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Esterified sterols and their contribution to the total sterols in edible mushrooms

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ABSTRACT

Mushrooms are a valued source of sterols, which can be present in free form and esterified to fatty acids. In addition to the principal ergosterol, several minor sterols occur. Ergosterol and ergosteryl ester were determined directly by gas chromatography coupled to mass spectrometry (GC/MS) in the trimethylsilylated lipid extract of each six samples of button mushrooms, oyster mushrooms, king trumpet mushrooms and shiitake. Free ergosterol was highest in button mushrooms 415–544 mg/100 g dry matter (d.m.) and lowest in oyster and king trumpet mushrooms (<350 mg/100 g d.m.). On the other hand, concentrations of ergosteryl esters were significantly lower (5–26 mg/100 g d.m.) in all mushroom samples. The distribution of minor sterols (most importantly ergosta-7,22-dienol, ergosta-5,7-dienol, ergosta-7-enol) between free and esterified form was determined after group separation with solid phase extraction. Higher proportions of the minor sterols were found esterified to fatty acids and ergosta-7-enol was found predominantly (70%) in this form in oyster mushrooms and shiitake. The minor sterols ergosta-8,24(24^1)-dienol, ergosta-8-enol and 7-dehydro-poriferasterol could be tentatively identified in the samples based on GC/MS data. In addition, four sterols were found only in the steryl ester fraction of the mushroom samples.

1. Introduction

Edible basidiomycetes are a class of mushrooms which are commonly consumed due to their characteristic taste (Chang and Miles, 2004; Teichmann et al., 2007; Villares et al., 2012). The bulk composition of edible basidiomycetes is comparable with vegetables in that they are also low in fat and calories but rich in water (Teichmann et al., 2007). In 2014, the annual intake of fresh mushrooms in Germany amounted to 1.7 kg/capita which is more than for example the one of peas and spinach (1.1 and 1.3 kg/capita) (Bundesministerium für Ernährung und Landwirtschaft, 2016). About 85% (~1.5 kg) of the intake originated from button mushrooms (Bund Deutscher Champignon- und Kulturpilzanbauer (BDC) e.V, 2015). One characteristic attribute that distinguishes mushrooms from vegetables is their notably higher content of sterols (Mattila et al., 2002; Piironen et al., 2003). Ergosterol, the major sterol in most edible basidiomycetes, is a valuable dietary precursor of vitamin D_2 and a natural antioxidant (Mattila et al., 2002; Phillips et al., 2011; Shao et al., 2010).

The concentration of ergosterol (and in few cases of other sterols) has been determined in different mushroom species sampled in retail markets in Portugal, Spain, Finland, Sweden, Singapore and USA (Barreira et al., 2014; Jasinghe and Perera, 2005; Mattila et al., 2002; Phillips et al., 2011; Teichmann et al., 2007; Villares et al., 2014). Typically, the total sterol content is determined after alkaline saponification of (extracted) lipids. Hence, this value includes ergosterol and other sterols present in free or esterified form. The most prevalent substance class of esterified sterols is esters with fatty acids (steryl esters). However, information on steryl esters and their contributions to the total sterol content in edible mushrooms is not obtained using typical analytical protocols.

Separate analysis of free and esterified sterols has been recognized as important due to the different physiological effects and properties of both compound classes. Studies on free and esterified sterols in plant oils and fats showed, that the ratio of free and esterified sterols was varying between individual sterols, oil sources and even within one oil source (Phillips et al., 2002; Verleyen et al., 2002b). Intake of phytosterols (which are structurally closely related to ergosterol) is connected with a decrease of the serum cholesterol level in humans (MacKay and Jones, 2011). Especially esterified sterols are commonly used as an

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additive in phytosterol-enriched food products (functional food), since they are easily dispersible in the food matrix, which is not the case for free sterols (Moreau et al., 2002). However, the liberation efficiency of the sterols (=active form) from steryl esters in humans depends on the exact structure of the steryl ester (Lubinus et al., 2013). In addition, Verleyen et al. noticed a different behavior of free and esterified sterols during oil refining (Verleyen et al., 2002a). Most commonly, both fractions are separated chromatographically using column chromatography or solid phase extraction cartridges. Subsequently, the sterol composition of both fractions can be analyzed individually (most commonly by gas chromatography), usually after alkaline saponification of the steryl esters (Abidi, 2001). With this procedure, the information on the fatty acids esterified to sterols can not be assessed, which prompted researchers to determine steryl esters in their intact form (Barnsteiner et al., 2012; Evershed and Goad, 1991).

Very few studies only explicitly reported on the steryl ester content of edible basidiomycetes. Villares et al. (2014) tentatively detected non-polar steryl conjugates and semi-quantified non-polar ergosteryl derivatives by HPLC-UV in button mushroom, oyster mushroom and shiitake from the Spanish market at ~100 mg/100 g d.m. or less (~5–20% contribution to the total ergosterol content). A lower quantity (~3% of the free ergosterol content) or no ergosteryl esters at all were determined in button mushrooms from Germany (Hammann and Vetter, 2016) and Canada (Shao et al., 2010). Finally, Yuan et al. detected low levels of esterified ergosterol (13–34 mg/100 g d.m., ~10% of total ergosterol) in different tissues of shiitake from China but found >50% of ergosterol esterified in the gills of poplar mushroom samples (*Agrocybe aegerita*) (Yuan et al., 2008). These sparse results indicated that steryl esters may represent a varying share of the total sterol content. In addition, virtually no data was available about steryl esters other than those of ergosterol.

The goal of this study was to determine the concentrations of esterified sterols and their contribution to the total sterol content in four species of edible basidiomycetes regularly consumed in Germany. Furthermore, we were interested to study whether this ratio between esterified and free sterols was rather constant or varied in individual samples. Samples of button mushrooms (*Agaricus bisporus*), oyster mushroom (*Pleurotus ostreatus*), king trumpet mushroom (*Pleurotus eryngii*) and shiitake (*Lentinus edodes*), originating from different suppliers (Table 1), were bought in retail markets in Germany and analyzed for their content of free and esterified ergosterol and further minor sterols.

2. Material and methods

2.1. Chemicals and standards

n-Hexane (HPLC grade) was from Th. Geyer (Renningen, Germany). Ethyl acetate (puriss., >99.5%), linoleic acid (puriss.), silica gel (silica gel 60, for column chromatography), pyridine (>99.9%, distilled prior to use), absolute ethanol (>99.8%) and *Candida rugosa* lipase (activity ≥700 U/mg solid) were from Sigma-Aldrich (Steinheim, Germany). Technical grade ethanol (distilled prior to use) and concentrated sulfuric acid were from BASF (Ludwigshafen, Germany). Helium (purity 99.9990%) was from Westfalen (Münster, Germany). Potassium hydroxide (>85%) was from Carl Roth (Karlsruhe, Germany) while α-cholestane (98%) and ergosterol (>97.5%) were from Acros Organics (Geel, Belgium). The silylating agent (N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and trimethyl-chlorosilane (TMCS), 99:1 (v/v)) was from Supelco (Bellefonte, PA, USA). Cholesterol (puriss.) was from Merck (Darmstadt, Germany). Pentadecanoic acid cholesteryl ester (15:0-CE) and ergosteryl esters (ErE) were prepared as described previously (Hammann and Vetter, 2016; Hammann et al., 2015).

2.2. Samples and sampling procedure

Three different packages (100–400 g or several lose mushrooms) of white button mushrooms (*Agaricus bisporus*), shiitake (*Lentinus edodes*), king trumpet mushrooms (*Pleurotus eryngii*) and oyster mushrooms (*Pleurotus ostreatus*) were bought in various retail markets in Stuttgart, Germany in January and February 2016. From every package, two portions of about 30 g mushrooms were taken (usually 2–3 individual mushrooms). One package of king trumpet mushrooms consisted of only two single mushrooms (about 40 g each) and here the portions consisted of only one single mushroom. Button mushrooms were visually assigned to development stages 4 and 5 according to the scale of Hammond and Nichols (Hammond and Nichols, 1975). Development stages of other mushrooms were not determined.

2.3. Sample preparation and lipid extraction

The individual portions (=samples) taken from the packages (Section 2.2) were immersed in liquid nitrogen for one minute and crushed and ground with a pestle. Ground and frozen mushroom samples were stored in 100 mL screw-cap polypropylene jars in the dark at −18 °C for a maximum of 3 h before being lyophilized (0 °C, 0.1 mbar, 3 days, protected from light) using a LYOVAC GT 2 system (Leybold-Heraeus, Hürth, Germany). Lyophilized samples were ground again with a pestle to a homogenous powder and stored in the dark at −18 °C. The total sample weight was usually around 2 g of lyophilized mushroom powder, which was then used for extraction.

Lipids were cold-extracted from each sample in duplicate according to the method of Shao et al. (2010) with *n*-hexane as described before (Hammann and Vetter, 2016). In brief, 0.2 g of lyophilized and ground mushroom was extracted three times with 6 mL *n*-hexane. The extracts were combined and brought to a final volume of 1 mL *n*-hexane. With every batch of samples, a blank containing no sample but 9 μg ergosterol and 1 μg linoleic acid ergosteryl ester was extracted identically. No internal standard could be used because other sterols behave differently during sample cleanup than ergosterol (which is less stable). To account for this, we extracted blanks with the target analytes (i.e. ergosterol and ergosteryl ester) with every extracted batch. After filtration with a 0.45 μm syringe filter (Carl Roth, Karlsruhe, Germany) aliquots of these solutions were trimethylsilylated or fractionated by solid phase extraction (SPE) according to Hammann et al. (2015). In the latter case, SPE fractions of free sterols were also trimethylsilylated while steryl esters were either analyzed directly or after saponification and trimethylsilylation.

Table 1
Common and latin names as well as country of production of the mushroom samples in this study.

Common name	Latin name	Country of production (number of samples)
Button mushroom	*Agaricus bisporus*	Germany (2), Netherlands (2), Poland (2)
Oyster mushroom	*Pleurotus ostreatus*	Poland (4), Germany (2)
King trumpet mushroom	*Pleurotus eryngii*	South Korea (4), Germany (2)
Shiitake	*Lentinus edodes*	Germany (4), Poland (2)