Quantitative Analysis of Vitamin D2 and Ergosterol in Yeast-Based Supplements Using High-Performance Liquid Chromatography with Ultraviolet Detection

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ABSTRACT: Owing to ergosterol content, after UV irradiation yeast become a well-known source of ergocalciferol (vitamin D2). Additionally, pharmaceutical yeast-based supplements may represent a suitable option for treating hypovitaminosis, especially in patients adhering to a vegan diet. Using the high-performance liquid chromatography-ultraviolet (HPLC-UV) methodology our study sought to analyse three commercially available yeast-based vitamin D2 supplements while comparing the effect of UV-C irradiation (254 nm) on yeast biomass derived from the brewing process and pure ergosterol. The two compounds were precisely separated under the described conditions in an efficient and quick manner with a retention time (Rt) of 4.152 ± 0.018 minutes for vitamin D2 and 5.097 ± 0.013 minutes for ergosterol. However, when approaching the quantitative analysis, based on our findings, it appears that the pharmaceutical supplements deviate from the declared amount of substance indicated on the label. 15 minutes of UV-C irradiation generates vitamin D2 in yeast biomass with a conversion rate of 1.78%. Also, high content of ergosterol, beside vitamin D2 formation after irradiation, may trigger the appearance of secondary products such as tachysterol.

KEYWORDS: Vitamin D2, ergosterol, yeast-based supplements, HPLC-UV.

Introduction

Vitamin D has a significant role for maintaining bone health and evidence suggests that it is also implied in regulating both innate and adaptive immune responses. Adequate circulating levels of serum 25-OH vitamin D of at least 30 ng/mL are necessary for these processes and maintaining optimal concentrations is essential, as deficient levels are correlated with a spectrum of immune-related pathologies, encompassing autoimmune conditions and infectious diseases [1].

Vitamin D comprises two primary forms: vitamin D3 (cholecalciferol), sourced from animal-derived foods or synthesized in the epidermal basal skin layer via the action of ultraviolet (UV) radiation on 7-dehydrocholesterol, and vitamin D2 (ergocalciferol), derived from plant-based sources [2].

Among the naturally rich vegetables, mushrooms are frequently regarded as a valuable source of vitamin D, particularly in the form of vitamin D2. Additionally, it is important to note that the primary natural precursor of vitamin D in fungi and yeast is ergosterol, which can undergo conversion to vitamin D2 through UV-B (280-315 nm) or UV-C (100-280 nm) irradiation [3-5].

In Romania, the prevalence of vitamin D deficiency is notably elevated, with seasonal fluctuation in serum 25-OH vitamin D levels across population regardless age or gender, and exhibiting peak concentrations in September and nadirs in March [6].

Addressing hypovitaminosis may entail recourse to sun exposure, dietary strategies such as the consumption of foods fortified with vitamin D and incorporation of foods naturally rich in vitamin D, or pharmaceutical supplementation [2].

A preferred food ingredient which has received approval from the European Food Safety Authority (EFSA) for its efficacy in enriching bakery products with vitamin D is Saccharomyces cerevisiae (S. cerevisiae) also known as baker’s yeast. The process involves the exposure of S. cerevisiae to UV-B radiation, resulting in the conversion of ergosterol to vitamin D2 [7].

Additionally, other strains, like S. cerevisiae brewing strain, are also characterized by high content of ergosterol, even after conditions of stressful fermentation, suggesting that they can be a source of vitamin D2 as well [8].

However, when addressing the avenues for therapeutic interventions in our country, the practice of fortifying food with vitamin D is not currently implemented, underscoring a notable absence of this nutritional intervention [9].

Moreover, numerous studies indicate that dietary intake alone, without concurrent sun exposure, fails to adequately fulfil the vitamin D...
requirements of numerous individuals, particularly those in high-risk populations [10].

Considering that prolonged sun exposure is limited by the risk of skin cancer [11], an important role in treating hypovitaminosis D belongs to pharmaceutical supplementation.

A special category to be considered among the demographic groups at risk for developing hypovitaminosis is represented by individuals adhering to vegetarian and vegan diets as they tend to exhibit a significantly vitamin D deficiency, compared to those consuming meat and fish [12].

As pharmaceutical supplements with vitamin D₁ originates from lanolin extracted from sheep's wool, vitamin D₂ serves as a plant-derived alternative suitable for vegan consumption [13]. In light of the preceding considerations, our study aims to employ the high-performance liquid chromatography-ultraviolet (HPLC-UV) methodology to analyse various commercially available pharmaceutical supplements containing vitamin D₂ originating from vitamin D-enriched yeast. Additionally, we seek to assess samples of UV-C exposed *Saccharomyces* yeast biomass derived from the brewing process and UV-C exposed ergosterol for comparative analysis.

**Materials and Methods**

For the pharmaceutical supplements’ analysis, we have chosen three commercially available products containing vitamin D₂. The total amount of vitamin D₂ / capsule according to the label was 5.6 µg for supplement no.1 (S1), 25 µg for supplement no. 2 (S2), and 50 µg for supplement no. 3 (S3). As claimed by the supplements’ manufacturers, the vitamin D₂’s origin is *Saccharomyces* (yeast)-based.

The following reference compounds were used: vitamin D₂ (ergocalciferol) (CAS No. 50-14-6, Sigma-Aldrich, Saint Louis, MO, USA) and ergosterol (CAS No. 57-87-4, Sigma-Aldrich, Saint Louis, MO, USA). Each reference compound was solubilized in 10 mL of ethanol at a concentration of 1 mg/mL. Dilutions were subsequently prepared to generate calibration curves for quantitative analysis.

The working samples were obtained by extracting the supplement powder from the capsules with ethanol. The samples were filtered through 0.2 µm syringe filters (Acrodisc MS Syringe Filters WWPTFE Membrane) prior to injection.

*Saccharomyces* yeast biomass derived from the brewing process was also purchased and analysed for its vitamin D₂ and ergosterol content. Approximately 4 grams were weighed for both control and UV exposed samples. The sample meant for UV exposure was irradiated at 254 nm wavelength (UV-C) for 15 minutes using a UV-lamp (254 nm) placed 10 cm above the yeast specimen.

Additionally, for comparative analysis, ethanolic solutions of 1 mg/mL ergosterol were also intended for UV exposure. The irradiation parameters remained the same as those employed for the yeast specimens.

The separation was performed on an ACQUITY Arc System. For data acquisition and processing we used the Empower 3 Software. LC separation was achieved using a CORTECS C18 column with the following characteristics: length of 50 mm, inner diameter of 4.6 mm and particle sizes of 2.7 µm. The chromatographic separation was accomplished using an isocratic mobile phase composed of 5% water (solvent A) and 95% acetonitrile (solvent B) both containing 0.1% formic acid with a flow rate of 0.8 mL/min. The volume injection of the sample was 5 µL. The separation process was operated at a column temperature of 40°C, while the samples were kept at 20°C. The maximum absorption wavelength chosen for vitamin D₂ was 265 nm, while for ergosterol it was 282 nm.

**Results**

In the conducted analysis, vitamin D₂ and ergosterol demonstrated high separation efficiency using the HPLC-UV method, a crucial aspect for accurate quantification in dietary supplements. The precision of the separation was highlighted by the distinct retention times (Rt) observed for each compound; vitamin D₂ was detected at a Rt of 4.152 ± 0.018 minutes, while ergosterol was slightly later, at 5.097 ± 0.013 minutes. This clear separation not only underscores the method's reliability but also ensures that interference between these structurally similar compounds is minimized, providing a solid foundation for further investigations into their effects (Figure 1).
The study established that the calibration curves for both vitamin D$_2$ and ergosterol exhibited linearity within specific concentration ranges, a critical factor for accurate quantification. For vitamin D$_2$, the curve was linear over the range of 0.964-494.000 µg/ml, while for ergosterol, linearity was observed from 0.966-496.000 µg/ml. The calibration equation for vitamin D$_2$ was determined to be $Y=1530X+13800$, and for ergosterol, it was $Y=843X-2800$. The high level of precision in these measurements was further underscored by the determination coefficients, with vitamin D$_2$ achieving an $R^2$ of 0.999 and ergosterol a slightly lower, yet still robust, $R^2$ of 0.999. These findings confirm the accuracy and reliability of the HPLC-UV method for quantifying these compounds in dietary supplements.

The quantitative data regarding the total amount of vitamin D$_2$ and ergosterol found by the proposed method in one capsule of pharmaceutical supplement, yeast specimens, or ergosterol samples, is presented in Table 1.

The results obtained for the vitamin D$_2$ determination were compared with the corresponding values stated on the label of the supplement.

<table>
<thead>
<tr>
<th>Product</th>
<th>Vitamin D$_2$ Label claim [µg/capsule]</th>
<th>Vitamin D$_2$ amount found by proposed method</th>
<th>% of the Label claim</th>
<th>Ergosterol amount found by proposed method</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>5.6</td>
<td>1.98 µg/capsule</td>
<td>35</td>
<td>130.50 µg/capsule</td>
</tr>
<tr>
<td>S2</td>
<td>25</td>
<td>49.45 µg/capsule</td>
<td>198</td>
<td>6.80 µg/capsule</td>
</tr>
<tr>
<td>S3</td>
<td>50</td>
<td>25.82 µg/capsule</td>
<td>52</td>
<td>159.00 µg/capsule</td>
</tr>
<tr>
<td>Brewing yeast (control)</td>
<td>-</td>
<td>2.16 ng/mg</td>
<td>-</td>
<td>262.26 ng/mg</td>
</tr>
<tr>
<td>UV-C exposed brewing yeast</td>
<td>-</td>
<td>2.47 ng/mg</td>
<td>-</td>
<td>244.85 ng/mg</td>
</tr>
<tr>
<td>UV-C exposed ergosterol</td>
<td>-</td>
<td>15.36 µg/mg</td>
<td>-</td>
<td>65.48 µg/mg</td>
</tr>
</tbody>
</table>

Considering that the range of tolerance values for vitamins recommended by the European Commission [14] is comprised between 80-150% of the declared amount, unfortunately none of our analysed supplements falls within the approved limits.
Vitamin D$_2$ was found in both analysed specimens of brewing yeast, with a value of 2.16 ng/mg in the control sample, and 2.47 ng/mg in the UV-C-exposed sample (Figure 2).

Considering the total amount of the vitamin and the consumption rate of ergosterol that occurs after irradiation, we can appreciate the conversion rate of ergosterol into vitamin D$_2$ in *Saccharomyces* yeast biomass derived from the brewing after 15 minutes of UV-C exposure as being 1.78%.

When analysing the UV spectra registered at 265 nm for the UV-C exposed ergosterol sample, a secondary peak is observed next to the one of vitamin D2.
After searching in the literature [15,16], we concluded that the secondary peak must belong to tachysterol.

As shown in Figure 3, vitamin D₂ and ergosterol are clearly identified with retention times of 4.152 ± 0.018 and 5.097 ± 0.013 minutes, respectively, while tachysterol, a product of ergosterol irradiation, emerges as a new peak, indicating its formation. This visualization underscores the transformation of ergosterol to both vitamin D₂ and tachysterol upon exposure to UV light.

**Discussions**

Nutritional yeasts, recognized for their substantial nutritional content, serve as a valuable source of essential amino acids such as lysine and methionine, which are typically limited in conventional dietary sources. These yeasts, subjected to heat deactivation during production to preserve their nutritional components, offer a broad spectrum of nutrients and boast a favourable carbohydrate to protein ratio. The methods employed in their harvesting, drying, and processing significantly influence the resultant product's nutritive quality [17].

According to the United States Food and Drug Administration (FDA), *Saccharomyces cerevisiae* cell wall and its fractionated products are generally acknowledged as safe [18].

Brewer's yeasts and baker’s yeasts represent specialized strains of *Saccharomyces cerevisiae* extensively employed in food processing applications. Renowned for their high content of amino acids, peptides, proteins, B-complex vitamins, lipids and trace minerals, these strains hold a pivotal position within the pharmaceutical industry due to their diverse biochemical profile. Furthermore, the biomass derived from brewer's and baker's yeasts exhibits low levels of sodium and fats, while being devoid of components that may be intolerable or contraindicated for certain individuals, such as sugar and gluten [17,19].

The synthesis of vitamin D₂ in yeast presents a notable advantage due to its straightforward approach. In the manufacturing of dietary yeast-based vitamin D₂, a singular final step is necessary and it involves UV exposure to facilitate the conversion of ergosterol into vitamin D₂ [20].

Although the UV-treatment of regular bread containing baker's yeast as an ingredient failed in increasing serum 25-OH vitamin D levels, the reason being attributed to limitations in vitamin bioavailability after the bakery process [21], the heightened ergosterol content of yeast still renders it suitable for the production of pharmaceutical supplements [20].

However, when it comes to quantification of vitamin D₂ from yeast-based supplements, our study has found some content-related inconveniences. Among the three pharmaceutical supplements analysed, none of them was in line with the declared vitamin amount.

Our study corroborates findings from other investigations, which have similarly observed the presence of content-related discrepancies in various vitamin D supplements available on diverse European pharmaceutical markets [22,23].

Within the European Union (EU), food supplements fall under the regulatory framework for foods by the Directive 2002/46/EC, with producers bearing responsibility for ensuring their quality. Although businesses involved in the production of food supplements in the EU must adhere to Hazard Analysis Critical Control Points (HACCP) guidelines, there exists no obligatory adherence to good manufacturing practices (GMP).

Moreover, only vitamins and minerals outlined in Annex I and II of the aforementioned Directive have minimum and maximum tolerance values and levels are determined through the procedure of the Standing Committee on Food Chain and Animal Health [22,24,25].

In the domain of regulating vitamin content, adherence to specified tolerances is imperative. According to guidance document provided by European Commission [14], the declared values must align with upper and lower tolerance limits, constituting a range of +50% and -35% relative to the declared amount, encompassing the uncertainty of measurement within the specified bounds. This mandates that the vitamin content falls within the scientifically stipulated range of 80-150% [14].

The underexplored potential of *Saccharomyces* yeast biomass, sourced from brewing processes, has evaded commercial attention despite its inherent value. Predominantly relegated to animal feed applications, its disposal can pose significant environmental concerns. Nonetheless, its versatility as a biomass resource holds promise for diverse applications. Biotechnological investigations have begun to probe its potential, encompassing its role in fermentative pathways for the synthesis of high-value compounds like ethanol, as well as its utility as a substrate for microbial cultivation and compound extraction. Moreover, there exists an interest in harnessing yeast constituents, including nucleotides, nucleic acids, and cell wall polysaccharides for industrial exploitation.

Of particular note is the prospect of brewer's yeast cells from the brewing industry to produce food-grade yeast extract, thereby offering novel avenues for flavour enhancement in food products [26].

Our study did find vitamin D₂ content in brewer yeast, and although the quantity is scarce, we concluded that 15 minutes of irradiation to 254 nm
can increase the content with 1.78% owing to conversion of the ergosterol into the vitamin. This result can suggest brewer yeast as a potential source of vitamin D₂ synthesis, probably with a prolonged irradiation time.

The Rt registered in our research were $4.152 \pm 0.018$ minutes for vitamin D₂ and $5.097 \pm 0.013$ minutes for ergosterol. Other Rt registered in literature when using the HPLC-UV method exhibit considerable variability, such as 8.2 minutes [27], 10.3 minutes [28], 16.5 minutes [29] for vitamin D₂ and 4.5 minutes [30], 7.0 minutes [31], 14.13 minutes [32] for ergosterol, the observed variability being likely attributable to distinctions in chromatographic conditions. Considering this, we can appreciate that our proposed method may be an efficient and quick procedure for vitamin D₂ and ergosterol determination.

"Overirradiation products" refer to secondary products that may arise following UV exposure of substances containing precursors of vitamin D. Among these, notable compounds include tachysterol and lumisterol [33].

The determination of tachysterol involved the comparison of the UV spectra from literature [15,16] and also the consideration of the wavelength range of UV utilized. Notably, UV lamps emitting wavelengths predominantly below 295 nm, particularly shorter wavelengths ranging from 248 to 254 nm, favour tachysterol production, whereas emissions exceeding 295 nm tend to yield more lumisterol [15,33].

In our research, tachysterol was observed only in the UV-exposed ergosterol samples. Structurally resembling vitamin D, tachysterol exhibits a rotated A-ring by 180 degrees and a shift in the triene region, facilitating the positioning of double bonds within both the A- and C-rings. Despite its structural similarity, tachysterol exhibits minimal chemical reactivity and boasts a substantial extinction coefficient. Consequently, its physiological role is postulated to impede excessive UV-induced previtamin D formation. Tachysterol demonstrates heightened photoproduction compared to vitamin D, thus serving as a principal alternative degradation pathway for vitamin D [33].

Nevertheless, scant information regarding the biological activity of tachysterol is available. It is generally characterized as chemically inert with negligible biological effects, although comprehensive risk assessments have only marginally considered this aspect in EFSA opinions [33].

The HPLC-UV method employed in our study for the determination of vitamin D₂ has been widely utilized in the assessment of various types of supplements. This methodology offers the distinct advantage of being both simpler and cost-effective [24].

**Conclusion**

Commercially available vitamin D supplements might suffer inaccuracies when it comes to the declared vitamin amount, and although the problem was reported in numerous other studies, it continues to be encountered. Among natural sources of vitamin D₃, *Saccharomyces* yeast biomass derived from the brewing process may be an option, and despite the low amount reached per one-gram, prolonged UV-C exposure might be the solution for increasing content.

In addition to vitamin D₂, high concentration of ergosterol content may trigger the formation of other secondary compounds, tachysterol being predominantly seen after irradiation at 254 nm wavelength.

**Conflict of interests**

None to declare

**References**


