JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Ergosterol Levels in Barley and Malt Varieties in the Czech Republic via HPLC

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Ergosterol is considered to be a suitable indicator of mold infestation in barley and malt. In this study ergosterol levels in different varieties of barley and malt produced in the Czech Republic were determined. A modified high-performance liquid chromatography (HPLC) method was statistically processed, validated (Effivalidation program), and applied to 124 samples of barley and malt. Ergosterol was isolated by extraction and saponification, and the quantification was performed using HPLC with diode array detection. The content of ergosterol ranged between the limit of detection (LOD) and 36.3 mg/kg in barley and between the LOD and 131.1 mg/kg in malt. Ergosterol is presumably connected with metabolites generated when barley grain is attacked by pathogens, and such barley often shows a high overfoaming (gushing) value. However, it was found that the content of ergosterol does not correlate with the degree of beer gushing.

KEYWORDS: Ergosterol; malt; barley; deoxynivalenol; gushing; HPLC

INTRODUCTION

The fundamental components that are essential for beer production are barley (*Hordeum vulgare* var. Nutans), hops, and water. High beer quality is ensured by the provision of exceptional premium raw materials. Barley quality can be influenced by the effects of unfavorable natural conditions such as drought, humidity, or frost. These factors might cause various stress reactions in plants. Ergosterol can be used as a signal molecule to elicit a plant's defensive reaction against fungi or simply bind to an unknown receptor and interfere in plant signal transduction (*1–3*).

Ergosterol is a provitamin of vitamin D_2, and its structure is similar to that of cholesterol (**Figure 1**). It is a secondary metabolite of parasitic fungi and can be used as an indicator for monitoring fungus incidence in barley and malt. The identification of specific secondary metabolites can provide useful monitoring information, but it is limited by the fact that fungal species do not always produce the same metabolites under all conditions (*4*). Ergosterol belongs to the most important fungus sterols because it is a constituent of their cell walls (*5*). Its content in fungi ranges widely from 0.2 to 8.0 mg/kg of dry weight. Ergosterol usually represents 60–70% of the sterols present in fungi. Ergosterol is partially ester-bound in plants. The content of extracted ergosterol can be influenced by the kind of extraction process used (*6*). For these reasons three extraction techniques for free, total free, and total ergosterol can be performed. Free ergosterol is extracted without saponification, total free ergosterol is obtained by saponification of the esters after extraction, and total ergosterol is accessed by saponification of the sample during extraction (*7, 8*). "Total free ergosterol" means ergosteryl esters. Free ergosterol is ergosterol that is not esterified, ergosteryl esters are esterified, and total ergosterol is free ergosterol plus ergosteryl esters.

The most frequent effect associated with micromycete occurrence is primary gushing (*9, 10*). Gushing is a common term that is used in the literature. It means beer overfoaming from a bottle or tin. The compounds that could produce primary gushing are not known as yet. It presupposes that compounds are produced as a metabolite from the stress of an organism (*11*). The reciprocal relationship between gushing and mycotoxin contamination of barley caused by the *Fusarium* species was the aim of the study in Schwarz's work (*10*). The authors monitored levels of various fungal metabolic products such as ergosterol or deoxynivalenol (DON) present in barley and in malt. They noted that for indication of contamination with fungal metabolites, ergosterol or DON is a good indicator, but it is unlikely that ergosterol or DON alone is itself responsible for the gushing. The gushing mechanism remains unknown, and thus prediction of gushing is, for many reasons, complicated. Recently, a sensitive method for DON, nivalenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol determination using MALDI TOF mass spectrometry was reported (*12, 13*).

There are a lot of methods used for the quantitative determination of ergosterol such as high-performance liquid chro-

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10.1021/jf703675q CCC: $40.75
Published on Web 05/14/2008

matography (HPLC) with ultraviolet (UV) detection (*4, 14, 15*). Jambunathan et al. studied the ergosterol content in grains of mold-susceptible and mold-resistant sorghum samples (*15*). They showed that there were distinctly higher concentration levels in the mold-susceptible samples. In the mold-resistant samples, the ergosterol concentration was only about 10% of the value of mold-susceptible samples in the mature grains. The other method reported for the quantitative determination of ergosterol was gas chromatography with mass spectrometry (GC-MS). For the GC methods a trimethylsilyl derivative or methyl ester is usually used to improve the peak detection (*16*). One can also find other methods in the literature such as LC-ACPI-MS-MS (*17*) and GC-MS-MS (*7, 18*).

The Czech Republic is a significant producer and exporter of barley. The aim of this study was the selection and implementation of a reliable method for the quantitative analysis of ergosterol at the Research Institute for Brewing and Malting (RIBM) and the validation of such an analytical method. The validated method will be used for the routine determination of ergosterol, to evaluate the levels of ergosterol in the most common varieties of barley grown in the Czech Republic, and to examine a possible correlation between ergosterol and gushing.

MATERIALS AND METHODS

Grain Samples. A total of 124 samples, 62 for barley and 62 for malt, were taken from the harvest in 2004. For this study, seven varieties of malt or barley were selected: Jersey, Kompakt, Malz, Nordus, Prestige, Scarlett, and Tolar. Barley samples were stored at laboratory temperature in the Research Institute for Brewing and Malting. All barley samples were cultivated at the Research Institute for Brewing and Malting, Kroměříž, Czech Republic. Before the analysis ~15 g of each sample was ground to particles of 0.4 mm or less using a mill.

Apparatus. HPLC was set up from a high-pressure pump P 2000, an injection cell with an injection loop, a diode array detector (DAD) (Thermo Electron Corp.), and a column (Nucleosil C18, 250 × 4.6 mm, 5 μm, Supelco). The other equipment was an analytical weighing machine (Mettler Toledo), a mill (SJ 500, Mezos, Hradec Králové, Czech Republic), an agitator (Kavalier LT2, Sázava-Votice, Czech Republic), a water bath (Heto Laboratory Equipment), and a vacuum evaporator (Veb MLW, Prüfgeräte-Werk).

Chemicals. Ergosterol standard was purchased from Sigma-Aldrich (St. Louis, MO). Potassium hydroxide (p.a.) was from PLIVA-Lachema a.s. (Brno, Czech Republic), and methanol (purity = 99.8%) and *n*-hexane (purity = 95%) were purchased from Chromservis (Prague, Czech Republic). Distilled water used to prepare all solutions was double-distilled in a quartz apparatus supplied by Heraeus Quartzschmelze (Hanau, Germany).

Ergosterol Analysis. The procedure for the extraction of ergosterol is a modification of the one described by Seitz et al. (*19*) and later modified by Jambunathan et al. (*16*). The procedure used in this work was as follows: For each analysis, 10 g of ground sample was treated with 50 mL of methanol in a closed vessel and then was shaken for 30 min. Then, 25 mL of cleaned extract was placed into a test tube and 3 g of KOH was added; the mixture was shaken until the KOH dissolved. To the extract was added 10 mL of *n*-hexane. The incubation temperature using a water bath was optimized, and a value of 65 ± 2–3 °C was found as optimal. The samples were incubated for 30 min and cooled to room temperature. Following the incubation and after the addition of 5 mL of distilled water, the *n*-hexane layer was transferred into a beaker. The extraction process using *n*-hexane was repeated three times using 10 mL of the solvent. The extracts were then transferred into a beaker. The joined extracts were evaporated until dry and redissolved in 5 mL of methanol. The extracted samples were analyzed using HPLC with DAD. To achieve ergosterol separation different separation columns were examined such as C-18 from MicroSolv Technology, type Cogent (150 × 4.6 mm, 5 μm), a column from Watrex, type Nucleosil (250 × 4 mm, 5 μm), and from Supelco, type Supelcosil (250 × 4.6 mm, 5 μm). The most suitable separation

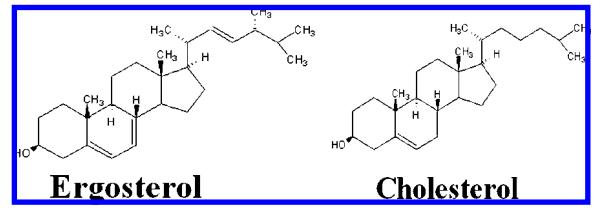

Figure 1. Comparison of cholesterol and ergosterol structures.

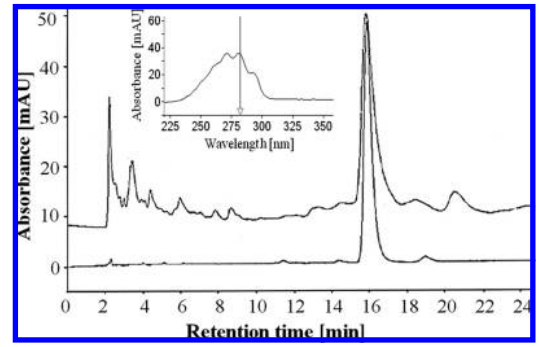

Figure 2. HPLC chromatogram for the analysis of ergosterol standard solution, 10 mg/L, and methanol extract sample in barley. Ergosterol was detected at 282 nm. Twenty microliters of sample was injected into an injection cell. All values represent the average of three determinations (*n* = 3).

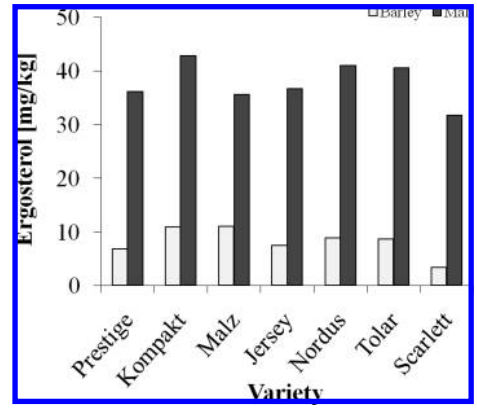

Figure 3. Ergosterol content in barley and malt plotted for different varieties.

was found to be on the column from Supelco, type Supelcosil (250 × 4.6 mm, 5 μm). The mobile phase was methanol, and 1.0 mL/min was the optimal flow rate. The column temperature was held at 25 °C. The absorbance maximum of eluted ergosterol was detected at 282 nm, and the peak purity was verified using the spectra recorded by DAD (**Figure 2**). The injected amount of sample was 20 μL. Each ergosterol analysis was repeated three times. A stock solution of standard ergosterol was prepared, and additional calibration solutions were prepared by a serial dilution with methanol. Quantification of ergosterol and precision of determination were based on the calibration curve over a range of ergosterol concentrations. Aliquots of the standard solution were diluted to concentrations of 1.0, 2.5, 5.0, 10.0, and 15.0 μg/mL and analyzed. The regression analysis of the calibration curve was carried out by plotting the peak areas against the concentration of ergosterol. The linearity of the calibration plot was demonstrated by a correlation coefficient (R^2) that was found to be >0.999. Some points of the calibration curve (1–2) were measured daily and, only if they were some deviation, was the standard curve repeated. Number of replicates in the test was 3, and number replicates of final analyses was 3, too. The limit of detection (LOD) and the limit of quantification (LOQ) were determined from signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively. Statistical analysis of the data was performed using the Effivalidation program purchased from EffiChem (Lysice, Czech Republic) (*20*).

Table 1. Values of Gushing and Concentrations of Ergosterol in Barley and Malt[a]

	gushing		ergosterol			gushing		ergosterol	
variety	barley, mL	malt, mL	barley, mg/kg	malt, mg/kg	variety	barley, mL	malt, mL	barley, mg/kg	malt, mg/kg
Malz	<LOD	<LOD	5.9	31.9	Jersey	<LOD	<LOD	7.7	<LOD
Malz	<LOD	3	7.3	7.5	Jersey	<LOD	<LOD	9.6	14.1
Malz	2	50	4.5	18.4	Jersey	<LOD	<LOD	<LOD	9.9
Malz	3	<LOD	2.4	71.5	Jersey	<LOD	<LOD	8.2	7.6
Malz	3	<LOD	7.9	37.3	Jersey	<LOD	<LOD	<LOD	29.3
Malz	18	24	22.2	53.7	Jersey	<LOD	<LOD	5.7	8.2
Malz	22	0	26.6	36.7	Jersey	<LOD	15	6.3	69.3
Kompakt	<LOD	<LOD	9.8	24.8	Jersey	<LOD	35	4.0	59.9
Kompakt	<LOD	<LOD	6.4	52.9	Jersey	<LOD	57	<LOD	30.8
Kompakt	1	<LOD	<LOD	47.8	Jersey	1	<LOD	7.5	6.1
Kompakt	1	<LOD	<LOD	<LOD	Jersey	1	56	30.5	40.2
Kompakt	1	34	15.4	11.6	Jersey	5	<LOD	5.8	109.7
Kompakt	1	52	<LOD	40.4	Jersey	8	94	<LOD	46.3
Kompakt	1	62	16.7	57.4	Jersey	10	53	15.3	28.1
Kompakt	6	D.L.	6.3	131.1	Jersey	12	9	11.8	84.6
Kompakt	10	25	36.3	94.5	Scarlett	<LOD	<LOD	9.9	58.1
Kompakt	10	93	18.1	18.6	Scarlett	1	<LOD	<LOD	<LOD
Prestige	<LOD	<LOD	10.2	11.2	Scarlett	1	62	<LOD	47.1
Prestige	<LOD	<LOD	7.4	32.5	Scarlett	1	<LOD	<LOD	<LOD
Prestige	<LOD	5	<LOD	48.1	Scarlett	1	62	<LOD	47.1
Prestige	<LOD	8	<LOD	29.8	Tolar	<LOD	<LOD	13.2	26.6
Prestige	<LOD	44	<LOD	46.9	Tolar	<LOD	1	5.5	21.9
Prestige	<LOD	44	9.1	52.1	Tolar	<LOD	<LOD	12.6	30.7
Prestige	1	<LOD	14.6	63.6	Tolar	<LOD	1	<LOD	86.2
Prestige	1	<LOD	2.9	51.0	Tolar	<LOD	<LOD	11.6	37.2
Prestige	2	<LOD	15.5	29.0	Nordus	<LOD	6	19.9	59.4
Prestige	2	<LOD	13.1	<LOD	Nordus	<LOD	6	20.0	59.4
Prestige	3	3	<LOD	<LOD	Nordus	12	<LOD	5.6	54.0
Prestige	8	<LOD	11.9	74.8	Nordus	2	<LOD	7.0	32.7
Prestige	8	2	4.1	48.5	Nordus	<LOD	<LOD	0.0	34.1
Prestige	8	74	5.6	14.9	Nordus	<LOD	3	3.7	24.1
Jersey	<LOD	<LOD	7.5	42.4	Nordus	<LOD	<LOD	17.0	71.3

[a] All values are the average of three determinations ($n = 3$).

Recovery of ergosterol was estimated by spiking 0.5 g of the barley sample (in-house standard sample) with a standard ergosterol solution (100 μg/mL). The amount of test solution that was added to the barley sample was 0.5 mL. The recovery of added ergosterol was found to be 91 ± 8% (mean ± standard deviation, $n = 3$, where the number of analyses, n, was equal to 3).

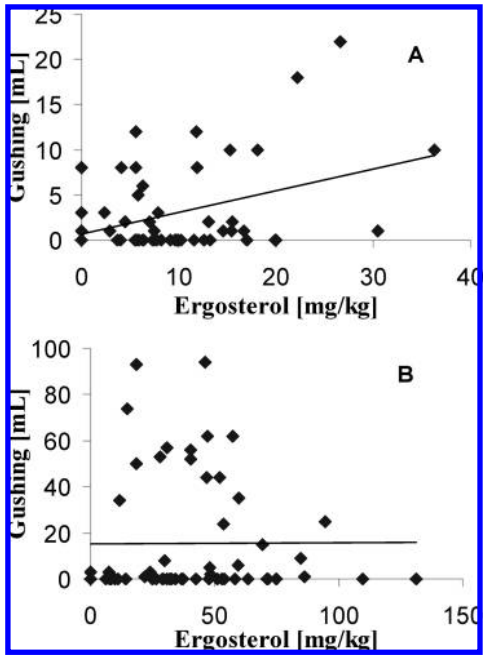

Figure 4. Relationship between gushing and the content of ergosterol in (**A**) barley and (**B**) malt. Each value represents the mean of three replicates ($n = 3$).

Gushing of Beer. The Carlsberg 3-day test (*21*) was used for the determination of gushing in malt. The method is based on the assumption that markers of overfoaming are soluble in water, active and soluble after boiling, and active under the conditions prevailing in beer. The method includes the replacement of 50 mL of non-overfoaming finished beer with the malt extract followed by a 3-day shaking under the defined conditions. The volume of the foamed beer after the consequent bottle opening expresses the gushing value (mL).

RESULTS AND DISCUSSION

Validation of the HPLC Method. The content of ergosterol was determined by HPLC-DAD, where the quantification of ergosterol was based on the calibration performed for ergosterol as described above. Under the optimized HPLC-DAD conditions, the LOD and the LOQ for ergosterol were 0.35 and 1.20 mg/kg, respectively. The coefficients of variation (CV) varied from 2.6 to 3.9%. The validation was done using the Effivalidation program. A relatively high uncertainty of 15.42% was found. Such a high uncertainty may be caused by the complexity of the matrix (barley and malt).

Ergosterol Content in Different Varieties of Barley and Malt. In general, the content of ergosterol found in the barley samples ranged from LOD to 36.3 mg/kg over all samples from the harvest in 2004. These contents are similar to those described in the literature (*4, 5, 12, 17*). All of the results are given in fresh weights.

Figure 3 shows the ergosterol content according to type of barley variety, Jersey, Kompakt, Malz, Nordus, Prestige, Scarlett, and Tolar. The highest ergosterol content present is for the Kompakt (36.3 mg/kg) variety, whereas the Scarlett variety showed the lowest content. According to the decreasing ergosterol concentration, we may order the varieties Kompakt

> Malz, Nordus, Tolar, Prestige, and Jersey > Scarlett. These results are consistent with the fact that the most fungus-resistant variety is Scarlett, and the content of ergosterol is proportional to the fungus infestation. This fact allows the selection of the most fungus-resistant varieties, which is important for agriculture. As for malt, the ergosterol level ranged between LOD and 131.1 mg/kg, Kompakt being the variety that exhibited the highest content of ergosterol (131.1 mg/kg).

The values of ergosterol content are much lower in barley than in malt. This was probably due to several factors, for example, (i) a high infestation of fungi in the malting process or other biochemical changes and (ii) the highest ergosterol content in malt perhaps being due to the release of fixed ergosterol during the malting process.

Ergosterol Correlation versus Gushing. The Carlsberg 3-day test was used for the determination of gushing in all samples of barley and malt (**Table 1**). The gushing values were calculated as the average of the results from three analyses ($n = 3$).

Understanding the relationship between the ergosterol content and gushing would contribute to the understanding of the overfoaming mechanism. Dependence between ergosterol concentration in barley and gushing in barley is plotted in **Figure 4**. The graphs (i) concentration of ergosterol in malt versus gushing in malt, (ii) ergosterol concentration in barley versus gushing in malt, and (iii) ergosterol concentration in malt versus gushing in barley were constructed. The statistical analyses of the relations were performed using Pearson correlation coefficients. The value of the correlation coefficient (R^2) between gushing and ergosterol in barley was 0.17 ($p > 0.05$, where p means significance), and that between gushing and ergosterol in malt was 0.21 ($p > 0.05$). As can be seen from the dispersion of the data (**Figure 4**) the correlation coefficient is quite low ($R^2 = 0.172$). Thus, no correlation between ergosterol and gushing was proved.

This study developed and statistically evaluated the HPLC method for ergosterol quantitative determination in barley and malt by using HPLC-DAD. The LOD and LOQ were determined. LOD was equal to 0.35 mg/kg, and LOQ was equal to 1.20 mg/kg. The developed methodology was established as an internal validated analytical method at the Research Institute for Brewing and Malting, and the method has been extensively applied for ergosterol analysis in barley and malt.

The ergosterol content was found to depend on the barley variety. The values of ergosterol content ranged from LOD to 36.3 mg/kg in barley and from LOD to 131.1 mg/kg in malt. It was proved that the most fungus-resistant variety was Scarlett, which presents the lowest ergosterol levels, whereas the Kompakt variety shows the highest ergosterol levels. The correlation between ergosterol content and the level of gushing was negligible. Although ergosterol remains the main indicator of fungus infestation, it was found that its presence and level in varieties grown in the Czech Republic are not related to gushing either in barley or in malt.

ACKNOWLEDGMENT

English has been kindly revised by Phil. G. Watson.

LITERATURE CITED

(1) Kašparovský, T.; Mikeš, V.; Havel, L.; Milat M. L.; Blein, J. P. Ergosterol as a signal molecule of plant defense reaction. In *Book of Abstracts of VIIth Workshop of Biochemists and Molecular Biologists*; Masaryk University: Brno, Czech Republic, 2003; s. 25-25.

(2) Vauthrin, S.; Mikes, V.;.; Milat, L. M.; Ponchet, M.; Maume, B.; Osman, H.; Blein, P. J. Elicitins trap and transfer sterols from micelles, liposomes and plant plasma membranes. *Biochim. Biophys. Acta* **1999**, *1419*, 335–342.

(3) Jasinghe, V. J.; Perera, C. O. Ultraviolet irradiation: the generator of vitamin D_2 in edible mushrooms. *Food Chem.* **2006**, *95*, 638–643.

(4) Abramson, D.; Gan, Z.; Clar, R. M.; Gilbert, J.; Marquardt, R. R. Relationships among deoxynivalenol, ergosterol and *Fusarium* exoantigens in Canadian hard and soft wheat. *Int. J. Food Microbiol.* **1998**, *45*, 217–224.

(5) Kadakal, C.; Nas, S.; Ekinci, R. Ergosterol as a new parameter together with patulin in raw apple juice produced from decay apple. *Food Chem.* **2004**, *90*, 95–100.

(6) Yuan, J. P.; Wang, J. H.; Liu, X.; Kuang, H. C.; Zhao, S. Z. Simultaneous determination of free ergosterol and ergosteryl esters in *Cordyceps sinensis* by HPLC. *Food Chem.* **2007**, *105*, 1755–1759.

(7) Nielsen, K. F.; Madsen, J. Ø. Determination of ergosterol on mouldy building materials using isotope dilution and gas chromatography–tandem mass spectrometry. *J. Chromatogr., A* **2000**, *898*, 227–234.

(8) Hippelein, M.; Rügamer, M. Ergosterol as an indicator of mould growth on building materials. *Int. J. Hyg. Environ. Health* **2004**, *207*, 379–385.

(9) Marasas, W. F. O. Fumonisins: history, world-wide occurrence and impact. In *Fumonisins in Food, Advances in Experimental Medicine and Biology*; Jackson, L. S., DeVaries, W., Bullerman, L. B., Eds.; Plenum Publishing: New York, 1996; Vol. 392, pp 1–17.

(10) Schwarz, B. P.; Beattie, S.; Casper, H. H. Relationship between *Fusarium* infestation of barley and the gushing potential of malt. *J. Inst. Brew.* **1996**, *102*, 93–96.

(11) Havlová, P.; Kosă, K. Prediction of gushing in beer from barley; Research Project Final Report; RIBM, Malting Institute Brno, **2005**.

(12) Blechová, P.; Havlová, P.; Gajdošová, D.; Havel, J. New possibilities of matrix-assisted laser desorption ionization time of flight mass spectrometry to analyze barley malt quality. Highly sensitive detection of mycotoxins. *Environ. Toxicol.* **2006**, *21*, 403–408.

(13) Elosta, S.; Gajdošová, D.; Hégrová, B.; Havel, J. MALDI TOF mass spectrometry of selected mycotoxins in barley. *J. Appl. Biomed.* **2007**, *5*, 39–47.

(14) Schwarz, B. P.; Casper, H. H.; Beattie, S. Fate and development of naturally occuring *Fusarium* mycotoxins during malting and brewing. *J. Am. Soc. Brew. Chem.* **1995**, *53*, 121–127.

(15) Jambunathan, R.; Kherdekar, S. M.; Vaidya, P. Ergosterol concentration in mold-susceptible and mold-resistant *Sorghum* at different stages of grain development and its relationship to flavan-4-ols. *J. Agric. Food Chem.* **1991**, *39*, 1866–1870.

(16) Olsson, J.; Borjesson, T.; Lunstedt, T.; Schnurer, J. Detection and quantification of ochratoxin A and deoxynivalenol in barley grains by GC-MS and electronic nose. *Int. J. Food Microbiol.* **2002**, *72*, 203–214.

(17) Headley, J. V.; Peru, K. M.; Verma, B.; Robarts, R. D. Mass spectrometric determination of ergosterol in a prairie natural wetland. *J. Chromatogr., A* **2002**, *958*, 149–156.

(18) Dong, Y.; Steffenson, B. J.; Mirocha, Ch. J. Analysis of ergosterol in single kernel and ground grain by gas chromatography−mass spectrometry. *J. Agric. Food Chem.* **2006**, *54*, 4121–4125.

(19) Seitz, L. M.; Mohr, H. E.; Burroughs, R.; Sauer, D. B. Ergosterol as an indicator of fungal invasion in grains. *Cereal Chem.* **1977**, *54*, 1207–1217.

(20) http://www.effichem.com/.

(21) Vaag, P.; Riis, P.; Knudsen, A. D.; Pedersen, S.; Meiling, E. A simple and rapid test for gushing tendency in brewing materials. *Proc. Eur. Brew. Conv. Congr. Oslo* **1993**, *24*, 155–162.

Received for review December 17, 2007. Revised manuscript received March 10, 2008. Accepted March 11, 2008. The study was carried out with the support of the Grant Agency of the Czech Republic, Project 525/06/0663.

JF703675Q