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Comparison of Analysis of Vitamin D_3 in Foods Using Ultraviolet and Mass Spectrometric Detection

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A method for analysis of vitamin D_3 in commonly fortified foods and in fish, which contains endogenous vitamin D_3, was developed by combining the best aspects of two official methods. The ethyl ether/petroleum ether extraction procedure from AOAC 992.26 was combined with the chromatographic separation and use of an internal standard (vitamin D_2) from AOAC 2002.05 to produce a method that was applicable to a variety of food samples. Results for skim milk, orange juice, breakfast cereal, salmon, a diluted USP reference standard (vitamin D_3 in peanut oil), and processed cheese are presented. Results indicated that UV detection was adequate in most cases, but the absence of interfering species must be determined in each food by mass spectrometry. Selected ion monitoring (SIM) atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) was shown to produce statistically indistinguishable results compared to UV detection for the skim milk, orange juice, multigrain cereal, and salmon samples. The processed cheese exhibited interferences that precluded quantification of vitamin D_3 by UV detection, and therefore, only SIM APCI-MS data for that sample were valid.

KEYWORDS: Vitamin D; cholecalciferol; ergocalciferol; APCI-MS; mass spectrometry; atmospheric pressure chemical ionization; LC-MS; liquid chromatography

INTRODUCTION

According to the NIH Office of Dietary Supplements, "Laboratory and animal evidence as well as epidemiologic data suggest that vitamin D status could affect cancer risk. Strong biological and mechanistic bases indicate that vitamin D plays a role in the prevention of colon, prostate, and breast cancers." (*1*). Recent reports (*2*−*4*) have shown that vitamin D may have a protective effect against prostate cancer, breast cancer, colorectal cancer, Hodgkin's and non-Hodgkin's lymphoma, and other types of cancer. Unfortunately, studies have shown that a relatively high proportion of the population has inadequate or deficient levels of vitamin D (*5*−*8*). These findings have led to increased attention and debate over how much vitamin D is considered adequate (*8*−*10*), and what is the best source from which to obtain it. This discussion has naturally given rise to increased interest in knowing the amount of vitamin D in the foods that we consume, which is essential to epidemiological studies and controlled feeding trials.

Very few foods naturally contain a substantial amount of vitamin D; natural sources include fish (*11*−*13*), cod liver oil (*14*), and certain mushrooms (vitamin D_2) (*15*). Instead, most dietary vitamin D comes from fortified foods, in which manufacturers add specific levels of the nutrient during processing. According to the Code of Federal Regulations (CFR) Title 21, the U.S. Food and Drug Administration (FDA) has approved (or required in the case of infant formula) vitamin D fortification of milk and milk products (21 CFR 131), enriched corn meal, farina, and rice (21 CFR 137), macaroni and noodle products (21 CFR 139), margarine (21 CFR 166), fruit juices, meal replacement bars, and cheese (21 CFR 172.380), Olestra-containing foods (21 CFR 172.867), and infant formula (21 CFR 107.100). These foods, therefore, require analysis of their vitamin D content to ensure that the levels in the food are within the limits imposed by the CFR (*16, 17*).

There are 7,413 foods listed in the USDA National Nutrient Database for Standard Reference, SR21 (*18*). Of the foods in the database, 641 have values listed for vitamin D content. Of those, only 47 foods have concentrations determined from independent analytical data. The remainder of the values were calculated from the manufacturer's label claim, taken from tabulated values in the literature, estimated from the ingredient list, imputed from the concentration in a similar food, or based on the manufacturer's analysis. Of the 47 foods having data obtained from independent chemical analysis, just 27 had nonzero values for vitamin D. Thus, only 27 of the 7,413 foods in the USDA National Nutrient Database have nonzero levels of vitamin D determined from independent analytical data, indicating a paucity of objective analytical data for the vitamin D content of foods commonly consumed as part of the American diet. Additional discussion of the need for vitamin D values in the USDA National Nutrient Database is found elsewhere (*19*).

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Part of the reason for the lack of analytical data is that the methods used for analysis of vitamin D are labor-intensive, tedious, and time-consuming. A recent review provided a summary of applications to milk and infant formula (*20*). Most methods for vitamin D analysis have several common characteristics, beginning with a saponification procedure to break down complex lipids such as triacylglycerols, which interfere with the analysis, into fatty acids (*20, 21*). Then, most methods employ a liquid/liquid extraction to isolate vitamin D. Next, analysis is typically carried out using at least two chromatography steps: (i) a normal-phase high performance liquid chromatography (NP-HPLC) semipreparative, or cleanup, separation followed by (ii) a reversed-phase (RP) HPLC analytical separation that is used for quantification. Since it was believed that vitamin D_2 is much less physiologically active than vitamin D_3 (*22*), most manufacturers fortify with vitamin D_3. Because most foods contain no vitamin D_2 either naturally or by fortification, vitamin D_2 is an appropriate and often used internal standard for the determination of vitamin D_3. However, in cases where the presence of vitamin D_2 is suspected or possible, its absence must first be verified experimentally by analysis of a sample with no vitamin D_2 internal standard added.

In this study, a combined method was used to address the shortfall of available information on vitamin D content in foods. As part of a collaborative effort between six laboratories, we undertook analysis of foods recognized as important dietary sources of vitamin D by the National Food and Nutrient Analysis Program (NFNAP). The results of the interlab comparison have recently been published (*23*) and discussed elsewhere (*24*). The samples were (1) skim milk; (2) orange juice; (3) a multigrain ready-to-eat cereal; (4) canned Alaskan red sockeye salmon; (5) a USP standard diluted in peanut oil; and (6) processed cheese slices. Here, we show a comparison of UV data to mass spectrometry data and point out the benefits and pitfalls of vitamin D analysis for these foods using the combined method.

MATERIALS AND METHODS

Prior to analysis of the samples described herein, we tested two officially approved extraction and analysis methods for vitamin D, AOAC Official Method 992.26 (*25*) and AOAC Official Method 2002.05 (*26*). Like many other methods, these procedures used either an ether/petroleum ether extraction (AOAC 992.26) or a heptane extraction (AOAC 2002.05). Hexane is also a very common extraction solvent (*20*). Orange juice samples produced an intractable emulsion layer when heptane was used as the extraction solvent. The same samples produced clean distinct layers with no emulsion when ether/petroleum ether was used. For other samples, the two extraction solvent systems provided comparable results. Since we wanted a single method that would be applicable to the widest range of samples, we selected the ether/petroleum ether extraction procedure from AOAC 992.26 for analysis of the samples in the collaborative study. However, AOAC 992.26 is an external standard method; therefore, the procedure was modified to include 1.0 mL of 0.5 μg/mL vitamin D_2 added as an internal standard prior to saponification or extraction of vitamin D_3, which is similar to the 0.8 μg/mL used in AOAC 2002.05.

The NP-HPLC procedure from AOAC Method 992.26 used a flow rate of 2 mL/min for the separation of vitamin D from other species, followed by a column wash at 4 mL/min to elute remaining peaks. The RP-HPLC procedure used a flow rate of 3 mL/min. However, AOAC Method 2002.05 used a flow rate of 1.5 mL/min for the NP-HPLC semipreparative step and 1.3 mL/min for the RP-HPLC separation used for quantification. Because AOAC 2002.05 used lower flow rates and therefore less solvent and lower backpressure, we incorporated the NP and RP HPLC separations from this method for our analysis. Thus, our procedure incorporated the best features of two Official Methods, the ether/petroleum ether extraction from AOAC 992.26 combined with the internal standard, NP-HPLC and RP-HPLC separations from AOAC 2002.05, to give a single versatile method that was applicable to a wide variety of samples.

Chemicals and Samples. HPLC or spectrophotometric grade solvents were purchased from Fisher Scientific, Inc. (Fairlawn, NJ) and were used without further purification. The petroleum ether was low-boiling (30−60 °C, Fisher #E139-S4). Homogenized control composites (*27*) of commercially available foods plus a diluted USP standard were prepared by the Food Analysis Laboratory Control Center in the Biochemistry Department at Virginia Polytechnic Institute and State University. These samples were used as control materials and analyzed in a collaborative study comparing results among laboratories using standard methods (*23*). These samples were (1) skim milk commercially fortified with vitamin D_3; (2) orange juice without pulp, fortified with calcium and vitamins A, B1 (thiamin), C, D_3, and E; (3) a multigrain ready-to-eat cereal fortified with vitamin D_3; (4) canned Alaskan red sockeye salmon; and (5) processed American cheese. Additionally, a USP vitamin D_3 standard (USP #1711504), diluted 1:100 in peanut oil, as well as the peanut oil diluant blank, were analyzed. The original USP standard as received was specified to contain 0.2% cholecalciferol, or 8,000,000 IU/100 g, in peanut oil. A 0.1511 g portion of the standard was combined thoroughly with 99.8780 g of peanut oil to give a sample that was 0.0003021% vitamin D_3 by mass, which equated to 12,084.36 IU/100 g (1 μg = 40 IU; 0.0003021% = 0.0003021 g/100 g = 302.1 μg/100 g = 12,084 IU/100 g). All samples were stored at −60 ± 5 °C under nitrogen and in darkness prior to analysis.

Extraction. Samples were extracted using the ethyl ether/petroleum ether extraction given in AOAC method 992.26 (*25*), as recently reported (*23*). The method was modified to include 1.0 mL of 0.5 μg/mL vitamin D_2 in ethanol added as an internal standard to most samples (milk, orange juice, cereal, processed cheese, and blank). Salmon contained a higher level of endogenous vitamin D_3; therefore, 4.0 mL of 0.5 μg/mL vitamin D_2 were added to those samples as the internal standard. The USP diluted standard contained a higher level of vitamin D_3 (12,000 IU/100 g ≅ 3.0 ug/mL); therefore, 6.0 mL of 0.5 μg/mL vitamin D_2 were added to those samples as the internal standard. Five subsamples of each material were analyzed, except for cereal, which was based on four replicates.

The sample weight for most samples (except salmon and the diluted USP sample) was calculated to contain approximately 12 IU of vitamin D_3, based on the label claim. The sample was weighed in a 250 mL Erlenmeyer flask with a ground glass neck, 1.0 mL of the internal standard (or amount given above) was added by volumetric pipet, and 400 mg ascorbic acid (as antioxidant) was added. Fifteen milliliters of ethanol was added, and the sample was swirled thoroughly to mix. The requisite amount of KOH was added, as a solid to milk and orange juice samples, and as a 1 M solution to solid samples. The sample sizes and amounts of KOH were as follows: (1) skim milk, ∼30 mL = ∼29.5 g, 7.5 g of KOH; (2) orange juice, 30 mL = ∼30.5 g, 7.5 g of KOH; (3) cereal, ∼9 g, 135 mL of 1 M KOH; (4) salmon ∼ 10 g, 135 mL of 1 M KOH; (5) processed cheese, ∼9 g, 135 mL of 1 M KOH; (6) diluted USP standard, ∼1 g, 135 mL of 1 M KOH; (7) blank peanut oil, ∼1 g, 135 mL of 1 M KOH. The flask was swirled until the solid KOH was dissolved in the liquid sample, or the solid sample was thoroughly suspended in liquid. The mixture was put onto a refluxing condenser and lowered into a water bath at 75 °C. After 30 min, the sample was removed and placed into ice water to rapidly cool to room temperature.

The sample was transferred to a 500 mL separatory funnel, with a 5 mL ethanol rinse. One hundred thirty milliliters of ethyl ether was added to the funnel, which was stoppered and shaken vigorously for at least 1 min. Next, 130 mL of petroleum ether was added to the funnel, which was again stoppered and shaken vigorously for at least 1 min. The shaking during extraction needed to be sufficiently vigorous to ensure complete extraction of vitamin D_3. The samples were allowed to stand to separate. Swirling aided the separation of the two layers. The lower layer was drained and discarded. Fifty milliliters of deionized (D.I.) water was added to the flask, which was stoppered and shaken for >30 s. The samples were allowed to stand to separate. The lower layer was drained to waste. Another 50 mL of D.I. water was added to the flask, which was stoppered and shaken for >30 s. The samples

were allowed to stand to separate and the lower layer was drained to waste. Fifteen milliliters of ethanol were added to the flask and shaken, and then a third wash of 50 mL D.I. water was added to the flask, stoppered, shaken, and allowed to separate. The lower layer was drained to waste. The remaining ether layer was collected in a 500 mL flat-bottom round flask. This was then decanted into a clean 500 mL round-bottom flask. This flask was put onto a rotary evaporator (Buchi, Flawil, Switzerland) and taken to dryness with the water bath at 45 °C. Fifty milliliters of acetone was added to the flask, and it was again taken to dryness.

The sample was then dissolved in 10 mL of ethyl ether, with swirling, and transferred to a prerinsed 50 mL centrifuge tube. The round-bottom flask was rinsed with two more 10 mL portions of ethyl ether, which were combined in the centrifuge tube. The ether solution was evaporated to dryness under ultra high purity N_2 on an N-Evap evaporator (Organomation, Northborough, MA). The sample was reconstituted in 1.0 mL of hexane. Even with a large amount of KOH, high fat samples such as salmon and processed cheese did not saponify completely, resulting in an oily extract. This condition did not interfere with the analyses since the oily mixture contained mostly diacylglycerols (based on unpublished full-scan MS analysis), which did not hinder the chromatography of vitamin D.

High Performance Liquid Chromatography. The two chromatographic separations from AOAC Official Method 2002.05 (*26*) were used. The first was a normal-phase preparative HPLC separation on a 25.0 cm × 4.6 mm, 5 μm, silica column (Inertsil, GL Sciences, Torrance, CA). It was conducted on an Agilent 1200 system consisting of a quaternary pump with a membrane degasser, an autosampler with extended volume injection option, a diode array detector (DAD) SL, and a 35900E analog-to-digital converter for acquisition of signal from an Alltech ELSD 800 (Alltech Associates, Deerfield, IL) evaporative light scattering detector (ELSD). Two solvent programs were used, which were the same except that one included a column wash after vitamin D eluted. The method without the column wash was used to analyze a pure vitamin D retention time standard that was run each day to establish the elution time of the collected analyte peak, while the method with the column wash was used for food sample extracts. Both programs began with isocratic mobile phase 1, composed of 0.5% isopropanol (IPA)/2.0% methyl-*t*-butyl ether (MTBE)/48.75% cyclohexane/48.75% *n*-heptane. For standards, only isocratic mobile phase 1 was used for 25 min. For samples, isocratic mobile phase 1 was used for 25 min, after which the column was washed with mobile phase 2, consisting of 20% IPA/80% *n*-heptane. The gradient for samples was as follows: 0 to 25 min, 100% mobile phase 1; 25 to 35 min, linear gradient to 100% mobile phase 2; 35 to 55 min, 100% mobile phase 2; 55 to 75 min, linear gradient back to mobile phase 1; and 75 to 85 min, equilibrate in 100% mobile phase 1. It was important to allow sufficient time for the column to re-equilibrate between runs, to avoid inconsistent retention times. The flow rate was 1.3 mL/min throughout. The injection volume was 450 μL, which allowed two injections plus waste for each 1.0 mL sample. Flow after the DAD went to the fraction collector or ELSD. The diverter valve on the fraction collector sent eluate to the ELSD except during the fraction time window. The vitamin D eluted at ~17.5 min, and the fraction was collected from 16 to 19 min in a 13 mm test tube. The fraction time was adjusted slightly, on the basis of the elution time of analyte in a standard solution. Fractions were taken to dryness by inserting the test tube into a 50 mL long-neck round-bottom flask on a rotary evaporator. The fractions were reconstituted in 650 μL of mobile phase 3, consisting of 20% methanol (MeOH)/80% acetonitrile (ACN). The reconstituted fraction was transferred to two autosampler vials containing limited volume inserts, which allowed four 100 μL injections plus waste for each fraction collected.

The reversed-phase HPLC was carried out using a Thermo Separation Products (San Jose, CA) chromatograph consisting of a P4000 quaternary pump with membrane degasser, AS3000 autosampler, UV6000 DAD, and a UV2000 dual channel detector operated in single channel mode at 265 nm. The full-scan spectra were obtained from 190−400 nm, with a bandwidth of 1 nm and an acquisition rate of 1 Hz. The single channel detection at 265 nm on the DAD was performed at 10 Hz with a 9 nm bandwidth. The UV 2000 detector had a risetime of 1 s, and the acquisition rate was 10 Hz. The solvent system was isocratic mobile phase 3 for 20 min on an Inertsil ODS-2 column, 25.0 cm × 4.6 mm and 5 μm particle size (Inertsil, GL Sciences, Torrance, CA) at a flow rate of 1.3 mL/min.

Quantification was based on integration of the areas under the peaks in the UV 265 nm chromatogram from the DAD. DAD UV results were then compared to results obtained by the mass spectrometer.

Mass Spectrometry. Tandem sector quadrupole mass spectrometry was used as an auxiliary detection method on the RP-HPLC system. The mass spectrometer was a TSQ 7000 mass spectrometer (Finnigan MAT, now Thermo Fisher Scientific Corp., San Jose, CA) operating in Q3 SIM mode, using the $[M + H]^+$ at *m/z* 397.3 and the $[M + H - H_2O]^+$ ion at *m/z* 379.3 for the vitamin D_2 internal standard, and the $[M + H]^+$ at *m/z* 385.3 and the $[M + H - H_2O]^+$ ion at *m/z* 367.3 for vitamin D_3, with a scan time of 0.5 s per ion and 1.0 *m/z* peak width. The total area for each analyte was the sum of the integrated areas of the $[M + H]^+$ and the $[M + H - H_2O]^+$ ions. The RP-HPLC was coupled to the TSQ 7000 via an APCI source, with the vaporizer heater at 250 °C, the sheath and auxiliary gases at 40 psi and 10 mL/min, respectively, and the corona current at 5.0 μA. Flow after the DAD was split via a tee, with 0.63 mL/min going to the APCI source of the TSQ7000 mass spectrometer, and 0.67 mL/min going to waste or to a second mass spectrometer. In some experiments (e.g., RP-HPLC analysis of processed cheese), data were also obtained on an LCQ Deca XP ion trap mass spectrometer (Thermo Electron Corp., now Thermo Fisher Scientific Corp., San Jose, CA), in parallel, operated in full scan mode. In those experiments, APCI was performed on the ion trap mass spectrometer using the same parameters as those listed for the TSQ7000 instrument.

Calculations. Internal standard quantification was based on the response factor, *RF*, determined from the ratio of the integrated area from vitamin D_3 to that of vitamin D_2 in a standard solution composed of equal amounts, 0.8 ug/mL each, of vitamin D_2 and D_3: RF = (area D_{3std}/area D_{2std}). The response factor from the standard was then applied to the food samples. The calculated amount of vitamin D_3 in a sample was given from the equation:

$$\text{Vitamin D}_3\ (\mu\text{g}) = \frac{\left(\frac{\text{area D}_3}{\text{area D}_2}\right) \times \mu\text{g D}_2\text{IS}}{RF}$$

Each sequence of runs was set up as a bracketed sequence to run two replicates of the 0.8 ug/mL standard solution, followed by eight sample runs, followed by one more standard run, followed by eight more sample runs, finished with two more standard runs. Thus, each sequence of runs had five standard runs and 16 sample runs. The samples were quantified using the average response factor for the five standard runs obtained on the same day as the sample runs. The value determined for each sample extraction replicate was based on the average of eight analytical (RP-HPLC) runs, and the value assigned to each food was the average of five replicate extractions, unless otherwise noted in **Table 1**.

Statistical Analysis. There were four primary potential sources for variance: (1) sample to sample variance, (2) saponification/extraction to saponification/extraction variance, (3) NP-HPLC fraction to NP-HPLC fraction variance, and (4) RP-HPLC run to RP-HPLC run variance. The samples obtained from FALCC were aliquots from large homogeneous lots and therefore were theoretically all the same sample within a sample type (*27*). Therefore, the first type of variance, sample to sample variance within a particular food control material, was considered negligible. Due to the length and complexity of the sample preparation method, the saponification/extraction to saponification/extraction variance was expected to be the primary source of variance in the results. The variance in the last two potential sources was also assessed. Single factor analysis of variance (one-way ANOVA) was performed for comparison of the average vitamin D_3 contents of extracts, using Microsoft Excel with optional data analysis tools installed. The Student's *t*-test, performed using Microsoft Excel (Microsoft Corp., Redmond, WA), was used to determine whether the results from NP-HPLC fractions of the same extract were statistically significantly different. The maximum or minimum values from the eight

Table 1. Quantification of Food Samples by Ultraviolet (UV) Detection and Selected Ion Monitoring Mass Spectrometry (MS) Detection[a]

	skim milk	orange juice	cereal	salmon	spiked peanut oil	processed cheese
			UV Detection			
IU/100 g mean	43.1	51.7	135	887	11220	847
std dev	1.7	4.4	12	16	360	74
%RSD	4.0%	8.6%	9.3%	1.8%	3.2%	8.7%
			MS Detection			
IU/100 g mean	45.7	54.0	142.9	889	12100	289
std dev	3.2	4.7	8.7	79	980	27
%RSD	6.9%	8.7%	6.1%	8.9%	8.1%	9.5%
	$n = 5$	$n = 5$	$n = 4$	$n = 5$	$n = 5$	$n = 5$
label claim or literature value	41.1[a]	40.8[b]	138[c]	763 ± 33[d]	12084[e]	190[c]

[a] First nonsignificant figure shown. [b] Label claim based on observed approximate density of 1.028 g/mL. [c] Label claim based on observed approximate density of 1.037 g/mL. [d] Label claim based on weight. [e] Literature value taken from National Nutrient Database SR21 (*18*) for "Fish, salmon, sockeye, canned, drained solids with bone", NDB #15087. [f] Concentration calculated from the value provided by supplier, mass of standard used, and mass of peanut oil diluant (approximate 1:100 dilution of commercial USP sample).

individual RP-HPLC runs for each of the five sample replicates were tested as outliers using the Q-test at the 95% level ($Q_{95\%} = 0.526$, $n = 8$).

RESULTS

Figure 1 shows the NP-HPLC separation of the extract from skim milk. Panel **A** of this figure shows the sizes of the peaks of interest, relative to full scale. Panel **B** shows the peak of interest at 17.626 min, labeled with an arrow, which was collected by a fraction collector. **Figure 2** shows the NP-HPLC separation of extracts of (**A**) orange juice, (**B**) Alaskan red sockeye salmon extract, (**C**) multigrain cereal extract, and (**D**) processed cheese. Three-minute fractions (16:00 to 19:00) centered on the peak of interest (~17.5 min) were taken. The chromatogram of the Alaskan salmon extract was comparatively simple and indicated that the fraction from this sample contained almost exclusively vitamin D. The chromatograms of orange juice and cereal were more complex and indicated that portions of other nearby peaks were included in the three minute fraction collected for these samples. The processed cheese chromatogram indicated that a variety of components coeluted during the time interval collected and that the peak of interest was smaller than other peaks that contributed to the collected fraction. This fraction gave results that differed from those of fractions from other materials.

The collected fractions were evaporated to dryness and reconstituted in the mobile phase used for RP-HPLC. The RP-HPLC system separated vitamin D_2 from vitamin D_3 and the resulting peaks were integrated separately for quantification using three different detectors. Typical results obtained for skim milk from the three detectors are shown in **Figure 3**. **Figure 3A** shows the total ion current chromatogram (TIC) for the sum of the four ions used for SIM analysis by MS. **Figure 3B**,**C** show the chromatograms for the two sets of ions used for vitamin D_2 and D_3, respectively. **Figure 3D** shows the output from an older dual channel UV detector operated in single channel mode at 265 nm, while **Figure 3E** shows the detector output for the DAD in full scan mode from 190 to 400 nm. **Figure 3F** shows the single channel output (265 nm) from the DAD that was used for quantitative analysis. The dual channel detector (**Figure 3D**) produced the same chromatographic profiles as the DAD detector operated in single channel mode (**Figure 3F**), except that the magnitude of the signal from the DAD was much larger due to its larger path length and the diminished sensitivity of the dual channel detector due to its age. This indicated that the dual channel detector was adequate for quantitative analysis of vitamin D in most samples, as long as the absence of interfering species was confirmed.

The average UV response factor across all runs used for quantification of all samples reported here was 1.0260 ± 0.0089 ($n = 119$), RSD = 0.87%, indicating that vitamin D_3 gave a slightly higher response than vitamin D_2 at 265 nm. The average APCI-MS response factor across all runs used for quantification of all samples reported here was 1.2479 ± 0.1392 ($n = 119$), RSD = 11.16%, showing that vitamin D_3 gave ~25% more signal by APCI-MS than an equal amount of vitamin D_2.

From the internal standard calculation given in the previous section, the amount of vitamin D_3, in micrograms, in the samples was determined. This was divided by the sample weight and scaled to 100 g to give a value for μg/100 g, which was then converted to IU/100 g since these are the units reported in the National Nutrient Databank. The calculated results were independent of the reconstituted volume of the sample or the injection volume. This was beneficial for samples containing large amounts of fat, such as salmon and the USP reference sample. For these samples, an oily residue was produced by saponification and extraction, instead of a solid residue, which gave a volume of more than 1.0 mL when 1.0 mL of hexane was added. In such samples, the triacylglycerols were decomposed; therefore, they no longer interfered with the chromatography of vitamin D, but some diacylglycerols and other oily partial decomposition products remained, which did not affect the calculation of the amounts of vitamin D_3 in the samples.

The amount of vitamin D_3 calculated for five replicate extractions of skim milk was 43.1 ± 1.7 IU/100 g (1 IU = 0.25 μg) from UV (265 nm) detection, for a RSD = 4.0%, as shown in **Table 1**. The value obtained from the simultaneous SIM APCI-MS analysis was 45.7 ± 3.2 IU/100 g, for RSD = 6.9%. The label claim value was 41.1 IU/100 g, on the basis of the observed approximate density of skim milk of 1.028 g/mL. These values indicated that the amount of vitamin D_3 in the skim milk met the label claim, with a small expected overage to account for storage and shelf life. The mean values obtained by MS detection were statistically indistinguishable ($p = 0.16$) from those obtained by UV detection, on the basis of a Student's *t*-test (two-sample assuming unequal variances).

Typical chromatograms used for the quantification of the other samples are shown in **Figure 4**. The determined amounts of vitamin D_3 in orange juice (**Figure 4A**), multigrain breakfast cereal (**Figure 4B**), Alaskan red sockeye salmon (**Figure 4C**), diluted USP sample (**Figure 4D**), and processed cheese (**Figure 4E**) are given in **Table 1**. The amount of vitamin D_3 in orange juice was 51.7 ± 4.4 IU/100 g as determined by UV detection, which was statistically indistinguishable ($p = 0.45$) from the value of 54.0 ± 4.7 IU/100 g as determined by MS detection (**Table 1**). Both methods indicated that this orange juice contained ~25% more vitamin D_3 than the label claim (40.8 IU/100 g). Similarly, the results for multigrain breakfast cereal by UV were within the range given by the standard deviation for the value determined by MS (**Table 1**) ($p = 0.35$). In this case, the label claim was within the range determined by both techniques. The amounts of vitamin D_3 determined by both UV and MS detection for Alaskan sockeye salmon were 887 ± 16 and 889 ± 79, respectively, and were statistically indistinguishable ($p = 0.97$). These values can be compared to the value of 763 ± 33 IU/100 g for a similar salmon product listed in the

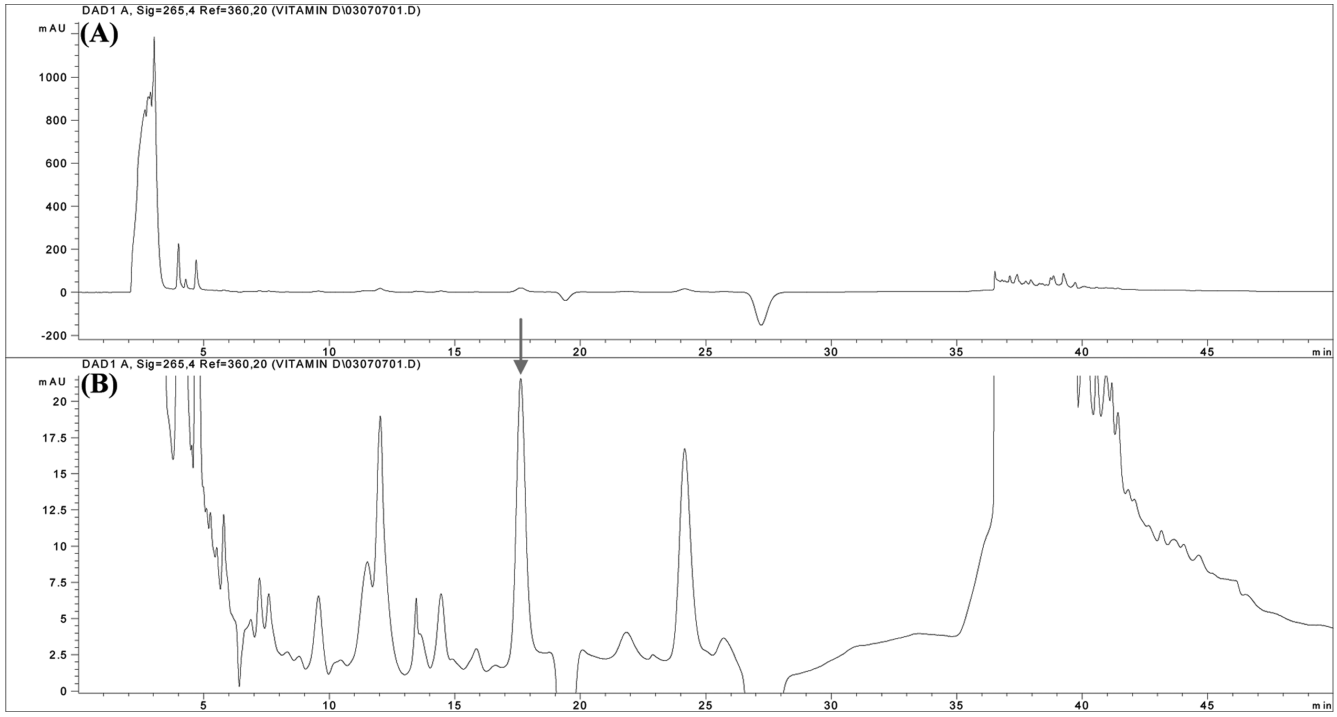

Figure 1. Separation of skim milk control sample by NP-HPLC on silica column. (**A**) Full-scale chromatogram showing the relative sizes of all peaks; (**B**) chromatogram showing peaks magnified for clarity. The vitamin D peak at 17.626 min (indicated by arrow) was collected and further analyzed by RP-HPLC.

current release of the USDA National Nutrient Database for Standard Reference, SR21 (*18*).

The diluted USP standard was the only sample for which a theoretical known value could be calculated. The original concentration was listed as 0.2% cholecalciferol, or 8,000,000 IU/100 g, and the diluted standard as analyzed was 0.0003021% vitamin D_3 (12084 IU/100 g). The assayed concentration for the diluted USP sample obtained by UV detection, 11,220 IU/100 g, **Figure 4D**, was lower than the value obtained by MS detection, 12,100 IU/100 g, although this difference was not statistically significant at the 95% confidence level ($p = 0.12$), on the basis of the Student's *t*-test. Although the MS data appeared to be more accurate than the UV results, the lack of significant figures in the value given for the USP standard precluded a rigorous comparison.

The results for processed cheese showed the greatest discrepancy in the comparison of UV versus MS results. The size of the vitamin D_3 peak in **Figure 4E** indicated that processed cheese contained a very large amount of vitamin D_3. The calculated value from this peak was 847 ± 74 IU/100 g, which was more than four times the label claim value. The MS results in **Figure 5** provided insight into the reason for the very large peak that eluted at the time expected for vitamin D_3. The SIM ion chromatogram for the ions used for quantification of vitamin D_2 showed the peak at 12.22 min (12.12 min by UV, which preceded the MS detector in series) in **Figure 5**, as expected, but also showed a large peak at 12.87 min that overlapped the vitamin D_3 peak at 12.80 min. While these peaks were easily distinguished by MS, they were overlapped and indistinguishable by UV detection, leading to dramatic overestimation of the amount of vitamin D_3. Consequently, the MS data were used for quantification of the amount of vitamin D_3 in processed cheese. The value determined by SIM APCI-MS was 289 ± 27 IU/100 g, which was much closer to the label value (190. IU/100 g), and was near the upper end of the range based on CFR 21 172.380 (c)(5), which allows 81 IU per 30 g (270. IU/100 g), within the variability of the analytical method. The additional amount above the label claim likely includes overage necessary to account for product storage and shelf life.

The results given above indicated that for most samples, quantification based on UV detection was perfectly adequate, and in most cases produced lower standard deviation than the results obtained by SIM APCI-MS. However, the results indicated that some samples (i.e., processed cheese) produced erroneous results by UV detection, which could go unrecognized without the use of MS as a confirmatory technique.

Using orange juice #NFY0603U2 RP-HPLC runs as an example, the average limit of detection by UV detection, LOD_{UV}, was 1.19 ± 0.39 IU/100 g ($n = 8$) or 0.0147 ± 0.0048 μg/mL injected, while the LOD_{MS} was 4.7 ± 1.3 IU/100 g ($n = 8$) or 0.058 ± 0.016 μg/mL injected, at 3 times the peak-to-peak noise for a typical peak width of 0.7 min by UV and 1.0 min by MS. The average limit of quantitation by UV detection, LOQ_{UV}, was 4.0 ± 1.3 IU/100 g ($n = 8$) or 0.049 ± 0.016 μg/mL, while the LOQ_{MS} was 15.7 ± 4.4 IU/100 g ($n = 8$) or 0.194 ± 0.055 μg/mL, at 10 times the peak-to-peak noise for typical peak widths.

Further Statistical Analysis. The three primary sources for analytical variability were (1) extraction to extraction variability, (2) NP-HPLC fraction to fraction variability, and (3) RP-HPLC run to run variability. The relative magnitudes of the variance from each factor can be seen using orange juice data as an example. **Table 2** shows the values for quantification from each individual run for orange juice, divided into groups of four that each represent the four RP-HPLC runs that resulted from the collection of one fraction from the NP-HPLC system. Each sample extract gave enough of the sample for two NP-HPLC runs; therefore, two fractions were collected (indicated as #1 and #2 in **Table 2**). The run to run variability by UV detection was very low, with an average RSD of 0.71% across all 10 fractions. Furthermore, the Student's *t*-test indicated that the average values from the two fractions from each extract were

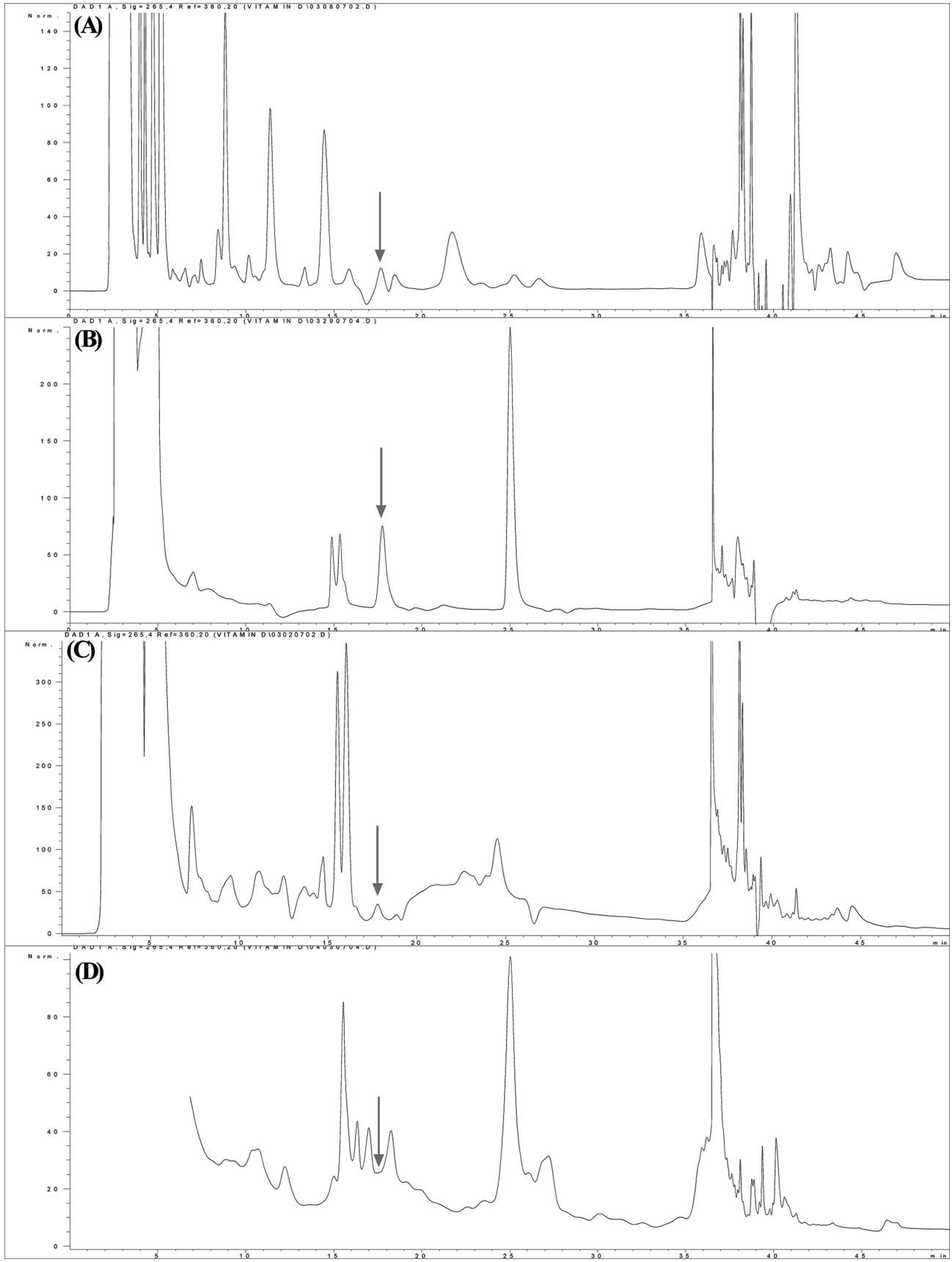

Figure 2. NP-HPLC chromatograms of food sample extracts. (**A**) Orange juice; (**B**) Alaskan red sockeye salmon; (**C**) multigrain cereal; (**D**) processed American cheese.

statistically indistinguishable ($p > 0.05$) by UV detection for all except one extract. Extract NFY0603U1 gave values of 55.27 ± 0.21 and 57.86 ± 0.34 for the two fractions, which were statistically different ($p \ll 0.05$, $t = 13.12$, $t_{crit} = 2.57$). This explained why the RSD for this sample in **Table 1** (2.49%) was higher than those of the other orange juice extracts, although

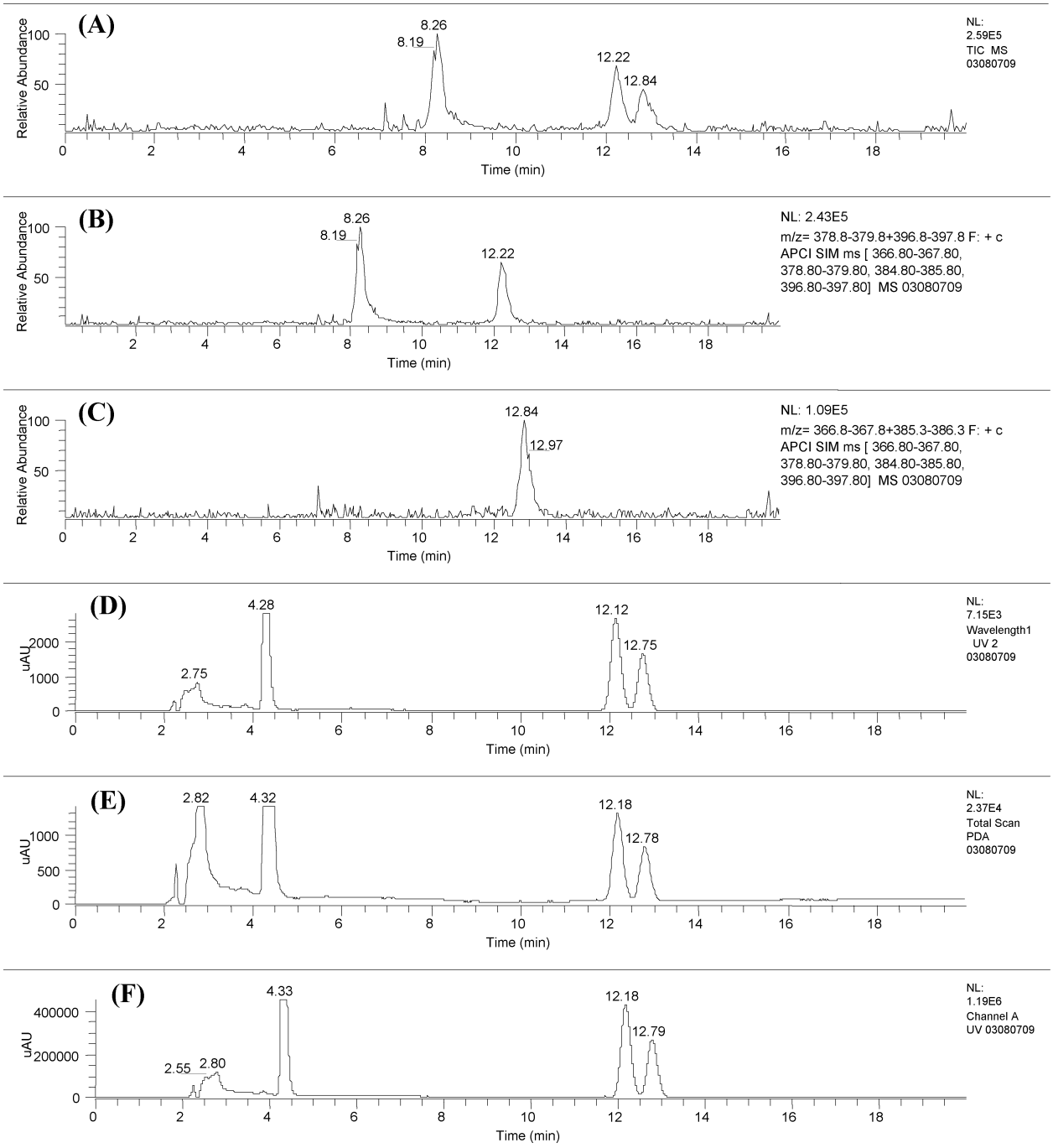

Figure 3. Mass spectrometry and UV RP-HPLC chromatograms of the skim milk extract fraction shown in **Figure 1**. (**A**) Total ion current (TIC) chromatogram; (**B**) ion chromatogram of *m*/*z* 379.3 and *m*/*z* 397.3 corresponding to vitamin D2; (**C**) ion chromatogram of *m*/*z* 367.3 and *m*/*z* 385.3 corresponding to vitamin D3; (**D**) single channel UV detector at 265 nm; (**E**) total scan (190−400 nm) chromatogram from the diode array detector (DAD); (**F**) single channel (265 nm) chromatogram from DAD.

each of the two fractions for that sample had very low RSD (0.37% and 0.58%). Analysis of variance indicated that the mean square variance between groups (fractions) was 71.64, whereas the within group variance was 0.17. This indicated that the run to run variability was less than the fraction to fraction variability. The agreement between pairs of fractions indicated that the fraction to fraction variability was less than the extract to extract total variability, which was seen in **Table 1** to be 8.6% RSD for the orange juice extracts. Thus, analysis of variance indicated that the variability could be attributed to, in decreasing order, (1) extraction to extraction variability, (2) NP-HPLC fraction to fraction variability, and (3) RP-HPLC run to run variability. RP-HPLC run to run variability by UV detection was usually below 1%. Similar trends were observed for all other fractions and extracts.

The data by mass spectrometry showed poorer reproducibility. The RP-HPLC run to run variability was much higher, with an average RSD of 17%. The fraction to fraction values showed an average RSD of 9.4%. Thus, the run to run variability was at least as large as the fraction to fraction variability. **Table 1** indicates that the extract to extract variability was 8.7%. ANOVA indicated that the mean square variance within groups (run to run) was 95.8, while between group (fraction to fraction) mean square variance was 100.

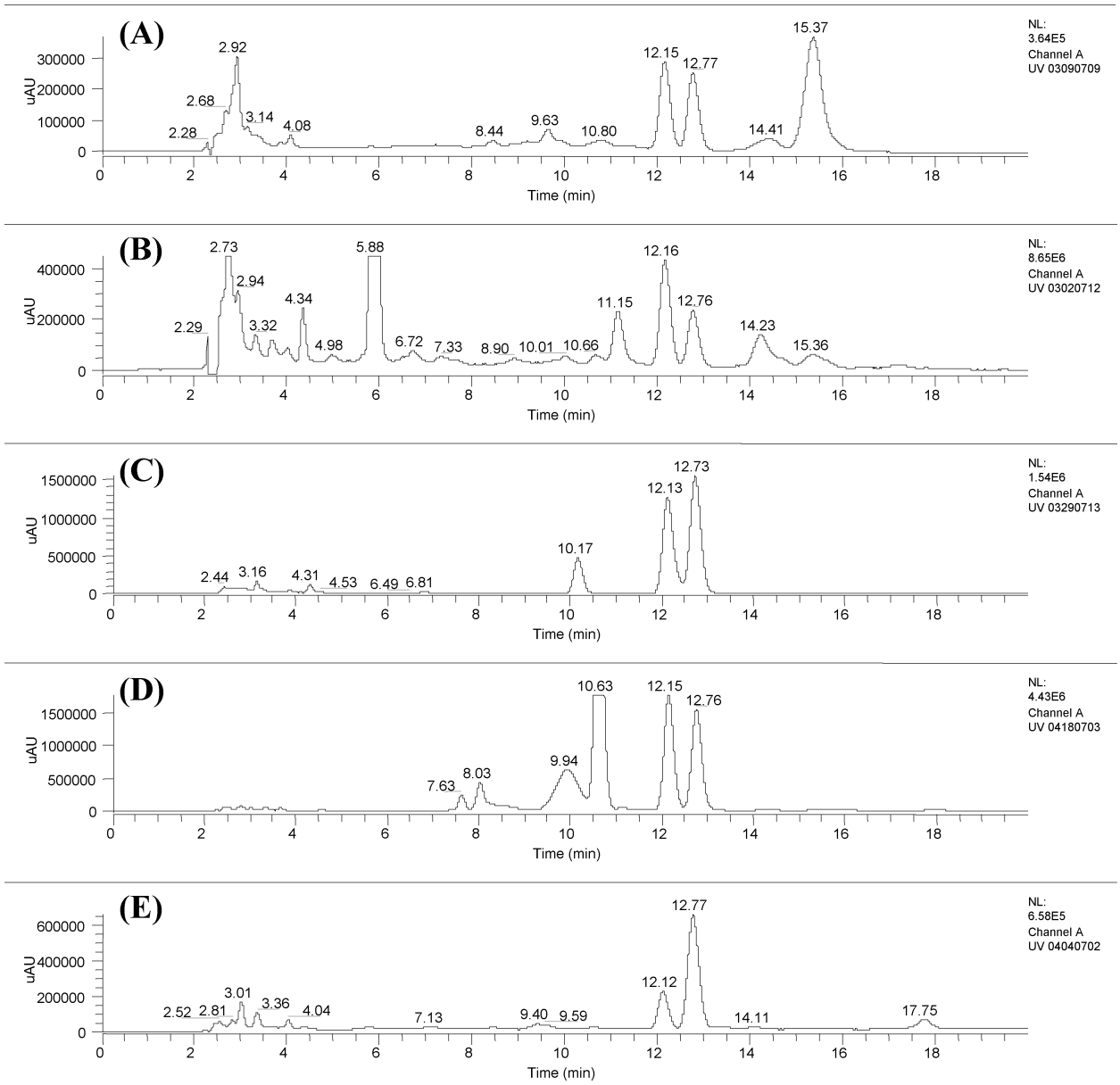

Figure 4. RP-HPLC separation with DAD single channel UV detection at 265 nm. (**A**) Orange juice extract fraction (see **Figure 2A**); (**B**) multigrain cereal extract fraction (see **Figure 2C**); (**C**) Alaskan red sockeye salmon extract fraction (see **Figure 2B**); (**D**) diluted USP standard; (E) processed cheese slice extract fraction (see **Figure 2D**). The vitamin D2 internal standard eluted at 12.1 to12.2 min and vitamin D3 eluted at 12.7 to12.8 min.

DISCUSSION

Difficulty with the orange juice extract, **Figure 2A**, led to the choice of the ether/petroleum ether extraction solvent pair. Before analysis of the control composite samples, both AOAC 2002.05 and AOAC 992.26 were tested on a variety of commercially obtained foods ranging from milk to diet supplement drinks and from orange juice to infant formula. We found that the samples that were substantially aqueous produced an intractable emulsion layer when the heptane extraction from AOAC 2002.05 was used. Other laboratories participating in the collaborative study that used a heptane extraction (*23*) added ethanol to take the emulsion back into solution. However, all samples gave good extracts when the ether/petroleum ether solvent pair from AOAC 992.26 was employed. Since we were seeking a method that would be widely applicable to the largest variety of samples, we chose the ether/petroleum extraction solvent pair, which worked well for all samples tested.

Table 1 shows that the mean values obtained by SIM APCI-MS were statistically indistinguishable from the results obtained by UV detection for most samples. In most cases, the standard deviation obtained from UV data was less than that obtained from the MS data, indicating a benefit to the use of UV data. Given the much greater cost of MS instruments versus UV detection, the results indicated that the cheaper instrument produced comparable results with much less cost. Furthermore, the much higher levels of routine and periodic maintenance required for the MS detector tilted the comparison of detectors in favor of the UV instrument. Thus, for most laboratories where routine analysis of large numbers of samples is required, the HPLC-UV instrument could be the best choice.

However, the processed cheese sample dramatically demonstrated the shortcoming of UV detection and highlighted the need for MS detection for some foods. The UV chromatographic profile of processed cheese in **Figure 4E** appeared to show a clean chromatogram with good resolution of vitamin D_2 and vitamin D_3. There was no indication of an overlapped peak, such as a shoulder or other chromatographic feature, to indicate the presence of coeluting molecules. However, the MS data

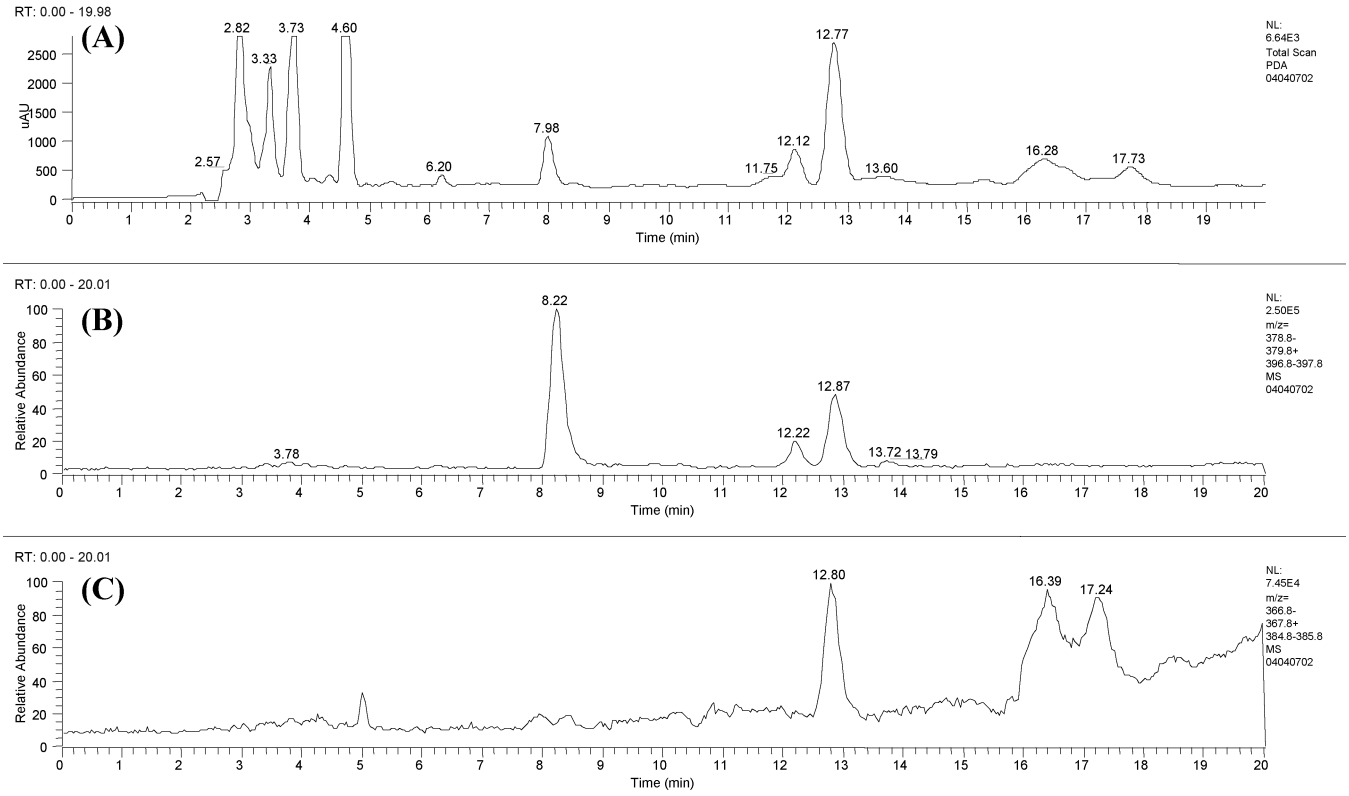

Figure 5. RP-HPLC separation of processed cheese extract fraction with detection by UV/vis spectroscopy and mass spectrometry. (**A**) Total scan DAD chromatogram; (**B**) ion chromatogram of *m*/*z* 379.3 and *m*/*z* 397.3 corresponding to vitamin D2; (**C**) ion chromatogram of *m*/*z* 367.3 and *m*/*z* 385.3 corresponding to vitamin D3.

Table 2. Quantification of Individual Runs (Rows) and Fractions (Columns) of Orange Juice Extracts by Ultraviolet (UV) Detection and Selected Ion Monitoring Mass Spectrometry (MS) Detection (IU/100g)[a]

NFY060	3TZ#1	3TZ#2	3U0#1	3U0#2	3U1#1	3U1#2	3U2#1	3U2#2	3U3#1	3U3#2
					UV Detection					
	46.07	45.18	48.97	49.12	55.25	58.24	55.41	55.24	51.51	51.81
	46.68	45.57	48.99	48.95	55.22	58.04	55.19	55.45	51.50	51.68
	45.36	46.14	49.86	49.67	55.55	57.59	55.13	54.87	51.51	51.58
	45.48	44.34	49.80	50.00	55.05	57.55	55.29	55.65	51.64	51.54
mean	45.90	45.31	49.40	49.44	55.27	57.86	55.26	55.30	51.54	51.66
SD	0.61	0.76	0.49	0.49	0.21	0.34	0.12	0.33	0.07	0.12
%RSD	1.3%	1.7%	1.0%	0.98%	0.37%	0.58%	0.22%	0.60%	0.13%	0.24%
					MS Detection					
	48.3	43.8	58.7	54.4	61.5	65.6	79.3	49.9	56.4	51.5
	47.4	49.9		53.9	42.3	61.1	47.9	52.6	49.1	77.1
	64.3	55.6	46.6	54.3	52.2	53.2	71.0	55.4	61.7	50.9
	38.3	37.4	46.4	41.0	46.9	57.2	51.7	74.4	53.3	46.5
mean	50	46.7	50.6	50.9	50.7	59.3	62	58	55.1	57
SD	11	7.8	7.0	6.6	8.3	5.3	15	11	5.3	14
% RSD	22%	17%	14%	13%	16%	9.0%	24%	19%	9.6%	25%

[a] First nonsignificant figure shown. [b] Value Q-tested out at the 95% confidence level.

conclusively indicated that there were interfering species present that produced ions homologous with vitamin D_2. Since we obtained full-spectrum UV data (190−400 nm) in addition to the single wavelength detection, it was possible to examine the full spectra across the peaks in question. The full UV spectrum at 12.8 min was noticeably different from that of the vitamin D_3 calibration standard (not shown). The spectrum did not have an absorbance maximum at 265 nm, as expected for vitamin D_3, but instead the maximum occurred at 249 nm, and the spectral profile was clearly distinguishable from that of the authentic analyte. Thus, even in the absence of MS data, full-scan UV data indicated the presence of one or more interfering species. Furthermore, **Figure 5A**, which is a chromatogram of the total absorbance of full-spectrum UV scans for processed cheese, showed a peak immediately preceding and overlapped with vitamin D_2, which was not evident in the single channel chromatogram in **Figure 4E**. Obviously, some of the species that produced peaks in the full scan UV chromatogram did not have absorbance maxima at 265 nm and therefore were not predominant in the single channel chromatogram. These data suggest that the absence of interfering species must be confirmed for each sample before the data from UV detection alone can be considered reliable. Mass spectrometry proved most conclusive for discrimination of the analyte from interference, but full scan UV spectra also indicated the presence of an overlapped substance. Therefore, even the lower cost DAD UV detection

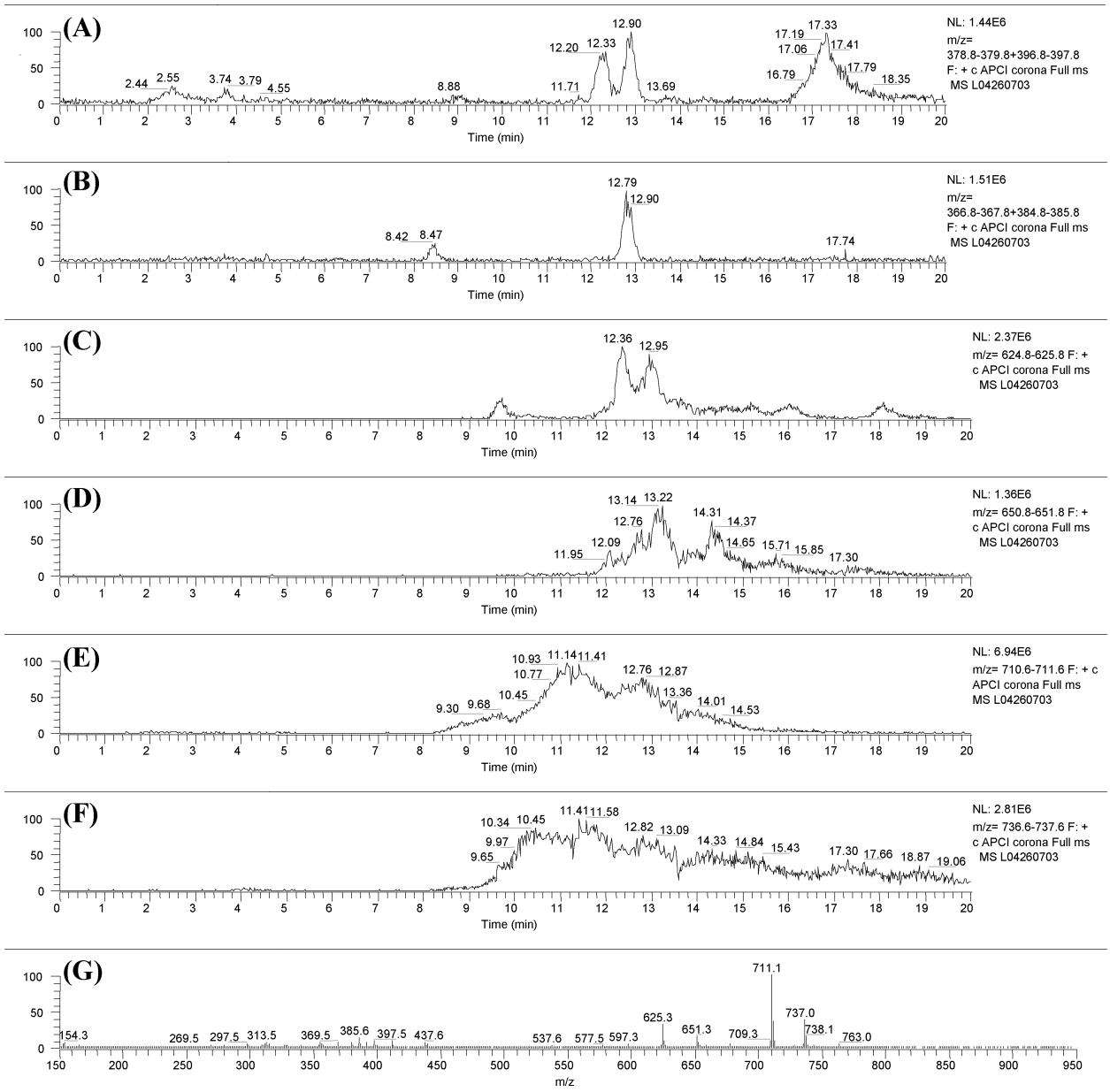

Figure 6. Extracted ion chromatograms (EICs) and mass spectrum from RP-HPLC APCI-MS of processed cheese. (**A**) EIC of *m*/*z* 379.3 and *m*/*z* 397.3 for vitamin D2; (**B**) EIC of *m*/*z* 367.3 and *m*/*z* 385.3 for vitamin D3; (**C**) EIC of *m*/*z* 625.3; (**D**) EIC of *m*/*z* 651.3; (**E**) EIC of *m*/*z* 711.1; (**F**) EIC of *m*/*z* 737.1; (**G**) mass spectrum averaged across the vitamin D3 peak in **Figure 6A**.

can be useful to indicate the presence of interfering substances, as long as full scan data are acquired in combination with the targeted single channel detection. Because of this, the dual channel detector operated in single wavelength mode is considered the least reliable, the DAD UV detector operated in combination full-scan and single wavelength modes is considered more reliable, and detection by SIM APCI-MS proved most conclusive.

SIM APCI-MS cannot, however, be considered the best possible detection method. It was beneficial that the interfering species that coeluted with vitamin D_3 produced ions at *m*/*z* 379 and *m*/*z* 379 that were homologous with vitamin D_2 and therefore produced a large peak from the SIM analysis using the four ions selected for the internal standard and the analyte (**Figures 5B** and **6A**). However, if the interfering molecule was not structurally similar, SIM analysis using these ions would not have been conclusive. Instead, full-scan MS detection is more reliable to detect all potential interfering species. However, full scan MS usually provides a poorer S/N than SIM analysis, due to the much wider range of masses scanned during the duty cycle of the mass spectrometer. At the very least, both the $[M + H]^+$ and the $[M + H - H_2O]^+$ ions should be used for SIM analysis since the ratio of these provides some confirmation of the purity of the analyte. In processed cheese, the interfering molecule that eluted at the same time as vitamin D_3 (**Figures 5B** and **6A**) gave a much larger dehydrated protonated molecule ion at *m*/*z* 379, relative to the protonated molecule at *m*/*z* 397, than authentic vitamin D_2, whereas vitamin D_3 (**Figures 5C** and **6B**) gave a dehydrated protonated molecule ion at *m*/*z* 367 in essentially the same proportion as the calibration standard.

Dimartino (*28*) recently reported the analysis of vitamin D in processed cheese, in which the value appeared to be statistically indistinguishable from the value reported here, on the basis of a manual interpolation of the figure. However, since no specific value for the sample was provided, the report cannot be discussed in detail. The author used SIM APCI-MS for the analysis of cheese and other foods and mentioned that only one

ion each for vitamin D_2 and vitamin D_3 could be used because of the presence of interfering species.

One approach that has been previously reported is the use of dual parallel mass spectrometers for simultaneous analysis of the eluate from a single chromatographic system (*29*). In the past, this has been used to obtain information from complementary ionization techniques, such as APCI and electrospray ionization (ESI) simultaneously from the same analyte. However, the approach that was applied here was the combination of SIM APCI-MS analysis, which is best for quantification, with full scan APCI-MS for qualitative analysis.

The benefit of full scan spectra can be seen in **Figure 6**. **Figure 6G** shows a mass spectrum across the vitamin D_3 peak from processed cheese, obtained on an ion trap mass spectrometer in parallel with SIM analysis on the tandem sector quadrupole instrument. **Figures 6A** and **B** show extracted ion chromatograms (EICs) extracted out of the full scan data that represent the same ions (*m*/*z* 367.3 and *m*/*z* 385.3 for vitamin D_3, and *m*/*z* 379.3 and *m*/*z* 397.3 for vitamin D_2) as those used for SIM analysis, similar to **Figures 5B** and **C**. The remainder of the EICs in **Figure 6** correspond to the major ions observed in the full scan spectrum in **Figure 6G**. The multiple peaks in these chromatograms indicated the likely presence of several isobaric isomers or homologues. MS/MS was performed to obtain product ion spectra of the *m*/*z* 711.1 precursor and other ions, but they did not fragment efficiently in the ion trap instrument and therefore did not produce usable MS/MS spectra. Nevertheless, the full scan spectra provided conclusive evidence of several overlapped molecules that were not evident or differentiable in the SIM APCI-MS data or UV data.

In summary, the statistical treatment of the UV versus APCI-MS data showed that the UV data provided lower RSD values between runs, between fractions, and between extracts. However, if data from a sufficient number of replicates was obtained, the average APCI-MS values were not statistically different from the average values obtained by UV. The reasons for this difference can be seen in **Figure 3**. The signal-to-noise ratio in APCI-MS ion chromatograms was lower than that in the UV chromatograms, due to the fact that the signal obtained by APCI-MS depended on the ionization efficiency of molecules in the atmospheric pressure chemical ionization source, whereas the UV signal depended on the molar absorptivity.

ANOVA indicated that the run to run variance from UV data was so low that reliable quantification was possible using fewer runs than we analyzed. However, mass spectrometry required the largest number of runs possible in order to produce average values that were statistically the same as those obtained by UV detection.

The results presented here demonstrated that SIM APCI-MS and UV detection produced very comparable results, except in the case where interfering species were present that skewed the results by UV detection. Thus, the results by UV detection were useful for quantification as long as it had been confirmed, using mass spectrometry, that there were no substantial interferences present that coeluted with the analytes.

ABBREVIATIONS USED

ACN, acetonitrile; ANOVA, analysis of variance; APCI-MS, atmospheric pressure chemical ionization−mass spectrometry; CFR, code of federal regulations; DAD, diode array detector; ESI, electrospray ionization; ELSD, evaporative light scattering detector; EIC, extracted ion chromatogram; IPA, isopropanol; MeOH, methanol; MTBE, methyl *tert*-butyl ether; NFNAP, National Food and Nutrient Analysis Program; NP-HPLC, normal-phase high performance liquid chromatography; RP, reversed-phase; RSD, relative standard deviation; SIM, selected ion monitoring; TIC, total ion current chromatogram; UV, ultraviolet; USP, United States Pharmacopeia.

SAFETY

Diethyl ether is highly flammable, and extra care must be taken to avoid sparks or sources of ignition.

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