Analysis of Ergosterol in Cereals without Saponification

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Summary

Ergosterol (ERG) is a sterol found in fungal cell membranes and is not produced by most plants and animals. Thus, ERG is a potential biomarker of fungal invasion in grain. Previously, we developed an analytical method for ERG in grains by refluxing samples with methanol-alkali, and performed a single laboratory validation of the method. However, the method is not suitable for screening purpose, which needs to be rapid and simple. Therefore a simplified method was developed for analysis of ERG in grains. In this simplified method, ERG was first extracted with methanol by horizontal shaking for 1 hr. After filtration, ERG was partitioned into hexane, and then analyzed with reverse phase high performance liquid chromatography (HPLC). Recoveries of ERG obtained with this shaking method were compared with recoveries obtained with the refluxing method. In maize, recovery of ERG at 3 mg/kg using this new shaking method was 84 % of that of the refluxing method and the relative standard deviation (RSD) of the shaking method and the refluxing method were 1.5 % and 16 %, respectively. In wheat, recovery of ERG at 12 mg/kg by the shaking method was 108 % of that of the refluxing method, and the RSD of the shaking method and the refluxing method were 2.8 % and 5.9 %, respectively. This study demonstrates this simplified protocol for ERG analysis in grains to be a suitable method for screening purposes.

Key words: ergosterol, cereal, without saponification, validation

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Introduction

Many foods and feeds such as grains, vegetables, and fruits consumed by human and animals, are potentially at risk of contamination by a wide range of fungi1) and their byproducts6). Fungal contamination on foods and feeds results in the deterioration of appearance, reduction of taste and flavor, and also may result in the presence of toxic secondary metabolites (mycotoxins)10).

Mycotoxins are produced by various fungi12) and more than several hundreds of these secondary metabolites have been identified1). Toxicity of these mycotoxins are diverse from acute4) to chronic toxicity5, 5), with effects, such as vomiting4), skin irritation6) and carcinogenicity5, 7). Contamination of mycotoxins is known to occur in a wide range of agricultural products such as grains9), fruits9), beverages9, 10) and meat products11). Contamination in grains such as maize and wheat continues to be a serious problem worldwide12, 13). As a result, many countries have established regulations to prevent and/or limit the levels of mycotoxins in agricultural products14, 15). Analytical methods for individual mycotoxins such as aflatoxins16), ochratoxin A17),

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Mycotoxins, specifically fusarium toxins\(^1\) and patulin\(^2\) have been developed to monitor their levels and to ensure compliance with these regulated limits.

Although analytical methods have been developed for individual mycotoxins, a single method to analyze for many or all mycotoxins in agricultural commodities is not available. Recently, multi-toxin detection methods using LC-MSMS have been developed\(^3,4\). However, these methods are still not capable of analyzing all mycotoxins. To screen for possible mycotoxin contamination, methods that can detect fungal invasion generally are very useful. To place grains on culture plates and detect fungi is one of the most popular approaches for this purpose\(^5\). However, this approach is time consuming and not helpful when the fungus is not alive, but the mycotoxins may be present. Another approach is to analyze general fungal metabolites\(^6,7\). Detection of ergosterol (ERG) is also a potential alternative to detect fungal invasion in grain\(^8\).

ERG is a precursor of vitamin D\(^9\) and a component of fungal cell membranes\(^10\). ERG is produced by most fungi, but not produced by most plants\(^11,12\). Because ERG is necessary for fungal growth, the dry weight of the fungus is highly correlated with ERG amounts, and as a result, this correlation has been used to estimate biomass in soil\(^13\). Other than in soil, ERG has been considered as a biomarker of fungal invasion in agricultural commodities such as maize\(^14\), wheat\(^15\) and apple\(^16\). In addition, when ERG in maize is less than 3 mg/kg the quality of maize is acceptable, while the quality is questionable when ERG is over 8 mg/kg\(^17\). Thus, if we have a method suitable to analyze ERG in grain, the method may be useful to evaluate the quality of the grain.

In most analytical methods for ERG, ERG is extracted by saponification and after some clean-up, ERG was analyzed by HPLC\(^18,19\), because a part of ERG exists as estered form in fungal membrane\(^20\). We recently developed and validated an analytical method of ERG in a variety of grains using saponification and silica gel column clean-up\(^21\). However, the saponification step is time and labor consuming. Therefore, saponification is not a suitable method for screening purposes, which requires rapid and simple steps. Therefore, we developed a simple analytical method of ERG in grains which is suitable for screening purposes.

**Materials and Methods**

**Chemicals and Sample**

ERG, 3β,22E-ergosta-5,7,22-trien-3-ol, standard purchased from ACROS (Geel, Belgium) was dissolved with methanol (2,000 mg/L) and stored at \(-30^\circ\)C. For extraction and clean-up, GR grade chemicals, and for HPLC, HPLC grade solvents were used. All these chemicals were purchased from Kanto Chemical Co. Ltd (Tokyo, Japan) and used without further purification.

Wheat and rice samples were purchased at local markets. The maize sample was imported from the USA. The buckwheat sample was cultured in our university experimental field. All cereal samples were analyzed by the saponification method which we reported previously\(^22\) and no ERG was detected from rice samples, but some ERG was detected in wheat (3.0 mg/kg), maize (7.0 mg/kg) and buckwheat (4.7 to 25 mg/kg).

**Preparation of mouldy grain samples**

Each cereal (100 g) was placed in 300 ml Erlenmeyer flasks and 15 ml of water was added, and then autoclaved at 121 \(^\circ\)C for 15 min. These samples were inoculated with *Aspergillus oryzae* Maru 59 or *Penicillium verrucosum* NRRL5589 and cultured for about 1 week at 20 \(^\circ\)C in the dark. After autoclaving, samples were dried in an oven at 60 \(^\circ\)C overnight and then milled through 1 mm
mesh (Variable Speed Rotor-Mill pulverisette 14, FRITSCH, Germany). ERG in each sample was measured by the saponification method\textsuperscript{30}, and stored at −30 °C until used.

**Preparation of samples for analysis**  To prepare maize samples, *A. oryzae* cultured maize (200 g) and *P. verrucosum* cultured maize (100 g) were mixed well and the concentration of ERG was measured by the saponification method. The mixture was then diluted to 8.0 mg/kg of ERG with non-inoculated maize to make samples used in this study. In the case of wheat, *A. oryzae* cultured wheat (100 g) and *P. verrucosum* cultured wheat (200 g) were mixed well, then diluted to 13 mg/kg of ERG with non-inoculated wheat. Polished and unpolished rice samples were prepared exactly the same way as wheat. Unpolished rice samples were prepared to 5.0 and 15 mg/kg and polished rice samples to 4.0 and 14 mg/kg of ERG. To prepare buckwheat samples, a naturally contaminated sample (25 mg/kg of ERG) was diluted to 8.0 mg/kg of ERG with less contaminated buckwheat (4.7 mg/kg of ERG). The naturally contaminated buckwheat at 25 mg/kg was also used for this study. All prepared samples were stored at −30 °C until used.

**HPLC analysis**  ERG was analyzed by HPLC with UV detection (Shimadzu LC-10 series, Shimadzu Co. Ltd., Kyoto, Japan). The HPLC system consisted of two LC-10AD pumps, an SPD-M10Avp photo diode array detector, a SIL-10A auto sampler, a SCL-10A system controller and a CTO-10ASvp column oven. Data were handled with LC Solution (Shimadzu).

ERG was separated with an ODS column (Inertsil ODS-SP, 4.6 mm i.d. × 150 mm, GL Sciences, Tokyo, Japan) and an ODS guard column (Inertsil ODS-SP, 4 mm i.d. × 10 mm, GL Sciences). The columns were maintained at 40 °C. The injection volume of samples was 10 µl for each analysis. Mobile phase A was a mixture of methanol and water (80 : 20, v/v), and mobile phase B was a mixture of methanol and ethanol (70 : 30, v/v). The total flow rate was 1.0 ml/min. The ratio of the mobile phase components was 1 : 1 for the first 5 min, then the ratio of mobile phase B was increased to 70 % in the next 5 min, then 90 % in the next 3 min. This last ratio was kept for 5 min. ERG was detected by UV absorption at 282 nm.

**Study of extraction of ERG without Saponification**  We evaluated 3 steps in the analytical method for ERG, namely the 1) effect of shaking time length for extraction rate, 2) effect of KCl for the liquid-liquid partition, 3) necessity of silica gel column clean-up for sample clean-up.

Shaking time length was studied at 15, 30, 60, 90 and 120 min and amounts of ERG in extract were measured. For this study, a wheat sample (13 mg/kg of ERG) and a buckwheat sample (25 mg/kg of ERG) were used.

To determine the effect of KCl on extraction during the liquid-liquid partition step, methanol extracts of wheat and maize were prepared by shaking. These extracts were spiked with ERG at a level of 500 µg/L and mixed well. Ten ml of these ERG spiked extracts were subjected to liquid-liquid partition using 10 ml hexane with and without the addition of 10 ml of 3 % (w/v) KCl aqueous solution. After the two layers were separated, 5 ml of the hexane layer was collected into a vial and evaporated under a gentle N\textsubscript{2} gas stream. Dried samples were dissolved in 1.0 ml of methanol then analyzed by HPLC.

To study the necessity of silica gel column clean-up for analysis, extracts of each grain were prepared by the shaking method with KCl solution liquid-liquid partition. Half of each extract was cleaned up using a SPE column (Strata Si-1 Silica, phenomenex, USA), following the method of Miyagawa et al.\textsuperscript{30}. The other half of
each extract was dried under a gentle N\textsubscript{2} gas stream. Both samples were analyzed by HPLC and the ERG quantities and chromatogram patterns were compared.

**Comparison of two analysis methods** Extraction amount and relative standard deviation (RSD) of ERG from various grains by two analytical methods for ERG were compared. The method using saponification for ERG extraction reported by Miyagawa et al.\textsuperscript{35} was compared to the new method presented here.

In the method with saponification for extraction, grain sample (10 g), NaOH (5 g) and methanol (40 ml) were placed in a 300 ml flat-bottom flask and refluxed for 60 min at a rate of about one drop per second. After cooling to room temperature, the extract was filtered through filter paper (No. 2, Whatman, Maidstone, UK) and 10 ml of filtrate was transferred into a 200 ml separatory funnel. Ten ml of 3 % (w/v) KCl aqueous solution was added to the funnel and mixed, and then 10 ml hexane was added and mixed. The mixture was shaken vigorously for 3 min and the phases allowed to separate. After separation, 3 ml of hexane layer was passed through a silica gel column which had been preconditioned with 3 ml hexane. ERG was then eluted from the column with 1.5 ml methanol.

In the newly developed method, grain sample (10 g) and methanol (40 ml) were placed in 100 ml Erlenmeyer flasks and shaken using a horizontal shaker (SA-31, Yamato Scientific Co. Ltd., Tokyo) at 320 rpm for 60 min. After filtering (No. 2, Whatman, Maidstone, UK), 10 ml of filtrate was transferred into a 200 ml separatory funnel, and 10 ml of 3 % (w/v) KCl aqueous solution was added and mixed. Ten ml hexane was then added and the mixture was shaken vigorously for 3 min. After separation of the phases, 5 ml of the hexane layer was aliquoted into a vial and evaporated under N\textsubscript{2} gas stream. The residue was dissolved with 1.0 ml of methanol.

**Results and Discussion**

**Optimization of extraction time** The amounts of ERG extracted after 15 to 120 min extraction periods are shown in Table 1. For the wheat sample, the amount of ERG extracted at 15 min extraction time was the same as that of 60 min. However, for the buckwheat sample, the amount of ERG analyzed became stable at the 30 min or longer extraction periods.

From these result, the shaking period was fixed for 60 min to minimize the effect from variable matrix conditions and for convenience of analysis.

<table>
<thead>
<tr>
<th>Extraction time (min)</th>
<th>Ratio of extraction amount (%)\textsuperscript{a}</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Buckwheat</td>
</tr>
<tr>
<td>15</td>
<td>86</td>
</tr>
<tr>
<td>30</td>
<td>98</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>90</td>
<td>101</td>
</tr>
<tr>
<td>120</td>
<td>102</td>
</tr>
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</table>

\textsuperscript{a}Ratio of extraction by time, against 60 min (N = 1)
Clean-up condition of extract for HPLC analysis

The effect of KCl addition upon liquid-liquid partition was studied using maize and wheat samples (Table 2). In both samples, when KCl aqueous solution was added before liquid-liquid partition, the extraction of ERG into hexane was higher than without KCl. ERG in methanol partitioned efficiently into hexane by single liquid-liquid partition when KCl aqueous solution was added. The addition of water without KCl to methanol resulted in an emulsion at liquid-liquid partition.

Compared to extraction by saponification, the extract by simple shaking appeared clear and contained few interfering materials for HPLC analysis. Therefore the need of a SPE column clean-up after the liquid-liquid partition step was also studied. First, without silica gel column clean up, the hexane solvent in the sample solution needed to be changed to methanol before the sample was injected into the HPLC. Although evaporation of hexane under N₂ gas stream is typically used, this step may cause the reduction of recovery of ERG. So for each matrix at several ERG concentrations, the recovery of ERG was checked to determine if the levels decreased. As shown in Fig. 1, ERG in grain without an SPE column clean-up was sufficiently analyzed with no significant improvement in the chromatogram near the ERG peak.

From these results, the clean-up step of ERG following the shaking method was fixed to one step liquid-liquid partition with KCl solution.

Comparison of performance of two analysis methods

In Table 3, the ratio of extraction amounts of ERG by the shaking and the saponification methods are shown. Recovery of ERG by the shaking method varied with grains and ERG levels. However, the extraction amounts of ERG using the shaking method were equal to more than 72 % of those of the saponification method and in most cases were close to or more than 100 % of those of the saponification method. In this study, there are no large differences between the recoveries of ERG using the shaking method and that of using the saponification method. According to de Sio et al., the ester form of ERG constitutes only up to 15 % of the total ERG. Because most of the ERG in moldy grain samples exist as free ERG, the shaking method should be sufficient for ERG estimation in moldy grains and for surveying for fungi contamination for grains.

The RSD of ERG amounts by the shaking and the saponification methods are shown in Table 4. Using the saponification method, the RSD of ERG from maize were slightly higher than other matrixes, but RSDs of ERG from other grain samples were in the range 5-10 % regardless of ERG levels and matrixes. On the other hand, using the shaking method, the RSD of ERG in grain samples were 1.5-6.2 % except buckwheat samples. In general, the RSDs of ERG by shaking method were better than that of the saponification method.

According to Miyagawa et al., using the saponification method, the RSD of ERG from maize with low contamination (2.2 mg/kg of ERG) and high contamination (16 mg/kg of ERG) were 4.8 % and 5.8 % (N = 6), respectively. Thus, there was no significant difference of the RSD of ERG by the saponification method and by this shaking method. The comparable RSD range supports this simplified method being
Fig. 1. Chromatograms of ERG by shaking method with and without SPE column
(a) Maize sample at 8.0 mg/kg of ERG by shaking method with SPE column
(b) Maize sample at 8.0 mg/kg of ERG by shaking method without SPE column
(c) Polished rice sample at 4.0 mg/kg of ERG by shaking method with SPE column
(d) Polished rice sample at 4.0 mg/kg of ERG by shaking method without SPE column
permissible for screening purpose.

The minimum detection limit (LOD) of ERG by the shaking method was studied adding ERG to uncontaminated rice samples. The LOD of unpolished rice was 0.2 mg/kg. The LOD by the saponification method was reported to be 0.1-0.3 mg/kg, so there were no significant differences of LOD between these two methods.

When ERG in maize is less than 3 mg/kg, the quality of maize is acceptable\(^\text{29}\). When over 8 mg/kg, the potential of fungal invasion and unacceptable quality of maize is high\(^\text{29}\). Pietri et al.\(^\text{29}\) also studied the relationship between the amount of ERG and mycotoxin contamination and concluded that when ERG in maize is less than 3 mg/kg the level of mycotoxin contamination is acceptable. Both results indicate that when ERG level are less than 3 mg/kg, the risk of fungal invasion or mycotoxin contamination in the grains is low and the quality is acceptable. For screening purpose, the analytical method requires both simplicity and accuracy, for which this simplified method without saponification and column clean-up appears suitable for this use.

### Conclusion

A simplified method to extract ERG from grains without saponification was developed. ERG was extracted with methanol by horizontal shaking for 1 hr. After filtration, ERG was partitioned into hexane with

<table>
<thead>
<tr>
<th>Sample/ERG level</th>
<th>Ratio of extraction amount (%) (^\text{a})</th>
<th>Low(^b)</th>
<th>Medium(^c)</th>
<th>High(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>84</td>
<td>109</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>98</td>
<td>126</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Unpolished rice</td>
<td>72</td>
<td>86</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Polished rice</td>
<td>98</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buckwheat</td>
<td>75</td>
<td></td>
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\(^{a}\) Amount of ERG by shaking / amount of ERG by saponification \(\times 100\) (\(N=6\))

ERG contents analyzed by the saponification method were grouped into three levels as follow: Low\(^b\) (3.0-5.0 mg/kg), Medium\(^c\) (8.0 mg/kg for maize and 7.0 mg/kg for wheat), and High\(^d\) (13-25 mg/kg)

<table>
<thead>
<tr>
<th>Sample/ERG level</th>
<th>RSD (%) (^\text{a})</th>
<th>Low(^b)</th>
<th>Medium(^c)</th>
<th>High(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>16</td>
<td>13</td>
<td>5.9</td>
<td>1.5</td>
</tr>
<tr>
<td>Wheat</td>
<td>8.0</td>
<td>5.1</td>
<td>8.7</td>
<td>3.2</td>
</tr>
<tr>
<td>Unpolished rice</td>
<td>6.5</td>
<td>9.1</td>
<td></td>
<td>1.7</td>
</tr>
<tr>
<td>Polished rice</td>
<td>6.7</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Buckwheat</td>
<td>6.2</td>
<td></td>
<td></td>
<td>13</td>
</tr>
</tbody>
</table>

\(^{b,c,d}\): Same as Table 3 (\(N=6\))
KCl aqueous solution, and then analyzed with reverse phase HPLC.

Following this shaking method, extraction efficiencies of ERG and the RSDs were different among the grains and contamination levels of ERG. However, no significant differences in efficiencies and RSDs between the shaking and the saponification methods were observed. The analytical method for screening purposes of fungal invasion should be rapid and easy to perform. In this study, a simplified method is presented and shown to be suitable for screening purposes.

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References

穀物中のエルゴステロールの分析法の簡易化について

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エルゴステロール (ERG) は真菌の細胞膜の構成成分の一種であり、多くの植物や動物は ERG を産生しないことから、ERG は穀物中の栄養素のバイオマーカーとして考えられている。以前、私達はメタノールとアルカリ溶液を用いたクエン酸法による穀物中の ERG の分析法を開発し、その妥当性を確認した。しかし、クエン化による分析法は時間やコスト、手間がかかるため、迅速さが求められるスクリーニングには適していない。そのため、私達は穀物中の ERG の分析法の簡易化を試みた。ERG はメタノールで一時間振とう抽出し、ろ過後、ERG は水溶液に於てヘキサンに転溶し、逆相 HPLC で分析した。振とう法による ERG の回収率をクエン化法による ERG の回収率で比較した。3 mg/kg の ERG のトウモロコシサンプルにおける振とう法による回収率をクエン化法による回収率の 84 % で、振とう法による相対標準偏差 (RSD) は 1.5 % であった。また、12 mg/kg の ERG の小麦サンプルにおける振とう法の回収率はクエン化法に対して 108 % で、振とう法の RSD 値は 2.8 % であった。今回開発した振とう法による穀物中の ERG の分析法は、スクリーニング目的として用いるには十分適用可能であると考えられる。

キーワード：エルゴステロール、穀物、クエン化法、分析法妥当性確認