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DETERMINATION OF ERGOSTEROL IN CELLULAR FUNGI BY HPLC. A MODIFIED TECHNIQUE.

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Abstract

Ergosterol is an important membrane sterol in almost all eumycotic fungi and has been postulated to be strongly associated with living cytoplasmic fungi in the soil. However, ergosterol is not produced by all fungi and the ergosterol concentrations are known to vary between the same species depending on the physiological state of the fungus. In this research we studied a modification to an ergosterol extraction technique which has the advantage of having a protocol with few steps of purification. This technique also includes an internal loss marker to evaluate the efficiency of extraction.

Key words: ergosterol; HPLC quantification; soil fungi

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Resumen

El ergosterol es un lípido de la familia de los esteroles componente importante de la membrana celular, presente en casi todos los hongos pertenecientes a los llamados "hongos verdaderos" (eumycotas). Se ha postulado que se halla asociado fuertemente con el citoplasma fúngico en el suelo, dando idea de la subsistencia del mismo. Sin embargo, el ergosterol no es producido por todos los hongos y las concentraciones de ergosterol se sabe que varían entre la misma especie en función del estado fisiológico del hongo. En este trabajo de investigación, se ha realizado una modificación a las técnicas de extracción de ergosterol ya conocidas, las cuales entre otras cosas, no tienen en cuenta las pérdidas de masa del esteroide a lo largo de los pasos de purificación. Esta innovación posee además la ventaja de tener un protocolo con pocos y sencillos pasos de purificación. Además, agrega la utilización de un marcador de pérdida para poder evaluar de esta manera la eficiencia de la extracción y así tener un resultado más cercano al real.

Palabras clave: ergosterol; cunatificación en HPLC; hongos del suelo

Introduction

Ergosterol is an important membrane sterol in almost all eumycotic fungi [1]. A number of techniques have been used to estimate biomass fungal: direct microscopic counting of hyphae [2,4] (the most widely accepted and commonly used method), fluorescence microscopy [5], leaf clearing and staining [5,6] and ATP assay [7] . Various serious sources of error exist in all of these techniques. A disadvantage of all these techniques is that they use conversion factors by means of which biomass either is often under or overestimated [8,9]. Biochemical markers of biomass, principally glucosamine and ergosterol, have also been used [10,11].

The glucosamine assays has been questioned, as this compound is not restricted to fungal cells and it is refractory thus persisting after the death of the mycelium [10 and 12]. Therefore, at best, glucosamine can only be an indicator of both living and dead hyphae.

Ergosterol is an important membrane sterol in almost all eumycotic fungi [13] and has been postulated to be strongly associated with living cytoplasmic fungi in the soil [14]. However, ergosterol is not produced by all fungi and the ergosterol concentrations are known to vary between the same species depending on the physiological state of the fungus [15,17].

Ergosterol has been widely used to quantify biomass in several studies of soils and mycorrhizal systems [18,19,7] and to determine biomass associated with decaying leaves in freshwaters [15,16,20].

Sung et al. (1995) [21] found good correlations between mycelial dry weight and ergosterol content for some species of ectomycorrhizal fungi, but also that in other species, ergosterol decreased as the mycelial culture aged.

Age of mycelium used, extraction procedures, incubation temperatures have been shown to influence ergosterol content [22]. However, we believe that these studies support the use of ergosterol as a measure of fungal biomass in the soil. We found soil ergosterol concentration to be positively correlated to both total and living hyphal lenghts in soil with a high degree of significance, but only a moderate degree of linear correlation. Therefore, a moderate degree of error will be involved in estimation of living fungal biomass from soil ergosterol content alone.

Our method is a modification to an extraction ergosterol technique [18,23] which has the advantage of having a protocol with few steps of purification. For example, in one of the most recent publications [24], we appreciate that the authors considered a lot of steps, which were carried out with several errors and greater extraction times. As a fundamental point of our method we developed a procedure of extraction of ergosterol different from those already published, taking into account the losses caused by the extraction. In addition, we had in mind the possible oxidation of

ergosterol, so we added from the beginning of the technic an antioxidant such as BHT thus avoiding the use of nitrogen.

The basic procedures involve sample extraction, saponification, partitioning, purification, determination and quantification of ergosterol.

Material and Methods

We collected different samples of wet soil from San Luis (Potrero de los Funes and Quebrada de la Bolsa), Argentina, with vegetation cover of ferns (*Bryopteris* sp., *Cheilantes* sp., and *Anemia* sp.). The samples (2 g of each; with 24% humidity) were saponificated with 2g KOH in 25 mL of methanol (HPLC grade), in the presence of 100 µL of corticosterone (loss marker) and 100 µL of butylated hydroxytoluene (BHT) as antioxidant. After the saponification (1 h reflux to 70°C), they were left to cool. The supernatant was placed in a funnel and 20 mL of methanol was added twice over the precipitate. We joined the three aliquots in the funnel with 30 mL of dry n-hexane (previously filtered the 60mL of methanol though Millex-HV 0.45 µm- Millipore S.A., Molsheim, France). This extraction was repeated twice. Fractions were joined and taken to dryness in a Rotavapor. The samples were stored (4°C in darkness) until HPLC analysis. Each sample was re-dissolved in 200 µL methanol. For HPLC analysis we used a P100 isocratic pump (Spectra Physics), a UV-100 detector (Spectra Physics) and a Data Jet integrator (Spectra Physics), a flow rate of 1.0 mL/min. The wavelength was set at 290 nm for determinate ergosterol and 254nm for corticosterone. Thus, each sample was injected twice in the HPLC. The column used was a Supelcosil LC18 (Supelco; 5 µm, 250 x 4.6 mm).

Methanol:acetonitrile, 80:20 v/v (HPLC-grade) was the mobile phase. Under these conditions the retention time was 13.4±0.2 min. (Figure 1).

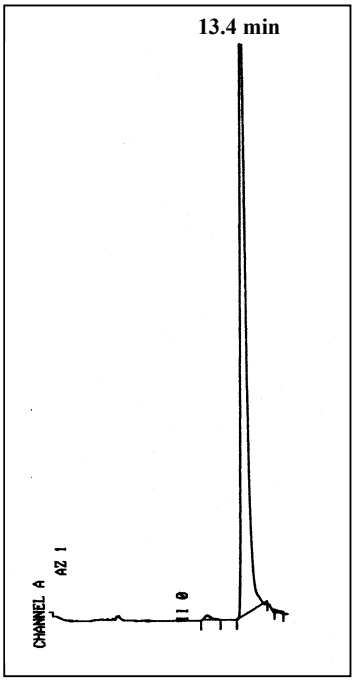

Figure 1. Ergosterol profile isolated from cellular fungi. Spectra Physics HPLC equipment was used with an isocratic Pump P100 incorporated. The absorbance spectrum was plotted at 290nm with a UV 100 Spectra Physics detector, chart speed was 0.5 $cm.min^{-1}$ integrated with a Spectra Physics Data Jet Integrator. A Rheodyne injector supplied with a 20µL loop was used. The column used was a Supelcosil C18 (Supelco; 5 µm, 250 x 4.6 mm) and, as mobile phase, HPLC-grade methanol : acetonitrile (80:20 v/v). The retention time in these conditions was 13.4 min ± 0.2 min at a flow rate of 1.0 $mL.min^{-1}$ and an attenuation of 128. A similar profile was obtained for the steroid used to calculate the losses but, in this case the absorbance spectrum was plotted at 254nm.

Stock calibration solutions of corticosterone and BHT were prepared in methanol.

Ergosterol was recalculated from peak areas of internal standard (IS). The relative response obtained from the ratio of IS to ergosterol peak areas was 0.99, whereas the relative recovery (the ratio of absolute recoveries of IS and ergosterol) was 0.88.

The linearity of the method was tested for corticosterone and ergosterol. Increasing amounts (5 to 20 µg) of analytes were injected for each determination.

The regression equations were calculated by the method of standardized principal component, and the coefficient of correlation was determined by linear regression.

Results and discussion

Calibration curves for peak areas vs. quantity of ergosterol were linear from 5 to 20 µg (Figure 2). The minimum detectable amount of ergosterol and IS was 2 µg. By injecting 20µL of the reconstituted extract we obtained 0.1 $\mu g.g^{-1}$ ergosterol. A relative response factor of IS compared with that of ergosterol was calculated and found to be 0.99 for the entire range. Absolute recoveries of ergosterol were calculated and were different depending on the sample considered. Thus, we incorporated a relative recovery according to the sample studied.

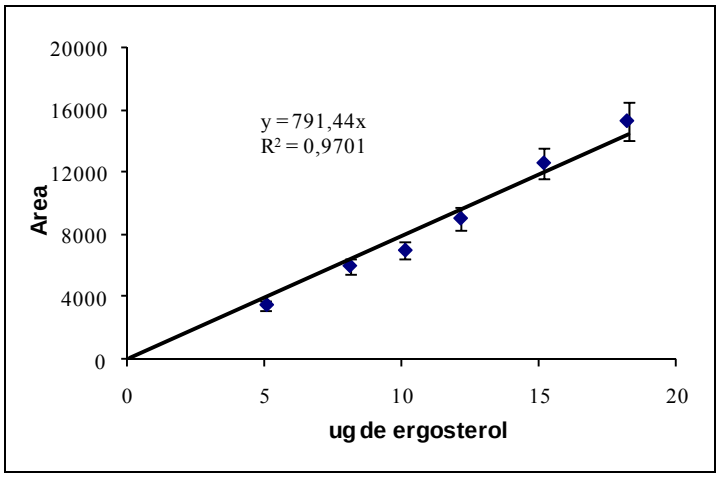

Figure 2. Calibration plot of µg ergosterol (Std 1.015 mg/mL). Ergosterol was determined by HPLC Spectra Physics equipment with UV 100 detector and a Supelcosil C18 column at room temperature. The mobile phase was HPLC grade methanol : acetonitrile (80:20 v/v). The elution time was 13.4 min ± 0.2 min. at a flow rate of 1.0 mL min^{-1}. The chat speed was 0.5 $cm.min^{-1}$.

The correlation of the losses measured with the standard of ergosterol and the standard of corticosterone was 98.9% with 0.1% SD.

These determinations were conducted on pooled ergosterol samples and thus reflect the entire process including the solid-phase extraction.

Our HPLC ergosterol method was optimized taking into account the losses produced in the various extraction steps. We used corticosterone as an internal standard, since it allowed us to correct the values obtained for ergosterol. Various different treatments were used for ergosterol determination. The best result in the recovery of ergosterol was 100% methanol in the

saponification process and n-hexane in the extraction process. A change of solvent in the process of saponification (to ethanol for example) gave a lower percentage of recovery in the first extraction (67%) and a lower percentage (9%) in a second extraction with the same solvent. The efficiency of the method proposed was already 80% in the first extraction (Table 1). Using this technique, we quantified ergosterol in soil samples (with *Bryopteris*) and showed the presence of fungal mycelium, as an indirect measure of fungal biomass.

We determined ergosterol in a range (0.18-0.43) µg /g dry soil depending on the sample. So, our method to quantify ergosterol showed to be efficient and able to be carried out in only a few steps, also implemented, correction by loss. It should be noted that there were no overlapped peaks in the HPLC procedure.

Table 1. Percentages of mass recovered from a total of 50.75 µg of standard ergosterol. Treatment with either ethanol or methanol involves saponification. Retention times were 13.4 min ± 0.2 min at a flow rate of 1.0 $mL.min^{-1}$ for all the determinations at the same conditions specified in Figure 2. Values are mean ± SDM of three independent experiments performed in duplicate.

Sample	Solvent Extraction	First extraction	Second extraction	Total recovery
Ergosterol + methanol	n-hexane	80 ± 7 %	8 ± 1 %	88 ± 8 %
Ergosterol + methanol	n-heptane	15 ± 3 %	1.3 ± 0,9 %	16 ± 4 %
Ergosterol + methanol	ether-ethilic	53 ± 2 %	4,8 ± 0,5 %	58 ± 3 %
Ergosterol + ethanol	n-heptane	67 ± 3 %	9 ± 1 %	76 ± 4 %

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