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Vitamin D3 quantification in a cod liver oil-based supplement

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ABSTRACT

A reliable, accurate and reproducible method to quantify vitamin D3 (Vit. D3) in oily dietary supplements was developed after three Vit. D3 intoxications were diagnosed as reasonably resulting from a dietary administration of a cod liver oil based supplement. Liquid chromatography coupled to mass spectrometry operating in atmospheric pressure chemical ionization conditions (LC–APCI) and by using a deuterium labelled internal standard resulted to be an effective technique to reach the analytical aim.

Due to the complexity of the oily matrix, the new analytical approach required a solid phase extraction step prior to analysis. The amount of Vit. D3 declared on the label of the cod liver oil based supplement for each soft capsule is 1.5 μg. Consequently, the method was developed to quantify Vit. D3 amounts in the range 1–5 μg/mL. To improve reliability of obtained data, both MS and MS/MS acquisition methods were employed. The method was evaluated by measuring the characteristic parameters such as linearity, precision, accuracy and robustness and cross checked against a certified pharmaceutical preparation. The LC–APCI–MS and MS/MS methods were applied in order to assess the Vit. D3 content in the dietary supplements taken by the intoxicated patients, found about three order of magnitude higher than that declared. The Vit. D3 content of other batches of the same commercial product was found as declared.

1. Introduction

Cholecalciferol, commonly referred as vitamin D3 (Vit. D3) or the sunshine vitamin, is recognized not only for its importance in promoting bone health in children and adults but also for other health benefits, including reducing the risk of chronic diseases such as cancer, autoimmune or cardiovascular diseases [1]. Vit. D3 regulates blood calcium and phosphorus levels by promoting their absorption from food in the intestines, and reabsorption of calcium in the kidneys, which enables normal mineralization of bone. Vit. D3 is synthesized in the skin (the main source for humans) or ingested through the diet as a biologically inactive molecule. To exert its physiological effects, cholecalciferol requires two successive hydroxylations, at first on position 25 in the liver to be transformed in 25-hydroxyvitamin D3 [25(OH)D3], and then on position 1 in the kidneys, obtaining the biologically active molecule 1,25-dihydroxyvitamin D3 [1,25$(OH)_2$D3] [2].

Vit. D3 deficiency is recognized as a worldwide problem for both children and adults [3–5]. Because of the growing concern about sun exposure and skin cancer, both children and adults avoid either sun exposure or use sun protection devices which may expose them to the risk of Vit. D3 deficiency. Thus, their only source of Vit. D3 is from diet or supplements [6].

In adults, the 5–20 μg/day (200–800 IU) Vit. D recommended dietary allowance may prevent osteomalacia in the absence of sunlight exposure, but an additional intake is needed to prevent osteoporosis and secondary hyperparathyroidism.

The presence of Vit. D3 is limited in most diets, as significant amounts occur only in fatty fish and specific fish oils, liver and fat from aquatic mammals and egg yolks of Vit. D3 fed hens. In the United States, manufacturers add Vit. D3 to many foods, including fluid milk, calcium-fortified orange juice, margarine, breakfast cereals and sometimes dairy products other than milk, such as yogurt and cheese. In adults, new evidence suggests that Vit. D3 plays a fundamental role in maintaining innate immunity [7] and

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has been demonstrated to be involved in the prevention of certain disease states including infections [8,9], autoimmune diseases (multiple sclerosis [7,10–12], rheumatoid arthritis [13]), some forms of cancer (breast, ovarian, colorectal, prostate) [14–17] and type 2 diabetes mellitus [18–20]. Since vitamin D may have toxic effects above certain threshold concentration [21], a full understanding of the role of Vit. D3 intake in promoting health, and also a safe use of dietary vitamin D supplements, require accurate and reliable Vit. D3 content data [2].

Symptoms of intoxication are hypercalcemia, hypercalciuria and often hyperphosphatemia. Due to the lipophilic characteristic of the cholecalciferol, vitamin D hypervitaminosis is difficult to treat, since the molecule has very slow elimination kinetics.

Three cases of hypercalcemia with acute kidney insufficiency caused by Vit. D3 intoxication were recently reported to the Italian National Surveillance System of Natural Health Products coordinated by the Italian National Health Institute and studied by the Unit of Pharmacovigilance and Phytovigilance of Florence University. All patients had used the dietary supplement *Merluzzovis*, containing cholecalciferol in a cod liver based supplement, as a self-medication for at least 6 months. The samples of the three batches were analysed, in order to quantify the Vit. D3 content present in the dietary supplement preparation.

The food supplement *Merluzzovis* was stated to contain cod liver oil with the following amounts of vitamins for each soft capsule: 1.5 μg of Vit. D3, 0.24 mg of vitamin A, 5 mg of vitamin E, 14 μg of vitamin K, 3.6 mg of vitamin F. The suggested doses were not consistent with the detected poisoning effect, and a higher concentration of Vit. D3 in the dietary supplement was hypothesized.

Many analytical methods have been proposed for the quantification of Vit. D3 in plasma and serum [22] but in the present case it must be taken into account that all the compounds contained in the cod liver oil based supplement exhibit chemical properties similar to those of vitamin D, constituting a potential source of interferences.

Vit. D3 analysis in foods is generally performed by LC–UV and, more recently, by UHPLC–UV; this technique is robust, inexpensive and widely used in many laboratories. However, food matrices usually require complicated and time consuming sample preparation such as saponification and solvent extraction. Vitamin D can be directly extracted from the food samples with organic solvents such as methyl dichloride, hexane or chloroform; therefore, the recoveries reached with direct extraction were lower than those obtained by saponification [23,24]. The officially approved extraction and analytical methods for Vit. D3 in dairy and fatty foods are available (AOAC Official Method 992.26 and AOAC Official Method 2002.05 [25]). These procedures, as well as other published methods, include a liquid/liquid extraction step using nonpolar solvents (diethyl ether/petroleum ether extraction in AOAC 992.26 method or heptane in AOAC 2002.05 method) which were ineffective in the present case: in fact, the addition of nonpolar solvent to a totally oily matrix resulted only in a sample dilution without extraction from other matrix components.

Anyway also after saponification and solvent extraction processes, some problems were described in LC separation, due to the presence of lipid residues. Therefore a purification step of the extracted sample is recommended [26].

The use of solid phase extraction (SPE) is less common; however a C18 cartridge together with polar eluents as methanol or silica cartridge with an apolar eluent as hexane has been used [27]. Conventional C18 cartridges are responsible for retention and elution of fat-soluble vitamins, and polar solvents used in the elution do not fully release the vitamin D retained by the cartridge [28]. Since the analysed matrix is oily, the use of C18 SPE is inappropriate. In the present study, as will be discussed below, a normal phase cartridge was used and, in agreement with the literature, SPE-NH_2 was used successfully in the analysis of steroids in complex matrices [29].

The aim of this study was the development of a reliable, accurate and reproducible method for Vit. D3 quantification not in plasma or serum, but in oily dietary supplements.

All analytical methods for quantitation of Vit. D3 reported in the literature based on non-specific detectors, should be supported by a different confirmatory technique. On the other hand LC–MS based methods are commonly used to improve the reliability in terms of specificity and sensibility. Electrospray ionization (ESI) is reported by many authors as an effective approach for plasma and serum, to detect and quantify hydroxylated metabolites of vitamin D. However ESI as described in literature, shows very low sensitivity to vitamin D3 reasonably for the lack of protonation sites reactive in solution. On the contrary, atmospheric pressure chemical ionization (APCI) was found to be a suitable ionization method for the characterization of steroids and vitamin D3 [28,30].

We evaluated a different analytical approach for Vit. D3 quantitation in an oily dietary supplement based on a solid phase extraction procedure and APCI–LC–MS with the addition of a deuterium labelled internal standard in order to overcome possible ion suppression phenomena and assure the necessary specificity and the reliability. The proposed method was validated by testing its linearity, accuracy, precision and robustness.

2. Materials and methods

2.1. Chemicals and supplies

The analytical reference standard of cholecalciferol (Vit. D3) was purchased from Sigma–Aldrich (Milan, Italy). The deuterium labelled internal standard (IS) 6,19,19-$^2H_3$-cholecalciferol, 99.6% purity was provided by Isotec (Sigma–Aldrich). LC–MS grade water, methanol and acetonitrile were supplied by Mallinckrodt Baker (Deventer, The Netherlands). Analytical grade 2-propanol, *n*-hexane and ethyl acetate were supplied by Sigma–Aldrich. Supelclean™ LC-NH_2 SPE Bulk Packing, empty columns and polyethylene frits (3 mL) were supplied by Supelco (Sigma–Aldrich).

2.2. Samples

Five samples from four different production batches of dietary supplements (*Merluzzovis*) were analysed. The samples whose expiration date was 2011 were sent to our laboratory from the intoxicated patients; the remaining samples were purchased in a herbalist's shop. To each sample a laboratory code was assigned as follows:

Code	Batch of production	Expiration date
(A)	No. 041658	04/2011
(B)	No. 020428	01/2011
(C)	No. 020428	01/2011
(D)	No. I0837409	09/2014
(E)	No. 0237208	09/2013

A pharmaceutical form of Vit. D3 (*DIBASE*® 300,000 U.I./mL, Abiogen Pharma, Ospedaletto, Pisa, Italy) was also analysed (1 μg = 40 U.I.) for a cross check of the procedure.

2.3. Preparation of standard solutions

Stock solutions of analytical standards, Vit. D3 and IS, at 1.0 μg/μL were prepared in *n*-hexane and stored in the dark at 4 °C for no longer than two months. Working solutions were freshly prepared for each experiment by diluting stock solutions in *n*-hexane up to a concentration of 100 ng/μL (solution 1 for Vit. D3 and solution 2 for the IS).

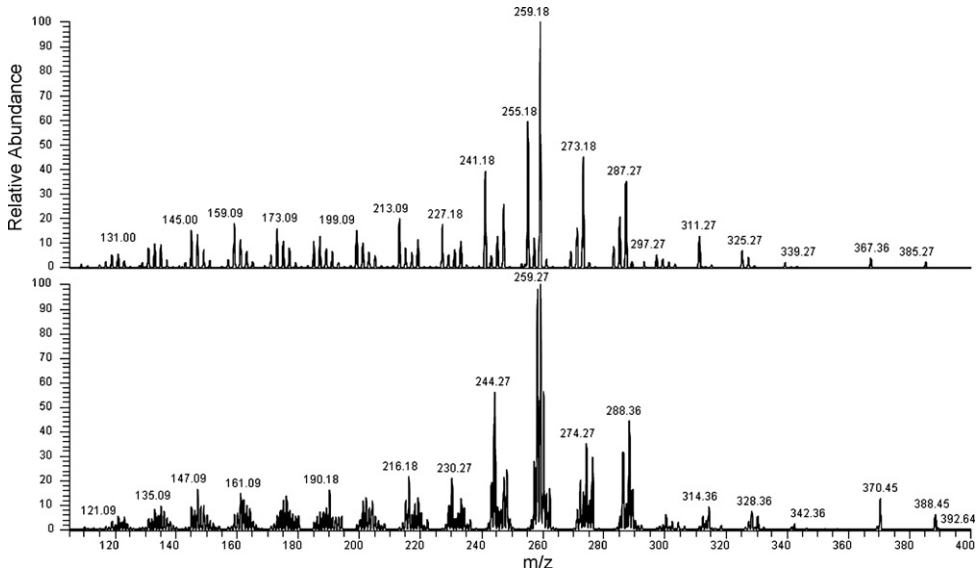


Fig. 1. MS/MS spectra of ion *m*/*z* 385 (top) and of ion *m*/*z* 388 (down) corresponding to vitamin D and its trideuterated isotopologue, respectively.

Six levels for each calibration curve were prepared by adding scalar volumes of solution 1 (i.e. 0, 10, 20, 30, 40 and 50 μL) to a constant volume of solution 2 (30 μL corresponding to 3 μg of IS), drying under a nitrogen stream and redissolving in 1 mL of 2-propanol. Each calibration level was analysed in triplicate by MS and MS/MS experiments. The calibration curves obtained covered the range 1–5 μg/mL.

2.4. Sample preparation

At least three soft capsules for each batch of *Merluzzovis* and a *DIBASE*® ampoule were processed.

The whole content of a *Merluzzovis* soft capsule was taken, added of 30 μL of solution 2, than diluted to 1 mL with *n*-hexane (*Merluzzovis* sample solutions).

Initial trials evidenced the saponification step was ineffective for the quantitation of Vit. D3 [28] and for samples A, B and C the dilution procedure yielded response factors exceeding the upper limit of the calibration range. Therefore, different aliquots of sample oil were processed to fall within the range of calibration curves.

The final procedure for samples A, B and C consisted in a dilution of whole content of a soft capsule to 10 mL with *n*-hexane; 20 μL of this solution were added to 30 μL of solution 2 then diluted up to 1 mL with *n*-hexane (*Merluzzovis* A, B and C sample solutions).

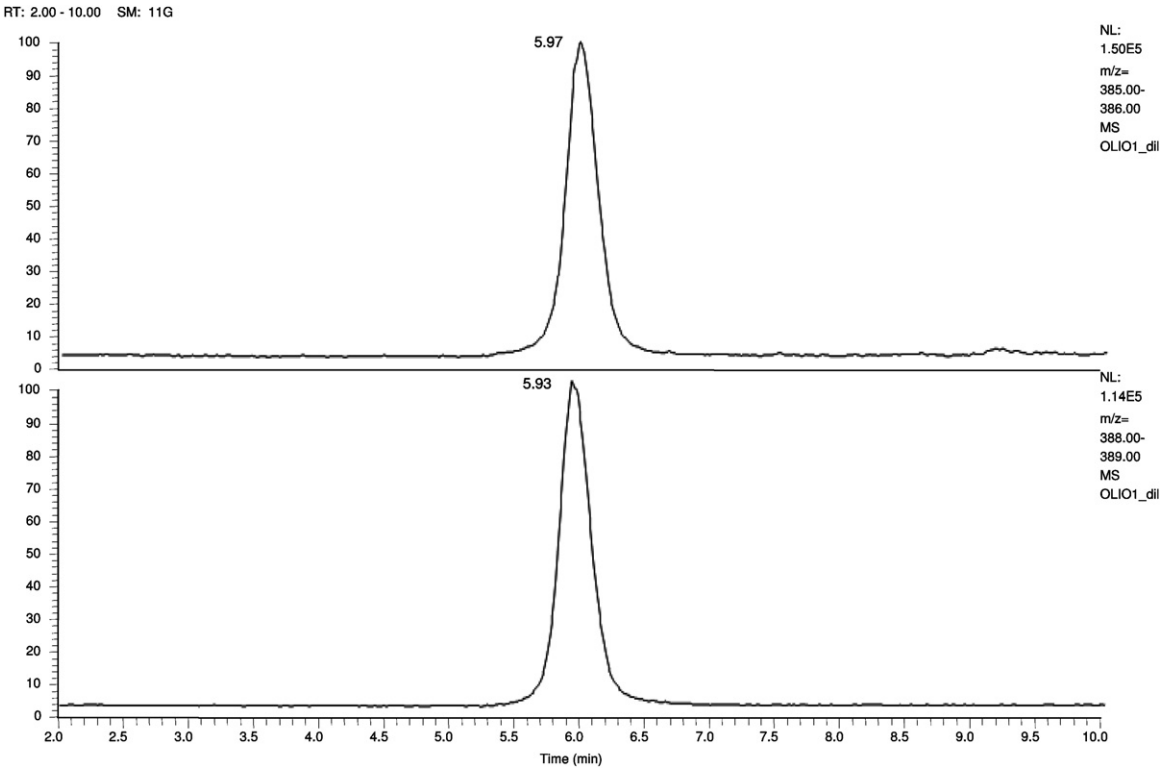
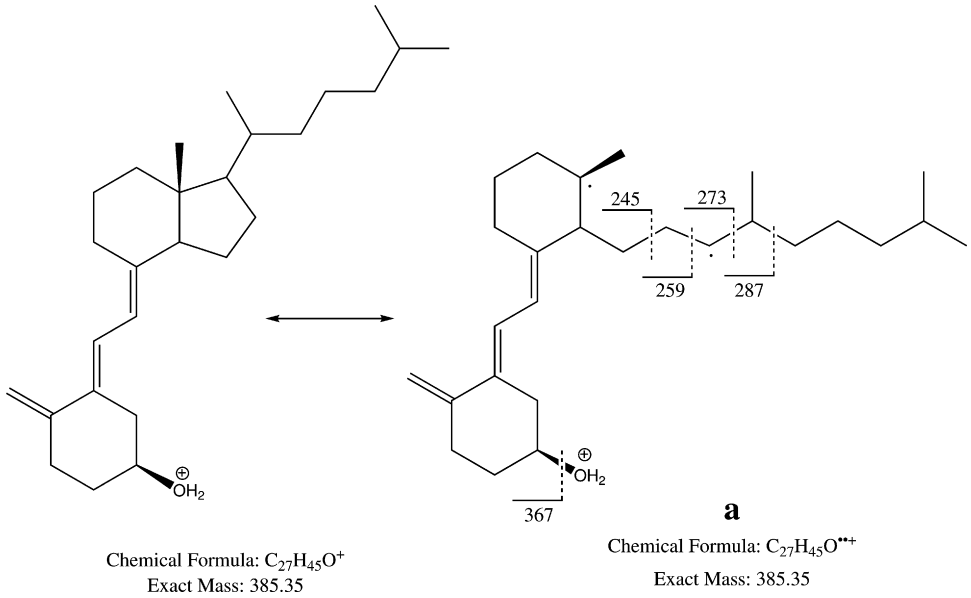


Fig. 2. Extracted ion chromatograms of *m*/*z* 385 and 388, recorded for a *Merluzzovis* sample by MS acquisition.



Scheme 1. Supposable mechanism for the origin of the fragment ion at *m/z* 259.

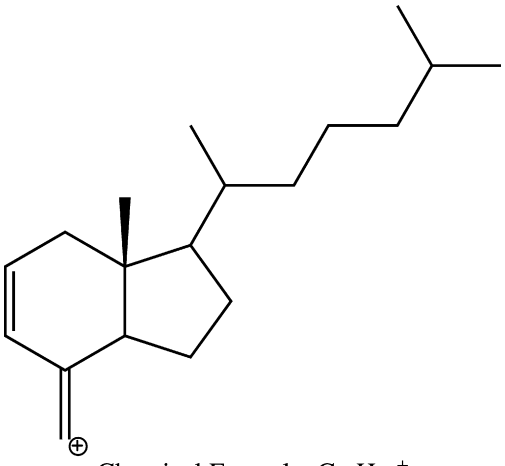
For further validating the quantitation data, the whole content of *DIBASE*® ampoule was diluted up to 50 mL with *n*-hexane; 20 μL of *n*-hexane diluted solution were added of 30 μL of solution 2 then diluted up to 1 mL with *n*-hexane (*DIBASE*® sample solution). This dilution was chosen to fall within the range of the calibration curve on the basis of the concentration declared on label.

2.5. Extraction procedure

The sample solutions (1 mL) were processed on 200 mg Supelclean™ LC-NH_2 SPE cartridges.

The extraction procedure was performed as follows:

1. the cartridge was conditioned with 2 mL of *n*-hexane;
2. the sample solution was loaded;
3. the cartridge was washed with 2 mL of *n*-hexane (eluates were discarded);
4. Vit. D3 was eluted with 1 mL of ethyl acetate.



Scheme 2. Proposed structure of ion at *m/z* 259.

The eluate was collected and evaporated to dryness under a gentle N_2 stream, then the residue was reconstituted in 1 mL of 2-propanol and analysed (in triplicate) by LC–MS as described below.

2.6. LC–MS analysis

The samples and the calibration solutions were analysed on a Surveyor LC system coupled to a LTQ MS equipped with an APCI source (ThermoFisher, San Josè, CA, USA).

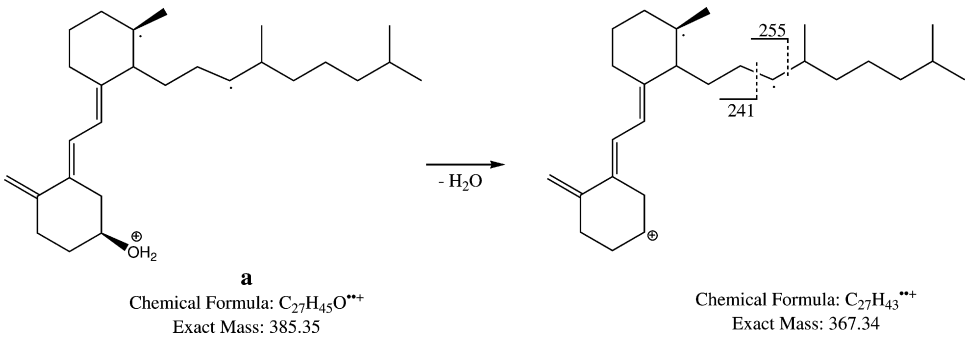
The analytical column was a Pursuit XRs RP C-18 50 mm × 2 mm, 5 μm (Varian, Palo Alto, CA, USA) operating at 0.5 mL/min flow rate. Solvent A was methanol:water 9:1 (v/v) and solvent B was acetonitrile. The LC pump delivered the following mobile phase gradient: 10% B was maintained for 1 min then to 50% B in 8 min, and to 100% B in 5 min. Initial conditions were restored in a minute and the column re-equilibrated for 5 min [31]. In these conditions, the retention time of cholecalciferol and its internal standard was about 6.3 min.

The APCI source operated in positive ion mode, using the following setting: temperature 350 °C, capillary temperature 200 °C, sheath gas flow 20 (arbitrary units), aux gas flow 5 (arbitrary units), sweep gas flow 4 (arbitrary units), capillary voltage 34 V, tube lens 65 V, source voltage 6 kV, source current 5 μA.

The mass spectrometer was calibrated and the resolution adjusted to a 1 *m/z* over the *m/z* 50–2000 range before each analytical batch. All voltages, ion optics and other instrumental parameters were optimized by infusing a 10 μg/mL standard solution of cholecalciferol in 2-propanol.

The analyses were performed by using both MS and MS/MS data. In the former case a *m/z* range 150–1000 was employed.

In the MS/MS acquisition method, the protonated molecules of cholecalciferol (*m/z* 385) and its internal standard (*m/z* 388) were isolated and collided, then the product ion spectra were recorded in the range *m/z* 105–450. The collision induced dissociation (CID) parameters were set as follows: isolation width *m/z* 2, 30 ms activation time, *q* 0.25 and wideband activation. The MS/MS spectra of Vit. D3 and its IS are reported in Fig. 1. The supplementary RF voltage values employed to activate the precursor ion fragmenta-



Scheme 3. Proposed mechanism for the origin of the fragment ion at *m/z* 241.

Table 1
Summary data of calibration curves obtained by MS and MS/MS methods.

[Vit. D3]/[IS] abundance ratios	Equation	R^2
m/z 385/388	$Y = 0.0377731 + 0.349957X$	0.9996
m/z 259/259	$Y = 0.0503981 + 0.638449X$	0.9966
m/z 241/244	$Y = 0.0444658 + 0.453367X$	0.9955

tion were 1.25 V for the ion at *m/z* 385 and 1.75 V for the ion *m/z* 388.

2.7. Data processing

A linear regression analysis was applied to obtain the calibration curve. The peak area ratio (PAR) of Vit. D3 to the IS was calculated and then regressed over the corresponding amount of cholecalciferol added for each calibration point by the Xcalibur Quan Browser software (version 2.5, ThermoFisher). In the chromatograms obtained in MS the signals of the ions at *m/z* 385 and *m/z* 388 (due to protonated molecules of Vit. D3 and IS, respectively) were used to calculate the PAR values, whereas for MS/MS experiments the collisionally generated fragments at *m/z* 259 (T1 385 → 259 Vit. D3), versus *m/z* 259 (T1 388 → 259 IS) and *m/z* 241 (T2 385 → 241 Vit. D3) versus *m/z* 244 (T2 388 → 244 IS).

3. Results and discussion

As reported in Section 1, most of the methods devoted to the quantification of Vit. D3 and its metabolites have been developed for plasma and serum samples analyses. In the present case the completely different matrix (cod liver oil) necessarily requires the development of a different analytical procedure and for this aim either different extraction methods or different mass spectrometric approaches were tested. As exhaustively described either in Section 1 or in Section 2 the most effective extraction procedure was found to be the treatment of the cod liver oil with the NH_2 cartridges.

The extracted samples so employed were analysed by LC–MS and LC–MS/MS.

Table 2
Precision evaluation of MS and MS/MS methods by quintuplicate analysis on *Merluzzovis* samples A.

Batch A	MS (μg/mL)	MS/MS T1 (μg/mL)	MS/MS T2 (μg/mL)
1	2.52	2.50	2.45
2	2.73	2.37	2.67
3	2.79	2.72	2.81
4	2.91	2.68	2.58
5	2.71	2.43	2.76
Average	2.73	2.54	2.65
SD	0.14	0.15	0.15
RSD	5.1%	6.1%	5.5%

First of all some attempts in ESI conditions were performed but they led to negative results. Either in positive or negative ion modes poor spectra have been obtained and only in ESI+ a weak signal at *m/z* 385, corresponding to the protonated molecule of Vit. D3 is present, with an abundance two or three times than that of the chemical background. This result can be explained by two different phenomena:

(i) low yield of the condensed phase protonation of Vit. D3;
(ii) high yield of protonation reaction of other components co-eluting with Vit. D3 (matrix effect).

For these reasons, some further experiments were performed in APCI conditions, operating in positive ion mode. In this case abundant $[MH]^+$ of Vit. D3 are produced with a signal-to-chemical background ratio close to 100.

Some attempts were done to optimize the solution vaporizer temperature: identical results were obtained for temperature range 330–370 °C. Consequently all the experiments were performed with a vaporizer temperature of 350 °C. The APCI spectrum shows practically only the peak due to protonated molecule; some fragments are detectable at *m/z* 367 and *m/z* 259, but their intensity is lower than 5%. An analogous behavior is observed for the IS. The results obtained in APCI conditions indicate that gas phase protonation of Vit. D3 occurs in high yield, differently to what observed in solution (i.e. ESI conditions). This difference can be ascribed to kinetic effects, independent from thermodynamic data. The MS/MS spectrum of the protonated molecule of Vit. D3 is reported in top of Fig. 1. It has been obtained by collisions performed by resonance using a supplementary RF voltage of 1.25 V.

It must be considered that collisionally experiments performed with ion trap are quite different from those performed by triple quadrupole. In fact the energy deposition present in ion trap experiments is a step-by-step phenomenon and consequently the fragmentation pathways at low critical energy are favoured.

The most abundant fragment ion is *m/z* 259 and for its origin the mechanism reported in Scheme 1 can be proposed. It consists on the cleavage of the cyclopentane ring between the two tertiary carbon atoms with the formation of the open structure **a** of the Scheme 1.

The ion **a** at *m/z* 259 shows the primary water loss leading to the fragment at *m/z* 241 (see Fig. 1 top). However the collision of $[MH]^+$ of the deuterate derivative shows that this hypothesis is not correct (see Fig. 1 down). In fact the ion at *m/z* 259 above discussed remains unchanged indicating that in its structure any deuterium atom is present. In other words it does not contain the cyclohexanol moiety as hypothesized in Scheme 1. To justify its structure it must be considered a double H transfer on the double bond linking the hexanol structure with the open part of the molecule. The cleavage of the C–C bond and the charge localization of the hydrocarbon substructure lead to the formation of the ion at *m/z* 259 (Scheme 2)

Table 3
Method accuracy evaluated analysing three points of the calibration curve (at low, medium and high concentrations) by MS and MS/MS methods.

Sample	MS			MS/MS T1			MS/MS T2		
	Low (μg/mL)	Medium (μg/mL)	High (μg/mL)	Low (μg/mL)	Medium (μg/mL)	High (μg/mL)	Low (μg/mL)	Medium (μg/mL)	High (μg/mL)
1	1.10	3.14	5.09	1.03	2.95	5.10	0.97	2.99	5.16
2	1.15	3.08	4.61	1.10	2.82	4.69	1.07	2.99	4.88
3	1.09	2.86	4.56	0.98	2.76	5.11	1.02	2.87	5.13
Average	1.11	3.03	4.75	1.03	2.84	4.97	1.02	2.95	5.05
SD	0.03	0.14	0.29	0.06	0.10	0.24	0.05	0.07	0.15
RSD	2.7%	4.8%	6.1%	5.6%	3.4%	4.8%	4.6%	2.3%	3.0%
Accuracy	88.8%	99.1%	95.0%	96.5%	94.8%	99.3%	98.2%	98.3%	98.9%

Table 4
Method robustness evaluated analysing in triplicate three samples prepared as above described by MS and MS/MS methods.

Batch A	MS			MS/MS T1			MS/MS T2		
	10 μL (μg/mL)	20 μL (μg/mL)	30 μL (μg/mL)	10 μL (μg/mL)	20 μL (μg/mL)	30 μL (μg/mL)	10 μL (μg/mL)	20 μL (μg/mL)	30 μL (μg/mL)
Average	1.47	2.73	3.57	1.46	2.54	3.43	1.52	2.65	3.34
SD	0.03	0.14	0.18	0.04	0.15	0.16	0.11	0.15	0.16
RSD	2.1%	5.1%	5.1%	2.8%	6.1%	4.8%	7.2%	5.5%	4.8%
mg Vit. D3/softcapsule	1.47	1.37	1.19	1.46	1.27	1.14	1.52	1.33	1.11
Average (mg Vit. D3/softcapsule)		1.34			1.29			1.32	
SD (mg Vit. D3/softcapsule)		0.14			0.16			0.20	
RSD		10.6%			12.3%			15.4%	

either for Vit. D3 or for the IS. The other two abundant ions at *m*/*z* 241 and at *m*/*z* 255 observed in the MS/MS spectrum of the $[MH]^+$ of Vit. D3 are in the case of the IS shifted to *m*/*z* 244 and to *m*/*z* 258 so proving that they originate from the "open" structure **a** after water loss (see Schemes 1 and 3).

Then, on the basis of the above discussed data, the ions at *m*/*z* 259, 241 and 259, 244 were chosen as product ions for the Vit. D3 quantification through MS/MS experiments.

The extracted ion chromatograms corresponding to Vit. D3 and its IS, recorded for a *Merluzzovis* sample by MS (Fig. 2) showed only one intense signal, indicating a satisfying specificity of detection.

As a further check of the absence of interferences, cholecalciferol content of the analysed samples was calculated using three different calibration curves obtained by MS and MS/MS acquisitions for the collisionally generated fragment ions above described.

The estimated Vit. D3 concentration calculated from all calibration curves were consistent as indicated in Tables 2 and 3 for both precision and accuracy. This indicates that all the quantitation procedures were equivalent in this oily matrix.

3.1. Validation of the analytical method

The day-by-day variation was evaluated by analysis of two independent solutions using MS and MS/MS methods. The results of these analyses were used to evaluate the linearity range of quantitation and the limit of quantitation (LOQ), set to the lowest point of calibration curve. A good linearity was obtained with R^2 always larger than 0.99 both in MS and MS/MS conditions (Table 1).

The precision of the determination was evaluated in both MS acquisition methods through the calculated relative standard deviation (RSD %) of replicated *Merluzzovis* sample solutions A.

The precision results are reported in Table 2.

To evaluate the accuracy of the determination, three points of the calibration curve (at low, medium and high concentrations) were processed by the SPE extraction above described and then analysed in triplicate using both acquisition methods (Table 3).

The robustness was evaluated by processing 10 μL and 30 μL volumes, instead of 20 μL of sample solution A, with the same extraction procedure as for the other samples. The robustness, expressed by RSD% reported in Table 4, indicates that different amounts of Vit. D3 in the oily sample were correctly estimated in all MS acquisition methods.

3.2. Samples analysis

The first dietary supplement analysed was the sample coded A (see Section 2) received from an intoxicated patient.

Vit. D3 content evaluation in this sample was carried out by processing five soft capsules in both MS and MS/MS conditions using the calibration curves previously described. The results are reported in Table 2. Quantitative data obtained from each MS/MS transition are comparable and in agreement with data obtained in MS mode. Since the data obtained from experiments in MS and MS/MS were in good agreement, it was decided to perform analysis only by the MS method for batches B and C. Moreover, it was decided to confirm the results on two new different *Merluzzovis* batches directed purchased in a herbalist's shop (batches D and E) and to evaluate the accuracy of the method on a certified pharmaceutical preparation form (*DIBASE*®).

MS analysis of batches A, B, C, D, E and pharmaceutical form were then performed in triplicate and the obtained results are listed in Table 5. For batches D and E, the content of Vit. D3 was about 1 μg for each soft capsule, a value lower than the declared amount; a larger amount of cholecalciferol was revealed in batches A, B and C.

Table 5
MS analysis (in triplicate) on *Merluzzovis* soft capsules of batches A, B, C, D, E and *DIBASE*®.

Sample	A (μg/soft capsule)	B (μg/soft capsule)	C (μg/soft capsule)	D (μg/soft capsule)	E (μg/soft capsule)	*DIBASE*® (μg/ampoule)
Average (μg)	1366.60	1274.33	1285.00	1.01	1.05	8045.83
SD (μg)	70.36	84.83	158.47	0.15	0.05	307.29
RSD	5.1%	6.7%	12.3%	14.6%	4.4%	3.8%

The data obtained from the analysis of the pharmacological form are shown in Table 5. The amount of cholecalciferol in the drug is 8.0 mg/ampoule with a calculated accuracy of 85% and a RSD of 3.8%. This value is in good agreement with the amount reported on the drug label (7.5 mg/ampoule), thus confirming the validity of the method in terms of accuracy and precision.

4. Conclusions

A LC–MS method has been developed and optimised to analyse oily Vit. D3 based preparations. The LC separation was achieved in 20 min. Quantitative performances of this method, making use of a deuterium labelled internal standard were satisfying in terms of precision, accuracy, linearity and robustness. Reduced noise chromatograms were obtained after SPE extraction which was proven to be fast and effective in extracting Vit. D3 from oily matrices. The MS detection afforded high specificity and sensitivity suitable for determination of Vit. D3 in drugs and in dietary supplement samples in which Vit. D3 concentration should be expected to be close to the RDA or higher.

The average amount of Vit. D3 estimated in a single soft capsule containing cod liver oil of batches A, B and C was about 1.31 ± 0.05 mg (about three orders of magnitude higher than the declared amount).

The measured content of Vit. D3 in the pharmacological preparation *DIBASE®* agreed with the concentration reported on label, and confirmed the validity of the developed method. These results call for a strict control of the Vit. D3 concentration in dietary supplements, as the risk of hypervitaminosis is elevated in patients subjected to controlled drug administration or Vit. D3 rich diets.

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