Measurement of a Genotoxic Hydrazine, Agaritine, and Its Derivatives by HPLC with Fluorescence Derivatization in the *Agaricus* Mushroom and Its Products

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***Agaricus blazei* MURRILL mushroom products are sold as so-called health foods in Japan. However, a part of *Agaricus* is known to contain hydrazines. A sensitive and specific method for analyzing a genotoxic hydrazine, agaritine, and its derivatives was developed to assess the safety of *Agaricus* products. β-*N*-(γ-L(+)-Glutamyl)-4-(hydroxymethyl)phenylhydrazine (agaritine, AGT), 4-hydrazinylbenzylalcohol (HMPH), 4-hydrazinylbenzoic acid (CPH), 4-methylphenylhydrazine (MPH) and phenylhydrazine (PH) were converted to their corresponding fluorescent derivatives with 3,4-dihydro-6,7 dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl chloride (DMEQ-COCl) as the fluorescence derivatization reagent. The detection limits (S/N=3) for CPH, AGT, PH and MPH were 422, 45.3, 16.5 and 138 fmol, respectively, in a 20 μl injection volume. Recoveries, achieved by adding known AGT amounts to the *Agaricus* sample and *Agaricus* products, ranged from 92.8 to 102%. By using this method which does not require partial purification of the *Agaricus* sample, the amounts of AGT in several types of foods were found to be 112—1836 μg/g dry weight.**

Kew words *Agaricus*; agaritine; fluorescence derivatization; mushroom; 3,4-dihydro-6,7 dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl chloride (DMEQ-COCl)

Agaricus blazei MURRILL (known as the Himematsutake mushroom or the Kawariharatake mushroom) products are sold as so-called health foods in Japan. *Agaricus bisporus*, the Western mushroom, belongs to the same group as *Agaricus* and is sold under the name "Mushroom".

However, aqueous and ethanol extracts from *A. bisporus* showed mutagenicity in the Ames test.[1,2] A part of *Agaricus* is known to contain aromatic hydrazines. The most abundant is considered to be β-*N*-(γ-L(+)-glutamyl)-4-(hydroxymethyl)phenylhydrazine (agaritine, AGT), the concentration of which is approximately 165—475 mg/kg[3] or 212—229 mg/g in fresh mushrooms and 14.9 mg/kg in canned mushroom products.[4] Direct mutagenicity was not influenced by baking the mushrooms at 225 °C for 10 min.[5]

On February 13, 2006, the Ministry of Health, Labour and Welfare of Japan requested the Food Safety Commission to assess the safety of products containing *A. blazei* MURRILL.[6] The possibility of so-called health foods containing *A. blazei* MURRILL impairing liver function has already been pointed out. An animal experiment using rats was conducted at the National Institute of Health Sciences (NIHS) from 2003 to 2005. Among the results obtained, tumor-promoting activities were observed in animals fed a so-called health food product containing *A. blazei* MURRILL. Therefore, the Ministry of Health, Labour and Welfare of Japan demanded a cessation of sales and voluntary recall of the product from K-Company. Thus, concern is increasing in Japan about the unhealthy influence of ingesting *A. blazei* MURRILL and its products. Moreover, AGT is considered to be converted to a stronger carcinogenic derivative, when it is metabolized.[7] Therefore, it is important to establish an analytical method for AGT and its derivatives. To date, AGT content has been determined by UV spectrometry. Recently, a specific analysis of AGT using LC-MS/MS was reported.[8]

In this study, to assess the safety of *Agaricus* products, we developed a sensitive and specific method for measuring the genotoxic AGT and its derivatives in mushrooms and *Agaricus* products by HPLC with fluorescence derivatization using 3,4-dihydro-6,7 dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl chloride (DMEQ-COCl).

Experimental

Samples Mushrooms and *Agaricus* health food products were purchased in the Tokyo Metropolitan area and by mail order in Japan.

DMEQ-COCl was obtained from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were of reagent grade or of the highest grade commercially available.

β-*N*-(γ-L(+)-Glutamyl)-4-(hydroxymethyl)phenylhydrazine (agaritine, AGT) and 4-hydrazinylbenzylalcohol (HMPH) were synthesized. 4-hydrazinylbenzoic acid (CPH), 4-methylphenylhydrazine (MPH) and phenylhydrazine (PH) were obtained from commercial sources. These compounds were converted to their corresponding fluorescent derivatives with DMEQ-COCl as the fluorescence derivatization reagent. The structures of phenylhydrazine derivatives are presented in Fig. 1.

Sample Preparation Powders of freeze-dried mushrooms or *Agaricus* products (0.5 g) were extracted with 30 ml of methanol by shaking for 3 h. After filtration with a syringe filter (0.45 μm), the extract was diluted 10 or more fold with milli Q, and the sample solution was then added to the fluorescence reagent.

Fluorescence Derivatization Procedure A sample solution (0.1 ml) was placed in a screw-capped test-tube, and 0.1 ml of 7 mM DMEQ-COCl in dimethylformamide (DMF) was then added. The tube was tightly capped and heated at 37 °C for 60 min in the dark. After cooling, 0.8 ml of 30% (v/v) methanol was added to the reaction mixture. The resulting solution (20 μl) was subjected to chromatography.

HPLC The HPLC apparatus (L-7100, Hitachi, Tokyo, Japan) was equipped with an auto sampler (MIDAS, Spark HOLLAND, Emmen, Netherlands) and a fluorescent spectrometer (RF-10AXL, Shimadzu, Kyoto, Japan). The fluorescent spectrometer was operated at an excitation wavelength of 392 nm and an emission wavelength of 462 nm, since the excitation maximum for each hydrazine-DMEQ-CO derivative was obtained between 390 nm and 394 nm and the emission maximum between 461 nm and 464 nm. An XBridge™ shield RP18 column (4.6 mm i.d.×150 mm, particle size 3.5 μm; Waters, Massachusetts, U.S.A.) was used, since this column is known to be suitable for separation of basic compounds.

Two solvents were used for gradient elution at a flow rate of 0.7 ml/min: solvents A (0.2 M Tris–HCl (pH 8.0)/methanol=70/30) and B

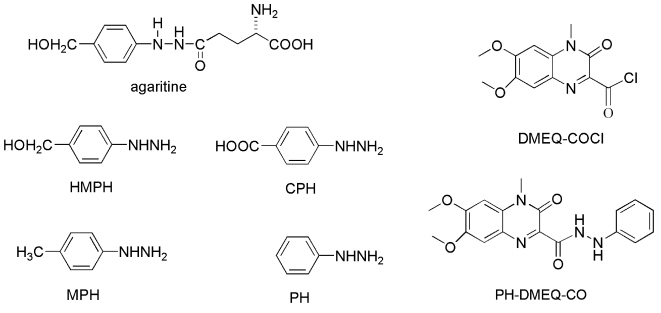

Fig. 1. Structures of Phenylhydrazines, the Fluorescence Derivatization Reagent and the Fluorescent Derivatives

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(methanol/water=90/10). The gradient conditions were as follows: 0—14 min, 0% solvent B; 14—15 min, from 0% to 40%; 15—24 min, from 40 to 80%; 24—34 min, 80%. The eluate was transferred to a fluorescence detector.

Results and Discussion

A typical chromatogram of the CPH-, AGT-, PH- and MPH-DMEQ-CO derivatives obtained under these HPLC conditions is shown in Fig. 2. The horizontal axis shows the retention time of the eluate, the vertical axis the intensity. The fluorescence derivatization reaction was performed without water soluble carbodiimide (WSC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and pyridine, since the reaction efficiencies decreased when WSC and pyridine were used (data not shown). Among four fluorescent derivatives, the conformation of PH-DMEQ-CO as a representative was confirmed by ^{1}H- and ^{13}C-NMR analyses (JEOL spectrometer, Akishima, Japan) and electrospray tandem mass spectrometry (API 3000, Applied Biosystems, Foster, U.S.A.).

When pyridoxamine, allantoin, guanine, aniline, arginine, histidine, glutamic acid and poly-arginine, all of which have amino group, were converted into the corresponding fluorescent compounds and applied to the column, each chromatogram corresponded to the same blank chromatogram. Thus, the reaction conditions without WSC and pyridine and the HPLC condition of pH 8.0 were considered to be suitable for the detection of compounds containing a hydrazino group, but not an amino group.

When HMPH was reacted with DMEQ-COCl, the HMPH-DMEQ-CO peak was observed at the same retention time as that of AGT-DMEQ-CO. Moreover, 1-acetyl-2-phenylhydrazine reacted with DMEQ-COCl, and the peak was observed at the same retention time as that of PH-DMEQ-CO. Therefore, it is suggested that the fluorescence derivatization reaction results in the amide bond being cleaved, and the hydrazino group of the compound and DMEQ-COCl thereby yield its fluorescent derivative.

The calibration curves (Fig. 3) obtained by plotting CPH, AGT, PH and MPH concentrations versus the peak areas showed good linearity with a straight line passing near the origin. The detection limits (S/N=3) for CPH, AGT, PH and MPH were 422, 45.3, 16.5 and 138 fmol, respectively, in a 20 μl injection volume.

Table 1 shows the results of three replicate analyses of five mushroom samples and three *Agaricus* products. The amounts of AGT in several types of foods were found to be

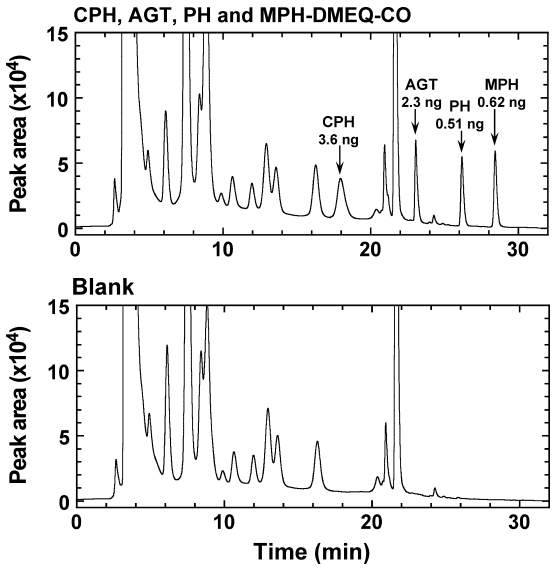

Fig. 2. A Typical Chromatogram of the DMEQ-CO-Derivatives of CPH, AGT, PH and MPH

Peaks and concentrations (ng/20 μl injection volume) in parentheses: CPH (3.6); agaritine (AGT) (2.3); PH (0.51); MPH (0.62).

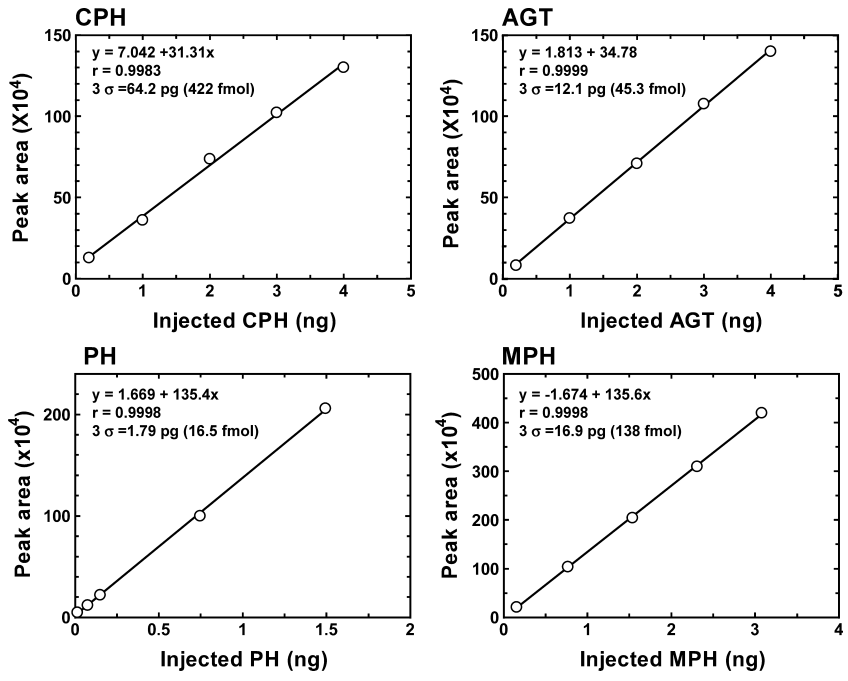

Fig. 3. Calibration Graphs for the Determination of CPH, AGT, PH and MPH

Table 1. Results of Three Replicate Analyses of Five Mushroom Samples and Three *Agaricus* Products

Samples	Mean±S.D. (μg/g)			
	CPH	AGT	PH	MPH
Sample A of *A. blazei* Murrill	N.D.[a)]	685±46	N.D.	N.D.
Sample B of *A. blazei* Murrill	N.D.	112±10	N.D.	N.D.
Sample C of *A. blazei* Murrill	N.D.	731±21	N.D.	N.D.
Lentinula edodes (Shiitake mushroom)	N.D.	Trace	N.D.	N.D.
A. bisporus	N.D.	1836±157	N.D.	N.D.
Product A of *A. blazei* Murrill	N.D.	1791±41	N.D.	N.D.
Product B of *A. blazei* Murrill	N.D.	124±9.7	N.D.	N.D.
Product C of *A. blazei* Murrill	N.D.	N.D.	N.D.	N.D.

a) N.D. (not detected).

Table 2. Recoveries of AGT from *A. blazei* Murrill and *Agaricus* Products Spiked with Known Amounts of AGT ($n=3$)

	Samples	Content (μg/0.5 g)	Added (μg)	Found (μg)	Recovery (%)[a)]
1	Sample B of *A. blazei* Murrill	56.0	400	427	92.8
2	Product A of *A. blazei* Murrill[b)]	896	950	1825	97.7
3	Product C of *A. blazei* Murrill[c)]	N.D.[d)]	100	102	102

a) Recovery (%)=[(found−content)/added amount]×100. *b*) The supernatant (30 ml MeOH/0.5 g) from product A of *A. blazei* Murrill was diluted 100 fold with milli Q, and the test solution was then added to the fluorescence reagent. *c*) The supernatant (30 ml MeOH/0.5 g) from product C of *A. blazei* Murrill was diluted 10 fold with milli Q, and the test solution was then added to the fluorescence reagent. *d*) N.D. (not detected).

112—1836 μg/g dry weight. The AGT value of *A. bisporus* (1836 μg/g dry) is the same as that of product A of *A. blazei* Murrill (1791 μg/g dry). The AGT one-day intake of product A was estimated to be 8955 μg according to the label.

The validity of the procedure was demonstrated by determining the recoveries of AGT from *A. blazei* Murrill and *Agaricus* products spiked with known amounts of AGT. Recoveries ranged from 92.8 to 102% (Table 2).

This is the first report describing a sensitive and specific simultaneous analysis method for CPH, AGT, PH and MPH in *A. blazei* Murrill and its products, not requiring partial sample purification by HPLC, with fluorescence derivatization using DMEQ-COCl. The fluorescence derivatization method in this study is superior to the LC/MS method in terms of simplicity, since partial purification using a pre-column method is not necessary.

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