

Isolation and Purification of Ergosterol and Ergosterolperoxide from an Edible and Medicinal Higher Ascomycete Mushroom *Xylaria striata* by High-speed Countercurrent Chromatography

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Abstract

Phytosterols, which serve as structural components of biological membranes of plants, are the most abundant dietary supplement. To date, numerous studies have demonstrated that phytosterols can block cholesterol absorption sites in the human intestine, thus helping to reduce cholesterol absorption in humans. However, phytosterols standards are either not commercially available or very expensive, mainly because their structural complexity and diversity complicate their fractionation and isolation from plant extracts by conventional separation techniques. High-speed countercurrent chromatography (HSCCC) is based on continuous liquid-liquid partitioning, which enables one to eliminate irreversible adsorption on solid supports. This technique has been extensively used for natural product isolation. It is well suited to the effective large-scale separation of phytosterols and their derivatives, achieving high purities and yields of up to several hundred milligrams per run within several hours. The present study successfully used an effective HSCCC method to isolate and purify Ergosterol and Ergosterol peroxide from an edible and medicinal higher ascomycete mushroom, *Xylaria striata*. This method's optimal conditions are as follows: using *n*-hexane-ethylacetate-ethanol-water (3:1:2:0.8, v/v) as the two-phase solvent system; the rotation speed was set to 850 rpm (forward); the flow rate of the lower phase was 3mL/min. Ergosterol with 96% purity and Ergosterol peroxide with 97% purity were obtained. The chemical structures of these compounds were identified by ¹H-NMR and ¹³C-NMR. In addition, the antimicrobial activities of these compounds for nine kinds of plant pathogens fungi are evaluated. Ergosterol exhibited excellent fungistatic activities against *Valsa mali*, *Sclerotinia sclerotiorum*, *Fusarium graminearum*, and *Helminthosporium maydwas*. Ergosterol peroxide also exhibited strong antifungal activity against *Helminthosporium maydwas*.

1. Introduction

Sterols play an essential role in diverse biological processes in eukaryotes, such as membrane biosynthesis, cellular signaling, and energy storage [1]. Adding phytosterols to diets can decrease serum cholesterol levels and may be beneficial in preventing colon cancer, breast cancer, and prostate cancer [2]. Hence, many natural phytosterols like Ergosterol, β -sitosterol, and stigmasterol et al. have become a source of active compounds in food, cosmetics, and clinic fields. Ergosterol is one of the most abundant sterols found in mushrooms of different species [3]. It is a precursor of vitamin D₂ and shows various healthy beneficial effects like anti-cancer [4,5]. Antihyperlipidemic and

anti-inflammatory [6,7]. An important derivative of Ergosterol is Ergosterol peroxide which also presents more bioactivities: antiangiogenic and antitumor, anti-inflammatory, antibacterial, and anti-atherosclerosis [8-11]. Good biological activity prompts great market demand for pure compounds. Hence, valid methods for separating and purifying Ergosterol and Ergosterol peroxide are necessary.

Although column chromatography is the most common method to separate phytosterol, sample loss caused by irreversible adsorption is very uneconomical and the biggest challenge in industrial applications [12]. However, as a support-free all-

liquid chromatographic technique, high-speed countercurrent chromatography (HSCCC) can perfectly eliminate the loss of samples caused by irreversible adsorption and denaturation in a solid support matrix [13]. HSCCC does not involve solid phases but instead relies on partitioning the solute between two non-mixable solvents (stationary and mobile phases) experiencing complicated hydrodynamic motion in a rotating coiled tube. In the case when one phase is eluted from the coil through the head end, hydrodynamic equilibrium allows one to maintain the original equilibrium volume ratio of the two phases and thus to retain a specific volume of the other phase permanently while both phases experience extreme agitation as the coil rotates. Accordingly, solutes locally introduced at the coil inlet are partitioned between the two phases and are chromatographically separated based on the related distribution coefficients. This technique is well suited for preparative separation, offering the advantages of low solvent consumption, high repeatability, high recovery, and high solute loading capability. It can offer remarkable sample recovery in contrast with conventional methods. Therefore, since its invention, this technique has been widely used for the separation of numerous ingredients from natural products, natural medicines, functional foods, etc [14].

Xylaria striata Pat.1887 belongs to the genus *Xylaria*. It is considered a precious medicinal fungus, edible and delicious when fresh, according to traditional Chinese medical literature. Our research group has extensively screened biology activities compounds from edible and medicinal mushrooms. We found high contents of Ergosterol and Ergosterol peroxide in the

fruiting body of *Xylaria striata*. Hence, this mushroom may be a valuable resource for producing these two sterols. According to our literature investigation, there were few reports of HSCCC separations of Ergosterol [15,16]. Significantly, no reports about HSCCC separations of Ergosterol peroxide. In the present work, we reported a simple HSCCC method to simultaneously obtain Ergosterol and Ergosterol peroxide from an edible and medicinal higher Ascomycete mushroom, *Xylaria striata*. Additionally, the antimicrobial activities of these two compounds to nine kinds of plant pathogens fungi have been evaluated. In addition, this article also focuses on the issues of the related limitations and development prospects of this technique, which is expected to provide useful information to scientists working in the field of phytosterol research, acting as an essential reference.

2. Protocol

Wild fruiting bodies of *Xylaria striata* (Figure 1) were collected from the trunk base and stumps of *Sophora japonica* in the town of Qingyi, Mianyang in January 2015. It was identified by Prof. X. S. He of Southwest University of Science and Technology. And the voucher specimen was kept in the Microbiology Laboratory of Southwest University of Science and Technology, Mianyang, Sichuan Province. *Xylaria striata* can easily form fruit bodies directly in the agar culture medium. The optimized culture methods of artificial cultivation fruiting bodies are referred to the literature [17]. The procedure for the isolation and purification of Ergosterol and Ergosterol peroxide using HSCCC is described below, and the materials and equipments are referenced in the Table of Materials.



Figure 1: The fruiting bodies of *Xylaria striata*.

3. Preparing the Sample

3.1 Remove the exterior soil of the fresh mushroom by washing them with demineralized water.

3.2 Dry the mushroom materials in the shade at 30 ± 2 °C for 24

h, then in a drying oven at 60 ± 2 °C for 12 hours.

3.3 Powdered the dried materials using a grinder and passed through a sieve of 1mm mesh size for further use.

4. Extracting the Sample and Enriching the Target Fraction

4.1 Decoct the powder samples (380 g) three times with 80 % ethanol at 95 °C(2 h for each time) and combine the filtration solutions.

Note: We determined that 80% ethanol has a higher extraction rate for Ergosterol and Ergosterol Peroxide from *Xylaria striata* through preliminary experiments,

4.2 Concentrate the extraction solutions to dryness under reduced pressure at 50 °C. Suspend the residue in pure water and fractionate successively with petroleum, ethyl acetate, and n-butanol, respectively. About 15 g concentrated ethyl acetate fraction was obtained.

4.3 Elute the column chromatography with petroleum ether-ethyl acetate (10:1, 7:1, 5:1, 3:1, and 1:1, v/v) to obtain 3.2 g crude sample (target fraction) monitored by TLC, using p-anisaldehyde as a post-chromatographic derivatizing agent.

Note: The target fraction, including Ergosterol and Ergosterol Peroxide, was isolated by column chromatography.

5. Separation Using HSCCC Technology

5.1 Selection of Solvent System

5.1.1 Dissolve 10 mg crude sample in a 20 mL pre-equilibrated two-phase solvent system and mix thoroughly for several minutes.

5.1.2 Detect the content of steroids in two-phase by HPLC. Record the peak areas of target compounds in the upper phase (stationary phase) as AU and lower phase (mobile phase) as AL. Note: The choice of solvent system is mainly evaluated according to the partition coefficients (K) of the target compounds, which were determined by HPLC analysis. The K values were calculated using the equation: $K = AU/AL$. The optimum K for HSCCC should be $0.5 \leq K \leq 2.0$.

5.2 Preparation of the Two-Phase Solvent System and Sample Solution

5.2.1 Mix the selected two-phase solvent in a separatory funnel. Shake the funnel vigorously and equilibrate thoroughly at room temperature until the two phases separate.

5.2.2 Degass the separated two phases using ultrasonication for 15min, respectively.

5.2.3 Prepare the sample solution for HSCCC separation: Dissolve 500mg of crude sample in 20mL mixture solution of lower phase and upper phase (1:1, v/v) and filter through 0.45mm membrane filter before injection into the HSCCC system.

5.3 HSCCC separation procedure

5.3.1 Fill the clean coil column with the upper phase. When the column temperature is maintained at 25°C, set the rotation of the apparatus at 850 rpm and elute the lower phase through the column at a flow rate of 3 mL/min.

Note: After the lower phase front emerged, the hydrodynamic equilibrium of the solvent system was reached. The retention ratio of the stationary phase (Sf) was calculated as follows:

$$S_f(\%) = \frac{V_T - V_S}{V_T - V_L} \times 100\%$$

Where V_S is the volume of the stationary phase flowing out, V_T is the total volume of the coils, and V_L is the sample loop volume.

5.3.2 Inject the sample solution through the injection valve into the column. Set the UV absorbance at 220nm. Collect the peak fractions manually according to the chromatogram and TLC analysis.

5.3.3 Evaporate the fractions containing the target compounds under reduced pressure.

5.4 Identification of Compounds

5.4.1 Dissolve the yielded samples in methanol for subsequent purity analysis by HPLC whose conditions were as below: Zorbax Eclipse XDB C-18 column; mobile phase was methanol; the flow rate was 0.8 mL/min, and the detection wavelength was 282 nm for Ergosterol, 220 nm for Ergosterol peroxide, respectively.

5.4.2 Identify the chemical structures of each target compound by 1H-NMR and 13C-NMR.

6. Antimicrobial Activity

6.1 Add 1 mL sample solution to 24 mL PDA medium, and pour the mixture immediately into a 9 cm diameter Petri dish. When the medium is solidification, put the activated fungal cakes (6 mm in diameter) in the center of the petri dish. Furthermore, inoculate the dish at 28 °C.

Note: The same fungal cakes were inoculated in the negative control, while 1 mL 20 % methanol solution instead of sample solution was added.

6.2 Measure the colony diameter to calculate the inhibition rate using the crossing method when the mycelium in control thoroughly covers the petri dish.

Note: The inhibitory effects of steroids against nine kinds of plant pathogens fungi were evaluated using the growth rate method. The plant pathogenic fungi used in the test include *Valsa mali*, *Sclerotinia sclerotiorum*, *Geotrichum candidum* Link ex Pers, *Fusarium graminearum*, *Physoalospora nasei*, *Helminthosporium maydis*, *Fusarium moniliforme*, *Fusarium oxysporum f.sp. vasinfectum* and *Alternaria ternate f.sp.mali*. Carbendazim WP is selected as the positive control.

7. Representative Results

After using n-hexane-ethyl acetate-ethanol-water (3:1:2:0.8, v/v) as the two-phase solvent system, two sterols were obtained in the separation within 4 hours (Figure 2). Ergosterol (Fraction A, 180mg) was obtained from a 500 mg crude sample. Ergosterol

peroxide (Fraction C, 30mg) was obtained from a 500 mg crude sample after repeated HSCCC separation of fraction B. From the raw material (380 g) to the product, the total recovery of the two

sterols (0.21/0.5*3.2 g) is 0.35 %. The HPLC analysis revealed that the purity of Ergosterol was 96% and Ergosterol peroxide was 97%, respectively (Figure 3).

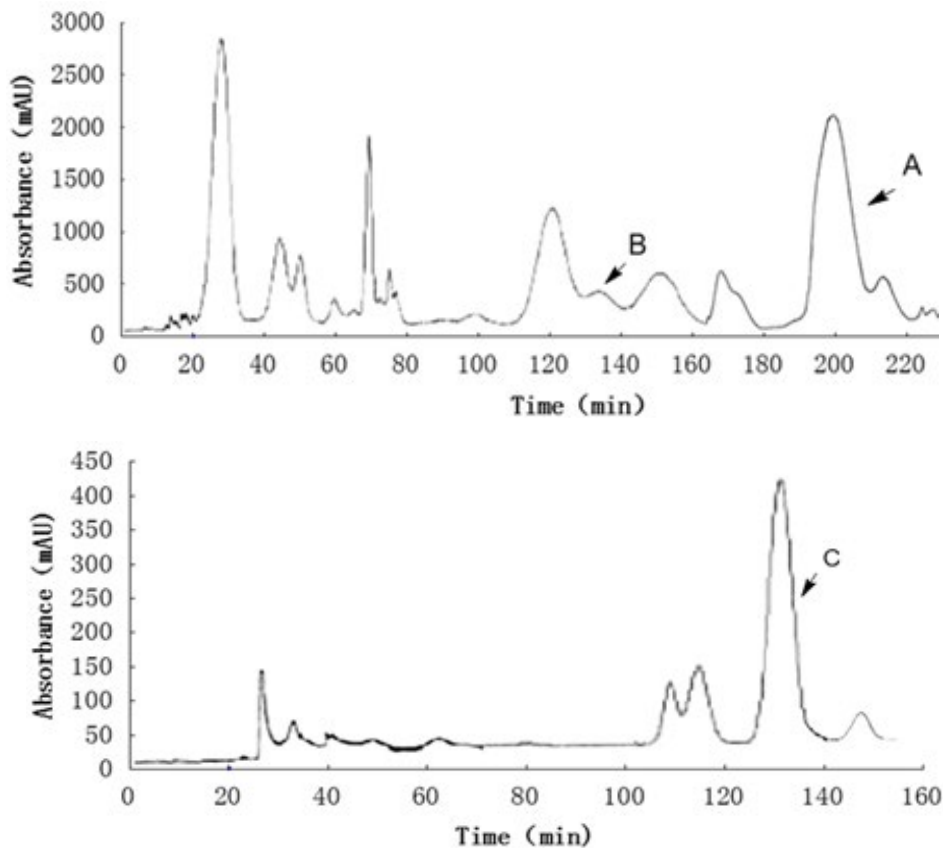


Figure 2: HSCCC chromatogram (Parameters: Two-phase solvent system, n-hexane-ethyl acetate-ethanol-water (3:1:2:0.8, v/v). The stationary phase was the upper phase, and the mobile phase was the lower phase. The rotation speed was 850rpm, injection volume was 20ml, detection wavelength was 220nm, flow rate was 3mL/min, separation temperature was 25°C)

- (1) HSCCC chromatogram of the target fraction. A: Ergosterol, B: Ergosterol peroxide.
- (2) Repeated HSCCC separation of fraction B. C: Ergosterol peroxide.

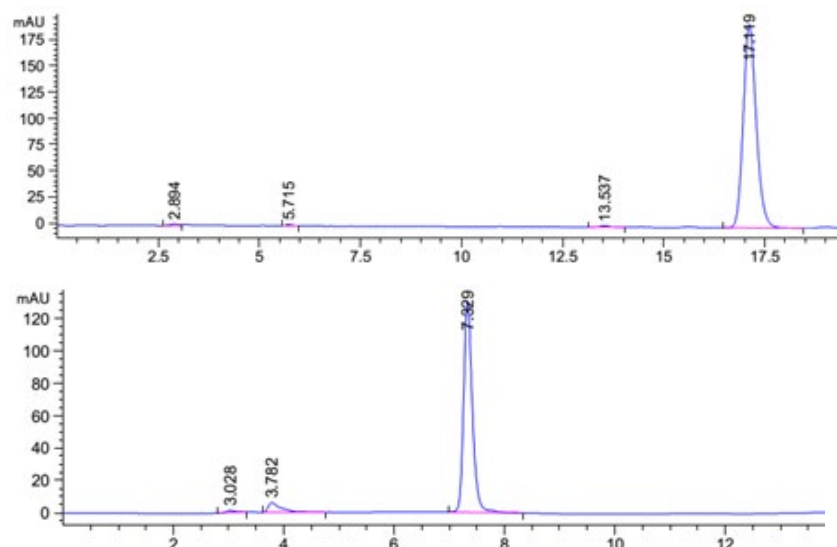


Figure 3: The HPLC chromatogram of Ergosterol (A) and Ergosterol peroxide (B)

The identification of two steroids was implemented by ¹H-NMR and ¹³C-NMR data.

Peak fraction A: White needle-shaped crystals, ¹³C-NMR(CDCl₃, 125MHz)δ141.3 (C-8), 139.7 (C-5), 135.4 (C-23), 132.1 (C-22), 119.3 (C-6), 116.1 (C-7), 70.4 (C-3), 55.7 (C-17), 54.3 (C-14), 46.2 (C-9), 42.8 (C-13), 42.8 (C-24), 40.1 (C-20), 39.1 (C-12), 38.3(C-1), 37.2 (C-10), 33.1 (C-4), 33.1 (C-25), 28.2 (C-16), 28.4 (C-2), 23.1 (C-15), 21.0 (C-11), 21.1 (C-27), 20.0 (C-26), 19.7 (C-21), 17.1 (C-28), 16.2 (C-19), 12.1 (C-18); ¹H-NMR(CDCl₃, 600MHz,)δ0.63 (s, 3H, H-18), 0.95 (s, 3H, H-9), 3.52-3.64 (m, 1H, H-3), 5.08-5.29 (m, 1H, H-22), 5.08-5.29 (m, 1H, H-23), 5.34 (m, 1H, H-7), 5.54 (dd, J= 5.4, 6.2 Hz, 1H, H-6). Comparing the data with the literature, Peak fraction A was identified as Ergosterol [18].

Peak fraction C: White needle-shaped crystals, ¹³C-NMR (CDCl₃, 125 MHz)δ135.5 (CH, C-6), 135.3 (CH, C-22), 132.3 (CH, C-23), 130.8 (CH, C-7), 82.3 (C, COOC), 79.5 (C, COOC), 66.4 (CH, C-3), 56.3 (CH, C-17), 51.6 (CH, C-14), 51.2 (CH, C-9), 44.7 (C, C-13), 42.7 (CH, C-24), 39.7 (CH, C-20), 39.3 (CH₂, C-12), 36.9 (C, C-10, and CH₂, C-4), 34.7 (CH₂, C-1), 33.0 (CH, C-25), 30.1 (CH₂, C-2), 28.6 (CH₂, C-16),

23.3 (CH₂, C-15), 20.8 (CH₃, C-21), 20.6 (CH₂, C-11), 19.9 (CH₃, C-26), 19.6 (CH₃, C-27), 18.1 (CH₃, C-19), 17.5 (CH₃, C-28), 12.8 (CH₃, C-18); ¹H-NMR(CDCl₃, 600MHz): δ0.72 (3H, s, H-18), 0.78 (3H, d, J= 6.5 Hz, H-26), 0.82 (3H, d, J= 6.5 Hz, H-27), 0.85 (3H, s, H-19), 0.87 (3H, d, J= 6.7 Hz, H-28), 0.96 (3H, d, J=6.7Hz, H-21), 3.94 (1H, m, H-3), 5.12 (1H, dd, J= 8.1, 15.1 Hz, H-22), 5.17 (1H, dd, J= 7.7, 15.1 Hz, H-23), 6.22 (1H, d, J= 8.1 Hz, H-7), 6.47 (1H, d, J= 8.3 Hz, H-6). Comparing the data with the literature, Peak fraction C was identified as Ergosterol peroxide [19].

The antimicrobial results of Ergosterol and Ergosterol peroxide are shown in Table 1. When the concentrations of the two compounds and positive control in the medium were 40ug/mL, the Ergosterol exhibited excellent fungistatic activities against *Valsa mali*, *Sclerotinia sclerotiorum*, *Fusarium graminearum* and *Helminthosporium maydwas*. The Ergosterol peroxide also exhibited strong antifungal activity against *Helminthosporium maydwas*. Moreover, these two compounds have a better inhibitory effect on *Sclerotinia sclerotiorum* than the positive control Carbendazim.

Test fungi	Inhibition rate (%)		
	Ergosterol	Ergosterol peroxide	Carbendazim
<i>Valsa mali</i>	100.0 ± 0.0 a	40.4 ± 5.5 e	100.0 ± 0.0 a
<i>Sclerotinia sclerotiorum</i>	100.0 ± 0.0 a	60.4 ± 5.05 b	41.9 ± 2.4 c
<i>Geotrichum candidum Link ex Pers</i>	81.2 ± 0.43 c	56.2 ± 6.23 b	56.0 ± 2.2 b
<i>Fusarium graminearum</i>	100.0 ± 0.0 a	30.8 ± 4.8 cd	100.0 ± 0.0 a
<i>Physalospora nasei</i>	88.5 ± 4.7 b	14.5 ± 1.7 ef	100.0 ± 0.0 a
<i>Helminthosporium maydwas</i>	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
<i>Fusarium moniliforme</i>	36.4 ± 2.2 e	4.2 ± 0.5 f	100.0 ± 0.0 a
<i>Fusarium oxysporum f.sp. vasinfectum</i>	54.7 ± 5.9 d	22.0 ± 3.5 de	36.1 ± 1.5 d
<i>Alternaria ternate f.sp.mali</i>	13.8 ± 2.3 g	37.3 ± 1.9 c	6.9 ± 1.1 f

Note: Three replicates, ± represents the standard deviation of mean. Different letter indicates significant difference between compounds (p < 0.05).

Table 1: Inhibitory effect of two steroids against nine kinds of pathogenic fungi

8. Discussion

8.1 Purification Parameter Screening

The solvent systems for the purification of Phytosterols in HSCCC include n-hexane-ethylacetate-acetonitrile, n-heptane-ethylacetate-acetonitrile, n-hexane-ethanol-H₂O, and n-hexane-ethylacetate-methanol/ethanol-H₂O₁₄. According to the literature reports above, we tested eight solvent systems and measured the K-values summarized in Table 2.

Selecting an appropriate two-phase solvent system plays a vital role in a successful separation by HSCCC. It depends on three conditions:

- The solvent system's settling time (stratification) should be less than the 30s.

- The stationary phase retention (Sf) should be greater than 45%.
- The target compound's partition coefficient (K) is generally 0.5 to 2.

In tested solvent systems, the settling time of the solvent system composed of n-hexane-ethanol-water (3:4:1, v/v) was so long that it was abandoned first. The solution system n-hexane-ethylacetate-n-butanol-ethanol-water(3.5:0.3:0.5:2.5:0.3) was chosen to purify Ergosterol by Huang¹⁵. However, we found that our sample was not easy to dissolve in it. When the solvent systems, n-hexane-ethylacetate-acetonitrile(5:1:5) and n-heptane-ethylacetate-acetonitrile(5:1:5) were tested, the separation factor of Ergosterol was greater than 2, which would result in eluting in broad peaks and extended time. In the solvent system composed

of n-hexane-ethylacetate-methanol-H₂O(3:1:2.8:0.8), the K values were higher than two, and the Sf was 63%. After using ethanol to replace methanol, K values became more suitable, and Sf achieved 75%. Fortunately, according to continually decreasing the ratio of ethanol, the K value gradually declined along with the solvent polarity. When a ratio of n-hexane-ethylacetate-ethanol-H₂O (3:1:2:0.8) was tested, the stationary phase retention improved, and the K values of the two target compounds were in the range of 0.5 to 2.0. The differences between the two K values were significant enough can improve the resolutions to each other.

We also investigated other parameters like the rotation speed of the coiled column, the mobile phase flow rate, and the column temperature to obtain the best HSCCC performance. The optimum HSCCC conditions could be summarized as follows: A flow rate of 3 mL/min, a rotation speed of 850 rpm, and a temperature of 25 °C were used.

Limitations and Development of HSCCC used in the Purification Of Phytosterols

Although HSCCC is an emerging technology that offers many advantages over traditional separation methods, its utilization for the separation of phytosterols is hindered by several drawbacks:

Complexity of Solvent Systems Selection

Solvent system selection is not guided by system-based theories but based on empirical regularity. Thus, selecting a suitable solvent system for high separation efficiency requires many prior references. However, there is less literature to refer to as HSCCC is not as widely known as LC techniques such as HPLC. Even if a referable similar solvent system is found, the risk of instability remains when the sample source changes, just like the problem we had met.

Time-Consuming

Not only us, but most HSCCC separations also reported thus far have been achieved with relatively long timeframes.

Sample Quantity Limit

The current procedures limit the HSCCC sample loading to several decagrams or a few hundred milligrams, whereas loadings of several hectograms or kilograms are urgently needed, as they can significantly improve yields and meet the applicable to industrialization. Despite the above shortcomings, HSCCC can offer remarkable sample recovery and directly purify the final product from the crude plant extracts. As an efficient separation and purification technique, HSCCC is expected to draw the interest of many researchers and will undoubtedly become a mainstream technique for Phytosterol separation.

Disclosures

The authors have no conflicts of interest and nothing to disclose.

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