# Quantitation of Ergosterol in River Sediment by Liquid Chromatography

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## Abstract

**The ergosterol content of a river sediment can be used as an indicator of fungal activity. A method is developed for the extraction and determination of ergosterol in river sediment as part of a study to assess the correlation between fungal activity and biodegradation of pyrene, which is an environmental pollutant. This method is based on saponification and the liquid–liquid extraction of ergosterol by ethyl acetate. Quantitation and detection are performed isocratically by liquid chromatography on a 5-µm Hypersil C18 column with methanol–acetonitrile (80:20, v/v) as the mobile phase and detection at 282 nm. The detection limit is 50 ng/mL ergosterol, which is equivalent to 0.1 µg/g. The recovery of ergosterol at a concentration level in the range of 2 to 12 µg/mL is 91.7% ± 3.1% without interferences. This method is applied in order to successfully quantitate the ergosterol content in a river sediment with or without a fungus supply.**

## Introduction

The microbial biomass of river sediment appears to be composed primarily of fungi and bacteria. One approach to estimate the separate fungal and bacterial biomass is to measure compounds specific to either group. Ergosterol is the primary sterol in the cell membranes of filamentous fungi and is either absent or a minor component in most higher plants (1). Ergosterol has been used as a marker to quantitate fungal biomass in soils and leaf litter (2,3). The determination of ergosterol has also been used to investigate fungal invasion in cereal grains, seeds, and rice (4,5).

In general, procedures used to extract ergosterol are dependent on the substrates, whether it be extraction with various alcohols or solid-phase or microwave-assisted extraction (4,6–8). The extraction of ergosterol in sediments has not been reported previously in literature, except for salt marsh soils (9).

This study describes a reversed-phase liquid chromatography (LC) isocratic method with UV detection for the quantitative determination of ergosterol. The proposed method was validated and applied to the extraction and the quantitative determination of ergosterol in a river sediment contaminated by pyrene. The effect of a fungus supply was assessed in view of a pyrene–bioremediation study.

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## Experimental

### Chromatographic systems

The LC system consisted of a Waters (Milford, MA) 600E multisolvent delivery system with an in-line degasser, a Rheodyne injection valve (Interchim, Montluçon, France) equipped with a 25-µL loop, a Shimadzu (Kyoto, Japan) SPD-6AV UV detector, and a Shimadzu CR-3A Chromatopac integrator.

The LC determination was performed isocratically at room temperature. The analytical column was a 5-µm Hypersil C18 column (250- × 4.6-mm i.d.) supplied by Interchim with a guard column packed with a 5-µm Lichrospher 100-RP18 from Merck (Darmstadt, Germany). UV detection was performed at 282 nm.

The mobile phase consisted of a methanol–acetonitrile mixture (80:20, v/v). The flow rate was 1.5 mL/min. All solvents were filtered through Millipore (Bedford, MA) membrane filters with a 0.45-µm pore size as well as the sample extracts.

### Chemicals and reagents

Ergosterol and ergocalciferol standards were from Sigma-Aldrich (Steinheim, Germany). Analytical-reagent-grade methanol, ethanol, acetonitrile, ethyl acetate, and sodium chloride were from SDS (Villeurbanne, France) and LC-grade high-quality water from Stillplus HP system (Oxon, U.K.). Potassium hydroxide was from Fluka (Glossop, U.K.).

### Standard working solution

For ergosterol, a 0.2-g/L stock standard solution was prepared in methanol and stored in glass bottles in the dark at 4°C.

### Sediment sample

The sediment (pH = 7) used for the recovery experiments and biodegradation test was collected on the bank of the Ain river around the Port Galland site near Lyon, France in April 1997 by

the Centre d'Etude du machinisme Agricole du Génie Rural des Eaux et Forêts (CEMAGREF, an agricultural and environmental engineering research group). Sediment samples were sieved through a 5-mm grid, thoroughly mixed, and stones and vegetation were removed. Samples were stored in the dark at 4°C in glass bottles with Teflon-lined caps.

The content of particles smaller than 50 µm in this sediment was 16.78% (w/w, dried matter) and 55% between 500 and 200 µm. The organic matter content was 1.04% (w/w) and water 44.47% (w/w).

### Fortification of sediment

The sediments (2 g) were spiked with a standard intermediate solution of ergosterol at four fortification levels in order to give concentrations of 4, 8, 16, and 24 µg/g (2–12 µg/mL) of ergosterol in the sediment. After mixing, the fortified sediments were kept at room temperature for a 24-h equilibration before extraction and LC analysis.

### Extraction procedure

Two grams of fresh sediment were treated with 20 mL of methanol and saponified under reflux for 30 min in ethanolic potassium hydroxide (3 g/5 mL). After filtration on the crucible, the mixture was washed twice with 10 mL methanol. The combined filtrates were reduced in a rotary evaporator to 5 mL. After the addition of a 30-mL NaCl solution (10%), ergosterol was extracted by 20, 10, and then another 10 mL of ethyl acetate. The combined extracts were reduced to dryness in a rotary evaporator at 40°C and then redissolved in 4 mL methanol. The sediments were extracted in darkness in order to avoid the photodecomposition of ergosterol.

### Statistical analysis

The proposed LC method for ergosterol quantitation and its extraction from sediment have been validated according to the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP), Committee recommendations (10). Determinations of linearity, precision, recovery efficiency, selectivity, and sensitivity were carried out.

The parameters of validation were statistically treated to obtain the linearity test and correlation coefficients. Experimental results were compared with tabulated values assessed by the ANOVA test with a 0.05 test level (11).

### Quantitation of ergosterol in sediment samples

Two samples of 8.7 kg sediments were distributed in boxes (42 cm × 37 cm × 12 cm). Pyrene (870 mg) was added to each sediment box. Box 1 was stored for two months in a laboratory at room temperature. Box 2 was stored in the same conditions but inoculated with *Phialophora alba* because of its ability to degrade pyrene. The water content of the samples was maintained at 44.47% (w/w) during the experiment.

Sediment sampling was carried out by using a plastic corer in such a way as to reflect the various sediment strata. Sediment

**Table I. Effect of Mobile Phase Composition on the Retention Time of Ergosterol**

| Mobile phase composition | $t_R$ (min) | Flow rate (mL/min) |
|---|---|---|
| Methanol–water (95:5, v/v) | 37.2 | 1 |
| Methanol–acetonitrile (70:30, v/v) | 16 | 1 |
| Methanol–acetonitrile (60:40, v/v) | 17.2 | 1 |
| Methanol–acetonitrile (50:50, v/v) | 11.7 | 1.5 |
| Methanol–acetonitrile (70:30, v/v) | 10.3 | 1.5 |
| Methanol–acetonitrile (80:20, v/v) | 8.5 | 1.5 |

**Table II. Cumulative Percentages of Ergosterol Extracted with Ethyl Acetate**

| Ethyl acetate volume | Percentage ergosterol extracted |
|---|---|
| 20 mL | 55.8 |
| (20 + 10) mL | 96.5 |
| (20 + 10 + 10) mL | 101.8 |
| (20 + 10 + 10 + 5) mL | 101.8 |

**Table III. Validation Data for Ergosterol in Standard Solutions and in Spiked Sediments**

| Ergosterol ($t_R$ = 8.5 min) | Standard curve | Spiked sediments | Tabulated values |
|---|---|---|---|
| Linearity range (µg/mL) | 2–12 | 2–12 | |
| Slope (*b*) | 10257.7 | 9403.2 | |
| Intercept (*a*) | 1886.1 | 2511.3 | |
| Correlation coefficient | 0.9998 | 0.9983 | |
| **Student's t-test** | | | |
| Method proportionality test (hypothesis *a* =0) | 1.46 | 1.93 | $t(0.05;10) = 2.22$ |
| **Cochran test** | | | |
| Variance homogeneity | 0.766 | 0.56 | $C(0.05;4;2) = 0.767$ |
| **F Snedecor test** | | | |
| Linear model verification | 2.09 | 3.26 | $F(0.05;2;8) = 4.46$ |
| **F Snedecor test** | | | |
| Slope null hypothesis proof ($b = 0$) | 4729.13 | 2991.9 | $F(0.05;1;10) = 4.93$ |
| **Precision:RSD (%)** | | | |
| Intraday variability | 1.92 | 3.86 | |
| Interday variability | 2.06 | 4.87 | |

**Table IV. Accuracy for Ergosterol Determination in Sediment**

| Concentration added (µg/mL) | Concentration found (µg/mL) (Mean ± SD) | RSD (%) | Recovery (%) |
|---|---|---|---|
| 2 | 1.78 ± 0.15 | 8.75 | 89.17 |
| 4 | 3.93 ± 0.03 | 0.73 | 98.22 |
| 8 | 7.26 ± 0.28 | 3.83 | 90.69 |
| 12 | 11.10 ± 0.18 | 1.62 | 92.54 |

samples ($n$ = 3) in the two boxes were collected from at least three places immediately after preparation (t = 0) and after 60 days of incubation. At each time, 5 g of sediment was dried at 103°C to constant weight for 24 h.

## Results and Discussion

### Optimization of LC eluent composition

The LC method proposed by Grant and West (12) was used initially for the determination of ergosterol. The retention time of 37.2 min for ergosterol that was obtained with the reported eluent mixture of methanol–water (95:5, v/v) was too long for routine analysis. The retention time was reduced by replacing the water in the initial eluent mixture with acetonitrile. An acceptable retention time (8.5 min) was achieved with methanol–acetonitrile (80:20, v/v) as the mobile phase with a flow rate of 1.5 mL/min (Table I).

### Ergosterol extraction efficiency

Ergosterol was extracted with hexane from the sediment by a procedure similar to that described by Rössner (13). The efficiency of this extraction from our matrix was unsatisfactory (recovery < 50%). Thus, we tested the solubility of ergosterol in a solvent with a high-polarity index such as ethyl acetate. The extraction efficiency was increased. Therefore, by analyzing consecutive extractions of the same assay with a different volume of ethyl acetate, we optimized the method (Table II). The addition of a 30-mL NaCl solution (10% in water) was necessary to aid partitioning between the alcohol and ethyl acetate phases.

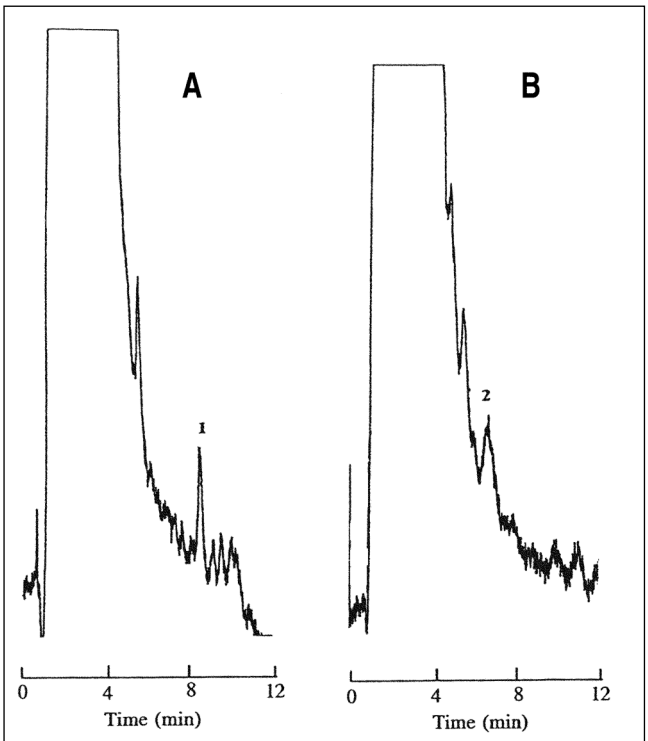


**Figure 1.** Chromatograms obtained for (A) native sediment and (B) UV irradiated sediment: (1) ergosterol and (2) ergocalciferol.

### Validation of the analytical method

*Linearity*

The standard calibration curve was established by plotting the peak area versus the ergosterol concentration. Four working solutions with concentrations of 2, 4, 8, and 12 µg/mL were injected in triplicate. A least-squares linear-regression analysis was performed to determine the slope, $y$-intercept, and the correlation coefficient of the standard plots. Results are summarized in Table III.

*Precision*

In order to evaluate the precision of the method, intra- and interday reproducibilities were investigated. For intraday variability, a 2-µg/mL solution of ergosterol was injected six times during the same day under the same experimental conditions. For interday variability, the same concentration was carried out six times for three different working days. The results are shown in Table III.

*Recovery*

Validation results for the linearity of the extraction procedure in the fortification range of 4 to 24 µg/g are listed in Table III. Recoveries of ergosterol from spiked samples were calculated by comparing the peak areas of the sediments spiked with the four concentration levels and then submitting them to the extraction procedure with those obtained from corresponding standards. The average recovery was 91.7% ± 3.1%. The accuracy of the method was very satisfactory according to the low relative standard deviations (Table IV).

*Selectivity*

In order to confirm the identity of ergosterol, the sediment extract was exposed to UV light. It is well-known that ergosterol decomposes to ergocalciferol, lumisterol, and tachysterol (14). The LC chromatogram showed a disappearance of the ergosterol peak and the appearance of a new peak ($t_R$ = 6.5 min) identified as ergocalciferol (vitamin D) (Figure 1). The sediment extract did not give an interfering peak with ergosterol.

*Sensitivity*

The limit of detection was deemed to be when the signal-to-noise ratio was 3:1. For 2 g dried sediment samples, it was estimated to 50 ng/mL (0.1 ppm in the sediment). The limit of quantitation was defined as the lowest concentration that could be determined with acceptable accuracy and precision, which resulted in a peak area twice that of the detection limit.

### Application of the method

The validated method was applied to investigate fungal activity in the sediment during a 60-day period in order to conduct a biodegradation kinetic study of pyrene in the river sediment with or without a *Phialophora alba* supply. In a preliminary study, we have demonstrated the efficiency of selected sediment fungi's ability to degrade pyrene in order to evaluate their potential use in sediment remediation processes. Micromycetes were isolated from polycyclic-aromatic-hydrocarbons-contaminated sediment. They were investigated for pyrene degradation (10 mg/L) in a

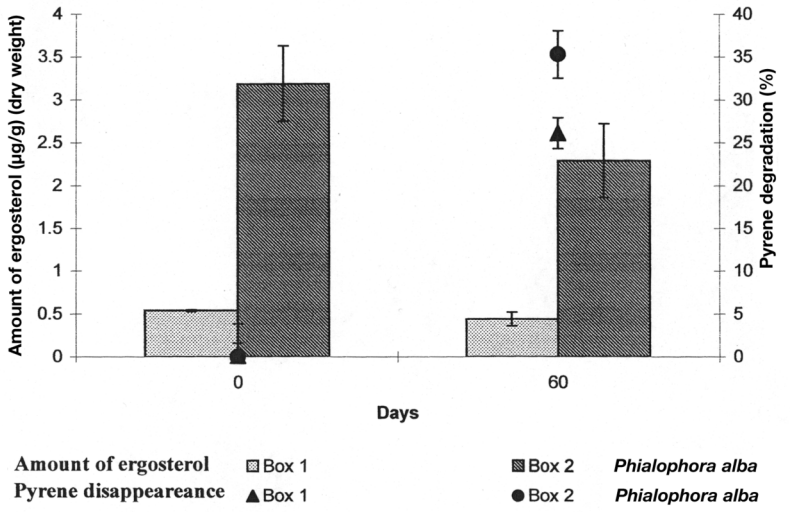


**Figure 2.** Ergosterol content and pyrene degradation in sediment boxes with or without *Phialophora alba* at t = 0 and t = 60 days. Box 1: sediment + pyrene; Box 2: sediment + pyrene + *Phialophora alba*.

liquid synthetic medium for two days. Among the 41 strains isolated, ten of these highly degraded pyrene. The best performance was observed with *Phialophora alba* (Moniliaceae) (15).

In the boxes, ergosterol concentrations were expressed as micrograms per gram of dry weight sediment. They were calculated from the mean of three assays (Figure 2).

For box 1, the ergosterol concentration at t = 0 and 60 days was 0.54 and 0.44 µg/g, respectively. After two months, the rate of pyrene disappearance was 26.1% ± 1.8%.

The ergosterol concentration was greater in sediment samples with *Phialophora alba* (3.19 µg/g at t = 0 and 2.29 µg/g at t = 60 days). Therefore, this difference between the two boxes reflected a fungus supply, but no increase was observed at t = 60 days (Figure 2). Nevertheless, the degradation of pyrene was 35% ± 2.8% after 60 days. A considerable variation in the ergosterol concentration was apparent in the sediment, with or without a fungus supply. In a further study, the efficiency of another fungus on pyrene-contaminated sediment should be explained by the ergosterol content in order to evaluate its potential use for bioremediation.

## Conclusion

The proposed LC procedure is rapid, sensitive, specific, reproducible, and provides good separation and recovery of ergosterol. Sample manipulation is very simple, and the extraction procedure requires small volumes of solvent and reduced sample handling. The method may be suitable for application for the determination of fungi biomass in sediments.

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