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Short communication

Simplified and rapid method for extraction of ergosterol from natural samples and detection with quantitative and semi-quantitative methods using thin-layer chromatography

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Abstract

A new and simplified method for extraction of ergosterol (ergosta-5,7,22-trien-3β-ol) from fungi in soil and litter was developed using pre-soaking extraction and paraffin oil for recovery. Recoveries of ergosterol were in the range of 94–100% depending on the solvent to oil ratio. Extraction efficiencies equal to heat-assisted extraction treatments were obtained with pre-soaking extraction. Ergosterol was detected with thin-layer chromatography (TLC) using fluorodensitometry with a quantification limit of 8 ng. Using visual evaluation of images of TLC plates photographed in UV-light the quantification limit was 16 ng.
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1. Introduction

Fungal biomass is used as a biomarker for assessing soil fertility and for monitoring the ecological impact of environmental pollution [1–3]. The principal membrane sterol of most fungi, ergosterol (ergosta-5,7,22-trien-3β-ol), is commonly used for estimating *living* fungal biomass.

In this paper, we describe our work with simplifying the procedure for extraction of ergosterol from soil and litter. Most methods for the extraction of ergosterol from natural samples are based primarily on heat assisted liquid–liquid extraction techniques with microwave ovens [4,5] and water baths [6,7]. We compared both treatments with pre-soaking extraction, which runs over a longer period but requires less labor and equipment than heat-assisted extractions. In the literature, we have not found examples of pre-soaking being used for ergosterol extraction.

Highly volatile organic solvents are primarily used in liquid–liquid extraction [6,8,9]. This procedure is fairly complex: the volatile solvents must be shaken and refluxed as many as three times, and are then evaporated to dryness. We attempted to simplify this procedure by substituting volatile solvents with paraffin oil. The use of paraffin reduced the number of shakings to one and removed the evaporation step.

The extracts were quantified fluorodensitometrically with thin-layer chromatography. For the purpose of developing an inexpensive detection method a semi-quantitative method relying on visual detection instead of using a TLC scanner was also developed.

2. Experimental

2.1. Materials and sample preparation

All solvents and reagents used in the experiments were of analytical grade. The reference standard of sterols (ergosterol, 98% purity) was purchased from Sigma–Aldrich, Denmark. Paraffin oil (Superfoss—purum pH EUR) was purchased from VWR International, Denmark.

Ergosterol was spiked in 1.50 M KOH 96% ethanol (SE) for the recovery experiment. SE, water, and paraffin oil (total volume of 8.50–9.00 ml) were mixed in ratios of 1:1:1, 4:4:1, and 8:8:1, respectively, in 15 ml disposable centrifuge tubes (Elkay polystyrene). Oil was dispensed with a macro transferpettor (Brand, 200–1000 μl). The tubes were shaken vigorously by hand for 300 s with tubes tied to a

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rack. The recovered oil from the 4:4:1 and 8:8:1 mixtures were then diluted with paraffin oil 3:1 and 7:1 to have comparable ergosterol concentrations to the 1:1:1 mixtures. Oil was transferred to 1.5 ml centrifuge vials and quantified immediately.

For extraction, 2.50 g fresh soil or 0.50 g fresh organic materials (OM) were put into 15 ml Pyrex specimen tubes (20 × 150 mm) or 15 ml disposable centrifuge tubes and mixed with 4.00 ml 1.50 M KOH in 96% ethanol. For comparing extraction efficiencies, three types of natural samples were extracted: (1) fresh *Psalliota campestris*; (2) sandy clay soil incubated in a microcosm for 26 days at 20 °C; and (3) litter from pine forest. The extraction treatments were: (1) batch heating in an 85 °C water bath for 30 min; (2) individual heating of tubes in a microwave oven (2450 MHz, 510 W output) for 2 × 70 s with 15 min intermission; and (3) pre-soaking at 21 °C for 14 h. After the treatments, 4.00 ml of demineralized water and 1000 μl of paraffin oil were added to each tube. The tubes were shaken by hand for 300 s and centrifuged for 2 min at 2900 × *g* (Sigma 4-10). Oil was transferred to 1.5 ml centrifuge vials and stored 12 h at −18 °C before quantification.

2.2. Chromatography

Paraffin oil from extracts and spikes samples were applied on RP-18 F_{254s} coated TLC glass plates (Merck 1.15423 & 1.15685) with a micro tranferpettor (Brand, 2.5–10 μl) set on 5 μl. Positions of the first track were $x = 15$ mm, $y = 12$ mm with spots spaced 10 mm apart. The application time for a 20 × 10 cm plate was approximately 7 min. After application the spots dried for 4 min before development.

The 20 × 10 plates were developed in 50 ml of eluent in a flat bottom developing chamber (CAMAG). The plates were developed twice in the same direction in pentane (19 min development time) followed by acetone (12 min development time). Drying time between the pentane and acetone developments was 1 min. After pentane development the plates dried for 5 min before being stored for 1 h at −18 °C. Plates were derivatized by dipping the plates for 2 s in a chemical reagent (500 mg iron chloride, 900 ml demineralized water, 50 ml acetic acid, 50 ml sulphuric acid) followed by air drying for 5 min. The plates were then heated for 3 min at 120 °C on CAMAG's Plate Heater III and quantified fluorodensitometrically on CAMAG's TLC Scanner 3 after a period of 1 min. Fluorescence was induced with a Hg lamp ($\lambda_{excitation} = 366$ nm) with the optical cut-off filter K400 ($\lambda_{emission} > 400$ nm). The slit dimension was set at 6.00 mm × 0.45 mm. Savitsky-Golay 7 was used for data filtering and lowest slope for baseline correction in order to integrate the area.

In order to find the fluorodensitometric quantification range ergosterol was applied in exponentially increasing amounts from 4 to 1000 ng ($n = 2$). Extracts from natural samples were quantified on the basis of two standard concentrations ($n = 2$) applied on each plate.

The Rf values for ergosterol, β-sitosterol, and cholesterol were measured. Spectrum scans on ergosterol from a natural extract of decomposing hay and a spiked oil sample were compared on CAMAG's TLC scanner 3.

In the semi-quantitative methods plate materials and methods for application and development were similar to the quantitative methods. Two detection methods were compared based on absorption and fluorescence, respectively. Ergosterol was applied in exponentially increasing amounts from 8 to 1000 ng ($n = 1$). For absorptive detection (henceforth called the absorption method), plates were derivatized in 10% ethanolic molybdatophosphoric acid and dried for 5 min before being heated to120 °C for 1 min on a CAMAG Plate Heater III. Plates were photographed in daylight with a Sony Cybershot DSC-F717 digital camera (1/80 s, F2.3). For fluorescent detection (henceforth called the fluorescence method), plates were derivatized with sterol agent as described in the quantitative method and photographed (1/4 s, F2.3) with the digital camera with a CAMAG Type 2 UV blocking filter in a CAMAG viewing box. Fluorescence was induced with 366 nm UV-light. Images shot under UV-light were subsequently cropped, inverted and subjected to auto levels in Adobe Photoshop.

Using the fluorescence method natural substrates extracted with the pre-soaking extraction method were compared to standards. The natural extracts were also quantified fluorodensitometrically for comparison.

3. Results and discussion

Using paraffin oil for recovery was a great simplification in comparison to common reflux methods relying on volatile solvents and yielded similar recoveries [5,6]. Recoveries of ergosterol from spiked solutions in paraffin oil ranged from 94 to 100% (Table 1). High ratios of ethanol to oil resulted in the lowest recoveries, whereas low ethanol to oil ratios resulted in the highest recoveries.

Ergosterol extraction efficiency using pre-soaking treatment was comparable to heat assisted treatments on soil substrates, higher for pine litter and lower for *P. campestris* (Table 2). These results indicate that presoaking is a valid treatment that can replace heat assisted extraction treatments.

The fluorodensitometric quantification method quantified ergosterol from 8 to 1000 ng ($R^2 = 0.999$, polynomial

Table 1
Recoveries of ergosterol from 96% ethanol to paraffin oil using different mixtures of ethanol:water:paraffin oil (v:v:v; $n = 3$)

Concentration (μg ergosterol ml^{-1} ethanol)	1:1:1 Recovery % (±S.D.)	4:4:1 Recovery % (±S.D.)	8:8:1 Recovery % (±S.D.)
3.2	100 (1)	102 (2)	94 (3)
12.5	100 (1)	99 (2)	97 (3)
50	100 (0)	100 (2)	96 (2)

Table 2
Comparison of extraction treatments with means and ±S.D.

Substrate	n	Soaking ($\mu g\,g^{-1}$ DW)	Waterbath ($\mu g\,g^{-1}$ DW)	Microwave ($\mu g\,g^{-1}$ DW)
Litter from pine forest	4	8.2 a (0.3)	6.7 b (0.3)	6.7 b (0.6)
Sandy clay soil	4	1.6 (0.1)	1.6 (0.3)	1.6 (0.2)
Psalliota campestris	3	4600 a (240)	5000 ab (250)	5300 b (400)

A matrix of 1.50 M KOH in 96% ethanol was used as extraction solvent. Different letters signify significant differences ($P < 0.05$, Fisher's LSD). DW: dry weight.

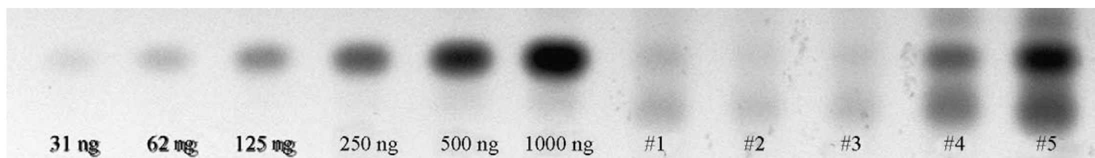

Fig. 1. TLC plate photographed with the fluorescence method. Standards are indicated with ng and natural substrates with # (see substrates in Table 3).

Table 3
The quantitative graduation of natural extracts determined with the fluorescence method

Quantification method	#1	#2	#3	#4	#5
	Soil amended with sawdust + dog food (2.3 g)	Soil below straw mulch (2.3 g)	Soil below hay mulch (2.3 g)	Straw mulch (0.4 g)	Hay mulch (0.2 g)
Fluorence (ng range)	[31–62]	<31	<31	[125–250]	[500–1000]
Fluorence ($\mu g\,g^{-1}$)	[2.7–5.4]	<2.7	<2.7	[60–120]	[400–800]
Fluorodensitometry (ng)	42	17	24	162	565
Fluorodensitometry ($\mu g\,g^{-1}$)	3.7	1.5	2.2	79	467

Fluorodensitometric quantification is included for comparison. 1000 µl paraffin oil were used for extractions. The amounts of substrates used are shown in brackets.

regression). Amounts <8 ng were not quantifiable. Using a volume of 500 µl of oil for extraction the lowest concentrations quantified were 0.5 µg and 1.8 µg erg g^{-1} DW for soil and litter, respectively. These values are sufficient for detecting ergosterol in soil and litter samples [10]. The Rf values of sterols were 0.45 for ergosterol, 0.35 for β-sitosterol, and 0.28 for cholesterol and were adequate for separation. Spectra from ergosterol extracted from decomposing hay were similar to that from ergosterol spiked in oil indicating that there were no substances interfering with ergosterol.

Ergosterol was detected with the absorption method from 32 ng from images shot in daylight. Using the fluorescence method ergosterol was detected from 16 ng and the exponentially increasing steps to 1000 ng were clearly distinguishable (Fig. 1). Using 2.3 g soil (DW) and 1000 µl of paraffin oil for extraction the lowest concentration gradient was <2.7 µg ergosterol g^{-1} soil (Table 3). Ergosterol was stored for 70 days without any breakdown. Ergosterol spiked in oil to a concentration of 50 $\mu g\,ml^{-1}$ had similar intensities (t-test, $P < 0.01$, $n = 3$) after 20 and 70 days of storage at −18 °C, respectively, compared to 1 h. Thus, paraffin oil was well suited for conserving ergosterol. The optimum drying time between pentane and acetone development was 1 min. The intensities of the ergosterol spots were significantly lower after 8 min or more of drying compared to 1 min (Fig. 2). The intensities decreased with a higher rate on tracks with 62 ng than tracks with 250 ng.

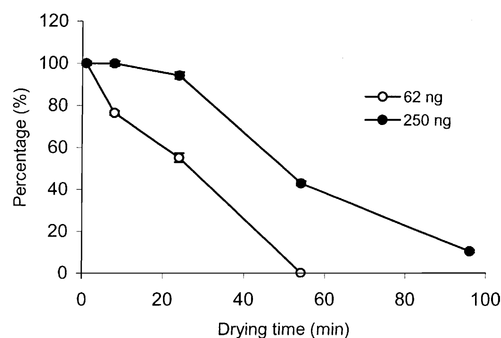

Fig. 2. Ergosterol decomposition on TLC plates as a function of time (1, 8, 24, 56 and 96 min).

Developed non-derivatized plates were stored for 10 days without any loss in the intensities of ergosterol. On plates that prior to storage were developed in pentane and acetone but not derivatized, the intensities of ergosterol in tracks applied with 25 and 50 ng were similar (t-test, $P < 0.01$, $n = 3$) after 1 and 10 h of storage, respectively, at −18 °C.

4. Conclusion

A new and simplified extraction method was developed combining presoaking with oil recovery. The extraction efficiency and recovery were equal to that of heat assisted

extraction treatments. Fluorodensitometric quantification was suitable for measuring ergosterol in soil and organic materials and the sensitivity was adequate for measuring ergosterol from natural samples. The method also holds the advantage that it can be modified to semi-quantitative use, which is useful in circumstances where no chromatographic instrumentation is available.

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References

[1] U.B. Jensen, S. Elmholt, R. Labouriau, Biol. Agric. Horticulture 18 (2000) 113.
[2] M. Khan, J. Scullion, Environ. Pollut. 110 (2000) 115.
[3] D.E. Padgett, M.A. Mallin, L.B. Cahoon, Environ. Monit. Assess. 65 (2000) 547.
[4] H.J. Montgomery, C.M. Monreal, J.C. Young, K.A. Seifert, Soil Biol. Biochem. 32 (2000) 1207.
[5] J.C. Young, J. Agric. Food Chem. 43 (1995) 2904.
[6] N. Eash, P. Stahl, T.B. Parkin, D. Karlen, Soil Sci. Soc. Am. J. 60 (1996) 468.
[7] M.O. Gessner, A.L. Schmitt, Appl. Environ. Microbiol. 62 (1996) 415.
[8] K.F. Nielsen, J.O. Madsen, J. Chromatogr. A 898 (2000) 227.
[9] P.D. Stahl, T.B. Parkin, Soil Biol. Biochem. 28 (1996) 847.
[10] R.G. Joergensen, Soil Biol. Biochem. 32 (2000) 647.