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Research Article

Extract–filter–shoot liquid chromatography with mass spectrometry for the analysis of vitamin D_2 in a powdered supplement capsule and standard reference material 3280

An "extract–filter—shoot" method for the analysis of vitamin D_2, ergocalciferol, in a dry powdered dietary supplement capsule containing rice flour excipient and in a National Institute of Standards and Technology standard reference material 3280 is reported. Quantification of vitamin D_2 was done by atmospheric pressure chemical ionization mass spectrometry using selected ion monitoring, two transitions of selected reaction monitoring, and extracted ion chromatograms from full scans. UV detection was used for the quantification of Vitamin D_2 in the dry powder capsule, whereas interfering species rendered UV detection unreliable for standard reference material 3280. Average values for standard reference material 3280 ranged from 8.27 ± 0.58 to 8.33 ± 0.57 μg/g using internal standard calibration and response factor approaches, compared to the previous National Institute of Standards and Technology internal value for vitamin D_2 of 8.78 ± 0.11 μg/g, and the recently updated reference value of 8.6 ± 2.6 μg/g. The powdered supplement capsule was found to contain 28.19 ± 0.35 to 28.67 ± 0.90 μg/capsule for a capsule labeled to contain 25.00 μg. The triacylglycerol composition of the rice flour excipient in the powdered supplement capsule determined by atmospheric pressure chemical ionization mass spectrometry is also reported.

Keywords: Cholecalciferol / Ergocalciferol / Vitamin D
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1 Introduction

In recent years, vitamin D deficiency or insufficiency has been associated with an increasing number of disease states [1–3], although the relationships between vitamin D and a variety of diseases are often not definitive, and are a topic of ongoing study and debate [4–8]. There has been increasing attention given in the popular press to the need to maintain adequate levels of this important nutrient, leading to an increasing awareness in the general population. Unfortunately, few foods are naturally rich in vitamin D, among which are fatty fish, such as salmon, which are a rich source of vitamin D_3, or cholecalciferol, and UV-irradiated mushrooms, which are a good source of vitamin D_2, or ergocalciferol.

Because of the paucity of natural food sources of vitamin D, the U.S. Food and Drug Administration (FDA) has long allowed (or required) fortification of some foods, as previously described [9]. Among these are milk, cheese, fruit juices, corn meal and rice, infant formula, and others. Most Americans who do not get adequate vitamin D from sun exposure obtain their daily recommended intake from fortified milk [10]. Since the number of vitamin D fortified foods is limited, while the need for vitamin D is great, many consumers have turned to dietary supplements to complement the amount of vitamin D they get from their daily food intake.

Both vitamin D_2 and vitamin D_3 are available as dietary supplements, in the form of oil-filled gelcaps, compressed powder tablets, powder-filled capsules, and others. In most dietary supplements, vitamin D_3 typically comes from synthetic vitamin D_3, vitamin D_3 extracted from lanolin, or vitamin D_3 "molecularly distilled" from fish oil. Even for synthetic

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Abbreviations: **ACN**, acetonitrile; **APPI**, atmospheric pressure photoionization; **CA**, certificate of analysis; **CAD**, charged aerosol detector; **CPST**, compressed powder supplement tablet; **DCM**, dichloromethane; **ELSD**, evaporative light scattering detector; **FA**, fatty acid; **FSVs**, fat-soluble vitamins; **IU**, international units; **MeOH**, methanol; **NIST**, National Institute of Standards and Technology; **PSC**, powdered supplement capsule; **SIM**, selected ion monitoring; **SSD**, statistically significantly different; **SRM**, selected reaction monitoring; **SRfM**, standard reference material; **USDA**, United States Department of Agriculture

vitamin D, there is a popular perception that vitamin D_3 is an animal product, so is undesirable for those pursuing a vegan or vegetarian lifestyle. For those consumers, "vegetarian" dietary supplements containing vitamin D_2 are available.

Clinical analyses predominantly focus on quantification of the circulating 25-hydroxy metabolites of vitamin D, which serve as the primary biomarkers for the nutrient. But it is also important to gain knowledge of the amounts of vitamin D in foods and supplements consumed that lead to those metabolites. Unfortunately, there are substantial methodological challenges to vitamin D analysis, as previously described [11]. Most analyses involve saponification followed by LLE, and then either a semi-preparative LC separation or SPE as a cleanup step, and finally the analytical separation. Detection is typically done using either UV absorbance (usually at or near the absorbance maximum of 265 nm) [12, 13] or mass spectrometric detection [14], often using selected reaction monitoring (SRM) for a high degree of specificity. The sample preparation and preparative chromatography or SPE steps are time consuming, labor and resource intensive, and lend themselves to substantial uncertainty and less than optimal reproducibility.

To address these issues, we reported a "dilute-and-shoot" method for the analysis of vitamin D_3 in gelcaps containing dietary oils as the excipient [15]. The method was ideally suited to those samples, since the gelcap contents were completely soluble in the dilution solvent, so extraction efficiency or recovery was not an issue. Using that method, all saponification, extraction, and semipreparative chromatography steps were eliminated, saving substantial time and resources versus conventional methods for vitamin D_3 analysis. Furthermore, by eliminating the saponification step, we were able to fully characterize the excipient oils, which would normally have been broken down by saponification and eliminated during the extraction process. This allowed us to identify, for the first time, short-chain triacylglycerols that were associated with vitamin D from fish oils that were not present in supplements containing vitamin D from other sources. That report employed three mass spectrometers in parallel for a "triple parallel mass spectrometry" approach, and was later expanded to a "quadruple parallel mass spectrometry" approach [16].

Here we report the modification and extension of the previous method to analysis of vitamin D_2 in a dry powdered supplement capsule (PSC) and in a compressed powder supplement tablet (CPST). Since it was necessary to filter insoluble excipient components in the powder suspensions prior to injection, and since the sample was not soluble (hence "extract" is more accurate than "dilute"), the previous dilute-and-shoot method became the "extract–filter–shoot" method reported here. But since the powders are not fully soluble in the dilution solvent, it is now necessary to prove the degree to which vitamin D is released from the powder matrices. To accomplish this, we used standard reference material (SRfM) 3280 from the National Institute of Standards and Technology (NIST) as the CPST, since this material initially had a certified value for vitamin D_2, with an LC–MS value from NIST that served as a measure of the accuracy of the technique. However, the certified value was recently changed to a reference value. The results herein may provide insights into the reasons for that change.

2 Materials and methods

2.1 Chemicals and samples

Methanol (MeOH), Optima LC–MS-grade #A456–4, acetonitrile (ACN), Optima LC–MS-grade #A955–4, and methylene chloride (dichloromethane, DCM), Optima grade #D151–4, from Fisher Scientific (Pittsburgh, PA, USA) were used. Ammonium formate #516961, synthetic crystalline cholecalciferol #1357, and synthetic crystalline ergocalciferol #5750 were from Sigma–Aldrich (St. Louis, MO, USA). Deionized (D.I.) water was obtained from a Millipore Milli-Q® purification system (Millipore, Bedford, MA, USA).

A bottle of dry, vegetarian 1000 IU (25 μg) PSCs with a "Best By" date of 05/2012 was purchased from an online supplier of herbal supplements and was kept refrigerated until the time of analysis. Analysis was begun at the end of the "Best By" period to determine the amount of vitamin D present at the end of the recommended storage period. One old batch of SRfM 3280 tablets that had originally been used for the determination of the certified value for water-soluble vitamins [17] was used, and a fresh batch of SRfM 3280 from the same lot was purchased from the NIST on 07/10/2012 (mm/dd/yyyy) and kept refrigerated until analysis (batch certificate of analysis (CA) issue date 05/09/2012, updated 07/31/2013, original CA date 01/14/2009). Batches of 12, 15, or 30 tablets were ground to a fine uniform powder using a Retsch RM100 (Verder Scientific, Newton, PA, USA) rotary mortar and pestle. Moisture was determined for five ~1.5 g (~1 tablet) aliquots using an oven at 80°C for 4 h. The dry weight fraction of the CPSTs was determined to be 0.9864 ± 0.0006, in agreement with the NIST value of 0.9863 ± 0.0051 in the CA.

The two halves of each PSC were carefully separated over the mouth of tared 100 mL volumetric flasks, the capsule halves were held inside the mouth of the volumetric flask, and the backs of the capsules were tapped with a thin spatula to release all powder into the flask. The average ($n = 5$) sample weight was 0.43934 ± 0.00847 g ($\pm$ 1.93%). The powder was covered with ~40 mL of the dilution solvent, 60% MeOH/40% DCM, with swirling, to minimize exposure to oxygen. 2.0 mL of 25.0 μg/mL vitamin D_3 internal standard (IS) was added (= 2000 IU/100mL), and the flask was filled to the mark with dilution solvent. The flask was partially inverted and very vigorously (manually) shaken for ~1 min 20 s (slow count to 60). Just prior to transferring the samples to autosampler vials, they were vigorously shaken again for a slow 30 count, and the suspension was immediately poured into the barrel of a 5 mL glass syringe (Popper & Sons, New Hyde Park, NY, USA) with a 25 mm, 0.45 μm PTFE syringe filter (Agela Technologies, Newark, DE, USA) attached. The syringe plunger was inserted, and ~3.0 mL of solution was

discharged to waste, and ~1.5 mL of clear filtered solution was transferred to an amber autosampler vial (National Scientific, Rockwood, TN), and the remainder was discharged to waste. Syringe filters were prerinsed with ≥5 mL of dilution solvent immediately prior to use.

Ground SRfM 3280 tablet powder was prepared and filtered the same as the capsule powders, except that ~1.5 g (~1 tablet) of powder was used. Average (n = 5) sample weights were 1.54765 ± 0.02955 g (1.91%) for the first sequence (12 older tablets from the same lot, ground), 1.54058 ± 0.01507 g (±0.98%) for the second sequence (30 fresh tablets from the same lot, ground, also used for moisture determination), and 1.54465 ± 0.00850 (±0.55%) for the third and fourth sequences (15 fresh tablets from the same lot, ground). The final filtered CPST solutions were clear orange/yellow.

Four calibration standard solutions were routinely prepared for analysis of all supplements, containing 0.125 μg/mL (500 IU/100 mL), 0.25 μg/mL (1000 IU/100 mL), 0.50 μg/mL (2000 IU/100 mL), and 1.25 μg/mL (5000 IU/100 mL), which all contained 0.50 μg/mL vitamin D_3 IS. The lower three standards were used for samples with <1000 IU label amounts (i.e., SRfM 3280), and the higher three standards were used for samples with ≥ 1000 IU label amounts (i.e., capsules).

Bracketed sequences were run that consisted of alternating sets of three calibration standards (low, medium, high) and three replicates of each sample prepared, giving five sets of calibration standards and five samples in triplicate, followed by a sixth set of standards, followed by one column cleanup run. An additional low standard run was performed at the beginning of each sequence as a troubleshooting run, since most instrument errors and sequence start failures, if any, occurred in this run. This gave 19 standard runs, 15 sample runs, and a column cleanup run, for 35 runs per sequence.

A solution of 50 mM ammonium formate in H_2O/ACN 1:4 was made by diluting 200 mL of 250 mM ammonium formate in D.I. water with 800 mL ACN, which served as the electrolyte solution to promote ion formation by ESI-MS.

2.2 Instrumentation

2.2.1 High-performance liquid chromatography

An Agilent 1200 system was used that consisted of the solvent module with membrane degasser (G1379B), quaternary pump (G1311A), autosampler (G1329A), thermostatted column compartment (G1316A), diode array detector (DAD) SL (G1315C), and two-channel 24-bit analog-to-digital converter (ADC) (35900E). Two Inertsil ODS-2 columns in series, 25 cm × 4.6 mm, 5 μm particles (GL Sciences, Torrance, CA, USA), joined by a circularly bent 7 cm piece of 0.007 in. i.d. stainless-steel tubing, were used. Columns were maintained at 10°C throughout using the Agilent column temperature controller.

For capsule powder samples, two options were demonstrated, the full-length run that was previously described was used [15, 16], and a second method was used that had only the initial isocratic methanol segment for 28 min. For SRfM 3280 samples, the initial isocratic methanol segment was extended to 65 min, to allow other fat-soluble vitamins to elute, in addition to vitamin D. The gradient for triacylglycerol analysis was eliminated from the method used for those samples.

Additional conditions and parameters, including those for the DAD, evaporative light scattering detector (ELSD), and corona charged aerosol detector (CAD), have been described recently [15].

2.2.2 Mass spectrometry

After the DAD, a series of five Valco tees was used to split the flow to go to: (1) the atmospheric pressure photoionization (APPI) mass spectrometer, (2) ELSD, (3) corona CAD, (4) atmospheric pressure chemical ionization (APCI) mass spectrometer in full-scan mode, (5) ESI mass spectrometer, and (6) an APCI mass spectrometer in selected ion monitoring (SIM), SRM, and full-scan modes. The lengths and flow rates of the flow-splitting tees, as well as the conditions used for each of the four mass spectrometers, have been described recently [16] and are repeated for convenience in the Supporting Information. Quantification of triacylglycerols (TAGs) was performed using APCI-MS on the TSQ Vantage EMR (Thermo Scientific, San Jose, CA, USA), since we previously showed that this gave the most accurate results without response factors [16]. The other mass spectrometers were used only for qualitative purposes.

2.2.3 Calculations

Raw integrated areas were pasted into Microsoft Excel spreadsheets with the Analysis Toolpak installed. For the PSCs, areas were divided by the weight of the powder sample times the average weight of the sample set, so all samples could be compared on an equal weight basis, in μg/capsule and IU/capsule, where 1 IU = 40 μg vitamin D_2. For SRfM 3280, areas were divided by the weight of the powder, divided by the determined dry weight factor (0.9864), and expressed in μg/g for comparison to the CA. For thorough comparison, results were calculated by the IS, external standard (ES), internal standard response factor (iRF), and external standard response factor (eRF) approaches, as previously described [15].

Statistical significance for data using the same detector (UV or MS) was calculated using a paired t-test (two-tailed) for sample means, and statistical significance between different detectors was calculated using a two-sample t-test (two-tailed) assuming unequal variance.

Extracted ion chromatograms (EICs) of vitamin D and TAGs that were extracted out of the total ion current chromatograms (TICs) included the 1x ^{13}C isotope for quantification to increase signal without loss of specificity, as recently described [16]. The LOD and LOQ values were calculated as

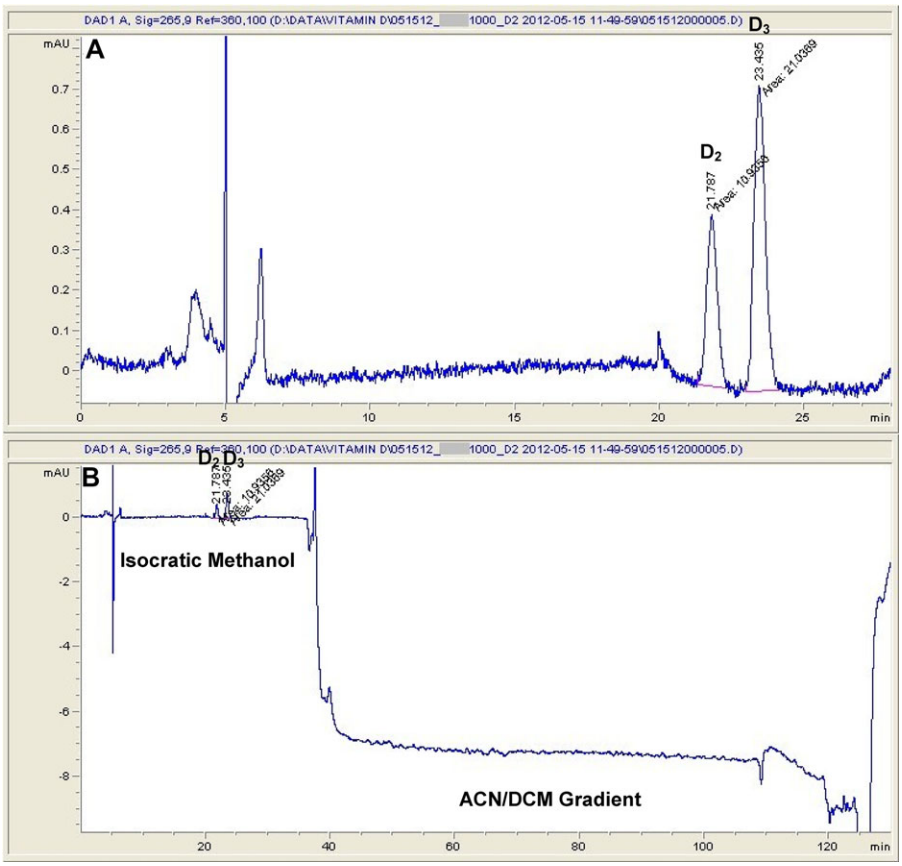

Figure 1. Agilent 1200 UV DAD chromatograms of dry powder 1000 IU vitamin D_2 supplement capsule. (A) 0–28 min and (B) full-length chromatogram. See Figure S7 for 3D chromatogram of 20–25 min. Brand masked for anonymity. Figure labels added for clarity. Higher resolution version provided as Figure S1 in Supporting Information.

previously described [15] and are given in the Supporting Information.

The following abbreviations are used for TAG analysis (C:db, carbons:double bonds): myristic acyl chain (a.c.), M, 14:0; palmitoleic a.c., Po, 16:1; palmitic a.c., P, 16:0; linolenic a.c., Ln, 18:3; linoleic a.c., L, 18:2; oleic a.c., O, 18:1; stearic a.c., S, 18:0; gadoleic a.c., G, 20:1; arachidic a.c., A, 20:0; behenic a.c., B.

3 Results

3.1 Powdered supplement capsules (PSCs)

3.1.1 Vitamin D_2 in the PSCs by UV detection

The UV chromatogram of the initial portion of the chromatographic run, employing isocratic methanol for 28 min, is shown in Fig. 1A, and the full chromatogram, incorporating the acetonitrile/dichloromethane gradient, is in Fig. 1B. Larger, higher resolution versions of Figs. 1–6 are provided in Supporting Information Figs. S1–S6. The signal-to-noise ratio (S/N), peak shapes, and resolution all indicate excellent separation and sensitive UV detection of vitamin D_2 (21.8 min) and vitamin D_3 (23.4 min). The 3D chromatogram of full-scan spectra for these two peaks is shown in Fig. S7. For comparison, similar chromatograms for the calibration standard having 0.25 μg/mL (1000 IU/100 mL) are given in Fig. S8. These data indicate that the PSC samples gave chromatograms and spectra that were in very good agreement with pure standards.

The full-scan UV data gave spectra that corresponded well to literature spectra, and provided a good indication that there were no overlapping species that interfered with UV detection. The absence of interferents was further confirmed by the mass spectrum in Fig. 2G. The absence of interfering species allowed: (1) the UV data to be used for quantification, taking advantage of the lower %RSD that is a characteristic of UV results versus MS results, and (2) the ES method to be used, in addition to the IS and iRF methods. This is why we repeatedly emphasize [15, 16, 18] the importance of obtaining both full-scan UV and full-scan MS spectra, in addition to any targeted approach used, to prove the purity of peaks and to determine whether UV data can be trusted (whether quantification bias is expected). For the three sequences of samples given in Table 1, the coefficients of determination, R^2, from the calibration curves were >0.9990 for all IS and ES calibration curves.

The three rows in Table 1 represent the same five aliquots run in triplicate, for $n = 15$, of samples that were run initially and then stored in a refrigerator and rerun using two different chromatographic methods. Using the IS method results as an example, the values obtained were 0.2699 ± 0.114,

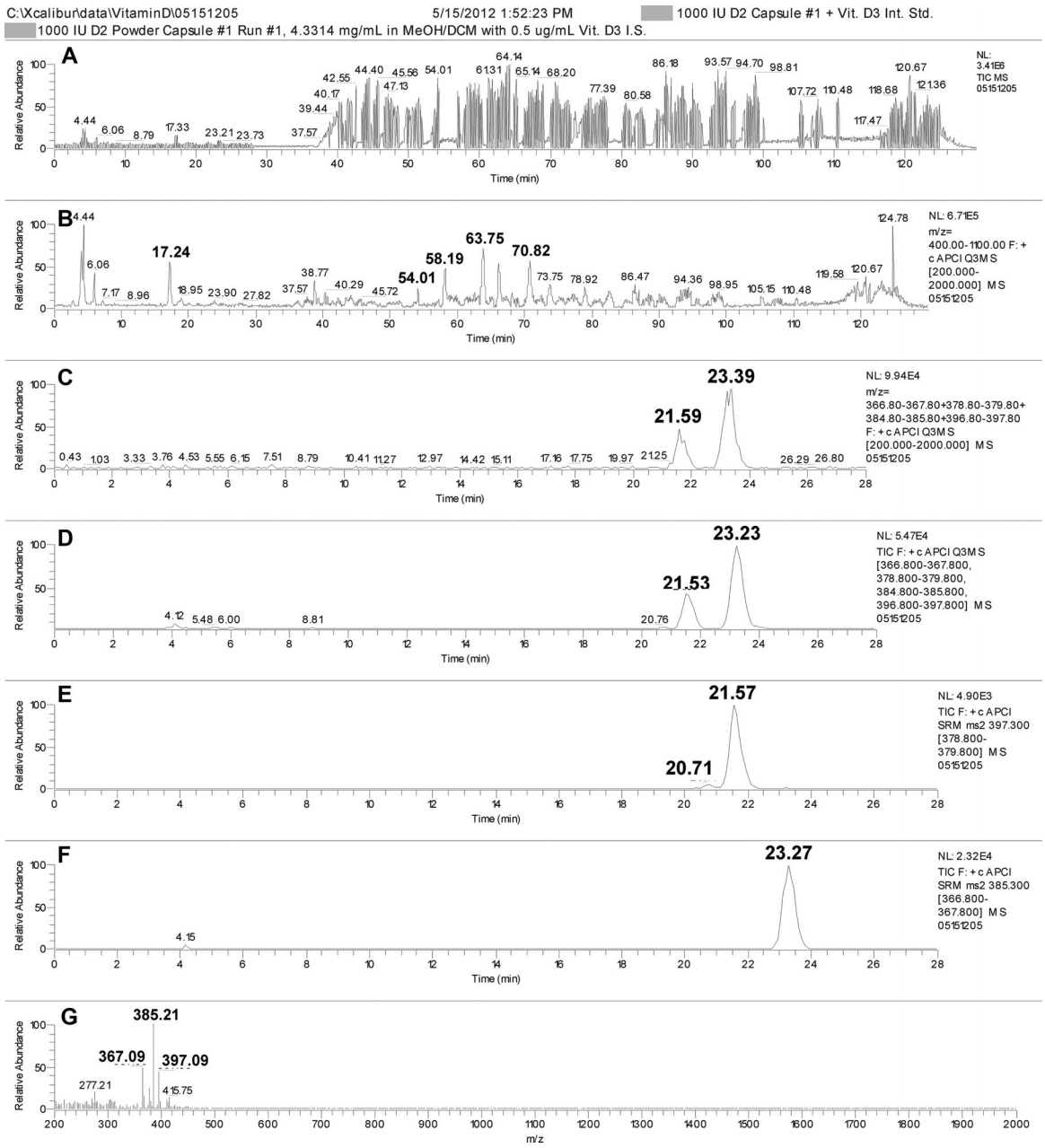

Figure 2. TSQ Vantage EMR APCI-MS data for dry powder 1000 IU vitamin D_2 supplement capsule. (A) total ion current chromatogram (TIC); (B) extracted ion chromatogram (EIC) of *m/z* 400–1100; (C) EIC of ions associated with vitamin D_2 and D_3; (D) selected ion monitoring (SIM) chromatogram of vitamin D ions; (E) selected reaction monitoring (SRM) chromatogram of *m/z* 397.3 → 379.3 for vitamin D_2; (F) SRM chromatogram of *m/z* 385.3 → 367.3 for vitamin D_3; and (G) mass spectrum across peaks in (C). Brand masked for anonymity. Figure labels added or enlarged for clarity. Original higher resolution version provided as Figure S2 in Supporting Information.

0.2811 ± 0.0108, and 0.2808 ± 0.0120 μg/mL, respectively, or 1080 ± 45, 1125 ± 43, and 1123 ± 48 IU, respectively. Using all three approaches for quantification (IS, ES, iRF), the initial analysis was statistically significantly different (SSD) from the later two analyses ($p = 6.98E\text{-}5$ and $p = 1.03E\text{-}4$), although the differences were less than 4% (3.98 and 3.88%) for the first versus the second and first versus the third analyses, respectively. The second analysis versus the third analysis gave very similar results using the long chromatographic separation versus the short separation for all three approaches (IS, ES, iRF), and these were not SSD.

3.1.2 Vitamin D_2 in PSCs by MS

Table 2 lists all results for vitamin D_2 analysis by MS. The preferred methods for analysis are SIM or SRM. Figure 2 shows chromatograms used for quantification by EIC (Fig. 2C), SIM (Fig. 2D), and MRM1 ($[M+H]^+ \rightarrow [M+H-H_2O]^+$) for

Table 1. UV detection (at 265 nm) results for vitamin D_2 in powdered supplement capsule (PSC) labeled to contain 25 μg (1000 IU) vitamin D_2, which were dissolved in 100 mL, to give 0.25 μg/mL. Third sequence performed using short runs only

Date	Int. std. (μg/mL)	± SD ($n = 5$)	% RSD	Ext. std.	± SD ($n = 5$)	% RSD	iRF	± SD ($n = 5$)	% RSD
051512	0.2699[a)]	0.0114	4.21%	0.2675	0.0106	3.96%	0.2712	0.0110	4.04
060812	0.2811	0.0108	3.86%	0.2822	0.0101	3.56%	0.2796	0.0104	3.71
062012	0.2808	0.0120	4.28%	0.2839	0.0119	4.20%	0.2805	0.0114	4.08
Average	0.2773		Average	0.2779		Average	0.2771		
SD	0.0064		SD	0.0090		SD	0.0051		
% RSD	2.30%		% RSD	3.24%		% RSD	1.86%		
Date	Int. std. (IU)	± SD ($n = 5$)	% RSD	Ext. std.	± SD ($n = 5$)	% RSD	iRF	± SD ($n = 5$)	% RSD
051512	1080[b)]	45	4.21%	1070	42	3.96%	1085	44	4.04
060812	1125	43	3.86%	1129	40	3.56%	1118	42	3.71
062012	1123	48	4.28%	1136	48	4.20%	1122	46	4.08
Average	1109		Average	1112		Average	1108		
SD	25		SD	36		SD	21		
	LOD (IU)	LOQ (IU)		LOD	LOQ		LOD	LOQ	
051512	47[b)]	157		36	120		45	151	
060812	55	183		38	126		52	175	
062012	40	134		45	152		38	127	

a) Values in μg/mL for PSC in 100 mL.
b) Values in IU per capsule, where 1 IU = 0.025 μg.

vitamin D_2 (Fig. 2E) and vitamin D_3 (Fig. 2F). The average values found for vitamin D_2 using the IS calibration curve approach by SIM, MRM1, and MRM2 were 0.2835 ± 0.0041, 0.2844 ± 0.0042, and 0.2855 ± 0.0070 μg/mL, respectively. These were not SSD, and these were not SSD from the corresponding value by UV using the IS method, given above.

We have previously reported, and these data confirm, that the ES method should not be used for MS data, due to a general decrease in the raw areas over the course of the sequence, primarily due to buildup of residue on the corona needle. Dramatically different values by ES versus IS and iRF approaches and large %RSD emphasize this point. Those data are included here to prove that the ES method by MS should be avoided. This is further proven by the high LOD and LOQ values in Table S1 (μg/mL in Table S1A, IU/capsule in Table S1B) for the ES approach. Eliminating the acetonitrile/dichloromethane gradient in the third sequence of runs eliminated this problem and produced values very similar to the internal standard (IS and iRF) approaches (third row in each section of Table 2), also reflected in the much lower LOD and LOQ values (Table S1). Nevertheless, use of the ES method by APCI-MS is discouraged.

The average values obtained for SIM, SRM1, and SRM2 by MS using the iRF approach were 0.2819 ± 0.0035, 0.2835 ± 0.0052, and 0.2867 ± 0.0090 μg/mL, respectively, which were not SSD. The only SSD value found in IS versus iRF results from SIM or SRM was between the second and third sequences for the iRF method by SRM2, which differed by 6.4%. This difference is typical for run-to-run variability by MS, so such a difference between separate sample sequences constitutes good agreement. Thus, any of the six internal standard methods for quantification by IS or iRF using SIM, MRM1, or MRM2 gave good agreement by MS. These methods indicated that the PSCs contained 12–15% in excess of the label amount, which is consistent with the U.S. code of federal regulations (CFR) 21 CFR 101.9(g)(4)(i), which requires content at least equal to the declared value, and 21 CFR 101.9(g)(6), which allows a reasonable excess in line with current good manufacturing practices.

The approach of using untargeted analysis and then extracting out the $[M+H]^+$ and $[M+H-H_2O]^+$ ions for quantification using EICs produces higher %RSD, since the proportion of the duty cycle of the mass spectrometer spent at the analyte masses, and therefore the signal-to-noise ratio, is so much less (scanning the full range m/z 200–2000 in 1 s in the first segment of the run versus scan times of 0.5 s at only the analyte masses in SIM and SRM modes). The IS values in Table 2 obtained using the peaks in the EICs were all three SSD from each other, whereas the iRF values were not.

3.1.3 Rice flour TAGs by MS

Although the PSC samples did not contain liquid oil like the supplements we previously analyzed [15, 16], the rice flour powder did have enough oil associated with it that it could be observed. But since the amount of oil was so much lower, the TAG peaks were not clearly visible in the TICs (Figs. 2A, 3A and D), but could be seen using EICs (Figs. 2B, 3B and E). This allowed fewer TAGs to be identified, compared to the earlier report on rice bran oil [15].

Table 3 shows the diacylglycerol (DAG), TAG, and calculated fatty acid (FA) compositions determined from LC–APCI-MS of the rice-flour-containing PSCs. The composition

Table 2. Quantification of vitamin D_2 in powdered supplement capsule having a 25 μg label amount diluted to 100 mL (= 0.25 μg/mL) by APCI-MS using selected ion monitoring (SIM), two transitions of selected reaction monitoring (SRM) experiments, and extracted ion chromatograms (EICs) from full-scan MS data. Individual values represent an average of 5 samples, each run in triplicate

Date	Int. std.	± SD ($n = 5$)	%RSD	Ext. std.	± SD ($n = 5$)	%RSD	iRF	± SD ($n = 5$)	%RSD
SIM weight-normalized results (μg/mL)									
051512	0.2788	0.0238	8.52%	0.0501	0.1163	232.18%	0.2788	0.0202	7.24
060812	0.2862	0.0294	10.27%	0.2804	0.1200	42.81%	0.2813	0.0229	8.16
062012	0.2855	0.0149	5.21%	0.2864	0.0174	6.07%	0.2857	0.0150	5.24
Average	0.2835			0.2056			0.2819		
SD	0.0041			0.1347			0.0035		
%RSD	1.45%			65.53%			1.25%		
% of Label	113.4%			82.3%			112.8%		
SRM1 weight-normalized results (μg/mL)									
051512	0.2893	0.0401	13.86%	0.1015	0.1215	119.79%	0.2895	0.0375	12.95
060812	0.2817	0.0107	3.79%	0.2629	0.0935	35.58%	0.2810	0.0103	3.65
062012	0.2821	0.0133	4.70%	0.2823	0.0207	7.34%	0.2802	0.0131	4.69
Average	0.2844			0.2155			0.2835		
SD	0.0042			0.0993			0.0052		
%RSD	1.49%			46.06%			1.83%		
% of Label	113.7%			86.2%			113.4%		
SRM2 weight-normalized results (μg/mL)									
051512	0.2889	0.0326	11.28%	0.1030	0.1242	120.63%	0.2861	0.0313	10.93
060812	0.2902	0.0195	6.70%	0.2485	0.0990	39.84%	0.2960	0.0196	6.63
062012	0.2775	0.0096	3.46%	0.2784	0.0209	7.52%	0.2781	0.0093	3.34
Average	0.2855			0.2100			0.2867		
SD	0.0070			0.0938			0.0090		
%RSD	2.45%			44.69%			3.13%		
% of Label	114.2%			84.0%			114.7%		
EIC weight-normalized results (μg/mL)									
051512	0.2645	0.0439	16.59%	-0.4756	0.1781	-37.44%	0.2517	0.0336	13.33
060812	0.3367	0.0500	14.85%	0.3777	0.0925	24.50%	0.2807	0.0152	5.43
062012	0.2902	0.0132	4.54%	0.2819	0.0203	7.18%	0.2751	0.0135	4.91
Average	0.2971			0.0614			0.2692		
SD	0.0366			0.4674			0.0154		
%RSD	12.32%			761.92%			5.72%		
% of Label	118.9%			24.5%			107.7%		

of the FAs calculated from the rice flour TAG composition for these samples was markedly different from the liquid rice bran oil we recently reported [15], and the TAGs had higher uncertainties due to their poorer S/N compared to liquid oils. The primary differences are that the oleic and linoleic acid percentages are essentially reversed compared to rice bran oil, with linoleic acid predominating. Unfortunately, there is a paucity of well-documented data on the FA composition of rice four, compared to rice bran oil. The data at the National Food Institute of the Technical Institute of Denmark (http://www.foodcomp.dk/v7/fcdb_details.asp?FoodId=0222), given in Table S2, show a FA composition that reflects more L than O, with 40.4% L and 28.9% O (converted to mole percentage), for a ratio of L/O of 1.40. The amount of O reported there is less than in Table 3, whereas the amount of P is higher. Those data were sourced from a 1978 book [19]. The USDA National Nutrient Databank for Standard Reference, Standard Release (SR) 26 gives values, in g, for the FA content in white and brown rice flour. When those data are converted to a mole percentage composition (Table S2), the brown flour FAs are more similar to the results in Table 3 than the white rice flour composition. In brown rice flour, the most abundant three FAs (P, O, and L) give mole percentages of 20.04, 38.40, and 36.96%, for P, O, and L, respectively, which has a ratio of L/O of 0.96, compared to 1.17 from Table 3. White rice flour has calculated values of 29.06, 33.50, and 29.06% for P, O, and L, respectively, giving a ratio of L/O of 0.87. Clearly there is high variability in the sparse literature results for rice flour FAs.

Furthermore, there is limited utility in comparing literature values for rice flour to these results for rice flour triacylglycerols, since saponification of whole rice flour includes fatty acids from phospholipids, free fatty acids, triacylglycerols, and other saponifiable lipids. We are not aware of any reports on rice flour triacylglycerols (e.g. SCOPUS search of rice flour triacylglycerols).

Unfortunately, it was not informative to saponify the PSC powder for FA determination, since the PSCs contained "magnesium stearate," of which commercial formulations

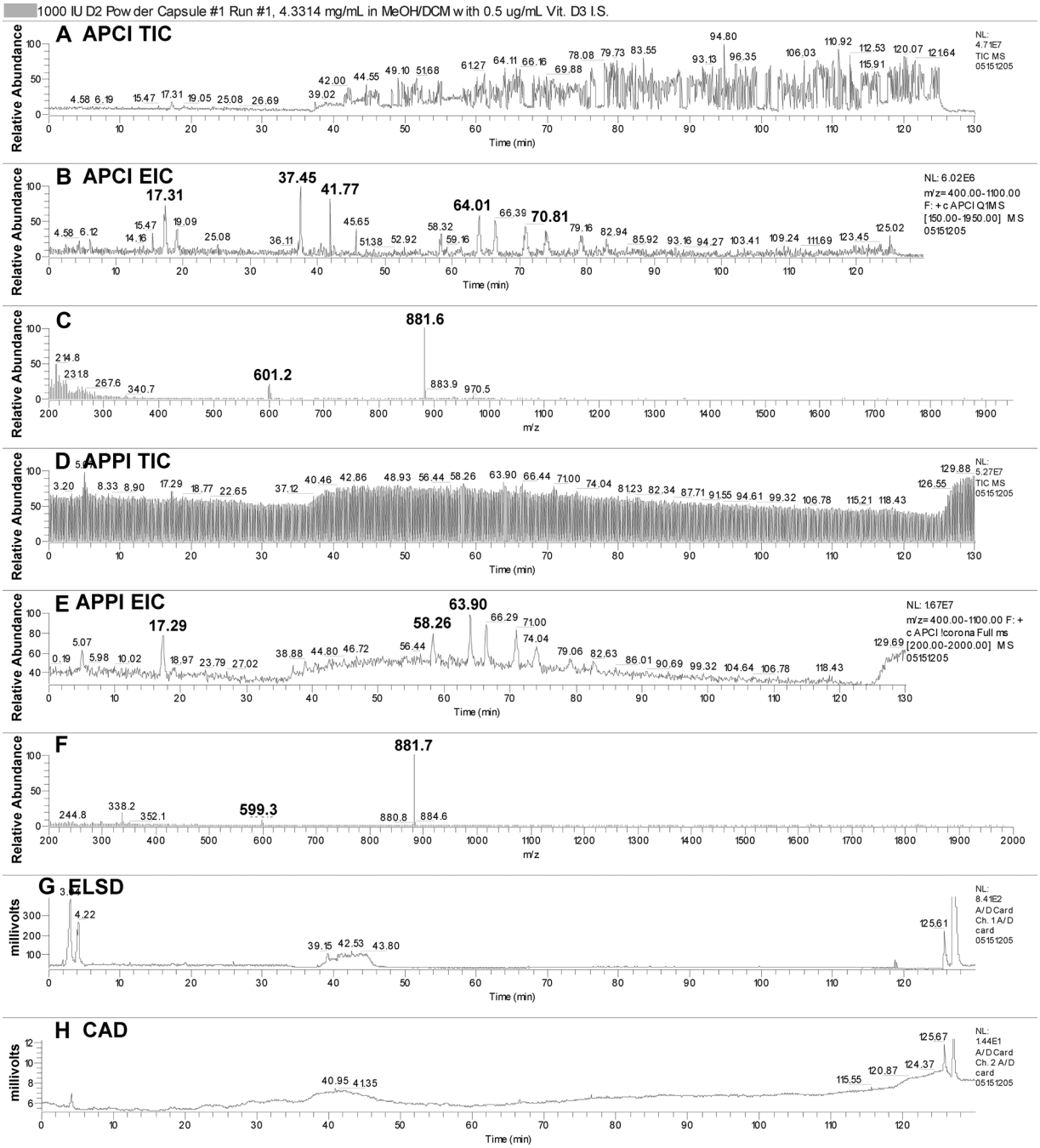

Figure 3. APCI, APPI, ELSD, and corona CAD chromatograms and mass spectra of vitamin D_2 powdered capsule. (A) TSQ 7000 APCI-MS total ion current chromatogram (TIC); (B) TSQ 7000 extracted ion chromatogram (EIC) of *m/z* 400–1100; (C) APCI-MS mass spectrum across peak of LLO at 64.01 min; (D) LCQ Deca XP APPI-MS TIC; (E) LCQ Deca XP EIC of *m/z* 400–1100; (F) APPI-MS mass spectrum of LLO at 63.90 min; (G) ELSD chromatogram; and (H) corona CAD chromatogram. Brand masked for anonymity. Figure labels added or enlarged for clarity. Original higher resolution version provided as Figure S3 in Supporting Information.

contain magnesium stearate and magnesium palmitate, as well as small amounts of other FA salts [20]. The FA composition from FAME analysis of the PSC powder (not shown) revealed 49.68% P and 34.79% S, clearly indicating the dominance of "magnesium stearate" over rice flour TAGs in the PSC excipient. Since GC–FID-derived RFs could not be used as recently described [16], only quantification of TAGs by APCI-MS on the most sensitive instrument was performed, because we previously showed [16] that this gave the most accurate results without RFs.

Table 3. Relative percentage of diacylglycerol (DAG) and triacylglycerol (TAG) molecular species by APCI-MS, and the fatty acid (FA) composition calculated from the TAG composition

DAG	Average (%)	SD (%)	TAG	Average (%)	SD (%)
LL1	5.00	4.88	LnLnL	0.05	0.02
LL2	3.26	2.43	LLLn	0.56	0.14
OLn1	4.93	1.32	LLL	7.09	1.96
OLn2	2.18	0.74	OLLn	0.97	0.26
OL1	22.17	3.90	PLLn	0.83	0.33
OL2	8.75	1.64	LLO	18.01	4.88
PL1	9.91	1.46	OOLn	0.54	0.13
PL2	3.26	1.02	PoOL	0.18	0.11
OO1	18.35	4.26	LLP	15.67	3.35
OO2	6.23	1.33	POLn	0.36	0.09
OP1	10.22	1.95	PoPL	0.42	0.15
OP2	3.84	1.09	LLG	0.11	0.03
SL1	1.03	0.42	PPLn	0.10	0.05
SL2	0.86	0.29	OOL	14.33	2.81
			OOPo	0.22	0.06
Sum	100.00		LLS	1.06	0.25
DAG/TAG	9.43	±2.11	POL	13.57	3.02
			SOLn	0.92	0.25
			OLG	0.23	0.08
			PPL	2.51	0.50%
Fatty acids from TAGs			OOO	7.98	2.16
FA	Average (%)	SD (%)	PLG	0.20	0.06
Po	0.27	0.08	SLO	1.16	0.36
P	16.40	1.44	OOP	6.80	1.75
Ln	1.46	0.23	SLP	0.63	0.16
L	42.62	3.39	OOG	0.18	0.06
O	36.59	2.58	POP	1.99	0.71
S	2.06	0.39	LOA	0.33	0.12
A	0.36	0.12	OOS	1.14	0.48
G	0.24	0.06	PPP	0.13	0.05
			SSL	0.14	0.07
Sum	100.00		PLA	0.22	0.12
			POS	0.68	0.20
			SOG	0.00	0.01
			OOA	0.30	0.14
			SLA	0.00	0.01
			POA	0.22	0.11
			SSO	0.15	0.07
			OOB	0.00	0.01
			Sum	100.00	

3.2 SRfM 3280

3.2.1 Vitamin D_2 in SRfM 3280 by UV detection

Since SRfM 3280 contained no triacylglycerols, but did contain other fat-soluble vitamins (FSVs), the acetonitrile/dichloromethane gradient was eliminated from the LC method, and isocratic methanol was extended to 65 min to elute FSVs. Although only vitamin D_2 was the subject of our analysis, the method was demonstrated to elute all FSVs to allow us to expand the analysis to other analytes in the future.

The chromatographic peaks for vitamin D_2 analyte in the compressed-powder SRfM 3280 tablet were not well-resolved symmetrical peaks like those from the powdered supplement capsule and calibration standards (Fig. 4C). There was abundant evidence for several overlapping species. The vitamin D_2 peak appeared as a shoulder on a larger peak, which both rose from the tail of a much larger peak (Figs. 4A and B), and required discernment to manually integrate. Figure 4 shows typical peaks that had to be integrated in UV chromatograms to accomplish quantification. It is easy to see that improper integration of the vitamin D_2 peak could easily result in higher values for vitamin D_2 in SRfM 3280.

Surprisingly, with careful integration, the quantification by UV detection (Table S3) agreed fairly well with the results by MS (see Section 3.2.2) and with the original value obtained by NIST by LC–MS (not the original certified value, which included UV data) and the updated reference value. The values in Table S3 are slightly lower than the reference value, since the tail of the peak overlapped the following peak and was not included in the integration. As expected, IS and iRF approaches to quantification gave better values than the ES method. Nevertheless, because of the observed interferent peaks, UV detection was deemed unacceptable for quantification of vitamin D_2 in SRfM 3280 using the extract–filter–shoot method, and the results were relegated to the Supporting Information.

3.2.2 Vitamin D_2 in SRfM 3280 by MS

Since the interfering species that were chromatographically unresolved from vitamin D_3 shared no ions in common with the analyte, quantification by MS was much more straightforward than by UV detection. This is demonstrated by the ion retention times and peak profiles in EICs, e.g. Figs. 5I and J, as well as by the EICs of the interfering species (showing different elution profiles than the analytes) and APCI-MS mass spectra and APPI-MS, MS/MS, and MS^3 mass spectra shown in Figs. S9 and S10 in the Supporting Information.

The results in Table 4 show that all of our average values except the external standard approach applied to EICs from full-scan spectra are in good agreement with both the original value of 8.78 ± 0.11 μg/g obtained by NIST using LC–MS (which was lower than the values of 9.56 ± 0.47 and 9.06 ± 0.06 μg/g obtained by other labs using LC with UV detection) [17], and the new reference value of 8.6 ± 2.6 μg/g [21]. Of course, we have repeatedly indicated [15, 16] that the external standard method, especially for EICs from full-scan MS, is the least preferred approach to quantification, compared to SIM and SRM methods, and should not be relied upon.

The percentage that our value represents compared to the original NIST LC–MS value [17] and the updated reference value [21] are shown in Table 4. These show that our average values were between 94 and 101% of the NIST values using

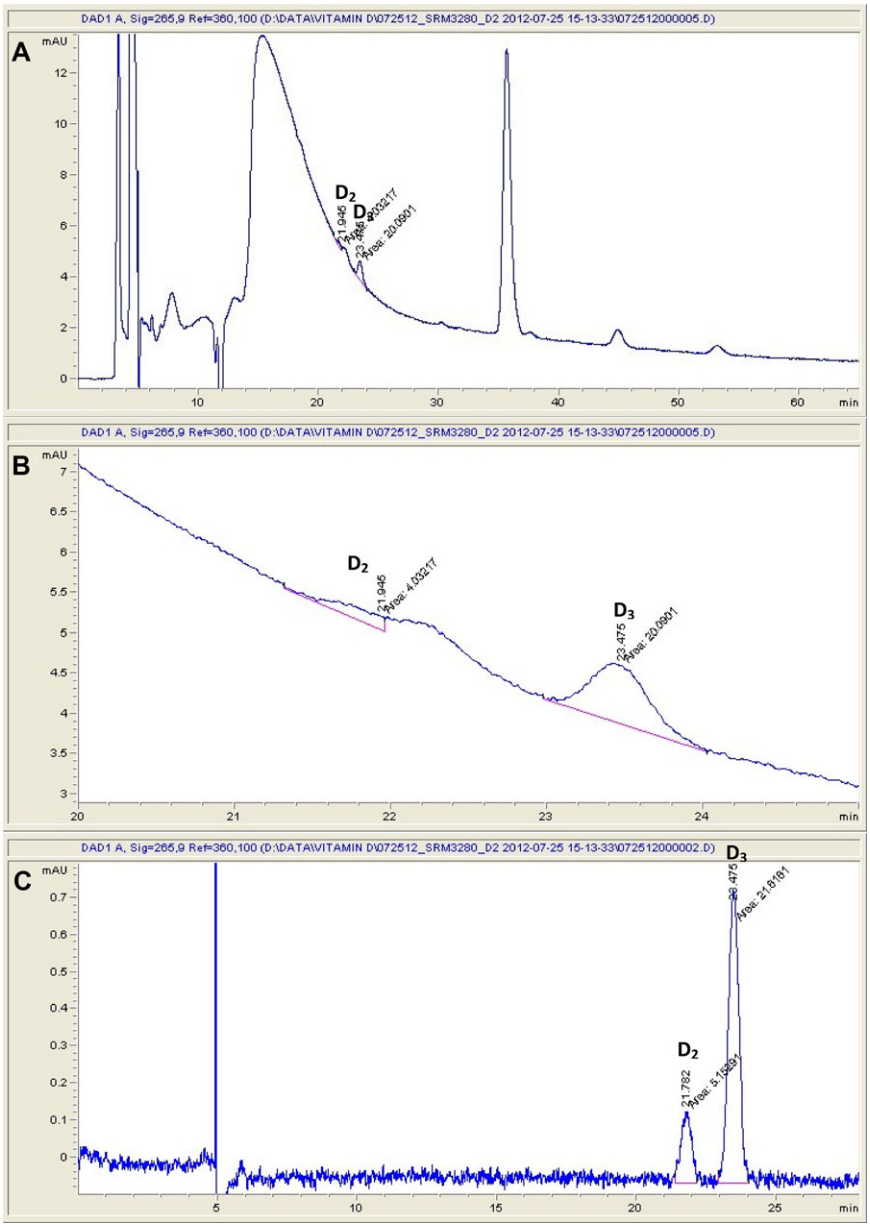

Figure 4. Chromatograms of UV detection at 265 nm for NIST SRfM 3280 and low calibration standard. (A) Full-length chromatographic run from 0–65 min isocratic methanol for NIST SRfM 3280; (B) expanded range from 20–25 min showing elution of vitamin D_2 and D_3 from SRfM 3280; and (C) chromatogram of 0.125 μg/mL (500 IU/100 mL) calibration standard. Figure labels added for clarity. Original higher resolution version provided as Figure S4 in Supporting Information.

the preferred methods for quantification, SIM, and SRM by IS or iRF approaches, and also for the ES approach (except using EICs from full-scans), which is not recommended for quantification as a matter of principle. Thus, Table 4 indicates that the extract–filter–shoot method for quantification provided good agreement to the NIST internal value and the reference value, whereas values from other labs by UV detection were higher [17].

3.2.3 Other fat-soluble vitamins in SRfM 3280

Although the purpose of this report is validation of the extract–filter–shoot method for ergocalciferol based on analysis of a NIST SRfM, we also modified the method to allow for analysis of other FSVs, vitamins A, E, and K. The isocratic methanol portion of the method was extended from 28 to 65 min, and the gradient for elution of TAGs was eliminated. Figure 6 shows EICs and mass spectra associated with retinol (vitamin A, Figs. 6B and F), vitamins D_2 and D_3 (Figs. 6C and G), α-tocopheryl acetate (acetate form of vitamin E, Figs. 6D and H), and phylloquinone (vitamin K_1, Figs. 6E and I) over the 65 min run. These figures show the same masses as those used by NIST, seen in Fig. B1 of the CA [21]. Figure 6D compared to Fig. 6A indicates that vitamin E predominated the TIC for this analysis. The implications of the large amount of α-tocopheryl acetate on quantification are discussed below. Figure 6 shows that the modified chromatographic method used with the extract–filter–shoot analysis of SRfM 3280 was capable of eluting well-resolved peaks for the four FSVs. Full validation of the other FSVs is beyond the scope of this

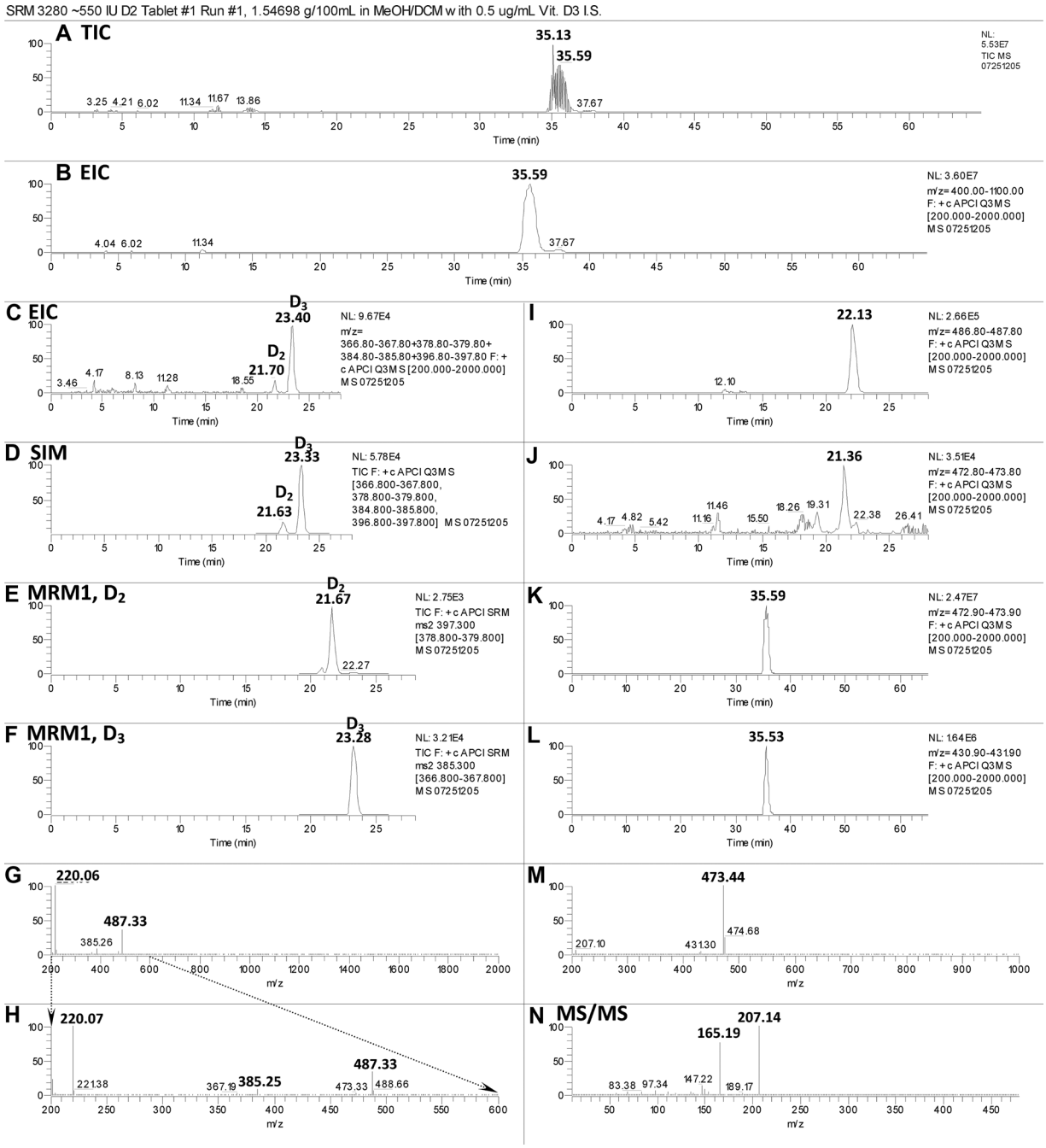

Figure 5. Chromatograms and APCI-MS mass spectra of vitamin D, vitamin E acetate, and related compounds in NIST SRfM 3280. (A) Total ion current chromatogram (TIC); (B) extracted ion chromatogram (EIC) of *m/z* 400–1100; (C) EIC of ions associated with vitamins D_2 and D_3; (D) selected ion monitoring (SIM) scans corresponding to vitamin D_2 and D_3; (E) selected reaction monitoring (SRM) scans corresponding to vitamin D_2; (F) SRM scans corresponding to vitamin D_3; (G) average mass spectrum across vitamin D_3 peak at 23.4 min in C); (H) expanded range of mass spectrum (G) showing *m/z* 200–600; (I) EIC of *m/z* 487.3 seen in H), from 0–28 min; (J) EIC of *m/z* 473.3 seen in H); (K) EIC of *m/z* 473.4 for α-tocopheryl acetate across full run length; (L) EIC of *m/z* 431.4; (M) average mass spectrum across peak at 35.59 min in K); and (N) average MS/MS spectrum of *m/z* 473.4 at 35.59 min. Figure labels added or enlarged for clarity. Original higher resolution version provided as Figure S5 in Supporting Information.

Table 4. Quantification of vitamin D_2 in NIST SRfM 3280 by APCI-MS using selected ion monitoring (SIM), two transitions of selected reaction monitoring (SRM) experiments, and extracted ion chromatograms (EIC) from full-scan MS data. Values represent an average of 5 samples in triplicate ($n = 15$). Values given on a 1g basis, adjusted using moisture factor of 0.9864

Weight (g)	Ref. val.[a)]	Date	Int. std.	± SD	Ext. std.	± SD	iRF	±SD
SIM 1g weight-normalized, moisture-adjusted results (μg/g)								
1.54765	8.6	061512	8.83[c)]	0.43	9.12	0.39	8.81	0.45
1.54058	8.6	071112	7.47	0.42	8.79	0.78	7.67	0.41
1.54465	8.6	072512	8.29	0.28	8.15	0.16	8.29	0.29
1.54465	8.6	073012	8.50	0.23	8.69	0.65	8.48	0.24
	± 2.6							
NIST LC–MS	8.78[b)]	Average:	8.27	96.2%[d)]	8.69	101.0%	8.31	96.7%
	± 0.11	SD:	0.58	94.2%[e)]	0.40	98.9%	0.48	94.7%
SRM1 1g weight-normalized, moisture-adjusted results (μg/g)								
1.54765	8.6	061512	9.01	0.49	9.28	0.43	8.78	0.52
1.54058	8.6	071112	7.62	0.37	8.70	0.72	7.69	0.39
1.54465	8.6	072512	8.31	0.30	8.13	0.24	8.28	0.30
1.54465	8.6	073012	8.37	0.19	8.48	0.58	8.33	0.20
	± 2.6							
NIST LC–MS	8.78	Average:	8.33	96.9%	8.65	100.6%	8.27	96.2%
	± 0.11	SD:	0.57	94.9%	0.48	98.5%	0.45	94.2%
SRM2 1g weight-normalized, moisture-adjusted results (μg/g)								
1.54765	8.6	061512	9.09	0.57	9.43	0.43	9.29	0.66
1.54058	8.6	071112	7.30	0.40	8.52	0.81	7.38	0.39
1.54465	8.6	072512	8.26	0.26	8.11	0.33	8.15	0.27
1.54465	8.6	073012	8.48	0.14	8.57	0.56	8.45	0.15
	± 2.6							
NIST LC–MS	8.78	Average:	8.28	96.3%	8.66	100.7%	8.32	96.7%
	± 0.11	SD:	0.75	94.3%	0.55	98.6%	0.79	94.7%
MS EIC 1g weight-normalized, moisture-adjusted results (μg/g)								
1.54765	8.6	061512	8.81	0.80	8.95	0.82	9.08	0.89
1.54058	8.6	071112	7.64	0.28	7.18	0.94	7.53	0.25
1.54465	8.6	072512	9.26	0.61	7.75	0.44	9.24	0.72
1.54465	8.6	073012	9.11	0.23	7.48	0.86	8.99	0.24
	± 2.6							
NIST LC–MS	8.78	Average:	8.70	101.2%	7.84	91.2%	8.71	101.3%
	± 0.11	SD:	0.73	99.1%	0.78	89.3%	0.79	99.2%

a) NIST reference value from certificate of analysis updated July 31, 2013.
b) NIST value by internal LC–MS given in initial certificate of analysis supplemental materials.
c) First nonsignificant figure shown.
d) Percentage of our average to NIST reference value.
e) Percentage of our average to NIST internal LC–MS value.

report, but Fig. 6 serves to illustrate that the extract–filter–shoot method can easily be adapted to analysis of other FSVs by LC–MS.

4 Discussion

4.1 Vitamin D analysis by UV detection

For the UV data, the small differences between samples that were run initially (first row in Table 1) and those that were run later were less than typical intralaboratory %RSD for samples analyzed using a wet-chemistry approach [15]. The values obtained from initial IS and iRF analyses, row 1 Table 1, represented more than 96% of the values obtained upon repeated analyses by UV. By MS, there was no evident trend relating to the order in which the sequences were analyzed. This indicated that storage of the samples in the dilution solvent for over a month was neither beneficial (extracting more vitamin D from the powder) nor detrimental (producing breakdown or loss of the vitamin D).

The data in Table 1 for the PSCs demonstrated that the ES method gave values that were in good agreement with the IS and iRF values using UV detection. As a matter of principle, the ES method is the least preferred method, when IS and iRF options are available. Nevertheless, the results by the ES approach should be routinely calculated, for use as a diagnostic tool. When the ES results agree well with the IS

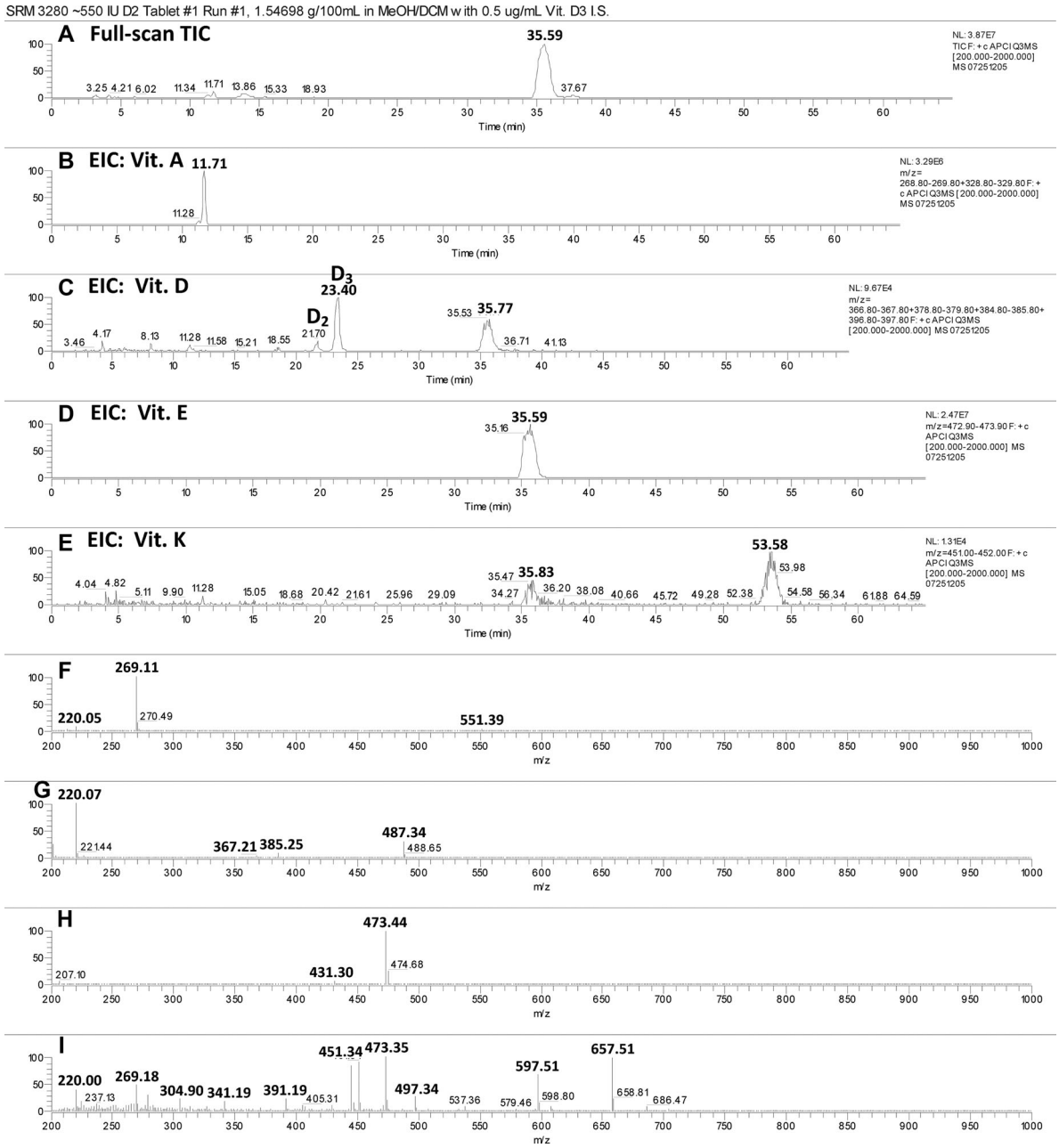

Figure 6. Chromatograms and mass spectra of vitamins A, D, E, and K. (A) TIC; (B) EIC of *m/z* 269.3 and *m/z* 329.3, $[M+H]^+$, for all *trans*-retinyl acetate (major) and 13-*cis*-retinyl acetate (minor); (C) EIC of $[M+H]^+$ and $[M+H-H_2O]^+$ of vitamins D_2 and D_3; (D) EIC of *m/z* 473.4 for α-tocopheryl acetate (vitamin E acetate); (E) EIC of *m/z* 451.5 for vitamin K (phylloquinone); (F) average mass spectrum of retinyl acetate at 11.71 min in B); (G) average mass spectrum of vitamin D_3 at 23.40 min in (C), with overlapping possible vitamin E homolog; (H) average mass spectrum across vitamin E acetate peak at 35.59 in D); and (I) average mass spectrum across vitamin K peak ($[M+H]^+ = m/z$ 451.34) in (E), with overlapping interferent(s). Figure labels added or enlarged for clarity. Original higher resolution version provided as Figure S6 in Supporting Information.

and iRF results, it provides a good indication of the absence of interfering species. Conversely, when the ES results do not agree well with the IS and iRF results, this provides an immediate indication of the likelihood that an interferent is present. Another useful diagnostic tool is the standard deviation in the raw integrated areas of the internal standard. We have previously mentioned [15, 16] that %RSD in the IS peak areas across all standards and samples should be very low, since they all ideally contain the exact same amount of IS. The sequences in Table 1 had %RSD of 1.06, 1.00, and 1.18% for the raw areas of the IS across all samples and standards for the three rows, respectively. This, along with the clean, symmetric peaks in the UV chromatograms of samples, indicated that there was nothing in the samples that interfered

with the IS area or affected results. The very low uncertainty in the vitamin D_3 IS raw areas used for quantification of vitamin D_2 is similar to the value of 1.15% in the raw areas of the vitamin D_2 IS that was used for quantification of vitamin D_3 in the earlier report [15]. These data, taken with the good agreement to the values for SRfM 3280 by LC–MS, indicate that the extract–filter–shoot approach for analysis of the PSCs was very effective, while at the same time saving time and resources.

In contrast to the PSCs, SRfM 3280 showed multiple overlapping species in the time range for vitamin D_2 and D_3. Based on Fig. 4B, it is easy to envision how improper integration of the vitamin D_2 peak, or mistakenly integrating the larger overlapping peak at ~22.2 min, could result in higher values for vitamin D_2 by UV detection. On the other hand, species that overlapped vitamin D_3 could lead to a larger area for the IS, and concomitant reduction in the calculated amount of vitamin D_2, due to an increased denominator in the D_2/D_3 peak area ratio. For instance, although the vitamin D_3 peak in Fig. 4B does not show the obvious overlap that the vitamin D_2 peak shows, the mass spectrum in Fig. 5G (expanded in Fig. 5H) shows peaks that are larger than the *m/z* 385.2 and 367.2 analyte peaks. Thus, quantification by UV detection could be a balance between compounds overlapping vitamin D_2 versus those overlapping vitamin D_3, and depend on the molar absorptivities of the interferents.

The European Committee for Standardization (CEN) Vitamin Working Group and Grocery Manufacturers' Association (GMA) labs that used UV detection for SRfM 3280 did show higher values and a much larger spread of values than the internal value obtained by NIST using LC–MS [17]. Of course, the argument can be made that the saponification, LLE, and preparative chromatography would eliminate the overlapping species, but that is not necessarily true. We have demonstrated several cases, such as orange juice [22] and cheese [23], in which overlapping species can accompany vitamin D through the saponification/extraction/preparative chromatography steps. This is not surprising, since a molecule that coelutes, due to its very similar polarity to ergocalciferol, will likely extract efficiently into the same solvents and behave similarly by preparative chromatography. Thus, some or all of the same species that coeluted as in Fig. 4 could be present and coelute even after several sample preparation steps. Therefore, the extra time, resources, and chemicals needed for laborious saponification/extraction/preparative separation do not provide substantial benefit compared to the extract–filter–shoot method reported here.

4.2 Vitamin D analysis by MS detection

The PSC with rice flour presented no particular challenge to vitamin D_2 analysis, as indicated by the simple, clean chromatograms in Figs. 1 and 2. SRfM 3280, on the other hand, had overlapping peaks, as described above, Figs. 5G and H.

As Figs. 5B and D indicate, vitamin E, as α-tocopheryl acetate, was present at a much higher level than vitamin D_2. Based on the amounts in the CA, vitamin E was present in a ~2500-fold larger amount than ergocalciferol. Thus, even if α-tocopheryl acetate were 99% pure, the 1% "impurity" containing isomeric or homologous molecules would be present in 25-fold excess to vitamin D_2. Figure 5J shows peaks associated with *m/z* 473.3, two of which occur at 21.36 and 22.38 min, overlapping vitamin D_2. These are isobaric with vitamin E acetate, Fig. 5K, eluted at 35.59 min, and likely represent minor isomers. The species at 21.36 min (Fig. 5J) has a peak height of 0.14% that of α-tocopheryl acetate (Fig. 5K). Figure 5I shows an ion chromatogram for *m/z* 487.3, which has a peak at 22.13 min, overlapping vitamin D_3, with a height almost ten times larger than the *m/z* 473.3 at 21.36. The mass difference of 14 amu suggests that it is a methylated homolog of α-tocopheryl acetate, and the peak height in Fig. 5I indicates that it is ~1.1% of the peak height shown in Fig. 5K. Based on the MS/MS spectrum of vitamin E in Fig. 5N, it appears that the vitamin E homologs produced no ions near those of vitamin D, and pose no threat of interference with the ions used for quantification of vitamin D_2. Data such as these highlight the diagnostic utility of MS data versus UV data, and show the benefit of acquiring full-scan spectra in addition to targeted analysis using only SIM and SRM.

4.3 TAG analysis

Classical methods for lipid extraction typically employ chloroform and methanol, such as the 1:2 MeOH/$CHCl_3$ ratio used by Folch et al. [24] and the 2:1 MeOH/$CHCl_3$ ratio used by Bligh and Dyer [25]. But as Sheng et al. demonstrated [26], both of these classic combinations of MeOH and $CHCl_3$ provided excellent, practically indistinguishable, recovery compared to a variety of other solvent combinations. Those data showed that any ratio of MeOH/$CHCl_3$ between or near the compositions of those classic approaches might be expected to similarly produce excellent recovery of lipids.

Others have demonstrated that dichloromethane, in a wide range of proportions, can be used instead of chloroform to obtain the same or better results with less toxicity and fewer regulatory issues [27–29]. Furthermore, according to the contour plots by Jeon et al. [30], the dilution solvent we used, 60:40 MeOH/DCM (= 1.5:1), is close to the optimal proportion of 1:1 MeOH/DCM that provided the best overall results for extraction of lipids from algal biomass. Of course, the dietary supplement powders we analyzed are not nearly as complex as some of the samples analyzed in the referenced reports, and so represent much less of a challenge for extraction of the small amount of lipids present. The most important factor, though, is that using our dilution/extraction solvent, we were able to achieve values for vitamin D_2 within standard deviations of the original NIST LC–MS value and the updated reference value in a SRfM having a known amount of the analyte.

One thing we were not able to do is to apply response factors by comparison to GC–FID, as we recently showed [16], to optimize the match between LC–MS and GC–FID results. The presence of "magnesium stearate" in an amount larger than the oil in the rice flour (based on the relative peak areas in the GC–FID analysis, not shown) dramatically skewed the FA composition toward stearic and palmitic acids. One way to overcome this problem would be to collect the eluates over the time range that TAGs eluted and submit those to FAME analysis. A time-controlled electronically actuated valve has been implemented to accomplish this, and will be undergoing testing in the near future. Even without RFs, the un-adjusted TAG composition is close to the "true" composition, and the degree of difference has been well described in our recent report [16]. Reporting TAG compositions without RFs is the *de facto* standard approach to TAG quantification in the literature, and only a few reports describe the use of RFs. Thus the composition presented above serves as a useful contribution to the sparse literature on rice flour TAGs.

5 Concluding remarks

The "dilute-and-shoot" analysis that we previously reported has now been expanded to an "extract–filter–shoot" method, to make it applicable to dry powdered supplements in addition to the oil-filled gelcap supplements we previously analyzed. The new method required validation by analysis of a known material to prove that vitamin D was released from the powder. Analysis of NIST SRfM 3280 gave values that were 94 to 101% of the original NIST LC–MS values and the updated reference value. These data provided insights into reasons that the UV data in the original CA may have been too high, and why UV results had a much larger spread of values than the LC–MS data from NIST.

Although the use of multiple mass spectrometers in parallel is not feasible for all laboratories, our results indicate that a minimum configuration of UV detection combined with a single tandem mass spectrometer is completely adequate, as long as full-scan UV and full-scan MS spectra are acquired for peak purity assessment, in addition to a targeted approach using SIM and/or SRM. The use of "multiple parallel mass spectrometry" in our lab simply allows a higher degree of confidence in results, and provides additional options for qualitative and quantitative analysis.

This "extract–filter—shoot" method eliminates hours of laborious sample preparation steps and reduces the use of solvents, materials, and resources, while yielding results in good agreement with the NIST LC–MS value and the updated reference value. Thus, the "extract–filter–shoot" method represents an efficient and time-saving alternative to existing methods for analysis of vitamin D_2 in powdered or compressed powder dietary supplements.

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The author declares no conflict of interest.

6 References

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