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Distribution of Exogenous δ-Tocopherol between the Membrane Lipids and Triacylglycerols of a Cod Muscle–Triacylglycerol Model System

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Membranes of muscle foods are more susceptible to oxidation than triacylglycerols. Hence, directing a lipid-soluble antioxidant into the membranes may reduce the oxidative deterioration of muscle tissue. The objective of this research was to use a model system of cod muscle and triacylglycerol to study the distribution of exogenous δ-tocopherol between the membranes and triacylglycerol fractions of muscle. When ethanol was the carrier solvent, more tocopherol was incorporated into the membranes than when oil was the carrier. Addition of tocopherol to the muscle before the triacylglycerol was added allowed more antioxidant to be incorporated into the membranes than for the case when the oil was added before the antioxidant. When the triacylglycerol was solid, the amount of tocopherol incorporated into the membranes was higher than if the triacylglycerol was liquid and the amount of tocopherol incorporated into the membranes was less dependent on the order of tocopherol and triacylglycerol addition. There was a competition between the membrane lipids and triacylglycerol for uptake of the δ-tocopherol. In some circumstances, some of the tocopherol did not enter either the membrane lipid or triacylglycerol phase.

KEYWORDS: Cod; triacylglycerol; oil; δ-tocopherol; membranes; ethanol

INTRODUCTION

Muscle foods are an important source of food protein. A major portion of these are sold as minced muscle products. The lipids of minced muscle products are highly susceptible to oxidative deterioration (*1*). Among the muscle lipids, the membrane phospholipids are more susceptible to oxidation than the neutral triacylglycerols (*2*). This may be due to the high degree of unsaturation of the membrane fatty acids as well as the large surface area of membranes per unit weight of lipid (*3*, *4*). Lipid oxidation can be retarded by using exogenous antioxidants (*5*). The presence of an antioxidant at the location where oxidation is initiated or propagated may be important in determining the antioxidant efficiency. Hence, directing a lipid-soluble antioxidant into the membrane lipids in preference to the triacylglycerols may help to reduce the oxidative deterioration of muscle tissues. Earlier studies with aqueous suspensions of membranes and triacylglycerols (*6*) have shown very little exchange of tocopherol between the lipid fractions when tocopherol resided in one of the fractions. When partitioning of added δ-tocopherol was studied between the membrane lipid and triacylglycerol fractions of chicken leg muscle (*7*) at a low total muscle lipid content (3−5%), the tocopherol was present in approximately the same concentration in both lipid fractions.

The objective of this research work was to determine the factors that affect the distribution of a lipid-soluble antioxidant between the triacylglycerols and the polar membrane lipids of minced muscle and to develop means of incorporating a lipid-soluble antioxidant selectively into the membrane lipids of the muscle. A model system comprising minced cod muscle and triacylglycerols was used. δ-Tocopherol was chosen as the exogenous lipid-soluble antioxidant as it is a minor component of muscle lipids (*8*) compared to α-tocopherol, and hence it is easy to detect its concentration and distribution among the various muscle fractions.

MATERIALS AND METHODS

Materials. Fillets of Atlantic cod (*Gadus morhua*) were purchased from a local fish distributor in Gloucester, MA, and transported to the laboratory on ice. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents were of ACS grade, and all solvents were of HPLC grade. Canola oil and beef fat were purchased from a local supermarket.

Methods. *Muscle Treatment.* The white muscle tissue from cod fillets was separated and was used for all treatments and analyses. Cod muscle (*G. morhua*) was chosen as it primarily contains membrane lipids with almost no triacylglycerols (*9*). Canola oil or beef fat was added as a relatively stable exogenous triacylglycerol that could adjust the total lipid content of the cod−triacylglycerol system to any desired level. Cod white muscle was minced twice with a KitchenAid grinder model KSM 90 (KitchenAid Inc., St. Joseph, MI) through a $^{3}/_{16}$-in. diameter

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Table 1. Distribution of δ-Tocopherol between Membrane, Oil, and Press Juice Fractions of the Cod–Canola Oil Model System[a]

order of addition	TOH (μg)[b]/ system (g)	% oil added to the system	% TOH recovery in aq phase[f]	δ-TOH concn[e] (ppm)		
				system	membrane	oil
TOH added first	2.5[c]	0	24	343 ± 40[c]	114 ± 31	0
TOH added first followed by oil	2.5[d]	6.1 ± 0.6	15	36 ± 4[d]	74 ± 8	13 ± 1
oil added first followed by TOH	2.5[d]	6.3 ± 1.7	ND[g]	38 ± 9[d]	40 ± 13	34 ± 4

[a] $n = 3$, where n = number of replicates for each experiment (number of analyses within each experiment = 1). [b] Amount of δ-tocopherol added (μg)/system (g), where the system is either cod or (cod + canola oil). [c] Cod system; concentration is based on total lipid content of cod muscle. [d] Cod + canola oil system; concentration is based on total lipid content of cod muscle + canola oil. [e] δ-TOH concentration = concentration of TOH determined using HPLC. [f] % TOH recovery in aq phase: TOH concentration was determined in the aqueous phase using HPLC (aqueous phase was separated by centrifugation). Recovery of TOH was calculated on the basis of the assumption that the concentration of TOH in the separated aqueous fraction was the same as that of the nonseparated aqueous fraction. [g] Not determined.

sieve. Triacylglycerol and tocopherol were added to the muscle and chopped in a Robot Coupe R301 Ultra Chopper (Robot Coupe USA Inc., Ridgeland, MS) at 10 °C for 3 min. When tocopherol was added first, the mixture of tocopherol and muscle was chopped for 1 min followed by a 2 min chopping after the triacylglycerol was added. When triacylglycerol was added first, the mixture of triacylglycerol and muscle was chopped for 0.5 min followed by a 2.5 min chopping with the tocopherol. Either ethanol or oil was used as the tocopherol carrier solvent. The temperature of chopping was maintained below 17 °C by cooling the chopper (along with its contents) at −20 °C for 5 min after each 1.5 min of chopping.

Isolation of the Membranes. The membranes from the cod muscle system were separated using a method similar to that of Sigfusson and Hultin (*6*). The term cod muscle system refers to either the cod muscle plus tocopherol mixture or the cod muscle plus triacylglycerol plus tocopherol mixture. The cod muscle system was mixed with 4 volumes of cold 0.1 M *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) buffer (pH 7.5) containing 0.2% w/v ascorbate and homogenized at speed 4 for 40 s using a Kinematica Polytron PT 10-35 homogenizer (Brinkmann Instruments, Westbury, NY). The pH of the homogenate was adjusted to 7.5, and the homogenate was centrifuged at 10000*g* for 20 min at a sample temperature of 7−9 °C using a Beckman L5-65B ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The supernatant obtained was recentrifuged at 130000*g* for 30 min. The crude membrane obtained as the sediment from this last centrifugation was suspended in HEPES buffer at pH 7.5 and was used for lipid, protein, phospholipid, and tocopherol determinations.

The membrane protein content was determined by means of the biuret reaction (*10*). The yield of the membranes was estimated as a percentage of the phospholipid recovered in the membrane fraction compared to the phospholipid in the initial muscle tissue. By this estimation, the yield of the membranes obtained by centrifugation was in the range of 18−25%. The tocopherol recovery in the membranes of the muscle was estimated on the basis of the assumption that the concentration of tocopherol in the membrane fraction obtained by centrifugation was the same as that of the unrecovered fraction of membranes in the muscle tissue.

Isolation of Triacylglycerols. Triacylglycerols were separated using the method followed by Sigfusson et al. (*7*). Fifty grams of the muscle−triacylglycerol system was centrifuged at 130000*g* for 1 h using an ultracentrifuge. After centrifugation, the top layer of oil was collected in a centrifuge tube and recentrifuged at speed 6 for 10 min in and IEC Clinical tabletop centrifuge (International Equipment Co., Needham Heights, MA). The oil collected was weighed, and the yield was determined on the basis of the amount of triacylglycerol added. Recovery of tocopherol in the oil fraction was estimated on the basis of the assumption that the concentration of tocopherol in the separated fraction of triacylglycerol was the same as that of the unseparated fraction (*11*).

Separation of Press Juice. The press juice fraction from the muscle was separated according to a method similar to that of Undeland et al. (*12*). Minced muscle was packed in a centrifuge tube and was centrifuged at 130000*g* for 1 h in an ultracentrifuge. The press juice separated on top of the sediment was collected and used for analysis. The tocopherol content of the press juice was determined after extraction with a 1:1 (v/v) chloroform/methanol mixture. The yield of the press juice was determined by expressing it in terms of the total moisture content of the muscle. The moisture content of the muscle was determined by using a Cenco Moisture Balance (CSC Scientific Co. Inc., Fairfax, VA). The yield of the press juice was 30−40% of the total moisture content of the muscle. The recovery of tocopherol in the press juice fraction was estimated on the basis of the assumption that the separated press juice fraction and the unseparated aqueous fraction had the same concentrations of tocopherol.

Lipid, Phospholipid, and δ-Tocopherol Determination. Total lipid contents of the minced muscle and the membrane suspensions were determined by extraction with a 1:1 (v/v) chloroform/methanol mixture for 3 h using a 0.5% NaCl solution for phase separation (*6*). The chloroform layer was separated and used for total lipid and phospholipid determination. Total lipid content was determined gravimetrically by drying the chloroform layer on a hot plate at low setting to avoid overheating. The phosphorus content of the lipid fraction was determined spectrophotometrically using the method of Anderson et al. (*13*). The δ-tocopherol content of the membrane sample was determined from the chloroform phase using HPLC (Hewlett-Packard, series 1100) as described by Petillo et al. (*14*).

Preparation of δ-Tocopherol in Various Carrier Solvents. δ-Tocopherol was prepared in carrier solvents of ethanol or canola oil. The concentration of tocopherol added to the cod−triacylglycerol system was maintained at ∼300 ppm based on the total lipid content. The amount of carrier solvent (ethanol or oil containing tocopherol) added to the muscle tissue was ∼0.8−1.0% of the weight of the cod muscle−triacylglycerol system. For example, in order to add 300 ppm of δ-tocopherol to a 100 g system containing 7% fat (7 g of fat), 52.5 mg of δ-tocopherol was dissolved in 25 mL of ethanol and 1 mL of this ethanolic tocopherol was added the system. This corresponds to 300 ppm of tocopherol based on the lipid content. The amount of carrier solvent added corresponds to 1% of the weight of the system.

Statistical Analysis. All experiments were done at least thrice. Analysis within each experiment was done once. Statistical analyses were done using the general linear model (GLM) procedure (Jandel Scientific, San Rafael, CA).

RESULTS

Partitioning of δ-Tocopherol among Various Muscle Fractions. When tocopherol dissolved in ethanol (ethanolic tocopherol) was added to the cod muscle at 343 ppm, based on the total lipid content of the muscle (**Table 1**), tocopherol was recovered from both the membrane fraction (114 ppm based on the membrane lipid content) and the press juice (24% of the added tocopherol). If oil was added at 6% of the cod muscle weight after the tocopherol had been incorporated, the added oil was able to extract some of the tocopherol from the membrane and the press juice fractions, thus decreasing the level of tocopherol in both fractions. After the addition of oil, the tocopherol concentration in the oil fraction was 13 ppm, and the amounts of tocopherol in the membrane and press juice fractions were 74 ppm and 15% of the added tocopherol, respectively. The concentration of tocopherol in the total lipid of the cod−canola oil system with 6% added oil was 36 ppm

Table 2. Effect of the Order of Addition of Ethanolic Tocopherol and Oil to Cod Muscle on Concentration of Tocopherol in Lipid Fractions at 4 and 9.5% Oil Contents[a,b]

TOH added before[c]/after oil addition	% oil in system	TOH added (μg)/ system (g)	δ-TOH concn (ppm)		
			system[d]	membrane	oil
before	4.2 ± 0.0	17.1 ± 0.0	345 ± 4	422 ± 43a	158 ± 8b
before	9.7 ± 0.4	38.3 ± 0.1	366 ± 13	1129 ± 104a	229 ± 13b
after	4.1 ± 0.1	16.9 ± 0.4	332 ± 46	280 ± 18	281± 21c
after	9.3 ± 0.3	38.2 ± 0.1	394 ± 20	295 ± 98	370 ± 18c

[a] $n = 3$, where n = number of replicates for each experiment (number of analyses within each experiment = 1). [b] Letters a–c indicate which values in each column differed significantly ($p < 0.05$). [c] TOH = δ-tocopherol in the carrier solvent ethanol. [d] Cod–canola oil mixture system.

Table 3. Amount of Oil Recovered by Centrifugation for Various Amounts of Oil Added to Cod Muscle Tissue[a]

% oil added to cod muscle[b]	% oil recovered by centrifugation[c]
4.2 ± 0.2	34 ± 9
6.0 ± 0.4	36 ± 13
9.6 ± 0.3	50 ± 12
23.3 ± 0.7	73 ± 3

[a] $n = 5$, where n = number of replicates for each experiment. [b] Percentage of oil added to the muscle based on the cod muscle weight. [c] Percentage of oil recovered by centrifugation based on the amount of oil added initially to the cod muscle.

when tocopherol was added at a concentration of 343 ppm of the lipid of the lean muscle.

If the order of addition of tocopherol and oil to the cod muscle was reversed by adding the oil first followed by the tocopherol in ethanol, the concentration of δ-tocopherol determined in the membrane fraction was lower (40 versus 74 ppm) and that in the oil fraction was higher (34 versus 13 ppm) than for the reverse order of addition with the same amount (2.5 μg) of tocopherol. Thus, the order of tocopherol and oil addition to the cod muscle affected the distribution of tocopherol between the lipid fractions.

Effect of the Order of Addition of Tocopherol and Oil on Distribution of δ-Tocopherol at Two Triacylglycerol Concentrations. The effect of the order of addition of tocopherol and oil to the cod muscle on the distribution of δ-tocopherol between the two muscle lipid fractions was studied at about 4 and 9.5% oil levels (based on the cod muscle weight) (**Table 2**). In one case, ethanolic tocopherol was mixed with the cod muscle first followed by oil. In the second case, oil was mixed with the cod muscle first followed by ethanolic tocopherol. The tocopherol concentration in the cod−canola oil system was maintained at ~330 ppm (based on the total lipid content) by adding 17 and 38 μg of tocopherol/g of the cod for the 4 and 9.5% oil levels, respectively. The typical yield of the separated triacylglycerol was 30−50% of the amount added (**Table 3**).

Irrespective of the level of oil used, the addition of ethanolic tocopherol to the cod muscle before the oil resulted in a higher membrane tocopherol concentration than for the reverse order of addition. When ethanolic tocopherol was added first, the concentrations of tocopherol in the membranes were 422 and 1129 ppm, respectively, for 4 and 9.5% oil levels ($p < 0.01$) compared to the membrane tocopherol concentrations of 280 and 295 ppm ($p > 0.05$) when ethanolic tocopherol was added last.

The concentration of tocopherol in the oil fraction was also affected by the order of addition. Irrespective of the level of added oil, the addition of tocopherol to the cod muscle before the oil resulted in a lower tocopherol concentration in the oil fraction than for the reverse order of addition. Also, when tocopherol was added to the cod muscle before the oil, the concentration of tocopherol in the oil fraction was lower than that of the membranes for both oil levels. When the tocopherol was added to the cod muscle after the oil, the concentration of tocopherol in the oil fraction was equal to or greater than that in the membrane fraction.

Effect of the Physical State of the Triacylglycerol on Incorporation of δ-Tocopherol into Membranes. The effect of the physical state of the triacylglycerol on δ-tocopherol incorporation into the membrane and triacylglycerol fractions of the muscle was studied by replacing canola oil with beef fat (**Table 4**). Tocopherol concentration in the cod−triacylglycerol system was maintained at ~300 ppm (based on the total lipid content) by adding ~20 μg of tocopherol/g of the system at ~6% triacylglycerol (endogenous δ-tocopherol content of canola oil and beef fat was found to be negligible). The order of addition of ethanolic tocopherol and triacylglycerol to the cod muscle was also studied.

When the triacylglycerol was oil, the addition of ethanolic tocopherol before the oil resulted in higher tocopherol incorporation into the membrane (818 ppm) compared to that incorporated (291 ppm) with the reverse order of addition. However, when beef fat was the triacylglycerol, the amount of tocopherol incorporated into the membrane was high (1002 and 1011 ppm) irrespective of the order of tocopherol and triacylglycerol addition. Thus, the amount of tocopherol incorporated into the membrane was dependent on the order of addition of tocopherol and triacylglycerol only when the triacylglycerol was liquid, suggesting that solid fat does not easily incorporate the lipid-soluble antioxidant.

Incorporation of δ-Tocopherol into Membranes of a High-Fat Product. To simulate a high-fat product, triacylglycerol at 25% of the initial cod muscle weight was added (**Table 5**). Ethanolic tocopherol and triacylglycerol were added to the cod muscle in two different ways. In the first case, cod muscle was mixed with 25% oil followed by the addition of ethanolic tocopherol. This was used to simulate the addition of tocopherol directly to a high-fat product. The amount of tocopherol added to the cod−oil system was 61 μg/g, which corresponds to a tocopherol concentration of 311 ppm at the 25% oil level (based on the total lipid content). When the tocopherol was added to this high-fat product, there was no detectable tocopherol in the membrane fraction. The tocopherol incorporated into the oil fraction was 415 ppm. When oil at a concentration of 25% is mixed with the muscle tissue, some of it might form an emulsion with the muscle proteins. The formation of an emulsion may not only restrict the uptake of tocopherol by the emulsified oil but also limit the recovery of oil from the emulsion by the centrifugation process. This may be the reason for the higher tocopherol concentration (415 ppm) in the separated oil fraction than the theoretical amount that should have been found if the tocopherol was evenly distributed throughout the added oil.

Table 4. Effect of the Physical State of the Triacylglycerol on δ-Tocopherol Incorporation into Membranes[a,b]

TAG[c] added	TOH added before/after TAG addition	TOH[d] (μg)/ system(g)	% TAG in system	δ-TOH concn (ppm)		
				system[c]	membrane	TAG
oil	before	23.4 ± 0.1	5.7 ± 0.4	370 ± 22	818 ± 39a	193 ± 16b
oil	after	23.2 ± 0.5	5.7 ± 0.4	362 ± 31	291 ± 16a	367 ± 17b
beef fat	before	19.9 ± 0.1	7.1 ± 0.3	256 ± 7	1002 ± 214	ND[f]
beef fat	after	20.1 ± 0.1	6.6 ± 0.3	276 ± 11	1011 ± 177	ND

[a] $n = 3$, where n = number of replicates for each experiment (number of analyses within each experiment = 1). [b] Letters a and b indicate which values in each column differed significantly ($p < 0.05$). [c] TAG = triacylglycerol (canola oil or beef fat). [d] Amount of δ-tocopherol added (μg)/system (g), where the system is cod + canola oil. TOH = δ-tocopherol in the carrier solvent ethanol. [e] Based on the total lipid content of the cod–triacylglycerol system. [f] Not determined.

Table 5. Manipulating the Order of Addition of Tocopherol and Triacylglycerol (TAG) To Incorporate δ-Tocopherol (TOH) into Membranes at High Triacylglycerol Concentration[a]

order of addition				tocopherol concn (ppm)			
					membrane[d]		
step 1, % oil added	step 2, TOH added in	step 3, % TAG added	TOH (μg)[b]/ system (g)	system[c]	after step 2	after step 3	TAG (after step 3)
25	EtOH[e]		61 ± 0.1	311 ± 17	NDT[f]		415 ± 59
5	EtOH	20% oil	61 ± 0.5	350 ± 39	869 ± 39	361 ± 59	320 ± 10
5	EtOH	20% beef fat	59 ± 4.5	302 ± 15	ND[g]	729 ± 176	178 ± 23

[a] $n = 3$, where n = number of replicates for each experiment (number of analyses within each experiment = 1). [b] Amount of δ-tocopherol added (μg)/system (g), where the system is a cod + TAG mixture. [c] Based on the total lipid content of the cod–triacylglycerol system. [d] Based on the membrane lipid content. [e] Ethanol. [f] Nondetectable. [g] Not determined.

Table 6. Effect of Antioxidant Carrier Solvent on δ-Tocopherol Incorporation into Membranes[a,b]

% oil added	carrier solvent	TOH (μg)[c] / system (g)	tocopherol concn (ppm)		
			system	membrane	oil
7	ethanol[d]	23.2 ± 0.5	362 ± 31	367 ± 17a	291 ± 16
7	oil[e]	23.3 ± 0.1	334 ± 0.4	108 ± 30a	295 ± 42

[a] $n = 3$, where n = number of replicates for each experiment (number of analyses within each experiment = 1). [b] Letter a indicates which values in each column differed significantly ($p < 0.05$). [c] Amount of δ-tocopherol added (μg)/system (g), where the system is cod + canola oil. [d] Ethanolic tocopherol was added to cod muscle containing 7% oil. [e] Tocopherol was dissolved in 2% oil. This mixture was added to cod muscle containing 5% oil.

In the second case, ethanolic tocopherol was added to cod muscle containing 5% added oil. After thorough mixing, an additional amount of oil/fat (20% based on original muscle weight) was added. The 5% oil level was to duplicate a moderately lean product. The tocopherol concentration was 300−350 ppm based on the final total lipid contents. In other words, the same amount of ethanolic tocopherol was added whether directly to a product with 25% added oil or first to a product with 5% oil followed by an additional amount of triacylglycerol (59 μg of tocopherol per gram of minced cod with 25% added triacylglycerol). When the antioxidant was added to the system with 5% oil, the concentration of tocopherol in the membrane fraction was 869 ppm. When an additional 20% oil was added and mixed, the tocopherol concentration in the membrane decreased to 361 ppm. The tocopherol concentration in the oil fraction was 320 ppm. When the 20% oil was substituted with 20% beef fat, the final tocopherol concentration in the membrane was 729 ppm, whereas the concentration of tocopherol in the triacylglycerol fraction was 178 ppm.

Effect of Carrier Solvent on Tocopherol Incorporation into Membranes. Ethanol and oil were used as the antioxidant carrier solvents (**Table 6**). δ-Tocopherol in ethanol was added to minced cod muscle to which 7% canola oil had first been added and mixed. When oil was used as the carrier solvent, it was added to the cod muscle in two stages. In the first stage, oil without tocopherol was added and mixed with cod muscle at 5% of the muscle weight. In the second stage, 2% oil (based on the initial muscle weight) containing the appropriate amount (23 μg) of tocopherol was added and mixed. This was to maintain the total amount of added oil at 7% but to change the tocopherol carrier solvent from ethanol to oil. For tocopherol added to cod mince containing similar amounts of oil, a higher concentration of tocopherol was incorporated into the membrane (367 ppm) with ethanol as the carrier than with oil as the carrier (108 ppm) (**Table 6**). The concentration of δ-tocopherol in the oil fraction was essentially the same with either carrier.

DISCUSSION

Lipid-soluble antioxidants are added to food tissues at a specific concentration based on the total lipids. However, it is not generally known how much of the antioxidant actually goes into the different lipid fractions. This becomes especially problematic when the carrier is a water-miscible solvent such as ethanol. δ-Tocopherol is almost insoluble in water, but it has considerable solubility in ethanol, which could be used as a carrier solvent for the antioxidant. However, as soon as the relatively small amount of ethanol comes in contact with the aqueous phase of the minced muscle, the ethanol would be greatly diluted and no longer able to keep the tocopherol soluble. The question we wished to address in this work was what factors affect the distribution of a lipid-soluble antioxidant, δ-tocopherol, between the two major lipid fractions in muscle, that is, the triacylglycerols and the polar lipids of the membranes.

When tocopherol was added in ethanol to lean minced cod fish muscle, which has <1% total lipid with most of it occurring in the membrane lipids, only about one-third of the tocopherol was recovered in the isolated membrane fraction. This recovery was estimated on the basis of the concentration found in the isolated membrane versus what it should have been if all of the tocopherol had been taken up into the membrane lipid fraction. At least a portion of the tocopherol did not go into the membrane fraction because 24% was recovered in the press juice. It is possible that when the ethanolic solution of tocopherol under-

Table 7. Effect of Order of Addition of Ethanolic Tocopherol and Oil to Cod Muscle on Recovery of Tocopherol in Lipid Fractions at 4, 6, and 9.5% Oil Contents[a]

TOH[c] added before/after oil addition	% oil in system	TOH added (μg)[d]/ system (g)	% recovery of TOH[b]	
			membrane	oil
before	4.2	17.1	15	39
before	6.0	23.4	23	47
before	9.7	38.3	21	58
after	6.3	2.5	9	84
after	4.1	16.9	9	83
after	5.7	23.2	8	91
after	9.3	38.2	5	90

[a] $n = 3$, where n = number of replicates for each experiment (number of analyses within each experiment = 1). [b] Percentage recovery of TOH based on the amount of TOH added to the cod muscle. [d] TOH = δ-tocopherol in carrier solvent ethanol. [d] Amount of δ-tocopherol added (μg)/system (g), where the system is cod + canola oil.

went rapid dilution on addition to the aqueous minced muscle, some of it formed hydrophobic droplets or micelles that simply remained in the aqueous phase. One-third of the tocopherol was not accounted for in either the aqueous phase or the membrane fraction (assuming that the isolated aqueous phase and the membranes were representative of their respective fractions). It is possible that some of the tocopherol interacted with hydrophobic sites of the myofibrillar or other insoluble proteins.

To estimate recoveries, it was necessary to assume that the antioxidant that was present in the separated oil and isolated membranes was representative of the oil and the membranes that were not separated. It would seem that this assumption would be most tenuous in the case of the membranes. Nevertheless, we did see a pattern of distribution of the tocopherol when added in ethanol between the membrane and oil fractions based on the concentration of oil added to the system (**Table 7**). When ethanolic tocopherol was added to the muscle before the oil, the fraction of tocopherol in the membrane preparation was relatively constant (15−23%) over a range of oil content of ∼4−10%, whereas the oil showed a steady increase in recovery from 39% at 4% oil to ∼58% at 10% oil. Over the same range of oil contents, there was a consistent distribution of tocopherol from 5 to 9% in the membranes and from 83 to 91% in the oil when ethanolic tocopherol was added after the oil had been put into the system. The total estimated recoveries of tocopherol in the two lipid fractions ranged from 91 to 99%. These data indicate that there was a competition for the added tocopherol between the oil and membrane fractions and that when the oil was added first, it could compete much better for the tocopherol than if it was added after the ethanolic tocopherol. Oil can extract some tocopherol previously taken up by the membranes.

The method of estimation of the amount of membranes isolated was based on phospholipid content. The amount of phospholipid associated with the membrane fraction obtained from a given amount of muscle tissue was compared to the initial phospholipid content of that muscle tissue. The estimation was based on the assumption that essentially all of the phospholipid in the muscle tissue is in the membrane fractions. This may not be entirely accurate because a considerable amount of phospholipid has recently been found in the Z-disk of the muscle tissue of some species (*15*). Any phospholipid in the muscle tissue that was not part of the membrane would lead to an underestimation of the recovery of the membranes from the tissue. The Z-disk phospholipid should be removed during the first centrifugation at 10000*g* for 20 min. The membrane fraction was obtained at centrifugations between 10000*g* for 20 min and 130000*g* for 30 min.

When δ-tocopherol was added to a system of lean cod fish muscle tissue and exogenously added triacylglycerols, there was a competition between the lipids of the membranes and the oil for the available antioxidant. The results of this competition depended on several factors. With an increasing concentration of oil and a constant amount of membrane lipids, a larger amount of the antioxidant was shunted into the oil phase. In the absence of any oil in the system before the addition of ethanolic δ-tocopherol, more antioxidant was incorporated into the membranes than if oil was added before the ethanolic δ-tocopherol. However, the recovery of tocopherol in the lipid fractions was less if the antioxidant was added before the oil. Presumably, this was due to agglomeration of the tocopherol in the aqueous phase, which occurred upon dilution of the ethanol carrier and insolubilization of the antioxidant. Alternatively, the tocopherol may have bound to other components in the aqueous phase such as the proteins. A higher amount of δ-tocopherol was incorporated into the membrane fractions when it was added in ethanol than when it was added in oil. Presumably, the more hydrophilic character of the ethanol favored the interaction with the polar surface of the membrane lipids as opposed to the more hydrophobic oil droplets. If the triacylglycerol was in a solid state, that is, as a fat, incorporation of the tocopherol into that fraction was small.

Regardless of other factors, at a given concentration of triacylglycerol and a given amount of tocopherol, higher concentrations of tocopherol were incorporated into the membrane fraction when the antioxidant was added in ethanolic solution before the addition of oil. If tocopherol was added to minced muscle with a moderate amount of oil to which more oil was to be added, the incorporation of the antioxidant into the membrane was greater if the tocopherol was added before the excess oil. Presumably, this occurred because of the decreased competition for the antioxidant in the presence of the lower amount of oil.

The data presented in this paper can give some strategic approaches for incorporating a lipid-soluble antioxidant selectively into the oil and/or membrane fractions of minced muscle tissue. This should give better protection to the unstable membrane lipids. The information may also be used to selectively incorporate different lipid-soluble antioxidants into different fractions of a muscle food. For example, one lipid-soluble antioxidant may be added into lean tissue in a relatively polar solvent favoring incorporation into the membrane lipid fractions. A second antioxidant may then be incorporated preferentially into the triacylglycerol phase by addition in oil.

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