

RESEARCH ARTICLE

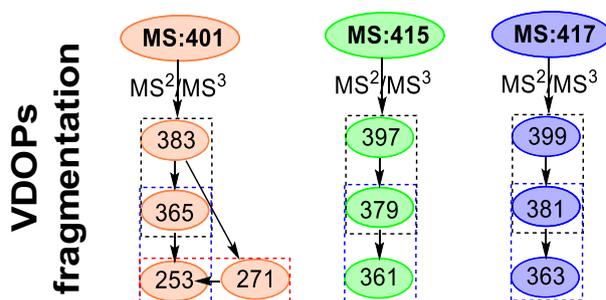
Identification of Vitamin D3 Oxidation Products Using High-Resolution and Tandem Mass Spectrometry

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Abstract. In a successful fortification program, the stability of micronutrients added to the food is one of the most important factors. The added vitamin D3 is known to sometimes decline during storage of fortified milks, and oxidation through fatty acid lipoxidation could be suspected as the likely cause. Identification of vitamin D3 oxidation products (VDOPs) in natural foods is a challenge due to the low amount of their contents and their possible transformation to other compounds during

analysis. The main objective of this study was to find a method to extract VDOPs in simulated whole milk powder and to identify these products using LTQ-ion trap, Q-Exactive Orbitrap and triple quadrupole mass spectrometry. The multistage mass spectrometry (MS^n) spectra can help to propose plausible schemes for unknown compounds and their fragmentations. With the growth of combinatorial libraries, mass spectrometry (MS) has become an important analytical technique because of its speed of analysis, sensitivity, and accuracy. This study was focused on identifying the fragmentation rules for some VDOPs by incorporating MS data with *in silico* calculated MS fragmentation pathways. Diels–Alder derivatization was used to enhance the sensitivity and selectivity for the VDOPs' identification. Finally, the confirmed PTAD-derivatized target compounds were separated and analyzed using ESI(+)-UHPLC-MS/MS in multiple reaction monitoring (MRM) mode.

Keywords: Vitamin D3 oxidation, Mass spectrometry, Mass Frontier, Fragmentation tree, Multiple reaction monitoring

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Introduction

Fortification is the practice of increasing the content of an essential micronutrient, i.e., vitamins and minerals in a food to improve health benefits and reduce dietary deficiencies within a population [1]. In a successful fortification program, the stability of micronutrients added to a food is an important

factor and determination of the actual level of the fortificant has always been a consideration. In terms of vitamin D3 fortification, the search for any degradation products in fortified food, most notably in dairy products, can result in the discovery of the mechanism of vitamin D3 degradation which can play an important role to minimize it.

Determination of vitamin D and its degradation products has been a challenge due to the low amount of their contents even in vitamin D enriched products. Any degradation products will be less abundant since there may be several breakdown products and isomers of each breakdown product [2, 3]. Therefore, any method to extract vitamin D3 compounds has to ensure that both nonpolar and polar breakdown products are

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captured and they are not altered by the extraction process. Previous studies have demonstrated the extraction and determination of vitamin D metabolites such as 25-hydroxyvitamin D in biological samples [3, 4]. However, the information on compounds related to vitamin D degradation products in foods is limited. In food systems, vitamin D can comprise a large class of isomerization products under various conditions, which were characterized in our previous study [5]. These isomers have the same molecular formula, and structural differences among them are primarily related to the variation of the double bonds across the triene system [5].

Another form of vitamin D degradation, oxidation of vitamin D, was reported by limited number of studies [6–9]. A self-initiated autoxidation of isotachysterol, an isomer of vitamin D, under atmospheric oxygen was reported by Jin [6]. Most of the reported vitamin D oxidation products are structurally similar to vitamin D except for having one or more additional oxygen atoms. In fact, it has proven difficult to differentiate among these structurally similar unknown compounds with classical analytical methods [10, 11]. Therefore, considering the ever-increasing rise in interest in these compounds, a more sophisticated method for the identification and confirmation of these products is clearly needed.

Nuclear magnetic resonance (NMR) spectroscopy has proven useful in the characterization of the structures of unknown compounds. However, NMR could be applied only to reasonably pure compounds and it might be insufficient for the characterization of structurally related compounds in a complex matrix without purification [12, 13]. In contrast, liquid chromatography (LC) is rather well suited for the analysis of complex mixtures and reversed-phase liquid chromatography (RP-HPLC) methods are widely used for the separation of vitamin D and its metabolites [12, 14, 15].

HPLC methods with UV detection have limitations in distinguishing compounds with very similar structures as they often exhibit similar UV absorption characteristics. Therefore, LC-MS has been the more extensively applied hyphenated technique [13]. Mass spectrometry (MS) can determine the analyte's elemental composition as well as provide structural information, such as accurate mass-to-charge ratio (m/z), isotope abundance, and fragmentation patterns [16, 17].

Tandem mass spectrometry (MS/MS) alone has some limitations such as being unable to explain all fragmentation pathways even when structures are known, while multistage MS^n analysis can link product ions to specific precursor ions to generate fragmentation pathways. MS^n analysis can select the product ions of the initial fragmentation step and subject them to another fragmentation reaction, which can reveal additional information about the dependencies between the fragments. The resulting fragment ions can again be selected as precursor ions for further fragmentation [17]. Previous studies successfully used MS^n to characterize structurally fragmented ions and fragmentation mechanisms of flavonoids, oligosaccharides, and sugar nucleotides [18–20]. Despite the growing popularity of versatile ion trap instruments, the analysis of MS^n spectra remains difficult due to the lack of generic software tools.

Recently, *in silico* prediction tools such as Mass Frontier were used to generate virtual MS^2 and MS^n spectral libraries in complex matrices [17, 21–23].

The objective of this study was to extract vitamin D3 oxidation products (VDOPs) in simulated whole milk powder. For identification of unknown VDOPs, some experiments with a high-resolution MS/MS instrumentation using a Q-Exactive Orbitrap mass spectrometer were carried out. Further, MS^n spectra were generated by an ion trap mass spectrometer. Data were organized and stored by Mass Frontier software to show the fragmentation pathways of VDOPs by mass spectral trees. 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) derivatization was also used to enhance the sensitivity and selectivity of mass spectrometry analysis. Finally, PTAD-derivatized VDOPs were separated and identified using UHPLC-MRM-MS/MS.

Experimental

Chemicals

Vitamin D3 (cholecalciferol, 99%), PTAD, formic acid, and pyrogallol were obtained from Sigma-Aldrich (Auckland, New Zealand). Copper (Cu) cube (1 cm^3) was obtained from Delta Educational (Auckland, New Zealand). Bond Elut (2 g) aminopropyl solid phase extraction (SPE) cartridge were from Agilent Technologies (CA, USA). Sodium caseinate, whey protein, and anhydrous milk fat (AMF) were supplied by the Fonterra Co-operative Group Ltd. (Palmerston North, New Zealand).

Sample Preparation

Laboratory-scale simulated whole milk powders (SWMP) were prepared with the main milk components. The formulation (w/w) was as follows: 3.5% milk proteins, 4.8% lactose, 3.5% milk fat, and 88.2% water. As for milk proteins, the ratio of the sodium caseinate/whey protein isolate was 8:2 (w/w) in all samples. The proteins and lactose were reconstituted in Milli-Q water at 40 °C, and the solutions were stirred for 1 h. Emulsions were prepared by adding the milk fat to the oil-free phase, heating to 50 °C, and allowed to cool to ambient temperature before homogenizing using a laboratory-scale homogenizer (model IKA T25 Ultra Turrax, Malaysia) at 24,000 rpm for 2 min. Simulated whole milk was fortified with high concentration of vitamin D3 standard solution in ethanol (10 mg/g), and freeze-dried powders were stored for 12 months at 40 °C.

In addition, 10 mL of vitamin D3 standard solution in isooctane (10 mg/mL) was oxidized under air in the presence of a copper cube for 5 h to generate vitamin D3 oxidation products in a sample without fat matrices.

Vitamin D3 Oxidation Product Extraction, Separation, and Derivatization

The extraction of VDOPs in samples of oxidized vitamin D3 solution and stored SWMP involved the following steps: liquid-liquid extraction (LLE) (only for SWMP), SPE,

derivatization using PTAD, chromatographic separation, and detection by MS. For liquid-liquid extraction of simulated whole milk powder, the method of Abernethy [24] with some modifications was used. To 1 g of milk powder, 5 mL of water was added in a centrifuge tube and the sample was suspended by vortex mixing and allowed to stand for 10 min. To the centrifuge tube, 25 mL of methanolic pyrogallol solution (1%, w/v), 10 mL of iso-octane, and 5 mL of water were added. The tubes were vigorously mixed and then centrifuged for 5 min at 2000×g. An aliquot of the upper layer (5 mL) was transferred to a test tube and dried under nitrogen. The sample of vitamin D3 standard solution, which was oxidized under air, was also dried under nitrogen. Dried samples were dissolved in 3-mL hexane for SPE as a cleanup step.

SPE optimization was carried out to achieve a selective SPE extraction that eliminates interfering compounds to the greatest extent possible without loss of analytes [25, 26]. Selective SPE was performed on a Bond Elut (2 g) aminopropyl (NH₂) SPE cartridge under the optimized conditions. The cartridge was conditioned with hexane (5 mL) prior to sample loading. The cartridge was washed subsequently with hexane (10 mL) and hexane/ethyl acetate 90:10 (v/v) (10 mL). Fraction 1 (F1), vitamin D3, was eluted using hexane/ethyl acetate 80:20 (v/v) (10 mL). Finally, fractions 2 and 3 (F2 and F3), VDOPs, were eluted with hexane/ethyl acetate 60:40 (v/v) (10 mL) and acetone (10 mL), respectively. To derivatize vitamin D3 and its oxidation products, fractions were dried under nitrogen and dissolved in 1-mL ethyl acetate containing 0.5 mg/mL PTAD reagent and kept at room temperature for 1 h. Finally, all fractions were dried under nitrogen and dissolved in methanol for further analysis.

Instrumentation

High-Resolution Mass Spectrometry Ultra-High Performance Liquid Chromatography (Accela 1250 UHPLC) instrumentation with high-resolution MS/MS using the Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Auckland, New Zealand) equipped with an Ion Max electrospray ionization source (ESI) in positive mode was used. Profile positive MS scans were acquired, with full MS in the mass range of 200–2000 *m/z* using the Orbitrap mass spectrometer with mass resolution of 70,000 (FWHM at *m/z* 200). The top 5 positive MS² were acquired with mass resolution of 17,500 (FWHM at *m/z* 200). The source settings were sheath gas flow rate, 50 arbitrary units, auxiliary gas flow rate, 13 arbitrary units, spray voltage 4.00 kV, capillary temperature 263 °C, and heater temp 425 °C.

MSⁿ Analysis Multistage mass spectra were obtained using a linear ion trap (LTQ) mass spectrometer, equipped with a survey HPLC system (Thermo Fisher Scientific, CA, USA). The MS operating parameters were as follow: source voltage, 4.6 kV; sheath gas flow rate, 15 arbitrary units; auxiliary gas flow rate, 5 arbitrary units; capillary voltage and capillary temperature, 47 V and 275 °C, respectively. The

normalized collision energy was set to 30%, and the activation *Q* was 0.25 for collision-induced dissociation (CID) mass spectra. For stepwise fragmentation experiments, Data Dependent Ion Tree was chosen using a maximum breadth of 5 and depth of 4. In this mode, the acquisition software probed the MS spectra to select the most intense parent ions for MSⁿ analysis. The method allowed for the fragmentation of the five highest peaks of the MS², MS³, and MS⁴ spectra for a subsequent scan.

Quadrupole Mass Spectrometry Multiple reaction monitoring (MRM) analyses were carried out using 6460C triple quadrupole mass spectrometer system (Agilent, USA) equipped with an electrospray ionization (Agilent Jet Stream ESI) source in positive mode. Analyst software (MassHunter, version B.07.00, USA) was used for instrument control and data processing. MRM was performed at a collision energy of 25 eV for all compounds, and MRM transitions monitored were (560 → 298) for vitamin D3 and (576 → 298, 576 → 314, 590 → 298, 592 → 298) for vitamin D3 oxidation products.

Liquid Chromatography Liquid chromatography analysis was carried out using a 1290 Infinity series UHPLC (Agilent, USA). The LC column used was Kinetex PFP 150 × 4.6 mm, 5-μm core-shell reverse-phase (Phenomenex, USA) at 30 °C. The mobile phases were (A) 0.1% formic acid in water and (B) methanol, containing 2 mmol/L of ammonium acetate. The gradient started from 75% B and moved to 100% B in 4.8 min and held until 6 min, then returned to the initial conditions in 10 min. The flow rate was 0.6 mL/min, and the injection volume was 5 μL.

Data Analysis The Xcalibur data system (version 3.0, Thermo Fisher Scientific, CA, USA) was used for data processing of both LTQ-ion trap and Orbitrap mass spectrometry. Based on the accurate mass, the elemental composition of the compounds of interest was calculated using the elemental composition tool within the Xcalibur software with 10 ppm mass tolerance. Parameter settings for the proposed elemental compositions for all the compounds detected were carbon (C 0–40), hydrogen (H 0–50), and oxygen (O 0–4), while the PTAD derivatives of these compounds contained nitrogen (N 0–3) as well. The double-bond equivalent (DBE) parameter, which was set (–1 to +100), was used as an indicator of the stability (degree of conjugation) and likelihood of the calculated elemental composition.

All library building and searching operations were also conducted using the Mass Frontier 7.0 software (HighChem, LLC, Slovakia). Mass Frontier software used candidate compound structures as input and generated spectra which were compared to the measured spectra. This software generates fragments based on rule-based predictions, produces mass spectral trees, and calculates fragmentation pathways.

Results and Discussion

Extraction of VDOPs from Simulated Whole Milk Powder

LLE followed by a SPE is the main method for the extraction of cholesterol oxidation products (COPs) [27–30]. Many studies preferred saponification of the fat-containing food as the principal enrichment and cleanup procedure [25, 31, 32]. However, artifact formation was observed during saponification in the extraction process of COPs [33, 34]. Solid phase extraction using different cartridges such as silica, Florisil, aminopropyl, or reversed-phase cartridges has been proposed as an alternative to saponification for the isolation and enrichment of COPs by several studies [28, 35–39]. Therefore, in the present study, LLE extraction without saponification was used to prevent additional degradation of VDOPs or formation of artifacts during saponification.

After solvent extraction, vitamin D3 and its oxidation products were purified using solid phase aminopropyl which allowed the removal of vitamin D3 and nonpolar compounds. After PTAD reagent was added to the fractions, the sample solutions had a pink color; however, this color disappeared during the incubation period. F1 showed the fastest apparent decolorization in the first 5 min, while pink color has remained in F2 and F3 after 1 h of derivatization. Fast decolorization of F1 indicated the high concentration of vitamin D3 in this fraction (Data not shown).

Mass Spectrometry Analysis of VDOPs

Introduction It is believed that hydroperoxides derived from oxidation of unsaturated fatty acids play a significant role to facilitate cholesterol oxidation [40, 41]. In this study, the hypothesis was that vitamin D3 might have the same oxidation process as influenced by lipoxidation reactions of unsaturated fats. As illustrated in Scheme 1, vitamin D3 (1) autoxidation can start at an allylic carbon, such as C-1, by abstraction of hydrogen following the addition of an oxygen molecule forming 1-hydroxy-vitamin D3 (2). In addition, 1-keto-vitamin D3 (3) might be formed by dehydration of 1-hydroxy-vitamin D3 in the presence of radicals. In addition, the hydroperoxides derived from oxidation of unsaturated fatty acids may play a significant role to facilitate vitamin D3 oxidation at double bonds to form vitamin D3 epoxy such as 7,8-epoxy-vitamin D3 (4), 1-hydroxy-7,8-epoxy-vitamin D3 (5), and 1-keto-7,8-epoxy-vitamin D3 (6) (Scheme 1).

After VDOP extraction, analytes were identified using various mass spectrometry systems. The analysis of VDOPs was divided into several parts including the generation of possible fragments to interpret the fragmentation pathways of unknown VDOPs using the Mass Frontier software: the identification of VDOPs using MS² and MSⁿ analysis with a Q-Exactive Orbitrap and a LTQ-ion trap mass spectrometry, respectively. Structural elucidation was further aided by calculating elemental compositions by exact mass measurements. Finally, PTAD-

derivatized VDOPs were separated and verified using the MRM in a quadrupole LC-MS/MS.

Accurate Mass Screening

Accurate mass analysis of target compounds using the Q-Exactive Orbitrap mass spectrometer was possible due to the high mass resolution potential of this instrument. The ability to determine the *m/z* of an ion within 5 ppm allows the determination of a unique elemental composition based on the mass defect of the constituent atoms from theoretical calculation. The ability to closely match the theoretical mass with the observed mass greatly increases the reliability of identification.

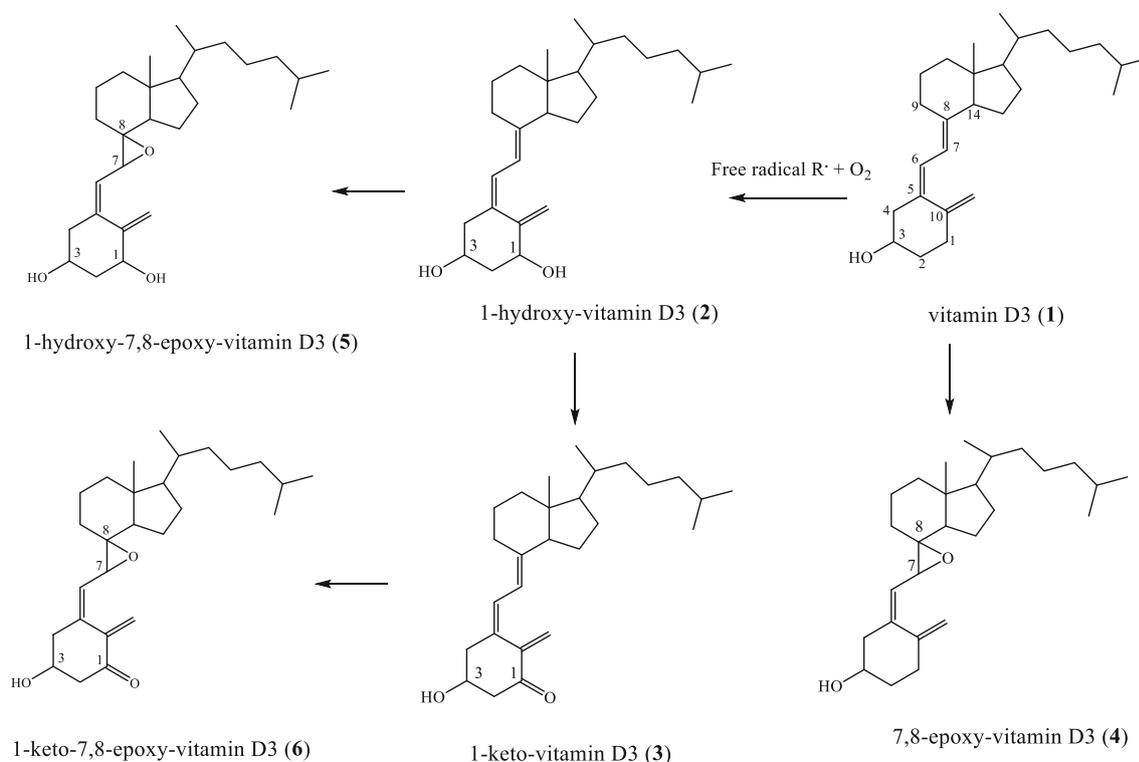
Tables 1 and 2 show the results of proposed VDOPs and their PTAD-derivatized analogues, including the accurate mass of their protonated molecules, the most probable elemental composition, the DBE, and the mass tolerance in ppm. Since milk is a complex matrix and the identification of low abundance VDOPs might be affected by isobaric interferences, a pure solution of vitamin D3 was oxidized to identify VDOPs in a simple matrix.

Analysis of VDOPs with Orbitrap allowed assigning the elemental formulas to both the precursor ions and the product ions; however, the MS/MS prediction alone was not sufficient for a successful assignment of a suitable structure to a molecular formula. Additional computational approaches were also required in this step and the use of fragment libraries with accurate mass information was likely to improve the match between measured data and candidate structures. The Mass Frontier software was used for this purpose as discussed in the next section.

Determination of Fragmentation Patterns

While MS/MS is the dominant technique for interpreting fragmentation patterns to find structural information, using MS/MS alone has intrinsic limitations because product ions found in the MS/MS spectrum may be derived from intermediary ions instead of being produced directly from the precursor ion. Therefore, many fragment ions in MS/MS cannot be explained through fragmentation pathways even if structures are known [17, 42, 43]. In order to obtain maximum structural information, samples were analyzed in both MS/MS (MS²) and MSⁿ mode in this study. MSⁿ measurements were performed to gain information on fragment ions generated in the linear ion trap. Since the ion trap lacked capabilities to do high mass accuracy, the Q-Exactive Orbitrap with a reasonable high mass accuracy was used to associate elemental compositions for VDOPs as described in the previous section.

Data structures, which are defined by graph theory to organize and store data including the fragmentation process of an analyte of interest and/or MSⁿ spectra generated by an ion trap mass spectrometer, are referred as trees. Typically, the graphs are called fragmentation trees, if these show the fragmentation pathways of a compound. In contrast, mass spectral trees refer to the sequential stages and relationships of mass spectral



Scheme 1. Proposed formation of vitamin D3 oxidation products

acquisition in MSⁿ processes [44–46]. Therefore, MSⁿ trees can reveal both the dependency of precursor ion/product ion and the product ion/product ion within the same MSⁿ stage or between different MSⁿ stages. For both fragmentation and

mass spectral trees, computational methods are required to organize specific information such as an implication of the fragmentation relationship between precursor ions and product ions. Since it was not possible to acquire reference mass spectra

Table 1. Results of MS/MS Screening of Vitamin D3 and Its Oxidation Products Obtained by Q-Exactive Orbitrap

Compound	Accurate mass of [M + H] ⁺	Chemical formula of [M + H] ⁺ ions	DBE ^a	Mass error (ppm)
VD3 ^b	385.34750 [§]	C ₂₇ H ₄₅ O	5.5	2.84
	367.33664 ^h	C ₂₇ H ₄₃	6.5	3.05
	259.20634 ^h	C ₁₉ H ₃₁	5.5	2.69
	259.24450 ^h	C ₁₈ H ₂₇ O	5.5	3.01
OH-D3 ^c	241.19676 ^h	C ₁₈ H ₂₅	6.5	2.78
	401.34255 [§]	C ₂₇ H ₄₅ O ₂	5.5	2.84
	383.33173 ^h	C ₂₇ H ₄₃ O	6.5	2.31
EPX-D3 ^d	365.32118 ^h	C ₂₇ H ₄₁	7.5	2.47
	401.34371 [§]	C ₂₇ H ₄₅ O ₂	5.5	5.73
	383.33289 ^h	C ₂₇ H ₄₃ O	6.5	5.34
	365.32229 ^h	C ₂₇ H ₄₁	7.5	5.50
Keto-EPX-D3 ^e	415.32302 [§]	C ₂₇ H ₄₃ O ₃	6.5	5.65
	397.31246 ^h	C ₂₇ H ₄₁ O ₂	7.5	5.92
	379.30169 ^h	C ₂₇ H ₃₉ O	8.5	5.66
	361.29107 ^h	C ₂₇ H ₃₇	9.5	5.79
OH-EPX-D3 ^f	417.33869 [§]	C ₂₇ H ₄₅ O ₃	5.5	5.67
	399.32811 ^h	C ₂₇ H ₄₃ O ₂	6.5	5.89
	381.31738 ^h	C ₂₇ H ₄₁ O	7.5	5.73
	363.30669 ^h	C ₂₇ H ₃₉	8.5	5.67

^aDouble-bond equivalent parameter; each ring or a double bond increases the DBE value by 0.5

^bVitamin D3

^c1-hydroxy-vitamin D3

^d7,8-epoxy-vitamin D3

^e1-keto-7,8-epoxy-vitamin D3

^f1-hydroxy-7,8-epoxy-vitamin D3

[§]Precursor ions

^hProduct ions

Table 2. Results of MS/MS Screening of PTAD-Derivatized Vitamin D3 and Its Oxidation Products Obtained by Q-Exactive Orbitrap

Compound	Accurate mass of [M + H] ⁺	Chemical formula of [M + H] ⁺ ions	DBE ^a	Mass error (ppm)
VD3 ^b	560.38672 ^g	C ₃₅ H ₅₀ O ₃ N ₃	12.5	3.66
	298.12106 ^h	C ₁₆ H ₁₆ O ₃ N ₃	10.5	2.45
	280.11067 ^h	C ₁₆ H ₁₄ O ₂ N ₃	11.5	2.23
OH-D3 ^c	576.38135 ^g	C ₃₅ H ₅₀ O ₄ N ₃	12.5	2.63
	314.11636 ^h	C ₁₆ H ₁₆ O ₄ N ₃	10.5	1.29
	296.10562 ^h	C ₁₆ H ₁₄ O ₃ N ₃	11.5	1.83
EPX-D3 ^d	576.38453 ^g	C ₃₅ H ₅₀ O ₄ N ₃	12.5	3.58
	298.12096 ^h	C ₁₆ H ₁₆ O ₃ N ₃	10.5	3.16
	280.11067 ^h	C ₁₆ H ₁₄ O ₂ N ₃	11.5	2.77
Keto-EPX-D3 ^e	590.36182 ^g	C ₃₅ H ₄₈ O ₅ N ₃	13.5	5.03
	298.12109 ^h	C ₁₆ H ₁₆ O ₃ N ₃	10.5	3.99
	280.11050 ^h	C ₁₆ H ₁₄ O ₂ N ₃	11.5	3.98
	413.30710 ^h	C ₂₇ H ₄₁ O ₃	7.5	5.27
	395.29754 ^h	C ₂₇ H ₃₉ O ₂	8.5	4.99
	377.28747 ^h	C ₂₇ H ₃₇ O	9.5	3.59
OH-EPX-D3 ^f	592.37708 ^g	C ₃₅ H ₅₀ O ₅ N ₃	12.5	4.35
	298.12137 ^h	C ₁₆ H ₁₆ O ₃ N ₃	10.5	3.76
	280.11060 ^h	C ₁₆ H ₁₄ O ₂ N ₃	11.5	4.55
	415.32740 ^h	C ₂₇ H ₄₃ O ₃	6.5	3.48
	397.31477 ^h	C ₂₇ H ₄₁ O ₂	7.5	3.88
	379.30282 ^h	C ₂₇ H ₃₉ O	8.5	3.50

^aDouble-bond equivalent parameter; each ring or a double bond increases the DBE value by 0.5

^bVitamin D3/PTAD

^c1-hydroxy-vitamin D3/PTAD

^d7,8-epoxy-vitamin D3/PTAD

^e1-keto-7,8-epoxy-vitamin D3/PTAD

^f1-hydroxy-7,8-epoxy-vitamin D3/PTAD

^gPrecursor ions

^hProduct ions

for VDOPs and their PTAD derivatives, *in silico* prediction tools such as Mass Frontier were used to generate much larger virtual MS² and MSⁿ spectral libraries.

After solid phase extraction, fraction 1 (F1) of sample showed a molecular ion with *m/z* 385.3477 which represented the presence of vitamin D3. As shown in the MS² spectrum of vitamin D3 (Figure 1a), the most significant product ions were fragments *m/z* 367 (a dehydrated vitamin D3 ion) and *m/z* 259. As for *m/z* 259, a structure without any oxygen atom was reported by previous studies [10, 26]. However, the Mass Frontier software suggested a structure containing one atom of oxygen was possible for fragment *m/z* 259 (Figure 1b). The elemental formulas of both structures were also suggested by accurate mass analysis for *m/z* 259 (Table 1). In order to confirm the proposed structure of *m/z* 259, MS³ on the ion trap was used to refragment this fragment ion, which could only lose water and generate the dehydrated fragment with *m/z* 241 from a hydroxylated precursor (Figure 1a, MS³: 259). The formation of the dehydrated fragment form *m/z* 259 confirmed that the proposed structure of this fragment created by Mass Frontier was correct (Figure 1b). In addition, the fragment *m/z* 367 (a dehydrated vitamin D3 ion) did not generate the fragment *m/z* 259 after refragmentation reasonable given the lack of oxygen in the precursor, while the fragment *m/z* 241 was generated instead (Figure 1a, MS³: 367).

Fractions 2 and 3 (F2 and F3) of the oxidized solution of vitamin D3 after solid phase extraction showed molecules with accurate mass of *m/z* 401.3426 in the Q-Exactive Orbitrap mass analysis, which represented the formation of VDOPs with one

extra oxygen. Both fractions showed similar chemical formulas related to these VDOPs and their product ions (Table 1). MSⁿ spectra of both fractions were also similar and are illustrated in Figure 2a. The MS² spectrum of the compounds with a precursor ion of *m/z* 401 [M + H]⁺ showed product ions with *m/z* 383 [M + H - H₂O]⁺ and 365 [M + H - 2H₂O]⁺ as a result of the loss of one and two water molecules, respectively. In addition, these compounds showed product ions with *m/z* 271 and 253; however, they had two hydrogen atoms less than those found in vitamin D3 mass spectra (Figure 2a, MS³: 383 and Figure 1a, MS²:385, respectively). This is because these fragments resulted from a fragment with *m/z* 383 (a dehydrated ion of *m/z* 401) instead of the precursor ion with *m/z* 385 (as would be the case for vitamin D3). Collectively, this data can confirm the presence of VDOPs with one additional oxygen atom.

The proposed structures and fragmentation trees of these VDOPs with an additional oxygen, 7,8-epoxy-vitamin D3 and 1-hydroxy-vitamin D3, are illustrated in Figure 2b, c, respectively. Since both VDOPs had similar fragmentation patterns and no [M + H-16]⁺ fragment ion was observed in the case of 7,8-epoxy-vitamin D3, the identification of these compounds without using any commercial standards or a method to distinguish between them was impossible.

Fractions 2 and 3 (F2 and F3) also showed compounds with accurate masses of *m/z* 415.3226 and 417.3383, respectively. The MS² and MSⁿ spectra of VDOP with two additional oxygen atoms with *m/z* 415 [M + H]⁺ showed the fragments with *m/z* 397 [M + H - H₂O]⁺, 379 [M + H - 2H₂O]⁺, and 361 [M + H - 3H₂O]⁺ (Figure 3a). The MS³ of fragments with *m/z* 397

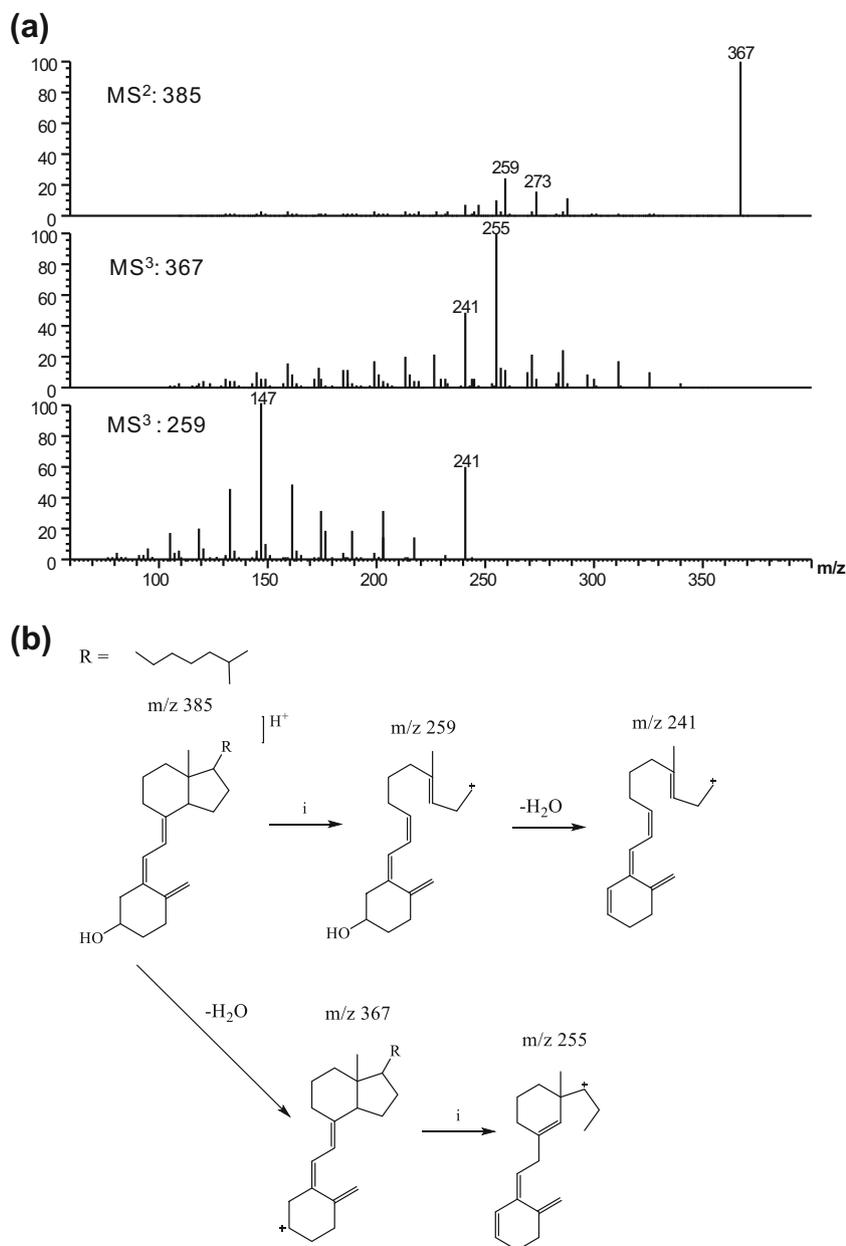


Figure 1. MSⁿ spectra (a) and fragmentation tree (b) of vitamin D3. “i” indicates inductive cleavage; the “-” symbols and the molecules above the arrows represent the neutral loss of these molecules

gave the fragment ions m/z 379 and 361 which resulted from the loss of one and two water molecules, respectively. The fragmentation tree of VDOP with m/z 415 is illustrated in Figure 3b.

Additionally, another type of VDOP with two oxygen atoms but a different precursor ion was obtained with m/z 417 $[M + H]^+$. MS², MS³, and MS⁴ spectra of this compound gave the product ions with m/z 399 $[M + H - H_2O]^+$, 381 $[M + H - 2H_2O]^+$, and 363 $[M + H - 3H_2O]^+$ (Figure 3c). The structure and fragmentation trees of the VDOP with m/z 417 are displayed in Figure 3d. Since the quality of the mass spectra is reduced with each additional fragmentation reaction, analysis is usually limited to a few fragmentation reactions beyond MS².

In order to detect the low abundant vitamin D oxidation products in complex matrices, more sensitive analyses were required. The sensitivity of vitamin D3 and its oxidation products' detection can be significantly improved using Diels–Alder derivatization with PTAD. Additionally, a further increase of the ionization efficiency of vitamin D metabolites was achieved after PTAD derivatization as previous studies reported [4, 47, 48]. Derivatization with PTAD also shifted the m/z ratios of the precursor ions to higher molecular weight, thus reducing interfering low m/z background ions. Underivatized vitamin D compounds showed a large number of product ions, due to the availability of low-energy fragmentation pathways. On the other hand, the product ion spectrum of PTAD-derivatized vitamin D3 and its oxidation products exhibited

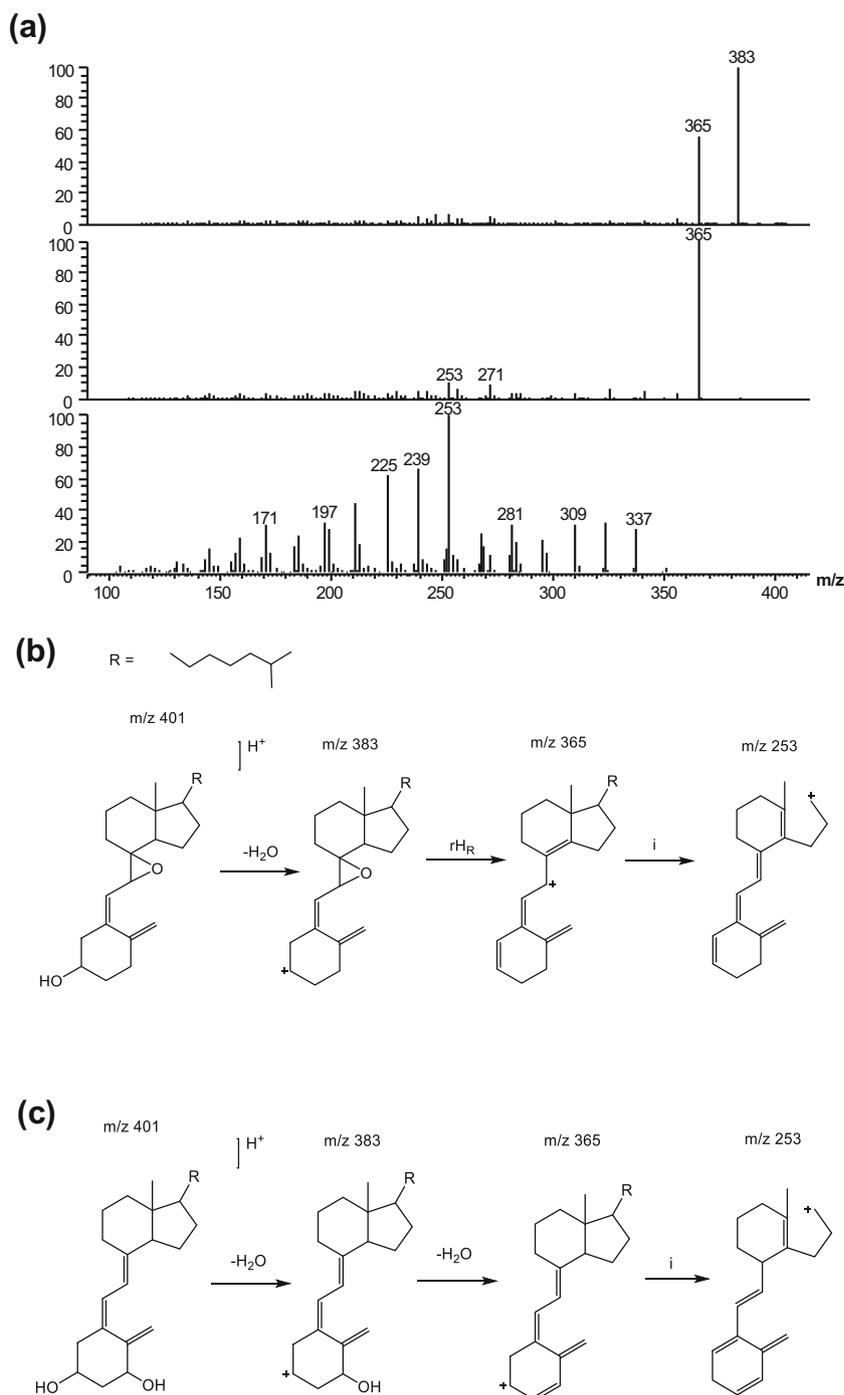


Figure 2. MSⁿ spectra of VDOPs with one additional oxygen atom with *m/z* 401 (a) and proposed fragmentation trees of 7,8-epoxy-vitamin D3 (b) and 1-hydroxy-vitamin D3 (c). “i” indicates inductive cleavage; “rH_R” stands for the charge-remote rearrangement; the “-” symbols and the molecules above the arrows represent the neutral loss of these molecules

only one or two major fragment ions, which was beneficial for sensitive MRM analysis in UHPLC-MS/MS.

It is clear that VDOPs, a class compound with very similar structures, follow similar fragmentation pathways. PTAD derivatization was used to not only increase the sensitivity of these compounds for mass spectrometry but also to distinguish between VDOPs with similar molecular weight due to the different fragmentation patterns after derivatization. This is

another advantage of using derivatization before analysis as previously discussed for vitamin D3 isomerization products in our previous study [5]. Apart from that, the lack of commercially available standards of VDOPs created the need of other methods for their confirmation, and PTAD derivatization could be a way to confirm VDOPs formation. Thus, in this study, the application of Diels–Alder derivatization was carried out for the identification of VDOPs.

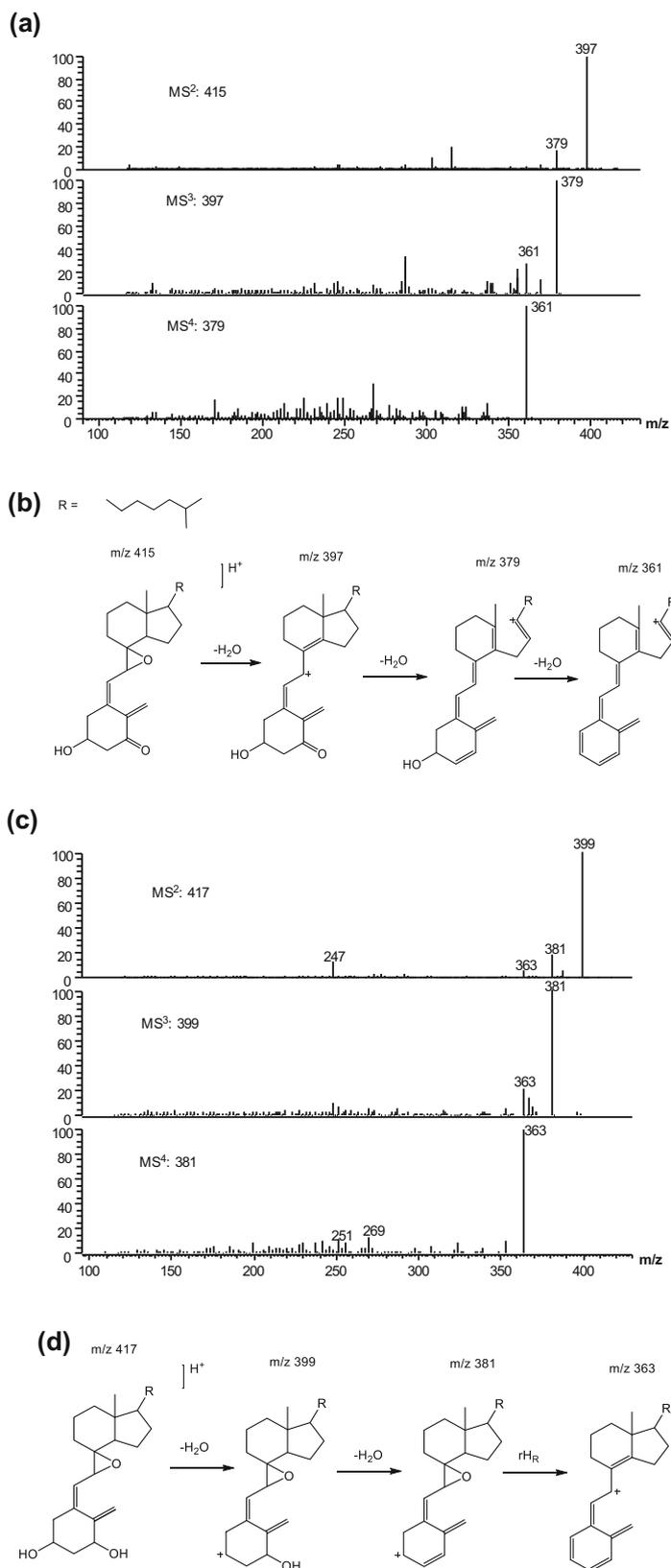


Figure 3. MSⁿ spectra of VDOPs with two additional oxygen atoms with *m/z* 415 (a) and *m/z* 417 (c) and proposed fragmentation trees of 1-keto-7,8-epoxy-vitamin D3 (b) and 1-hydroxy-7,8-epoxy-vitamin D3 (d). “i” indicates inductive cleavage; “rH_R” stands for the charge-remote rearrangement; the “-” symbols and the molecules above the arrows represent the neutral loss of these molecules

Diels–Alder reaction is a selective reaction between dienes and dienophile compounds, and the triene system is preferred for this reaction because it has two possible dienes available. A reaction scheme for the derivatization of vitamin D3 with PTAD to generate a Diels–Alder adduct (vitamin D3/PTAD) is available in the Supplementary data (Scheme S1). Then, VDOPs with the intact triene system could be possible candidates for PTAD derivatization.

The MS² spectra of PTAD-derivatized vitamin D3 and VDOPs are illustrated in Figures 4, 5, and 6. As it is shown in the MS² spectrum of vitamin D3/PTAD adduct with *m/z* 560 (Figure 4a), the most intense product ions were fragments *m/z* 298 and its dehydrated fragment (*m/z* 280). The fragmentation pathways of the vitamin D3/PTAD adduct showed the formation of these two product ions as specified in Figure 4. Fragments *m/z* 383 [M + H-PTAD(2H)]⁺ and its dehydrated ion (*m/z* 363) were also derived (Figure 4). These fragment ions and their structures were previously reported for the vitamin D3/PTAD adduct by Abernethy [24].

As shown in Figure 2 and Table 1, both fractions 2 and 3 (F2 and F3) in sample extracts gave VDOPs with one extra oxygen with similar molecular formulas and product ions after MS analysis. In fact, MS spectra yielded insufficient data to distinguish between VDOPs with *m/z* 401 because no specific diagnostic ions were present to differentiate these compounds. In contrast, PTAD derivatives of these VDOPs showed different fragmentation patterns and helped with their identification. While MS² spectra of VDOPs with *m/z* 401 showed similar product ions, different MS² spectra were achieved for PTAD derivatives of these compound (*m/z* 576) (Figure 5a, c).

While MS² spectrum of F2 showed product ions with *m/z* 298 and 280 (Figure 5a), F3 displayed a fragment with *m/z* 314 as the most significant product ion (Figure 5c). Different mass spectra and fragmentation pathways demonstrated different

types of VDOPs with one extra oxygen were present in F2 (7,8-epoxy-vitamin D3/PTAD) and F3 (1-OH-vitamin D3/PTAD).

The MS² spectra of PTAD-derivatized vitamin D3 plus two more oxygen atoms with *m/z* 590 and 592 are shown in Figure 6a, c, respectively. Both VDOPs gave fragment ions *m/z* 298 and 280 as the most significant product ions. However, these VDOPs can be distinguished by the product ions which were generated from loss of PTAD(2H). As shown in Figure 6a, the compound with *m/z* 590 [M + H]⁺ showed the fragments *m/z* 413 [M + H-PTAD(2H)]⁺, 395 [M + H-PTAD(2H)-H₂O]⁺, and 377 [M + H-PTAD(2H)-2H₂O]⁺. However, the compound with *m/z* 592 [M + H]⁺ displayed the same fragments with two mass units larger (415 [M + H-PTAD(2H)]⁺, 397 [M + H-PTAD(2H)-H₂O]⁺, and 379 [M + H-PTAD(2H)-2H₂O]⁺) (Figure 6c). The two mass unit difference can confirm the formation of different types of VDOPs with two oxygen atoms (*m/z* 415 and 417).

As shown in Figure 6a, c, while VDOP with *m/z* 590 showed two fragments as a result of the loss of the oxygen atom (*m/z* 379 [M + H-PTAD(2H)-H₂O-O]⁺ and 363 [M + H-PTAD(2H)-H₂O-2O]⁺, respectively), the compound with *m/z* 592 gave only one fragment by losing the oxygen atom (*m/z* 363 [M + H-PTAD(2H)-2H₂O-O]⁺). It can be concluded that while VDOP with *m/z* 590 was 1-keto-7,8-epoxy-vitamin D3/PTAD, the another VDOP (*m/z* 592) can be 1-hydroxy-7,8-epoxy-vitamin D3/PTAD (Figure 6).

MRM Analysis of PTAD-Derivatized VDOPs Using UHPLC-QQQ-MS/MS

Multiple reaction monitoring (MRM), also known as selected reaction monitoring (SRM), is a targeted MS approach accessible on triple quadrupole (QQQ) mass instruments [49, 50]. In this technique, the precursor ions are selected in the first

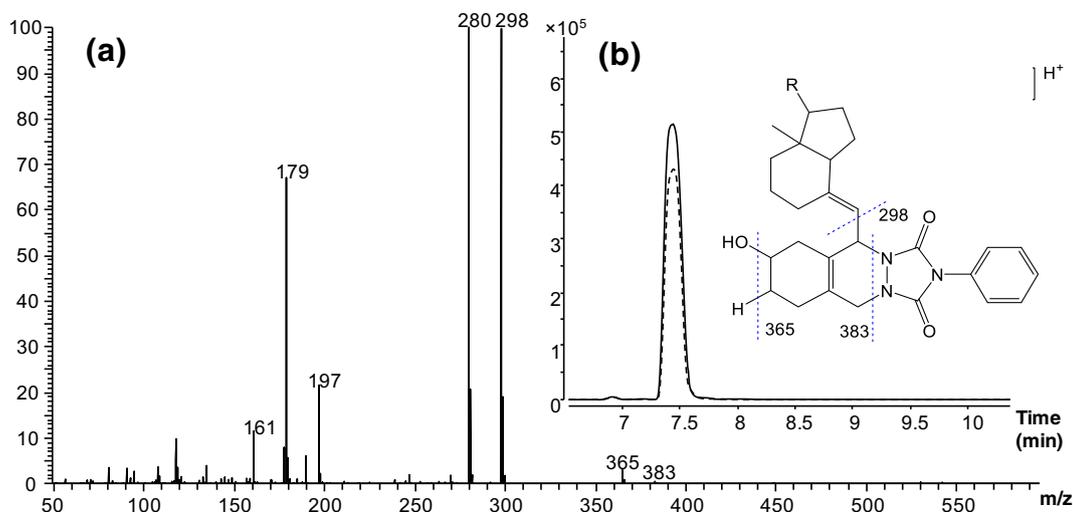


Figure 4. MS² spectra (a) and MRM chromatograms (b) of vitamin D3/PTAD adduct at *m/z* 560 → 298 in F1 of the oxidized vitamin D3 solution (—) and stored SWMP (---)

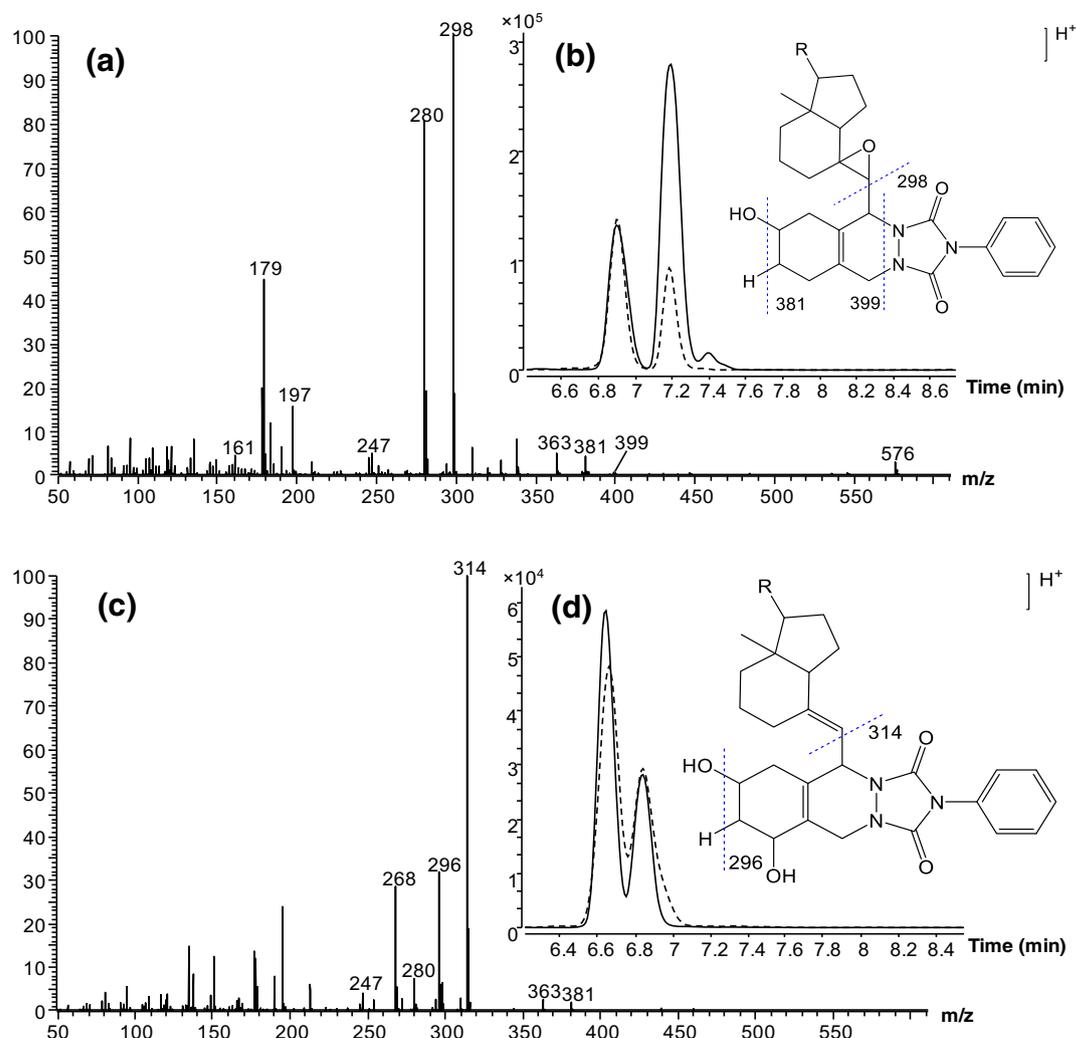


Figure 5. MS² spectra (a) and MRM chromatograms (b) of VDOP-PTAD adduct at m/z 576 → 298 in F2 and MS² spectra (c) and MRM chromatograms (d) of VDOP-PTAD adduct at m/z 576 → 314 in F3 of the oxidized vitamin D3 solution (—) and stored SWMP (---)

quadrupole and undergo fragmentation through CID dissociation in the second quadrupole to generate product ions. The pair of m/z values for a given precursor and product ion is referred to as a MRM transition and the success of MRM analysis relies on the selection of suitable targets and their optimal MRM transitions. Monitoring of specific transition for each analyte yields a superior signal-to-noise ratio with significantly higher sensitivity and selectivity.

Vitamin D3 and its oxidation products were extracted from stored simulated whole milk powder (SWMP). The extraction method was based on the liquid-liquid extraction followed by the solid phase extraction. Separated fractions (F1, F2, and F3) were derivatized with PTAD and identified by UHPLC-MRM-MS/MS. A simple matrix of oxidized solution of vitamin D3 was also analyzed with the same method to verify VDOPs formation. The MRM chromatograms of PTAD-derivatized vitamin D3 and its oxidation products extracted from the

oxidized solution of vitamin D3 and stored SWMP stored are shown in Figures 4, 5, and 6.

Fraction 1 (F1) of samples gave a peak at retention time of about 7.5 min for vitamin D3/PTAD adduct (Figure 4b). This is the same retention time found for PTAD-derivatized vitamin D3 standard. Fractions 2 and 3 (F2 and F3) of samples were analyzed for MRM transitions m/z 576 → 298 and 576 → 314. While F2 only showed peaks for the transition m/z 576 → 298, two peaks were detected in F3 when the transition m/z 576 → 314 was monitored (Figure 5b, d), respectively.

As it is shown in Figure 5b, F2 showed two peaks for 7,8-epoxy-vitamin D3/PTAD at retention times of about 6.9 and 7.2 min. Both samples of vitamin D3 solution and SWMP showed similar retention times for this compound which confirmed the presence of this VDOP in both simple and more complex samples. When the transition m/z 576 → 314 was monitored, 1-hydroxy-vitamin D3/PTAD was eluted at the

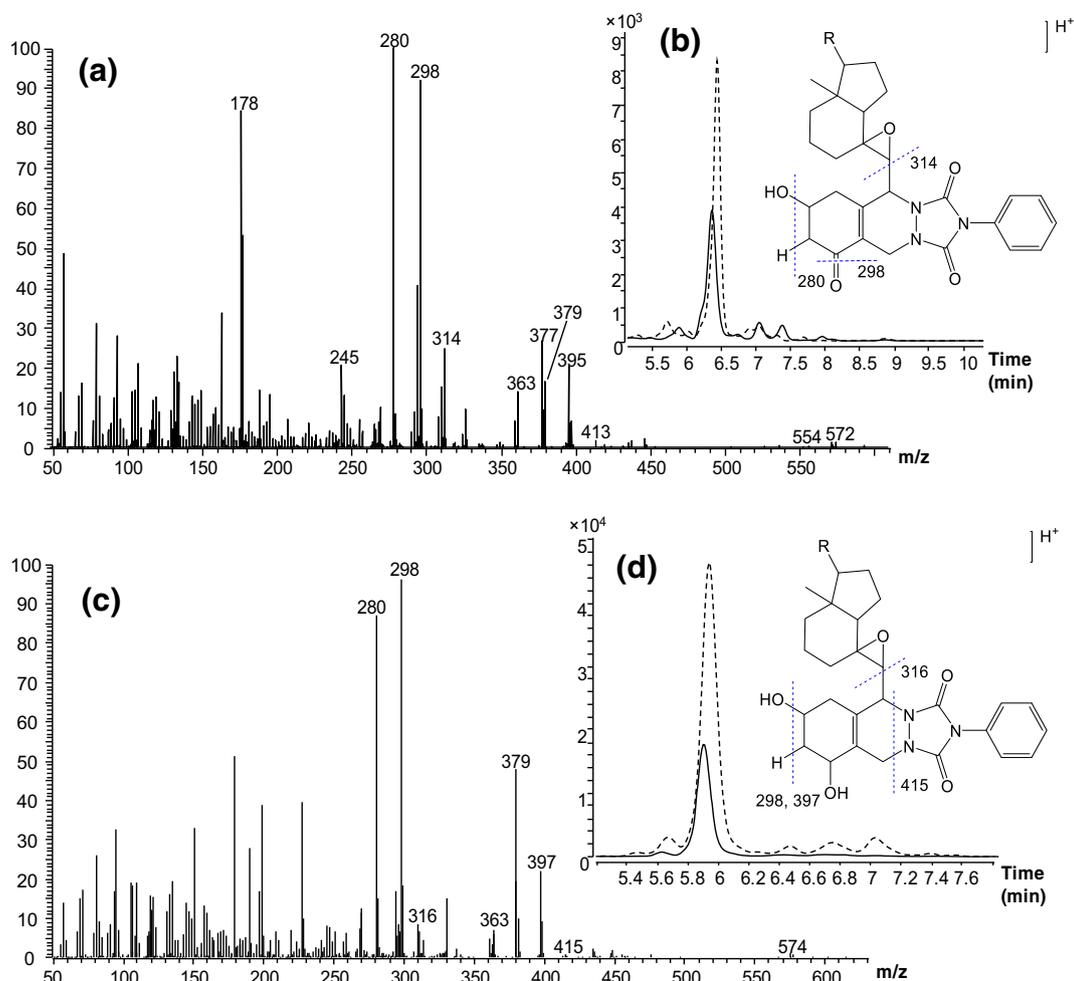


Figure 6. MS² spectra (a) and MRM chromatograms (b) of VDOP-PTAD adduct at m/z 590 → 298 in F2 and MS² spectra (c) and MRM chromatograms (d) of VDOP-PTAD adduct at m/z 592 → 298 in F3 of the oxidized vitamin D3 solution (—) and stored SWMP (---)

retention times of about 6.6 and 6.8 min in F3 (Figure 5d). In addition, the commercial standard of PTAD-derivatized 1-hydroxy-vitamin D3 was analyzed which showed similar peaks with similar retention times. According to previous studies, derivatization of vitamin D metabolites with PTAD produces two epimers, 6S and 6R, because the reagent reacts with the *S-cis*-diene moiety from both the α and the β sides [4, 47]. As a result, two peaks were separated for each VDOP in the MRM ion chromatograms provided that the two epimers are fully separated during the chromatography of these compounds.

The MRM transition m/z 590 → 298 and 592 → 298 were monitored in F2 and F3 for the detection of 1-keto-7,8-epoxy-vitamin D3/PTAD and 1-hydroxy-7,8-epoxy-vitamin D3/PTAD, respectively. PTAD-derivatized 1-keto-7,8-epoxy-vitamin D3 was observed in F2 at a retention time of about 6.4 min (Figure 6b), while F3 eluted 1-hydroxy-7,8-epoxy-vitamin D3/PTAD at a retention time of about 5.9 min (Figure 6d). Both of these VDOPs were available in the oxidized solution of vitamin D3 and stored SWMP.

Apart from that, SWMP was also prepared without vitamin D3 addition and stored in the same conditions. When the MRM transitions of VDOPs were monitored for this sample, no peaks were observed. Therefore, it could be concluded that the observed peaks were related to vitamin D3 compounds.

Conclusions

The present method for the isolation of VDOPs was adapted based on the extraction of cholesterol oxidation products in dairy products. Liquid-liquid extraction without saponification was used to minimize the risk of generation of artifacts. Further, solid phase extraction using aminopropyl cartridges was applied as a cleanup step to separate different classes of lipid and vitamin D3 from VDOPs. Different mass spectrometry analysis schemes have been developed and optimized to analyze the unknown VDOPs. The sensitivity of the VDOPs'

detection was significantly improved using Diels–Alder derivatization with PTAD.

The lack of standardized mass spectra libraries and limited publicly available data with respect to authentic MSⁿ spectra limited LC-MS/MS fragmentation studies of unidentified vitamin D3 compounds. The bottleneck of the compounds identification could not be overcome without mass spectra prediction tools. Fragmentation trees and MSⁿ spectral trees can give reliable methods to identify vitamin D3 unknown compounds such as VDOPs. Methods based on high accurate MS² and MSⁿ analysis have been developed for the identification and determination of vitamin D3 and its oxidation products.

In this study, the Mass Frontier software was used to calculate fragmentation pathways of VDOPs which was helpful in the annotation of compounds detected using ion trap mass spectrometry. The precursor-product ion relationships observed in MSⁿ spectra not only allowed to efficiently identify VDOPs, it also allowed to assign elemental formula and structure to each relevant fragment ion. This new method could help in comparing MSⁿ data and in the annotation and identification of known and unknown vitamin D3 compounds.

Finally, the MS/MS step was carried out by MRM which enabled us to identify these compounds with high selectivity at very low concentrations of a few parts per billion. For the first time, oxidized vitamin D3 products have been detected in real fortified whole milk powder, and this provides compelling support that the mode of degradation of vitamin D is in fact oxidation. The high selectivity and sensitivity obtained demonstrated the suitability of the proposed method for the determination of these compounds in more complex matrices such as food systems.

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References

- Allen, L., DeBenoist, B., Dary, O., Hurrell, R.: Guidelines on food fortification with micronutrients, *World Health Organization and Food and Agricultural Organization of the United Nations*. http://www.who.int/entity/nutrition/publications/guide_food_fortification_micronutrients.pdf (2006)
- Japelt, R.B., Jakobsen, J.: Vitamin D in plants: a review of occurrence, analysis, and biosynthesis. *Front. Plant Sci.* **4**, 136 (2013)
- Lipkie, T.E., Janasch, A., Cooper, B.R., Hohman, E.E., Weaver, C.M., Ferruzzi, M.G.: Quantification of vitamin D and 25-hydroxyvitamin D in soft tissues by liquid chromatography–tandem mass spectrometry. *J. Chromatogr. B.* **932**, 6–11 (2013)
- Aronov, P.A., Hall, L.M., Dettmer, K., Stephensen, C.B., Hammock, B.D.: Metabolic profiling of major vitamin D metabolites using Diels–Alder derivatization and ultra-performance liquid chromatography–tandem mass spectrometry. *Anal. Bioanal. Chem.* **391**, 1917 (2008)
- Mahmoodani, F., Perera, C.O., Fedrizzi, B., Abernethy, G., Chen, H.: Degradation studies of cholecalciferol (vitamin D3) using HPLC–DAD, UHPLC–MS/MS and chemical derivatization. *Food Chem.* **219**, 373–381 (2017)
- Jin, X., Yang, X., Yang, L., Liu, Z., Zhang, F.: Autoxidation of isotachysterol. *Tetrahedron.* **60**, 2881–2888 (2004)
- King, J.M., Min, D.B.: Riboflavin-photosensitized singlet oxygen oxidation product of vitamin D2. *J. Am. Oil Chem. Soc.* **79**, 983–987 (2002)
- Min, D.B., Boff, J.M.: Chemistry and reaction of singlet oxygen in foods. *Compr. Rev. Food Sci. Food Saf.* **1**, 58–72 (2002)
- Yamamoto, K., Imae, Y., Yamada, S.: Biomimetic oxidation of vitamin D by iron-sulfur model cluster and dioxygen system. *Tetrahedron Lett.* **31**, 4903–4906 (1990)
- Adamec, J., Jannasch, A., Huang, J., Hohman, E., Fleet, J.C., Peacock, M., Ferruzzi, M.G., Martin, B., Weaver, C.M.: Development and optimization of an LC-MS/MS-based method for simultaneous quantification of vitamin D2, vitamin D3, 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3. *J. Sep. Sci.* **34**, 11–20 (2011)
- Kobold, U.: Approaches to measurement of vitamin D concentrations–mass spectrometry. *Scand. J. Clin. Lab. Invest.* **72**, 54–59 (2012)
- Elipse, M.V.S.: Advantages and disadvantages of nuclear magnetic resonance spectroscopy as a hyphenated technique. *Anal. Chim. Acta.* **497**, 1–25 (2003)
- Lee, J.S., Kim, D.H., Liu, K., Oh, T.K., Lee, C.H.: Identification of flavonoids using liquid chromatography with electrospray ionization and ion trap tandem mass spectrometry with an MS/MS library. *Rapid Commun. Mass Spectrom.* **19**, 3539–3548 (2005)
- Clarke, N.J., Rindgen, D., Korfmacher, W.A., Cox, K.A.: Systematic LC/MS metabolite identification in drug discovery. *Anal. Chem.* **73**, 430A–439A (2001)
- Wu, Y.: The use of liquid chromatography–mass spectrometry for the identification of drug degradation products in pharmaceutical formulations. *Biomed. Chromatogr.* **14**, 384–396 (2000)
- Kind, T., Fiehn, O.: Seven golden rules for heuristic filtering of molecular formulas obtained by accurate mass spectrometry. *BMC Bioinformatics.* **8**, 105 (2007)
- Vaniya, A., Fiehn, O.: Using fragmentation trees and mass spectral trees for identifying unknown compounds in metabolomics. *TrAC Trends Anal. Chem.* **69**, 52–61 (2015)
- Fabre, N., Rustan, I., de Hoffmann, E., Quetin-Leclercq, J.: Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. *J. Am. Soc. Mass Spectrom.* **12**, 707–715 (2001)
- Shi, P., He, Q., Song, Y., Qu, H., Cheng, Y.: Characterization and identification of isomeric flavonoid O-diglycosides from genus Citrus in negative electrospray ionization by ion trap mass spectrometry and time-of-flight mass spectrometry. *Anal. Chim. Acta.* **598**, 110–118 (2007)
- Waridel, P., Wolfender, J., Ndjoko, K., Hobby, K.R., Major, H.J., Hostettmann, K.: Evaluation of quadrupole time-of-flight tandem mass spectrometry and ion-trap multiple-stage mass spectrometry for the differentiation of C-glycosidic flavonoid isomers. *J. Chromatogr. A.* **926**, 29–41 (2001)
- Gerlich, M., Neumann, S.: MetFusion: integration of compound identification strategies. *J. Mass Spectrom.* **48**, 291–298 (2013)
- Neumann, S., Böcker, S.: Computational mass spectrometry for metabolomics: identification of metabolites and small molecules. *Anal. Bioanal. Chem.* **398**, 2779–2788 (2010)
- Kasper, P.T., Rojas-Chertó, M., Mistrik, R., Reijmers, T., Hankemeier, T., Vreeken, R.J.: Fragmentation trees for the structural characterisation of metabolites. *Rapid Commun. Mass Spectrom.* **26**, 2275–2286 (2012)
- Abernethy, G.A.: A rapid analytical method for cholecalciferol (vitamin D3) in fortified infant formula, milk and milk powder using Diels–Alder derivatization and liquid chromatography–tandem mass spectrometric detection. *Anal. Bioanal. Chem.* **403**, 1433–1440 (2012)
- Rose-Sallin, C., Huggett, A.C., Bosset, J.O., Tabacchi, R., Fay, L.B.: Quantification of cholesterol oxidation products in milk powders using [2H7] cholesterol to monitor cholesterol autoxidation artifacts. *J. Agric. Food Chem.* **43**, 935–941 (1995)
- Trenery, V.C., Plozza, T., Caridi, D., Murphy, S.: The determination of vitamin D3 in bovine milk by liquid chromatography mass spectrometry. *Food Chem.* **125**, 1314–1319 (2011)
- Georgiou, C.A., Kapnissi-Christodoulou, C.P.: Qualitative and quantitative determination of COPs in cyprid meat samples using HPLC determination of the most effective sample preparation procedure. *J. Chromatogr. Sci.* **51**, 286–291 (2012)

28. Guardiola, F., Codony, R., Rafecas, M., Boatella, J.: Comparison of three methods for the determination of oxysterols in spray-dried egg. *J. Chromatogr. A*. **705**, 289–304 (1995)
29. Nielsen, J.H., Olsen, C.E., Duedahl, C., Skibsted, L.H.: Isolation and quantification of cholesterol oxides in dairy products by selected ion monitoring mass spectrometry. *J. Dairy Res.* **62**, 101–113 (1995)
30. Ulberth, F., Rössler, D.: Comparison of solid-phase extraction methods for the cleanup of cholesterol oxidation products. *J. Agric. Food Chem.* **46**, 2634–2637 (1998)
31. Calderón-Santiago, M., Peralbo-Molina, Á., Priego-Capote, F., de Castro, L., Dolores, M.: Cholesterol oxidation products in milk: processing formation and determination. *Eur. J. Lipid Sci. Technol.* **114**, 687–694 (2012)
32. Sander, B., Addis, P., Park, S., Smith, D.: Quantification of cholesterol oxidation products in a variety of foods. *J. Food Prot.* **52**, 109–114 (1989)
33. Dionisi, F., Golay, P., Aeschlimann, J., Fay, L.: Determination of cholesterol oxidation products in milk powders: methods comparison and validation. *J. Agric. Food Chem.* **46**, 2227–2233 (1998)
34. Park, P., Guardiola, F., Park, S., Addis, P.: Kinetic evaluation of 3 β -hydroxycholest-5-en-7-one (7-ketocholesterol) stability during saponification. *J. Am. Oil Chem. Soc.* **73**, 623–629 (1996)
35. Chen, B., Chen, Y.: Evaluation of the analysis of cholesterol oxides by liquid chromatography. *J. Chromatogr. A*. **661**, 127–136 (1994)
36. Hwang, K., Maerker, G.: Quantitation of cholesterol oxidation products in unirradiated and irradiated meats. *J. Am. Oil Chem. Soc.* **70**, 371–375 (1993)
37. Johnson, C.B.: Isolation of cholesterol oxidation products from animal fat using aminopropyl solid-phase extraction. *J. Chromatogr. A*. **736**, 205–210 (1996)
38. Lai, S., Gray, J.I., Zabik, M.E.: Evaluation of solid phase extraction and gas chromatography for determination of cholesterol oxidation products in spray-dried whole egg. *J. Agric. Food Chem.* **43**, 1122–1126 (1995)
39. Penazzi, G., Caboni, M., Zunin, P., Evangelisti, F., Tiscornia, E., Toschi, T.G., Lercker, G.: Routine high-performance liquid chromatographic determination of free 7-ketocholesterol in some foods by two different analytical methods. *J. Am. Oil Chem. Soc.* **72**, 1523–1527 (1995)
40. Guardiola, F.: Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence, and Biological Effects. AOCS Press, Champaign (2002)
41. Iuliano, L.: Pathways of cholesterol oxidation via non-enzymatic mechanisms. *Chem. Phys. Lipids*. **164**, 457–468 (2011)
42. Kind, T., Fiehn, O.: Advances in structure elucidation of small molecules using mass spectrometry. *Bioanal. Rev.* **2**, 23–60 (2010)
43. Rudewicz, P., Straub, K.M.: Rapid structure elucidation of catecholamine conjugates with tandem mass spectrometry. *Anal. Chem.* **58**, 2928–2934 (1986)
44. Böcker, S., Rasche, F.: Towards de novo identification of metabolites by analyzing tandem mass spectra. *Bioinformatics*. **24**, 49–55 (2008)
45. Hufsky, F., Scheubert, K., Böcker, S.: Computational mass spectrometry for small-molecule fragmentation. *TrAC Trends Anal. Chem.* **53**, 41–48 (2014)
46. Sheldon, M.T., Mistrik, R., Croley, T.R.: Determination of ion structures in structurally related compounds using precursor ion fingerprinting. *J. Am. Soc. Mass Spectrom.* **20**, 370–376 (2009)
47. Burild, A., Frandsen, H.L., Poulsen, M., Jakobsen, J.: Quantification of physiological levels of vitamin D3 and 25-hydroxyvitamin D3 in porcine fat and liver in subgram sample sizes. *J. Sep. Sci.* **37**, 2659–2663 (2014)
48. Higashi, T., Miura, K., Kitahori, J., Shimada, K.: Usefulness of derivatization in high-performance liquid chromatography/tandem mass spectrometry of conjugated vitamin D metabolites. *Anal. Sci.* **15**, 619–623 (1999)
49. Gallien, S., Duriez, E., Domon, B.: Selected reaction monitoring applied to proteomics. *J. Mass Spectrom.* **46**, 298–312 (2011)
50. Picotti, P., Aebersold, R.: Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions. *Nat. Methods*. **9**, 555–566 (2012)