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ARTICLE

Effect of γ-Irradiation on Agaritine, γ-Glutaminyl-4-hydroxybenzene (GHB), Antioxidant Capacity, and Total Phenolic Content of Mushrooms (*Agaricus bisporus*)

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Fresh mushrooms (*Agaricus bisporus*) were irradiated at doses of 1, 3, and 5 kGy to assess the effect of γ-irradiation on the major aromatic compounds agaritine (β-*N*-(γ-L-(+)-glutamyl)-4-(hydroxymethyl)phenylhydrazine) and GHB (γ-glutaminyl-4-hydroxybenzene) as well as on the total phenolic content and antioxidant capacity. Up to 3 kGy, agaritine was not affected. At 5 kGy, a significant reduction ($p = 0.05$) from 1.54 (0 kGy) to 1.35 g/kg dry weight (DW) was observed. γ-Glutaminyl-4-hydroxybenzene decreased by 22% at 1 kGy and by 31% at 5 kGy. Additionally, agaritine standard solutions at concentrations of 10^{-4} and 5×10^{-5} mol/L were irradiated to compare the effect on agaritine content in aqueous solutions and in the sample matrix. A rapid decay was observed, 50% at 750 Gy (10^{-4} mol/L) and 400 Gy (5×10^{-5} mol/L). The total phenolic content and antioxidant capacity were not significantly ($p = 0.05$) influenced by irradiation.

KEYWORDS: *Agaricus bisporus*; mushrooms; γ-irradiation; agaritine; γ-glutaminyl-4-hydroxybenzene (GHB); antioxidant capacity; total phenolics

INTRODUCTION

The most commonly cultivated white button mushrooms, *Agaricus bisporus*, have been part of the human diet for many years. The highly perishable nature of mushrooms remained a problem for the worldwide production (*1*−*3*). The quality loss is recognized by color change, cap opening, stipe elongation, cap diameter increase, weight loss, and texture changes. Browning is mostly induced by polyphenol oxidase (PPO) but could also be caused by bacterial contamination through *Pseudomonas tolaasii* or molds. Frequent methods to extend the shelf life of fresh mushrooms are low-temperature storage, controlled-atmosphere packaging, and chemical treatments (*3*−*5*). In addition, ionizing radiation has been investigated as a preservation method by several authors. A dose of 2 kGy, applied in combination with a storage temperature of 10−15 °C, was found to reduce microbial counts and to extend shelf life from 2 to 10 days (*1*, *3*). γ-radiation has also been shown to successfully delay the quality loss of mushrooms and to reduce the activity of PPO, the key enzyme of the browning process in mushrooms (*1*, *3*, *4*, *6*−*10*). Research has predominately been focused on quality parameters and enzymatic changes in the browning process thus far; therefore, the effect of γ-radiation on individual compounds in mushrooms is widely unclear.

The main aromatic compounds of mushrooms are agaritine and its derivatives, which belong to the chemical class of hydrazines. Hydrazines are natural products existing in considerable amounts in a number of mushroom species, including *A. bisporus*. Agaritine was found to contribute to the formation of toxic aryldiazonium ions (*11*). The potential carcinogenic and mutagenic effects of edible mushrooms and the possible contribution of agaritine have been the subject of intensive research. Lifetime administration of fresh mushrooms for 3 days a week induced tumors in a number of tissues in Swiss mice. However, agaritine itself, despite some evidence of toxicity, failed to produce tumors (*12*). Recent studies have demonstrated that agaritine is, to a greater extent, exerting indirect toxic effects as it quickly metabolizes, involving an initial loss of the γ-glutaminyl group, to the more mutagenic 4-(hydroxymethyl) phenylhydrazine (HMPH), which, in turn, is oxidized to the highly mutagenic 4-(hydroxymethyl)benzenediazonium ion (HMBD) (*13*).

γ-Glutaminyl-4-hydroxybenzene (GHB) (**Figure 1**) is the principal phenolic compound in mushrooms (*9*). Phenolic substances belong to the group of secondary plant products, being able to exert antioxidant properties by acting as reducing agents, hydrogen donating antioxidants, and singlet oxygen quenchers (*14*). In the presence of oxygen and tyrosinase, γ-glutaminyl-4-hydroxybenzene is easily hydroxylated to γ-L-glutaminyl-3,4-dihydroxybenzene (GDHB) (**Figure 1**) and oxidized to γ-L-glutaminyl-3,4-benzoquinone (GBQ), which further leads to the formation of polymerized melanin-like compounds (*15*). GHB and its derivatives occur in substantial amounts only in *Agaricus* species (*16*).

The aim of our investigations was directed to the effect of γ-radiation on the individual compounds agaritine and GHB. A decay of agaritine might contribute to the formation of toxic compounds. In addition, the antioxidant activity should be

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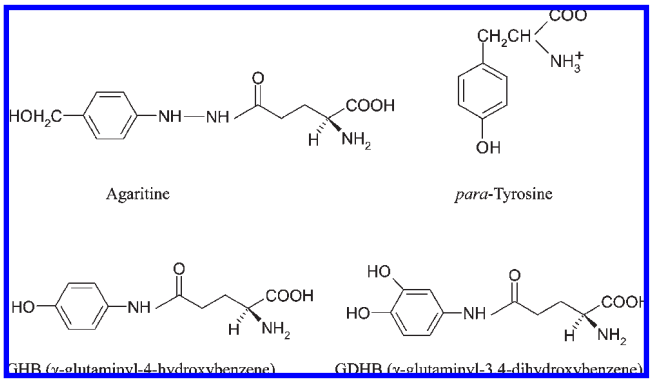

Figure 1. Chemical structures of compounds detected in mushroom extracts.

assessed by measuring the total phenol content and the antioxidant capacity (TEAC).

MATERIALS AND METHODS

Chemicals. All chemicals used were purchased of the highest quality grade available: methanol (>99.5%), methanol ROTISOLVHPLC ultra gradient grade, sodium carbonate (Roth, Karlsruhe, Germany), potassium dihydrogen phosphate (>99%), *ortho*-phosphoric acid (85%), glacial acetic acid, 2,2′-azinobis-(3-ethyl-benzothiazoline)-6-sulfonic acid (ABTS), potassium peroxodisulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma-Aldrich, Buchs, Switzerland), and Folin−Ciocalteu's reagent; gallic acid = 98% (Sigma-Aldrich, Steinheim, Germany). Agaritine (*β*-*N*-(*γ*-L-(+)-glutamyl)-4-(hydroxymethyl)phenylhydrazine) was provided by Hendrik Frandsen from the Danish Institute for Food and Veterinary Research, Søborg, Denmark. For sample preparation, water was distilled and further purified using a Direct-Q3 UV purification system (Millipore, Bedford, MA).

Sampling. To prevent fluctuations in the content of the components because of substrate cultivation, fruiting conditions, developmental stage, and age of the fresh mushrooms (*2*), 1 kg of *A. bisporus* mushrooms (Don Juan Selection, Hungary) from the same cultivation and harvest were purchased at a local store.

Processing and *γ*-Irradiation. Each mushroom was cut into fourths and distributed to four beakers. One part (∼250 g) of mushrooms was left unirradiated, and the other parts were irradiated at 1, 3, and 5 kGy under air at ambient temperature. To exclude enzymatic oxidation, the samples were immediately freeze-dried after irradiation. Then, they were ground to a fine powder using a small blender. The powder was stored in an exsiccator in the dark until analysis.

Sample irradiation was performed using a Cobalt-60-*γ*-ray irradiator type "Gammacell 220" (Nordion International, Inc., Kanata, Ontario, Canada) at a dose rate of 34 Gy/min. Irradiation of agaritine was carried out in aqueous solution (10^{-4} and 5×10^{-5} mol/L) under air at ambient temperature using doses of 100−1500 Gy.

Analysis of Agaritine and GHB. *Sample Preparation.* Dried mushrooms were worked up according to Schulzová et al. (*17*). A total of 1 g of dry mushrooms was mixed with 50 mL of methanol/water (9:1, v/v), shaken for 1 h, and then filtered. The residue was extracted again. The overall recovery found by Schulzová et al. (*17*) was 91.1%. In general, the agaritine content in the second extract was under the detection limit of high-performance liquid chromatography (HPLC) (<2 ppm agaritine). The filtrate was evaporated to dryness. The obtained residue was dissolved in 20 mL of distilled water. The solution was filtered through a FP 30/0.2 cellulose acetate filter (Whatman Ltd., Maidstone, Kent, U.K.) prior to injection onto the HPLC column. All prepared mushroom extracts were used within 1 day. The number of measurements (*N*) corresponds to the number of extracts.

HPLC−UV Measurements. Measurements were carried out on a Hewlett-Packard 1050/1100 series HPLC system (Agilent, Palo Alto, CA) equipped with a quaternary pump (G1311A), a mobile phase degassing unit (G1322A), a diode array detector (series 1050), and an autosampler (G1311A) adapted with a 20 *μ*L sample loop. The column used was a 250 × 4.6 mm inner diameter, 5 *μ*m, Li-Chrospher 100 RP-18, with a 4 × 4 mm inner diameter guard column of the same material (Merck, Darmstadt, Germany). The substances were eluted isocratically (0.05 mol/L KH_2PO_4/H_3PO_4 at pH 3.3, with a flow rate of 1 mL/min) and detected at 237 nm. A calibration curve for agaritine was established between 1×10^{-5} and 5×10^{-4} mol/L ($N = 9$), and the concentrations of agaritine in aqueous solutions and in the sample extracts were determined.

HPLC−ESI−MS Measurements. The presence of agaritine, GHB, GDHB, and *para*-tyrosine (**Figure 1**) was confirmed by liquid chromatography−mass spectrometry (LC−MS). The HPLC 1100 series system (Agilent, Palo Alto, CA) consisted of a G1312A binary pump, a G1322A mobile phase vacuum degassing unit, a G1313A autosampler adapted with a 10 *μ*L sample loop (10 *μ*L injection volume), an ACE 3 C18 column, 100 × 2.1 mm inner diameter, 3 *μ*m particle size (Advanced Chromatography Technologies, Aberdeen, Scotland), a guard column of the same stationary phase material, and an HCT+ ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. For data acquisition, Hystar 3.1 software for chromatography and hyphenated techniques was used. Data evaluation was carried out using the software DataAnalysis, version 3.3 (Bruker Daltonics). Isocratic elution was performed with 0.1% glacial acetic acid in water at a flow rate of 0.2 mL/min. Mass spectrometric analysis was performed in the negative ion mode with the following settings: capillary voltage, +4.5 kV; drying gas (nitrogen), 10 L/min at 300 °C; nebulizing gas (nitrogen), 40 psi; skimmer, −40 V; capillary exit, −100 V; and trap drive, 38. Analyses were first carried out in the scan mode (*m*/*z* 100−700) and then in the MRM mode, where only the deprotonated molecular ions of interest and previously observed in the scan mode were isolated and fragmented and the fragments were recorded.

Total Phenol Content and Antioxidant Capacity Assay. *Sample Preparation.* The extraction procedure was conducted as described by Chun et al. (*18*). A total of 2 g of freeze-dried mushrooms were mixed with 50 mL of methanol/water (8:10, v/v) and shaken for 1 h at room temperature. The mixture was filtered and evaporated under reduced pressure at 40 °C. The concentrate was dissolved in 10 mL of absolute methanol and then diluted to 20 mL with distilled water. Finally, the solution was centrifuged at 4000*g* for 10 min, and the clear supernatant decanted into a 25 mL bottle (sample extract).

Total Phenolic Content (FC Test). The total phenolic content was determined following the method of Chun et al. (*18*). A total of 0.4 mL of the sample extract was added to a 10 mL volumetric flask filled with 7 mL of distilled water. A reagent blank, using distilled water instead of the sample, was prepared for the spectrometric measurements. The Folin−Ciocalteu reagent (0.5 mL) was added to the mixture and shaken. After 3 min, 1 mL of 7% Na_2CO_3 solution was added and the mixture was diluted to the volume of 10 mL with distilled water. The solution was kept in the dark for 1 h. Subsequently, the absorbance was measured at 760 nm against the blank using a Lambda 650 UV/vis spectrometer (Perkin-Elmer, Vienna, Austria).

The total phenolic content was calculated from a calibration curve of gallic acid (200−2000 mg/L) prepared at the same time and expressed as milligrams of gallic acid equivalent (GAE) per liter.

Antioxidant Capacity (TEAC Assay). The TEAC assay was carried out applying the method of Re et al. (*19*). A total of 0.192 g of 2,2′-azinobis-(3-ethyl-benzothiazoline)-6-sulfonic acid (ABTS) was dissolved in 50 mL of distilled water to a concentration of 7 mM. The addition of 0.033 g of dipotassium peroxodisulfate (final concentration of 2.45 mM) generated the production of the $ABTS^{\bullet+}$ radical cation. This $ABTS^{\bullet+}$ solution was diluted with distilled water at 2:25 (v/v). The Trolox standard (2.5 mM) was prepared in methanol, and the dilution series ranged from 0.25 to 2 mM. An absorbance reading was taken directly after the addition and mixing of 100 *μ*L of sample or Trolox standards to 2 mL of $ABTS^{\bullet+}$ solution. Measurements were performed at the maximum at 734 nm versus water using the same spectrometer as for the total phenolic content test.

The antioxidant capacity of the sample was calculated from the Trolox standard curve by plotting the absorbance versus the concentration and expressed as Trolox equivalent per liter. The concentration of antioxidants giving the same percentage change in absorbance of $ABTS^{\bullet+}$ as that of 1 mM Trolox is defined as TEAC (*20*).

Statistical Analysis. Statistical analysis was carried out using SPSS, version 16.0. Differences between the means of the applied doses were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. p values = 0.05 were regarded as significant. The total phenol content and TEAC correlation was performed using Pearson's correlation.

RESULTS AND DISCUSSION

Qualitative Analysis of Mushroom Extracts by HPLC−UV and HPLC−ESI−MS. The chemical structures of the detected compounds are presented in **Figure 1**. A typical HPLC-UV chromatogram of a mushroom extract is illustrated in **Figure 2**. Agaritine (peak 1) represents the main peak, being eluted after 5.4 min. It was identified by comparison to the UV spectrum and retention time of the agaritine standard. The same is valid for *para*-tyrosine (peak 2) eluting at 6.1 min. However, the comparison of the UV spectrum with the *para*-tyrosine standard exhibited small differences, indicating that this peak is superimposed. Therefore, it was excluded from quantitative analysis. The third compound (peak 3) at 9.4 min showed the same UV spectrum as that given for GHB in the literature (*21*). Because there was no reference compound available, HPLC−ESI−MS analysis was carried out. The compounds agaritine (267.3 g/mol), tyrosine (181.2 g/mol), as well as GHB (238.2 g/mol) were identified by their mass/charge ratios. This analysis also showed the presence of the 3,4-dihydroxybenzene compound GDHB (254.2 g/mol). Because for this substance neither a reference standard nor a UV spectrum was available, it could not be identified by HPLC−UV. Isolation and fragmentation of the deprotonated molecular ions $[M - H]^-$ of agaritine (266.0), GHB (236.9), and GDHB (253.1) showed their common fragment, the glutamyl group at m/z 127.9. It should be mentioned that MS analysis did not indicate the presence of the genotoxic metabolites of agaritine, HMPH, and HMBD.

Quantitative Analysis of Mushroom Extracts by HPLC−UV. Agaritine was quantified on the basis of an external HPLC−UV calibration curve (R^2 = 0.996). Its content ranged from 1.47 to 1.59 g/kg of dry weight (DW) for unirradiated mushrooms. These values are slightly lower than the findings for other *A. bisporus* mushrooms: 2.1 g/kg of DW, Sharman et al. (*22*); 2.1−6.9 g/kg of DW, Fischer et al. (*23*). An irradiation treatment did not result in a significant change of the agaritine content at doses up to 3 kGy. Accordant with the Food Irradiation Clearance Data Base (*24*), the irradiation of mushrooms is approved between 1 and 3 kGy. In this database, some countries do not specify their products (using "fresh fruits and vegetables") and some do not indicate the dose range; therefore, it can not be excluded that higher doses may be applied. At a dose of 5 kGy, agaritine showed a statistically significant decrease from 1.54 ± 0.04 (0 kGy) to 1.35 ± 0.09 g/kg of DW (5 kGy) (**Figure 3**; N = 7). Therefore, toxic degradation products (*11*, *13*) might be formed.

The main phenolic compound, GHB, in mushrooms was found at a concentration of 6.4 g/kg of DW in a fully developed fruit body (*25*). In the present study, GHB could not be quantified because no reference compound for a calibration curve was available. The influence of γ-irradiation on the GHB content was measured by comparing the peak areas in the HPLC−UV chromatograms at different dose levels. Results showed a significant decrease of GHB when irradiated at 1, 3, and 5 kGy (**Figure 4**; N = 7). The concentrations of γ-glutaminyl-4-hydroxybenzene (GHB) in mushrooms irradiated at 1 kGy were by 22% lower than in unirradiated mushrooms. Use of 5 kGy decreased the content by 31% compared to the untreated control.

Irradiation of Agaritine in Aqueous Solution. Because mushrooms are high in moisture (~92%) (*2*), the effect of irradiation on agaritine in mushrooms was compared to that in aqueous solutions in the presence of air. Although an indirect radiolysis effect, i.e., reactions of $^{\bullet}$OH radicals formed upon water radiolysis, was to be expected in both samples, the agaritine concentration in mushrooms was hardly influenced by irradiation. In aqueous solutions, however, a strong decrease of agaritine at both concentrations, 5×10^{-5} and 10^{-4} mol/L, was observed. In the former, the 50% decay was achieved with 400 Gy, and in the latter, the 50% decay was achieved with 750 Gy. Obviously, the matrix of the mushroom makes the individual components less susceptible to irradiation modifications. Newly formed irradiation products did not appear in the HPLC−UV chromatograms at 237 nm.

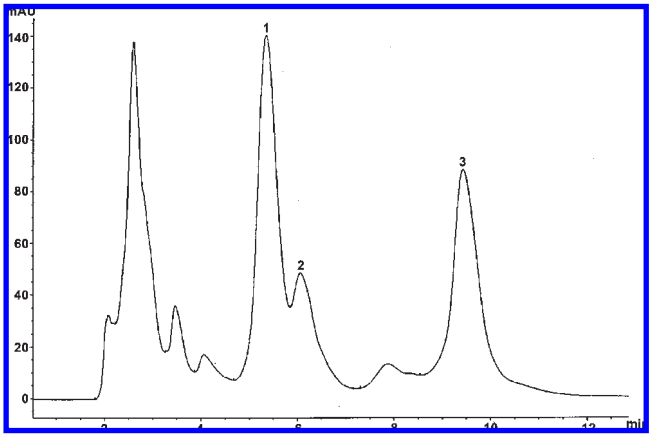

Figure 2. HPLC−UV chromatogram of a mushroom extract at 237 nm: peak 1, agaritine; peak 2, *para*-tyrosine; peak 3, GHB.

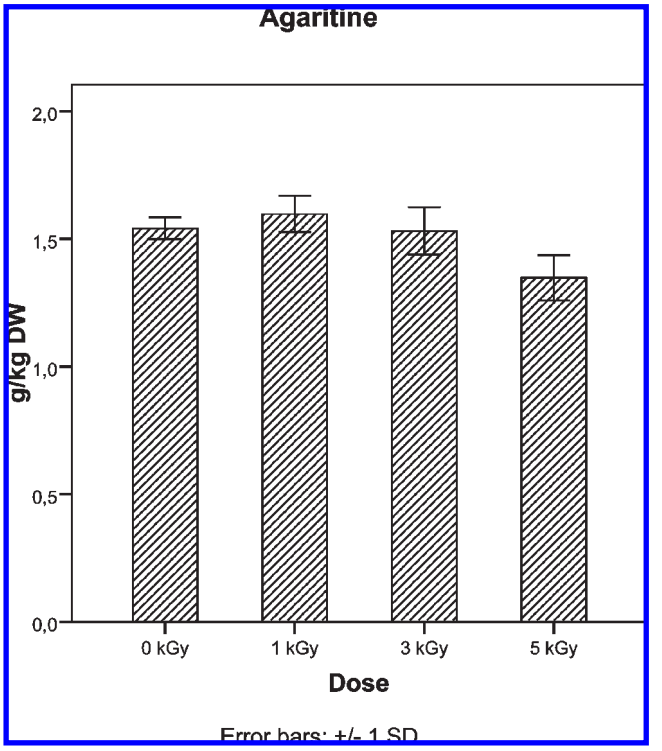

Figure 3. Mean values (N = 7) of agaritine (g/kg of DW) in mushroom extracts at different doses.

Total Phenol Content. Generally, mushrooms contain low levels of total phenols in comparison to other fruits and vegetables (*18*) because phenolic compounds, such as flavonoids and phenolic acids, are absent or present only in very low concentrations in *A. bisporus* (*2*, *26*). Therefore, it can be assumed that the

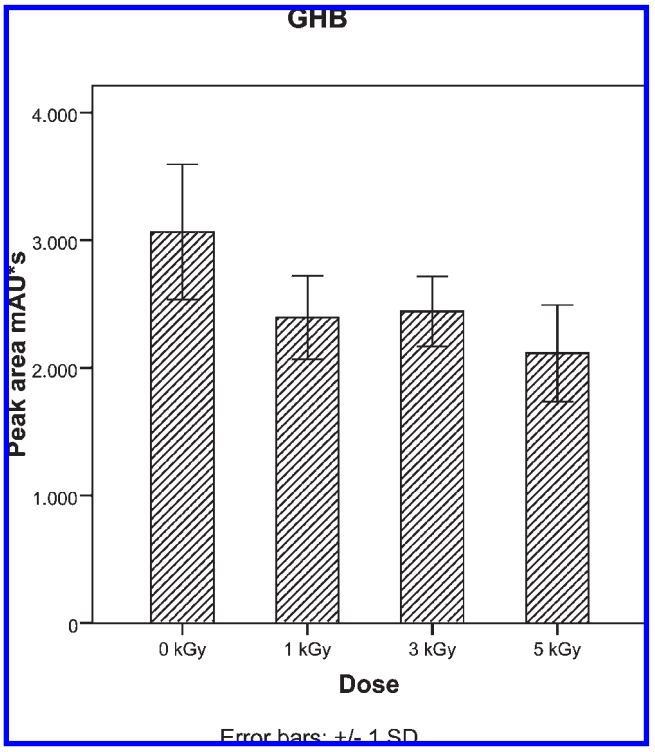

Figure 4. Mean values ($N = 7$) of GHB (peak areas) in mushroom extracts at different doses.

main part of phenols expressed by GAE results from melanogenous phenols (e.g., GHB and *para*-tyrosine) and vitamins (e.g., vitamin C). The results obtained (2.42 ± 0.25 g of GAE/100 g; **Table 1**) are in accordance with the literature data. In a recent study on the radical-scavenging properties of three different *A. bisporus* strains, values of the same order of magnitude were reported: 3.1−4.9 g of GAE/100 g (*27*). Investigations with respect to shelf life extension of fresh mushrooms by irradiation have been conducted by Beaulieu et al. (*10*) and Benoît et al. (*4*). Concerning the total phenolic content, their results are contradictory. Whereas, in the publication of Benoît et al. (*4*), an increase of total phenols directly after irradiation was not indicated, Beaulieu et al. (*10*) reported a significant enhancement (30%). In our studies, influences of the browning process on phenolic compounds were avoided because all mushroom samples had been immediately freeze-dried after irradiation to eliminate enzymatic activity. The content of total phenols, expressed as GAE values, did not show distinct changes in irradiated mushrooms. The values were slightly decreasing; however, variations were not statistically significant (**Table 1**). Because GHB concentrations (peak areas in this study) were found to decrease with increasing dose (**Figure 4**), it might be assumed that other phenolic compounds are formed during irradiation. It has to be mentioned that irradiation of aqueous systems containing aromatic compounds can implicate the formation of phenols. The hydroxylation of phenylalanine in protein-containing food, for example, results in the formation of *ortho*-, *meta*-, and *para*-tyrosine. The former two could act as a marker for an irradiation treatment (*28*). In the case of GHB, the generation of GDHB might be expected. MS analysis indicated the presence of this compound in irradiated mushrooms. However, because of the lack of an authentic standard, unequivocal identification of GDHB was not possible.

Antioxidant Capacity. In contrast to the results of the total phenol content test, a slight statistically non-significant increase of the antioxidant activity with dose was observable (**Table 1**).

Table 1. Total Phenol Content (GAE) and Antioxidant Capacity (TEAC)

	gallic acid equivalent (g/100 g of DW)	Trolox equivalent (mg/100 g of DW)
dose (kGy)	mean ± SD	mean ± SD
0	2.42 ± 0.25	5.43 ± 0.30
1	2.32 ± 0.14	5.40 ± 0.35
3	2.34 ± 0.20	5.62 ± 0.35
5	2.19 ± 0.16	5.64 ± 0.25

There is a controversial discussion whether total phenols and antioxidant activity correlate well in mushrooms. Phenols are regarded as main contributors to the antioxidant activity, the measurements of which are based on radical scavenging. However, not all phenols are effective radical-scavenging antioxidants (*29*, *30*), because the antioxidant capacity depends upon various factors, such as the number and location of hydroxyl groups on the aromatic ring, as well as their mutual positions. This might explain the rather poor correlation coefficient (0.633, $p < 0.01$) found for total phenol content and TEAC in this study. In conclusion, γ-irradiation of mushrooms has no effect on agaritine below 3 kGy; however, it seems to have a slight but significant effect on agaritine at a dose of 5 kGy and GHB at all doses. Changes in the total phenolic content or antioxidant activity were not significant. The relatively high radiation resistance of GHB and especially of agaritine in mushrooms is quite surprising considering that the irradiation doses (up to 5 kGy) were high for a system containing about 90% water and that agaritine in aqueous solution decreased by 50% at doses < 1 kGy. The fact that these compounds and parameters were analyzed with respect to γ-irradiation for the first time makes further investigations necessary, in particular, regarding the degradation of GHB.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis-(3-ethyl-benzothiazoline)-6-sulfonic acid; agaritine, β-*N*-(γ-L-(+)-glutamyl)-4-(hydroxymethyl)phenylhydrazine; FC, Folin−Ciocalteu; GAE, gallic acid equivalent; GDHB, γ-L-glutaminyl-3,4-dihydroxybenzene; GHB, γ-L-glutaminyl-4-hydroxybenzene; GBQ, γ-L-glutaminyl-3,4-benzoquinone; HMBD, 4-(hydroxymethyl)benzenediazonium ion; HMPH, 4-(hydroxymethyl)phenylhydrazine; PPO, polyphenol oxidase; TEAC, Trolox equivalent antioxidative capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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