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Analysis

Fast comprehensive analysis of vitamin D and triacylglycerols in dietary supplements using multiple parallel mass spectrometers

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Summary

New, faster methods have been developed for analysis of vitamin D and triacylglycerols that eliminate hours of wet chemistry and preparative chromatography, while providing more information than classical methods for analysis. Unprecedented detail is provided by combining liquid chromatography with the power of three or four mass spectrometers used simultaneously, in parallel, for definitive analysis of the vitamins and lipids in dietary supplements.

Introduction

Vitamin D has become an area of intense interest around the world because of the variety of disease states that have been correlated with vitamin D deficiency, and the increasing realization of just how many people have either insufficient or deficient levels of vitamin D. Vitamin D insufficiency or deficiency is especially a problem at higher latitudes and in the wintertime, as well as in countries where cultural conventions lead to minimization of skin exposure [1]. At low latitudes under a summer sun, the skin produces enough vitamin D in just a few minutes to more than meet minimum daily requirements. Because of this, some scientists advocate waiting at least a few minutes before applying sunscreen, to allow the body to naturally meet its vitamin D needs, while others warn against any sun exposure at all, due to concerns over skin damage from sun exposure.

A steady stream of articles in the popular press and on television have made consumers aware of the importance of maintaining adequate levels of vitamin D. Because of this and a variety of factors, many people have opted to take multivitamin/multimineral (MVMM) or other supplements as part of their daily routines. Dietary supplement use has steadily gone up, based on data in the U.S. from the National Health and Nutrition Examination Survey (NHANES) I, II, III and IV, and their use increases with increasing age [2]. According to analysis of the 2003–2006 NHANES data published in 2011, 54% of adults had taken some type of dietary supplement in the last month [2]. As dietary supplement use has increased, there is an increasing need for improved methods for analysis of the supplements taken. This is particularly true for vitamin D supplements, because the methods for vitamin D analysis are typically time-consuming, laborious, and involve both preparative and analytical chromatography steps [3].

Part of the problem with the vast majority of existing methods is that the first step is a saponification step to break down all triacylglycerols (TAGs), which are seen as an interferent. Thus, no information on these is obtained. We have worked to develop fast, thorough analytical methods for both vitamin D and TAGs. Some dietary supplement gelcaps are made of vitamin D dissolved in a dietary oil. A "dilute-and-shoot" method that eliminates virtually all sample prep and provides a comprehensive analysis of both the vitamin D and bulk excipient oil is ideal for those samples [4]. Some supplements are made of either a compressed powder or a fluffy powder excipient with vitamin D (and other fat-soluble vitamins) mixed in. For those samples, steps must be taken to ensure that the vitamin D is extracted efficiently. We used National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 3280 to prove the complete extraction [5]. Provided below is an overview of the benefits and complications of vitamin D analysis, and how new, fast, comprehensive methods have been applied to address the issues associated with classical methods for vitamin D analysis.

Multiple parallel mass spectrometry

I use liquid chromatography (LC) coupled to three types of atmospheric pressure ionization (API) mass spectrometry (MS), because each provides different and complementary information and has different strengths and weaknesses. Atmospheric pressure chemical ionization (APCI) MS is the simplest and perhaps the most versatile, since almost all molecules are ionized by this technique. But two shortcomings are that acetonitrile in the LC mobile phase causes accumulation of a "blob" of polymerized material on the end of the corona needle leading to diminished sensitivity over the course of a sequence of samples, and it does not give abundant intact protonated molecules for some molecules, such as saturated TAGs and some oxidation products. Electrospray ionization (ESI) MS gives intact ammonium adducts of most molecules (especially oxidation products), but barely responds to vitamin D, if at all. Also, each ESI source behaves slightly differently, and different instruments form different amounts of other adducts, such as those derived from acetonitrile and dichloromethane, and some sources readily experience charge saturation. It also requires an electrolyte to enhance ionization. Atmospheric pressure photoionization (APPI) is a non-contact ionization source, so it does not suffer from loss of sensitivity, and ionizes almost all classes of molecules effectively. However, it does require a dopant to enhance ionization, and it exhibits a much larger differential response to polyunsaturated TAGs than APCI. Thus, no single API technique is ideal, and I like

to compare and contrast the data from each of the different approaches. Also, vitamin D is present at a much lower level (<1 μg/mL) than the TAG excipient (~ mg/mL), so I use an older, less sensitive APCI-MS instrument for TAG analysis of large gelcaps (~500 mg), and a newer, more sensitive APCI-MS instrument for vitamin D analysis in all samples and TAG analysis in smaller gelcaps (~100 mg).

Thus, I have employed four mass spectrometers simultaneously in a 'quadruple parallel mass spectrometry arrangement' to address all of the issues mentioned above. I also perform quantification of vitamin D using selected ion monitoring (SIM), which is ~2 to 100 times more sensitive, but less specific than the selected reaction monitoring (SRM) MS/MS that I also perform, as well as using extracted ion chromatograms (EICs) from full scans. I further compare quantification from internal standard, external standard, and response factor approaches. I also perform these three approaches for quantification using UV data (@265 nm), to take advantage of the lower noise and lower %RSD from UV data. But sometimes, peaks that appear sharp and resolved by UV are shown to contain substantial interferents by MS. A corona charged aerosol detector (CAD) and evaporative light scattering detector (ELSD) both going to two analog-to-digital converters are also included, for comparison. The arrangement of instruments used for the quadruple parallel mass spectrometry recently reported [5] is in **Figure 1**. The instruments are coordinated via a series of contact closure switches, which have recently been updated to wireless communication.

Dietary supplements

There are three main sources for vitamin D in supplements: synthetic, from lanolin, and from 'molecularly distilled' fish oil. The benefit of the "dilute-and-shoot" approach is that all non-polar components are analyzed without breaking down TAGs. This allowed us to identify three short-chain (SC) TAGs present only in the supplements that contained vitamin D from fish oil. **Figure 2** shows the chromatograms and a mass spectrum of the three SC-TAGs found. We found those same three SC-TAGs in a different, as far as we know unrelated, brand that also used vitamin D from fish oil. Perhaps the manufacturers sourced their vitamin D from the same supplier. Despite decades of vitamin D analysis, this had not been noticed before, because the first step in conventional methods is to break down all TAGs.

Figure 2 is an example of using the "dilute-and-shoot" method for a liquid gelcap sample having interfering species that co-eluted with vitamin D. CyCyCa (dicaprylic, capric TAG) and vitamin D_2 co-eluted at 19.9 min, but the mass spectrum proved that they did not have ions in common that interfered with quantification by MS, and, since the short-chain TAGs (identified by MS) did not contain chromophores, should not skew UV result dramatically.

Figure 2 also exhibits the common characteristic of TAGs that polyunsaturated TAGs give a protonated molecule, $[M+H]^+$, base peak, while TAGs with few sites of unsaturation give diacylglycerol-like ions, $[DAG]^+$, as base peaks with little to no $[M+H]^+$. We later extended the length of the chromatographic run, because we kept identifying TAGs containing longer and longer fatty acids (FAs), up to 28 carbons long from the excipient oils. Figure 2 also shows ESI-MS data for TAGs, obtained in parallel with APCI-MS from the same injection. The same three SC-TAGS gave good signal by ESI-MS, unambiguously confirming their presence. ESI-MS gives ammonium adducts, $[M+NH_4]^+$, as base peaks for TAGs regardless of the degree of unsaturation. The ESI-MS chromatogram of all TAGs showed different relative peak sizes, though, demonstrating that the ESI source on that instrument was more susceptible to charge saturation than APCI, or ESI on our other instruments (we later switched that instrument over to APPI-MS).

Figure 3 shows the ion chromatograms from an "extract-filter-shoot" analysis of a dry powdered supplement, and demonstrates how the different approaches to quantification (EICs, SIM, SRM) are compared and contrasted in our reports. The figure shows that the signal-to-noise (S/N) for the EIC is poorer than for SIM and SRM (and it degrades substantially over the course of the sequences of runs), even though the raw signal is higher (9.9E4). It shows that SIM gives higher signal than SRM (5.47E4 vs. 2.32E4), but anything that gives ions in common with vitamin D and elutes at the same time could interfere, which rarely occurs. SRM gives less total signal, since it employs MS/MS (not an entirely efficient process), but it is more specific for the same reason. Mass spectrometers each have different characteristics, so some exhibit more difference in response between SIM and SRM than that shown in Figure 3.

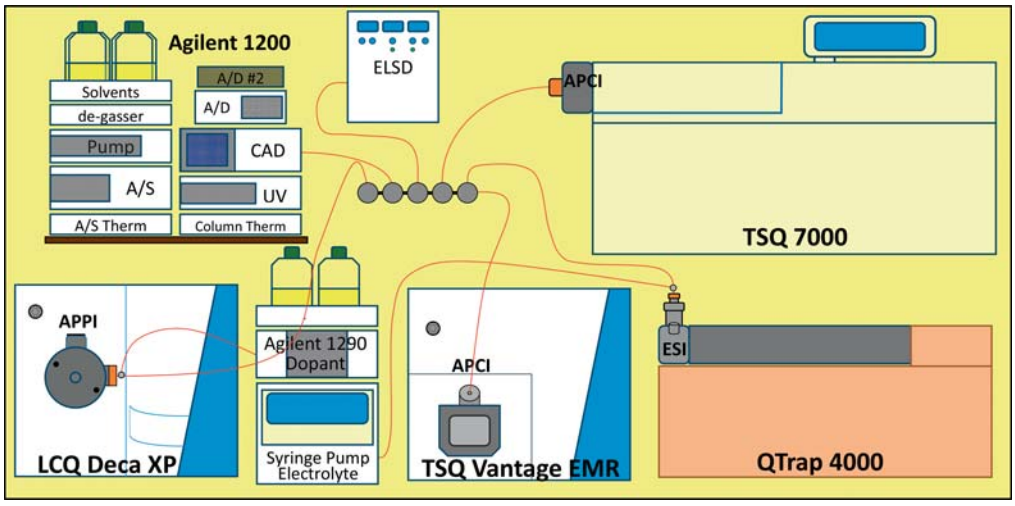

Figure 1. Instrument arrangement for 'quadruple parallel mass spectrometry' using liquid chromatography with atmospheric pressure chemical ionization (APCI) mass spectrometry (MS), electrospray ionization (ESI) MS, and atmospheric pressure photoionization (APPI) MS.

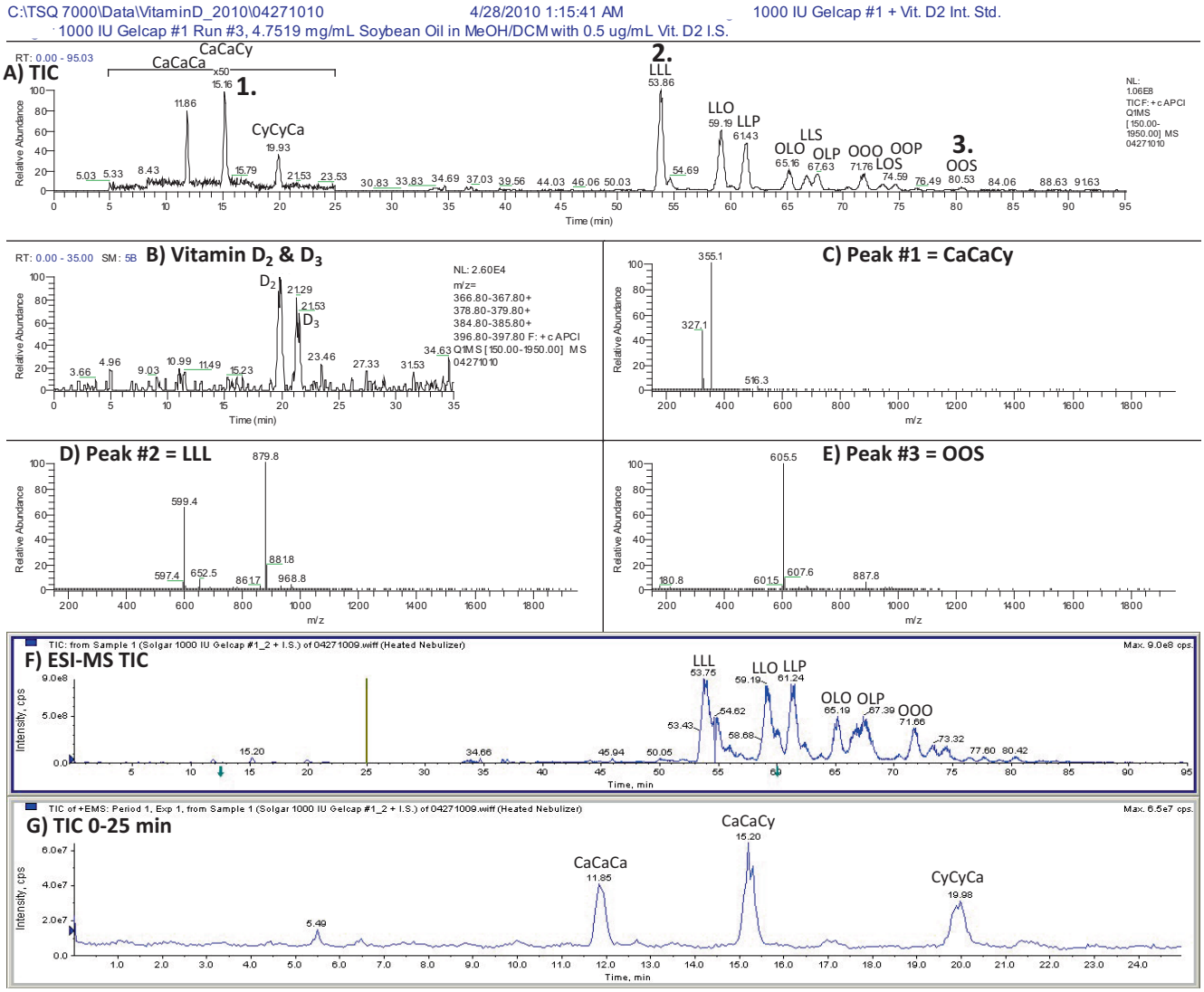

Figure 2. Soybean oil-containing gelcap triacylglycerols by atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) and electrospray ionization (ESI) MS (exhibiting some charge saturation). A) APCI-MS total ion current chromatogram (TIC) with 5–25 min × 50; B) extracted ion chromatogram (EIC) of vitamin D masses; C) avg. mass spectrum at 15.16 min = CaCaCy; D) avg. mass spectrum at 53.86; E) avg. mass spectrum at 80.53; F) ESI-MS TIC; G) expanded ESI-MS TIC from 0 to 25 min.

Figure 3 shows a clean mass spectrum for vitamin D_2 and D_3, indicating that the UV data can be trusted. The figure also shows the APCI-MS and APPI-MS TICs for TAGs that came from the rice bran powder excipient using the arrangement in Figure 1. Despite the fact that the excipient was a powder, not an oil, we were able to obtain and compare complete TAG compositions from rice bran oil by APCI-MS, APPI-MS, and ESI-MS using the single-flask "extract-filter-shoot" approach. To show that the vitamin D was completely extracted from the dry powder-containing supplements, we analyzed NIST SRM 3280, and got 94–101% extraction efficiency.

All of the information about the TAGs that has been described above would not be possible using conventional methods for analysis of vitamin D. All the information, especially regarding short-chain TAGs that come along with 'molecularly distilled' fish oil and very-long chain fatty acid-containing TAGs would be lost by the saponification step historically employed.

Moving forward, we are testing the application of short "dilute-and-shoot" and "extract-filter-shoot" methods to fortified foods, as well as natural foods, such as mushrooms. Natural foods represent the biggest challenge, since the vitamin D may be sequestered in cells that need to be disrupted. The goal is not to force the method onto samples for which it is not ideally suited, but to find other types of samples to which it ideally applies. This is because, for every type of sample we find that the methods work, they greatly simplify analysis of those samples, and save time, labor, resources, and money. So, while the methods will not apply to every food, it represents a substantial advantage, providing more information with less time and effort, for those to which it does apply.

Conclusion

I like to say: "I do these experiments so you don't have to." In other words, not everyone can assemble and coordinate the number of instruments that I have in my lab. These were not bought all at once, but accumulated over time, and I have managed to keep old instruments running long after they were retired from other labs. By doing these experiments simultaneously in parallel from the

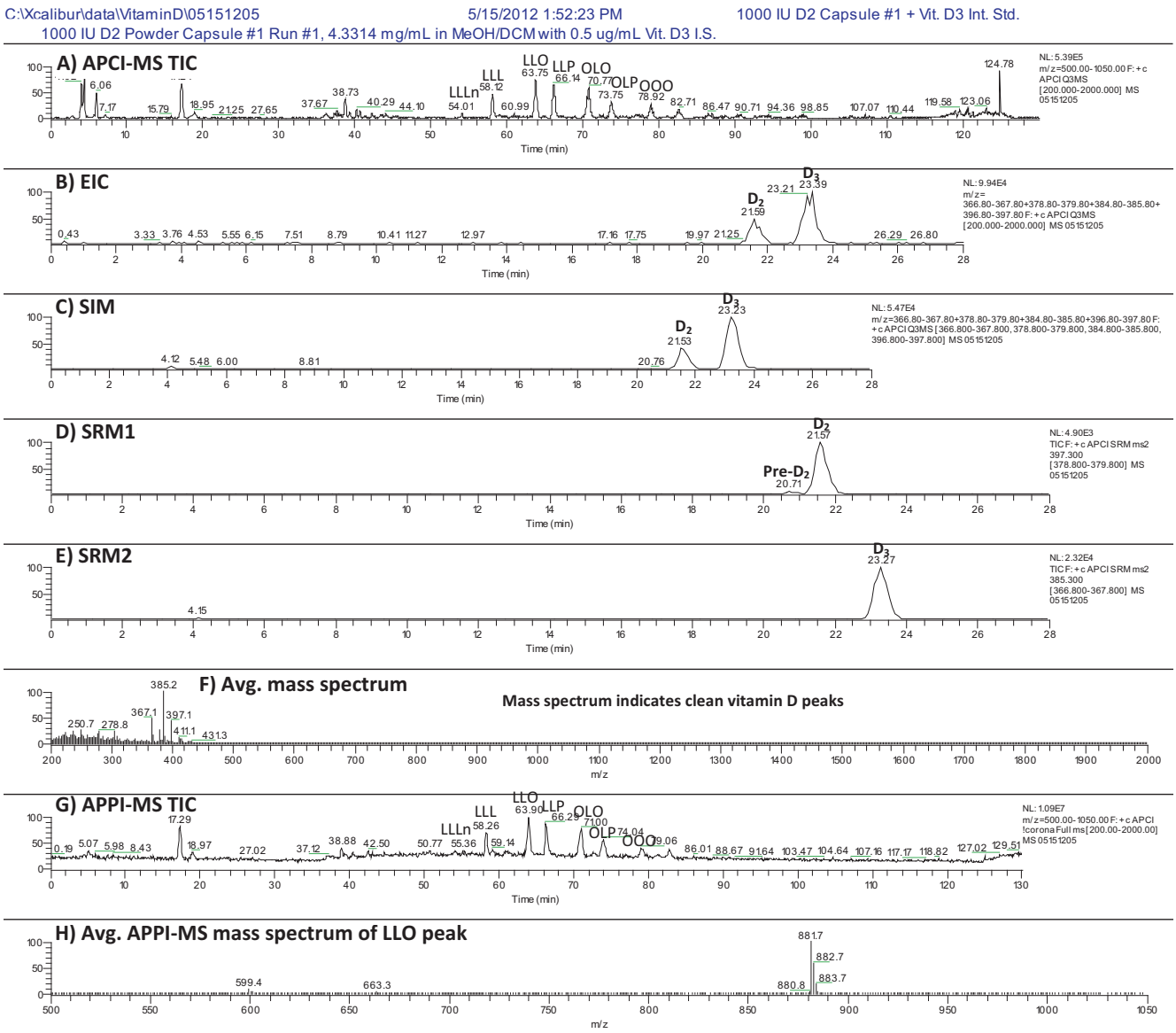

Figure 3. Atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) mass spectrometry (MS) data from "extract-filter-shoot" analysis of vegetarian (vitamin D_2) dietary supplement containing rice bran powder. Triacylglycerols (TAGs) are from rice bran oil from bran powder. A) APCI-MS total ion current chromatogram (TIC); B) extracted ion chromatogram (EIC) of vitamin D_2 and D_3 masses; C) selected ion monitoring (SIM) chromatogram; D) selected reaction monitoring (SRM chromatogram) for vitamin D_2 ions; E) SRM chromatogram for vitamin D_3 ions; F) average APCI-MS mass spectrum across vitamin D_2 and D_3 peaks; G) APPI-MS TIC; H) avg. APPI-MS mass spectrum across dilinoleoyl, oleoyl triacylglycerol (LLO).

same injection, I can more effectively compare and contrast strengths and weaknesses to allow others to draw their own conclusions about what is best for their analyses, and what to expect from such choices.

If I had to make due with one single instrument, however, the data show that APCI-MS is most versatile, and suffers the least from non-linear response effects compared to other API techniques, while still providing maximum information. If acetonitrile can be eliminated from the LC system, one of the major shortcomings of this technique can be eliminated. One other take-home message that has been demonstrated over and over is that UV data alone cannot be trusted! I have seen beautiful, sharp, well-resolved peaks for vitamin D that hid interfering species that dramatically skewed quantitation. UV data can only be trusted if MS data prove that the peak is pure. In short: "Don't trust. Verify."

References

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