JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Reinvestigation of the Effect of Heat Pretreatment of Corn Fiber and Corn Germ on the Levels of Extractable Tocopherols and Tocotrienols

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We previously reported that heat pretreatment of corn fiber (150 °C, 1 h) caused a tenfold increase in the levels of extractable γ-tocopherol. The current study was a reinvestigation of the previous effect, using improved methods (HPLC with fluorescence detection, diode-array UV detection, and mass spectrometry) for tocol analysis. Heat pretreatment did not cause an increase in the levels of any of the tocopherols or tocotrienols in corn fiber oil, but lowered the levels of three of the tocols and had no effect on the levels of the other two tocols. Heat pretreatment of corn germ had a similar effect. UV and mass spectra indicated that the peak that we had identified as γ-tocopherol in our previous report was probably a mixture of oxidation products of triacylglycerols. Thus, heat treatment of corn germ or other corn-oil containing fractions at high temperatures leads to decreases in γ-tocopherol, γ-tocotrienol, and δ-tocotrienol and to the production of triacylglycerol oxidation products.

KEYWORDS: Heat; tocopherol; tocotrienol; roasting; oxidation; hydroperoxide.

INTRODUCTION

Previously, we reported that heat pretreatment at 150 °C for 1 h of corn fiber caused a tenfold increase in the levels of extractable γ-tocopherol (*1*). In the previous report we used HPLC with UV detection at 280 nm to quantitatively analyze γ-tocopherol, and γ-tocopherol standard was used to identify the retention time and to construct a calibration curve for quantification. Others have recently attempted to study the effect of heat treatment of rice bran (*2*), rice germ (*3*), and safflower seeds (*4*) and have reported little or no increase in the levels of tocopherols and tocopherols; however, some have noted significant increases in extractable levels of other phytochemicals, such as oryzanols in rice bran. The current study re-evaluates the effect of heat pretreatment of corn fiber on the levels of tocopherols and tocotrienols, using HPLC with more accurate detection methods, including fluorescence, UV diode-array detection, and mass spectrometry. Because tocopherols and tocotrienols fluoresce under excitation at about 296 nm and emission at about 326 nm, methods that employ this type of detection (*5*, *6*) are more selective and about 30-fold more sensitive than those that employ UV detection.

MATERIALS AND METHODS

Samples of yellow dent #2 corn kernels (Pioneer H3361) were grown at the University of Illinois and harvested in 2005. Wet-milled corn fiber (also called coarse fiber or pericarp fiber) was supplied by a commercial corn wet mill. Dry-milled corn germ was supplied by a commercial corn dry mill. Samples of corn kernels and corn fiber were milled to 20 mesh (<0.85 mm) with a Wiley Mill (Thomas Scientific, Philadelphia, PA). Samples (20−30 g) of dry-milled corn germ were ground for 10 s with a model 203B coffee mill (Krups) and passed through a 20-mesh sieve (<0.85 mm).

Heat Pretreatment and Extraction. Two sets of triplicate 4-g samples (control and 150 °C heat pretreatment) were prepared in 50-mL screw-cap tubes. The heat-pretreatment samples were positioned horizontally with material spread out along the tube wall and no cap in an oven set at 150 °C for 1 h (Precision Thelco Laboratory Oven, Winchester, VA). After heating, the heat-pretreatment sets were placed horizontally on the bench top to cool to touch. Following cooling, all samples of heat pretreatment and control were extracted with hexane as follows: 40 mL of hexane was added, the tube was capped, inverted 30 times to mix, and placed on a Burrell Wrist-Action Shaker (Burrell Corp., Pittsburgh, PA) for 1 h, stopping to remix at 30 min. After the extraction, the solids were separated from the solvent by vacuum filtration using a Buchner funnel and Whatman GF/A paper (5.5 cm). The solvents were collected, and the solids were discarded. From each sample, three separate extractions were performed, and two HPLC separate injections were made from each extract.

Tocopherol and Tocotrienol HPLC Analyses (method A). Tocopherols and tocotrienols were quantitatively determined using a modified version of the previously published method (*5*). The HPLC was a Hewlett-Packard model 1100 with autosampler, and detection was accomplished by an HP model 1100 fluorescence detector (Agilent Technologies, Avondale, PA), with excitation at 294 nm and emission at 326 nm. The column was 100 × 3 mm i.d., 7 μm, DIOL column (Chrompack, Raritan, NJC), and the flow rate was 0.5 mL/min. The binary gradient consisted of the following: solvent A, hexane/THF 98/2, v/v, and solvent B, isopropyl alcohol. Gradient timetable: at 0−40 min, 100% A; 40−45 min, 100−95% A; 50−51 min, 95−100% A; 51−60 min, 100% A. The minimum detectable limits of tocols was

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10.1021/jf061422g
Published on Web 09/26/2006

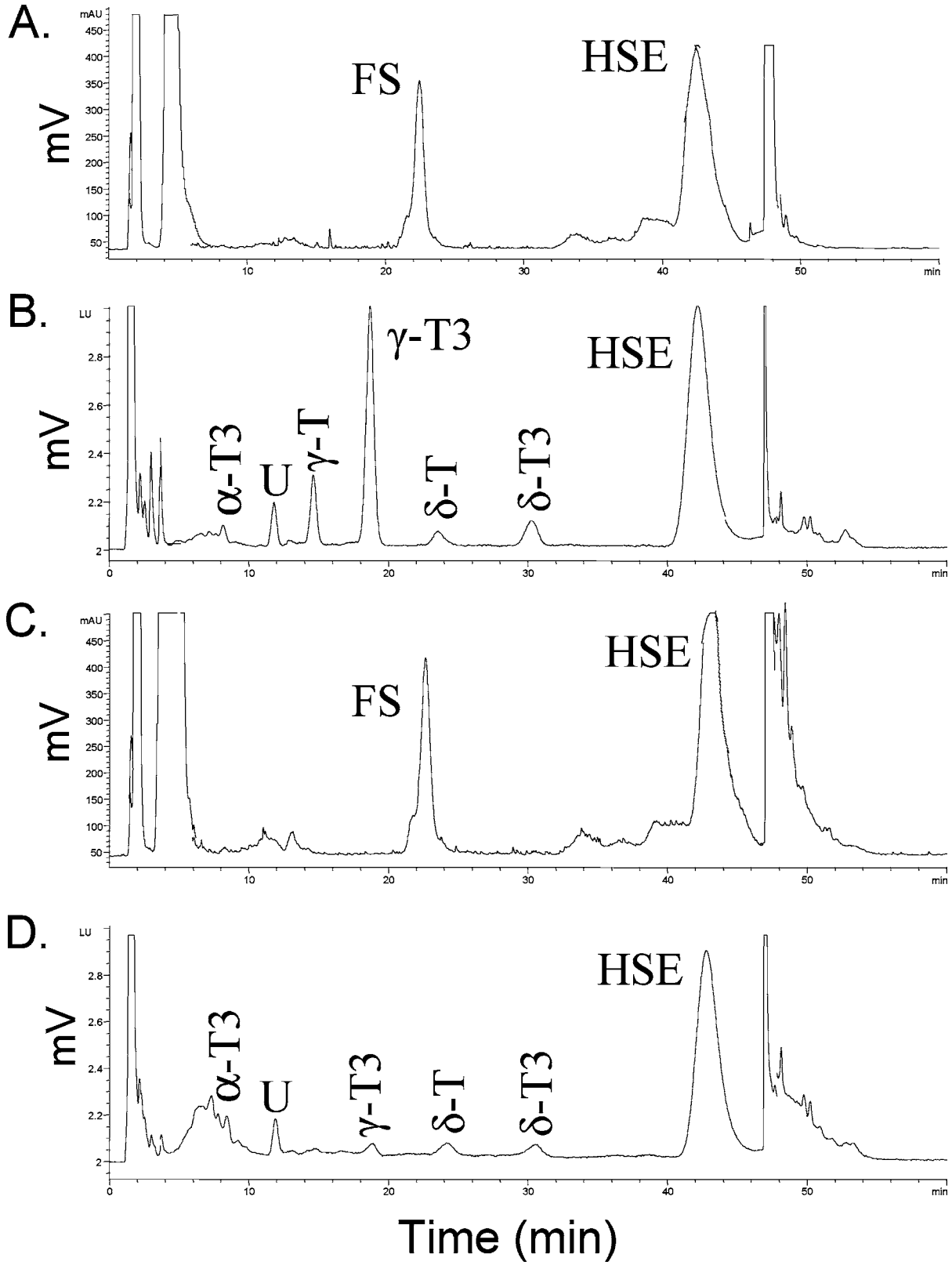

Figure 1. Chromatogram (method A) showing the tocopherols and tocotrienols in an extract of corn fiber, before and after heating of the fiber. (**A**) Unheated, evaporative light scattering detector (ELSD); (**B**) unheated, fluorescence detection; (**C**) heated 150 °C, ELSD; (**D**) heated 150 °C, fluorescence detection. Abbreviations: FS, free sterol; HSE, hydroxycinnamate steryl ester; T, tocopherol; T3, tocotrienol; U, unknown.

about 0.1 ng per injection, and a standard curve was constructed with α-tocopherol in the range of 1−200 ng per injection; this curve was used to quantify all tocopherols. According to AOCS Official Method Ce 8-89, α-tocopherol can be used as a standard for all four tocopherols and all four tocotrienols as long as this is clearly stated (*6*). The structures and retention times of the tocopherols and tocotrienols were confirmed by purchasing gelcap supplements of tocopherols (Bio E Gamma Plex, Soloray Inc., Park City, UT) and tocotrienols (Tocopherol Complex, Solgar, Leonia, NJ) at a local vitamin store; α-tocopherol (αT) (M + 1, *m*/*z* 431.4), α-tocotrienol (αT3) (M + 1, *m*/*z* 425.3), γ-tocopherol (γT) (M + 1, *m*/*z* 416.3), γ-tocotrienol (γT3) (M + 1, *m*/*z* 411.2), δ-tocopherol (δT) (M + 1, *m*/*z* 402.3), and δ-tocotrienol

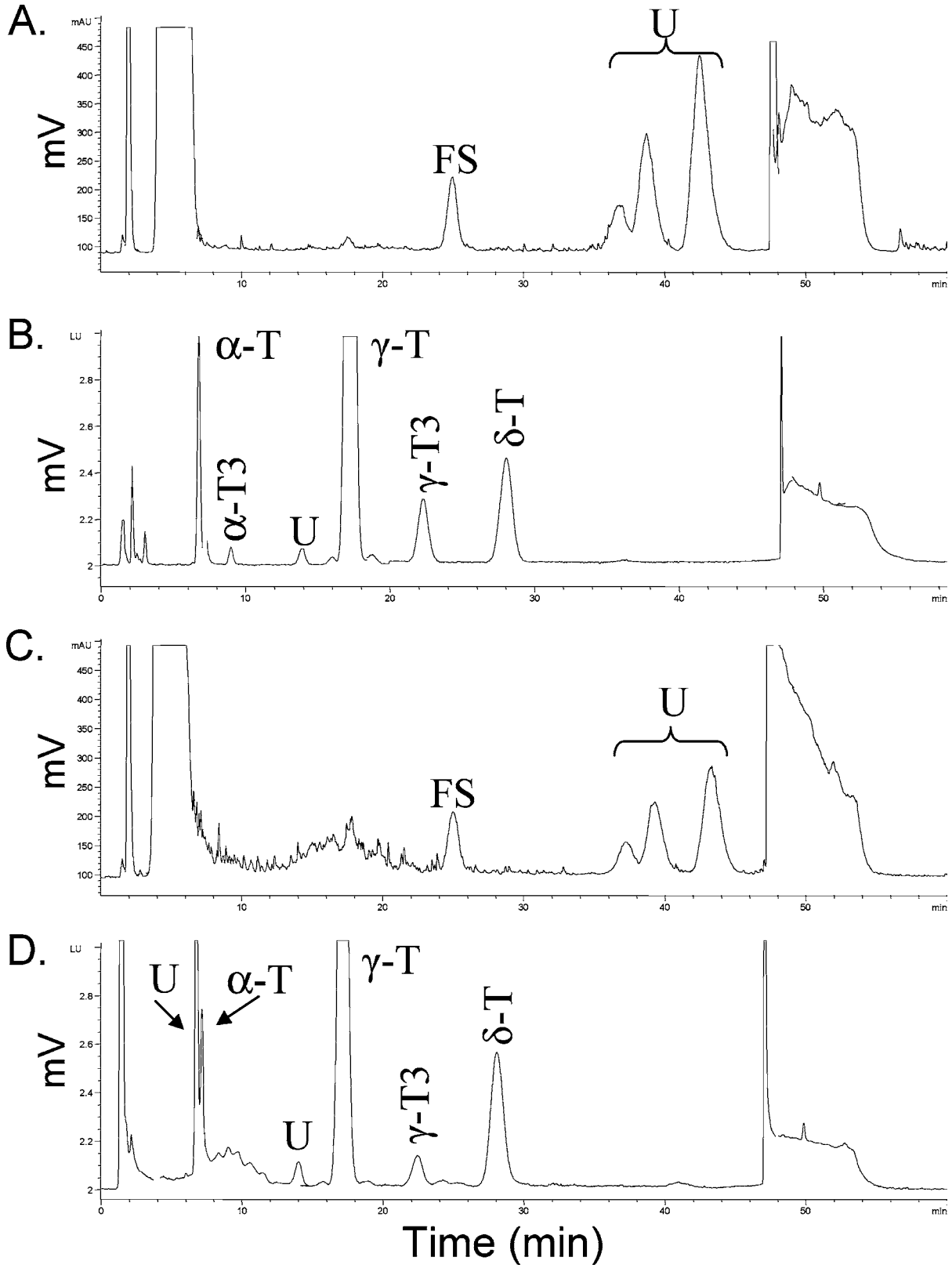

Figure 2. Chromatogram (method A) showing the tocopherols and tocotrienols in an extract of corn germ, before and after heating the germ. (**A**) Unheated, ELSD; (**B**) unheated, fluorescence detection; (**C**) heated 150 °C, ELSD; (**D**) heated 150 °C, fluorescence detection.

(δT3) (M + 1, *m/z* 397.1), were confirmed by LC-MS, performed with an Agilent 1100 MSD equipped with an atmospheric pressure chemical ionization (APCI) interface operated in the positive mode (drying gas at 6.0 L/min, nebulizer pressure at 60 psi, drying gas temperature at 350 °C, vaporizer gas temperature at 325 °C, capillary voltage at 4000 V, corona current at 4.0 μA, and fragmentor at 80 v).

Nonpolar Lipid Class HPLC Analyses (method B). Nonpolar lipids (including phytosterol fatty acyl esters, hydroxycinnamate phytosterol esters, triacylglycerols, and free fatty acids) were quantitatively analyzed by an updated version of a normal phase HPLC method with evaporative light-scattering detection (*7*). These analyses were performed on a Hewlett-Packard model 1050 HPLC, with

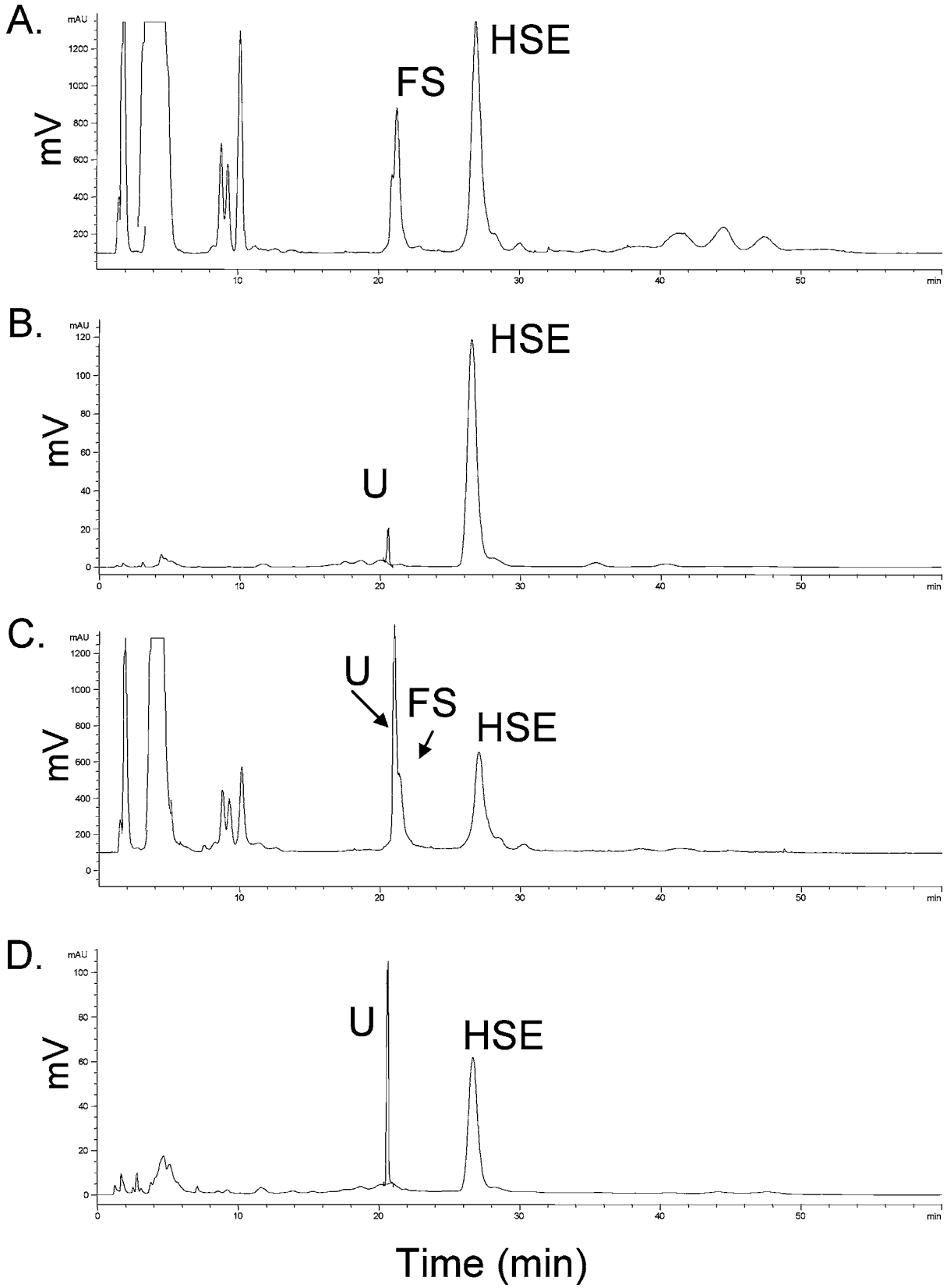

Figure 3. Chromatogram (method B) showing the nonpolar lipids in an extract of corn fiber, before and after heating of the fiber. (**A**) Unheated, ELSD; (**B**) unheated, UV 280; (**C**) heated 150 °C, ELSD; (**D**) heated 150 °C, UV 280.

autosampler, and detection by both an HP model 1050 diode-array UV−visible detector (DAD) (Agilent Technologies, Avondale, PA) and an Alltech-Varex MKII evaporative light-scattering detector (ELSD) (Alltech Associates, Deerfield, IL), operated at 40 °C and a nitrogen gas flow rate of 1.7 L per min. The diol column and flow rates were the same as above. The ternary gradient consisted of solvent A, hexane/acetic acid (1000/1), solvent B, hexane/isopropyl alcohol (100/1). Gradient timetable: at 0−8 min, 100% A; 8−10 min, 100−75% A; 10−40 min, 75% A; 40−41 min, 75−100% A; 41−60 min, 100% A.

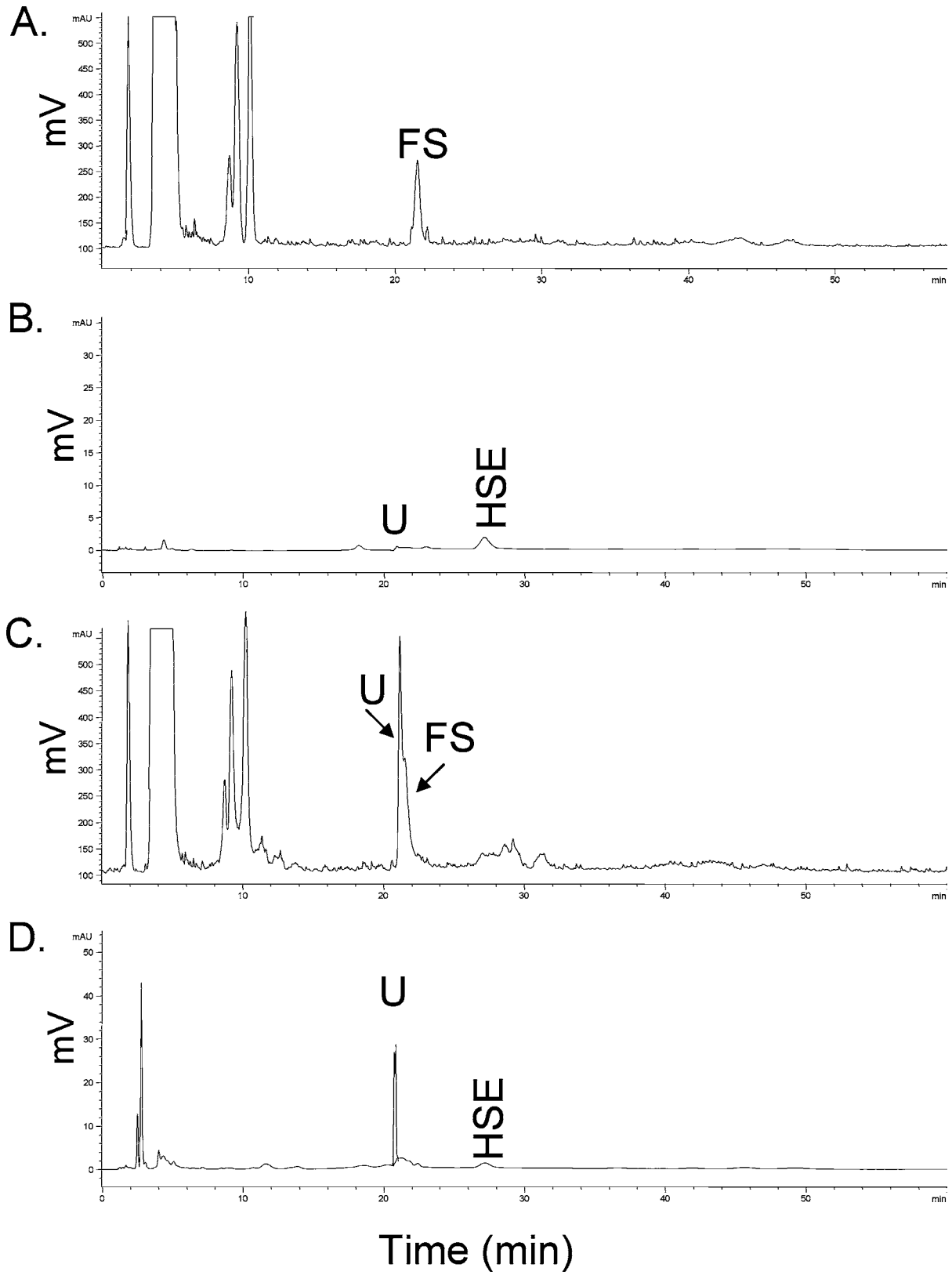

Figure 4. Chromatogram (method B) showing the nonpolar lipids in an extract of corn germ, before and after heating of the germ. (**A**) Unheated, ELSD; (**B**) unheated, UV 280; (**C**) heated 150 °C, ELSD; (**D**) heated 150 °C, UV 280.

Nonpolar Lipid Reverse Phase Molecular Species HPLC Analyses (method C). This method was recently developed to separate molecular species of triacylglycerols and other nonpolar lipids. The HPLC was the same Hewlett-Packard model 1100 with autosampler and Agilent 1100 MSD equipped with an atmospheric pressure chemical ionization interface, described above. The column was a 150 × 2.1 mm i.d., 3 μ, Prevail RP18 (Alltech Associates, Deerfield, IL). The binary gradient had a constant flow rate of 0.5 mL/min, with solvent A, methanol/acetonitrile/dichloromethane/acetic acid (500/470/30/2, v/v/v/v); solvent B, isopropyl alcohol. Gradient timetable: 0−20 min, 100−95% A; 20−40 min, 95−50% A; 40−50 min, 50% A; 50−51 min, 50−95% A; 51−60 min, 100% A.

All experiments were performed at least twice with triplicate samples each time. The data presented are means ± SD.

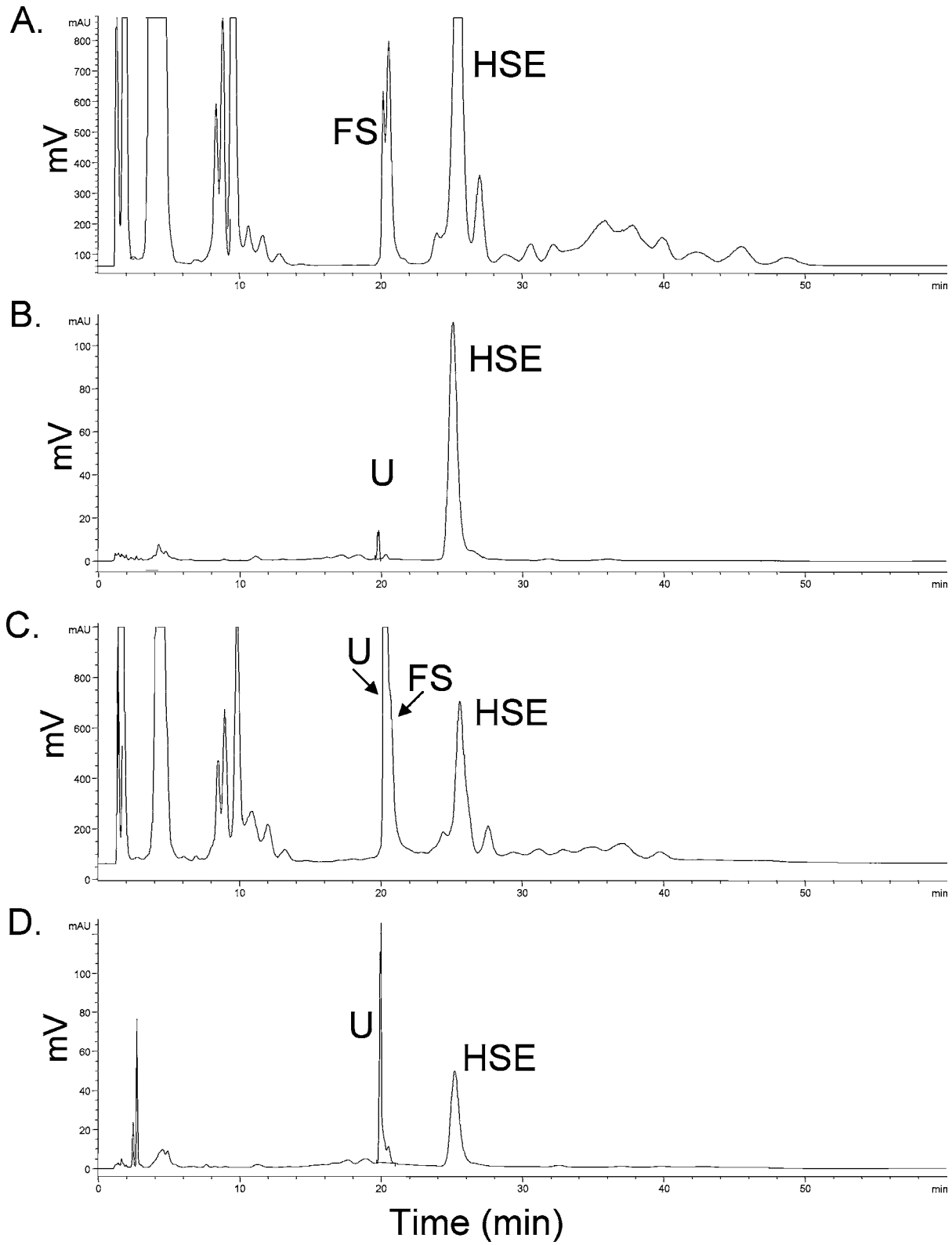

Figure 5. Chromatogram (method B) showing the nonpolar lipids in a sample of corn fiber oil, before and after heating of the corn fiber oil. (**A**) Unheated, ELSD; (**B**) unheated, UV 280; (**C**) heated 150 °C, ELSD; (**D**) heated 150 °C, UV 280.

RESULTS AND DISCUSSION

Previously, we reported that heat pretreatment of corn fiber (150 °C for 1 h) increased the levels of extractable γ-tocopherols from a level of about 0.3% to about 3% of the oil (*1*). In the previous experiment, γ-tocopherol was quantitatively analyzed using a normal phase HPLC method (*7*) with a UV detector at 280 nm. Recently, several more sensitive methods have been published for the analysis of the four tocopherol and four tocotrienol isomers using normal phase HPLC and fluorometric detection (*5*, *6*). Using a sensitive normal phase HPLC-fluorescence detection method we identified the three expected tocopherols and three expected tocotrienols in a hexane extract of corn kernels (**Table 1**). Our tocol analysis for the corn kernel extract was consistent with the results of two previous reports

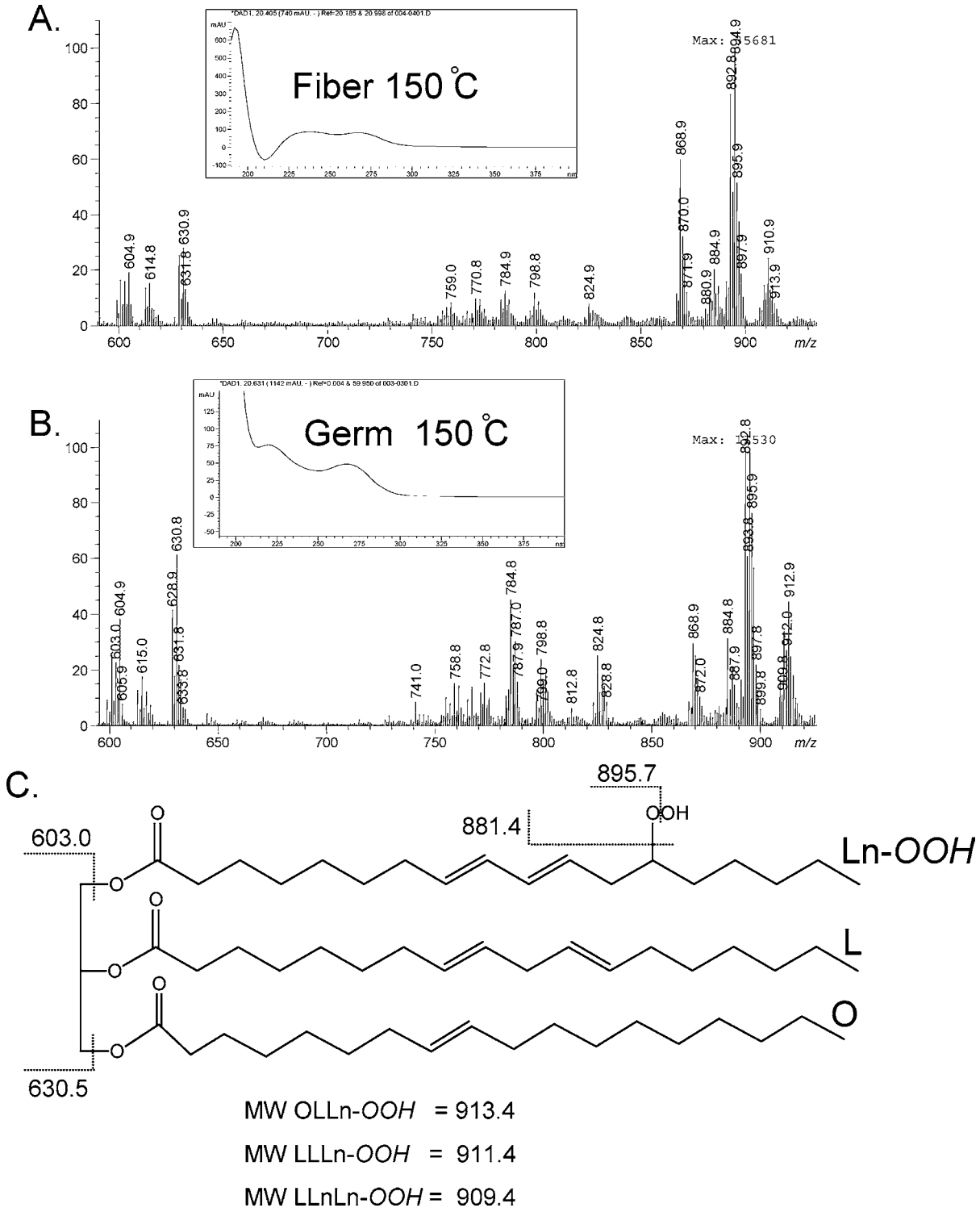

Figure 6. Mass spectra and UV spectra of the unknown peaks (U) from an extract of heated corn fiber (**Figure 3**) and an extract of heated corn germ (**Figure 4**), and evidence that its structure is a triacylglycerol hydroperoxide. Abbreviations: OLLn–OOH, the hydroperoxide of the triacylglycerol, glycerol–oleate–linoleate–linolenate; LLLn–OOH, the hydroperoxide of the triacylglycerol, glycerol–linoleate–linoleate–linolenate; LLnLn, the hydroperoxide of the triacylglycerol, glycerol–linoleate–linolenate–linolenate.

(*8*, *9*). This experiment revealed two unusual observations. First, α-tocopherol was detected in the kernel and bran samples, but it was absent in the corn fiber sample. Second, δ -tocotrienol was present in the corn fiber sample but absent in the kernel and bran samples. These differences may be due to the fact that different cultivars were used to produce the two germ samples, and/or compositional changes may have occurred during storage due to oxidation or UV-induced changes.

The tocopherol/tocotrienol HPLC method with fluorescence detection was then used to reevaluate our previously reported heat pretreatment induction of the levels of γ-tocopherol (**Figure 1** and **Table 1**). Unlike our previous observations, heat pretreatment of corn fiber did not increase the extractable levels of any tocopherols. In fact, heating decreased the levels of γ-tocopherol, γ-tocotrienol, and δ-tocotrienol, and it had little or no significant effect on the levels of δ-tocopherol and

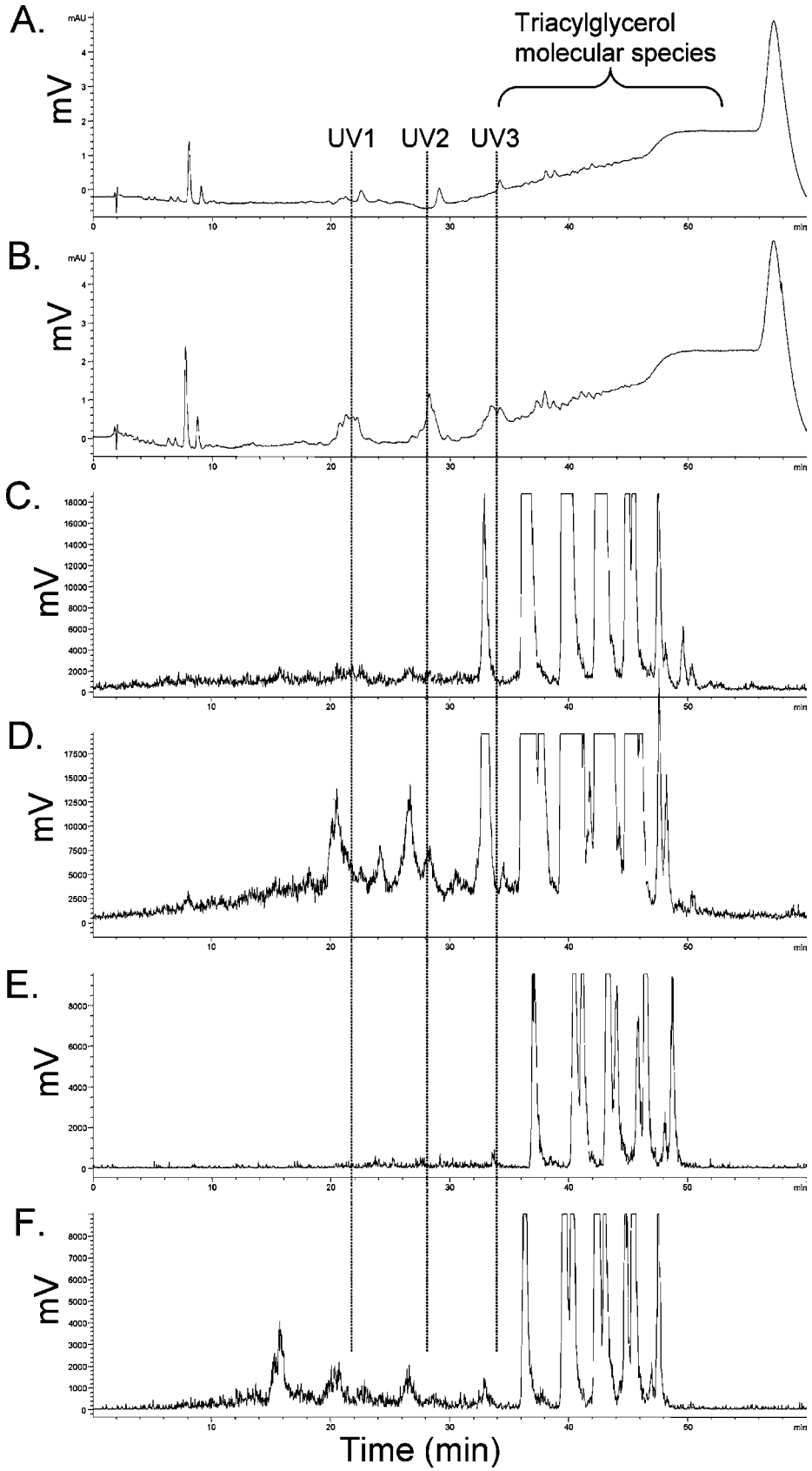

Figure 7. Chromatograms (method C) showing the nonpolar lipids in an extract of corn germ, before and after heating of the germ. (**A**) UV 280 nm, control; (**B**) UV 280 nm, 150 °C, 1 h; (**C**) mass spectrometric detection, SIM, *m*/*z* 850–909, control; (**D**) mass spectrometric detection, SIM, *m*/*z* 850–909, 150 °C, 1 h; (**E**) mass spectrometric detection, SIM, *m*/*z* 909–915, control; (**F**) mass spectrometric detection, SIM, *m*/*z* 909–915, 150 °C, 1 h.

α-tocotrienol (**Table 1**). Also, unlike our previous observation that corn fiber oil obtained from unheated corn fiber had high levels of γ-tocopherol (about 0.3%, on the basis of co-chromatography with γ-tocopherol standards), these new and more accurate analyses revealed that corn fiber oil contained relatively low levels of γ-tocopherol (79 mg/kg, 0.0079%) and

Table 1. Tocopherols and Tocotrienols in Extracts from Control and Heat-Pretreated Oil from Corn Fiber, Corn Germ, and Ground Corn

sample, solvent, °C	% oil extracted	αT (mg/kg)	βT (mg/kg)	γT (mg/kg)	δT (mg/kg)	αT3 (mg/kg)	βT3 (mg/kg)	γT3 (mg/kg)	δT3 (mg/kg)
corn kernel extract	2.70a ± 0.06	386.7 ± 2.3	0	1066.7 ± 26.7	72.2 ± 3.7	138.0 (3.5	0	214.8 ± 2.8	0
corn fiber, unheated	2.13 ± 0.02	0	0	79.0 ± 19.5	35.7 ± 1.2	15.3 ± 2.1	0	378.3 ± 104.2	70 ± 2.7
corn fiber, heated, 150 °C, 1 h	2.25 ± 0.02	0	0	0	36.3 ± 6.1	17.3 ± 1.5	0	26.0 ± 2.7	35.0 ± 9.9
corn germ, unheated	12.88 ± 0.33	218.0 ± 45.2	0	2756.3 ± 95.8	214.8 ± 2.1	15.0 ± 3.3	0	110.7 ± 2.8	0
corn germ, heated, 150 °C, 1 h	13.93 ± 1.11	101.7 ± 10.8	0	1059.7 ± 39.3	251.0 ± 17.1	0	0	46.0 ± 2.4	0

[a] Data presented are means ± SD.

instead the major tocol component was identified as γ-tocotrienol (378 mg/kg).

Because of its economic importance, as the source of all commercial corn oil, we decided to investigate the effect of heating of corn germ on the levels of extractable tocols (**Figure 2** and **Table 1**). Very high levels of γ-tocopherol (2756 mg/kg) and moderate levels of α- and δ-tocopherols and α- and γ-tocotrienols were measured in the extract from unheated germ. Heating the corn germ at 150 °C for 1 h before extraction decreased the level of α-tocopherol (53% decrease), γ-tocopherol (62% decrease), α-tocotrienol (100% decrease), and γ-tocotrienol (58.4% decrease) and slightly increased (17%) the levels of δ-tocopherol.

After analyzing the results of these current experiments and then comparing them with our previous observation of a heating-induced increase in the levels of γ-tocopherol (*1*), it seems reasonable to hypothesize that heating generates an unknown extractable compound and this compound has a retention time identical to that of γ-tocopherol in our previously used nonpolar lipid HPLC system. Like γ-tocopherol, the unknown has strong UV 280-nm absorption, but our current results indicate that it is not γ-tocopherol, or any other tocopherol or tocotrienol. Also, in our current HPLC-fluorescence detection system, we monitored the chromatograms at UV 280 and did not see any peaks which were generated or elevated by heat-pretreatment. It is possible that with the tocol HPLC system, that uses a different mobile phase, the unknown lipid elutes either before or after the tocols and is masked by the other lipids in the extracts.

In the next set of experiments, we used our previous HPLC method (now called method B) to investigate the effect of the heating of corn fiber (**Figure 3**) and corn germ (**Figure 4**) on the levels of nonpolar lipids in the extracts. As we previously observed, with unheated corn fiber, a small peak was detected at 20 min (UV 280 nm) and the peak area increased about fivefold after heating the fiber (**Figure 3**). With unheated corn germ, no 20-min peak was detected, but after heating there was a distinct UV 280-nm absorbing peak at 20 min (**Figure 4**). Finally, we decided to heat a small sample of previously extracted corn fiber oil (**Figure 5**). We also observed a small peak at 20 min in the unheated corn fiber oil, and the peak increased more than tenfold after heating the oil (**Figure 5**). The unknown 20-min peak in the UV chromatogram was just before the 21-min peak of free sterols (FS) in the evaporative light scattering detection (ELSD) chromatogram. Interestingly, after heat pretreatment, a new shoulder appeared at about 20 min in the ELSD chromatograms. This shoulder indicates that sufficient mass of the 20-min UV-absorbing peak is generated by heat pretreatment to make it detectable with the ELSD. These results indicate that the unknown artifact peak that was incorrectly identified as γ-tocopherol in our previous report (*1*) is also present in extracts of heated corn germ and in heated corn fiber oil. We then decided to use UV spectroscopy and mass spectrometry to try to identify this peak in the extracts of heated corn fiber and heated corn germ.

The UV spectra of the unknown UV-absorbing peaks "U" in the extracts of corn fiber (**Figure 3**) and corn germ (**Figure 4**) had one UV maximum at about 220−230 nm and one at about 270 nm (**Figure 6**). A maximum at 230 nm is indicative of two conjugated carbon−carbon double bonds, which are formed when a hydroperoxide is produced from linolenic acid (*10*). The source of the 270-nm maximum is not known. The mass spectra of both peaks failed to identify any easily discernible molecular ions, but it did reveal major ions at *m*/*z* 630.0 and 894.9 for heated corn fiber and at *m*/*z* 630.9, 894.8, and 912.9 for heated corn germ. The *m*/*z* 894.9 ion could be created by the loss of water from the hydroperoxide of the triacylglycerol LLLn−OOH (glycerol−linoleate−linoleate−linolenate) (MW 911.4). Neff and Byrdwell (*11*) reported that the most abundant ion produced by the LnLnLn−OOH was that due to M − H_2O + 1. The two major linolenic acid-containing molecular species in corn oil are LLLn (1% of the total molecular species, MW 877.4) and LnLO (representing 2% of the TAG molecular species, MW 879.4) (*12*).

In the final experiment, a reversed-phase HPLC system was used to try to characterize the unknown UV-absorbing peak which eluted at 20 min in **Figures 3**−**5**. In this HPLC system, the standard heat treatment of corn germ caused the appearance of three new UV-absorbing peaks (labeled UV1, UV2, and UV3) in the chromatogram (**Figure 7**). As in **Figure 6**, the mass spectra of these three peaks were complex (not shown). However, when the chromatograms of the mass spectra obtained by selective ion monitoring (SIM) for two ranges were observed, some new peaks appeared after heat treatment in the range of *m*/*z* 909−915 (the range of the M + 1 ions of the molecular species of three triacylglycerol hydroperoxides shown in **Figure 6**) and the range of *m*/*z* 850−909 (the range of the "M − OH + 1" and "M − OOH + 1" ions of three triacylglycerol hydroperoxides shown in **Figure 6**). It seems reasonable to suggest that the three unknown UV 280-absorbing peaks (UV1, UV2, and UV3) in **Figure 7** may represent the same compounds that eluted together in the 20-min peak (U) in **Figure 6,** and these peaks probably are composed of triacylglycerol oxidation products.

In conclusion, the evidence in this study indicates that the heat-induced component that we identified as γ-tocopherol in our previous report (*1*) was not γ-tocopherol; instead, it probably was one or more triacylglycerol oxidation products, including triacylglycerol hydroperoxides and other oxidation products. Thus, heat treatment of corn germ or other corn-oil containing fractions at high temperatures leads to decreases in γ-tocopherol, γ-tocotrienol, and δ-tocotrienol and to the production of triacylglycerol hydroperoxides.

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Received for review May 19, 2006. Revised manuscript received August 17, 2006. Accepted August 17, 2006.

JF061422G