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Quadruple parallel mass spectrometry for analysis of vitamin D and triacylglycerols in a dietary supplement

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ABSTRACT

A "dilute-and-shoot" method for vitamin D and triacylglycerols is demonstrated that employed four mass spectrometers, operating in different ionization modes, for a "quadruple parallel mass spectrometry" analysis, plus three other detectors, for seven detectors overall. Sets of five samples of dietary supplement gelcaps labeled to contain 25.0 μg (1000 International Units, IU) vitamin D_3 in olive oil were diluted to 100 mL and analyzed in triplicate by atmospheric pressure chemical ionization (APCI) mass spectrometry (MS), atmospheric pressure photoionization (APPI) MS and electrospray ionization (ESI) MS, along with an ultraviolet (UV) detector, corona charged aerosol detector (CAD), and an evaporative light scattering detector (ELSD), simultaneously in parallel. UV detection allowed calculation by internal standard (IS), external standard (ES), and response factor (RF) approaches, which gave values of 0.2861 ± 0.0044, 0.2870 ± 0.0059, and 0.2857 ± 0.0042 μg/mL, respectively, which were not statistically significantly different. This indicated an average amount of vitamin D_3 of 14.5% over the label amount. APCI-MS analysis by selected ion monitoring (SIM) and two transitions of selected reaction monitoring (SRM) provided values of 0.2849± 0.0055, 0.2885± 0.0090, and 0.2939 ± 0.0097 μg/mL, respectively, relative to vitamin D_2 as the IS. The triacylglycerol (TAG) composition was determined by APCI-MS, APPI-MS and ESI-MS, and the fatty acid (FA) compositions calculated from the TAG compositions were compared to the FA composition determined by gas chromatography (GC) with flame ionization detection (FID) of the FA methyl esters (FAME). APCI-MS provided the FA composition closest to that determined by GC-FID of the FAME. A previously reported approach to TAG response factor calculation was employed, which brought all TAG compositions into good agreement with each other, and the calculated FA compositions into excellent agreement with the FA composition determined from GC-FID of the FAME.

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1. Introduction

Cholecalciferol, vitamin D_3, and ergocalciferol, vitamin D_2, continue to be of interest to researchers and consumers alike, due to the fact that study after study has appeared that indicates deleterious effects of dietary deficiency in this vitamin, collectively referred to as vitamin D. While these two components are the forms of the vitamin normally consumed, it is the 25-hydroxy vitamin D metabolite formed in the liver (and elsewhere) that acts as the primary biomarker for the nutrient. Because of structural differences between the native molecules and their circulating metabolites, and differences in sample matrices between vitamin D-containing foods and biological fluids such as serum, which are used to assess its biological adequacy, the methods for analysis of the dietary form of the nutrient are often very different from those used to quantify its metabolite(s).

Biological samples can be quickly and routinely analyzed using radioimmunoassays (RIA) or competitive protein binding assays (CPBA), although lack of specificity leading to cross-reaction between forms of the metabolites and other issues makes these types of analysis less than ideal, but nevertheless useful for high throughput clinical screening. For foods and supplements, on the other hand, vitamin D is the analyte, and an entirely different approach is used. For analysis with the greatest level of specificity and high sensitivity, liquid chromatography with mass spectrometric detection is used. LC-MS is usually considered the "gold-standard" for vitamin D analysis due to its ability to use specific precursor-product fragmentation pathways in selected reaction monitoring (SRM) experiments to differentiate forms of the nutrient and its metabolites.

The coupling of liquid chromatography to mass spectrometry is accomplished nowadays using atmospheric pressure ionization (API) interfaces, the most common of which are atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), and atmospheric pressure photoionization (APPI). Vitamin D, however, does not respond well to ESI, so APCI has become the preferred

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ionization technique for LC-MS of vitamin D. Only very limited reports are available describing the use of APPI-MS for vitamin D analysis [1,2], since this is not the default source supplied with most mass spectrometers. Furthermore, although a few classes of molecules respond better to APPI than APCI, APCI is more of a universal ionization technique, since a very wide range of classes of molecules respond well to this ionization process, without the need for the addition of dopant, normally associated with APPI.

Prior to LC-MS analysis, extensive sample extraction and cleanup procedures are still generally required before samples are ready for the final chromatographic separation and analysis. In 1999, Eitenmiller and Landen [3] summarized the methods commonly used at that time, and similar approaches continue to be used today. Advances in LC have led to the use of ultra-high performance liquid chromatography (UHPLC) for the chromatographic separation [4–6], but preliminary sample preparation often continues to employ various combinations of saponification and either liquid/liquid extraction (LLE) or solid phase extraction (SPE) prior to analysis. Unfortunately, these sample preparation steps are often the most labor-intensive part of the analytical process. While automated SPE equipment is commercially available and is used in some cases, only a fraction of the methods describing LC-MS of vitamin D employ automated sample preparation. Thus, while great advancements have been made in LC and MS instrumentation, especially in regard to increased mass spectrometer sensitivity, the sample preparation steps often still require substantial commitments of time and expertise by qualified chemical technicians.

To address the issue of saponification and extraction, we recently reported a "dilute-and-shoot" approach that eliminated the saponification and extraction steps altogether, giving greatly simplified sample preparation [7]. While this approach is not applicable to all sample types, for those samples to which it does apply, it allows a substantial reduction in sample preparation time and chemical resources used. Of equal importance is the fact that components in the sample that were previously regarded as interfering species, such as triacylglycerols (TAG) that required isolation and removal, are now able to be analyzed along with the vitamin D analyte. This allows a holistic analysis of the entire samples, instead of targeted analysis of a single analyte to the exclusion of other components.

Since many components are analyzed using such an approach, it is beneficial to employ multiple ionization techniques, because some classes of compounds respond better to different types of ionization, and different ionization techniques provide different types of mass spectra. Specifically, ESI produces mainly molecular adduct ions from TAG, with the addition of a suitable electrolyte, which are ideal for further structural characterization by MS^n, whereas APCI usually produces substantial diacylglycerol-like fragments, $[DAG]^+$, that provide an immediate indication of the constituent fatty acids making up the TAG, while at the same time yielding varying amounts of intact protonated molecule, $[M+H]^+$, depending on the degree of unsaturation in the TAG. APPI can also be used for TAG analysis [8–13], and gives spectra that appear similar to spectra obtained by APCI-MS. In order to compare the results obtained by these disparate ionization techniques, many reports have demonstrated analysis of the same samples using different combinations of these ionization sources, almost always by sequentially re-analyzing samples after changing the ionization source between runs. A database search of "mass spectrometry" and "APPI" and "APCI" returns 100 citations, a search of "mass spectrometry" and "APPI" and "ESI" returns 102 citations, and a search of "mass spectrometry" and "APCI" and "ESI" returns 450 citations! Including APPI, APCI and ESI together produces 64 citations, but only 4 seem to be applications to lipids or triacylglycerols (found by including ("lipid*" or "triacylglycerol*" or "triglyceride*") as keywords) [9,13–15]. Clearly there is substantial interest in acquisition of data from two or more ionization techniques in order to obtain the complementary information that these API methods provide. Obviously there are too many citations to list here, even though these searches are not comprehensive.

Very few reports, on the other hand, have employed a "parallel mass spectrometry" approach, in which two or three mass spectrometers, as well as other detectors, have been attached in parallel to the same effluent stream [16,17,7,18,19]. In addition to allowing a direct comparison between ionization types, this approach reduces the number of runs necessary to acquire the complementary data, and eliminates all run-to-run variability in chromatographic runs, which can complicate interpretation of the data from complex samples.

Described here is the first report of four mass spectrometers in parallel, for a "quadruple parallel mass spectrometry" experiment, also in parallel with a UV detector, evaporative light scattering detector (ELSD), and corona charged aerosol detector (CAD), for seven detectors overall. ESI-MS, APPI-MS, and high- and low-sensitivity APCI-MS are used for a holistic analysis of dietary supplements containing olive oil and vitamin D using a dilute-and-shoot approach. A new meta-analysis of sample results over two months using numerous methods for quantification is presented. We also incorporate, for the first time, the $1 \times {}^{13}C$ isotopic variant for TAG analysis to increase signal, without loss of specificity. This work also provides the first report of the application of a previously reported approach to determining response factors from GC with flame ionization detection (FID) to APPI-MS of TAG. Finally, we demonstrate a novel modification of the previously reported approach to compensate for charge saturation in ESI-MS.

2. Materials and methods

2.1. Chemicals and samples

Fisher Optima LC-MS grade methanol (MeOH), #A456-4, and acetonitrile (ACN), #A955-4, were used (Fisher Scientific, Pittsburgh, PA, USA). Fisher Optima grade methylene chloride (dichloromethane, DCM), #D151-4, was used. The following solvents and reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA): ammonium formate #516961, synthetic crystalline cholecalciferol #1357, synthetic crystalline ergocalciferol #5750, boron trifluoride 14% in methanol, and ACS reagent grade sodium chloride and sodium hydroxide. GC fatty acid methyl ester (FAME) reference standards GLC-68B, GLC-68D, GLC-14B, and methyl tricosanoate (C23:0) were obtained from Nu-Chek Prep (Elysian, MN, USA). Methyl pentacosanoate (C25:0) was from Santa Cruz Biotechnology (Dallas, TX, USA), and methyl hexacosanoate (26:0) was from Matreya, LLC (Pleasant Gap, PA, USA). Deionized (D.I.) water was obtained from a Millipore Milli-Q® purification system (Millipore, Bedford, MA, USA).

Dietary supplement gelcaps containing 25 μg, or 1000 International Units (IU), vitamin D_3 in olive oil, with the vitamin D_3 from lanolin, were ordered from an online supplier of vitamins. Samples were received on 1/19/2010 unrefrigerated, and were stored refrigerated upon receipt, as would be typical for the average consumer. Sample weights for the primary samples used in this report were 0.10524 g, 0.10414 g, 0.09610 g, 0.10191 g, 0.10288 g, for an average of 0.10212 ± 0.00343 g (3.36% RSD). Gelcap samples were prepared and bracketed sequences were run as previously described [7].

2.2. Instrumentation

2.2.1. High performance liquid chromatography

The HPLC conditions, solvent gradient, and parameters, including those for the DAD, ELSD, and CAD have been described

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