Chemical Data Collections 25 (2020) 100311

Contents lists available at ScienceDirect

Chemical Data Collections

journal homepage: www.elsevier.com/locate/cdc

Data Article

Vitamin D_3 in fish oil extracted with limonene from anchovy leftovers

Antonino Scurria[a], Claudia Lino[a], Rosa Pitonzo[b], Mario Pagliaro[a], Giuseppe Avellone[c,*], Rosaria Ciriminna[a,*]

[a] Istituto per lo Studio dei Materiali Nanostrutturati, CNR, via U. La Malfa 153, 90146 Palermo (Italy)
[b] ATeN Center, Università di Palermo, viale delle Scienze, 90128 Palermo (Italy)
[c] Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche, Università di Palermo, via Archirafi 32, 90123 Palermo (Italy)

ARTICLE INFO

Article history:
Received 19 August 2019
Revised 1 November 2019
Accepted 3 November 2019
Available online 13 November 2019

Keywords:
Vitamin D
Fish oil
Anchovy
Limonene
Cholecalciferol

ABSTRACT

Aiming to identify and assess the amount of fat-soluble vitamins in anchovy oil extracted with *d*-limonene from anchovy fillet leftovers via the UHPLC–HESI–MS advanced mass spectrometry analytical technique, this study shows that only vitamin D_3 is present in anchovy by-product oil. The sum of the quantities of the three isomers of vitamin D_3 amounts to 0.0815 μg vitamin D_3 per g oil, namely a 81.5 μg/kg content, in good agreement with the typical amounts of vitamin D_3 in fish oils (ranging from 18 to 350 μg/kg).

Specifications Table [please fill in right-hand column of the table below]

Subject area	Biochemistry, Spectroscopy
Compounds	Vitamin D_3
Data category	Spectral
Data acquisition format	Mass spectra
Data type	Raw, analyzed
Procedure	Brief description of procedure (e.g., transformation, simulation, etc.)
Data accessibility	Fat-soluble vitamins in anchovy oil extracted with *d*-limonene from anchovy fillet leftovers were recovered according to slight modification of published methods. Screening of the oil by HPLC-MS shows that only vitamin D_3 is present in anchovy by-product oil, whereas the study of the mass spectrum suggests the presence of three vitamin D_3 isomers.

1. Rationale

The abundant by-products from anchovy, about 32% of the wet fish weight, are a potential source of protein powder, fish oil, and minerals [1]. We have recently described the extraction of fish oil rich in omega-3 lipids from European anchovy (*Engraulis encrasicolus*) fillet discards using orange oil-derived *d*-limonene as extraction solvent [2]. Using a natural and edible solvent with multiple health beneficial properties [3], the process shifts the omega-3 lipids extraction from fish to fish

* Corresponding authors.
E-mail addresses: beppe.avellone@unipa.it (G. Avellone), rosaria.ciriminna@cnr.it (R. Ciriminna).

https://doi.org/10.1016/j.cdc.2019.100311
2405-8300/

waste. The latter is an urgent global need to end the contribution of omega-3 dietary supplement production to overfishing [4].

The capital investment in the low energy extraction set up, we have further shown along with Chemat and Fabiano-Tixier, is chiefly limited to purchase of limonene, and the operational cost mostly consists of labor and electricity necessary to separate the oil from the agro-solvent [5]. Ending the latter study we emphasized how, besides being a source of essential omega-3 lipids, fish and fish products are commonly regarded as important natural food sources of vitamin D, and vitamin D_3 in particular.

We briefly remind that, according to IUPAC-approved terminology, the term "vitamin D" is used as a general term to describe all steroids that exhibit qualitatively the biological activity of calciol (cholecalciferol) [6]. From the biochemical point of view, the most important vitamin D isomers are calciol (cholecalciferol, vitamin D_3), calcidiol (25-hydroxycholecalciferol), [7] and calcitriol (1α,25-dihydroxycholecalciferol, the biologically active form of vitamin D, first identified in 1971) [8].

Produced by the body in the form of cholecalciferol from cholesterol in response to the exposure of the skin to ultraviolet UVB solar radiation (280–315 nm, namely 5% of the overall UV photons present in the terrestrial radiation from the midday sun) [9], vitamin D_3 is critical for bone and mineral metabolism being the main hormone of bone metabolism. However, vitamin D is also a regulator of inflammation playing an important role in the immune system [10], including affecting a key mechanism in the body's immune system involved in the development of several autoimmune diseases (vitamin D causes dendritic cells to produce more of a molecule hindering the activation of T cells) [11].

Adequate vitamin D level (serum 25-hydroxyvitamin D >50 nmol/L) is currently observed in less than 50% of the world population (at least in winter) [12]. Prevention of its deficiency requires longer sunlight exposure, consumption of fish and other foodstuffs containing either vitamin D_3 or vitamin D_2 (ergocalciferol), or the assumption of vitamin D dietary supplements [12].

Providing proof of concept, this work shows how to identify and assess the amount of fat-soluble vitamins in anchovy oil extracted with *d*-limonene from anchovy fillet leftovers. Subsequent measurements applied to series of extraction batches are likely to reveal variations in the amounts of bioactive compounds, as it happens with the lipid profile of anchovies catched in different seasons of the year [13].

2. Procedure

Vitamins in anchovy oil were extracted according to the method used by Sanchez-Machado and co-workers for the HPLC analysis of α-tocopherol in macroalgae [14], with a slight modification reported by Stancheva et al. for retinol, alpha-tocopherol and fatty acid content analysis in fish [15].

All fat-soluble vitamins, Vitamin A (all-*trans*-retinol), Vitamin D_2 (ergocalciferol), Vitamin D_3 (cholecalciferol), Vitamin E (α-tocopherol) and Vitamin K_1 (phylloquinone), were supplied by Sigma-Aldrich (St. Louis, Missouri). All solvents, including methanol and water (LC–MS grade), were purchased from Biosolve B.V. (Valkenswaard, The Netherlands). Formic acid (98–100% purity) was obtained from VWR International (Roden, The Netherlands). A sample of fresh fish oil (0.5 g) obtained from a one year old sample of frozen fillet leftovers was obtained as previously described in the literature.[1] A potassium methoxide solution was prepared dissolving 700 mg of extrapure KOH pellets (Merck, Germany) in 25 mL of methanol (Sigma Aldrich, ≥99,8% pure), whereas a 1 wt% solution of L(+) ascorbic acid was prepared dissolving 165 mg of ascorbic acid (Honeywell Fluka 99.7%) in 10 mL of methanol.

The fish oil (500 mg) in a glass flask was added with 2 mL ascorbic acid (1 wt% solution) followed by 5 mL of MeOK solution. The resulting mixture was stirred using a Vortex for 30 s after which the flask was immersed in a silicone bath at 80 °C under reflux for 30 min. The sample was rapidly cooled in an ice-water mixture and then added with 5 mL of *n*-hexane. The hexane layer was separated from the solid saponifiable fraction and eventually dried under a N_2 flux. Prior to injection, a sample of unsaponifiable fraction was brought to dryness and the residue dissolved in 600 µL of MeOH. Stock standard solutions of all five vitamins were prepared in methanol and stored at 4 °C in amber glass bottles to protect them from direct light exposure.

The UHPLC–HESI–MS system was a UHPLC (Dionex UltiMate3000 Rapid Separation LC) system by Thermo Fischer Scientific equipped with an autosampler and controlled by Chromeleon 7.2 software (Thermo Fisher, Bremen, Germany) and Dionex Softron (Germering, Germany). The UHPLC system was coupled to a Orbitrap mass spectrometer instrument (Q Exactive, Thermo Scientific), equipped with an heated electrospray (HESI) ion source. A novel method for identification of vitamin compounds was applied using the following conditions for electrospray conditions for analysis in positive ion mode of vitamins A, D_2, D_3, E and K_1: sheath gas flow rate 35 (arbitrary units); auxiliary gas unit flow rate 15 (arbitrary units); spray voltage 3.5 kV; S lens RF level 50; capillary temperature 280 °C; auxiliary gas heater temperature 300 °C. The UHPLC column was a Sinergy Hydro RP 100 Å, 150 × 2.1 mm, 2.5 µm. The column temperature was set at 25 °C and the injection volume at 1.0 μL. The mobile phase composition was the following: formic acid/water 0.1% v/v (eluent A), formic acid/ methanol 0.1% v/v (eluent B), at a flow rate of 150 μL•min^{-1}. The selected gradient was: 0–5.5 min, 35% B; 5.5–16 min, linear increase to 70% B; 16–18 min, linear increase to 78% B; 18–19 min, linear increase to 96% B; 19–44 min, hold 100% B; 46–47 min, linear decrease 55% B; 47–52 min, hold 35% B, total run time was 52 min.

The MS was operated in electrospray positive mode and the analyses were conducted in two acquisition modes: Full-Scan and SIM. The resolution power in full scan was 70,000 FWHM (at $m/z = 200$) and the scan range was 200–500 m/z.

Table. 1
Molecular mass and retention time of vitamin compounds researched in anchovy oil by UHPLC–HESI/Orbitrap mass spectrometry.

Compound	Molecular Formula	Theoretical mass, m/z $[M+H]^+$	Retention time, min
Vitamin A	$C_{20}H_{28}$	269.22638 $[M–H2O+H]^+$	23.01–23.30
Vitamin D_2	$C_{28}H_{44}O$	397.34649 $[M+H]^+$	26.92–27.18
Vitamin D_3	$C_{27}H_{44}O$	385.34649 $[M+H]^+$	27.50–28.18
Vitamin E	$C_{29}H_{50}O_2$	431.38836 $[M+H]^+$	27.89–30.15
Vitamin K_1	$C_{31}H_{46}O_2$	451.35706 $[M+H]^+$	33.15–33.40

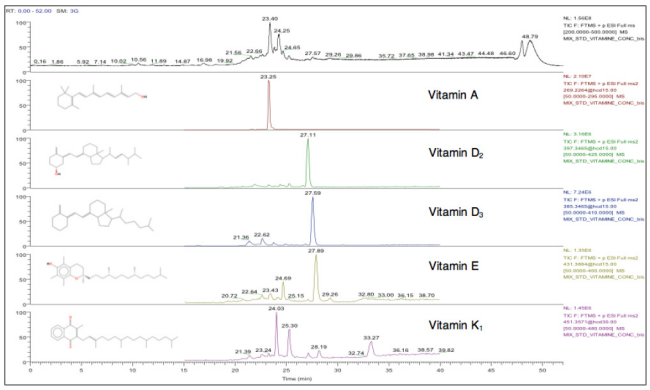

Fig. 1. UHPLC–HESI–MS(+) SIM profile of a solution containing the five vitamins of Table 1.

Scan rate was set at 1 scan•s^{-1} and the automatic gain control (AGC) target at 10^5 ions for a maximum injection time of 100 ms. For targeted SIM analyses, with a 15 s time window, a mass inclusion list containing expected retention times of target vitamin analytes was built and applied. The resolution power was 17,500 FWHM (at m/z 200) and the isolation window 2.0 m/z. Data were analyzed with Qual Browser Xcalibur 3.0 (Thermo Fisher Scientific) and identification of individual vitamin compounds supported by compound accurate mass and retention time (if a reference standard was available, see Table 1).

For LC-MS method development, all the parameters were optimized based on repetitive injections of reference standards. Standard solutions were prepared by taking into account possible matrix effects, by dissolving vitamin compounds into formic acid/ methanol 0.1% v/v. Each standard was injected 5 times consecutively, at one concentration level (namely 1 ppm). Experiment repeatability assessed through RSD% was found on average to have RSD $\leq$2.5%. In order to avoid carry over effects or artefact formation, blank runs were carried out every three analyses. The mobile phase composition for LC gradient elution was suitably varied both in terms of solvent and concentration. In general, all the parameters were tuned in order to get the best signal from the MS detector.

3. Data, value and validation

Preliminary screening of the oil by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) shows that only vitamin D_3 was present in anchovy by-product oil (data not shown). Indeed, neither the peaks for the other four vitamins corresponding to the expected retention times of five vitamins (A, D_2, D_3, K_1, and vitamin E) researched in the oil, nor their theoretical mass ions listed in Table 1 were observed. As shown in Fig. 1, using the conditions developed for the present study, all five commercial vitamins studied were nicely separated, with an overall 40 min elution time for the five vitamins.

The vitamin analysis shown in Fig. 1 at levels well above the S/N ratios is new, and required thorough optimization of the analytical parameters. For instance, the use of 0.1% formic acid at the specific gradient program in the Experimental section turned out to be of fundamental importance for improving the parameters of efficiency (peak shape) and sensitivity. In other words, the mobile phase composition was a crucial parameter affecting not only the chromatographic resolution to reach selectivity, efficiency and reproducibility but also the quantitative transfer of target analytes to the mass spectrometer through the ESI interface.

The overall amount of vitamin D_3 in anchovy oil was assessed by testing four different concentrations of this fat-soluble vitamin. The calibration lines, produced in ESI (+) mode, was linear over the 0.7–4.2 ppm range tested (correlation coefficient $r = 0.9931$).

The LC/MS chromatogram in Fig. 2 shows the expected peak at 27.59 min and two other peaks at 28.80 and 35.10 min corresponding to the fragments ions selected by ion monitoring (SIM, the MS technology which allows the mass spectrometer to detect specific compounds with very high sensitivity) with the same ion selected ($m/z = 385.34649$). Under the

Table. 2
Vitamin D_3 isomer and amount in anchovy oil extracted with *d*-limonene from fillet discards.

Compound	Molecular Formula	Accurate mass of $[M+H]^+$	Retention time, min	Concentration in oil, μg/g
Vitamin D_3 Isomer 1	$C_{27}H_{44}O$	385.34653	28.16	0.018
Vitamin D_3 Isomer 2	$C_{27}H_{44}O$	385.34592	29.82	0.035
Vitamin D_3 Isomer 3	$C_{27}H_{44}O$	385.34604	35.04	0.0285

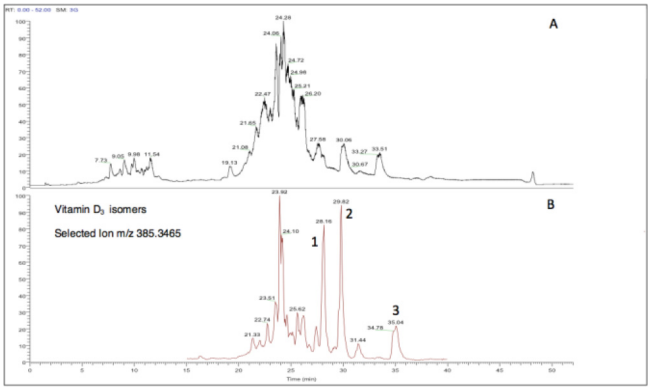

Fig. 2. A: Full scan chromatogram of unsaponifiable fraction of anchovy oil; B: SIM chromatogram of unsaponifiable fraction.

Fig. 3. Fish oil extracted from anchovy by-products with *d*-limonene exposed to Palermo sunlight on 26 April 2019. A video of the anchovy oil exposed for about 15 min to Palermo's sunlight irradiation on a clear, cloudless day as of late April 2019 is available at the URL: https://photos.app.goo.gl/Npfiemw7WcyhvG7H7.

experimental conditions applied, only a few parent ions were detected (Fig. 2). The mass spectrum of Vitamin D_3 yielded a protonated molecule $[M+H]^+$ at $m/z = 385.34612$ and a fragment $[M–H_2O+H]^+$ at $m/z = 367.33571$ as more abundant ions.

Given the limited fragmentation observed, the study of the mass spectrum suggests formation of three vitamin D_3 isomers. A new mass spectrometry study [16] aimed to investigate which vitamin D_3 isomers [17] are present in the anchovy by-product fish oil will be reported in the future. The results of recovery studies in Table 2 demonstrate good recovery capacity of the new extraction method for vitamin D_3. The sum of the quantities of the three isomers of vitamin D_3 indeed amounts to 0.0815 μg vitamin D_3 per g oil which translates into a 81.5 μg/kg content, in good agreement with the typical amounts of vitamin D_3 in fish oils (ranging from 18 to 350 μg/kg) [18].

When vitamin D_3 in solution is irradiated with sunlight, it converts to 5,6-*trans*-vitamin D_3 and suprasterol I and suprasterol II [19]. Due to the absence of conjugated double bonds in their structure, the latter suprasterol photoproducts do not absorb light above 250 nm [19]. We exposed the anchovy oil kept in a transparent glass flask to Palermo's sunlight on a clear cloudless day (Fig. 3) for about 15 min from 14:30–14:45 GMT +1 on April 26, 2019, and, indeed, the orange oil became clearer.

In general, fish and fish products are the most important food sources of vitamin D_3 [20]. Given the public health concerns arising from the concomitant global deficiency of vitamin D [12] as well as of omega-3 [21] nutrients in the population of most world's countries, it is likely that, following optimization of the method, new dietary supplements might soon use fish oil extracted from blue fish by-products via this straightforward process based on edible and health beneficial limonene

derived from waste orange peel [2,5]. The same Orbitrap technique can be used to identify impurities and contaminants out of several comprising fish oils. We therefore propose its use as a quality control tool for companies that will commercialize anchovy by-products fish oil as nutraceutical ingredient.

Acknowledgements

This study is dedicated to Dr. Giuseppina Luvarà, European Commission, Directorate General for Environment, for all she has done to promote the implementation of REACH regulation in European countries. We thank Agostino Recca Conserve Alimentari Srl (Sciacca, Italy) for kindly providing the leftovers of fresh anchovy fillets.

References

[1] G. Gencbay, S. Turhan, Proximate composition and nutritional profile of the black sea anchovy (*Engraulis encrasicolus*) whole fish, fillets, and by-products, Aquatic Food Products Technol. 25 (2016) 864–874.
[2] R. Ciriminna, A. Scurria, G. Avellone, M Pagliaro, A circular economy approach to fish oil extraction, ChemistrySelect 4 (2019) 5106–5109.
[3] R. Ciriminna, M. Lomelli, P. Demma Carà, J. Lopez-Sanchez, M. Pagliaro, Limonene: a versatile chemical of the bioeconomy, Chem. Commun. 50 (2014) 15288–15296.
[4] R. Ciriminna, F. Meneguzzo, R. Delisi, M. Pagliaro, Enhancing and improving the extraction of omega-3 from fish oil, Sustain. Chem. Pharm. 5 (2017) 54–59.
[5] R. Ciriminna, A. Scurria, A.S. Fabiano-Tixier, C. Lino, G. Avellone, F. Chemat, M. Pagliaro, Omega-3 extraction from anchovy fillet leftovers with limonene: chemical, economic and technical aspects, ACS Omega 4 (2019) 15359–15363.
[6] Joint commission on biochemical nomenclature, Pure Appl. Chem. 54 (1982) 1511.
[7] P. Biondi, J. Pepe, F. Biamonte, M. Occhiuto, M. Parisi, C. Demofonti, V. Baffa, S. Minisola, C. Cipriani, Oral calcidiol is a good form of vitamin d supplementation, Clin. Cases Miner. Bone Metab. 14 (2017) 207–208.
[8] M.F. Holick, H.K. Schnoes, H.F. DeLuca, Identification of 1,25-Dihydroxycholecalciferol, a form of vitamin D_3 metabolically active in the intestine, Proc. Natl. Acad. Sci. U.S.A. 68 (1971) 803.
[9] IARC working group on the evaluation of carcinogenic risk to humans, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, No. 100D (2012) pp.35–90.
[10] M. Wacker, M.F. Holick, Vitamin D-Effects on skeletal and extraskeletal health and the need for supplementation, Nutrients 5 (2013) 111–148.
[11] L. Saul, I. Mair, A. Ivens, P. Brown, K. Samuel, J.D.M. Campbell, D.Y. Soong, N. Kamenjarin, R.J. Mellanby, 1,25-Dihydroxyvitamin D_3 restrains $CD4^+$ t cell priming ability of $CD11c^+$ dendritic cells by upregulating expression of CD31, Front. Immunol. 10 (2019) 600.
[12] N. van Schoor, P. Lips, Worldwide Vitamin, D. Status In, in: Vitamin D, Volume : Health, Disease and Therapeutics, 2, Elsevier, Amsterdam, 2018, pp. 15–40.
[13] Y. Kaya, H. Turan, Fatty acid composition of anchovy (*Engraulis encrasicolus L.*) oil produced in sinop-turkey, J. Fisheriessciences.com. 1758 (2) (2008) 693–697.
[14] D.I. Sánchez-Machado, J. López-Hernández, P. Paseiro-Losada, High-performance liquid chromatographic determination of alpha-tocopherol in macroalgae, J. Chromatogr. A 976 (2002) 277–284.
[15] M. Stancheva, B.T. Galunska, D. Dobreva, A. Merdzhanova, Retinol, alpha-tocopherol and fatty acid content in bulgarian black sea fish species, Grasas Aceites 63 (2012) 152–157.
[16] An excellent research tool devoted to the LC-MS analysis of vitamin D is online at the URL: www.LCMSofVitaminD.com.
[17] F. Mahmoodani, C.O. Perera, G. Abernethy, B. Fedrizzi, D. Greenwood, H. Chen, Identification of vitamin D_3 oxidation products using high-resolution and tandem mass spectrometry, J. Am. Soc. Mass Spectrom. 29 (2018) 1442.
[18] Z. Coppes Petricorena, in: P.C.K Cheung, B.M Mehta (Eds.), Chemical Composition of Fish and Fishery Products In Handbook of Food Chemistry, Springer, Berlin, 2015, pp. 403–435.
[19] A.R. Webb, B.R. Decosta, M.F. Holick, Sunlight regulates the cutaneous production of vitamin D_3 by causing its photodegradation, J. Clin. Endocrinol. Metab. 68 (1989) 882–887.
[20] V. Venugopal, in: J.M Mérillon, K.G Ramawat (Eds.), Nutrients and Nutraceuticals from Seafood In Bioactive Molecules in Food, Reference Series in Phytochemistry, Springer, Cham, 2018, pp. 1–45.
[21] K.D. Stark, M.E. Van Elswyk, M.R. Higgins, C.A. Weatherford, N. Salem Jr, Global survey of the omega-3 fatty acids, docosahexaenoic acid and eicosapentaenoic acid in the blood stream of healthy adults, Prog. Lipid Res. 63 (2016) 132–152.