Biochemical Mechanisms in Absorption, Retention and Metabolism of Dietary Astaxanthin in Atlantic salmon, *Salmo salar*

Noppawan Chimsung

A Thesis Submitted in Fulfillment of the Requirements for the Degree of
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I hereby certify that this work has not already been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ กลไกทางชีวเคมีในการดูดซึม สะสมและเมทาบอลิสมสารแอสตาแซนทินของปลาแอตแลนติกแซลมอน

ผู้เขียน นางนพวรรณ ฉิ้มสังข์

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บทคัดย่อ

แอสตาแซนทิน (3, 3′-dihydroxy-β,β-carotene-4, 4′ dione) เป็นสารสีที่เสริมลงไปในอาหารเพื่อให้เนื้อปลาแซลมอนมีสีสวยตามความต้องการของผู้บริโภค แอสตาแซนทินมีราคาแพงและมีการสะสมในเนื้อปลาได้ตั้งแต่ 5-15% ของปริมาณที่เสริมลงไป ทั้งนี้เนื่องจากสารแอสตาแซนทินถูกดูดซึมที่ระบบทางเดินอาหารของปลาได้น้อย ส่งผลให้ประสิทธิภาพการใช้สารแอสตาแซนทินในอาหารของปลาต่ำ การศึกษาครั้งนี้จึงมุ่งเน้นศึกษากลไกการดูดซึม ขนส่งและสะสมสารแอสตาแซนทินในปลาแซลมอน โดยศึกษาถึงวัตถุดิบในอาหารบางตัวที่อาจมีผลต่อการดูดซึมสารแอสตาแซนทินรวมทั้งกลไกทางธรรมชาติของวัตถุดิบเหล่านี้ต่อการดูดซึมของสารแอนตาแซนทินที่เกิดขึ้นที่ลำไส้ รวมไปถึงการขนส่ง เน้นการศึกษาถึงผลของคอเลสเตอรอลต่อการดูดซึมสารแอสตาแซนทินที่เซลล์ลำไส้ และการขนส่งสารแอสตาแซนทินในไลโปโปรตีนแต่ละชนิด ซึ่งการศึกษาดังกล่าวนี้ จำเป็นต้องมีการพัฒนาวิธีการศึกษาเพื่อไปสู่เป้าหมายที่วางไว้ เทคนิคด้านชีวเคมีรวมทั้งเซลล์โมเดล ถูกนำมาประยุกต์ใช้เพื่อการศึกษาในปลาแซลมอน

การทดลองที่ 1 ศึกษาถึงผลของวัตถุดิบในอาหารบางตัว ได้แก่ วิตามินอี คอเลสเตอรอล สเตอรอลจากพืช ไฟเบอร์ ลูทีนและซีอาแซนทิน ที่อาจมีต่อการดูดซึมสารแอสตาแซนทินของปลาแซลมอน ทำการทดลองในปลาขนาด 778$\pm$78 กรัม จำนวน 88 ตัว โดยเลี้ยงในน้ำทะเลภายใต้สภาวะควบคุมที่เหมาะสมกับการเจริญเติบโต อาหารทดลองมีระดับของสารแอสตาแซนทินเท่ากันหมด คือ 40 มิลลิกรัมต่ออาหาร 1 กิโลกรัม แตกต่างกันที่มีการเติมวัตถุดิบอาหารที่ต้องการทดสอบ ได้แก่ คอเลสเตอรอล 2 % ของน้ำหนักอาหาร วิตามินอี 450 IU ต่ออาหาร 1 กิโลกรัม ไฟเบอร์จากรำข้าวสาลี 5% ของน้ำหนักอาหาร ลูทีน 40 มิลลิกรัม ต่ออาหาร 1 กิโลกรัม ซีอาแซนทิน 40 มิลลิกรัม ต่ออาหาร 1 กิโลกรัม และสเตอรอลจากพืช 2% น้ำหนักอาหาร หลังจากปลากินอาหารทดลอง 26 วัน ทำการเก็บตัวอย่างเลือดปลา และแยกพลาสมา เพื่อวิเคราะห์หาปริมาณของสารแอสตาแซนทินในพลาสมา พบว่า ไม่พบสารแอสตาแซนทินในตัวอย่างพลาสมาของปลาที่กินอาหารกลุ่มควบคุม ซึ่งไม่การเติมสารแอสตาแซนทินในอาหาร และปลาที่กินอาหารที่เติมคอเลสเตอรอล 2% ของน้ำหนักอาหาร มีปริมาณของสารแอสตาแซนทินในพลาสมาสูงกว่าปลากลุ่มอื่นอย่างมีนัยสำคัญ ส่วนวัตถุดิบอาหารชนิดอื่นไม่ว่า จะเป็นวิตามินอี ไฟเบอร์ ลูทีน ซีอาแซนทิน และสเตอรอลจากพืช ไม่ส่งผลต่อระดับของสารแอสตาแซนทินในพลาสมาของปลาแซลมอนแต่อย่างใด

การทดลองที่ 2 พัฒนารูปแบบที่เหมาะสมในการศึกษาการดูดซึมสารแอสตาแซนทินที่ลำไส้ของปลา โดยการใช้เซลล์ Caco-2 ซึ่งเป็นเซลล์ลำไส้ของมนุษย์ที่นิยมนำมาศึกษาการดูดซึมสารและได้รับการยอมรับว่าเป็นโมเดลที่ดีที่สุด ในการพัฒนาเทคนิคพบว่า Dimethylsulfoxide หรือ DMSO เป็นตัวทำละลายที่สามารถละลายสารแอสตาแซนทินในอาหารเลี้ยงเซลล์และทำหน้าที่ในการขนส่งสารผ่านเยื่อเซลล์เข้าไปในเซลล์ได้อย่างมีประสิทธิภาพ

เทคนิคการแยกเซลล์ลำไส้ของปลาแซลมอนถูกพัฒนาขึ้นเพื่อใช้ศึกษา ทั้ง *in vitro* และ *in vivo* ในการดูดซึมสารแอสตาแซนทินที่ลำไส้ของปลาแซลมอน การศึกษา *in vitro* มีวิธีการศึกษา โดยให้ปลากินอาหารเม็ดสำเร็จรูปที่ไม่มีการเติมสารแอสตาแซนทิน ก่อนที่จะผ่าตัดเอาชิ้นส่วนของลำไส้ส่วนไส้ติ่งของปลา เพื่อทำการแยกเซลล์ นำเซลล์ที่แยกได้นี้ไปบ่มด้วยอาหารเลี้ยงเซลล์ที่มีเฉพาะสารแอสตาแซนทิน เปรียบเทียบกับการมีสารแอสตาแซนทินร่วมกับคอเลสเตอรอล ส่วนการศึกษา *in vivo* มีวิธีการศึกษาโดยให้ปลาแซลมอนกินอาหารทดลองที่มีการเสริมสารแอสตาแซนทิน 40 มิลลิกรัมต่ออาหาร 1 กิโลกรัม และ อาหารที่มีสารแอสตาแซนทิน 40 มิลลิกรัมต่ออาหาร 1 กิโลกรัม ร่วมกับ คอเลสเตอรอล 2% ของน้ำหนักอาหาร ก่อนทำการเก็บตัวอย่างเลือดเพื่อแยกพลาสมา และแยกเซลล์ลำไส้จากส่วนของไส้ติ่งของปลา เพื่อวิเคราะห์หาปริมาณของสารแอสตาแซนทินในพลาสมาและเซลล์ลำไส้ ผลการศึกษาทั้ง *in vitro* และ *in vivo* บ่งชี้ว่า กระบวนการดูดซึมสารแอสตาแซนทินเป็นแบบ passive transport และการเสริมคอเลสเตอรอลไม่มีผลต่อการการดูดซึมสารแอสตาแซนทินที่ลำไส้ของปลาแซลมอน ซึ่งในการศึกษาครั้งนี้เป็นการรายงานการศึกษาครั้งแรกของการใช้เทคนิคการแยกเซลล์ลำไส้ของปลาเพื่อศึกษาการดูดซึมแคโรทีนอยด์

การที่ระดับของสารแอสตาแซนทินในพลาสมาของปลาที่กินอาหารที่มีสารแอสตาแซนทินและเสริมด้วยคอเลสเตอรอล 2% ของน้ำหนักอาหาร มีค่าสูงกว่าอย่างมีนัยสำคัญเมื่อเทียบกับปลาที่กินอาหารที่เสริมด้วยสารแอสตาแซนทินแต่เพียงอย่างเดียวนั้น อาจเนื่องจากคอเลสเตอรอลมีผลต่อกระบวนการขนส่งสารแอสตาแซนทินโดยไลโปโปรตีน การศึกษาถึงกระบวนการขนส่งสารแอสตาแซนทินโดยไลโปโปรตีน และผลของคอเลสเตอรอลต่อการขนส่งสารแอสตาแซนทินได้ดำเนินการทดลองโดย ทำการแยกชนิดของไลโปโปรตีนจากพลาสมาของปลาที่กินอาหารแต่ละสูตร โดยใช้เทคนิค sucrose density gradient ซึ่งเป็นเทคนิคที่ดัดแปลงมากจากการศึกษาในมนุษย์ ส่วนของ apolipoprotein ในไลโปโปรตีน นั้นทำการแยกโดยใช้เทคนิค SDS-PAGE ผลการทดลองพบว่า เทคนิคนี้สามารถแยกไลโปโปรตีนของปลาแซลมอนได้ โดยไลโปโปรตีนชนิดต่าง ๆ คือ VLDL (ความหนาแน่น (d) 1.031-1.043 กรัมต่อมิลลิลิตร), LDL (ความหนาแน่น 1.043-1.050 กรัมต่อมิลลิลิตร), HDL (ความหนาแน่น 1.050-1.103 กรัมต่อมิลลิลิตร) และ protein-rich fraction (PR) (ความหนาแน่น 1.103-1.157 กรัมต่อมิลลิลิตร) apolipoprotein ของ HDL พบเฉพาะน้ำหนักโมเลกุล 25kDa ส่วน VLDL พบ 260 และ 240 kDa และ LDL พบ 240 และ 76 kDa ส่วนปริมาณของสารแอสตาแซนทินนั้น พบกระจายอยู่ในไลโปโปรตีนแต่ละชนิด ทั้งนี้ PR fraction เป็นไลโปโปรตีน

หลักที่ทำหน้าที่ขนส่งสารแอสตาแซนทิน ตามด้วย HDL, VLDL และ LDL ตามลำดับ การเสริมคอเลสเตอรอลลงไปในอาหารมีผลต่อการเพิ่มระดับของสารแอสตาแซนทินในไลโปโปรตีนชนิด VLDL อย่างมีนัยสำคัญ

จากการศึกษาครั้งนี้ได้ข้อมูลใหม่เกี่ยวกับผลของวัตถุดิบอาหารบางตัวต่อการดูดซึมสารแอสตาแซนทิน รวมทั้งกลไกทางชีวเคมีของการดูดซึมสารแอสตาแซนทินที่ลำไส้และผลของคอเลสเตอรอลต่อการขนส่งสารแอสตาแซนทินโดยไลโปโปรตีน ข้อมูลจากการศึกษา *in vitro* สรุปได้ว่า ในการดูดซึมสารแอสตาแซนทินที่ลำไส้นั้น เป็นกระบวนการ passive transport เนื่องจากพบปริมาณของแอสตาแซนทินในเซลล์เพิ่มมากขึ้นตามปริมาณของสารแอสตาแซนทินที่เพิ่มขึ้นในอาหารเลี้ยงเซลล์ การเสริมคอเลสเตอรอลในอาหารให้แก่ปลาทดลอง ไม่มีผลในการเพิ่มการดูดซึมสารแอสตาแซนทินที่ลำไส้ แต่จะมีผลต่อการขนส่งสารแอสตาแซนทินโดยไลโปโปรตีน โดยพบว่า PR fraction มีบทบาทสำคัญในการขนส่งสารแอสตาแซนทินในกระแสเลือด ตามด้วย HDL, VLDL และ LDL ส่วนคอเลสเตอรอลในอาหารส่งผลต่อให้มีการเพิ่มปริมาณของสารแอสตาแซนทินใน VLDL การศึกษาในอนาคตควรมีการศึกษาถึงการยึดเกาะของสารแอสตาแซนทินกับโปรตีนที่อยู่ในส่วนของ PR fraction โดยการพัฒนาเทคนิคการแยก Ax-bond protein นอกจากนี้ ควรมีการศึกษาถึงเมตาบอลิสมของคอเลสเตอรอล ซึ่งการศึกษาเหล่านี้จะมีประโยชน์ในการเพิ่มประสิทธิภาพของการดูดซึม เมตาบอลิสม และสะสมของสารแอสตาแซนทินของปลามากขึ้น

Thesis Title **Biochemical Mechanisms in Absorption, Retention and Metabolism of Dietary Astaxanthin in Atlantic salmon, *Salmo salar***

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Abstract

Astaxanthin (Ax; 3, 3′-dihydroxy-β,β-carotene-4, 4′ dione) is a major carotenoid pigments used in Atlantic salmon feeds to achieve the red coloured flesh desired by consumers. The cost of adding Ax to diets is high and the retention of Ax in the muscle ranges from 5 to 15% of the total carotenoid. The absorptive processes which occur in the gut can potentially constitute a major limitation in the effective utilisation of carotenoids in salmonid fish. A series of experiments were designed to study the effects of several dietary factors that may affect the bioavailability of Ax from diets of Atlantic salmon and the nature of interactions between carotenoids and other dietary factors occurring during intestinal absorption and transport. Experiments were also directed to study the effect of cholesterol on cellular Ax uptake in the intestine and plasma transport of Ax by lipoproteins. It was also necessary to develop methodologies to achieve these goals. Biochemical methods involving cell culture models used for vertebrate animals were modified and applied to these studies in salmon.

The first experiment was designed to investigate the dietary factors that might enhance or interfere with Ax absorption in salmon including interfering factors such as certain carotenoids (zeaxanthin and lutein), plant sterols, fiber, and potential enhancing compounds such as cholesterol and vitamin E. Two hundred and eighty eight salmon (778±78g) were reared in sea water under controlled conditions and fed practical experimental diets. The experimental diets were supplemented with 40 mg Ax/kg, in addition to various dietary factors, including cholesterol (2%), vitamin E (450 IU/kg), wheat bran fiber (5%), lutein (40 mg/kg), zeaxanthin (40 mg/kg) and phytosterol (2%). After 26 days of feeding, blood was collected and separated to determine the plasma Ax concentration. Ax was not detected in the plasma of fish fed the non-pigmented control diet. Fish fed diet containing 2 % cholesterol significantly improved Ax absorption which

was reflected in higher the Ax concentration in plasma of Atlantic salmon. Other supplements including vitamin E, wheat bran fiber, lutein, zeaxanthin and phytosterols in diet had no significant effect on plasma Ax absorption.

The second study was undertaken to find a suitable cell culture model to study the intestinal Ax absorption. A human colon adenocarcinoma cell line (Caco-2) that has proved to be the best model for the absorption study was used because no intestinal cell line was available for fish. In developing the technique, it was necessary to first have a vehicle for delivery of carotenoids to the apical surface of cells. The cell culture model involving Caco-2 cells was successful to demonstrate the absorption of Ax by solubilizing in dimethlysulfoxide (DMSO).

An enterocyte isolation technique was modified and applied for *in vitro* and *in vivo* investigation of Ax uptake. Atlantic salmon were maintained on non-pigmented diet and pyloric caeca removed to isolate enterocytes from this tissue. Fresh cells were incubated with Ax-enriched media with and without a cholesterol supplement. For *in vivo* study, salmon enterocytes were isolated from pyloric caecae of fish fed experimental diets containing 40 mg Ax per kg diet, either with cholesterol (2% of the diet) or without cholesterol supplement. Blood and pyloric caeca were collected at specified time after the last meal. This *in vivo* model indicated that cellular Ax can be detected in enterocytes isolated from pyloric caecae after different times of feeding either from fish fed Ax supplemented diet only or diet containing Ax plus cholesterol (2%). It was evident that relatively high levels of cellular Ax were present when cells were exposed to a higher concentration of this carotenoid. Both *in vitro* and *in vivo* experiments clearly demonstrated that cellular uptake of carotenoids is a passive absorption process and there is no significant effect of cholesterol on cellular Ax uptake. Most of the *in vitro* absorption studies with fish have used intestinal tissue section or isolated enterocytes from various intestinal regions to investigate nutrient absorption in gut. This is the first study to report carotenoid absorption using an enterocyte isolation technique.

A significantly higher level of plasma Ax in fish fed Ax supplemented diet with 2% cholesterol as compared with fish fed diet containing only Ax supplement indicated that the effect of dietary cholesterol on Ax utilization may occur via the lipoprotein transport process. A comprehensive study was undertaken to investigate whether the distribution of Ax among the

lipoproteins in Atlantic salmon plasma was affected by dietary cholesterol supplementation. The lipoprotein separation was carried out according to the methods used in human lipoprotein investigations with some modifications. Plasma was fractionated using sucrose density gradients. This technique avoided contamination by albumin and scattering effects. Separation of protein by SDS-PAGE according to their electrophoretic mobility allowed for the isolation of apolipoproteins in each fraction. It also allowed identification of various lipoproteins including VLDL (d 1.031-1.043g/mL), LDL (d 1.043-1.050g/mL), HDL (d 1.050-1.103g/mL) and a protein-rich fraction (d 1.103-1.157g/mL). The application of this technique showed that only apolipoproteins of 25kDa in HDL fraction were present in detectable amounts. The amount of other apolipoproteins in HDL of plasma was too low to be identified by this method. The 260 and 240 kDa molecular weight protein in the VLDL fraction and 240 and 76 kDa molecular weight protein in the LDL fraction were found. Ax was distributed in each lipoprotein fractions. Protein-rich lipoprotein (PR) fraction appears to be a main carrier of Ax in the blood of Atlantic salmon, followed by HDL, VLDL and LDL. Cholesterol supplementation in the diet significantly increased the Ax concentration in VLDL.

In summary, the present investigation has provided new information on the effects of certain dietary factors on Ax absorption by Atlantic salmon as well as biochemical mechanisms involved in the uptake of Ax by intestinal mucosal cells (enterocytes) and the effect of cholesterol supplementation on Ax absorption and transport in fish blood. *In vitro* study on the cellular uptake of Ax by enterocytes provided evidence that when cells were exposed to a higher concentration of Ax, they have the ability to absorb relatively high concentrations of Ax. This indicates that cellular uptake of carotenoids is the passive transport process. Dietary cholesterol has a significant effect on the Ax transport process in the blood. However, there is no interaction of Ax and cholesterol on intestinal absorption of this carotenoid. The lipoprotein study showed Ax transport and distribution of Ax in each lipoprotein classes with the high levels in HDL followed by VLDL and LDL. The highest concentration of Ax was found in the protein-rich lipoprotein fraction which plays an important role as a main carrier of Ax in blood for metabolism by other tissues. The cholesterol content of the diet had a significant effect on Ax concentration in VLDL, which may explain the effect of higher concentrations of Ax retained by increasing dietary cholesterol levels.

Further research is needed to identify the protein that binds Ax in the protein-rich lipoprotein fraction by developing new methods to separate Ax-bound protein in this fraction as well as cholesterol metabolism of Atlantic salmon. Advanced knowledge in these areas would be useful to improve efficiency of carotenoid absorption, metabolism and retention in the flesh of Atlantic salmon.

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Chapter 1

Introduction and Literature Review

Salmonid fishes such as Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) represent some of the most widely cultured fish worldwide. Farming of Atlantic salmon in sea cages was developed in Norway during the early 1970's and shortly after that was established in countries around the North-Atlantic, Pacific Canada, Chile and Australia. The world aquaculture production of Atlantic salmon reached 1.5 million metric tons in 2009 (Table 1-1). Norway is the largest producer followed by the United Kingdom, Chile and Canada (Torrissen *et al*, 2011).

Atlantic salmon is an anadromous fish that spawns in freshwater and alevins (~ 2 cm) emerge from eggs, subsisting off the attached yolk sac until they reach the fry stage when they are ready to accept exogenous food. In the natural environment, they spend their early life stages in freshwater and undergo physiological and behavioral changes, a process called smoltification that prepares them for their life at sea. From rivers, they migrate to sea and return after 1-2 years to their native rivers and streams to spawn. Farmed salmon are cultured in freshwater hatcheries under controlled temperature and photoperiod on well-balanced diets and reach a characteristic physiological smolt stage (50–80 g) within one year. Smolts transferred to sea cages reach harvest size (~ 4 kg) in 10–15 months. Salmon are cultured in freshwater tanks and raceways in flow-through systems prior to transfer to sea. In the marine environment, they are farmed in sea cages where they depend solely on formulated feeds. In the natural environment, fry take much longer period (2-3 years) to reach the smolt stage before their migration from rivers to the ocean. The life cycle of Atlantic salmon is summarized in Figure A-1 (Appendix).

Although Atlantic salmon is the most successfully farmed salmonid, the nutrient requirements of this species are not well defined, and the available information is based on studies conducted on young fish (NRC, 1993, 2011). They require the same nutrients (protein, amino acids, essential fatty acids, vitamins and minerals) as other fish species for normal growth, reproduction, and immune and metabolic functions. Nutrient requirements of rainbow trout and other salmonids have been used to predict the requirements of certain micronutrients such as

amino acids and vitamins for feed formulation when this information is not available for Atlantic salmon (NRC, 1993, 2011; Storebakken, 2002).

Early research based on purified diets showed that juvenile Atlantic salmon reared in seawater required 45 % protein (Lall and Bishop, 1977). Salmon fry and juvenile fish perform better on high protein diet (~50 %) and grower diets containing 42–48 percent protein (Storebakken, 2002). The quantitative dietary essential amino acids requirement established for rainbow trout is also used for Atlantic salmon. Lysine, methionine and arginine (or threonine) are the most limiting amino acids in salmon feeds when fishmeal levels are reduced and plant protein sources are increased. The optimum protein level in feeds depends upon dietary energy content and the ratio of essential to non-essential (or indispensable to dispensable) amino acids. Atlantic salmon have no specific requirements for dietary carbohydrates. The following two types of carbohydrate are derived from the feed ingredients of plant origin used in salmon feeds: starch and non-soluble polysaccharides (NSP). Atlantic salmon has poor ability to regulate blood glucose when carbohydrate load is excessive (Hemre *et al*., 1995). NSP are not available to fish (reviewed by Stone, 2003). The efficiency of energy utilization is improved by reducing dietary protein content and increasing dietary lipid, thereby reducing the digestible protein (DP) to digestible energy (DE) ratio. The ratios of DP to DE for maximum growth have been measured using practical diets: fingerlings, 23 g/MJ; smolts, 20 g/MJ; grower (0.2 –2.5 kg), 19 g/MJ; and grower (2.5–4 kg), 16–17 g/MJ (Storebakken, 2002).

Dietary lipids supply energy and essential fatty acids (EFA). Increasing levels of dietary fat (up to 24 %) increases the efficiency of protein utilization. The EFA requirement of Atlantic salmon can only be met by supplying the long-chain highly unsaturated fatty acids, eicosapentaenoic acid (EPA), 20:5n-3, and/or docosahexaenoic acid (DHA), 22:6n-3. Based on total body and tissue fatty acid composition data, the estimated EFA requirement of salmon is 1 % of the diet for 20:5n-3 and 22:6n-3 fatty acids combined (Ruyter *et al*., 2000). Marine fish oils (MFO) have been traditionally used in salmon feeds; however, the overexploitation of marine resources has resulted in limited supply of this oil supplement. Recent research has shown that it is possible to replace the major proportion of MFO with vegetable oils (VO) and still maintain optimum growth and feed utilization over the major part of the life cycle (reviewed by Bell and Koppe, 2011). Finishing diets based on MFO can be used to achieve the desired level of EPA and

DHA in the final product. To date, no significant effects of either partial or full replacement of MFO with vegetable oils (canola, rapeseed and flaxseed oils) on flesh quality of fish have been observed

Qualitative and quantitative requirement values of most fat-soluble (A, D, E and K) and water-soluble (thiamin, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid, vitamin B_{12} and vitamin C) vitamins established for other salmonids have been used for the feed formulation of Atlantic salmon with some exceptions (NRC, 1993, 2011). The minimum requirement of vitamin E for juvenile salmon has been estimated as 60 mg/kg dry feed (Hamre and Lie, 1995), a value higher than that for other salmonids (reviewed by Gatlin III, 2002). Most essential elements required by terrestrial animals are also considered essential for Atlantic salmon, and thus requirements have been reported for phosphorus, magnesium, iron, copper, manganese, zinc, selenium and iodine (Lall and Milley, 2008). Mineral deficiency signs in salmon and other fish include reduced bone mineralization, anorexia (potassium), lens cataracts (zinc), skeletal deformities (phosphorus, magnesium, zinc), fin erosion (copper, zinc), nephrocalcinosis (magnesium, selenium toxicity), tetany (potassium), thyroid hyperplasia (iodine), muscular dystrophy (selenium) and hypochromic microcytic anemia (iron).

Atlantic salmon feeds are formulated for various stages of freshwater (starter, grower, smolt transfer) and marine (grower and broodstock) production cycle. Freshwater feeds contain 45–54 % protein and 16–24 % lipid. The protein content is decreased after salmon fry reach fingerling size. Seawater transfer feeds are used for salmon going through parr-smolt transformation. These diets contain salt, betaine, amino acids, nucleotides and other supplements to improve the osmotic adaptation of smolts to seawater and for better survival. Smolts are fed marine grower feeds after the seawater acclimation is complete. Generally, the protein content is reduced from 45–48 % to 36–42 % and lipid content increased from 24 to 30–40 % during their seawater growout phase to market-size salmon.

Table 1-1 World production of Atlantic salmon in 2011 by country[1, 2]

Countries	Metric ton	%
Norway	944,600	65
United kingdom	141,800	10
Chile	129,500	9
Canada	118,000	8
Faroe Island	42,100	3
Australia	33,000	2
United States	18,000	1
Ireland	17,800	1
Others	1,400	0

From: [1]Lassen *et al.* (2011) cited by [2]Torrissen *et al.* (2011)

Carotenoids and pigmentation in salmonids

One of the distinguishing features of salmonids is the pink to red colored flesh imparted by carotenoid, which contributes to their elite image (Torrissen 1986). The most important salmon quality criterion, next to freshness of the product is the flesh color because of consumer's preferences for pigmented flesh. Pigmentation of flesh results from the absorption and deposition of astaxanthin (Ax) and canthaxanthin (Cx) obtained from dietary sources. Salmonids, like other vertebrates, are unable to synthesize carotenoid *de novo* and depend entirely on carotenoids in their feed. Astaxanthin (3,3′-dihydroxy-*β*,*β*-carotene-4,4′-dione) is the predominant carotenoid in wild salmonids (Schiedt *et al*., 1986; Scalia *et al*., 1989) and is derived mainly from ingested zooplankton. Flesh pigmentation of farmed salmonids is achieved by dietary supplementation of either Ax or Cx (*β*,*β*-carotene-4,4′-dione) in the range of 35-75 mg/kg diet. The dietary supplementation of astaxanthin accounts for approximately 15-20% of the feed cost and 6-8% of the total production cost of the salmon industry (Torrissen, 1995). More recently feedings practices and pigmentation strategies have reduced the amount of carotenoid supplements in fish feeds thus its cost. The retention of total carotenoids in flesh is only 5-15 % of total carotenoids (Buttle *et al*., 2001; Torrissen *et al*., 1989). They suggested that carotenoid retention in fish flesh

is affected by the efficiency of absorption from the digestive, transport, biochemical mechanisms involved in tissue uptake of specific carotenoid in various tissues and excretion rate in fish.

Carotenoids are classified by their structure as carotenes and xanthophylls and they both share a common C_{40} polyisoprenoid structure containing a series of centrally located, conjugated double bonds (Deming and Erdman 1999). Carotenes (e.g. β-carotene, Figure 1-1) are relatively non-polar hydrocarbons, while xanthophylls (e.g. zeaxanthin, β,β-carotene-3,3'-diol) are more polar, containing oxygen either as a hydroxyl or keto group contained in the end group. Some of the naturally occurring carotenoids are chiral, bearing from 1 to 5 asymmetric carbon atoms (Pfander 1992). Most carotenoids are yellow, orange, and red in colour and their conjugated double bond structure is related to their physiological function including the absorption of light during photosynthesis, and protecting cells from sunlight (Demmig-Adams *et al*. 1996).

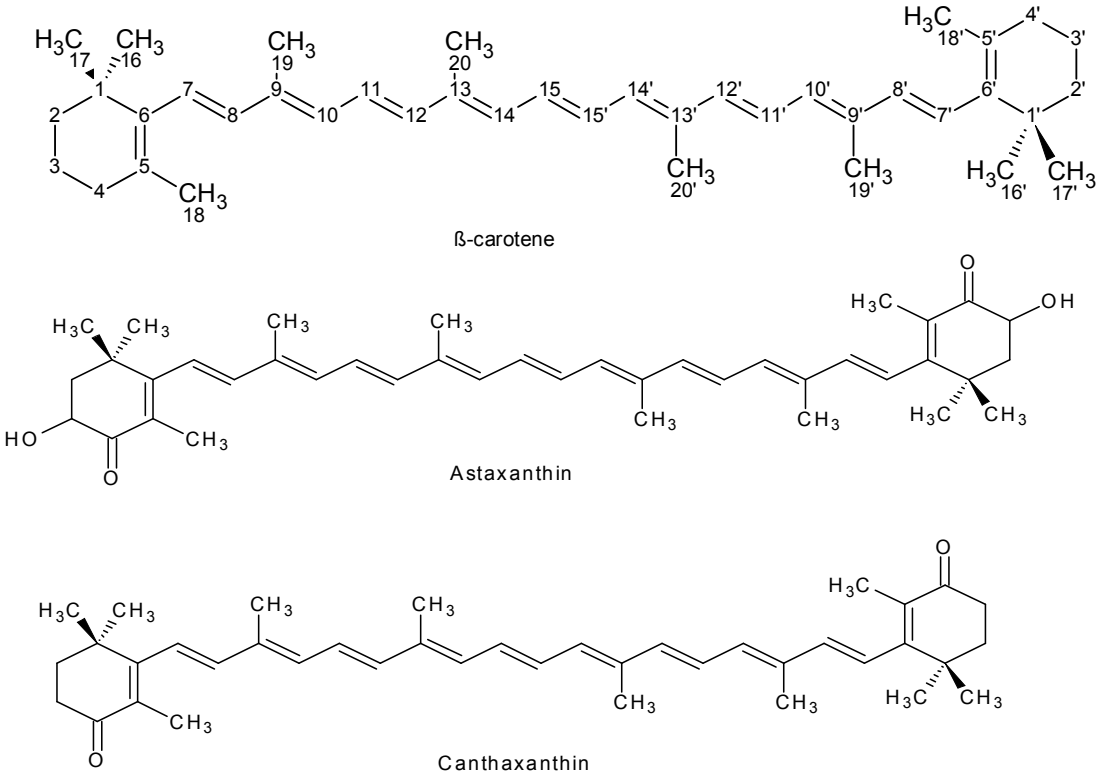


Figure 1-1 Structure of ß-carotene, astaxanthin and canthaxanthin.

Unlike other fish species, salmonids primarily deposit ingested carotenoids in muscle tissues, however, juvenile and sexually mature fish also deposit carotenoids in their skin and gonads respectively (Shiedt *et al*. 1985). In wild Atlantic salmon, astaxanthin is the principal carotenoid of muscle and eggs (Craik and Harvey, 1987). Farmed salmonids are fed feeds containing synthetic Ax or Cx for flesh pigmentation. A combination of Ax and Cx supplementation of salmonid diets have resulted in a higher total carotenoid deposition in the flesh than either Ax or Cx alone (Torrissen, 1989). Ax seems to be more efficiently absorbed and deposited in salmonids than Cx (Foss *et al*., 1984; Torrissen, 1986; Storebakken *et al*., 1987). Free Ax has a better pigmenting efficacy compared with its dipalmitate form (Storebakken *et al*., 1987).

Astaxanthin is distributed in the fish body in either free or esterified form. Ax in integument is in the diester form but Ax bound to protein is found in blood, flesh and various organs. The concentration of Ax found in Atlantic salmon flesh is 4-10 mg/kg in farmed fish, while the levels in wild Atlantic salmon range from 3-11 mg/kg (Torrissen *et al*., 1995). Flesh pigment levels of 4-5 mg/kg are considered to be the minimum to impart acceptable flesh coloration (Torrissen, 1989).

Several investigations have demonstrated the importance of dietary Ax supplementation on broodstock nutrition. Addition of Ax above 10 mg/kg is necessary for proper egg quality, hatchability and survival of newly hatched Atlantic salmon fry (Torrissen and Christiansen, 1995). Ax supplementation in rainbow trout broodstock diets appears to be effective in improving egg quality and lowering of egg mortality during embryonic development (Ahmadi *et al*., 2006). Furthermore, Bazyar Lakeh *et al*. (2010) showed that dietary supplement of Ax (>12.5 mg/kg) in broodstock diets improves specific growth rates of rainbow trout larvae. An investigation during the first feeding period of Atlantic salmon fry found Ax was important for both growth and survival. A minimum dietary Ax concentration of 5.1 mg/kg supported optimum growth and survival of Atlantic salmon fry (Christiansen *et al*., 1995).

Astaxanthin is also considered to play an important role in the innate immune function of fish. Salmon eggs infected with M74, a causative agent for lethal egg yolk syndrome which appears during the swim up stage, showed lower incidence of this yolk syndrome when Ax

concentration in eggs was optimum (Pickova *et al*., 1998). Ax also increased the serum complement and lysosome activity, nonspecific cytotoxicity of peripheral blood lymphocyte and phagocytic activity of rainbow trout head kidney phagocytes (Amar *et al*., 2001).

Source of dietary astaxanthin

The carotenoids used in salmon feeds consist of two commonly used compounds produced by commercial synthesis (Ax and Cx) and natural sources of Ax from algae, yeast and zooplankton. Carotenoids are known to exist in different geometric forms (*cis* and *trans* isomers). These isomers can be interconverted by light, thermal energy or chemical reactions, however the majority of carotenoids exists in the all trans configuration. Astaxanthin has three optical (R/S isomers and 272 possible geometrical (E/Z) isomers, however, the most important isomers are the following: all E-, 9Z-, 13Z- and 15Z-. The major natural commercial pigment sources of Ax include fresh water green algae, *Haematococcus pluvialis* (Sommer *et al*., 1991; Choubert and Heinrich, 1993; Barbosa *et al*., 1999) and the red yeast, *Phaffia rhodozyma* meal. *Phaffia* yeast contains Ax at a minimum 3 g/kg (Sanderson and Jolly, 1994). Several high yielding Ax strains have been developed in industrial scale fermentors which contain more than 10 g Ax/kg (Johnson, 2003) and 18 g Ax/kg in a laboratory scale fed batch culture system (Kusdiyantini *et al*., 1998). *Haematococcus* contains β-carotene, Ax (free, monoester and diester), Cx and lutein (Sommer *et al*., 1991; Choubert and Heinrich, 1993). In *H. pluvialis*, Ax and Cx are the major red carotenoid pigments in amounts as high as 86.4% of total carotenoids (Choubert and Heinrich, 1993). However, the carotenoid concentration may vary among strains and under different culture conditions. Barbosa *et al*. (1999) found both Ax and lutein in their cultured algal stains, however, Ax was the major red carotenoid with Ax concentration as high as 95.5% of total carotenoids.

Bioavailability of carotenoids from both yeast and natural algal sources has some limitations because cell wall disruption is essential to allow their enzymatic digestion and absorption from the digestive tract of fish. Studies conducted in rainbow trout showed that Ax retention in the muscle of fish fed diet containing *Haematococcus* was 1.5% versus 3.1% for fish fed a mixture of synthetic Ax and Cx (Choubert and Heinrich, 1993). Similar results were also reported by Sommer *et al*. (1991). They found that synthetic Ax was deposited with a greater

efficacy than the algal source. The Ax levels at 100 days for juvenile and adult trout fed algal pigments were 58% and 55%, respectively, of the values measured for synthetic Ax at a similar level of supplementation. The lower efficacy of the algal carotenoid may be explained by the presence of esterified forms of Ax, which are not efficiently utilized by trout. In addition, the algal biomass contained a variety of other carotenoids such as β-carotene, zeaxanthin and lutein which may be less effective than Ax for flesh pigmentation. It is also possible that the algal carotenoids may not have been completely bioavailable due to incomplete break down of the algal cell walls (Sommer *et al*., 1991). They reported that rainbow trout fed diet supplemented with the mechanically ground *Haematococcus* algae had a higher Ax serum concentration and was at the same extent as fish fed synthetic Ax supplemented diet (Barbosa *et al*., 1999). Similar results were found in rainbow trout fed mechanically ruptured red yeast, *Phaffia* (Johnson *et al*., 1980). Enzymatic cell wall disruption improved the utilization of Ax from red yeast in diets for rainbow trout (Storebakken *et al*., 2004).

Metabolism of carotenoids in salmonid fishes

The large difference of approximately 30-50% reported by several researchers between the apparent digestibility and retention of carotenoids suggests that higher levels of carotenoids are absorbed compared to that deposited by salmonids (Torrissen *et al.* 1989; No and Storebakken 1991). Early work of Hardy *et al.* (1990) suggested that the absorbed Cx was metabolized in the liver and metabolic product(s) were excreted in the bile. This may partly explain the discrepancy between absorption and retention of carotenoids in salmonid tissues. Salmonid fishes are capable of transforming the 4(4´)-ketocarotenoids, Ax and Cx into retinol and 3, 4-didehydroretinol (vitamin A_1 and A_2) (Schiedt *et al.*, 1985; Al-Khalifa and Simpson, 1988; White *et al.*, 2003). Although carotenoids are enzymatically cleaved to produce vitamin A in the gastrointestinal tissue, this transformation may occur at a low rate and be quantitatively insignificant (Schiedt *et al.* 1985). Torrissen *et al*. (1990) suggested that the differences observed between absorption and retention may only be explained by metabolism and excretion of absorbed carotenoids. However, White *et al*. (2003) demonstrated significant conversion of Ax to vitamin A. Page and Davies (2006) reported that both Ax and Cx had much lower amounts in intestinal tissue extracts in Atlantic salmon which they attributed to potential metabolites. They suggested that it is possible

that carotenoids may be oxidatively degenerated in the digestive tract but this hypothesis was not tested.

Generally, the gastrointestinal epithelium and liver are considered the most important organs for catabolic transformation of carotenoids despite the fact that reductive metabolites have been detected in the kidney, spleen, gonads, skin and retina. The quantitative importance of the various organs for metabolic transformation has not been investigated and requires the identification of genes and enzymes of different metabolic pathways and identification of metabolic products. It appears that metabolic products are formed rapidly because the reductive Ax metabolite, idoxanthin (3, 3´, 4´-trihydroxy-ß, ß-carotene-4-one) was detected in the plasma within 6 hours after force-feeding Atlantic salmon with radioactively labelled Ax (Aas *et al.*, 1999). This metabolite is formed by reduction of one of the keto-groups of Ax, whereas 4´-hydroxyechinenone (4´-hydroxy-ß, ß-carotene-4-one) is a similar reductive metabolite of Cx (Figure 2). Among salmonids, species differences exist in accumulation of idoxanthin in the blood and other tissues. Studies conducted on Arctic charr (*Salvelinus alpinus*) show that 40 to 60 % of the total carotenoids in the muscle was idoxanthin (Aas *et al.*, 1997; Hatlen *et al*., 1997) as compared to 10 % in Atlantic salmon (Bjerkeng *et al*., 2000). However, idoxanthin concentration in rainbow trout muscle is detectable at very low concentrations (Bjerkeng *et al*., 1997). These workers have isolated 4´-hydroxyechinenone in the muscle of Atlantic salmon 0+ and 1+ smolts fed a diet supplemented with Cx (Aas *et al.*, 1997). It appears that the (4´*S*)-isomer accumulates selectively (81 % 4´*S*- and 19 % 4´*R*-isomer, of the total amount of 4´-hydroxyechinenone) suggesting that Cx is reduced selectively by enzymes. A selective reduction to Cx the (4´*S*)-hydroxyechinenone has been also reported in the skin of rainbow trout (Bjerkeng *et al*., 1992). The concentration of idoxanthin was higher in small fish than larger fish. This indicates that the metabolic capacity to transform absorbed astaxanthin decreases with age and size in salmonid fishes (Aas *et al*., 1997; Schiedt *et al*., 1989). Bjerkeng *et al*. (1996) reported that idoxanthin content of Atlantic salmon muscle was partly genetically determined and the heritability coefficient was 0.4. It has also been suggested that stress has causes increased metabolism of carotenoids in salmonid fishes (Schiedt *et al*., 1989).

Alternative metabolic pathways of carotenoid metabolism have also been investigated mainly in rats and humans but not in fish. It has long been known that carotenoids are substrates

for lipoxidase-type enzymes (Chichester & Nakayama 1965). Lipoxygenase isolated from trout convert polyunsaturated fatty acids (PUFA) from muscle into PUFA hydroxyperoxides (German and Kinsella 1985), which may cause discoloration of fish flesh (Tsukuda 1972), most likely by bleaching of carotenoids following free-radical quenching (Krinsky and Deneke 1982). However, no studies have investigated the induction of the lipoxygenase enzyme system due to dietary carotenoid inclusion. Caris-Veyrat *et al*. (2001) showed that dioxygen produced a number of ß-apo-carotenals. In addition, ß-apo-carotenals, with different carbon chain lengths, can be produced via a number of enzymatic reactions, co-oxidation by lipoxygenase, autooxidation, or direct reaction with free radicals (reviewed by Yeum and Russell 2002). The cytochrome P450 enzymes, CYP26A1, CYP26B1 and CYP26C1, enable the catabolism of retinoic acid to 4-hydroxy-retinoic acid, 4-oxo-retinoic acid and 18-hydroxy-retinoic acid (White *et al*. 2000). The formation of 4-oxo-retinoic acid could be the precursor to some of the 4-oxo-metabolites recovered in humans and rats (Wolz *et al*. 1999, Kistler *et al*. 2002). Urlacher *et al*. (2005) demonstrated that ß-ionone could be converted into 4-hydroxy-ß-ionone via cytocrhome P450 monooxygenases in bacteria. Since cytochrome P450 enzyme systems were not induced in rainbow trout (Page and Davies 2002), they concluded that the inherent activity (due to vitamin A in the basal diet) may be sufficient to metabolize the dietary carotenoid load delivered due to the low hepatic carotenoid uptake (Page and Davies 2003).

Bjerkeng and his colleague (1997) have investigated the distribution of *E/Z* and *R/S* optical isomers and concluded that these isomers may have significant impact on selective metabolic transformation. When rainbow trout were fed diets high or low in all-*E*-astaxanthin (97 % vs. 64 % of total Ax), a selective accumulation of 13*Z*-astaxanthin was found in the liver (48-59 % of total Ax), whereas 9*Z*-astaxanthin comprised only 2.5 % of the total of the blood (Østerlie *et al*., 1999). This indicates that a selective metabolic transformation of the various geometrical *E/Z* isomers occurs most likely in liver , Some selective accumulation of (3*S*, 3´*S*)-astaxanthin has been also observed in skin and kidney (Østerlie *et al*., 1999). In the skin, the selective accumulation may be related to the esterification of Ax, a metabolic process similar to the one that occurs in the gastrointestinal tract. The selective accumulation of (3*S*, 3´*S*)-zeaxanthin, an Ax metabolite in the skin of rainbow trout further indicates this metabolic transformation (Bjerkeng *et al*., 1992).

Comprehensive studies have been conducted by the Halifax researchers to investigate the biochemical mechanisms involved in accumulation of carotenoids in muscle and other tissues of Atlantic salmon. It has long been recognised that carotenoids are primarily associated with the protein, not the lipid fraction of muscle tissue (Henmi *et al*., 1989). Since Ax is soluble in lipid, a method was developed to enable the determination of Ax binding proteins in fish muscle tissue (Saha *et al*., 2005). The ultracentrifugation method along with the use of sodium cholate for dispersion of astaxanthin complexes were developed to separate unbound astaxanthin by filtration over a 30 kDa filter. After fractional extraction of the muscle proteins, the various fractions that bound astaxanthin could be identified. The fractional extraction of protein-bound astaxanthin using different brine concentrations and pH also indicated that a water soluble extract had the highest affinity to Ax, i.e. the highest ratio between Ax and protein. This indicated a sarcoplasmaprotein was involved in the binding of astaxanthin. By using fractional extraction of muscle proteins from Atlantic salmon, Atlantic halibut and haddock, it was shown that certain fractions had a greater ability to bind to Ax than other fractions, but the same protein fractions from all three species were able to associate with Ax (Saha *et al*., 2006). Matthews *et al*., (2006) provided further evidence that the primary binding protein of astaxanthin in the muscle of Atlantic salmon was ∞-actinin. They also showed that ∞-actinin isolated from Atlantic halibut also combined with astaxanthin in about the same stoichiometric ratio. In summary, these findings suggest that the Ax-binding capacity of the muscle protein does not limit the deposition of Ax in the muscle but a high metabolic transformation rate may limit the retention of ingested Ax.

Methods for evaluating carotenoid absorption: An *in vitro* model of cellular uptake

The absorption mechanism for carotenoids may be similar to that as other lipids. The following major steps may be involved in carotenoid absorption within the mammalian gastrointestinal tract: 1) release of carotenoids from food matrix; 2) solubilization of carotenoids into mixed lipid micelles in the lumen; 3) cellular uptake of carotenoids by intestinal mucosal cells (enterocytes); 4) incorporation of carotenoids into chylomicrons (CM) and; 5) secretion of carotenoids and their metabolites associated with CM into the lymph (van Het Hof *et al*., 2000). In fish, carotenoid absorption mechanisms remains to be investigated. Information is needed regarding the absolute efficiencies of carotenoid absorption, the nature of luminar and

intracellular factors regulating the process of their absorption, the mechanism of intracellular transport of these carotenoids and their incorporation into CM, and the nature of interactions between carotenoids and other dietary factors occurring during intestinal absorption.

Human carcinoma cell culture model

Human colon adenocarcinoma cell line or Caco-2 is a cell line originating from human colonic carcinoma and has proved to be the best model to study the intestinal absorption (Sambruy *et al*., 2001). They have some similar morphological and functional characteristics to the epithelial intestinal mucosal cells. The Caco-2 cell line has been extensively used to study absorption of several nutrients including proteins (Lenaerts *et al*., 2007; Mayjune *et al*., 2009; Rubio and Clemente, 2009), vitamins (Quick and Ong, 1990; Cross *et al*., 1997; Anwar *et al*., 2006; Reboul *et al*., 2006), minerals (Garcia *et al*., 1996; Ekmekcioglu *et al*., 1999; Etcheverry *et al*., 2002; Viadel *et al*., 2006; Cámara *et al*., 2007), glucose (Harris *et al*., 1992; Johnton *et al*., 2005; Chang *et al*., 2007), and lipids and fatty acids (Trotter *et al*., 1996; Ho and Storch, 2001; Nakano *et al*., 2009). This cell line has been also used to estimate carotenoid bioavailability from various sources (Garrett *et al*., 1999; Garrett *et al*., 2000; Liu *et al*., 2004; Chitchumroonchokchai *et al*., 2004). Other studies include comparison of the uptake and secretion between carotene and xanthophyll (During *et al*., 2002; O'Sullivan *et al*., 2007), evaluation of the intestinal transport rate compared to vitamin A (During and Harrison, 2007) and factors which may affect the intestinal carotenoid absorption (Gracía-Casal *et al*., 2000; During and Harrison, 2005).

In vitro studies have been hampered by difficulties in delivering the carotenoid, a hydrophobic compound to cells in culture conditions and also by the instability and insolubility of carotenoids, and cytotoxicity arising from solvents used (O'Sullivan *et al*., 2004). Several techniques have been used to solubilise carotenoids before addition to cell culture media. Organic solvents such as tetrahydrofuran (THF), dimethylsulfoxide (DMSO), ethanol and n-hexane and emulsifiers such as polyoxyethylene sorbitan monopalmitate (Tween 40) and polyoxyethylene sorbitan monooleate (Tween 80) have frequently been used to solubilize carotenoids and as delivery vehicles for carotenoids absorption studies.

During *et al*. (1998) compared two methods for delivering ß-carotene to TC7 clone of human intestinal cell line Caco-2. The same amounts of ß-carotene was dissolved in either tetrahydrofuran (THF) or Tween 40 before adding the solution to the cell culture media, Dulbecco's Modified Eagle Medium (DMEM). They concluded that Tween 40 gave better results than THF, in terms of recovery in the medium and cellular uptake of ß-carotene. It appears that ß-carotene solubilised in THF formed a solid-state precipitate when added to the cell medium (aqueous phase). This caused poor uptake and recovery of THF solubilised ß-carotene. In contrast, cells incubated with ß-carotene in Tween 40 exhibited a consistent yield of metabolic residue and a significant increase in enzyme activity. During and Harrison (2005) used Tween 40 to deliver carotenoids (ß-carotene, lutein and lycopene) to facilitate their intestinal absorption. Tween has also been applied to determine the mechanism of carotenoid and retinoid intestinal absorption which focuses on membrane transport mechanisms through the Caco-2 cell model (During and Harrison, 2007).

O'Sullivan *et al*. (2004) developed a system for delivering lipophilic compounds such as tocopherols and the mixture of carotenoids (astaxanthin, lutein, canthaxanthin, lycopene, α-carotene, ß-catotene). These compounds were solubilized in three different organic solvents, Tween 40, Tween 80 and THF before administering them to Caco-2 cells. Their findings were in agreement with those reported by During *et al*. (2002) in that Tween 40 and Tween 80 resulted in higher solubility of carotenoids and tocopherols and delivered substantially more to the cells than THF.

Dimethylsulfoxide (DMSO) is an amphipathic molecule with a highly polar domain and two apolar methyl groups, which makes it soluble in both aqueous and organic media (Santos *et al*., 2003). It is frequently used as a cryoprotectant of cell suspensions during cryopreservation. Concentrations of DMSO between 5-15% have been widely used, however, a range of 7.5 to 10% is more common (Freshney, 2005). DMSO is also a highly effective solvent for carotenoid extraction. In studies related to the isolation of intracellular Ax from *Phaffia rhodozyma*, use of DMSO during the cell disruption process resulted in higher recovery of Ax as compared to the use of Na_2CO_3, vortex agitator or ultrasonic waves (da Fonseca *et al*., 2011). In an earlier study, Calo *et al*. (1995) used ethanol and DMSO to extract carotenoid extraction from several *P. Rhodozyma* mutants. They found that both DMSO and ethanol were highly efficient in Ax

extraction, however, ethanol had the advantage of having a low toxicity to animals as well as no residue of DMSO in the final product.

DMSO has been widely used as a solvent in biological studies and as a vehicle for drug delivery (Krishna *et al*., 2001; Demirbas and Stavchansky, 2003; Liu *et al*., 2004; Ebert *et al*., 2005; Elsby *et al*., 2008). In studies related to carotenoid uptake, Liu *et al*. (2004) developed a Caco-2 cell culture model to assess the uptake of carotenoids. Synthetic carotenoid standards (lutein, zeaxanthin and β-carotene) were dissolved in DMSO and diluted to an average concentration of 1-8 μM in cell culture media (final concentration of DMSO was 2%). Carotenoid uptake by Caco-2 cells after 2 and 14 days of plating were studied. Lutein, zeaxanthin and β-carotene uptake were detected after 1 h and reached a stable level after 4 h incubation period. Their model successfully used DMSO as a carrier of carotenoids to the Caco-2 cells. No negative side effects of DMSO on cell cultured at 2% DMSO were observed. Previous researchers have reported a high efficiency of DMSO to transepithelial transport of lipophilic drugs in Caco-2 cell culture (Krishna *et al*., 2001; Demirbas and Stavchansky, 2003). Artursson (1990), however, demonstrated that when Caco-2 cells monolayers are exposed to more than 1% DMSO, the integrity of the monolayer is lost due to a rapid increase in osmolarity in the media. In order to avoid this problem, 4% (w/v) human serum albumin (HAS) was used as a co-solvent in the basolateral side to facilitate the solubilization of highly lipophilic compounds. This reduced the risk of cellular damage leading to structural changes in membranes that might affect the paracellular and/or the transcellular pathways, and yield incorrect permeability measurements. The cytoprotective action of 4% HAS (w/v) showed that increasing the concentration of DMSO to 10% (v/v) in the cell culture media did not significantly change the effect on the permeability of compounds through the Caco-2 cell monolayer (Demirbas and Stavchansky, 2003).

Fish intestinal cell culture model

Studies on fish intestinal nutrient uptake have used *in vitro* techniques with either tissue (Al-Khalifa and Simpson, 1988; White *et al*., 2003) or isolated epithelial intestinal cells (Soengas and Moon, 1998) to incubate them in a media containing nutrient supplements. Some investigators have carried out experiments with isolated enterocyte and labeled substrates to study the fatty acid metabolism in fish (Pérez *et al*., 1999; Tocher *et al*., 2002; Tocher *et al*., 2004;

Oxley *et al*., 2005; Bogevik *et al*., 2008). *In vitro* intestinal uptake of carotenoids in an inverted rainbow trout and Atlantic salmon intestine have been also reported (White *et al.*, 2003). Fish intestinal section, pyloric caecae, mid and hind intestine were exposed to a micelle media containing 5 mg/L solubilized carotenoid (canthaxanthin and astaxanthin) for 1h and then the tissue carotenoid content was determined. Their results showed that the Ax tissue concentration between intestinal sections of the proximal intestinal region (pyloric caecae and mid intestine) had significantly higher values compared to hind section of the intestine. The carotenoid content of pyloric caecae after exposure to micellar media containing similar carotenoid concentrations was significantly lower for Cx compared to Ax in trout caecae. However, the Ax content of the mid or hind intestine between rainbow trout and Atlantic salmon were not significantly different when they were exposed to micellar Ax containing media. Al-Khalifa and Simpson (1988) reported a similar finding when everted rainbow trout intestine, ileum and duodenum section were suspended in incubation media containing labeled Ax. They found that Ax uptake from the duodenum (pyloric caecae) was higher than the ileum which further confirmed that the absorption of carotenoids occured mainly along the proximal intestine of salmonids.

Freshly isolated primary cell cultures have been used to study nutrient metabolism, the effects of enzyme inducers, and to investigate biochemical mechanisms involved and to determinate changes in structural activity (Kilemade and Quinn, 2003). The first step for isolation of compounds involved disruption of various tissues isolated from different organs of experimental animals. Loosely organized lymphoid tissue was dispersed mechanically to yield cell suspensions, whereas in other tissues, the simplest method was to finely chop the tissues and allow migration of cells out of different pieces on growth surfaces. Generally, tissue pieces were subjected to different enzyme in a suspension of single cells or clump of cells. Commercially available enzymes were either used alone or in combination with different enzymes, depending upon the organ involved (Bols and Lee, 1991).

Progress has been made towards the development of fish intestinal cell isolation techniques. Typically, intestinal epithelial cells have been isolated from different segments of the fish intestine and subjected to enzymatic digestion (Dópido *et al*., 2004). Certain tissues from intestine have been also digested with collagenase enzymes for preparation of single cell suspensions (Soengas and Moon, 1998; Pérez *et al*.,1999; Tocher *et al*., 2002; 2004). Pérez *et al*.

(1999) described a method for the isolation of enterocytes from intestinal tissues including foregut and pyloric caecae of rainbow trout. Intestinal tissue were initially cleaned and incubated with buffer solution containing 1.5mM EDTA before palpating them with fingers. The luminal content was filtered and dissociated cells were collected and re-suspended in cell media, Minimum Essential Medium (MEM) containing collagenase enzyme with constant stirring to isolate single cells. Finally, enterocytes were washed twice and resuspended in MEM. This procedure allowed higher viability rates of cells (89%) and almost complete absence of mucus.

Dópido *et al.* (2004) successfully developed a procedure for the isolation of epithelial cells along the intestine of gilthead seabream (*Sparus aurata*) using a dissociation method based on intracellular type solution. They used different sections intestine including, pyloric caecae, anterior intestine and posterior intestine. After dissection, the contents were removed by gentle wash with cold saline solution. Each intestinal segment was then filled and incubated at room temperature in a hyperosmolar (intracellular-like) solution. The intestinal segments were gently palpated with fingers to prevent the loss of cells. The luminal solution containing cells free from the epithelia were filtered through a 100 µm nylon mesh. The cell pellets were resuspended in a Ringer-type solution with collagenase (0.1 mg/mL), incubated while shaking for 15 min, then the cell suspension was filtered through a 60µm nylon mesh and centrifuged. Cell pellets were resuspended in Dulbecco's modified eagle medium. After isolation of enterocytes, their viability was measured using the trypan blue exclusion method and the release of the enzyme lactate dehydogenase (LDH) to culture media as well as activities of other intestinal enzymes (e.g. sucrase, maltase, alkaline phosphatase, 5-nucleotidase, leucine aminopeptidase and γ-glutamyl transferase). This technique showed that the use of intracellular-like solution allowed for isolation of cell preparations with high purity and viability from three intestinal regions. Isolated cells consisted mostly of enterocytes (>95%) and a small proportion of mucous (goblet) cells. After isolation, cells tended to adopt round shapes with similar diameters of enterocytes (5µm) and mucous cell (8µm). Enterocyte preparations exhibited a clear heterogeneity in the distribution of enzyme activities along the digestive tract. Sucrase, maltase and leucine aminopeptidase activities were at the highest level in the posterior intestine, whereas the anterior intestine had the lowest levels. However, alkaline phosphatase and 5-nucleotidase activities were at the lowest level in pyloric caecae and posterior intestine and highest in the anterior region. Finally, γ-glutamyl transferase activities were similar in enterocyte preparations from the three intestinal sections.

They emphasized that isolated cells from the intestinal epithelia of gilthead seabream exhibited segment-specific heterogeneity which was likely to reflect differences in the functional role of enterocyte populations in each intestinal segment. Thus isolated cells are viable and suitable for further cytological and molecular studies to elucidate the mechanism of ionic-osmoregulation, digestion and nutrient transport processes.

Tocher *et al*. (2002; 2004) reported a method to isolate enterocytes from various regions of the intestine including pyloric caecae. In their enterocyte isolation technique, fish were killed, the whole intestinal tract removed, pyloric caecae dissected and adhering adipose tissue removed. The intestine was slit open and the luminal contents rinsed with buffer solution. The caecae and mid-gut tissues were finely chopped, incubated with buffer containing 0.1% (w/v) collagenase enzyme at 20˚C for 45 min in a shaking water bath. After enzymatic digestion, intestinal tissues were filtered through a 100µM nylon gauze and cells collected, and washed with buffer solution containing 1% (w/v) fatty acid free bovine serum albumin. The enterocytes were resuspended in Medium 199 containing 10mM HEPES, 2mM glutamine before incubation with the solution.

Pérez *et al.* (1999) investigated the uptake and esterification of radiolabelled fatty acids using isolated enterocytes from rainbow trout to study intestinal metabolism. They employed various radiolabelled fatty acids solubilized in MEM containing the bile salt, sodium taurocholate in cell suspension. The incubation period was 1min and 15 min. The enterocyte solutions were washed and their lipids extracted. The amount of radioactivity present in the lipid was determined for cellular uptake. They found that the amount of radioactivity recovered in the enterocytes was increased by incubating the cells with radiolabelled fatty acids and these radiolabelled fatty acids taken up by the isolated enterocytes were extensively esterified into cellular lipids. These investigators suggested that isolated enterocytes can be used to study the mechanisms involved in intestinal fatty acid absorption of fish. In a later study with rainbow trout, Tocher *et al*. (2002) added labelled fatty acids to a cell suspension culture flask. They measured the fatty acyl desaturation and elongation as well their oxidation in isolated intestinal enterocytes isolated from a study involving interaction between water temperature and dietary lipid composition of diet particularly the effects on regulation of fatty acid metabolism (Tocher *et al*., 2004).

In a recent study, enterocytes isolated from the pyloric caecae of rainbow trout were used to measure the fatty acid uptake and efficiency of utilisation from copepod oil (Oxley *et al*., 2005). From the same group, Bogevik *et al*. (2008) employed the method of Tocher *et al*. (2004) to isolate enterocytes from Atlantic salmon pyloric caecae to study the utilization and metabolism of palmityl and oleoyl fatty acids and fatty alcohols. The labelled fatty acid (FA) were added to media and distributed into enterocyte suspension culture flask. Ethanol was the preferred mode as a carrier for the delivery of FA to enterocytes. This *in vitro* study proved their hypothesis that the uptake of fatty acid alcohols in copepod oil wax esters was lower than the uptake of FA in triacylglycerols by salmon enterocytes.

The uptake and metabolism of glucose in enterocytes isolated from black bullhead, *Ictalurus melas* have been studied by Soengas and Moon (1998). These investigators collected the whole intestine, excised them and then divided it into an anterior (approximately two-thirds of the length) and a posterior (the remaining third) section. The gut was weighed, slit longitudinally, spread on an ice-cooled plate and rinsed twice with Hanks' medium. The mucosa was gently scraped using the edge of a glass slide and transferred immediately to a Petri dish containing modified Hanks'medium plus 1 mmol/L EGTA and 0.05 mg/mL collagenase (from *Clostridium histolyticum*, type IV). This suspension was gently aspirated to disperse the cells into the medium and then filtered through 250 and 72µm filters. The filtered cell suspension was centrifuged, washed and re-suspended in Hanks' solution. The cellular passive D-glucose uptake was measured within 5h. There was no indication of decreased viability and cells maintained their distinctive microvillar structure on a portion of the membrane throughout this period.

Cellular intestinal carotenoid and cholesterol uptake

The mechanism involved in the absorption of carotenoids has been subject of limited investigation. A recent review on the mechanisms of dietary carotenoid absorption in perfused rat intestine suggested that carotenoid absorption by intestinal cells may be a simple diffusion or passive process (Yonekura and Nagao, 2007). Studies using Caco-2 cells to determine the intestinal uptake of carotenoids in human enterocytes showed that the absorption mechanism involved not only a passive diffusion but also a receptor protein of the cell membrane. There is sufficient evidence to indicate that scavenger receptor class B type I (SR-BI) is involved in the

transport of ß-carotene (During and Harrison, 2005; During and Harrison, 2007), lutein (Reboul *et al*., 2006) and lycopene (Moussa *et al*., 2008) in the apical membrane of human enterocytes. Human SR-BI is a 509 amino acid, 82kDa glycoprotein (Rhainds and Brissette, 2004). Current literature suggests that SR-BI may be involved at the duodenal and jejunal apical membranes for cholesterol uptake (Hui *et al*., 2008). A SR-BI receptor has also been characterized in the head kidney cell line of Atlantic salmon and the TO cells have 494 amino acids and 80kDa glycoprotein. High level of gene expression of SR-BI have been found in the midgut and lowest levels in the hindgut section of the digestive tract (Kleveland *et al*., 2006). However, there is no specific study related to the function of SR-BI in the intestinal uptake of nutrients in salmon.

Several investigators have suggested that intestinal absorption of carotenoids and cholesterol may follow a common mechanistic pathway (During *et al*., 2005). In the human digestive tract, cholesterol passing through the small intestine is first emulsified by bile acids in the lumen of the duodenum. After emulsification, the cholesterol appears to be specifically removed from the micelles as part of the absorption process. Cholesterol is absorbed principally in the duodemun and jejunum, but bile acids are not absorbed to an appreciable degree at these sites. However, bile acids are subsequently absorbed by specific bile acid transporters in the ileum. Since cholesterol enters into enterocytes from bile acid micelles, it appears likely that cholesterol monomers but not cholesterol aggregates, are absorbed. Dietary cholesterol esters are not absorbed and must be hydrolyzed to free cholesterol before absorption. After absorption, the free cholesterol is re-esterified in the enterocyte by the action of acyl coenzyme A (cholesterol acyltransferase). The resulting cholesterol ester is packaged into chylomicrons and secreted from the basolaterol aspects of the enterocyte into the lamina propria, thereafter to enter the lymph (Hernandez *et al*., 2000).

It appears that the mechanism of cholesterol transport through enterocytes is not clear. Cholesterol intestinal cellular uptake is viewed as energy-independent, passive diffusion process down through a concentration gradient from the intestinal lumen across the brush border membrane to the cytosol of the enterocyte (Thurnhofer and Hauser, 1990). It was also reported that the uptake process may be a facilitated process that involves the participation of the scavenger receptor class B type I (SR-BI) and cluster determinant 36 (CD36) (During *et al*., 2005). There are few reports about the interference of cholesterol in carotenoid metabolism using

liposome, a model membranes. Socaciu *et al*. (2000) studied competitive carotenoid and cholesterol incorporation into lipid bilayer. Their models used liposomes made from single or mixed phospholipids. Liposomes were incorporated with either dietary carotenoids (ß-carotene, lutein and zeaxanthin) alone or with cholesterol to realize a 1:1 and 1:3 molar ratio between these molecules. When carotenoids were incorporated into liposomes together with high cholesterol concentration, carotenoid incorporation was reduced. Only membranes with low cholesterol concentration allow carotenoids to be incorporated into membranes effectively. It is considered that, cholesterol causes a fluidizing effect in the membrane core and increased oxygen transport across the membrane. In cholesterol rich membrane, carotenoids are incorporated into cell plasma membranes which have high cholesterol content and can act as a barrier for the flow of some carotenoids, depending on their polarity ie. lutein and zeaxanthin, the more polar carotenoids which fit better into the membrane structure than those of ß-carotene. (Socaciu *et al*., 2000).

Effects of dietary factors on carotenoid absorption

Carotenoids are hydrophobic compounds and are not easily solubilized in the aqueous environment of the gastrointestinal tract of fish. The solubilization of dietary carotenoids in the digestive tract of salmon is a limiting factor for their absorption. An understanding of processes involved in the absorption, transport and deposition of carotenoids is important to determine the bioavailability of carotenoids from various dietary sources. The term “bioavailability” is used to refer to how much of the consumed carotenoid is accessible for utilization for normal physiological function, metabolism, or storage. The absorption encompasses the movement of carotenoids from the mucosal cells into the lymphatic blood system (Canene-Adams and Erdman, 2009). Extensive studies conducted on humans and experimental animals has shown that several dietary factors influence carotenoid bioavailability. These factors include dietary fat, fiber (cellulose and hemicelluloses), pectin, sucrose polyesters (false fats), plant sterols and vitamin E. An interaction between different carotenoids when consumed from the same diet could have an inhibitory effect on the absorption, metabolism and transport of each other (Canene-Adams and Erdman, 2009). Effects of most of these dietary factors on Ax and Cx absorption have not been investigated in salmonid fishes. Commercial salmonid feeds contain ingredients from plant and marine sources that contain fiber, xanthophylls, chitin, certain antinutritional compounds and

higher levels of vitamin E than terrestrial vertebrate diets. These sources have an impact on bioavailability of Ax and Cx supplements.

In addition, feed intake and water temperature may influence the apparent digestibility coefficient (ADC) of Ax. Atlantic salmon (~ 500 g) were found to have approximately 10 % lower ADC of Ax at 8ºC compared to 12ºC (Ytrestøyl *et al*., 2005). A low ADC and retention of Ax occurred despite the high level of feed intake. This led to a reduction in the muscle concentration of Ax in the rapidly growing salmon (Ytrestøyl *et al*., 2006).

Lipid

Among the dietary factors that affect carotenoid absorption in fish, dietary lipid composition and level of incorporation have shown some positive effects on Ax and Cx absorption. It appears that the lipophilic nature of carotenoids make dietary lipids necessary for dissolution of Ax or Cx in the intestinal lumen (Torrissen, 2000) and their dispersion in lipid and bile enhances the uptake from the digestive tract. Torrissen *et al*. (1990) reported that Cx absorption was enhanced by high dietary lipid levels in Atlantic salmon. Jensen *et al*. (1998) found that Ax deposition was significantly improved by increasing the dietary fat level from 27% to 32% in 0.8-0.9 kg female rainbow trout. Bjerkeng *et al*. (1999) reported higher Ax concentrations in Atlantic salmon fed diets supplemented with capelin or Peruvian oil high in polyunsaturated fatty acids as compared to Atlantic salmon fed diets supplemented with herring or sand eel oils. Olsen *et al*. (2005) studied the effect of several lipid sources and bile acids on blood Ax concentration in dorsal aorta cannulated Atlantic salmon. The salmon were fed diets supplemented Ax and herring oil, soy lecithin, lard, or herring oil supplemented with taurocholate. Soy lecithin caused a poorer uptake of Ax compared to herring oil, whereas lard had a positive effect on the uptake. Dietary supplementation with taurocholate did not significantly affect the blood concentration of Ax. Thus, it seems that alteration of the mixed micelle structure in the intestine does not improve the Ax uptake. The authors suggested that a positive effect of lard on the blood concentration of Ax may be due to the increased concentrations of 16:0, 18:1n-9 or 18:2n-6 fatty acids or the lower content of the 20:1n-9 and 22:1n-9 fatty acids in lard compared to herring oil, which caused an increased solubility of Ax in the intestinal micelles.

Cholesterol

Cholesterol is an essential nutrient for several marine crustaceans species (Teshima *et al*., 1997). Limited research, however, has focused on the potential need for a dietary supply of cholesterol in fish primarily due to the fact that vertebrates can synthesize cholesterol from sterol precursors (Sealey *et al*., 2001). Cholesterol is essential for morphogenesis of cell membranes, growth and differentiation. It is a precursor of endogenous bile acids and steroid hormone (Vlahcevic *et al*., 1994). Investigations conducted on possible nutritional effects of cholesterol in Atlantic salmon were reported by Bjerkeng *et al*. (1999). They studied the effects of dietary cholesterol (CHOL) and short chain fatty acid (SCFA) on growth, organ indices, macro nutrient digestibility and fatty acid composition of fish body. Salmon (initial weight, 0.7 kg) held in seawater (7°C) for 175 days were fed one of six diets: 1. no CHOL/SCFA supplement; 2. with 0.5% SCFA; 3. with 2% SCFA; 4. with 1.0% CHOL; 5. with 1% CHOL and 0.5% SCFA; and 6. with 1.0 % CHOL. There was no significant effect of cholesterol on growth, mortality, digestibility and neither short chain fatty acid nor cholesterol in the diets had any effect. Cholesterol supplement slightly increased hepatosomatic index and hepatic cholesterol concentration, however, it did not affect the fillet or gut tissue cholesterol concentration.

Buttle (2000) observed that increasing the cholesterol levels in the diet of Atlantic salmon to a level from 1 to 3% resulted in an increase in plasma Cx concentration. Maximum Cx plasma concentrations of 3.6 µg/mL were found in salmon fed 1% cholesterol in the diet compared to 1.5-2 µg/mL in control fish fed no cholesterol supplemention in the diet. They also observed that Ax plasma concentration was 0.65 µg/mL in Atlantic salmon fed 3.8% cholesterol in the diet compared with 0.62 µg/mL in fish fed diets containing no cholesterol supplement. Ax level in the flesh was 2.76 mg/kg in salmon fed 3.8% cholesterol compared to 2.32 mg/kg in fish fed Ax alone. The mechanism by which a high dietary cholesterol level may increase carotenoid absorption in salmon remains to be investigated.

In rats, a high level of dietary cholesterol supplement induced an increase in the enzyme activity of 7-∞- hydroxylase in liver allowing these animals to efficiently convert excess dietary cholesterol to bile acids (Horton *et al*., 1995). The inclusion of the bile acid, taurocholate, in the diet of ferret (Lakshman *et al*., 1996) and rat (Schweigert *et al*., 2002) significantly increased the

absorption and tissue accumulation of ß-carotene but did not increase blood Ax levels in salmon (Olsen *et al*., 2005). The ability of fish to regulate cholesterol by increasing bile acid production has not been investigated. Cholesterol is also an important determinant of apolipoprotein B synthesis and may promote the transport of Ax by increasing lipoprotein formation (Kumar *et al*., 1992). An increase in dietary cholesterol can also affect the cellular uptake and recycling of lipoproteins in the liver through the down-regulation of hepatic LDL-receptor activity (Turley, 1999).

Phytosterol

Phytosterols are plant sterol and their structures are similar to that of cholesterol except that they contain an additional hydrophobic carbon chain attached at C-24 position (Figure 1-2). These molecules are more hydrophobic than cholesterol and poorly soluble both in water and the oil phase (Rozner and Garti, 2006). Phytosterol isolation on a large scale is based on two major raw materials, tall oil and vegetable oils. There is a high concentration of phytosterol in vegetable oils, including corn, sunflower, safflower and soybean oils at 952, 725, 444 and 221 mg/100g edible portion, respectively (reviewed by Rozner and Garti, 2006). Phytosterols are classified into the two categories that of sterols and stanols. Sitosterol is the most abundant form in food, followed by campesterol and stigmasterol (Ostlund, 2002).

The major use of phytosterols is related to pharmaceuticals, cosmetics and functional food ingredient applications for humans. Phytosterols lowers the total plasma or serum cholesterol and low density lipoprotein (LDL) cholesterol without any consistent effects on high density lipoprotein (HDL) cholesterol and triglycerides (Ostlund, 2002). An intake of 1.5-3.0 g phytosterols/day caused a reduction of 8-15% blood LDL-cholesterol levels (reviewed by Fernandes and Cabral, 2007). The mechanism involved in reducing blood cholesterol is linked to the inhibition of cholesterol absorption in the intestine including reduction in bile salt cholesterol micellar formation and increase in the competition at the brush border membrane of gut for cholesterol uptake (Ling and Jones, 1995). There is also some evidence that high levels of phytosterol in margarine spreads may reduce plasma carotenoid concentration. Weststrate and Meijer (1998) compared the effects of different sources of plant sterols (sitostanol ester, esterified sterols from soybean, shea nut or rice bran) on plasma carotenoid levels and found that a small

quantity (ca. 3 g/day) of either plant sterol esters or sitostanol esters significantly reduced plasma carotenoid concentrations. Gylling *et al*. (1999) reported a 25% reduction in plasma ß-carotene concentrations in humans when they consumed 2.6 g sitostanol ester fortified spread/day. Clifton *et al*. (2004) also reported a 3-26 % decrease in human plasma carotenoid (ß-carotene, α-carotene, lutein and lycopene) concentration when they consumed a diet enriched with phytosterol esters for 12 weeks.

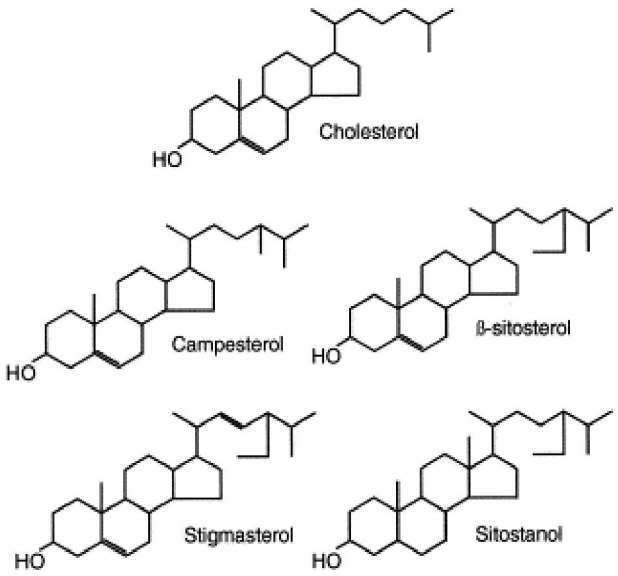


Figure 1-2 Chemical structures of cholesterol and certain unsaturated (campesterol, ß-sitosterol, stignasterol) and saturated (sitostanol) plant sterols. (adapted from Moghadasian, 1999)

Utilization of phytosterols as well as their effects on carotenoid absorption and metabolism has not received much attention. Recent trends towards higher use of feed ingredients of plant origin to replace the fishery products has increased the concentration of phytosterols in salmonid feeds which is likely to have some effect on carotenoid metabolism of fish.

Fiber

Fiber includes indigestible plant matter such as cellulose, hemicellulose, lignin, pentasanes and other complex carbohydrates found in feedstuff. Most fish can tolerate up to 8%

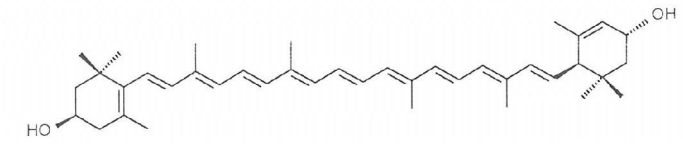
fiber in their diets, but higher concentrations (8-30%) depress growth (NRC, 2011). Practical fish diets may contain 3-6% crude fiber. Studies conducted on humans and monogastric animals show that the absorption and metabolism of carotenoids are affected by different types of fiber. It has been reported that in healthy humans dietary pectin reduced the plasma ß-carotene concentration (Rock and Swendseid, 1992; Riedl *et al*., 1999). Incorporation of hemicellulose, lignin and pectin in the diets of chicken also reduced the bioavailability of ß-carotene (Erdman *et al*., 1986). In studies conducted with humans, Riedl *et al*. (1999) showed that water-soluble dietary fibers (pectin, guar, and alginate) strongly decreases the absorption of ß-carotene and Cx. Water-insoluble type fibers (cellulose, hemicellulose and lignin) decreased the absorption of lycopene and lutein. They concluded that the differences in molecular structure and polarity of the carotenoids may be responsible for fiber and carotenoid interaction and their effects on bioavailability of carotenoids. Dietary fiber may also increase fecal excretion of bile acids causing a decrease in absorption of fats and fat-soluble substances including carotenoids and cholesterol (Castenmiller and West, 1998).

Other carotenoids

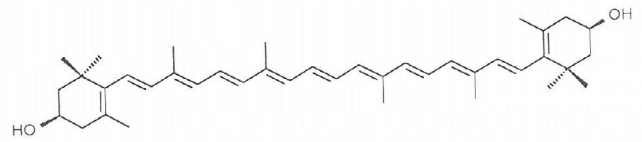
Several feeding studies conducted on experimental animals and humans have indicated an interaction between carotenoids during intestinal absorption and metabolism (Castenmiller and West, 1998). Much of the evidence suggests an interaction between β-carotene and oxycarotenoids such as Cx and lutein, and between the hydrocarbon carotenoids β-carotene and lycopene. The interactions between carotenoids might occur at the various stages of the absorption stage, i.e. a competition for incorporation (solubilization) within the fat globules and/or mixed micelles in the intestinal lumen, within the enterocyte during intracellular transport, during metabolism (cleavage) and assembly of chylomicrons, as well as during post-absorptive metabolism, such as plasma lipoprotein transport (van den Burg, 1999).

Lutein and zeaxanthin (xanthophylls) are abundant in various organs and tissue of many fishes, and reports show that Ax can transform to these xanthophylls in Atlantic salmon (Schiedt, 1998). The structures of these xanthophylls are similar to that of Ax (Figure 1-3). Fish feed producers use some feed ingredients that contain significant amounts of the yellow pigments lutein and zeaxanthin, which may interfere with intestinal absorption or tissue metabolism of Ax

(Olsen and Baker, 2006). These investigators evaluated the absorption efficiency, deposition, retention and possible interaction of Ax and lutein in diets fed to Atlantic salmon. Experimental diets contained between 52 to 55 mg/kg Ax supplement with an increase in the lutein level from 0.7 mg/kg (control diet) to 11 and 23 mg/kg in other two test diets. Increasing the dietary lutein content did not appear to affect the Ax content in either blood or muscle. They concluded that dietary lutein, at levels up to at least 23 mg/kg of diet, had no affect on deposition of Ax in Atlantic salmon.



Lutein (3R, 3'R, 6'R)-ß,ε-Carotene-3,3'-diol



Zeaxanthin (3R, 3'R)-ß,ß-Carotene-3,3'-dione

Figure 1-3 Lutein and zeaxanthin structure

Vitamin E

Vitamin E activity is derived from at least eight naturally occurring derivatives of dihydrochromanol that are differentiated by the degree of methyl substitution in the ring (α, ß, γ and δ) and the presence of unsaturated bonds in the phytyl side chain (tocopherol and tocotrienol). In biological systems, vitamin E functions as an antioxidant to protect the cellular lipid components by donation of a phenolic hydrogen to a free radical. Carotenoids, in association with others antioxidants including vitamin E, are known to increase their activity against free radicals to reduce cellular oxidation. Although, it is recognized that vitamin E protects carotenoids from oxidation during their absorption and transport in fish, to date, no systematic study has been undertaken in this area.

Pozo *et al*. (1988) reported the effects of increasing α-tocopherol levels in commercial rainbow trout feeds. A solution of α-tocopherol in diethyl ether was sprayed on feed pellets formulated to contain 50 mg Cx per kg of feed. After 60 days of feeding, they found an increase in the deposition of Cx in rainbow trout flesh. In a study with female rainbow trout (0.8–0.9 kg), Jensen *et al*. (1998) raised fish for 6 months on 13 different feeds varying in fat content (27% or 32%), Ax (40, 70 or 100 mg /kg feed) and vitamin E (α-tocopherol; 100, 300 or 600 mg *all-rac*-a-tocopheryl acetate/kg feed). They found a significant increase in contents of fat, Ax and α-tocopherol in fillets with the increasing levels of each feed supplement. Furthermore, Ax deposition increased with increasing dietary fat level but not α-tocopherol levels. Similar results have been reported in Atlantic salmon that the deposition of Ax in the muscle was not affected by dietary vitamin E levels (Sigurgisladottir *et al.,* 1994; Bell *et al*., 2000). However, Bjerkeng *et al*. (1999) found an improvement in Ax deposition and flesh coloration of Atlantic salmon by increasing dietary α-tocopheryl acetate concentrations from 200 to 800 mg/kg in diets containing 30 mg Ax/kg of diet. It appears that there is a need to further investigate the effects of dietary vitamin E and Ax levels on Ax metabolism in fish using a diet of known nutrient composition to establish the interaction between these two important and costly feed supplements.

Carotenoid transport and lipoproteins

Carotenoids and lipids are absorbed in the intestinal mucosa in both animals and human before being transported by lipoproteins (Wang, 1994; Parker, 1996) and may be transferred or exchanged between lipoprotein groups in blood circulation (Salvador *et al*., 2007). Plasma lipoproteins are synthesized mainly in the liver and intestines. Particles formed by the non-covalent association of lipids with specific proteins are called apolipoproteins or apoproteins (Myant, 1990). The most abundant lipids are triacylglycerols, free cholesterol, cholesterol esters and phospholipids. Apolipoproteins which are only weakly associated with a particular lipoprotein are easily transferred to another lipoprotein (Lehninger *et al*., 1993). Lipoprotein structure includes an outer surface coat and the core (Figure 1-4). Hydrophobic components are found in the lipoprotein core, whereas components having both a hydrophobic and hydrophilic region (cholesterol, phospholipid and protein) make up the outer surface coat. Phospholipids have their polar part oriented to the aqueous surface, while the apolar part interacts with the hydrophobic core of the lipoprotein. Free cholesterol appears to form part of the lipoprotein

envelope, with the carbon 3 hydroxyl oriented to the polar environment, and the remaining apolar part of the molecule embedded in the hydrophobic portion of the monolayer formed by the phospholipids. Hydrophilic amino acids of apolipoproteins are oriented to the outside, while hydrophobic amino acids interact with the apolar part of the lipoprotein structure (Grummer and Carroll, 1988). All cells rely on lipid, including cholesterol, as building blocks for membranes that cell use to control internal water and water soluble compounds to maintain their internal structure and protein enzymatic systems.

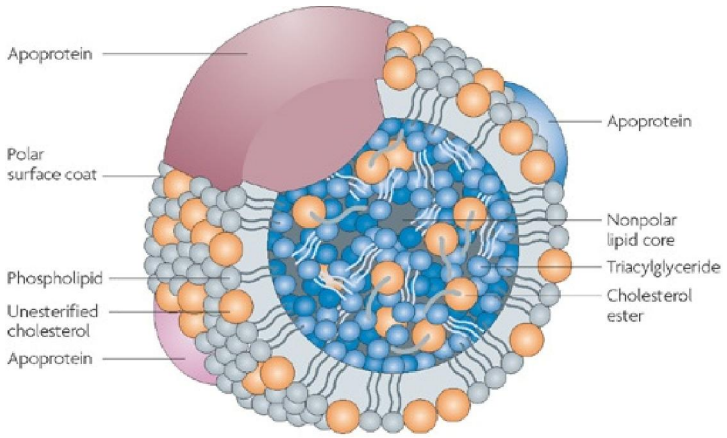


Figure 1-4 The general structure of lipoprotein
From: Wasan *et al*. (2008)

Characteristics of major plasma lipoproteins

Lipoprotein varies in composition and physiological function. It can be classified according to density and decreasing size; chylomicrons, VLDL (very low density lipoprotein), IDL (intermediate density lipoprotein), LDL (low density lipoprotein) and HDL (high density lipoprotein) (Babin and Vernier, 1989). Chylomicrons are the largest lipoproteins (>100 nm in diameter). They are synthesized by the intestine to transport dietary triglyceride and cholesterol from the site of absorption in the intestinal epithelium to various cells of the body. The triglycerides of these particles are hydrolyzed within the plasma compartment by the action of

lipoprotein lipase, which is attached to endothelial surface. Fatty acids liberated during hydrolysis are used as an energy source by various cells or are taken up by adipocytes and stored as triglycerides. Lipoprotein particles generated by the action of lipoprotein lipase on chylomicrons are referred to as chylomicron remnants. They are enriched in cholesterol and are rapidly cleared by the liver. VLDL (d<1.006g/mL) are 30 to 80 nm particles that transport triglycerides and cholesterol from the liver for redistribution to various tissue. Within the plasma compartment, triglycerides of VLDL are hydrolyzed to free fatty acids by lipoprotein lipase generating a series of smaller, cholesterol-enriched lipoproteins including IDL (d=1.006-1.019 g/mL) and LDL (d=1.019-1.063 g/mL), a product of VLDL catabolism approximately 20 nm in size. They are the major cholesterol transporting lipoproteins in the plasma. HDL (d=1.063-1.21 g/mL) may appear in plasma from several sources, including the liver and intestine. In addition, HDL or HDL precursors appear to be produced within the plasma compartment during lipolytic processing of chylomicrons by the generation of phospholipid protein disks arising from the surface of the lipolyzed chylomicron. HDL is the smallest (5-12nm in diameter) of the lipoproteins. They are involved in a process referred to as reverse cholesterol transport, a postulated pathway whereby HDL acquires cholesterol from peripheral tissues and transport the cholesterol, directly or indirectly, to the liver for excretion (Mahley *et al*., 1984). The characteristics of the major classes of lipoproteins in human plasma are summarized in Table 1-2

Table 1-2 Characteristics of the major classes of lipoproteins in human plasma

	chylomicrons	VLDL	IDL	LDL	HDL
Density (g/mL)	<0.95	<1.006	1.006-1.019	1.019-1.063	1.063-1.210
Particle diameter (nm)	75-1,200	30-80	25-35	18-25	5-12
Particle mass (kDa)	400,000	10,000-80,000	5,000-10,000	2300	174-360
Protein (%)[a]	1.5-2.5	5-10	15-20	20-25	40-55
Phospholipids (%)[a]	7-9	15-20	22	15-20	20-35
Free cholesterol (%)[a]	1-3	5-10	8	7-10	3-4
Triacylglycerols (%)[b]	84-89	50-65	22	7-10	3-5
Cholesteryl esters (%)[b]	3-5	10-15	30	35-40	12
Major apolipoproteins	A-I, A-II, B-48, C-I, CII, C-III, E	B-100, C-I, C-II, C-III, E	B-100, C-I, C-II, C-III, E	B-100	A-I, A-II, C-I, CII, C-III, D, E

[a]Surface components, [b]Core lipids

From: Voet *et al*. (2008)

Apolipoproteins

Apolipoproteins are carrier proteins that combine with lipids to form lipoproteins and transport lipids through the lymphatic and circulatory systems. Since lipid components of lipoproteins are not soluble in water, apolipoproteins and other molecules such as phospholipids with amphipathic (detergent-like) properties surround lipids and form lipoprotein particle with water-soluble properties to transport them in blood. Apolipoproteins also serve as enzymes cofactors, ligands for interaction with lipoprotein receptors in tissues and lipid transport carriers that regulate the metabolism of lipoproteins and their metabolism in tissues (Lehninger *et al*., 1993). There are six major classes of apolipoproteins, A (apoA-I and apoA-II), B (apoB48 and

apoB100), C (apoC-I, apo-II and apoC-III), D, E, and H, and several subclasses. Several genetic polymorphisms of apolipoproteins have been reported in humans (Voet *et al*., 2008).

ApoA-I circulates in plasma primarily as a component of HDL. It is also present on chylomicrons but is rarely found in significant amounts on chylomicron remnants, VLDL or their remnants, or LDL. ApoA-I has two major sites of synthesis; the intestine and the liver. The intestinal derived apoA-I enters the circulation associated with chylomicrons but they are rapidly transferred to HDL particles during hydrolysis of chylomicrons. Hepatic apoA-I enters the circulation and is probably associated with nascent HDL particles having little or no core of cholesteryl ester. ApoA-I is a single polypeptide of 243 amino acids (28 kDa) (Mahley *et al*., 1984). ApoA-II is the second most abundant protein component of HDL and may be associated with other lipoproteins in smaller amounts. The major site of synthesis of apoA-II is the liver. The apoA-II subunits have 77 amino acids (17 kDa) (Mahley *et al*., 1984). Apolipoprotein B is a primary apolipoprotein of chylomicrons, VLDL, IDL and LDL. It is heterogeneous and exists primarily in two forms: apoB-100 and apoB-48. ApoB-100 is synthesized by the liver and obligatory constituent of VLDL, IDL and LDL. In humans, apoB-48 is synthesized by the intestine and found in chylomicrons and chylomicrons remnants (Mahley *et al*., 1984). They range in molecular weight from 8 to 270 kDa (Morrisett *et al*., 1977).

Apolipoprotein C is found as three low molecular weight apolipoproteins, designated as apoC-I, apoC-II and apoC-III. They are surface components of chylomicrons, VLDL. They range in molecular weight from 7 to 9 kDa. It appears that the liver is the major site of synthesis of the apoC proteins, with the intestine contributing a minor portion. ApoC are diverse in their metabolic functions and share the common property of redistribution among lipoprotein classes. In the fasting state, the C apolipoproteins are mainly associated with HDL. During absorption of dietary fat by the intestine with the production of chylomicrons or during the active synthesis of VLDL by the liver, the C apolipoproteins preferentially redistribute to the surface of the triglyceride-rich chylomicrons and VLDL. In the reverse manner, as the triglyceride core of the VLDL and chylomicrons is hydrolyzed and depleted by the action of lipoprotein lipase, excess surface components (phospholipid, unesterified cholesterol and apolipoprotein units) are generated and the C apolipoproteins along with the other excess surface components are transferred to HDL. Thus, the C apolipoproteins are associated with equilibrium that occurs in the

dynamic metabolic remodeling of plasma lipoproteins (Mahley *et al*., 1984). ApoC-I is the smallest of the C apolipoproteins and consists of 57 amino acids in a single polypeptide chain with a calculated molecular weight 7 kDa. ApoC-I has been shown to activate lecithin cholesterol acyltransferase (LCAT). Thus, it has the potential to participate in the esterification of the cholesterol that is transferred to HDL as a part of the excess surface components generated during lipolysis of VLDL and chylomicrons or that is transferred to HDL from cells (Mahley *et al*., 1984). ApoC-II is a single polypeptide chain consisting of 79 amino acids with a calculated molecular weight 9 kDa. The primary metabolic function of this lipoprotein appears to be associated with its ability to act as a cofactor in activating lipoprotein lipase. ApoC-II has also been reported to activate LCAT (Mahley *et al*., 1984). ApoD occurs mainly in HDL. The molecular weight of the protein is estimated at 19 to 20 kDa (Morrisett *et al*., 1977). ApoE or the arginine-rich protein occurs in VLDL, HDL, IDL and chylomicrons. They have a molecular weight 33 kDa (Morrisett *et al*., 1977). ApoH previously known as β_2-glycoprotein I, is a multifunctional apolipoprotein and involved in phospholipid binding with positively charged amino acids of protein. It binds to cardiolipin, involved in agglutination and also inhibits the release of serotonin from platelets and other proteins.

Apolipoprotein synthesis in the intestine is regulated by the lipid content of the diet whereas, its synthesis is controlled by several factors. They include changes in diet composition, alcohol consumption, hormones (insulin, thyroxine, glucagon, estrogens, androgens etc.) and certain drugs (statins, fibric acids and niacin). Generally, apoB is considered as an integral apolipoprotein whereas others are peripheral apolipoproteins in humans.

Fish lipoprotein and apolipoprotein

As in mammals, fish lipoproteins are classified as chylomicrons, VLDL, LDL, IDL and HDL (Babin, 1987; Babin and Vernier ,1989). Previous work on fish serum and plasma lipoprotein isolation was conducted by ultracentrifugation using the same density intervals as those used for fractioning human lipoproteins. Each class of fish lipoprotein showed the same components of mammalian lipoproteins, however, the composition of plasma lipoproteins were different (Table 1-3). Rainbow trout lipoproteins (chylomicrons, VLDL and LDL) have more surface constituents than their human counterparts and are therefore relatively smaller. However,

in fish HDL, the proportions of core and surface constituents are similar to human HDL (Babin and Vernier, 1989).

Table 1-3 Characteristics of the lipoproteins class in trout plasma

	chylomicrons	VLDL	IDL	LDL	HDL
Density (g/mL)	<1.015	<1.015	1.015-1.040	1.040-1.085	1.085-1.210
Particle diameter (nm)	80-800	20-50	12-21		6-11
Protein, %	4.5	12.8	29.5		44.7
Lipid, %	95.5	87.2	70.5		55.3
Major apolipoproteins	25 (A-I), B240, B260, 76, 13 (A-II), 9-11 (C)			B240, 76	25 (A-I), (A-II

From: Babin and Vernier (1989)

The lipid composition of lipoproteins in trout and humans is summarized in Table 1-4. Trout VLDL were slightly higher in phospholipid but had less triglyceride than humans. Both human and trout LDL and HDL show substantially low in cholesteryl ester and more triglyceride was distributed in trout than humans particularly the high proportions of neutral lipid (cholesteyl ester and triglyceride). Trout lipoprotein also showed a higher content of phospholipids compared to human lipoprotein (Table 1-4). Lie *et al*. (1993) fractionated Atlantic salmon serum lipoproteins and analyzed their lipid composition (Table 1-5). Each fraction contained different levels of triacylglycerol, cholesterol and protein. HDL had the highest protein concentration whereas the cholesterol content of LDL was relatively high and highest proportion of triacylglycerol was found in VLDL.

Table 1-4 The composition (%) of trout and human lipoproteins

Component	VLDL		LDL		HDL	
	Trout	Human	Trout	Human	Trout	Human
Cholesteryl ester	15.1	14.9	15.6	38.0	7.7	15.0
Free cholesterol	6.9	6.4	6.7	9.0	3.4	2.9
Triglyceride	41.9	49.9	26.9	11.2	15.5	8.0
Phospholipid	26.5	18.6	27.1	22.1	26.5	22.7
Protein	9.6	7.7	24.7	20.9	46.9	51.9

From: Chapman *et al*. (1978)

Table 1-5 Composition (%) of the different lipoprotein in Atlantic salmon serum

Component	VLDL	LDL	HDL
Triacylglycerol	62	31	13
Cholesterol	16	21	13
Protein	23	48	74

From: Lie *et al*. (1993)

The lipoprotein distribution in fish such as rainbow trout depends on age and the degree of sexual maturity (Fremont and Marion, 1982; Babin and Vernier, 1989). In adult rainbow trout, HDL is the major lipoprotein class at 1393 mg/100 mL of serum (Chapman *et al*., 1978). It increases progressively in the course of sexual maturation reaching 2500 mg/100 mL of serum during spermiation or ovulation, whereas VLDL and LDL are predominant in juvenile trout (Fremont and Marion, 1982). In Japanese eel (*Anguilla japonica*) VLDL is the main component of lipoprotein in plasma (Ando and Matsuzaki, 1997).

Apolipoprotein of several fish has been also investigated ie. rainbow trout (Chapman *et al*.,1978; Skinner and Rogie *et al*., 1978; Babin, 1987; Babin and Vernier, 1989) chum salmon (Ando and Hatano, 1988a,b) European seabass (Santulli *et al*., 1996; Santulli *et al*., 1997) and haddock (Nanton *et al*., 2006) but document on Atlantic salmon apolipoprotein was not obtained.

In these investigations lipoprotein fractions were isolated by flotation at various densities and multiple apolipoprotein bands identified by electrophoresis. Santulli *et al*. (1996) investigated the apolipoprotein composition of European sea bass (*Dicentrarchus labrax*) lipoproteins. The molecular weights of apolipoproteins were estimated by electrophoresis on a polyacrylamide linear gradient gel. They found a varying number of apolipoproteins in each lipoprotein fractions. The eighteen apolipoproteins from VLDL showed a molecular weight ranging from 6 to 263 kDa. In LDL, nineteen apolipoproteins were identified with molecular weight ranging from to 8 to 263 kDa. HDL fraction contained 15 electrophoretic bands with a calculated molecular weight ranging from 9 to 242 kDa. Apolipoproteins with high molecular weight (from 216 to 263 kDa) considered apoB-like were present in LDL and VLDL. The most abundant apolipoproteins in HDL was apoAI-like (27 kDa) and apoAII-like (12 kDa).

The apolipoprotein components of haddock serum were analyzed by Nanton *et al*. (2006). They observed a large apoB-like protein in VLDL. LDL contained apoB-like protein and AI-like protein. ApoAI-like protein was a major protein in HDL and it also contained an apoAII-like protein. Skinner and Rogie (1978) isolated and characterized apolipoprotein of rainbow trout (*Salmo gairdneri*). They found that the patterns observed on electrophoresis showed resemblance to those obtained with humans. The major structural subunit of chylomicrons VLDL and LDL corresponds to apoprotein B of human. The major components of trout HDL migrated in approximately the same positions as apoA-I and A-II of human HDL. Rainbow trout HDL contained 25 and 13 kDa apolipoprotein (Babin, 1987; Babin and Vernier, 1989). A study on chum salmon apolipoprotein reported that the protein composition of HDL fraction had two subunits with molecular weights of 24 and 12 kDa (Ando and Hatano, 1988a). Different methods in isolation and characterization of apolipoprotein may be the reason for changes of molecular weight of apolipoprotein in several studies. The molecular weights of apolipoprotein in each lipoprotein of salmonid fishes are summarized in Table 1-6.

Table 1-6 Molecular weight (kDa) of apolipoprotein in each lipoprotein of salmonid fish

Fish species	VLDL	IDL+LDL	LDL	IDL	HDL
Rainbow trout[1]	310		280		300
	88		84		72
	27		54		54
	15		45		36
			26		22
			16		10
Rainbow trout[2]	260	260	240	260	55
	240	240	76	240	40
	76	76			25
	25				13
	13				
	9-11				

From: [1]Chapman *et al*. (1978), [2]Babin (1987)

The role of lipoproteins in carotenoids transport

It is well known that digestion and release of carotenoids from the food matrix requires several steps during the digestion and absorption process. A proposed mechanism for digestion, absorption and metabolism of carotenoids in the gastrointestinal tract is summarized in Figure 1-5 (Lee *et al*., 1999), and involves the following steps: (1) digestion of food to release carotenoids; (2) micelle formation with lipids and bile salts; (3) uptake of carotenoids through intestinal brush border; (4) intracellular metabolism and transport through enterocytes for subsequent processing and (5) incorporation into chylomicrons and secretion into the lymph for uptake by hepatic and extrahepatic tissues (6-9). Carotenoids are transported in the plasma of vertebrates exclusively by lipoproteins (Chapman, 1980).

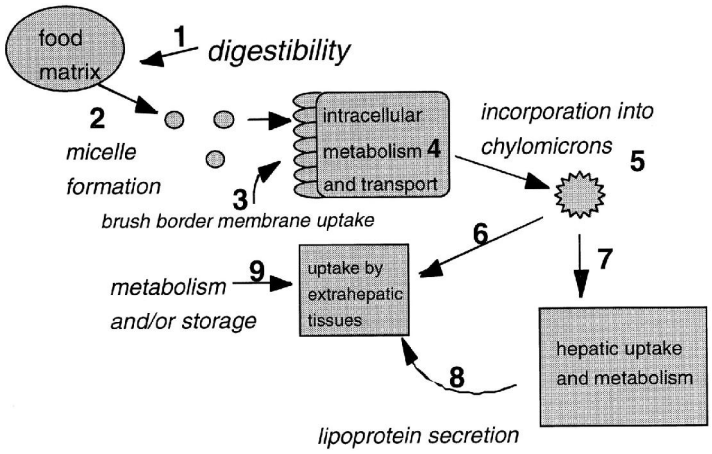


Figure 1-5 Digestion, absorption and metabolism of carotenoids.
From: Lee *et al.*(1999)

There is limited information on the mechanism of carotenoid transport in fish, however, it is considered similar to humans and other terrestrial animals. In humans, chylomicrons transport carotenoids from the intestinal mucosa to the bloodstream via the lymphatics. The molecular orientation of carotenoids within the chylomicron is not known. It is thought that hydrocarbon carotenoids (e.g. carotenes and lycopene) are distributed exclusively in the hydrophobic core of the particle, whereas carotenoids with polar functional groups (e.g. xanthophylls) may exist partly at the surface. Such orientation is likely to affect their transfer to other lipoproteins during circulation or their uptake by extrahepatic tissues during lipoprotein lipase hydrolysis of chylomicron triglycerides (Parker, 1996).

The rates of transfer of carotenoids of various structural types between different lipoproteins particles in the presence of human plasma lipid transfer factors have not been extensively investigated. The relative distribution of several individual carotenoids among VLDL, LDL and HDL was determined by separation after sequential flotation ultracentrifugation and quantification by HPLC (Clevidence and Bieri, 1993). The hydrocarbon carotenoids were found primarily in LDL, whereas more polar carotenoids were evenly distributed between LDL and HDL. The distribution of carotenoids among the various lipoprotein classes therefore appears to be determined by the physical characteristics of individual carotenoids and the lipid composition of the lipoproteins.

Carotenoid transport in fish

After carotenoids are absorbed from the gastrointestinal tract, they are transported in blood by lipoproteins throughout the body to various tissues where they may be deposited and/or metabolized/excreted. The exact mechanism by which carotenoids are distributed and interact with various tissues (i.e. uptake and deposition) is poorly understood. Limited studies have been undertaken to investigate the transport mechanism of carotenoids in fish. Ando *et al.* (1985; 1988a, b) found that different proportions of carotenoids were bound non-covalently to serum carotenoid-carrying-lipoproteins, specifically to HDL, very high density (VHDL) and LDL fractions in chum salmon (*Oncorhynchus Keta*) during spawning migration. The triacylglycerol-rich chylomicron fraction of plasma is considered to be more indicative of carotenoid uptake than serum or plasma levels because newly absorbed carotenoids are found primarily in chylomicrons however, they only represent a small fraction of total plasma carotenoids due to the rapid rate of catabolism of chylomicrons and uptake by the liver in mammals (Deming and Erdman, 1999).

Chavez *et al*. (1998) fractionated serum lipoproteins by density-gradient ultracentrifugation and found that 1.2, 1.8 and 0.7 μg of Cx/mL serum in VLDL, LDL and HDL respectively. Choubert *et al*. (1992) reported that the following distribution of canthaxanthin in various lipoprotein fractions in rainbow trout (300g) fed 80 mg Cx/kg of diet: VLDL, 13.9%; LDL, 15.2%; HDL, 60.4% and VHDL 10.5%. In a later study, they found a similar distribution pattern of Ax in rainbow trout (250g), 0.7, 16.8, 66.3 and 16.1 % in VLDL, LDL HDL and VHDL respectively (Choubert *et al*., 1994). Aas *et al*. (1999) forced fed a single dose of ^{14}C-astaxanthin to large Atlantic salmon (800-1200g) and found that 57.5% of radiolabeled Ax was in the high density protein fraction (HDPF), 38.0% in HDL and 6.1% in LDL. They suggested that HDPF fraction may be albumin.

In humans, albumin is the major plasma protein and its main function is the regulation of colloidal osmotic pressure of numerous endogenous and exogenous compounds including free fatty acids, hormones, bilirubin and drug (De Smet *et al*., 1998). Although, salmonid blood contains albumin (Maillou and Nimmo, 1993a; Maillou and Nimmo, 1993b), its role in transport of cartotenoids and nutrients is not clear (Metcalf and Gemmell, 2005). Rainbow trout albumin has a molecular weight of 70 kDa with the similar electrophoretic mobility as human albumin,

(Maillou and Nimmo, 1993a; Maillou and Nimmo, 1993b). It comprises over 50% of the total serum protein. The distribution of carotenoids in lipoproteins in cultured salmonids are summarized in Table 1-7.

Table 1-7 Carotenoids in lipoproteins of salmonids

Carotenoid	VLDL	LDL	HDL	Fish species
Canthaxanthin	1.2 μg/mL	1.8 μg/mL	0.7 μg/mL	Rainbow trout[1]
	13.9%	15.2-29.1%	60.4%	Rainbow trout[2]
Astaxanthin	0.7%	16.8%	66.3%	Rainbow trout[3]
	-	6.1%	38.0% HDPF (albumin?) 57.5%	Atlantic salmon[4]

From: [1]Chavez *et al*. (1998); [2] Choubert *et al*. (1992); [3]Choubert *et al*. (1994); [4]Aas *et al*. (1999)

Exchange and redistribution of carotenoids between lipoproteins have been observed in humans and animals (Tyssandier *et al.*, 2002). The distribution of carotenoids among lipoproteins are determined in part by enzymes such as lecithin cholesterol acyltransferase (LCAT) and transfer proteins such as cholesteryl ester transfer protein (CETP) (Romanchik *et al.*, 1995). Such exchange mechanisms of carotenoids among lipoprotein in fish has received limited attention. *In vitro* studies conducted in rainbow trout showed that β-carotene and several xanthophylls (astaxanthin, canthaxanthin, lutein and β-cryptoxanthin) may transfer between lipoproteins (Tyssandier *et al*., 2002). The transfer was bidirectional from HDL to VLDL and *vice versa*.

March *et al.* (1990) reported that plasma astaxanthin concentrations in rainbow trout reached a peak 24 h after administration of a single dose of astaxanthin, ranging from 1 to 7 μg/ml. These results were in agreement with those Choubert *et al.* (1987) and Guillou *et al.* (1992), while somewhat higher serum values of 9.04 μg/ml were found by Choubert *et al*. (1994). Whole plasma pharmacokinetics may not be the most practical method to assess carotenoid status, as plasma (or serum) concentrations are not only a measure of the absorption of

carotenoids, but also a measure of the exchange from tissue storage, bioconversion, and excretion (Castenmiller and West, 1998).

Chavez *et al*. (1998) suggested that the high amounts of triglycerides and cholesterol in VLDLs in rainbow trout make them the natural carriers of canthaxanthin. These authors propose that the high amount of phospholipid in lipoproteins is responsible for enhanced binding of canthaxanthin, with the amphophilic nature stabilizing the lipoprotein for carotenoid incorporation. From their *in vitro* carotenoid/lipoprotein saturation studies, Chavez *et al*. (1998) found that the phospholipid content of individual lipoproteins correlated highly to the level of carotenoid in the saturated lipoprotein, and could be used to predict the total amount of carotenoid in the saturated lipoprotein. However, no data is available on the potential astaxanthin saturation in individual lipoprotein fractions, but should provide similar values.

Choubert *et al*. (1994) observed a plateau in plasma carotenoid concentration over time with continuous feeding and they suggested that it was either due to the inability of rainbow trout to absorb additional carotenoid, or saturation of the lipoprotein binding sites by carotenoids. However, research in human subjects has shown that lipoprotein fractions that transport carotenoids in the blood evidently have ample binding capacity for carotene, even when carotene intake is high (Matthews-Roth and Gulbrandsen, 1974). Despite the fact that potential gastrointestinal absorption capacity and the limiting factors regulating serum carotenoid levels are not known, it is possible that factors that affect chylomicrons and other lipoproteins formation may affect carotenoid uptake and transport.

Both Storebakken and Goswami (1996) and Kiessling *et al*. (1995) found a strong correlation between dietary astaxanthin and plasma astaxanthin concentration. Storebakken and Goswami (1996) also found a high correlation between plasma astaxanthin concentration and retention in flesh (mg astaxanthin per kg body weight increase), as was the correlation between plasma astaxanthin concentration and flesh carotenoid level. Storebakken and Goswami (1996) concluded that plasma astaxanthin concentrations may be used as an indicator of astaxanthin availability in salmon. Maltby *et al*. (2003) noted that the route of administration (intraperitoneal injection vs. oral delivery) had a significant influence over plasma carotenoid levels.

We propose to further investigate albumin-like proteins that may be involved in carotenoid transport and metabolism. Plasma will be separated by density using ultracentifugation method (Chung *et al*., 1980; Poumay and Ronveaux-Dupal, 1985) on NaCl/KBr- gradient and an iodixanol-gradient. Proteins from various fractions will be separated by PAGE under non-denaturing conditions.

Cholesterol may increase the supply of carotenoids to the muscle by increasing synthesis and secretion of lipoproteins in the intestine and liver, as well as the recycling and uptake of lipoprotein remnants (chylomicron, LDL) in the liver. This experiment will provide some clues regarding the effect of dietary cholesterol on carotenoid transport in the plasma. The experiment will also measure bile acid production (cholesterol 7-alpha hydroxylase activity) and fecal carotenoid and cholesterol excretion.

Objectives

The thesis examines the effects of several dietary factors that may affect bioavailability of Ax from diets of Atlantic salmon and the biochemical mechanisms involved in absorption and transport to tissues. In the preliminary study, various dietary lipid and lipid soluble (cholesterol, phytosterol, other carotenoids and vitamin E) and plant components that might either enhance or reduce Ax absorption were considered. It was clear from the initial study that cholesterol supplementation had a significant positive effect on Ax absorption. Therefore, subsequent experiments were directed to determine the effect of cholesterol on cellular Ax uptake in the intestine and plasma transport of Ax by lipoproteins. It was necessary to develop methodologies to achieve these goals. Biochemical methods involving cell culture models used for vertebrate animals were modified to be applied for these studies in salmon.

The main objectives of the present research were:

1. Determination of the dietary factors that might enhance or interfere with Ax absorption in salmon including interfering factors such as certain carotenoids (zeaxanthin and lutein), plant sterols and fiber, and potential enhancing compounds such as cholesterol and vitamin E.

2. Evaluation of an *in vitro* mammalian cell culture model and its application to fish, in order to better understand the mechanisms involved in the intestinal absorption of Ax.
3. Separation of lipoprotein to investigate the distribution of Ax among various lipoprotein and albumin-like high density protein in Atlantic salmon plasma.
4. Examination of the specific role of cholesterol in Ax transport including the distribution of Ax in various lipoprotein fractions that may affect uptake of Ax in muscle and other extrahepatic tissues.

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Chapter 2

Influence of various dietary factors on astaxanthin absorption in Atlantic salmon (*Salmo salar*)

Introduction

Astaxanthin (3, 3′-dihydroxy-β,β-carotene-4, 4′ dione) and canthaxanthin (β,β-carotene-4, 4'-dione) are the major carotenoid pigments used in salmonid feeds to achieve the red coloured flesh desired by consumers. Beyond their use to achieve flesh pigmentation of salmonids, astaxanthin (Ax) and canthaxanthin (Cx) have other physiological functions such as precursor of vitamin A (Schiedt *et al.* 1985; Al-Khalifa and Simpson 1988; Guillou *et al.* 1989) and an antioxidant (Christiansen *et al.,*1995). Ax may also potentially serve as *in vivo* inhibitors of oxidative stress and involved in reproduction particularly skin coloration during sexual maturation (Torrissen, 1990). Despite these important physiological functions most of the studies have been directed to develop strategies to improve flesh pigmentation of salmon by Ax and Cx supplementation to meet the color quality for marketing and consumer acceptance.

Carotenoids constitute a major portion of the cost associated with salmon feed production (*ca.* 20%). However, the amount of Ax utilized for flesh pigmentation does not exceed 10 and 15 % in Atlantic salmon (Torrissen *et al*., 1989) and rainbow trout (Storebakken and No, 1992), respectively. Several factors that affect the utilization and retention of carotenoids include pigment source, losses during feed processing and storage, concentration, duration of feeding, diet composition and genetic differences (Torrissen *et al.* 1990; Metuslach *et al.* 1996). However, retention of dietary carotenoids in muscle depends on intestinal absorption, metabolism, tissue uptake and excretion (Christiansen *et al*., 1993; Bjerkeng *et al*., 1999), the biochemical mechanisms involved in these processes are not clear and have been subject to limited investigation. Low absorption from the intestinal tract, high excretion (30-70 % fecal loss) and catabolism in liver are considered major factors for low retention of Ax and Cx in salmonid flesh. The uptake of Ax and Cx from the intestine is slow, approximately 18-24 h post-prandial (March *et al*., 1990; Choubert *et al*., 1994). The blood clearance rate of these carotenoids is also slow and

depends on the transport and cellular uptake of carotenoids from lipoproteins (Guillou *et al*., 1992). Species differences also appear to exist in pigmentation retention efficiencies between Atlantic salmon, rainbow trout, Coho salmon (*Oncorhynchus kisuth*) and arctic char (*Salvelinus alpinus*). Ax appears to be better utilized than Cx in trout (Storebakken and Choubert, 1991), while the reverse has been observed in Atlantic salmon (Buttle *et al*., 2001).

Information on Ax and Cx absorption is mostly derived from a limited number of studies using different techniques (reviewed by Schiedt *et al*., 1998). The comparison of the peak carotenoid absorption levels in the blood of salmon fed various diets has been used to provide an indication of carotenoid availability (Kiessling *et al*., 2003; Storebakken and Goswami, 1996). This more rapid, less resource-intensive method for the estimate of relative carotenoid availability is based on the observation that peak astaxanthin concentrations in the blood are highly correlated with both dietary and flesh astaxanthin levels (Storebakken and Goswami, 1996). The absorption process for carotenoids may be similar to that for other lipids. Extensive studies conducted on laboratory animals suggest that the following major steps are involved in carotenoid absorption within the mammalian gastrointestinal tract: 1) release of carotenoids from food matrix; 2) solubilization of carotenoids into mixed lipid micelles in the lumen; 3) cellular uptake of carotenoids by intestinal mucosal cells (enterocytes); 4) incorporation of carotenoids into chylomicrons (CM) and 5) secretion of carotenoids and their metabolites associated with CM into the lymph (van Het Hof *et al*., 2000; Yeum and Russell, 2002).

It appears that once the intestinal barrier for carotenoid absorption is avoided by intraperitoneal administration of Ax in rainbow trout, higher uptake of Ax in plasma, muscle, kidney and liver was achieved (Ytresoyl and Bjerkeng, 2007). Advanced knowledge of the absorption and metabolism of Ax are necessary to improve the retention of this pigment in salmonid flesh particularly the absolute efficiencies of carotenoid absorption, the nature of luminar and intracellular factors regulating the process of their absorption, the mechanism of intracellular transport of Ax and Cx. Certain dietary factors known to influence carotenoids absorption in animals such as vitamin E, cholesterol, fiber, lutein, zeaxanthin and phytosterol (Yeum and Russell, 2002) are also supplied by the ingredients used to formulate fish feeds. They are also likely to affect gastrointestinal absorption of Ax and metabolism in salmonids. A small improvement of Ax deposition and colouration of Atlantic salmon by increasing dietary

vitamin E (Bjerkeng *et al*.,1999). A previous work demonstrated that after fish fed carotenoid supplemented diet and fish plasma fractionated for lipoprotein, carotenoid was found in all lipoprotein fractions (Choubert *et al*., 1992; 1994; Chavez *et at*., 1998). Since lipoproteins are containing phospholipids, proteins and cholesteryl esters, cholesterol may insert a role in increasing synthesis and secretion of lipoproteins in intestine and liver (Vance, 1999). Plant sterols, which present at high level in vegetable oil has similar structure to cholesterol showed effective effect in reducing blood cholesterol in human (Fernandes and Cabral, 2007). The study in chicken and human showed that absorption and metabolism of carotenoids were affected by dietary fiber (Erdmann *et al*., 1986; Riedl *et al*., 1999). Lutein and zeaxanthin, the carotenoids of similar structure with Ax have been reported abundant in fish tissue and organ (Czeczuga, 1975). Ax can be converted to ether zeaxanthin in rainbow trout and tilapia (Matsuno, 1991) or both xanthophylls in Atlantic salmon (Schiedt, 1998).

The main objective of this study was to determine the dietary factors that may enhance or interfere with the Ax absorption including certain carotenoids (zeaxanthin and lutein), plant sterols and fiber, and enhancing compounds such as cholesterol and vitamin E. The effect of these dietary factors was measured using plasma Ax concentration method that provides a good indicator of carotenoid bioavailabilty (Choubert *et al*., 1994; Kiessling *et al*., 2003; Storebakken and Goswami, 1996).

Materials and Methods

Fish, rearing conditions and diets

Aquaria facilities at NRC's Marine Research Station, Halifax, Nova Scotia were used for the fish feeding trials. Two hundred and eighty-eight Atlantic salmon (initial wt ca. 778 ± 78g) were distributed between 16, 1000L fiberglass tanks with flow-through (12 L/min), filtered seawater (30 μm; salinity, 28-30 ppt) and a light regime of 12h light:12h dark. Water quality was monitored daily with water temperature maintained at 10°C and dissolved oxygen levels at 90-95% saturation. Each diet was hand-fed to apparent satiation to duplicate tanks containing 18 fish per tank twice daily (0900h and 1600h) on weekdays and once daily (1000h) on the weekend. Prior to starting the experiment, fish were held on a commercial non-pigmented diet (6.5 mm Shur-Gain Feeds, Truro, NS, Canada).

Eight practical fish meal–based experimental diets were produced at the research station with levels of all nutrients and energy carefully controlled in accordance with known salmonid requirements (NRC 1993). The composition of the basal and experimental diets used in this study can be found in Tables 2-1 and 2-2. Diet 1 served as the control and did not contain Ax. The remaining 7 diets were produced from the same basal diet with the addition of 40 mg Ax/kg diet. Dietary factors tested included cholesterol, vitamin E, wheat bran, lutein, zeaxanthin and phytosterol. The basal ingredients were mixed using a Hobart mixer (Model H600T, Rapids Machinery Co., Troy, OH, USA). Micronutrients and the test ingredients were pre-mixed using a twin-shell mixer (Paterson-Kelly, East Stroudsburg, PA, USA) prior to being added to the basal mixture. Individual diets were pelleted using laboratory pellet mill (California Pellet Mill Co., San Francisco, CA, USA), dried for 1h at 80°C and stored at -20°C until use.

The initial weight of the fish was estimated by randomly weighing 5 fish per tank after a 24-h fast. After 26 days of feeding the diet and allowing for 24 h between the last meal and blood sampling (Rehulka, 2000), fish were anaesthetized, their weight recorded and blood was taken from the caudal vein. Blood samples were spun down using an IEC Centra CL3R centrifuge at 4,000 rpm for 10 min. at 4°C, carefully transferred plasma to pre-labelled Eppendorf tubes, placed on dry ice immediately after collecting plasma, then transferred to -80°C for storage until analysis.

Table 2-1 Composition of experimental diets

Ingredient	g/kg
Herring meal[a]	480
CPSP-G[b]	40
Soybean meal[c]	100
Wheat middlings[d]	103
Dried whey[e]	50
Gelatin[f]	20
Pre-gelatinized starch[g]	40
Vitamin premix[h]	17
Mineral premix[i]	10
Herring oil[j]	140
Supplement premix[k]	*

[a]Corey Feed Mills Ltd., Fredericton NB, Canada

[b]Concentre proteique soluble de poisson (soluble fish protein concentrate) (Sopropêche, France)

[c]Bunge Canada, Oakville, ON, Canada

[d]Dover Mills, Halifax, NS, Canada

[e]Farmers Co-operative Dairy Ltd., Truro, NS, Canada.

[f]US Biochemical, Cleveland, OH, USA.

[g]National Starch and Chemical Co., Bridgewater, NJ, USA.

[h]Vitamin premix (per kilogram diet); vitamin A (retinyl acetate), 6,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU vitamin E (dl-alpha tocopheryl acetate), 150 IU; vitamin K_3, (menadione sodium bisulfite complex) 30 mg; thiamin (thiamin mononitrate), 40 mg; riboflavin, 50 mg; pantothenate (d-calcium pantothenate), 150 mg; biotin (d-biotin), 1mg; folic acid, 15 mg; vitamin B_{12}, 0.1 mg; niacin, 200 mg; pyridoxine (pyridoxine HCl), 20 mg; ascorbic acid (ascorbic acid mono phosphate, stay C), 200 mg; inositol, 100 mg; BHT, 15 mg; 20 g choline chloride added directly to the main ingredient mixture.

[i]Mineral premix (per kilogram diet); manganous sulfate (32.5%Mn), 40 mg; ferrous sulfate (20.1% Fe), 30 mg; copper sulfate (25.4% Cu), 5 mg; zinc sulfate (22.7% Zn), 75 mg; cobalt chloride (24.8% Co), 2.5mg; sodium selenite (45.6% Se), 1 mg; sodium fluoride (45.2% F), 4 mg.

[j]stabilized with 0.06% ethoxyquin, Comeau Seafood, Saulnierville, NS, Canada.

[k]Supplement premix formulated to provide levels indicated in table 2-2

Table 2-2 Amount of various carotenoids and various dietary supplements used in the experimental diets

Supplement	Experimental diets							
	1	2	3	4	5	6	7	8
Carophyll pink® [a] (mg Ax/kg)	0	40	40	40	40	40	40	40
Cholesterol[b] (%)	0	0	2	0	0	0	0	0
Vitamin E[c] (IU/kg)	0	0	0	450	0	0	0	0
Wheat bran[d] (%)	0	0	0	0	5	0	0	0
Lutein[e] (mg/kg)	0	0	0	0	0	40	0	0
Zeaxanthin[f] (mg/kg)	0	0	0	0	0	0	40	0
Phytosterol[g] (%)	0	0	0	0	0	0	0	2

[a]Carophyll pink (10% Ax), DSM Nutritional Products Ltd., Cambridge, ON, Canada

[b]Sigma-Aldrich Chemical Co., St. Louis, MO, USA

[c]dl-alpha tocopheryl acetate; Rovimix E-50 SD, DSM Nutritional Products Ltd., Cambridge, ON, Canada

[d]Wheat bran, Planet Organic Market, Halifax, NS, Canada

[e]Lutein (5%), DSM Nutrition Products Ltd., Switzerland

[f]Zeaxanthin (5%), DSM Nutrition Products Ltd., Switzerland

[g]Phytosterol, Isolated from crude soybean oil by Dr. Nimal Ratnayake, Health Canada, Ottawa, Ontario

Analysis

The diets were analysed for proximate composition: moisture after drying at 105°C for 24h (AOAC, 1990), ash after incineration at 550°C for 18 h (AOAC, 1990), crude protein (% nitrogen × 6.25) was measured by the Dumas method (Ebeling, 1968) using a Leco nitrogen determinator (Model FP-528, Leco Corporation, St. Joseph, MI., USA) and lipid using a modified Folch method (Folch *et al.*, 1957). Carotenoid content in the diet was extracted using the method described by Schierle and Hardi (1994). Carotenoid was extracted from the plasma according to the method of Kiessling *et al*. (2003). The HPLC was performed using an Agilent HP1100 with a DAD detector on a Waters μPorasil column (3.9 mm × 30 cm). Authentic carotenoid standards (Sigma) were used to verify the retention time and quantify.

For the cholesterol analysis of the plasma, lipid was first extracted from the plasma using a modified Folch method (Folch *et al*., 1957). The lipid was then analyzed for cholesterol content using 5-α cholestane (Sigma) as an internal standard and the method described by Kovacs (Kovacs *et al.,* 1979). The analysis was done by GC/FID (Agilent 6890) on a Supelco SAC-5 capillary column (30 m × 0.25 mm × 0.25 μm).

Statistical analysis

Mean values of data were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test to determine significant difference between individual treatments when $p<0.05$ (Zar, 1984).

Results

Dietary carotenoid concentration

The salmon readily accepted all diets during the 26 day trial. The proximate analysis of the diets is summarized in Table 2-3. Experimental diets were in range of 49.1 to 52.4% for protein and 21.9 to 24.7% for lipid. These diets were formulated to supply an Ax level of 40 mg/kg, whereas the control diet did not contain Ax. The Ax content, as well as lutein and zeaxanthin, of the experimental diets is summarized in Table 2-4. Analysis of the diets showed that there was no Ax detected in the control diet and the dietary Ax concentrations ranged between 37.9 to 42.1 mg/kg for the various experimental diets. Lutein and zeaxanthin were also analysed in the diets. In the diet supplemented with lutein at 40 mg/kg, the analysis confirmed the level to be 39.6 ± 4.22 and absent in all other diets. Analyzed zeaxanthin concentration was 41.0 ± 0.94 mg/kg in the diet supplemented with 40 mg/kg and absent in all other diets.

Table 2-3 Proximate analysis of experimental diets[a]

Diet[a]	Moisture (%)	Ash[b] (%)	Protein[b] (%)	Lipid[b] (%)
1	7.3 ± 0.03	10.1 ± 0.13	52.4 ± 0.18	22.2 ± 0.13
2	7.3 ± 0.02	10.2 ± 0.08	49.3 ± 0.12	22.1 ± 0.11
3	7.0 ± 0.03	10.1 ± 0.15	49.8 ± 0.18	24.0 ± 0.09
4	7.0 ± 0.31	9.9 ± 0.06	49.4 ± 0.53	21.9 ± 0.09
5	7.2 ± 0.06	10.4 ± 0.23	50.2 ± 0.14	22.6 ± 0.54
6	7.3 ± 0.02	10.2 ± 0.12	50.1 ± 0.05	23.2 ± 0.36
7	7.4 ± 0.06	10.0 ± 0.02	49.1 ± 0.31	23.4 ± 0.20
8	7.5 ± 0.01	10.3 ± 0.18	50.8 ± 0.38	24.7 ± 0.11

[a]Mean ± SE, n=3

[b]Expressed on a dry-matter basis

Table 2-4 Astaxanthin, lutein and zeaxanthin content of experimental diets[1]

Diet	Astaxanthin (mg/kg)	Lutein (mg/kg)	Zeaxanthin (mg/kg)
Control, no Ax (Diet 1)	ND	ND	ND
Ax 40 mg/kg (Diet 2)	40.7 ± 0.28	ND	ND
Ax 40 mg/kg + 2% cholesterol (Diet 3)	39.3 ± 1.54	ND	ND
Ax 40 mg/kg + 450 IU vitamin E (Diet 4)	37.9 ± 0.52	ND	ND
Ax 40 mg/kg + 5% fiber (Diet 5)	42.1 ± 0.46	ND	ND
Ax 40 mg/kg + 40 mg/kg lutein (Diet 6)	39.3 ± 3.04	39.6 ± 4.22	ND
Ax 40 mg/kg + 40 mg/kg zeaxanthin (Diet 7)	39.2 ± 3.35	ND	41.0 ± 0.94
Ax 40 mg/kg + 2% phytosterol (Diet 8)	40.7 ± 2.86	ND	ND

[1] Mean ± SE, n=3

[2]ND=not detected

Effect of dietary factors on astaxanthin absorption

The mean values for plasma Ax, lutein and zeaxanthin concentration are presented in Table 2-5 and 2-6, respectively. The fish fed the non-pigmented control diet did not show carotenoids in fish plasma. The average plasma Ax concentration of other fish groups ranged from 2.17 to 4.26 mg/L. The plasma Ax level of fish fed the diet supplemented with 2% cholesterol reached 4.26±0.57 mg/L and was significantly higher ($p<0.05$) than those from fish fed the other diets. Statistical differences were not observed ($p>0.05$) between other groups of fish. The plasma lutein and zeaxanthin concentration was detected only in fish fed certain carotenoids supplemented diet (Table 2-6). Plasma lutein concentration was at levels of 3.71 ± 0.55 mg/L. In fish fed diets containing lutein and zeaxanthin, the plasma Ax concentration was 1.93 ± 0.30 mg/L. Thus supplementation of these carotenoids had no significant effect on Ax absorption. Unlike findings on terrestrial vertebrates, the results of this study also showed that experimental diets supplemented with phytosterol, wheat bran and vitamin had no significantly effect the plasma Ax concentrations as compared with the other diets.

Table 2-5 Plasma astaxanthin content of Atlantic salmon fed various dietary supplements

Diets	Plasma Ax concentration[1, 2] (mg/L)
Control, no Ax (Diet 1)	ND[3]
Ax 40 mg/kg (Diet 2)	2.76 ± 0.32 [a]
Ax 40 mg/kg + 2% cholesterol (Diet 3)	4.26 ± 0.57 [b]
Ax 40 mg/kg + 450 IU vitamin E (Diet 4)	2.79 ± 0.31 [a]
Ax 40 mg/kg + 5% fiber (Diet 5)	2.60 ± 0.15 [a]
Ax 40 mg/kg + 40 mg/kg lutein (Diet 6)	2.37 ± 0.31 [a]
Ax 40 mg/kg + 40 mg/kg zeaxanthin (Diet 7)	2.70 ± 0.26 [a]
Ax 40 mg/kg + 2% phytosterol (Diet 8)	2.13 ± 0.21 [a]

[1] Mean ± SE, n=10 fish

[2]Means within each column not sharing a common superscript are significantly different ($p<0.05$)

[3]ND=not detected

Table 2-6 Plasma lutein and zeaxanthin content of Atlantic salmon fed various dietary supplements

Diet	Plasma lutein [1] (mg/L)	Plasma zeaxanthin[1] (mg/L)
Control, no Ax (Diet 1)	ND [2]	ND
Ax 40 mg/kg (Diet 2)	ND	ND
Ax 40 mg/kg + 2% cholesterol (Diet 3)	ND	ND
Ax 40 mg/kg + 450 IU vitamin E (Diet 4)	ND	ND
Ax 40 mg/kg + 5% fiber (Diet 5)	ND	ND
Ax 40 mg/kg + 40 mg/kg lutein (Diet 6)	3.71 ± 0.55	ND
Ax 40 mg/kg + 40 mg/kg zeaxanthin (Diet 7)	ND	1.93±0.30
Ax 40 mg/kg + 2% phytosterol (Diet 8)	ND	ND

[1] Mean ± SE, n=10 fish

[2]ND=not detected

Plasma cholesterol level

The plasma cholesterol level of experimental fish is shown in Table 2-7. The level of cholesterol in the plasma of fish fed the diet supplemented with cholesterol at 2% differed significantly from that of fish fed all other experimental diets. The plasma of fish fed 2% cholesterol supplemented diet showed 5.30 ± 0.56 mg cholesterol/g plasma whereas the other experimental diet were very close in concentration with an average of 3.53 mg cholesterol per g of plasma. The cholesterol supplementation of the experimental diets caused 1.5 times higher plasma cholesterol levels than the average cholesterol concentration of fish fed other experimental diets. They ranged from 3.42 ± 0.21 to 3.74 ± 0.29 mg cholesterol/g plasma. None of the dietary supplements including phytosterols had a significant effect on plasma cholesterol concentration.

Table 2-7 Plasma cholesterol content of Atlantic salmon fed various dietary supplements

Diet	Plasma cholesterol concentration[1, 2] (mg/g)
Control, no Ax (Diet 1)	3.52 ± 0.38 [a]
Ax 40 mg/kg (Diet 2)	3.42 ± 0.24 [a]
Ax 40 mg/kg + 2% cholesterol (Diet 3)	5.30 ± 0.56 [b]
Ax 40 mg/kg + 450 IU vitamin E (Diet 4)	3.56 ± 0.26 [a]
Ax 40 mg/kg + 5% fiber (Diet 5)	3.51 ± 0.19 [a]
Ax 40 mg/kg + 40 mg/kg lutein (Diet 6)	3.53 ± 0.30 [a]
Ax 40 mg/kg + 40 mg/kg zeaxanthin (Diet 7)	3.74 ± 0.29 [a]
Ax 40 mg/kg + 2% phytosterol (Diet 8)	3.42 ± 0.21 [a]

[1] Mean ± SE, n=10 fish

[2]Means within each column not sharing a common superscript are significantly different ($p<0.05$)

Discussion

The carotenoid retention in the flesh of salmonids is affected by the efficiency of absorption from the digestive tract, transport capacity, deposition mechanisms in the various tissue and metabolism and rate of excretion (Torrissen *et al*., 1989). The present study determined the effects of synthetic Ax and certain dietary supplement to a control diet of Atlantic salmon (ca. 800g) on the Ax absorption by Atlantic salmon. Various dietary factors examined included, cholesterol, vitamin E, fiber, lutein, zeaxanthin and phytosterol. The plasma Ax concentrations of salmon fed the aforementioned dietary supplements were measured.

Previous research has shown that plasma Ax concentration can be used as an indicator of Ax availability from salmonid feeds. Storebakken and Goswami (1996) found a high correlation between the plasma Ax concentration of Atlantic salmon and the dietary Ax concentration (r^2=0.90) and also flesh carotenoid level (r^2=0.95). A high correlation between Ax concentration in blood with Ax content and retention in fish muscle has been also observed (Kiessling *et al*, 2006). They concluded that the level of Ax in blood may be an indicator of long-term muscle deposition and that it could be used as a rapid scanning technique to study the effect of dietary

treatment on long-term deposition of Ax in muscle. Maximum plasma Ax concentration can be attained approximately 24 hr after oral administration (March *et al*., 1990).

The absorption of carotenoids requires several steps including: a) disruption of the food matrix to release carotenoids, b) dispersion in lipid emulsion particles, c) solubilization into mixed bile salt micelles, d) uptake by the cells of intestinal mucosa, and e) incorporation into lipoproteins. In this study, salmon diets containing Ax and cholesterol supplement at a level of 2% wt. showed a significant increase (1.5 fold) in plasma Ax concentration compared to fish fed other experimental diets. The retention of carotenoid pigments into the flesh of salmon was shown to be higher when cholesterol was added to provide a level of between 1 and 3 % in the feed (Buttle, 2000). The mechanism by which a high dietary cholesterol level may work to increase carotenoid absorption in salmon is unknown. In rats, the high basal level of cholesterol 7-alpha hydroxylase was induced by dietary cholesterol allowing these animals to convert excess dietary cholesterol to bile acids efficiently (Horton *et al*., 1995). The inclusion of the bile acid, taurocholate, in the diet of ferret (Lakshman *et al*., 1996) and rat (Schweigert *et al*., 2002) significantly increased the absorption and tissue accumulation of ß-carotene, but did not increase blood Ax levels in salmon (Olsen *et al*., 2005). The ability of fish, which are both hyperlipidemic and hypercholesterolemic (Babin and Vernier, 1989), to regulate cholesterol by increasing bile acid production is unknown. Babin and Vernier (1989) also reported that juvenile rainbow trout transports three times more lipid (1940 vs. 685 mg/dL) and cholesterol (303 vs. 106 mg/dL) in plasma than rats and these values can reach 12 times higher than rats. Most of the plasma cholesterol is in the esterified form.

Cholesterol is also an important determinant of apolipoprotein B synthesis and may promote the transport of Ax by increasing lipoprotein formation (Kumar *et al*., 1992). An increase in dietary cholesterol can also affect the cellular uptake and recycling of lipoproteins in the liver through the down-regulation of hepatic LDL-receptor activity (Turley, 1999). Cholesterol and carotenoids have also been observed to compete for incorporation into lipid bilayers. Cholesterol is favoured for incorporation due to its easy-to-fit structure compared to the large carotenoid molecules (Socaciu *et al*., 2000). The close interaction of these lipid-soluble molecules in the gastrointestinal tract, entercocytes, plasma or tissues may also play a role in enhancing Ax absorption.

Phytosterols or plant sterols have been used as pharmaceuticals and anti-cholesterol additives for humans. An intake of 1.5-3.0 g phytosterols /day reduced blood levels of LDL-cholesterol by 8-15% (reviewed by Fernandes and Cabral, 2007). High concentrations of phytosterols are found in vegetable oils such as corn, sunflower, safflower and soybean oils with levels of 952, 725, 444 and 221 mg/100g edible portion, respectively (reviewed by Rozner and Garti, 2006). The results of this study showed that the diet supplemented with 2% phytosterol in salmon feed did not significantly affect the plasma cholesterol or Ax concentrations as compared with the other diets. The levels of plasma cholesterol and Ax were comparable for all dietary factors tested with the exception of the 2% cholesterol supplemented diet.

The structures of phytosterols are similar to that of cholesterol except that phytosterols contain an additional hydrophobic carbon chain that is attached at the C-24 position. The molecules are more hydrophobic than cholesterol and poorly soluble both in water and oil phases (Rozner and Garti, 2006). The fact that the phytosterol did not affect the Ax level in the plasma may be due to the physical property of its insoluble nature in the powder form. Some of the more effective form of phytosterols used in reducing blood cholesterol are the fat-soluble sterol derivatives (Fernandes and Cabral, 2007). Although, results presented here do not show an effect of phytosterols on plasma cholesterol and Ax contents, additional research is needed to confirm these findings. The physical properties and composition of diets, factors that affect digestion of lipid in ingredients from plant by-products and micelle formation may also influence the interaction between phytosterols and carotenoid including their absorption forms in the digestive tract. In recent years significant amount of research has been focussed on the use of high levels of plant products as alternatives to fish meal and fish oil. There are concerns that this may give rise to interferences in dietary cholesterol absorption and metabolism which will also affect carotenoid uptake from the digestive tract of salmonids.

Lutein and zeaxanthin are abundant in various organs and tissues of many fishes and it has been reported that Ax can be transformed to both xanthophylls in Atlantic salmon (Schiedt, 1998). Their structures are similar with that of Ax. Fish feed producers use feed ingredients that contain significant amounts of yellow pigment from the carotenoids lutein and zeaxanthin. It has been reported that they may compete with Ax during intestinal or tissue absorption (Olsen and Baker, 2006). In this study, lutein or zeaxanthin did not influence the absorption of Ax into the

plasma. This result is consistent with that of Olsen and Baker (2006) who concluded that dietary lutein at levels up to 23 mg/kg of diet did not appear to affect the deposition of Ax in Atlantic salmon.

Addition of 5% wheat bran had no significant affect on the salmon plasma Ax concentration. Studies in humans and chickens have shown that absorption and metabolism of carotenoids can be affected by different types of fiber. Rock and Swendseid (1992) observed in healthy females that an increase in plasma ß-carotene concentration was significantly reduced by dietary pectin which was similar to those reported by Riedl *et al*. (1999). Most practical diets for fish contain 3-6% crude fiber derived from ingredients of plant origin. Fiber refers to indigestible plant matter such as cellulose, hemicellulose, lignin, pentasanes and other complex carbohydrates found in feedstuff. Most fish can tolerance up to 8% fiber in their diets, whereas higher concentrations (8-30%) can depress growth (NRC, 1993). Wheat bran contains the water-insoluble fibers hemicellulose and lignin (Riedl *et al*.1999). Erdmann *et al*. (1986) investigated the bioavailabilty of ß-carotene in chicken and suggested that various types of dietary fiber (hemicellulose, lignin and pectin) would reduce its bioavailability. Riedl *et al.* (1999) investigated the interaction of five dietary fibers with the absorption of carotenoids in humans. They examined pectin, guar and alginate as representative of the water-soluble dietary fiber component, cellulose (containing hemicelluloses and lignins) as a water-insoluble type and lastly the dietary fiber wheat bran. Their results showed that water-soluble dietary fibers (pectin, guar, and alginate) caused a strong decrease in the relative absorption of ß-carotene compared with water-insoluble fibers and this also tended to occur with Cx. All dietary fibers tested caused a strong decrease in relative absorption of lycopene and lutein and suggested that the differences in molecular structure and polarity of the carotenoids might lead to the different effects observed with the various types of dietary fiber. Additional research is required to further assess the effects of different types of cellulose, hemicellulises, pectin etc. on Ax absorption in salmon as well as the influence of higher levels of fiber from commonly used feed ingredients in salmonid feeds.

Vitamin E or tocopherols act as inter- and intracellular antioxidants to maintain homeostasis of labile metabolites in the cell and tissue plasma. Both vitamin E and carotenoids can act as antioxidants. In this study, an experimental diet was formulated to supply 450 IU/kg vitamin E in an Ax supplemented diet. We found that fish fed this diet with the high level of

vitamin E did not have an enhanced Ax concentration in the plasma. These results are in agreement with those of Torrissen (1985) who worked with low dietary Ax concentrations (3-12 mg/kg) and also with Sigurgisladottir *et al.* (1994) who fed Atlantic salmon diets containing approximately 80 mg Ax/kg diet with different levels of vitamin E. Bjerkeng *et al.* (1999), however, found a small improvement in carotenoid deposition and coloration of Atlantic salmon with increasing dietary vitamin E levels. Their results showed a 14% enhancement of Ax deposition when the dietary Ax level of 30 mg/kg was supplemented with increased dietary levels of α-tocopheryl acetate ranging from 200 to 800 mg/kg. Some of the differences in the results of various studies may be due to the differences in lipid and fatty acid composition of experimental diets used by investigators.

Conclusions

It is widely recognized that increasing the retention of pigments in salmonids has major economic importance for the aquaculture industry. The present study examined several dietary factors in Atlantic salmon and it has found that only cholesterol improves the Ax absorption which was reflected in higher plasma Ax concentration. However, other dietary supplements including vitamin E, fiber, lutein, zeaxanthin and phytosterol did not show a beneficial effect. Additional studies are necessary to better define the role of cholesterol on Ax absorption and transport including the distribution of Ax in various lipoprotein fractions. Basic knowledge in this area may provide some clues to improve Ax absorption but also identify the biochemical mechanisms involved in intestinal carotenoid metabolism of fish.

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Chapter 3

In vitro assessment of intestinal uptake of astaxanthin using human colon adenocarcinoma cell (Caco-2) model

Introduction

The mechanisms for carotenoid absorption are complex and several reviews have comprehensively discussed the present knowledge and methodological constraints to investigate various biochemical mechanisms and suitability of animal models (Parker, 1996; Castenmiller and West, 1998; Yeum and Russell, 2002; During and Harrison, 2005). Although the bioavailabilty of carotenoids, including the release of carotenoids from the food matrix and solubilization of carotenoids into mixed lipid micelles in the lumen, have been investigated in terrestrial invertebrates, the cellular uptake of carotenoids by intestinal mucosal cells (enterocytes) is considered a complex process. In order to better understand the efficiency of carotenoid absorption, the nature of luminar and intracellular factors regulating the process of their absorption, particularly cellular uptake and intracellular transport mechanisms, are needed.

Human colon adenocarcinoma cell line or Caco-2 is a cell line originating from human colonic carcinoma and has proved to be the best model for studies involving intestinal absorption of protein, micronutrients and carotenoids. These cells have some morphological and functional characteristics similar to the epithelial intestinal mucosal cells (Sambruy *et al*., 2001). The Caco-2 cell line has been extensively used to study absorption of several substances such as proteins (Lenaerts *et al*., 2007; Mayjune *et al*., 2009; Rubio and Clemente, 2009), vitamins (Quick and Ong, 1990; Cross *et al*., 1997; Anwar *et al*., 2006; Reboul *et al*., 2006) minerals (Garcia *et al*., 1996; Ekmekcioglu *et al*., 1999; Etcheverry *et al*., 2002; Viadel *et al*., 2006; Cámara *et al*., 2007), glucose (Harris *et al*., 1992; Johnton *et al*., 2005; Chang *et al*., 2007), lipids and fatty acids (Trotter *et al*., 1996; Ho and Storch, 2001; Nakano *et al*., 2009). In addition, this cell line has been used to estimate carotenoid bioavailability from various sources (Garrett *et al*., 1999; Garrett *et al*., 2000; Liu *et al*., 2004; Chitchumroonchokchai *et al*., 2004). Other studies include comparison of the uptake and secretion between carotene and xanthophylls (During *et al.,* 2002;

O'Sullivan *et al*., 2007), evaluation of the intestinal transport rate compared with vitamin A (During and Harrison, 2007) and factors which may affect the intestinal carotenoid absorption (Gracía-Casal *et al*., 2000; During and Harrison, 2005). Although many publications have shown that Caco-2 cells can be used to investigate the mechanism of transport and metabolism of carotenoids within intestinal epithelial cells, few studies have focused on the mechanism of Caco-2 astaxanthin absorption.

The main objective of this study was to first examine cellular uptake of Ax through a Caco-2 cell culture model. After adaption of this cell culture model, these techniques were modified and refined to demonstrate the mechanism of astaxanthin absorption in Atlantic salmon intestinal cells.

Materials and Methods

Preparation of stock Ax in DMSO and Ax-enriched media

A stock solution of Ax (Sigma-Aldrich, St Louis, MO, USA) was prepared by dissolving 3.0 mg Ax in 1 mL of sterile dimethyl sulfoxide, DMSO (ATCC, Manassas, VA) and warming in water bath at 50-55°C for 15 min. The solution was filtered through a 0.2µm DMSO safe Acrodisc® syringe filter (Pall, Ann Arbou, MI, USA) and stored in a sterile amber vial. The concentration of Ax determined by reverse phase HPLC (described below) using a standard calibration curve.

The media, Minimum Essential Medium (MEM, Fisher Scientific, USA) containing micelles (0.5mM sodium taucholate, 45mM glycerol and 1.6mM oleic acid) and 4µM BHT (Sigma-Aldrich, St Louis, MO, USA) was sterilized by filtering through a 0.22µm Millex®-GV syringe filter (Millipore Inc., Bedford, MA, USA). The media was transferred by pipette to 15 mL disposable sterilized tubes (Fisherbrand, Fisher Scientific, USA) and the Ax stock solution added to give an Ax concentration of approximately 4µM and 8 µM taking care that the final concentration of DMSO did not exceed 0.6% in the enriched media. The Ax concentration in the media was determined using a slightly modified method from During *et al*. (2002). Briefly 100 µL of medium and 300 µL of 2- propanol/dichloromethane (2:1, v/v) were placed in a tube and

vortexed for 1 min then centrifuged for 1 min using a microcentrifuge. The supernatant was removed, brought to dryness under N_2, the residue dissolved in 150 µL methanol/TBME (70:30, v/v) and filtered through a 0.2µM PTFE syringe filter for HPLC analysis as described below.

Analytical methods

Caco-2 cells culture

Caco-2 cells (ATCC #HTB-37^{TM}, Rockville, MD) were activated from a frozen state, transferred and grown in 75T-flasks (Corning Incorporated, NY, USA). They were maintained in media until they reached confluency then were split and seeded in culture conditions as described below. The Caco-2 cells were seeded for 2 days following methods of Liu *et al.* (2004). They were seeded in six-well flat bottom plates at a density of $1x10^6$ cells/well. The cells were incubated at 37°C and 5% CO_2 and used after 24h seeding. The cells were maintained in MEM supplemented with 20% FBS, 2mM L-glutamine, 10 µL/mL of 100mM sodium pyruvate, 10 µL/mL of MEM non essential amino acids solution (100x) and antibiotics (50 units/mL penicillin plus 50 µg/mL streptomycin).

The culture media in each experimental well plate was removed and the cells washed three times with pre-warmed PBS. The cells were treated with 1 mL carotenoid enriched media and incubated at culture conditions for 2, 4, 8, 16 and 24h. The treatment media was collected at the specified times and the cells washed 3 times with 1 mL of 5mM sodium taurocholate in PBS at 22°C (Garrett *et al*., 1999). Cells were scraped into 1 mL cold PBS. For protein analysis, 100µL of cells suspension was collected and 100µL of 0.1N NaOH was added. The cell samples were stored at -80°C (protected from light) until further analysis (O'Sullivan *et al.*, 2007).

Method for cellular carotenoid and protein analysis

The Ax content of cells was determined according O'Sullivan *et al.* (2007). Frozen cell samples were thawed and briefly vortexed. The cell suspension was sonicated for 30s on ice with a hand held sonic probe then 300µL samples extracted twice with 2mL hexane-ethanol-acetone (50:25:25, v/v). Supernatant layers were removed, pooled and dried under N_2. The residues were

reconstituted in 150µL methanol/TBME (70:30, v/v), filtered through a 0.2µM PTFE syringe filter and analyzed by reverse phase HPLC. The cell residue in 0.1N NaOH was analyzed for protein content using a bicinchoninic acid assay (BCA1, Sigma-Aldrich, St Louis, MO, USA).

Results

Ax uptake by 2-day Caco-2 cells was measured at different incubation times. The results are given in Figure 3-1. The cellular uptake of Ax was detected after 4h incubation with the lower concentration of Ax enriched media (4.1µM Ax in media) while at the higher concentration (8.6µM) it was detected earlier after 2h of incubation. The dose-response of Ax uptake by Caco-2 cells increased with increasing Ax concentration in the media and reached a stable level after 8h incubation.

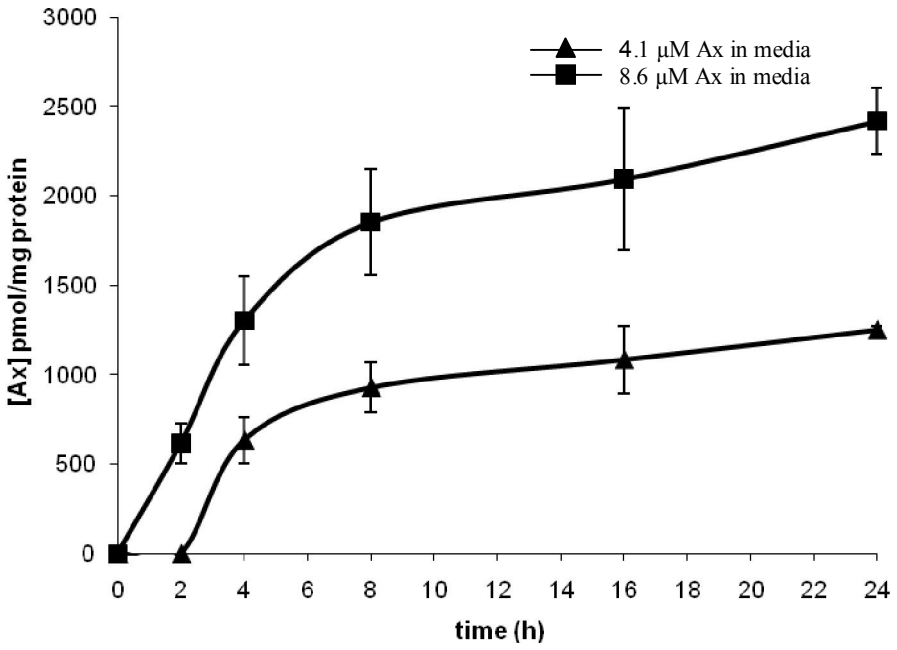

Figure 3-1 Ax uptake by Caco-2 cells after incubation with media containing Ax for given times (n=3)

The Ax level in the culture media after the given incubation periods is shown in Figure 3-2. The Ax content decreased with increasing incubation time. At 16h of incubation, both experimental treatments had similar Ax concentrations in the culture media, which is 1.21µM and 1.13µM compared to the initial Ax concentrations in the culture media of 4.1µM and 8.6µM, respectively. The levels slightly decreased after 24h incubation.

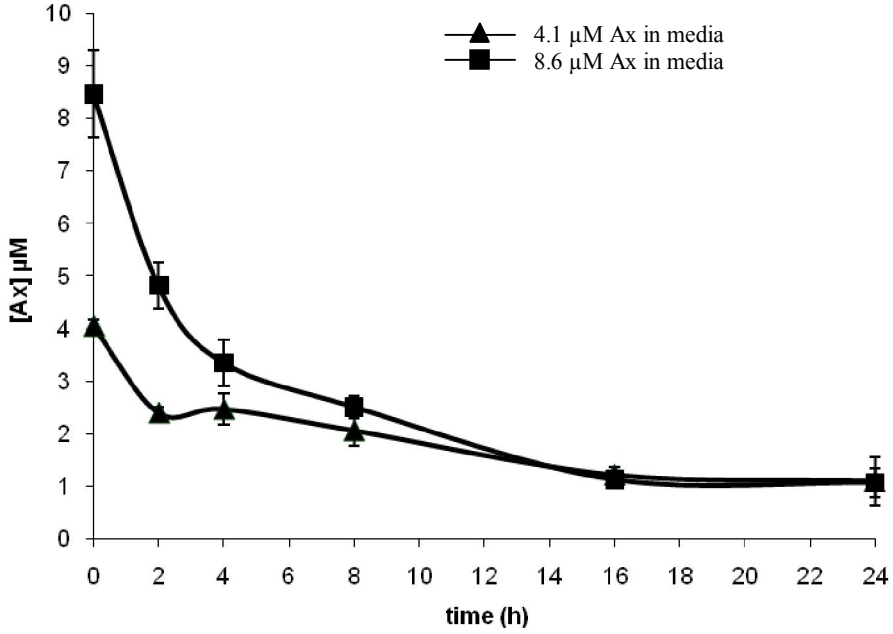

Figure 3-2 Ax concentration in media after uptake by Caco-2 cells after given times (n=3)

Discussion

The Caco-2 cell line exhibits many morphological and biochemical characteristics of enterocytes or intestinal absorptive cells, including polarization and expression of several brush border enzymes. It has been demonstrated that the Caco-2 cell monolayer shows barrier properties similar to those observed in the small intestine (Puyol *et al*., 1995). There are several reports demonstrating the use of Caco-2 on cellular carotenoid uptake (Garrett *et al*., 1999; Garrett *et al*., 2000; Gracía-Casal *et al*., 2000; During *et al*., 2002; Liu *et al*., 2004;

Chitchumroonchokchai *et al*., 2004; During and Harrison, 2005; During and Harrison, 2007; O'Sullivan *et al*., 2007).

A few investigations have used Caco-2 as a model to approximate the cellular metabolism of Ax. In this study, the technique from Liu *et al*. (2004) was modified for use as an experimental model to elucidate cellular Ax uptake. Liu *et al*. (2004) compared the uptake of carotenoids (ß-carotene, zeaxanthin and lutein) by using different seeding periods with Caco-2 cells. Their results showed that the kinetics and dose-response of carotenoid uptake showed a similar pattern in Caco-2 cells after plating for 2 and 14 days. They also compared the cellular uptake from different sources of carotenoids, ingested and synthetic Ax. Ax was dissolved in DMSO before adding to cell culture media and incubating a monolayer of the cell line.

The present study used the model involving 2-day seeding of Caco-2 cells to demonstrate the absorption of synthesized Ax solubilised in DMSO at two different concentrations. The cellular Ax concentration detected were comparable to the results of Liu *et al*. (2004) in terms of the intracellular carotenoid concentration of zeaxanthin and ß-carotene after incubation at similar concentrations in the carotenoid enriched media. However, the uptake of Ax by Caco-2 cells reached a saturated level after 8h incubation while Liu *et al*. (2004) reached a saturated level of carotenoids after 4h of incubation. This difference could have been due to the structural differences of carotenoids in the study or undergoing in cell culture process. Sambuy *et al*. (2005) found that differences between laboratories in culture-related conditions and Caco-2 cell lines made it extremely difficult to compare published results. They cited factors such as seeding density, cell differentiation, medium composition, as well as the different passage number of the Caco-2 cells or the influence of line sub-types (clonal Caco-2 cell line i.e. Caco-2/TC7 or parental Caco-2/ATCC) potentially affect the variability of results from different laboratories.

Several studies have used the Caco-2 cell culture model to assess individual carotenoid uptake. In doing so, it is necessary to have vehicle for delivery of carotenoids to the apical surface of cells. Since most carotenoids are hydrophobic in nature, different methods have been used to solubilize the carotenoid before addition to the cell culture media or a specific form of water-soluble carotenoid (such as water miscible beadlets of ß-carotene) (Garrett *et al*., 1999) or a

complex of crystalline carotenoid with Captisol® (commercial brand of sulfobytyl ether ß-cyclodextrin) (Lockwood *et al*., 2003) were used.

During *et al*. (1998) compared two methods for delivering ß-carotene to TC7 clone of human intestinal cell line Caco-2. The same weight of ß-carotene was dissolved in either tetrahydrofuran (THF) or Tween 40 before being added to cell culture media, DMEM. They found that Tween 40 gave a better recovery from the media and cellular uptake of ß-carotene than THF. This may be due to solid-state formation of ß-carotene when solubilized in THF which preticipated when added tp the cell medium (aqueous phase). The results showed poor uptake and recovery of ß-carotene when solubilised in THF. In contrast, cell incubated with ß-carotene in Tween 40 exhibited a consistent yield of metabolic residue and a significant increase in enzyme activity. During and Harrison (2005) used this Tween 40 method to deliver carotenoids (ß-carotene, lutein and lycopene) to examine intestinal absorption. The Tween 40 method has also been used to define the mechanisms of intestinal absorption of dietary carotenoids and retinoids which focused on membrane transport mechanisms through the Caco-2 cell model (During and Harrison, 2007).

In the current study, crystalline Ax was dissolved in DMSO before being used in the cell culture system. DMSO is an amphipathic molecule with a highly polar domain and two apolar methyl groups, making it soluble in both aqueous and organic media (Santos *et al*. 2003). It is frequently used as a cryoprotectant in cell suspension cryopreservation. Concentrations of DMSO present in the cell media have ranged between 5-15% but 7.5-10% is more commonly used (Freshney, 2005). Several studies have demonstrated the use of DMSO as a solvent in biological studies and as a vehicle for drug therapy (Krishna *et al.*, 2001; Demirbas and Stavchansky, 2003; Liu *et al.*, 2004; Ebert *et al.*, 2005; Elsby *et al.*, 2008).

The solubility of Ax in DMSO is 50mg/mL (Sigma product information). The stock solution of Ax was prepared by dissolving crystalline Ax in DMSO and sterilized by filtration before adding to cell culture medium. The final concentration of DMSO in media did not exceed 0.6%. This level is significantly lower than that used by Liu *et al*. (2004). In this study, there was no sign of negative side effects to culture cell from either the Ax or DMSO.

Conclusion

Using this model, 2 day seeded Caco-2 cells and Ax prepared in DMSO for sterilization and then diluted in the culture media was used as an effective model to study Ax uptake. This model will be used in the subsequent study related to Ax uptake using salmon enterocytes.

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Chapter 4

The assessment of intestinal uptake of astaxanthin using Atlantic salmon enterocyte model: *in vitro* and *in vivo* studies

Introduction

The color of Atlantic salmon flesh is recognized as one of the most important quality criteria for consumers. The bright red colouration is caused by deposition of carotenoid pigment. Astaxanthin (Ax) is the predominant carotenoid found in wild Atlantic salmon (Schiedt *et al*., 1981). Salmonids cannot synthesize carotenoid themselves but rather they absorb carotenoids from their diet and deposit it in their tissue (Torrissen *et al*., 1989). In the case of farmed salmon, Ax and canthaxanthin (Cx), either alone or in combination, are predominantly used in salmonids feeds to achieve the red coloured flesh desired by consumers (Torrissen *et al*., 1989; Storebakken and No, 1992). The cost of adding these carotenoids to diets is high (up to $250/ton) (Bjerkeng *et al*., 1999). However, the apparent digestibility coefficients (ADC) of Ax in Atlantic salmon typically range between 45-60% with retention of Ax in the muscle usually only 5-15% of the total carotenoid (Torrissen *et al*., 1989; Buttle *et al*., 2001; Bjerkeng and Berge, 2000). Thus the absorptive processes which occur in the gut can potentially constitute a major limitation in the effective utilisation of carotenoids in salmonid fish.

The absorption mechanism for carotenoids has been hypothesized to be similar to that of lipids. The following major steps have been suggested to be involved during carotenoid absorption within the mammalian gastrointestinal tract: 1) release of carotenoids from the food matrix; 2) solubilization of carotenoids into mixed lipid micelles in the lumen; 3) cellular uptake of carotenoids by intestinal mucosal cells (enterocytes); 4) incorporation of carotenoids into chylomicrons (CM) and 5) secretion of carotenoids and their metabolites associated with CM into the lymph (van Het Hof *et al*., 2000). In fish, the mechanism for carotenoid absorption remains to be investigated. Information is needed regarding the absolute efficiencies of carotenoid absorption, the nature of lumina and intracellular factors that regulate the process of their absorption, the mechanism of intracellular transport of these carotenoids and their incorporation

into CM, and the nature of interactions between carotenoids and other dietary factors occurring during intestinal absorption.

Intestinal carotenoid uptake was investigated using *in vitro* and *in vivo* methods to assess tissue and cellular behavior. Other researchers have used an everted intestine technique to study *in vitro* carotenoid uptake (Al-Khalifa and Simpson, 1988; White *et a*l., 2003). The everted intestine of rainbow trout and an Atlantic salmon intestinal section were exposed to a micellar medium containing Ax and Cx. Carotenoid analysis of sections of the intestine showed that the tissue Ax concentration between intestinal sections of the proximal intestinal region (pyloric caeca and mid intestine) had significantly higher mean values compared to the hind intestine. The carotenoid concentration in the pyloric caeca of trout following exposure to micellar medium containing similar carotenoid concentrations was significantly less for Cx compared to Ax. However, the Ax concentration of rainbow trout compared to Atlantic salmon mid or hind intestines were not significantly different when exposed to micellar Ax supplemented media (White *et al*., 2003).

The application of isolated enterocytes in intestinal metabolism studies in fish have been investigated. Isolated fish enterocytes from various regions of the intestine including pyloric caeca were used to determine fatty acid desaturation and oxidation in Atlantic salmon (Tocher *et al*., 2002) and rainbow trout (Tocher *et al*., 2004). Bogevik *et al*. (2008) employed Tocher's method to study the utilization and metabolism of fatty acids and alcohols in Atlantic salmon. Pérez *et al.* (1999) determined the uptake and esterification of radiolabelled fatty acids using isolated enterocytes from rainbow trout and concluded that the isolated enterocytes can be used for studying mechanisms of intestinal fatty acid absorption in fish.

Intestinal carotenoid uptake using isolated enterocytes has not been reported. In order to further knowledge of salmon intestinal carotenoid uptake and also to clarity the role of cholesterol, we have determined the intestinal absorption of Ax by using both *in vitro* and *in vivo* models was determined. For the *in vitro* study, a freshly isolated enterocyte suspension was incubated with Ax-enriched media with and without an enhancing factor. Whereas in the *in vivo* study, intestinal cells were isolated from fish fed diets containing only Ax and those having Ax supplemented with cholesterol. The Ax content in fish enterocytes were analyzed by HPLC. A

better understanding of intestinal Ax uptake and absorption and the effects of cholesterol on enhancing carotenoid absorption in both *in vitro* and *in vivo* models were developed from this study.

Materials and Methods

Application of salmon enterocyte model: *In vitro* study

Fish and enterocyte isolation

Atlantic salmon juveniles were housed in the aquaria facilities at NRC's Marine Research Station, Halifax, Nova Scotia. Ten of Atlantic salmon (1.2-2.5 kg) were distributed between 16 – 1000L fiberglass tanks with flow-through (12 L/min), filtered seawater (30 μm; salinity, 28-30 ppt) and a light regime of 12h light:12h dark. Water quality was monitored daily with water temperature maintained at 12°C and dissolved oxygen levels at 90-95% saturation. Fish were held on a commercial non-pigmented diet (6.5 mm Shur-Gain Feeds, Truro, NS, Canada) fed twice daily (0900 and 1600h) on weekdays and once daily (1000h) on weekends. Prior to enterocyte isolation, salmon were maintained in a small tank for 2 days without feeding according to the suggestion of Dópido *et al*. (2004). They were sacrificed with an over-dose of tricaine methane sulfonate (MS-222) and immersed in 70% ethanol for a few seconds before an *in vivo* operation to remove the intestinal section (Tong *et al*., 1997, Ye *et al*., 2006).

Salmon enterocytes were isolated according to Tocher *et al*. (2002). The intestinal tract was removed and pyloric caeca dissected, cleaned of adhering adipose tissue, slit open and luminal contents rinsed away with solution A (calcium and magnesium-free Hanks balanced salt solution (HBSS, Invitrogen, NY, USA) containing 10 mM HEPES (Sigma-Aldrich, St Louis, MO, USA). The cleaned caeca was finely chopped with scissors and incubated with 20 mL of solution B (HBSS containing calcium, magnesium and 10 mM HEPES) containing 0.1% (w/v) collagenase type IV (Sigma-Aldrich, St Louis, MO, USA) in a 250 mL culture flask. The flask was incubated in a refrigerated incubator shaker (Innova 4330, New Brunswick Scientific, Enfield, CT, USA) at 60 rpm and 20°C for 45 min. The digested intestinal tissues were filtered through a 100 μm nylon gauze. The cells were collected by centrifugation at 500g for 5 min. The

cell pellet was washed with solution B containing 1% w/v bovine serum albumin (BSA) (Invitrogen, NY, USA) and centrifuged again as above. The washing was repeated with an additional 20 mL solution B without BSA and re-centrifuged. The enterocytes pellet was resuspended in Medium 199 containing 10mM HEPES, 2 mM glutamine and antibiotics (50 units/mL penicillin plus 50 µg/mL streptomycin) (MP Biomedicals, Canada).

The digestive enzyme, alkaline phosphatase, was determined in the isolated enterocytes using a kit to spectrophotometrically measure the rate of nitrophenyl phosphate hydrolysis by alkaline phosphatase-SL (Genzyme Diagnostics, Charlottetown, PE, Canada). Cell viability was determined using trypan blue (Dópido *et al*., 2004). Cell size was measured using Countess ® Automated cell counter (Invitrogen, NY, USA). Cellular protein content was measured using a bicinchoninic acid test kit (BCA1, Sigma-Aldrich, St Louis, MO, USA).

Cellular uptake of carotenoid

Preparation of stock Ax in DMSO and Ax-enriched media

A stock solution of astaxanthin (Sigma-Aldrich, St Louis, MO, USA) was prepared by dissolving 3.0 mg Ax in 1 mL of sterile dimethyl sulfoxide, DMSO (ATCC, Manassas, VA) and warming in water bath at 50-55°C for 15 min. The solution was filtered through a 0.2µm DMSO safe Acrodisc® syringe filter (Pall, Ann Arbou, MI, USA) and stored in a sterile amber vial. The concentration of Ax determined by normal phase HPLC (described below) using a standard calibration curve.

The media, Medium 199 (M199, Invitrogen, NY, USA) containing micelles (0.5mM taucholate, 45mM glycerol and 1.6mM oleic acid) and 4µM Butylated Hydroxytoluene, BHT (Sigma-Aldrich, St Louis, MO, USA) as an antioxidant was sterilized by filtering through a 0.22 µM Millex®-GV syringe filter (Millipore Inc., Bedford, MA, USA). Media was pipetted into 15mL disposable sterilized tubes (Fisherbrand, Fisher Scientific, USA) before adding the Ax stock solution and sonicating it on ice (Sonic Dismembrator model 100, Fisher Scientific, USA) for six times for 15 sec. The enriched media had a final concentration of DMSO (ATCC, Manassas, VA) not exceeding 0.3%.

Astaxanthin uptake experiments were carried out in duplicate at 20°C according to Oxley *et al.* (2005). The experiments were completed within 6h after fish were sacrificed. The salmon enterocyte density was adjusted to be 1 to 1.5x10^6 cells/mL. Aliquots (2mL) of cell suspension in Ax supplemented media were seeded into 25 cm^2 cell culture flasks (Corning Incorporated, NY, USA). The concentration of Ax in media was 7.33±0.22µM. Cells were incubated in a refrigerated incubator shaker (Innova 4330, New Brunswick Scientific, Enfield, CT, USA) at 60 rpm and 20°C for 0, 10, 30, 60, 90 and 120 min. Before each Ax uptake experiment, enterocytes viability was tested.

The effect of cholesterol on Ax uptake by Atlantic salmon enterocytes was investigated using freshly isolated salmon enterocytes in M199 media were supplemented with 4µM and 8µM Ax with and without 15 µM cholesterol (Sigma-Aldrich, St Louis, MO, USA). The experimental protocol followed was similar to that of the previous salmon enterocyte study. After incubation, cells were harvested according to Oxley *et al.* (2005). The cell suspension was transferred from the cell culture flask to a 1.8 mL microcentrifuge tube (Progene, St-Laurent, QC, Canada) and centrifuged at 500 x g for 5 min. The supernatant was transferred and the cell pellet washed with chilled solution A, then centrifuged at 500g for 5 min and resuspended in chilled solution A. The cell suspension was kept at -80°C until further analysis.

Application of salmon enterocyte model: *In vivo* study

Fish - sampling and enterocyte isolation

Juvenile Atlantic salmon (average weight 1.2-2.5 kg) were reared under similar conditions as described above. Each diet was hand-fed to duplicate tanks containing 12 fish per tank to apparent satiation twice daily (0900h and 1600h) on weekdays and once daily (1000h) on the weekend. Prior to feeding experimental diets, fish were held on a commercial non-pigmented diet (6.5 mm Shur-Gain Feeds, Truro, NS).

Three practical fish meal–based experimental diets were produced at the research station with levels of all nutrients and energy carefully controlled in accordance with known salmonid nutrient requirements (NRC, 1993). The composition of the basal and experimental diets used in

this study can be found in Table 4-1. Diet 1 contained no added Ax and served as the control. The remaining 2 diets were produced from the same basal diet with diet 2 having 40 mg Ax/kg diet and diet 3 as 40 mg Ax/kg diet plus 2% cholesterol. The basal ingredients were mixed using a Hobart mixer (Model H600T, Rapids Machinery Co., Troy, OH, USA). Micronutrients and the test ingredients were pre-mixed using a twin-shell mixer (Paterson-Kelly, East Stroudsburg, PA, USA) prior to being added to the basal mixture. Individual diets were steam pelleted using laboratory type pellet mill (California Pellet Mill Co., San Francisco, CA, USA), dried for 1h at 80°C and stored at -20°C until use.

Table 4-1 Composition of experimental diets.

Ingredient	**g/kg**
Herring meal[a]	450
CPSP-G[b]	40
Soybean meal[c]	100
Wheat middlings[d]	83
Dried whey[e]	70
Gelatin[f]	20
Pre-gelatinized starch[g]	40
Vitamin premix[h]	17
Mineral premix[i]	10
Herring oil[j]	170

[a]Corey Feed Mills Ltd., Fredericton NB, Canada
[b]Concentre proteique soluble de poisson (soluble fish protein concentrate) (Sopropêche, France)
[c]Bunge Canada, Oakville, ON, Canada
[d]Dover Mills, Halifax, NS, Canada
[e]Farmers Co-operative Dairy Ltd., Truro, NS, Canada.
[f]US Biochemical, Cleveland, OH, USA.
[g]National Starch and Chemical Co., Bridgewater, NJ, USA.
[h]Vitamin premix (per kilogram diet); vitamin A (retinyl acetate), 6,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU vitamin E (dl-alpha tocopheryl acetate), 300 IU; vitamin K_3, (menadione sodium bisulfite complex) 30 mg; thiamin (thiamin mononitrate), 40 mg; riboflavin, 50 mg; pantothenate (d-calcium pantothenate), 150 mg; biotin (d-biotin), 1mg; folic acid, 15 mg; vitamin B_{12}, 0.1 mg; niacin, 200 mg; pyridoxine (pyridoxine HCl), 20 mg; ascorbic acid (ascorbic acid mono phosphate, stay C), 200 mg; inositol, 100 mg; BHT, 15 mg; 20 g choline chloride added directly to the main ingredient mixture.
[i]Mineral premix (per kilogram diet); manganous sulfate (32.5%Mn), 40 mg; ferrous sulfate (20.1% Fe), 30 mg; copper sulfate (25.4% Cu), 5 mg; zinc sulfate (22.7% Zn), 75 mg; cobalt chloride (24.8% Co), 2.5mg; sodium selenite (45.6% Se), 1 mg; sodium fluoride (45.2% F), 4 mg.
[j]stabilized with 0.06% ethoxyquin, Comeau Seafood, Saulnierville, NS, Canada.

After 4 weeks of feeding experimental diets, fish were fasted for 24h before being fed to satiation. At 3, 6 and 24h after last meal, four fish fed diets 2 and 3 were sacrificed with an over-dose of MS-222. Blood and pyloric caeca were collected from individual fish in each tank. Blood was collected from the caudal vein using a 10 mL non-heparinised disposable syringe and transferred to a heparinized vacuum tube on crushed ice before being centrifuged at 4,000 rpm for 10 min at 4°C (IEC Centra CL3R). Plasma was carefully transferred to an Eppendorf tube and samples for Ax analysis were immediately placed on dry ice then stored at -80°C until further analysis. Four salmon at each sampling hour were sampled. Pyloric caeca from individual fish was rapidly cleaned and processed for enterocyte isolation as described in *in vitro* study. All isolations were carried out under yellow light. The enterocyte pellet was re-suspended in solution A (calcium and magnesium-free HBSS containing 10 mM HEPES). The cell suspension was collected and kept at -80°C until further analysis. The Ax concentration in the cell was expressed as mg protein of cell.

Analytical methods

Proximate analysis

The diets were analysed for proximate composition as follows: moisture after drying at 105°C for 24h (AOAC, 1990), ash after incineration at 550°C for 18h (AOAC, 1990), crude protein (% nitrogen × 6.25) was measured using a Leco nitrogen determinator (Model FP-528, Leco Corporation, St. Joseph, MI., USA) and lipid using a modified Folch method (Folch *et al.*, 1957).

HPLC analysis for carotenoid

Carotenoid content in the diet was extracted using the method described by Schierle and Hardi (1994) and from the plasma according to the method of Kiessling *et al.* (2003). Carotenoids content in cell samples were extracted according to O'Sullivan *et al.* (2007). Frozen cells were thawed, sonicated with ultrasonic probe (Misonix sonicator-XL-2000 series) for 30 sec on ice and briefly vortexed before solvent extraction. The cellular Ax content was calculated based on cellular protein content analyzed using a bicinchoninic acid test kit (no. BCA1, Sigma). The

analytical method to determine the Ax content in media was according to During *et al*. (2002). All extractions were performed under yellow light. The HPLC was performed using an Agilent HP1100 with a DAD detector on a Waters μPorasil column (3.9 mm × 30 cm). Authentic standard carotenoid (Sigma) were used to verify the retention time and quantify.

Statistical analysis

All data were tested for normality of distribution. The regression analysis and analysis of variance was conducted using SPSS (Statistical Products Service and Solutions) with a significant level of $p<0.05$.

Results

In vitro study

A sufficient amount of cells were isolated from the salmon enterocytes for the Ax uptake study. The cell size was around 8-10 μm. Preliminary results showed that the viability of isolated enterocytes decreased with increasing incubation time with cell viability declining greatly after 2h incubation to levels less than 80% of initial levels to viabilities of less than 5% after 24h incubation. The kinetics of Ax uptake using isolated salmon enterocytes at different incubation times up to 2h is shown in Figure 4-1. The enterocyte viability was >85% at the time of termination.

Figure 4-1 shows that cellular uptake of Ax was detected at 30 min after incubation with Ax enriched media. The dose-response of Ax uptake by enterocytes increased with increasing incubation time. The cellular Ax content was highest after 120 min incubation with a concentration of 508.47 ± 225.50 pmol/mg protein. The Ax content in fish enterocytes was similar to that using Caco-2 as a model from a previous study. Caco-2 had 616.12±137.87 pmol/mg protein after 2h incubation.

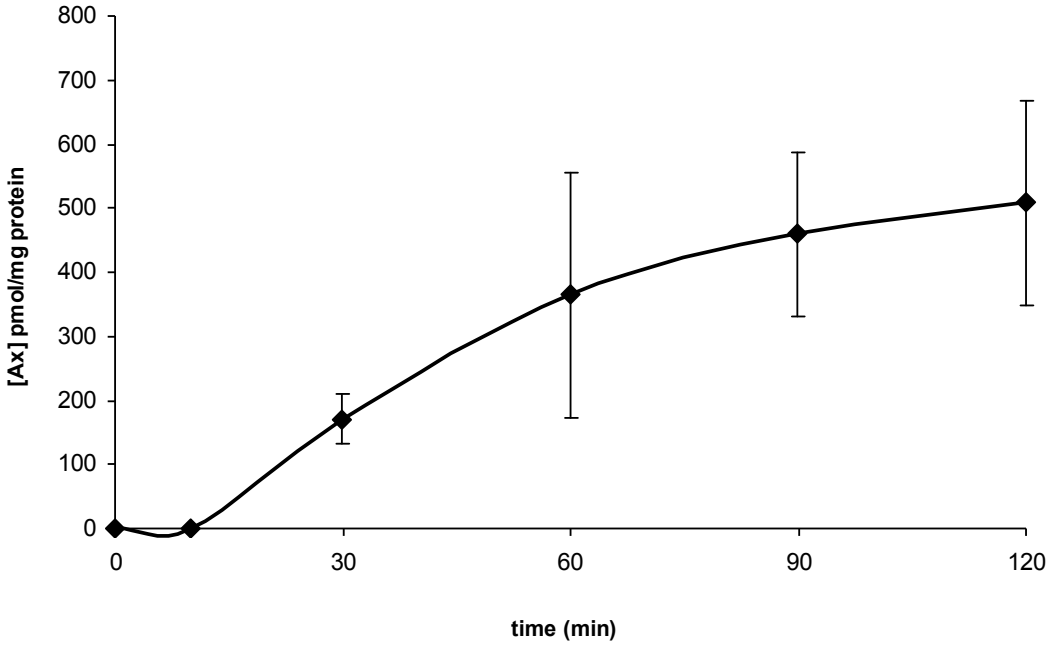

Figure 4-1 Ax concentration in salmon enterocytes after incubation with Ax-enriched media (7.3±0.2μM) at indicated time (n=2)

The effect of cholesterol on Ax uptake by Atlantic salmon enterocytes

The uptake of Ax by salmon enterocytes incubated with 4 and 8μM Ax with or without cholesterol in cell culture media was examined and the results are presented in Figure 4-2. Even though it appears that a slightly higher cellular Ax content was present in cholesterol supplemented media, there was no statistically difference ($p>0.05$) observed between treatments.

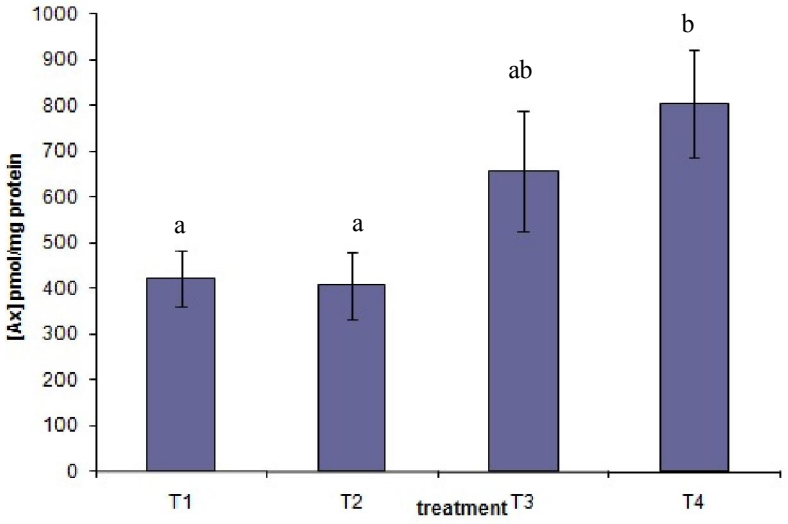

Figure 4-2 Ax in salmon enterocytes after incubation with media (T1, 3.3µM Ax; T2, 3.3µM Ax plus15µM cholesterol; T3, 6.7µM Ax and T4, 6.7µM Ax plus15µM cholesterol) (n=4)

In vivo study

Diet composition

Table 4-2 shows the proximate composition of the experimental diets along with their Ax content. The diets contained between 42.9 to 43.8% protein and 25.0 to 25.8% lipid. Experimental diets were formulated to have an astaxanthin level of 40 mg/kg whereas the control diet had no added Ax. Analysis of the diets showed that, as expected, there was no Ax detected in diet 1 (control diet) and the dietary Ax concentrations were 38.9 ± 0.6 and 39.0 ± 1.5 mg/kg for diets 2 and 3, respectively.

Table 4-2 Proximate analysis of experimental diets[a]

Experimental diets	Moisture[b] (%)	Ash[b] (%)	Protein[b] (%)	Lipid[b] (%)	Energy[b] (%)	Ax (mg/kg)
Diet 1 control	7.7±0.1	7.6±0.1	42.9±0.2	25.0±0.2	22.6±0.1	ND[c]
Diet 2 40mg Ax /kg diet	7.5±0.1	7.6±0.4	43.8±0.3	25.5±0.3	22.7±0.1	38.9±0.6
Diet 3 40mg Ax /kg diet plus 2%cholesterol	7.6±0.1	8.5±0.0	43.8±0.1	25.8±0.1	22.7±0.1	38.9±1.5

[a]Mean ± SE; n=3 replicates
[b]Expressed as fed-basis
[c]ND = not detected

Intestinal Ax uptake

Pyloric caeca was removed from salmon at given times and the enterocytes isolated for Ax analysis. The results, given in Figure 4-3 show the Ax content of the enterocytes, expressed as cellular protein concentration, for enterocytes collected at the given times after last feed. The concentration of Ax in enterocytes at 3h in fish fed diet 2 (40 mg/ kg diet) and diet 3 (40 mg/kg diet plus 2% cholesterol) were 28.52±3.38 and 28.92±2.80 pmol/mg protein, respectively. The level of Ax in enterocytes slightly deceased at 6h after feeding in fish fed both diets 2 and 3 to 25.06±3.34 and 26.59±0.77 pmol/mg protein, respectively. After 24h when fish were fed their last meal, Ax showed a little increase from the levels at 6h. The average concentration of Ax in enterocytes after 24h were 29.15±2.93 and 30.25±2.72 pmol/mg protein in fish fed diets 2 and 3, respectively.

Results were compared between samples collected from fish fed diets 2 and 3 at the same sampling hour and on the same day. Significant difference was considered to be $p<0.05$ using t-test analysis. The concentration of Ax in isolated enterocytes at each time after feeding fish fed diet 3 was slightly higher than fish fed diet 2 at any sampling time. Statistical analysis showed the difference to be insignificant between fish fed diets 2 and 3 ($p>0.05$).

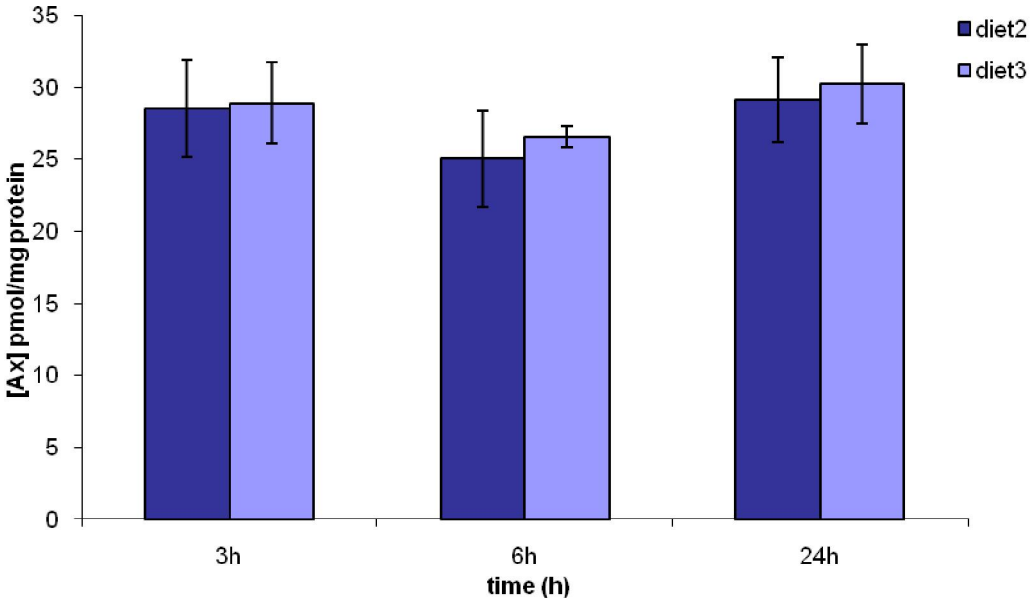

Figure 4-3 Ax concentration in isolated salmon enterocyte at given times after meal (n=4)
(diet 2 contained 40 mg/ kg diet; diet 3 contained 40 mg/kg diet plus 2% cholesterol)

Plasma Ax level

Blood collected from individual fish before sampling the pyloric caeca and the Ax content of the plasma was analyzed. The data are presented in Figure 4-4. Plasma Ax concentration showed a higher level for fish fed diet 3 compared to fish fed diet 2. The plasma Ax at 3h after feeding was slightly higher than other hours for fish fed diet 3. The plasma Ax level after 3h of fish fed diets 2 and 3 were 2.78±0.42 and 3.69±0.15 mg/L, respectively. The concentration of Ax in plasma of fish fed diets 2 and 3 were 2.82±0.65 and 3.21±0.26 mg/L, respectively at 6h versus 2.58±0.74 mg/L and 2.68±0.32 mg/L, respectively at 24h after last meal. The paired comparison data were made within the same sampling hour after fish were fed both diets. Although fish plasma Ax concentration presented higher levels for fish fed diet 3 than those fed diet 2, these differences were not significant ($p>0.05$).

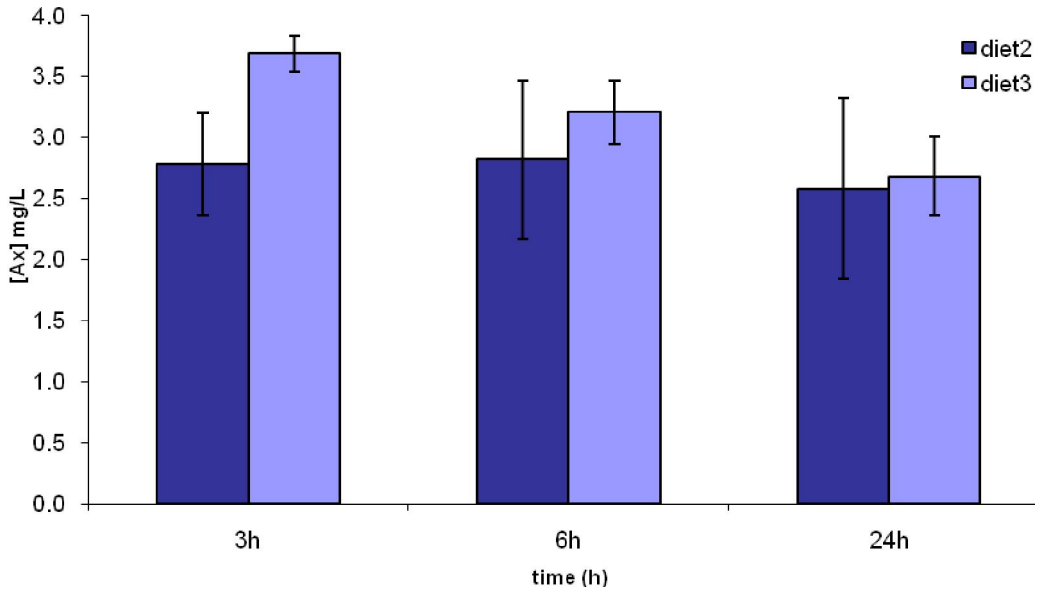

Figure 4-4 Ax concentration in salmon plasma at given times after last meal (n=4)
(diet 2 contained 40 mg/ kg diet; diet 3 contained 40 mg/kg diet plus 2% cholesterol)

The possible correlations between cellular and plasma Ax concentrations were analyzed. The relationship between Ax in cell versus plasma of fish fed diet 2 is shown in Figure 4-5 and Figure 4-6 for diet 3. Neither fish fed diet 2 nor diet 3 were seen relative level of Ax in cell and plasma. The R^2 value was fairly low, 6.4% and 3.5% for diets 2 and 3, respectively. In addition, the data of Ax level in the cell and plasma from fish fed both diets were pooled and analyzed for correlation (Figure 4-7). No interaction was observed (R^2=0.6%). It was concluded that cellular Ax uptake and Ax in plasma was independent.

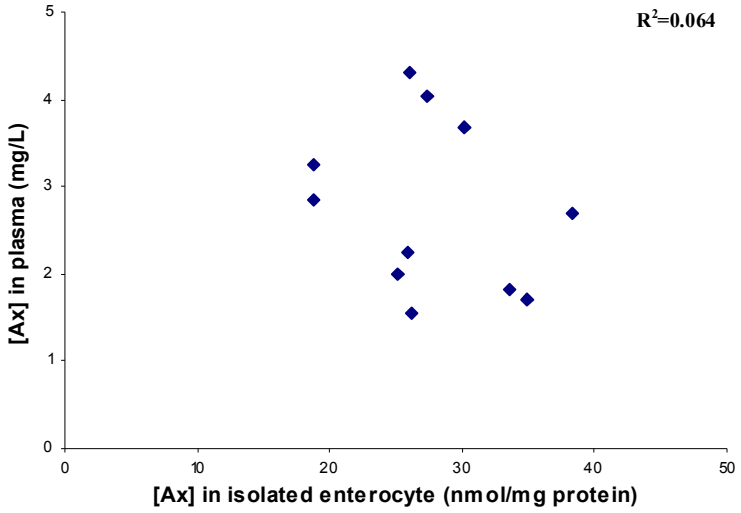

Figure 4-5 Relationship between Ax concentration in plasma and isolated enterocyte of fish fed diet 2 (n=11)

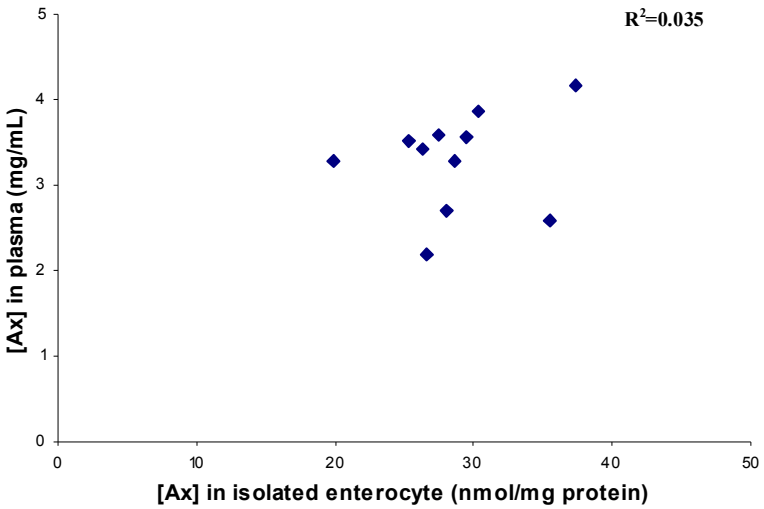

Figure 4-6 Relationship between Ax concentration in plasma and isolated enterocyte of fish fed diet 3 (n=11)

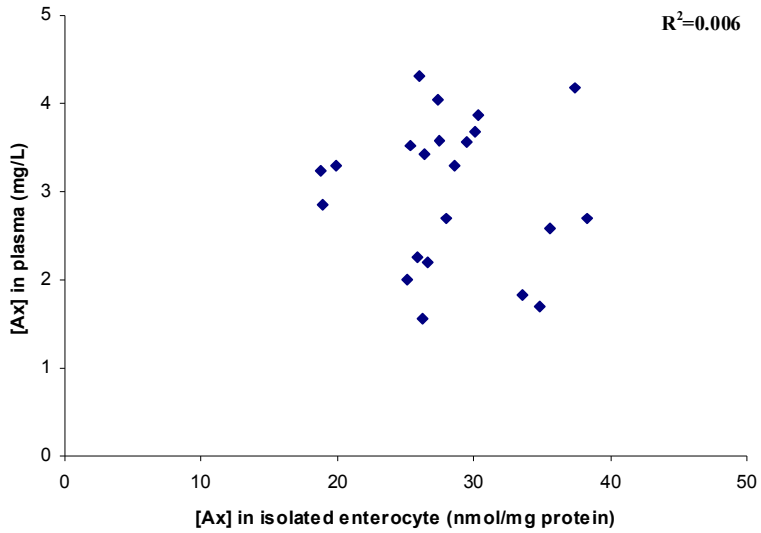

Figure 4-7 Relationship between Ax concentration in plasma and isolated enterocyte of fish fed diet 2 and 3 (n=22)

The activity of alkaline phosphatase from isolated salmon enterocytes in this study was measured and found to be 37.2±7.1 unit per mg protein (n=6). Alkaline phosphatase enzyme is expressed by active and mature mucosal enterocytes and is indicative of enterocyte functional activity (Harpaz and Uni, 1999). Harpaz and Uni (1999) measured the activity of alkaline phosphatase enzyme in various intestinal sections of silver carp, tilapia and bass. They showed that the foregut of tilapia and bass had the highest levels but in silver carp the midgut region was highest. The highest activity found in the foregut of tilapia was 30 units per mg protein. Activity of alkaline phosphatase enzyme in the pyloric caeca of bass and tilapia was 20 and 5 units per mg protein, respectively. Our results agree with those of Harpaz and Uni (1999) in terms of the activity of this enzyme.

Discussion

Astaxanthin uptake by enterocytes

The *in vitro* model developed in this study using Atlantic salmon was successful in showing cellular uptake of Ax from culture media by detecting cellular Ax after incubation. White *et al*. (2003) using an everted pyloric caeca of rainbow trout detected Ax in tissue after exposure to 5mg/L Ax supplemented media for 1h at 20°C. This current study detected Ax in salmon enterocytes after 30 min of incubation. The dose-response of Ax uptake by enterocytes increased with increasing incubation time and reached the highest concentration at 120 min incubation.

The uptake of carotenoid by enterocytes is considered to occur by simple diffusion. Mechanisms of absorption by the intestinal cells are not completely understood. In humans, carotenoids are incorporated into mixed micelles in the intestinal lumen and are readily absorbed by the enterocytes. The mixed micelles are formed by the action of bile salts, phospholipids, dietary lipid and their hydrolysis products (Yonekura and Nagao, 2007). In this *in vitro* fish enterocyte study a micellar nature was produced in the cell culture media by adding 0.5mM of sodium taurocholate, 45mM glycerol and 1.6mM oleic acid following the Caco-2 cell model used by O'Sullivan *et al*. (2007). Yonekura and Nagao (2007) described the structure of micelle having a disk-like shape with an approximate diameter of 4-60nm, consisting of an outer shell of bile salts surrounding a core formed by hydrophilic lipids. The carotenoids are assimilated into the micellar core and remain there until their uptake by enterocytes. The steps involved in carotenoid transfer from mixed micelles to the enterocytes are not clear.

Yonekura and Nagao (2007) reviewed mechanisms of intestinal absorption of dietary carotenoids. They concluded from the published literature on perfused rat intestines that carotenoid absorption by intestinal cells is a simple diffusion or passive process. Recent research has used human enterocytes, Caco-2 cells to evaluate intestinal carotenoid uptake (Garrett *et al.*, 1999; Garrett *et al.*, 2000; Gracía-Casal *et al.*, 2000; During *et al.*, 2002; Liu *et al.*, 2004; Chitchumroonchokchai *et al.*, 2004; During and Harrison, 2005; O'Sullivan *et al.*, 2007; During and Harrison, 2007). They found that absorption mechanism involves not only passive diffusion

but also that a receptor protein in the cell membrane is involved in the cellular carotenoid uptake. The literature reported the involvement of the scavenger receptor class B type I (SR-BI) in the transport of ß-carotene (During and Harrison, 2005; During and Harrison, 2007), lutein (Reboul *et al*., 2005) and lycopene (Moussa *et al*., 2008) in the apical membrane of human enterocytes. Rhainds and Brissette (2004) reported that human SR-BI is a 509 amino acid, 82kDa glycoprotein. Current literature suggests that SR-BI may be involved at the duodenal and jejunal apical membranes for cholesterol uptake (Hui *et al*., 2008). Kleveland *et al*. (2006) confirmed the characterization of SR-BI in Atlantic salmon from the head kidney cell line, salmon TO cells. It is a 494 amino acid, 80kDa glycoprotein. They also found SR-BI gene expression in a selection of salmon tissue to be at a high level in the midgut and lowest in the hindgut. However, there seems to be no specific study related to the function of SR-BI in the intestinal uptake of nutrient in salmon.

Fish intestinal nutrient uptake studies have been mainly directed to *in vitro* techniques in tissue (White *et al*., 2003) and isolated epithelial intestinal cells (Flik *et al*., 1990; Vilella *et al*., 1995; Soengas and Moon, 1998; Larsson *et al*., 1998) incubated with nutrient supplemented media. Some studies have been carried out with labeled substrates to study fatty acid metabolism (Tocher *et al*., 2002; Tocher *et al*., 2004; Bogevik *et al*., 2008). White *et al.* (2003) investigated i*n vitro* intestinal carotenoid uptake using an everted intestine from rainbow trout and Atlantic salmon exposed to micelle solubilised carotenoid for 1h before the extraction and measurement of the carotenoid content in tissue. They found no significant difference in Ax concentration between rainbow trout and Atlantic salmon mid or hind intestine exposed to micelle Ax. The Ax concentration in trout pyloric caeca and mid intestine was higher compared to the hind intestine. This finding is in agreement with that of Al-Khalifa and Simpson (1988). They found that the uptake capacity of the duodenum (pyloric caeca) was higher than the ileum in rainbow trout. This finding clearly shows absorption of carotenoids occurs mainly along the proximal intestine of salmonids.

In vitro model was conducted by isolating enterocytes from the pyloric region of salmon and then incubating the freshly isolated enterocytes with two different concentrations of Ax with or without a cholesterol supplement in cell culture media. There was no clear evidence of the affect of cholesterol supplementation to Ax uptake using the *in vitro* model.

A novel approach was taken in this investigation, where an *in vivo* study was developed. The main aim of this *in vivo* study was to determine the effects of supplemented dietary cholesterol on Ax in enterocytes. Thus, the cellular Ax level was determined in isolated enterocytes from pyloric caeca of fish fed diet containing Ax with or without a supplement of 2% cholesterol. Slightly higher cellular Ax content of enterocytes in cholesterol supplemented media was found, however, no statistically differences ($p>0.05$) were observed between treatments.

There are few reports about the role of cholesterol in carotenoid metabolism using a cell model. Socaciu *et al*. (2000) studied competitive carotenoid and cholesterol incorporation into the lipid bilayer. Their models used liposomes made from single or mixed phospholipids. Liposomes were incorporated with the dietary carotenoids (ß-carotene, lutein and zeaxanthin) or with cholesterol. When carotenoids were incorporated into liposomes together with cholesterol, carotenoid incorporation was reduced. Cholesterol is favored for incorporation due to its easy-to-fit structure compared to the large carotenoid molecules.

Carotenoids are known to be associated with plasma lipoproteins and in salmonids, Ax and Cx are reported to be transported by lipoproteins (Ando *et al*., 1985; Ando and Hatano, 1988; Choubert *et al*., 1992, 1994). Chimsung *et al*. (2012) found a positive effect of dietary cholesterol in increasing salmon plasma Ax. Significantly increased Ax levels were found in plasma when fish were fed pigmented diet containing 2% cholesterol. Cholesterol appears to have a role in forming part of the lipoprotein envelope and promoting the transport of astaxanthin by increasing lipoprotein formation (Kumar *et al*., 1992). Turley (1999) reported that an increase in dietary cholesterol can also affect the cellular uptake and recycling of lipoproteins in the liver through the down-regulation of hepatic LDL-receptor activity. However, both the *in vitro* or *in vivo* studies, in the present investigation there was no clear evidence of a positive effect of cholesterol on cellular Ax uptake. Unfortunately, this may partly be due to a high variation in enterocyte Ax uptake data associated with individual fish variation. Development a fish intestinal cell line would provide a much better tool to study intestinal nutrient uptake.

Plasma carotenoid concentration can serve as a good indicator of dietary carotenoid availability to salmon. It can provide a rapid screening test to identify new carotenoid sources and optimal applications in the feed to be tested in more detailed pigmentation studies (Storebakken

and Goswami, 1996). In addition, an important criterion for screening of Ax availability is that the results express the efficiency of carotenoid transfer from the feed to the flesh. The use of plasma Ax content to predict availability fulfills this criterion. The study in Atlantic salmon showed a high correlation between carotenoid levels in the plasma and flesh (R=0.95).

Several studies have reported a relationship between dietary Ax level and blood carotenoid concentration. Choubert *et al.* (1994) found a high correlation between dietary Ax concentration and serum carotenoid levels in rainbow trout (R=0.99). A linear relationship (R=0.90) between plasma Ax and dietary Ax was also found in Atlantic salmon (Storebakken and Goswami, 1996). While Kiessling *et al.* (2003) found a similar linear relationship (R^2=0.97) between dietary levels and blood concentration for both Ax and Cx.

Conclusion

Both *in vitro* and *in vivo* studies have consistently demonstrated that there was no significant effect of cholesterol on Ax uptake by salmon enterocytes. The plasma Ax concentration did not show a correlation with the level of Ax uptake by enterocytes. Future research using these models would greatly benefit from the development of a fish intestinal cell line. If fish to fish differences could be eliminated, further refinement and control of parameters would help clarify the effect of cholesterol in Ax uptake using the fish enterocyte model.

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Chapter 5

The transport of astaxanthin in Atlantic salmon *(Salmo salar)* plasma

Introduction

Astaxanthin (Ax; 3,3′-dihydroxy-β,β-carotene-4,4′ dione) and canthaxanthin (Cx; β,β-carotene-4,4′ dione) are the two major carotenoids widely used in Atlantic salmon diets to achieve the distinctive red color of the flesh. The retention of dietary Ax in the flesh of salmonid fishes is approximately 10 to 15 % (Torrissen *et al*., 1998). Its retention depends on intestinal absorption, transport and metabolism in fish (Christiansen *et al*., 1993; Aas *et al*., 1999), however, the biochemical mechanisms involved in these processes are not clear and have been subject to limited investigations. Low absorption from the gastrointestinal tract, high excretion in feces (30-70 %) and catabolism in liver are considered major factors for low retention of Ax and Cx in salmonid flesh. Lipid soluble Ax is primarily bound to the protein in muscle tissue (Henmi *et al*., 1989; Saha *et al*., 2005). The primary binding protein of Ax in the muscle of Atlantic salmon is α-actinin (Matthews *et al.,* 2006). It appears that the Ax-binding capacity of the muscle proteins do not limit the deposition of Ax in the muscle; however, a high metabolic transformation rate may affect the retention of astaxanthin. Species differences in pigmentation retention also exist between Atlantic salmon, rainbow trout, coho salmon and Arctic char. Astaxanthin appears to be better utilized in trout than Cx, while the reverse has been observed in Atlantic salmon.

Among the dietary factors, lipid levels (Torrissen *et al*., 1998), cholesterol levels (Buttle, 2000; Chimsung *et al*., 2012) and high levels of polyunsaturated fatty acids in certain fish oils (Bjerkeng *et al*., 1999) increase the absorption of carotenoids. When Ax was administered intraperitoneally, a high plasma concentration of Ax was observed in Atlantic salmon and rainbow trout (Maltby, 2003; Ytrestøyl and Bjerkeng, 2007a, b). The major steps involved in carotenoid absorption in fish are considered similar to mammals and involvee the following steps: 1) disruption of food the matrix and molecular linkages; 2) uptake in lipid droplets; 3) formation and uptake in micelles; 4) uptake in enterocytes and; 5) incorporation for transport into chylomicrons (van het Hof *et al*., 2000). Uptake of Ax and Cx from the intestine is slow,

approximately 18-24 h (March *et al*., 1990; Choubert *et al*., 1994). The blood clearance rate of these carotenoids depends on the transport and cellular uptake of carotenoids from blood lipoproteins (Guillou *et al*., 1992). Carotenoids are absorbed in the intestinal mucosa of animals and humans (Wang *et al.,* 1996) before being transported by lipoproteins. They can also be transferred or exchanged between lipoprotein groups in blood circulation (Salvador *et al*., 2009; Tyssandier *et al.,* 2002). These plasma lipoproteins are formed by the noncovalent association of lipids with specific proteins, apolipoproteins or apoproteins (Myant, 1990).

Generally, lipoproteins are classified according to their density as very low density lipoprotein (VLDL, density (d) <1.006 g/mL), low density lipoprotein (LDL, d 1.006-1.063 g/mL), and high density lipoprotein (HDL, d 1.063-1.21 g/mL). LDL is the predominant lipoprotein type in human plasma (Babin, 1987). However, in fish such as rainbow trout lipoprotein distribution depends on the degree of sexual maturity of fish (Fremont and Marion, 1982). In adult rainbow trout, HDL is the major lipoprotein class at 1393 mg/100 mL of serum (Chapman, *et al*., 1978). It increases progressively in the course of sexual maturation reaching 2500 mg/100 mL of plasma during spermiation or ovulation, whereas VLDL and LDL are predominant in juvenile trout (Fremont and Marion, 1982). In Japanese eel (*Anguilla japonica*) VLDL is the main component of lipoprotein in plasma (Ando and Matsuzaki, 1997).

Fish are both hyperlipidemic and hypercholesterolemic in comparison with mammals (Babin and Vernier, 1989). The most abundant lipids in lipoptroteins are triacylglycerols, free cholesterol, cholesterol esters and phospholipids. Our recent work showed that cholesterol supplementation (2% by wt) was more effective for increasing the plasma Ax concentration than these control diet containing herring oil alone (Chimsung *et al*., 2012). Changes in dietary composition can change the density profile and composition of serum or plasma lipoprotein. Lecithin seems to have a selective action on cholesterol transport by serum lipoproteins. It reduces high levels of LDL and at the same time, it increases the level of HDL that eliminate of excess cellular cholesterol (Wójcicki *et al*, 1995). Cholesterol may play a role in increasing synthesis and secretion of lipoproteins in intestine and liver (Vance, 1999). The role of dietary cholesterol in regulating lipoprotein metabolism in humans (Demacker *et al*., 1988; Clifton and Nestel, 1996), monkey (McGill *et al*., 1981; Stucchi *et al.*, 1998), hamster (McAteer *et al*., 2003)

and chicken (Mol *et al*., 1982; Loo *et al.,* 1990) have been carried out, however, such a relationship between lipoproteins and dietary cholesterol levels in fish has not been studied.

The present study was designed to investigate the distribution of astaxanthin among the lipoproteins and albumin-like protein in Atlantic salmon fed diets supplemented with astaxanthin with different levels of cholesterol. The lipoprotein separation was carried out according to the methods used in human lipoprotein investigations.

Materials and Methods

Fish, rearing conditions and experimental diets

Atlantic salmon (*Salmo salar*) with an average weight of 1 kg were randomly distributed to 6, 1000L fiber-glass tanks (25 fish/tank). Fish were maintained in these flow-through (12 L/min) tanks with filtered seawater (30 µm; salinity, 28-30 ppt) and a light regime of 12 h light:12 h dark. Water quality was monitored daily with water temperature maintained at 12°C and dissolved oxygen levels at 90-95% saturation. Each diet was hand-fed to apparent satiation to duplicate tanks containing 25 fish per tank twice daily (0900 h and 1630 h) on weekdays and once daily (1000 h) on the weekend. Prior to starting the experiment, fish were held on a commercial non-pigmented diet (6.5 mm Shur-Gain Feeds, Truro, NS).

Three isonitrogenous and isoenergetic diets were formulated based upon the published digestible energy values of feed ingredients for salmonids and all the essential nutrients supplied according to the recommendation of NRC (1993). The three experimental diets consisted of a control diet (Diet 1; no Ax supplement), diet 2 contained 40 mg/kg Ax and diet 3 40 mg/kg Ax plus 2% cholesterol supplement. Vitamins and minerals were pre-mixed with ground wheat as a filler using a twin-shell blender (Paterson-Kelly Co., East Stroudsburg, PA, USA) prior to adding them to the main ingredient mixture (Table 5-1). All diets were homogenized using a Hobart mixer (Model H600T, Rapids Machinery Co., Iowa, USA) and steam-pelleted into 6 mm pellets using a laboratory pellet mill (California Pellet Mills, San Francisco, USA). They were then dried in an air-convection drier at 80°C for 1 h, screened and stored in a freezer at –20°C until needed.

Fish sampling

After 12 weeks of feeding experimental diets, a blood collection was made carefully controlling the time between last feed and collection to be 24 h. All work was carried out under yellow light. Five fish from each tank were randomly selected and anaesthetized with 40 mg/L of MS222. Individual fish weights were recorded and blood (about 7 mL/fish) was taken from the caudal vein by syringe, transferred to a heparinized tube and held on crushed ice until centrifugation (IEC Centra CL3R; 4,000 rpm for 10 min at 4°C). Plasma for lipoprotein fractionation was held at 4°C and processed within 24 h of collection (Nanton *et al*., 2006) whereas those for Ax determination were immediately frozen on dry ice and stored at -80°C until further analyses.

Table 5-1 Composition of experimental diets

Ingredient	g/kg
Herring meal[a]	450
CPSP-G[b]	40
Soybean meal[c]	100
Wheat middlings[d]	83
Dried whey[e]	70
Gelatin[f]	20
Pre-gelatinized starch[g]	40
Vitamin premix[h]	17
Mineral premix[i]	10
Herring oil[j]	170

[a]Corey Feed Mills Ltd., Fredericton NB, Canada
[b]Concentre proteique soluble de poisson (soluble fish protein concentrate) (Sopropêche, France)
[c]Bunge Canada, Oakville, ON, Canada
[d]Dover Mills, Halifax, NS, Canada
[e]Farmers Co-operative Dairy Ltd., Truro, NS, Canada.
[f]US Biochemical, Cleveland, OH, USA.
[g]National Starch and Chemical Co., Bridgewater, NJ, USA.
[h]Vitamin premix (per kilogram diet); vitamin A (retinyl acetate), 6,000 IU; vitamin D3 (cholecalciferol), 3,000 IU vitamin E (dl-alpha tocopheryl acetate), 300 IU; vitamin K3, (menadione sodium bisulfite complex) 30 mg; thiamin (thiamin mononitrate), 40 mg; riboflavin, 50 mg; pantothenate (d-calcium pantothenate), 150 mg; biotin (d-biotin), 1mg; folic acid, 15 mg; vitamin B12, 0.1 mg; niacin, 200 mg; pyridoxine (pyridoxine HCl), 20 mg; ascorbic acid (ascorbic acid mono phosphate, stay C), 200 mg; inositol, 100 mg; BHT, 15 mg; 20 g choline chloride added directly to the main ingredient mixture.
[i]Mineral premix (per kilogram diet); manganous sulfate (32.5%Mn), 40 mg; ferrous sulfate (20.1% Fe), 30 mg; copper sulfate (25.4% Cu), 5 mg; zinc sulfate (22.7% Zn), 75 mg; cobalt chloride (24.8% Co), 2.5mg; sodium selenite (45.6% Se), 1 mg; sodium fluoride (45.2% F), 4 mg.
[j]stabilized with 0.06% ethoxyquin, Comeau Seafood, Saulnierville, NS, Canada.

Plasma lipoprotein fractionation

Plasma lipoproteins were obtained using a sucrose density gradient ultracentrifugation following the procedure of Boren *et al*. (1994) with modifications of McLeod *et al*. (1996). Briefly 1µL of 0.5M EDTA, 1µL of 15% (w/v) phenylmethylsulfonylfluoride (PMSF) in DMSO and 4µM BHT were added to 5mL of fish plasma. The density was adjusted to 12.5% by adding sucrose. Dulbecco's phosphate buffer saline (PBS; Sigma, D8537) was placed in the bottom of an open polycarbonate tube (Beckman Coulter, Thinwall, Ultra-Clear™) before gently underlayering it with the sample (prepared as described above), followed by 25% and 47% sucrose solutions containing 0.1% sodium azide (see Figure 5-1). The samples were centrifuged for 64 h at 15°C and 43,000 rpm using a Beckman Counter TM Optima TM LE-80K equipped with a Beckman 70.1Ti using slow acceleration and no deceleration.

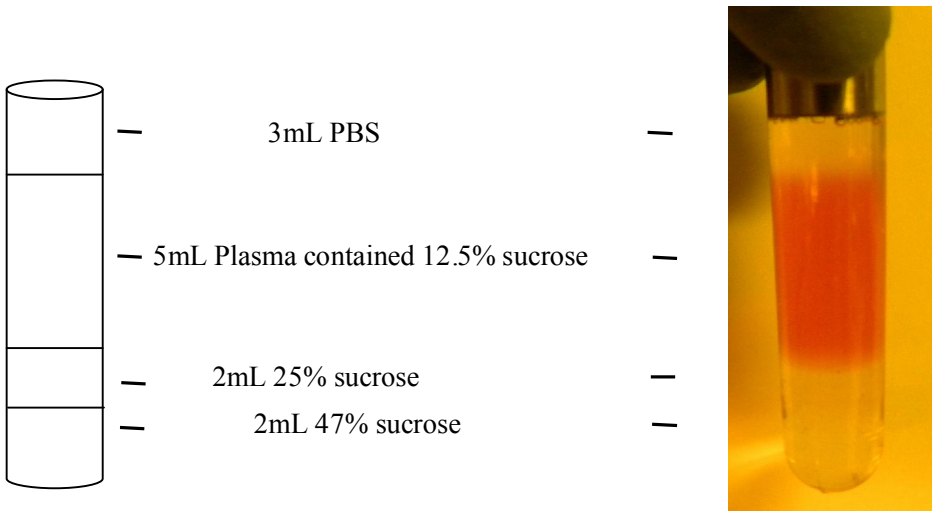

Figure 5-1 The solution profile of ultracentrifuge tube before density gradient ultracentrifugation

After centrifugation, 13 fractions were carefully collected from the top of each tube and stored at -80°C until further analyses. In addition, the density of each fraction obtained after centrifugation using these sucrose solutions was measured with a portable density meter (Model DMA 35, Anton Parr, Graz, Austria).

Separation of apolipoproteins by polyacrylaminde gel electrophoresis

Apolipoproteins within each lipoprotein fraction of salmon plasma were separated and characterized by molecular weight using sodium dodecyl sulphate polyacrylaminde gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Linear gradients of 4-20% precast gels (Bio-Rad, Mini-PROTEAN®TGX™) were used for the separation. Standard proteins (Bio-Rad, Precision Plus™ protein standards) of known MW were run simultaneously. The protein bands were stained with 0.1% Coomassie Brilliant Blue solution (Bio-Rad, Coomassie Brilliant Blue R250 staining solution #161-0436). Gels were scanned in a GS-800 Calibrated Densitometer (Bio-Rad). The approximate MW of each electrophoretic band was determined by comparison with the SDS-PAGE protein standards.

Analytical methods

Proximate composition of the experimental diets were completed as follows: moisture content after drying in a mechanical convection oven Precision Model STM 80) at 105°C for 24 h, ash was determined after incineration at 550°C for 18 h, crude protein (% nitrogen × 6.25) was measured using a Leco nitrogen determinator (Model FP-528, Leco Corporation, St. Joseph, MI, USA) and lipid using a modified Folch method (Folch *et al.*, 1957). Dietary cholesterol content was analyzed following Kovacs *et al*. (1979). Plasma and lipoprotein cholesterol concentrations were measured enzymatically with a commercial kit (Calbiochem, #428901). Protein content in each sample fractions was measured using a bicinchoninic acid assay kit (Sigma, BCA1).

Extraction of carotenoids from plasma was performed according to Kiessling *et al*. (2003). Ax content in lipoprotein fractions was determined using the method described by Chavez *et al*. (1998) and Salvador *et al*. (2009). Experimental diets were finely ground and pre-treated with a trypsin and pepsin digestion by sonication in an ultrasonic bath at 50°C for 45 min. The lipid containing astaxanthin was extracted following the Bligh and Dyer method (1959). Astaxanthin was analysed by normal phase HPLC.

The HPLC system consisted of an HP 1100 (Hewlett Packard, USA) equipped with an automated sample injector, quaternary pump, temperature-controlled column compartment and

diode array detector. Separation was achieved using a Waters µPorasil column (300 X 3.9 mm, 125 Å, 10 µm; Milford, MA, USA) with a guard column and an isocratic elution of 86% hexane: 14% acetone as the mobile phase. The flow rate was 1.5 mL min^{-1}, column temperature 25°C and typical injection volume 10 µL. Peaks were detected at 476 nm and quantified using an authentic Ax standard (DSM Ltd., Basel, Switzerland) following the procedure of Schierle and Hardi (1994).

Results

Diet composition

Three experimental diets were formulated to supply 0 (control diet, diet 1) and 40 mg astaxanthin per kg of diet (diet 2) and diet 3 with cholesterol supplemented. Astaxanthin analysis of these diets showed that Ax concentrations of diets 2 and 3 were 39.1 ± 2.3 mg/kg and 38.7 ± 3.1 mg/kg, respectively (Table 5-2). As expected, no Ax was detected in diet 1. The cholesterol content of Diets 1, 2 and 3 were 0.31, 0.40 and 2.69 %, respectively. The crude protein and lipid contents of these diets ranged from 42.9 to 43.3% and lipid from 24.2 to 24.5% respectively. The average moisture (10 %) and ash (6.1 %) contents of the three experimental diets were approximately the same (Table 5-2).

Table 5-2 Chemical composition of experimental diets

Diet	Moisture (%)	Ash[a] (%)	Protein[a] (%)	Lipid[a] (%)	Astaxanthin[a] (mg/kg)	Cholesterol[a] (%)
1	10.1 ± 0.1	6.1 ± 0.1	43.3 ± 0.1	24.5 ± 0.2	ND	0.31±0.0
2	10.0 ± 0.1	6.0 ± 0.0	43.2 ± 0.1	24.2 ± 0.2	39.1 ± 2.3	0.40±0.0
3	9.9 ± 0.2	6.1 ± 0.2	42.9 ± 0.2	24.5 ± 0.2	38.7 ± 3.1	2.69±0.0

[a]mean ± SE; n=3

[b]expressed on as fed-basis

ND = not detected

Plasma astaxanthin and cholesterol concentration

The average plasma Ax concentration ranged from 0.19 to 6.09 mg/L (Table 5-3). Fish fed Ax and cholesterol supplemented diet (diet 3) had the highest level of plasma Ax concentration ($p<0.05$) as compared to fish fed diets 1 and 2. In fish fed diet 1, negligible amount of Ax was detected in their plasma. Fish fed diet 2, Ax supplemented diet had the same concentration of plasma cholesterol with fish fed diet 1, control diet ($p>0.05$). Fish fed diet 3, which Ax and cholesterol supplementation diet significantly ($p<0.05$) increase the level of plasma cholesterol compare to those 2 groups of fish. The level of cholesterol in plasma of fish fed diet 3 was over 2 times of fish fed diet 1 and 2 (Table 5-3).

Table 5-3 Astaxanthin and cholesterol content of fish plasma

Diet	[Ax] (mg/L) [1, 2]	[Cholesterol] (mg/L) [1, 2]
Diet 1 Control (no Ax)	0.19 ± 0.03^{a}	354.7 ± 33.60^{a}
Diet 2 Ax 40 mg/kg	4.33 ± 0.51^{b}	411.9 ± 97.00^{a}
Diet 3 Ax 40 mg/kg + 2% cholesterol	6.09 ± 0.64^{c}	1026 ± 27.89^{b}

[1] mean ± SE; n=3 replicates

[2] Means within each column not sharing a common superscript are significantly different ($p<0.05$) significance level at $p<0.05$

Lipoprotein classification

Plasma samples from three groups of fish were fractionated under the sucrose density gradient conditions of McLeod *et al*. (1996) for 64h. The gradient was able to generate the orange color band of fish plasma, especially easily noticed in the plasma of fish fed diet 2 and 3. Fractionated plasma sample were collected to13 fractions, approximate 1 ml each from the top of each tube. The density of each fraction was shown in Figure 5-2. The density increased from the top to the bottom of the tube from 1.031 to 1.157 g/mL.

The density of each fractions from sucrose gradient fractionation were incomparable with fractionated fish plasma with salt density gradient technique from the previous study in fish (Chapman *et al*., 1978; Choubert *et al*., 1992; Choubert *et al*., 1994; Chavez *et al*., 1998). Edelstein *et al*. (1984) did compare the role of salts and sucrose density gradient ultracentrifugation in the fractionation of human serum lipoprotein. They mentioned that the sucrose gradient has the benefit that contamination by albumin is eliminated with no scattering effect.

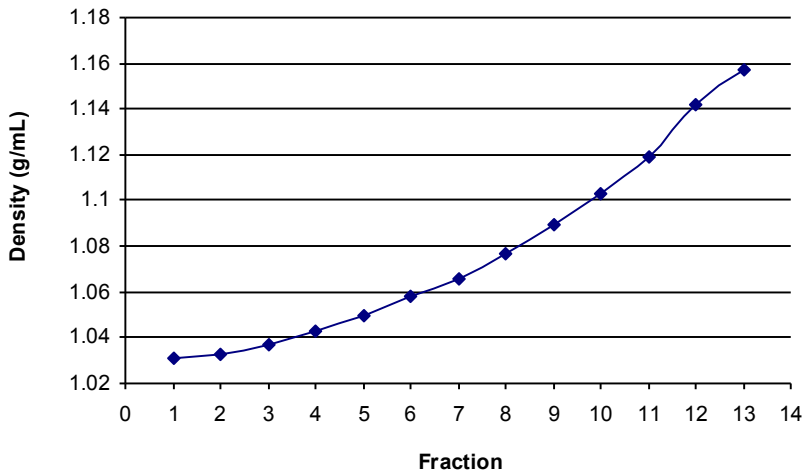

Figure 5- 2 The profile of density in each lipoprotein fraction (range 1.031-1.157 g/mL) VLDL (1.031-1.043 g/mL), LDL (1.043-1.050 g/mL), HDL (1.050-1.103 g/mL), protein rich (1.103-1.157 g/mL) measured with portable densitometer, DMA35 (Anton Paar)

The fish apolipoprotein components of VLDL, LDL and HDL in each sample fraction separated using SDS-PAGE method (Laemmli, 1970) are shown in Figure 5-3. Separated protein in each lane provided the desired concentration of protein for staining with Coomassie Brilliant Blue. Lanes labeled marker shown a broad range of apparent molecular weights (Bio-Rad, Precision plus protein^{TM} standards) 10 to 250 kDa. The protein band from each lane of sample were compared with a series of protein marker.

Salmon plasma lipoprotein classes were classified by matching the molecular weight of apolipoprotein in plasma lipoprotein of rainbow trout (Babin, 1987). The results presented that apoproteins of VLDL contained protein of approximately 260, 240, 76, 25 and 13 kDa. Apolipoproteins of LDL contained a band of approximately 240 and 76 kDa and apolipoprotein of HDL observed with approximately molecular weights of 55, 40, 25 and 13 kDa.

The present results (Figure 5-3) were identified that fraction 1-4 were VLDL, contained a band of 260 and 240 kDa. Fraction 5 was LDL can be observed with molecular weight of 240 and 76 Kda. Fractions 8-10 were identification to HDL with the largest and darkest staining component corresponded to a molecular weight of about 25 kDa. The cross contamination between LDL and HDL was found in fractions 6 and 7. Fractions 11-13 were a protein rich fraction. They were a viscosity fraction containing several protein bands and darkness related to the high concentration of protein.

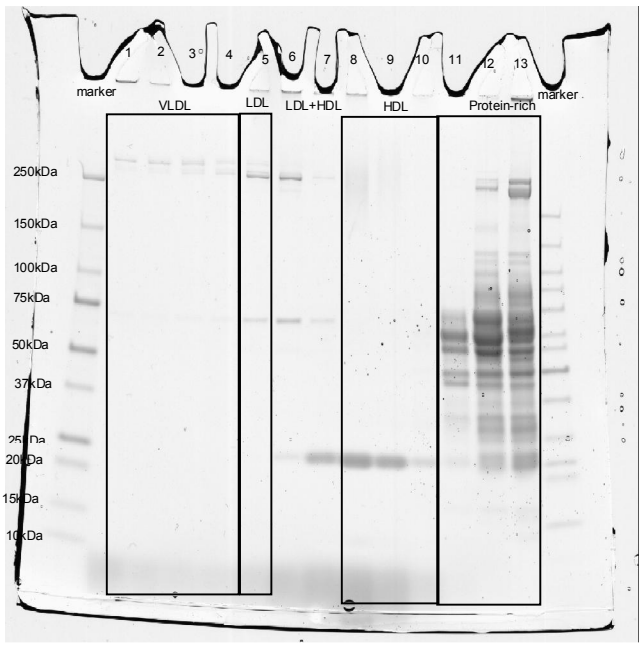

Figure 5-3 Electrophoretogram of Atlantic salmon lipoprotein fractionated by sucrose gradient and separated by SDS-PAGE on a 4-20% linear gradient gel (Laemmli, 1970). Lanes labeled: Bio-Rad molecular weight standard, Lane 1-13; lipoprotein fraction 1-13

Ax lipoprotein

The results of Ax concentration in each fractionated fish plasma in fish fed different diets are shown in Figure 5-4. Lipoprotein fraction F1 to F4 (VLDL class) and F5 (LDL class) in fish fed diet 3 had higher Ax content ($p<0.05$) than fish fed diets 1 or 2, whereas Ax content in other factions were not significantly different ($p>0.05$).

The summary of Ax distribution in each lipoprotein class was presented in Table 5-4. The data were used to calculate the percentage of Ax binding in each lipoprotein class. The highest percentage Ax binding occurred in protein rich (PR) fraction in all 3 groups of fish. Fish fed diets 1 and 2 had similar pattern of Ax distribution in each lipoprotein class as PR>HDL>VLDL>LDL. Fish fed diet 3 had Ax content in PR>VLDL>HDL>LDL.

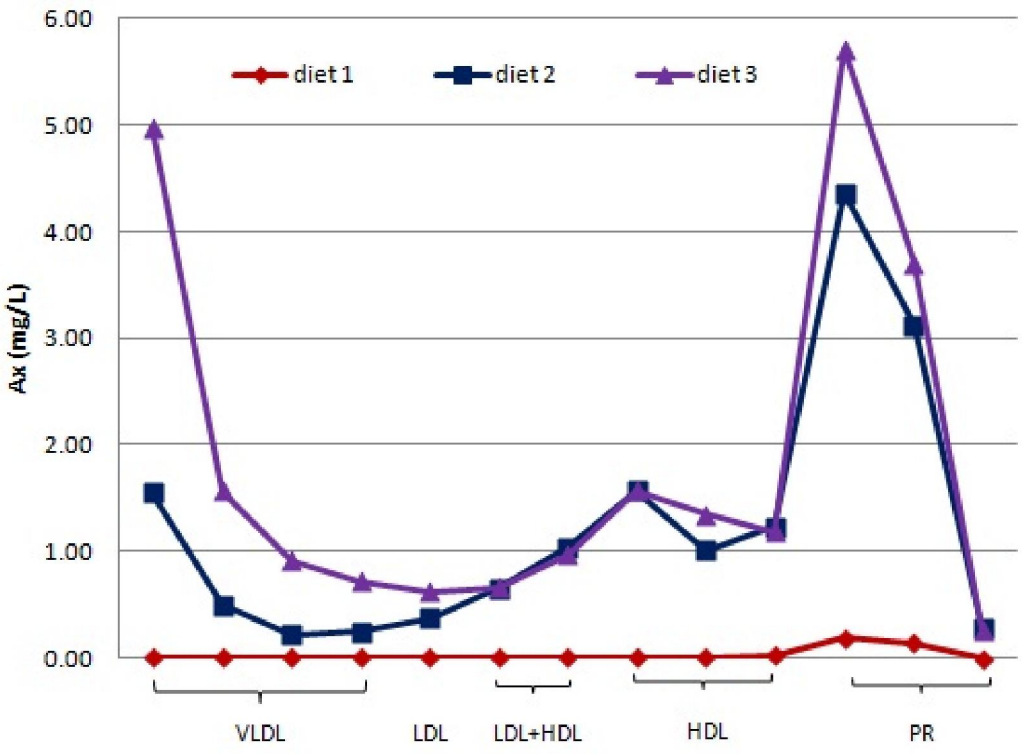

Figure 5-4 Ax concentration in plasma lipoprotein fraction in fish fed different diet (diet 1, control diet (no Ax or cholesterol supplement); diet 2, 40 mg Ax/kg diet; diet 3, 40 mg Ax/kg diet supplemented with 2% cholesterol)

Table 5-4 Distribution of Ax (%) in each lipoprotein class

Treatment	VLDL	LDL	HDL	Protein–rich (PR)	Comparison of Ax
Diet 3	33.8	2.56	16.94	39.96	PR>VLDL>HDL>LDL
Diet 2	15.6	2.29	23.59	48.05	PR>HDL>VLDL>LDL
Diet 1	7.14	1.79	17.86	69.64	PR>HDL>VLDL>LDL

Lipoprotein cholesterol concentration

The cholesterol content in fish lipoprotein class was determined using a commercial test kit (Calbiochem). The results of cholesterol concentration in each fractionated fish plasma in fish fed different diets are shown in Figure 5-5. Lipoprotein VLDL class (F1 to F4) of fish fed diet 3 contained higher cholesterol content ($p<0.05$) than those of fed diets 1 or 2. Otherwise, there were no significantly difference ($p>0.05$) in cholesterol content found in other lipoprotein class among 3 groups of fish. The protein-rich (PR) fraction contained low cholesterol concentration.

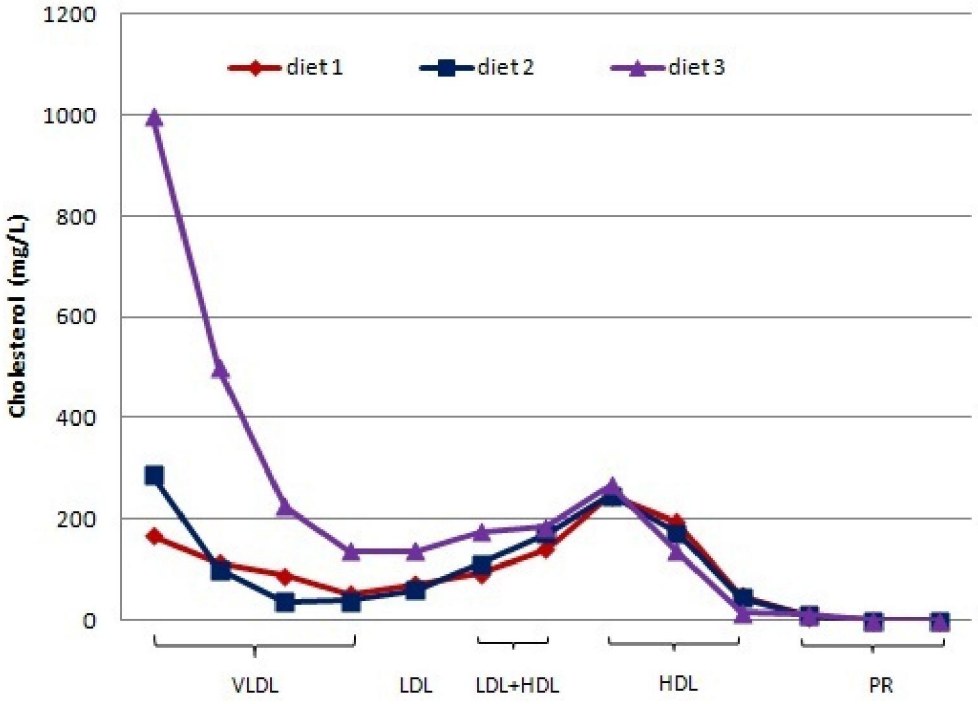

Figure 5- 5 Cholesterol concentration in plasma lipoprotein fraction in fish fed different diet (diet 1, control diet (no Ax or cholesterol supplement); diet 2, 40 mg Ax/kg diet; diet 3, 40 mg Ax/kg diet supplemented with 2% cholesterol)

The cholesterol content in each lipoprotein class was used to calculate the percentage (Table 5-5). Fish fed diet 3 had the highest percentage of cholesterol in VLDL followed by HDL, LDL and PR. Fish fed diets 1 and 2 had similar pattern of cholesterol distribution. The cholesterol levels of VLDL and HDL in fish fed diet without cholesterol supplementation remained the same. In the group of fish fed cholesterol supplemented diet, VLDL contained highest cholesterol content compared to other lipoprotein class.

Table 5-5 Distribution of cholesterol (%) in each lipoprotein class

Treatment	VLDL	LDL	HDL	Protein –rich (PR)	Comparison of protein
Diet 3	66.68	4.91	15.09	0.37	VLDL>HDL>LDL>PR
Diet 2	36.12	4.65	36.38	0.65	HDL>VLDL>LDL>PR
Diet 1	34.36	5.86	40.12	0.62	HDL>VLDL>LDL>PR

Protein concentration in each lipoprotein fraction

The total protein concentration in each lipoprotein fraction was determined using a commercial kit from Sigma. The results are presented in Figure 5-6. They were only F1-F2 (VLDL) in fish fed diet 3 with a higher protein content ($p<0.05$) than those of fish fed diets 1 or 2. Otherwise, there were no significant difference ($p>0.05$) in total protein content of other lipoprotein class among 3 groups of fish. All groups of fish had similar pattern of protein distribution in each lipoprotein class. Protein rich lipoprotein (PR) was a majority protein containing lipoprotein class, contained protein over 70%. This value was the highest level followed by HDL, VLDL and lowest concentration of protein found in LDL class (Table 5-6).

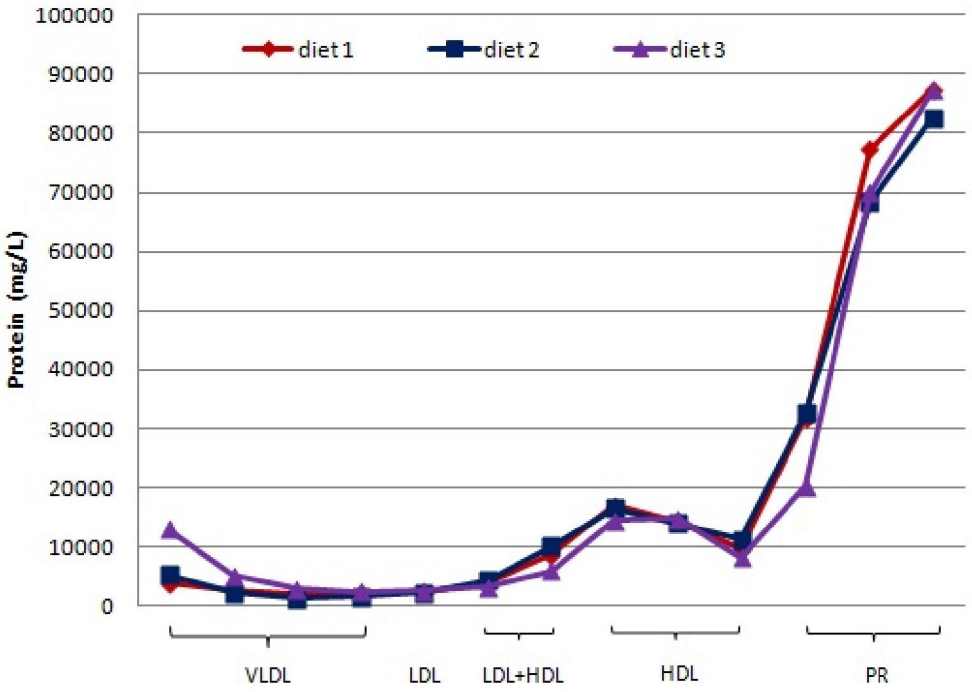

Figure 5-6 Protein content of each lipoprotein fraction
(diet 1, control diet (no Ax or cholesterol supplement); diet 2, 40 mg Ax/kg diet; diet 3, 40 mg Ax/kg diet supplemented with 2% cholesterol)

Table 5-6 Distribution of protein (%) in each lipoprotein class

Treatment	VLDL	LDL	HDL	Protein –rich (PR)	Comparison of protein
Diet 3	9.28	1.03	14.99	71.09	PR>HDL>VLDL>LDL
Diet 2	4.14	0.88	16.53	72.72	PR>HDL>VLDL>LDL
Diet 1	4.04	0.93	15.53	74.77	PR>HDL>VLDL>LDL

Discussion

Plasma astaxanthin and cholesterol content of Atlantic salmon were in a similar pattern to those observed in a previous study (Chimsung *et al*., 2012). Dietary supplementation of experimental diet with 40mg Ax/kg and 2% cholesterol significantly ($p<0.05$) increased the plasma Ax and cholesterol concentration as compared to diets containing either 40 mg Ax/kg or no added Ax. In the diet supplemented with 2% cholesterol, the Ax level in the plasma was 1.5 times that of the average of diets containing only Ax. Even as, the cholesterol level in plasma of fish fed diets containing supplemental cholesterol approximately twice as high as than two other experimental diets which were not supplemented with cholesterol. Atlantic salmon are hypercholesterolemic and their total cholesterol level in plasma fluctuates under different physiological conditions e.g. starvation, sexual maturation (Farrell and Munt, 1983) or dietary composition (Farrell *et al*., 1986; Jordal *et al.*, 2007; Chimsung *et al*., 2012).

In vertebrates, cholesterol serves many metabolic functions including cell membrane formation, growth and differentiation as well as precursors of endogenous bile acid and steroid hormone synthesis (Vlahcevic *et al*. 1994). These metabolic functions of cholesterol are also widely recognized in fish, however, limited research has been conducted on the effects of dietary cholesterol on endgonesous and exogenous cholesterol metabolism on fish tissues. The effect of dietary cholesterol on plasma cholesterol level of Atlantic salmon has been reported by Farrell *et al*. (1986). Fish fed the 3% cholesterol supplement in place of herring oil had significantly ($p<0.05$) higher total plasma cholesterol levels than fish on the herring oil in the control diet. Sealey *et al*. (2001) found a different phenomenon when supplemented the lower level of cholesterol, at 1% by weight in the diet for hybrid striped bass (*Morone chrysops* x *M. saxatilis*). The 1% of dietary cholesterol supplementation decreased concentrations of liver and plasma free fatty acids and liver phospholipids while increasing concentrations of liver triglycerides and plasma phospholipids. However, it did not increase the relative amounts of cholesterol in plasma. They suggested the inability of juvenile hybrid striped bass to utilize dietary sources of this compound. The dietary lipid levels (low; 310g, medium; 380g and high; 470 g of lipid/kg diet) (Hemre and Sandnes, 1999) as well as different sources of dietary lipid (capelin oil, palm oil and sunflower oil) (Torstensen *et al*., 2000) shown no effect on regulation of plasma cholesterol level in Atlantic salmon. However, Jordal *et al.* (2007) found that Atlantic salmon fed 100% fish oil

had a significantly higher amount of total cholesterol in plasma compared with salmon fed 100% vegetable oil blend (55% rapeseed oil, 30% palm oil and 15% linseed oil) when rearing in fresh water.

The absorption process for carotenoids is considered similar to lipids in humans and laboratory animals, however, the mechanisms of carotenoid absorption in fish is not fully understood. In a series of experiments conducted before, it has been shown that astaxanthin absorption within the gastrointestinal tract involves disruption of the food matrix and molecular linkages prior to absorption. The mechanism of Ax uptake in lipid droplet, involves formation and uptake of Ax in micelles and gut enterocytes and incorporation for transport into chylomicrons and other lipoproteins. The biochemical mechanism by which dietary cholesterol level may affect carotenoid absorption in salmon is unknown. In rats, dietary cholesterol induced higher cholesterol 7-alpha hydroxylase activity, an enzyme involved in the conversion of cholesterol to bile acids, and may allow animals to efficiently convert excess dietary cholesterol to bile acids (Horton *et al*., 1995). The inclusion of the bile acid, taurocholate, in the diet of ferret (Lakshman *et al.*, 1996) and rat (Schweigert *et al*., 2002) significantly increased the absorption and tissue accumulation of β-carotene. Olsen *et al.* (2005) found 20% increase of blood astaxanthin levels in Atlantic salmon when fish were fed the diet supplemented with taurocholic acid. The ability of fish, which are hypercholesterolemic (Babin & Vernier, 1989), to regulate cholesterol by increasing bile acid production is unknown.

Atlantic salmon plasma was fractionated using sucrose density gradients according to McLeod *et al*. (1996). This technique avoided contamination by albumin and scattering effects (Edelstein *et al*., 1984). Separation of protein by SDS-PAGE according to their electrophoretic mobility allowed for the isolation of apolipoproteins in each fraction. It also allowed identification of various lipoproteins including VLDL (d 1.031-1.043g/mL), LDL (d 1.043-1.050g/mL), HDL (d 1.050-1.103g/mL) and a protein-rich fraction (d 1.103-1.157g/mL). The application of this technique showed that only apolipoproteins of 25kDa in HDL fraction were present in detectable amounts. The amount of other apolipoproteins in HDL of plasma was too low to be identified by this method. We found 260 and 240 kDa molecular weight protein in the VLDL fraction and 240 and 76 kDa molecular weight protein fraction in the LDL fraction. These findings are in agreement with earlier reports on rainbow trout apolipoprotein (Babin, 1987;

Babin and Vernier, 1989). In the serum of haddock, a white fleshed marine fish, the apolipoprotein components consisted of a large apo B-like protein and AI-like protein in VLDL (Nanton *et al*. , 2006). They observed that apo AI-like protein was a major protein in HDL and it also contained an apo AII-like protein. In the salmonid fish, rainbow trout, HDL contained 25 and 13 kDa apolipoprotein (Babin, 1987; Babin and Vernier, 1989). A study in chum salmon found that the protein composition of the HDL fraction was composed of two subunits with molecular weights of 24 and 12 kDa (Ando and Hatano, 1988).

In this study, the lipoprotein densities of Atlantic salmon plasma ranged from 1.031 to 1.157 g/mL using sucrose gradient, thus these values do not agree with earlier reports on other fish. In previous studies, different techniques were used to isolate lipoproteins from either fish plasma or serum and their density was adjusted with appropriate salt solutions for isolation by ultracentrifugation prior to identification. In addition, the classification of lipoprotein classes was made according to the density intervals applied to human lipoprotein (VLDL, density (d) <1.006 g/mL, LDL, d 1.006-1.063 g/mL and HDL, d 1.063-1.21 g/mL) (Chapman *et al*., 1978; MacFarland *et al*., 1990; Choubert *et al*., 1992; Choubert *et al*., 1994; Chavez *et al*., 1998).

The protein-rich high density fraction had a high concentration of Ax and contained multiple bands of protein. Aas *et al*. (1999) forced fed Atlantic salmon with a single dose of ^{14}C-Ax and reported that the highest radioactivity of radiolabeled Ax was present either in the non-lipoprotein fraction or highest density fraction after separation of plasma by ultracentrifugation. They speculated that albumin-like protein might constitute the major proportion of protein in this fraction. Ando and Hatano (1988) reported incorporation of Ax into vitellogenin protein in mature chum salmon. Additional studies are necessary to identify specific proteins in these protein-rich fractions that bind Ax which involved in transport and metabolism of this carotenoid.

The composition of fish lipoprotein has been reported by several investigators (McKay *et al*., 1985; Fainaru *et al*., 1988; MacFarland *et al*., 1990; Lie *et al*., 1993; Santulli *et al*., 1996; Caballero *et al*., 2006; Nanton *et al*., 2006). In Atlantic salmon each fraction of lipoprotein contained triacylglycerol, cholesterol and protein at different levels. Lie *et al*. (1993) found that HDL contained high levels of protein while, high cholesterol content was present in the LDL and VLDL had a high proportion of triacylglycerol. The proportion and composition of lipids in each

of the lipoprotein fraction may depend on the nutritional status of fish, stage of sexual maturation and other factors (Lie *et al*., 1993). Recently, Caballero *et al*. (2006) reported that lipoprotein lipid content and fatty acid composition of sea bream was affected by incorporation of vegetable oils in their diet. VLDL was more affected by dietary fatty acid, followed by LDL and HDL. No significant effects of the dietary α-tocopherol on the fatty acid composition of the different lipoproteins was observed in Atlantic salmon (Lie *et al*., 1993), however, the dietary fatty acid composition had a major influence on the fatty acid composition of the core neutral lipids of the lipoprotein. MacFarlane *et al*. (1990) found that starvation altered the distribution of lipids in all lipoprotein fractions of sea bass. In VLDL, there was a decrease in the concentration of triglyceride and cholesterol esters, triglycerides and phospholipids also decreased in LDL. The composition of HDL was affected least by the changes in blood phospholipid levels. Quantitatively, the starvation of sea bass had little influence on apolipoproteins isolated from individual lipoproteins.

Astaxanthin was detected in all lipoprotein fractions of Atlantic salmon plasma. For all three experimental diets, the highest concentration of Ax was found in the protein-rich (PR) fraction. It appears that when dietary Ax concentration is low, plasma Ax is not transferred from the PR fraction to VLDL or HDL in salmon. Aas *et al*. (1999) found that 42% and 58 % of the radiolabeled carotenoids were present in lipoprotein and a high density protein fraction and suggested that Ax binding was likely to be associated with albumin. In humans, albumin is the major plasma protein. Its main function is the regulation of the colloidal osmotic pressure of numerous endogenous and exogenous compounds including free fatty acids, hormones, bilirubin and drugs (De Smet *et al*., 1998). Although albumin is present in salmonids, its role in nutrient transport in fish is unclear (Metcalf and Gemmell, 2005). Albumin comprises over 50% of the total serum, has a molecular mass of 70kDa and has the same electrophoretic mobility as human albumin (Maillou and Nimmo, 1993a; Maillou and Nimmo, 1993b). The several bands of protein present in the high density fraction in our study cannot be identified to a specific protein. Separation of the Ax-bound protein present in these fractions by other techniques such as ion-exchange chromatography would allow for further characterization of these proteins distributed in the PR fractions of salmon plasma.

In humans, chylomicrons transport carotenoids from the intestinal mucosa to the blood stream via the lymphatics and then to the liver and VLDL and LDL for transport of carotenoids from the liver to peripheral tissues (Tyssandier *et al*., 2002). In rainbow trout, HDL is a major transporter of carotenoids followed by LDL (Choubert *et al*., 1992; Choubert *et al*., 1994; Tyssandier *et al*., 2002). They observed the lowest concentration of carotenoids in VLDL. However, Aas *et al*. (1999) did not observe the same pattern in Atlantic salmon and found the highest Ax concentration in the high density protein fraction of fish plasma. Our results showed that only fish fed diet supplemented with Ax had a higher proportion of Ax in HDL compared to VLDL and LDL. Fish fed diet supplemented with cholesterol changed the profile of Ax distributed in plasma lipoprotein. There was an increase of Ax in VLDL fraction.

The effect of dietary cholesterol on Ax distribution in lipoprotein classes can be explained by two hypotheses. First, dietary cholesterol increases the amount of VLDL in plasma. A study conducted on chicken has shown that supplementation of 1% cholesterol in diet not only enhance serum cholesterol levels but also induced changes in the density profile and composition of the serum lipoprotein. Separation of serum lipoproteins by density gradient ultracentrifugation showed that in the chickens on diets which contained cholesterol, there was a shift in the lipoprotein density pattern from the LDL fraction to IDL and VLDL fractions. Compositional analyses of the lipoprotein classes showed that the amount of VLDL and IDL was increased and furthermore they contained additional cholesterol (Mol *et al*., 1982).

The second hypothesis suggests that a transfer of carotenoids occurs between the fish lipoproteins class. Little is known about how the distribution of carotenoids among lipoproteins are established or maintained. For the major lipid classes (triglyceride, cholesterol, cholesteryl ester and phospholipid), the distribution among lipoproteins is governed in part by enzymes such as lecithin cholesterol acyltransferase (LCAT) and transfer proteins such as cholesteryl ester transfer protein (CETP) (Romanchik *et al*, 1995). *In vitro* studies conducted by Tyssandier *et al*. (2002) in rainbow trout showed that β-carotene and several xanthophylls (astaxanthin, canthaxanthin, lutein and β-cryptoxanthin) can transfer between lipoprotein. The transfer is bidirectional from HDL to VLDL and *vice versa*. The presence of enzyme inhibitors would affect the transfer of carotenoids between lipoprotein class.

In the present study, changes were observed in the distribution of Ax in fish fed diet containing Ax supplemented with cholesterol. Lower Ax levels were found in the HDL and protein rich fractions compared to fish fed diet with Ax only. It is possible that dietary cholesterol increased the substrate and enhanced the activity of enzymes such as CETP, a hydrophobic plasma glycoprotein that mediates transfer and exchange of neutral and phospholipid between lipoprotein classes and may be involved in carotenoid metabolism as well. Rainbow trout has a considerable high activity of the CETP in plasma with activities more than double that of man (Ha and Barter, 1982).The presence of CETP inhibitor in medium of an *in vitro* rainbow trout study changed the concentration of catotenoids between VLDL and HDL after incubation. The inhibitor reduced the amount of carotenoid recovered from the VLDL fraction and consequently increased that in the HDL fraction (Tyssandier *et al*., 2002). The increased expression of CETP in mice liver and in peripheral tissues in response to increased dietary cholesterol has been reported (Jiang *et al*., 1992). Future work should consider determination of enzyme activity to explain the effect of dietary cholesterol on Ax transportation.

An increase in dietary cholesterol can also affect the cellular uptake and recycling of lipoproteins in the liver through the down-regulation of hepatic LDL-receptor activity (Turley, 1999). Cholesterol and carotenoids have also been observed to compete for incorporation into lipid bilayers. Cholesterol is favoured for incorporation due to its easy-to-fit structure compared to the large carotenoid molecules (Socaciu *et al*., 2000). The close interaction of these lipid-soluble molecules in the gastrointestinal tract, entercocytes, plasma or tissues may also play a role in enhancing astaxanthin absorption.

Conclusion

Dietary cholesterol plays a significant role in the Ax transport process in blood. However, there is no interaction of Ax and cholesterol on intestinal absorption of this carotenoid. The lipoprotein study on Ax transport showed distribution of Ax in each lipoprotein class with the highest levels in HDL followed by VLDL and LDL. The highest concentration of Ax was found in the protein-rich lipoprotein fraction (PR) which plays an important role as a main carrier of Ax in blood for metabolism by other tissues. The cholesterol content of the diet had a significant

effect on Ax concentrations in VLDL, which may explain the effect of higher concentration of Ax retained by increasing dietary cholesterol levels.

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Chapter 6

General discussion

The biochemical mechanisms involved in the absorption and metabolism of Ax are considered important to understand the utilization of carotenoids by salmonid fishes. Limited numbers of studies have been conducted to measure the absorption of Ax and Cx in salmonid fishes and different techniques have been used by investigators to determine the absorption and digestibility values of these carotenoids (reviewed by Schiedt, 1998). It appears that the comparison of the peak carotenoid absorption levels in the blood of salmon fed various diets provides a reasonable indication of carotenoid availability (Kiessling *et al*., 2003; Storebakken and Goswami, 1996). This rapid method that measures peak astaxanthin concentrations in the blood requires fewer resources to estimate the relative carotenoid bioavailability and also shows a high correlation between the Ax concentrations of the diet and flesh (Storebakken and Goswami, 1996). In laboratory animals methodologies used to measure carotenoid absorption include techniques such "balance method" involving their intake and excretion, the total plasma "carotenoid response", stable-isotope dilution methods and the use of cell culture models (Yeum and Russell, 2002). In the present investigation, total plasma carotenoid response was used to screen the dietary constituents and certain supplements widely used in salmonids that may affect Ax absorption. In order to determine whether a bottle neck exists in carotenoid uptake from enterocytes of salmon intestine, a cell culture model was used. Other techniques used for laboratory animals were considered to be complex and costly.

Carotenoid bioavailability and metabolism are influenced by a number of dietary and physiological factors. The absorption process for carotenoids is considered similar to that for other lipids, however, to date some reports have shown that lipid level increased the absorption of Ax and Cx, however the effects of lipid composition (e.g. phospholipid, glycolipids, cholesterol) have not been investigated. van Het Hof *et al*. (2000) suggested that the following major steps may be involved during carotenoid absorption within the mammalian gastrointestinal tract: 1) release of carotenoids from food matrix; 2) solubilization of carotenoids into mixed lipid micelles in the lumen; 3) cellular uptake of carotenoids by intestinal mucosal cells (enterocytes); 4) incorporation of carotenoids into chylomicrons (CM) and; 5) secretion of carotenoids and their

metabolites associated with CM into the lymph. In salmonid diets, the release of Ax from the food matrix may not be an issue because synthetic form of Ax is supplemented in the diet unless algae or yeast are used as carotenoid supplements for flesh pigmentation. When *Hematococcus pluvalis* or *Phaffia* yeast are used as Ax supplements, a processing step is necessary to rupture cells to release Ax because digestive enzyme have limited ability to hydrolyze algal cell and the major proportion of Ax is excreted. In the initial study (Chapter 2), we have considered the luminar and intracellular factors that may affect the process of Ax absorption and transport.

The solubilization of dietary carotenoids in the digestive tract of salmon is considered a limiting factor for their absorption. The lipophilic nature of carotenoids makes dietary lipids necessary for dissolution of Ax or Cx in the intestinal lumen (Torrissen, 2000). Changes in the lipid composition of the diet can significantly influence carotenoid absorption in salmonids (Bjerkeng, 2000). Compounds with aqueous solubility lower than 100 µg/ml often present dissolution problems for absorption. In self-emulsifying drug delivery systems this problem is overcome by the use of oil containing triglycerides and ethoxylated non-ionic surfactants (Pouton, 1997). In the current study all diets contained high amounts of lipid which allowed dissolution of carotenoids in the lumen of fish.

Among various dietary supplements used to determine their effect on Ax absorption, cholesterol supplementation produced a positive response on plasma Ax concentration. Diets containing Ax and cholesterol supplement at a level of 2% of the diet showed a significant increase (1.5 fold) in plasma Ax concentration compared to the control diet which also supplied some cholesterol (2%). The mechanism by which a dietary cholesterol supplementation may increase carotenoid absorption in salmon is not known. In rats, the high basal level of cholesterol 7-alpha hydroxylase induced by dietary cholesterol allowed these animals to efficiently convert excess dietary cholesterol to bile acids (Horton *et al*., 1995). The inclusion of the bile acid, taurocholate, in the diet of ferret (Lakshman *et al*., 1996) and rat (Schweigert *et al*., 2002) significantly increased the absorption and tissue accumulation of ß-carotene, but did not increase blood Ax levels in salmon (Olsen *et al*., 2005). The ability of fish, which are both hyperlipidemic and hypercholesterolemic (Babin and Vernier, 1989), to regulate cholesterol by increasing bile acid production is unknown. Babin and Vernier (1989) also reported that juvenile rainbow trout transport three times more lipid (1940 vs. 685 mg/dL) and cholesterol (303 vs. 106 mg/dL) in

plasma than rats and these values can reach the values of 12 times higher than rats in this species. Cholesterol is also an important determinant of apolipoprotein B synthesis (major structural protein in VLDL and chylomicrons) and may promote the transport of astaxanthin by increasing lipoprotein formation (Kumar *et al*., 1992).

In recent years, significant amounts of vegetable oil and oilseed proteins are incorporated into salmonid feeds as alternate sources of protein and lipid. The vegetable oils such as corn, sunflower, safflower and soybean oils may contain 952, 75, 444 and 221 mg of total phytosterols /100g oil respectively (reviewed by Rozner and Garti, 2006). An experimental diet was supplemented with 2 % phytosterol to insure that the amount of phytosterol was in the range of value supplied by 10-15 % vegetable oils in Atlantic salmon commercial feeds. This diet did not significantly affect the plasma cholesterol or Ax concentrations as compared with diets containing Ax or cholesterol alone or their combinations. The structures of phytosterols are similar to that of cholesterol except that phytosterols contain an additional hydrophobic carbon chain that is attached at the C-24 position. Phytosterol molecules are more hydrophobic than cholesterol and they are poorly soluble both in water and oil phases (Rozner and Garti, 2006). It is likely that the physical properties of phytosterols particularly low solubility when incorporated in a dry powder form, phytosterols had no affect on Ax absorption and plasma Ax level. Some of the more effective forms of phytosterols used in reducing blood cholesterol are the fat-soluble sterol derivatives (Fernandes and Cabral, 2007). The physical properties and composition of diets, factors that affect digestion of lipid in ingredients from plant by-products and micelle formation may also influence the interaction between phytosterols and carotenoid including their absorption in the digestive tract. Additional research is needed to confirm these findings as well as to determine the effects of various vegetable oils and their lipid components on Ax absorption from salmonid feeds.

Lutein or zeaxanthin showed no significant effect on the absorption of Ax in plasma. This is consistent with the findings of Olsen and Baker (2006) who concluded that dietary lutein at levels up to 23 mg/kg of diet did not appear to affect the deposition of Ax in Atlantic salmon. Lutein and zeaxanthin are abundant in various organs and tissues of many fishes and it has been reported that Ax can be transformed to both these xanthophylls in Atlantic salmon (Schiedt, 1998). Their structures are similar to that of Ax. Fish feed producers use feed ingredients that

contain significant amounts of yellow pigment from the carotenoids, lutein and zeaxanthin. It has been reported that they may compete with Ax during intestinal or tissue absorption (Olsen and Baker, 2006). The present investigation focused on the absorption aspects of carotenoid in Atlantic salmon at a specified level of lutein and zeaxanthin. However, additional dose response studies are needed to further investigate their effects on Ax deposition in muscle of salmon.

Unlike studies conducted on humans and chickens, addition of fiber as wheat bran (5%) had no significant effect on the salmon plasma Ax concentration. Fiber refers to indigestible plant matter such as cellulose, hemicellulose, lignin, pentasanes and other complex carbohydrates found in feedstuff. Most practical diets for fish contain 3-6% crude fiber derived from ingredients of plant origin and fish can tolerance up to 8% fiber in their diets, whereas higher concentrations (8-30%) can depress growth (NRC, 1993). Wheat bran contains the water-insoluble fibers hemicellulose and lignin (Riedl *et al*., 1999). The differences in dietary fiber composition as well as the molecular structure and polarity of the carotenoids used in animal experiments may have shown different responses in fish as compared to monogastric animals. Additional research is required to further assess the effects of different types of cellulose, hemicellulises, pectin etc. on Ax absorption in salmon as well as the influence of higher levels of fiber from commonly used feed ingredients in salmonid feeds.

Diets containing a high level of vitamin E did not affect the Ax concentration in the plasma of fish fed this experimental diet. These results are in agreement with those of Torrissen (1985) who fed low dietary Ax concentrations (3-12mg/kg) and Sigurgisladottir *et al.* (1994) who fed Atlantic salmon diets containing approximately 80 mg Ax/kg diet with different levels of vitamin E. However, Bjerkeng *et al.* (1999) found a small improvement in carotenoid deposition and coloration of Atlantic salmon with increasing dietary vitamin E levels. Their results showed a 14% enhancement of Ax deposition when the dietary Ax level of 30 mg/kg was supplemented with increased dietary levels of α-tocopheryl acetate ranging from 200 to 800 mg/kg. Some of the differences in the results of various studies may be due to the differences in lipid and fatty acid composition of experimental diets used by investigators. It appears that vitamin E as a biological antioxidant protects Ax in tissue rather than influences Ax absorption.

The intestinal absorption of carotenoids in humans and animals may occur via a passive diffusion process (Parker, 1996). Although the mechanism of carotenoids uptake from the digestive tract of fish has not been investigated, a similar passive uptake process may occur in fish. Studies on human intestinal carotenoids absorption have widely used a Caco-2 cell line as a suitable model to investigate the cellular metabolism of carotenoids (Garrett *et al.*, 1999; Garrett *et al*., 2000; Gracía-Casal *et al*., 2000; During *et al*., 2002; Liu *et al*., 2004; Chitchumroonchokchai *et al*., 2004; During and Harrison, 2005; During and Harrison, 2007; O'Sullivan *et al*., 2007). The method to enrich cell culture media with carotenoids and delivery to the apical surface of intestinal cells represents a problem because carotenoids are hydrophobic substances. Several compounds such as DMSO (During *et al.*, 1998; Liu *et al.*, 2004), THF (During *et al.*, 1998), ethanol, Tween 40 (During *et al.*, 1998; During and Harrison, 2005) have been used to solubilise carotenoids to administer them in cell culture media. However, a few studies have focused on Ax uptake in a cell culture model. The objective of the study in Chapter 3 was to find a suitable technique to deliver Ax to cells. The method of Liu *et al.* (2004) was modified and adapted for this investigation. Their method was developed to study the uptake of β-carotene, zeaxanthin and lutein by using a 2 day Caco-2 cells seeding and DMSO to solubilise and to deliver carotenoids to the cell. DMSO is an amphipathic molecule with a highly polar domain and two apolar methyl groups, which makes it soluble in both aqueous and organic media (Santos *et al*. 2003).

For the modification of this technique, the stock solutions of Ax were prepared by dissolving crystalline Ax in DMSO followed by filtering under sterilized conditions before adding this solution to the cell culture medium. The comparison of cellular uptake between two different concentrations (average 4.1μM and 8.6 μM) was conducted such that the final concentration of DMSO in media did not exceeded 0.6%. No sign of negative side effects to the cultured cells from either Ax or DMSO were observed. The results were compatible with the results from Liu *et al*. (2004) in terms of the amount of intracellular zeaxanthin and ß-carotene detected after incubation with the same concentration of carotenoid enriched media. The uptake of Ax by Caco-2 cells reached a saturated level after 8 h incubation.

The techniques such as seeding density, cell differentiation, medium composition, as well as the different passage number of Caco-2 cells and the influence of line sub-types (clonal Caco-2

cell line i.e. Caco-2/TC7 or parental Caco-2/ATCC) have affected results obtained from different laboratories (Sambuy *et al*., 2005). Nevertheless this model involving 2 day Caco-2 cells seeding and dissolving Ax in DMSO was found to be an effective model to study Ax uptake in salmon. This model also has been used for the salmon enterocytes study with minor modifications (Chapter 4). This study involved the use of freshly isolated cells from fish pyloric caeca, enterocyte pellet resuspension in different kinds of cell culture media (use Medium 199 instead of Minimum Essential Medium, MEM).

Another approach to study the nutrient absorption involves the determination of their concentrations in everted salmonid intestine following exposure to solutions of carotenoids and vitamin A (Al-Khalifa and Simpson, 1988; White *et al*., 2003). Al-Khalifa and Simpson (1988) found that the uptake capacity of the duodenum (pyloric caeca) was higher than the ileum in rainbow trout which showed that the absorption of carotenoids occurs mainly along the proximal intestine of salmonids. In this study we used the cell culture model and enterocyte uptake of Ax to investigate whether this approach would be effective in demonstrating the effects of dietary cholesterol on Ax uptake (Chapter 4). To date, no such studies have been conducted in this area. Since pyloric caeca is the main site of carotenoids absorption (Al-Khalifa and Simpson, 1988; White *et al*., 2003), Ax cellular uptake study was performed using the intestinal cell isolation method of Tocher *et al*. (2002).

In a novel approach undertaken in this investigation, an *in vivo* study was conducted to determine the effects of dietary cholesterol on Ax uptake in enterocytes. The cellular Ax level was determined in isolated enterocytes from pyloric caeca of fish fed diet containing Ax with or without a supplement of 2% cholesterol. A slightly higher cellular Ax content of enterocytes in cholesterol supplemented media was shown, however, no statistical differences ($p>0.05$) were observed between the two dietary treatments. Our *in vitro* model involved the isolation of enterocytes from the pyloric region of salmon followed by incubation in the freshly isolated enterocytes with two different concentrations of Ax with or without a cholesterol supplement in cell culture media. There was no clear evidence of the effect of cholesterol supplementation to Ax uptake using the *in vitro* model.

Limited studies conducted on the role of cholesterol in carotenoid metabolism using a cell model show the competitive carotenoid and cholesterol incorporation into the lipid bilayer (Socaciu *et al*., 2000). In this model, liposomes made from single or mixed phospholipids were used. Liposomes were incorporated with the dietary carotenoids (ß-carotene, lutein and zeaxanthin) or with cholesterol. When carotenoids were incorporated into liposomes together with cholesterol, carotenoid incorporation was reduced. Cholesterol is favored for incorporation due to its easy-to-fit structure compared to the large carotenoid molecules. Turley (1999) suggested that an increase in dietary cholesterol can also affect the cellular uptake and recycling of lipoproteins in the liver through the down-regulation of hepatic LDL-receptor activity. However, in both the *in vitro* or *in vivo* studies in the present investigation, there was no clear evidence of a positive effect of cholesterol on cellular Ax uptake. This may be partly due to a high variation in enterocyte Ax uptake data associated with individual fish variation. Development of a fish intestinal cell line would provide a much better tool to study intestinal Ax and nutrient uptake.

It is clear from the above discussion that Ax absorption within the gastrointestinal tract involves disruption of the food matrix prior to absorption. The mechanism of Ax absorption in the presence of lipid involves formation of micelles, uptake by gut enterocytes and incorporation into chylomicrons and further transport by lipoproteins. A comprehensive study was undertaken to investigate whether the distribution of Ax among the lipoproteins in Atlantic salmon plasma was affected by dietary cholesterol supplementation. The lipoprotein separation was carried out according to the methods used in human lipoprotein investigations with some modifications.

Atlantic salmon plasma was fractionated using sucrose density gradients according to McLeod *et al*. (1996). This technique avoided contamination by albumin and separation of protein by SDS-PAGE according to their electrophoretic mobility allowed the isolation of apolipoproteins in each fraction. Various lipoproteins were isolated including VLDL (d 1.031-1.043 g/mL), LDL (d 1.043-1.050 g/mL), HDL (d 1.050-1.103 g/mL) and a protein-rich fraction (d 1.103-1.157 g/mL). Only apolipoproteins of 25 kDa in the HDL fraction were present in detectable amounts and other apolipoproteins in HDL of plasma were relatively low to be identified by this method. VLDL was present in the 260 and 240 kDa molecular weight protein fraction and 240 and 76 kDa molecular weight protein fraction in the LDL fraction. These results

are in agreement with earlier reports on rainbow trout apolipoprotein (Babin, 1987; Babin and Vernier, 1989).

The lipoprotein densities of Atlantic salmon plasma ranged from 1.031 to 1.157 g/ mL using sucrose gradient, which did not agree with earlier studies conducted on fish. In previous studies, different techniques were used to isolate lipoproteins from either fish plasma or serum and their density was adjusted with appropriate salt solutions for isolation by ultracentrifugation prior to identification. In addition, the classification of lipoprotein classes was made according to the density intervals applied to human lipoprotein (VLDL, density (d) <1.006 g/mL, LDL, d 1.006-1.063 g/mL and HDL, d 1.063-1.21 g/mL) (Chapman *et al*., 1978; MacFarland *et al*., 1990; Chavez *et al*., 1998).

Astaxanthin was detected in all lipoprotein fractions of Atlantic salmon plasma. In fish fed the three experimental diets, the highest concentration of Ax was found in the protein-rich (PR) fraction. It appears that when dietary Ax concentration is low, plasma Ax in salmon is not transferred from the PR fraction to VLDL or HDL. Aas *et al*. (1999) found that 42% and 58 % of the radiolabeled carotenoids were present in lipoprotein and a high density protein fraction which suggested that Ax binding was likely to be associated with albumin. In humans, albumin is the major plasma protein. Its main function is the regulation of the colloidal osmotic pressure of numerous endogenous and exogenous compounds including free fatty acids, hormones, bilirubin and drugs (De Smet *et al*., 1998). Although albumin is present in salmonids, its role in nutrient transport in fish is unclear (Metcalf and Gemmell, 2005). Albumin comprises over 50% of the total serum in salmonids, has a molecular mass of 70 kDa and has the same electrophoretic mobility as human albumin (Maillou and Nimmo, 1993a, b). The several bands of protein present in the high density fraction in our study could not be identified as a specific protein. Separation of the Ax-bound protein present in these fractions by other techniques such as ion-exchange chromatography would allow further characterization of these proteins distributed in the PR fractions of salmon plasma.

Certain changes were observed in the distribution of Ax in fish fed the diet containing Ax supplemented with cholesterol. Lower Ax levels were found in the HDL and protein rich fractions compared to fish fed diet with Ax only. The effect of dietary cholesterol on Ax

distribution in lipoprotein classes can be explained by two hypotheses. First, dietary cholesterol increases the amount of VLDL in plasma. A study conducted on chicken has shown that supplementation of 1% cholesterol in diet not only enhanced serum cholesterol levels but also induced changes in the density profile and composition of the serum lipoprotein (Mol *et al*., 1982). Separation of serum lipoproteins by density gradient ultracentrifugation showed that in the chickens on diets containing cholesterol, there was a shift in the lipoprotein density pattern from the LDL fraction to IDL and VLDL fractions. Compositional analyses of the lipoprotein classes showed that the amount of VLDL and IDL was increased and furthermore they contained additional cholesterol (Mol *et al*., 1982).

The second hypothesis suggests that a transfer of carotenoids occurs between the fish lipoprotein classes. Little is known about how the distribution of carotenoids among lipoproteins is established or maintained in fish and laboratory animals. For the major lipid classes (triglyceride, cholesterol, cholesteryl ester and phospholipid), the distribution among lipoproteins is governed in part by enzymes such as lecithin cholesterol acyltransferase (LCAT) and transfer proteins such as cholesteryl ester transfer protein (CETP) (Romanchik *et al*., 1995). *In vitro* studies conducted by Tyssandier *et al*. (2002) in rainbow trout showed that β-carotene and several xanthophylls (astaxanthin, canthaxanthin, lutein and β-cryptoxanthin) can transfer between lipoprotein. The transfer is bidirectional from HDL to VLDL and *vice versa*. The presence of enzyme inhibitors would affect the transfer of carotenoids between lipoprotein classes.

It is also possible that dietary cholesterol increased the substrate and enhanced the activity of enzymes such as CETP, a hydrophobic plasma glycoprotein that mediates transfer and exchange of neutral and phospholipid between lipoprotein classes and may be involved in carotenoid metabolism as well. Rainbow trout has a considerable high activity of the CETP in plasma with activities more than double that of humans (Ha and Barter, 1982).The presence of CETP inhibitor in medium of an *in vitro* rainbow trout study changed the concentration of catotenoids between VLDL and HDL after incubation. The inhibitor reduced the amount of carotenoid recovered from the VLDL fraction and consequently increased that in the HDL fraction (Tyssandier *et al*., 2002). The increased expression of CETP in mice liver and in peripheral tissues in response to increased dietary cholesterol has been reported (Jiang *et al*.,

1992). Future work should consider the determination of enzyme activity to explain the effect of dietary cholesterol on Ax transport in fish blood.

It is clear from the above discussion that systematic studies to better estimate the absorption efficiency of natural and synthetic carotenoids and the factors affecting their bioavailability are necessary. In recent years, higher proportions of plant protein and oil supplements are being incorporated in salmonid feeds due to decrease in global supply of fish meal and fish oil. Use of plant products changes the fatty acid and amino acid composition of diets, increases the xanthophylls, plant sterol and fiber content of the diet and contributes to antinutritional factors that are known to interfere in absorption of nutrients. The research in this thesis has characterized dietary cholesterol as an important factor that influences carotenoid absorption and transport of Ax in Atlantic salmon. Further studies in this area based on the basic knowledge generated from this thesis will be beneficial to develop nutritional strategies to enhance flesh pigmentation of Atlantic salmon.

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Chapter 7

Summary and Conclusions

It is widely recognized that advanced knowledge of the absorption and metabolism of Ax are necessary to improve the retention of this pigment in salmonid flesh. Limited research efforts have been directed to study the mechanism of carotenoid absorption mechanisms. This thesis examines the effects of several dietary factors that may affect the bioavailability of Ax from diets of Atlantic salmon and the nature of interactions between carotenoids and other dietary factors occurring during intestinal absorption and transport.

The preliminary experiment was designed to investigate the dietary factors that might enhance or interfere with Ax absorption in salmon including interfering factors such as certain carotenoids (zeaxanthin and lutein), plant sterols, fiber, and potential enhancing compounds such as cholesterol and vitamin E. The results clearly showed that supplementation with 2 % cholesterol in the diet significantly improved the Ax absorption which was reflected in higher Ax concentration in plasma of Atlantic salmon. Other supplements including vitamin E (450IU/kg), wheat bran fiber (5% w/w), lutein (40 mg /kg diet), zeaxanthin (40 mg/kg diet) and phytosterols (2% w/w) in diet had no significant effect on plasma Ax absorption. Subsequent experiments were directed to study the effect of cholesterol on cellular Ax uptake in the intestine and plasma transport of Ax by lipoproteins. It was necessary to develop methodologies to achieve these goals. Biochemical methods involving cell culture models used for vertebrate animals were modified and applied to studies in salmon.

In vitro study of the cellular intestinal absorption in humans using human colon adenocarcinoma cell line (Caco-2) has proved to be the best model for the absorption study. However, no intestinal cell line is available for fish. Most of the *in vitro* absorption studies with fish have used intestinal tissue section or isolated enterocytes from various intestinal regions to investigate nutrient absorption in gut. This is the first study to report carotenoid absorption using an enterocyte isolation technique. In order to develop this method, it was necessary first to have a vehicle for delivery of carteonoids to the apical surface of cells. A cell culture model was developed involving Caco-2 cells to demonstrate the absorption of synthetic Ax by solubilizing it

in dimethlysulfoxide (DMSO). Subsequently, enterocyte isolation technique was modified for the isolation of enterocytes for *in vitro* and *in vivo* to determine the Ax uptake. This *in vivo* model indicated that cellular Ax can be detected in enterocytes isolated from pyloric caecae after different times of feeding either from fish fed Ax supplemented diet only or diet containing Ax plus cholesterol (2% w/w). It was evident that relatively higher levels of cellular Ax were present when cells were exposed to a higher concentration of this carotenoid. Both *in vitro* and *in vivo* experiments clearly demonstrated that cellular uptake of carotenoids is a passive absorption process and there is no significant effect of cholesterol on cellular Ax uptake.

The significantly higher level of plasma Ax in fish fed Ax supplemented diet with 2% cholesterol as compared with fish fed diet containing only Ax supplement. Which indicated that the effect of dietary cholesterol on Ax utilization may occur via the lipoprotein transport process. Atlantic salmon plasma lipoprotein was fractionated using a modified sucrose gradient ultracentrifugation method developed for the color bands of different density plasma solutions. The identification lipoprotein class was achieved by comparing the molecular weight of apolipoprotein, which identified using SDS-PAGE and matching with the apolipoprotein of rainbow trout. We found that Ax was distributed in each lipoprotein fractions. Protein-rich lipoprotein (PR) fraction appears to be a main carrier of Ax in the blood of Atlantic salmon, follow by HDL, VLDL and LDL. Cholesterol supplementation of in the diet significantly increased the Ax concentration in VLDL.

In conclusion, the present investigation has provided new information on the effects of certain dietary factors on Ax absorption by Atlantic salmon as well as biochemical mechanisms involved in the uptake of Ax by intestinal mucosal cells (enterocytes) and the effect of cholesterol supplementation on Ax absorption and transport in fish blood. *In vitro* study on the cellular uptake of Ax by enterocytes provided evidence that when cells were exposed to a higher concentration of Ax, they have the ability to absorb relatively high concentration of Ax. This indicates that cellular uptake of carotenoids is the passive transport process. Dietary cholesterol has a significant effect on the Ax transport process in the blood. However, there is no interaction of Ax and cholesterol on intestinal absorption of this carotenoid. The lipoprotein study on Ax transport showed distribution of Ax in each lipoprotein classes with the high levels in HDL followed by VLDL and LDL. The highest concentration of Ax was found in the protein-rich

lipoprotein fraction (PR) which plays an important role as a main carrier of Ax in blood for metabolism by other tissues. The cholesterol content of the diet had a significant effect on Ax concentrations in VLDL, which may explain the effect of higher concentration of Ax retained by increasing dietary cholesterol levels.

The future research directed in the following areas will advance knowledge to improve efficiency of carotenoid absorption, metabolism and retention in the flesh of Atlantic salmon:

- Identify the protein that binds Ax in the protein-rich lipoprotein (PR) fraction (fractions 11 to 13) by developing new methods to separate Ax-bound protein in this fraction.
- Determine shift in Ax distribution in lipoproteins associated with dietary cholesterol intake (graded levels), particularly VLDL. This will allow for the development of strategies to incorporate cholesterol in salmonid feeds that now contain high amounts of plant products.
- Examine the effects of alternate plant protein sources and dietary cholesterol on Ax binding with the PR fraction, lipoprotein metabolism and Ax uptake in muscle tissues of salmon.
- Study the role of dietary cholesterol in bile acid synthesis, micelle formation in gut and tissue uptake.

Appendix

Appendix 1: Flow diagram of Salmon enterocytes isolation (Modified method from Tochor *et al*., 2002)

Fast salmon for 48h, sacrifice with
an overdose of TMS

↓

Weigh fish

↓

Fish is spray by 70% ethanol for a few seconds and operate *in vivo*
(Perform in laminar flow hood)

Isolate fish enterocytes from pyloric caeca

Remove intestinal tract
Dissect pyloric caecae, clean/remove adhering adipose tissue

↓

Slit Open and clean with solution A (10 mL/5 times)

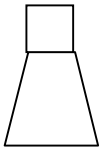
↓

Chop to 2-3 mm

↓

Weigh **2 g** of tissue and add 20 mL solution B +collagenase (1mg/mL)

↓



X 6 flask

Incubate at 20°C /45 min
Gently shaking at 60 rpm

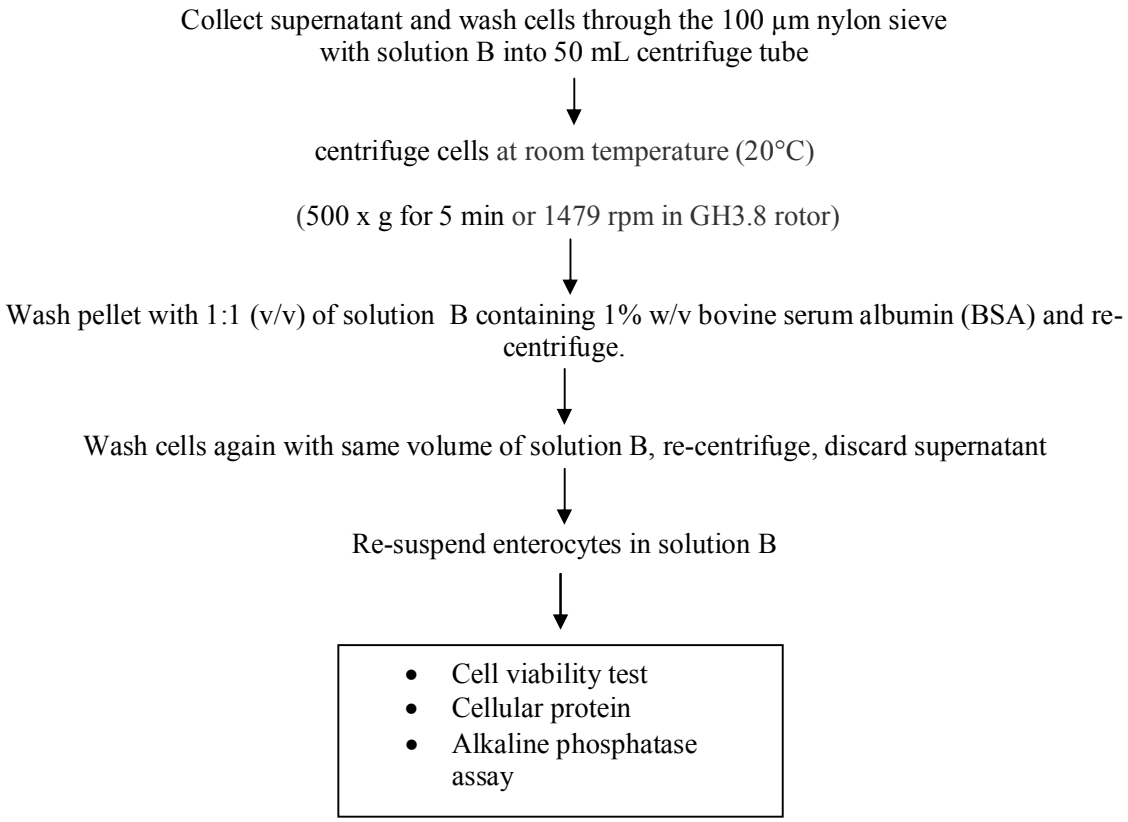
↓

collect supernatant and wash cell through 100µm sieve

↓

► add 10 mL solution B
► gently mix for 15 sec
► collect supernatant and wash cell through 100µm sieve
► repeat 2-3 times

↓



Appendix 2: Method for cellular carotenoid analysis

Sample extraction (O'Sullivan *et al.,* 2007)

1. Samples are thawed, sonicated for 30 sec on ice and briefly vortex
2. 300µL of samples are extracted twice with 2 mL hexane-ethanol-acetone (50:25:25, v/v)
3. Extractions are centrifuged at 3000 rpm for 5 min
4. Supernatant layers are removed, pooled and dried down under N_2. Re-dissolve in Hex:Acetone (86:14) transfer to 2mL vial, dried under N_2 again
5. The residues are reconstituted in 150 µL Hex:Acetonitrile (86:14, v/v) and directly analyzed by normal phase HPLC system

HPLC method

1. µPoracil silica column, 39x300 mm
2. Isocratic Hex : Acetone (86/14) as the mobile phase.
3. Flow rate at 1.5 mL/min at 25C for 12.5 min
4. Each carotenoid is quantified from its peak area by comparison with a standard reference curve established with different amounts of the respective standard carotenoid

Appendix 3: Method for cell culture media carotenoid analysis

Sample extraction (During et al., 2002)

1. 100 µL of medium and 300 µL of 2- propanol:dichloromethane (2:1, v/v) are placed in a tube and vortexed for 1 min
2. Centrifugation for 1 min using a microcentrifuge
3. The resultant supernatant are removed into 2mL HPLC vial, dried under N_2
4. The residue is re-dissolved in 150 µL methanol:TBME, 70:30, v/v)
5. Filter the extracted solution through 0.2µM filter, put into inserted HPLC vial and analyzed by RPLC

HPLC condition

1. YMC carotenoid C_{30} reverse phase column, 20x250 mm (CT99S052520WT)
2. Methanol : TBME (90:10 isocratic elution) as the mobile phase under method
3. Flow rate at 0.2 mL/min
4. Each carotenoid is quantified from its peak area by comparison with a standard reference curve established with different amounts of the respective standard carotenoid

Appendix 4: Preparation standard curve of carotenoids

Standard astaxanthin (Ax)

Standard astaxanthin obtained from DSM Ltd. are used for quantification. The standard curve is obtained by dissolving approximately 8 mg of crystalline astaxanthin with 10 mL Chloroform, mixed well and added hexane up to 100 mL in volumetric flask. The series of dilutions of the solution is made to derived the standard curve. All dilution series are determined at the 470 nm (Schierle and Hardi, 1994).

The standard astaxanthin concentration were calculated by using

$$\text{astaxanthin concentration (mg/L)} = \frac{\text{absorption} \times 10000}{2100}$$

When

2100 = E(1%/1cm) = standard absorption of a 1% Ax solution (weight/volumn) in a 1 cm cuvette at 470 nm in n-Hexane

Standard Lutein and Zeaxanthin

Standard lutein and zeaxanthin obtained from DSM Ltd. are used for quantification follow by Schierle and Hardi (1994). The standard curve are obtained by dissolving approximately 1.5 mg of crystalline with 10 mL acetone and 10 mL ethanol, mixed well and added hexane up to 100 mL in volumetric flask. The series of dilutions of the solution is made to derived the standard curve. All dilution series are determined at the specific wavelength.

The standard lutein concentration are calculated by using

$$\text{lutein concentration (mg/L)} = \frac{\text{absorption} \times 10000}{2529}$$

When

2529 = E(1%/1cm) = standard absorption of a 1% lutein solution (weight/volume) in a 1 cm cuvette at 446 nm in n-hexane

The standard zeaxanthin concentration were calculated by using

$$\text{zeaxanthin concentration (mg/L)} = \frac{\text{absorption} \times 10000}{2450}$$

When

2450 = E(1%/1cm) = standard absorption of a 1% zeaxanthin solution (weight/volume) in a 1 cm cuvette at 450 nm in n-hexane

The series of dilution standard solution, in n-Hexane 1 µL are injected into the normal phase HPLC

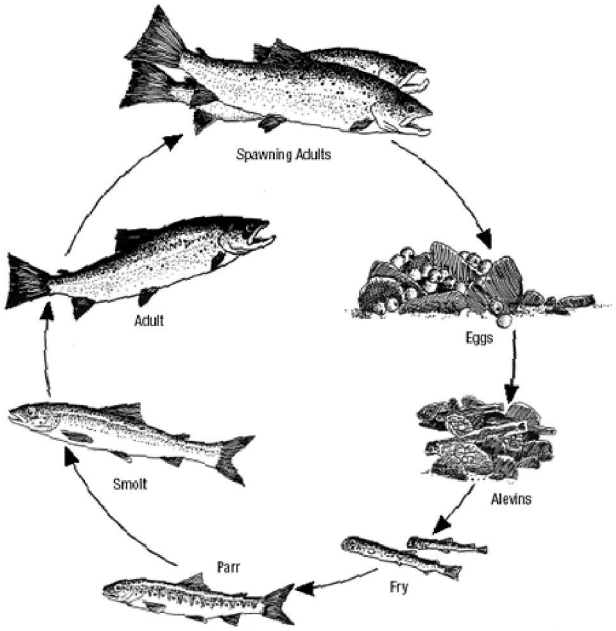
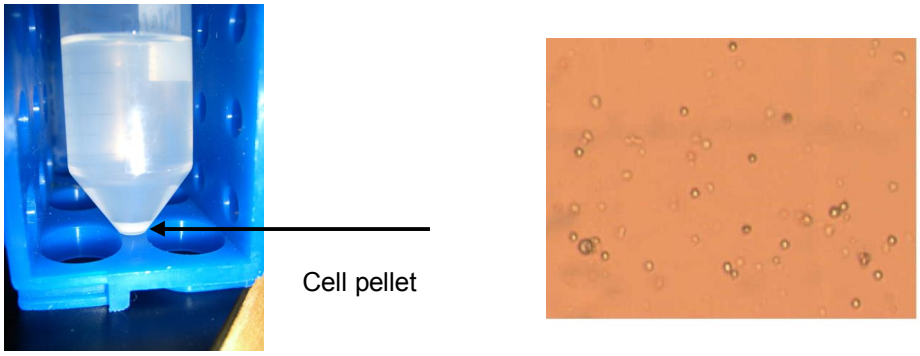
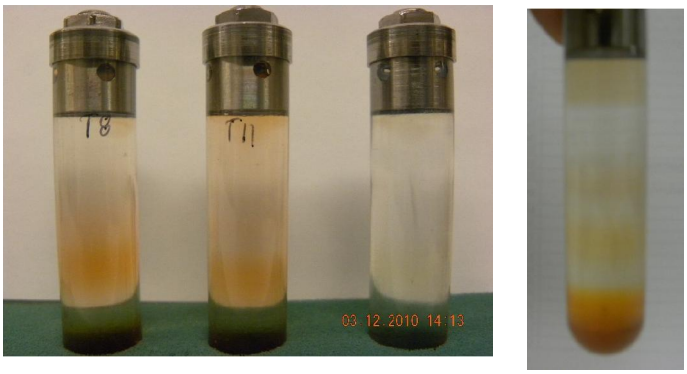


Figure A-1 Life cycle of Atlantic salmon (http://www.pc.gc.ca/apprendre-learn/prof/sub/eco/itm5/fi-lr2/salmon-saumon_e.asp)



(A) (B)

Figure A-2 The pellet (A) and (B) cells of isolated salmon enterocyte



A **B** **C** **D**

Figure A-3 Density profile of lipoprotein after density gradient ultracentrifugation of pooled plasma from salmon fed Ax-supplement diet without and with added cholesterol.

(A) Ax supplemented diet;

(B) Ax supplemented diet with 2% cholesterol;

(C) Control diet (no Ax or cholesterol supplement); and

(D) Density profile of pigmented plasma

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Chimsung, N., Tantikitti, C., Milley, J. E., Verlhac, V. and Lall, S. P. 2012. Effect of various dietary factors on astaxanthin absorption in Atlantic salmon (*Salmo salar*). *Aquacult. Res*. In press.