Table II. Vitamin E Analyses of Corn

samples	range, USP units/kg	mean, USP units/kg	ref
15 inbreds	9.0–37.7[a]	21.4[a]	Weber, 1984
11 samples from 5 geographical regions	3.2–12.0[a]	8.9[a]	Coret et al., 1983
4 inbreds	31.7–35.5[a]	34.0[a]	Contreras-Guzman et al., 1982
4 inbreds	1.9–12.2[c] 12.2–16.8[d]	6.2 15.0	Gavrilovic, 1982
17 samples from 7 states	17.0–52.2[b]	29.7[b]	Bunnell et al., 1968
10 samples from 7 states	16.1–31.4[b]	22.8[b]	Herting and Drury, 1969

[a] Calculated from data of cited paper by using the formula 1.49 × mg of (α-tocopherol/kg + 0.1 × mg of γ-tocopherol/kg). [b] Calculated by using 1.49 × mg of α-tocopherol/kg. [c] 1980 crop year. [d] 1981 crop year.

variations among samples from different geographical regions (see Table II, Cort et al., 1983; Bunnell et al., 1968; Herting and Drury, 1969).

Most feed tables for use in the formulation of livestock feeds list the vitamin E content of corn from 20–26 USP units per kg (National Research Council, 1980, 1979, 1978, 1977, 1975). The average from the present study falls slightly above this range. However, as can be seen from Table II, many other reported averages are lower. Furthermore, an average value does not indicate the very great variation which may occur among different samples of corn. Hence, the use of the average vitamin E values listed in current feed tables may at times significantly overestimate the vitamin E contribution of corn to a diet.

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Registry No. α-Tocopherol, 59-02-9; γ-tocopherol, 7616-22-0.

LITERATURE CITED

Bieri, J. G.; Evarts, R. P. *J. Nutr.* 1974, *104*, 850–857.
Bunnell, R. H.; Keating, J. P.; Quaresimo, A. J. *J. Agric. Food Chem.* 1968, *16*, 659–664.
Contreras-Guzman, E.; Strong, F. C.; da Silva, W. S. *J. Agric. Food Chem.* 1982, *30*, 1113–1117.
Cort, W. H.; Vicente, T. S.; Waysek, E. H.; Williams, B. D. *J. Agric. Food Chem.* 1983, *31*, 1330–1333.
Gavrilovic, B. *Stocarstvo* 1982, *36*, 409–420.
Herting, D. C.; Drury, E. E. *J. Agric. Food Chem.* 1969, *17*, 785–790.
National Research Council. "Nutrient Requirements of Sheep"; National Academy of Sciences: Washington, D.C., 1975.
National Research Council. "Nutrient Requirements of Poultry"; National Academy of Sciences: Washington, D.C., 1977.
National Research Council. "Nutrient Requirements of Dairy Cattle"; National Academy Press: Washington, D.C., 1978.
National Research Council. "Nutrient Requirements of Swine"; National Academy Press: Washington, D.C., 1979.
National Research Council. "Recommended Dietary Allowances"; National Academy of Sciences: Washington, D.C., 1980.
Scott, M. L. In "The Fat Soluble Vitamins"; Deluca, H. F., Ed.; Plenum: New York, 1978; Chapter 3.
The United States Pharmacopoeia, Twentieth Revision; United States Pharmacopoeial Convention, Inc.: Rockville, MD, 1980.
Weber, E. J. *J. Am. Oil Chem. Soc.* 1984, *61*, 1231–1234.

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Isolation of N^2-[γ-L-(+)-Glutamyl]-4-carboxyphenylhydrazine in the Cultivated Mushroom *Agaricus bisporus*

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N^2-[γ-L-(+)-Glutamyl]-4-carboxyphenylhydrazine (GCPH) has been isolated from *Agaricus bisporus*, the cultivated mushroom of commerce of the Western hemisphere. The compound was purified by using a combination of cation-exchange and reverse-phase high performance liquid chromatography and the structure confirmed by using fast-atom bombardment (FAB) mass spectrometry and FAB/tandem mass spectrometry. The average level of GCPH in the four samples analyzed was 42 ± 3 μg/g of wet mushroom.

INTRODUCTION

The mushroom of commerce in the Western hemisphere, *Agaricus bisporus*, has been shown to contain up to 400 ppm of agaritine "N^2-[γ-L-(+)-glutamyl]-4-(hydroxymethyl)phenylhydrazine" (GHPH), (Levenberg, 1961; Kelly et al., 1962). We subsequently found agaritine in locally purchased mushrooms in levels up too 700 ppm (Ross et al., 1982).

Although GHPH does not induce tumors in mice (Toth et al., 1981; Toth and Sornson, 1984) "over 50 hydrazine derivatives have induced tumors in laboratory animals" (Toth, 1984). The aim of our continuing studies is to identify biosynthetic hydrazine precursors or products of agaritine that are present in this commercial mushroom.

Shütte et al. (1972) have examined the incorporation of isotopically labeled compounds into agaritine by *Agaricus bisporus*. They found incorporation of radioactivity into agaritine from shikimic acid, glutamic acid, and *p*-aminobenzoic acid at levels of 0.14%, 0.68%, and 4.1%, respectively. LaRue (1977) subsequently postulated a biosynthetic scheme leading to GHPH invoking *p*-hydrazinobenzoic acid (HB) and N^2-[γ-L-(+)-glutamyl]-4-carboxyphenylhydrazine (GCPH) as intermediates.

We recently reported that HB is present in *Agaricus bisporus* at an average level of 10.7 ± 2.0 μg/g of mushroom (wet weight) (Chauhan et al., 1984). We now report the successful isolation, identification, and quantitation

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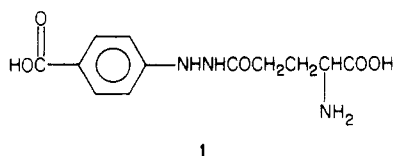


of GCPH in *Agaricus bisporus*.

EXPERIMENTAL SECTION

Materials. Compound GCPH was synthesized as previously described (Chauhan and Toth, 1984). All solvents used for solvent extraction were glass distilled. The solvents used for HPLC were glass distilled and UV spectroscopic grade.

Mushrooms. The cultivated mushroom *Agaricus bisporus* was purchased through a local distributor between April, 1984 and Sept, 1984 from Camsco Mushroom Co., Inc. (West Chicago, IL). The estimated time from harvest to experimental use was 24–48 h.

Analysis of *Agaricus bisporus* for GCPH. A 100-g sample of the commercial mushrooms was homogenized with methanol (100 mL) in a Waring blender at room temperature for 15 min. After centrifugation, the supernatant was stored overnight at −20 °C and recentrifuged. The slightly yellowish pale clear supernatant was passed through a 5 × 30 mm silica gel column. The sample was filtered through a Millex GS 0.22-μm filter (Millipore Corp., Bedford, MA) and 20-μL samples were analyzed by using HPLC as described below. Synthetic GCPH had a retention time (system A) of 6.2 min and GHPH had a retention time of 5.4 min (system A). The fraction eluting between 5.5 and 6.5 min was collected, concentrated, and rechromatographed on HPLC system B. Synthetic GCPH had a retention time of 13.4 min in system B.

High Performance Liquid Chromatography (HPLC). Analysis of GCPH was carried out on a Waters Associates (Milford, MA) HPLC system. HPLC was performed by using a M 6000 A solvent delivery system, equipped with a U6K injector and Model 660 solvent programmer. A Gilson Holochrome Model HM ultraviolet–visible range spectrophotometer (Middleton, WI) was used with a 10-mL quartz flow cell. All chromatography solvents were vacuum degassed immediately prior to use. A Hewlett–Packard 3390A integrator (Avondale, PA) was used.

System A. Column, Partisil M9 10/25 SCX, 9.4 mm i.d. × 250 mm (Whatman Inc., Clifton, NJ); flow rate, 3.0 mL/min; mobile phase, 0.01 M $NH_4H_2PO_4$ in deionized water; detector, 254 nm; pressure, 1050 psi; sample volume, 20 μL; optical density, 0.02–0.05 range.

System B. Column, Partisil M9 10/25 C_8, 9.4 mm i.d. × 250 mm (Whatman Inc., Clifton, NJ); flow rate, 1.0 mL/min; mobile phase, methanol and water (80:20 v/v); detector, 254-nm pressure; 950 psi; sample volume, 40 μL; optical density, 0.01–0.02 range.

Recovery of GCPH from *Agaricus bisporus* Samples. The percent recovery of GCPH from the mushroom was determined by adding known amounts of synthetic GCPH to the methanolic mushroom extracts and analyzing by HPLC as described above.

Confirmation of GCPH. Confirmation of the identity of GCPH following purification by HPLC was performed by fast atom bombardment mass spectrometry (FAB MS) and FAB tandem mass spectrometry (FAB MS/MS) with synthetic GCPH as a reference standard.

The mass spectra were obtained with a Kratos MS-50 (Manchester, U.K.) triple analyzer mass spectrometer which has been described elsewhere (Gross et al., 1982). The instrument consists of a high-resolution MS-I of Nier-Johnson geometry followed by an electrostatic analyzer as MS-II. The FAB ion source was of the standard Kratos design and was equipped with an Ion Tech atom gun. The sample was dissolved in a droplet of either a 1% H_2SO_4 acidified glycerol solution for positive ion studies or triethanolamine for negative ion experiments and placed on the copper probe tip of an insertion probe. The bombardment was done with 8 keV xenon atoms.

The described ions were accelerated to 8 keV for acquisition of mass spectra. Collisional activation decomposition (CAD) or MS/MS spectra of daughter ions from selected parents were obtained after ion activation in the third field-free region by using helium as the target gas. The helium pressure was increased until 50% suppression of the ion beam was observed. The MS/MS spectra were then taken by voltage scanning of the final analyzer and the data were accumulated by a DS-55 data system. The signal averaged spectra (20 20-s scans) were processed by using software written in this laboratory.

Table I. Quantities of GCPH in Commercial Samples of *Agaricus bisporus*

batches	μg GCPH/g mushroom
batch 1	36.9 ± 1.8
batch 2	48.5 ± 3.0
batch 3	44.4 ± 2.4
batch 4	38.3 ± 2.2

Table II. Recovery of GCPH from Mushroom Extracts[a]

μg GCPH added/g mushroom	I	II	III	% recovery av
10	33.8	29.8	35.7	33.1
50	48.2	46.5	47.3	47.3
150	80.1	79.5	76.8	78.8

[a] Minimum detection limit based on calculation ~1.85 μg/g.

RESULTS AND DISCUSSION

Compound GCPH was isolated from a methanolic extract of *Agaricus bisporus*, purified, and quantified via HPLC by using a combination of cation-exchange and reverse-phase columns. An independently synthesized sample of GCPH was used as a standard, and the isolated GCPH had a mean concentration of 42 ± 3 μg/g of wet mushroom (Table I). We have previously reported that GHPH is present in *Agaricus bisporus* in levels up to 700 ppm wet weight (Ross et al., 1982), two of the biosynthetic precursors originally postulated by LaRue (1977), HB (Chauhan et al., 1984), and now GCPH occur as much lower amounts.

The data in Table I were adjusted for recovery. The recoveries, shown in Table II, varied considerably with concentration. This may be due, as we have previously shown with HB (Chauhan et al., 1984), to irreversible adsorption of GCPH on the small silica column used for the initial cleanup. (The reverse-phase column employed also has a silica backbone).

Although electron impact (EI) and FAB MS were inadequate to confirm the presence of GCPH, confirmation was achieved employing FAB MS/MS. An attempt was made to obtain EI spectra after derivatizing the extract with diazomethane, but the results were unsatisfactory. Even after extensive treatment of the synthetic GCPH with diazomethane, only a small amount of the sample had been converted to the diester. Apparently, the glutamic acid portion of molecule was largely nonderivatized as was revealed by a study of the mass spectrum.

The positive ion FAB spectrum showed the expected $(M + H)^+$ ion, m/z 282, but many ions of comparable abundance were also observed: m/z 225, 229, 235, 245, 247, 252,

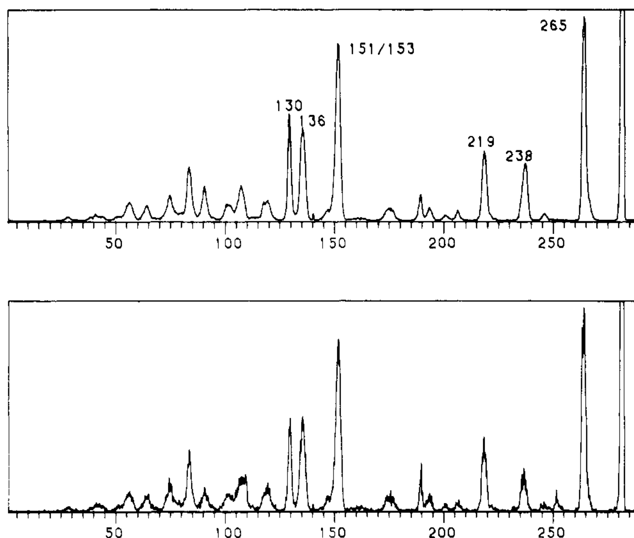


Figure 1. Positive ion CAD spectra of $(M + H)^+$, m/z 282: (top) standard; (bottom) isolated sample.

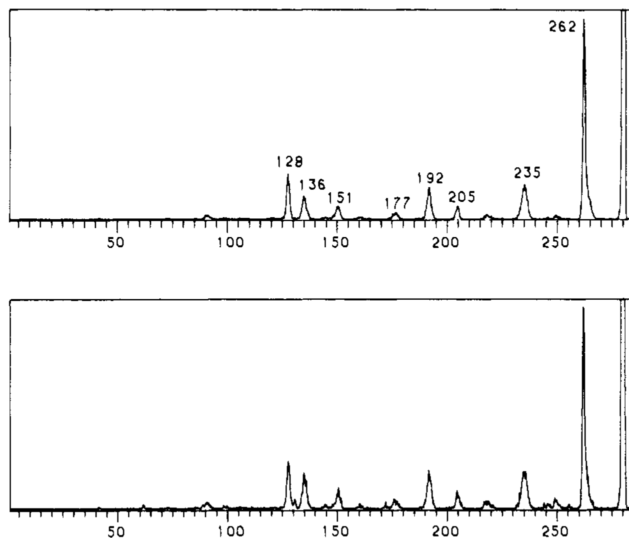


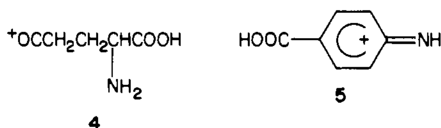
Figure 2. Negative ion CAD spectra of $(M - H)^-$, m/z 280: (top) standard; (bottom) isolated sample.

268, 290, 304, and 321. The FAB spectrum of a second fraction also showed an abundant m/z 282, $(M + H)^+$, and many interferences, some of which were different than those in fraction GCPH. The exact origins of these ions are not known, but they may arise, at least in part, from the various salts which were present in the HPLC reverse-phase eluant. For example, m/z 304 may be $(M + Na)^+$.

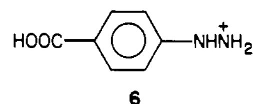
The negative ion FAB spectrum provides more convincing evidence for the presence of GCPH. The most abundant ion was m/z 280, $(M - H)^-$, along with approximately five ions of minor abundance (20–40% of the abundance of m/z 280). Nevertheless, neither the positive nor negative ion spectra were unequivocal evidence for the presence of GCPH.

To establish the existence of GCPH, the FAB MS/MS technique was employed. This enabled us to obtain a spectrum of the daughter ions from collisionally activated $(M + H)^+$ and $(M - H)^-$. The spectra of daughter ions from the $(M + H)^+$ and $(M - H)^-$ parents desorbed from the synthetic standard and from the extract were virtually identical, thereby confirming the identity of GCPH in the mushroom extract (Figures 1 and 2). One difference was the presence of an enhanced abundance of m/z 190 in the spectrum of $(M + H)^+$. This fragment, corresponding to loss of 92, indicates that some glycerol background ions were selected along with $(M + H)^+$. The spectrum of $(M - H)^-$ from the extract showed very low abundance ions m/z 131 and 172 which also come from fragmentation of a matrix ion coselected with the $(M - H)^-$ and which are more abundant in isolates containing lower amounts of GCPH.

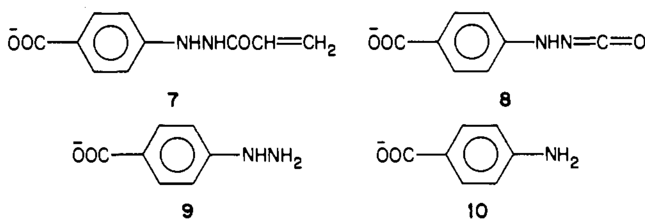
The fragmentation of $(M + H)^+$ is reasonable and consistent with the assigned structure. The higher mass ions, m/z 265, 238, and 219, are formed by losses of ammonia, CO_2, and NH_3 + formic acid. The three other abundant ions can be rationalized by protonation and cleavage at the hydrazide bond. Protonation at the nitrogen adjoining the carbonyl followed by simple cleavage gives **4** (m/z 130).



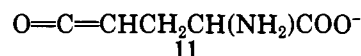
Cleavage of the N–N bond gives **5** (m/z 136). Hydrogen rearrangement and cleavage of the NH–CO bond yield **6**. The peak representing **6** is broadened and shifted to slightly lower mass probably because of the overlapping presence of another ion m/z 151.



With regard to the spectrum of fragments from $(M - H)^-$, we see an abundant loss of water to produce m/z 262. It is likely that proton removal occurs from the aromatic carboxylic acid, and the water loss involves the other acid moiety located remote from the charge site. There appears to be competitive loss of NH_3, but the peak for this loss cannot be resolved from that for H_2O elimination. Losses of CO_2 and HCOOH produce the next lower mass peak, a doublet centered at m/z 235. The ions m/z 205, 177, 151, and 136 may represent a series of fragments produced by eliminations occurring remote from the charge site (Jensen et al., 1985) to give structures **7–10**, respectively. The ion



m/z 192 must be a radical anion. Proton removal from the other carboxylic acid and remote charge-site fragmentation gives **11** (m/z 120). Compound GCPH is currently under study for carcinogenic action in mice.



Registry No. 1, 69644-85-5.

LITERATURE CITED

Chauhan, Y.; Nagel, D.; Issenberg, P.; Toth, B *J. Agric. Food Chem.* **1984**, *32*, 1067.

Chauhan, Y. P.; Toth, B. *J. Agric. Food Chem.* **1984**, *32*, 676.

Gross, M. L.; Chess, E. K.; Lyon, P. A.; Crow, F. W.; Evans, S.; Tudge, H. *Int. J. Mass Spectrom. Ion Phys.* **1982**, *42*, 243.

Jensen, N. J.; Tomer, K. B.; Gross, M. L. *J. Am. Chem. Soc.*, in press.

Kelly, R. B.; Daniels, E. G.; Hinman, J. W. *J. Org. Chem.* **1962**, *27*, 3229.

LaRue, T. *Lloydia* 1977, *40*, 307.
Levenberg, B. *J. Am. Chem. Soc.* 1961, *83*, 503.
Ross, A.; Nagel, D.; Toth, B. *Food Chem. Toxicol.* 1982, *20*, 903.
Schütte, H. R.; Liebish, H. W.; Miersch, O.; Senf, L. *An. Quim.* 1972, *68*, 899.
Toth, B. *Environ. Carcinog. Rev.* 1984, *C2*, 51.
Toth, B.; Raha, C. R.; Wallcave, L.; Nagel, D. *Anticancer Res.* 1981, *1*, 255.
Toth, B.; Sornson, H. *Mycopathologia* 1984, *85*, 75.

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HPLC Determination of the Cholesterol Content of Egg Noodles as an Indicator of Egg Solids

W. Jeffrey Hurst,* M. D. Aleo, and Robert A. Martin, Jr.

An HPLC method is presented which allows for the determination of cholesterol content which serves as an indicator of egg solids. The method involves the extraction of the noodles with methanolic KOH and the removal of interfering substance by the use of a commercially available Sep-pak. The final HPLC determination uses a reversed-phase column with a mobile phase of hexane/isopropyl alcohol and detection at 205 nm. The method is accurate and precise and is suitable for use in both product monitoring and nutritional labeling schemes.

INTRODUCTION

Presently, the method for the determination of egg solids in noodles is based on the analysis of lipid phosphorus (AOAC, 1980) with a calculation to arrive at egg solids. The method employed is cumbersome, time-consuming, and sometimes yields sporadic results. Additionally, the amount of lipid extracted varies with the organic solvent used (Hubbard et al., 1976). On the other hand, cholesterol measurement itself is straightforward. When the cholesterol content of the egg used to make the noodles is known, it is simple to measure the cholesterol in noodles and calculate the amount of egg based on the fact. The information about egg content in noodles is important from both a production and regulatory viewpoint.

Cholesterol analysis can be accomplished by a variety of methods including GLC and enzymatic techniques (Shen et al., 1982; Newkirk and Sheppard, 1981; Henry et al., 1971). HPLC has been used (Henry et al., 1971; Heffmann and Hunter, 1979; Kiuchi et al., 1975; Orgen et al., 1980) in the analysis of cholesterol in foods with the use of benzoate derivatives (Newkirk and Sheppard, 1981) while in the biological area the analysis was accomplished with direct detection at 205 nm (Duncan et al., 1979). The use of enzymes is possible but the enzyme used attacks the common sterol site (Roschlau et al., 1981) causing all sterols to mimic cholesterol. Since plants contain other sterols such as stigmasterol and sitosterol a direct analysis of cholesterol is not possible by enzymatic means.

In this paper, a method is described for the analysis of cholesterol in egg noodles by using a nonaqueous reversed-phase (NARP) HPLC technique with detection at 205 nm.

MATERIALS AND METHODS

HPLC. The HPLC apparatus consisted of an M6000A solvent delivery system (Waters Assoc.), an Alltech C_{18} column (4.0 mm i.d. × 25 cm) (Alltech Assoc.), a variable wavelength detector, a data unit, and miscellaneous ancillary equipment. The detector used was a Model 100-40 variable wavelength at 205 nm (Altex Inst. Co.). Data acquisition was performed with a Shimadzu ITG-4A Data Unit. The HPLC mobile phase consisted of hexane: IPA (99.9/0.1) (v/v) at a flow rate of 2.0 mL/min. All solvents were LC grade. The mobile phase must be thoroughly degassed prior to use or an unstable base line results.

Samples and Standards. Samples of egg noodles and dried whole egg solids (DWES) were obtained from commercial sources. The cholesterol standard was obtained from Pfansteihl Laboratories, sitosterol from Aldrich Chemical Co., and stigmasterol from Sigma Chemical Co. The [^{14}C] cholesterol used in recovery studies was obtained from New England Nuclear (0.02 μCi). The scintillation cocktail reagent was J. T. Baker β-count.

Liquid Scintillation Counting. Two-milliliter portions of the impinging, wash, and elution solutions were collected and placed into 4-mL capacity scintillation vials containing 2 mL of scintillation cocktail. The combined vials were then counted for 2 min each with a Tracor Model 6811 liquid scintillation system. Recovery data were then calculated based on total counts obtained from each vial.

Preparation of Samples. Samples are prepared for analysis by grinding in a Mouli grater or equivalent to insure sample homogeneity. Samples are then passed through a 25 mesh sieve prior to initial weighing. For egg noodles accurately weigh 1.0 g of prepared sample while for dried whole egg solids weigh 100 mg to ± 0.1 mg.

The sample is weighed into a 250-mL round-bottom flask. Samples are refluxed for 30 min with 50 mL of 2.0 N methanolic KOH. The resulting suspension is transferred while hot into a 250-mL separatory funnel. The round-bottom flask was washed with two 25-mL portions of distilled water, which are then added to the separatory funnel; the solution is allowed to cool to room temperature. Ten milliliters of 10% (w/v) NaCl is added and the resulting solution is extracted with two 100-mL portions of

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