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ARTICLES

Detection and Quantification of Provitamin D_2 and Vitamin D_2 in Hop (Humulus lupulus L.) by Liquid Chromatography–Diode Array Detection–Electrospray Ionization Tandem Mass Spectrometry

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In this work, ergosterol and ergocalciferol were identified for the first time in hop. In addition, in this article, a simple and reliable analytical methodology for analysis of these compounds in different commercial forms of hop is presented. The performance of the method was assessed by the evaluation of parameters such as absolute recovery (higher than 70%), repeatability (lower than 3 %), linearity ($r^2 > 0.9988$) and limits of detection (ranging from 0.034 for ergocalciferol to 0.058 mg/L for ergosterol) and quantification (ranging from 0.113 for ergocalciferol to 0.195 mg/L for ergosterol). On the basis of standard additions applied with the optimized procedure and high-performance liquid chromatography with diode array detection, it appears that the Nugget hop plant (crop 2006) contains 1.84 ± 0.09 μg/g of ergosterol and 1.95 ± 0.05 μg/g of ergocalciferol. The identity of the compounds was confirmed by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry in the positive ion mode. The presence of ergosterol here reported should have great potential for the assessment of hop as related to the fungal contamination proportion and hence the quality of this raw material.

KEYWORDS: Hop; sterols; ergosterol; ergocalciferol; vitamin D; HPLC-DAD; HPLC-MS/MS

INTRODUCTION

The hop plant (*Humulus lupulus L.*) is a dioecious plant of the Cannabacea family, cultivated in most temperate zones of the world (*1*). The major market for this perennial climbing plant is based on its female inflorescence, which has been used for centuries in the brewing industry. Resins, sequestered in lupulin glands within the cones, contain different types of molecules, such as α-acids, which contribute to the bitter taste and the aroma of beer (*1–3*).

Growing hops are liable to attack by both fungi and viruses. Severe attacks can result in the partial or complete loss of a crop and those hops that are salvaged will probably receive a low valuation (*4*). Pesticide treatment of hops is strictly controlled. Fungicide use is heavily restricted because the presence of these chemicals in the cones results in the crop being unsuitable for sale to the brewing industry. The most widespread pathologies are caused by the fungus *Pseudoperonospora humuli* and *Podosphaera macularis*, responsible for downy mildew and powdery mildew, respectively (*2, 4, 5*). Downy mildew is most severe in areas of heavy spring rainfall, when the combination of moisture and temperature (15–25 °C) are favorable for infection and disease development. However, at least 30 different fungal species are linked to hop diseases or disorders (*2*).

Ergosterol, belonging to the sterol family, is a cell membrane component largely restricted to fungi, thus making it an ideal index molecule for these micro-organisms (*6*). Selected species of the order *Peronosporales*, which are unable to epoxidize squalene and thus synthesize sterols, are able to metabolize exogenous cycloartenol to lanosterol and in some organisms to fucosterol, ergosterol, and cholesterol (*7*). However, ergosterol can also be present as a minor constituent in higher plants, suggesting that can be used as a measure of fungal growth in plant materials (*8*). Plants have a variety of more than 40 well-identified and studied sterols, which are termed phytosterols and are predominantly present in oilseed plants. The most abundant phytosterols are sitosterol, campesterol, and stigmasterol. Other phytosterols like avenasterol and cycloartenol are synthesized

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earlier in the biosynthetic pathway and as sterol precursors, the phytosterols usually occur in relatively small amounts. Phytosterols are, with respect to their physiological function and their chemical structure, similar to the major and only animal-produced sterol, cholesterol (*5, 9, 10*).

Ergosterol (provitamin D_2) in plant tissue is converted to ergocalciferol (vitamin D_2) by UV irradiation and it is assumed to have the same biological activity as vitamin D_3. Intake of an adequate amount of vitamin D is essential in preventing rickets in children and osteomalacia in adults. Vitamin D_2 is the form that has been generally used in food and pharmaceutical supplementation (*11*). In nature, food sources of vitamin D_2 are very limited (*9*). A few plants, such as wild mushrooms, are known to contain small amounts of vitamin D_2 (*11, 12*).

A survey on the literature will show that there are several methods available for the analysis of ergosterol and ergocalciferol. However, high-performance liquid chromatography (HPLC) and gas chromatography (GC) are currently the most common techniques used for analysis of these compounds (*6, 8, 11, 13–15*). Limitations caused by incomplete resolution of the different molecular species in complex mixtures can be overcome by coupling chromatographic separation to mass spectrometry (*6, 13, 14*). Because of the high selectivity and sensitivity of MS detection, both gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI–MS) have been applied in ergosterol and ergocalciferol analysis (*6, 13, 14*). However, until nowadays, electrospray ionization technique (ESI) has not been applied very much in the study of these compounds.

The aim of the present work was the development and optimization of a suitable methodology for the identification and quantification of ergosterol and ergocalciferol in hop plant by high-performance liquid chromatography with diode array detection (HPLC–DAD) and tandem mass spectrometry using electrospray ionization (HPLC–ESI–MS/MS). This methodology was applied to investigate the presence of ergosterol and ergocalciferol in different varieties of hop plant and different forms of commercial hop products (pellets type 45, ethanolic extract, and supercritical CO_2 extract). To the best of our knowledge, the presence of these compounds in hop has never been reported.

MATERIALS AND METHODS

Chemicals. Ergosterol (≥95%) and ergocalciferol (≥98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetic acid (glacial) 100% anhydrous and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Petroleum ether (80–100 °C) and potassium hydroxide were purchased from Pronalab (Lisbon, Portugal). High-purity water from a Millipore Simplicity 185 water purification system (Millipore Iberian S.A., Madrid, Spain) was used for all chemical analyses and glassware washing.

A stock standard solution (ca. 500 mg/L) of each compound was prepared in methanol/acetic acid (99:1, v/v). An intermediary mixed standard solution was prepared by diluting the stock standard solutions in methanol to give a concentration of ca. 50 mg/L for each compound. All standard solutions were stored in the dark at 4 °C.

Raw Materials. *Premiant* (bittering type, crop 2006) and *Saaz* (aroma type, crop 2006) varieties of hop plant were a kind gift from the Hop Research Institute (Zatec, Czech Republic). *Strisselspalter* (Alsace, France, aroma type, crop 2006) was kindly supplied by UNICER–Bebidas de Portugal SGPS S.A. *Nugget* (bittering type, crop 2006) variety was kindly supplied by the Bralupulo company (Bragança, Portugal).

Three commercial hop products from different origins commonly used by brewers were also used in this work: a commercial ethanol extract produced from hops (*Hallertauer Taurus*,crop 2004) using pure fermentation alcohol (Hopsteiner, Germany), a commercial supercritical CO_2 extract (*Warrior*, crop 2004), which is obtained under high-pressure and low temperature (John I. Haas, USA), and commercial pellets type 45 (Hopsteiner, Germany) from an aroma hop variety (*Strisselspalter*, crop 2006).

Sample Preparation. The content of vitamin D in most food samples is generally low. Vitamin D is found in very few food sources, including milk (fortified), cheese, eggs, liver, salmon, and fortified margarine. However, this type of vitamin is usually found in the presence of larger amounts of other substances (e.g., sterols, triglycerides, phospholipids) that may cause interference (*15*). The majority of the methods for determination of ergosterol and ergocalciferol consist of three main steps: extraction from the sample, clean up of the extract, and subsequent analysis by HPLC.

The method for extraction and quantification of ergosterol and ergocalciferol in the different hop matrices was based on that developed by Seitz et al. (*16*) with some modifications. The different hop plant varieties and hop pellets were coarsely ground in a mortar and pass through a No. 20 sieve (0.85 mm, Cole-Parmer, USA) whereas the hop extracts were directly extracted with methanol. An aliquot (2.0 g) was extracted with 25.0 mL of methanol for 15 min in a blender and filtered through a filter paper Whatman 40. The residue in the filter paper was transferred to a blender to which was added 25.0 mL of methanol, and the mixture was shaken for 15 min. The mixture was filtered again, and the combined extracts were stirred by vortexing. The clear light green methanolic extract (50.0 mL) was then saponified with 1 g of potassium hydroxide (KOH) during the night (12 h) at room temperature, under slow constant stirring in an automatic shaker. The mixture was then transferred to a 100 mL separating funnel to which was added 50.0 mL of petroleum ether. After being shaken (2 min.), the mixture was allowed to settle and the upper petroleum ether fraction was collected. This process was repeated twice with 50.0 mL of petroleum ether and this solvent was evaporated to dryness using a rotary evaporator at room temperature. The residue was redissolved in 10.0 mL of pure methanol and filtered through a 0.45 μm regenerated-cellulose syringe filter (Schleicher & Schuell, Microscience), and an aliquot of the solution was injected into the HPLC for the analysis.

As the sample preparation process for vitamin analysis has a direct impact on accuracy, precision, and quantification limits and is often the rate-determining step for many analytical methods, the optimization of the methodology described above was fundamental in this work. For the method optimization, other procedures were tested as follows: the volume of methanol used on the extraction was studied by using 25.0 and 50.0 mL and a two-stage extraction with 25.0 mL; overnight cold saponification and hot saponification at 70 °C were also compared; finally, to verify the yield of the liquid–liquid extraction with petroleum ether, we tested different number of extraction steps.

HPLC–DAD Analysis. The HPLC system (Jasco Corporation, Tokyo, Japan) consisted of a low pressure quaternary gradient unit (model LG-1580–04) with an in-line DG-1580-54 degasser and a model AS-950 auto-sampler. The system is equipped with a photodiode array detector (model MD-1510 UV/vis multiwavelength detector). Separations were achieved on a LiChrocart (Merck, Darmstadt, Germany) RP-C_{18} column (125 mm × 3.0 mm, 3 μm) using an isocratic mobile phase consisting of methanol/water (98/2, v/v) at a flow rate of 0.4 mL/min. A guard column (LiChrocart RP-C_{18} 4.0 mm × 4.0 mm, 5 μm) was placed in front of the analytical column. The selected detection wavelengths of ergosterol and ergocalciferol were 280 and 264 nm, respectively. A total of 100 μL was injected into the column kept at room temperature. At these conditions, ergocalciferol eluted at 13.7 min and ergosterol at 17.3 min (**Figure 1**). Analytes in each sample were identified by comparing their retention times and UV–vis spectra with those of authentic compounds. Peak purity was checked to exclude any contribution from interfering peaks.

HPLC–ESI–MS/MS Analysis. Identification of ergosterol and ergocalciferol in samples was confirmed by HPLC coupled online with electrospray ionization (ESI) tandem mass spectrometry. The HPLC system (Finnigan-Thermo Electron Corporation, San Jose, CA) consisted of a low-pressure quaternary pump (model Finnigan Surveyor Plus) and an auto-sampler (model Finnigan Surveyor Plus with 200-

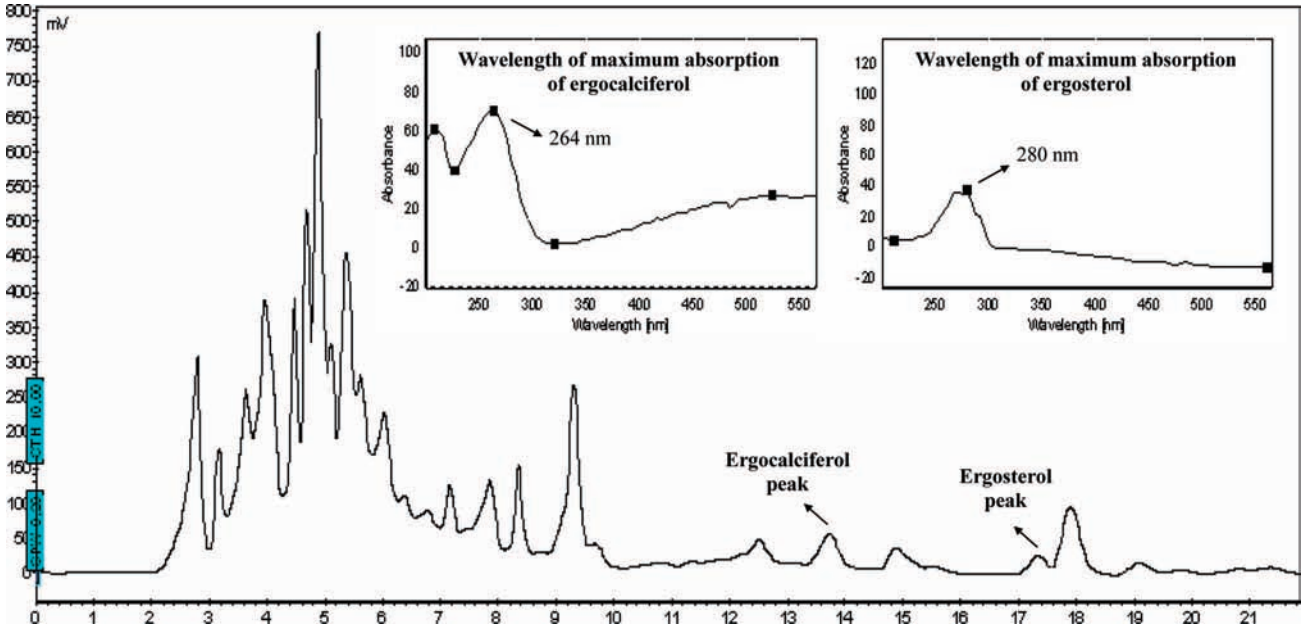

Figure 1. Chromatogram obtained in the HPLC–DAD analysis of a hop plant extract (variety *Nugget*). Inset: UV spectrum of ergocalciferol and ergosterol.

Table 1. Recovery Factors (%) of Ergosterol and Ergocalciferol According to the Volume of Solvent (Methanol) Used for the Extraction from the Hop Plant ($n = 2$)[a]

	hop spiked with ergosterol			hop spiked with ergocalciferol		
params	assay 1	assay 2	assay 3	assay 1	assay 2	assay 3
calibration curve equation (from direct injection; $y = a + bx$)		$y = 0.08 + 2.09\,x$			$y = 0.18 + 3.89\,x$	
standard addition curve equation ($y = a + bx$)	$y = 0.79 + 1.50\,x$	$y = 0.81 + 1.56\,x$	$y = 0.84 + 1.63\,x$	$y = 0.88 + 2.39\,x$	$y = 0.95 + 2.65\,x$	$y = 0.98 + 2.76\,x$
recovery factor (average ± standard deviation; %)	70.9 ± 1.3	73.4 ± 0.7	76.8 ± 1.0	61.1 ± 0.9	67.9 ± 0.7	70.3 ± 0.7

[a] Assay 1, one single extraction with 25.0 mL; assay 2, one single extraction with 50.0 mL; assay 3, two-step extraction with 25.0 mL.

vial capacity sample). Separations were achieved on a LiChrocart (Merck, Darmstadt, Germany) RP-C_{18} column (125 mm × 3.0 mm, 3 μm) using an isocratic mobile phase consisting of methanol/water (98/2, v/v) at a flow rate of 0.4 mL/min. An aliquot of the total extract (25 μL) was injected into the column kept at room temperature.

A quadropole ion trap mass spectrometer (Finnigan LCQ Deca XP Plus, San Jose, CA) equipped with a ESI source in the positive ion mode and Xcalibur software version 1.4 (Finnigan, San Jose, CA) were used for data acquisition and processing.

Optimal operating parameters of the ESI interface and quadropole/ion trap were found by infusing standard solutions of ergosterol and ergocalciferol in the mobile phase at 3 μL/min using a Finnigan syringe pump. The optimum conditions of the interface were applied as follows: source voltage, 5.0 kV for ergosterol and 6.0 kV for ergocalciferol; source current, 80.0 μA; capillary voltage, 15.0 V; capillary temperature, 275 °C; tube lens offset, −15 V; sheath gas (N_2) flow rate at 80 arbitrary units; auxiliary gas (N_2) flow rate at 20 arbitrary units; collision energy for fragmentation, 30 V. During the chromatographic run, mass spectra of the eluate were recorded from m/z 0 to m/z 1500 and fragmentations experiments were carried out on eluting substances. For structure elucidation, the pseudomolecular ions were fragmented by CID (collision-induced dissociation). Nonspecific fragmentation of all ions in the ion source was achieved by collision with air molecules during the acceleration between ion source and trap (source CID). Fragmentation of selected ions was achieved by induced collision of the preisolated ion with the nitrogen (N_2) damping gas in the ion trap (trap CID).

RESULTS AND DISCUSSION

Determination of Ergosterol and Ergocalciferol in Different Forms of Hop. *(a) Method Optimization.* For an accurate quantification of ergosterol and ergocalciferol in the different forms of hop it was necessary to take into consideration any losses of the compounds that can occur during the experimental procedure. For these experiments, only one variety of hop plant was used (Nugget, bittering type, crop 2006), since there is no significant differences of the matrices. The recovery factors were calculated by standard addition from reverse phase HPLC/DAD. The hop plant was spiked with ergosterol and ergocalciferol standards at different levels ranging from 0.5 to 2.5 mg/L.

Volume of Solvent (Methanol) Used for the Extraction of Ergosterol and Ergocalciferol. The volume of methanol used on the extraction was tested by using 25.0 (assay 1), 50.0 mL (assay 2), and a two-stage extraction with 25.0 mL (assay 3). Duplicate analyses were performed for each volume of solvent. The other experimental conditions used on the extraction of ergosterol and ergocalciferol from the hop plant were the same as those described in the sample preparation section. As shown in **Table 1**, 50.0 mL of methanol seems to be almost sufficient for the extraction of both compounds from the different forms of hop products. However, as can be seen in **Table 1**, a two-step solid/liquid extraction with 25.0 mL of methanol (assay 3)

Table 2. Recovery Factors (%) of Ergosterol and Ergocalciferol According to the Saponification Conditions of the Hop Plant ($n = 2$)

	hop spiked with ergosterol		hop spiked with ergocalciferol	
params	hot saponification (70 °C)	overnight cold saponification	hot saponification (70 °C)	overnight cold saponification
calibration curve equation (from direct injection; $y = a + bx$)	$y = 0.16 + 2.04\,x$		$y = 0.38 + 3.78\,x$	
standard addition curve equation ($y = a + bx$)	$y = 0.65 + 1.29\,x$	$y = 0.84 + 1.63\,x$	$y = 0.70 + 2.04\,x$	$y = 0.98 + 2.76\,x$
recovery factor (average ± standard deviation; %)	63.2 ± 1.0	76.8 ± 2.1	54.1 ± 0.9	70.3 ± 0.8

Table 3. Recovery factors (%) of ergosterol and ergocalciferol according to the number of liquid/liquid extractions in the sample preparation of the hop plant ($n = 2$)[a]

	hop spiked with ergosterol			hop spiked with ergocalciferol		
params	assay 1	assay 2	assay 3	assay 1	assay 2	assay 3
calibration curve equation (from direct injection; ($y = a + bx$)	$y = 0.19 + 2.04\,x$			$y = 0.35 + 3.79\,x$		
standard addition curve equation ($y = a + bx$)	$y = 0.78 + 1.46\,x$	$y = 0.83 + 1.59\,x$	$y = 0.84 + 1.60\,x$	$y = 0.88 + 2.39\,x$	$y = 0.97 + 2.76\,x$	$y = 0.93 + 2.78\,x$
recovery factor (average ± standard deviation; %)	69.8 ± 1.3	77.2 ± 1.9	77.8 ± 0.4	63.7 ± 0.3	69.8 ± 2.8	70.3 ± 1.0

[a] Assay 1, one single extraction with 50.0 mL; assay 2, two-step extraction with 50.0 mL; assay 3, three-step extraction with 50.0 mL.

increases the recovery factors for 2–4%. Usually, a better recovery is obtained by using two equal volumes of solvent than by using one larger volume of solvent.

Saponification Conditions of Ergosterol and Ergocalciferol Esters. Saponification has been used almost exclusively as the initial extraction step for analysis of ergosterol and ergocalciferol. Alkaline hydrolysis or saponification is an efficient procedure for removing neutral lipids, mainly triglyceride components of food matrices. The hydrolysis reaction attacks ester bonds and releases the fatty acids from the glycerol or glycerides and phospholipids, and from esterified sterols and carotenoids. This reaction also frees indigenous vitamin D from any combined form that may exist (e.g., lipoprotein complexes) (*15*).

To study the influence of alkaline hydrolysis or saponification conditions on the recovery of ergosterol and ergocalciferol, we tested two different kinds of saponification: overnight cold saponification and hot saponification at 70 °C. Overnight cold saponification consists of the sample treatment (50 mL of sample) with 1 g of KOH during the night (12 h) at room temperature, under slow constant stirring in an automatic shaker. Hot saponification consists of the saponification of 50 mL of sample with 1 g of KOH under reflux at 70 °C for about 30 min. Duplicate analyses were performed for each test. The other experimental conditions used on the extraction of ergosterol and ergocalciferol from the hop plant were the same as described in the sample preparation section.

As shown in **Table 2**, the recovery factors of the studied compounds after the overnight cold saponification (70.3 ± 0.8% for ergocalciferol and 76.8 ± 2.1% for ergosterol) were higher than that found after hot saponification (54.1 ± 0.9% for ergocalciferol and 63.2 ± 1.0% for ergosterol). The observed increase in ergosterol and ergocalciferol recovery factors was already expected, because the use of high temperatures during the saponification step may result in the degradation of these compounds (*15*). As we said before, overnight cold saponification consists of the sample treatment with ethanolic or methanolic KOH at room temperature, under slow constant stirring for about 12 h. This procedure prevents thermal isomerisation of vitamin D to previtamin D that could occur in hot saponification (*15*, *17*). In agreement with S. Perales et al., isomerisation losses of less than 5% could occur under cold conditions versus about 10–20% under hot conditions (*15*).

Liquid–Liquid Extraction. Too concentrate and clean up the extract from the saponified digest, solid-phase extraction is the most common approach. Other less commonly applied purification procedures use semipreparative LC and liquid–liquid extraction (*12*). In this work, the liquid–liquid extraction was chosen for the sample clean-up.

Once saponification has been completed, the unsaponifiable fraction is usually extracted with organic solvents that are not miscible in the aqueous phase, mainly *n*-hexane and other solvents such as petroleum ether, diethyl ether, pentane, or mixtures of these substances. However, some authors have preferred diethyl ether and petroleum ether because they are less prone to form emulsions (*18*). In this work, petroleum ether (80–100 °C) was used because it is cheaper than diethyl ether. To verify the yield of the liquid–liquid extraction with petroleum ether, we tested different numbers of extraction steps: one single extraction with 50.0 mL (assay 1), a two-step extraction with 50.0 mL per step (assay 2), and a three-step extraction with 50.0 mL per step (assay 3). Duplicate analyses were performed for each test. The other experimental conditions used on the extraction of ergosterol and ergocalciferol from the hop plant were the same as those described in the sample preparation section. As can be seen in **Table 3**, a two-step liquid–liquid extraction with 50.0 mL of petroleum ether (assay 2) increases the recovery factors for 6–7%. A third extraction (assay 3) seems to be unnecessary because the recovery factors (70.3 ± 1.0% for ergocalciferol and 77.8 ± 0.4% for ergosterol) were not significantly different than those obtained with two extractions (69.8 ± 2.8% for ergocalciferol and 77.2 ± 1.9% for ergosterol). Because the distribution coefficient (K) is a ratio, unless K is very large, not all of a soluble will reside in the organic layer in a single extraction. Usually, a better recovery is obtained by using two or three extractions of the aqueous layer with organic solvent than the recovery that would be obtained using only one single large extraction.

(b) Method Performance. Different commercial forms of hop (hop plant, pellets, ethanolic extract, and supercritical CO_2

Table 4. Repeatability, Recovery Factor (%), Standard Addition Curve Equation, Correlation Coefficient (r^2), Limit of Detection (LOD) and Quantification (LOQ) of the Developed Methodology for the Determination of Ergosterol and Ergocalciferol in Hop Plant Samples (*Nugget* variety; $n = 2$)

compd	repeatability [a] C.V. (%)	recovery factor (%)	standard addition curve ($y = a + bx$)	correlation coefficient (r^2)	LOD (mg/L)	LOQ (mg/L)
ergosterol	3.0	76.7 ± 2.9	$y = 0.82 + 1.62\,x$	0.9988	0.058	0.195
ergocalciferol	2.2	70.3 ± 1.9	$y = 1.00 + 2.75\,x$	0.9990	0.034	0.113

[a] Coefficient of variation (C.V.), or relative root mean square, is the square root of the appropriate mean square from the analysis of variance, divided by the mean and multiplied by 100.

Table 5. Ergosterol and ergocalciferol contents (average $\pm$ standard deviation; μg/g) in the different commercial forms of hop ($n = 2$).

	commercial hop products						
compd	hop plant (*Premiant*)	hop plant (*Saaz*)	hop plant (Strisselspalter)	hop plant (*Nugget*)	pellets type 45	ethanolic extract	CO_2 extract
ergosterol (μg/g)	<LOD	<LOD	<LOD	1.84 ± 0.09	<LOD	<LOD	<LOD
ergocalciferol (μg/g)	<LOD	<LOD	<LOD	1.95 ± 0.05	<LOD	<LOD	<LOD

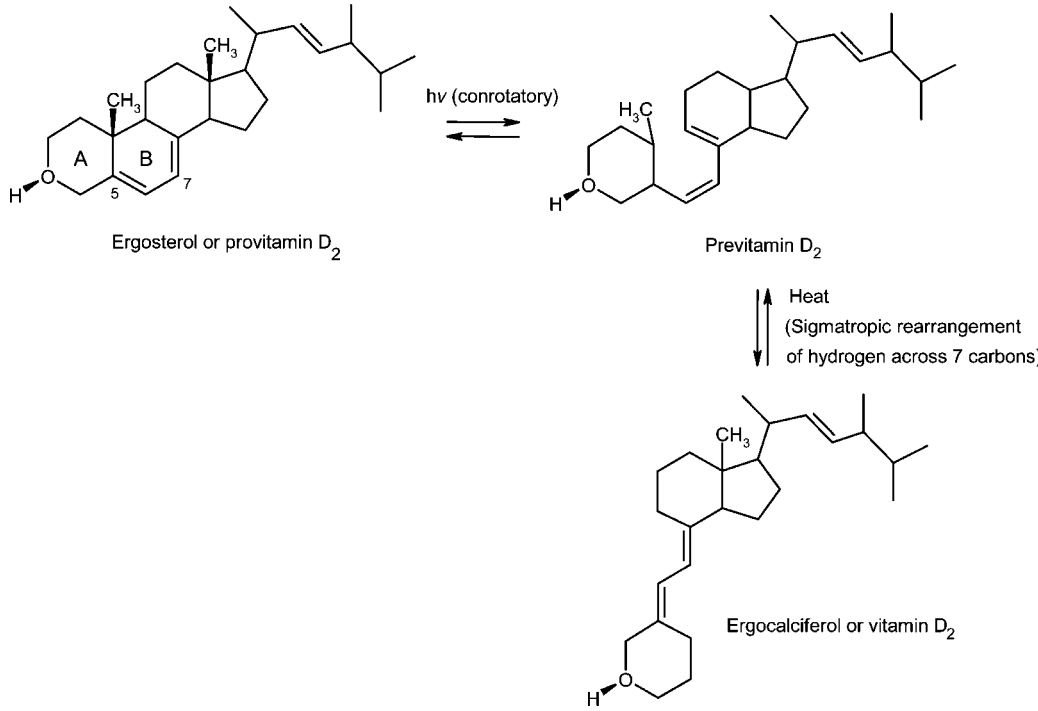

Figure 2. Conversion of ergosterol (provitamin D_2) to ergocalciferol (vitamin D_2) (*12*).

extract) were examined in order to study the matrix effect. These hop samples were spiked at a level of 0.5 mg/L (in duplicate), extracted, and analyzed following the previously described experimental procedure. No significant differences on ergosterol and ergocalciferol recovery at the 95% probability level ($\alpha = 0.05$) were found for the different hop samples spiked at the same level (data not shown). After these results, it is seems to be that the quantification process is not affected by the differences in hop matrices and method calibration can be performed by spiking different hop samples.

Hop plant (*Nugget* variety) was also spiked at different levels ranging from 0.5 to 2.5 mg/L (4 standard additions) to construct method calibration lines by plotting the instrument response as a function of the increment of the standard (**Table 4**).

Quality parameters such as recovery values, repeatability and limits of detection (LOD) and quantification (LOQ) were evaluated (**Table 4**). For this purpose, hop plant samples (*Nugget* variety) were previously fortified with ergosterol and ergocalciferol (0.5 mg/L) and treated following the described experimental conditions. Analysis of some unspiked hop samples gave a response at the retention time of the compounds studied in this work; their contributions to the blank were subtracted to estimate spiking recoveries. The recovery and repeatability of the method was assessed by analyzing six spiked hop samples in the same day (ca. 0.5 mg/L) of each compound. The coefficient of variation (C.V. %) was lower or equal than 3%, showing a closeness of the agreement between the results of successive measurements. On the other hand, absolute recoveries, those measured against ergosterol and ergocalciferol standard solutions directly injected into the chromatographic column, are higher than 70% for both compounds. The lowest recovery was found for ergocalciferol ($70.3 \pm 0.8\%$), which is known to be more sensitive to degradation during the sample preparation (*12, 15*).

LOD and LOQ were evaluated on the basis of the signal obtained in the analysis of unfortified hop samples ($n = 4$), following the recommendations of the American Chemical Society (*19*). LOD and LOQ were expressed, respectively, as the concentration of the analyte that produced a signal-to-noise ratio of three and ten (**Table 4**). The LOD and LOQ were slightly lower for the determination of ergocalciferol than for ergosterol, showing that the developed methodology has a better sensitivity for ergocalciferol.

(c) Application of the Methodology to the Analysis of Ergosterol and Ergocalciferol in Hop Samples. The optimized

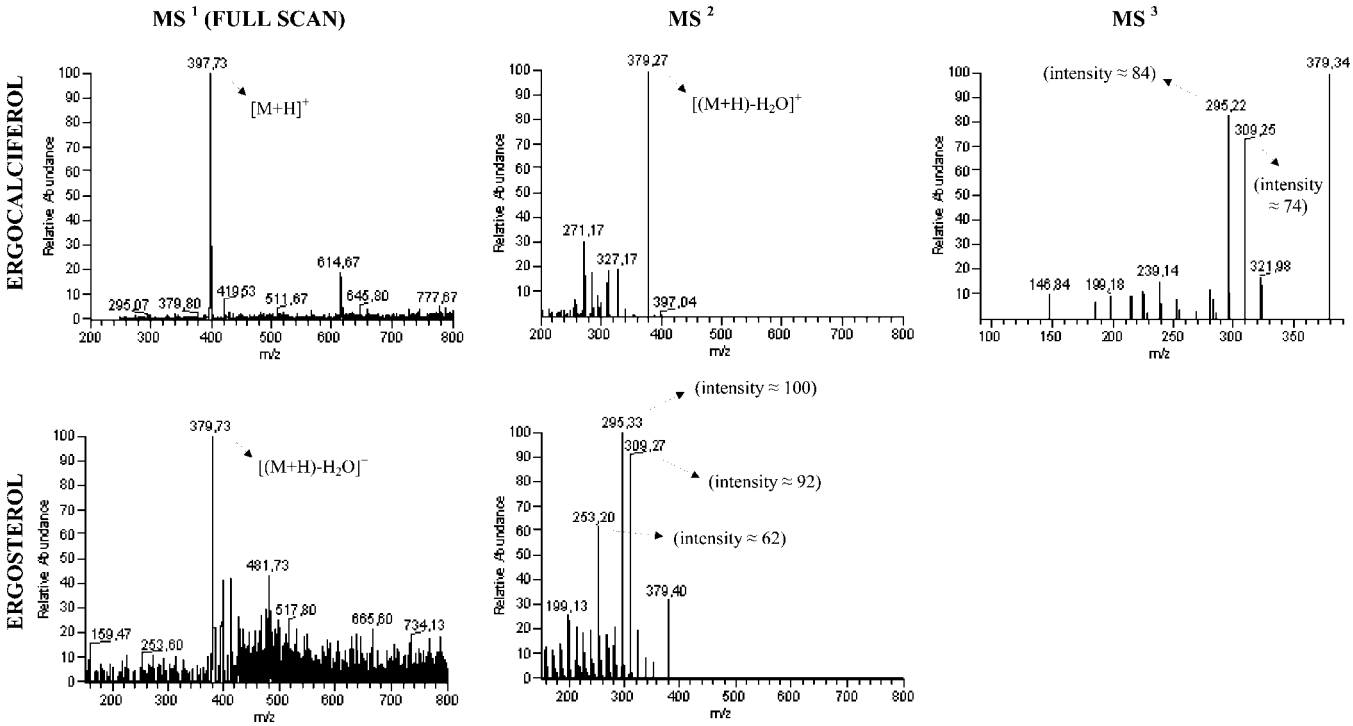

Figure 3. ESI mass spectra showing the parent and product ions and their corresponding *m*/*z* values of ergosterol and ergocalciferol standards (1.0 mg/L).

procedure (overnight cold saponification, two-stage extraction with 25.0 mL of methanol, and a two-step liquid/liquid extraction with 50.0 mL of petroleum ether) was applied to the determination of ergosterol and ergocalciferol in the different commercial forms of hop: hop plant (*Premiant*, *Saaz*, *Strisselsplater*, and *Nugget* varieties), pellets type 45, ethanolic extract, and supercritical CO_2 extract. The identification of the peaks was carried out by comparing their retention times with standards and by the method of standard addition, as well as by comparing the UV spectra in samples and standards by using photodiode array detection.

The different commercial forms of hop were analyzed in duplicate for each compound. The values obtained for the studied compounds in the different hop samples are shown in **Table 5**. As reported in this table, the presence of ergosterol and ergocalciferol was detected for only one variety of the hop plant (*Nugget*, crop 2006). The content of ergosterol and ergocalciferol found in this hop variety was approximately 1.84 $\pm$ 0.09 μg/g and 1.95 $\pm$ 0.05 μg/g, respectively. To the best of our knowledge, the presence of these compounds has never been reported in hop plant. Until nowadays, ergosterol was found in several matrices like leaves of aquatic and land plants, algaes, lichens, mushrooms, corn and wheat grains, paddy rice, and raw apple juice produced from decayed apples (*6*, *8*, *11*, *14*, *20*, *21*). As the presence of this compound is generally associated with a fungus contamination of the sample, the results obtained suggest that the hop plant (variety *Nugget*) analyzed in this work was infected with a fungus. As previously discussed, hop is extremely susceptible to fungal deterioration or viral contamination because of the climatic conditions that prevail during harvesting, drying, and storage. The presence of ergosterol reported here should have great potential for the assessment of hop products as related to the fungal contamination proportion and hence the quality of this raw material.

On the other hand, the presence of ergocalciferol (vitamin D_2) in the *Nugget* variety can probably be correlated with the plant sterol, ergosterol (provitamin D_2) by UV radiation from sunlight (*11*, *22*). As illustrated in **Figure 2**, vitamin D_2 synthesis consists of two basic stages. At the first stage, previtamin D_2 is formed from initial provitamin D_2 photochemically, and at the second stage, it converts into vitamin D_2 by thermoisomerization (*12*). The action of sunlight upon the precursors is a result of the presence of the endocyclic diene functionality in the B ring of provitamin D_2, which facilitates a ring opening of the B-ring (an electrocyclic reaction) and a rearrangement to a stable hexatriene (a sigmatropic rearrangement of hydrogen) (*12*).

The sun emits ultraviolet radiation in the UV-A, UV-B, and UV-C bands, but because of absorption in the atmosphere's ozone layer, more than 90% of the ultraviolet radiation that reaches the Earth's surface is UV-A. Mau et al. (1998) studied the effect of UV irradiation on the conversion of ergosterol to vitamin D_2 in edible mushrooms and found that the conversion was highest under UV-B (wavelength 280–320 nm) compared to UV-C (wavelength 190–280 nm) (*22*). The effect of UV-A (wavelength 315–400 nm) on the conversion of ergosterol in mushrooms to vitamin D_2 was studied by Jasinghe et al. (2005) (*11*). In this work, remarkably high levels of vitamin D_2 were obtained by UV-A irradiation of mushrooms for 2 h.

Provitamin D_2 and vitamin D_2 were detected in a hop plant (variety *Nugget*, crop 2006) that was harvested and dried in Bragança (northeast of Portugal). This region is very susceptible to a high exposure to ultraviolet radiation from the sun during the summer and spring. These undesirable climatic and environmental conditions probably induce the conversion of provitamin D_2 to vitamin D_2.

(d) Confirmation of Ergosterol and Ergocalciferol in Hop by HPLC–ESI–MS^n. Identification of ergosterol and ergocalciferol in the hop plant (*Nugget* variety) was confirmed by HPLC online coupled with electrospray ionization tandem mass spectrometry (HPLC–ESI–MS^n). The combination of retention time and MS^n spectral information of ergosterol and ergocalciferol in this hop sample with those of pure standards can be a useful tool for a reliable identification and confirmation of these compounds in this raw material.

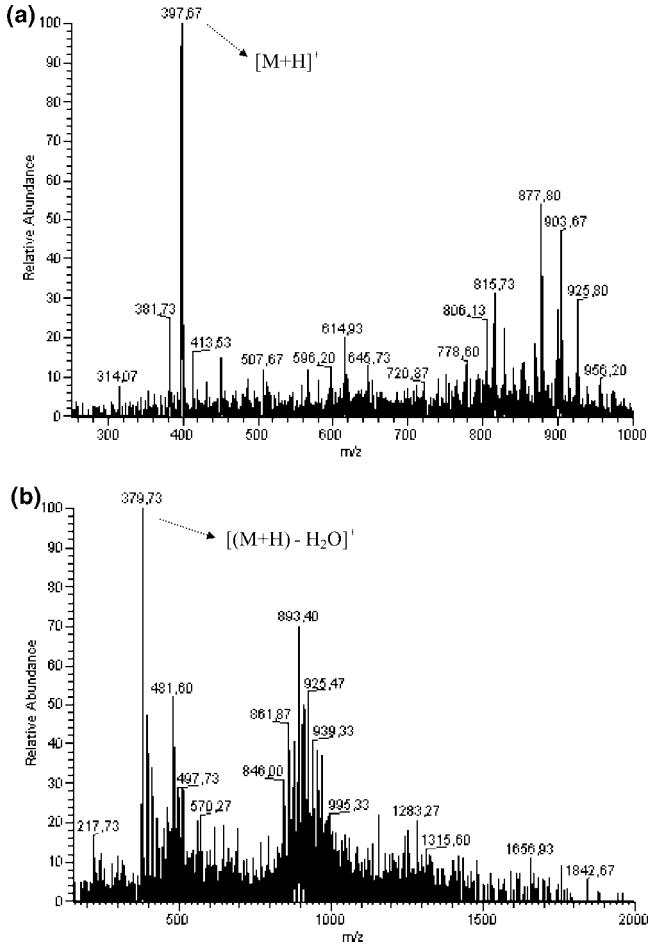

Figure 4. ESI (+) MS data for the hop plant (*Nugget* variety): (a) experimental mass spectra for ergocalciferol ($[M + H]^+$ of *m*/*z* 397.7) and (b) experimental mass spectra for ergosterol ($[(M + H) - H_2O]^+$ of *m*/*z* 379.7).

The positive ion full-scan ESI-MS spectrum of ergosterol and ergocalciferol standards (1.0 mg/L) is shown in **Figure 3**. Ergocalciferol mass spectra was characterized by the presence of the parent ion $[M + H]^+$ with *m*/*z* corresponding to 397.7. Regarding to the ergosterol mass spectra, an abundant ion at *m*/*z* 379.7 was observed. This most abundant signal corresponds to the dehydrated molecule $[(M + H) - H_2O]^+$. The susceptibility for dehydratation appears to be dependent on the ring conformation and, hence, the proximity of adjacent hydrogen atoms. The loss of water from ring A of ergosterol is also enhanced by the Δ^5 or especially the $\Delta^{5,7}$ unsaturation (*13*) (**Figure 2**).

To gain structural information on the ion *m*/*z* 379.7 (for ergosterol) and *m*/*z* 397.7 (for ergocalciferol), ESI–MS–MS studies were performed. The collision-induced dissociation (CID) of the ion at *m*/*z* 379.7, caused by its interaction with the nitrogen damping gas in the ion trap, resulted in three main fragments: *m*/*z* 253.2 (intensity ≈ 62), *m*/*z* 295.3 (intensity ≈ 100), and *m*/*z* 309.3 (intensity ≈ 92). Regarding the ergocalciferol, the most abundant ion originating from the CID of the ion at *m*/*z* 397.7 is at *m*/*z* 379.3 ($[(M + H) - H_2O]^+$). This radical cation at *m*/*z* 379.3 was isolated and further fragmented. As can be seen in **Figure 3**, the fragments were quite similar to those of ergosterol.

As reported in **Figure 4**, RP–HPLC–ESI (+) mass spectra of ergosterol and ergocalciferol for hop plant (*Nugget*variety) were characterized by the presence of the protonated molecules $[M + H]^+$ at *m*/*z* 379.7 and *m*/*z*397.7, respectively. On the other hand, the observed fragments of these molecules were similar to those of the standards (data not shown), confirming the identification of both compounds.

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