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Modified Microwave-Assisted Extraction of Ergosterol for Measuring Fungal Biomass in Grain Cultures

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Ergosterol is a measure for fungal biomass. The recovery rates using a previously described microwave-assisted-extraction (MAE) method for ergosterol analysis tended to be low for grain cultures (pure culture in sterilized 40% moisture content grain) inoculated with *Fusarium graminearum*. An improved MAE method for measuring ergosterol in grain cultures was developed and compared. Modification to the original MAE included alterations in duration of microwave exposure and extraction solvents. Four autoclaved grains (wheat, rice, barley, and corn) were inoculated with *F. graminearum* or spiked with ergosterol at concentrations from 0.88 to 100 μg/g and extracted with both methods. The ergosterol recovery rates were significantly different ($p < 0.05$) for the two methods in assaying both the spiked and grain culture samples. The modified method provided greater recovery rates than the previously reported MAE method for the spiked samples and *F. graminearum* grain cultures.

KEYWORDS: Ergosterol; microwave-assisted extraction; grain cultures; recovery; HPLC

INTRODUCTION

Ergosterol can be used to quantitatively measure fungal biomass in grains (*1*−*3*). The conventional liquid−liquid extraction is labor-intensive and time-consuming and requires large volumes of reagents and large sample sizes (*4*−*6*). The microwave-assisted-extraction (MAE) method was developed by Young (*6*), to overcome the problems associated with the traditional extraction method. Young (*6*) indicated that ergosterol values derived from the MAE method were comparable to those obtained from the conventional liquid−liquid extraction after assaying fungal hyphae and spores, mushrooms, filtered air, artificially contaminated corn, naturally contaminated grain dust, and soil.

Montgomery et al. (*4*) investigated the ergosterol recovery in soil matrices using a modified MAE protocol from the method of Young (*6*), obtaining an average recovery of 62 ± 11% of ergosterol from mycelial mats added to soils and 90 ± 6% of ergosterol added in solutions at a concentration of 1 μg/g. The radiation duration of 35 s in Young's method (*6*) was changed to two stages, 18 and 17 s, with 15 min cool down in between, because of "rupturing of test tubes under high temperature and pressure conditions".

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The Montgomery et al. (*4*) method was used to analyze grain culture samples inoculated with *Fusarium graminearum*, but the resulting concentrations were unreasonably low and the repeatability was not satisfactory for these high-concentration samples. The objective of this study was to develop an MAE method suitable for analyzing the ergosterol contents of grain culture samples. The recovery rates of spiked ergosterol from four grain matrices were compared using the reported method (*4*) and the modified method. The ergosterol values of grain culture samples were also compared for both methods.

MATERIALS AND METHODS

Experimental Designs. The recovery experiment from spiked material was a random complete block design (RCBD) with split−split plot arrangement, in which the extraction methods (the modified method from this project and the reported MAE by Montgomery et al. (*4*)) were treated as whole plots, a concentration as a subplot, and a grain matrix as a sub-subplot (*7*). The dependent variable of interest to be investigated in the study was the ergosterol recovery rate. The main independent variable was the extraction method. Grain matrices included 40% moisture content of autoclaved corn, rice, barley, and wheat. The ergosterol was spiked to cover a range of concentrations from 0.88 to 100.00 μg/g. This range was chosen because it fits into the linearity of the calibration plot of ergosterol standards. In addition, the incomplete solubility of ergosterol in propan-2-ol at concentrations higher than 100.00 μg/g was another reason for choosing this range.

For the grain culture experiment, RCBD with factorial arrangement (*8*) was employed to compare ergosterol recovery for the two extraction methods and the two incubation temperatures. A total of 2 g of each grain culture sample was divided into two equal subgroups: 1 g was extracted with the modified method, and the other 1 g was extracted

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Table 1. Extraction Protocol and the Differences between the Two Methods

procedures	reported method (*4*)	modified method
1 g of sample extracted with	2 mL of methanol and 0.5 mL of 2 M NaOH in a 27 mL capacity test tube	2 mL of methanol, 2 mL of propan-2-ol, and 1 mL of 2 M NaOH in a 50 mL test tube
the combination was placed at the center of the microwave and exposed for	18 s	10 s
the sample was removed from the oven and cooled for	15 min	3 min
the sample was returned to the oven and irradiated for an additional	17 s, cooled for 15 min, ending up with a total microwave treatment of 35 s and total cycle time of 30 min	10 s, cooled for 3 min, with the cycle repeated twice, ending up with a total microwave treatment of 40 s (4 × 10 s) and total cycle time of 15 min
the contents were neutralized with	1 M HCl, and then 2 mL of methanol was added	1 M HCl, and then 2 mL of distilled water was added
the contents were vortexed, and ergosterol was extracted with	6 mL (3 times, 2 mL each time) of pentane	9 mL (3 times, 3 mL each time) of pentane

with the reported MAE of Montgomery et al. (*4*). Three replications were performed for all of the treatments.

Analysis of variance (ANOVA) with least-squares mean (LSM) multiple comparison was employed to test the treatment differences at the significance level of 0.05. Three replications were performed for all of the treatments.

Materials. Ergosterol (above 99% purity, Fluka, Steinheim, Switzerland) was used as the calibration standard and in the spiking experiment. Methanol, HCl, NaOH, high-performance liquid chromatography (HPLC)-grade propanol, and hexane from EMD Science (Gibbstown, NJ), as well as pentane (Baker, Phillipsburg, NJ), were used in the study. Wheat kernels were obtained from Casselton Agricultural Station, North Dakota State University, in June 2003. Barley kernels were obtained from Busch Agriculture Resources, Inc., Fargo, ND, in June 2003. Long-grain white rice was purchased (Fargo, ND) in June 2003. *Fusarium*-free corn was obtained from the University of Nebraska in 1998. Grain samples were determined to be free of *Fusarium* infection and were frozen until used.

Preparation of Autoclaved Grain Samples, Spiking and Recovery. A total of 25 g of the corn, rice, barley, or wheat whole kernels was put inside half-pint Mason jars (Keri brand, Goodman's, Inc., Miami, FL), adjusted to 40% moisture with distilled water, then allowed to equilibrate overnight (>16 h), and autoclaved for 15 min at 121 °C. The grains were freeze-dried and ground into fine powder by a grain grinder (3600 Laboratory Mill, Perten Instruments, Huddinge, Sweden). A total of 2 g of grains at each treatment was spiked with standard ergosterol dissolved in 2 mL of propan-2-ol at each concentration. The ground grain samples were mixed well with the ergosterol solution and evaporated to dryness at 50 °C under a stream of nitrogen gas. Recoveries were calculated by the comparison of UV response peaks from the spiked samples, with the external ergosterol standards at corresponding concentrations (*9, 10*).

Preparation of *F. graminearum*-Inoculated Grain Cultures. *F. graminearum* strain R-9826 (*Fusarium* Research Center, Pennsylvania State University, University Park, PA) was used to inoculate the grain samples. Carboxymethycellulose broth medium (*11*) was used to stimulate and produce conidiation of *F. graminearum*. The broth was filtered with four layers of sterile cheesecloth to remove the mycelia and then diluted with sterile distilled water to make the conidia suspension. The concentration of the conidia suspension was determined by a hemocytometer and adjusted to 10^4 conidia/mL with sterile distilled water.

Grain samples were prepared as described above. The jars were sealed with prepunched lids containing two layers of Whatman no. 1 filter paper, placed under the lids to facilitate air exchange, and autoclaved for 15 min at 121 °C. After cooling, 1 mL of the *F. graminearum* (10^4 macroconidia/mL) inoculum was added aseptically into each jar and mixed well by shaking. Cultures were incubated at 15 or 25 °C at 80% humidity for 2 weeks with a 12 h/day fluorescent light (20 W) cycle in an environmental chamber (Caron, Model 6030, Marietta, OH). Each jar was shaken by hand for 30 s once every day to ensure even growth during the incubation period. Growth of cultures was stopped by submerging them in 20 mL of chloroform (EMD, Gibbstown, NJ) and allowing them to dry overnight inside a hood. The cultures were freeze-dried (Labconco freeze-dry system 4.5, Kansas City, MO), ground into fine powder (3600 Laboratory Mill, Perten Instruments, Huddinge, Sweden), and stored at −18 °C until analyzed.

Extraction. A Goldstar (Goldstar LTD, Seoul, Korea) microwave oven operating at 2450 MHz and 750 W maximum output was used to irradiate the samples. The differences between the reported method (*4*) and modified method are shown in **Table 1**. A "safety note" to consider with microwave extraction is to seal containers as indicated and to not exceed the irradiation times specified in the method.

Reported Extraction Method (4). A total of 1 g of sample was extracted with 2.0 mL of methanol and 0.5 mL of 2 M aqueous NaOH in a 27 mL test tube. The test tube was sealed tightly and inserted into a screw-capped high-density polyethylene (HDPE) plastic bottle (16.5 × 7 cm, Nalgene, Lima, OH). The bottles were tightly sealed with the corresponding HDPE screw caps, and the samples were microwave-irradiated for 18 s, removed from the oven, and cooled for 15 min. The bottles were then returned to the oven and irradiated for an additional 17 s, taken out from the oven, and cooled for 15 min, and the tubes were removed from the plastic outer bottle. The contents inside the test tubes were neutralized with 1 M HCl by measuring the pH values with a pH meter (model 520A, Orion Research, Inc., Boston, MA), and then methanol (2 mL) was added. The contents were vortexed, and ergosterol was extracted with 2 mL of pentane, 3 times. The pentane extracts were combined and evaporated to dryness at 50 °C under a stream of nitrogen gas. Methanol (1 mL) was added to the tubes to redissolve the ergosterol for HPLC analysis.

Modified Extraction Method. A total of 1 g of sample was suspended in methanol (2 mL) and propan-2-ol (2 mL). Samples were treated with 2 M NaOH (1 mL) in a 50 mL capacity test tube. One tube containing the mixture was put inside a HDPE plastic bottle (16.5 × 7 cm, Nalgene, Lima, OH) and tightly sealed with the corresponding HDPE screw caps. The bottle was placed at the center of the microwave and irradiated for 10 s. After cooling for 3 min, the tube was irradiated for an additional 10 s. Then, the same cycle was repeated twice, ending up with a total microwave treatment of 40 s (4 × 10 s) and total cycle time of 15 min. Then, the contents were neutralized with 1 M HCl, and distilled water (2 mL) was added. The contents were vortexed and extracted with 9 mL of pentane (3 times, 3 mL each time). The pentane extracts were combined and evaporated to dryness at 50 °C under a stream of nitrogen gas. Methanol (1 mL) was added to the tubes to redissolve the ergosterol for HPLC analysis.

Ergosterol Analysis. Samples were filtered through a 0.2 μm filter and then analyzed with Waters HPLC (2690 separation module, Waters Corporation, Milford, MA) using a mobile phase of hexane/propan-2-ol (90:10, v/v) at 1.2 mL/min. Separation was achieved with a Waters spherisorb silica column (4.6 × 250 cm), with detection of ergosterol at 282 nm using a Waters photodiode array (PDA, model 996, Waters Corporation, Milford, MA) detector. Data acquisition and processing were performed by Millennium software (3.20 version, Waters Corporation,

Table 2. Comparison of Two Methods for Ergosterol Recovery[a] from Spiked Autoclaved Grains

matrix[b]	spiked ergosterol concentration (μg/g)	recovery (%) reported method (*4*)	recovery (%) modified method
barley	100.00	37.80 ± 6.51 a	99.47 ± 2.40 c
	10.00	50.87 ± 9.62 a	99.50 ± 2.32 c
	5.00	47.77 ± 3.95 a	100.63 ± 1.11 c
	2.50	68.16 ± 6.84 b	98.40 ± 0.96 c
	1.25	65.20 ± 9.78 b	95.57 ± 1.96 c
	0.88	80.66 ± 10.29 bc	99.46 ± 2.70 c
wheat	100.00	49.87 ± 1.48 a	100.43 ± 1.93 c
	10.00	33.20 ± 8.32 a	100.17 ± 1.75 c
	5.00	39.10 ± 8.67 a	99.33 ± 0.87 c
	2.50	62.57 ± 16.16 b	99.40 ± 0.98 c
	1.25	78.06 ± 8.86 b	99.87 ± 2.25 c
	0.88	82.40 ± 5.84 b	99.47 ± 2.89 c
corn	100.00	43.13 ± 4.17 a	100.73 ± 1.87 c
	10.00	48.40 ± 6.91 a	97.70 ± 1.90 c
	5.00	45.73 ± 2.38 a	99.20 ± 1.01 c
	2.50	45.13 ± 3.49 a	99.37 ± 1.10 c
	1.25	81.12 ± 3.60 b	98.57 ± 0.85 c
	0.88	80.43 ± 11.55 b	101.57 ± 1.75 c
rice	100.00	38.10 ± 3.90 a	98.60 ± 1.99 c
	10.00	50.90 ± 3.15 a	99.10 ± 1.83 c
	5.00	40.07 ± 2.37 a	97.50 ± 1.23 c
	2.50	54.27 ± 9.43 b	96.70 ± 1.80 c
	1.25	75.48 ± 5.71 b	96.20 ± 2.57 c
	0.88	82.72 ± 5.98 bc	99.87 ± 3.02 c

[a] Means ± standard deviations. The same letters in columns and rows indicate no significant difference, while different letters indicate a significant difference ($p < 0.05$, $n = 3$). [b] A 40% moisture content of autoclaved grains.

Milford, MA). The method for determining the sample concentrations in the study was external standard calibration (*12*). The linear calibration plot that was used to quantify the grain culture samples had a range between 0.88 and 100 μg/mL, with an injection volume of 10 μL.

RESULTS AND DISCUSSION

Limit of Detection (LOD) and Limit of Quantification (LOQ). The experimental method LOD for wheat, barley, corn, and rice was 0.31 μg/g, and the experimental LOQ for the four grains was 0.88 μg/g. The LOD and LOQ in the study were set at a signal-to-noise of more than 3 and 10, respectively (*10, 12*). The ergosterol retention time was confirmed by the UV spectrum at 282 nm, similar to that observed by Seitz et al. (*3*). Schwadorf and Muller (*5*) also used 282 nm as the output absorbance wavelength in their normal-phase HPLC method. At ergosterol concentrations below 0.88 μg/g, the interference became significant because the recovery rates were calculated at over 110%. Hence, the LOQ was 0.88 μg/g.

Comparison of Ergosterol Recovery from Spiked Samples. The ergosterol recovery comparisons are shown in **Table 2**. Ergosterol recovery rates from spiked autoclaved grains were significantly different ($p < 0.001$). Overall, the modified method provided greater recovery rates than the reported MAE method (*4*).

For the reported method, the higher the ergosterol concentration, the lower the recovery rate (**Table 2**). The limiting factor for recovery might be the amount and kinds of solvents used to extract ergosterol. Montgomery et al. (*4*) evaluated ergosterol recoveries from samples at concentrations of 1 μg/g. They found that the recovery was 90% (±6%). In the current study, with grain matrices, the following modifications were made to improve recovery at higher concentrations. A total of 2 mL of propan-2-ol in addition to methanol prior to the microwave irradiation was added. Ergosterol had a higher solubility in propan-2-ol than in methanol when making ergosterol stock solutions. The enhanced solubility may have promoted an increased removal of ergosterol from the spiked samples. The extraction efficiency of the modified method may have been further promoted by the addition of water in place of methanol, which may have forced partioning of the ergosterol into pentane during final extraction (*1, 2*). When the amount of 2 M NaOH was increased from 0.5 to 1 mL, and the amount of pentane increased from 6 to 9 mL (3 mL, 3 extraction times). A further increase in ergosterol recovery was observed in spiked samples containing higher ergosterol concentrations during preliminary studies. Davis and Lamar (*1*) also noticed that "losses of ergosterol undergoing extraction were subsequently attributed to incomplete partitioning to hexane from the 10% KOH methanol-aqueous phase by use of two hexane extractions". The amount and extraction times of pentane were increased in the study to completely partition ergosterol from the NaOH methanol-aqueous phase to pentane, especially in higher concentration samples; thus, recovery rates were improved.

Table 3. Ergosterol Concentrations[a] (μg/g) of Grain Culture Samples

media[b]	temperature[c] (°C)	concentration by the reported method(*4*)	concentration by the modified method
rice	25	2921.82 ± 259.03 a	9718.29 ± 196.43 e
	15	879.24 ± 51.02 cd	1877.73 ± 88.53 b
corn	25	1425.96 ± 138.15 c	3168.85 ± 59.05 a
	15	699.78 ± 99.88 d	1348.09 ± 26.22 c
wheat	25	3659.97 ± 108.94 g	8630.22 ± 165.81 f
	15	800.16 ± 200.43 cd	1672.81 ± 29.18 b
barley	25	2148.80 ± 371.08 b	4145.79 ± 100.06 h
	15	450.35 ± 85.15 d	1208.53 ± 23.69 c

[a] Means ± standard deviations. The same letters in rows and columns indicated no significant difference, while different letters indicated a significant difference ($p < 0.05$, $n = 3$). [b] A 40% moisture content of autoclaved grains, inoculated with *F. graminearum* and incubated for 14 days. [c] Temperature of incubation.

The ergosterol recovery results had no significant difference among the four grain matrices for either method. This indicated that the different grain matrices evaluated did not influence ergosterol recovery.

Comparison of the Modified Method to the Reported Method for *F. graminearum*-Inoculated Grain Cultures. The results, summarized in **Table 3**, indicated that the modified method recovered a significantly greater concentration of ergosterol from grain culture samples in comparison to the reported method ($p < 0.001$). Also, there was a significant difference ($p < 0.001$) between the ergosterol concentrations obtained at the two different incubation temperatures; 25 °C yielded more ergosterol than 15 °C, as was expected.

CONCLUSION

This study investigated the ergosterol recovery rates from spiking four autoclaved grains over a range of concentrations. Ergosterol recovery was not affected by grain matrix but was significantly influenced by the ergosterol concentration for the previously reported method (*4*). Higher spiking concentrations in the method of Montgomery et al. (*4*) provided less recovery. The modified method recovered more ergosterol in grain culture samples compared to the reported method ($p < 0.05$). In summary, the modified method demonstrated a significant improvement for measuring fungal biomass in autoclaved grains inoculated with *F. graminearum*.

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