from light, a resistance to elevated temperature and ammonia, and a provitamin A precursor (*3*). These possible functions remain to be clarified.

Astaxanthin is the predominant carotenoid in rainbow trout and in other wild salmonids. However, canthaxanthin is in use in aquaculture for rainbow trout muscle pigmentation. Gobantes et al. (*4*) showed that canthaxanthin was cleared more rapidly from the plasma of rainbow trout than astaxanthin. This indicates that the metabolic transformation rate of canthaxanthin is higher than that of astaxanthin. Torrissen (*5*) found that a combination of astaxantin and canthaxanthin in the fish diet led to a higher total carotenoid level in the flesh than either of the two carotenoids alone.

As carotenoids are lipid-soluble compounds, they are transported by lipoproteins in plasma. Chylomicrons are responsible for the transport of carotenoids from the intestinal mucosa to the bloodstream, via the lymphatics, and then to the liver. Very low density lipoproteins (VLDLs) and low-density lipoproteins (LDLs) are apparently responsible for the transport of carotenoids from the liver to the peripheral tissues. The role of high-

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Table 1. Experimental Diet Composition[a]

ingredients	%
fish meal	38.4
proteins	10.0
vegetal flour	32.0
vitamins[b]	2.0
minerals[b]	2.0
sodium alginate	1.0
chromium oxide	1.0
soybean lecithin[c]	1.6
fish oil	12.0
canthaxanthin	80 mg/kg

[a] Amounts are expressed in percentages. [b] Choubert et al. (*47*). [c] Soybean lecithin from GERBLÉ, Norvatis Consumer Health S.A., Barcelona, Spain (97% phospholipids of which 20% is phosphatydilcholine).

density lipoproteins (HDLs) in carotenoids transport is unknown (*6*).

Carotenoids combine within phospholipids both the hydrophilic (water affinity) phosphate chain and the hydrophobic fatty acids chains. They are therefore surface-active and play an important role as emulsifying agents, thus promoting the absorption of lipids and lipid-soluble compounds (*7*).

Besides, lecithin seems to have a selective action on cholesterol transport by serum lipoproteins: it reduces the high levels of LDLs, and at the same time, it increases the level of the HDLs that favor the elimination of excess cellular cholesterol. It also helps the digestion and absorption of fat-soluble vitamins, namely, A, D, E, and K. Its contribution is very useful for the conformation of cellular membranes, especially in the brain, heart, kidneys, bone marrow, and liver (*8*).

Digestibility constitutes a total measure of the carotenoids' intestinal absorption. It measures indirectly the quantity of carotenoids presumably absorbed by the intestinal membrane by proportioning pigments contained in feces (*9*). Apparent digestibility coefficients for astaxanthin and canthaxanthin in trout increased with the dietary lipid level, resulting in a higher carotenoids level in the flesh (*10*).

The aim of the present work was to investigate the effect of dietary phospholipids (soybean lecithin) on canthaxanthin transport, digestibility, and muscle deposition in rainbow trout (*Oncorhynchus mykiss*).

MATERIALS AND METHODS

Fish and Diets. The fish were farmed in 60 L tanks in a recirculated water system, with a volume change of 4 L/min. Three groups of 12 immature trout (*O. mykiss*) with a mean body weight of 130 g were used.

The trout were fed for 12 days a diet supplemented with canthaxanthin (β,β-carotene-4,4′-dione; Rhône Poulenc Animal Nutrition, Commentry, France; **Table 1**). The form of administration of the canthaxanthin supplement was varied: pure canthaxanthin (80 mg/kg feed) plus lecithin and fish oil (hereafter designated AC), pure canthaxanthin (80 mg/kg feed) plus lecithin (hereafter designated LC), and pure canthaxanthin (80 mg/kg feed) alone (hereafter designated PC).

As a carrier, a commercial preparation of soybean lecithin phospholipids (GERBLE, 97% phospholipids, Norvatis Consumer Health S.A., Barcelona, Spain) was used. A total of 1 mg of canthaxanthin plus 200 mg of soybean lecithin was dissolved in 1 mL of dichloromethane. The mixture was centrifuged for 15 min at 5000 rpm (KS-8000 KUBOTA, Fujioka, Japan) to remove the undissolved canthaxanthin. The solvent was evaporated under a nitrogen atmosphere, and the sample was resuspended in 1 mL of fish oil through heating (70 °C) and agitation cycles in a vortex for 1 h. The resulting dissolution was filtered through a short column of anhydrous sodium sulfate that retained the undissolved canthaxanthin. The filtrate was centrifuged for 15 min at 5000 rpm in a desk centrifuge, and the supernatant was taken out and incorporated into the fish feed.

Lipoprotein Isolation and Characterization. Blood samples were taken from the trout caudal vein with nonheparinized syringes and maintained for 24 h at 4 °C for clotting. All samples were then centrifuged at 4000 rpm for 10 min Serum samples were pooled for isolation of the lipoproteins. Serum lipoproteins were separated by density-gradient ultracentrifugation according to the procedure described by Vieira et al. (*11*). A total of 3.6 g of potassium bromide (KBr) was weighed, and 10 mL of a 1:1 dilution of serum in a 0.16 M NaCl solution was added to each centrifuge tube. Gentle stirring was necessary to dissolve the salt without denaturing the lipoproteins. Extra saline solution was gently layered over the serum. Lipoproteins were isolated using an ultracentrifuge (Kontron T-2190, Kontron Instruments, Schlieren, Switzerland), using a TFT-50.38 rotor, at 4 °C and 244500*g*, for 19.5 h. Lipoprotein bands were collected by aspiration.

The amount of total cholesterol in each lipoprotein was determined using a cholesterol enzymatic kit (Merckotest, Merck, Darmstadt, Germany) according to the CHOD-iodide method (*12*). Triglycerides were estimated with an enzyme kit (Triglyceride UV, Sigma Diagnostics, St. Louis, MO) (*13*). Phospholipid phosphorus was analyzed according to the method in Böttcher et al. (*14*). Protein was measured following the Lowry method using bovine serum albumin (1 mg/mL) as a standard (*15*).

Muscle Lipid and Canthaxanthin Extraction and Determination. After blood collection, the rainbow trout were sacrificed by a sharp blow to the head. The latero-dorsal muscle was taken off. Muscle lipids were extracted according to the Folch et al. method (*16*).

Total muscle cholesterol was assayed according to the method of Martensson (*17*) using cholesterol (1 mg/mL) in chloroform as a standard. The reaction solution was prepared by mixing acetic anhydride and sulfuric acid (10:1 v/v). The sulfuric acid should be added slowly to acetic anhydride, previously cooled to 4 °C, resulting in a colorless mixture.

A total of 100 μL of the lipid extract and the different samples of the standard (10, 20, 30, 40, and 50 μL) were evaporated under nitrogen and resuspended with 100 μL of chloroform. A total of 3 mL of an acetic anhydride/sulfuric acid solution (10:1 v/v) was added to all tubes, and its absorbance was measured with a spectrophotometer (Uvikon 941 Plus, Kontron Instruments, Schlieren, Switzerland) at 620 nm after 10 min at room temperature.

Canthaxanthin extraction from lipoproteins and muscle was carried out by the procedure described by Chávez et al. (*18*). Canthaxanthin absorption was measured with a spectrophotometer (Uvikon 941 Plus, Kontron Instruments, Schlieren, Switzerland). Canthaxanthin concentrations were calculated assuming its $E_{1\ cm}^{1\%}$ value in hexane at 474 nm to be 2250 (*19*).

Canthaxanthin Digestibility. The apparent digestibility coefficient (ADC) of canthaxanthin was estimated following the method described by Choubert (*20*) using chromium oxide (Cr_2O_3) as a marker. Feces were collected through an automatic feces collector (*21*). There are two possible methods to measure ADC: a direct or an indirect method. In the direct method, the total ingested and the total excreted carotenoids are measured (*22*). In the indirect method used in this experiment, carotenoids are measured comparatively to a marker, a reference substance, usually chromium oxide (*23*, *24*). The ADC was calculated according to the following equation (*25*):

$$\mathrm{ADC} = 100 - [(\%\ \text{feed marker}/\%\ \text{feces marker}) \times (\%\ \text{feces canthaxanthin}/\%\ \text{feed canthaxanthin})] \times 100 \quad (1)$$

Statistical Analysis. A statistical analysis for serum and muscle samples was performed through a Student *t* analysis using the Microsoft Excel program. Digestibility data were processed through ANOVA (independent group analysis) followed by the Tukey's multiple comparison test (*26*). The signification level was $p \leq 0.05$.

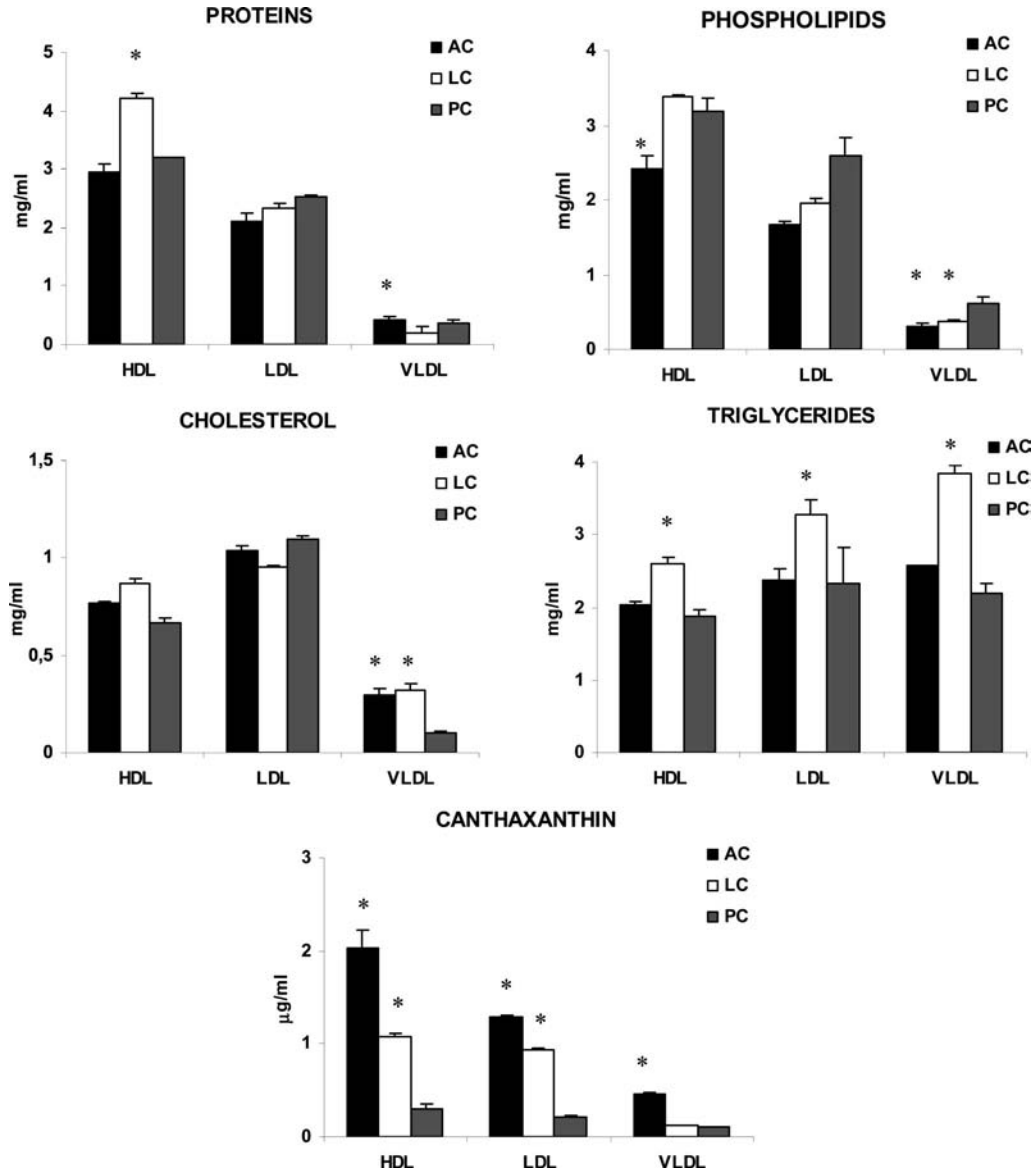

Figure 1. Protein, phospholipid, cholesterol, triglyceride, and canthaxanthin concentrations in different lipoprotein fractions of trout serum fed with each experimental diet. AC: Pure canthaxanthin (80 mg/kg feed) plus lecithin and fish oil. LC: Pure canthaxanthin (80 mg/kg feed) plus lecithin. PC: Pure canthaxanthin (80 mg/kg feed) alone. ∗ indicates $p < 0.05$.

RESULTS AND DISCUSION

Lipoproteins. *Isolation.* Ultracentrifugation after 19.5 h revealed three lipoprotein bands with the following densities: 1.021 g/mL for the VLDL, 1.053 g/mL for the LDL, and 1.115 g/mL for the HDL. Lipoproteins were identified and classified according to the density intervals applied to human serum (VLDL, $d < 1.006$ g/mL; LDL, 1.006–1.063 g/mL; HDL, 1.063–1.21 g/mL; and VHDL, $d > 1.21$ g/mL) (*27*, *28*).

Lipoprotein Characterization. Independently of the given diet, HDLs prevail over the other lipoproteins in immature trout. This is especially remarkable in the LC diet, with canthaxanthin and lecithin (64% HDL, 34% LDL, and 2% VLDL). These values are in accordance with those obtained by Chapman et al. (*29*) and Babin (*30*), where prevalent lipoproteins in trout serum were HDL followed by LDL and finally VLDL. Also, in the LC diet, the amount of proteins was the highest (**Figure 1**). LeBlanc et al. (*31*) noted that VLDL and LDL decreased in rats fed a diet containing lecithin, while HDL increased. Comparing the two diets with lecithin, AC and LC, the amount of LDL in both diets was very similar, while HDL was more abundant in the LC and AC diets, respectively.

Cholesterol was transported mainly by LDL ($p < 0.05$), followed by HDL and VLDL (**Figure 1**). There are many studies showing the emulsifier capacity of soybean lecithin in humans, rats, and crustaceans (*32*–*36*). Essential fatty acids of lecithin facilitate the solubilization and transport of cholesterol. Large doses of dietary phosphatidylcholine have been shown to inhibit the intestinal absorption of cholesterol in both humans and rats and to stimulate the capacity of the organism to eliminate cholesterol (*37*, *38*). After oral administration of soybean lecithin to 32 patients with primary hyperlipidemia for a period of 30 days, it was shown that their total serum cholesterol and LDL concentration decreased in 33% and 38% of the cases, respectively. Simultaneously, HDL concentration increased in 46% of the cases, and the triglyceride concentration fell in 33% of the cases (*8*).

In our experiment, HDL and VLDL cholesterol decreased more if lecithin was associated with fish oil compared with lecithin alone. It appeared that the combined effect of lecithin and fish oil decreased cholesterol more than the two of them separately. However, cholesterol was transported mainly by LDL independently of the type of administered diet.

The higher amount of phospholipids was transported by HDL independently of the given diet (**Figure 1**). Some studies underlined that lecithin is absorbed partially intact in the intestine and then is incorporated into HDL. On the other hand, lecithin is a good substrate for lecithin—cholesterol acyltransferase, which is associated with the irreversible production of HDL_2 and HDL_3. An increase of these lipoproteins leads to an increase of serum cholesterol elimination by means of bile acids excretion (*31*, *39*).

The major amount of triglycerides was found in VLDL (**Figure 1**) independently of the given diet. It is known that VLDL is the main carrier for triglyceride transport in trout and humans (*12*). The LC diet led to higher triglyceride amounts in all lipoprotein classes. This can be explained by the fact that there are two pathways involved in the conversion of phospholipids in triglycerides. Phosphatidylcholine can be hydrolyzed via receptor-mediated stimulation of a specific phosphatidylcholine-phospholipase C, to produce diacylglycerol, or by the stimulation of phospholipase D to produce phosphatidate. The latter can then be converted to lysophosphatidate by the action of an A-type phospholipase, or to diacylglycerol by phosphatidate phosphohydrolase (*40*).

Lecithin is also essential for maintaining liver health and is essential for transporting triglycerides out of the liver. Lecithin needs to be in balance with cholesterol and is active in cholesterol transport too (*41*). Some studies showed that lecithin plays an important role in triglyceride transport out of the intestinal mucosa by providing phospholipids for the chylomicron envelope and by supporting mucosal protein biosynthesis (*42*). Mathur et al. (*43*) suggested that intestinal cell exposure to phosphatidylcholine mobilized cellular triacylglycerol secretion by increasing the number of apoB-containing lipoprotein particles being secreted, and this occurred when the phosphatidylcholine concentration was 250 μM or more.

A noticeable amount of canthaxanthin was observed in the serum of trout fed the AC diet, mainly in HDL ($p < 0.05$) and followed LDL and VLDL (**Figure 1**). This amount of canthaxanthin decreased when the canthaxanthin carrier was not fish oil. The serum amount of trout fed lecithin (LC diet) was higher than that of trout fed canthaxanthin in the pure form (PC; $p < 0.05$). Canthaxanthin was transported by lipoproteins that had a great number of phospholipids for each diet.

The chemical composition of trout serum lipoproteins is given as percentage of weight in **Table 2**. The major lipoprotein class was HDL, rich in polar components such as proteins and phospholipids, likewise the LDL fraction. On the other hand, the VLDL fraction was rich in apolar components, mainly triglycerides. These results are similar to those reported by Chapman et al. (*29*), and the discrepancies in the amount of cholesterol and triglycerides in all lipoprotein classes may be due to rearing conditions. The LDL fraction transported the major proportion of cholesterol in serum. This result may be explained by the fish oil used, which contributed to an increase in the presence of fatty acids in serum.

Digestibility. According to our data, a higher canthaxanthin ADC was observed in trout fed the AC diet (66.4 ± 0.4%), in which canthaxanthin was associated with lecithin and fish oil, than in the others fed the LC or PC diet. There was no statistical difference ($p > 0.05$) between canthaxanthin ADC in trout fed the LC (56.6 ± 0.4%) and PC (54.9 ± 0.8%) diets. Canthaxanthin ADCs found in the literature in fish differ from one study to another: 45–71% (*5*) and 5–40% (*44*, *45*). Rearing conditions, feces collection, and analytical procedures may explain these discrepancies (*46*).

Table 2. Chemical Composition of Trout Serum Lipoproteins (Percentage by Weight)[a]

	HDL			
	AC	LC	PC	
proteins	36.17	38.13	35.78	46.9
phospholipids	29.60	30.54	35.80	26.5
cholesterol	9.34	7.83	7.50	11.1
triglycerides	24.86	23.49	20.91	15.5
canthaxanthin	0.02	0.01	0.003	
	LDL			
	AC	LC	PC	
proteins	29.31	27.46	29.69	24.7
phospholipids	23.19	22.96	30.29	27.1
cholesterol	14.41	11.14	12.87	22.3
triglycerides	33.07	38.44	27.15	26.9
canthaxanthin	0.02	0.01	0.002	
	VLDL			
	AC	LC	PC	
proteins	11.50	4.43	11.34	9.6
phospholipids	8.51	7.92	18.81	26.5
cholesterol	8.32	6.73	3.13	22
triglycerides	71.66	80.92	66.72	41.9
canthaxanthin	0.013	0.003	0.003	

[a] The column head ∗ indicates data from Chapman et al. (*29*). AC: Pure canthaxanthin (80 mg/kg feed) plus lecithin and fish oil. LC: Pure canthaxanthin (80 mg/kg feed) plus lecithin. PC: Pure canthaxanthin (80 mg/kg feed) alone.

Table 3. Phospholipids, Cholesterol, and Canthaxanthin in Muscle of Trout Fed the Different Experimental Diets[a]

	AC[b]	LC[b]	PC[b]
cholesterol[c]	9.09 ± 0.84[c]	8.16 ± 1.15[c]	6.79 ± 0.58
phospholipids[c]	7.50 ± 0.82[c]	7.52 ± 0.44[c]	6.38 ± 0.29
canthaxanthin[d]	4.33 ± 0.35[c]	3.93 ± 0.42[c]	2.77 ± 0.37

[a] AC: Pure canthaxanthin (80 mg/kg feed) plus lecithin and fish oil. LC: Pure canthaxanthin (80 mg/kg feed) plus lecithin. PC: Pure canthaxanthin (80 mg/kg feed) alone. [b] Mean ± SE, $n = 12$; means in a row not followed by the * are significantly different ($p < 0.05$). [c] In mg/g muscle. [d] In μg/g muscle.

Muscle. In muscle (**Table 3**), the cholesterol amount was higher in trout fed the AC diet ($p < 0.05$) than in those fed the LC or PC diet. Conversely, the total cholesterol transported by lipoproteins was not affected, indicating that there was no relationship between cholesterol lipoprotein transport and the administered diet.

According to Torrissen et al. (*10*), when trout are fed a high lipid diet, muscle lipid depositions can vary. In our experiment, the highest increase of cholesterol took place in the muscle of trout fed the AC diet, containing fish oil. Thus, if trout are fed with a great amount of fish oil, cholesterol deposition in muscle increases due to a large amount of cholesterol in fish oil.

The phospholipid level in trout muscle was not significantly modified with the type of diet administered. The greater concentration of phospholipids was associated with the LC and AC diets ($p < 0.05$) with an extra contribution of phospholipids than with PC diet.

Muscle canthaxanthin deposition was affected by the diet ($p < 0.05$). Muscle canthaxanthin deposition from trout fed the AC diet was higher than those fed the LC or PC diet. A high level of canthaxanthin was obtained after only 12 days. A higher amount would be expected with a longer period of administration. Further experiments will clarify this assertion.

Conclusions. Extra phospholipid contributions in the diet produced an increase in the total circulating lipoproteins. The

present work demonstrated that dietary phospholipids have a positive effect on canthaxanthin transport, its digestibility, and muscle deposition in nonmature rainbow trout. Also, it was shown that phospholipids associated with fish oil in the diet have a greater effect than phospholipids alone. These results were obtained after a 12 days of experimentation. Further experiments, based on industrial practices, will confirm these findings.

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