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Stability of agaritine – a natural toxicant of *Agaricus* mushrooms

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*Agaritine (*N*-(γ-*L*-(+)-glutamyl)-4-hydroxymethylphenylhydrazine) is a phenylhydrazine derivative found in the cultivated* Agaricus *mushroom which is claimed to give rise to carcinogenic products when metabolized. The stability of a synthetic sample of agaritine was tested in water and methanol. In tap water kept in open vials, agaritine was totally degraded within 48 h. Since agaritine degradation was less pronounced in closed than in open vials, and slower in Milli Q water and, in particular, in Milli Q water purged with N_2, the degradation seems to be oxygen-dependent. The antioxidant dithiothreitol reduced the degradation. Four or possibly five ultraviolet-absorbing compounds were formed during degradation, but these have not yet been identified. Whereas the rate of degradation was similar at temperatures between 4 and 22°C, it was quicker at an acidic than at a neutral pH. The latter observation was confirmed in experiments where agaritine was incubated in simulated gastric fluid (pH 1.2). The importance of the degradation when performing toxicological studies with agaritine is discussed.*

Keywords: agaritine, stability, *Agaricus*

Introduction

Their special, delicious flavour has made mushrooms greatly appreciated ingredients in a variety of meals throughout time. Besides their attractive organoleptic properties, the popularity of mushrooms as dietary constituents is based on their low energy content, which is due to a typically low amount of lipids, and that they are a good source of dietary fibre and minerals (Hajšlová 1995).

Unfortunately, many wild and even some cultivated edible mushrooms contain toxic constituents (Benjamin 1995). Among the latter group are species belonging to the genus *Agaricus*, including the cultivated mushroom of commerce *A. bisporus* and closely related species. The toxic compounds identified in fruit bodies of the cultivated mushroom are agaritine (*N*-(γ-L-(+)-glutamyl)-4-hydroxymethylphenylhydrazine), related phenylhydrazine derivatives and the 4-hydroxymethylbenzenediazonium ion (Levenberg 1961, Ross *et al.* 1982, Chauhan *et al.* 1984, 1985). Of these compounds, agaritine is most prevalent, usually occurring in quantities between 200 and 450 mg kg^{-1} fresh weight, whereas 4-(carboxy)-phenylhydrazine, *N*-(γ-L-(+)-glutamyl)-4-(carboxy)-phenylhydrazine and the 4-(hydroxymethyl)benzenediazonium ion have been found in much smaller quantities, about 10–11, 16–42 and 0.6–4 mg kg^{-1} fresh weight, respectively (Ross *et al.* 1982, Chauhan *et al.* 1984, 1985, Toth *et al.* 1997).

Life-long feeding of Swiss albino mice with fresh, dry-baked or freeze-dried *A. bisporus* resulted in tumour development in various tissues of the experimental animals (Toth and Erickson 1986, Toth *et al.* 1997, 1998). When the phenylhydrazines occurring in the mushroom in cancer tests on mice were administered orally as pure compounds in drinking water or by gavage, all, except agaritine, induced tumours (Toth *et al.* 1982, Toth 1986, McManus *et al.* 1987). The fact that agaritine did not induce tumours (Toth *et al.* 1981a) has puzzled many investigators since agaritine is the most prevalent phenylhydrazine in the mushroom and, furthermore, it is believed to be metabolized to the very reactive 4-hydroxymethylbenzenediazonium ion via 4-(hydroxymethyl)phenylhydrazine (Fischer *et al.* 1984). In addition, agaritine (isolated

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from commercially purchased *A. bisporus*) did not induce tumours in Swiss albino mice when given as five weekly subcutaneous injections (Toth and Sornson 1984).

During the course of a study quantifying the amount of agaritine present in wild species of the genus *Agaricus*, we recently made an observation that may contribute to the interpretation of results obtained in toxicological studies performed with agaritine. It turned out that agaritine solutions (tap water [TW], TW containing 2 mM dithiotreitol, Milli Q water, Nitrogen purged Milli Q water, methanol), particularly aqueous solutions, were rather unstable in the presence of oxygen. We report here on these findings and on the stability of agaritine in simulated gastric fluid.

Materials and methods

Chemicals and samples

Fresh cultivated mushrooms of the species *Agaricus bitorquis* (one of the *Agaricus* species cultivated in the Czech Republic) were purchased at the open market in Prague. The agaritine content of the purchased samples was analysed as described below and was between 168 and 272 mg kg^{-1} fresh weight. Water used for preparation of solutions was distilled and further purified using the Milli Q RG purification system (Millipore, Germany). Methanol used for extraction was purchased from Merck (Darmstadt, Germany). The NaH_2PO_4 and H_3PO_4 used as a mobile phase in the HPLC analysis, and the sodium chloride and hydrochloric acid used in simulated gastric fluid were of analytical grade and supplied by Lachema (Brno, Czech Republic). Pepsin was supplied by Léciva (Prague, Czech Republic) and dithiothreitol (dtt) by Sigma-Aldrich (Steinheim, Germany). The agaritine standard was synthesized by Dr Henrik Frandsen, Danish Veterinary and Food Administration, according to the method of Wallcave *et al.* (1979), but incorporating some important modifications (Frandsen 1998). The standard was stored under argon and protected from light in the freezer. The synthesis was financed by the Nordic Council of Ministers. The synthesized agaritine was > 85% pure as shown at wavelengths 200, 237 and 280 nm by HPLC. The purity factor of the agaritine peak, reported by the Hewlett Packard ChemStation peak purity software, was 999.9. The quantity of the main impurity, peak B in figure 2, did not change during the experiments reported here.

Sample preparation

Agaritine standard for identification and quantification. A fresh stock solution of agaritine in methanol (0.02 mg ml^{-1}) was prepared weekly and stored in the refrigerator. Before HPLC analysis, 10 ml stock solution was evaporated to dryness and the remainder dissolved in 10 ml Milli Q water.

Agaritine solutions for stability studies. (1) TW, (2) TW added to 2 mM dtt, (3) Milli Q water, (4) nitrogen purged Milli Q water and (5) methanol were used. Solutions (concentration of 0.3 mg ml^{-1}) were stored in open or closed vials at ambient temperature (22°C) and at 4°C and analysed for agaritine content after 3, 24, 48 or 120 h. The chosen concentration was similar to one of the concentrations used in chronic toxicity studies.

Agaritine standard in simulated gastric fluid. Agaritine (0.3 mg ml^{-1}) was dissolved in a simulated gastric fluid composed of 2.0 g sodium chloride, 3.2 g pepsin and 7 ml hydrochloric acid (11.6 M) in 1000 ml distilled water, giving a pH of 1.2 (*US Pharmacopeia* 1980). The final concentration of agaritine was 0.02 mg ml^{-1}.

Fresh mushroom extracts. Fresh mushrooms (20 g) were homogenized in 100 ml methanol for 10 min in an Ultra Turax (Janke a Kunkel, IKA-Werk, Germany). The crude extract was shaken for an additional 30 min, after which the crude particles were removed by filtration. The volume of this crude extract was adjusted to 200 ml with methanol. A 10-ml aliquot of the extract was evaporated to dryness and the residue dissolved in 2 ml distilled water. This solution was filtered through a microfilter (25 mm Filter Unit, 5.0 µm PTFE, pp, ThermoQuest, USA) into a vial and 20 µl immediately injected onto the HPLC column.

Mushroom extracts at various pH. Fresh mushrooms (20 g) were mixed with 100 ml distilled water of various pH (pH 1.5 and 4.5 was prepared by adding hydrochloric acid; pH 6.8 was the natural pH of

distilled water) and homogenized for 10 min in an Ultra Turax. The homogenate was shaken for 30 min and a crude extract prepared by filtration. The volume of the filtrate was adjusted to 200 ml. This solution was filtered through a microfilter into a vial and a 20-µl aliquot injected onto the HPLC column.

Mushroom extracts in simulated gastric fluid. Fresh (20 g) or cooked (20 g sliced mushrooms boiled in 250 ml water for 20 min) mushrooms were mixed with 100 ml simulated gastric fluid and homogenized for 10 min in an Ultra Turax. The homogenate was shaken for 30 min and a crude extract prepared by filtration. The volume of the filtrate was adjusted to 200 ml. This solution was filtered through a microfilter into a vial and a 20-µl aliquot injected onto the HPLC column.

Identification and quantification

HPLC was performed using a Hewlett-Packard HP 1100 liquid chromatograph (Hewlett-Packard, Wallbron, Germany) equipped with diode array detector (DAD) and termostated autosampler. Separation was carried out on column (250 × 4 mm), LiChrospher 100 RP-18 (5 µm) (Merck, Darmstadt, Germany) with precolumn (4 × 4 mm), LiChrosopher 100 RP-18 (5 µm). The mobile phase was 0.05 M NaH_2PO_4 buffer (pH 3.3) at a flow rate 1 ml min^{-1}. The column temperature was 25°C. For identification of agaritine, the retention time, DAD spectra and peak purity software function were used. Agaritine was quantified by comparing the peak area of the analysed sample with the peak area for known amounts of the pure standard. The agaritine was detected at 237 nm when not otherwise stated. The detector response was linear in the range 0.2 µg ml^{-1} to 2 mg ml^{-1}. Under the experimental conditions described for sample preparation, the limit of detection for agaritine was 2 mg kg^{-1} mushrooms and the repeatability of measurement as the relative standard deviation (RSD) was 4.2%.

Results

Figure 1 show the stability of agaritine (0.3 mg ml^{-1}) over 5 days when dissolved in TW, a 2 mM TW solution of dtt (TW + dtt) or in methanol (MeOH) and kept in closed (figure 1a) and open (figure 1b) vials at ambient temperature, respectively. The data in figure 1 are the means from two parallel experiments. As can be seen, a successive drop in agaritine content occurred during storage in all tested media. However, the stability of aqueous solutions was significantly lower than that of methanolic solutions. Agaritine solutions were more stable in closed vials (figure 1a).

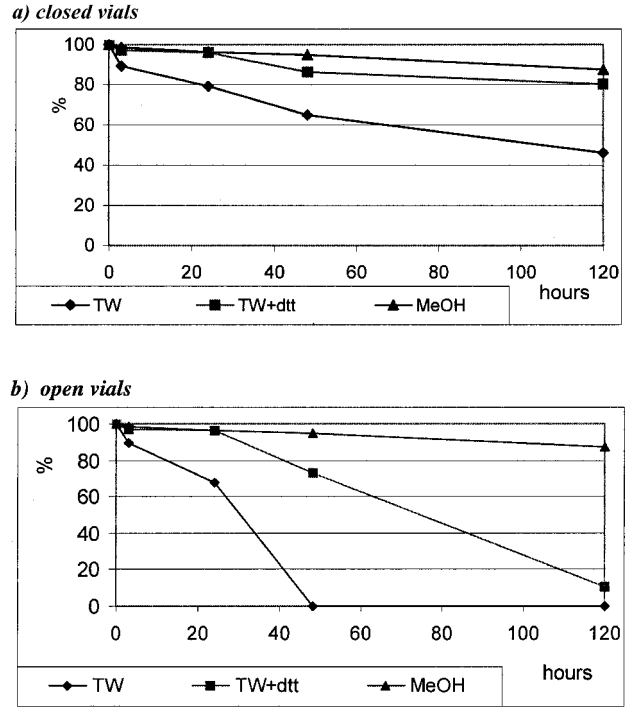

Figure 1. Degradation of agaritine (0.3 mg ml^{-1}) in various media (TW, tap water; dtt, 2 mM dithiotrcitol in TW; MeOH, methanol) in (a) closed vials and (b) open vials.

The quickest degradation of agaritine occurred in TW. Already after 48 h, no agaritine could be detected in TW solutions stored in open vials. In closed vials, slightly < 50% of the compound remained after 120 h. Addition of dtt to the TW improved the stability of the analyte, particularly when the solution was stored in closed vials. However, also in the presence of dtt, nearly all agaritine was degraded after 120 h in open vials (around 11% remaining). Agaritine was most stable in methanol, around 87–88% of the compound remained after 120 h both when stored in open and closed vials.

A separate experiment analysed the stability of agaritine (0.3 mg ml^{-1}) in Milli Q water and Milli Q water

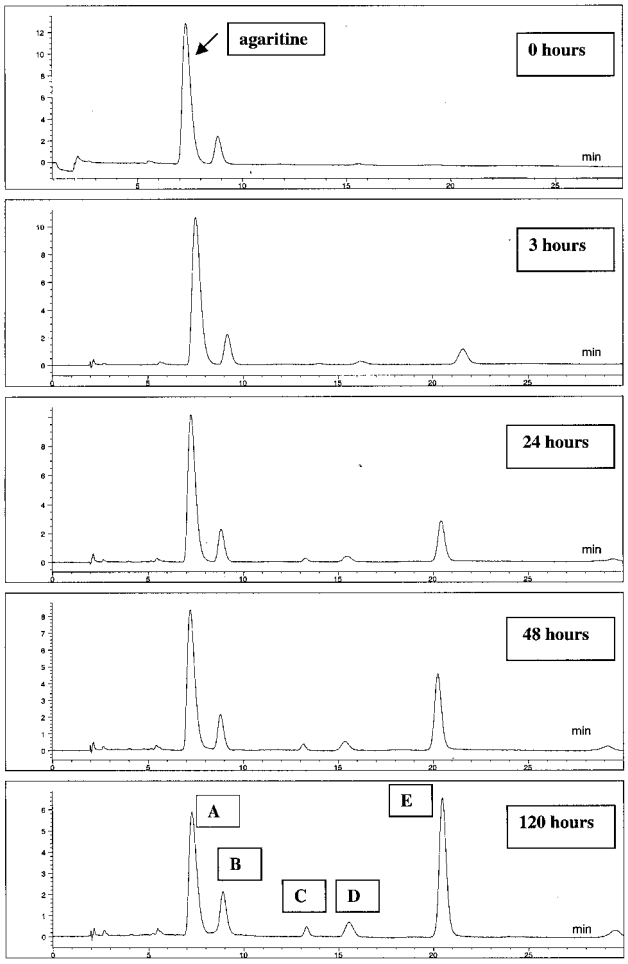

Figure 2. Degradation of agaritine in tap water (0.3 mg ml^{-1}, closed vials) during storage at room temperature. HPLC conditions: column, LiChrospher 100 RP-18 250 × 4 mm, 5 μm; mobile phase, 0.05 M NaH_2PO_4 phosphate buffer (pH 3.3); flow, 1 ml min^{-1}; detection, DAD 280 nm.

purged with N_2 (data not shown). In both solutions, agaritine was degraded to a similar degree as in methanol >75% remaining after 120 h. The stability was slightly higher in Milli Q water purged with N_2, but this difference was not statistically significant (*t*-test, $\alpha = 95\%$).

Figure 2 shows agaritine breakdown in TW in closed vials at ambient temperature as documented by HPLC/DAD analysis (trace at 280 nm shown) of aliquots taken within a 120-h storage period. At the end of the experiment, only 37% of the original content of target analyte remained (figure 3, compound A). The drop in concentration of agaritine

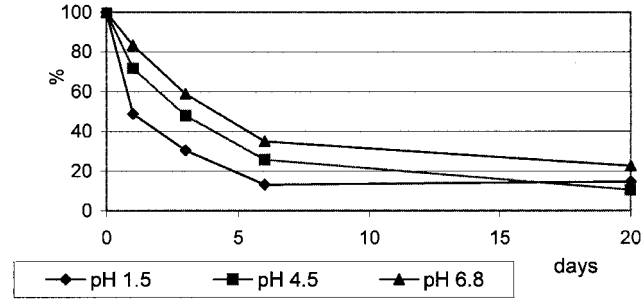

Figure 3. Degradation of agaritine at various pHs (the agaritine content in fresh mushrooms was 197.7 mg kg^{-1}).

with storage time was accompanied by the simultaneous occurrence of unknown degradation products (compounds B–E in the chromatogram). The amount of compound B (an impurity in the standard coming from the agaritine synthesis) remained more or less constant during the incubation (reduction from 13.6 to 11.9%). However, the concentrations of compounds C–E progressively increased during incubation. At the end of the incubation, the solution contained higher concentrations of compound E (43%) than of agaritine. The degradation products have not yet been identified. At detection wavelengths 200 and 237 nm (data not shown), only compound E could be detected, but the signal was weak. In addition, a minor product was indicated at a retention time of 4 min when the detection was made at 200 nm.

The influence of the temperature on the stability of aqueous solutions of the agaritine standard was also studied, as was the stability of aqueous and methanolic extracts of agaritine from *A. bitorquis*. In these studies, the solutions were kept in closed vials. There was a similar stability of agaritine in solutions of temperatures between 4 and 22°C, but again the compound was more stable in methanol extracts than in aqueous extracts (data not shown).

An important aspect of possible risks connected with consumption of the cultivated mushroom is whether or not agaritine is stable when entering the stomach. Figure 3 shows the influence of pH on the degradation rate of this compound in aqueous extracts of *A. bitorquis* stored at ambient temperature in closed vials. Agaritine was degraded more quickly at pH 1.5 than at pH 4.5, especially during the first days of incubation. It was relatively more stable at pH 6.8. After an incubation of 24 h, >50% of the compound was degraded at pH 1.5, but only around 18% was at pH 6.8. The recovery of agaritine from mushrooms

Table 1. Degradation of agaritine in simulated gastric fluid (percentage of original content).

Time (days)	Pure solution of agaritine	Agaritine extract from fresh mushrooms	Agaritine extract from cooked mushrooms
0	100.0	100.0	100.0
1	83.4	50.1	82.1
2	73.6	36.9	56.7
5	60.7	27.2	38.6
8	53.8	11.7	21.4
16	32.1	n.d.	n.d.
22	14.2	n.d.	n.d.

Original content of agaritine in standard solution (100%) was $0.02\,mg\,ml^{-1}$, whereas it was $230.2\,mg\,kg^{-1}$ in the 'fresh mushrooms' and $183.9\,mg\,kg^{-1}$ in the 'cooked mushrooms', respectively.
n.d., Not determined.

was not affected by the pH. It was comparable for all tested conditions, with a RSD of 4.2%.

A reduced stability of agaritine at low pH was confirmed in a study where agaritine was exposed to simulated gastric fluid having a pH 1.2. In this study, the stability of the agaritine standard in simulated gastric fluid was compared with the stability of the compound in simulated gastric fluid extracts of fresh and cooked mushrooms. The concentration of agaritine in the extracts of fresh and cooked mushroom was, however, approximately 10 times higher than the concentration in the standard. The breakdown of agaritine was quicker both in extracts of fresh and cooked mushroom than in pure gastric fluid (table 1).

Discussion

The studies presented here reveal that the stability of agaritine in solution, particularly aqueous solution, is highly dependent on the oxygen tension in the solution. In agreement with this finding, we recently observed that agaritine is substantially more stable in argon purged Milli Q water stored under argon at room temperature than in (1) TW (all solutions stored in open vials), (2) TW + 2 mM dithiotreitol (and stored in closed vials) and (3) 50% ethanol (and stored in closed vials) (Andersson and Gry 2002). Whereas < 20% agaritine remained in the solutions surrounded by ambient air after a 100-h incubation, very little agaritine had been degraded during the same period under argon (Andersson and Gry 2002). Hence, to avoid poor accuracy of analytical results when measuring the agaritine content of a material, quantification should be carried out as soon as possible after extraction of the sample. Furthermore, the extraction should preferably be done with methanol. Oxygen should be excluded as far as possible.

Many toxicological studies performed with extracts of the cultivated mushroom and with agaritine *per se* set out to explore whether this constituent in *A. bisporus* and *A. bitorquis* can give rise to adverse effects on living systems and they have given variable and not easily interpretable results (Andersson and Gry 2002). Since few, if any, studies have carefully taken care of protecting agaritine solutions from oxidative degradation, it is highly likely that agaritine degradation has contributed to the variable results. Thus, in some experimental studies the exposure to agaritine might have been much lower than intended. Further, the experimental material may have contained unidentified degradation products of agaritine with unknown toxic potential. Studies are underway to identify the degradation products formed in oxygenated neutral and acidic solutions using LC/MS for identification. Although presumed precursors and degradation products of agaritine (4-aminobenzoic acid, 4-hydrazinobenzoic acid, γ-glutaminyl-4-hydroxybenzen, γ-glutamyl-4-formylphenylhydrazine, 4-hydroxymethylphenylhydrazine, 4-hydroxymethylbenzendiazonium ion, 4-methylphenylhydrazine, γ-glutamyl-4-carboxyphenylhydrazine) have been looked for by LC/MS methods in standard solutions of agaritine and mushroom extracts stored for up to 4 weeks, only 4-methylphenylhydrazine has yet been confirmed.

Our studies with extracts of *A. bitorquis* indicate that agaritine is less stable in an acidic solution than in solutions having a neutral pH. The results confirm earlier studies (Levenberg 1964, Baumgartner *et al.* 1998). The reduced stability is unlikely to be a result of co-extracted enzymatic activity as the γ-glutamyltransferase activity detected in *A. bisporus* has a sharp optimum at pH 7.0 (Gigliotti and Levenberg 1964). The agaritine degradation at acidic conditions reported by others has been claimed to result in irreversible alterations of the molecule (Levenberg 1964, Baumgartner *et al.* 1998). In studies with simulated gastric fluid having a pH of 1.2, we noted that agaritine breakdown is faster in mushroom extracts (containing approximately $200\,mg\,l^{-1}$) than in the standard (with $20\,mg\,l^{-1}$). It is possible that it is the

higher concentration of agaritine in the mushroom extracts that results in the quicker degradation. To explore whether enzymatic activity in the mushroom contributed to the quicker degradation of agaritine in extracts, we boiled the mushrooms shortly before extraction to inactivate the native enzymes. Rather surprisingly, the rate of degradation was still faster in the mushroom extracts than in pure solution (table 1). It is possible that a mushroom component co-extracted with the phenylhydrazine could have catalysed the degradation of agaritine in this study.

The different stability of agaritine at various pHs is of importance when extrapolating data obtained in experimental animals to man. The pH of the human stomach varies considerably, but it is commonly around pH 2. The pH of the rodent stomach, on the other hand, is higher, usually around pH 5.

Acknowledgements

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References

Andersson, H. C., and Gry, J., 2002, Phenylhydrazines in the cultivated mushroom (*Agaricus bisporus*)—occurrence, biological properties, risk assessment and recommendations. *TemaNord* (accepted).

Baumgartner, D., Hoesch, L., and Rast, D. M., 1998, The biogenesis of β-*N*-(-glutamyl)-4-hydroxymethylphenylhydrazine (agaritine) in *Agaricus bisporus*. *Phytochemistry*, **49,** 465–474.

Benjamin, D. R., 1995, *Mushrooms Poisons and Panaceas* (New York: Freeman).

Chauhan, Y., Nagel, D., Gross, M., Cerny, R., and Toth, B., 1985, Isolation of *N*-[γ-L-(+)-glutamyl]-4-carboxyphenylhydrazine in the cultivated mushroom *Agaricus bisporus*. *Journal of Agricultural and Food Chemistry*, **33,** 817–820.

Chauhan, Y., Nagel, D., Issenberg, P., and Toth, B., 1984, Identification of p-hydrazinobenzoic acid in the commercial mushroom *Agaricus bisporus*. *Journal of Agricultural and Food Chemistry*, **32,** 1067–1069.

Fischer, B., Lüthy, J., and Schlatter, C., 1984, Gehaltstimmung von Agaritin im Zuchtchampignon (*Agaricus bisporus*) mittels Hochleistungsflüssigchromatographie (HPLC). *Zeitschrift für Lebensmittel Untersuchung und Forschung*, **179,** 218–223.

Frandsen, H., 1998, Synthesis of L-glutamic acid 5-(2-(4-hydroxymethyl)phenylhydrazide) (Agaritine). *Nordic Seminar of Phenylhydrazines in the Cultivated Mushrooms (Agaricus bisporus)*, edited by J. Gry and H. C. Andersson. *TemaNord*, **539,** p. 38. (Copenhagen: Nordic Council of Ministers).

Hajšlová, J., 1995, *Mushrooms Toxins. Natural Toxic Compounds of Food*, edited by J. Davídek (Boca Raton: CRC Press).

Levenberg, B., 1961, Structure and enzymatic cleavage of agaritine, a phenylhydrazine of L-glutamic acid isolated from Agaricaceae. *Journal of American Chemical Society*, **83,** 503–504.

Levenberg, B., 1964, Isolation and structure of agaritine, a γ-glutamylsubstituted arylhydrazine derivative from Agaricacaea. *Journal of Biological Chemistry*, **239,** 2267–2273.

McManus, B. M., Toth, B., and Patil, K. D., 1987, Aortic rupture and aortic smooth muscle tumors in mice. Induction by *p*-hydrozinobenzoic acid hydrochloride of the cultivated mushroom *Agaricus bisporus*. *Laboratory Investigations*, **57,** 78–85.

Ross, A. E., Nagel, A. E., and Toth, D. L., 1982, Occurrence, stability and decomposition of β-*N*-(γ-L-(+)-glutamyl)-4-hydroxymethylphenylhydrazine (agaritine) from the mushroom *Agaricus bisporus*. *Food Chemistry and Toxicology*, **20,** 903–907.

Toth, B., 1986, Carcinogenicity by *N*2-[γ-L-(+)-glutamyl]-4-carboxyphenylhydrazine of *Agaricus bisporus* in mice. *Anticancer Research*, **6**, 917–920.

Toth, B., 1995, Mushroom toxins and cancer [Review]. *International Journal of Oncology*, **6**, 137–145.

Toth, B., and Erickson, J., 1986, Cancer induction in mice by feeding of the uncooked cultivated mushroom of commerce *Agaricus bisporus*. *Cancer Research*, **46,** 4007–4011.

Toth, B., Erickson, J., Gannett, P. M., and Patil, K., 1997, Carcinogenesis by the cultivated baked *Agaricus bisporus* mushroom in mice. *Oncology Reports*, **4**, 931–936.

Toth, B., Gannett, P., Visek, W. J., and Patil, K., 1998, Carcinogenesis studies with the lyophilized mushroom *Agaricus bisporus* in mice. *In Vivo*, **12,** 239–244.

Toth, B., Nagel, D., and Ross, A., 1982, Gastric tumorigenesis by a single dose of 4-(hydroxymethyl)benzenediazonium ion of *Agaricus bisporus*. *British Journal of Cancer*, **46,** 417–422.

Toth, B., Patil, K., and Hwan-Soo Jae, 1981b, Carcinogenesis of 4-(hydroxymethyl)benzendiazonium ion (tetraflouroborate) of *Agaricus bisporus*. *Cancer Research*, **41,** 2444–2449.

Toth, B., Raha, C. R., Wallcave, L., and Nagel, D., 1981a, Attempted tumor induction with agaritine in mice. *Anticancer Research*, **1**, 255–258.

Toth, B., and Sornson, H., 1984, Lack of carcinogenicity of agaritine by subcutaneous administration in mice. *Mycopathologia*, **85,** 75–79.

United States Pharmacopeia, 1980, 20th revn (Rockwill), p. 1105.

Wallcave, L., Nagel, D. L., Rohan, C. R., Hae, S.-H., Bronczyk, S., Kupper, R., and Toth, B., 1979, An improved synthesis of agaritine. *Journal of Organic Chemistry*, **44,** 3752.