1 # Vitamin D-enriched extracts obtained from shiitake mushrooms
2 (*Lentinula edodes*) by supercritical fluid extraction and UV-irradiation

3 **Diego Morales[1*], Alicia Gil-Ramirez[1], Fhernanda R. Smiderle[2], Adriana J. Piris[1], Alejandro Ruiz-Rodriguez[1],**
4 **Cristina Soler-Rivas[1]**

5 [1]Department of Production and Characterization of Novel Foods, CIAL – Research Institute in Food Science
6 (UAM+CSIC), C/ Nicolas Cabrera 9, Campus de Cantoblanco, Universidad Autonoma de Madrid, 28049
7 Madrid, Spain.
8 [2]Department of Biochemistry and Molecular Biology, Federal University of Parana, Campus Centro
9 Politecnico, CP 19046, 81531-980, Curitiba-PR, Brazil.

10 *Corresponding author: diego.morales@uam.es

11 **Running title:** Vitamin D enriched extracts from *L. edodes*

12 **Keywords:** supercritical fluid extraction, cholesterol, ergocalciferol, UV-irradiation, vitamin D_2

13 **Abbreviations:** SFE, Supercritical fluid extraction; WS-UV, Wide-spectrum ultraviolet light; GC-FID-MS, Gas
14 chromatography coupled to flame ionization detector and mass spectrometry; HPLC-DAD, High
15 performance liquid chromatography coupled to diode array detection; HPLC-MS, High performance liquid
16 chromatography coupled to mass spectrometry.

17 **Abstract**

18 The combination of supercritical fluid extraction followed by UV-irradiation resulted in an interesting
19 strategy to obtain ergosterol- and Vitamin D_2-enriched extracts from *Lentinula edodes* to design novel
20 functional food. Extractions carried out following a specific experimental design pointed out that extraction
21 yields and ergosterol concentrations were more influenced by pressure than by the extraction temperature
22 although, high temperatures (55-75 ºC) might induce transformation of ergosta-7,22-dienol and fungisterol

into ergosterol. After extraction, the ergosterol-enriched extracts should be dissolved in methanol or ethanol and irradiated (25 °C, 4 cm) to partially transform ergosterol into vitamin D_2. Irradiation at 365 nm was less effective than at 254 nm. The fastest transformation was obtained using a UV lamp covering the complete UV spectrum for a maximum of 1 h. However, this lamp also induced vitamin D_4 formation although in lower amounts than vitamin D_2 or $lumisterol_2$ while with irradiation at 254 nm most of ergosterol was transformed into vitamin D_2.

1. Introduction

Edible mushrooms contain many different hypocholesterolemic compounds such as β-glucans, fungal sterols, specific strain-dependent compounds, etc. that could be extracted and utilized to design bioactive ingredients for novel functional foods. Several studies indicated that these molecules might modulate cholesterol homeostasis by inhibiting the endogenous cholesterol biosynthesis and impairing exogenous cholesterol absorption (Gil-Ramirez & Soler-Rivas, 2014; Guillamon et al., 2010). However, there might be many other indirect mechanisms involved in the regulation of serum cholesterol levels since, for instance, mushroom extracts containing eritadenine, an inhibitor of the *S*-adenosyl-L-homocysteine hydrolase (a key enzyme in the hepatic phospholipid metabolism) lowered total cholesterol levels *in vivo* (Sugiyama, Akachi, and Yamakawa, 1995). Vitamin D might also indirectly affect cholesterol levels (besides the other already known calcium- or parathyroid-related metabolic pathways) since low vitamin D status was associated with high total cholesterol levels and an increased risk of developing hyperlipidemia (Vitezova et al., 2015; Skaaby et al., 2012).

The different vitamin D structures are synthetized from pro-vitamins D that are temporarily transformed by UV irradiation into previtamins D. The latter intermediate can generate tachysterols, lumisterols or vitamins D depending on light and temperature (Wittig, Krings, and Berger, 2013). The main vitamins D found in foods are ergocalciferol (or vitamin D_2), cholecalciferol (or vitamin D_3) and 22,23-dihydroergocalciferol (or vitamin D_4). The vitamin D form found in blood serum is generated in liver by cholecalciferol hydroxylation originating 25-hydroxycholecalciferol. However, some reports preferred to

48 name it as 25-hydroxyvitamin D since ergocalciferol can also be bioavailable yielding 25-
49 hydroxyergocalciferol and improving the levels of total 25-hydroxyvitamins D (Keegan et al., 2013).
50 Mushrooms contain mainly vitamin D_2 (with traces of the other vitamins D (Keegan et al., 2013)) but
51 their levels are largely dependent on environmental conditions. Those picked from the woods usually
52 showed higher levels than indoor cultivated mushrooms. However, they all contain ergosterol (and other
53 derivative sterols) because it is a constitutive compound in fungal hyphae as well as an ergocalciferol
54 (vitamin D_2) precursor. Transformation of ergosterol into vitamin D_2 takes place during the development of
55 fruiting bodies exposed to light however, it can be reproduced *in vitro* by exposing their caps or gills to UV-
56 light (Jasinghe and Perera, 2005; Mau, Chen, and Yang, 1998; Slawinska et al., 2016). *Pleurotus* sp. fruiting
57 bodies increased their vitamin D_2 content from almost 0 to approx. 60 or 200 μg/g depending on the
58 analyzed strain after 2 h UV-B irradiation (Huang, Lin, and Tsai, 2015). Lower irradiation times yielded lower
59 transformation but still 30 min exposure increased vitamin D_2 levels in *A. bisporus* and *L. edodes* up to
60 respectively 119.21 and 59.89 μg/g (Slawinska et al., 2016). Exposure of gills facing UV-A source induced 4.4
61 fold more transformation of ergosterol into vitamin D_2 than cap exposure (Jasinghe and Perera, 2005) being
62 UV-B irradiation more effective than UV-A or UV-C.
63 Recent works indicated that ergosterol enriched fractions obtained from mushrooms using supercritical
64 fluid extractions (SFE) were able of lowering cholesterol levels in hypercholesterolemic mice (Caz et al.,
65 2016) however, they might be even more effective if ergosterol is partly transformed into vitamin D_2 as
66 noticed on hypercholesterolemic patients treated with statins after vitamin D supplementation (Qin et al.,
67 2015). Therefore, in this work a new method to extract ergosterol using SFE and convert it into vitamin D_2 is
68 described. The UV irradiation was carried out once the extract was generated and not on the mushroom
69 fruiting body, as has traditionally been done.

70 **2. Material and Methods**

71 *2. 1. Biological material and reagents*

72 Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies were purchased from Glucanfeed S.L. (La Rioja, 73 Spain) and stored in darkness at -20 ºC until further use. Obtained powder showed a particle size lower 74 than 0.5 mm and a moisture content lower than 5 %.

75 Solvents as hexane (95 %), chloroform (HPLC grade) and methanol (HLPC grade) were obtained from 76 LAB-SCAN (Gliwice, Polland) and absolute ethanol and sea sand from Panreac (Barcelona, Spain). Potassium 77 hydroxide, ascorbic acid and BHT (2,6-Di-tert-butyl-*p*-cresol) as well as hexadecane, ergosterol (95 %), 78 ergocalciferol (99 %) (vitamin D_2) and cholecalciferol (98 %) (vitamin D_3) were purchased from Sigma-79 Aldrich Química (Madrid, Spain). The CO_2 (99,99 % purity) was supplied by Air-Liquid España, S.A. (Madrid, 80 Spain). All other reagents and solvents were used of analytical grade.

81 *2.2. Supercritical fluid extractions (SFE)*

82 Supercritical fluid extractions with CO_2 were carried out in a plant (TharTechnology, Pittsburgh, PA, USA, 83 model SF2000) comprising a 2 L cylinder extraction cell and two different separators (S1 and S2) of 0.5 L 84 capacity each with independent control of temperature and pressure. The extraction vessel had a ratio of 85 5.5 height/diameter. A detailed explanation of the experimental device can be found elsewhere (Garcia-86 Risco et al., 2011). The extraction cell was filled with shiitake powder (253 g) and washed sea sand (1100 g) 87 in a ratio of 1:1 (v/v). The temperature of separators 1 and 2 was set to 40 ºC for all the experimental 88 assays and the pressure of S1 and S2 was maintained at 60 bar for all the extractions. The CO_2 flow was set 89 at 3.6 kg/h and during the total extraction time (3 h) it was recirculated. The compounds extracted in both 90 separators were washed with ethanol and immediately submitted to concentration until dryness on a 91 rotary vacuum evaporator. The dried extracts were stored at -20 ºC until further analysis. Extracted dry 92 matter content was measured to calculate the extraction yields. Extraction yields were expressed as 93 percentage of dry matter (in grams) obtained from 100 g of dry raw material utilized for extraction.

94 In order to optimize the extraction method to obtain sterol-enriched fractions, parameters such as 95 extraction pressure and extraction temperature were tested following a central composite design (2^2 + star 96 design). All the experiments (see Table 1) were fully randomized to provide protection against the effect of 97 lurking variables. Values for extraction temperature and pressure ranged respectively from 33.8 to 76.2 ºC

98 and 48.2 to 401.8 bar, with star points corresponding to 33.8 and 76.2 °C in the case of temperature, and
99 48.2 and 401.8 bar in the case of pressure.

100 *2.3. UV-Irradiation of SFE extracts*

101 Sterol-enriched extracts obtained by SFE and lyophilized shiitake mushrooms were submitted to UV-
102 irradiation using two different lamps: a Höhensonne 100 quartz lamp from Original Hanau (Hanau,
103 Germany) that emits UV radiation covering a wide light spectrum (200 – 700 nm) since it coupled an IR rod
104 (WS-UV); and a VL-4.LC lamp from Vilber Lourmat (Eberhardzell, Germany) that can irradiate specifically at
105 254 (UV-C) or 365 nm (UV-A).

106 Powdered *L. edodes* fruiting bodies (50 mg) were mixed with 3 ml of different solvents (water, methanol
107 and ethanol) in 2.5 cm diam. x 8 cm height cylindrical vessels, and exposed uncovered to the radiation
108 under vigorously shaking at a distance of 26 (Höhensonne lamp) or 14 (VL-4 lamp) cm for different
109 incubation times (0, 15, 30, 60 and 120 min). Other distances to the lamp were also tested and therefore,
110 the vials were placed at 4, 14 and 24 cm far from the UV source. Similarly, the fractions obtained after SFE
111 extractions (12 mg) were dissolved and treated as previously mentioned for the powdered fruiting bodies.

112 *2.4. GC-FID-MS analysis*

113 Fungal sterols from both shiitake mushrooms and SFE fractions (irradiated and non-irradiated) were
114 extracted following the procedure described by Gil-Ramirez et al. (2013). The unsaponified fractions
115 obtained (6 mg/mL) were injected into an Agilent 19091S-433 capillary column (30m x 0.25 mm ID and 0.25
116 µm phase thickness). The column was connected to a 7890A System gas chromatograph (Agilent
117 Technologies, USA) including a G4513A auto injector and a 5975C triple-Axis mass spectrometer detector.
118 The injector and detector conditions as well as the column temperature program were those described by
119 Gil-Ramirez et al. (2013). Ergosterol was used as standard to validate the GC method, using hexadecane (10
120 % v/v) as internal standard.

121 GC-MS database identified the obtained peaks in concordance with previous studies (Gil-Ramirez et al.,
122 2013; Jasinghe and Perera, 2005; Teichmann et al., 2007). The major detected sterols were ergosterol

123 (ergosta-5,7,22-trien-3β-ol) (RT= 12.6 min), ergosta 7,22-dienol (RT= 12.8 min), ergosta-5,7-dienol (RT= 13.1
124 min) and ergosta-7-enol (fungisterol) (RT= 13.3 min).

125 *2.5. HLPC-DAD and HPLC-MS analyses*

126 The unsaponified fractions obtained as previously described from both shiitake mushrooms or irradiated
127 and non-irradiated SFE fractions were injected (20 μl) into a Varian HPLC, model 920-LC Galaxy, with a
128 diode array (PAD) detector. Reverse phase chromatographic separation was performed with a Carotenoid
129 C30 analytical column (250 x 4.6 mm, 5 μm) from YMC Europe (Dinslaken, Germany). Solvents utilized as
130 mobile phase were 85 % methanol (v/v) (A) and ethanol (B). They were mixed following the gradient: 5 % B
131 during 2 min, up to 40 % B in 5 min and then maintained 15 min, up to 90 % B in 5 min and maintained 5
132 min more. The flow rate was 1 mL/min and the oven temperature 50 ºC. The absorbance changes were
133 followed by a UV-VIS DAD and 265 nm was selected for quantification. Vitamin D_2 (R.T. = 16.2 min) and
134 vitamin D_3 (R.T. = 16.5 min) were injected as standards obtaining chromatograms similar to Wittig, Krings
135 and Berger (2013). Nevertheless, to identify vitamin D-related structures, samples were also injected (using
136 the above described method and column) into an Agilent 1100 series liquid chromatograph equipped with
137 a PDA detector and directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) via an
138 atmospheric pressure chemical ionization (APCI) interface. The selected parameters and conditions were:
139 positive ionization mode, capillary voltage, -3.5 kV; drying temperature, 350 ^{o}C; vaporizer temperature, 400
140 ^{o}C; drying gas flow rate, 5 L/min; corona current, 4000 nA; nebulizer gas pressure, 60 psi. Full scan was
141 acquired in the range from *m/z* 50-2200.

142 Samples were injected in duplicate and ergocalciferol (vitamin D_2) was used as standard for the
143 quantitative determination of vitamins D and derivative compounds.

144 *2.6. Statistical data analyses*

145 The one-way ANOVA as well as the Durbin-Watson statistic tests were used to determine the statistical
146 significance of the extraction pressure and temperature on the percentage of ergosterol extracted.
147 Significance was set at *P*<0.05. Calculations were made using StatGraphics Centurion XVII.I (Statpoint
148 Technologies, Inc, Virginia, USA) software.

149 The rest of experimental data was analyzed for statistical significance by one-way ANOVA followed by
150 Tukey's multiple comparison test (α= 0.05) using Prism GraphPad 5.03 software (GraphPad Software Inc.,
151 San Diego, CA, USA).

152 **3. Results and discussion**

153 *3.1. SFE extraction of sterols-enriched fractions*

154 Fruiting bodies from *Lentinula edodes* were submitted to supercritical CO_2 extractions without co-solvent
155 since previous results carried out with both *L. edodes* and *A. bisporus* stated that mixtures including 5, 10 or
156 15% ethanol (v/v) yielded extracts with higher dry matter but lower sterol contents (Gil-Ramirez et al.,
157 2013; Kitzberger et al., 2007 and 2009). Moreover, the use of other solvents such as dichloromethane or
158 ethyl acetate did not improve the extracted amounts (Kitzberger et al., 2007). Thus, two extraction
159 variables such as pressure and temperature were tested following the previously described experimental
160 design (Table 1) to estimate the optimal combinations.

161 To study the level of significance of each factor an analysis of variance (ANOVA) was performed for two
162 selected responses (extraction yield and percentage of ergosterol extracted). To be able of describing the
163 effects of the different factors and interactions in the response, only the significant factors were chosen (95
164 % confidence level).

165 The ANOVA analysis of the extraction yield showed that only the factor *extraction pressure* had a P-value
166 less than 0.05, indicating that it was significantly different from zero at the 95 % confidence level. The
167 Response Surface Plot for the extraction yield (% w/w) (Figure 1a) indicated that the extraction yields were
168 much more influenced by the extraction pressure, being the highest of 3.28 %, at the conditions of 350 bar
169 and 70 ^{o}C. These results were in concordance with those Kitzberger et al. (2009), which reported that high
170 pressures combined with the increase of the operational temperature led to the enhancement of the
171 extraction yield of shiitake oil.

172 However, results obtained with conditions such as 225 bar and 75 ºC allowed recovery yields of approx.
173 2.3 % (w/w) being higher than those obtained in previous reports (approx. 1 %) where lower temperatures

174 and/or similar or higher pressures were selected (Kitzberger et al. 2009). Mazzutti et al. (2012) reported
175 yields of 1.19 % when working with *Agaricus brasiliensis* at 300 bar and 50 °C. Previous results using
176 *Agaricus bisporus* as raw material achieved yields of approx. 0.6 % at extraction temperatures of 40 °C
177 regardless of the pressure selected (90, 180 and 300 bar) (Gil-Ramirez et al., 2013).
178 In the case of ergosterol percentage (the grams of ergosterol extracted from 100 g of mushroom powder),
179 both the extraction pressure and the extraction temperature had P-values less than 0.05, indicating that
180 they were significantly different from zero at the 95 % confidence level. The regression equation fitting to
181 the data was:
182 [Ergosterol] = 48.4066 – 1.42215*Temperature + 0.03441*Pressure + 0.01362*Temperature^2 –
183 0.00001*Temperature*Pressure + 0.00011*Pressure^2

184 The Response Surface Plot obtained by a graphical representation of the fitted equation (Figure 1b)
185 showed the behavior of the response as a function of the different factors values. An increase in both the
186 extraction temperature and pressure led to an increase in the percentage of ergosterol in the extracts. The
187 experimental condition of 350 bar and 70 °C provided the highest ergosterol percentage (18 % w/w)
188 corresponding to 180 mg/g dw (dry weight). This may be explained by the combined effect of a high CO_2
189 density (0.83 g/cm^3) plus the vapor pressure at these conditions (Ghoreishi et al., 2012; Kamali et al., 2015).
190 The ergosterol content of the obtained fractions ranged from 87 mg/g dw (at 85 bar 55°C) up to 170.6 mg/g
191 at 350 bar 70°C.
192 Fraction containing 18 % ergosterol (w/w) included other ergosterol derivatives such as fungisterol (2 %),
193 ergosta 7,22-dienol (1.7 %) and ergosta 5,7-dienol (0.2 %). Increase of pressure did not largely influence the
194 extraction of the other fungal sterols however, an increase of temperature might induce the transformation
195 of ergosta-7,22-dienol and fungisterol into ergosterol. In extractions obtained at 225 bar, an increase in
196 temperature from 35, 55 to 75 °C led to a rise in the ergosterol levels of respectively 76, 79 and 82 % of the
197 total extracted sterols, and to a decrease in ergosta7,22-dienol levels of respectively 11, 10 and 8 % and of
198 12, 10 and 9 % in the case of fungisterol. On contrary, levels of ergosta-5,7-dienol remained constant but
199 they only represented the 1 % of the total extracted sterols.

200 *3.2. Effect of the UV-lamp utilized for SFE-extracts irradiation*

201 The powdered *L. edodes* strain utilized in this study contained ergosterol but no ergocalciferol (vitamin
202 D_2) (Figure 2). When the mushroom powder was directly exposed to UV-C irradiation (254 nm), ergosterol
203 levels decreased 16 % while vitamin D_2 increased. However, when the powder was suspended in methanol,
204 stirred and irradiated, ergosterol levels were reduced 27 % and vitamin D_2 levels increased 6.6 folds
205 compared with initial values. These results indicated that irradiation of mushroom powder within a
206 medium was more effective than dry irradiation and even more than direct fruiting body irradiation (if
207 compared with levels previously reported). Fresh *L. edodes* fruiting bodies irradiation for 1 h but at 310 nm
208 (UV-B) induced the formation of 0.004 mg/g vitamin D_2 (Mau, Chen, and Yang, 1998) and 2 h incubation
209 yielded 0.015 mg/g (Huang, Lin, and Tsai, 2015). If they were placed with their gills facing the UV source for
210 30 min at 20 °C, 0.029 mg/g vitamin D_2 were generated while irradiation of dried fruiting bodies induced up
211 to 0.06 mg/g (Slawinska et al., 2016).

212 Thus, the irradiation of the ergosterol-enriched extracts was carried out using methanol as solvent for
213 two reasons: firstly, because after the above described comparison, the use of an organic solvent was more
214 effective than using none or water to induce vitamin D_2 biosynthesis. The higher solubility of sterol and
215 vitamin D derivatives in organic mixtures might positively influence transformation yields. Secondly,
216 because the oily fractions obtained after SFE extraction were easier solubilized in organic solvents rather
217 than in aqueous mixtures.

218 The SFE extract selected for irradiation studies was the one obtained after SFE extraction at 350 bar and
219 70 ºC due to its higher ergosterol concentration compared to the ones obtained under other experimental
220 conditions. This extract initially contained 216.9 mg/g fungal sterols where ergosterol represented 82.9 %
221 (w/w) of total sterols, ergosta-7,22-dienol and fungisterol were present in lower concentrations (respect.
222 7.7 and 7.6 %), and ergosta-5,7-dienol was also detected but in very low quantities (1.8 %) (Table 2). When
223 the extract was solubilized and irradiated with the lamp able to supply a wide spectrum of UV light (WS-
224 UV), a large reduction in the ergosterol level was noticed until its complete degradation after 2 h incubation
225 (Figure 3). This compound was rapidly transformed into vitamin D_2 since its level was increasing with the
226 irradiation time until 1 h. Afterwards, the UV-light exposure seemed detrimental also for the vitamin

227 perhaps, the heat generated by the IR rod coupled to the UV-lamp was excessive. IR irradiation was
228 complementary used because heat enhanced UV effect enhancing the transformation of previtamins D into
229 vitamins D rather than into tachysterols or lumisterols (Wittig, Krings, and Berger, 2013). Levels of the other
230 derivative sterols were also influenced and drastically reduced after 1 h irradiation. They were not expected
231 to be transformed into any other compound due to the UV-light therefore, their reduction might also
232 suggest a negative influence of the heat generated by the IR rod.

233 Irradiation with UV-A (365 nm) induced almost no transformation of ergosterol into vitamin D_2.
234 However, the procedure still yielded more vitamin than described in other reports where the irradiation
235 was carried out directly on mushroom fruiting bodies. SFE extract irradiation for 1 h yielded 0.24 mg/g
236 vitamin D_2 and after 2 h the amount increased up to 1.13 mg/g while the largest amount obtained after 2 h
237 of fruiting bodies UV-A irradiation (with gills facing the source) was 0.023 mg/g (Jasinghe and Perera, 2005).
238 Nevertheless, UV-C irradiation (254 nm) of SFE extracts was more effective than UV-A because after 2 h UV-
239 C irradiation almost half of the ergosterol content was transformed into vitamin D_2 that doubled its levels.
240 Levels of the other ergosterol derivatives remained constant within the irradiation period independently of
241 the wavelength utilized (Table 2).

242 Apparently, the UV-lamps utilized also stimulated production of different vitamins D and photoisomers
243 (Table 3). According to HPLC-MS analysis, generated compounds were in concordance with those
244 previously reported by Witting, Krings, and Berger (2013). Previtamin D_2 formation could be noticed after
245 15 and 60 min when irradiated with respect. UV-C and the WS-UV lamps. But probably, it was further
246 transformed since its levels were reduced further in time concomitantly with an increase of its derivatives
247 such as vitamin D_2, lumisterol$_2$ and tachysterol$_2$. Lumisterol$_2$ was after vitamin D_2 the second previtamin D_2-
248 derivative generated. Its levels were only approx. 3 fold lower than vitamin D_2 after 120 min irradiation in
249 extracts irradiated with UV-C or after 60 min when irradiation was carried out with WS-UV lamps. The
250 transformation of previtamin D_2 into tachysterol$_2$ was not stimulated since only low concentrations could
251 be noticed after 60 min WS-UV irradiation. However, vitamin D_4 levels increased up to 60 min when the
252 irradiation was carried out with the WS-UV lamp and was almost not detected with UV-C irradiation.

253 Previtamin D_4, tachysterol$_4$ and lumisterol$_4$ were also increased although at lower concentrations than
254 vitamin D_4 during 60 min with WS-UV irradiation.

255 Since irradiation with WS-UV lamp was promoting generation of other structures (vitamin D_4 -related)
256 and the aim of the work was to increase the level of vitamin D_2 but avoiding the complete ergosterol
257 transformation (because of its hypocholesterolemic properties) the WS-UV lamp was discarded and the UV-
258 C lamp was selected to carry out further experiments.

259 *3.3. Effect of the solvent and temperature utilized for SFE-extracts irradiation*

260 Transformation of ergosterol from SFE extract into vitamin D_2 under UV-C light was also studied using
261 different solvents. After 1 h irradiation (at 50 °C), vitamin D_2 was slightly synthetized if water was used as
262 reaction medium (Figure 4). Addition of methanol up to different percentages improved the transformation
263 ratio but up to levels significantly lower than when irradiation was carried out using 100 % organic solvents
264 such as methanol or ethanol suggesting that solubilization of the SFE extract was essential for the proper
265 vitamin D_2 generation.

266 Because of the detrimental effect noticed on sterol content and vitamin D_2 transformation after
267 prolonged WS-UV irradiation, the effect of temperature was also studied on SFE extracts irradiated with the
268 UV-C lamp to ensure that the observed degradation could be due to the heat generated by the IR rod and
269 to estimate the optimal temperature needed to enhance the vitamin D_2 generation. However, no significant
270 differences were found after 1 h UV-C irradiation if temperatures were maintained from 25 up to 50 °C
271 (Figure 5). Other tested temperatures (1h) such as 30, 40 and 60 °C did not significantly improve speed of
272 vitamin D generation (as also noticed by Wittig, Krings, and Berger (2013) after irradiation of Oyster
273 mushrooms fruiting bodies). However, after 2 h irradiation of the SFE extract at 50 °C, a slightly higher
274 amount of vitamin D_2 was obtained than at 25 °C.

275 *3.4. Effect of the distance to UV source*

276 The optimal distance to the UV source was also investigate because previous studies placed mushroom
277 fruiting bodies at many different distances (from 10 up to 30 cm) with no further testing to study whether

278 they were the more adequate position for irradiation. For instance, Huang, Lin, and Tsai (2015) irradiated
279 Oyster mushrooms with a UV-B lamp at 25°C, for 2 h, at 19 cm far from the light and obtained 69 μg/g
280 vitamin D while Wittig, Krings, and Berger (2013) irradiated the same mushrooms with a similar UV-B lamp
281 at 20 and 30 °C at 10 cm from the light and obtained larger amounts (80 μg/g) with only 10 min irradiation.
282 Differences between both experiments could be due to the fact than the latter mushrooms were placed
283 closer to the UV lamp. Thus, SFE extracts were dissolved in methanol and placed at three different positions
284 from the lamp (Figure 6). Results indicated that the transformation of ergosterol into vitamin D_2 was
285 enhanced when the vials were placed closer to the UV source since at 4 cm distance almost 5 fold more
286 vitamin D_2 was obtained than at 24 cm after 1 h exposure.

287 **4. Conclusions**

288 Fractions containing up to 18 % (w/w) ergosterol and other ergosterol derivatives can be obtained by
289 supercritical fluid extractions from *Lentinula edodes*. They can be further processed to induce partial
290 transformation of this provitamin D_2 into vitamin D_2 by UV-light irradiation. Then, the SFE extracts should
291 be dissolved in organic solvents such as methanol or ethanol, exposed at room temperature under WS-UV
292 or UV-C rather than UV-A light and as closer as possible to the UV source. WS-UV irradiation also induced
293 vitamin D_4 formation although in lower amounts than vitamin D_2 or lumisterol$_2$. However, if WS-UV lamp is
294 couple to IR rod, exposures longer than 1 h are not encouraged due to detrimental effect on ergosterol and
295 vitamin D_2 contents. This procedure to generate vitamin D_2 enriched extracts is more than 100 folds more
296 effective than direct fruiting body irradiation followed by extraction.

297 **Acknowledgments**

298 This research was supported by national R+D program from the Spanish Ministry of Science and Innovation
299 (project AGL2014-56211-R) and the regional program from the Community of Madrid, Spain (S2013/ABI-
300 2728). F.R.S. thanks to the Brazilian funding agency CNPq (*Conselho Nacional de Desenvolvimento Cientifico*
301 *e Tecnologico*).

302 **Conflict of interest** None.

303 **References**

304 Caz, V., Gil-Ramirez, A., Santamaria, M., Tabernero, M., Soler-Rivas, C., Martin-Hernandez, R., Marin,
305 F.R., Reglero, G., & Largo, C. (2016) Plasma cholesterol-lowering activity of lard functionalized with
306 mushroom extracts is independent of Niemann-Pick C1-like 1 protein and ABC sterol transporters gene
307 expression in hypercholesterolemic mice. *Journal of Agricultural and Food Chemistry, 64,* 1686-1694

308 Garcia-Risco, M. R., Vicente, G., Reglero, G., & Fornari, T. (2011). Fractionation of thyme (*Thymus*
309 *vulgaris L.*) by supercritical fluid extraction and chromatography. *Journal of Supercritical Fluids, 55,* 949–
310 954.

311 Ghoreishi, S.M., Kamali, H., Ghaziaskar, H.S., & Dadkhah, A.A. (2012). Optimization of supercritical
312 extraction of linalyl acetate from lavender via Box-Behnken design. *Chemical Engineering and Technology,*
313 *35*, 1641–1648.

314 Gil-Ramirez, A., Aldars-Garcia, L., Palanisamy, M., Jiverdeanu, R.M., Ruiz-Rodriguez, A., Marin, F.R.,
315 Reglero, G., & Soler-Rivas, C. (2013). Sterols enriched fractions obtained from *Agaricus bisporus* fruiting
316 bodies and by-products by compressed fluid technologies (PLE and SFE). *Journal of Innovative Food Science*
317 *and Emerging Technologies 18,* 101-107.

318 Gil-Ramirez, A., Ruiz-Rodriguez, A., Marin, F. R., Reglero, G., & Soler-Rivas, C. (2014). Effect of
319 ergosterol-enriched extracts obtained from *Agaricus bisporus* on cholesterol absorption using an in vitro
320 digestion model. *Journal of Functional Foods, 11*, 589-597

321 Gil-Ramirez, A., & Soler-Rivas, C. (2014). The use of edible mushroom extracts as bioactive ingredients to
322 design novel functional foods with hypocholesterolemic activities. Chapter 2. In G. Pesti (Ed.), *Mushrooms:*
323 *Cultivation, Antioxidant Properties and Health Benefits* (pp. 43-73). New York: Nova Science Publishers, Inc.
324 ISBN: 978-1-63117-521-3.

325 Guillamon, E., Garcia-Lafuente, A., Lozano, M., D'Arrigo, M., Rostagno, M.A., Villares, A., & Martinez, J.A.
326 (2010) Edible mushrooms: role in the prevention of cardiovascular diseases. *Fitoterapia, 81,* 715-723.

327 Huang, S.J., Lin, C.P., & Tsai, S.Y. (2015). Vitamin D2 content and antioxidant properties of fruit body and
328 mycelia of edible mushrooms by UV-B irradiation. *Journal of Food Composition and Analysis, 42,* 38-45.

329 Jasinghe, V.J., & Perera, C.O. (2005). Distribution of ergosterol in different tissues of mushrooms and its
330 effect on the conversion of ergosterol to vitamin D_2 by UV irradiation*.* *Food Chemistry, 92,* 541–546.

331 Kamali, H., Aminimoghadamfarouj, N., Golmakani, E., & Nematollahi, A. (2015). The optimization of
332 essential oils supercritical CO2 extraction from *Lavandula hybrida* through static-dynamic steps procedure
333 and semi-continuous technique using response surface method. *Pharmacognosy Research, 7,* 57–65.

334 Keegan, R.J.H., Lu, Z., Bogusz, J.M., Williams, J.E., & Holick, M.F. (2013). Photobiology of vitamin D in
335 mushrooms and its bioavailability in humans. *Dermato-Endocrinology, 5,* 165-176.

336 Kitzberger, C.S.G., Lomonaco, R.H., Michielin, E.M.Z., Danielski, L., Correia, J., & Ferreira, S.R.S. (2009)
337 Supercritical fluid extraction of shiitake oil: Curve modeling and extract composition. *Journal of Food*
338 *Engineering, 90,* 35–43.

339 Kitzberger, C.S.G., Smania, A., Pedrosa, R.C., & Ferreira, S.R.S. (2007). Antioxidant and antimicrobial
340 activities of shiitake (*Lentinula edodes*) extracts obtained by organic solvents and supercritical fluids.
341 *Journal of Food Engineering, 80,* 631-638.

342 Mau, J.L., Chen, P.R., & Yang, J.H. (1998). Ultraviolet irradiation increased vitamin D2 content in edible
343 mushrooms. *Journal of Agricultural and Food Chemistry, 46,* 5269-5272.

344 Mazzutti, S., Ferreira, S.R.S., Riehl, C.A.S., Smania ,A. Jr., Smania, F.A., & Martinez, J. (2012). Supercritical
345 fluid extraction of *Agaricus brasiliensis*: Antioxidant and antimicrobial activities. *Journal of Supercritical*
346 *Fluids, 70,* 48–56.

347 Qin, X.F., Zhao, L.S., Chen, W.R., Yin, D.W., & Wang, H. (2015). Effects of vitamin D on plasma lipid
348 profiles in statin-treated patients with hypercholesterolemia: A randomized placebo-controlled trial.
349 *Clinical Nutrition, 34,* 201-206.

350 Skaaby, T., Hysemoen, L.L., Pisinger, C., Jorgensen, T., Thuesen B.H., Fenger, M., & Linneberg, A. (2012).
351 Vitamin D status and changes in cardiovascular risk factors: a prospective study of a general population.
352 *Cardiology, 123*, 62-70.

353 Slawinska, A., Fornal, E., Radzki, W., Skrzypczak, K., Zalewska-Korona, M., Michalak-Majewska, M.,
354 Parfieniuk, E., & Stachniuk, A. (2016). Study on vitamin D2 stability in dried mushrooms during drying and
355 storage. *Food Chemistry, 199,* 203–209.

356 Sugiyama, K., Akachi, T., & Yamakawa, A. (1995). Hypocholesterolemic action of eritadenine is mediated
357 by a modification of hepatic phospholipid metabolism in rats. *The Journal of Nutrition, 125,* 2134-2144.

358 Teichmann, A., Dutta, P.C., Staffas, A., & Jägerstad, M. (2007). Sterol and vitamin D2 concentrations in
359 cultivated and wild grown mushrooms: Effects of UV irradiation. *LWT – Food Science and Technology*, 40,
360 815-822.

361 Vitezova, A., Voortman, T., Zillikens, M.C., Jansen, P.W., Hofman, A., Uitterlinden, A.G., Franco, O.H., &
362 Kiefte-de Jong, J.C. (2015). Bidirectional associations between circulating vitamin D and cholesterol levels:
363 The Rotterdam Study. *Maturitas, 8,* 411-417.

364 Wittig, M., Krings, U., & Berger, R.G. (2013). Single-run analysis of vitamin D photoproducts in oyster
365 mushroom (*Pleurotus ostreatus*) after UV-B treatment. *Journal of Food Composition and Analysis, 31*, 266-
366 274.

Table 1: Central composite design (2^2 + star design) of supercritical extractions from *Lentinula edodes*

Run	Variables	
	Temperature (°C)	**Pressure (bar)**
1	33.8	225.0
2	70.0	100.0
3	55.0	225.0
4	55.0	225.0
5	76.2	225.0
6	40.0	100.0
7	40.0	350.0
8	55.0	225.0
9	55.0	401.8
10	70.0	350.0
11	55.0	48.2

Table 2: Fungal sterols content in the SFE extract irradiated for 2 h using different UV-lamps

Lamp	Time (min)	Ergosterol (mg/g)	Ergosta7,22-dienol (mg/g)	Fungisterol (mg/g)	Ergosta5,7-dienol (mg/g)	Total sterols (mg/g)
UV-C (254 nm)	0	179.89±11.72^{a}	16.70±0.82^{a}	16.50±0.79^{b}	3.80±0.23^{a}	216.89
	15	174.04±2.00^{a}	8.93±0.55^{b}	17.46±0.23^{b}	3.34±0.60^{a}	203.77
	30	175.82±1.33^{a}	13.71±0.21^{ab}	23.90±2.44^{a}	7.13±2.42^{a}	220.57
	60	159.26±1.50^{a}	11.03±0.03^{b}	18.41±1.72^{ab}	3.74±0.69^{a}	192.44
	120	88.75±8.82^{b}	10.29±0.53^{b}	22.78±3.45^{a}	3.90±1.04^{a}	125.72
UV-A (365 nm)	0	179.89±11.72^{a}	16.70±0.82^{a}	16.50±0.79^{a}	3.80±0.23^{a}	216.89
	15	181.34±4.18^{a}	11.77±3.13^{a}	24.51±4.65^{a}	4.55±0.77^{a}	222.17
	30	185.51±4.19^{a}	13.26±2.38^{a}	24.08±3.53^{a}	4.68±1.07^{a}	227.53
	60	182.87±2.19^{a}	13.22±2.19^{a}	22.86±3.30^{a}	3.63±0.61^{a}	222.58
	120	175.41±0.24^{a}	11.03±0.86^{a}	21.62±0.94^{a}	3.79±0.54^{a}	211.86
WS-UV	0	179.89±11.72^{a}	16.70±0.82^{a}	16.50±0.79^{b}	3.80±0.23^{a}	216.89
	15	141.74±5.45^{b}	9.85±0.13^{b}	17.95±0.70^{b}	3.31±0.68^{a}	172.85
	30	121.02±6.00^{b}	16.55±2.25^{a}	24.44±1.27^{a}	1.79±0.36^{a}	163.80
	60	74.40±3.60^{c}	9.74±1.71^{b}	17.42±0.22^{b}	1.26±0.04^{a}	102.83
	120	n.d.^{d}	n.d.^{c}	2.34±0.30^{c}	n.d.^{b}	2.34

n.d. = not detected

$^{a-d}$ Different letters denote significant differences (P < 0,05) between different times of exposure for the same compound and the same lamp.

Table 3: Vitamins D and photoisomers detected in the SFE extract during 2 h irradiation using different UV-lamps. Previtamin D_2 (PRE_2), tachysterol$_2$ (T_2), lumisterol$_2$ (L2), vitamin D_2 (V_2), provitamin D_4 (PRO_4), previtamin D_4 (PRE_4), tachysterol$_4$ (T_4), lumisterol$_4$ (L_4) and vitamin D_4 (V_4).

Lamp	Time (min)	PRE_2 (mg/g)	T_2 (mg/g)	L_2 (mg/g)	V_2 (mg/g)	PRO_4 (mg/g)	PRE_4 (mg/g)	T_4 (mg/g)	L_4 (mg/g)	V_4 (mg/g)
UV-C (254 nm)	0	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{b}	n.d.^{a}	n.d.^{a}	n.d.^{b}	n.d.^{a}
	15	n.d.^{a}	n.d.^{a}	2.53±0.86^a	20.54±4.02^b	n.d.^{b}	n.d.^{a}	n.d.^{a}	0.38±0.42^b	n.d.^{a}
	30	n.d.^{a}	n.d.^{a}	9.34±0.00^a	26.81±0.97^b	1.13±0.00^a	n.d.^{a}	n.d.^{a}	1.67±0.00^a	n.d.^{a}
	60	0.10±0.15^a	n.d.^{a}	12.38±3.72^a	42.38±8.84^b	n.d.^{b}	n.d.^{a}	0.27±0.22^a	0.16±0.22^b	n.d.^{a}
	120	n.d.^{a}	n.d.^{a}	32.74±24.58^a	95.10±10.23^a	n.d.^{b}	0.58±0.77^a	2.03±2.07^a	0.70±0.36^a	0.13±0.19^a
UV-A (365 nm)	0	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}
	15	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.26±0.37^a	n.d.^{a}
	30	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.55±0.40^a	n.d.^{a}
	60	0.41±0.58^a	n.d.^{a}	n.d.^{a}	0.24±0.34^a	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.22±0.31^a	n.d.^{a}
	120	0.33±0.46^a	n.d.^{a}	0.68±0.96^a	1.13±0.31^a	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.68±0.37^a	n.d.^{a}
WS-UV	0	n.d.^{c}	n.d.^{a}	n.d.^{c}	n.d.^{c}	n.d.^{a}	n.d.^{a}	n.d.^{b}	n.d.^{b}	n.d.^{c}
	15	2.18±0.00^a	n.d.^{a}	27.78±0.09^b	65.95±10.33^b	0.03±0.04^a	0.63±0.04^a	1.01±0.57^b	1.32±0.55^a	1.05±0.32^c
	30	1.43±0.00^b	0.11±0.16^a	35.89±4.63^a	64.75±1.34^b	n.d.^{a}	0.83±1.18^a	1.34±0.08^a	1.36±0.02^a	3.43±0.16^b
	60	n.d.^{c}	2.09±1.16^a	45.24±3.97^a	103.45±10.02^a	n.d.^{a}	1.65±0.04^a	2.18±0.04^a	1.53±0.08^a	7.97±0.44^a
	120	n.d.^{c}	0.82±0.31^a	2.45±0.11^c	22.10±0.97^c	n.d.^{a}	n.d.^{a}	n.d.^{b}	0.20±0.12^b	0.38±0.19^c

n.d. = not detected

$^{a-c}$ Different letters denote significant differences (*P* < 0,05) between different times of exposure for the same compound and the same lamp.

Figure 1: Response Surface Plot for a) extraction yield and b) ergosterol concentration obtained after SFE extractions (expressed in % w/w).

Figure 2: Levels of ergosterol and vitamin D_2 (mg ergosterol or vitamin D_2 / g mushroom powder) in powdered Shiitake fruiting bodies non-irradiated and irradiated with UV-C for 1h at 50 °C. Irradiation was carried out directly on mushroom powder (dry irradiation) or suspended in methanol. $^{a\text{-}c}$ Different letters denote significant differences (*P* < 0.05) between values of the same series.

Figure 3: Effect of UV light using different lamps on ergosterol (dashed line) and vitamin D_2 (solid line) levels during 2h irradiation. Concentration is expressed in mg ergosterol or vitamin D_2 / g SFE extract.

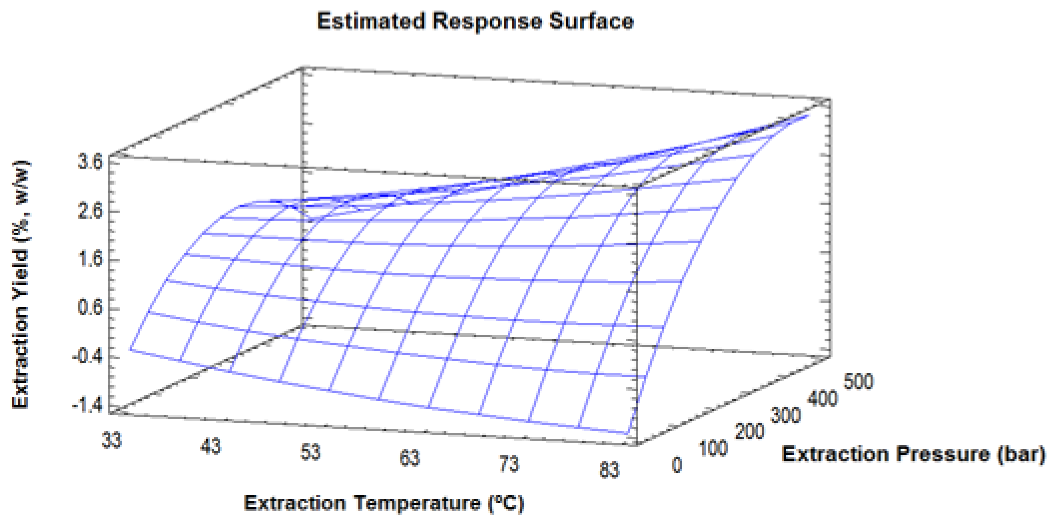
Figure 4: Influence of the different solvents (utilized to dissolve SFE extract for UV irradiation) in vitamin D_2 levels. $^{a\text{-}b}$ Different letters denote significant differences (*P* < 0.05).

Figure 5: Influence of the different temperatures (utilized during SFE extract UV irradiation) in vitamin D_2 levels. $^{a\text{-}c}$ Different letters denote significant differences (*P* < 0.05) between different irradiation times at the same temperature (25 °C or 50 °C).

Figure 6: Influence of the different distances from the UV lamp (utilized during SFE extract UV irradiation) in vitamin D2 levels. $^{a\text{-}c}$ Different letters denote significant differences (*P* < 0.05).

Figure 1:

a)



b)

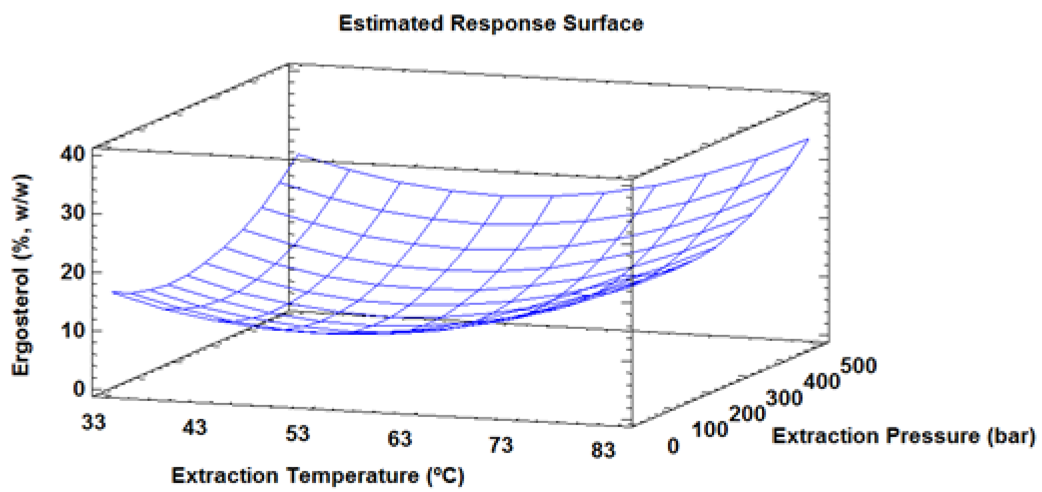


Figure 2:

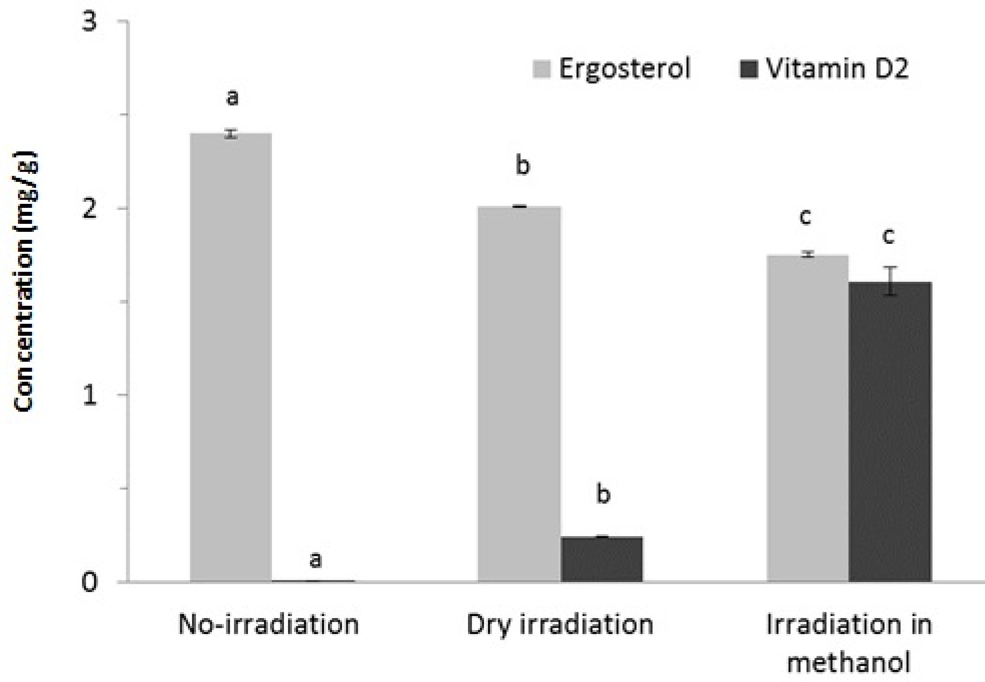


Figure 3:

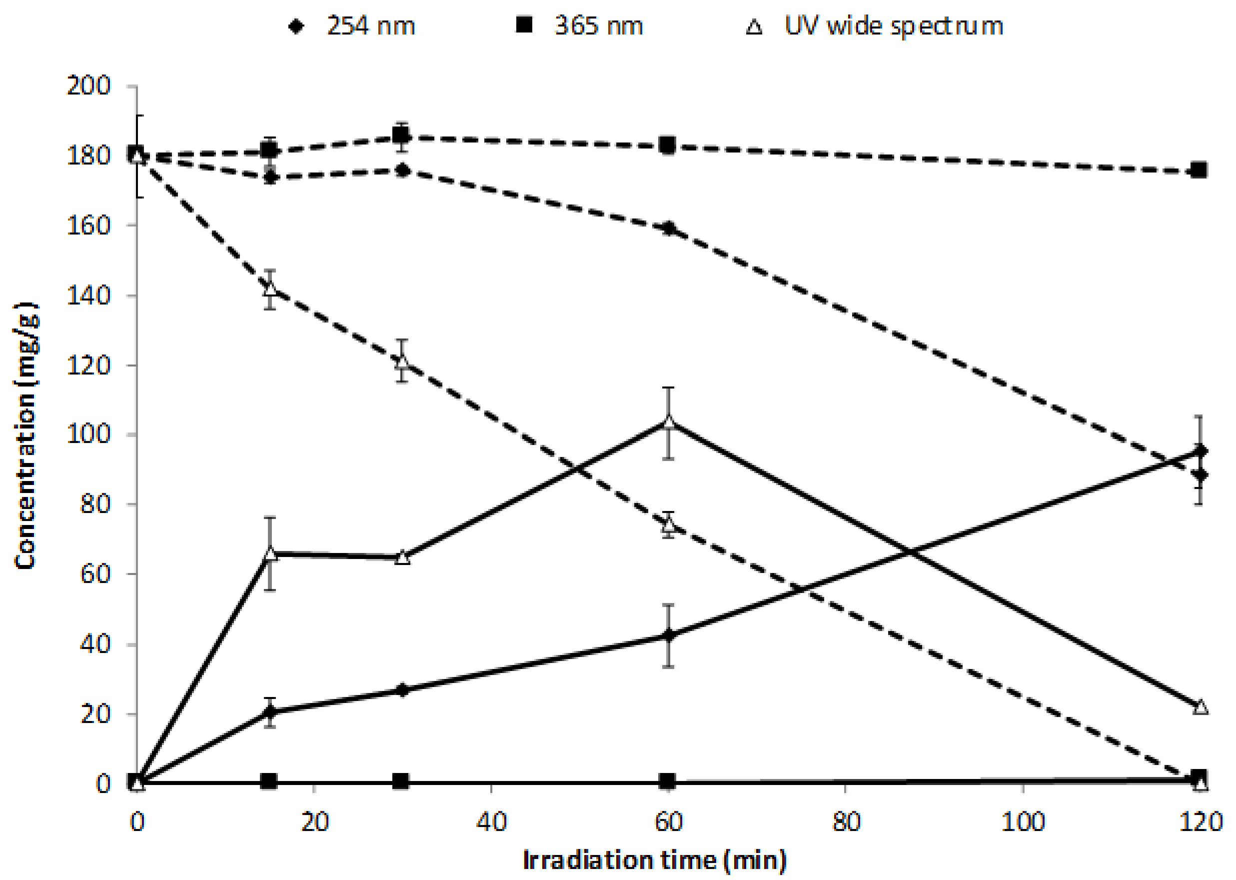


Figure 4:

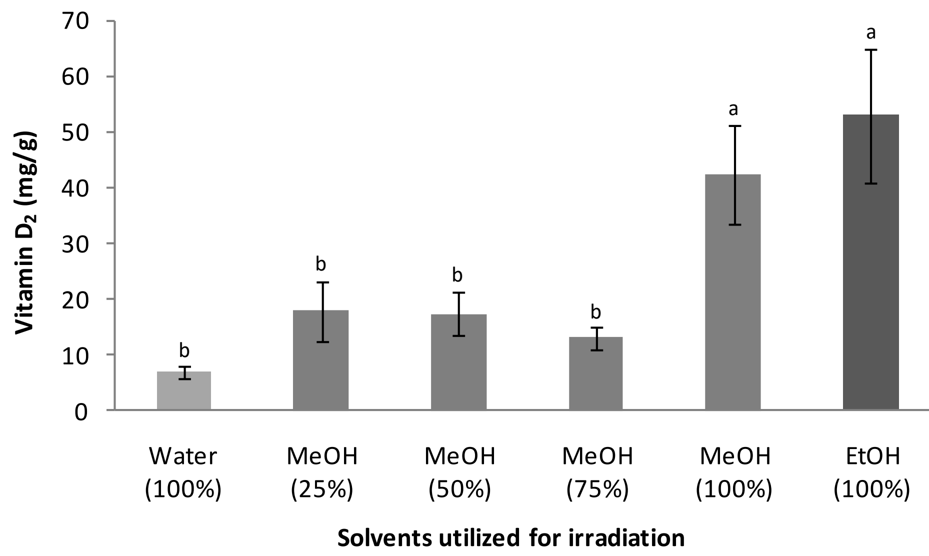


Figure 5:

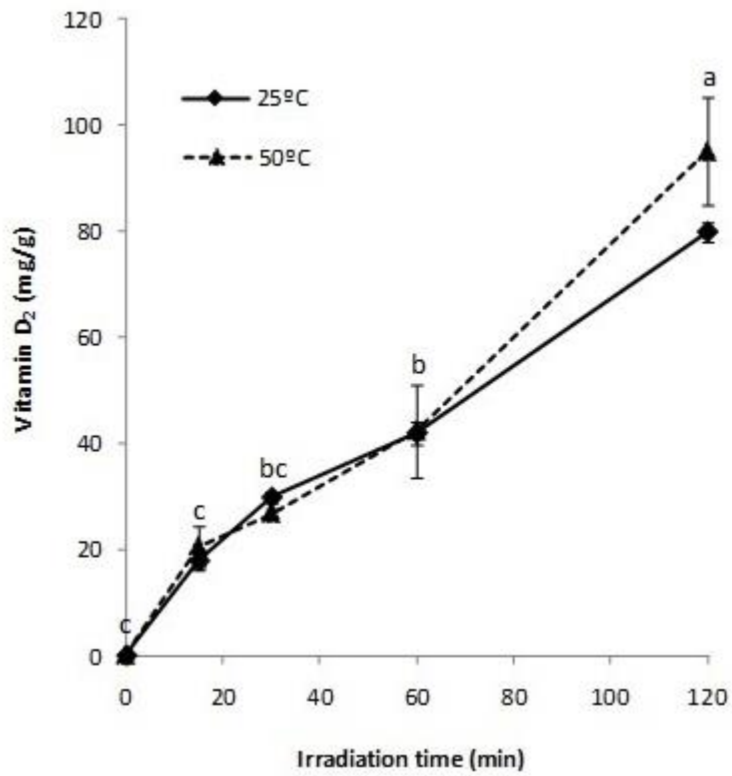


Figure 6: