JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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# Main Phenolic Compounds of the Melanin Biosynthesis Pathway in Bruising-Tolerant and Bruising-Sensitive Button Mushroom (*Agaricus bisporus*) Strains

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Supporting Information

**ABSTRACT:** Browning is one of the most common postharvest changes in button mushrooms, which often results in economic losses. Phenolic compounds, which are associated with browning, were extracted from the nonbruised and bruised skin tissue of various button mushrooms with a sulfite-containing solution and analyzed with UHPLC-PDA-MS. In total, 34 phenolic compounds were detected. Only small differences in the total phenolic content between bruising-tolerant and -sensitive strains were observed. The contents of γ-L-glutaminyl-4-hydroxybenzene (GHB) and γ-L-glutaminyl-3,4-dihydroxybenzene (GDHB) correlated with bruising sensitivity; for example, $R^2$ values of 0.85 and 0.98 were found for nonbruised brown strains, respectively. In nonbruised skin tissue of the strains with brown caps, the GHB and GDHB contents in sensitive strains were on average 20 and 15 times higher, respectively, than in tolerant strains. GHB and GDHB likely participate in the formation of brown GHB−melanin, which seemed to be the predominant pathway in bruising-related discoloration of button mushrooms.

**KEYWORDS:** *Agaricus bisporus, bruising, enzymatic browning, GHB, GDHB, UHPLC-PDA-MS*

## INTRODUCTION

Due to picking, handling, and storage of the button mushroom, *Agaricus bisporus*, discoloration reactions are initiated that result from enzyme-catalyzed oxidation (polyphenol oxidase, PPO) of phenolics into their respective quinones.[1] These quinones undergo further oxidative polymerization leading to melanins, which are high molecular mass dark brown or black pigments.[2] This so-called enzymatic browning reaction decreases the commercial value of mushrooms. Four pathways for melanin synthesis in *A. bisporus* have been proposed (Figure 1).[3] One hypothesized pathway starts from chorismate (**35**) and branches into three different melanin pathways. Chorismate, which is derived from the shikimate pathway, is converted via several enzymatic steps, including γ-glutamyltransferase (GGT, EC 2.3.2.2), into γ-L-glutaminyl-4-hydroxybenzene (GHB) (**19**), which has been identified as the main phenolic in *A. bisporus* fruiting bodies, spores, and skin.[1,4−6] In the presence of PPO (EC 1.10.3.1) and oxygen, GHB is readily oxidized into the corresponding diphenol (γ-L-glutaminyl-3,4-dihydroxybenzene, GDHB) (**15**) and subsequently into *o*-quinone (γ-L-glutaminyl-3,4-benzoquinone, GBQ) (**39**), which polymerizes into GHB−melanins.[7] Furthermore, amino−phenolics might react with different kinds of melanin following the same PPO-mediated and autopolymerization steps, that is, PAP−melanin, formed from *p*-aminophenol (**6**),[1] and DOPA−melanin formed from L-3,4-dihydroxyphenylalanine (L-DOPA) (**13**).[8] The fourth putative pathway starts from catechol (**24**) and leads to catechol melanins, involving similar reactions as described for the other three melanin types (Figure 1).[8] Catechol has been identified in mushroom cap tissue as well.[9]

Due to the poor solubility of melanins,[10] no good methods are available to analyze them. Therefore, most studies on discoloration focus on analysis of their precursor phenolics. Most often, HPLC analysis combined with UV measurements and/or mass spectrometry (MS) is used to identify and quantitate individual phenolics that are involved in the browning reaction. It was shown that a decrease in whiteness during 7 days of storage of *A. bisporus* var. *albidus* mushrooms resulted in increases of chorismate (**35**), prephenate (**40**), and *p*-aminobenzoic acid.[11] Unfortunately, the concentration of GHB was not determined in this study. In the fruiting body of *A. bisporus* strain A-6 (ATCC 382581) the concentration of GHB increased during maturation, and the highest concentration was found in the lamellae (21.3 mg/g dry weight) of a 120 mm cap diameter mushroom.[12] In the cap skin of a browning-sensitive strain, the contents of tyrosine (**18**), GHB (**19**), and GDHB (**15**) were nearly 7-fold higher than in a less sensitive *A. bisporus* strain.[5] GHB contents from peel samples (epidermis and 2 mm cap tissue) were similar among wild

Received: May 10, 2013
Revised: July 30, 2013
Accepted: August 1, 2013
Published: August 1, 2013

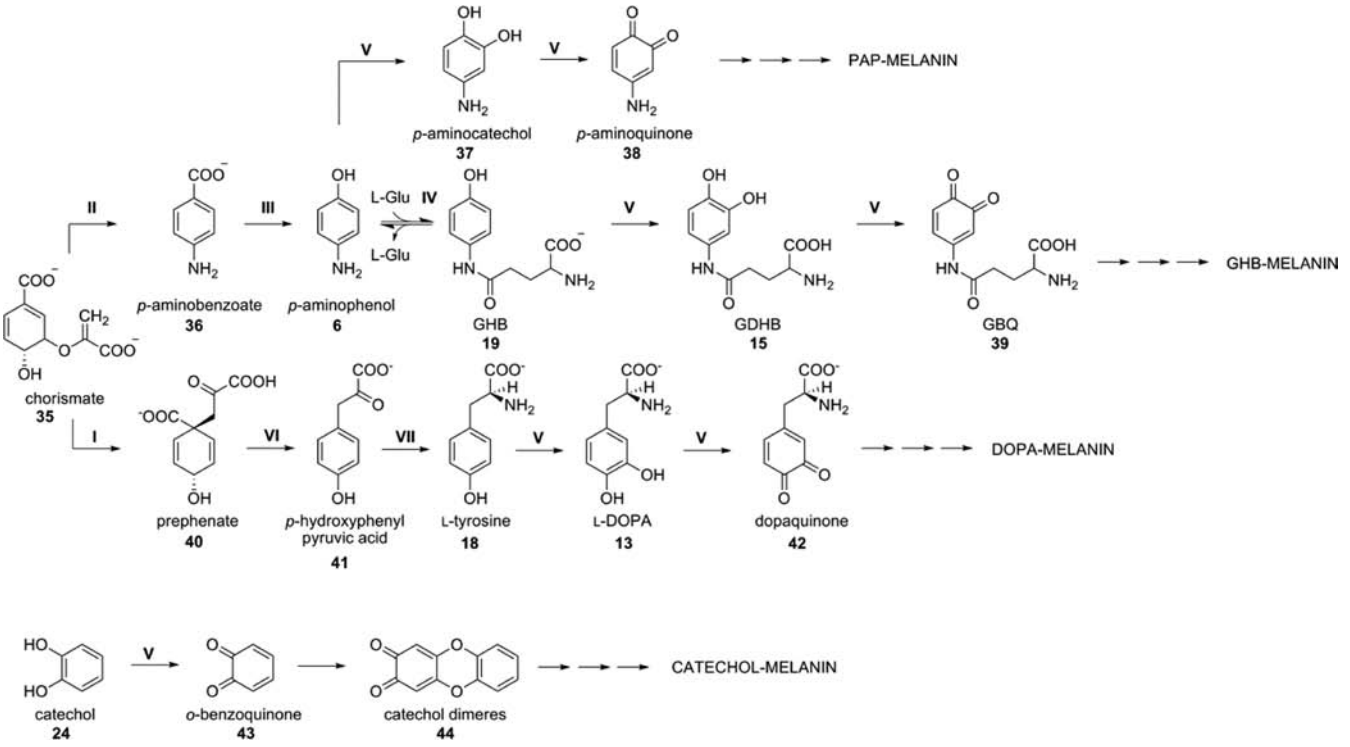


**Figure 1.** Putative melanin biosynthesis pathways of *A. bisporus*. Phenolic compounds are indicated by Arabic numbers, which correspond with Table 2. Enzymes involved in the pathway are indicated by Roman numerals: I, chorismate mutase; II, 4-aminobenzoate synthase; III, 4-aminobenzoate hydroxylase; IV, γ-glutamyltransferase; V, polyphenol oxidase; VI, prephenate dehydrogenase; VII, (4-hydroxy)phenylpyruvate aminotransferase. GHB, γ-L-glutaminyl-4-hydroxybenzene; GDHB, γ-L-glutaminyl-3,4-dihydroxybenzene; GBQ, γ-L-glutaminyl-3,4-benzoquinone; L-DOPA, L-3,4-dihydroxyphenylalanine. Adapted from Jolivet et al.[1]

strains that differed in cap color.[13] A significant difference in GHB content was found between wild and commercial strains.[13]

The contents of L-phenylalanine and L-tyrosine were highest in stage 3 *A. bisporus* mushrooms (closed mushrooms with a cap diameter of 30−40 mm).[14] In another study,[15] an increase in L-tyrosine and L-phenylalanine contents in button mushrooms during storage was observed. In addition to the compounds discussed and shown in the pathway, other phenolic compounds have been described in *A. bisporus* mushrooms.[16−18] However, these reports were not always mutually confirming. The differences between studies might be related to the mushroom strain used, their developmental stage, environmental factors, harvesting conditions, extraction solvents, and detection methods.[19] In summary, there is a need for a systematic study that compares multiple mushroom strains with respect to the composition of phenolics (not restricted to just a few representatives) and discoloration by bruising.

Here, an UHPLC-PDA-MS method is described to analyze the phenolics of the melanin biosynthesis pathways to provide a comprehensive analysis of the individual biosynthetic intermediates in skin tissue of *A. bisporus* button mushrooms. This study aimed to reveal the most important fluxes through the four melanin pathways. Previously, *A. bisporus* strains with differential bruising sensitivities were identified.[20] In the present study, the phenolics in skin tissue of the cap of bruising-sensitive and bruising-tolerant button mushroom strains were analyzed to correlate bruising sensitivity to phenolic composition. Our hypothesis is that these results, together with gene expression analysis, will help in determining the key pathways and parameters for bruising tolerance of *A. bisporus* strains.

## MATERIALS AND METHODS

**Materials.** The phenolic standards and other materials used were all HPLC grade compounds or solvents. *p*-Aminophenol, catechol, *p*-coumaric acid, L-DOPA, L-phenylalanine, and L-tyrosine were from Sigma-Aldrich (Steinheim, Germany). UHPLC-MS grade Milli-Q (MQ), acetonitrile, and formic acid were from Biosolve BV (Valkenswaard, The Netherlands). Chemicals used for the synthesis of GHB were obtained from commercial sources without further purification unless stated otherwise. The solvents used were peptide synthesis grade and stored on molecular sieves (4 Å).

**Mushroom Strains and Cultivation.** Mushrooms were grown as described previously.[20] Nine *A. bisporus* strains (Table 1) were grown in seven replicates. The strains were randomly distributed over the growing room. Strains used originated from either the Laboratory of Plant Breeding at Wageningen University or the *Agaricus* resource program culture collection.[21] To minimize biological variation, all samples were collected during the same growing experiment under controlled pre- and postharvest conditions.[20]

**Mushroom Bruising and Sampling.** Mushroom bruising and discoloration analyses were performed as described previously.[20] The bruised skin tissue of the cap was collected directly after taking the picture and immediately frozen in liquid nitrogen. As a control, samples of the skin tissue of nonbruised mushrooms were taken at the same time point. Samples were stored at −80 °C until use. The samples were ground with a mortar and pestle with liquid nitrogen, and for each experiment the amount of mushroom powder needed was weighed. Weighed samples were handled with liquid nitrogen and stored at −80 °C.

**SNP Analysis of *A. bisporus* Strains.** A dendrogram was obtained for the *A. bisporus* strains based on single nucleotide polymorphism (SNP) analysis. Mycelium of each strain was grown on MMP plates (1% malt extract, 0.5% mycological peptone, 1.5% agar) covered with cellophane. DNA was isolated from ground freeze-dried mycelium with the Wizard Magnetic DNA Plant System (Promega Benelux b.v.,

**Table 1. Bruising Sensitivity of Nine *A. bisporus* Mushroom Strains**

| strain[a] | abbrev | classification | WI diff | SD | Tukey[b] |
|---|---|---|---|---|---|
| commercial hybrid 3 (white) | CH3 | tolerant | 13.51 | 4.43 | a |
| commercial hybrid 1 (white) | CH1 | tolerant | 14.66 | 2.88 | a |
| commercial hybrid 2 (white) | CH2 | tolerant | 15.45 | 3.30 | a |
| traditional white strain 4 | TW4 | sensitive | 22.45 | 5.37 | b |
| traditional off-white strain 7 | TO7 | sensitive | 22.95 | 4.39 | b |
| wild brown strain 2 | WB2 | tolerant | −0.37 | 4.06 | a# |
| wild brown strain 18 | WB18 | tolerant | 0.13 | 6.56 | a# |
| wild brown strain 17 | WB17 | sensitive | 15.67 | 4.86 | b# |
| wild brown strain 16 | WB16 | sensitive | 20.31 | 4.81 | b# |

[a]Strain names are according to Weijn et al.[20]. [b]Tukey was calculated for white and off-white strains separately from the brown strains, which is indicated by #.

Leiden, The Netherlands). SNP analysis was based on the comparison of 117 randomly chosen SNPs with on average 2 SNPs per scaffold. SNP analysis was done according to the KASPAR method (Van Haeringen Laboratorium B.V., Wageningen, The Netherlands). This information was used to construct the dendrogram. As SNP analysis was not performed for commercial hybrids 1 and 3, the position of these two strains was based on LTR-AFLP analysis, which has shown that commercial hybrids 1, 2, and 3 are very closely related to each other (personal communication with Patrick Hendrickx, Plant Research International). The similarity matrix was made with the simple matching method making use of the NTSYSpc2.1 program.[22] For cluster analysis SAHN was used in which the following parameters were used: UPGMA was used as the clustering method and FIND was chosen as the option for "in case of ties". All other parameters were used with the standard settings.

**GHB Synthesis.** GHB (**19**) was synthesized according to a literature procedure.[23] Boc-Glu(OH)-OBn (1.01 g, 2.98 mmol) was dissolved in $CH_2Cl_2$ (30 mL) followed by the addition of (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (1.32 g, 2.98 mmol), *p*-aminophenol (390 mg, 3.58 mmol), and *N*,*N*-diisopropylethylamine (DIPEA) (1.04 mL, 5.96 mmol). The dark-colored reaction mixture was stirred for 3 h at room temperature and concentrated under vacuum. The residue was taken up in EtOAc (30 mL), and the organic layer was subsequently washed with aqueous 13.6% (w/v) $KHSO_4$ (3 × 20 mL), aqueous 5% (w/v) $NaHCO_3$ (3 × 30 mL), and aqueous saturated NaCl (3 × 20 mL). Then the EtOAc layer was dried ($Na_2SO_4$) and evaporated to dryness. The residue was purified by silica column chromatography using Silicycle SiliFlash P60 silica gel (particle size 40−63 μm) (Screening Devices b.v., Amersfoort, The Netherlands) with a column size of 4 cm (diameter) × 15 cm (height), collecting 10 mL fractions. Purified fractions were pooled after evaporation of the solvent under vacuum, and Boc-GHB-OBn was obtained as an off-white solid in 50% yield (683 mg).

In a two-step procedure the C-terminal benzyl ester was deprotected using $H_2$ and Pd/C as a catalyst, and finally the N-terminus was deprotected using 95% (v/v) TFA in $H_2O$. Therefore, Boc-GHB-OBn (**2**) (350 mg, 0.82 mmol) was dissolved in THF/$H_2O$ (10 mL), and 10% Pd/C (15 mg) was added to the solution. After flushing with $H_2$(g), the reaction mixture was stirred for 5 h and pressurized with $H_2$, using a hydrogen balloon. Then, the catalyst was filtered off using Celite, and the filtrate was evaporated to dryness. Subsequently, the residue was dissolved in 95% (v/v) TFA in $H_2O$ and stirred for 30 min at room temperature. The product was precipitated as its TFA salt, using methyl *tert*-butyl ether (MTBE)/hexane (30 mL, 1:1) and obtained as an off-white solid. After centrifugation (5 min, 2000*g*, room temperature), the supernatant was decanted. The pellet was suspended a second time in MTBE/hexane (30 mL, 1:1) and centrifuged (5 min, 2000*g*, room temperature), and the supernatant was decanted. After the pellet had been dried using a $N_2$ flow, the product was dissolved in *t*-BuOH/$H_2O$ (30 mL, 1:9) and lyophilized, yielding the TFA salt of GHB as a white powder in 73% yield (200 mg).

**GHB Analysis.** Reaction progression was monitored using thin layer chromatography (TLC).[24] TLC was performed on a Merck precoated silica gel 60F254 glass plate. Compound spots were evaluated by UV quenching at 254 nm and ninhydrin staining.[25] Analytical HPLC was performed on a HPLC system (Shimadzu, Kyoto, Japan) equipped with a UV−vis detector operated at 220/254 nm using a Dr. Maisch ReproSil-Pur C18-AQ column with pore size = 120 Å and particle size = 5 μm, 250 × 4.6 mm (Screening Devices b.v.), at a flow rate of 1 mL/min from 100% buffer A (0.1% v/v TFA in $CH_3CN/H_2O$ 95:5 v/v) to 100% buffer B (0.1% v/v TFA in $CH_3CN/H_2O$ 5:95 v/v) in 20 min. The product purity was confirmed by HPLC with a single peak (at 6.55 min, peak area = 97.5% at 220 nm) and only two slight contaminations (10.58 min, peak area 2.1%; and 15.43 min, peak area 0.4%). ESI-MS was performed on an LCMS-QP8000 electrospray ionization mass spectrometer (Shimadzu) operating in a positive ionization mode. The calculated mass of GHB ($C_{11}H_{14}N_2O_4$) is 238.10, with ESI-MS *m*/*z* $[M + H]^+$ 239.00 found.

**UHPLC-PDA-MS Analysis.** Ten milligrams of mushroom powder was weighed into a 1.5 mL dark Eppendorf tube using liquid nitrogen to cool the samples. To the sample was added 500 μL of a sulfite solution (0.5% (w/v) sodium metabisulfite ($Na_2S_2O_5$) in 1% (v/v) acetic acid solution). Each sample was vortexed for 1 min and put in an ultrasonic water bath for 10 min followed by centrifugation (5 min, 14500*g*, 4 °C). The supernatants obtained were transferred to a new dark 1.5 mL Eppendorf tube. A second extraction was performed with the sulfite solution, and the supernatants were combined. The samples were filtered with a Spartan 13/0.2 RC 0.2 μm filter (Whatman's Hertogenhosch, The Netherlands) and stored at −20 °C until use.

Samples were injected in an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, autosampler, and photodiode array detector (PDA) using a Hypersil gold aQ column of 150 mm × 2.1 mm i.d., particle size = 1.9 μm (Thermo Scientific), at 20 °C. The eluents used were 0.1% (v/v) formic acid in MQ (eluent A) and 0.1% (v/v) formic acid in acetonitrile (eluent B). The elution program was 0−6 min, 100% A; 6−14 min, 100−90% A with 0−10% B; 14−15 min, 10−100% B; 15−20 min, 100% B; 20−21 min, 0−100% A; 21−30 min, 100% A. The flow rate was 300 μL/min, and the injection volume was 5 μL. The PDA detector was set to measure between 200 and 600 nm. $MS^n$ analysis was performed on an LTQ-XL mass spectrometer (Thermo Scientific) using electrospray ionization (ESI) and detection in the positive and negative mode, with a source voltage of 4.7 kV and an ion transfer tube temperature of 260 °C. The instrument was tuned to optimize the ionization process and sensitivity using L-tyrosine for the positive mode and catechol for the negative mode. A full-scan mass spectrum over a range of *m*/*z* values of 100−500 was recorded. From each branch of the melanin biosynthesis pathway (Figure 1) two standard compounds were chosen, except for the catechol−melanin branch for which only catechol was used. The other standard compounds were *p*-aminophenol, GHB, L-DOPA, and L-tyrosine. L-Phenylalanine and *p*-coumaric acid were chosen as standards because chorismate can also branch to phenylpyruvic acid, which serves as a precursor for L-phenylalanine, which can be converted into coumaric acid (via cinnamic acid) in the phenylpropanoid pathway. Standard compounds were dissolved in 0.5% (w/v) $Na_2S_2O_5$ in 1% (v/v) acetic acid. Calibration curves were run at 254 nm. For analysis, 254 nm was chosen, matching the narrower window of absorption of L-phenylalanine compared to the other standard compounds.[26]

**UHPLC-PDA-MS Data Analysis.** Data analysis was performed with Xcalibur (version 2.1.0) (Thermo Scientific). A background subtraction of an MQ sample was used for each sample. Peak integration was done at 254 nm with the ICIS algorithm. Peak

parameters were set as follows; baseline window at 500, area noise factor at 10, and peak noise factor at 20. For all other settings the defaults were used. The automatic peak integration was checked to verify if the baseline was followed correctly and if peaks were integrated correctly; otherwise, peak integration was performed manually. Peaks were considered for analysis when the peak area exceeded 0.5% of the total peak area of that particular sample. In this way, 34 peaks were quantitated with a high reliability and reproducibility. Results are represented as the average of four determinations, unless stated otherwise. The data were combined from two independent extracts, which both were analyzed in duplicate. Peak area was calculated per gram of fresh weight (g FW) skin tissue. Heat map analysis was done with Squared Pearson correlation for similarity and UPGMA for clustering in GeneMaths XT (version 2.12) (Applied Maths, Sint-Martens-Latem, Belgium). Identification of phenolic compounds was performed on the basis of the standard compounds and the molecular mass of the parent ion as well as $MS^n$ and UV−vis spectra data. After comparison with the standards, unidentified peaks were compared with other phenolic compounds from the same melanin pathway and compared to literature data. Metabolite databases were also used for confirmation of peak identity.[27,28] For conversion of peak area to concentration the corresponding standard compound was used. When this was not possible, a standard compound from the same melanin pathway (Figure 1) was used.

**Statistical Analysis.** Statistical analysis was performed using SPSS (version 21.0) (PASW statistics, IBM, Amsterdam, The Netherlands). Comparison between strains was calculated with one-way ANOVA with the Tukey post hoc comparison test. A value of $p < 0.05$ was considered to be significant.

## RESULTS AND DISCUSSION

**Quantitation of Bruising Sensitivity.** Bruising discoloration of nine white, off-white, and brown mushroom strains was quantitated by comparing the color of the nonbruised control area with that of the bruised area. The resulting whiteness index difference is shown in Table 1 (WI diff). Mushrooms with a white/off-white cap were statistically analyzed separately from brown cap mushrooms, because the difference in background color could not be entirely compensated for with computer image analysis. The results supported our previous findings,[20] as the strains showed the expected bruising sensitivity and tolerance. In particular, WB2 and WB18 showed very high levels of bruising tolerance (WI ≈ 0), and the bruised skin area was hardly visible to the eye. The variation in WI diff between tolerant and sensitive strains was larger for the brown strains (WI diff from −0.37 to 20.31) than for the white strains (WI diff from 13.51 to 22.95).

**Genetic Relationship of the Strains.** As bruising tolerance and the presence of phenolic compounds might be correlated to the genetic origin of the strain, a similarity matrix analysis was performed on the basis of single nucleotide polymorphism (SNP) analysis (Figure 2). The results indicated that the genetic origin of the tolerance trait of the three commercial strains originated from the same genetic background. The sensitive white strains TW4 and TO7 are genetically related and have a similar WI difference. The same is found for the sensitive brown strains, WB16 and WB17. The tolerant brown strains WB2 and WB18 do not cluster together and, therefore, it might be that different genetic backgrounds underlay the bruising tolerance trait.

**Identification of Phenolic Compounds from *A. bisporus* Skin Tissue.** Two representative elution patterns of extracted phenolic compounds are shown in Figure 3. All seven standard compounds were found in the mushroom skin tissue (Table 2). To identify peaks additional to the standard

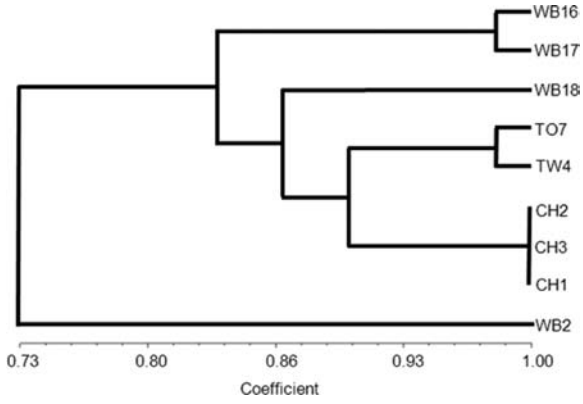


**Figure 2.** Dendrogram of the *A. bisporus* strains analyzed, based on SNP analysis. Abbreviations of the strains correspond with Table 1.

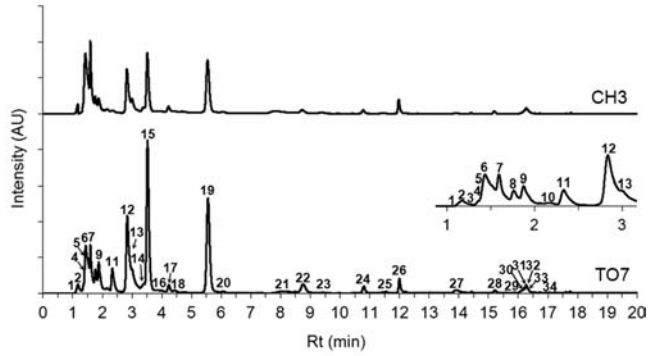


**Figure 3.** Elution profile of the skin tissue phenolics of two representative, nonbruised white *A. bisporus* strains. (Inset) Zoom in from 1 to 3 min of TO7.

compounds, their UV−vis and MS data were compared with those of the standard compounds and other phenolics from the melanin pathways and, subsequently, with phenolic metabolites in general. This resulted in the identification of *p*-hydroxybenzaldehyde (**3**), ergothioneine (**7**), agaritine (**12**), GDHB (**15**), agaritane (**25**), agaritinic acid (**26**), and tryptophan (**28**). Sulfo adducts might be formed as a result of the extraction procedure, which leads to an additional 80 Da (corresponding to the mass of $SO_3$) of the parent ion compared to the respective unsulfonated compound.[29] This approach led to the identification of sulfo-GDHB (**9**). In total, 15 of the 34 peaks present in the chromatograms were identified. This covered almost all compounds hypothesized in the pathways (*p*-aminophenol, GHB, GDHB, L-tyrosine, L-DOPA, L-phenylalanine, *p*-coumaric acid, and catechol), except for the precursors at the start of the melanin pathways and the quinones, which are highly reactive species. The identities of the other compounds remain unknown.

**Quantitation of Phenolic Compounds.** After peak identification, the total phenolic content of both the nonbruised and bruised skin tissue of the nine strains was compared. A reasonable correlation between bruising sensitivity and total phenolics in the nonbruised tissue was found ($R^2 = 0.84$ for the white strains and $R^2 = 0.75$ for the brown strains). The correlation between total phenolics in bruised skin tissue and the bruising sensitivity was very weak, or no link at all was found ($R^2 = 0.68$ for the white strains and $R^2 = 0.32$ for the brown strains). The total content of phenolics did not differ significantly between bruised and nonbruised tissues (Figure 4).

**Table 2. Identification of Peaks in Extracts of Mushroom Skin Tissue, Based on MS $[M + H]^+$ and UV−Vis Data**

| peak | $t_R$(min) | MS ($m/z$) | MS$^{2a}$ ($m/z$) | UV−vis $\lambda_{max}$ | tentative identification |
|---|---|---|---|---|---|
| 1 | 1.05 | 123 | 82 | 204, 215 | |
| 2 | 1.14 | 179 | 161, 123 | 276, 229 | |
| 3 | 1.22 | 123 | 81, 79, 96, 93 | 276, 232 | *p*-hydroxybenzaldehyde |
| 4 | 1.32 | 205 | 76, 130, 84 | 216, 255 | |
| 5 | 1.39 | 205 | 76, 130, 84 | 262, 217 | |
| 6 | 1.43 | 110 | | 271, 235 | *p*-aminophenol |
| 7 | 1.57 | 230 | 186, 127 | 258, 229 | ergothioneine |
| 8 | 1.73 | 118 | 72, 59, 88 | 243 | |
| 9 | 1.85 | 335 | 129, 205, 235 | 249, 297 | sulfo-GDHB |
| 10 | 2.15 | 256 | 130, 206, 300, 289, 278, 98 | 262, 217 | |
| 11 | 2.24 | 241 | 210, 130, 84 | 246, 217 | |
| 12 | 2.74 | 268 | 232, 121 | 240 | agaritine |
| 13 | 2.93 | 198 | 138, 135, 162 | 279, 236 | L-DOPA |
| 14 | 3.19 | 132 | 86 | 245, 218 | |
| 15 | 3.38 | 255 | 126, 130, 192, 238, 108, 84 | 249, 285 | GDHB |
| 16 | 3.77 | 150 | 88, 70 | 217, 245, 282 | |
| 17 | 4.07 | 150 | 88, 70 | 260, 230 | |
| 18 | 4.20 | 182 | 146, 136, 123, 119 | 274, 231 | L-tyrosine |
| 19 | 5.30 | 239 | 110, 129, 193, 176, 222, 84 | 246 | GHB |
| 20 | 5.74 | 285 | N/D | 235, 306 | |
| 21 | 7.64 | 332 | N/D | 262 | |
| 22 | 8.32 | 166 | 120, 130, 135, 93 | 257, 220 | L-phenylalanine |
| 23 | 9.38 | 193 | N/D | 217, 226, 255 | |
| 24 | 10.41 | (109) | (negative mode) | 274, 233 | catechol |
| 25 | 11.22 | 252 | N/D | 247 | agaritane |
| 26 | 11.71 | 280 | 130, 249, 240, 216 | 248, 298 | agaritinic acid |
| 27 | 13.69 | 259 | 232, 121 | 245, 216 | |
| 28 | 14.92 | 205, 188 | 146, 134, 92, 76 | 279, 229 | tryptophan |
| 29 | 15.57 | 157 | 106, 116, 125, 97 | 204, 254 | |
| 30 | 16.21 | 145 | 114, 60, 99 | 216, 238, 269 | |
| 31 | 16.26 | 215 | 187, 154, 86 | 269, 235 | |
| 32 | 16.30 | 145 | 60, 115, 95 | 276, 236 | |
| 33 | 16.39 | 165 | 119, 147, 123, 165 | 293, 241 | *p*-coumaric acid |
| 34 | 17.10 | 150 | 88, 70 | 216, 262 | |

[a]The ions are written in order of intensity; the first one is the base peak. N/D, not determined.

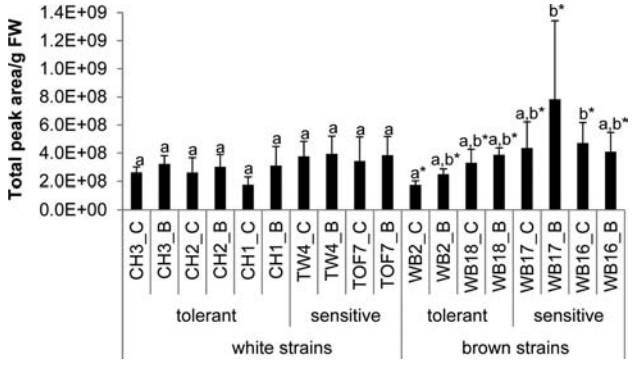


**Figure 4.** Total phenolics content in the *A. bisporus* strains analyzed. The total phenolic content is based on total peak area per gram fresh weight (FW). Abbreviations of the strains correspond with Table 1. C, nonbruised; B, bruised skin tissue. Tukey was calculated based on log transformed data and for the white and off-white strains separately from the brown strains, which is indicated by #.

Also, the samples were compared on the basis of peak area per gram FW for each peak individually. This was done to identify compounds that are different between nonbruised and bruised samples and different between tolerant and sensitive strains. None of the phenolics was specifically present in nonbruised or bruised tissue, or in tolerant or sensitive strains, or in brown or white strains. However, a correlation between the content of specific phenolic compounds and bruising sensitivity was observed, as is clarified below.

**Differences between Bruising-Sensitive and -Tolerant Strains before Bruising.** When the elution profiles of the extracts from the nonbruised skin tissue of white tolerant and sensitive strains were compared (Figure 3), a clear difference in the intensity of the individual phenolics was observed. The same was found for the brown strains. The results of all strains before bruising were summarized in a heat map-based comparison using an unsupervised clustering of the samples, and the peaks detected are shown in order of elution (Figure 5A). Clustering of the *A. bisporus* strains showed a division into sensitive and tolerant strains. It is striking that GDHB (**15**) and GHB (**19**) were most abundant in the sensitive strains, whereas their direct precursor in the GHB−melanin biosynthesis pathway, *p*-aminophenol (**6**), showed similar abundance in sensitive and tolerant strains. Furthermore, the GHB and GDHB contents seemed to vary among the tolerant strains. Albeit in less abundance, sulfo-GDHB (**9**) showed a similar pattern of accumulation as GHB and GDHB. GDHB and sulfo-GDHB were calculated as GHB equivalents, and this might lead to underestimation of the content. On average, sulfo-GDHB

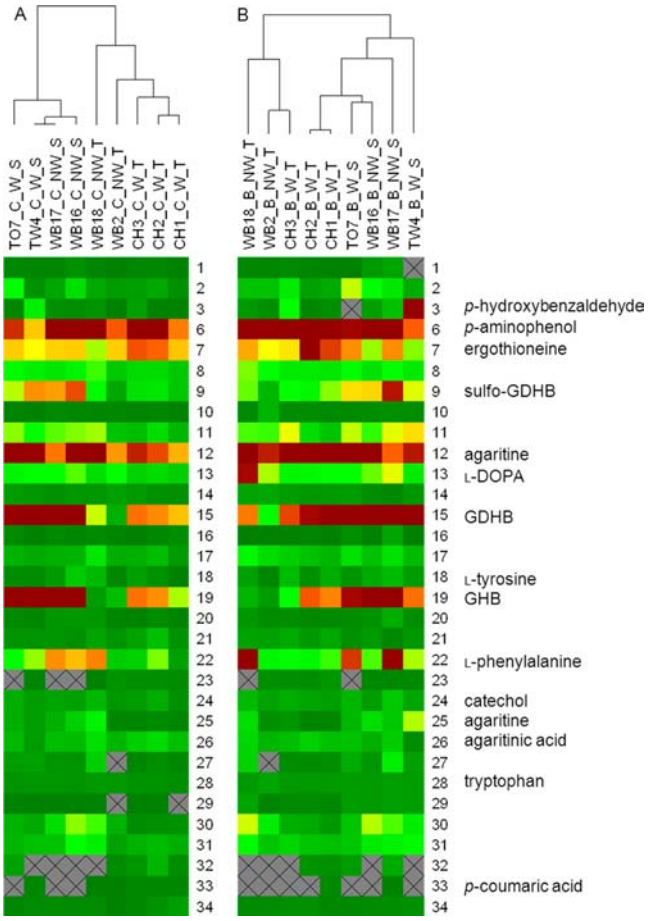


**Figure 5.** Heat map analysis of nonbruised (A) and bruised (B) skin tissue samples. Results are based on peak area per gram FW. The lowest peak area is shown in dark green and the highest peak area in dark red. A gray square with a cross indicates peaks that could not be integrated. C, nonbruised skin tissue; B, bruised skin tissue; W, white strain; NW, brown (nonwhite) strain; T, tolerant; S, sensitive.

(**9**) was 2.5 times higher in the sensitive white strains than in the tolerant white strains, whereas GDHB and GHB were 3 and 2.5 times higher, respectively (Figure 6). For the brown strains, an even more pronounced difference was found between sensitive and tolerant strains for sulfo-GDHB, GDHB, and GHB: 5, 15, and 20 times, respectively (Figure 6). For both the white and brown strains a significant difference was found between the tolerant and sensitive strains for the sum of the three phenolics (Figure 6). The difference between bruising-sensitive and -tolerant strains could, therefore, be related to the presence of phenolic compounds in the skin before bruising, in particular, sulfo-GDHB, GDHB, and GHB. These compounds generally comprise between 36 and 52% of the total peak area for the white and brown sensitive strains and, therefore, are likely to have a major impact on melanin formation. The very tolerant strain WB2 had an extremely low content (<10%) of these three compounds.

**Difference between Bruising-Sensitive and -Tolerant Strains after Bruising.** Also, the bruised skin tissues of the nine strains were analyzed (Figure 5B). Unsupervised clustering of the bruised samples resulted in two main clusters, but these were not based on the bruising sensitivity as was found for the nonbruised skin tissue. The tolerant commercial hybrid 1 and 2 strains were more closely related to the sensitive strains than to

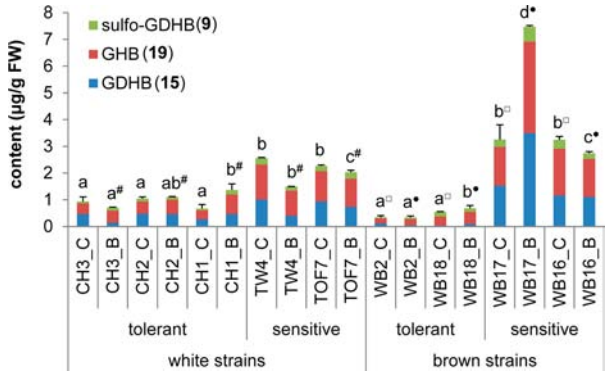


**Figure 6.** Contents (µg/g FW as equivalents of GHB) of GHB, GDHB, and sulfo-GDHB in sensitive and tolerant *A. bisporus* strains. The data are shown of one extract, which was analyzed in duplicate. The standard deviation was calculated on the basis of the sum of GHB, GHDB, and sulfo-GDHB. Tukey was calculated on the basis of the sum of the three compounds, and for the control and bruised white and off-white strains separately, and for the control and bruised brown strains separately, which are indicated by $^{\#}$, $^{\square}$, $^{\bullet}$, respectively.

the other tolerant strains. After bruising, GHB, GDHB, and sulfo-GDHB were higher in the sensitive strains than in the tolerant strains, similar to our observations with the nonbruised samples (Figure 6). A significant difference was found between the tolerant and sensitive brown strains for the sum of the three phenolics (Figure 6). Again, it is striking that strain WB2 still has very low concentrations of GHB, GDHB, and sulfo-GDHB.

**Dynamic Flux of Phenolics during Bruising.** On the basis of this study, it is tempting to speculate which phenolic conversions occur in response to bruising. Bruising-induced discoloration is likely caused by polymerization of phenolics, mediated by enzymes and subsequent autopolymerization into melanins.[30−33] We did not detect any peaks with an absorption at 400 nm (corresponding to brown color) in our LC traces, suggesting that not all phenolics were detected by the method used. It was expected that the total content of phenolics was lower in bruised samples compared to nonbruised tissues. Surprisingly, these levels were found to be similar. This suggests that the phenolic compounds from which melanins are formed are immediately replenished via de novo synthesis, indicating a dynamic flux of compounds from a reservoir of precursors, such as *p*-aminobenzoate (**36**) and chorismate (**35**), which we could not detect with the method used. The observation that *p*-aminophenol (**6**) increased upon bruising in almost all bruised samples (on average 1.3 times) supports this hypothesis. Another explanation for the similar levels of total phenolics in bruised and nonbruised samples might be that only a very small fraction of phenolics is responsible for discoloration, but this would not be in accordance with the increased levels of *p*-aminophenol observed.

**GHB and GDHB as Markers for Bruising Sensitivity.** The main phenolics involved in discoloration by bruising were identified to be GHB (**19**) and GDHB (**15**). Therefore, it can be concluded that the melanin formed upon bruising with the highest impact on discoloration is likely GHB−melanin. The content of sulfo-GDHB, GHB, and GDHB was the highest in the nonbruised and bruised skin tissue of bruising-sensitive strains of both white and brown strains. Particularly in sensitive brown strains, their content could comprise up to ∼50% of the total phenolics content. The concentrations of sulfo-GDHB, GHB, and GDHB in mushroom skin tissue appeared to be


dx.doi.org/10.1021/jf4020558 | J. Agric. Food Chem. 2013, 61, 8224−8231

relevant predictors for bruising sensitivity: the higher their concentrations, the more sensitive the strains. Good correlations between bruising sensitivity in nonbruised skin tissue of white strains and content of sulfo-GDHB, GDHB, and GHB were found with $R^2$ of 0.78, 0.81, and 0.73, respectively. Even better correlations were found for the nonbruised tissue of brown strains: $R^2$ of 0.94, 0.98, and 0.85 for sulfo-GDHB, GDHB, and GHB, respectively. On the basis of these correlations, it can be concluded that these phenolics can be used as markers to predict bruising sensitivity of a strain. Others have found a 7-fold higher phenolic content in the skin (combined quantity of tyrosine, GHB, and GDHB) of a browning-sensitive strain than in a less sensitive strain.[5] This study compared only two strains as in our study several strains were compared and a more complete overview of the available phenolics in the skin tissue is given. In our study L-tyrosine (**18**) did not show a correlation with bruising sensitivity, but GHB and GDHB did show a good correlation.

**Rate-Limiting Step in Tolerant Brown Strains.** GHB and GDHB concentrations in samples correlated mutually, but not with the precursor of GHB, *p*-aminophenol, which was found in high quantities in all strains. This suggests that the conversion of *p*-aminophenol into GHB, and not the GHB to GDHB conversion, is rate-limiting in bruising- tolerant strains. This might be caused by mutations affecting the expression of, for example, γ-glutamyltransferase or mutations altering this enzyme's activity.[1] The very tolerant strains WB2 and WB18 contained less GHB and GDHB than the somewhat less tolerant commercial white strains CH1, CH2, and CH3. Therefore, WB2 and WB18 have a very interesting genetic background for creating offspring with reduced GHB−melanin formation. Future research should therefore concentrate on introducing this genetic background in white commercial strains.

## ■ ASSOCIATED CONTENT

### Supporting Information

Standard compounds used for UHPLC-PDA-MS analysis (Table S1), peak area of phenolic compounds (Table S2), and reaction scheme of GHB synthesis (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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### Funding

This project is supported by the governmental grant TTI Green Genetics, Dutch Board for Horticulture, Bromyc, Lutèce, Sylvan, Banken Mushrooms, the Greenery and WeBe Engineering. J.C.S. performed the synthesis of GHB and is funded by The Netherlands Organization of Scientific Research (NWO) ECHO Grant 700.57.014.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

Mark Sanders (Laboratory of Food Chemistry, Wageningen University) is thanked for his help with the UHPLC-PDA-MS setup. The similarity matrix was made by Patrick Hendrickx (Plant Research International).

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