Studies on the Influence of the Solvent on the Contribution of Single Maillard Reaction Products to the Total Color of Browned Pentose/Alanine Solutions—A Quantitative Correlation Using the Color Activity Concept

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As recently reported, thermal treatment of an aqueous solution of xylose, alanine, and furan-2-carboxaldehyde (mixture I) led to the formation of a variety of Maillard reaction products, among which four compounds, contributing the most to the brown color, could be differentiated from the less color-active compounds by using the color dilution analysis (CDA) and, thus, be proposed for identification experiments. To study the influence of the solvent on the colored compounds, this screening procedure was applied in the present investigation on colorants formed in the Maillard mixture heated in water/methanol (mixture II). In addition to the four colorants identified in mixture I, three additional compounds were evaluated with high color intensities in mixture II. To evaluate the color impact of these color-active compounds more exactly, their absolute color contribution was measured by correlating quantitative data to the color detection threshold. On the basis of the color activity values (CAVs) obtained, it could be shown that the presence of methanol conversely influenced the color contribution of 1- and 3-deoxyosone-derived compounds and that additional compounds were involved in the overall color of mixture II. By application of this concept, 13.4 or 16.5% of the total color of Maillard mixture I or II, respectively, was shown to be caused by four or seven colorants of known structures.

Keywords: *Color activity value; Maillard reaction; colored compounds; nonenzymatic browning*

INTRODUCTION

Browned colors formed by the Maillard reaction consist of complex mixtures of colored compounds. Despite extensive model studies, surprisingly little is known about the key chromophores evoking this brown colorization.

On the basis of the finding that aqueous conditions destabilize certain colorants in Maillard reactions, Ledl et al. (1983) used methanol instead of water to gain insights into the structures of possible labile colorants, for example, labile colored hemiacetal structures could be isolated as their more stable methyl acetals. It is, however, as yet very unclear how the spectrum of colorants formed is influenced by the solvent methanol and which compounds significantly contribute to the color of the Maillard mixtures.

The first attempt to rank colored Maillard reaction products by their color impact was recently made by application of color dilution analysis (CDA) on a heated aqueous solution of xylose, alanine, and furan-2-carboxaldehyde (Hofmann, 1998a). Compounds **1a**/**1b**−**4a**/**4b** (Figure 1) contributing most to the brown color of the reaction mixture could be differentiated from the less color-active compounds and were, therefore, identified (Hofmann, 1998a). This screening technique ranks the colorants on the basis of their relative color intensities in aqueous solution but gives no insight into their absolute contribution to the total color of the browned Maillard mixture.

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In the past 15 years, the calculation of so-called odor activity values as the ratio of concentration to odor threshold of individual odorants has been successfully used to elucidate the contribution of single odor-active compounds to the overall aroma of processed flavors generated by thermal treatment of carbohydrate/amino acid mixtures (Schieberle and Hofmann, 1996a−c) as well as of foods such as wheat and rye bread crust (Schieberle and Grosch, 1994) or fresh strawberry juice (Schieberle and Hofmann, 1997).

To establish the contribution of key colorants in evoking the total color of browned carbohydrate/amino acid mixtures, it might be, therefore, a promising approach to determine the exact amounts of the colorants formed in the Maillard mixtures and to rank these compounds by their color contribution by application of a dosage/activity relationship. Using this concept, the influence of the solvent on the contribution of the key colorants to the total color might be clarified.

The objectives of the present investigation were, therefore, to compare the most intense colorants formed in a methanol-containing xylose/alanine/furan-2-carboxaldehyde Maillard system with those earlier identified in the methanol-free, aqueous reaction mixture on the basis of exact quantitative data. By application of a dosage/activity relationship, these colorants should then be ranked by their color activities to clarify the influence of the solvent on the contribution of these compounds to the total color of the browned Maillard mixtures.

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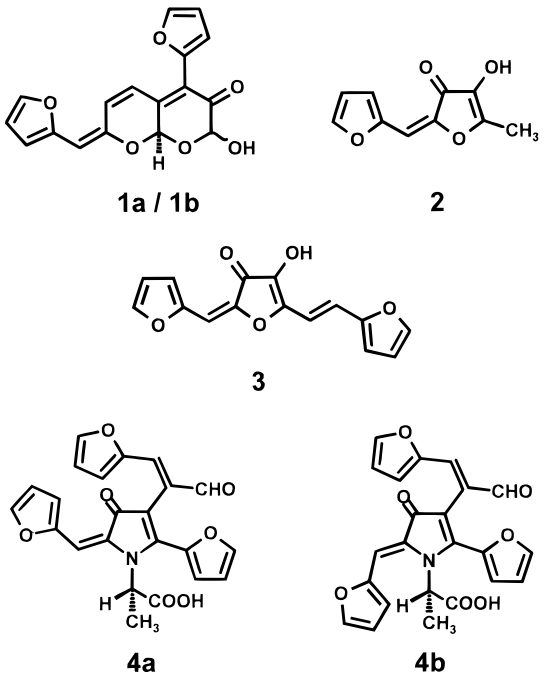

Figure 1. Structures of (1*R*,8a*R*)- and (1*S*,8a*R*)-4-(2-furyl)-7-[(2-furyl)methylidene]-2-hydroxy-2*H*,7*H*,8a*H*-pyrano[2,3-*b*]-pyran-3-one (**1a**/**1b**), 2-[(2-furyl)methylidene]-4-hydroxy-5-methyl-2*H*-furan-3-one (**2**), 2-[(2-furyl)methylidene]-4-hydroxy-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one (**3**), (*S*)-4-[(*E*)-1-formyl-2-(2-furyl)ethenyl]-5-(2-furyl)-2-[(*E*)-(2-furyl)methylidene]-2,3-dihydro-α-amino-3-oxo-1*H*-pyrrole-1-acetic acid (**4a**), and its 2-[(*Z*)-(2-furyl)methylidene] isomer (**4b**).

EXPERIMENTAL PROCEDURES

Chemicals. The following compounds were obtained commercially: D-xylose, L-alanine, furan-2-carboxaldehyde (Aldrich, Steinheim, Germany). Furan-2-carboxaldehyde was distilled twice at 30 °C under high vacuum prior to use. Solvents were of HPLC grade (Aldrich). DMSO-d_6 and CD_3-OD were obtained from Isocom (Landshut, Germany).

Preparation of Reference Colorants. The following compounds were prepared as recently reported: (1*R*,8a*R*)-4-(2-furyl)-7-[(2-furyl)methylidene]-2-hydroxy-2*H*,7*H*,8a*H*-pyrano-[2,3-*b*]pyran-3-one and its (1*S*,8a*R*)-diastereomer (**1a**/**1b**; Hofmann, 1998a), 2-[(2-furyl)methylidene]-4-hydroxy-5-methyl-2*H*-furan-3-one (**2**; Hofmann, 1998a), 2-[(2-furyl)methylidene]-4-hydroxy-5-[(*E*)-(2-furyl)methylidene]methyl-2*H*-furan-3-one (**3**; Hofmann, 1998a), (*S*)-4-[(*E*)-1-formyl-2-(2-furyl)ethenyl]-5-(2-furyl)-2-[(*E*)-(2-furyl)methylidene]-2,3-dihydo-α-amino-3-oxo-1*H*-pyrrole-1-acetic acid and the corresponding 2-[(*Z*)-(2-furyl)methylidene] isomer (**4a**/**4b**; Hofmann, 1997, 1998b), and (2*R*)-4-oxo-3,5-bis[(2-furyl)methylidene]tetrahydropyrrolo[1,2-*c*]-5(*S*)-(2-furyl)oxazolidine and its 5(*R*)-(2-furyl)oxazolidine diastereomer (**5a**/**5b**; Hofmann, 1998c).

(1*R*,8a*R*)- and (1*S*,8a*R*)-4-(2-Furyl)-7-[(2-furyl)methylidene]-2-methoxy-2*H*,7*H*,8a*H*-pyrano[2,3-*b*]pyran-3-one (6a/6b). Colorant **1a/1b** (0.5 mmol) was dissolved in methanol (10 mL), concentrated hydrochloric acid (5 μL) was added, and the mixture was refluxed for 10 min. The red colorant formed was isolated in 98% purity by preparative thin-layer chromatography on silica gel (20 × 20 cm; 0.5 mm; Merck, Darmstadt, Germany) using *n*-pentane/toluene/ethyl acetate (70:15:15, v/v) as the solvent. A red band at $R_f = 0.5$ was scraped off and dissolved in methanol (20 mL). After filtration, the solvent was removed in vacuo, affording **6a/6b** as a red crystalline residue (0.46 mmol, 92% in yield). NMR data are given for both diastereomers separated by slashes: ^{1}H NMR (500 MHz; DMSO-d_6; DQF−COSY; arbitrary numbering of the carbon atoms refers to formula **6a/6b** in Figure 2) δ 3.39/3.30 [s, 3H, H−C(18)], 5.11/5.05 [s, 1H, H−C(1)], 6.28/6.22 [s, 1H, H−C(8)], 6.49/6.47 [dd, 1H, $^3J_{11,10} = 3.54$ Hz, $^3J_{11,12} = 1.77$ Hz, H−C(11)], 6.49/6.55 [d, 1H, $^3J_{15,16} = 3.54$ Hz, H−C(15)], 6.56/6.59 [s, 1H, H−C(13)], 6.65/6.66 [dd, 1H, $^3J_{16,15} = 3.54$ Hz, $^3J_{16,17} = 1.77$ Hz, H−C(16)], 6.79/6.61 [d, 1H, $^3J_{10,11} = 3.54$ Hz, H−C(10)], 7.50/7.51 [d, 1H, $^3J_{6,5} = 5.4$ Hz, H−C(6)], 7.55/7.55 [d, 1H, $^3J_{5,6} = 5.4$ Hz, H−C(5)], 7.65/7.66 [d, 1H, $^3J_{12,11} = 1.77$ Hz, H−C(12)], 7.80/7.81 [d, 1H, $^3J_{17,16} = 1.77$ Hz, H−C(17)]; ^{13}C NMR (500 MHz; DMSO-d_6; DEPT-135, HMQC, HMBC; arbitrary numbering of the carbon atoms refers to formula **6a/6b** in Figure 2) δ 55.2/54.8 [CH_3, C(18)], 72.8/72.8 [CH, C(8)], 101.1/101.5 [CH, C(1)], 101.7/101.78 [CH, C(13)], 103.4/103.7 [C, C(3)], 110.8/109.7 [CH, C(10)], 110.9/111.0 [CH, C(11)], 113.8/113.9 [CH, C(16)], 115.3/115.2 [CH, C(15)], 123.4/123.4 [CH, C(5)], 136.8/136.7 [CH, C(6)], 144.1/143.7 [CH, C(12)], 145.4/145.4 [CH, C(17)], 149.4/149.4 [C, C(14)], 152.3/152.4 [C, C(7)], 152.7/152.5 [C, C(9)], 164.1/163.9 [C, C(4)], 194.3/194.3 [C, C(2)]; LC/MS *m*/*z* 295 (100, $[M + 1 - MeOH]^+$), 327 (29, $[M + 1]^+$); UV−vis (water, pH 7.0) $\lambda_{max} = 460$ nm, $\epsilon = 1.1 \times 10^4$ L mol^{-1} cm^{-1}.

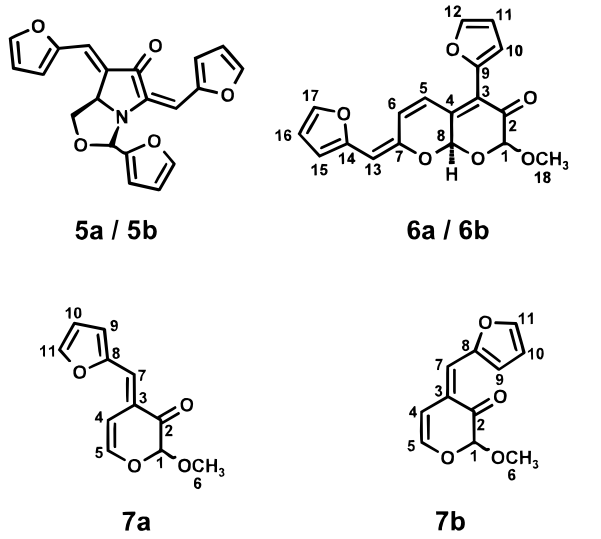

Figure 2. Structures of (2*R*)-4-oxo-3,5-bis[(2-furyl)methylidene]-tetrahydropyrrolo[1,2-*c*]-5(*S*)-(2-furyl)-oxazolidine and its 5(*R*)-(2-furyl)oxazolidine diastereomer (**5a**/**5b**), (1*R*,8a*R*)- and (1*S*,8a*R*)-4-(2-furyl)-7-[(2-furyl)methylidene]-2-methoxy-2*H*,7*H*,8a*H*-pyrano[2,3-*b*]pyran-3-one (**6a**/**6b**), and (*E*)- and (*Z*)-2-methoxy-4-[(2-furyl)methylene]-2*H*-pyran-3-one (**7a**/**7b**).

(*E*) and (*Z*)-2-Methoxy-4-[(2-furyl)methylene]-2*H*-pyran-3-one (7a/7b). Following a procedure of Ledl et al. (1983) with major modifications, a mixture of xylose (0.54 mol) and alanine (0.14 mol) in methanol (100 mL) was refluxed for 1 h, then furan-2-carboxaldehyde (1.0 mol) was added, and heating was continued for another 4 h. After cooling, the solvent was removed in vacuo, and the residue was taken up in water (500 mL) and was extracted with diethyl ether (5 × 100 mL). After concentration to ∼100 mL, the volatile fraction was removed by high-vacuum distillation (0.04 mbar) at 35 °C. The residue was taken up in ethyl acetate, and aliquots were then fractionated by column chromatography (35 × 450 mm) on silica gel (200 g, silica gel 60, Merck) conditioned with toluene. Elution with toluene (400 mL) affords a yellow fraction, which was further fractionated by flash chromatography on RP-18 material (Lichroprep 25−40 μm; 15.0 g; Merck) conditioned in methanol/water (80:20, v/v). Elution with 80 mL of the same solvent yielded a fraction containing a yellow colorant, which was extracted with ethyl acetate (4 × 30 mL). The combined organic layers were separated by thin-layer chromatography on silica gel (20 × 20 cm; 0.5 mm; Merck) using pentane/diethyl ether (95:5, v/v) as the mobile phase. Two yellow bands at $R_f = 0.54$ [(*E*) isomer, **7a**] and $R_f = 0.72$ [(*Z*) isomer, **7b**] were scraped off, and the material was suspended in ethyl acetate. After filtration, the solvent was removed and the yellow residues were immediately analyzed by NMR spectroscopy. The NMR data are given in Tables 2 and 3. Upon standing at room temperature, **7b** was converted rapidly

Table 1. Color Impact of the Complete Maillard Mixtures Heated in Water (I) or in Water/Methanol (II)[a]

Maillard mixture	CD_{total} factor
I	1450
II	900

[a] The reaction mixtures are detailed under Experimental Procedures.

Table 2. Assignment of ^{1}H-NMR Signals (360 MHz, DMSO-d_6) of (*E*)- and (*Z*)-2-Methoxy-4-[(2-furyl)methylene]-2*H*-pyran-3-one (7a/7b)

H at relevant C atom[a]	δ[b] (ppm) **7a**	**7b**	I[c]	M[c]	J[c] (Hz)	connectivity[d] with
CH_3(6)	3.47	3.45	3	s		
H−C(1)	5.19	5.14	1	s		
H−C(4)	6.64	5.96	1	d	5.8	H−C(5)
H−C(10)	6.72	6.69	1	dd	3.5, 1.4	H−C(9), H−C(11)
H−C(5)	6.73	6.59	1	d	5.8	H−C(4)
H−C(7)	7.01	6.80	1	s		
H−C(9)	7.12	7.75	1	d	3.5	H−C(10)
H−C(11)	7.98	7.91	1	d	1.4	H−C(10)

[a] Numbering of carbon atoms refers to formula **7a**/**7b** in Figure 2. [b] The ^{1}H chemical shifts are given in relation to DMSO-d_6. [c] Determined from 1D spectrum. [d] Observed homonuclear ^{1}H,^{1}H connectivities by DQF−COSY.

Table 3. Assignment of ^{13}C-NMR Signals (360 MHz, DMSO-d_6) of (*E*)- and (*Z*)-2-Methoxy-4-[(2-furyl)methylene]-2*H*-pyran-3-one (7a/7b)

H at relevant C atom[a]	δ[c] (ppm) **7a**	**7b**	DEPT[c]	heteronuclear ^{1}H,^{13}C multiple-quantum coherence[d] via ^1J(C,H)	via $^{2,3}J$(C,H)
C(6)	55.8	55.9	CH_3	CH_3(6)	H−C(1)
C(1)	98.6	99.2	CH	H−C(1)	CH_3−C(6)
C(4)	102.5	107.5	CH	H−C(4)	H−C(5), H−C(7)
C(10)	113.1	113.3	CH	H−C(10)	H−C(9), H−C(12)
C(7)	115.7	116.9	CH	H−C(7)	H−C(9)
C(9)	119.6	122.4	CH	H−C(9)	H−C(11), H−C(12)
C(3)	120.9	122.1	C		H−C(4), H−C(7)
C(5)	143.0	140.5	CH	H−C(5)	H−C(1), H−C(4)
C(11)	146.9	143.0	CH	H−C(11)	H−C(9), H−C(10)
C(8)	151.5	146.2	C		H−C(7), H−C(10), H−C(11)
C(2)	188.9	188.3	C		H−C(4), H−C(7)

[a] Numbering of carbon atoms refers to formula **7a**/**7b** in Figure 2. [b] The ^{13}C chemical shifts are given in relation to DMSO-d_6. [c] DEPT-135 spectroscopy. [d] Assignments based on HMQC (^1J) and HMBC ($^{2,3}J$) experiments.

into the predominating isomer **7a**: LC/MS (APCI$^-$) 205 (100, $[M + 1 - H_2]^-$), 190 (17, $[M + 1 - H_2 - CH_3]^-$); UV−vis (water, pH 7.0) $\lambda_{max} = 386$ nm, $\epsilon = 0.7 \times 10^4$ L mol^{-1} cm^{-1}.

Maillard Reaction Mixtures. Mixture I was a solution of xylose (66 mmol) and alanine (16 mmol) in phosphate buffer (90 mL; 1 mmol/L, pH 7.0) and was heated under reflux for 15 min; furan-2-carboxaldehyde (100 mmol) was then added, and heating was continued for another 60 min. Mixture II consisted of xylose (66 mmol) and alanine (16 mmol), dissolved in a mixture of phosphate buffer (60 mL; 1 mmol/L, pH 7.0) and methanol (30 mL), and was heated under reflux for 15 min; furan-2-carboxaldehyde (100 mmol) was then added, and heating was continued for another 60 min.

Quantification of Colorants in Maillard Reaction Mixtures. The pH of the cooled reaction mixture was adjusted to 3 with hydrochloric acid (1 mol/L), and the aqueous mixture was extracted with ethyl acetate (5 × 40 mL). The combined organic layers were dried over Na_2SO_4 and concentrated to ~150 mL at 25 °C under vacuo (100 mbar). To remove volatiles, the extract was then distilled under high vacuum (0.04 mbar) at 35 °C. The intense colored residue was dissolved in a minimum amount of ethyl acetate and was applied onto the top of a glass column (450 × 35 mm) filled with silica gel (150 g, silica gel 60, Merck), which was conditioned with toluene. Chromatography was performed using toluene (300 mL; fraction A), followed by toluene/ethyl acetate (90:10, v/v; 300 mL; fraction B), toluene/ethyl acetate (80:20, v/v; 300 mL; fraction C), toluene/ethyl acetate (70:30, v/v; 300 mL; fraction D), toluene/ethyl acetate (60:40, v/v; 300 mL; fraction E), toluene/ethyl acetate (50:50, v/v; 300 mL; fraction F), ethyl acetate (90:10, v/v; 300 mL; fraction G), ethyl acetate/methanol (90:10, v/v; 300 mL; fraction H), ethyl acetate/methanol (80:20, v/v; 300 mL; fraction I), and ethyl acetate/methanol (50:50, v/v; 300 mL; fraction J). The fractions, containing the colorants given in parentheses, A (**7a**/**7b**), C (**5a**/**5b**, **6a**/**6b**), D and E (**1a**/**1b**, **2**, **3**), and I (**4a**/**4b**) were collected and freed from the solvents under vacuo at 25 °C, and the colorants were taken up in methanol (1 mL). After membrane filtration, the fractions were analyzed by RP-HPLC. Identification of the colorants was performed by comparison of the LC/MS and the UV−vis spectra as well as the retention times (RP-HPLC; **1a**/**1b**, 26.5 min; **2**, 22.5 min; **3**, 30.4 min; **4a**/**4b**, 33.5/33.9 min; **5a**/**5b**, 22.3/21.2 min; **6a**/**6b**, 29.1/28.1 min; **7a**/**7b**, 27.0/28.0 min) with those obtained for the reference compounds. Quantification of the colorants was performed by comparing the peak areas obtained at the absorption maximum of each colorant (**1a**/**1b**, 460 nm; **2**, 350 nm; **3**, 425 nm; **5a**/**5b**, 460 nm; **4a**/**4b**, 414 nm; **6a**/**6b**, 460 nm; **7a**/**7b**, 386 nm) with those of defined standard solutions of each reference compound in acetonitrile.

Determination of Color Dilution (CD_{total}) Factors of the Maillard Mixtures. The Maillard mixtures were diluted with water until a color difference between an aliquot (5 mL) and two blanks (tap water; 5 mL) in a glass vial (1 cm i.d.) could just be visually detected using a triangle test. Using this procedure (Hofmann, 1998a), a color dilution (CD) factor could be defined for each reaction mixture.

Determination of Detection Thresholds. An aqueous solution, containing known amounts of the colorant, was diluted with water until no color difference between the diluted sample (5 mL) and two blanks containing tap water (5 mL) in a glass vial (1 cm i.d.) could be detected using a triangle test. The concentration of the colorant at which a difference between the diluted sample and the two blanks could just be visually detected is defined as the detection threshold.

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Kontron, Eching, Germany) consisted of two pumps (type 422), a gradient mixer (M 800), a Rheodyne injector (100 μL loop), and a diode array detector (DAD type 440) monitoring the effluent in a wavelength range between 220 and 500 nm. Separations were performed on a stainless steel column packed with RP-18 (ODS-Hypersil, 5 μm, Shandon, Frankfurt, Germany) in an analytical scale (4.6 × 250 mm, flow rate = 0.6 mL/min). For quantification of colored Maillard reaction products, the following solvent gradient was used: starting with a mixture (10:90, v/v) of acetonitrile and aqueous ammonium formiate buffer (20 mmol/L; pH 3.5), the acetonitrile content was increased to 100% within 40 min.

Liquid Chromatography/Mass Spectrometry (LC/MS). An analytical HPLC column (Nucleosil 100-5C18, Macherey and Nagel, Dürren, Germany) was coupled to an LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) using electrospray ionization (ESI) and negative atmospheric pressure chemical ionization (APCI$^-$). After injection of the sample (2.0 μL), analysis was performed using a gradient starting with a mixture (10:90, v/v) of acetonitrile and water and increasing the acetonitrile content to 100% within 15 min.

UV−Vis Spectrocopy. UV−vis spectra were obtained by means of a U-2000 spectrometer (Colora Messtechnik GmbH, Lorch, Germany).

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C, DEPT-135, DQF−COSY, TOCSY, HMQC, and HMBC experiments were performed on a Bruker AC-200, a Bruker AM-360, and an AMX-500 spectrometer (Bruker, Rheinstetten, Germany) using the acquisition parameters described recently (Hofmann, 1997). Tetramethylsilane (TMS) was used as the internal standard.

RESULTS

Thermal treatment of xylose, alanine, and furan-2-carboxaldehyde in water (mixture I) or in a water/methanol (2+1) mixture (mixture II) led to a rapid colorization of both solutions. To study the influence of the solvent on the total color intensity of the Maillard mixture, in a first experiment, the color dilution (CD_{total}) factor was determined in both mixtures. The complete Maillard mixtures were, therefore, diluted step by step with water until no color difference between the diluted sample and two blanks containing tap water could be just visually detected using a triangle test. As given in Table 1, a CD_{total} factor of 1450 was found for the browned aqueous mixture I, whereas a somewhat lower CD_{total} factor of 900 was determined for the methanol-containing mixture II, thereby indicating that the color intensity of Maillard mixtures is significantely influenced by the solvent.

To gain more insights into the influence of the solvent on the spectrum of single colorants and their color contribution, we, as recently performed for the aqueous mixture I, selected the most color-active compounds in mixture II by application of CDA (data not shown). The identification experiments were then focused on seven compounds, which were found with the highest CD factors. In addition to the colorants **1a**/**1b**−**4a**/**4b** (Figure 1), three additional compounds were among the most color-active, which were not formed in the absence of methanol.

One of these three compounds exhibited absorption maxima at 379 and 456 nm. LC/MS measurements revealed an intense $[M + 1]^+$ ion at *m*/*z* 350 and a loss of 18 and 96 to *m*/*z* 332 and 254, respectively, most likely corresponding to the elimination of one molecule of water and furan-2-carboxaldehyde. On the basis of spectroscopic data and retention times (RP-18) identical with those of the reference compound (Hofmann, 1998c), this red compound could be identified as a mixture of (2*R*)-4-oxo-3,5-bis[(2-furyl)methylidene]tetrahydropyrrolo-[1,2-*c*]-5(*S*)-(2-furyl)oxazolidine and its 5(*R*)-(2-furyl)-oxazolidine diastereomer (**5a**/**5b**), the structure of which is displayed in Figure 2.

LC/MS measurements of the second colorant exibiting an absorption maximum of 460 nm gave an $[M + 1]^+$ ion at *m*/*z* 327. The loss of 32, most likely corresponding to the cleavage of a molecule of methanol, indicated the same base peak at *m*/*z* 295 as found for compound **1a**/**1b** after cleveage of a molecule of water. As we recently reported on the conversion of **1a**/**1b** into the ethyl acetal by reaction in ethanol (Hofmann, 1998a), these data prompted us to study whether reaction of **1a**/**1b** with methanol generated the unknown orange colorant. The colored compound formed in a heated methanolic solution of **1a**/**1b** showed identical UV−vis and LC/MS data as well as the same retention times (RP-18) as the colorant detected in mixture II, leading to its identification as (1*R*,8a*R*)-4-(2-furyl)-7-[(2-furyl)methylidene]-2-methoxy-2*H*,7*H*,8a*H*-pyrano[2,3-*b*]pyran-3-one and its (1*S*, 8a*R*)-diastereomer (**6a**/**6b**, Figure 2).

The third colored compound, detected by CDA, exhibited an absorption maximum at 386 nm. LC/MS measurements using negative APCI ionization showed a $[M + 1 - H_2]^-$ ion at *m*/*z* 205 (100%) and a loss of 15, most likely corresponding to the cleavage of a methyl group, to an ion at *m*/*z* 190. These data encouraged us to study whether this yellow colorant has a methyl acetal structure. The Maillard mixture was, therefore, heated in anhydrous methanol, affording high amounts of a colorant showing UV−vis and LC/MS data identical with those of the colorant detected in mixture II. After isolation, its structure was characterized by several NMR techniques. The ^{1}H-NMR spectrum measured in DMSO-d_6 showed 16 resonance signals consisting of two sets of 8 signals each (Table 2). The major isomer showed singlets at 3.47 and 5.19 ppm, consistent with the chemical shifts expected for a methoxy group and an acetal proton, thereby confirming the proposed methyl acetal structure. Double-quantum-filtered homonuclear δ,δ correlation spectroscopy (DQF−COSY) revealed strongly coupled ^{1}H spin systems, demonstrating the presence of a furan ring system and two vicinal olefinic hydrogen atoms, which were deduced from the doublets at 6.64 and 6.74 ppm. Structural information obtained by heteronuclear correlation experiments (HMQC, HMBC), which are listed in Table 3, led to the identification of the yellow colorant as a mixture of (*E*)- and (*Z*)-2-methoxy-4-[(2-furyl)methylene]-2*H*-pyran-3-one (**7a**/**7b**; Figure 2). The configuration of the C(3)═C(7) double bond was deduced from the chemical shift differences of the furan proton H−C(9), which was downfield shifted by 0.63 ppm in **7b**, and the olefinic proton H−C(4), which was high-field shifted by 0.68 ppm in the isomer **7a**. In the (Z) configuration, the proximate furan hydrogen H−C(9) should be nearly coplanar with the carbonyl function and, due to the magnetic anisotropy of the carbonyl function, should be strongly deshielded, which was found for the isomer **7b**. This *peri* effect leading to abnormally strong deshielding of a hydrogen was recently reported for pyrrolinones (Hofmann, 1997, 1998c). On the other hand, the (E) orientation of the (2-furyl)methylidene branch brings the furan ring in the planarity of the olefinic proton H−C(4). Due to the anisotropy effect of the furan ring, this effect results in a downfield shift of the resonance signal, fitting well with the NMR data obtained for **7a**. This colorant was earlier reported by Ledl et al. (1983); however, neither ^{13}C-NMR data nor signal assignments for **7a**/**7b** were as yet available in the literature.

To gain more detailed insights into the influence of the solvent on the amounts of the most color-active compounds, the aqueous mixtures I and, in comparison, the methanol-containing mixture II were separated by HPLC and the colorants **1a**/**1b**−**7a**/**7b** were quantified by means of diode array detection using the reference compounds as external standards. The data, given in Table 4, showed that compounds **2** and **3** were formed with 142.5 and 163.8 mg/kg, respectively, by far the highest concentrations in the aqueous mixture. Colo-

Table 4. Concentrations of Selected Colored Compounds Formed in Maillard Mixtures I and II

	concn (mg/kg)	
colorant	I[c]	II[d]
1a/**1b**[a]	38.8	12.5
2[a]	142.5	65.0
3[a]	163.8	73.8
4a/**4b**[a]	23.8	37.5
5a/**5b**[b]	5.0	56.3
6a/**6b**[b]	nd[e]	73.8
7a/**7b**[b]	nd	26.2

[a] The structure of the colorant is displayed in Figure 1. [b] The structure of the colorant is displayed in Figure 2. [c] The reaction mixture was heated in water as detailed under Experimental Procedures. [d] The reaction mixture was heated in a water/methanol mixture (2+1) as detailed under Experimental Procedures. [e] nd, not detected.

Table 5. Detection Thresholds and Color Activity Values (CAV) of Selected Colored Compounds in Maillard Mixtures I and II

colorant	detection threshold[c] (mg/kg of water)	CAV[d] I[e]	CAV[d] II[f]
1a/**1b**[a]	4.1	10	3
2[a]	1.5	95	43
3[a]	2.5	66	30
4a/**4b**[a]	1.0	24	38
5a/**5b**[b]	6.2	<1	9
6a/**6b**[b]	4.1	<1	18
7a/**7b**[b]	3.5	<1	8

[a] The structure of the colorant is given in Figure 1. [b] The structure of the colorant is given in Figure 2. [c] The detection threshold was determined in water using a triangle test. [d] CAV is calculated from the ratio of the concentration to the visual threshold (in water). [e] The reaction mixture was heated in water as detailed under Experimental Procedures. [f] The reaction mixture was heated in water/methanol (2+1) as detailed under Experimental Procedures.

rants **1a**/**1b** and **4a**/**4b** were found in 4.2- or 6.9-fold lower amounts compared to **3**, whereas the red colorant **5a**/**5b** was found only in trace amounts. In mixture II, the highest amounts were found for compounds **3** and **6a**/**6b**, followed by **2** and **5a**/**5b**. In contrast to mixture I, the colorants **5a**/**5b**−**7a**/**7b** were formed among the colored main reaction products. The predominant formation of **6a**/**6b** and the decrease in the amounts of **1a**/**1b** is well in line with the rapid conversion of the hemiacetal into the methyl acetal in the presence of methanol. Comparing the quantitative data in both mixtures indicated that the colorant formation was significantly influenced by the solvent. For example, in the presence of methanol the formation of compounds **2** and **3**, involving the 1-deoxypentosone as a precursor, was unfavorable (Ledl and Severin, 1978; Hofmann, 1998a). On the other side, the amounts of colorants **1a**/**1b** and **4a**/**4b**−**7a**/**7b**, which were found to be formed via the 3-deoxypentosone pathway (Hofmann, 1998c,e), were significantly increased in the methanol-containing Maillard mixture.

Because the quantitative data alone do not allow an estimation of the importance of these colorants in evoking the total color of the browned Maillard mixtures, we, to gain insights into their color contribution, correlated the actual amount of each colorant in the Maillard mixture to its visual detection threshold by combining chemical−instrumental techniques and visual−sensory analysis. To determine the color impact of a single colored reaction product, we defined the color activity value (CAV; eq 1) of a colorant *x* as the ratio of its concentration to its detection threshold, as follows:

$$\mathrm{CAV}_x = \frac{\text{concentration}_x\ (\mu\text{g/kg})}{\text{detection threshold}_x\ (\mu\text{g/kg})} \qquad (1)$$

Colorants, the actual concentrations of which in a solution are at or above their detection thresholds, show, by definition, CAVs ≥ 1 and, consequently, contribute to the total color of the solution.

To calculate the CAVs of the colorants **1a**/**1b**−**7a**/**7b** in the mixtures I and II, we therefore determined the detection threshold of each colorant in water (Table 5). Aqueous solutions containing known amounts of each colorant were diluted step by step with water until no color difference between the diluted sample and two blanks containing tap water could just be visually detected using a triangle test. As given in Table 5, the lowest threshold was found for colorant **4a**/**4b**, with 1.0 mg/kg (water), followed by **2**, with 1.5 mg/kg (water). Colorant **5a**/**5b** showed a 6.2-fold higher detection threshold concentration.

Using the color activity concept, the colorants **1a**/**1b**−**7a**/**7b** were then ranked according to their color activities as given in Table 5. The highest color activity in the aqueous mixture I was found for colorant **2**, followed by colorant **3** showing a 1.5-fold lower CAV. Also, colorant **4a**/**4b** showed a high color activity, because its concentration was 24-fold above its detection threshold. Despite the higher concentration of colorant **1** in comparison to **4a**/**4b**, this colorant did not contribute as much as **4a**/**4b** to the color, because its detection threshold was >4-fold higher. These data demonstrate that the calculation of CAVs is a useful tool to rank colorants on the basis of their color contribution. Well documented by a CAV < 1, colorants **5a**/**5b**−**7a**/**7b** are not involved in the overall color of the aqueous mixture I. Also in mixture II, the highest CAVs were found for compound **2**, closely followed by **4a**/**4b** and **3**. In contrast to mixture I, colorant **1a**/**1b** contributed not much to the overall color of mixture II, whereas compounds **5a**/**5b**−**7a**/**7b** were significantly involved in the brown colorization. These data demonstrated that the solvent significantly influenced the color contribution of the single compounds, showing colorants **2** and **3** with the highest color activities in the aqueous mixture and compounds **2** and **4a**/**4b** in the methanol-containing mixture.

The calculated CAVs rank the colorants on the basis of their relative effectiveness in generating the overall color of the browned Maillard mixtures. To evaluate the percentual contribution of each colorant in the total color, the following calculation was performed: The overall color impact was measured by determination of the $\mathrm{CD}_{\mathrm{total}}$ factor, given in Table 1. For example, the $\mathrm{CD}_{\mathrm{total}}$ factor of 1450 in mixture I means that the detection threshold of the total browning was reached when the original volume of the Maillard mixture I was diluted by a factor of 1450. This indicated that the color of the nondiluted mixture was 1450-fold above its detection threshold. The CAV of each colorant corresponds, by definition, to the factor to which the actual concentration is above the detection threshold. The percentual color contribution of a single colorant *x* (eq 2) could, therefore, be calculated from the color activity value of the colorant (CAV_x) and the $\mathrm{CD}_{\mathrm{total}}$ factor of the complete Maillard mixture, which was defined with 100% color activity, as follows:

$$\text{color contribution of compound } x\ (\%) = \frac{\mathrm{CAV}_x}{\mathrm{CD}_{\mathrm{total}}} \times 100 \qquad (2)$$

As an example, the CAV of 95 for **1a**/**1b** documents that the actual concentration of **1a**/**1b** in mixture I is 95-fold above its detection threshold. Because the complete Maillard mixture, which covered 100% of the total browning, is 1450-fold above its detection threshold, using eq 2 it can be calculated that ~6.4% of the overall color of mixture I is caused by compound **2**. The data, given in Table 6, showed that colorants **2** and **3** contributed to 6.4 and 4.6%, respectively, at the most to the total color of the aqueous mixture. Also, compounds **1** and **4a**/**4b** are significantly involved in evoking

Table 6. Contribution of Selected Colored Compounds to the Total Color of Browned Maillard Mixtures I and II

	I[e]		II[f]	
colorant	color effectivity	contribution to total color (%)	color effectivity	contribution to total color (%)
1a/1b[a]	10	0.7	3	0.3
2[a]	95	6.4	43	4.8
3[a]	66	4.6	30	3.3
4a/4b[a]	24	1.7	38	4.2
5a/5b[b]	<1	0.0	9	1.0
6a/6b[b]	<1	0.0	18	2.0
7a/7b[b]	<1	0.0	8	0.9
$\sum (CAV_{\mathbf{1a/1b}} - CAV_{\mathbf{7a/7b}})$[c]	195	13.4	149	16.5
CD_{total} factor[d]	1450	100.0	900	100.0

[a] The structure of the colorant is given in Figure 1. [b] The structure of the colorant is given in Figure 2. [c] The color effectivity of the seven colorants **1a**/**1b**−**7a**/**7b** was calculated as the sum of their CAVs. [d] The color effectivity of the complete Maillard mixture was determined as the color dilution factor. [e] The Maillard mixture was heated in water as detailed under Experimental Procedures. [f] The Maillard mixture was heated in water/methanol (2+1) as detailed under Experimental Procedures.

the color of the browned Maillard mixture I, because the color contribution was estimated as 0.7 or 1.7%, respectively. The highest color contribution in mixture II was found for colorants **2** and **4a**/**4b**, covering 4.8 and 4.2% of the total color, respectively. In addition, compounds **5a**/**5b**−**7a**/**7b** were significantly involved in the bowning of mixture II, because in sum they caused 3.9% of the total color. The data obtained demonstrate that about 13.4 or 16.5% of the total color of the aqueous mixture I or the methanol-containing mixture II is covered by only four or seven colorants, respectively.

CONCLUSIONS

The data presented demonstrate that the color activity concept, correlating quantitative data with the color detection threshold of a compound, is a useful tool to characterize the key colorants in nonenzymatic browning and to measure their contribution to the total color of the heated Maillard mixtures. The fact that only four key colorants evoke 13.4% of the overall color of the aqueous reaction mixture indicates that the variety of different key types of chromophores might be limited in the Maillard reaction. The characterization of additional colorants and the determination of their color activities are, however, necessary to gain a complete insight into the complex network of nonenzymatic browning reactions of carbohydrates and amino acids.

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