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Original article

Liquid chromatography with ultraviolet and dual parallel mass spectrometric detection for analysis of vitamin D in retail fortified orange juice

William C. Byrdwell [a,*], Jake Exler [b], Susan E. Gebhardt [b], James M. Harnly [a], Joanne M. Holden [b], Ronald L. Horst [c], Kristine Y. Patterson [b], Katherine M. Phillips [d], Wayne R. Wolf [a]

[a] USDA, ARS, Beltsville Human Nutrition Research Center, Food Composition and Methods Development Laboratory, Beltsville, MD, United States
[b] USDA, ARS, Beltsville Human Nutrition Research Center, Nutrient Data Laboratory, Beltsville, MD, United States
[c] Heartland Assays Inc., Ames, IA, United States
[d] Virginia Polytechnic Institute and State University, Department of Biochemistry, Food Analysis Laboratory, Blacksburg, VA, United States

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ABSTRACT

Samples of vitamin D fortified orange juice obtained from retail food stores were analyzed for vitamin D_3 content using a method developed by combining the best features of two AOAC methods. Detection by ultraviolet absorption at 265 nm was compared to detection by selected ion monitoring (SIM) using atmospheric pressure chemical ionization (APCI) mass spectrometry (MS). Furthermore, an ion trap (IT) mass spectrometer was employed in a 'dual parallel MS' arrangement to simultaneously obtain qualitative APCI-ITMS data. The method was applied to 33 samples of 3 national American orange juice brands and 7 samples of 5 other American brands collected using a statistically designed sampling plan as part of the National Food and Nutrient Analysis Program to provide values for the USDA National Nutrient Databank for Standard Reference. Vitamin D_3 values ranged from 1.071 μg/100 g (43 IU/100 g) to 1.663 μg/100 g (67 IU/100 g), with an average across 55 samples analyzed, including duplicates, of 1.4 ± 0.1 μg/100 g (57 ± 5 IU/100 g). The average of the 38 non-zero uniquely identified samples, using the averages of duplicate sets, was 1.4 ± 0.1 μg/100 g (57 ± 5 IU/100 g), indicating that a typical 8 oz. (~240 mL = 240 cm^3) glass of orange juice provided 3.4 ± 0.3 μg (140 ± 10 IU) vitamin D_3.

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1. Introduction

Vitamin D deficiency has long been known to cause rickets (Rajakumar, 2003), but inadequate levels of vitamin D have more recently been implicated in a wide variety of diseases (Bischoff-Ferrari et al., 2006; Holick, 2006a; Zittermann, 2003), including some types of cancer (Garland et al., 2009; Holick, 2004b, 2006b; Mohr, 2009; Ordonez-Moran et al., 2005), cardiovascular disease (Holick, 2004a; Zittermann, 2006), diabetes, multiple sclerosis, and others (Gesek and Desmond, 2008; Janssens et al., 2009; Pappa et al., 2008; Shoenfeld et al., 2009).

In humans, most vitamin D originates from exposure to sunlight (Calvo et al., 2004), which causes conversion of 7-dehydrocholesterol (pro-vitamin D_3) to pre-vitamin D_3, by opening of the β-ring though scission of the 9,10 bond. Enthalpic isomerization then produces cholecalciferol, or vitamin D_3 (Holick et al., 1980). However, this is not the active form of the nutrient. Instead, vitamin D_3 moves to the liver, where it is acted on by the enzyme *25-hydroxylase*, to produce 25-hydroxy vitamin D_3. This is also not the most active form of the nutrient, but it is the metabolite that is most commonly measured as a biomarker for determination of vitamin D sufficiency or inadequacy (DeLuca, 2004). This intermediate moves to the kidneys, where it is acted on by other hydroxylase enzymes, to form the active form of the nutrient, 1,25-dihydroxyvitamin D, as well as other metabolites, such as 24,25-dihydroxyvitamin D (Koshy, 1982). Although still classified as a vitamin, it is now clear that vitamin D is actually a prohormone (DeLuca, 2004).

Unfortunately, few foods naturally contain vitamin D (Holick, 2006b; Lamberg-Allardt, 2006), among them are oily fish, such as salmon, mackerel and herring, as well as fish liver oils, such as cod liver oil, which has long been known as a good source of vitamin D. Mushrooms represent a natural source of ergosterol and vitamin

* Corresponding author at: USDA, ARS, BHNRC, FCMDL, 10300 Baltimore Ave., Bldg. 161, Beltsville, MD 20705, United States. Tel.: +1 301 504 9357; fax: +1 301 504 8314.
E-mail address: C.Byrdwell@ARS.USDA.gov (W.C. Byrdwell).

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D_2 (Mattila et al., 1994; Teichmann et al., 2007), where ergosterol is the precursor to vitamin D_2. The amount of vitamin D_2 may be greatly increased by irradiation of the mushrooms with UV light (Jasinghe and Perera, 2005, 2006; Roberts et al., 2008). For a time, it was believed that vitamin D_2 is much less effective than vitamin D_3 (Armas et al., 2004; Trang et al., 1998), but recent evidence calls that conclusion into question (Holick et al., 2008). Nevertheless, due to the paucity of natural sources for vitamin D, much of the nutrient in the diet comes from foods that are fortified with vitamin D.

The allowed amounts of vitamin D in fortified American foods are specified by the U.S. Food and Drug Administration (FDA), and are given in the Code of Federal Regulations (CFRs) Title 21. The FDA has approved vitamin D fortification of fruit juices (21 CFR 172.380) in a 2005 action that allows fortification at levels not to exceed 100 International Units, or 2.5 μg, (1 IU = 0.025 μg) per 240 mL in 100% fruit juices that are also fortified with greater than or equal to 33% of the reference daily intake of calcium per 240 mL. Fruit juice drinks may be similarly fortified at levels not to exceed 100 International Units per 240 mL in fruit juice drinks that are also fortified with greater than or equal to 10% of the reference daily intake of calcium per 240 mL.

Such stipulations of allowed levels in fortified foods beget the need for analysis to determine the actual amounts of the vitamin added to products. In the past, there have been problems reported with the levels of vitamin D_3 found in fortified milk (Chen et al., 1993; Holick et al., 1993; Murphy et al., 2001; Tanner et al., 1988; Patterson et al., 2010). Many samples had less than 80% of the listed label amount, while others had more than 120% of the label value. Unfortunately, although orange juice is approved for fortification, a broad study of the levels of fortification in a wide range of commercially available products of this commonly consumed beverage has not yet been reported. Tangpricha et al. (2003) reported that orange juice was an effective vehicle for vitamin D fortification, and showed that despite the acidity of orange juice, the nutrient was unchanged after 30 d at 4 °C. That report incorporated vitamin D at the level of 1000 IU per 240 mL juice, which is 1.67 times the newly recommended adequate intake (AI) for persons less than 70 years old (AI = 15.0 μg/d or 600 IU/d), and 1.25 times the AI for persons over 70 (AI = 20 μg/d or 800 IU/d) (Institute of Medicine, 2010).

We previously analyzed multiple replicates of a single orange juice sample (Byrdwell, 2009), developed as a control material, to demonstrate the applicability of the method to orange juice, and as part of a multi-lab validation process (Phillips et al., 2008). Here we extend that initial example to a survey of the vitamin D content of orange juice in a statistically selected pool of orange juice samples from across the United States. We have conducted an analysis of commercially available fortified orange juice to accurately determine the vitamin D_3 content for the National Food and Nutrient Analysis Program (NFNAP) to provide values for the USDA National Nutrient Databank for Standard Reference (SR) (USDA, 2010). Forty-seven orange juice samples were analyzed for this study, which included seven blinded samples of the control composite (CC) material which was characterized by several labs (Phillips et al., 2008). Twenty-five samples were analyzed in duplicate (twenty retail samples and five CC samples), for a total of 72 analyses.

2. Materials and methods

2.1. Orange juice samples and chemicals

A total of 40 retail orange juice samples were collected from 14 states across the U.S. between April and June of 2007 as part of the Nutrient Data Laboratory's (NDL) NFNAP (Haytowitz et al., 2008; Pehrsson et al., 2000, 2003), and were sent on ice packs to the Virginia Polytechnic Institute and State University Food Analysis Laboratory Control Center (FALCC) for processing. Twenty of the commercial samples, as determined by NDL and FALCC (based on their statistical sampling and analysis plan), were requested to be analyzed in duplicate by the USDA Food Composition and Methods Development Laboratory (FCMDL), to provide data for an assessment of sample extraction reproducibility. Therefore, 60 individual samples of retail orange juice were analyzed. Seven aliquots of control composite (CC) orange juice samples that had previously been analyzed by several laboratories (Byrdwell, 2009; Phillips et al., 2008) and given unique identification numbers by FALCC were included with the samples distributed to FCMDL, with no indication of which samples were controls. These 7 blinded control samples, 5 of which were analyzed in duplicate (n = 12), provided ongoing verification and validation of the analytical methodology, and allowed these results to be compared with the results previously obtained for those samples (Byrdwell, 2009). In total, 72 analyses were performed on 40 retail samples with 20 in duplicate (n = 60) and 7 blinded control samples with 5 in duplicate (n = 12).

All retail samples were commercially available half-gallon cartons of refrigerated orange juice, except one gallon-size sample. These included one sample that was not fortified with vitamin D. The samples were stored refrigerated (at 4–6 °C) in the sealed original containers until being subsampled for analysis, prior to the labeled expiration dates. Each carton was inverted 5–10 times, then 75–90 mL subsamples were dispensed into 125 mL wide-mouth tall straight-sided glass bottles with Teflon®-lined lids (I-Chem®, Rockwood, TN; product # 221-0125). Each subsample was given a unique identification number, and the aliquots were capped under nitrogen and stored at −60 °C until being distributed, frozen on dry ice, to FCMDL for analysis. All samples were extracted soon after receipt, and were kept at −20 °C until analysis.

Fisher Optima HPLC or LC-MS 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). Potassium hydroxide (KOH) and ascorbic acid, as well as the crystalline cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) that were used as standards, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

2.2. Extraction

Samples were extracted using the ethyl ether/petroleum ether extraction given in AOAC method 992.26, as recently reported (Byrdwell, 2009). The AOAC method was modified to include 1.0 mL of 0.5 μg/mL vitamin D_2 in ethanol added as an internal standard. This method was used since it is an AOAC method that had been subjected to multi-lab validation, and because we wanted one method that could be applied to a variety of samples from milk to orange juice to seafood and others. Approximately 30 mL of each sample was weighed in a 250 mL Erlenmeyer flask with a ground glass neck, 1.0 mL of the internal standard was added by volumetric pipette, and 400 mg ascorbic acid (as antioxidant) was added, and the extraction was carried out as previously described (Byrdwell, 2009).

2.3. Instrumentation

2.3.1. High performance liquid chromatography with ultraviolet detection

The two chromatographic separations from AOAC Official Method 2002.05 were used. The first was a preparative normal-phase HPLC separation on an Inertsil® Sil 100A 25.0 cm × 4.6 mm, 5 μm, silica column (GL Sciences, Torrance, CA), as reported earlier (Byrdwell, 2009). Two solvent programs were used, which were

the same except that the one used for orange juice samples included a column wash after vitamin D eluted, whereas the one for pure standard solutions did not. The vitamin D eluted at ~17.5 min, and a fraction was collected from 16 min to 19 min in a 13 mm test tube. 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 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, and UV6000 DAD. Full-scan spectra were obtained from 190 to 400 nm, with a bandwidth of 1 nm and an acquisition rate of 1 Hz. Single channel detection at 265 nm on the DAD was performed at 10 Hz with a 9 nm bandwidth. A backup UV2000 dual channel detector operated in single channel mode at 265 nm was used, but those data were not used. The solvent system was 40% acetonitrile/60% methanol for 20 min on an Inertsil® ODS-2 column, 25.0 cm × 4.6 mm, 5 μm particle size (GL Sciences, Torrance, CA) at a flow rate of 1.0 mL/min. Initially a Vydac® column was used, as called for in AOAC 2002.05, but after the first sample was analyzed, the column was changed to the Inertsil® ODS-2 column as discussed below (Section 3.1).

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 mass spectrometry.

2.3.2. Mass spectrometry

Tandem sector quadrupole mass spectrometry was used as a detector for 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 protonated molecule, $[M+H]^+$, at m/z 397.3 and the dehydrated protonated molecule, $[M+H-H_2O]^+$, 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 0.48 mL/min going to the APCI source of the TSQ7000 mass spectrometer, and 0.52 mL/min going to a second mass spectrometer. An LCQ Deca XP ion trap mass spectrometer (Thermo Electron Corp., now Thermo Fisher Scientific Corp., San Jose, CA) in full scan mode was operated in parallel with the TSQ 7000. APCI was performed on the LCQ Deca XP with a combination APCI/APPI source from Syagen, Inc. (Tustin, CA), in APCI-only mode, using the same parameters as given for the TSQ 7000 instrument.

2.4. Calculations

2.4.1. Sample quantification

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 μg/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 orange juice samples. The calculated amount of vitamin D_3 in a sample was given from the equation:

$$\text{Vitamin } D_3\ (\mu g) = \frac{(\text{Area } D_3/\text{Area } D_2) \times \mu g\ D_2 IS}{RF}$$

Runs were typically set up as bracketed sequences to run two replicates of the 0.8 μg/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. Longer sequences had an additional standard run between any additional sets of eight sample runs. The samples were quantified using the average response factor for the standard runs obtained in the same sequence as the sample runs. The value determined for each sample extraction replicate was typically based on the average of eight analytical (RP-HPLC) runs. Due to the large number of runs (72 extracts × 8 runs/extract = 576, plus standard runs) an occasional fraction or individual run was lost due instrument failure.

2.4.2. Statistical analysis

Microsoft Excel 2007 (Microsoft Corp., Redmond, WA) with the data analysis tool pack installed was used for determination of means and standard deviations, and for calculation of the student's t-test. Graphs were plotted using Sigma Plot 11 (Systat Software, Inc., San Jose, CA). Any suspect maximum or minimum values from the eight individual RP-HPLC runs for each sample was tested as an outlier using the Q-test at the 95% level ($Q_{95\%} = 0.526$, $n = 8$).

3. Results and discussion

3.1. Dual parallel mass spectrometry

Fig. 1A–C shows the full-scan ion trap MS analysis of the first orange juice sample extract, analyzed on the Vydac® 201TP54 column, as called for in the AOAC method. The background-subtracted mass spectrum, Fig. 1C, averaged across the vitamin D_3 peak at 13.2 in Fig. 1A clearly showed a substantial abundance at m/z 553.2, in addition to the protonated molecule and dehydrated protonated molecule for vitamin D_3 at m/z 385.2 and m/z 367.2, respectively. When m/z 553.2 was extracted out of the TIC, the elution profile of the interfering species could be clearly observed in the extracted ion chromatogram (EIC) in Fig. 2B. This overlapped interferent caused a shoulder on the vitamin D_3 in the UV chromatogram and caused the UV peak maximum to shift away from 265 nm (not shown).

We therefore changed the chromatography column to an Inertsil® ODS-2 column. Because of the high carbon load (18.5% vs. 8% for the Vydac® 201TP54) and thorough end-capping, the Inertsil® column retained vitamin D longer, and required a higher proportion of methanol in the solvent composition to elute the analytes. Fig. 1D–F represents a duplicate extraction of the same orange juice sample separated on the Inertsil® ODS-2 column, and shows that the previously interfering species eluted near 17 min on this column. The mass spectrum in Fig. 1F proved that no masses from any interfering species were visible in the mass spectrum at the retention time for vitamin D_3.

3.2. Results for quantification of vitamin D_3 by UV at 265 nm

Fig. 2 shows results for a typical orange juice extract using the final chromatographic method. The peaks in Fig. 2A and B were used for MS quantification of vitamin D_2 (internal standard) and vitamin D_3, respectively, based on the SIM ions shown in Fig. 2F. The peaks in Fig. 2D were used for quantification by UV detection.

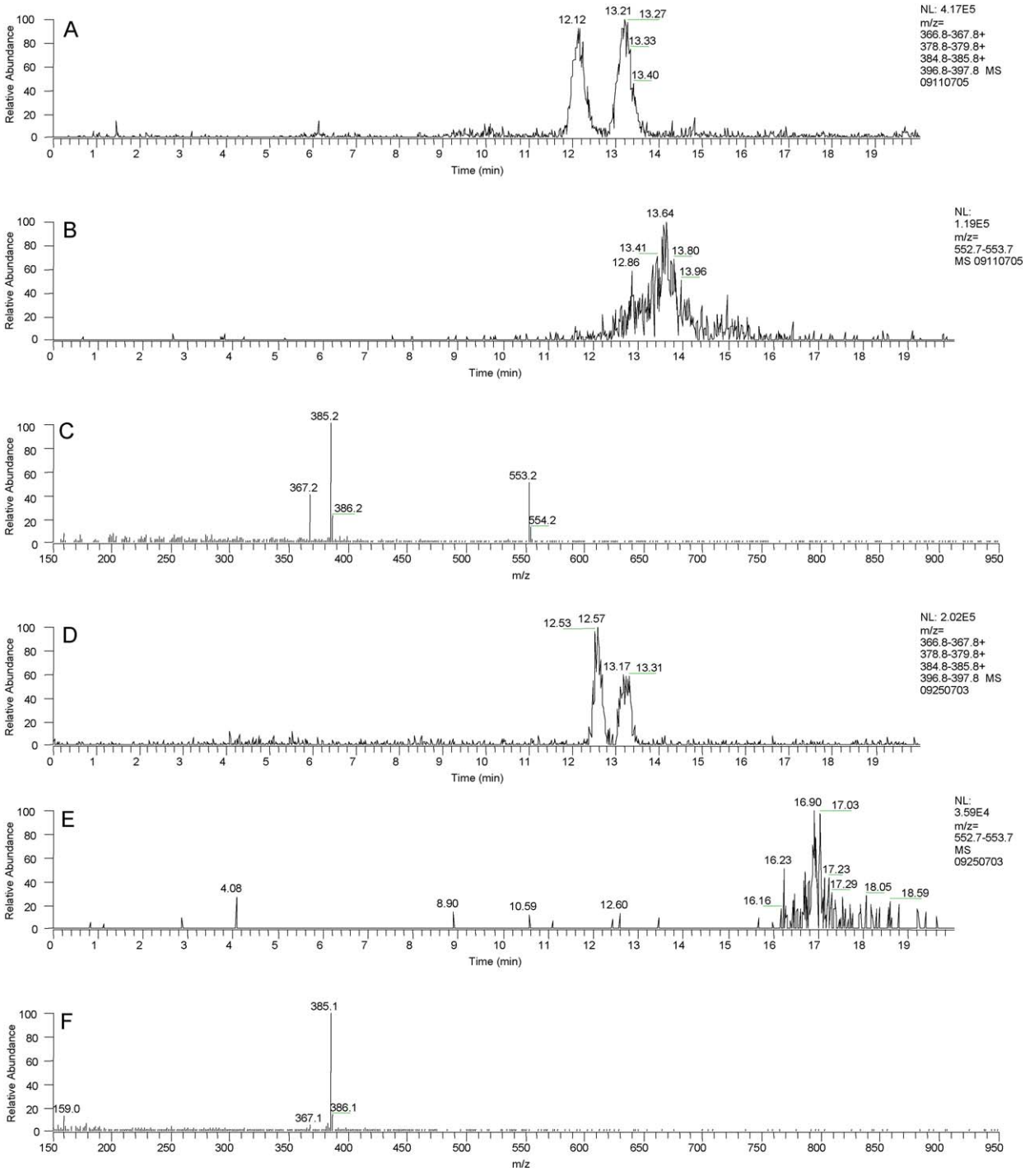

Fig. 1. (A–C) full-scan APCI-MS detection of orange juice analyzed on a Vydac® C18 column. (D–F) full-scan APCI-MS detection of orange juice analyzed on an Inertsil® ODS-2 C18 column. (A) Extracted ion chromatogram (EIC) of *m*/*z* 367.3, 385.3 for Vitamin D_3 and *m*/*z* 379.3, 397.3 for Vitamin D_2; (B) EIC of *m*/*z* 553.2 from interferent; (C) average full-scan mass spectrum across Vitamin D_3 peak in (A) at 13.2 min; (D) EIC of *m*/*z* 367.3, 385.3, 379.3, 397.3; (E) EIC of *m*/*z* 553.2; (F) average full-scan mass spectrum across Vitamin D_3 peak in (D) at 13.2 min.

The UV spectrum in Fig. 2E appeared the same as the spectrum of the pure vitamin D_3 standard run in the same sequence.

Fig. 3 shows the results for analysis of vitamin D in three national orange juice brands, three store brands, one other brand, and control samples. The label value (and FDA stipulated value) is 100 IU/240 mL (=100 IU/8 oz.), or 41.67 IU/100 g, which equals 1.0417 μg/100 g, assuming a density of 1.00 g/mL for orange juice (the approximate average density observed was 1.02 ± 0.01 g/mL, $n = 72$). Each data point represents the average of eight replicate analyses, unless otherwise indicated, with ±one standard deviation shown. This figure shows that all samples analyzed except one contained at least as much vitamin D_3 as indicated by the label. One sample of Store Brand F (marked by an asterisk) was labeled to contain vitamin D, but was found to contain no vitamin D_3 and so was excluded from the graph for clarity of scale. That sample was analyzed in duplicate initially, and then a second sample was obtained from the same location 11 months later and analyzed by a commercial laboratory, which also determined that it contained no vitamin D_3.

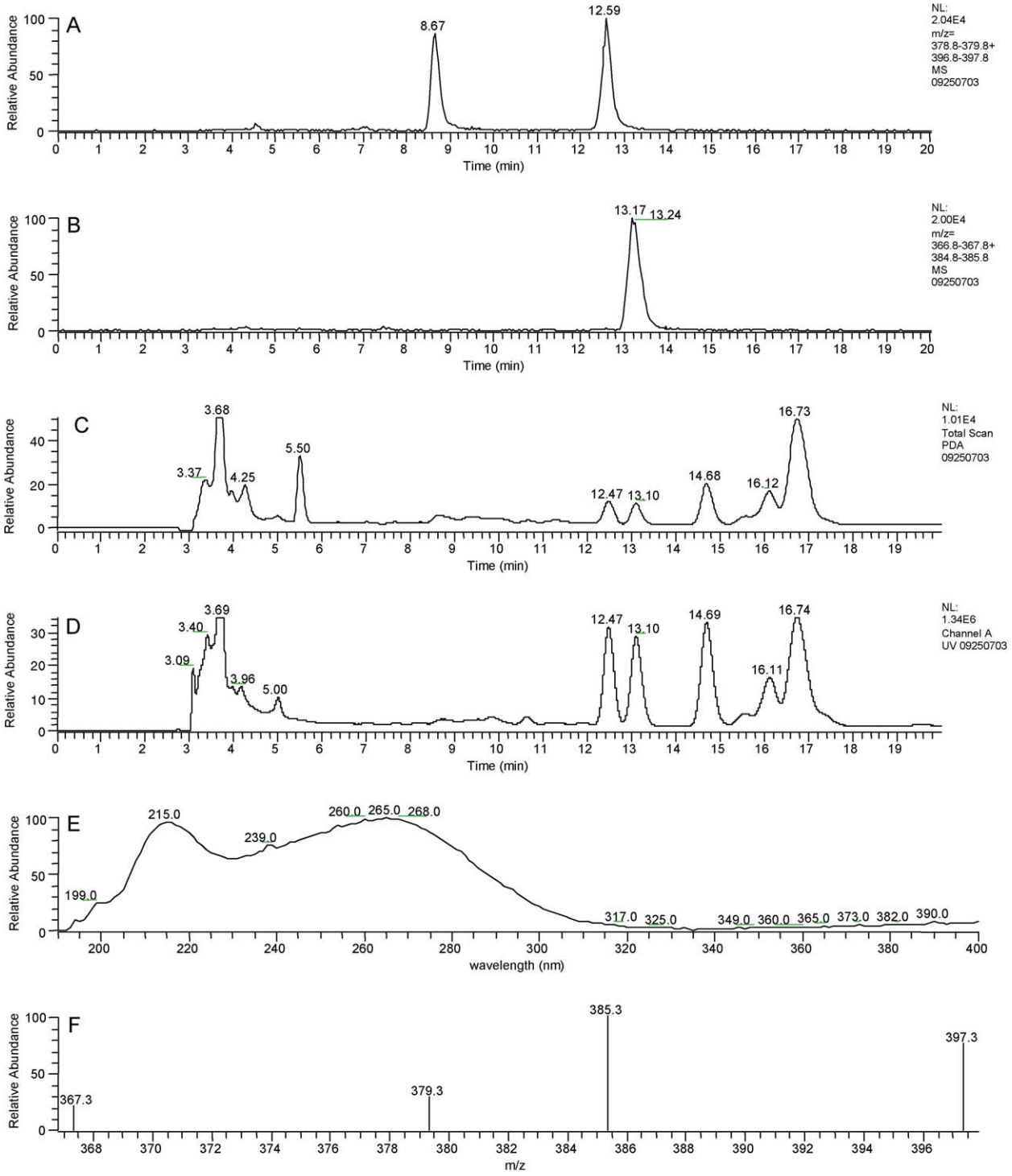

Fig. 2. UV and MS detection of orange juice analyzed on an Inertsil® ODS-2 column. (A) Extracted ion chromatogram (EIC) of *m*/*z* 379.3, 397.3 for Vitamin D_2; (B) EIC of *m*/*z* 367.3, 385.3 for Vitamin D_3; (C) UV photodiode array (PDA) total scan chromatogram; (D) single channel UV at 265 nm chromatogram; (E) UV spectrum across peak at 13.10 min; (F) selected ion monitoring (SIM) ion mass spectrum. See Fig. 1D–F for parallel full-scan APCI-MS run.

One 'other brand' sample which label indicated it did not contain vitamin D_3 was also excluded from the graph, since it was expected, and duplicate analysis confirmed, that it contained no vitamin D_3.

National Brand A averaged 1.44 ± 0.04 μg/100 g (58 ± 2 IU/100 g) for the nine samples analyzed, five in duplicate ($n = 14$). National Brand B gave an average value of 1.4 ± 0.1 μg/100 g (56 ± 4 IU/100 g) for the twelve samples analyzed, five in duplicate, excluding the first run ($n = 16$). The first replicate of the first sample of Brand B was excluded due to confirmation by MS, Fig. 2, of other compounds overlapped with the analyte peaks. National Brand C averaged 1.52 ± 0.06 μg/100 g (61 ± 3 IU/100 g) for the twelve samples analyzed, six in duplicate ($n = 18$). Store Brand D averaged 1.28 ± 0.03 μg/100 g (51 ± 1 IU/100 g) for two samples, one in duplicate ($n = 3$). Store Brand E gave a value of 1.12 ± 0.01 μg/100 g (45.0 ± 0.4 IU/100 g), and this was the only sample that had a label value of 20% (=80 IU/100 mL) of the dietary reference intake (DRI) (at that time) instead of 25%. One sample of Store Brand F gave an average of 1.440 ± 0.008 μg/100 g (57.6 ± 0.3 IU/100 g) from

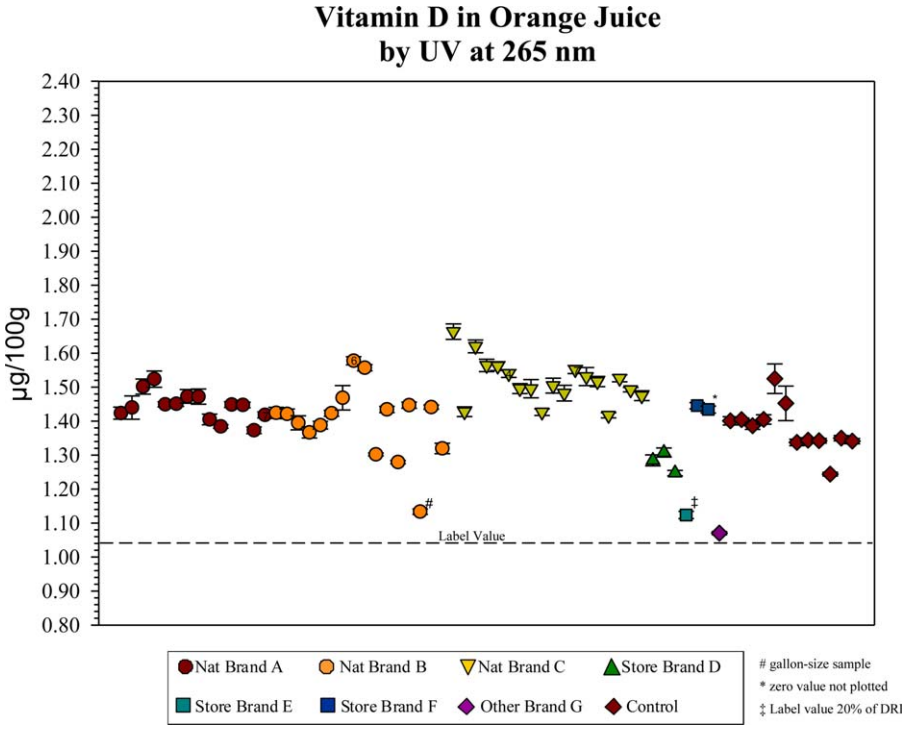

Fig. 3. Vitamin D_3 in retail orange juice samples, determined using UV detection at 265 nm. Numbers inside symbols represent replicates in average, if different from $n = 8$.

duplicate analyses. Another sample of Store Brand F gave values of 0.005 ± 0.003 μg/100 g (0.2 ± 0.1 IU/100 g) and 0.002 ± 0.002 μg/100 g (0.1 ± 0.1 IU/100 g), which indicated that it contained no vitamin D above the limit of detection previously reported (Byrdwell, 2009). Other Brand G contained 1.071 ± 0.005 μg/100 g (42.8 ± 0.2 IU/100 g). The values determined by UV detection in Fig. 3 have been incorporated into the USDA National Nutrient Databank SR 23 (USDA, 2010).

The standard deviations above are the sample-to-sample SD, except for the single samples, Brand E and Brand G, for which the run-to-run SD ($n = 8$) is given. The run-to-run standard deviations by UV detection were typically less than 1% RSD, with an average of 0.97% RSD for commercial samples ($n = 55$ for non-zero samples, excluding the first Brand B replicate run on the Vydac® column). The values of vitamin D_3 in fortified samples ranged from 1.071 ± 0.005 μg/100 g (42.8 ± 0.2 IU/100 g) to 1.66 ± 0.02 μg/100 g (66.5 ± 0.9 IU/100 g) for all brands.

The average content of vitamin D_3 across 55 retail samples analyzed that contained vitamin D, including duplicates, was 1.4 ± 0.1 μg/100 g (57 ± 5 IU/100 g), which excluded the first run due to overlap, duplicate runs of the sample labeled to be fortified that was not, and duplicate runs of the sample that was not labeled fortified. This means that the 55 samples analyzed had an average of 140 ± 10 IU/8 oz. When the duplicate sample that contained no vitamin D_3 is included, the average content was 1.4 ± 0.3 μg/100 g (60 ± 10 IU/100 g). For the best estimate of what a consumer might expect, the average of the 38 uniquely identified non-zero samples, using the average of duplicate sets (excluding zero values), was 1.4 ± 0.1 μg/100 g (57 ± 5 IU/100 g). This indicates that a typical 8 oz. glass of orange juice (~240 mL) provides 140 ± 10 IU (assuming $d = 1.00$ g/mL), for 34% of the former DRI of vitamin D_3.

These data reveal that all samples except one contained more than the label value of vitamin D_3, and that under-fortification was not a widespread problem. All samples contained enough additional vitamin D_3 to account for any potential losses during storage and shelf life, although such losses might be expected to be minimal (Tangpricha et al., 2003).

Blinded control samples were used as a test of analytical reproducibility, by comparison to consensus values previously obtained from analysis by three validated laboratories (Phillips et al., 2008). From that analysis, a value of 52 ± 6 IU/100 g was set as the validated range of values for the orange juice control samples. This was determined as the mean of the five values each from the three labs, plus or minus two standard deviations. That range was obtained using 'robust statistics' that employed a median absolute deviation (MAD) approach (Analytical Methods Committee, 2001). A conventional analysis using routine statistics gives an average and ±2 SD range of 1.3 ± 0.2 μg/100 g (52 ± 7 IU/100 g). Using either approach, all twelve blinded control sample analyses except one were within the validated range. The average value obtained for the blinded control samples was 1.38 ± 0.07 μg/100 g (55 ± 3 IU/100 g), which is well within the validated range, and is within one standard deviation of the three-lab average (Phillips et al., 2008). The average standard deviation for control samples by UV detection was 1.06% RSD ($n = 12$, 7 samples, 5 duplicates).

3.3. Results for quantification of vitamin D_3 by mass spectrometry

Fig. 4 shows the average values and standard deviations determined from SIM APCI mass spectrometry on the TSQ 7000. The values have been plotted in the same order on the same scale so this graph can be overlaid with Fig. 3 for a point-to-point comparison of values. As we reported previously (Byrdwell, 2009), the standard deviations observed by mass spectrometry were substantially higher than those obtained by UV detection at 265 nm. The average HPLC-MS run-to-run relative standard deviation was 6.11% RSD for retail samples and it was 9.27% for control samples. There were 11 samples for which the ranges given by the standard deviations by UV and MS did not overlap. Specifically, there were 11 samples for which the absolute difference between the mean values was greater than the sum of the standard deviations of the two different methods. The same was true for three control samples. One sample gave an anomalously large standard deviation due to a high value and a

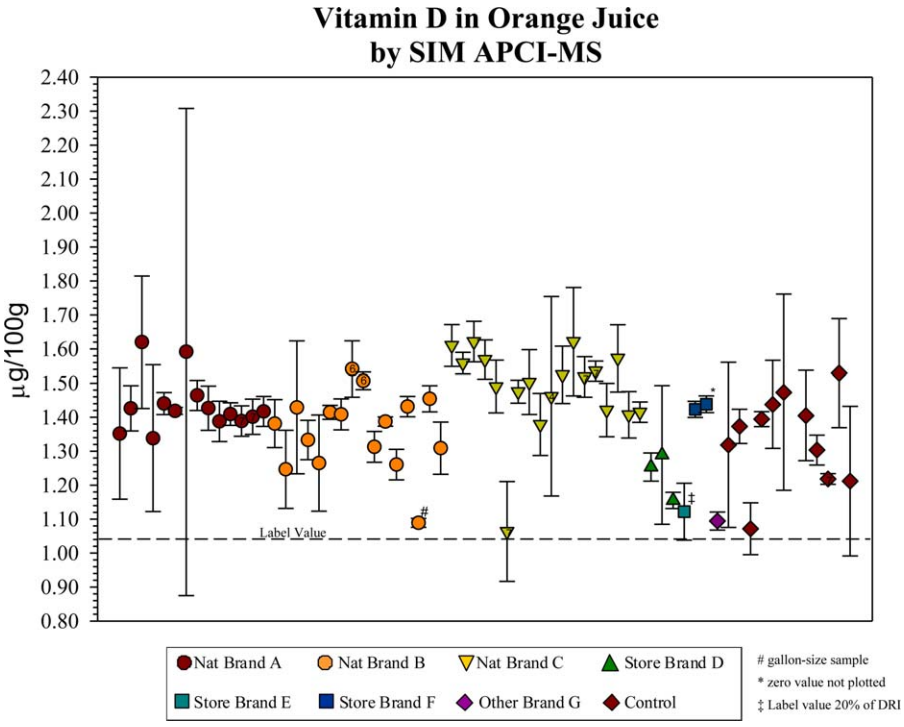

Fig. 4. Vitamin D_3 in retail orange juice samples, determined using selected ion monitoring APCI-MS. Numbers inside symbols represent replicates in average, if different from $n = 8$.

low value (out of 8 analytical replicates) that could not be *Q*-tested out. There were 22 samples for which the student's *t*-test indicated a statistical significance between the values obtained by MS versus that obtained by UV, though 6 values were very close to the $t_{critical}$.

The overall sample averages determined by mass spectrometry were statistically indistinguishable from the averages determined by UV detection, based on the student's *t*-test. National Brand A averaged 1.43 ± 0.08 µg/100 g (57 ± 3 IU/100 g) by APCI-MS. National Brand B gave an average value of 1.4 ± 0.1 µg/100 g (54 ± 5 IU/100 g) by MS. National Brand C averaged 1.5 ± 0.1 µg/100 g (59 ± 5 IU/100 g) for the seventeen samples analyzed. Store Brand D averaged 1.23 ± 0.07 µg/100 g (49 ± 3 IU/100 g). The Store Brand E sample gave a value of 1.12 ± 0.08 µg/100 g (45 ± 3 IU/100 g). The two fortified samples of Store Brand F gave an average of 1.43 ± 0.01 µg/100 g (57.2 ± 0.4 IU/100 g), while one sample was found to be unfortified. The Other Brand G was determined by MS to have 1.09 ± 0.03 µg/100 g (44 ± 1 IU/100 g). The average content of vitamin D_3 across 55 non-excluded retail samples (see Section 3.2), as determined by SIM APCI-MS, was 1.4 ± 0.1 µg/100 g (56 ± 5 IU/100 g). The average including the duplicate sample that was labeled to, but contained no vitamin D_3 was 1.4 ± 0.2 µg/100 g (55 ± 9 IU/100 g). The average of the 38 uniquely identified retail samples, using the average of duplicate sets (excluding zero values), was 1.4 ± 0.1 µg/100 g (56 ± 5 IU/100 g).

These data agree with the previous report (Byrdwell, 2009) that demonstrated that in most cases, MS data gave statistically indistinguishable results from UV data. It should be noted that the newest MS instruments offer several orders of magnitude better sensitivity, and that the standard deviations observed by MS would be expected to be smaller on newer instruments. The sensitivity of our instrument precluded the use of multiple reaction monitoring (MRM), which is considered more definitive, instead of selected ion monitoring. Because SIM does not involve collision cell fragmentation, it is often ~100 times more sensitive than MRM.

In summary, analysis of commercially available orange juice samples was conducted using a method obtained by combining the ether/petroleum ether extraction of AOAC 992.26 with the chromatographic system and internal standard of AOAC 2002.05. Quantification by both UV detection at 265 nm and by SIM APCI MS was performed. A second mass spectrometer operated in parallel with the first provided valuable qualitative confirmation of the purity of chromatographic peaks used for quantification. The overall brand averages of the retail brands determined by UV and by MS were statistically indistinguishable.

The method was applied to orange juice samples collected from across the country by the NFNAP sampling program. All samples except one were found by both UV and mass spectrometric detection to contain in excess of the amount listed on the label, with overage to account for anticipated storage and shelf life. Thus, the widespread variability that had previously been reported for milk samples was not encountered with orange juice samples. With the exception of one sample of one store brand, all commercially available orange juice brands tested contained levels of vitamin D_3 above the FDA stipulated amount, indicating that vitamin D fortified orange juice is a generally reliable source for adding this important nutrient to the diet.

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