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Effect of Gamma Irradiation on Selected Compounds of Fresh Mushrooms

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Abstract

Fresh mushrooms of the common cultivated species *Agaricus bisporus* were gamma-irradiated with doses of 1, 3, and 5 kGy, and freeze-dried afterwards. Total phenolic content and antioxidant activity were determined by Folin-Ciocalteu (FC) Test and Trolox equivalent antioxidative capacity (TEAC) assay, respectively. Analysis of selected, individual compounds was carried out using HPLC-mass spectrometry and HPLC-UV. Total phenolic content and antioxidant activity were not affected by gamma irradiation. The contents of agaritine and γ-L-glutaminyl-4-hydroxybenzene (GHB) were both decreased; those of agaritine at 5 kGy, from 1.54 g/kg (0 kGy) to 1.35 g/kg (5 kGy), and those of GHB already at 1 kGy by 22%. The amounts of phenylalanine and tyrosine did not show any changes. The results of nucleotides were inconsistent as a concentration decrease was only observed for adenosine 5′-monophosphate (AMP) and guanosine diphosphate (GDP). Gamma irradiation does not seem to greatly affect the contents of compounds in mushrooms, provided that enzyme activities after a radiation treatment are avoided.

Abstract (German)

Frische Zuchtchampignons (*Agaricus bisporus*) wurden mit Dosen von 1, 3, und 5 kGy bestrahlt und danach gefriergetrocknet. Der Gesamtphenolgehalt und die antioxidative Aktivität wurden mittels Folin-Ciocalteu (FC)- und Trolox equivalent antioxidative capacity (TEAC) Test bestimmt. Die Analyse der einzelnen Inhaltsstoffe wurde anhand der HPLC- Massenspektrometrie und der HPLC-UV durchgeführt. Der Gesamtphenolgehalt und die antioxidative Aktivität wurden von der Gamma-Strahlung nicht beeinflusst. Die Mengen an Agaritin und γ-L-Glutaminyl-4-Hydroxybenzen (GHB) nahmen ab; Agaritin bei einer Dosis von 5 kGy von 1.54 g/kg (0 kGy) bis 1.35 g/kg, GHB bereits bei 1 kGy um 22%. Die Konzentrationen von Phenylalanin und Tyrosin wurden nicht verändert. Die Ergebnisse der Nukleotide waren widersprüchlich, eine Abnahme der Menge wurde nur bei Adenosin 5′-Monophosphat (AMP) und Guanosin Diphosphat (GDP) beobachtet. Der Einfluss von Gamma-Strahlung auf die Konzentration der Inhaltsstoffe in Champignons dürfte vernachlässigbar sein, vorausgesetzt, die Enzymaktivitäten nach einer Strahlenbehandlung werden verhindert.

Contents

Chapter 1

Introduction and Objectives

The common cultivated white button mushrooms, *Agaricus bisporus*, have been part of the human diet for many years. Amounts consumed are increasing steadily, involving a worldwide growing production (Gautam et al., 1998; Mattila et al., 2001). However, the high perishable nature of mushrooms remains a problem for the progress of this industry (Beaulieu et al., 1992; Gautam et al., 1998). In fact, fresh mushrooms can only be stored for a few days until they lose freshness and quality. This quality loss is recognized by colour 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 from *Pseudomonas tolaasii* or molds. Frequent methods to extend the shelf-life of fresh mushrooms are low temperature storage, controlled-atmosphere packaging, and chemical treatments (Beaulieu et al., 1992; Benoît et al., 2000; Sapers et al., 2001). These common preservation methods revealed to be moderately successful, therefore, the use of ionizing radiation as additional/alternative process for shelf life extension had been intensively investigated.

International agencies including IAEA, FAO and WHO concluded that irradiation of any food commodity up to a dose of 10 kGy exhibits no health risks (WHO report, 1981; Diehl, 2002). Today, irradiation of "fresh fruits and vegetables" is approved in many countries, whereby in general no detailed information about the food product is given. "Mushrooms" are specifically quoted in five countries: Belgium, United Kingdom, Poland, Argentina and the Republic of Korea. The accepted dose range is 1 to 3 kGy (European Parliament and Council, 2006; IAEA, 2008).

Several reports about the preservative effect of low-dose gamma irradiation on mushrooms (*Agaricus bisporus*) exist. A dose of 2 kGy, applied in combination with a storage temperature of 10-15°C, was found to reduce microbial counts at the beginning

and throughout the storage of mushrooms. As a consequence, the shelf life was extended from 2 to 10 days (Gautam et al., 1998; Beaulieu et al., 1992). In addition, low dose irradiation (0.1-0.3 kGy) exerted a stimulatory effect on the growth of mushrooms, obtaining an increased yield (Roy et al., 2000).

Gamma irradiation has been shown to successfully maintain the quality of mushrooms by inhibiting cap opening, stipe elongation and weight loss, and by reducing polyphenol oxidase (PPO) activity (Wahid and Kovács, 1980; Roy and Bahl, 1984; Beaulieu et al., 1992; Lescano 1994; Gautam et al., 1998; Beaulieu et al., 1999; Benoît et al., 2000; Beaulieu et al., 2002). Concerning PPO, Sun et al., 2002, observed a decrease of its activity by 52% at a dose of 1 kGy. Irradiations above 5 kGy even led to a 93% reduction of the PPO activity.

Also adverse effects on the quality of mushrooms have been reported. Mau and Hwang, 1997, focused their investigations on volatile flavour compounds in fresh mushrooms. Whereas some aromatic compounds were not affected by irradiation, the amounts of eight-carbon compounds, like the major component 1-octen-3-ol, decreased significantly with increasing dose.

Extensive studies are available in relation to the browning process. Benoît et al., 2000 and Beaulieu et al., 2002, investigated the effect of dose rate (4.5 kGy/h and 32 kGy/h) and storage time on quality and browning of mushrooms *Agaricus bisporus*. They observed changes of some biochemical parameters including phenylalanine ammonia-lyase (PAL) activity, the key enzyme for the synthesis of phenols, PPO content, respiration, and whiteness. Irradiation significantly enhanced PAL activity and consequently total phenols content, especially between days 1 and 4, whereas PPO activity was lowered. The reduced respiration rate, indicating a lower metabolic activity, was linked to the delay of browning. On the basis of colouration, a dose of 2 kGy extended mushroom shelf life by 4 days with the lower dose rate and only 2 days with the higher dose rate.

Apart from the flavour compounds (Mau and Hwang, 1997) research has predominately focused on quality parameters and enzymatic changes in the browning process so far, thus the effect of gamma irradiation on individual compounds in mushrooms is widely unclear.

The main aromatic compounds of mushrooms are agaritine and its derivates, which belong to the chemical class of hydrazines. Hydrazines are natural products to be found

in considerable amounts in a number of mushroom species including *Agaricus bisporus*. Agaritine plays a key role in the fungal nitrogen metabolism due to the central position of its glutaminyl residue (Espin et al., 1999). The browning substrates accounting for melanogenesis in fungi are especially GHB (β-glutaminyl-4-hydroxybenzene) and tyrosine as well as their oxidative and/or hydrolysis product. GHB and its derivates occur only in *Agaricus* species, being contained there in substantial amounts (Jolivet et al., 1998). Phenylalanine also belongs to the group of melanogenous substrates as it constitutes the basic substance of phenols synthesis (Benoît et al., 2000).

The typical flavour of mushrooms is the sum of several small water-soluble substances, including 5′-nucleotides, free amino acids and soluble carbohydrates. The mixture of monosodium glutamate and 5′-nucleotides forms the basis of the EUC (equivalent umami concentrations) value, which is used to calculate the tasty properties of mushrooms (Tsai et al., 2007).

The aim of these investigations was mainly directed to the effect of gamma-irradiation on the individual compound agaritine, the melanogenous phenolics, and 5′-nucleotide flavour compounds. In addition, the antioxidant activity was assessed by measuring total phenols (FC) and the antioxidative capacity (TEAC). Since the content of mushroom components may vary considerably, because their concentration is highly affected by substrate cultivation, fruiting conditions, developmental stage and age of the fresh mushroom sample (Mattila, 2001), mushrooms from the same cultivation and harvest were purchased to provide exactly equal conditions for all chemical analyses. All samples were immediately freeze-dried after irradiation, together with the reference sample, and stored until analysis.

Figure 1.1: Mushrooms

(Source: http://www.worldcommunitycookbook.org/season/guide/photos/mushrooms.jpg

Chapter 2

Background

2.1 Basic Principles of Radiation Chemistry

Radiation chemistry addresses chemical effects generated in matter by the absorption of ionizing radiation. It is claimed to have had its beginning with the discovery of X-rays by Roentgen in 1895 and of radioactivity by Bequerel one year later (Spinks and Woods, 1990: 1-3).

2.1.1 Parameters of Radiation Chemistry

2.1.1.1 Radiation-Chemical Yield

Radiation chemistry is interested in measuring the chemical alterations induced by high energy particles or photons which deliver energy onto the irradiated material. The extent of the chemical effect is expressed by the radiation chemical yield, the so-called G-value. The G-value of a reaction refers to the number of specified chemical events in an irradiated substance produced per 100 eV of energy absorbed from ionizing radiation (Henglein et al., 1976: 20).

$$G = 100 \times \frac{Number\ of\ produced/\ degraded\ molecules}{Absorbed\ radiation\ energy \quad [eV]} \tag{2.1}$$

Since, generally, three ion pairs and approximately the same or double amount of excited molecules are produced per 100 eV of absorbed radiation energy, the G-value is usually less than 10 (Henglein et al., 1969: 20).

The G-value has the SI (Système International) units of μmol J^{-1}, whereas 1 molecule per 100 eV equates to 0.1036 μmol J^{-1} (Mincher and Curry, 2000; Spinks and Woods, 1990: 8).

2.1.1.2 Units of Radioactivity and Radiation Dose

2.1.1.2.1 Activity of an Isotope

The activity of an isotope does not remain constant; it decreases as the isotope ages. This relation is given by

$$A = A_0 \times e^{-kt} \tag{2.2}$$

where A_0 is the activity at time t = 0. The decay constant (k) is correlated to the half time $\tau_{1/2}$ by the expression $k = \ln 2/\tau_{1/2}$. The half time of cobalt-60, the most widely used gamma-radiation source, is 5.26 years. The unit of radioactivity is Becquerel (Bq); a Becquerel is 1 disintegration per second. The former unit was curie (Ci) (1 curie = 37 gigabecquerel) (European Nuclear Society, 2008).

2.1.1.2.2 Radiation Dose

The dose determines the amount of energy carried from the radiation source to the absorbing material. There are three subtypes indicating that term more precisely.

- Ion dose
- Absorbed dose, absorbed dose rate
- Dose equivalent

The ion dose is a measuring variable of the radiation on the basis of its ability to generate ionization in air. The unit of the ion dose is 1 Coulomb (C) kg^{-1}. The former unit was roentgen (1 roentgen = 2.58×10^{-4} C kg^{-1} (Spinks and Woods, 1990: 74).

The absorbed dose is the amount of energy absorbed per unit mass of irradiated material. It is expressed by the SI unit Gray, which is ascertained as energy absorption of 1 J kg^{-1}. The former unit name was rad (1 Gray = 100 rad) (Spinks and Woods, 1990: 72).

The absorbed dose rate is defined as the absorbed dose rate per unit time. The unit of the absorbed dose rate is Gray per unit time, e.g., 1 Gray s^{-1} (100 rad s^{-1}) (Spinks and Woods, 1990: 72).

The dose equivalent is defined for routine radiation-protection applications. It is the product of the absorbed dose in tissue multiplied by a "radiation weighting factor" (RW) or quality factor (Q) to account for the dependence of the harmful biological effects on the type of radiation being absorbed. It is expressed in Sievert, 1 Sv = 1 J kg^{-1} × radiation weighting factor, or traditionally in rem. One Sievert is equal to 100 rem (Health

physics society, 2008). Concerning X- and gamma radiation, beta radiation, electrons and positrons, Q is 1 (European Nuclear Society, 2008).

2.2 Absorption of High-Energy Radiation

High-energy rays that are frequently applied in radiation chemistry include high- frequent electromagnetic waves, namely γ- and X rays, further charged particles of high velocity such as electrons, protons, deuterons, α- particles and uncharged neutrons. The energies of these rays easily exceed the energies required for ionization of a single atom or molecule. Since their absorption induces the production of many ions amongst other things, they are named ionizing rays (Henglein et al., 1969: 4).

Due to the wave-particle dualisms, electromagnetic waves may be interpreted as particles named "photons" or "quants". A photon moves with speed of light c, possessing the energy E = h $\times$ ν, E = h $\times$ c/λ, whereas ν expresses frequency, λ the wavelength of radiation and h, the Planck constant [h = 4.136×10^{-15}eV sec]. Therefore applies:

$$E = 1,24 \times 10^3/\lambda \ \ [nm] \tag{2.3}$$

and correlations between E and λ can be calculated (Henglein et al., 1969: 4).

Table 2.1: Energy ranges

Type of radiation	**Wavelength [nm]**	**Energy [eV]**
Visible light	780-400	1.6-3.1
UV	400-100	3.1-12.4
X-,γ-rays	<10-10^{-4}	10^4-10^7

(Henglein et al., 1969)

2.2.1 Absorption of X- and γ-Radiation

Absorption of X- and γ-rays follows an exponential regularity. These rays are prone to release the greater part of their energy through a single collision, which means they are completely absorbed by a material layer but the remainder pass through with their entire energy. The number of absorbed photons (N) in a thin material layer of thickness x [m or cm] is directly proportional to the number of incident photons (N_0),

$$N = N_0 \, e^{-\mu x} \tag{2.4}$$

where μ is the linear attenuation coefficient [cm^{-1} or m^{-1}] (Spinks and Woods, 1990: 23). μ depends on material characteristics and on the energy hν of the photons. This dependency is described by three different elementary processes:

- Photoelectric Effect
- Compton Scattering
- Pair Production

The Photoelectric Effect: In this type of interaction a photon transfers its entire energy hν to a single electron, thereby stimulating it with its kinetic energy E_k.

$$E_k = h\nu - E_B \tag{2.5}$$

E_B is the binding energy of the electron in the atomic nucleus. It increases with a high atomic number (Henglein et al., 1969: 50).

The Compton Scattering: In the Compton Effect a photon collides with an electron so that the electron is set in motion. The photon does not vanish; it is deflected with reduced energy hν'. The energy of the Compton-electron is given by (Henglein et al., 1969: 52-53):

$$E_k = h\nu - h\nu' - E_B \tag{2.6}$$

The photon and the electron share the energy of the incident photon. The Compton process prevails for a wide range of energy in low atomic number materials. In water, Compton scattering prevails from 30 keV to 20 MeV (Spinks and Woods, 1990: 42-44).

The Pair Production: This process occurs at photon energies above 1.02 MeV. At this, a photon is completely altered into two particles, an electron and a positron, in the vicinity of an atomic nucleus. According to the Einstein relation $E = mc^2$ between energy and mass, the rest mass of the electron corresponds to 0.51 MeV. The $2 \times 0.51 = 1.02$ MeV exceeding energy of the photon occurs as kinetic energy of the electron and the positron

$$E_{k(e^- + e^-)} = h\nu - 2\, m_0 c^2. \tag{2.7}$$

Both particles will be stopped in matter, in which they initiate chemical reactions through ionization and dissociation. At the end the positron combines with an electron, the two particles vanish and an annihilation radiation is emitted by two photons of 0.51 MeV each (Henglein et al., 1969: 54-55).

The Half-thickness value: Due to the fact that γ-rays do not possess a definite range in matter, the half-thickness value is applied. The Half-thickness value is the thickness

of a material that is needed to reduce the number of incident photons by one half (Spinks and Woods, 1990: 23-24).

2.2.2 Absorption of Charged Particles

High speed charged particles passing a medium interact with the molecules, lose their energy, and are gradually stopped. This process is described by the specific energy loss ($\frac{-dE}{dx}$), which indicates the reduction of the kinetic energy E of a charged particle per unit path length dx. The specific energy loss is also termed stopping power of the medium or linear energy transfer (LET). It may be splitted into three parts:

$$\frac{-dE}{dx} = (\frac{-dE}{dx})_I + (\frac{-dE}{dx})_K + (\frac{-dE}{dx})_S \tag{2.8}$$

The first expression of the equation is the most important one. It describes the energy loss per unit path length through ionization and excitation of molecules, e.g. through interaction of charged particles with bound electrons. The second term describes the specific energy loss due to elastic collisions with atomic nuclei. This part accounts for less than 1% of the first term. The third element implies the specific energy loss through electromagnetic radiation that is produced by the deceleration of high energy electrons (>10 -100 MeV) particularly through particles of high atomic numbers (Bremsstrahlung) (Henglein et al., 1976: 64-65).

The specific energy loss by ionization and excitation is given by the Bethe-equation:

$$(\frac{-dE}{dx})_I = \frac{2\pi e^4 z^2 M}{Em} \; N_{e,c} \; ln \; \frac{4Em}{I_m M} \tag{2.9}$$

E	...	particle energy
x	...	distance travelled by the particle
e	...	charge on the particles
z	...	number of charges on the particle
M	...	particle mass
m	...	electron mass
$M_{e,c}$	...	number of electrons per cubic centimetre
I_m	...	mean excitation energy of all electrons in the absorbing material

According to Bloch the mean excitation potential rises with increasing atomic number

Z, thus existing a linear relationship between I_m and Z,

$$I_m = K \times Z \tag{2.10}$$

where K is a constant of 8.8 eV for heavy elements. For light elements, a mean K value of 11.5 is used (Henglein et al., 1969: 65-66).

Material characteristics: The electron density $N_{e,c}$ and the mean excitation energy I_m occur as material characteristics. Since changes in I_m have little effect on the stopping power of a material, it is basically depending on the number of electrons per cubic centimetre in the material (Henglein et al., 1969: 67).

Particle characteristics: The decisive term of the Bethe-equation is 1/E, and $1/v^2$ respectively. Thus, the specific energy loss declines with increasing velocity and energy of a particle. Particles of higher charge z and mass M will be stopped more intensively than a particle with the same kinetic energy and low z and M (Henglein et al., 1969: 69).

Range of high energy particles: The length of path or true range of a particle (R) is given by the integral of the reciprocal specific energy loss inside of E_0 and 0, whereas E_0 expresses the initial energy of the particle (Henglein et al., 1969: 70-71):

$$R = \int_0^{E_0} \frac{dE}{(\frac{-dE}{dx})} \tag{2.11}$$

2.3 Water Radiolysis

The radiation of water and aqueous solutions leads to the decomposition of water and to the production of reactive radical species. In aqueous solutions, the solute is “indirectly” affected by radiation, implying that the radiation exclusively interacts with the solvent (in many times water) and the solute is altered by produced reactive species (Spinks and Woods, 1990: 243-244).

The major reactions in water radiolysis are:

$$H_2O + h\nu \rightarrow H_2O^{\bullet +} + e^-_{aq} \tag{2.12}$$

$$H_2O^* + h\nu \rightarrow H^\bullet + OH^\bullet \tag{2.13}$$

The ionisation of the water molecule is described in the first equation. Naturally, the electron is not hydrated. The hydration or salvation process occurs as soon as the electron

has slowed down to thermal energy (about 10^{-12} sec after radiation absorption). Imaginably, $e^-_{\ aq}$ is an electron surrounded by shells of water molecules. The excited water molecule in the second equation dissociates into H^- and OH^- radicals. These primary radicals are cumulatively produced in so-called spurs being located close together. However, they easily escape from the spurs and diffuse to the dissolved molecules of the solution. The $H_2O^{\bullet+}$- ions do not participate in reactions; they are converted with adjacent water molecules directly after production (Henglein et al., 1969:132-133):

$$H_2O^{\bullet+} + H_2O \rightarrow H_30^+ + OH^{\bullet} \tag{2.14}$$

Before diffusion, radicals interact and generate molecular products:

$$H_30^+ + e^-_{aq} \rightarrow H^{\bullet} + H_2O \tag{2.15}$$
$$H^{\bullet} + OH^{\bullet} \rightarrow H_2O \tag{2.16}$$
$$H^{\bullet} + H^{\bullet} \rightarrow H_2 \tag{2.17}$$
$$OH^{\bullet} + OH^{\bullet} \rightarrow H_20_2 \tag{2.18}$$
$$e^-_{aq} + OH^{\bullet} \rightarrow OH^- \tag{2.19}$$
$$e^-_{aq} + e^-_{aq} \rightarrow H_2 + 2OH^- \tag{2.20}$$
$$e^-_{aq} + H^{\bullet} \rightarrow H_2 \tag{2.21}$$

Some radicals and molecular products escape the spurs, diffuse away and disperse homogeneously. Homogenous distribution is achieved at 10^{-7} seconds after radiation absorption, and the radicals/molecular products present at that time are called “primary products” of water radiolysis (Spinks and Woods, 1990: 256-257):

$$H_2O + h\nu \rightarrow H_2, H_2O_2, e^-_{aq}, H^{\bullet}, OH^{\bullet}, H_30^+, OH^- \tag{2.22}$$

The radical and molecular yields (μmol J^{-1}) in irradiated water at pH 3-11 are the following (Spinks and Woods, 1990: 260):

$$G(e^-_{aq}) = 0,28$$
$$G(H^{\bullet}) = 0,062$$
$$G(OH^{\bullet}) = 0,28$$
$$G(H_2O_2) = 0,073$$
$$G(H_2) = 0,047$$

The presence of air (oxygen) advances the disintegration of water. Oxygen is easily at-

tacked by hydrated electrons and hydrogen atoms (Henglein et al., 1969: 154):

$$e^-_{aq} + 0_2 \rightarrow 0^{\bullet -}_2 \tag{2.23}$$

$$H^\bullet + O_2 \rightarrow HO^\bullet_2 \tag{2.24}$$

$$HO^\bullet_2 \rightarrow H^+ + 0^{\bullet -}_2 \tag{2.25}$$

The hydroxyl radical is the main oxidizing radical in aqueous solutions. Both the peroxylradical and its anion, the superoxide ion, are moderately oxidizing (Henglein et al., 1969: 154).

2.4 Effects of Radiation in Solids

Exposure of solutions to energy-rich radiation leads to the production of free radicals, electrons and ions. These species exist only for a split second, until they diffuse together and deactivate each other. By contrast, the intermediate products in materials of high viscosity such as glasses or crystalline solids have considerably longer life span due to their limited mobility. Glass and many other solids become coloured when exposed to radiation. This colouration is caused by electrons in traps and can be observed many days after radiation, although being bleached by the action of light and heat (Henglein et al., 1969: 263).

2.4.1 Types of Traps in Crystals

There are two types of traps breaking into the regular arrangement of crystals - the Frenkel- and the Schottky defect. In the Frenkel defect, one ion is in a metastable location between normal lattice sites that is called an interstitial position. Besides, some lattice sites are unoccupied, being named vacancies. In case a positive ion is sliding into an interstitial position, a cation vacancy is generated. In the Schottky defect, the same amount of anion and cation vacancies exists. Vacancies differ from “holes”. Holes result from the ejection of electrons from some atoms in the crystal. In case the electron is removed from a negative ion, a positive hole remains, accordingly, a negative hole from a positive ion (Henglein et al., 1969: 264).

Traps are able to migrate within the crystal: e.g. an interstitial ion can move to an adjacent interstitial position. A vacancy moves by filling its free space with a neighboured ion, whereas a new vacancy evolves at the original place of the ion. Furthermore, a positive hole migrates as a negative ion delivers its electron to an adjacent ion and itself becomes a positive hole. Migrations afford energy (Henglein et al., 1969: 264).

2.4.2 Irradiation of Crystals

Irradiation results in delivering of electrons from the crystal lattice. As most of the electrons will reunite with their mother ion, some will be caught in traps, in an anion vacancy. This forms an F-centre, a so-called colour centre absorbing in the visible range. The loss of the electron makes the positive holes migrate to a cation vacancy constituting a V-centre. V-centres are detected in the UV-range. Heating of the irradiated crystal leads to the discharge of the electrons from the traps and their recombination with the mother ion. The F- and V-centres vanish (Spinks and Woods, 1990: 455-456).

The same mechanism is observed by irradiated glass. However, the greater complexity of glass enables the production of more colour centres. Therefore, irradiation of glass results in overlapping absorption bands and not in discrete bands as it does in crystals (Spinks and Woods, 1990: 457-458).

2.5 Radiation Preservation of Foods

Food irradiation is a process by which food is exposed to a controlled source of ionizing radiation in order to extend shelf life and diminish food losses, enhance microbiological safety, and/or replace the application of chemical fumigants and additives (Shea, 2000).

The effect of irradiation on food is depending on the dose of the ionizing radiation. However, the dose absorbed by the food differs from the amount of energy delivered from the radiation source. Intensity of the radiation source and time of exposure determine the absorbed dose (Morehouse, 1998).

The mechanism behind the preservative effect of ionizing radiation is basically on account of DNA damaging, directly or indirectly by oxidative radicals. As a consequence, living cells become inactivated and microorganisms, insect gametes, and plant meristems cannot reproduce. Differences in the sensitivity of microbes are explained by variations in their chemical and physical structure (Farkas, 2006). Complex organisms are more prone to radiation damage than organisms with a simple structure. Therefore, viruses are unlikely affected by irradiation, and spores and cytes comprising little DNA are rather resistant (Shea, 2000). Other factors influencing the radiation process include temperature, moisture content, composition of the medium, presence or absence of oxygen and fresh or frozen state (Farkas, 2006).

Although irradiation is able to inactivate microorganisms, it does not substitute adequate food production, handling or preparation. With the applied radiation doses ($\leq$ 10 kGy), it is impossible to destroy all pathogens. As mentioned before microbes differ in their sensitivity which means they require different treatments. Food irradiation is neither a method to increase food quality nor can it avoid contamination after irradiation (ADA, 2000).

Reliable numbers of the real amount of irradiated foods worldwide do not exist. In 2006, according to IAEA estimations, about 500 000 tons of over 60 kinds of foodstuffs were irradiated for preservation purposes in over 60 countries.

2.5.1 Legislation

2.5.1.1 EU

The European Parliament and Council have passed two directives, 1999/2/EG and 1999/3/EG, on the approximation of laws regarding ionizing radiation of foods and food ingredients. Their main purpose was to suppress the differences of state regulatory inhibiting free movement of foods within the European Union.

The framework directive 1999/2/EG contains the admission requirements for irradiation of foods and food ingredients, the types of radiation sources, labelling of irradiated foods, and the maximal radiation dose.

The admission of food irradiation is only acceptable if the process

- is technologically reasonable and necessary;
- presents no health threat and is conducted under the recommended conditions;
- benefits the consumer;
- is not used as a replacement for hygienic and health measurements or for good manufacturing and agricultural practices.

Furthermore, food irradiation may only be geared to the following purposes:

- Reduction of pathogens in foods by disturbing them;
- Reduction of food spoilage due to delay or stoppage of spoilage processes and destruction of spoilage promoting organisms;

- Reduction of food losses through early maturation, pullulation or germination;
- Liberation of foods from plant pests.

The authorized ionizing radiation sources include γ-rays from radionuclide ^{60}Co and ^{137}Cs, X-rays from machine source with energies up to 5 MeV, and electrons from energy source up to 10 MeV.

The implementing directive 1999/3/EG sets the establishment of a community list of irradiated foods and food ingredients. It includes a list of foods allowed to be treated with ionizing radiation and their maximum doses. So far, dried aromatic herbs, spices and vegetable seasonings with a maximum absorbed dose of 10 kGy are found on the list. Additionally, each EU member state has its own national permissions for irradiation of other products. For example, Belgium allows the irradiation of poultry, frozen shrimps, strawberries, fish and many more products. These national admissions can be maintained until the positive list of the 1999/3/EG directive is expanded (Diehl, 2002).

2.5.1.2 Austria

In Austria, the EU directives were implemented into national law in 2000. Currently, the radiation legislation, BGB1.II Nr. 327, only allows the irradiation of dried aromatic herbs, spices and vegetable seasonings at a maximum absorbed dose of 10 kGy.

2.5.2 Applications

Food irradiation has a wide range of potential uses. The fact that radiation does not initiate temperature rise in the product and application through packaging materials is also possible gives an advantage over heat treatment (Farkas, 2006).

Irradiation is most useful in four areas:

- Control of sprouting
- Control of insects
- Pasteurization
- Sterilization

Control of sprouting: sprouting of potatoes, garlic and onions leads to rapid quality loss on stored products. The prevalent use of chemical agents could be replaced by a

radiation dose of 100 Gy. The reason for its non-application in a broader scale is the high cost (Ehlermann and Delincée, 1998).

Control of insects: high amounts of stored food products deteriorate due to insect damage. The killing of eggs, larvae and full-grown insects is already achieved by a dose below 1 kGy. Additional doses can inhibit reproduction. Concerning insect control irradiation is widely used as a substitute for fumigation, e.g. of coca beans. Irradiation of fruits for quarantine purposes (e.g. to prevent spreading of fruit flies) may facilitate fruit trade worldwide (Ehlermann and Delincée, 1998).

Pasteurization: radiation doses ranging from 1 to 10 kGy induce a partial killing of microorganisms comparable to heat pasteurization. Thus, a definite shelf life extension of irradiated foods is achieved. Dried herbs and spices are usually highly contaminated with numerous pathogens. Although they do not deteriorate themselves due to their little water content, they are able to infect other foods such as soups or salads when seasoning. Since application of ethylenoxid poses a health threat and is prohibited in most countries, presently decontamination of herbs and spices is the most frequent use of food irradiation. Poultry and egg products, meat and other food items frequently contain pathogens such as salmonella, campylobacter, listeria and escherichia coli which cause food poisoning. Radiation is an effective treatment against these microbes. Shelf life of fruits can also be extended. It is therefore possible to transport subtropical fruits by sea which is cheaper than by air. Another benefit is that harvesting can be done when fruits are ripe and have developed its full flavour (Ehlermann and Delincée, 1998).

Sterilization: sterilization is the killing of all microorganisms in food requiring a radiation dose of 30 kGy or more. The high dosage causes changes in flavour, odours and texture in most foods. As a consequence, sterilization by radiation in the food area is only used for sterile foods in hospitals or for space travel. Sterile food in hospitals is treated with 75 kGy. Radiation sterilization is widely used in other fields, e.g. medicine where heat-sensitive products such as syringes, canulas or artificial joint are sterilized (Ehlermann and Delincée, 1998).

Table 2.3 shows selected samples of irradiated foods and the effect of ionizing radiation.

Table 2.3: Area of application, products and dose ranges

Effect	**Dose in kGy**	**Products**
	Low dose up to 1kGy	
Control of sprouting	0.05-0.15	Potatoes, onions, garlic, ginger, yams
Control of insects and parasites	0.15-0.75	Grains, legumes, fresh and dried fruits, dried fish, raw fish, dried meat, pork, ham
Delay of physiological processes	0.25-1	Fresh fruits and vegetables (bananas, champignons,..)
	Medium dose 1-10kGy	
Extension of shelf-life	1-3	Fresh fish, strawberries
Reduction of spoilage and pathogenic microorganisms	1-10	Fresh and frozen seafood, raw and frozen poultry and meat,egg products, camembert, herbs, dried vegetables, thickening agent
Improved technological properties	2-7	Grapes (increased juice yield), dried fruits (improved rehydratation)
	High dose ≥ 10kGy	
Industrial sterilization	30-75	Meat, poultry, seafood, prepared foods, sterile foods
Decontamination of ingredients and additives	10-50	Spices, enzyme preparations

(Ehlermann and Delincée, 1998)

2.5.3 Nutritional Considerations

Irradiation induces energy absorption in foods, initiating chemical reactions. These changes increase with rising radiation doses, but not in a linear way. This is explained by the emerging interactions of the generated intermediates and other food compounds. For example, free radicals will be caught by scavengers at low doses. High radiation doses produce such a high number of radicals that scavenger will be expended soon

and free radicals will steeply increase, leading to an enhanced formation of radiolytic products. Reaction mechanisms in foods are rather complex, since foods are composed of a multitude of chemical compounds. These compounds can partly exert protective effects on each other (Elmadfa et al., 1999).

Chemical changes due to radiation treatment are similar to those occurring from heat treatment or other preservation methods (Morehouse, 1998). Generally at approved dose levels, the major constituents of food (protein, lipid, carbohydrate) are modified insignificantly, as well as minerals or trace elements. Micronutrients, especially vitamins cause the largest nutritional concern in respect of irradiation. Vitamin A, C and E belong to the more radiosensitive vitamins, showing significant losses at high doses of irradiation (Shea, 2000). Reduced levels of Thiamin (vitamin B_1), the most affected vitamin, have been found in meat products (Smith and Pillai, 2004). Irradiation treatment of heat-sensitive vitamins does not result in greater losses than from heat processing. Exclusion from oxygen has been proven to diminish storage loss (Shea, 2000).

2.5.4 Safety

2.5.4.1 Radiologic Safety

Irradiation dose not make the food itself radioactive. The radiation sources approved for use such as γ-, X-rays or electrons contain too low energy to induce nuclear reactions. Therefore, both food and packaging material can only be radioactive due to natural background radiation (Shea, 2000).

2.5.4.2 Toxicologic Safety

Radiolytic products: Numerous investigations have been made to identify radiolytic products and to estimate their health risk. Most of the detected compounds are regarded as being safe; apart form two product groups that have indicated to present a possible health risk. One group are the benzene and its derivates occurring from the irradiation of beef. Since benzenes could only be detected in small numbers, several health institutions considered them as no cause of concern. The other suspicious group of compounds are alkylcyclobutanones (ACBs). ACBs are generated by the irradiation of triglycerides in fat and are termed unique radiolytic products as they cannot be found in raw or heat processed foods. Their genotoxic potential has been studied using different assays. Their controversial results led to the conclusion that genotoxicity of ACBs is not regarded as being established yet (Smith and Pillai, 2004).

Feeding studies and human clinical studies: Several hundreds animal feeding studies have been conducted so far. Lack of experimental design makes about half of them negligible (SCF, 2003). Collected parameters included subchronic and chronic changes in metabolism, histopathology, and function of most systems; reproductive effects; growth; terato-genticity; and mutagenicity (Shea, 2000). Some adverse effects were reported, mostly at radiation doses above 10 kGy. The few conducted human clinical studies did not show any effect (SCF, 2003).

Chemiclearance: This term was used for the toxicological analysis and safety clearance of irradiated foods in 1981. It is based on the awareness that similar composed foods respond to irradiation in a similar way. Therefore, findings of a class of irradiated foods can be extrapolated to other foods of the same class, and unnecessary feeding test of every single irradiated food can be prevented (SCF, 2003).

2.5.4.3 Microbiologic Safety

Microorganisms in food can be divided into three groups. The so-called desirable microorganisms ferment foods and make them tasteful. Spoilage microorganisms deteriorate colour, odour, and texture, creating unpalatable food, but they do not induce human disease. Against it, pathogens such as invasive and toxigenic bacteria, toxigenic moulds, viruses and parasites are the cause for many food-borne illnesses. Viruses, spores, prions, and generated toxins are resistant to radiation. Very high radiation resistance might also be true for bacteria, fungi and moulds requiring further investigations. Regarding bacteria, differences in radiation sensitivities between gram-negative, pathogens and gram-positive ones exist, whereby gram-positive bacteria are the most resistant ones. Food generally contains a much higher number of spoilage microorganisms than pathogens, this proportion remaining after irradiation. Therefore, food spoils probably earlier than becomes pathogenic. A substantial problem present sporulating toxin-producing bacteria, being 10 times more resistant to radiation than nonspore forming ones. The most toxic example is Clostridium botulinum type E, withstanding nonsterilizing doses of radiation. However, other nonsterilizing food processing technologies are confronted with the same problem. Mycotoxins cause concerns as well. Studies give evidence that they even increase when being irradiated (Shea, 2000).

2.5.5 Detection Methods

Scepticism of consumers towards food irradiation still persists in many countries. The main issue consumer organisations are demanding is the right-to-know if a food is

irradiated or not. Since the acceptance in Europe is rather low, the EU has taken a leading position in the establishment of detection methods (Delincée 2002).

The Belfast ADMIT (Analytical Detection Methods for Irradiation Treatment of Foods) meeting has set up a list of criteria for the "ideal" method. It mainly includes the discrimination between irradiated and non-irradiated products, specificity, reliability and other practical criteria such as simplicity, low cost, speed, applicability to a wide range of food types, easy standardization, and cross calibration (Mc Murray et al., 1996).

The different detection methods may be categorized into physical, chemical and biological techniques:

Physical techniques: The two most important techniques are the electron spin resonance (ESR) and the thermoluminiscence (TL), both being standardized methods adopted by the European Committee for Standardization (CEN). The ESR is based on the detection of free radicals. It is a highly sensitive and specific method for food containing bone (meat, fish), cellulose (pistachio nut shells, paprika powder, fresh strawberries) and crystalline sugar (dried figs, dried mangoes, dried papayas and raisins). Since free radicals in soft and wet foodstuffs are very short-lived, ESR can only be applied for dry and solid components. The thermoluminiscence method measures the energy released from trapped charge carriers when heat is applied. In food, the energy is stored in crystalline lattices of silicate minerals. Isolation of silicate minerals and their controlled heating leads to characteristic TL glow curves of irradiated products. TL is a standard technique for the detection of herbs and spices, shellfish including shrimps and prawns, fresh and dehydrated fruits and vegetables, and potatoes. A drawback is the laborious preparation since minerals should be as free of organic material as possible. The mechanism behind photostimulated luminiscence (PSL) is similar to the one of TL, only using optical stimulation instead of heat to release the energy. It is used for screening purposes of shellfish, herbs, spices and seasonings (EN, 1996-2002).

Chemical techniques: Two methods have emerged as being the most advanced methods, both involving fat. The first one is based on the detection of 2-alkylcyclobutanones formed by irradiation of triglycerides. Gas chromatography (GC) is used for separation and mass spectrometry (MS) for detection. A striking advantage is the fact that 2-alkylcyclobutanones do not occur in non-irradiated food. The principle of the second method is rather similar. Radiolytic hydrocarbons are produced during irradiation process of fat, being separated by adsorption chromatography prior to use of gas chromatog-

raphy and detected by mass spectrometry or flame ionization detector. In opposite to 2-alkylcyclobutanones, hydrocarbons are also found in non-irradiated products, but in low concentrations (Delincée, 1998).

Biological techniques: The DEFT (direct epifluorescent filter technique)/ APC (aerobic plate count) assay and the LAL (Limulus Amoebocyte Lysate) test are methods to estimate the ratio of dead and alive microorganisms in foods, offering information of the hygienic status of the food (Delincée, 1998). Among the DNA methods, the Comet assay has emerged to be applicable to a variety of foods. It is based on DNA fragmentation through radiation. This fragmentation is examined by a micro-gel electrophoresis of lysed cells generating a characteristic comet trait (Haire, 1997). However, these mentioned techniques are not radiation specific. They are used for screening purposes of herbs and spices (DEFT/APC), poultry (LAL), and food products of animal and plant origin (DNA comet assay) (EN, 2001-2004).

2.6 Mushrooms (*Agaricus bisporus*)

2.6.1 Nutritional Quality

The species *Agaricus bisporus*, commonly termed mushroom, is the premier cultivated edible mushroom throughout the world. The favourable nutritional and medicinal value is due to its chemical composition. This chemical composition depends on cultivation techniques, maturity at harvest and methods of analysis, and, therefore, can vary significantly (Beelman et al., 2003).

One crucial determinant of the nutritional value is the dry matter/moisture content of mushrooms, ranging from 87.2% to 93.5%. Although a variation of 6.3% seems low, it may account for a 1.97 times higher nutrient content (Kurtzman, 1997). The energy value with approximately 30 kcal/100g is rather low. The carbohydrates as the main constituent of the dry matter are comprised of polysaccharides such as glucans, mono- and disaccharides (such as trehalose), sugar alcohols (such as mannitol), glycogen, and chitin. Chitin, a N-actely-glucosamine polymer of the fungal cell, forms the major component of dietary fibre. It is supposed to exert beneficial effects in humans. Other polysaccharides e.g. β-glucans have been found to exhibit antitumor, antimutagenic, and anticancer activities (Mattila et al., 2002). However, the structure of β-glucans in *Agaricus bisporus* has not been elucidated yet. Alkali extracts of this species indeed have shown antitumor activity (Beelman et al., 2003).

Apart from the favourable fibre content, mushrooms are also good sources of protein in comparison to other vegetables, containing almost all essential amino acid (Mattila et al,. 2002). The crude fat content represents a small part of the mushrooms. Ergosterol, accounting for 15% of the fat, is the precursor of vitamin D_2. Unfortunately, only traces of vitamin D_2 can be found in cultivated mushrooms, since cultivation techniques without sunlight impede the conversion from ergosterol to ergocalciferol (vitamin D_2). The observed hypocholesteremic properties of *Agaricus bisporus* do not originate from ergosterol (Beelman et al., 2003).

Concerning vitamins, mushrooms contain remarkable levels of riboflavin, niacin and folates as well as small amounts of vitamin C and thiamine. The major mineral elements are potassium, phosporus and magnesium with potassium being the most abundant. Low sodium concentrations promote mushrooms as functional foods. They can also be a good source of copper and selenium, the latter depending on compost substrates (Mattila et al., 2001).

The characteristic taste of edible mushrooms is the umami or palatable taste, a general food flavour that is induced or enhanced by monosodium glutamate (Tsai et al., 2007). A series of sensory evaluations attributed the typical flavour by the majority to mannitol, alanine, aspartic and glutamic acids, glycine, threonine, 5′-guanosine monophosphate (5′-GMP), 5′-inosine monophosphate (5′-IMP) and 5′-xanthosine monophosphate (5′-XMP) (Tseng and Mau, 1999).

2.6.2 Health Benefits

As mentioned above antitumor activity and hypocholesteremic properties of mushrooms have been observed. However, not only these two health effects have emerged during investigations. The most well described property of mushrooms is their antioxidant activity which prevents cell damage induced by reactive oxygen species (ROS). Phenolic compounds such as tyrosine, γ-L-glutaminyl-4-hydroxybenzene (GHB) and DOPA have been identified as the main contributors to this effect. Ergothioneine (2-mercapto-L-histidine betaine) and the activities of free radical processing enzymes seem to play an important role in the antioxidative process as well (Dubost et al., 2007; Savoie et al., 2008). The former substance may also exert other functions including retarding of lipid peroxidation, protecting erytrocytes, and exhibiting anti-inflammatory properties (Clemens et al., 2007).

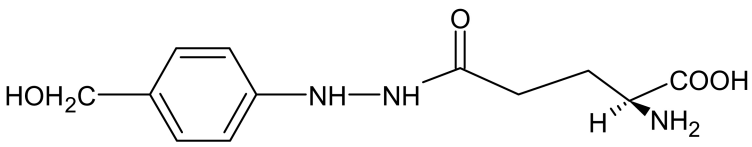
Mushrooms have also been shown to suppress aromatase activity, an enzym ac-

countable for the estrogen biosynthesis. Abnormal expression of aromatase may have a dominant role in tumor proliferation of human breast cancer. The compounds in mushrooms inhibiting the aromatase activity could be the major fatty acids, linoleic acid and conjugated linoleic acid (Chen et al., 2006).

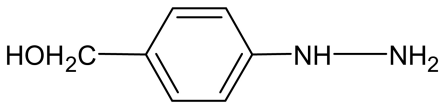
The potential genoprotective effect of mushrooms has been ascertained using the single-cell gel electrophoresis ("Comet") assay. Mushroom water extracts have shown to prevent hydrogen peroxide-induced damage to cellular DNA. The key mechanism behind this effect is enzymatic hydroxylation of tyrosine to L-DOPA and the further oxidation to dopaquinone. The enzym for this process has been identified as the tyrosinase (Shi et al., 2002).

2.6.3 Agaritine and Its Derivates

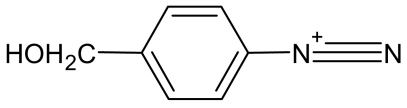
Phenylhydrazines have been found to be abundant in mushrooms. Agaritine [β-N-(γ-L(+)-glutamyl)-4-(hydroxymethyl)phenylhydrazine] is the most prevalent component which can be encountered in relatively high amounts, generally between 200 and 450 mg $kg^{-}1$ fresh weight with considerable variations (Schulzová et al., 2002). It might function as a growth factor in mushrooms (Beelman et al., 2003). The biogenesis of agaritine is traditionally explained via the shikimate-chorismate pathway, but has been revised recently. Agaritine is now assumed to be a product of lignin breakdown induced by fungus, diazotrophic activity of a bacterial commensal and γ-glutamination by a fungus (Baumgartner et al., 1998). Its instability in combination with enzymatic reactions leads to the formation of derivates including 4-(hydroxymethyl)phenylhydrazine (HMPH) and the 4-(hydroxymethyl)benzenediazoniumion (HMBD), both a result of the loss of the γ-glutaminyl group, the reaction being catalyzed by γ-glutaminyltransferase. Other notable hydrazines which have been supposed to be precursors of agaritine are 4-hydrazinobenzoic acid (HBA) and N-(γ-L(+)-glutamyl)-4-(carboxy)phenylhydrazine (GCPH) (Sharman et al., 1990).



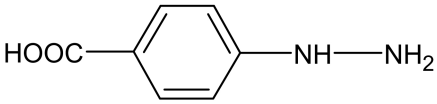
Agaritine



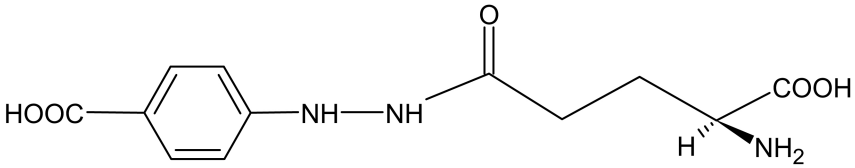
HMPH



HMBD



HBA



GCPH

Cumulative amount of data from animal experiments indicate harmful health effects of mushrooms and phenylhydrazines after chronically oral exposure. Studies conducted with swiss albino mice demonstrated a carcinogenic potency of HMPH, HMBD, HBA, GCPH and the raw mushrooms feeding itself by eliciting tumors in lungs, aorta, blood vessels, skin, subcutis, forestomach, glandular stomach, liver and bone (Toth and Gannett, 1993). Agaritine, previously believed to exert carcinogenic effects, did not induce cancer. It is rather regarded as an indirect toxic component since it is assumed to quickly metabolize to HMPH and HMBD (Hajšlova et al., 2002). Further carcinogenic studies on rats and mice feeding mushrooms itself could not emphasize the aforementioned results. There might also be an influence whether raw, cooked, dried or freeze-dried mushrooms are administered (Pilegaard et al., 1997).

In vitro experiments investigated the mutagenicity of ethanolic and aqueous extracts from mushrooms. The observed mutagenic response was further studied on agaritine and

its derivates, also analyzing different activation systems. The results attribute agaritine no contribution to the mutagenicity of mushroom extracts. This supports the assumption of agaritine being quickly metabolized to mutagenic products. Of HMPH, HMBD, HBA and agaritine, HMBD was shown to be the most mutagenic compound. The activation systems tyrosinase and rat hepatic cytosol seemed to enhance the mutagenicity of HMPH. The activation by tyrosinase indicates a potential role of phenols and quinones in the mutagenic process by operating as genotoxic radical producer. In turn, antioxidant systems such as glutathione and superoxide dismutase suppress the mutagenicity (Walton et al., 1997). Tyrosinase also appears to be able to metabolize agaritine and HMPH, the latter by generating genotoxic products (Walton et al., 2001).

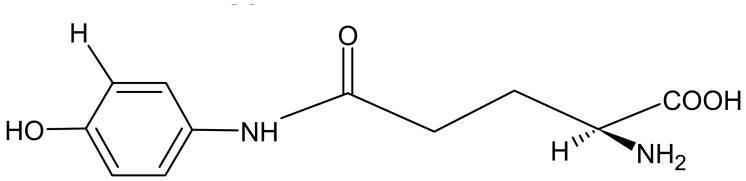
In vivo experiments attribute agaritine a weak genotoxic activitiy. Based on these results, the cumulative lifetime cancer risk of agaritine in mushrooms is calculated to be around 10^{-5} (Shepard and Schlatter, 1998).

Another potential biological function of agaritine and its derivates might be the inhibition of HIV proteases. Docking studies have shown strong binding interaction, especially of the derivates, with the HIV protease (Gao et al., 2007).

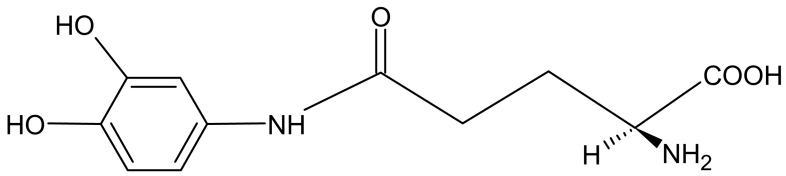
Agaritine may also act in the browning process of mushrooms. Due to its nucleophile nature, it easily reacts with o-quinones, and thereby prevents melanin formation (Espin et al., 1999). A second aspect concerning the browning is the inhibition of the monophenolase and diphenolase activities of mushroom polyphenol oxidase (PPO) by agaritine (Espin et al., 1998).

2.6.4 GHB and Phenolic Compounds

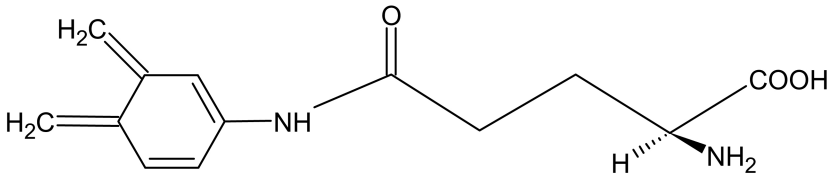
γ-L-glutaminyl-4-hydroxybenzene (GHB) is the only phenol in mushrooms occurring in any quantity. It originates from the shikimate-chorismate pathway and biosynthesis involves the γ-glutamyl transfer to 4-aminophenol by the γ-glutaminyltransferase as can be seen in the agaritine metabolism. Thus, GHB and agaritine may one another act as γ-glutamyl donor for their biosynthesis (Stüssi and Rast, 1981). The biological function of GHB has not been elucidated yet. Its oxidized product is supposed to act as an inhibitor of mitochondrial respiratory enzymes and protein synthesis in mushrooms. Another possible physiological role of GHB is as a precursor of melanin. GHB is easily oxidized to γ-L-glutaminyl-3,4-dihydroxybenzene (GDHB) and γ-L-glutaminyl-3,4-benzoquinone (GBQ), being able to polymerize to melanin. This reaction is catalyzed by the tyrosinase and oxygen (Joivet et al., 1999).



GHB



GDHB



GBQ

The tyrosinase, the main polyphenol oxidase in mushrooms, is the substantial enzyme in the enzymatic browning process. As described for GHB, this enzyme oxidizes phenolic compounds to quinones, and with it initializing a series of further reactions resulting in melanin. Its characteristic is the ability to catalyze two different reactions: monophenol hydroxylation to o-quinones and their oxidation to o-diphenols. The other main phenolic compound undergoing melanin formation besides GHB is tyrosine which is oxidized to L-DOPA. The total phenolic content of mushrooms depends on the stage of maturity and the tissue, and ranges between 3.4 and 10.7 mg g^{-1} dry weight for soluble phenols, respectively (Jolivet et al., 1998).

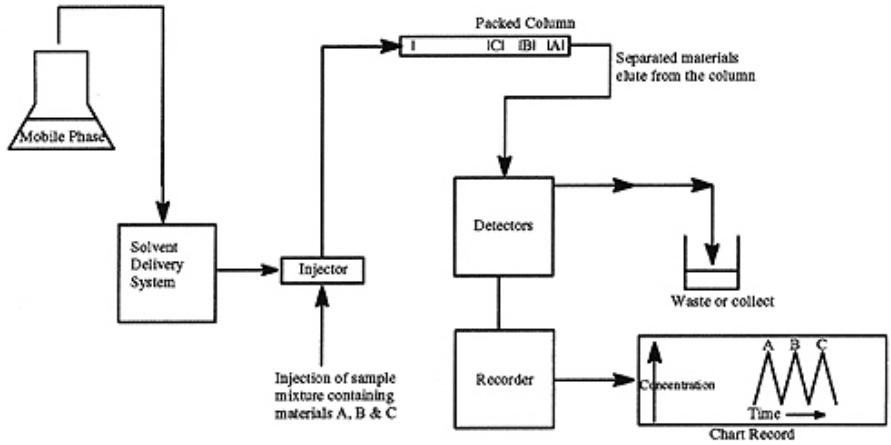
The possible function of GHB oxidation products to inhibit mitochondrial respiratory enzymes and protein synthesis has motivated for further investigations. The results propose a cytotoxic activity of two oxidation products against tumor cell lines which might be useful therapeutic agents to melanocarcinoma. However, this finding is controversial (Jolivet et al., 1998).

2.7 Basic Principles of Analytical Devices

2.7.1 HPLC

The high performance liquid chromatography (HPLC) is the most efficient chromatographic method. It is a favoured technique, applicable to a wide range of sample types and is, therefore, used in many fields of activity. Principally, liquid chromatography (LC) is a method to separate compounds of a solution according to different migration times of the solutes in a liquid flowing. These different migration times of the solutes are a result of various interactions between the solutes and the solid particles packed on a column (Lough and Wainer, 1996). The HPLC system consists of a high-pressure pump, an eluent delivery system, an injection device for sample application, a column, a detector and the data acquisition which is in most cases a computer (Fig. 2.1) (Lindsay, 1996).

Figure 2.1: Components of an HPLC Instrument



(Source: http://www.protein.iastate.edu/images/hplc.jpg, assessed 7.08.3008)

Pump: The pump is also termed the heart of the HPLC system. Its main task is the transport of the mobile phase through the column at high pressure and at a constant and puls-free flow rate. According to their function one can distinguish between to different types of pumps: those which deliver the flow rate at a constant pressure (constant pressure

pumps) and those that pump at a constant flow rate (constant flow pumps) (Lindsay, 1996).

Injection: Today, sample injection onto the HPLC column is made by the use of valve-type injectors. These injectors consist of a sample "loop" that can be fully flushed, a precondition for quantitative analysis. Modern equipments are provided with an auto-injector with a fixed sample loop. The minimum injection volume is 1 μl (Hanai, 1999).

Column: The column is the place where compounds are separated. Usually, it consists of stainless steel which is relatively inert against corrosion. Common metrics for columns are an external diameter of 6.35 mm; an internal diameter of 4.6 mm and a length up to 25 cm. Columns are packed with the stationary phase made up of small particles between 3 μm and 10 μm. The most frequently used packaging material is silica gel. It is composed of small, porous particles being either spheric or irregular shaped. These particles are produced in a way that particle size and pore diameter are narrowly distributed. Silica gel can be chemically modified by derivatisation of the silanol-groups in order to extend the applicability of silica gel to different modes of chromatography. Other packaging material that in opposite to silica gel withstands extreme pH-values includes hydrofluorcarbons, carbons, aluminium oxide and polymer resins (Lindsay, 1996).

The mode of chromatography is elected according to molecular weight, polarity and ionic character of the sample. Normal-phase signifies the stationary phase being more polar than the mobile phase. Thus, the stationary phase consists of a polar adsorbent (e.g. silica gel) and the mobile phase of a mixture of non-aqueous solvents. The reversed-phase implies a lower polarity of the stationary phase than of the mobile phase. This is the case when a hydrocarbonaceous phase covalently bound to silica gel and a hydro-organic mobile phase is used. In both modes, samples are eluted in order of their polarity (Lough and Wainer, 1996).

Elution: One can distinguish between two different types of elution. If the composition of the mobile phase remains constant, it is called isocratic elution. The term step-wise elution describes the predetermined change of the mobile phase composition during separation. It is used to shorten the retention time (Lindsay, 1996).

Detector: The main task of a detector is the identification of defined components of the mobile phase passing through the column. According to the detector, chemical

characteristics of the analyte are measured. The most important detectors include UV-visible absorbance detector, fluorescence detector, refractive index detector and electrochemical detector (Lough and Wainer, 1996).

UV-visible detector: The UV-visible detector is by far the most frequently used. Its principle is based on the absorption of UV or visible light by molecules of the solution (Hanai, 1999). Compounds being detected include alkenes, aromatic compounds and C, O, N and S containing molecules. The mobile phase should not be UV-active. The absorption of radiation by sample molecules is described by the Beer-Lambert law (Equ. 2.26) (Lindsay, 1996).

Fluorescence detector: The process of absorbing light involves the electron being raised to a higher energy level. This energy can return to the ground state by emitting light, either immediately (fluorescence) or delayed (phosphorescence). Generally, the energy difference between absorbed and emitted energy is rather low. For detection purposes, compounds can be modified in a way to make them fluorescent (Lough and Wainer, 1996).

Electrochemical detectors: Electrochemical detectors measure the electricity produced by oxidation and reduction of the sample. They are termed amperometric and coulometric detectors. Amperometric detectors oxidize or reduce only a small part of the sample molecules, the observed electricity is very small. However, modern amplifier intensifying the signal and a relatively high sensitivity benefit these detectors. The coulometric detector consists of many electrodes. The eluent is rather bypassing the electrodes unless the accurate potential is reacting with the electrochemical compounds. The different potential of the electrodes makes it possible to remove electrochemical compounds of no interest with one electrode. Although EC-detectors have a higher sensitivity than UV-visible detectors, their handling is rather complicated and therefore, the application is limited to a small field (Lindsay, 1996).

2.7.2 Mass Spectrometry

Mass spectrometry has emerged to an essential instrument in analytical laboratories. It is used in a variety of fields - structure elucidation of unknowns, environmental and forensic analytics, and quality control of drugs, flavours and polymers, to name only a few. It is an analytical technique that ionizes atoms or molecules from either organic or inorganic samples, separates these ions according to their mass-to-charge ratio (m/z) and detects them. A mass spectrometer system is composed of an ion source, a mass

analyzer and a detector. The process is conducted under vacuum conditions (Gross, 2004).

The ion source is the place where atoms or molecules are converted into ions and accelerated into the mass analyzer. Many different methods have been developed to perform this task determining a variety of samples. The traditional technique is electron ionization, generating ions by shooting electrons on neutrals. Chemical ionization comprises ion- molecules interactions between two reactants. Other ionization methods are field ionization and field desorption, fast atom bombardment, matrix-associated laser desorption/ionization and electrospray ionization. The last one was used for the measurements in this study, being employed for the analysis of large, non-volatile, chargeable molecules such as proteins and nucleic acid polymers (Gross, 2004).

The mass analyzer separates the ions according to its mass-to-charge ratio. Different types of mass analyzer vary in their physical principle. The classical instrument is the magnetic sector mass analyzer, where ions are deflected in a magnetic field, being separated by the momentum based on the Lorentz Force. The time-of-flight analyzer operates by accelerating the produced ions with electric pulses. All ions are dispersed at the same time into a field-free path of known length to bring about the heavier ions arriving later at the detector than the lighter ones. The linear quadrupole mass analyzer is composed of two pairs of parallel rod electrodes being applied the same potential which consists of DC (direct current) and RF (radiofrequency) components. Ions are separated due to their stability in the oscillating trajectories (Barker, 1999). The linear quadrupole ion trap mass analyzer basically follows the same principles as the linear quadrupole mass analyzer, additionally allowing ion storage. The fourier-transform (FT) instruments measures by detecting the current image in the detector plates. This current image is caused by the ion-cyclotron frequency in a magnetic field (Gross, 2004). Another important technique forms the tandem mass spectrometry (MS/MS). It is based on the prior isolation of the ion followed by fragmentation in order to obtain product ions and neutral fragments (Barker, 1999).

Among the detectors, the most frequently used are the secondary electron multipliers (SEM), measuring secondary electrons released from surfaces through ion impact. The discrete dynode electron multipliers work with 8-12 succesively arranged dynodes each causing an electric current, which is detected by a preamplifier. In the channel electron multipliers (CEM), the electric current is generated in a continuous tube. Based on the same principle are the microchannel plates, where millions of tubes are put together in a bundle. The most elementary detector is the Faraday cup, which forms a single electrode

able to receive the charge of the ion (Gross, 2004).

2.7.3 Absorption Spectrometry

2.7.3.1 Light Absorption in the Visible and UV Region

Ultraviolet (UV) and visible light belong to the electromagnetic spectrum where the energy is expressed as wavelength (nm) (Chap. 2.1 Basic Principles of Radiation Chemistry). Absorption of electromagnetic energy from leads to an increase of the energy content in organic molecules. Concerning UV and visible light absorption, electrons are excited and advanced to orbitals of higher energy levels. The energy of the absorbed light equals the energy level difference between excited state and the ground state. Structural differences and, thereby, differences in the excitability of electrons account for absorption characteristics of various molecules. Electrons do not remain in the excited state; the energy is used among other things for photochemical reactions (Görög, 1995).

2.7.3.2 Beert-Lambert Law

The Beer-Lambert law describes the relation between attenuation of light intensity induced by an absorbing solution, the concentration and the path length of that solution.

$$A = \frac{I_0}{I} = \varepsilon \times b \times c \tag{2.26}$$

A ... absorbance
I ... transmitted light intensity
I_0 ... intensity of the incident light
ε ... molecular extinction coefficient
b ... cell length
c ... solute concentration

The light transmittance is proportionate to the molecular extinction coefficient, depending on the character of the absorbing substance, the cell length and the solute concentration. Keeping the cell length constant, the equation can be used to calculate the concentration c by measuring the absorbance A. Therefore, the Lambert-Beer law is crucial for qualitative and quantitative determinations in spectrophotometry (Görög, 1995).

2.7.3.3 Main Parts of the Spectrophotometer

A spectrophotometer consists of a light source, a monochromator, a cell holder with cells and a detector. UV-visible spectrophotometers use two types of lamps- the tungsten fila-

ment bulb for the visible region and a deuterium lamp for the UV region. The monochromator is required to narrow the wavelength region of the light beam. The light beam passes through the cell filled with the solute. The cell is usually made of glass or quartz, having a standard path length of 10 mm. The cell holder is constructed in a way to enable the measurement of multiple solutions after another. Detection is achieved by converting the light into an electric signal. Most spectrophotometers are equipped with a photomultiplier. Modern double beam instruments function as follows: after passing through the monochromator, the light beam is split into two parts of the same intensity, one passing through the reference cell, the other through the sample cell. A mirror system is used to focus both beams alternately on the detector (Görög, 1995).

Chapter 3

Materials and Methods

3.1 Chemicals

All the chemicals used were purchased of highest purity available (Table 1). The agaritine (β-N-(γ-L(+)-glutamyl)-4-(hydroxymethyl) phenylhydrazine) standard was provided by Hendrik Frandsen from the Danish Institute for Food and Veterinary Research, Denmark. For sample preparation, water was distilled and further purified using Direct-Q3 UV purification system (Millipore).

3.2 Irradiation

Source: Sample irradiation was performed using a Co-60-gamma ray irradiator Type "Gammacell 220" (Nordion International Inc., Kanata Ont., Canada) at a dose rate of 34 Gy min^{-1} (Figs. 3.1 and 3.2).

Mushrooms: Fresh cultivated mushrooms of the species *Agaricus bisporus* (Don

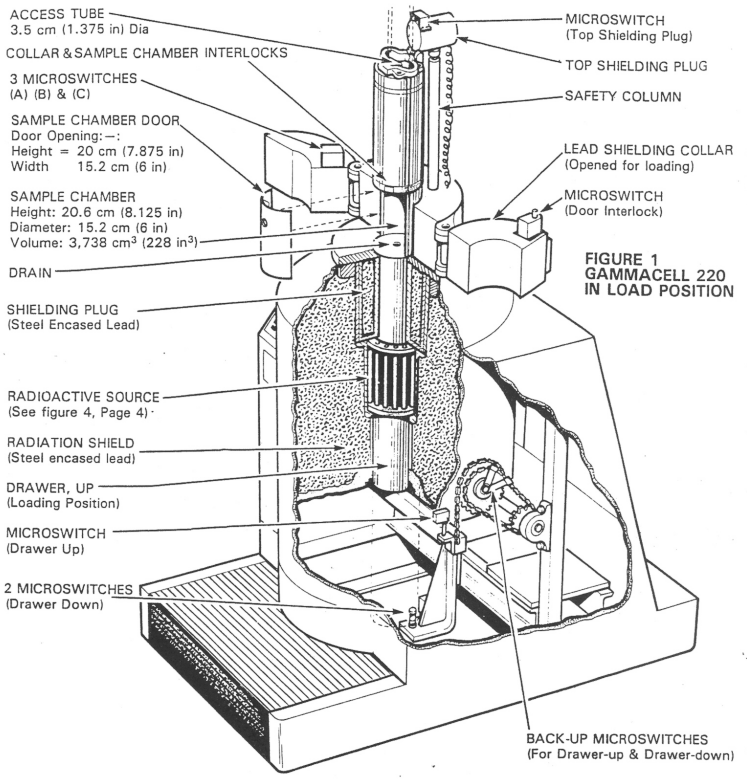
Figure 3.1: Gammacell 220

(© Isolde Sommer)

Table 3.1: Chemicals

Chemicals:	**Supplier:**
Methanol > 99,5%	Roth
Methanol ROTISOLV@HPLC ultra gradient grade	Roth
Potassium dihydrogene phosphate > 99%	Fluka
ortho-Phosphoric acid 85%	Fluka
Acetic acid (glacial)	Merck
Adenosine 5′-monophosphate monohydrate (AMP)	Sigma Aldrich
Guanosine 5′-monophosphate disodium salt (GMP)	Sigma Aldrich
Cytidine 5′-monophosphate (CMP)	Sigma Aldrich
Uridine 5′-monophosphate disodium salt (UMP)	Sigma Aldrich
Xanthosine 5′-monophosphate disodium salt (XMP)	Sigma Aldrich
Inosine 5′-monophosphate disodium salt (IMP)	Sigma Aldrich
β-Nicotinamide adenine dinucleotide hydrate (NAD)	Sigma Aldrich
Phenylalanine > 95%	Sigma Aldrich
o-,p-,m-Tyrosine	Sigma Aldrich
3,4-dihydroxyphenylalanine (DOPA)	Sigma Aldrich
Folin-ciocalteu's reagent	Sigma Aldrich
Sodium carbonate	Roth
Gallic acid ≥ 98%	Sigma Aldrich
2,2′-azinobis-(3-ethyl-benzothiazoline)-6-sulfonic acid (ABTS)	Fluka
Potassium peroxodisulfate	Merck
6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)	Fluka

Figure 3.2: Gammacell 220



(Source: Nordion International Inc.)

Juan Selection, Hungary) were purchased at a local store. Each mushroom of the total amount of 1 kg was cut into fourths and distributed to four beakers. One part (~250 g) of mushrooms was left unirradiated, the other parts were irradiated at 1, 3 and 5 kGy under air. Immediatley after irradiation the mushrooms were freeze-dried and ground to fine powder using a small blender. Until analysis, they were stored in an exsiccator in the dark.

Standard solutions: Radiation of pure agaritine, phenylalanine and tyrosine was carried out in aqueous solution in presence of air. The dose range was 100 to 1500 Gy.

3.3 HPLC

The HPLC-UV measurements were carried out on a Hewlett-Packard 1100/1050 series HPLC-system equipped with a diode array detector. The column used was a LiChrospher 100 RP-18 (250x4.6 mm, 5 μm), with a precolumn (4x4 mm), Merck. The flow rate of 1 ml min^{-1} in isocratic elution and the injection volume (20 μl) were kept constant during all measurements.

3.4 Analysis of Agaritine and Glutaminyl Derivates

Dried mushrooms were prepared according to Schulzová et al., 2002 with a few minor modifications. About 1 g dry mushrooms were mixed with 50 ml methanol-water (9:1 v/v), shaken for 1h and then filtered. The volume of the filtrate was adjusted to 100 ml with methanol and the residue dissolved in 20 ml distilled water *(agaritine extract)*. The solution was filtered through a cellulose acetate filter (FP 30/0.2 CA-S, Whatman) prior to injection onto the HPLC column. The mobile phase consisted of 0.05 M KH_2PO_4/H_3PO_4, pH 3.3. UV detection was at 237 nm and run time lasted for 15 min.

For identification of agaritine, an agaritine stock solution in water was prepared and directly injected onto the HPLC. A calibration curve ranging from $10^{-3} - 10^{-4}$ mol/l was made for quantification.

3.5 Analysis of Nucleotides and Amino Acids

Nucleotides and Amino acids were extracted as described by Tsai et al., 2007. Freeze-dried mushroom powder (500 mg) was extracted with 25 ml of distilled water. This suspension was shaken whilst being heated to boiling for 1 min. After boiling, the suspension was still shaken for another 15 min. Then it was cooled down and centrifuged at 4000 g for 15 min. The extraction was repeated once with 20 ml of distilled water. The combined was rotary-evaporated to dryness and the residue dissolved in 10ml distilled water. Filtration through a cellulose acetate filter was performed before injection onto the HPLC. The mobile phase for the nucleotides and amino acids analysis varied in the pH-value. The proportion of 0.05 M K_2PO_4/H_3PO_4 to Methanol was 99:1 with pH adjustment to 4.3 for nucleotides analysis *(nucleotide extract)* and 3.3 for amino acids analysis *(amino acids extract)*. UV detection wavelengths were 205 nm, 225 nm and 260 nm. The total run time was 25 min.

Each nucleotide and amino acid was identified by a standard solution of the authentic compound (AMP, GMP, CMP, UMP, XMP, IMP, NAD, tyrosine, phenylalanine) and quantified by a calibration curve.

3.6 Mass Spectrometric Analysis

With both solutions described above, HPLC-UV-MS measurements were performed. The system consisted of an Agilent 1100 series HPLC and a variable wavelength UV detector

coupled with an HCT plus ion trap mass spectrometer equipped with an electrospray ionization source. Data acquisition was achieved using HyStar 3.1, and data evaluation was carried out using DataAnalysis Version 3.3 from Bruker Daltonics. Separation was carried out on a ACE 3 C18 column (100x2.1 mm, 3 μm particle size) equipped with a precolumn of the same stationary phase material. 10 μl of the extract was injected into the chromatographic system and the mobile phase (0.1% acetic acid) was delivered at a flow rate of 0.2 ml min^{-1} in isocratic elution.

Table 3.2: MS parameters

Parameter	**Positive mode**	**Negative mode**
Capillary voltage	-4500 V	+4500 V
Nebulizer pressure	40 psi	40 psi
Dry gas flow	10 l min^{-1}	10 l min^{-1}
Dry temperature	300 °C	300 °C
Skimmer	+ 40 V	- 40 V
Capillary exit	+ 100 V	- 100 V
Octapole 1/2 DC	12/1.7 V	- 12/-1 V
Octapole RF	100 Vpp	100 Vpp
Lens 1/2	-5/-60 V	5/50 V
Trap drive	35	38
ICC smart target	50 000	50 000
Scan range	50-700 m/z	100-700 m/z

3.7 HPLC-Analysis of Irradiated Standard Solutions

For that purpose, stock solutions of agaritine, phenylalanine and tyrosine were prepared (10^{-3}mol l^{-1} and $5x10^{-4}$mol l^{-1}). Then, the irradiated solution was directly injected onto the HPLC. The mobile phase was a phosphate buffer (pH 3.3) for agaritine and a methanol-phosphate buffer (1:99 v/v) (pH 4.3) for phenylalanine and tyrosine. UV detection wavelengths were 237 nm for agaritine and 210 nm for phenylalanine and tyrosine.

The decrease of the standard solutions as well as the increase of new formed substances, DOPA from tyrosine, and o-,m-,p- tyrosine and DOPA from phenylalanine, were quantified by a calibration curve.

3.8 Folin-Ciocalteu (FC) Test and Trolox Equivalent Antioxidative Capacity (TEAC) Assay

3.8.1 Sample Preparation

The extraction procedure was conducted in the same way as Chun et al., 2005 had described. About 2 g of freeze-dried mushrooms were mixed with 50 ml methanol-water 8:10 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 absolute methanol and then made up to the final volume of 20 ml with distilled water. Finally, the solution was centrifuged at 4000 g for 10 min.

3.8.2 FC-Test

Total phenolic content was determined by the procedure according to Chun et al., 2005. About 0.4 ml of the sample was added to a 10 ml volumetric flask filled with 7 ml distilled water. A reagent blank using distilled water instead of the sample was prepared for the spectrophotometric 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. After 1 h keeping the reaction in dark, the absorbance was measured at 760 nm against the blank value using a Perkin Elmer UV/VIS Sprectrometer Lambda 650.

Total phenolic content was calculated from a standard curve of gallic acid (200 mg-2000 mg/l) prepared at the same time and expressed as mg of gallic acid equivalent (GAE) l^{-1}. Samples were analyzed in triplicate.

3.8.3 TEAC-Assay

Applying the method of Re et al., 1999, 0.192 g of ABTS was dissolved in 50 ml distilled water to a concentration of 7 mM. The addition of 0.0331 g of di-potassium peroxdisulfate (2.45 mM) introduced the production of the $ABTS^{\bullet+}$ radical cation. This $ABTS^{\bullet+}$ solution was diluted with distilled water at a ratio of 2:25. The Trolox standard (2.5 mM) was prepared in Methanol and dilution series ranging from 0.25 mM to 2 mM were established. Absorbance reading was taken directly after addition and mixing of 100 μl of sample or Trolox standards to 2 ml $ABTS^{\bullet+}$ solution. Measurements were done at the maximum at 734 nm versus water using the same spectrometer as described above.

All determinations were carried out three times.

The antioxidative activity of the sample was calculated from the Trolox standard curve by blotting absorbance versus concentration and expressed as Trolox equivalent l^{-1}.The concentration of antioxidants giving the same percentage change in absorbance of $ABTS^{\bullet+}$ as that of 1 mmol Trolox is defined as TEAC (Huang et al., 2005).

3.9 Statistical Analysis

Statistical analysis was carried out using SPSS Version 16.0. In all cases, the Kolmorogov-Smirnov test was applied to test for normal distribution. Differences between the means of the applied doses were analyzed by one-way analysis of variance (ANOVA), followed by Tukey post hoc test. P-values < 0.05 were regarded as significant. FC and TEAC correlation was performed using Pearson′s correlation.

Chapter 4

Results and Discussion

4.1 FC-Test

Originally the FC-test was used to determine tyrosine content in proteins (Folin and Ciocalteu, 1927). In the late nineties, it was established for quantifying total phenolics and antioxidants (Singleton et al., 1999). Since then it has become a routine assay, being frequently applied to determine the total phenol content of fruit and vegetables. It actually measures a sample′s reducing capacity, which can be caused by other compounds than phenols as well, e.g. vitamin C. The typical yellow colour of the FC-reagent is changed to green when reductants such as phenolic compounds are added. The dissociation of a phenolic proton results in a phenolate anion, which is able to reduce the FC-reagent. The detailed chemical nature of the FC reagent is not known, but it is believed that molybdenum is involved in the crucial electron transfer step (Huang et al., 2005):

$$\text{Mo (VI)} + \text{phenolate } (PhO^{-}) \rightarrow \text{Mo (V)} + \text{phenoxyl radical } (PhO^{\bullet})$$

Since the reaction proceeds only under basic conditions (pH 10), a sodium carbonate solution is needed for adjustment. The stop of the colour change makes the reaction end point. The change of absorbance is plotted against the antioxidant concentration which is expressed by gallic acid equivalent (GAE). The gallic acid calibration curve is given in Fig. 4.1

Figure 4.1: Gallic acid calibration curve, 760 nm

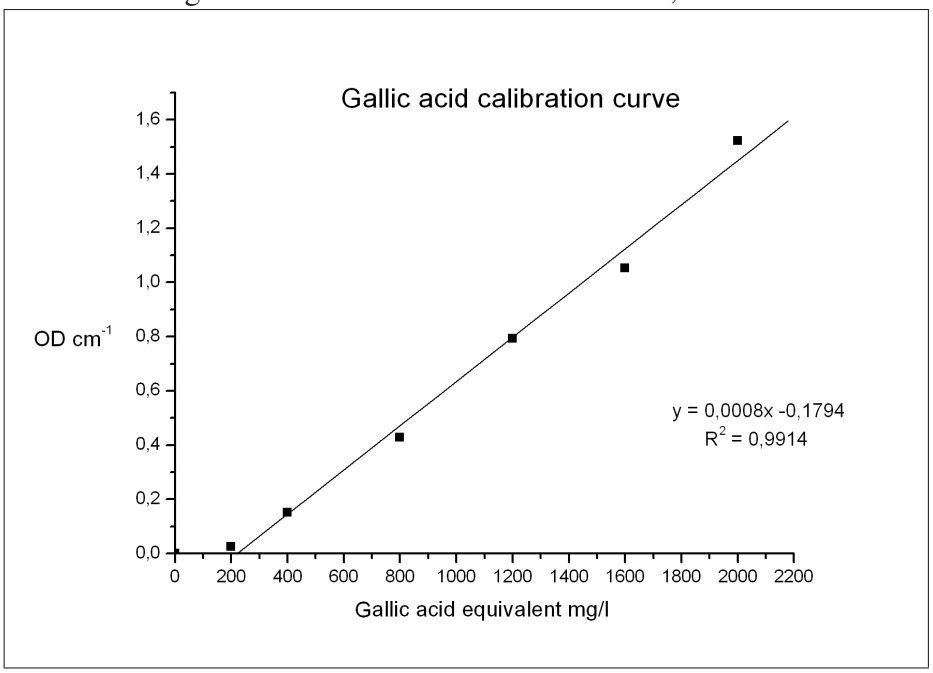


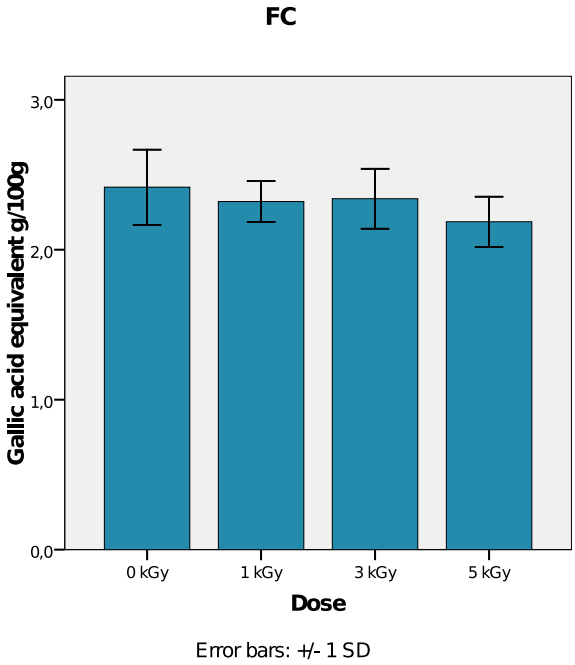
Table 4.1: FC - Descriptive statistics (Gallic acid equivalent g/100G dry weight (DW))

	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	9	2,42	2,75	2,03	0,25	2,53	10,36
1 kGy	9	2,32	2,62	2,13	0,14	2,31	5,86
3 kGy	9	2,34	2,61	2,00	0,20	2,30	8,55
5 kGy	9	2,19	2,44	1,81	0,16	2,19	7,64

The total phenols content of mushrooms exhibits significant natural variations. It is often given for 100 g fresh weight (FW). Chun et al., 2005, found 11.25 mg GAE/100g FW. Assuming the water content in mushrooms to be ~90%, this would correspond to about 0.1 g GAE/100g DW. For dried mushrooms the following values were published: 8 mg GAE/g DW (Dubost et al., 2007) and 0.63 mg GAE/g DW (Fu et al., 2002). Converted into the unit used in this work it corresponds to 0.8 g/100g and 0.063 g/100g. In a recent study on the radical scavenging properties of three different *Agaricus bisporus* strains higher values were reported: 3.1-4.9 g GAE/100g (Savoie et al., 2007). The obtained results for the mushrooms used in this study (Table 4.1) go conform to literature, lying between 0.8 g GAE/100 g DW and 3.1-4.9 g GAE/100g DW. It should be mentioned that phenolic compounds like flavonoids and phenolic acids are absent or in very low level present in *Agaricus bisporus* (Herrmann, 1974; Mattila et al., 2001). Therefore, it can be

assumed that the main part of phenols expressed by GAE is resulting from melanogenous phenols (e.g.GHB and tyrosine) and vitamins (e.g. vitamin C).

Figure 4.2: Mean values of total phenols (GAE) at different doses



The investigations in respect to shelf life extension of fresh mushrooms by irradiation (Benoît et al., 2000, and Beaulieu et al., 2002) have been discussed in detail in the introduction (Chap.1). Concerning the total phenols content, their results are contradictory. Whereas in the publication of Benoît et al., 2000, an increase of total phenols directly after irradiation was not indicated, Beaulieu et al., 2002 reported a significant enhancement (30%). In the framework of this study all mushroom samples had been immediately freeze-dried after irradiation to avoid enzymatic activity. For the GAE-values, being a function of irradiation dose, practically no change was observable. They are slightly decreasing, however, the statistical analysis showed no significant differences (Table 4.1, Fig. 4.2).

4.2 TEAC

The TEAC assay has been introduced to measure the total antioxidant activity of body fluid, food extracts and pure compounds. It is based on the formation of the ABTS [2,2′-azinobis-(3-ethyl-benzothiazoline)-6-sulfonic acid] radical cation. The method used in this study was a decolourization technique involving the direct production of the

blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium peroxodisulfate (Re et al., 1999). This $ABTS^{\bullet+}$ radical cation has absorption maxima at 654 nm, 734 nm, 815 nm and 415 nm. Antioxidants added to the radical cation lead to its reduction, to an extent that is depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction. Therefore, the absorbance, expressing the inhibition of the $ABTS^{\bullet+}$ radical, is a function of concentration and time and it is computed relative to the reactivity of the Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard, under the same conditions. The Trolox equivalent calibration curve is presented in Fig. 4.3.

Figure 4.3: Trolox equivalent calibration curve, 734 nm

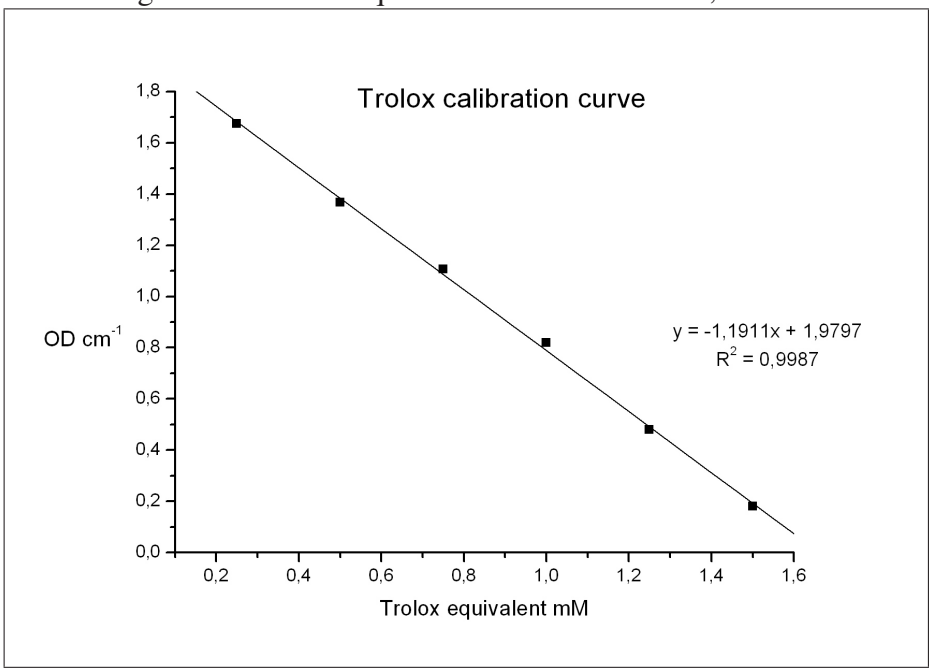


Table 4.2: TEAC - Descriptive statistics (Trolox equivalent mg/100g DW)

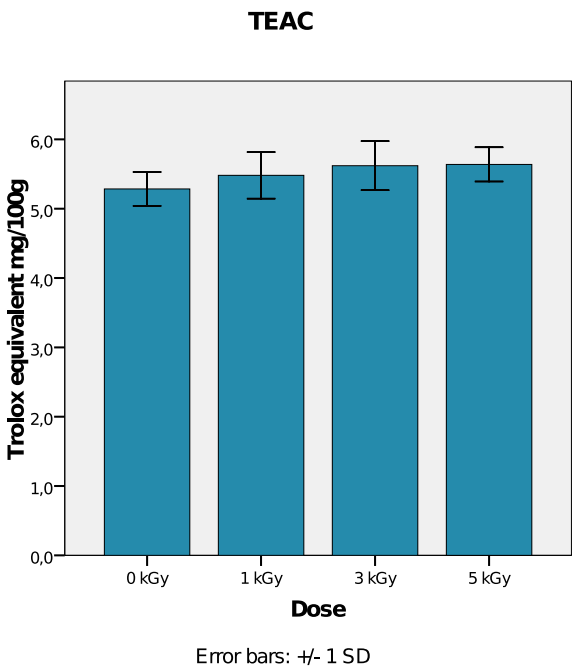
	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	9	5,43	5,82	4,86	0,30	5,37	5,52
1 kGy	9	5,40	5,76	4,79	0,35	5,56	6,45
3 kGy	9	5,62	6,19	5,17	0,35	5,49	6,29
5 kGy	9	5,64	6,05	5,27	0,25	5,62	4,37

The TEAC-values as a function of dose are summarized in Table 4.2 and Fig. 4.4. TEAC determinations of irradiated mushrooms have not been performed up to present.

In contrast to the FC-values a slight increase of the antioxidant activity with dose was observable. However, the results were not statistically significant.

For non irradiated mushrooms there are only few TEAC data available, and those are expressed as EC_{50} values, describing the effective concentration at which $ABTS^{\bullet+}$ radicals are scavenged by 50% (Savoie et al., 2008). Therefore, they are not directly comparable to the obtained results.

Figure 4.4: Mean values of antioxidant capacity (trolox equivalent) at different doses



4.3 Correlation between FC and TEAC

The similarity of chemistry among the FC- and the TEAC assays (both are based on electron transfer) enables linear correlations between the "total phenolic profiles" and the "antioxidant activity" (Huang et al., 2005). In this study, total polyphenols and antioxidant activity correlate with Pearson correlation coefficient 0.633, significance level $p<0.01$. This result indicates that the reducing components expressed by GAE may be of different nature than the antioxidative compounds represented by TEAC, and of different radiation sensitivity. Some "phenols" might be degraded by irradiation, whereas other antioxidants might be formed, i.e. liberated from a more complex structure. It has been shown for flavonoids, that glycosylation reduces the TEAC values. Luteolin has a TEAC of 2.09, whereas the mono- and diglucosides have clearly lower values, 1.74 and 0.79,

respectively (Rice-Evans et al., 1996). For non irradiated mushrooms, different results concerning relationship of GAE and antioxidative capacity measurements are available. Alvarez-Parrilla et al., 2007, performed correlation analysis of total phenolics (FC-test, total phenols expressed as caffeic acid equivalents) with the antioxidant capacity (FRAP, ferric reducing /antioxidant power assay) obtained for commercial and wild *Agaricus* species. They found a good correlation (R^2 = 0.9721, significance level p<0.002). Other authors reported a good relationship between total phenols and free radical scavenging activities (DPPH, 2,2-Diphenyl-1-picrylhydrazil radical) of various commercially available mushrooms in Korea (Fu et al., 2002). Savoie et al., 2008, investigated three different strains of *Agaricus bisporus* and found those with the highest phenols concentration to have the lowest antioxidative properties (TEAC). A similar characteristic for *Agaricus* strains was reported by Czapski, 2004.

4.4 Glutaminyl Derivates

4.4.1 Qualitative Analysis

Components of mushrooms were identified using HPLC-UV and HPLC-MS analyses. The extracts prepared for agaritine analysis (Chap. 3.1) were employed.

4.4.1.1 HPLC-UV

For HPLC-UV analysis, the detection wavelength at the peak maxima of agaritine (237 nm) was used (Fig. 4.5).

Figure 4.5: HPLC chromatogram, agaritine extract, 237 nm

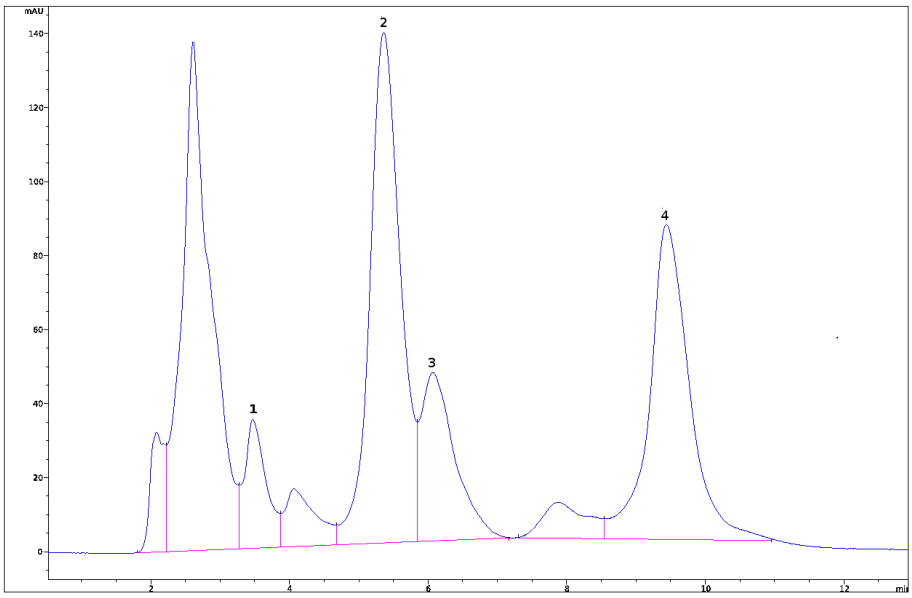


Figure 4.6: UV-peak 1, a.e*

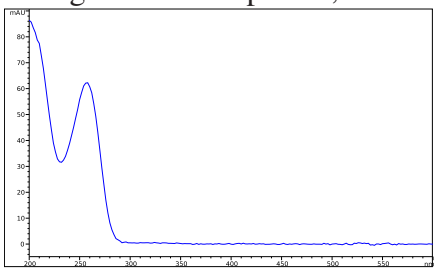


Figure 4.8: UV-tyrosine, a.e*

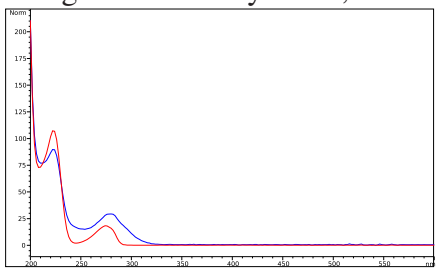


Figure 4.7: UV-agaritine

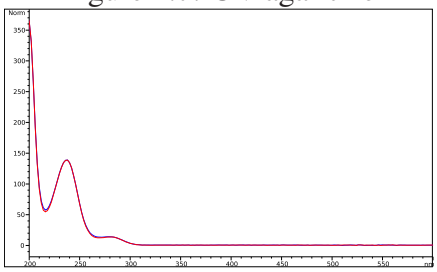
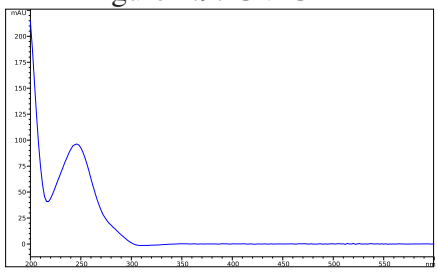


Figure 4.9: UV-GHB



*agaritine extract

Four compounds listed in Table 4.3 were taken for quantitative analysis.

Table 4.3: Elution of compounds from agaritine extract

Peak number	Compound	Retention time (min)
1	unknown	3,471
2	Agaritine	5,361
3	Tyrosine	6,070
4	GHB*	9,438

* γ-glutaminly-4-hydroxybenzene

UV-spectra The individual spectra of compounds (peak 1-4) are shown in Fig. 4.6-4.9. The red overlaid spectra signify the corresponding standard used to confirm the identity of the compound. The GHB (γ-glutaminly-4-hydroxybenzene) spectrum was compared with a spectrum pictured in the literature (Baumgartner, 1995: p.50), since no reference compound was available. The compound at the retention time 3.47 min (peak 1) could not be identified. It was included in the quantitative analysis, since its UV-spectrum displayed a mainly pure compound (absorption maximum: 255 nm). All substances eluting ahead of peak 1 are combined and do not show a clear UV-spectrum. Tyrosine was quantified at a different wavelength (225 nm).

4.4.1.2 HPLC-MS

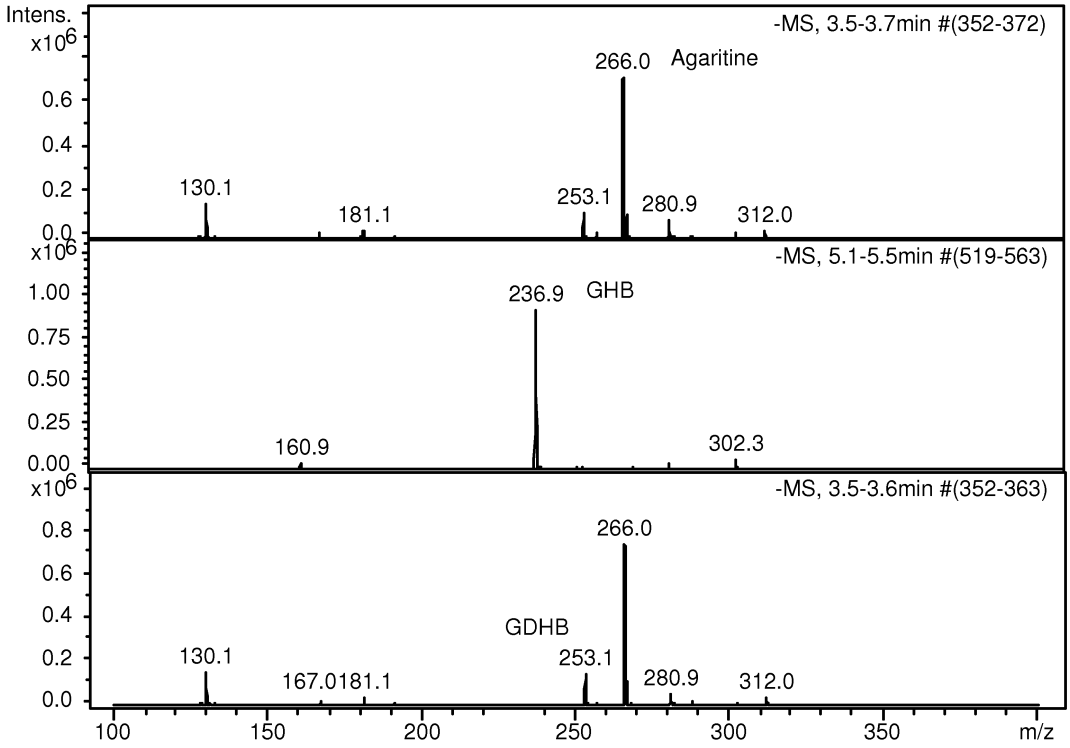
In order to confirm the results obtained from the HPLC-UV, mass spectrometric measurements were performed. Agaritine, GHB and its oxidation product, the 3,4-dihydroxybenzene compound GDHB, could be identified by their molecular masses $[M\text{-}H]^-$ (Fig 4.10). Their molecular weights are displayed in Table 4.4. Further, the presence of agaritinal and 2-hydroxy-4-imino-2,5-cyclohexadienone could be confirmed.

Table 4.4: Molecular weight of compounds, agaritine extract

Compound	Molecular weight (g/mol)
Agaritine	267,28
GHB	238,24
GDHB	254,20

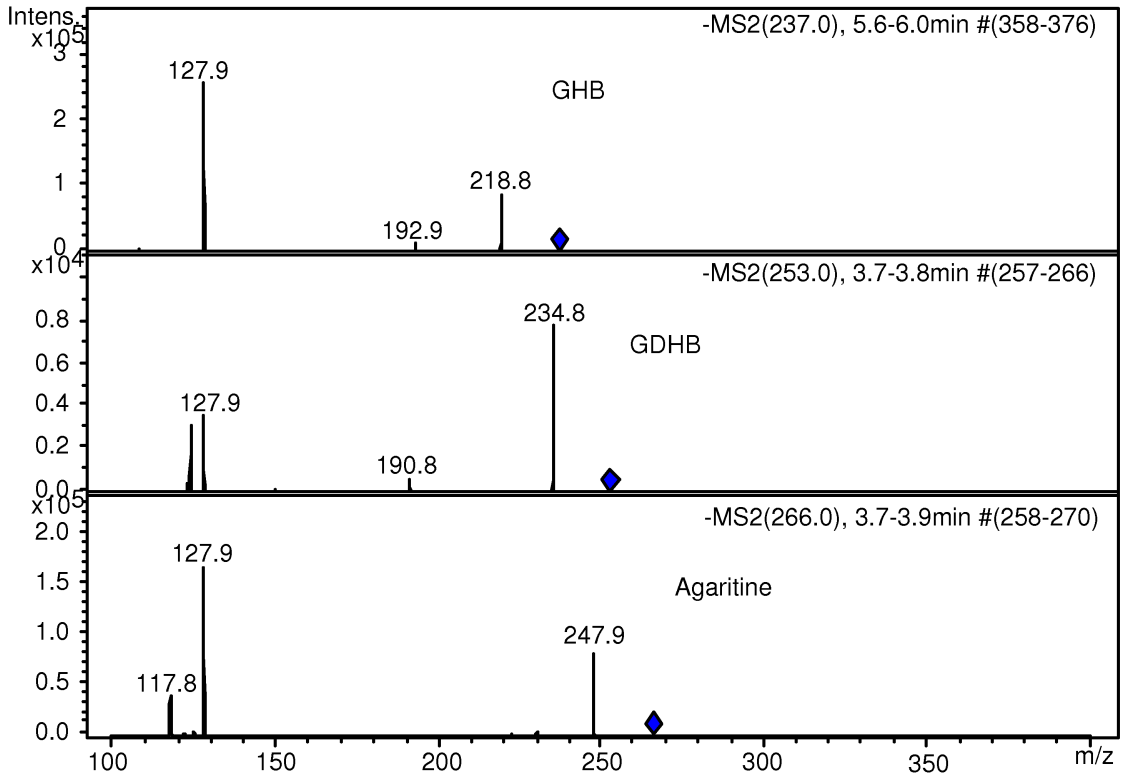
This is in agreement with Baumgartner et al., 1998, who determined several nitrogen-constituents in *Agaricus bisporus* including agaritine, agaritinal, GCPH, HBA, HMBD, GHB, GDHB, GBQ (s. List of Abbreviations) and 2-hydroxy-4-imino-2,5-cyclohexadienone.

Figure 4.10: Agaritine, GHB, GDHB: MS-spectra, negative mode



The common structural characteristic of agaritine, GHB, and GDHB, the glutaminyl residue m/z $[M\text{-}H]^-$ = 127.9, is displayed in the fragment spectra Fig. 4.11.

Figure 4.11: GHB, GDHB, Agaritine: MS-fragment spectra, negative mode



GDHB, agaritinal and 2-hydroxy-4-imino-2,5-cyclohexadienone were not detected in the HPLC chromatogram and, therefore, could not be further analyzed.

4.4.2 Quantitative Analysis

HPLC-UV
Quantitative analysis was performed by manual integration of the peak areas in the chromatogram.

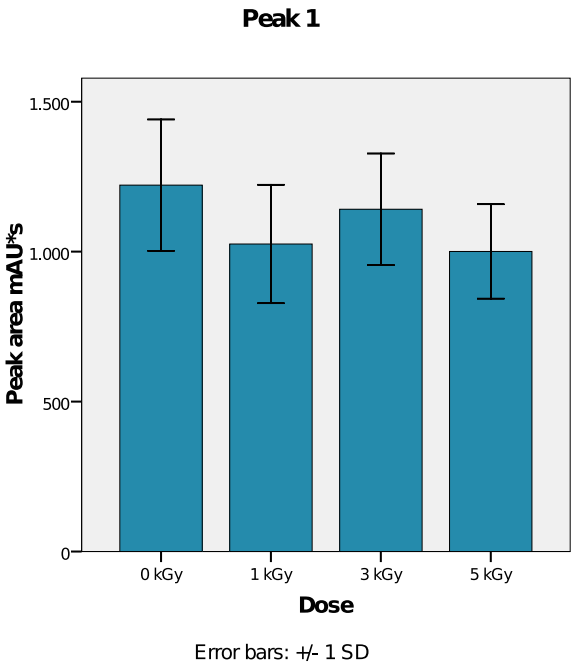
4.4.2.1 Peak 1

The peak areas as a function of dose are presented in Table 4.5 (Descriptive statistics) and Fig. 4.12.

Table 4.5: Peak 1 - Descriptive statistics (Peak area mAU*s), agaritine extract

	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	7	308,29	337	278	20,73	305	6,73
1 kGy	7	332,86	411	240	66,97	343	20,12
3 kGy	7	354,29	387	301	33,40	361	9,43
5 kGy	7	285,71	332	219	45,22	307	15,83

Figure 4.12: Mean values of peak 1 (peak areas) at different doses, agaritine extract



The unknown compound peak 1 shows a non-significant increase up to a dose of 3 kGy and a significant decrease from 3 kGy to 5 kGy. The high variation of the results might be partly explained by the insufficient separation in the HPLC chromatogram.

4.4.2.2 Agaritine

The agaritine content of mushrooms was determined by a standard calibration curve as it is shown in Fig. 4.13.

Figure 4.13: Agaritine standard calibration curve, 237 nm

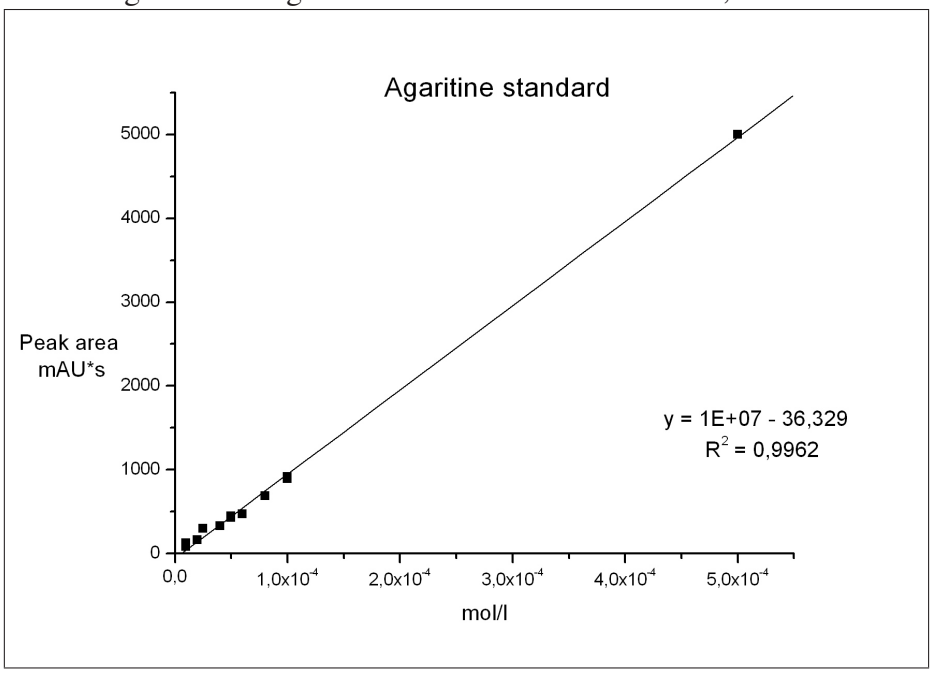
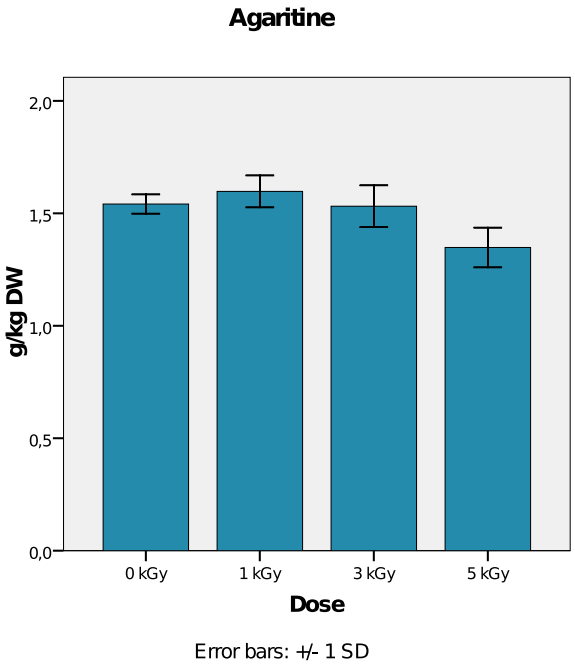


Table 4.6: Agaritine - Descriptive statistics (g/kg DW)

	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	7	1,54	1,59	1,47	0,04	1,55	2,77
1 kGy	7	1,60	1,68	1,52	0,06	1,58	2,77
3 kGy	7	1,53	1,64	1,41	0,09	1,54	6,07
5 kGy	7	1,35	1,44	1,18	0,09	1,38	6,56

Agaritine, the principal hydrazine in *Agaricus bisporus*, is of fundamental interest since many years (Espin et al., 1999). Its content in mushrooms was found in a range 94-629 mg/kg of fresh material and 2.1-6.9 g/kg in dried mushrooms by Fischer et al., 1984. These findings agree with other agaritine determinations. Toth and Gannett, 1993, measured 700 μg/g FW in their laboratory, corresponding to 700 mg/kg FW. Schulzová et al., 2002, reported similar contents, namely 200-450 mg/kg FW. Another research group, Sharman et al., 1990, determined a concentration of 2100 mg/kg DW. The obtained results can be well compared with the literature agaritine contents in dried mushrooms. A mean value of 1.54 g/kg DW in the unirradiated sample is slightly lower than those reported, but is in a realistic range (Table 4.6).

Figure 4.14: Mean values of agaritine (g/kg DW) at different doses



There is no report on agaritine investigations in irradiated mushrooms existing so far. Our analysis of agaritine resulted in non-significant changes between the doses 0 kGy, 1 kGy, and 3 kGy, and a statistically significant decrease from the dose 5 kGy (Fig. 4.14) The Tukey post hoc test details the significant difference between dose 5 kGy and all other doses (0 kGy, 1 kGy and 3 kGy), which is marked by * (Table 4.7). These findings suggest an influence of γ-irradiation on the agaritine content at doses above 3 kGy.

4.4.2.2.1 Irradiation of Agaritine in Aqueous Solution

Due to the fact that mushrooms tend to be high in moisture, ~92%, (Mattila et al., 2002), the effect of irradiation on mushrooms was compared with that in aqueous solutions in the presence of air. Although an indirect radiolysis effect, i.e. reactions of OH-radicals (Chap. 2.3 Water Radiolysis), was to be expected in both samples, the agaritine concentration in mushrooms was hardly influenced by radiation. In aqueous solutions, however, a strong decrease of agaritine at both concentrations of $5x10^{-5}$ mol l^{-1} and 10^{-4} mol l^{-1} was observed (Fig. 4.15, Fig. 4.16). At concentration of 10^{-4} mol l^{-1}, as can be read from the graphic, the 50% decay is achieved at a radiation dose of 750 Gy, in the $5x10^{-5}$ mol l^{-1} solution at 400 Gy.

Table 4.7: Tukey post hoc test of agaritine

Multiple Comparisons Value Tukey HSD						
		Mean			95% Confidence Interval	
(I) Dose	(J) Dose	Difference (I-J)	Std. Error	Sig.	lower bound	upper bound
0 kGy	1 kGy	-0.561	0.408	0.527	-0.169	0.056
	3 kGy	0.0099	0.408	0.995	-0.103	0.122
	5 kGy	0.1933*	0.408	0.000	0.081	0.306
1 kGy	0 kGy	0.0561	0.408	0.527	-0.056	0.169
	3 kGy	0.0660	0.408	0.389	-0.047	0.179
	5 kGy	0.2495*	0.408	0.000	0.137	0.362
3 kGy	0 kGy	-0.0099	0.408	0.995	-0.122	0.103
	1 kGy	-0.0660	0.408	0.389	-0.179	0.047
	5 kGy	0.1835*	0.408	0.001	0.071	0.296
5 kGy	0 kGy	-0.1933*	0.408	0.000	-0.306	-0.081
	1 kGy	-0.2495*	0.408	0.000	-0.362	-0.137
	3 kGy	-0.1835*	0.408	0.001	-0.296	-0.071

*The mean difference is significant at the 0.05 level

Figure 4.15: Agaritine (10^{-4} mol l^{-1}) decay as a function of dose

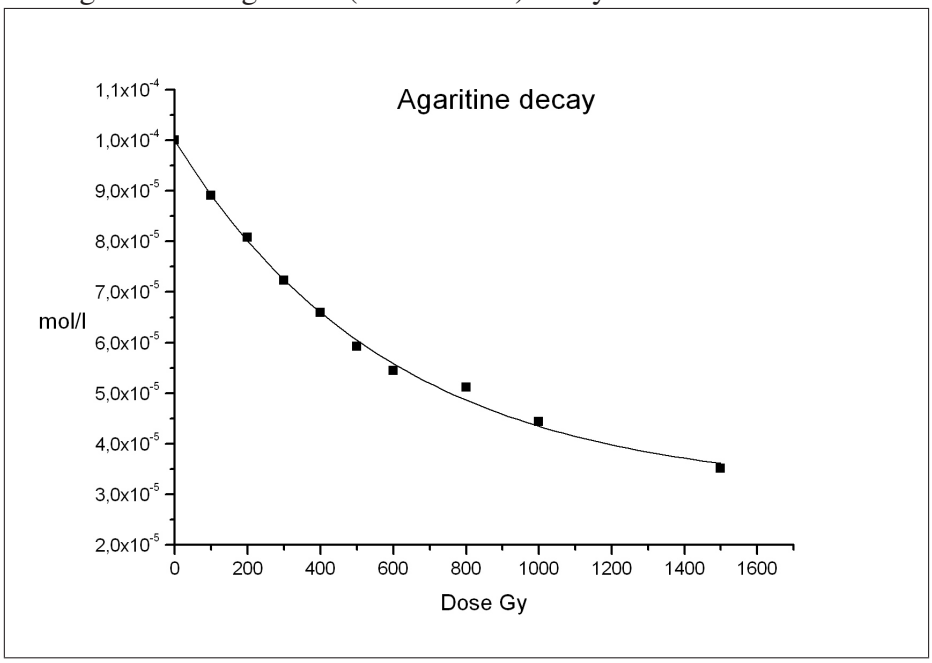
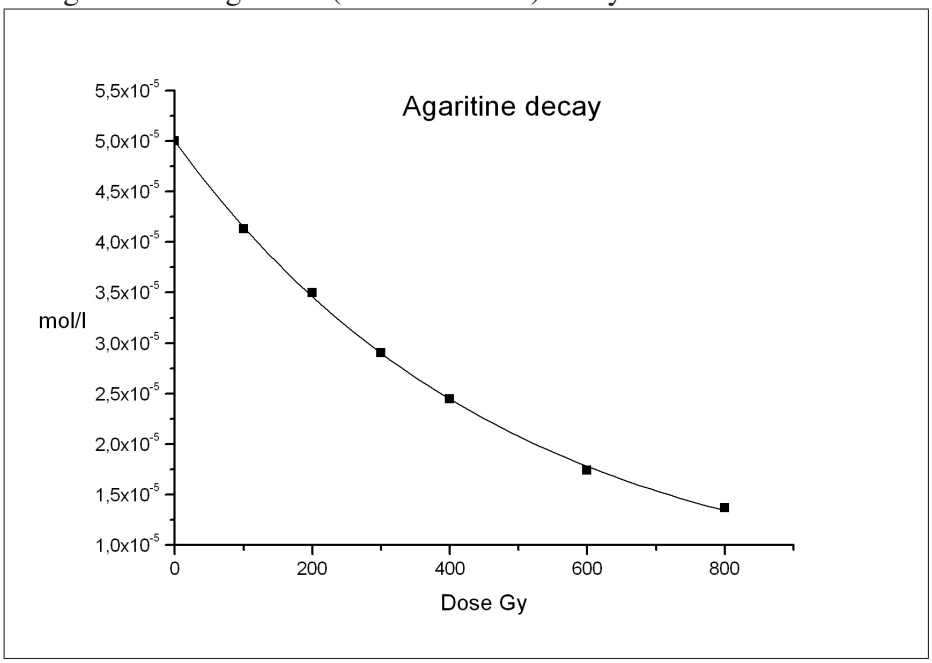


Figure 4.16: Agaritine ($5x10^{-5}$ mol l^{-1}) decay as a function of dose



New formed irradiation products, generated from bond cleavage of agaritine, were further expected. However, no other compounds apart from agaritine appeared in the HPLC chromatograms of both concentrations at the wavelength 237 nm.

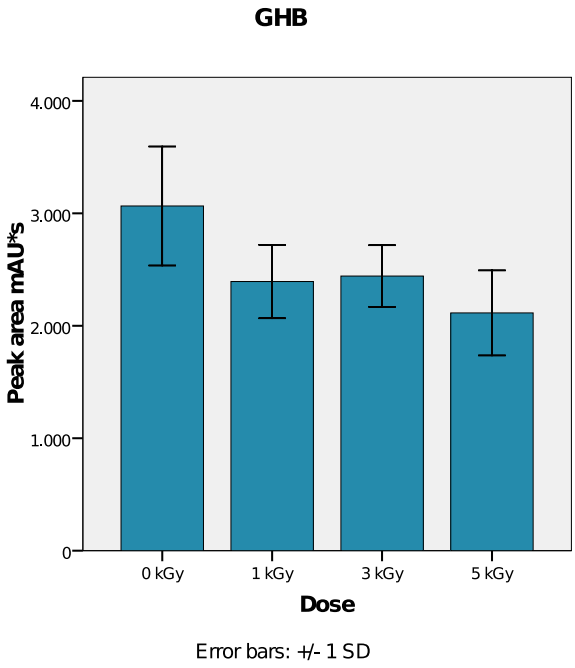
4.4.2.3 GHB

Table 4.8: GHB - Descriptive statistics (Peak area mAU*s)

	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	7	3065,29	3775	2293	528,82	3103	17,25
1 kGy	7	2393,43	2755	1944	326,59	2492	13,65
3 kGy	7	2442,43	2749	2120	273,63	2513	11,20
5 kGy	7	2114,43	2678	1784	377,98	1912	17,88

GHB is the main phenolic compound in mushrooms. Its content varies according to the developmental stage and the tissue of the mushroom, the highest amounts being present in the gills. According to Rast et al., 1979, a fully developed fruit body (cap diameter of 120 mm) contains GHB at a concentration of 6.4 mg/g DW. Taken the gills themselves, 21.3 mg/g DW were found. The obtained results are expressed in peak areas as GHB was not available as reference compound (Table 4.8). Quantification was, therefore, not possible.

Figure 4.17: Mean values of GHB (peak areas) at different doses



Up to now, examinations on effects of irradiation on GHB in mushrooms have not been carried out. However, Beaulieu et al., 1999, investigated the fate of GHB precursors including chorsimic acid, prephrenic acid, 4-aminobenzoic acid, 4-aminophenol, and tyrosine in irradiated mushrooms. There, irradiation with 2 kGy (at two different dose rates: 4.5 kGy/h and 32 kGy/h) had no influence on the amounts of chorsimic acid, prephrenic acid, and 4-aminobenzoic acid in mushrooms, whereas the content of 4-aminophenol was significantly higher in the control. Tyrosine will be discussed in Chapter 4.5.3.2.

In these investigations, a significant decrease of GHB was observed when irradiated at 1, 3 and 5 kGy (Fig. 4.17). The Tukey post hoc test underlined the trend shown in the graphic. There existed a statistically significant difference between the unirradiated sample and each sample irradiated at the mentioned doses. As there are only data about irradiation of GHB precursors in mushrooms and not GHB itself available, the results are not comparable.

4.5 Nucleotides and Amino Acids

4.5.1 Qualitative Analysis

Components of mushrooms were identified using HPLC-UV and HPLC-MS analyses. The extracts prepared for nucleotides (pH 4.3) and amino acids (pH 3.3) analyses (Chap. 3.5) were used.

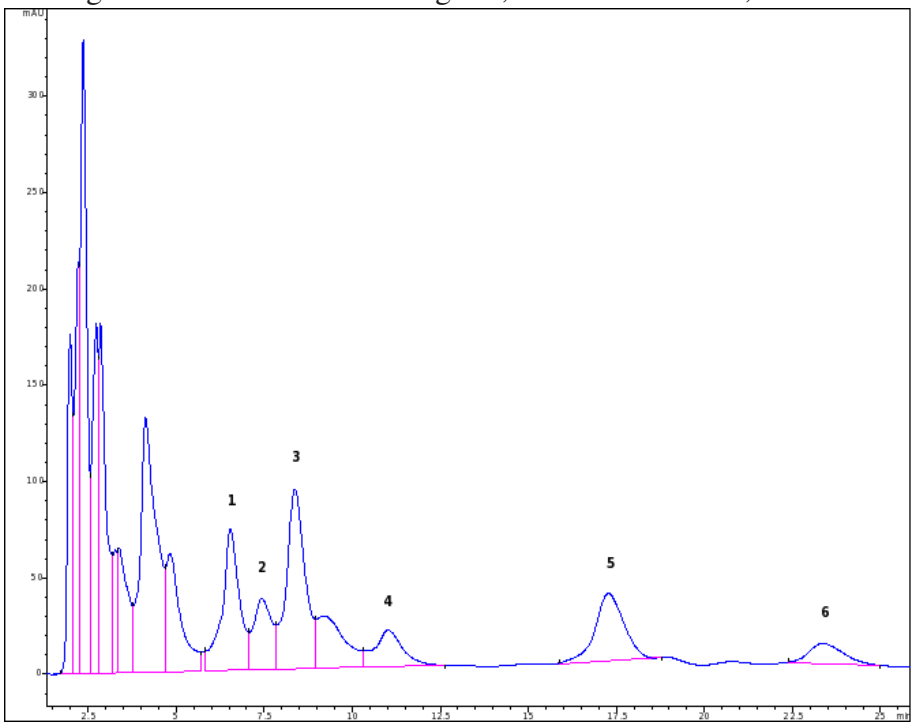
4.5.1.1 HPLC-UV

During our investigations, the pH-value of the mobile phase and the UV detection wavelength was adapted to chemical characteristics of the selected compounds.

4.5.1.1.1 Nucleotides, pH 4.3

The HPLC chromatogram obtained from the nucleotide extracts at pH 4.3 is presented in Fig. 4.18.

Figure 4.18: HPLC chromatogram, nucleotide extract, 260 nm



Six compounds were chosen (on the basis of clear UV spectra) for quantitative analysis; at least three of them could be definitely identified. These compounds are listed in Table. 4.9. The abbreviations MP and DP used in the following descriptions signify monophosphate and diphosphate. The letter in the front represents the nucleotide base.

Table 4.9: Elution of compounds from nucleotide extract

Peak number	Compound	Retention time (min)
1	unknown	6,536
2	unknown	7,427
3	*AMP	8,366
4	*GDP	11,013
5	*GMP	17,267
6	unknown	23,345

* A: adenine, G: guanine

UV-spectra The absorption spectra of peak 1-6 are summarized in Figs. 4.19-4.24. On the basis of the UV-spectra, four compounds could be identified. AMP and GMP are in conformity with their standards (overlaid in red). The similarity of the GDP (guanosine diphosphate) to the GMP spectrum and the fact that GDP was detected in mass spectrometric analysis was decisive to assign GDP to peak 5. The identification of peak 1 turned out to be more difficult. As can be seen in Fig. 4.19, the UMP (U: uridine) standard spectrum perfectly fits to its spectrum. Therefore, peak 1 is assumed to be identified as UMP, but not definitely, since UMP was not found in the mass spectrometric analysis.

Figure 4.19: UV-Peak 1, UMP , n.e.*

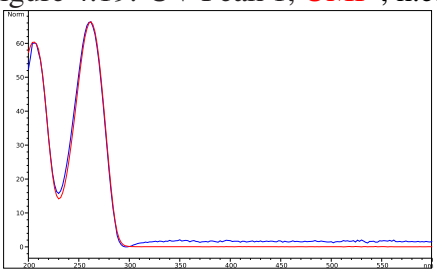


Figure 4.20: UV-Peak 2, n.e.*

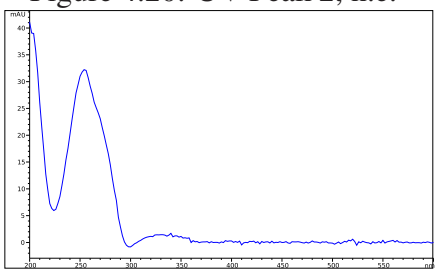


Figure 4.21: UV-AMP

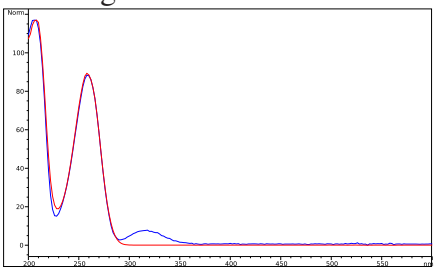


Figure 4.22: UV-GDP, GMP

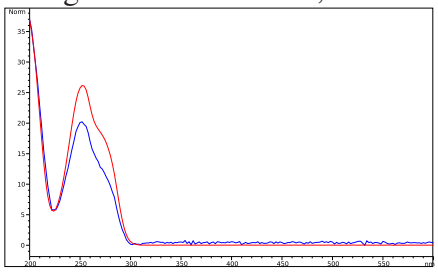


Figure 4.23: UV-GMP

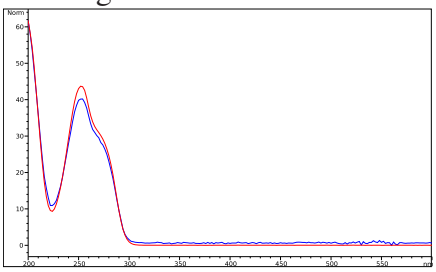
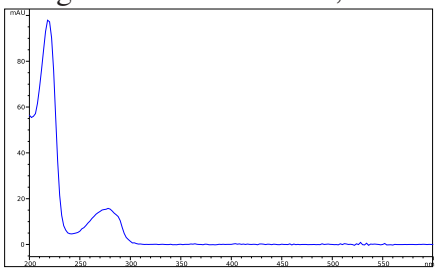


Figure 4.24: UV-Peak 6, n.e.*



* nucleotide extract

4.5.1.1.2 NAD and Amino Acids, pH 3.3

For this purpose, the extracts of amino acids analysis (Chap. 3.5) were used. Tyrosine was found in all extracts, but was been shown to be most pure and visible using the amino acids extracts. Since phenylalanine, tyrosine and NAD (nicotinamide adenine dinucleotide) vary in their peak maxima, three different detection wavelengths were used for HPLC analysis (Fig. 4.25). Their retention times are given in Table 4.10.

Figure 4.25: HPLC chromatograms, amino acids exctract, 205 nm, 225 nm, 260 nm

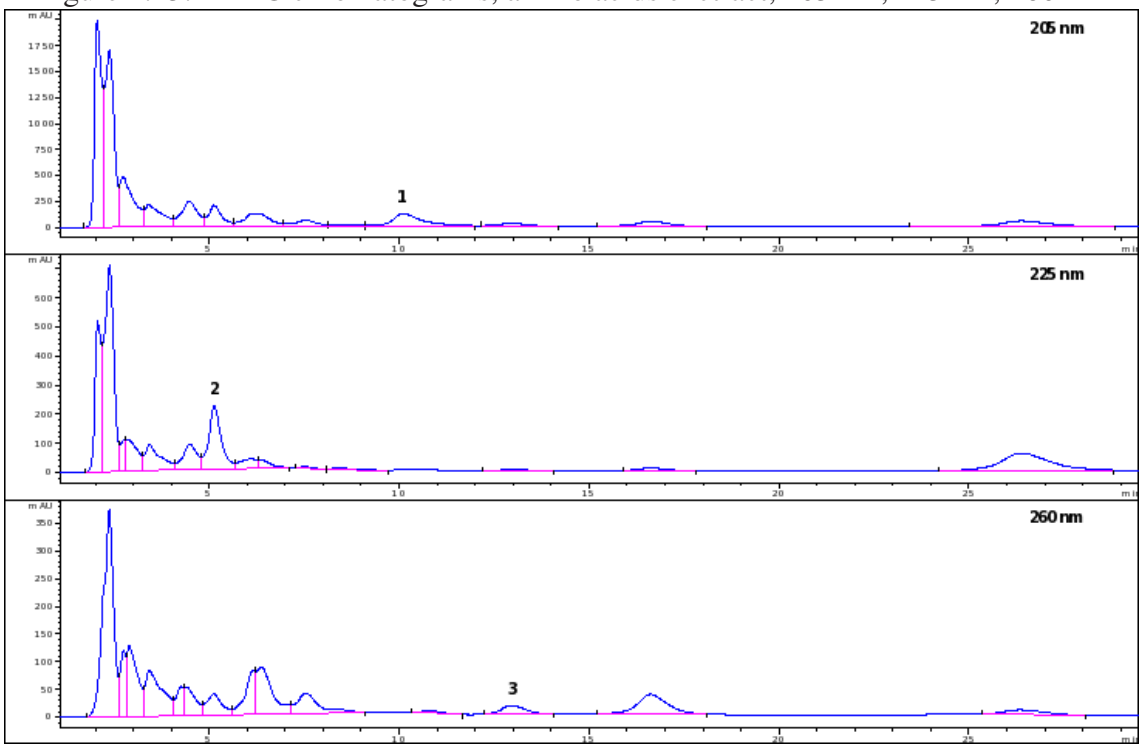


Table 4.10: Elution of compounds from amino acids extract

Peak number	Compound	Retention time (min)
1	Phenylalanine	10,114
2	Tyrosine	5,121
3	NAD*	12,960

*nicotinamide adenine dinucleotide

UV-spectra The absorption spectra of all three compounds are consistent with their standards (overlaid in red) (Figs. 4.26-4.28).

Figure 4.26: UV-phenylalanine

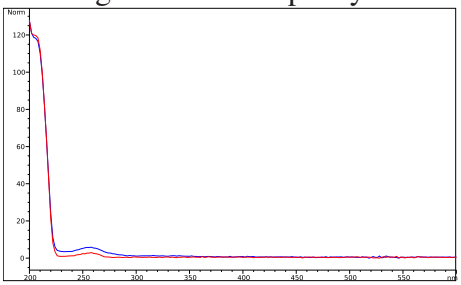


Figure 4.28: UV-NAD

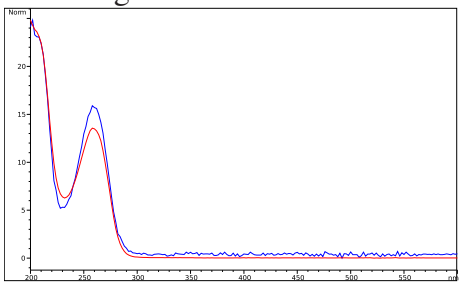
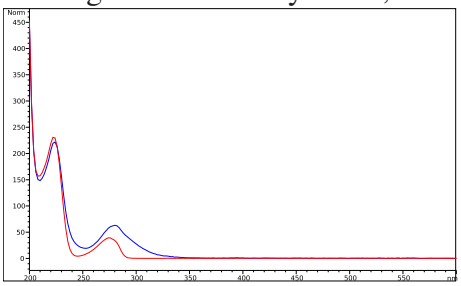


Figure 4.27: UV-tyrosine, a.a.e.*



* amino acids extract

4.5.1.2 HPLC-MS

The molecular weights of all compounds supposed to be detected in mass spectrometric analyses are listed in Table 4.11.

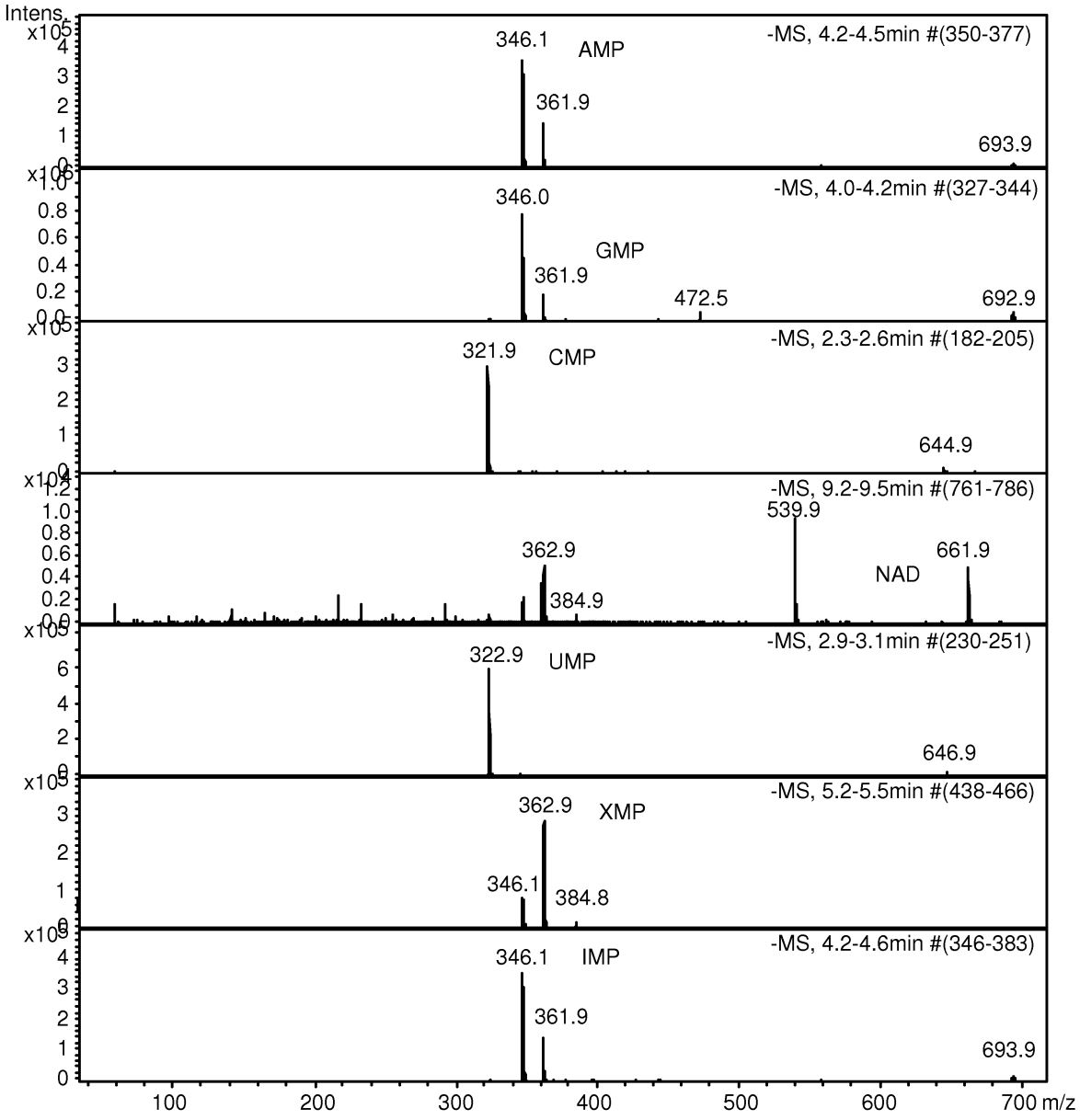
As this study was conducted on the basis of the description provided by Tsai et al., 2007, six different types of 5′-nucleotides were expected to be found, namely AMP, CMP, GMP, IMP, UMP, and XMP. Therefore, all nucleotide standards were injected under the same condition as the sample into the HPLC-MS. Since, from HPLC-UV analysis, NAD was assumed to be contained in the sample, its standard was added as well, Fig. 4.29.

Table 4.11: Molecular weight of nucleotide phosphate compounds

Compound	Molecular weight (g/mol)
AMP	347,22
GMP	363,22
*CMP	323,20
NAD	663,43
*UMP	324,19
*XMP	364,21
*IMP	348,21
GDP	443,20
Phenylalanine	165,19
Tyrosine	181,19

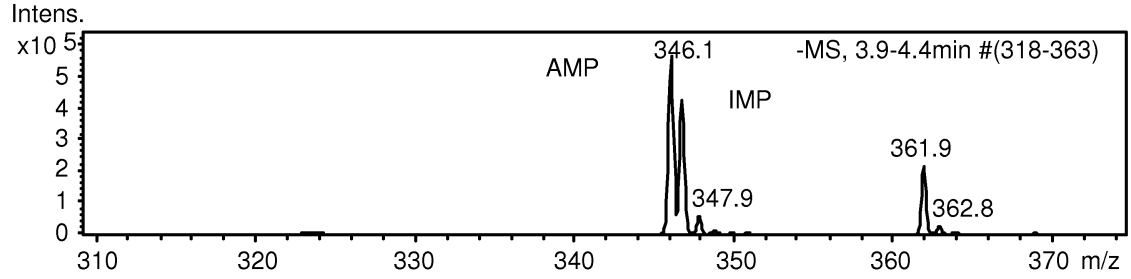
*C: cytidine, X: xanthosine, I: inosine, U: uridine

Figure 4.29: Nucleotide standards: MS-spectra, negative mode



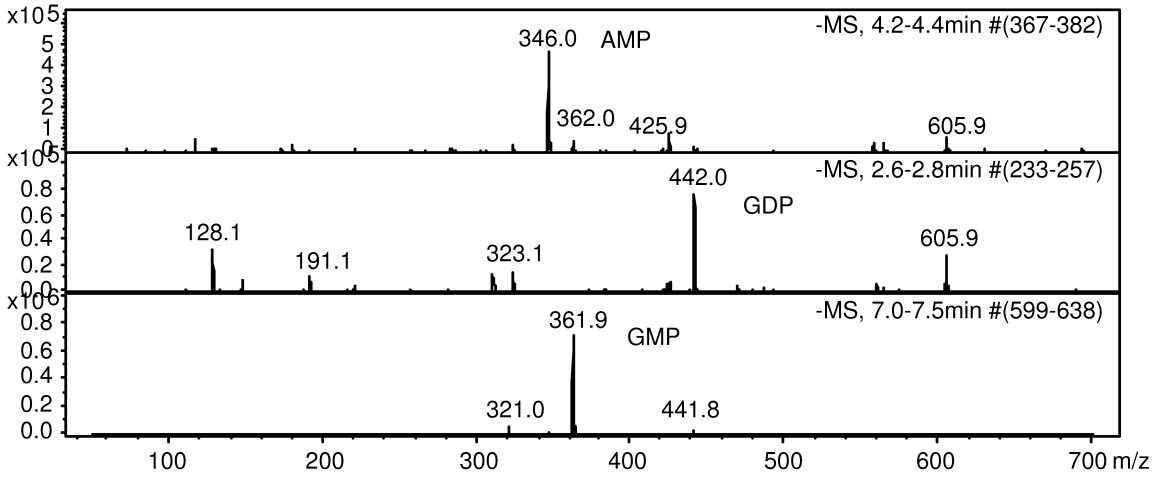
MS-spectra of AMP and IMP are identical. The reason for that is the coeluation of both compounds, the $[M\text{-}H]^-$ (negative mode) of IMP is observed in a spectrum together with the $[M\text{-}H]^-$ m/z 346 of AMP (Fig. 4.30).

Figure 4.30: AMP and IMP: MS-spectrum, negative mode



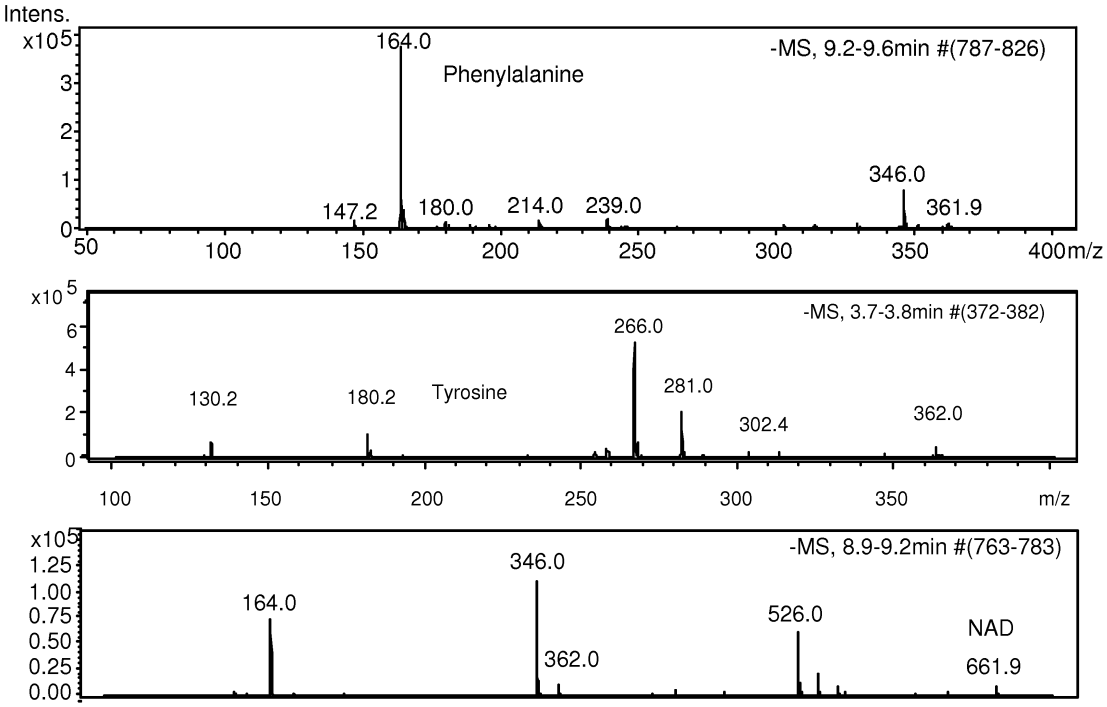
In the mushroom sample of this study, only two of the six nucleotides reported by Tsai et al., 2007, could be identified. Additionally, as the UV spectrum indicated GDP to be present in the sample, it was screened for this compound as well. Fig. 4.31 shows the MS-spectra of the detected nucleotides.

Figure 4.31: AMP, GMP, GDP: MS-spectra, negative mode



Mass spectrometric analysis of phenylalanine, tyrosine, and NAD (Fig. 4.32) could support the findings from HPLC-UV analysis. All three compounds were found, besides, NAD presence could be confirmed by standard injection. DOPA (3,4-dihydroxyphenylalanine), the oxidative product of tyrosine, was identified in the sample as well.

Figure 4.32: Phenylalanine, tyrosine, NAD: MS-spectra, negative mode



4.5.2 Quantitiative Analysis

HPLC-UV Quantitative analysis was performed by manual integration of the peak areas in the chromatogram obtained from nucleotide analysis, 260 nm (Fig. 4.18).

4.5.2.1 Nucleotides, pH 4.3

4.5.2.1.1 Peak 1

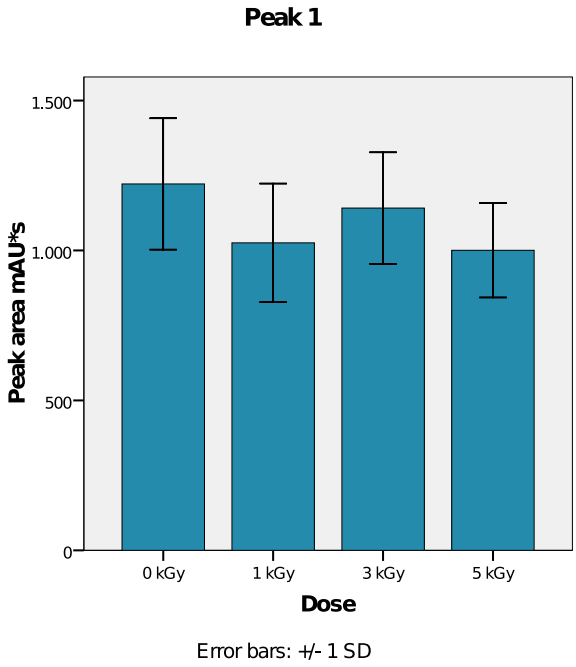
The mean values of peak areas as a function of dose are summarized in Table 4.12 and Fig. 4.33.

Table 4.12: Peak 1 - Descriptive statistics (Peak area mAU*s), nucleotide extract

	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	4	1222	1459	1027	219,04	1180	17,92
1 kGy	4	1025,50	1147	797,50	197,60	1132	19,27
3 kGy	4	1141,50	1293	934	185,94	1197,50	16,29
5 kGy	4	1000,67	1140	830	157,36	10326	15,73

Since peak 1 cannot be definitely claimed to be UMP, it is impossible to quantify this compound. For analysis, peak areas were compared.

Figure 4.33: Mean values of peak 1 (peak areas) at different doses, nucleotide extract



The graphic shows no tendency towards an increase or decrease of this unknown compound. Statistical analysis did not produce significant results as well. This is due to the high variation of measurements, which might be caused by insufficient separation of compounds in the HPLC chromatogram.

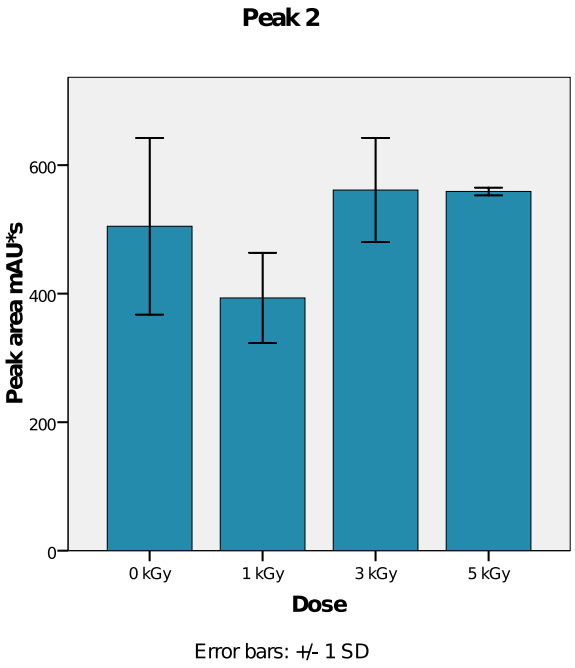
4.5.2.1.2 Peak 2

The mean values of peak areas as a function of dose are summarized in Table 4.13 and Fig. 4.34.

Table 4.13: Peak 2 - Descriptive statistics (Peak area mAU*s), nucleotide extract

	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	3	504,83	636	362	137,37	516,5	27,21
1 kGy	3	393,33	468	328	70,47	384	17,91
3 kGy	3	561,33	621	469	81,09	594	14,45
5 kGy	3	559	566	555	6,08	556	1,09

Figure 4.34: Mean values of peak 2 (peak areas) at different doses, nucleotide extract



Concerning peak 2, there is no evidence about the identity of this compound. Due to results from analysis, γ-irradiation does not seem to influence the concentration of peak 2. However, the high variation coefficient of the measurements has to be considered. High variation is also the reason for non-significant results of the one-way ANOVA.

4.5.2.1.3 AMP

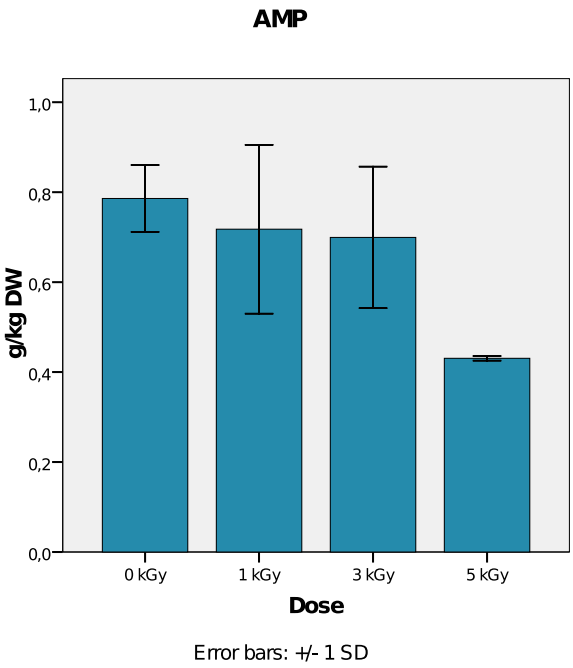
Table 4.14: AMP - Descriptive statistics (g/kg DW)

	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	3	0,79	0,86	0,72	0,07	0,78	9,46
1 kGy	3	0,72	0,88	0,51	0,19	0,76	26,15
3 kGy	3	0,70	0,87	0,57	0,16	0,66	22,51
5 kGy	2	0,43	0,43	0,43	0,01	0,43	1,20

AMP is a free 5′-nucleotide contributing to the umami taste intensity in mushrooms (Tsai et al., 2007). The AMP content in mushrooms has been measured by Tseng and Mau, 1999, and Tsai et al., 2007. The results are inconsistent: 0.26 mg/g DW and 2.04

g/kg DW. The obtained results lie in between, and can, therefore, be regarded as realistic values (Table 4.14).

Figure 4.35: Mean values of AMP (g/kg DW) at different doses



Irradiation of 5′-nucleotides has not been subject of research yet. In this study, a tendency to a decrease in the AMP content with increasing doses was observed. However, this interpretation has to be attended with caution due to high variation of the values. The results are not statistically significant and the value dose 5 kGy only includes two measurements (Fig. 4.35).

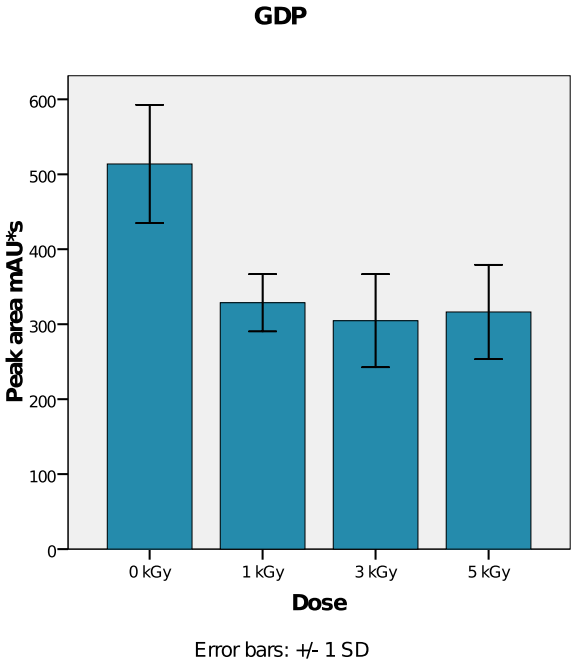
4.5.2.1.4 GDP

GDP was included in this analysis by reason that it is a dinucleotide, which, in addition to AMP and GMP, is another compound to examine the influence of γ-irradiation on mushrooms. Since it was not available as reference compound, peak areas were used for calculations. The results are summarized in Table 4.15 and Fig. 4.36.

Table 4.15: GDP - Descriptive statstics (Peak area mAU*s)

	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	2	468,75	480	457,50	15,91	468,75	3,39
1 kGy	3	328,67	369	293	38,21	324	11,63
3 kGy	3	304,67	365	241	62,07	308	20,37
5 kGy	3	316,33	370	247	62,99	332	19,91

Figure 4.36: Mean values of GDP (peak areas) at different doses



According to these results, there seems to be a definite effect of irradiation on GDP in mushrooms, and that concerns all doses applied. Statistically examined, GDP in the unirradiated sample showed a significant difference to all irradiated samples (1 kGy, 3 kGy and 5 kGy). It should be minded that there were only two measurements included in the analysis at dose 0 kGy.

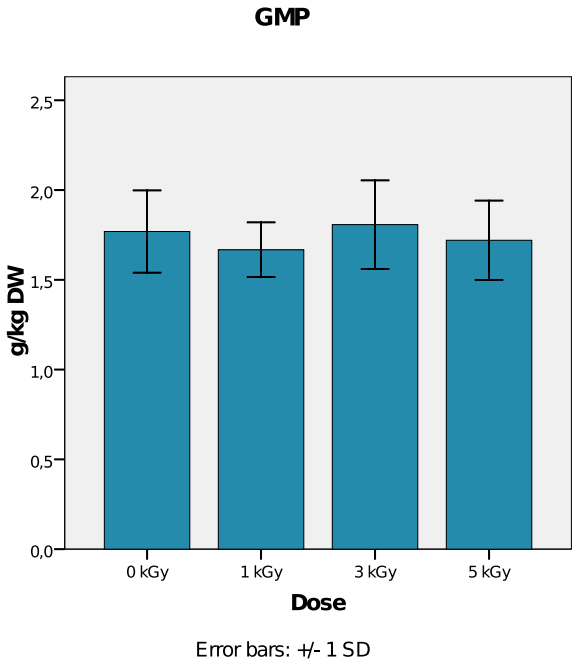
4.5.2.1.5 GMP

Table 4.16: GMP - Descriptive statistics (g/kg DW)

	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	4	1,77	2,02	1,46	0,23	1,80	12,94
1 kGy	4	1,67	1,90	1,57	0,15	1,60	9,15
3 kGy	3	1,81	1,95	1,52	0,25	1,95	13,64
5 kGy	4	1,72	2,00	1,54	0,22	1,67	12,85

GMP is the second 5′-nucleotide after AMP that was identified and analyzed. As for AMP, there are basically two literature sources providing information about the GMP concentrations in mushrooms (Tseng and Mau, 1999; Tsai et al., 2007). Tseng and Mau, 1999, found notably higher amounts of GMP, namely 1.52 g/kg DW, than Tsai et al., 2007, who assessed 0.13 mg/g DW. If we take the values of Tseng and Mau, 1999, for comparison, the obtained results are well located (Table 4.16).

Figure 4.37: Mean values of GMP (g/100g DW) at different doses



By contrast to AMP and GDP, no decrease in the GMP content of mushrooms irradiated at different doses is observed. Actually, no trend can be deduced at all. In addition,

this assessment is accompanied by high variations of the values. Statistical analysis produced non-significant results as well (Fig. 4.37).

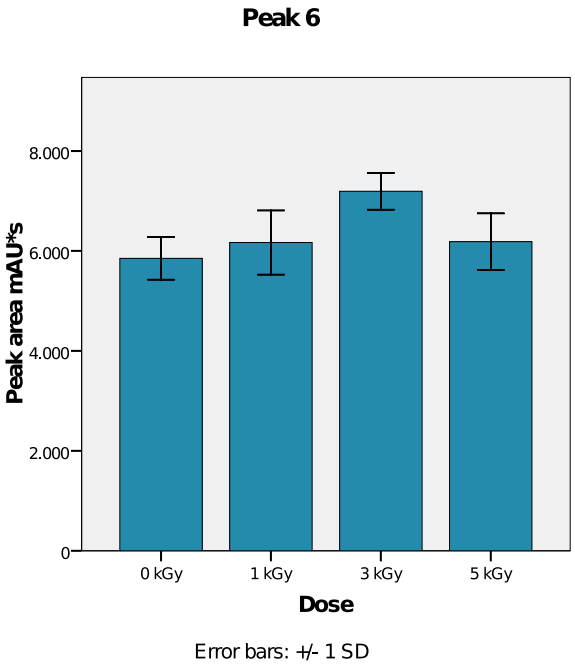
4.5.2.1.6 Peak 6

Peak 6 is another unknown compound, where the peak areas were used for analysis (Table 4.17, Fig. 4.38).

Table 4.17: Peak 6 - Descriptive statistics (Peak area mAU*s), nucleotide extract

	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	3	5852,67	6312	5468	426,93	5778	7,29
1 kGy	3	6169,33	6688	5451	642,22	6369	10,41
3 kGy	3	7194,67	7437	6771	368,17	7376	5,12
5 kGy	3	6185,67	6835	5790	566,80	5932	9,16

Figure 4.38: Mean values of peak 6 (peak areas) at different doses, nucleotide extract



The graphic of peak 6 is difficult to interpret. It seems that its content increases up to dose 3 kGy and afterwards decreases again. In fact, statistically analysis displayed a

significant difference between the unirradiated sample and dose 3 kGy. All other obtained results were not statistically significant.

4.5.3 NAD and Amino Acids, pH 3.3

4.5.3.1 Phenylalanine

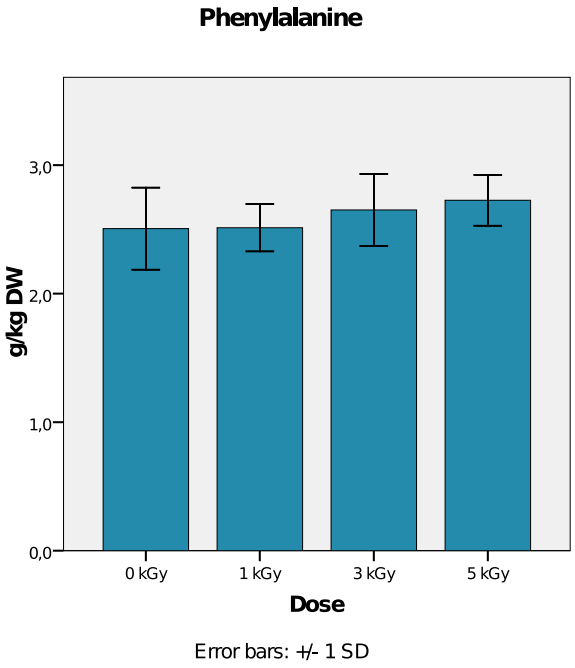
Table 4.18: Phenylalanine - Descriptive statistics (g/kg DW)

	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	3	2,60	2,95	2,35	0,31	2,49	12,08
1 kGy	4	2,51	2,75	2,33	0,18	2,49	7,34
3 kGy	3	2,76	2,99	1,41	0,22	2,74	7,89
5 kGy	4	2,72	2,96	2,48	0,20	2,73	7,26

When comparing the phenylalanine concentrations in mushrooms, one has to distinguish between total phenylalanine (bound in proteins) and free phenylalanine. For example, Mattila et al., 2002, found 107 mg/100g FW for total phenylalanine, which is not comparable with the results of this study, since the free phenylalanine content in dried mushrooms was analyzed. Although Oka et al., 1981, determined free phenylalanine (0.29 μmol/g), they did it on a fresh weight basis and this makes, together with a different data expression, a comparison impossible as well. The authors, Tseng and Mau, 1999, and Tsai et al., 2007 provided comparable values: 6.92 g/kg DW and 0.82 mg/g DW, respectively. According to them, the obtained results lie in a realistic range (Table 4.18)

Radiation effects on phenylalanine in mushrooms have not been reported yet. It was only measured indirectly, when contributing to the total phenolic content (Benoît et al., 2000, and Beaulieu et al., 2002). One might observe a slight increase in the phenylalanine content with increasing dose (Fig. 4.39). However, statistical analysis showed no significant changes.

Figure 4.39: Mean values of phenylalanine (g/kg DW) at different doses



4.5.3.1.1 Irradiation of Phenylalanine in Aqueous Solution

As for agaritine, the properties of phenylalanine in irradiated mushrooms were compared with those in aqueous solution. The irradiation effect on phenylalanine ($5x10^{-4}$ mol l^{-1}) in air saturated aqueous solution is demonstrated in Fig. 4.40. A fast degradation is observable and the 50% decay is achieved at a dose of 850 Gy. Concomitantly, ortho-, meta-, and para-tyrosine are formed (Fig. 4.41). In agreement with the literature (Krajnik et al., 1995), ortho-tyrosine was the principal product, followed by meta-tyrosine and, finally, para-tyrosine. Linear proportionality between tyrosine isomer formation and dose is observable up to 200 Gy. These hydroxylation products are a consequence of the reaction of OH-radicals with the substrate, and it is to be mentioned that the formation of meta- and especially ortho-tyrosine can be used as detection method for irradiated food (Mc Murray et al., 1996; Krach et al., 1997, 1999). In mushrooms, the phenylalanine content is practically not influenced by irradiation, it even might slightly increase. No tyrosine isomer could be found.

Figure 4.40: Phenylalanine decay ($5x10^{-4}$mol l^{-1}) as a function of dose

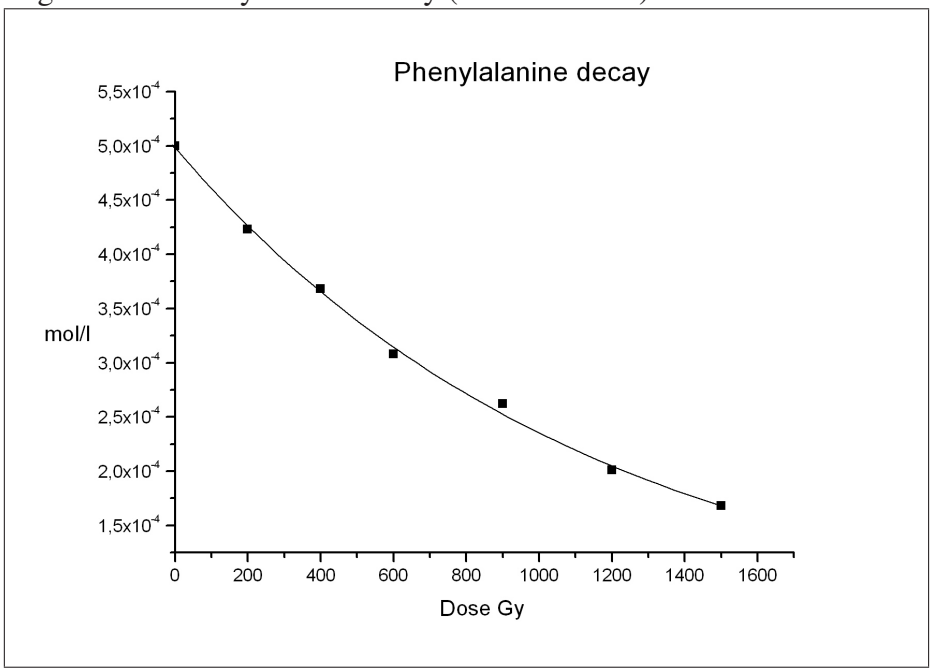
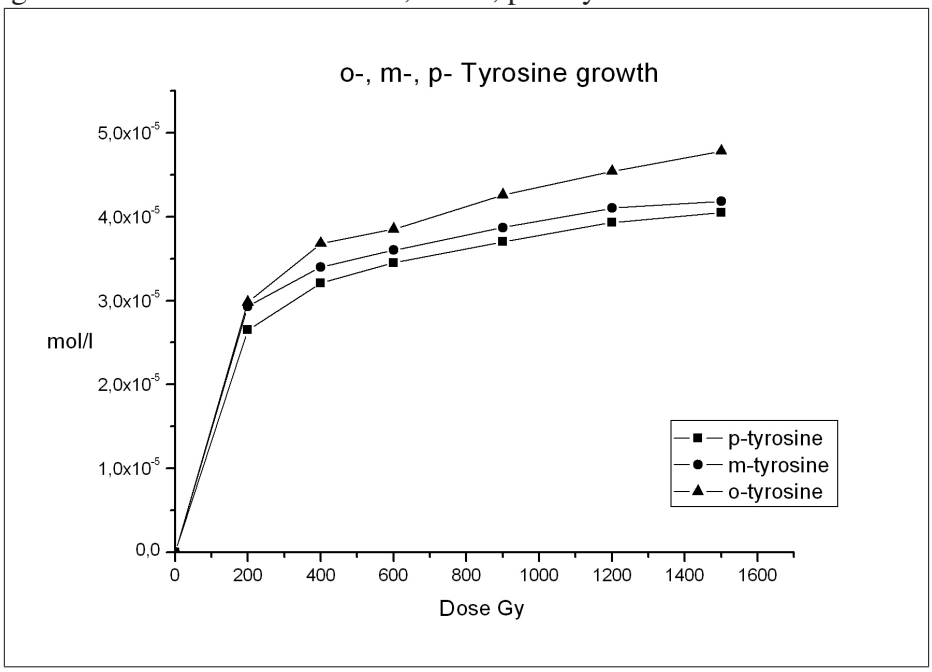


Figure 4.41: Formation of ortho-, meta-, para-tyrosine as a function of dose



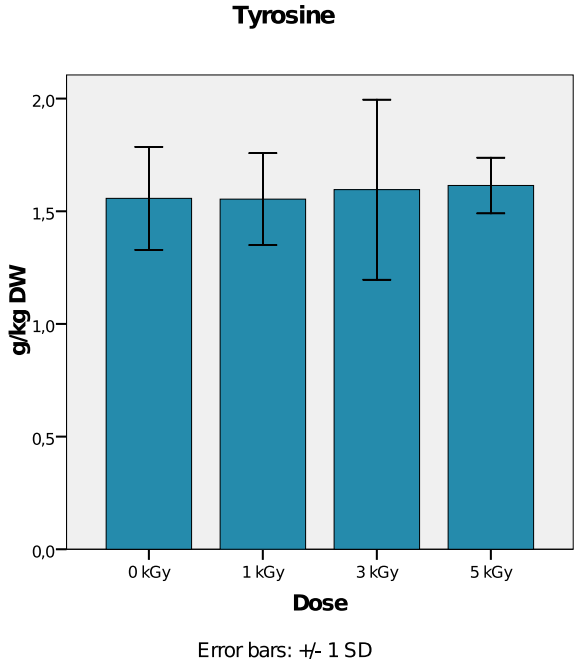
4.5.3.2 Tyrosine

Table 4.19: Tyrosine - Descriptive statistics (g/kg DW)

	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	3	1,65	1,84	1,49	0,17	1,61	10,53
1 kGy	4	1,55	1,85	1,38	0,20	1,49	13,12
3 kGy	4	1,74	2,00	1,47	0,26	1,75	15,01
5 kGy	3	1,61	1,73	1,49	0,12	1,62	7,67

The same difficulties as for phenylalanine arise when comparing tyrosine contents with values stated in literature. Information provided by Mattila et al., 2002, who found 283 mg/100g FW for total tyrosine concentration and Oka et al., 1981, who named 0.31 μmol/g FW of free tyrosine, is not comparable with the results in this study. If we take the results from Tseng and Mau, 1999, (1.11 g/kg) and Tsai et al., 2007, (1.28 mg/g DW), both assessing the free tyrosine content in dried mushrooms, the obtained result, 1.65 ± 0.17 g/kg DW, comes very close to them (Table 4.19).

Figure 4.42: Mean values of tyrosine (g/kg DW) at different doses



When describing the irradiation effect on GHB, it was already mentioned that tyrosine had been subject to irradiation investigations conducted by Beaulieu et al., 1999. Tyrosine constitutes a precursor of GHB and is one of the major phenolic compounds in mushrooms (Espin et al., 1999). Beaulieu et al., 1999 observed higher amounts of tyrosine in mushrooms irradiated at 2 kGy (dose rates: 4.5 kGy/h and 32 kGy/h) than in the control. The dose rate of 4.5 kGy/h even yielded substantially higher tyrosine concentrations. By contrast, the obtained results do not show any effect of irradiation at all (Fig. 4.42). This was confirmed by statistical analysis.

4.5.3.2.1 Irradiation of Tyrosine in Aqueous Solution

Radiation induced formation of DOPA from tyrosine in air saturated aqueous solution has been demonstrated earlier (Boguta and Dancewicz, 1981). Hydroxyl radical addition to p-Tyrosine preferentially yields 3,4-dihydroxyphenylalanine (3,4-DOPA), whereas o-Tyrosine hydroxylation generates 2,3-dihydroxyphenylalanine (2,3-DOPA) and 2,5-dihydroxyphenylalanine (2,5-DOPA) (Żegota et al., 2005). These observations were confirmed by irradiating para-tyrosine at concentrations of $5x10^{-4}$mol l^{-1} and 10^{-3} mol l^{-1} in air saturated aqueous solutions.

Fig. 4.43 and Fig. 4.44 show the tyrosine decay and DOPA growth at the mentioned concentrations. The 50% decay can only be assessed at the concentration of $5x10^{-4}$mol l^{-1}. There, it is achieved with the application > 1500 Gy. Comparing the result with that of $5x10^{-4}$mol l^{-1} phenylalanine (50% decay at a dose of 850 Gy), it is obvious that tyrosine is much more radiation resistant.

4.5.3.3 NAD

Table 4.20: NAD - Descriptive statistics (mg/100g DW

	Nr. of Measure-ments	Mean value	Maximum	Minimum	Standard deviation	Median	Variation coefficient %
0 kGy	3	78,20	86,16	69,31	8,46	79,13	10,82
1 kGy	3	61,55	65,06	57,77	3,66	61,81	5,94
3 kGy	4	72,88	77,21	68,51	4,78	72,89	6,55
5 kGy	4	72,59	78,13	67,32	5,77	72,46	7,95

NAD functions as coenzyme in mushrooms. The glutamate dehydrogenase, a key enzyme in the nitrogen metabolism, is NAD dependent (Kersten et al., 1999). Its concentration

Figure 4.43: Tyrosine decay (10^{-3} mol l^{-1}) and DOPA growth as a function of dose

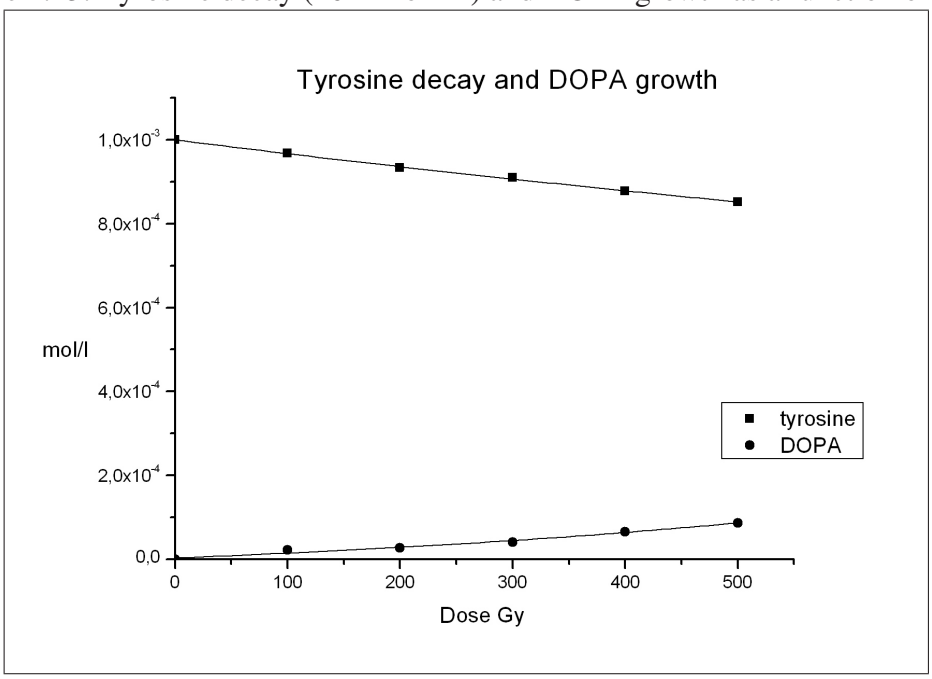
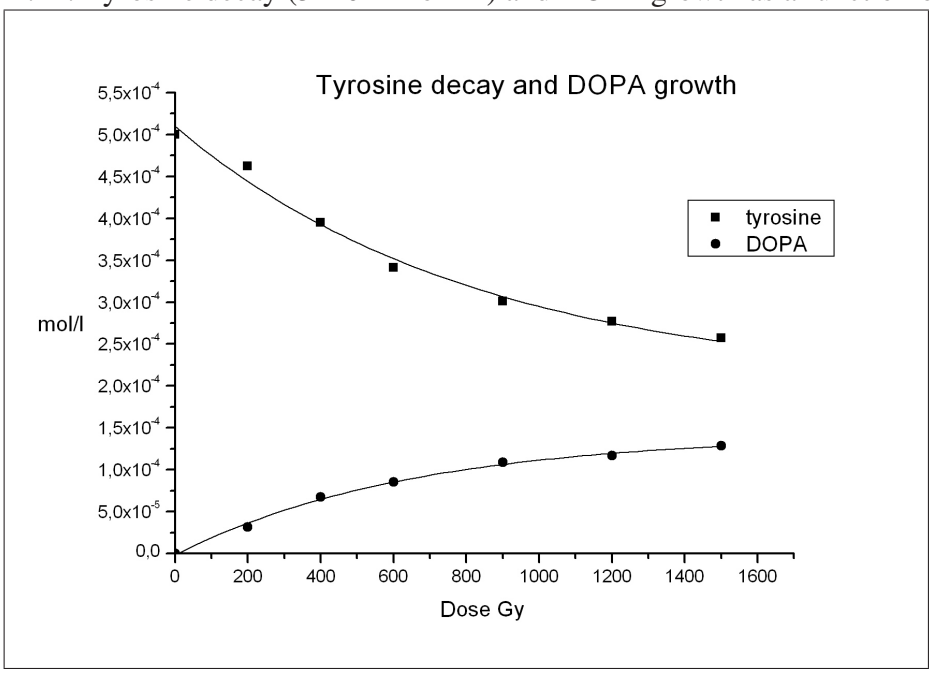
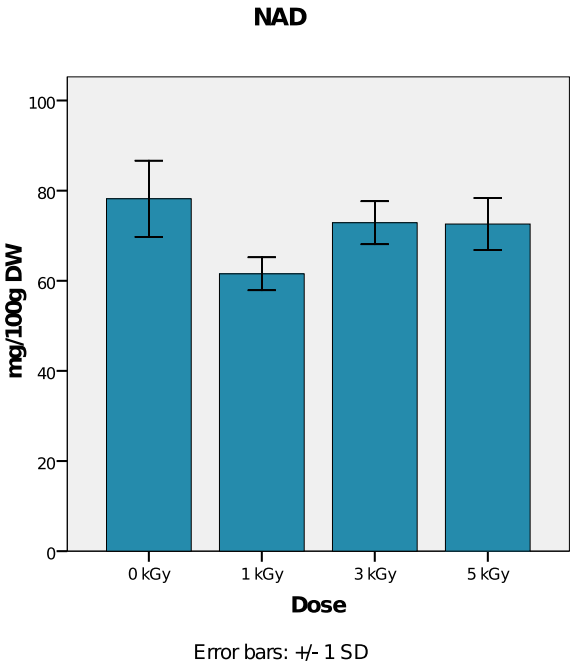


Figure 4.44: Tyrosine decay ($5x10^{-4}$ mol l^{-1}) and DOPA growth as a function of dose



in mushrooms has not been assessed yet, and the results are, therefore, not comparable (Table 4.20).

Figure 4.45: Mean values of NAD (mg/100g) at different doses



Analysis of NAD concentrations indicates at first a decrease with dose 1 kGy applied, and then again an increase at dose 3 kGy, which does not reach the level of 0 kGy (Fig. 4.45). Statistical analysis confirmed the significant difference between dose 0 kGy and dose 1 kGy. All other results were not significant.

Chapter 5

Conclusion

The high moisture content of mushrooms, which is supposed to be a favourable condition for the indirect effect of water radiolysis, let assume that gamma irradiation may have a significant impact on the content of individual compounds in mushrooms. However, apart from some minor effects, the study results did not confirm this assumption.

Concerning the concentration of total phenols in mushrooms, one might expect an increase when considering the enzymatic changes caused by irradiation. Beaulieu et al., 2002 explained the observed increase in the total phenolics content during storage by the enhanced activity of the PAL enzyme. Since this study was designed to measure the effect of irradiation on the concentrations of substances at a defined point of time (lyophilisation of mushrooms directly after irradiation), alterations might have been more of chemical than of enzymatic nature.

The TEAC assay has never been carried out with irradiated mushrooms yet and is, therefore, not comparable. Irradiation did not change the antioxidative activity of mushrooms in this study. There is a controversial discussion whether total phenols and antioxidative activity correlate well in mushrooms or not. These bioactive compounds are supposed to mainly contribute to the antioxidative activity. However, not all phenols are effective radical scavenging antioxidants (Huang et al., 2005), depending on various factors such as the number of hydroxyls bound to the aromatic ring and the site of bounding (Czapski, 2004). This might explain the rather poor correlation coefficient (0.633, $p<0.01$) found in this study.

The principal compound of interest, agaritine, showed a clear trend in this study; it decreased at dose 5 kGy, from 1.54 g/kg (0 kGy) to 1.35 g/kg. Due to the fact that gamma irradiation of mushrooms is only approved in a dose range from 1 to 3 kGy (IAEA, 2008), the agaritine decrease from dose 5 kGy does not play a role in the application of gamma irradiation for preservation purposes.

The concentrations of GHB in irradiated mushrooms were lower than in unirradiated

ones, being reduced at 1 kGy by 22%. Compared to the results of the TEAC-test, GHB, a major phenolic compound in mushrooms, does not seem to exert significant antioxidative effects as the antioxidative capacity did not change with increasing doses.
By contrast, the results of tyrosine go conform to the findings from the TEAC-test. Tyrosine does not seem to be affected by irradiation. Beaulieu et al., 1999, reported even higher tyrosine contents in irradiated samples, being explained by enzymatic change in the browning process.
The aromatic nature of phenylalanine makes it contribute to the browning process in mushrooms as well. Actually, it forms the basis of phenol production since phenylalanine deamination, catalyzed by PAL, to yield ammonia and trans-cinnamic acid is the initial step in phenol synthesis (Benoît et al., 2000). Whether gamma irradiation influences phenylalanine and, consequently, phenol contents in mushrooms or not, has never been investigated. The findings suggest no influence.
The inconsistent results of the nucleotide concentrations (AMP, GDP, GMP, and NAD) in mushrooms do not allow clear statement whether irradiation affects the concentrations of this class of compounds in mushrooms or not. As this was the first study to investigate nucleotides in irradiated mushrooms, no information of irradiation effects on nucleotides is provided by the literature.
The unknown compounds included in the analysis are difficult to discuss, since their chemical structures have remained unidentified. Apart from that, no clear trend could be deduced from the analysis of any of these compounds.

To conclude, the results of all selected compounds in mushrooms, there was hardly any effect of gamma irradiation observable. The fact that most of the compounds were analyzed in respect of gamma irradiation for the first time makes further investigations necessary. Additionally, it should be mentioned that gamma-irradiation with doses above 3 kGy is usually not applied for preservation purposes.

Chapter 6

Summary

The aim of this study was to investigate the effect of gamma irradiation on selected, individual compounds in the common cultivated white button mushroom, *Agaricus bisporus*. The selected substances basically included three different classes of compounds, namely hydrazines, melaenous phenols and nucleotides. For this purpose, four levels of irradiation (0 kGy, 1 kGy, 3 kGy, 5 kGy) were chosen in order to assess a trend in the concentrations of the substances in mushrooms. Examinations on the total phenolic content and antioxidative activity were carried out with FC-test and TEAC assay. The individual compounds were determined by mass spectrometry and HPLC analysis, using standards solutions for identification. Agaritine, phenylalanine, and tyrosine were irradiated in aqueous solution and analyzed for the purpose of comparison. Generally, the obtained results do not indicate a substantial influence of gamma irradiation on compounds in mushrooms. The biochemical tests, FC and TEAC, did not show any change in the levels of total phenols and antioxidative activity at the different doses. Associating the two tests, a rather poor correlation coefficient was found. The two main interesting compounds under investigation, the glutaminyl derivates agaritine and GHB, were both affected by irradiation, even though to a different extent. Agaritine concentrations decreased at the highest dose applied, GHB contents already at the lowest. Phenylalanine and tyrosine did not indicate alterations in their contents when being irradiated. Results of nucleotides were inconsistent, showing only decreased concentrations for AMP and GDP in irradiated samples. Analysis of compounds those were unidentified did not provide any definite results. These findings suggest a potential influence of gamma irradiation on a few individual compounds in mushrooms. However, the observed effect is much lower than expected. Additionally, it should be considered that doses above 3 kGy are usually not applied for preservation purposes.

Chapter 7

Zusammenfassung

Das Ziel der vorliegenden Arbeit war es, den Einfluss von Gamma-Strahlung auf bestimmte Inhaltsstoffe des Champignons, *Agaricus bisporus*, zu untersuchen. Die Inhaltsstoffe wurden aus drei verschiedenen Substanzklassen ausgewählt, den Hydrazinen, melaninbildenden Phenolen und Nukleotiden. Es wurden vier Bestrahlungsdosen angewandt (0 kGy, 1 kGy, 3 kGy, 5 kGy), um einen Trend der Konzentrationen dieser Inhaltsstoffe in Champignons erfassen zu können. Der Gesamtphenolgehalt und die antioxidative Kapazität wurden mittels FC- und TEAC-Test bestimmt. Die Bestimmung der Inhaltsstoffe wurde anhand von Massenspektrometrie und HPLC-Analyse durchgeführt. Für die Identifizierung der Substanzen wurden Standardlösungen verwendet. Agaritin, Phenylalanin und Tyrosin wurden zusätzlich in wässrigen Lösungen in Gegenwart von Luft bestrahlt, um das Verhalten dieser Substanzen in unterschiedlichen Medien zu vergleichen. Im Allgemeinen wurde kein deutlicher Einfluss der Bestrahlung auf die einzelnen Inhaltsstoffe festgestellt. Die biochemischen Tests, FC und TEAC, zeigten keine Veränderungen des Gesamtphenolgehaltes und der antioxidativen Aktivität bei Bestrahlung unterschiedlicher Dosen. Es wurde ein eher schlechter Korrelationskoeffizient zwischen den beiden Tests ermittelt. Die beiden interessantesten untersuchten Inhaltsstoffe des Champignons, Agaritin und GHB, zeigten einen Effekt der Strahlung auf ihre Konzentration, wenn auch in unterschiedlichem Ausmaß. Die Menge an Agaritin nahm erst bei der höchsten angewandten Dosis ab, während die an GHB bereits bei der niedrigsten Bestrahlungsdosis verringert war. Auf Konzentrationen von Phenylalanin und Tyrosin wirkte sich die Gamma-Strahlung nicht aus. Die Ergebnisse der Nukleotide waren widersprüchlich, nur AMP und GDP zeigten eine Konzentrationsabnahme in den bestrahlten Proben. Die Analyse der unbekannten Substanzen ergab keine eindeutigen Ergebnisse. Diese Ergebnisse weisen auf einen Einfluss von Gamma-Strahlung auf einige wenige Inhaltsstoffe im Champignon hin, wenn auch in geringem Ausmaß. Es sollte jedoch bedacht werden, dass Bestrahlungsdosen über 3 kGy in der Regel nicht angewandt werden.

Chapter 8

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List of Abbreviations

a.a.e	Amino acids extract
a.e	Agaritine extract
ABTS	2,2′-azinobis-(3-ethyl-benzothiazoline)-6-sulfonic acid
ACBs	Alkylcyclobutanones
ADMIT	Analytical detection methods for irradiation treatment of foods
Agaritine	β-N-(γ-L(+)-glutamyl)-4-(hydroxymethyl) phenylhydrazine
ANOVA	Analysis of variance
AMP	Adenosine 5′-monophosphate monohydrate
APC	Aerobic plate count
Bq	Bequerel
C	Carbon/Coulomb
CEN	European committee for standardization
CEM	Channel electron multipliers
Ci	Curie
CMP	Cytidine 5′-monophosphate
Co	Cobalt
Cs	Caesium
DC	Direct current
DEFT	Direct epifluorescent filter technique
DNA	Desoxyribonucleic acid
DOPA	3,4-dihydroxyphenylalanine
DW	Dry weight
EC	Electrochemical
e.g.	exempli gratia, “for example”
EG	Europäische Gemeinschaft
ESR	Electron spin resonance
EU	European union
EUC	Equivalent umami concentrations
eV	Electron volt

FAO	Food agriculture organisation
FC	Folin Ciocalteu
Fig.	Figure
FT	Fourier-transform
FW	Fresh weight
g	Gram
GAE	Gallic acid equivalent
GBQ	γ-L-glutaminyl-3,4-benzoquinone
GC	Gas chromatography
GCPH	N-(γ-L(+)-glutamyl)-4-(carboxy)phenylhydrazine
GDHB	γ-L-glutaminyl-3,4-dihydroxybenzene
GDP	Guanosine diphosphate
GHB	γ-L-glutaminyl-4-hydroxybenzene
GMP	Guanosine 5′-monophosphate disodium salt
Gy	Gray
h	Hours
HBA	4-hydrazinobenzoic acid
HIV	Humane immunodeficiency virus
HMBD	4-(hydroxymethyl)benzenediazoniumion
HMPH	4-(hydroxymethyl)phenylhydrazine
HPLC	High performance liquid chromatography
IAEA	International atomic energy association
IMP	Inosine 5′-monophosphate disodium salt
J	Joule
keV	Kilo electron volt
kGy	Kilogray
kg	Kilogram
l	Litre
LAL	Limulus amoebocyte lysate
LC	Liquid chromatography
LET	Linear energy transfer
m	Meta
M	Mole
mAU*s	Milli absorption units per second
mm	Millimetre
MeV	Mega electron volt
mg	Milligram

min	Minutes
ml	Millilitre
mM	Millimole
mmol	Millimole
mol	Mole
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
μl	Microlitre
μm	Micrometre
N	Nitrogen
NAD	β-Nicotinamide adenine dinucleotide hydrate
n.e.	nucleotide extract
nm	Nanometer
o	Ortho
O	Oxygen
p	Para
PAL	Phenylalanine ammonia-lyase
PPO	Polyphenol oxidase
Q	Quality factor
RF	Radiofrequency
ROS	Reactive oxygen species
RW	Radiation weighting factor
s	second
S	Sulfur
SEM	Secondary electron multipliers
Sv	Sievert
SI	Système International
TEAC	Trolox equivalent antioxidative capacity
TL	Thermoluminiscence
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UMP	Uridine 5′-monophosphate disodium salt
UV	Ultraviolett
UV/VIS	Ultrovilett/Visible
WHO	World health organisation
XMP	Xanthosine 5′-monophosphate disodium salt

Curriculum Vitae

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