

Do Bioactive Carotenoids Contribute to the Color of Edible Mushrooms?

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Abstract: Carotenoids are biologically active phytochemicals present as micro-components in fruits and vegetables, being responsible for their yellow, orange and red colors. The chromatographic behavior and the UV absorption spectrum provided by HPLC-DAD analysis constitute the clues for their identification. Mushrooms are of increasing importance in modern nutrition and medicine, due to the presence of metabolites with pharmacological potential. In this work, samples of wild and commercial mushroom species (*Agaricus bisporus*, *Amanita caesarea*, *Amanita rubescens*, *Boletus edulis*, *Cantharellus cibarius*, *Fistulina hepatica*, *Hydnum rufescens*, *Hygrophorus agathosmus*, *Pholiota nameko*, *Pleurotus ostreatus*, *Russula cyanoxantha*, *Suillus bellini*, *Suillus bovinus*, *Suillus granulatus*, *Suillus luteus*, *Tricholoma equestre* and *Tricholoma portentosum*) were screened by HPLC-DAD for the presence of carotenoids. By applying this methodology to 22 samples, comprising either lyophilized or fresh materials, only β -carotene was found and just in *C. cibarius* species. The occurrence of this pigment in other three of the analyzed species previously described raises some questions about the methodology used.

Keywords: Carotenoids, Edible mushrooms, HPLC-DAD.

1. INTRODUCTION

Mushrooms are macrofungi with a distinctive fruiting body that are consumed as a delicacy, particularly for their specific aroma and texture. They constitute an integral part of the normal human diet, presenting an increasing share, and both fresh and preserved fruiting bodies of plenty of species can be culinary-processed in different manners [1]. Mushrooms have also been reported as source of pharmacologically active compounds, to which activities like antimicrobial, antitumor, cytostatic, hypoglycemic, anti-inflammatory or antioxidant are attributed [2].

Carotenoids biosynthesis occurs in bacteria, fungi, algae, and plants. Animals are unable to synthesize them, so they obtain these molecules *via* ingestion [3]. In addition to very few bacterial carotenoids with 30, 45 or 50 carbon atoms, C₄₀-carotenoids constitute the majority of the known structures [4]. More than 600 carotenoids have been

identified in nature so far. Nevertheless, only about 40 are present in a typical human diet and about 20 of them have been identified in human blood and tissues. Close to 90% of the carotenoids in the diet and human body is represented by β -carotene, α -carotene, lycopene, lutein and cryptoxanthin [5].

Characteristic fungal carotenoids possess 4-keto groups, may be monocyclic, or exhibit 13 conjugated double bonds [4]. The conjugated double bounds are responsible for the typical colors of these molecules (yellow, orange, red and purple) because they absorb between 440 and 490 nm of the light spectrum [5]. In fungi, these molecules arise from the mevalonate biosynthetic pathway. Mevalonate is formed from acetyl-CoA *via* hydroxymethylglutaryl-CoA. The subsequent steps involve two kinase reactions yielding mevalonic acid pyrophosphate, which is then decarboxylated to isopentenyl pyrophosphate, the building unit for the synthesis of terpenoids [4].

The role of carotenoids in organisms is to protect cells against the lethal combination of light (photoprotectors) and oxygen (antioxidants). The high number of conjugated double bonds makes carotenoids more reactive and able to function as antioxidants, namely against oxygen radicals. The carotenoids associated to membranes can substantially stabilize them and modify their thickness, strength and

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fluidity, subsequently affecting many of their functions. These metabolites can also act as photoreceptors [5,6].

Information on carotenoids in mushrooms is scarce. Nevertheless, previous studies described the presence of β -carotene in *Cantharellus cibarius* [7-11], *Agaricus bisporus* [11,12], *Pleurotus ostreatus* [11,13], *Boletus edulis* [9,11], *Suillus bovinus* [11] and *Tricholoma equestre* [11], of lycopene in *C. cibarius* [7-9,11], *A. bisporus* [11,12], *B. edulis* [9,11], *P. ostreatus* [11], *S. bovinus* [11] and *T. equestre* [11], of lutein, α -carotene [7] and canthaxanthin [14] in *C. cibarius* and of γ -carotene, auroxanthin and neurosporene in *B. edulis* [15].

The climatic characteristics and flora diversity of North-eastern Portugal renders this to be one of the European regions with higher wild edible mushrooms variety, some of them with great gastronomic and economic impact. The mushroom species growing there are usually consumed fresh, dried, during the off-season, or canned. Despite their consumption and increasing exportation to foreign countries, the available data regarding the nutritive valorization of the species are still very scanty. A previous study [16] revealed the presence of volatile norisoprenoids (apocarotenals), such as β -ionone, *trans*-geranyl acetone and (*E,E*)-farnesyl acetone, in some wild mushroom species collected in this region. Those are odor active substances that can be formed from carotenoids degradation. β -Ionone can arise from the degradation of β -carotene, which contains two non-substituted β -ionone rings in their ends [17]. Acyclic tetraterpenoids, such as lycopene, can give rise to geranyl acetone and farnesyl acetone by oxidative degradation [17].

In the sequence of the chemical characterization that we have been developing on mushrooms collected in Trás-os-Montes region, namely focusing primary and secondary metabolites [16,18-23], a study was undertaken on the carotenoids of species commonly consumed in Portugal. For this purpose, samples of wild mushroom species (*Suillus bellini*, *Suillus luteus*, *Suillus granulatus*, *S. bovinus*, *Hygrophorus agathosmus*, *Amanita rubescens*, *Russula cyanoxantha*, *B. edulis*, *T. equestre*, *Fistulina hepatica*, *Amanita caesarea*, *Hydnum rufescens* and *C. cibarius*) and five commercially available ones (*C. cibarius*, *Pholiota nameko*, *A. bisporus*, *P. ostreatus* and *Tricholoma portentosum*) were analyzed by HPLC-DAD.

2. MATERIALS AND METHODS

2.1. Standards and Reagents

β -Carotene and lutein were supplied by Sigma (St. Louis, MO, USA). Ethyl acetate, acetonitrile, diethyl ether, acetone and hexane were obtained from Merck (Darmstadt, Germany). β -Apo-8'-carotenal was obtained from Fluka (Buchs, Switzerland). Neoxanthin and violaxanthin were purchased to CaroteNature GmbH (Lupsingen, Switzerland). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

2.2. Samples

The characterization of the analyzed samples is presented in Table 1. Seventeen samples (samples 1-17) of wild mushroom fruiting bodies were collected from different places in Trás-os-Montes (North-eastern Portugal).

Taxonomic identification followed that of several authors [24-29]. Representative voucher specimens were deposited at the herbarium of School of Agriculture from Polytechnic Institute of Bragança. Five samples (samples 18-22) were acquired in a local market. Samples 1, 2, 4, 6, 8-15 and 17 were freeze-dried in a Labconco 4.5 Freezone apparatus (Kansas City, MO) and stored in a desiccator until analysis. Fresh mushroom samples were kept refrigerated until analysis, which was performed within 4 days.

2.3. Extracts Preparation

For carotenoids characterization, ca. 50 g of fresh mushrooms or ca. 10 g of powdered lyophilized samples were used. The fresh ones were homogenized using a Turrax homogenizer at 9500 rpm, for 15 min. In both cases, the samples were picked with 200 μ L of internal standard (170 mg/L of β -apo-8'-carotenal). Due to the hydrophobic character of carotenoids, extraction was carried out with ether/hexane (1:1, v/v). The extraction was repeated five times with 100 mL of solvent, under stirring at 450 rpm for 10 min, at room temperature. The final combined extract was concentrated to dryness under reduced pressure and redissolved in 1 mL of acetone/hexane (1:1, v/v) for HPLC-DAD determination. Light exposure was minimized during samples' preparation to avoid photoisomerization [30].

2.4. Carotenoids Analysis by HPLC-DAD

A Beckman model 126 quaternary solvent system, equipped with System 32 Karat software and a 168 rapid-scanning UV-visible photodiode array detector, was used. The absorption spectra were recorded between 270 and 600 nm. The injection volume was 20 μ L. The analysis was performed using a Nova-Pack C18, 60 Å, 4 μ m particles (3.9 \times 300 mm) column (Waters). Elution was developed with ethyl acetate (solvent A) and acetonitrile/water (9:1, v/v, solvent B) at a flow rate of 1 mL/min. The following gradient was employed: 0-31 min (0-60% A); 31-46 min (60% A); 46-51 min (60-100% A); 51-55 min (100% A); 55-60 min (100-0% A); 60-65 min (0% A) [30].

Carotenoids were identified by comparison of their absorption spectra and retention times with those of commercial standards. The content of β -carotene was calculated on the basis of a calibration curve constructed using authentic standard ($y = 0.017x + 0.7596$).

3. RESULTS AND DISCUSSION

The chromatographic behavior and the UV absorption spectrum provided by HPLC-DAD analysis constitute the clues for the identification of carotenoids, being related with their structures. Both the wavelengths of maximum absorption and the shape of the spectrum are characteristic of the chromophore.

First we analyzed the lyophilized material of wild *S. bellini*, *S. luteus*, *S. granulatus*, *S. bovinus*, *H. agathosmus*, *A. rubescens*, *R. cyanoxantha*, *B. edulis*, *T. equestre*, *F. hepatica*, *A. caesarea*, *H. rufescens* and *C. cibarius* fruiting bodies (samples 1, 2, 4, 6, 8-15 and 17) (Table 1). However, only β -carotene was detected, in vestigial amounts and just in *C. cibarius* sample (sample 17), although it was also described in *B. edulis* [9,11], *S. bovinus* [11] and *T. equestre* [11]. In addition, despite other carotenoids are reported to

Table 1. Characterization of the Mushroom Samples

Sample	Species	Treatment	Origin	Orchard
1	<i>Suillus bellini</i>	Lyophilized	Bragança	<i>Pinus pinaster</i>
2	<i>Suillus luteus</i>	Lyophilized	Vinhais	<i>Pinus pinaster</i> + <i>Castanea sativa</i>
3	<i>S. luteus</i>	Fresh	Vinhais	<i>Pinus pinaster</i> + <i>Castanea sativa</i>
4	<i>Suillus granulatus</i>	Lyophilized	Bragança	<i>Pinus pinaster</i>
5	<i>S. granulatus</i>	Fresh	Bragança	<i>Pinus pinaster</i>
6	<i>Suillus bovinus</i>	Lyophilized	Grandais	<i>Pinus pinaster</i> + <i>Castanea sativa</i>
7	<i>S. bovinus</i>	Fresh	Grandais	<i>Pinus pinaster</i> + <i>Castanea sativa</i>
8	<i>Hygrophorus agathosmus</i>	Lyophilized	Bragança	<i>Pinus pinaster</i>
9	<i>Amanita rubescens</i>	Lyophilized	Bragança	<i>Quercus pyrenaica</i>
10	<i>Russula cyanoxantha</i>	Lyophilized	Bragança	<i>Quercus rotundifolia</i>
11	<i>Boletus edulis</i>	Lyophilized	Bragança	<i>Castanea sativa</i>
12	<i>Tricholoma equestre</i>	Lyophilized	Carrzeda de Ansiães	<i>Pinus pinaster</i>
13	<i>Fistulina hepatica</i>	Lyophilized	Bragança	<i>Castanea sativa</i>
14	<i>Amanita caesarea</i>	Lyophilized	Grandais	<i>Castanea sativa</i>
15	<i>Hydnum rufescens</i>	Lyophilized	Grandais	<i>Castanea sativa</i>
16	<i>H. rufescens</i>	Fresh	Grandais	<i>Castanea sativa</i>
17	<i>Cantharellus cibarius</i>	Lyophilized	Bragança	<i>Castanea sativa</i>
18	<i>C. cibarius</i>	Fresh	Commerce	
19	<i>Pholiota nameko</i>	Fresh	Commerce	
20	<i>Agaricus bisporus</i>	Fresh	Commerce	
21	<i>Pleurotus ostreatus</i>	Fresh	Commerce	
22	<i>Tricholoma portentosum</i>	Fresh	Commerce	

occur in *C. cibarius* [7-9,14], *B. edulis* [9,15], *S. bovinus* [11] and *T. equestre* [11], no other compound was noticed in the lyophilized samples.

Lyophilization is widely considered the appropriate processing technique to preserve biological samples that have to be stored before carotenoid analysis. However, carotenoids are degraded during lyophilization. Additionally, this procedure increases sample porosity, thus raising exposure of carotenoids to oxygen during storage [31]. As so, in order to check whether the absence of carotenoids in the samples was a consequence of the lyophilization procedure we searched for the presence of these compounds in the available fresh samples of *S. luteus*, *S. granulatus*, *S. bovinus*, *H. rufescens* and *C. cibarius* (samples 3, 5, 7, 16 and 18) (Table 1). Fresh material of other species, namely *P. nameko*, *A. bisporus*, *P. ostreatus* and *T. portentosum*, was also analyzed (samples 19-22) (Table 1).

The analysis of the extracts obtained from the several fresh mushrooms allowed the identification of β -carotene only in *C. cibarius* (sample 18) (Fig. 1). This pigment had already been reported in this mushroom [7-11], as well as in other species of the same genus, namely in *Cantharellus lateritius*, *Cantharellus tabernensis* [10] and *Cantharellus cinnabarinus* [32]. The amount of this compound in the fresh sample 18 was clearly higher than that in the lyophilized one (sample 17), corresponding to 51.2 $\mu\text{g}/100\text{g}$. The different

amounts may be related to the negative effect of the lyophilization procedure on carotenoids, but the variability between individuals cannot be excluded.

With respect to the remaining fresh samples (samples 3, 5, 7, 16, 19-22), although several peaks were detected no carotenoid-like spectrum was noticed.

It should be highlighted that the color of some mushroom species, such as *C. cibarius*, *P. nameko* and *S. bovinus*, was a contributing factor to carry out the present study. Like *C. cibarius*, the other two mushroom species present a slight orange color, thus suggesting the possible occurrence of β -carotene. However, this carotenoid was not identified in any of them.

Regarding *H. rufescens*, *S. granulatus* and *S. luteus*, as neither lyophilized nor fresh samples presented carotenoids, and considering that data for these matrices is not found in literature, it can be speculated the absence of these kind of compounds to be a characteristic of the species.

In what concerns to *S. bellini*, *H. agathosmus*, *A. rubescens*, *R. cyanoxantha*, *F. hepatica* and *A. caesarea*, only lyophilized material was analyzed. As no carotenoid was found, not only the negative impact of this drying procedure on these compounds [31], but also the possibility that their absence corresponds to a property of the species, have to be taken into consideration.

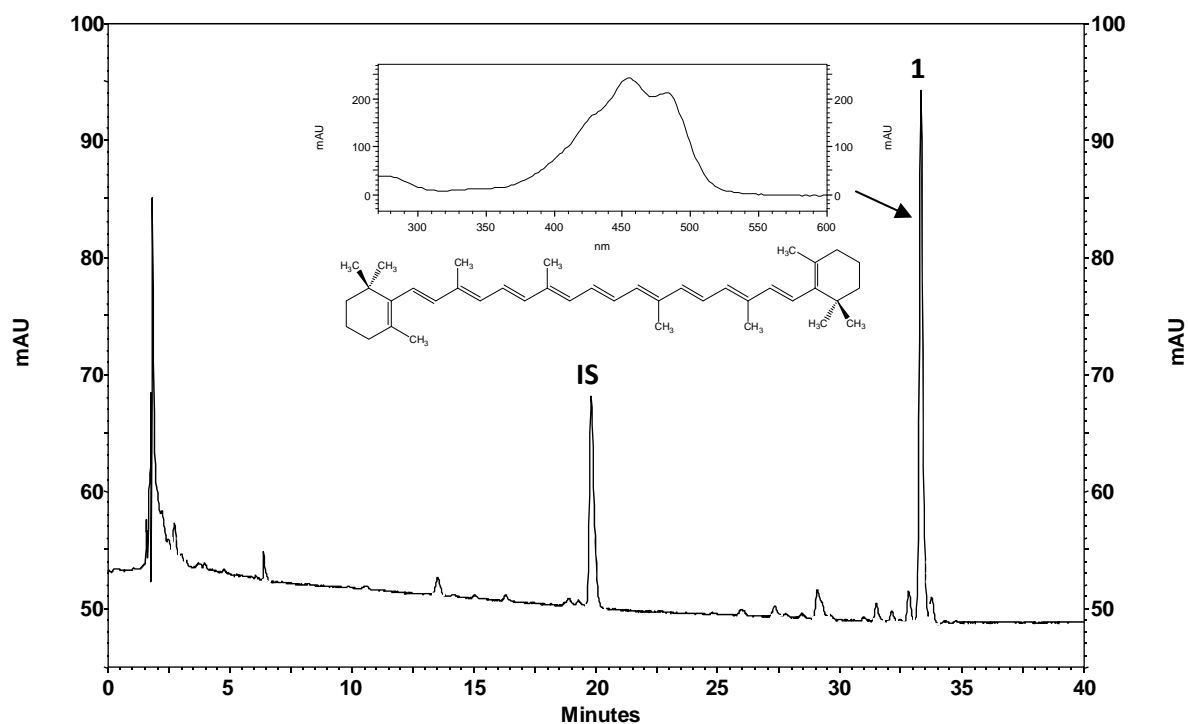


Fig. (1). HPLC-DAD Chromatogram of Carotenoids in Fresh *Cantharellus cibarius*. Detection at 447 nm. (1) β -Carotene; (IS) Internal Standard.

As referred above, β -carotene and lycopene were previously described in *B. edulis* [9,11] and *T. equestre* [11]. However, their identification was achieved by measuring the absorbance of a solution at 453, 505, 645 and 663 nm, a spectrophotometric method not as accurate as HPLC-DAD, which may suffer the interference of other compounds absorbing at the same wavelengths that could lead to the misidentification of carotenoids in the sample. The absence of γ -carotene, auroxanthin and neurosporene, also reported in this species [15], may be due to the use of a different extraction procedure. Nevertheless, the effect of the lyophilization process cannot be excluded.

As for the fresh samples, the absence of β -carotene in *T. portentosum* has already been reported [33], which was confirmed in the present study. Although β -carotene and lycopene were previously described in *A. bisporus* [12], *S. bovinus* [11] and *P. ostreatus* [11,13], they were not detected in our work. However, the three species were analyzed by the spectrophotometric method referred above for *B. edulis* and *T. equestre*. In addition, for *P. ostreatus* a different extraction procedure was also performed, followed by HPLC-UV with detection at 470 nm [13]. Both methodologies are not as precise as HPLC-DAD, lacking information on UV spectra. In addition, interferences may also occur, as already mentioned. In *P. nameko* no carotenoid was found, for which other pigments may be responsible for its orange color.

β -Carotene assumes a great biological importance. It is the precursor of vitamin A, decreases cancer risk, has a potent antioxidant ability that contributes to protection against cardiovascular diseases, as it inhibits LDL-cholesterol oxidation preventing atherosclerosis development,

reduces the photosensitivity, stimulates the immune system response, namely to HIV, and scavenges peroxy radicals, avoiding lipids oxidation and subsequent degradation [34-38]. Therefore, according to the results obtained, *C. cibarius* may contribute for beneficial effects in human organism.

CONCLUSIONS

The screening of carotenoids in both lyophilized and fresh samples of edible mushrooms species by HPLC-DAD did not confirm their presence in some of them, for which this kind of compounds was previously reported. This indicates that not always the results obtained by using less precise methodologies for the detection of carotenoids are confirmed when more sophisticated techniques are applied, despite the existence of chemical variability within the species, as each individual results from the cross-breeding of different hyphas, presenting a distinct genotype. Within the twenty two analyzed samples, only β -carotene was found and just in *C. cibarius* material, being responsible, at least partially, for the orange color of this species.

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CONFLICT OF INTEREST

There is no conflict of interest.

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