

## Original research article

## Vitamin D levels in fish and shellfish determined by liquid chromatography with ultraviolet detection and mass spectrometry

William C. Byrdwell<sup>a,\*</sup>, Ronald L. Horst<sup>b</sup>, Katherine M. Phillips<sup>c</sup>, Joanne M. Holden<sup>d</sup>, Kristine Y. Patterson<sup>d</sup>, James M. Harnly<sup>a</sup>, Jacob Exler<sup>d</sup>

<sup>a</sup> USDA, ARS, Beltsville Human Nutrition Research Center, Food Composition and Methods Development Laboratory, Beltsville, MD, United States

<sup>b</sup> Heartland Assays Inc., Ames, IA, United States

<sup>c</sup> Virginia Tech, Department of Biochemistry, Food Analysis Laboratory, Blacksburg, VA, United States

<sup>d</sup> USDA, ARS, Beltsville Human Nutrition Research Center, Nutrient Data Laboratory, Beltsville, MD, United States

## ARTICLE INFO

## Article history:

Received 26 July 2012

Received in revised form 21 December 2012

Accepted 4 January 2013

## Keywords:

Seafood

Vitamin D

Cholecalciferol

Ergocalciferol

APCI-MS

HPLC

Mass spectrometry

LC-MS

Food analysis

Food composition

## ABSTRACT

Vitamin D<sub>3</sub> (cholecalciferol) levels were determined in finfish and shellfish using UV detection at 265 nm (combined with auxiliary full scan UV detection) and selected ion monitoring (SIM) mass spectrometry (MS), using vitamin D<sub>2</sub> (ergocalciferol) as an internal standard. Analysis of standard reference material (SRM) NIST 1849 (Infant/Adult Nutritional Formula) was included to validate the method. Three-point calibration curves were employed, allowing values to be determined over a range of species, from those having little or no detectable vitamin D<sub>3</sub> (e.g., pollock, shrimp) to those with high levels (e.g., salmon with up to 33.23 µg/100 g). The limit of detection (LOD) and limit of quantitation (LOQ) calculated from the uncertainty and intercept of the calibration curves were 1.22 µg/100 g and 5.30 µg/100 g, respectively, based on all analyses ( $n = 27$  sequences). Use of response factors (RF) allowed quantitation at lower levels of vitamin D<sub>3</sub>, with an LOQ of <0.20 µg/100 g. The values obtained using the validated methodology agreed well with literature and tabulated database results for most species. However, much lower average vitamin D<sub>3</sub> concentrations were found for oysters (0.05 µg/100 g, raw) and clams (0.18 µg/100 g, cooked) compared to other reports for these products.

Published by Elsevier Inc.

## 1. Introduction

Vitamin D has been the subject of intense interest in the past few years because of an increasing number of health benefits that are potentially related to adequate vitamin D levels (Holick, 2011, 2012; Pilz et al., 2011), although the evidence for some claimed correlations has been found to be less than clear (Institute of Medicine Food and Nutrition Board, 2011). Vitamin D<sub>3</sub> (cholecalciferol) and vitamin D<sub>2</sub> (ergocalciferol) are the two forms of the class of molecules known as “vitamin D” that are most relevant to the diet. There are very few natural sources of vitamin D, with fatty fish being one of the most abundant. Because of this, a variety of foods have been approved by the U.S. Food and Drug Administration for vitamin D fortification, to increase the overall dietary intake of this important nutrient (Byrdwell, 2009).

Although fatty fish have long been known to be good sources of vitamin D<sub>3</sub>, there are still relatively few reports in the literature describing the determination of vitamin D in fish and other seafood using modern analytical techniques, specifically high performance liquid chromatography (HPLC) and mass spectrometry (MS). A few studies have described the vitamin D<sub>3</sub> content of individual fish species, such as menhaden oil (Scott and Latshaw, 1994), raw and smoked Atlantic mackerel (Aminullah Bhuiyan et al., 1993), Atlantic salmon (Horvli et al., 1998), and salted herring (Aro et al., 2005). Several reports have described the vitamin D<sub>3</sub> content of 3 to 8 different fish species (Bilodeau et al., 2011; Mattila et al., 1997, 1999; Takeuchi et al., 1984, 1986), and a few reports have given the content of a larger variety of species (Mattila et al., 1995; Ostermeyer and Schmidt, 2006). Of course, not all of the same species are discussed in these publications, so there are limited data on each species, with varying degrees of statistical treatment (multiple analytical or sample replicates with uncertainties). Furthermore, the values determined by different authors vary substantially, such that it is difficult to determine a single representative value for each species, and whether the variability is from differences in analytical methods or biodiversity in the

\* Corresponding author. USDA, ARS, BHNRC, FCMDL, 10300 Baltimore Avenue, Building 161, Beltsville, MD 20705, United States. Tel.: +1 301 504 9357; fax: +1 301 504 8314.

E-mail address: [C.Byrdwell@ARS.USDA.gov](mailto:C.Byrdwell@ARS.USDA.gov) (W.C. Byrdwell).

samples themselves. Thus, there is an ongoing need for additional data for the vitamin D content of various fish and shellfish species determined with validated methods correlated to known standard reference materials (SRM) to provide an objective measure of the accuracy of the results produced. Additionally, environmental and dietary factors can affect the vitamin D content of particular samples of a given species from different sources. A representative sampling plan that accounts for natural variability of the products as they occur in the food supply is therefore important to obtain a reliable mean and confidence interval for nutrient values in food composition databases that are used to estimate vitamin D intake in populations.

The primary source of food composition data in the United States is the U.S. Department of Agriculture (USDA) Nutrient Database for Standard Reference (SR) (U.S. Department of Agriculture, 2012). SR data are used in conjunction with dietary surveys such as the What We Eat in America component of the National Health and Nutrition Examination Survey (U.S. Department of Agriculture, 2012a) and software such as the University of Minnesota Nutrition Data System for Research (University of Minnesota, 2012) to estimate dietary intake. The accuracy of these estimates obviously depends on the quality and completeness of the food composition data, including accounting for any variability in the food supply. In 1997, the USDA initiated the National Food and Nutrient Analysis Program (NFNAP) (Haytowitz et al., 2008) to update data in the SR using robust representative statistical food sampling plans, validated analytical methods, and comprehensive analytical quality control, as discussed in previous communications (Haytowitz et al., 2008; Phillips et al., 2008; Pehrsson et al., 2000). Analytical data from the NFNAP either enhance, replace, or fill-in missing values for food components in the SR, or generate entries for foods not yet represented.

In this report, data are presented for a variety of fish and shellfish commonly consumed in the U.S. and sampled in 2007–2008, determined using an improved method that included modifications to a previously reported approach (Byrdwell et al., 2011; Phillips et al., 2008), involving a stronger saponification. The improved method is validated by analysis of SRM 1849 Infant/Adult Nutritional Formula from the National Institute for Standards and Technology (NIST, Gaithersburg, MD), using detection by UV and LC–MS.

## 2. Materials and methods

### 2.1. Samples

Fish and shellfish samples were purchased in February 2007 (Blacksburg, VA) and from February to May 2008 from one or two retail outlets in each of twelve U.S. cities according to a statistical plan developed for the NFNAP using the methodology previously described (Pehrsson et al., 2000). The sampling plan was designed to procure representative products based on availability and consumption in the U.S. retail market; therefore samples were not specified to originate from specific producers. Some samples were packaged and some were obtained in bulk from the seafood counter. For some products the country of origin was available and was documented. The amount of product for each species obtained from each outlet ranged from 0.5–4.5 kg.

The samples were procured, packaged, and shipped using methods described elsewhere (Trainer et al., 2010). Prepackaged samples were shipped in their original packages and bulk seafood was kept in its original wrapping, with each sample placed within a Ziploc® bag, with no more than two pounds per bag. Samples were frozen for 18–24 hours prior to shipping, and shipped on dry ice via overnight service to the Food Analysis Laboratory (FALCC) at Virginia Tech (Blacksburg, VA). Upon receipt, the product labels

and visual appearance of the samples were used to verify identity of the products. All samples were held frozen ( $-15 \pm 3^\circ\text{C}$ ) between receipt and preparation. Each product was prepared as described below within 4 weeks of receipt (median, 20 days; range 6–26 days).

For each product, the samples from 3 randomly grouped locations were combined and homogenized to create 4 triad composites per species. Single-location composites and/or a composite of all locations (national composite) were also prepared for some products. This compositing scheme was part of the overall statistical sampling and analysis plan. In cases where samples were not available at all locations (blue crab, lobster), composites of 4–5 locations were prepared. All of the composites were analyzed for a number of other nutrients to update data in the SR, and cooked and raw triad composites of shrimp and scallops were prepared for that purpose. The limited amount of sample for blue crab and did not allow raw and cooked composites, so only the latter were prepared.

The samples for each triad composite were prepared by combining and homogenizing approximately equal weights of the edible portion from each location, without sub-sampling of individual pieces. Each species was prepared and cleaned of inedible parts, then cut into pieces of  $\sim 1.25$  cm, frozen in liquid nitrogen and homogenized using a 6 L stainless steel industrial food processor (Robot Coupe Blixer®, Robot Coupe USA, Jackson, MS) while being kept frozen with liquid nitrogen. Subsamples (8–12 g) of the frozen composite were dispensed into 30-mL glass jars with Teflon™-lined lids, surrounded with aluminum foil, and stored in darkness at  $-60^\circ\text{C}$  prior to analysis.

A salmon control composite (Salmon CC) that was prepared previously for use as an analytical quality control material (Phillips et al., 2008) was also analyzed. The Salmon CC comprised approximately 15.8 kg of drained, canned red sockeye salmon that was homogenized (without liquid nitrogen) using a 30-quart stainless steel industrial food processor (Robot Coupe R30) and distributed among 960 30-mL glass jars with Teflon™-lined lids, with stirring to maintain homogeneity during dispensing. The homogeneity of the Salmon CC was validated as described in a previous publication (Phillips et al., 2008).

Samples were as analyzed as follows, with the numbers in parentheses being the number of samples, and the numbers in brackets being the number of those samples analyzed in duplicate: oyster (2)[2], crab (1), clam (3), mussel (2), shrimp (2), salmon (11)[1], cod (1), flounder (2), ocean perch (1), haddock (1), catfish (2)[2], rockfish (1), pollock (1), halibut (1), trout (1)[1], tuna (2), sardine (2)[2], herring (2)[2], swordfish (1)[1], and scallop (1).

Each sample, including duplicates, were analyzed using eight replicate chromatographic separations, with inclusion of blinded samples of the Salmon CC, using the methods described above. Multiple detectors were employed, including UV detection at 265 nm, full scan UV detection, selected ion monitoring (SIM) mass spectrometry (MS), and a corona charged aerosol detector (CAD). Results were quantified using UV at 265 nm and SIM MS, with the other detectors used for qualitative purposes.

### 2.2. Saponification and extraction

Samples were thawed to room temperature, weighed into 250 mL Erlenmeyer flasks with ground glass necks, and 1.0 mL of 0.5  $\mu\text{g/mL}$  vitamin D<sub>2</sub> was added as the internal standard (IS) to most samples, with 2.0 mL of 0.5  $\mu\text{g/mL}$  IS added to salmon, halibut, tuna, and swordfish, in which higher levels of vitamin D<sub>3</sub> were expected. Approximately 10 g of sample was used when not sample limited, otherwise approximately 5 g of sample was used. All results were calculated on a 100 g basis. Samples were saponified using KOH and extracted using the ethyl ether/petroleum ether extraction given in AOAC 992.26 (AOAC, 1999), as previously described (Byrdwell, 2009; Phillips et al., 2008).

Because of the high fat content of some fish samples, 60 mL of 50% KOH was used for the saponification. The extraction yielded a dry residue that was reconstituted in 1.0 mL hexane for injection onto the semi-preparative HPLC system described below.

### 2.3. Semi-preparative HPLC

HPLC or spectrophotometric grade solvents were purchased from Fisher Scientific, Inc. (Fairlawn, NJ) and were used without further purification. The extraction provided enough sample for two 450  $\mu$ L injections on the semi-preparative HPLC system comprised of an Agilent 1200 system having a quaternary pump with membrane degasser, autosampler with extended volume injection option, diode array detector (DAD) SL, and 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. Gradient conditions have been described in detail previously (Byrdwell, 2009). A 25.0 cm  $\times$  4.6 mm, 5  $\mu$ m, silica column (Inertsil, GL Sciences, Torrance, CA) was used for the semi-preparative separation. Vitamin D<sub>2</sub> and D<sub>3</sub> coeluted at  $\sim$ 23 min on this column. The fractions from two runs were collected separately and evaporated to dryness on a rotary evaporator, and each reconstituted in 650  $\mu$ L of mobile phase 3 consisting of 20% methanol (MeOH)/80% acetonitrile (ACN). Each reconstituted fraction was transferred to two autosampler vials containing limited volume inserts, which allowed four 100  $\mu$ L injections plus waste for each fraction collected.

### 2.4. Reversed-phase HPLC

The reversed-phase (RP) HPLC was carried out using a Thermo Separation Products (San Jose, CA) chromatograph consisting of a P4000 quaternary pump with membrane degasser, AS3000 autosampler, UV 2000 dual wavelength detector operated at 265 nm, UV6000 diode array detector (DAD) operated in both full scan mode and single channel mode at 265 nm at 10 Hz. The solvent system was isocratic mobile phase 3 for 40 min on an Inertsil ODS-2 column, 25.0 cm  $\times$  4.6 mm, 5  $\mu$ m particle size (Inertsil, GL Sciences, Torrance, CA) at a flow rate of 1.3 mL/min. The two fractions collected from semi-preparative HPLC allowed a total of eight (8) 100  $\mu$ L injections on the RP-HPLC system.

Quantification was based on manual integration of the areas under the peaks in the UV 265 nm chromatogram from the DAD and on the sum of the integrated areas in selected ion chromatograms from SIM MS of the protonated molecules,  $[M+H]^+$ , and dehydrated protonated molecules,  $[M+H-H_2O]^+$ . All results given below represent an average of eight runs, unless otherwise indicated.

A corona charged aerosol detector (CAD) was acquired after the first set of salmon and shrimp samples had been run. It was attached to an Agilent SS420X 24-bit analog-to-digital converter, and data were acquired using XCalibur software. However, since the CAD was not available for all samples, and did not produce consistent, reliable results compared to UV and MS, as mentioned in our recent report (Byrdwell, 2011), those data are not presented here.

### 2.5. Mass spectrometry

The mass spectrometer was a TSQ 7000 tandem sector quadrupole 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<sub>2</sub> 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<sub>3</sub>, with a scan time of 0.5 s per ion and 1.0  $m/z$  peak width. Source and other acquisition parameters have been described previously (Byrdwell, 2009).

### 2.6. Calculations

Since we analyzed seafood samples containing a wide range of vitamin D<sub>3</sub> concentrations, a three-point calibration curve was used, instead of only the single response factor (RF) approach that we have used previously (Byrdwell et al., 2011). Nevertheless, values were also calculated using response factors from the standard closest to the sample concentration. For those samples with low vitamin D content, which gave D<sub>3</sub>/D<sub>2</sub> area ratios below the lowest calibration standard, the results from the RF calculated from the lowest standard were calculated.

Bracketed sequences of runs were conducted that included alternating sets of standards and samples, with a minimum of four sets of calibration standards. Long sequences were run that were composed of three standards (3<sub>St</sub>), eight sample replicates (8<sub>sa</sub>), three samples, eight sample replicates, etc. abbreviated as 3<sub>St</sub> + 8<sub>sa</sub> + 3<sub>St</sub> + 8<sub>sa</sub> + 3<sub>St</sub> + 8<sub>sa</sub> + 3<sub>St</sub>. Shorter sequences included only two sets of eight sample replicates, but still included four standards, 3<sub>St</sub> + 8<sub>sa</sub> + 3<sub>St</sub> + 8<sub>sa</sub> + 3<sub>St</sub> + 3<sub>St</sub>, to ensure that at least four sets of standards were used. Quantification was based on the calibration standards run the same day as the sample replicates.

Areas were manually integrated and imported into a Microsoft Excel spreadsheet with the optional 'Analysis Toolpak' installed. Calibration curves were constructed using the 'linest()' function, and UV data were compared to MS data using the student's *t*-test and analysis of variance (ANOVA) tools. Comparisons of two replicates of the same sample or comparisons of the same sample by two different detectors (UV versus MS) were done using a paired *t*-test for sample means.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the conventional approach based on calibration curves. The LOD was calculated from the intercept of the calibration curve plus 3 times the standard deviation in the lowest standard ( $n=5$  per sequence), and the LOQ was the intercept plus 10 times the standard deviation in the lowest standard. The LOD and LOQ were calculated for every sequence of samples, and the averages calculated from all sequences ( $n=27$ ) are given here.

Data analysis using calibration curves required one modification to calculations compared to the response factors used previously. To avoid having negative values for samples that had very low levels of vitamin D<sub>3</sub>, a logic test was inserted into all calculations, such that unless the value from any sample,  $y = (\text{area D}_3/\text{area D}_2)$ , was greater than the intercept,  $b$ , that value was set to zero, specifically,  $\text{if}(y > \text{abs}(b), x = (y - b)/m, \text{else } 0)$ . By doing this, the concentration,  $x$ , calculated from the ratio of vitamin D<sub>3</sub> to the IS,  $y$ , from  $x = (y - b)/m$  was never negative.

Values below are given to the nearest 0.01  $\mu$ g/100 g or to the second uncertain figure, whichever is greater. Values in International Units (IU, where 1 IU = 0.025  $\mu$ g) that are calculated from those values are not further rounded, so rounding error is not compounded.

### 2.7. Quality control of sample analyses

Selected samples were assayed in duplicate, and blinded samples of the Salmon CC were routinely analyzed with samples, using the methods described above. When the sample numbers of the Salmon CC samples were later revealed, results for those samples were compared to previously validated tolerance limits (Phillips et al., 2008). Additionally, NIST SRM 1849 (Infant/Adult

Nutritional Formula) (National Institute of Standards and Technology, Gaithersburg, MD), which was not available at the time of our earlier report (Phillips et al., 2008), was analyzed and results were compared to the certified concentration for vitamin D<sub>3</sub> provided in the certificate of analysis (National Institute of Standards and Technology, 2010).

The Salmon CC and selected other samples were also analyzed by Heartland Assays, Inc. (Ames, IA) for comparison of results, according to the previously described method (Hollis, 2005).

### 3. Results and discussion

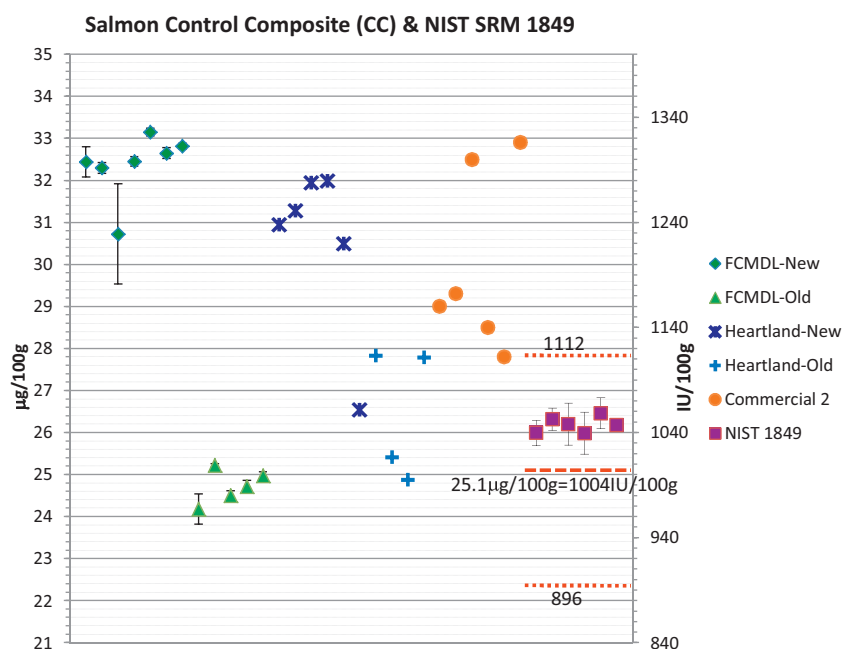
#### 3.1. Method improvement and validation

In our earlier analyses of salmon samples, we saponified the fish with 30 mL of 50% KOH, compared to 135 mL of 1 M KOH used for samples of cereal, and 7.5 g of solid KOH used for liquids, such as milk and orange juice (Byrdwell, 2009; Phillips et al., 2008). Even after saponification, however, a red oily residue resulted from salmon CC samples, instead of a dry pellet, indicating the remaining presence of some oil. Nevertheless, we were able to obtain reproducible results, as seen in Fig. 1 and in the previous reports. However, the salmon CC samples were the only control composite material that was developed by the collaborative effort that did not produce sufficiently consistent results from all participating laboratories to allow a consensus value and tolerance limits to be established (Phillips et al., 2008). That report mentioned that additional work on method development for fatty fish needed to be done.

We later undertook analysis of NIST SRM 1849, Infant/Adult Nutritional Formula. This sample was challenging for vitamin D analysis, and necessitated modification of our method to include even more rigorous saponification. The amount of KOH used for saponification was increased to 60 mL of 50% KOH, as had been used for margarine (Byrdwell, 2007), which is composed almost entirely of edible fat. When the modified method was applied to

NIST SRM 1849 very reproducible results were obtained, as seen in Fig. 1, which were close to the certified value stated in the certificate of analysis (National Institute of Standards and Technology, 2010), which was  $0.251 \pm 0.027$  mg/kg ( $=0.251 \pm 0.027$   $\mu$ g/g  $=25.1 \pm 2.7$   $\mu$ g/100 g  $=1004 \pm 108$  IU/100 g) (11% RSD), and well within the  $\pm 1\sigma$  uncertainty limits given in that document. The values in Fig. 1, determined using the three-point calibration curve from the UV data (same approach as 'UV (Cali.)' in Table 1), gave an average of  $0.262 \pm 0.002$   $\mu$ g/g ( $=26.2 \pm 0.2$   $\mu$ g/100 g  $=1047 \pm 7$  IU/g) (0.70% RSD) for the six samples analyzed (filled squares in Fig. 1). Each sample was analyzed in eight chromatographic replicates, having an average standard deviation of 1.3%, or error expressed as the square root of the sum of the squares of individual uncertainties of 3.4%. The response factor approach from UV data (same as 'UV (RF)' in Table 1) gave similar good agreement (well within the  $\pm 1\sigma$  uncertainty limits), with an average value ( $n=6$ ) of  $0.263 \pm 0.002$   $\mu$ g/g  $=26.3 \pm 0.2$   $\mu$ g/100 g  $=1052 \pm 9$  IU/100 g (0.82% RSD). The SIM MS results (same approach as 'MS (SIM)' in Table 1) were closest to the certified value, giving an average ( $n=6$ ) of  $0.250 \pm 0.004$   $\mu$ g/g  $=25.0 \pm 0.4$   $\mu$ g/100 g  $=1000 \pm 16$  IU/100 g (1.6% RSD). Because of the very low %RSDs of the averages, the value by SIM MS was statistically significantly different from the value by UV calibration curve ( $t=9.1$ ,  $P=2.6e-4$ ) and by UV response factor ( $t=10.$ ,  $P=1.4e-4$ ), but all values were close to the certified value and well within the  $\pm 1\sigma$  uncertainty limits. These results all represented good agreement to the certified value, and indicated that the improved method produced accurate results within stated confidence limits for a high-fat material having a known amount of vitamin D<sub>3</sub> using the UV calibration and response factor approaches and the MS SIM method.

Fig. 1 shows the values obtained for the salmon CC samples analyzed by three laboratories over the course of three years. FCMDL results showed that when the old method that employed lower amounts of KOH was used, the values obtained were lower (filled triangles in Fig. 1), giving an average of  $24.72 \pm 0.40$   $\mu$ g/mL (1.6% RSD). When the improved method was used, in which



**Fig. 1.** Vitamin D<sub>3</sub> levels (in  $\mu$ g/100 g) in salmon control composite (CC) samples determined by FCMDL before ("old") and after ("new") method improvement, old and new analyses by Heartland Assays (Ames, IA), and by a second commercial laboratory, as well as FCMDL results from improved method applied to National Institute of Standards and Technology (NIST, Gaithersburg, MD) standard reference material (SRM) 1849. The NIST SRM 1849 certificate of analysis gives a value of  $0.251 \pm 0.027$  mg/kg ( $=0.251 \pm 0.027$   $\mu$ g/g  $=25.1 \pm 2.7$   $\mu$ g/100 g  $=1004 \pm 108$  IU/g). FCMDL values each represent averages of 8 replicates (error bars give  $\pm 1\sigma$ ) using UV detection with three-point calibration; commercial labs are singlet values. Right axis in international units, IU/100 g, where 1 IU = 0.025  $\mu$ g.



**Table 1**

Vitamin D<sub>3</sub> in seafood samples, in µg/100 g, analyzed using UV at 265 nm from calibration curves (Cali.) and response factors (RF) and using selected ion monitoring (SIM) mass spectrometry using Vitamin D<sub>2</sub> as an internal standard.<sup>a</sup>

	[Rep.] <sup>b</sup>	UV (Cali.) Average ± SD	UV (RF) Average ± SD	MS (SIM) Average ± SD	Description
Catfish	1[1]	0.15 ± 0.08	0.34 ± 0.08	0.00 ± 0.00	AL, CA, MI – Raw
	1[2]	0.13 ± 0.02	0.22 ± 0.02	0.00 ± 0.00	AL, CA, MI – Raw
	2[1]	0.06 ± 0.01	0.17 ± 0.01	0.00 ± 0.00	AL, CA, MI – Baked
	2[2]	0.15 ± 0.08	0.26 ± 0.08	0.00 ± 0.00	AL, CA, MI – Baked
Clam	1	0.08 ± 0.01	0.15 ± 0.01	0.41 ± 0.03	Steamed
	2	0.01 ± 0.01	0.08 ± 0.01	0.00 ± 0.00	Raw
	3	0.00 ± 0.00	0.01 ± 0.00	0.10 ± 0.04	Canned, US Origin – (FL1, MO1, NY1)
Cod	1	0.90 ± 0.03	0.98 ± 0.03	1.38 ± 0.25	CO, CT, IN – Raw (8.9%) <sup>c</sup>
Crab	1	0.00 ± 0.00	0.05 ± 0.03	0.00 ± 0.00	Blue Crab, Steamed – AL, CA, CO, CT, FL
Flounder	1	2.36 ± 0.04	2.37 ± 0.04	2.28 ± 0.12	Sole – (WV, NC, VA) – Flatfish, Raw (0.57%) <sup>c</sup>
	2	6.73 ± 0.08	6.67 ± 0.08	6.30 ± 0.16	Sole – (WV, NC, VA) – Flatfish, Baked(0.86%) <sup>c</sup>
Haddock	1	0.59 ± 0.04	0.69 ± 0.04	0.07 ± 0.20	FL, NC, NY – Raw (16%) <sup>c</sup>
Halibut	1	5.73 ± 0.01	5.82 ± 0.01	5.55 ± 0.32	CO, CT, IN – Raw (1.6%) <sup>c</sup>
Herring	1[1]	2.82 ± 0.03	2.94 ± 0.02	2.28 ± 0.68	Herring snacks in wine sauce (4.2%) <sup>c</sup>
	1[2]	2.76 ± 0.03	2.87 ± 0.03	2.72 ± 0.42	Herring snacks in wine sauce (4.0%) <sup>c</sup>
	2[1]	3.39 ± 0.03	3.51 ± 0.03	3.13 ± 0.44	Herring snacks in wine sauce (3.4%) <sup>c</sup>
	2[2]	3.37 ± 0.06	3.45 ± 0.05	2.41 ± 0.53	Herring snacks in wine sauce (2.4%) <sup>c</sup>
Mussel	1	0.14 ± 0.15	0.20 ± 0.17	0.00 ± 0.00	Steamed
	2	0.08 ± 0.03	0.14 ± 0.03	0.00 ± 0.00	Raw
Ocean Perch	1	0.66 ± 0.03	0.73 ± 0.02	0.87 ± 0.12	AL, CA, MI – Raw (12%) <sup>c</sup>
Oyster	1[1]	0.07 ± 0.08	0.19 ± 0.11	0.75 ± 0.34	Steamed
	1[2]	0.07 ± 0.01	0.12 ± 0.01	0.04 ± 0.08	Steamed
	2[1]	0.00 ± 0.00	0.04 ± 0.04	0.00 ± 0.00	Raw
	2[1]	0.02 ± 0.02	0.07 ± 0.02	0.00 ± 0.00	Raw
	3	0.04 ± 0.04	0.23 ± 0.04	0.00 ± 0.00	Steamed – CT, IN, NC
Pollock	1	0.07 ± 0.02	0.21 ± 0.02	0.15 ± 0.21	MI, MO, NY – Raw
Rockfish	1	6.16 ± 0.03	6.11 ± 0.03	5.83 ± 0.34	AL, CA–Raw (0.77%) <sup>c</sup>
Salmon	1	32.44 ± 0.36	32.48 ± 0.35	31.2 ± 2.2	CC (0.11%) <sup>c</sup>
	2	25.99 ± 0.10	26.08 ± 0.10	25.2 ± 2.5	Sockeye, Baked (0.34%) <sup>c</sup>
	3	32.30 ± 0.13	32.34 ± 0.13	31.7 ± 1.9	CC (0.13%) <sup>c</sup>
	4	18.21 ± 0.07	18.24 ± 0.07	18.03 ± 0.71	Sockeye, Raw (0.19%) <sup>c</sup>
	5	14.32 ± 0.13	14.26 ± 0.12	13.3 ± 1.9	Wild- (CA, NC, OK) – Baked (0.46%) <sup>c</sup>
	6	11.29 ± 0.04	11.26 ± 0.04	12.0 ± 1.0	Wild- (CA, NC, OK) – Raw (0.19%) <sup>c</sup>
	6[H] <sup>d</sup>	11.43			Wild- (CA, NC, OK) – Raw
	7	30.7 ± 1.2	30.7 ± 1.2	30.19 ± 0.84	CC (0.19%) <sup>c</sup>
	8	32.45 ± 0.11	32.47 ± 0.11	31.9 ± 1.5	CC (0.04%) <sup>c</sup>
	9	7.80 ± 0.11	7.95 ± 0.10	7.62 ± 0.30	Chum/Pink – AL, NY (1.9%) <sup>c</sup>
	10	33.15 ± 0.09	33.23 ± 0.09	34.5 ± 2.4	CC (0.22%) <sup>c</sup>
	11[1]	32.65 ± 0.13	32.75 ± 0.13	32.0 ± 2.1	CC (0.30%) <sup>c</sup>
	11[2]	32.82 ± 0.07	32.91 ± 0.07	32.9 ± 3.1	CC (0.28%) <sup>c</sup>
Sardine	1[1]	3.62 ± 0.02	3.68 ± 0.02	3.36 ± 0.38	Canned (1.5%) <sup>c</sup>
	1[2]	3.68 ± 0.05	3.73 ± 0.05	3.73 ± 0.32	Canned (1.4%) <sup>c</sup>
	2[1]	2.89 ± 0.10	3.01 ± 0.10	1.54 ± 0.25	Canned (4.0%) <sup>c</sup>
	2[2]	3.02 ± 0.04	3.17 ± 0.04	2.37 ± 0.20	Canned (5.0%) <sup>c</sup>
Scallops	1	0.00 ± 0.00	0.12 ± 0.07	0.00 ± 0.00	Raw
Shrimp	1	0.03 ± 0.03	0.09 ± 0.08	0.00 ± 0.00	Unsp. Var. <sup>b</sup> (M-L) – (CO, CT, IN) – Raw
	2	0.00 ± 0.00	0.01 ± 0.02	0.00 ± 0.00	Unsp. Var. (M-L) – (CO, CT, IN) – Boiled
	3[1] <sup>e</sup>	0.00 ± 0.00	0.05 ± 0.03	0.00 ± 0.00	Unsp. Var. (M-L) – (CA, NC, OK) – Boiled
	3[2] <sup>e</sup>	0.00 ± 0.00	0.08 ± 0.01	0.00 ± 0.00	Unsp. Var. (M-L) – (CA, NC, OK) – Boiled
	4[1] <sup>e</sup>	0.38 ± 0.09	0.53 ± 0.09	0.44 ± 0.63	Unsp. Var. (M-L) – (CA, NC, OK) – Raw
	4[2] <sup>e</sup>	0.00 ± 0.00	0.04 ± 0.02	0.00 ± 0.00	Unsp. Var. (M-L) – (CA, NC, OK) – Raw
	5 <sup>e</sup>	0.01 ± 0.01	0.09 ± 0.07	0.00 ± 0.00	Unsp. Var. (M-L) – (CA, NC, OK) – Raw
	6 <sup>e</sup>	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	Unsp. Var. (M-L) – NC – Raw
	7 <sup>e</sup>	0.14 ± 0.10	0.30 ± 0.09	0.22 ± 0.17	Unsp. Var. (M-L) – CO – Raw
Swordfish	1[1]	9.74 ± 0.11	9.75 ± 0.10	9.86 ± 0.54	CO, CT, IN – Raw (0.14%) <sup>c</sup>
	1[2]	9.64 ± 0.05	9.72 ± 0.05	9.41 ± 0.64	CO, CT, IN–Raw (0.85%) <sup>c</sup>
Trout	1[1]	14.23 ± 0.03	14.03 ± 0.03	14.9 ± 1.6	Rainbow Trout – FL, MO, NY – Raw (1.4%) <sup>c</sup>
	1[2]	14.55 ± 0.05	14.39 ± 0.05	15.1 ± 2.1	Rainbow Trout – FL, MO, NY – Raw (1.1%) <sup>c</sup>
Tuna	1	8.06 ± 0.04	8.15 ± 0.04	8.10 ± 0.57	FL, MO, NY – Baked (1.2%) <sup>c</sup>
	2	1.10 ± 0.03	1.50 ± 0.03	2.09 ± 0.14	FL, MO, NY – Raw (37%) <sup>c</sup>

<sup>a</sup> Values and uncertainties are given to 0.01 µg/100 g, or to the second uncertain figure, whichever is greater.

<sup>b</sup> Abbreviations: [Rep.] = sample number followed by replicate number in brackets; N.D., not determined; Unsp. Var. = unspecified variety. States = standard abbreviations.

<sup>c</sup> % Difference between value from UV calibration curve versus that from UV response factor, for values >0.50 µg/100 g by calibration curve.

<sup>d</sup> Sample analyzed by commercial laboratory (Heartland Assays, Ames, IA).

<sup>e</sup> Samples analyzed using older FCMDL method.

saponification was more thorough and a dry residue was obtained, values for vitamin D<sub>3</sub> in the salmon CC samples were higher (filled diamonds in Fig. 1), giving an average of 32.36 ± 0.78 µg/mL (2.4% RSD). One of our sample values (point #3) had a high standard deviation due to partial loss of one semi-preparative LC fraction of one

sample in the rotary evaporator, which gave four higher values (first fraction) and four lower values (second fraction), each with low %RSDs, 31.84 ± 0.03 (0.10%) µg/mL and 29.61 ± 0.04 (0.14%) µg/mL, respectively. This highlighted the overall difficulty with the complexity of the method for vitamin D analysis, which included

semi-preparative LC, fraction collection, reconstitution, and re-injection onto the analytical LC system. Other than this sample, all values were close to each other and had low standard deviations for the 8 replicates.

Since the improved method produced accurate results for NIST 1849, we had confidence in the higher values for the salmon CC samples, and concluded that the lower values included a bias low due to incomplete saponification/extraction. In principle, incomplete extraction of vitamin D<sub>3</sub> could be expected to produce low values, but would not lead to high values.

The values from commercial lab 1 (Heartland Assays, Ames, IA) also showed a bimodal distribution, with most of the newer values ( $\times$  with vertical line in Fig. 1) being higher, and all of the older values (+ in Fig. 1) being lower. One of the newer values, however, was similar to the older values. The same method was used by that laboratory for all samples.

A second commercial laboratory also produced results with a bimodal distribution, but these did not correspond to the date of analysis. The first three analyses, conducted in 2009, gave two low values and one high value (filled circles in Fig. 1), and the second analyses, performed in 2011, similarly gave one higher value and two low values.

The results shown in Fig. 1 highlight several important points. The difference between FCMDL results from the original and modified method suggests that sample-to-sample variability apparently arose primarily from saponification/extraction method variability. The inter-laboratory results in Fig. 1 demonstrated again why it was not possible to come to a consensus value for the salmon CC. FCMDL results showed that when the samples were saponified thoroughly, both salmon CC and NIST 1849 samples produced consistent results (low %RSD). Our data indicated that when low values were obtained, it was probably due to incomplete saponification. Thus, it is beneficial to include NIST SRM 1849 as a method validation sample. Inclusion of results for a commercially available SRM in published studies allows comparison of data between published reports and helps to account for any contribution of analytical variability in the comparison of results for samples.

High fat samples were the only ones that were problematic. The methods that produced inconsistent, bimodal results for salmon often produced perfectly adequate results for most other samples, as seen in the earlier report (Phillips et al., 2008). The salmon CC also had higher levels of vitamin D<sub>3</sub> than all other salmon samples. Therefore, a method that produced consistent results for this material could be expected to produce good results for other, less challenging samples.

### 3.2. Application of the improved saponification method

All analyses of fish and shellfish samples employed rigorous saponification, except for some shrimp samples. The data for shrimp samples that were run before the method improvement are included because shrimp are not high in fat, so the original saponification was deemed appropriate, and the values obtained (<LOQ) were the same using the improved and the older method.

Results for the wide variety of fish and shellfish samples determined using the validated methodology are given in Table 1. In almost all cases, cooked samples contained higher amounts of vitamin D<sub>3</sub> by weight than the same raw sample. The exception was one shrimp sample that was below the LOQ. Salmon had the highest levels of vitamin D<sub>3</sub> of the samples analyzed, consistent with expectations based on literature results from a variety of sources. Semi-preparative (NP-HPLC) and analytical (RP-HPLC) separations for a typical salmon sample are shown in Figs. 2 and 3, respectively (representative of high vitamin D samples). Values ranged from  $7.80 \pm 0.11 \mu\text{g}/100 \text{ g}$  ( $7.62 \pm 0.30 \mu\text{g}/100 \text{ g}$  by MS) for chum to  $33.15 \pm 0.09 \mu\text{g}/100 \text{ g}$  ( $34.5 \pm 2.4 \mu\text{g}/100 \text{ g}$  by MS) for the CC samples

(see Supplemental Materials for salmon CC RP-HPLC figure). Sockeye salmon contained  $18.21 \pm 0.07 \mu\text{g}/100 \text{ g}$  ( $18.03 \pm 0.71 \mu\text{g}/100 \text{ g}$  by MS) for the raw sample, and  $25.99 \pm 0.10 \mu\text{g}/100 \text{ g}$  ( $25.2 \pm 2.5 \mu\text{g}/100 \text{ g}$  by MS) for the baked sample (Figs. 2 and 3). Wild salmon contained  $11.29 \pm 0.04 \mu\text{g}/100 \text{ g}$  ( $12.0 \pm 1.0 \mu\text{g}/100 \text{ g}$  by MS) for the raw sample, and  $14.32 \pm 0.13 \mu\text{g}/100 \text{ g}$  ( $13.3 \pm 1.9 \mu\text{g}/100 \text{ g}$  by MS) for the cooked sample. A duplicate analysis of the raw sample by Heartland Assays gave almost the identical value by UV for the raw sample, at  $11.43 \mu\text{g}/100 \text{ g}$  (singlet analysis). The UV and MS values above were statistically indistinguishable, due to the larger standard deviations associated with the MS data.

Trout contained the next highest levels of vitamin D<sub>3</sub>, giving values of  $14.23 \pm 0.03 \mu\text{g}/100 \text{ g}$  and  $14.55 \pm 0.05 \mu\text{g}/100 \text{ g}$  for the two samples analyzed. These two values are statistically significantly different ( $t = 14$ ,  $P = 1.0\text{e-}6$ ), due to the very low %RSD given by the UV detector for each sample. But as a practical matter, the relative difference of 2.3% between these values is not meaningful compared to the sample-to-sample variability inherent in the saponification/extraction. The results by MS of  $14.9 \pm 1.6 \mu\text{g}/100 \text{ g}$  and  $15.1 \pm 2.1 \mu\text{g}/100 \text{ g}$  were statistically indistinguishable from the values obtained by UV detection for the same two samples, respectively.

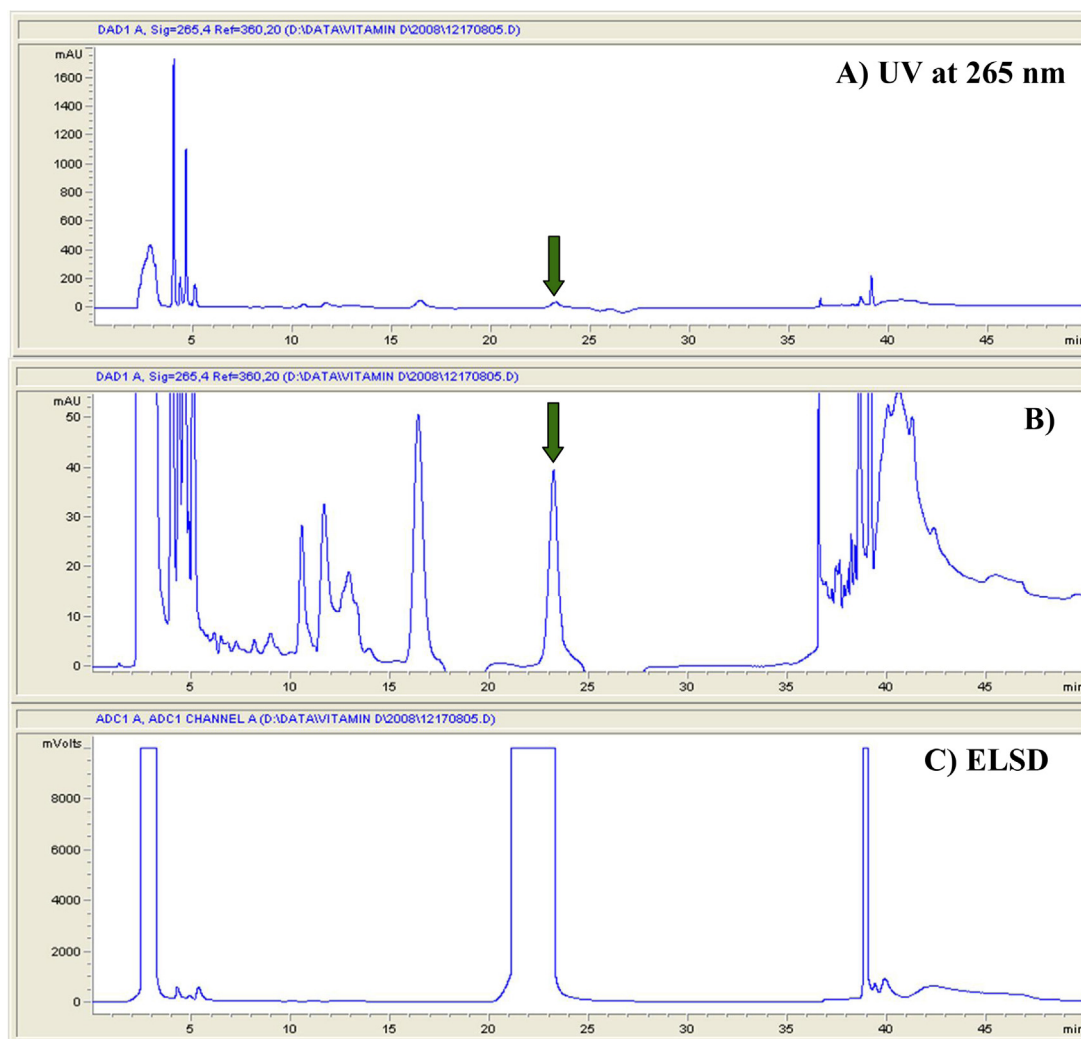
Swordfish contained the next highest amount of vitamin D<sub>3</sub>, having levels of  $9.74 \pm 0.11 \mu\text{g}/100 \text{ g}$  and  $9.64 \pm 0.05 \mu\text{g}/100 \text{ g}$  for duplicate analyses of the same sample. These two values were statistically indistinguishable, and they are indistinguishable from the results by MS, which were  $9.86 \pm 0.54 \mu\text{g}/100 \text{ g}$  and  $9.41 \pm 0.64 \mu\text{g}/100 \text{ g}$ , respectively.

Halibut and rockfish contained similar levels of vitamin D<sub>3</sub> in raw samples, at  $5.73 \pm 0.01 \mu\text{g}/100 \text{ g}$  and  $6.16 \pm 0.03 \mu\text{g}/100 \text{ g}$ , respectively. The results by MS were  $5.55 \pm 0.32 \mu\text{g}/100 \text{ g}$  and  $5.83 \pm 0.34 \mu\text{g}/100 \text{ g}$  for these two fish, respectively. The MS results for halibut were statistically the same as the UV results ( $t = 1.1$ ,  $P = 0.15$ ), while those for rockfish were not ( $t = 2.9$ ,  $P = 0.01$ ). Although statistically significantly different, the relative difference of 5.4% between UV and MS results for rockfish still represents good agreement between the two different detection techniques.

These values were similar to the amounts in cooked tuna and flounder (sole), which were  $8.06 \pm 0.04 \mu\text{g}/100 \text{ g}$  and  $6.73 \pm 0.08 \mu\text{g}/100 \text{ g}$ , respectively. As noted above, raw samples of tuna and flounder contained less vitamin D<sub>3</sub> by weight than the cooked samples. The amounts in raw samples were  $1.10 \pm 0.03 \mu\text{g}/100 \text{ g}$  and  $2.36 \pm 0.04 \mu\text{g}/100 \text{ g}$  in tuna and flounder, respectively. The results for cooked tuna by MS were indistinguishable from the UV results, but the MS results for raw tuna were statistically significantly different ( $t = 21$ ,  $P = 8.0\text{e-}8$ ). Similarly, the UV and MS results for raw flounder were statistically significantly different ( $t = 5.9$ ,  $P = 3.1\text{e-}4$ ), though the relative difference was only 10.9%.

Sardines and herring, all of which were cooked samples, contained similar amounts of vitamin D<sub>3</sub>, in the range 2.8 to  $3.7 \mu\text{g}/100 \text{ g}$ . One sample of sardines gave an average of  $3.65 \mu\text{g}/100 \text{ g}$  for duplicate sample analyses ( $3.62 \pm 0.02$  and  $3.68 \pm 0.05 \mu\text{g}/100 \text{ g}$ ), while another sample analyzed in duplicate gave an average of  $2.96 \mu\text{g}/100 \text{ g}$  ( $2.89 \pm 0.10$  and  $3.02 \pm 0.04 \mu\text{g}/100 \text{ g}$ ). These values were above the LODs calculated from the four sequences in which the four sample replicates were run, which ranged from  $0.97 \mu\text{g}/100 \text{ g}$  to  $1.15 \mu\text{g}/100 \text{ g}$ . But they were below the calculated LOQs in those sequences, which ranged from  $4.20 \mu\text{g}/100 \text{ g}$  to  $4.96 \mu\text{g}/100 \text{ g}$ . Since the UV detector was more sensitive than MS, three of four sardine samples were below the LODs for those sequences by MS, and two of four (sardine samples 2[1] and 2[2] in Table 1) were statistically significantly different from the corresponding UV results, while two were indistinguishable.

Similarly, one herring sample gave an average of  $3.38 \mu\text{g}/100 \text{ g}$  for duplicate sample analyses ( $3.39 \pm 0.03$  and  $3.37 \pm 0.06 \mu\text{g}/100 \text{ g}$ ) and another gave an average of  $2.79 \mu\text{g}/100 \text{ g}$  from duplicates



**Fig. 2.** UV and evaporative light scattering detector (ELSD) chromatograms of NP (semi-prep) HPLC of baked salmon ( $25.99 \pm 0.10 \mu\text{g}/100 \text{ g}$  vitamin  $\text{D}_3$ ). (A) UV at 265 nm, full scale; (B) UV at 265 nm scaled to show vitamin D peak (marked by arrow); (C) ELSD. One of two preparative runs for sample, 450  $\mu\text{L}$  injected.

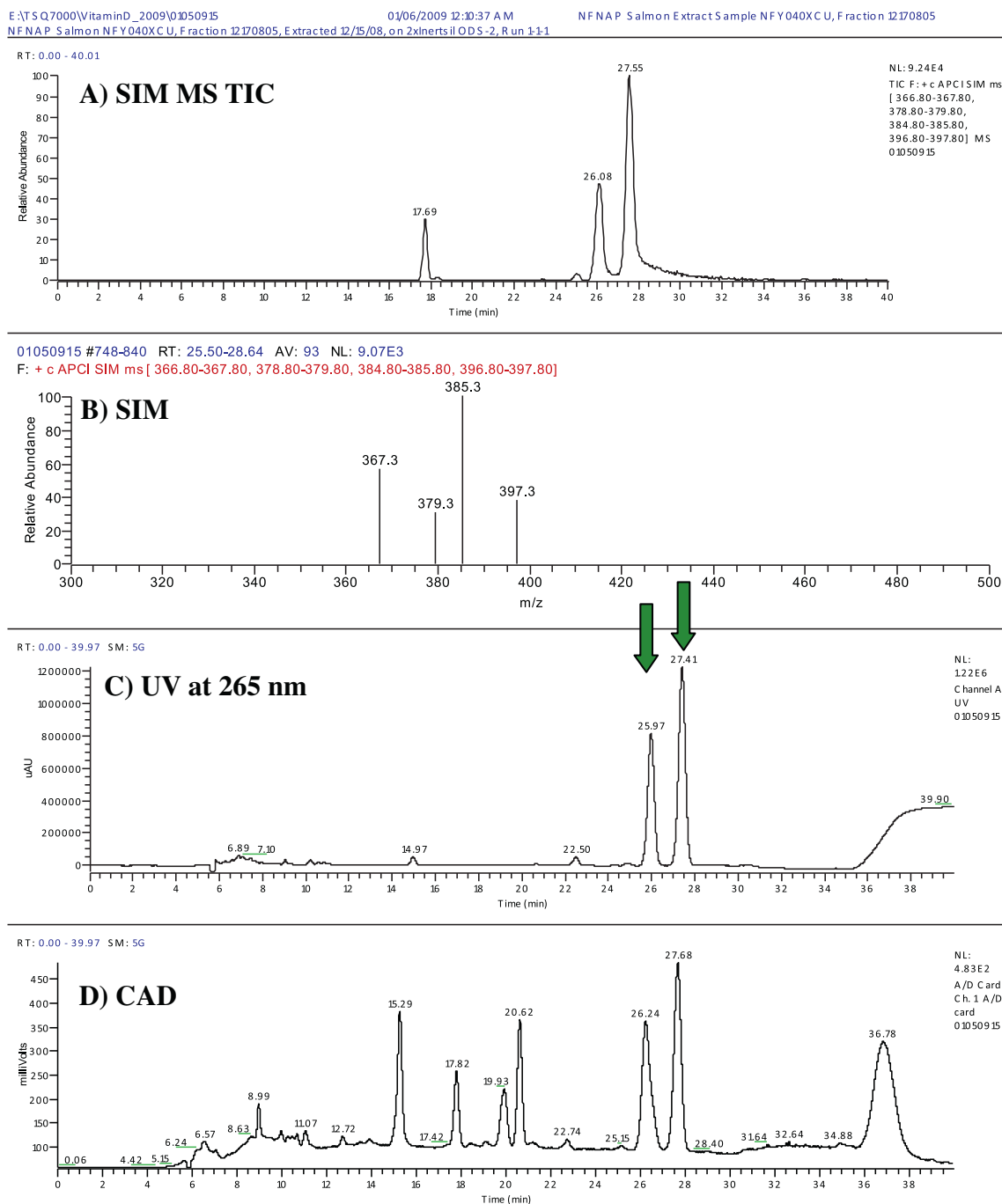
( $2.82 \pm 0.03$  and  $2.76 \pm 0.03 \mu\text{g}/100 \text{ g}$ ). Herring samples also gave values that were between the calibration curve LODs and the LOQs from the sequences in which they were run, which ranged from 0.92 to  $1.15 \mu\text{g}/100 \text{ g}$  for the LODs, and from 4.09 to  $4.96 \mu\text{g}/100 \text{ g}$  for the LOQs. Three of four values obtained by MS were again below the calculated LODs for the sequences in which the samples were run, and two were statistically significantly different from the corresponding UV results (herring samples 1[1] and 2[2]).

The average LODs and LOQ from all 27 sequences in which samples were run were  $1.22 \mu\text{g}/100 \text{ g}$  and  $5.30 \mu\text{g}/100 \text{ g}$  from UV data, and  $2.51 \mu\text{g}/100 \text{ g}$  and  $8.37 \mu\text{g}/100 \text{ g}$  for MS data. In practice, these values represented conservative estimates, since the values were based on the standard deviation in the lowest calibration standard, which was run-to-run variability, instead of the noise in actual UV chromatograms, which was quite low. This is demonstrated effectively by Fig. 4, which represents the RP-HPLC separation of the scallop sample. This sample had a low  $\text{D}_3/\text{D}_2$  integrated area ratio ( $=0.012$ ) that was less than the calibration curve intercept ( $=0.022$ ) calculated from the five sets of standards in that sequence, which would have given a negative value for vitamin  $\text{D}_3$ , if not for the Excel logic test implemented as described in the calculations section above. Thus, by the calibration curve approach, this sample gave a value of  $0.00 \mu\text{g}/\text{mL}$  vitamin  $\text{D}_3$ . Nevertheless, there was

clearly a peak in Fig. 4D, arising from a very low level of vitamin  $\text{D}_3$ , that could be manually integrated. A pragmatic estimation of noise from this detector is seen in Fig. 4C, in which a 1-min window of noise was integrated from the dead time region (this guaranteed that no peaks were present). Thus, the 'pragmatic S/N' from these integrated areas was 10.8, which represents the approximate 'real', instead of calculated, LOQ possible. However, very low values of vitamin  $\text{D}_3$  could not be quantified using calibration curves, due to the small non-zero intercepts.

For this reason, we calculated values for all samples based on response factors, as well as calibration curves, as given in Table 1. Since RFs use a simple comparison of the integrated areas of the  $\text{D}_3/\text{D}_2$  peaks in samples as a ratio to that in a standard (the lowest standard was used for all samples with low vitamin  $\text{D}_3$  levels), non-zero values were obtained by the RF approach. The eight replicates of the scallop sample shown in Fig. 4 gave a value of  $0.12 \pm 0.07 \mu\text{g}/\text{mL}$ . Obviously, the % RSD was higher at these very low levels.

Thus, while calibration curves were valuable for analyzing samples with a wide range of vitamin  $\text{D}_3$  values, they were not as effective as response factors for samples with very low levels. As a practical consideration, the practical LOQ based on an acceptable S/N of UV chromatographic peaks was less than half



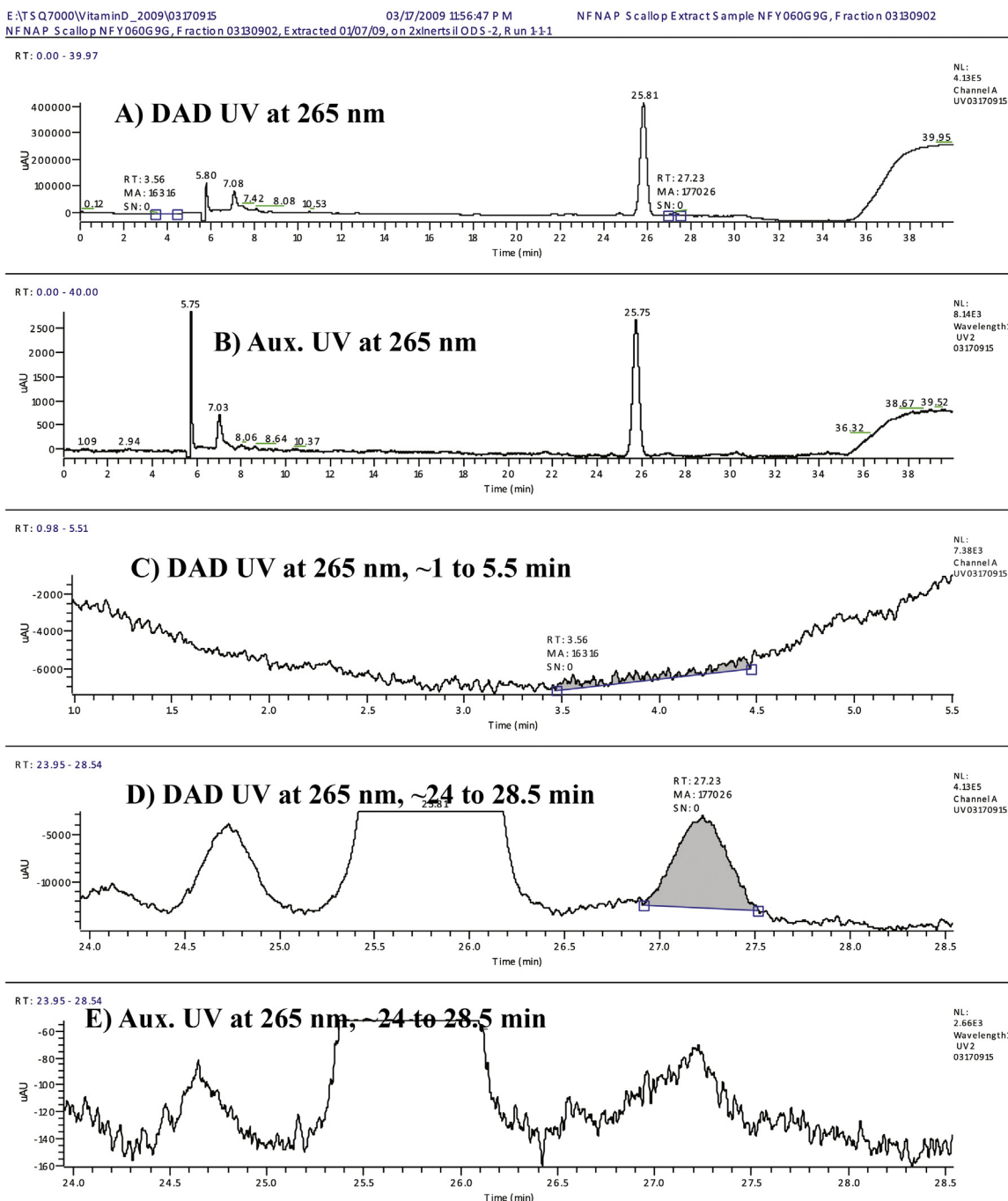
**Fig. 3.** Selected ion monitoring (SIM) MS, UV and corona charged aerosol detector (CAD) chromatograms of RP-HPLC separation of fraction collected (see Fig. 2) from separation of baked salmon. (A) Total ion current chromatogram (TIC); (B) SIM ion mass spectrum; (C) UV at 265 nm, with vitamin D<sub>2</sub> (1st) and vitamin D<sub>3</sub> (2nd) peaks marked by arrows; (D) corona CAD. One of eight replicate runs. 2.0 mL of 0.5 µg/mL Vitamin D<sub>2</sub> IS added. Vitamin D<sub>3</sub> = 25.99 ± 0.10 µg/100 g.

the level of the LOD calculated from the calibration curves. On the other hand, MS chromatograms were noisier, with lower S/N, and the observed LOQ was close to the calculated LOD. Thus, a practical estimate of LOQ by UV detection using calibration curves was approximately 0.5 µg/100 g, while for MS it was between 2.0 and 2.5 µg/100 g. Of course, newer, more sensitive MS instruments are expected to provide lower LODs and LOQs. On the other hand, using RFs the practical LOQ was less than 0.20 µg/100 g, as seen in Fig. 4. For samples with values higher than 0.5 µg/100 g in Table 1, the difference between the calibration curve and RF calculations is given in parentheses in the right column.

All other fish analyzed (catfish, cod, haddock, ocean perch, and pollock) and all shellfish (clam, crab, mussel, oyster, scallop and shrimp) were below the LOD and LOQ calculated from the calibration curves. Because of this, the %RSDs by MS for those samples were higher, and there was poorer agreement between MS and UV results, so only UV results are discussed.

Raw cod gave a value of 0.90 ± 0.03 µg/100 g; raw haddock was found to contain 0.59 ± 0.04 µg/100 g; and similarly, raw ocean perch was determined to have 0.66 ± 0.03 µg/100 g. The low %RSD values below for UV results between 0.5 µg/100 g to 1.0 µg/100 g, for eight replicates each, demonstrated that reproducible UV results could be obtained in this range, while MS results were less consistent.





**Fig. 4.** UV chromatograms from RP-HPLC for scallop showing: (A) full chromatogram from diode array detector (DAD) single channel at 265 nm; (B) full chromatogram from auxiliary dual wavelength detector at 265 nm; (C) DAD noise in dead time region, ~1–5.5 min, (integrated noise ~1 min, in grey) from chromatogram (A) above; (D) time range ~24.0–28.5 min from (A) showing typical integrated vitamin D<sub>3</sub> peak, in grey (peak width 0.62 min); (E) time range ~24.0–28.5 from (B).

### 3.3. Comparison to previous results

Mattila et al. (1995) have done extensive work to demonstrate that there are wide variations in vitamin D values obtained from fish samples of the same species, which vary by, among other things, location and season. Earlier work by Takeuchi et al. (1986) had shown differences in the vitamin D content in fish tissue, even between dorsal flesh and ventral flesh. Unfortunately, no correlation could be found between vitamin D content and age, weight, or gender of the species (Mattila et al., 1997). Furthermore, recent work has shown substantial variability for fish from the same locations caught the same day (Ostermeyer and Schmidt, 2006).

Wide ranges of values in fish continue to be observed using even the latest in LC-MS/MS technology employing multiple reaction monitoring (MRM) transitions, which provides a high degree of specificity for vitamin D (Bilodeau et al., 2011). Such variability demonstrates that no single value of vitamin D in a particular fish species is representative of the vitamin D content of that species. Instead, there is a need for multiple reports in the literature so that a general consensus can be arrived at regarding the range of values that can be expected for a species, rather than defining a single value.

For instance, the range of values that we obtained for samples of different varieties or species of salmon demonstrates that no single

value is representative of all salmon products. Our values (excluding the control composite) ranged from a low of 7.80  $\mu\text{g}/100\text{ g}$  for pink salmon chum to 18.21  $\mu\text{g}/100\text{ g}$  for raw sockeye salmon, whereas cooked samples ranged from 14.32  $\mu\text{g}/100\text{ g}$  for wild salmon to 25.99  $\mu\text{g}/100\text{ g}$  for baked sockeye. Values in the National Nutrient Databank for Standard Release version 24 (U.S. Department of Agriculture, 2012) ranged from as low as 1.2  $\mu\text{g}/100\text{ g}$  and 2.0  $\mu\text{g}/100\text{ g}$  for kippered king salmon (NDB No. 35168) and sockeye salmon (NDB No. 35167), respectively, to 21  $\mu\text{g}/100\text{ g}$  for canned sockeye (drained solids, NDB No. 15087). Ostermeyer and Schmidt (2006) reported a range of values from 4.2  $\mu\text{g}/100\text{ g}$  to 10.7  $\mu\text{g}/100\text{ g}$  for fresh salmon, and values from 4.9  $\mu\text{g}/100\text{ g}$  to 27.2  $\mu\text{g}/100\text{ g}$  for smoked salmon; Bilodeau et al. reported a range of 12.7–43.5  $\mu\text{g}/100\text{ g}$  for canned pink salmon (Bilodeau et al., 2011); Horvli et al. (1998) reported a value of  $30 \pm 10\text{ }\mu\text{g}/100\text{ g}$  for fillet of Atlantic salmon, which went as high as  $210 \pm 16\text{ }\mu\text{g}/100\text{ g}$  for fish fed vitamin D<sub>3</sub> fortified feed for 11 weeks. Our values are in the middle of the ranges reported by others, but the wide range of values for various species and preparations demonstrates the high degree of variability in the literature values available. Blinded control samples, duplicate sample analyses within our lab, plus duplicate analyses by different laboratories, provided confidence that the values obtained for individual samples analyzed were reproducible and reflected the content in the samples provided. Overall, the consistently relatively high values in salmon, compared to other fish, confirmed the common perception that salmon is a good source for dietary vitamin D.

Similarly, our results for trout, swordfish and tuna confirmed the generally accepted notion that these fish also represent good sources of dietary vitamin D. Raw farmed rainbow trout have a value in SR24 of 15.9  $\mu\text{g}/100\text{ g}$  (NDB No. 15240), whereas our sample of raw rainbow trout gave an average of 14.39  $\mu\text{g}/100\text{ g}$  from two sample replicates. Trout values in SR24 ranged from 3.9  $\mu\text{g}/\text{mL}$  (mixed species, raw, NDB No. 15114) to 19  $\mu\text{g}/\text{mL}$  (farmed, cooked, NDB No. 15241). The tabulated value given by Bourre and Paquette (2008) was 8.0  $\mu\text{g}/100\text{ g}$ . Mattila et al. (1995) reported an average value of 7.6  $\mu\text{g}/100\text{ g}$  between Autumn and Spring rainbow trout samples, and later, a range of 7.2  $\mu\text{g}/100\text{ g}$  to 15.3  $\mu\text{g}/100\text{ g}$  (Mattila et al., 1999). Rainbow trout analyzed by Ostermeyer and Schmidt (2006) gave values ranging from 3.8  $\mu\text{g}/100\text{ g}$  to 10.7  $\mu\text{g}/100\text{ g}$ .

Swordfish values for vitamin D in SR24 were  $13.9 \pm 4.0\text{ }\mu\text{g}/100\text{ g}$  in the raw fish (NDB No. 15110) and 16.6  $\mu\text{g}/100\text{ g}$  for the cooked (NDB No. 15111). Our average value from two sample replicates was 9.69  $\mu\text{g}/100\text{ g}$ .

Tuna values in SR24 ranged from 1.7  $\mu\text{g}/100\text{ g}$  for raw yellowfin tuna (NDB No. 15127) to 6.7  $\mu\text{g}/100\text{ g}$  for canned tuna in oil (drained solids, NDB No. 15119), while our values were 1.10  $\mu\text{g}/100\text{ g}$  for raw tuna and 8.06  $\mu\text{g}/100\text{ g}$  cooked. Bourre and Paquette (2008) gave a value of 5.0  $\mu\text{g}/100\text{ g}$ . Literature values for tuna include values from Takeuchi et al. (1984) of 18.73  $\mu\text{g}/100\text{ g}$  for fresh skipjack and 3.65  $\mu\text{g}/100\text{ g}$  for albacore, and later, values of 1.7  $\mu\text{g}/100\text{ g}$  to 6.9  $\mu\text{g}/100\text{ g}$  in skipjack dorsal and dark flesh, respectively, and values of 3.1  $\mu\text{g}/100\text{ g}$  to 5.9  $\mu\text{g}/100\text{ g}$  for dorsal and ventral flesh, respectively (Takeuchi et al., 1986).

For herring, there was great variability that fell into two ranges. The values for herring in SR24 were from Atlantic herring, and ranged from 2.2  $\mu\text{g}/100\text{ g}$  for kippered fish (NDB No. 15042) to 5.4  $\mu\text{g}/100\text{ g}$  for cooked herring (NDB No. 15040). Our average values, 2.79  $\mu\text{g}/100\text{ g}$  and 3.38  $\mu\text{g}/100\text{ g}$ , were for herring snacks in wine sauce, and were in the same range as the values in SR24. Values in the literature are mostly Baltic herring, and are substantially higher. Mattila et al. (1995) reported values of 17.1  $\mu\text{g}/100\text{ g}$  (Autumn + Spring average), and later reported 22.5  $\mu\text{g}/100\text{ g}$  and 22.9  $\mu\text{g}/100\text{ g}$  for raw and cooked small fish, respectively, and 31.9  $\mu\text{g}/100\text{ g}$  and 26.1  $\mu\text{g}/100\text{ g}$  for raw and cooked large fish, respectively (Mattila et al., 1999). Ostermeyer

and Schmidt gave values of 9.5 and 14.7  $\mu\text{g}/100\text{ g}$  for two sample pools ( $n = 5$  each) of herring caught in the same area on the same day. More recently, Aro et al. (2005) reported values between 16 and 24  $\mu\text{g}/100\text{ g}$  for salted herring fillets, and from 12 to 25  $\mu\text{g}/100\text{ g}$  for the pickled product. The tabulated value from Bourre and Paquette (2008) was 17.0  $\mu\text{g}/100\text{ g}$ .

Values for halibut also exhibited a wide range. In SR24, Atlantic halibut was given a value of 4.7  $\mu\text{g}/100\text{ g}$  (NDB No. 15036), while Greenland halibut was assigned a value of 27.4  $\mu\text{g}/100\text{ g}$  (NDB No. 15038). Bourre and Paquette gave a value of 4.3  $\mu\text{g}/100\text{ g}$ , while Ostermeyer and Schmidt determined values of 7.16 and 7.65  $\mu\text{g}/100\text{ g}$  for Greenland halibut. We quantified a value of 5.73  $\mu\text{g}/100\text{ g}$ . The consensus of values tends to indicate that the value of 27.4  $\mu\text{g}/100\text{ g}$  in SR24 may be an outlier.

Cod has values of 0.5  $\mu\text{g}/100\text{ g}$  for Pacific cod, 1.2  $\mu\text{g}/100\text{ g}$  for cooked or canned Atlantic cod (NDB No. 15016, 15017), and 4.0  $\mu\text{g}/100\text{ g}$  for dried and salted Atlantic cod (NDB No. 15018) in SR24. The value tabulated by Bourre and Paquette (2008) was 1.3  $\mu\text{g}/100\text{ g}$ . Our determined values of 0.90  $\mu\text{g}/100\text{ g}$  by calibration curve or  $1.38 \pm 0.25\text{ }\mu\text{g}/100\text{ g}$  by RF agree well with the database values. These values are all lower than the 6.9  $\mu\text{g}/100\text{ g}$  reported by Mattila et al. (1995) for cod from the Baltic Sea.

As seen from the above comparisons, the values for almost all fishes from the present study were within the ranges previously reported in databases and in the literature. There were differences, however, between our determined values and tabulated values for shellfish. Specifically, we found very little vitamin D in oysters, mussels, clams, scallops, and shrimp. For these comparisons, the values calculated from RFs are used, since the values are well below the LODs calculated from the calibration curves. We obtained values from 0.04  $\mu\text{g}/100\text{ g}$  to 0.23  $\mu\text{g}/100\text{ g}$  for raw and cooked oysters, respectively. This is in sharp contrast to the value of 8.0  $\mu\text{g}/100\text{ g}$  tabulated by Bourre and Paquette (2008). That value is equal to the amount given for trout in that same report (Bourre and Paquette, 2008), and is more than the values given for tuna, halibut, anchovy, and other fishes.

Based on the fact that values determined from multiple and duplicate analyses using UV with confirmation by MS, and use of methodology validated by analysis of SRM 1849, the higher vitamin D value for some shellfish in some literature reports appear to be overestimates. Every analysis of mollusks (clam, mussel, oyster and scallop) (Table 1) gave results of less than 0.25  $\mu\text{g}/100\text{ g}$  vitamin D<sub>3</sub>, showing mollusks to be consistently low in this nutrient. The tabulated value from Bourre and Paquette (2008) for mussel was 2.4  $\mu\text{g}/100\text{ g}$ . Since our data are validated and supported by multiple mutually confirmatory detection techniques, we believe that further analyses will confirm the low levels of vitamin D in mollusks, especially oysters.

Similarly, we found only low to trace levels of vitamin D in shrimp. The value given by Bourre and Paquette (2008) is 1.3  $\mu\text{g}/100\text{ g}$ . While this is not a high value, it is higher than all of our determinations. These data demonstrate the need for more published data on the vitamin D content of shellfish. Such data need to be based on multiple analytical replicates (we used  $n = 8$ ) and sample replicates, with both UV and MS data. Preferably, the method used to obtain such data should also be applied to SRM 1849 or similar standard reference material for which a certified value is given for vitamin D, with results within the confidence limits for that sample, to validate the method. We have previously shown that UV data by itself is not reliable for all samples (Byrdwell, 2009), and that MS data provides a valuable complement. Ideally, full scan MS data are obtained in combination with SIM or selected reaction monitoring (SRM) MS data. Unfortunately, the auxiliary mass spectrometer normally used in the 'dual parallel mass spectrometer' configuration (Byrdwell, 2009) was inoperative at the time that these samples were analyzed.

#### 4. Conclusion

Values were reported for vitamin D<sub>3</sub> in a wide range of seafood samples, including fish and shellfish that were determined using two approaches to UV detection at 265 nm (calibration curve and response factors), which is the 'industry standard' method employed for vitamin D analysis. Additional data based on quantification by SIM MS confirmed the vitamin D levels determined by UV detection, and full scan UV spectra provided qualitative assessment of peak purity. UV calibration curve and response factor results agreed quite well, although the response factor results may be preferred because they allowed a lower limit of quantification and gave values for species with very low vitamin D<sub>3</sub> levels. UV calibration curve, UV response factor, and MS SIM results all gave values within the acceptable  $\pm 1\sigma$  limits given by NIST for SRM 1849. Statistically significant differences between most same-sample results arose primarily because of the very low %RSDs of the UV results. Most such differences were negligible compared to sample-to-sample results. These results allow us to conclude that results based on a calibration curve are preferred if analysis time allows, since regression analysis provides a check of calibration standards. However, for samples with very low levels that could give negative values due to a small negative intercept, response factor results are preferred. Response factors may also be preferred if time does not allow use of repetitive runs of multiple calibration standards. MS survey scans should always be used to confirm that no interferents are present, to provide confidence in UV results. In cases where interfering species are present, MS results are preferred, although the standard deviations are higher, since the APCI is an inherently noisier process.

While most samples agreed with ranges found in the literature, vitamin D levels in mollusks and shrimp were substantially lower than in some other literature reports. The values for vitamin D in oysters especially indicate that extant values should be used with caution. There is a need for additional high quality data based on multiple mutually confirmatory detection methods that are correlated to a known reference material.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jfca.2013.01.005>.

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