JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 7027−7035 **7027**

Colorimetric Characterization for Comparative Analysis of Fungal Pigments and Natural Food Colorants

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Exogenous pigments produced by ascomycetous filamentous fungi belonging to the genera *Penicillium*, *Epicoccum*, and *Monascus*, preselected based on chemotaxonomic knowledge, have been extracted and characterized by quantitative colorimetry. The color characteristics of the fungal extracts were compared to water soluble natural colorants derived from sources currently in use. The tested fungal extracts also included some commercially available *Monascus* colorants. The *a** values for the fungal extracts were found to be both positive and negative, the *b** values were found to be positive, while the hue angles of the fungal color extracts ranged from 40 to 110 indicating the color distribution of fungal extracts over the red−orange−yellow region of the CIELAB color space. The fungal extracts exhibited additional color hues in the red spectrum and similar hues in the yellow spectrum as compared to the reference natural colorants. They were also found to be similar or brighter in terms of chroma to some of the reference natural colorants. Principal component analysis was performed to group and distinguish different colors based on the *a** and *b** values. The fungal color extracts could be grouped in accordance with the similarity or difference in the color to those of the existing natural colorants. The diversity of colors was not only found among different fungal genera and/or species but also within the same species on changing the media. There was a marked change in the color composition of the extracts resulting in relatively different hues. Our results, thus, indicate that there exists pigment-producing genera of ascomycetous fungi other than *Monascus* that produce color shades in the red and the yellow spectra in addition or similar to reference colorants. These color shades could add to the color palette of the natural colorants currently in use. In addition, the multivariate approach in distinguishing and classifying the colorants was shown to be a very useful tool in colorimetric comparison of colorants.

KEYWORDS: Fungal pigments; colorimetry; natural colorants; *Monascus* pigments; multivariate analysis

INTRODUCTION

The utilization of natural pigments in foodstuff has been increasing in recent years due to the marketing advantages of employing natural ingredients and due to consumer concerns about eventual harmful effects of synthetic pigments; Fast Green dye, widely used as a food colorant, has been shown to be an immunotoxic agent (*1*). Currently, natural pigments are derived from sources like plants (*2*−*4*), insects (*5*), and microorganisms (*6*−*10*). The latter is gaining much attention outside of the food industry in Europe and the United States because of the stability of the pigments produced (*11*), the possibility for in-house production, and the availability of cultivation technology that can be optimized for higher yields (*12*, *13*). The ultimate aim is the production of pigments by microbial biotechnology that would be independent of the external supply of agricultural raw materials and climatic conditions. The use of filamentous fungi, such as *Monascus*, as sources of food colorants has a long-term history in the Orient but is still forbidden in the West except for the successful production of β-carotene from the fungus *Blakeslea* (*14*). One unsuccessful attempt of a food colorant from fungi in the West was of the anthraquinone-based red fungal pigment Arpink Red (*14*, *15*), which received a temporary 2 year approval for distribution in the Czech Republic from 2004 until May 5, 2006. We believe that exploring fungal chemical diversity by a chemotaxonomic approach, whereby the preselection of a nontoxigenic and a nonpathogenic strain is ensured, is a worthwhile route for the identification of potential pigment-producing fungi as sources of color leads for the food industry (*15*). Most of the available literature about pigment-producing fungi for food use points toward *Monascus*, which produces pigments that are good food colorants because they are stable in the pH range of 2−10, heat stable to autoclaving, and exhibit different colors of yellow to red (*16*, *17*). There is a report (*18*) on characteristics and stability of pigments produced by *Monascus anka* where it was found that

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10.1021/jf062094n CCC: $33.50
Published on Web 08/29/2006

these pigments were stable under UV and fluorescent light but were very sensitive to sunlight. The same study also brought out that tartaric and citric acids were detrimental to the *Monascus* pigments and that the copper ions showed the most deleterious effect on color change of these pigments. Besides *Monascus* pigments, several characteristic noncarotenoid pigments are produced by filamentous fungi that exhibit a unique structural and chemical diversity with an extraordinary ranges of colors (*19*). Noncarotenoid pigments of filamentous fungi are secondary metabolites, many of which are structurally diverse polyketides. These polyketide pigments are often complex compounds that are at least partially synthesized by multifunctional enzymes called polyketide synthases (*20*). The structures of polyketides are known to have loose π-electrons as they often contain polyunsaturated functionality, i.e., ring systems, one or more carbonyl groups, carboxylic acid, and ester or amide functional groups exhibiting characteristic UV−vis spectra. Therefore, we assume that these polyketides may serve a potential source of new chromophores (color leads) that can be promising for food use.

Even though color is a perceptual phenomenon, a quantitative description of color is a key factor for both scientific investigations and applications of colorants in food products. Qualitative investigations of physical spectra and the absorption maxima do not always provide the correct color (*21*). Therefore, a more quantitative approach is needed to describe a color. One of the ways color can be described quantitatively is the use of extinctions of colored solutions at their respective absorption maxima according to the Lambert−Beer law. Another widely adopted way of describing a color is based on stimuli generated in the human eye by visible light of various wavelength and intensities. The so-called CIE (Commission International de l'Eclairage) system is based on the fact that light reflected from any colored surface can be visually matched by an additive mixture of the three primary colors: red, green, and blue (*21*).

Recently, there have been a couple of reports on describing the color characteristics of *Monascus* pigment derivatives (*22*, *23*) and their enhancement of photostability to improve their scope for industrial application. Moreover, the successful production of β-carotene from the fungus *Blakeslea* over a decade and the recent temporary authorization of a fungal food colorant in the Czech Republic (*14*, *15*) have put a large incentive to characterize the color of fungal pigments more than *Monascus* pigments for future food use. However, the lack of coproduction of volatile compounds in significant amounts that may alter the organoleptic characteristics of the food and toxicity are some of the key factors that need to be tested for the food application of fungal pigments.

In light of this, we used chemotaxonomic tools and a priori knowledge of fungal metabolites to preselect some potential pigment-producing, nonpathogenic, and nontoxigenic ascomycetous fungi. The present paper describes the color characteristics of fungal pigment extracts from such fungi using quantitative colorimetry in order to test our hypothesis that the hues and chroma of fungal pigments in the red and the yellow spectra are comparable to the existing natural food colorants; hence, fungi may serve as a new source of natural colorants for food use. We focus on the color of fungal pigments to emphasize their similarity or differences to the existing commercially available counterparts used as references in our study by multivariate data analysis based on their CIELAB color characteristics. The pigment compositions of some of the representative crude color extracts were also analyzed chromatographically to understand the difference in their color hues, and some of the components were identified by high-resolution liquid chromatography−diode array detection−mass spectrometry (LC-DAD-MS).

MATERIALS AND METHODS

Standard Colorants. Eight colorants, viz. annatto, beet root, carthamus, lutein, natural carotene, turmeric, carminic acid, and acid-stable carmine (7-β-D-glucopyranosyl-9,10-dihydro-5-amino-3,6,8-trihydroxy-1-methyl-9,10-dioxo-2-anthracenecarboxylic acid) (*5*), were kindly delivered by Chr. Hansen A/S (Hørsholm, Denmark). Four *Monascus* colorants were obtained from different suppliers from China and Japan as follows: *Monascus* Red 1 was provided by Feng sheng (China), *Monascus* Red 2 was provided by Yaegaki (Japan), *Monascus* Red 3 was provided by Riken Vitamin Co. Ltd. (Japan), and *Monascus* Yellow was provided by Kelongbio (China). The colorants were dissolved directly in distilled water (pH 6.8−7.0), except in the case of natural carotene where drops of acetone were added to completely dissolve the colorant, and were subsequently used for colorimetric characterization.

Fungi, Media, Cultivation Conditions, and Name Codes. All fungal isolates used in this study were procured from the IBT Fungal Culture Collection at BioCentrum-DTU, Technical University of Denmark (Kgs. Lyngby, Denmark). The fungal isolates were listed by the IBT numbers. All fungi were cultivated on three different solid media, namely, yeast extract sucrose (YES) agar, potato dextrose (PD) agar, and Czapek−Dox yeast autolysate (CYA) agar (*24*). Three of the pigment-producing fungi that exogenously produced intense coloration on solid media were also grown in liquid medium, Czapek−Dox (CZ) broth (*24*), with an initial pH adjusted to 6.5. The cultures were incubated in the dark at 25 °C for 7 days. Liquid cultivations were performed in 500 mL of baffled Erlenmeyer flasks containing 100 mL of the medium (CZ) at 150 rpm on a rotary shaker at 25 °C for 7 days. **Table 1** represents the identity of reference colorants and the source of fungal extracts viz. the name of fungi, the IBT number, and the media used for their incubation. Specific name codes have been assigned to each of the color extracts, and hence forth, they have been referred to by these codes in all of the figures and tables.

Extraction of Fungal Pigments. Extraction was carried out by a modified version of the microextraction method (*25*), where 6 mm plugs were extracted in two steps in a 2 mL vial for 30 min, first using 1 mL of ethyl acetate with 0.5% formic acid to break open the cell wall and extract relatively apolar metabolites. The extract so obtained was then transferred to a new 2 mL vial and evaporated in vacuo. The second extraction was performed using 1 mL of methanol or isopropanol based on our preliminary results indicating maximum pigment extraction from the specific pigment-producing fungus (data unpublished). Because the exact chemical nature of pigments varied from fungus to fungus, it was necessary to use the appropriate solvent for the specific strain. By doing so, we could extract the maximum color. However, the same solvent system was used to extract for the same strains cultured in different media. The second extract was then added to the vial with the residue from the previous extraction. It was then evaporated in a rotational vacuum concentrator (RVC; Christ Martin, Osterode, Germany). The residue was redissolved in 400 μL of methanol, in an ultrasonic bath (Branson 2510, Kell-Strom, Wethersfield, CT) for 10 min and filtered through a 0.45 μL PTFE syringe filter (SRI, Eatontown, NJ). A part of this extract was used for chromatographic analysis, and the rest was used for quantitative colorimetry. In case of the liquid medium CZ, where the pigment was mostly diffused in the media, the color extract was obtained as per the method used by Jung et al. (*22*).

Colorimetry. The absorbance values of reference colorants as well as the fungal color extracts were adjusted to 0.40 ± 0.04 at their respective absorption maxima with purified water, obtained from a Milli-Q system (Millipore, Bedford, MA), as a diluent. The absorption maxima were determined by scanning the extracts for their absorption spectra over the range of 350−700 nm, using a spectrophotometer (Agilent HP 8453, Agilent Technologies, Palo Alto, CA). The same extracts were then used for determining CIELAB color coordinates using Chromameter (Minolta CT 310, Konica Minolta, Mahwah, NJ).

Table 1. CIELAB Color Coordinates of Reference Water-Soluble Natural Colorants and Fungal Color Extracts

sample code	sample/fungal name	IBT no.	media	*L*	*a**	*b**	hue angle[a]	chroma[b]
A	acid-stable carmine			85.62	28.24	–0.78	358.42	28.25
B	carminic acid			93.94	9.22	29.18	72.5	30.60
C	beet root			83.36	30.30	–4.13	352.24	30.58
D	annatto			98.16	–9.98	39.76	104.9	40.99
E	turmeric			99.68	–10.57	27.79	110.82	29.73
F	carthamus			100.00	–5.98	15.49	111	16.60
G	lutein			93.92	–2.88	30.48	95.3	30.61
H	natural carotene			98.01	–6.62	40.10	99.3	40.64
I	*Monascus* red 1			87.27	16.34	24.73	56.6	29.64
J	*Monascus* red 2			86.92	18.14	20.35	48.3	27.26
K	*Monascus* red 3			85.62	21.89	18.20	39.7	28.46
L	*Monascus* yellow			94.97	–3.99	37.87	95.9	38.07
M	*P. purpurogenum* chemotype II	11180	CYA	87.85	10.69	22.64	64.8	25.03
M1	*P. purpurogenum* chemotype II	11180	YES	85.81	15.90	16.54	46.2	22.94
M2	*P. purpurogenum* chemotype II	11180	CZ	85.01	19.72	22.38	46.62	29.83
N	*Penicillium aculeatum*	14263	CYA	87.83	11.95	14.48	50.5	18.77
N1	*P. aculeatum*	14263	YES	88.44	10.51	21.76	64.3	24.16
O	*P. purpurogenum*	3951	YES	83.80	12.78	17.36	53.64	21.56
O1	*P. purpurogenum*	3951	CZ	84.9	16.21	22.06	53.8	27.37
P	*Monascus ruber*	7904	MEA	91.07	10.76	11.80	47.7	15.96
Q	*Penicillium atrovenetum*	5990	YES	97.50	–3.65	14.03	104.5	14.49
Q1	*P. atrovenetum*	5990	CZ	98.55	–3.08	9.35	108.23	9.84
R	*Penicillium herquei*	21731	YES	97.47	–3.99	15.61	104.3	16.11
R1	*P. herquei*	21731	CZ	98.17	–4.16	11.71	109.5	12.42
S	*E. nigrum*	7571	YES	97.12	–6.11	24.04	104.2	24.8
T	*E. nigrum*	7232	YES	94.87	–6.59	32.29	101.5	32.95
U	*E. nigrum*	7802	YES	96.69	–7.83	32.16	103.6	33.09
V	*E. nigrum*	7901	YES	96.48	–7.94	29.89	104.8	30.92
W	*E. nigrum*	41028	CYA	97.85	–7.71	28.81	104.9	29.82
W1	*E. nigrum*	41028	PD	97.68	–8.98	31.67	105.8	32.91

[a] Hue angle (h_{ab}) = $\tan^{-1}$ (b^*/a^*). [b] Chroma (C) = $[(a^*)^2 + (b^*)^2]^{1/2}$.

The CIELAB colorimetric system was interpreted as follows: L^* indicates lightness read from 0 (black) to 100 (white). The positive a^* value indicates the red color while the negative a^* value represents the green color. Similarly, positive and negative b^* values indicate the yellow and the blue colors, respectively. Chroma values denote the saturation or purity of color. In a color wheel, values close to the center, at the same L^* value, indicate dull or gray colors, whereas values near the circumference represent vivid or bright colors. Hue angle values represent the degree of redness, yellowness, greenness, and blueness; the maximum is at 0, 90, 180, and 270, respectively.

Chromatographic Analysis. High-resolution LC-DAD-MS was performed on an Agilent HP 1100 LC system with a DAD and a 50 mm × 2 mm i.d., 3 μm, Luna C 18 II column (Phenomenex, Torrance, CA). The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, United Kingdom) with a *Z*-spray electrospray ionization (ESI) source and a LockSpray probe and controlled by the MassLynx 4.0 software. The MS system was operated in the positive ESI mode using a water−acetonitrile gradient system starting from 15% acetonitrile, which was increased linearly to 100% in 20 min with a holding time of 5 min or starting from 5% acetonitrile for 2 min and increasing to 100% in 18 min and keeping it for 5 min. The water was buffered with 10 mM ammonium formate and 20 mM formic acid, and the acetonitrile was buffered with 20 mM formic acid. The instrument was tuned to a resolution >7000 (at half peak height). The method is well-established at our Center as described by Nielsen et al. (*26*).

Analysis of LC-DAD-MS Data. The presence of PP-R was detected in ESI^+ from the first scan function of the reconstructed ion chromatograms of *m*/*z* 426.4288 $[M + H]^+$ and confirmed by the fragments *m*/*z* 448.3817 $[M + H + Na]^+$ and 489.3471 $[M + H + Na + CH_3CN]^+$, and the presence of orevactaene was detected in ESI^+ from the same scan function as *m*/*z* 613.2708 $[M + H]^+$ and confirmed by the fragments *m*/*z* 595.3037 $[M + H - H_2O]^+$ and 635.2318 $[M + H + Na]^+$.

Principal Component Analysis (PCA) Analysis. The colorimetric data (comprised of a^* and b^* color coordinates) were analyzed by PCA using The Unscrambler 7.6 on Windows NT, 2000 and XP. Prior to statistical analyses, all variables were standardized to mean zero and variance one.

RESULTS AND DISCUSSION

Comparative Analyses of Colorimetric Values of Fungal Color Extracts and Water-Soluble Natural Colorants. The red and the yellow commercially available water- and oil-soluble natural colorants were used as references for comparing the red and the yellow fungal crude extracts. The absorbance for all colorants in the present study was adjusted to a value of 0.40 ± 0.04; as a result, the lightness values for all colorants were similar and varied in the range from 83 to 100 (**Table 1**). The a^* values for the fungal extracts were found to be both positive, ranging from 11 to 20, and negative, ranging from −3 to −9, while the b^* values were only positive with values ranging from 9 to 32 (**Table 1)**. The hue angles of all of the fungal extracts ranged from 46 to 110. This indicated the color of fungal extracts to be red to orange and yellow to light green-yellow on the basis of the CIELAB color system as explained in the Materials and Methods. To compare the hue angles and chroma, i.e., the purity or saturation of a color, of the red and the yellow colorants, two-dimensional polar scatter plots were made at the same lightness level of 87 ± 3 and 97 ± 2 for the two groups of colorants, respectively.

Hues. The hue angle values for the fungal extracts [M], [M1], [M2], [N], [N1], [O], [O1], and [P] ranged from 46 to 65 (**Table 1)**, signifying orange-red color (**Figure 1**), while the hue angle values of fungal extracts [Q], [Q1], [R1], [S], [T], [U], [V], [W], and [W1] ranged from 101 to 110 (**Table 1)**, signifying yellow to green yellow in color (**Figure 2**). Among the red reference colorants, the hue angle of acid-stable carmine [A] was 358.4, which is close to the hue angle value of beet root

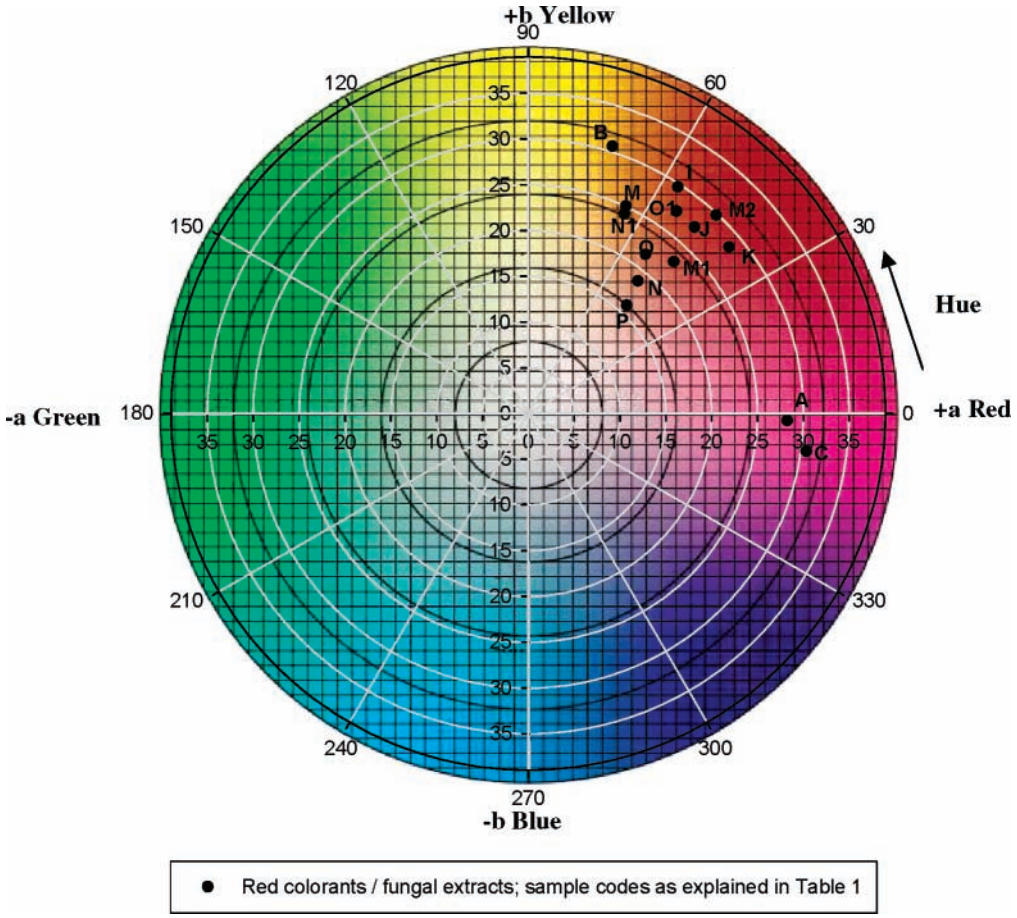

Figure 1. Polar scatter plot showing hue angles and chroma of standard red colorants and red fungal extracts at *L* values of 87.4 ± 3.3.

[C], which was 352.2, indicating light blue-red color; and the hue angle of carminic acid [B] was 72.5 indicating light orange color (**Table 1** and **Figure 1**). The commercially available red *Monascus* colorants [I, J, and K] had hue angles in the range of 40−57 (**Table 1**). These three *Monascus* colorants thus represented orange-red hues. Among the yellow reference colorants, the hue angle value of lutein [G] was 95.3 indicating the highest degree of yellowness followed by natural carotene [H] with a hue angle value of 99.3. The other yellow reference colorants viz. annatto [D], turmeric [E], and carthamus [F] had hue angles ranging from 105 to 111, indicating green-yellow color (**Table 1** and **Figure 2**). The red fungal extracts including commercially available *Monascus* colorants showed scarlet red or middle red as compared to the red reference colorants as their hue angles were in the range of 30−65. The color of the red fungal extracts was similar to color hues of the control red and orange *Monascus* pigments reported by Jung et al. (*22*). Thus, it can be inferred that there are several species of Penicillia that produce pigments of similar color hues like that of *Monascus*, and these could be interesting to explore further. Chemodiversity was exhibited among different species and/or chemotypes in terms of color as seen from the different hue angles of extracts [M], [N], and extracts [M1], [N1], and [O], respectively, representing various hues of orange-red color (**Table 1** and **Figure 1**). The diversity of colors was also seen among the same strains but grown on different media as exemplified by the red extracts [M], [M1], [N], and [N1] (**Table 1** and **Figure 1**). Among the yellow fungal extracts, the hue angle of the fungal extract [W] was 104.9, the same as for the reference colorant annatto [D]. Fungal extracts [Q], [S], [U], [V], and [W1] had relatively similar hue angles as compared to annatto [D] (**Table 1** and **Figure 2**). Thus, the yellowness of most of the yellow fungal extracts was comparable to the yellowness of annatto [D] with respect to the hue angle. The fungal extract [R1] was similar to the reference colorant turmeric [E] in terms of hue angle indicating a similar yellowness. The yellowness of the fungal extract [T] was comparable to the yellowness of natural carotene [H] as indicated by their similar hue angles (**Table 1** and **Figure 2**). There are not any previous data available on the CIELAB color characteristics of yellow fungal pigments for comparison, especially in the context of food use. This signifies the contribution of the present findings in this area and also shows that the yellow hues of fungal pigments may also prove to be worth considering for food use.

Chroma. Carminic acid [B] and beet root [C] had the highest chroma values of 31 (**Table 1**) representing the brightest color hues closely followed by *Penicillium purpurogenum* chemotype II IBT 11180 on CZ medium [M2], *Monascus* Red 1 [I] with chroma values of 30, and *Monascus* Red 3 [K] with a chroma value of 29 (**Table 1** and **Figure 1**). The fungal extracts [M], [O1], and *Monascus* Red 2 [J] were similar in brightness as acid-stable carmine [A] (**Figure 1**) as their chroma values were found to be in the range of 25−28 (**Table 1**). Fungal extracts [P] and [N] were found to be relatively dull or gray as compared to both reference colorants and other red fungal extracts as their chroma values were in between 15 and 20 (**Figure 1**). Among the yellow colorants, annatto [D] and natural carotene [H] had the highest chroma values of 41 representing the brightest color hues followed by *Monascus* Yellow [L] with a chroma value of 38 (**Table 1** and **Figure 2**). Fungal extracts *Epicoccum nigrum* IBT 7232 on YES [T], *E. nigrum* IBT 7802 on YES [U], *E. nigrum* IBT 7901 on YES [V], *E. nigrum* IBT 41028

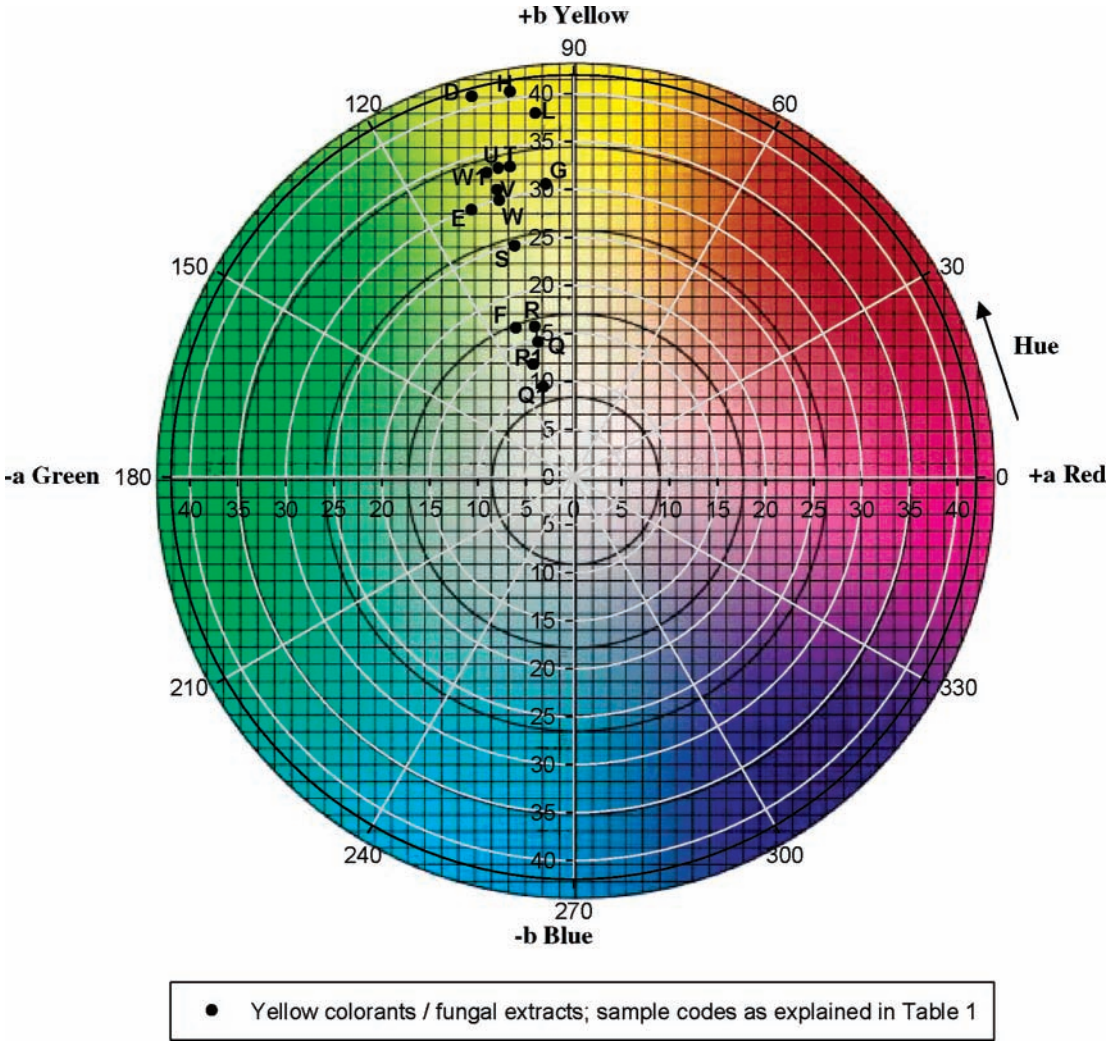

Figure 2. Polar scatter plot showing hue angles and chroma of standard yellow colorants and yellow fungal extracts at L values of 97.4 ± 1.8.

on CYA [W], and *E. nigrum* IBT 41028 on PD [W1] had chroma values in the range of 30−33 similar to the chroma values of the reference colorants turmeric [E] and lutein [G] and hence the brightness (**Table 1** and **Figure 2**). The chroma value of the yellow fungal extract [R] was comparable to the brightness of the reference colorant carthamus [F], while the fungal extracts [Q], [Q1], and [R1] were relatively dull and gray as compared to the other yellow fungal extracts and the yellow reference colorants as indicated by their lower chroma values ranging from 10 to 15 (**Table 1** and **Figure 2**).

Thus, the colors of fungal extracts in the present study including commercially available *Monascus* colorants provide additional hues in the red spectrum and similar hues in the yellow spectrum as compared to the reference colorants derived from contemporary sources. In terms of chroma, no significant difference was observed between fungal extracts and reference colorants. In some cases, the fungal extracts were found to be similar or brighter than some of the reference colorants, while as compared to some other reference colorants, they were found to be gray or dull.

Multivariate Analysis. In order to test the use and validation of a multivariate approach to group and distinguish different colors, PCA analysis was performed using a^* and b^* color coordinates. The red and the yellow fungal extracts formed quite distinct clusters based on a^* and b^* values while the red colorants beet root [C] and acid-stable carmine [A] were seen far to the right in the plot (**Figure 3**). Hence, PCA analysis was clearly able to discriminate these colors from the other red colorants. As discussed earlier, the color hue of beet root and carmine lied in the blue-violet-pink region of the CIELAB color space (**Figure 1**); therefore, it was located far away from the rest of the red colorants. The red fungal extracts clustered around the red *Monascus* colorants, signifying a similar color of the fungal extracts to the commercially available *Monascus* red colorants. The color palette of the fungal extracts including *Monascus* colorants was clearly shown to be in between the color range of the tested red natural colorants [A] and/or [C] and [B] in the red region of the CIELAB color space. In the yellow group of colorants, left-hand side region of the plot, the distinction between the colorants was more visible as the points were well-spread within the group in the same pattern as in the CIELAB color space (**Figures 1** and **2**). The PCA score plot thus exhibited an enlarged view of the points located in the CIELAB color space discriminating the red and the yellow colorants more explicitly by spreading the points in the space across the two components. Clearly, the PCA score plot based on a^* and b^* values (**Figure 3**) discriminates the color characteristics in agreement to that of the CIELAB color space (**Figures 1** and **2**) thus validating the grouping of colorants by the use of multivariate approach.

HPLC-DAD-MS Analysis of Crude Fungal Color Extracts. After colorimetric characterization, some representative red and yellow crude fungal extracts were analyzed by a LC system with a DAD coupled to a MS to determine the pigment

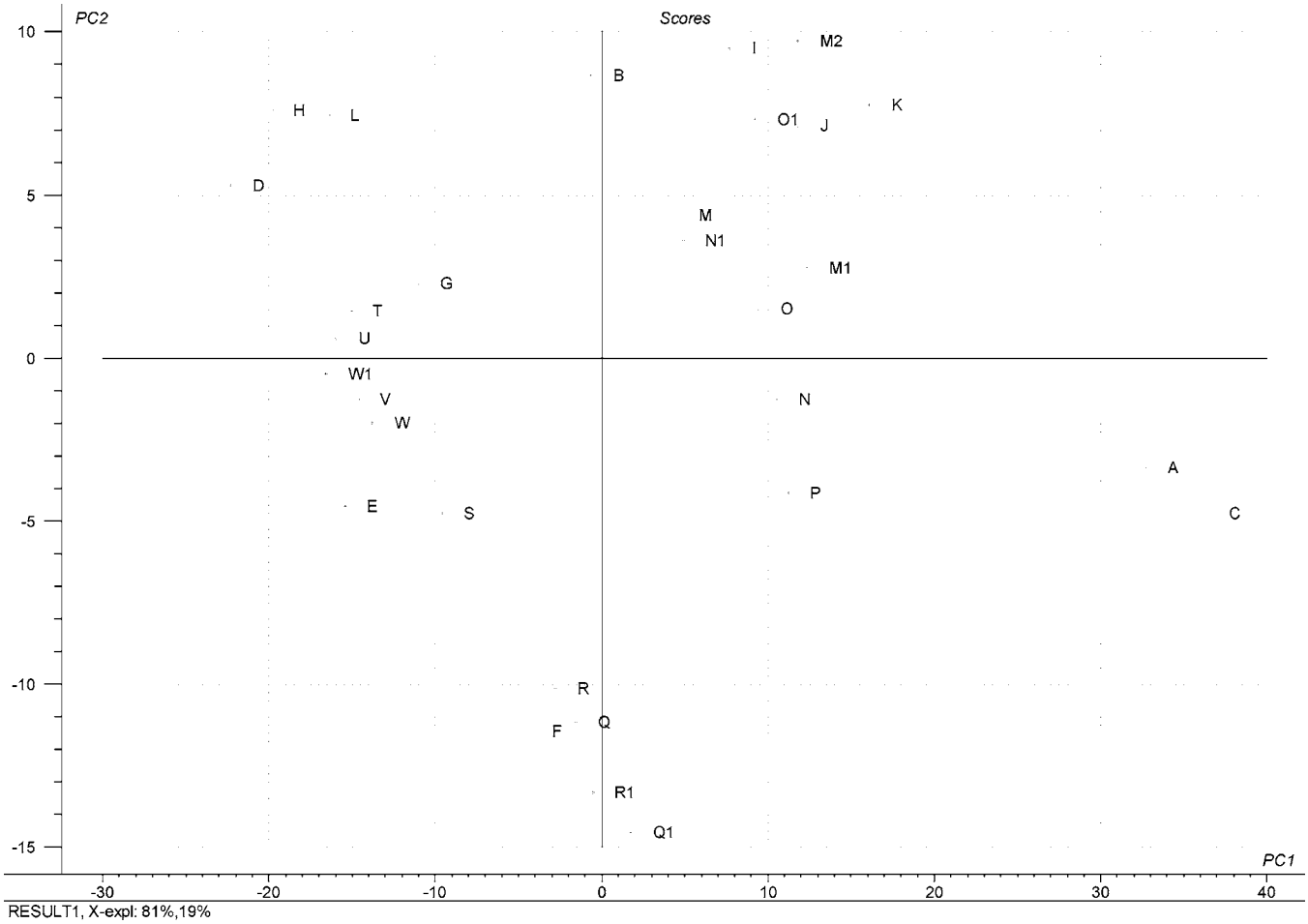

Figure 3. PCA score scatter plot showing the classification of colorants based on *a** and *b** values where sample codes are explained in **Table 1**.

composition and identify the color components of the extract. The retention times of only the major colored components detected between 400 and 700 nm contributing ≥10% of peak area (normalized as per the highest peak) were measured. Some of these major color components were identified based on their UV−vis and mass spectra (**Figure 4**). **Figure 5** depicts the structures of the characteristic pigments detected and identified.

Red Extracts. The red extracts [M] and [O] showed the presence of a single major component whereas extracts [M1] and [N1] had two components each, and the extract [N] had four different components (**Table 2**). This indicated that the color could result from either a single major color component or a mixture of colored components. In this case, it resulted from a mixture of components, and each of these color components contributed to the final color based on their typical absorption spectra exhibiting the difference in the color hues (M1 and N, N1 in **Figure 1**) in the red spectrum. If the color was due to a single major component, a slight change in the pattern of the absorption spectra including the area under the peak could result in the variation of color hues (M and O in **Figure 1**). However, it must be noted that the variation in the color hue due to the minor colored components in the crude extract was neglected as they were found to be insignificantly present in most of the colored extracts studied. The retention time of major peaks and their absorption spectra were found to be unique (**Table 2**) for each of the red extracts indicating their characteristic color composition. Both of these attributes of the color composition explained the chemodiversity in terms of color among different genera, species, and/or chemotypes also among the same strain grown on different media. The feature that different color hues results on different media is very significant considering the potential biotechnological production of such fungal food colorants. In this case, the cause of this effect might be due to a change in the color composition resulting from a derivative of a parent compound differing only in a slight change in the chromophore and having the same basic polyketide backbone; the results put a large incentive into investigating the metabolic pathways of pigments production in fungi, as such an understanding may lead to possibilities for directing the pathways into desired pigment production and hence a desired hue. One of the major components of the red extract M1 obtained from *Penicillium purpurogenum* chemotype II on YES media was identified to be PP-R, 7-(2-hydroxyethyl)monascorubramine (top panel of **Figure 4**), a structural homologue of the *Monascus* pigments, ankaflavin and monascorubrin. There have been reports where PP-V, a homologue of the red *Monascus* pigment, monascorubramine, and also PP-R were found to be produced by an unidentified *Penicillium* sp. (*27*−*30*). Also, the absorption spectra in the visible region of the major components of red fungal extracts (M, M1, N, N1, and O) from Penicillia (**Table 2**) showed a general pattern comprised of two absorption maxima at 412−430 and 512−528 nm except for the peak 2 in the extract N1 (**Table 2**), which had a single absorption maximum at 462 nm. These values are in agreement with the findings of Jung et al. (*22*), who reported the absorption maximum wavelengths of 422 and 508 for the red *Monascus* pigments monascorubramine and rubropunctamine and of 417−427 and 498−525 nm for their derivatives. Similar absorption spectral patterns with either a single absorption maximum value near 470 nm or two absorption maxima near 410 and 500 nm

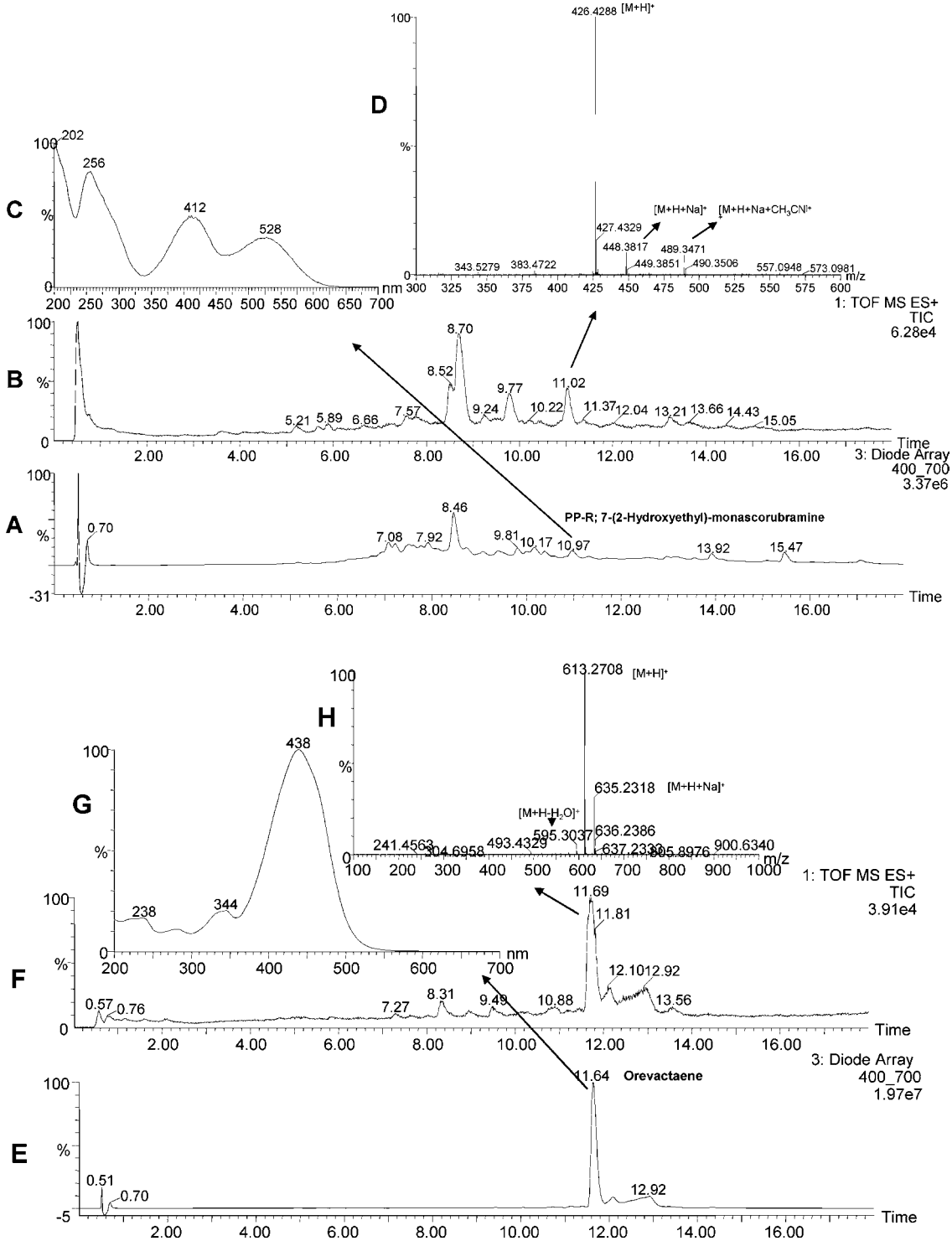

Figure 4. LC-UV-ESI+ chromatograms of some of the representative red and the yellow fungal color extracts. The top panel depicts chromatogram of the red fungal extract M1. (**A**) UV–vis chromatogram showing colored compounds only. (**B**) Total ion chromatogram (*m*/*z* 100–900) from positive ion electrospray. (**C**) UV–vis spectrum of PP-R. (**D**) Mass spectrum of PP-R from ESI+. The bottom panel depicts chromatogram of one of the yellow fungal extracts [V] from *Epicoccum nigrum* grown on YES media. (**E**) UV–vis chromatogram showing colored compounds only. (**F**) Total ion chromatogram (*m*/*z* 100–900) from positive ion electrospray. (**G**) UV–vis spectrum of orevactaene. (**H**) Mass spectrum of orevactaene from ESI+. The time delay between UV and MS signals is 0.05 min.

have also been shown by Juzlova et al. (*31*) for the ethanol extracts of *Monascus* pigments. This indicates that the red pigments produced by these *Penicillia* are either likely to be *Monascus* pigments or have similar chromophores as in *Monascus* pigments. Therefore, it is very likely that the red pigments produced by these *Penicillia* would also exhibit substitution reactions in their chromophores as shown by *Monascus* pigments. Hence, it should be possible to fermentatively produce newer hues in the red spectra by using different side chain precursors as in the case of *Monascus* pigment derivatives (*22*, *23*).

Yellow Extracts. The yellow extracts [S], [T], [U], [V], [W], and [W1] obtained from *E. nigrum* strains grown on one of the three media tested in this study showed relatively similar color

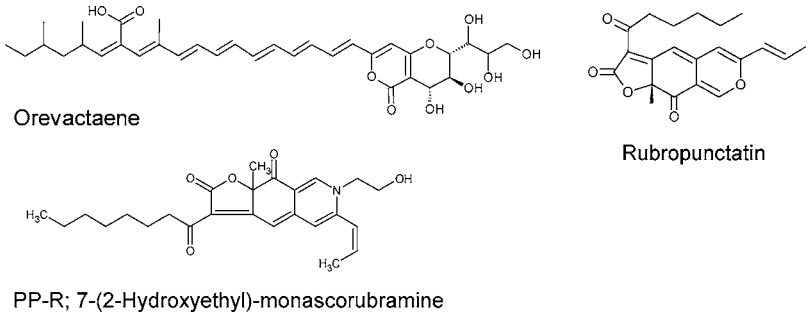

Figure 5. Characteristic structures of some fungal pigments detected and identified.

Table 2. LC-DAD Analysis Showing Pigment Composition of the Red and the Yellow Crude Fungal Extracts

sample code[a]	total no. of major peaks[b]	retention time of major peaks[b] (min)	UV−vis absorption wavelength (nm) (within brackets, % of UV-max; in bold, VIS region)
M (red)	1	7.88	210 (82), 250 (77), 278 (64), **422 (87)**, **518 (100)**
M1 (red)	2	7.08 (peak 1)	200 (35), 246 (65), 280 (63), **424 (83)**, **512 (99)**
		10.97[c] (peak 2)	202 (100), 256 (90), **412 (55)**, **528 (41)**
N (red)	4	7.85 (peak 1)	200 (100), 260sh[f], 320sh[f], **424 (19)**, **518 (32)**
		8.48 (peak 2)	204 (100), 282 (18), **424 (10)**, **524 (11)**
		8.91 (peak 3)	204 (100), 272 (57), **430 (24)**, **518 (33)**
		12.19 (peak 4)	202 (100), 250 (33), 278 (31), **430 (42)**, **518 (48)**
N1 (red)	2	6.93 (peak 1)	248 (58), 286 (25), **430 (70)**, **522 (100)**
		12.17 (peak 2)	238 (69), 288 (27), **400sh**,[f] **462 (100)**
O (red)	1	8.47 (peak 1)	248 (38), 280 (52), **424 (92)**, **520 (100)**
P (red)	2	13.09 (peak 1)	224 (55), 298 (20), **468 (100)**
		13.41[d] (peak 2)	216 (54), 246 (58), 288 (42), **460sh**,[f] **474 (100)**
Q (yellow)	3	10.73 (peak 1)	218 (100), 268 (45), **406 (43)**
		19.69 (peak 2)	222 (100), 260sh[f], 268 (66), **404 (56)**
		20.39 (peak 3)	218 (100), 268 (44), **404 (55)**
R (yellow)	3	8.93 (peak 1)	222 (58), 264 (28), 318 (100), 375sh,[f] **455 (8)**
		11.20 (peak 2)	220 (63), 260 (48), 316 (100), **442 (21)**
		11.51 (peak 3)	222 (82), 262 (58), 288sh,[f] 318sh,[f] 326 (100), **472 (18)**
S (yellow)	2	11.64[e] (peak 1)	200 (17), 280sh[f] 344 (20), **438 (100)**
		12.92 (peak 2)	230 (20), 280sh[f] 342 (20), **440 (100)**
T (yellow)	2	11.65[e] (peak 1)	238 (17), 280sh[f] 344 (20), **440 (100)**
		12.93 (peak 2)	218 (16), 280sh[f] **434 (100)**
U (yellow)	2	11.63[e] (peak 1)	238 (16), 280sh[f] 344 (20), **440 (100)**
		12.92 (peak 2)	238 (16), 280sh[f] 344 (19), **436 (100)**
V (yellow)	2	11.67[e] (peak 1)	238 (16), 280sh[f] 344 (20), **438 (100)**
		12.92 (peak 2)	232 (17), 280sh[f] 344 (20), **438 (100)**
W (yellow)	2	11.64[e] (peak 1)	236 (16), 280sh[f] 344 (20), **440 (100)**
		12.92 (peak 2)	232 (15), 280sh[f] 338 (19), **440 (100)**
W1 (yellow)	2	11.64[e] (peak 1)	238 (17), 280sh[f] 344 (20), **440 (100)**
		12.93 (peak 2)	200 (29), 238 (12), 280sh[f] 344 (39), **436 (100)**

[a] As in **Table 1**. [b] Contributing ≥10% of peak area. [c] Identified as PP-R. [d] Identified as rubropunctatin. [e] Identified as orevactaene. [f] sh, shoulder.

compositions comprised of two predominant colored compounds. Therefore, these yellow extracts represented relatively similar hues in the yellow spectrum (**Figure 2**). One of those two compounds was identified to be the polyketide, orevactaene, as shown from one of these extracts (bottom panel of **Figure 4**). This oxopolyene compound was previously reported (*32*) to be produced also by *E. nigrum.* As compared to the extracts from *Epicoccum* cultures, the color composition of extracts [Q] and obtained from *P. atrovenetum* and *P. herquei* respectively, was markedly different, comprised of three major colored components each. The absorption spectra in the visible region of those three major colored components of extracts (Q) and (R) showed two different patterns comprised of absorption maxima at 404−406 and 442−472 nm, respectively, and hence resulting in a relatively different yellow hue than the Epicoccal extracts (**Table 2** and **Figure 2**).

In conclusion, we have shown that there is a great potential in the extraordinary color range of pigments produced by ascomycetous fungi in the red and the yellow spectra, although there are definitely more color shades that need to be explored from the other parts of the visible spectrum. *Monascus* pigments have been known for a long time, but there exists other pigment-producing genera, the colors of which resemble some of the commercially available colorants in terms of hue and chroma in the yellow spectra. Also, fungal colorants including *Monascus* colorants seem to provide additional hues in the red spectra. In addition, a multivariate approach based on the CIELAB color values seems to be a very useful tool in the colorimetric characterization of colorants. An examination of the stability, including pH and heat stability, of a few of the most promising fungal pigment extracts characterized in the present study are underway in our laboratory.

ACKNOWLEDGMENT

We thank Alan Mortensen for fruitful discussions, and Annette Salskov-Iversen and Hans van den Brink are thanked for critically reading the manuscript.

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Received for review July 24, 2006. Accepted July 27, 2006. We thank Chr Hansen A/S and the Centre for Advanced Food Studies (LMC)−Food Graduate School for financial support of the project.

JF062094N