ELSEVIER

Journal of Chromatography A, 964 (2002) 129–135

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Simple method for reversed-phase high-performance liquid chromatographic analysis of fungal pigments in fruit-bodies of Boletales (Fungi)

Paolo Davoli[a,*], Roland W.S. Weber[b]

[a]*Dipartimento di Chimica, Università di Modena e Reggio Emilia, Via Campi 183, I-41100 Modena, Italy*
[b]*Lehrbereich Biotechnologie, Universität Kaiserslautern, Paul-Ehrlich-Strasse 23, D-67663 Kaiserslautern, Germany*

Received 28 January 2002; received in revised form 23 April 2002; accepted 14 May 2002

Abstract

A reversed-phase HPLC method has been developed for the analysis of hydroxylated pulvinic acid derivatives which are responsible for the pigmentation of fruit-bodies belonging to the order Boletales (Fungi). Variegatic, xerocomic and atromentic acid as well as variegatorubin were detected and separated in methanolic extracts of *Boletus permagnificus* and *Xerocomus parasiticus*, and the pigment profile of these species was compared. The identity of the pigments was confirmed by means of LC–atmospheric pressure chemical ionization (APCI) mass spectrometry.

Keywords: Fruits; *Boletus permagnificus*; *Xerocomus parasiticus*; Pigments; Variegatic acid; Variegatorubin; Xerocomic acid; Atromentic acid; Organic acids; Pulvinic acids; Hydroxypulvinic acids

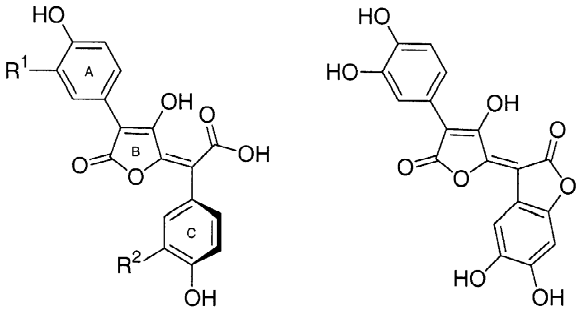
1. Introduction

The vivid yellow and red pigmentation of the fruit-bodies of several members of the Boletales (Fungi) is due to the presence of hydroxylated pulvinic acid derivatives [1]. Amongst them, variegatic acid (**1**) and, to a lesser extent, xerocomic acid (**2**) are responsible for the yellow colour, and variegatorubin (**4**) for the red colour, of many species of *Boletus*, *Xerocomus*, *Suillus* and related genera (Fig. 1). Indeed, the typical blueing reaction of many species after bruising is due to the oxidation of variegatic acid [2]. Many chemotaxonomic studies have described the occurrence of such pigments in diverse members of the order and the pigment composition has been used as a taxonomic character within the Boletales [1,3,4]. More recently, the traditional taxonomic arrangement based on morphological and chemical characters has been verified by comparisons of selected DNA sequences and is now generally accepted [5,6].

Initial research on the pigments from Boletales focused mainly on their isolation, often from a considerable amount of fungal material, and their subsequent spectroscopic elucidation [2,7–10]. Later studies on the occurrence of pigments were simply performed by a thin-layer chromatographic comparison of fruit-body extracts with reference stan-

*Corresponding author. Tel.: +39-59-205-5110; fax: +39-59-373-543.
E-mail address: davoli.chemistry@unimo.it (P. Davoli).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)00664-7



1 R^1=OH R^2=OH
2 R^1=OH R^2=H
3 R^1=H R^2=H
4

Fig. 1. Chemical structure of pigments from *B. permagnificus* and *X. parasiticus*: variegatic acid (**1**), xerocomic acid (**2**), atromentic acid (**3**), variegatorubin (**4**).

dards [11–15]. Since the determination exclusively on the basis of a comparison of TLC data or UV spectra is now considered rather questionable as the sole criterion for identification, especially in the field of fungal pigments [1], we are currently applying HPLC and related hyphenated techniques, such as LC–atmospheric pressure chemical ionization (APCI) MS, to the study of pigment profiles of fungal species [16–18]. In fact, HPLC techniques have facilitated the development of procedures for the separation of crude extracts in which pigments are contained and, when coupled to mass spectrometry, allow at the same time a rapid and reliable identification of the molecules under examination, especially in the case of minor or uncommon substances, for which a reference standard may not be readily available. Along this line, we have recently characterized the pigment profile of an intensely coloured species belonging to the Boletales, *Boletus permagnificus* Pöder, which had not been investigated so far, and its major pigments were identified as variegatorubin and variegatic acid by means of HPLC–APCI-MS [16].

Although some reports have dealt with the HPLC comparison of lichen extracts, many of which contain pulvinic acid analogues [19,20], to the best of our knowledge this analytical technique has never been applied to the chemical comparison of the pigment composition in different species of Boletales. Since an astonishing inter- and intraspecific chromatic variability exists within the Boletales and is often used as an important taxonomic argument, a quick and reliable method for the qualitative and quantitative characterisation of pigment profiles might contribute to a better definition of the species concept within this order, especially if coupled to modern genetic analysis.

In the current contribution, we report a newly developed HPLC method which allows an excellent separation of hydroxypulvinic acids and derivatives in crude extracts of fungal fruit-bodies.

2. Experimental

2.1. Fungal material

Fresh fruit-bodies of *Boletus permagnificus* Pöder were collected on 9 October 1999 in the vicinity of San Bachisio (Tempio Pausania, SS, Italy) in a *Quercus suber* L. (cork oak) forest. Fresh specimens of *Xerocomus parasiticus* (Bull.) Quél. were collected on 16–28 August 2001 in Forstgut Wiegersen near Buxtehude (Lower Saxony, Northern Germany) from fruit-bodies of *Scleroderma citrinum* Pers. growing in a *Quercus robur* L. (English oak) forest. No colour modifications were observed during storage and after subsequent lyophilization in either type of fruit-body. Voucher specimens have been deposited in the Herbarium, Department of Biotechnology, University of Kaiserslautern, under the accession numbers RW99101 and RW01003, respectively.

2.2. Sample preparation

Freeze-dried fruit-bodies (5 g, corresponding to 5–6 small specimens) were powdered in a mortar and extracted with 50 ml MeOH at room temperature under vigorous magnetic stirring for 1 h. After removal of the solid material by filtration and washing with methanol (50 ml), the coloured clear solution was concentrated in vacuo. The sticky residue was dissolved in distilled water (30 ml), sonicated and extracted with EtOAc (7×20 ml), until disappearance of all pigmentation from the aqueous phase; if necessary, 5 ml of a saturated NaCl solution was added to solve emulsions which occasionally

formed. Whereas the red colour could be easily extracted with EtOAc, addition of a few drops of concentrated HCl was required for a complete extraction of the yellow pigmentation from the aqueous phase. The pooled organic phases were dried on Na_2SO_4, filtered and rotary evaporated, thus affording a dark red residue in the case of *B. permagnificus* (179 mg) and a dark yellow one from *X. parasiticus* (264 mg). The crude extracts were then dissolved in MeOH to a final concentration of 5 mg/ml, filtered to remove the white gummy granules which were left undissolved, and 5-μl aliquots of the clear solutions were subjected to reversed-phase HPLC analysis.

2.3. HPLC analysis

The analyses were performed with a Hewlett-Packard 1090 Series II liquid chromatograph equipped with a photodiode array detection system, using a LiChrospher 100 RP-18 analytical column (5 μm; 125×4.0 mm; Merck, Darmstadt, Germany) at 40 °C. The mobile phase consisted of a two-step gradient of 0.085% (v/v) aqueous phosphoric acid and methanol: from 0 to 50% MeOH in 15 min, then from 50 to 100% MeOH in 5 min, at a flow-rate of 1.5 ml/min. Detection was performed at 400 and 500 nm, and UV–Vis absorption spectra of the pigments were recorded on-line with the photodiode array detection system.

2.4. LC–APCI-MS analysis

In order to confirm unambiguously the identity of the pigments, the crude extracts were subjected to LC–APCI-MS analysis with a Hewlett-Packard Series 1100LC-MSD instrument fitted with a LiChroCART Superspher 100 RP-18 analytical column (4 μm; 125×2.0 mm; Merck) at 40 °C. The mobile phase consisted of a linear gradient from 0.1% (v/v) formic acid to acetonitrile in 19.5 min at a flow-rate of 0.8 ml/min. Fragmentation was achieved in the APCI mode at either 70 or 100 V fragmentor voltages. The gas temperature was 350 °C, with the vaporizer set to 400 °C; drying gas was supplied at 5 l/min, with a nebulizing pressure of 50 p.s.i.g. (1 p.s.i.=6894.76 Pa). Spectra of a mass range between 50 and 800 (step size 0.1) were recorded in the negative (NI) as well as in the positive ionization (PI) mode.

3. Results and discussion

Chromatographic analysis of methanolic extracts of *B. permagnificus* and *X. parasiticus* by means of reversed-phase HPLC led to the separation of the different pigments responsible for the yellow and red colour of the fruit-bodies (Figs. 2 and 3). Three polar yellow pigments were detected, with absorption maxima around 260 and 390 nm in the UV–visible spectrum, showing a chromatographic behaviour rather different from the red pigment (λ_{max} 277, 501 nm), which also displayed a lower polarity (t_R 18.1 min) as compared to the yellow pigments (t_R 10.4–13.1 min).

The first yellow pigment (t_R 10.4 min; λ_{max} 262, 388 nm) showed the base peak at m/z 371 in the APCI mass spectrum using the NI mode, corresponding to a molecular mass of 372; when the fragmentor voltage was increased to 100 V, a conspicuous fragment peak at m/z 327 also appeared (Fig. 4a), thus suggesting the loss of a carboxylic group from the molecule. On the basis of these data, the compound was unambiguously identified as variegatic acid (**1**, Fig. 1), a yellow pigment widespread in the Boletales [1] and which represented the major yellow pigment in both species analyzed here (Figs. 2 and 3). The oxidation of variegatic acid is known to be responsible for the typical blueing reaction of many boletes [2], which has also been observed in *B. permagnificus* [21] and *X. parasiticus* [22]. Recently, the antioxidant activity and the mercaptan-capturing properties of variegatic acid have been investigated for possible applications as a deodorizing agent [23,24].

The UV–Vis spectrum of the second yellow pigment (t_R 11.7 min; λ_{max} 259, 387 nm) was similar to that of variegatic acid, but the APCI-NI mass spectrum differed by 16 units (Fig. 3b), showing the base peak with m/z 355 at 70 V, along with a weak fragment peak at 311 which became the base peak at 100 V. The structure of xerocomic acid (**2**, Fig. 1), which lacks a phenolic hydroxy group on ring C with respect to **1**, was therefore assigned. Whilst xerocomic acid was detected in *B. permagnificus*

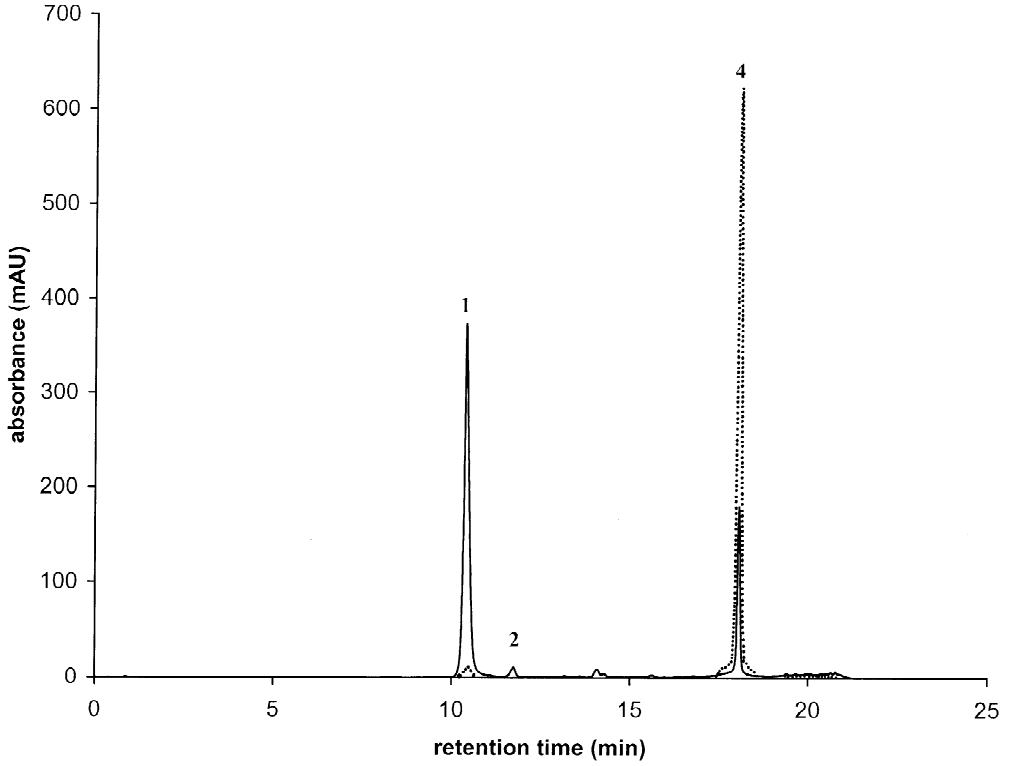


Fig. 2. HPLC chromatogram of a *B. permagnificus* extract recorded at 400 nm (solid line) and 500 nm (dashed line); approx. 25 μg of crude extract were injected; for chromatographic conditions, see text.

only in traces (Fig. 2), in *X. parasiticus* it represented the second major yellow pigment after variegatic acid (Fig. 3), which is by far the most abundant yellow pigment in the order Boletales. Xerocomic acid has been found in most species of the genus *Xerocomus*, as the name suggests, including *X. parasiticus* [12], but also from several species of *Boletus* and *Suillus* [1,8]. In the genus *Boletus* section Luridi, xerocomic acid is reported only in traces, from 15- to 100-fold less than variegatic acid [8,11]. In this respect, our results with *B. permagnificus* confirm the arrangement of this species within the section Luridi also on the basis of chemotaxonomic arguments.

A third, although minor, yellow pigment was also detected in *X. parasiticus*, with a slightly lower polarity (t_R 13.1 min) than xerocomic acid (Fig. 3). The UV–Vis spectrum (λ_{max} 257, 378 nm) and APCI-NI-MS data with a base peak of m/z 339 at 70 V being reduced to m/z 295 at 100 V (Fig. 4c) confirmed its identity as atromentic acid (**3**, Fig. 1). This is a yellow pigment very similar to **2** but differing in the absence of a phenolic hydroxy group on ring A. Atromentic acid is reported to occur in traces in most species which contain **1** and **2** [1], and has been found accordingly also in *X. parasiticus* [12].

The less polar red pigment (t_R 18.1 min) detected in both extracts (Figs. 2 and 3) had absorption maxima at 277 and 501 nm, suggesting the structure of variegatorubin (**4**, Fig. 1), which was confirmed by the APCI-NI mass spectrum showing the expected molecular ion at m/z 370, preceded by the base peak at 369 (Fig. 4d). Variegatorubin, an oxidation product of variegatic acid, represents the most widespread red pigment in the order Boletales, in particular in the genus *Boletus* section Luridi [1]. In *B. permagnificus*, variegatorubin represented the main pigment (Fig. 2), and its high concentration accounted for the intense red colour of the whole

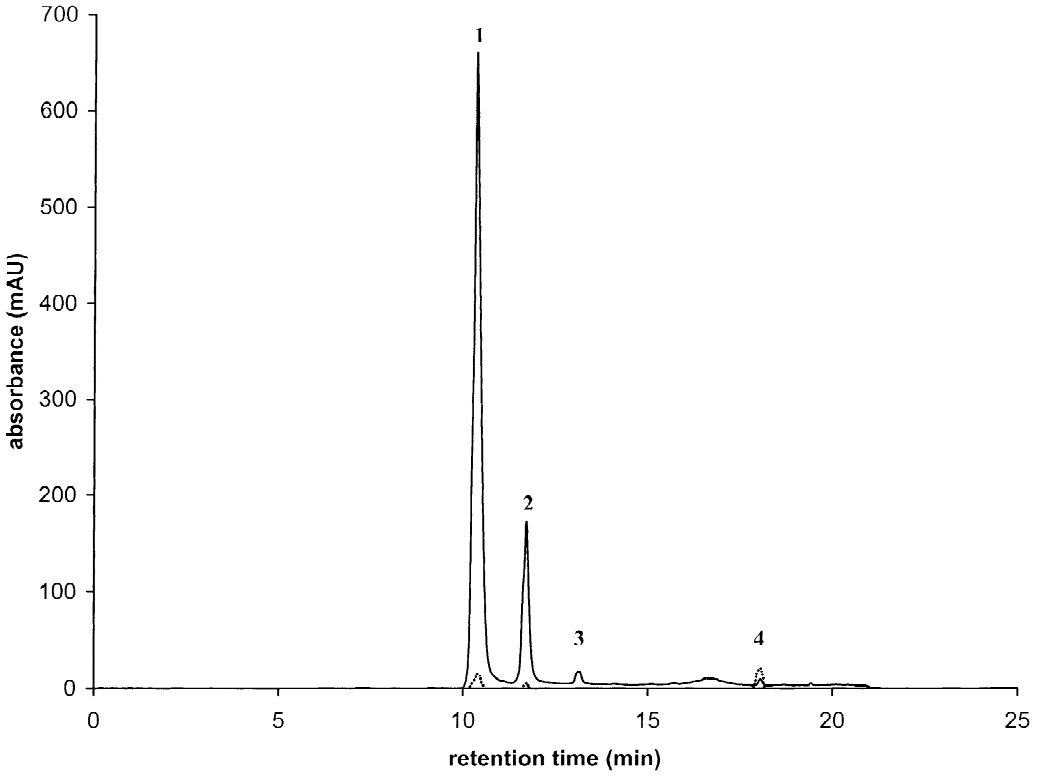


Fig. 3. HPLC chromatogram of a *X. parasiticus* extract (approx. 25 μg) recorded at 400 nm (solid line) and 500 nm (dashed line), using the same chromatographic conditions as in Fig. 1.

fruit-bodies [16]. On the contrary, **4** was only a trace pigment in *X. parasiticus* (Fig. 3), a species which has virtually no red pigmentation in its fruit-bodies.

On the basis of these results, the pigment profile of the two species examined here clearly differed not only by the expected predominance of variegatorubin in the bright red *B. permagnificus*, but also in the composition of the yellow pigments; in fact, although variegatic acid always represented the major yellow pigment, in *X. parasiticus* xerocomic acid partly contributed to the yellow pigmentation, whereas in *B. permagnificus* it was found only in trace amounts. Moreover, the latter lacked atromentic acid which was detected in the former, albeit in minor amounts. A broader range of species will have to be investigated in order to assess the suitability of the minor yellow pigments as chemotaxonomic markers between different genera and species within the Boletales.

4. Conclusions

The pigment profile of fruit-bodies of two distinct species of fungi belonging to the order Boletales was compared by means of reversed-phase HPLC analysis and their chemical identity was confirmed by LC–APCI-MS. In particular, a specific method for the separation of variegatic (**1**), xerocomic (**2**) and atromentic acid (**3**) as well as variegatorubin (**4**) in crude fungal extracts by reversed-phase HPLC was developed and applied to the qualitative determination of these pigments in fruit-bodies. The method is sufficiently sensitive to permit the analysis of hydroxypulvinic acids and derivatives in single fruit-bodies or part thereof, which should be of value especially in the case of rare species or herbarium material. Moreover, if coupled to APCI-MS, a rapid and reliable identification of each pigment is possible, in particular when a reference standard is not

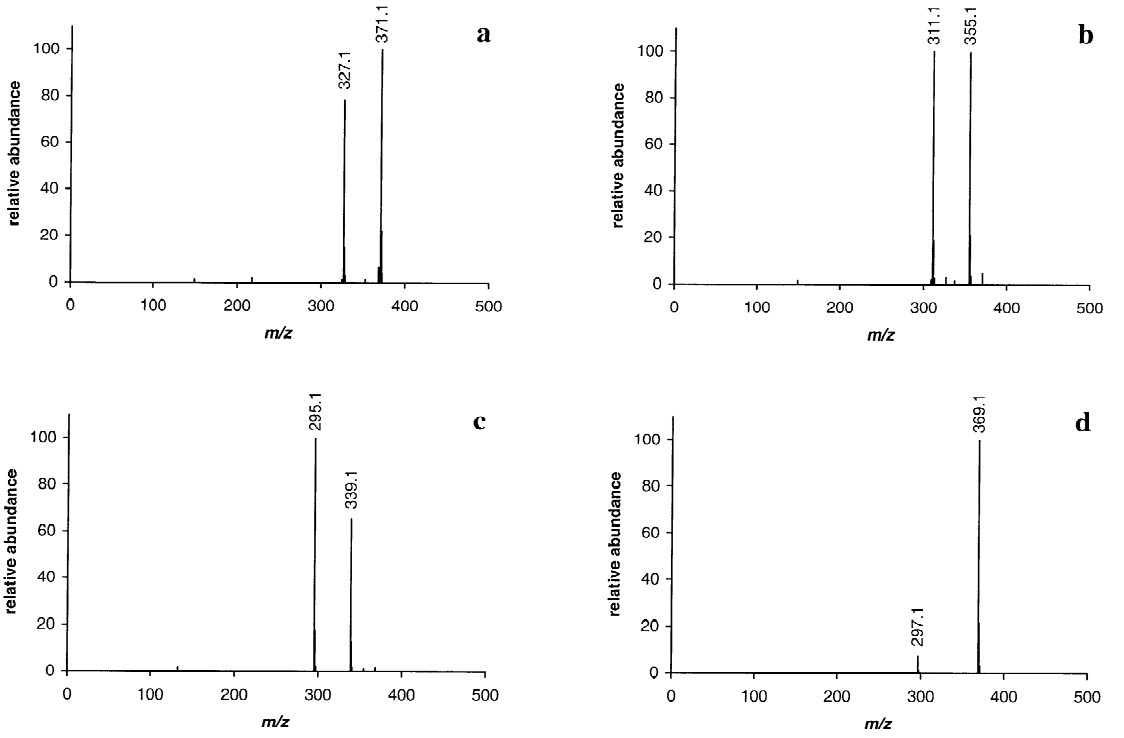


Fig. 4. APCI-NI mass spectra (100 V) of pigments from *B. permagnificus* and *X. parasiticus*; (a) variegatic acid (**1**); (b) xerocomic acid (**2**); (c) atromentic acid (**3**); (d) variegatorubin (**4**).

available, as for some less common analogues, such as gomphidic acid [9] or monochloro derivatives [25], which are known to occur sporadically in single members of the Boletales [1] and whose presence and distribution within the order could be more easily assessed. The HPLC analytical method described above therefore represents a suitable tool for future investigations including the individuality of fungal fruit-bodies, the physiology of pigment production, and chemotaxonomic aspects.

Acknowledgements

We thank Professors Timm and Heidrun Anke [Department of Biotechnology, University of Kaiserslautern, Germany and Institute of Biotechnology and Drug Research (IBWF), Kaiserslautern, Germany] for their encouragement and support. Dr Marco Contu (Olbia, SS, Italy) is acknowledged for sharing his local knowledge with us during our forays around Tempio Pausania, and for confirming the identification of our *B. permagnificus* specimens. We are most grateful to Anja Meffert (Department of Biotechnology, University of Kaiserslautern) for recording the HPLC–APCI-MS spectra.

References

[1] M. Gill, W. Steglich, in: W. Herz, H. Grisebach, G.W. Kirby, C. Tamm (Eds.), Progress in the Chemistry of Organic Natural Products, Vol. 51, Springer, Vienna, 1987, p. 1.
[2] P.C. Beaumont, R.L. Edwards, G.C. Elsworthy, J. Chem. Soc. C (1968) 2968.
[3] H. Besl, A. Bresinsky, Plant Syst. Evol. 206 (1997) 223.
[4] A. Kämmerer, H. Besl, A. Bresinsky, Plant Syst. Evol. 150 (1985) 101.
[5] L.C. Grubisha, J.M. Trappe, R. Molina, J.W. Spatafora, Mycologia 93 (2001) 82.
[6] D.S. Hibbett, R.G. Thorn, in: D.J. McLaughlin, E.G. McLaughlin, P.A. Lemke (Eds.), The Mycota, Vol. 7B: Systematics and Evolution. Springer: Berlin, 2001, p. 121.
[7] R.L. Edwards, G.C. Elsworthy, Chem. Commun. (1967) 373.

[8] W. Steglich, W. Furtner, A. Prox, Z. Naturforsch. B 23 (1968) 1044.
[9] W. Steglich, W. Furtner, A. Prox, Z. Naturforsch. B 24 (1969) 941.
[10] W. Steglich, W. Furtner, A. Prox, Z. Naturforsch. B 25 (1970) 557.
[11] J.A. Schmitt, Z. Pilzkunde 36 (1970) 77.
[12] A. Bresinsky, P. Orendi, Z. Pilzkunde 36 (1970) 135.
[13] H. Besl, A. Bresinsky, I. Kronawitter, Z. Pilzkunde 41 (1975) 81.
[14] H. Besl, A. Bresinsky, Z. Pilzkunde 43 (1977) 311.
[15] A. Bresinsky, H. Besl, Z. Mykol. 45 (1979) 247.
[16] P. Davoli, R.W.S. Weber, Micologia Italiana 30 (2001) 89.
[17] P. Davoli, R.W.S. Weber, Mycologist 16 (2002) in press.
[18] P. Davoli, R.W.S. Weber, Phytochemistry 60 (2002) 309.
[19] I. Yoshimura, Y. Kinoshita, Y. Yamamoto, S. Huneck, Y. Yamada, Phytochem. Anal. 5 (1994) 197.
[20] G.B. Feige, H.T. Lumbsch, S. Huneck, J.A. Elix, J. Chromatogr. 646 (1993) 417.
[21] R. Pöder, Sydowia 34 (1981) 149.
[22] J. Breitenbach, F. Kränzlin, Fungi of Switzerland, Vol. 3: Boletes and Agarics (1st part), Verlag Mykologia, Lucerne, 1991.
[23] O. Negishi, Y. Negishi, T. Ozawa, Food Sci. Technol. Res. 6 (2000) 186.
[24] O. Negishi, Y. Negishi, Y. Aoyagi, T. Sugahara, T. Ozawa, J. Agric. Food Chem. 49 (2001) 5509.
[25] R. Marumoto, C. Kilpert, W. Steglich, Z. Naturforsch. C 41 (1986) 363.