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Acidolysis Reactions Lead to Esterification of Endogenous Tocopherols and Compromised Oxidative Stability of Modified Oils

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For the first time, a possible mechanism responsible, in part, for the removal of endogenous antioxidants through the formation of tocopheryl esters during acidolysis reactions is proposed and confirmed. Tocopherols in the oils were found to react with carboxylic acids present in the medium, thus leading to the formation of tocopheryl esters that do not render any stability to the resultant modified oils as they lack any free hydroxyl groups on the phenolic ring of the molecule. Tocopheryl oleate, used as a standard, was syntheized through the reaction of acyl chloride of oleic acid with α-tocopherol (*m*/*z* 695.5 as evidenced by mass spectrometry). Subsequently, lipase-assisted esterification of α-, γ-, and δ-tocopherols with oleic acid was carried out, and corresponding tocopheryl esters were isolated. In a real acidolysis reaction system involving docosahexaenoic acid single-cell oil and capric acid, high-performance liquid chromatography−mass spectrometry analysis demonstrated the presence of several tocopheryl esters. These included tocopheryl esters of myristic acid, namely, α-tocopheryl myristate, *m*/*z* 641.1, γ-tocopheryl myristate, *m*/*z* 627.1, and δ-tocopheryl myristate, *m*/*z* 613.1, as well as those of palmitic acid, namely, α-tocopheryl palmitate, *m*/*z* 669.1, γ-tocopheryl palmitate, *m*/*z* 655.1, and δ-tocopheryl palmitate, *m*/*z* 641.1. The mixture also contained different species of tocopheryl oleates, namely, α-tocopheryl oleate, *m*/*z* 695.5, γ-tocopheryl oleate, *m*/*z* 681.1, and δ-tocopheryl oleate, *m*/*z* 667.2. Esters produced from reactions of docosahexaenoic acid and tocopherols were also detected, namely, α-tocopheryl docosahexaenoate, *m*/*z* 738.7, and δ-tocopheryl docosahexaenoate, *m*/*z* 710.7.

KEYWORDS: Tocopherols; tocopheryl esters; esterification; oxidative stability; normal phase high-performance liquid chromatography (HPLC)−mass spectrometry (MS); APCI, atmospheric pressure chemical ionization; acidolysis; lipase; structured lipids; capric acid (C10:0); docosahexaenoic acid single-cell oil (DHASCO)

INTRODUCTION

The stability of fats and oils depends on various factors but mainly their fatty acid composition, governed by the degree of unsaturation, the content of endogenous antioxidants, and the presence of oxygen as well as different storage and packaging conditions (*1*). Edible oils consist mainly of triacylglycerols (TAG; 95%). Non-triacylglecerols (also known as minor components or unsaponifiable matter) make up the remaining 5%. These minor components are naturally occurring compounds, some of which have antioxidative properties that give the oil the ability to protect itself against oxidation (*2*, *3*). The minor components of vegetable oils are primarily composed of phospholipids, tocopherols, tocotrienols, flavonoids and other phenolic compounds, pigments (carotenoids and chlorophylls), sterols, and free fatty acids, as well as mono- and diacylglycerols (*2*, *4*). Several classes of minor components might be present in each oil, and these contribute to its oxidative stability (*2*).

Tocols, both tocopherols and tocotrienols, include four naturally occurring homologues (α-, β-, γ-, and δ-), are present in various oils in different proportions and amounts and are known to increase the oxidative stability of foods. Among the tocopherol homologues, α-tocopherol has the highest vitamin E activity and occurs most abundantly in natural sources. Although α-tocopherol has been the center of vitamin E studies, γ- and δ-tocopherols are known to have stronger antioxidant activity in vitro. Generally, the antioxidant activity of tocopherols is in the order of δ- > γ- > β- > α-tocopherol (*5*). Results from in vitro studies have demonstrated that γ-tocopherol is 1.4 times as efficient as α-tocopherol in inhibiting the oxidation of polyunsaturated fatty acids (*6*). Nevertheless, α-tocopherol serves as a more powerful antioxidant than γ-tocopherol in vivo (*5*).

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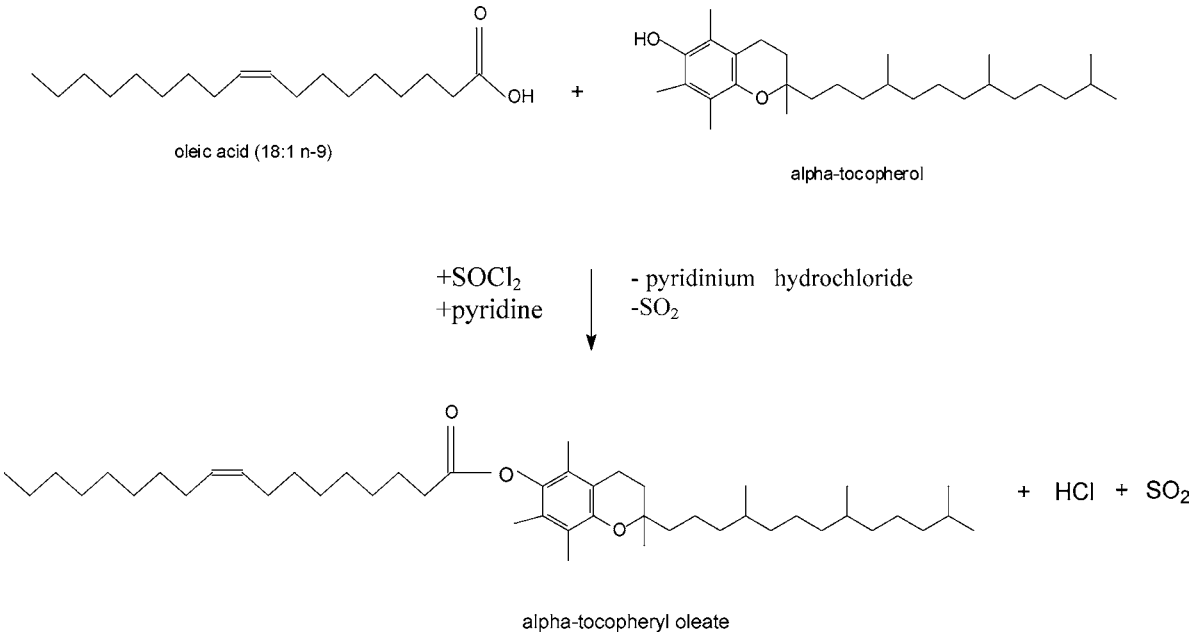

Figure 1. Synthesis of α-tocopheryl oleate from the reaction of oleic acid and α-tocopherol.

During the processing of oils and in the production of structured and specialty lipids many of the minor constituents are eliminated, leading to faster oxidative deterioration of the modified oils. In enzymic acidolysis of algal oils with capric acid, we found that the resultant oils were found to be less stable than their unmodified counterparts despite an increase in the degree of saturation of the products due to the incorporation of capric acid in the glycerol backbone of the molecules (*7*−*9*). Senanayake and Shahidi (*10*) reported that removal of the minor components, such as tocopherols, during modification of borage and evening primrose oils with n-3 fatty acids might play a significant role in the compromised oxidative stability of the resultant fats or oils as much lower amounts of tocopherols were present in the modified oils. Akoh and Moussata (*11*) reported a considerable loss of tocopherols in fish- and canola-based structured lipids (SL) containing caprylic acid. However, these studies did not investigate the exact mechanism by which the removal and loss of natural antioxidants during processing and synthesis of specialty lipids took place. This study aimed to investigate the reasons behind the compromised stability of the stuctured lipids so produced, particularly the formation of tocopheryl esters during the acidolysis of oils from the reaction of free carboxylic acids in the medium and tocopherols present in the oils. Model systems were also employed to demonstrate this viewpoint.

MATERIALS AND METHODS

Materials. *Pseudomonas* sp. (PS-30) was obtained from Amano Enzyme (Troy, VA). Oleic acid was purchased from Nu-Check (Elysian, MN). Tocopherols (α-, γ-, and δ-tocopherols) were purchased from Sigma Chemical Co. (St. Louis, MO). Docosahexaenoic acid (DHA) single-cell oil (DHASCO) containing 40% DHA was obtained from Martek Bioscience Corp. (Columbia, MD). All solvents used in these experiments were of analytical grade and were purchased from Fisher Scientific (Nepean, ON, Canada).

Methods. In general, oleic acid (100 mg) was mixed with α-, γ-, and δ-tocopherols at a mole ratio of oleic acid to tocopherol of 1:1, in a screw-capped test tube, and then 10% by weight of substrates of the most effective lipase (PS-30 from *Pseudomonas* sp.) and water (2% by weight of substrates and enzyme) were added in *n*-hexane (3.0 mL). The mixture was flushed with a stream of nitrogen, stirred, and then incubated for 48 h in an orbital shaker at 250 rpm at 45 °C. In another set of experiments, DHASCO was mixed with capric acid (CA) (10:0) at a mole ratio of 1:3 (DHASCO/CA), at 45 °C, over 24 h in the presence of 4% lipase from *Pseudomonas* sp. and 2% water content. The mixture was flushed with a stream of nitrogen, stirred, and then incubated for 24 h in an orbital shaker at 250 rpm at 45 °C.

Separation of Tocopheryl Esters after Acidolysis. After a given time period, the reaction was stopped by the addition of a mixture of acetone and ethanol (20 mL, 1:1, v/v). To neutralize the released and unused free fatty acids, the reaction mixture was titrated with a 0.5 M NaOH solution (using a phenolphthalein indicator) until the solution turned pink. The tocopheryl esters were then extracted into *n*-hexane (25 mL). The two layers (aqueous and *n*-hexane) were allowed to separate in a separatory funnel, and the lower aqueous layer was discarded. The *n*-hexane layer was passed through a bed of anhydrous sodium sulfate to remove any residual water. The *n*-hexane was evaporated using a rotary evaporator at 45 °C and the residue stored at −20 °C until further analysis.

Synthesis of α-Tocopheryl Ester. *Acyl Chloride Syntheses.* Acyl chloride was synthesized according to the method described by Taylor et al. (*12*) with some modifications (**Figure 1**). A 50 mL, three-necked flask equipped with a dropping funnel and a reflux condenser fitted with an inert gas (nitrogen) inlet tube that was attached to a mineral oil bubbler was used. The system was flushed with nitrogen, flame-dried, cooled to room temperature, and maintained under a positive nitrogen pressure. The mixture contained oleic acid (1.0 g) and 0.422 g of α-tocopherol. Distilled thionyl chloride ($SOCl_2$) (0.257 mL, 4.0 mmol) was added dropwise over a 30 min period. Pyridine (5−10 mL) was added to the mixture. The mixture was heated under reflux condition for 7 h. The resultant tocopheryl esters were extracted three times, each with diethyl ether. The mixture was thoroughly mixed and transferred into a separatory funnel. The two layers (aqueous and diethyl ether) were allowed to separate, and the lower aqueous layer was discarded. The diethyl ether layer was then passed through a bed of anhydrous sodium sulfate to remove any residual water. The diethyl ether was evaporated using a rotary evaporator at 45 °C, and the residue was recovered.

Silica gel thin layer chromatography (TLC) plates were evenly sprayed with 5% (w/v) boric acid and dried at 100 °C for 1 h. The mixture was separated on activated TLC plates, which were then developed using hexane/diethyl ether/acetic acid (70:30:1, v/v/v) for 40−50 min and then allowed to air-dry. The bands were located by viewing under a short-wavelength (254 nm) and a long-wavelength (365 nm) light (Spectroline, Co., Westbury, NY). The bands were scraped off and extracted with methanol/chloroform (1:1, v/v). The solvent (methanol/chloroform) was evaporated using a rotary evaporator

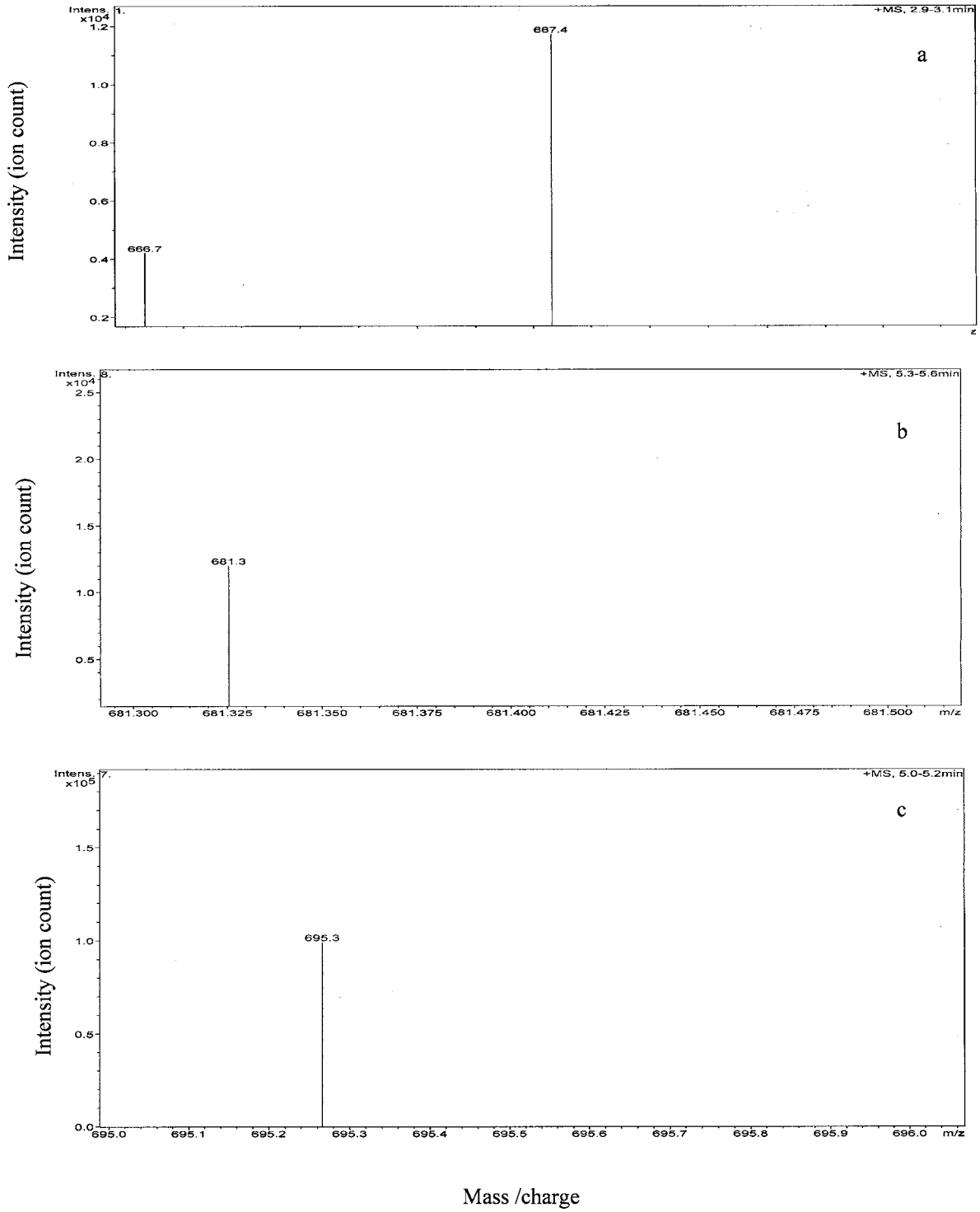

Figure 2. Mass spectrum obtained from HPLC-MS analysis of (**a**) δ-, (**b**) γ-, and (**c**) α-tocopheryl oleate from reactions of oleic acid and δ-, γ-, and α-tocopherols in the presence of lipase PS-30 from *Pseudomonas* sp.

at 45 °C, and the residue was recovered and stored at −20 °C until used for further analysis as a standard.

Normal Phase High-Performance Liquid Chromatography−Mass Spectrometry (HPLC-MS) Analysis of Tocopherols. Tocopheryl esters in samples were determined by HPLC-MS. The analysis was performed using an Agilent 1100 HPLC system (Agilent, Palo Alto, CA) with a UV diode array detector (UV-DAD). Separation was achieved on a Supelcosil LC-Si column (250 mm length, 4.6 mm i.d., 5 μm particle size, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) coupled with a Supelcosil LC-Si guard column. Tocopherols and tocopheryl esters were eluted using an isocratic solvent system containing hexane/2-propanol (95:5, v/v) at a flow rate of 1.0 mL/min. Twenty microliters of each sample was injected. Tocopherols were detected at 290 nm by UV detection. LC flow was analyzed on-line by a mass spectrometric detector system (LC-MSD-Trap-SL, Agilent) with a positive ion atmospheric pressure chemical ionization (APCI), which presents results as M + 1 under the conditions employed. The operating conditions used were 121 V for the fragmentor voltage, 350 °C for the drying temperature, 400 °C for the APCI temperature, 60 psi for the nebulizer pressure, and 7 L/min for the drying gas flow.

RESULTS AND DISCUSSION

It was thought that tocopherols present in the oils might react with carboxylic acids in the reaction medium during acidolysis

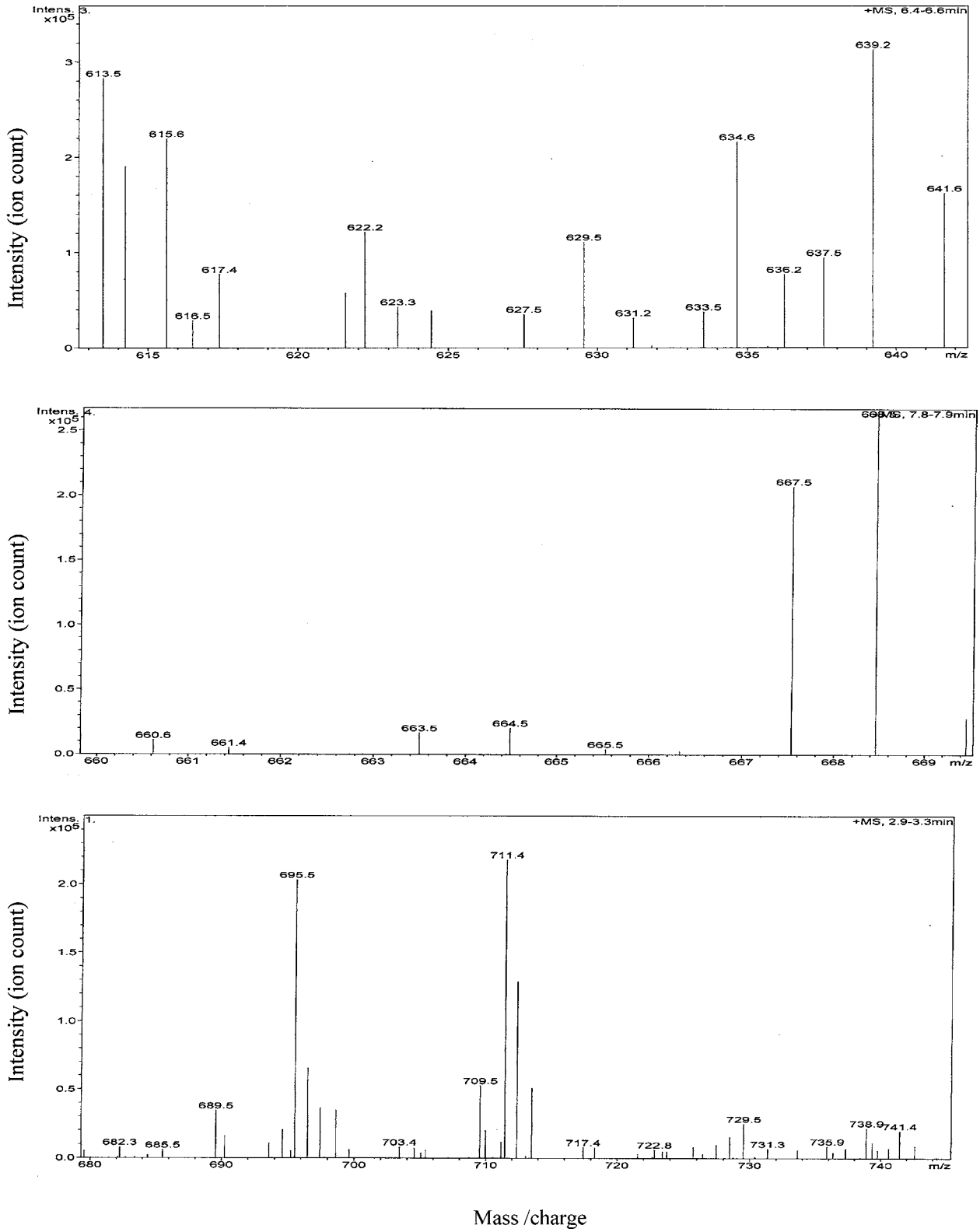

Figure 3. Mass spectrum obtained from HPLC-MS analysis of tocopheryl esters present in DHASCO-based structured lipids.

reaction, thus leading to the formation of tocopheryl esters that are not analyzed as free tocopherols and do not render any stability to the resultant modified oils. To examine this possibility, α-tocopheryl oleate was synthesized through the reaction of oleic acid and α-tocopherol following the formation of the corresponding acyl chloride in the presence of thionyl chloride and pyridine. The mass spectrum of the mixture, using normal phase HPLC-MS, showed the presence of a peak of α-tocopheryl oleate of *m*/*z* 695.5. In another set of experiments, reaction products of tocopheryl esters from model systems that used oleic acid and α-, γ-, and δ-tocopherols in the presence of enzymes were subjected to HPLC-MS analysis. The results obtained showed the presence of the corresponding esters as evidenced by *m*/*z* 695.5, 681.3, and 667.4 data, respectively, for each isolated compound (**Figure 2**).

The third step was designed to detect the molecular species of tocopheryl esters in a real acidolysis reaction system involving an algal oil and CA. For this purpose, DHASCO-based SL was

Table 1. Major Tocopheryl Esters Identified in the Acidolysis Reaction of DHASCO with CA[a]

tocopheryl ester	*m/z*
δ-tocopheryl myristate (δ-TMA)	613.5
γ-tocopheryl myristate (γ-TMA)	627.5
α-tocopheryl myristate (α-TMA)	641.6
δ-tocopheryl palmitate (δ-TPA)	641.1
γ-tocopheryl palmitate (γ-TPA)	655.1
α-tocopheryl palmitate (α-TPA)	669.1
δ-tocopheryl oleate (δ-TOA)	667.5
γ-tocopheryl oleate (γ-TOA)	681.2
α-tocopheryl oleate (α-TOA)	695.5
δ-tocopheryl docosahexaenoate (δ-TDHA)	710.7
α tocopheryl docosahexaenoate (α-TDHA)	738.7

[a] The fatty acids in the modified oil were capric acid (10.2%), myristic acid (10.0%), palmitic acid (7.90%), oleic acid (26.3%), and docosahexaenoic acid (37.1%) (*7*).

produced under optimum conditions as described by Hamam and Shahidi (*7*). DHASCO was selected because it had the highest content of total tocopherols among several algal oils that were examined earlier [1140 mg/kg compared to 790 mg/kg in arachidonic acid single-cell oil, ARASCO; and 730 mg/kg in the OMEGA-GOLD oil rich in DHA and DPA (22:5n-6)] (*13*). The results indicated that the original, unmodified oil was more stable than the modified counterpart when oils were subjected to accelerated oxidation under Schaal oven condition at 60 °C, despite a higher degree of saturation of the product. This was explained as being due to the removal of natural antioxidants from the oil (*7*), but the mechanism by which tocopherols were removed was not specified.

The mass spectrum obtained from HPLC-MS analysis of tocopheryl esters of the modified DHASCO with CA are shown in **Figure 3**, and major peaks are specified and summarized in **Table 1**. A number of tocopheryl esters were identified in the reaction products, and these included those of myristic acid esterified to tocopherols, notably, α-tocopheryl myristate, *m/z* 641.1, γ-tocopheryl myristate, *m/z* 627.1, and δ-tocopheryl myristate, *m/z* 613.1, as well as palmitic acid and tocopherol homologues, namely, α-tocopheryl palmitate, *m/z* 669.1, γ-tocopheryl palmitate, *m/z* 655.1, and δ-tocopheryl palmitate, *m/z* 641.1. The mixture also contained different species of tocopheryl oleates; these were α-tocopheryl oleate, *m/z* 695.5, γ-tocopheryl oleate, *m/z* 681.1, and δ-tocopheryl oleate, *m/z* 667.2. Esters produced from reactions of DHA and different tocopherols were also detected, namely, α-tocopheryl docosahexaenoate, *m/z* 738.7, and δ-tocopheryl docosahexaenoate, *m/z* 710.7. Thus, many species of tocopheryl esters were present, only major ones in the DHASCO-based structured lipids. The data on the oxidation stability of the oils upon storage under Schaal conditions at 60 °C for 48 h indicated that the conjugated dienes and thiobarbituric reaction substances (TBARS; μmol/g of oil) were 9.4 and 4.7 for the original oil and 30.8 and 16.5 for the corresponding modified oil, respectively (*7*). This confirms the assumption that the formation of tocopheryl esters from the reactions of carboxylic acids and tocopherols present in the oils is a possible route by which endogenous tocopherols are removed from the oils and a contributing factor responsible for the rapid oxidative deterioration of structured lipids.

ABBREVIATIONS USED

DHA, docosahexaenoic acid; DHASCO, docosahexaenoic acid single-cell oil; *m/z*, mass/charge; TLC, thin layer chromatography; CA, capric acid; HPLC-MS, high-performance liquid chromatography−mass spectrometry; APCI, atmospheric pressure chemical ionization.

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