JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Bioautography and Chemical Characterization of Antimicrobial Compound(s) in Commercial Water-Soluble Annatto Extracts

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Annatto preparations based on extracts of the seed of tropical bush *Bixa orellana* L consist of carotenoid-type pigments. Previous reports indicate that commercial annatto extracts have biological activities against microorganisms of significance to food fermentation, preservation, and safety. The objective of this study was to separate and identify the compound(s) responsible for the antimicrobial activity of annatto preparations. Commercial water-soluble annatto extracts were screened by thin-layer chromatography and bioautography followed by liquid chromatography/photodiode array/mass spectrometry (LC/PDA/MS) analysis of active fractions. Bioautography revealed two fractions with antimicrobial activity against *Staphylococcus aureus*. LC/PDA/MS analysis of both fractions revealed 9′-*cis*-norbixin (UV_{max} 460 and 489 nm) and *all-trans*-norbixin (UV_{max} 287, 470, and 494 nm) as the major components. Structure confirmation was achieved by ^1H NMR spectroscopy. Results indicate that 9′-*cis*-norbixin and *all-trans*-norbixin are responsible for the antimicrobial properties of annatto.

KEYWORDS: Annatto; achiote; bioautography; antimicrobial; carotenoids; natural colorant; norbixin; LC/PDA/MS

INTRODUCTION

The use of food additives to enhance the microbial safety of foods is of great interest to the food industry. Essential oils of spices such as cloves, garlic, mustard, and cinnamon are known to contain compounds that possess antimicrobial activities. Understanding how natural antimicrobials work and affect the growth of microorganisms can lead to new technologies for their use in maintaining the safety and quality of foods (*1*). The increasing use of natural extracts in foods suggests that, in order to find active compounds, a systematic study of medicinal plants is very important. Bioautography as a method to localize antimicrobial activity on a chromatogram has found widespread application in the identification of new antimicrobial agents (*2*, *3*).

Annatto extracts are natural coloring agents obtained from seeds of the tropical bush *Bixa orellana* L. The pericarp of annatto seeds is composed of up to 80% of the carotenoid *cis*-bixin IUPAC: methyl hydrogen 9′-*cis*-6,6′-diapocarotene-6,6′-dioate, also known as 9′-*cis*-bixin or α-bixin (*4*); the remaining 20% include *trans*- and *cis*-norbixin (6,6′-diapocarotene-6,6′-dioic acid and 9′-*cis*-6,6′-diapocarotene-6,6′-dioic acid), several apocarotenoids, volatile compounds (*5*), and other uncharacterized substances. When treated with alkali, bixin and all its isomeric forms are transformed to their water-soluble norbixin counterpart. The major coloring component of commercial water-soluble annatto extracts is 9′-*cis*-norbixin. Besides its uses in food as a natural colorant and spice, the medicinal uses of annatto include anecdotal treatment of diabetes (*6*), skin infections, burns, fever (*7*), measles (*8*), gonorrhea (*9*), diarrhea, and asthma (*10*). Previous reports indicate that commercial annatto extracts have biological activities against microorganisms of significance to food fermentation, preservation, and safety (*11*). However, there is no report describing the nature of the antimicrobial compound(s) in commercial annatto preparations. The studies presented here were designed to isolate and identify the main antimicrobial compound(s) present in water-soluble annatto extracts.

MATERIALS AND METHODS

Reagents. All reagents used were of recognized analytical grade unless specified otherwise. Acetic acid, acetone, acetonitrile, chloroform, and methanol were HPLC-grade. Deuterated methanol (CD_3OD) was used as a solvent for NMR analysis and sterile deionized (DI) water was used to prepare the annatto fractions.

Sample Preparation. A stock solution was prepared by adding 10 mL of annatto extract (double-strength water-soluble annatto) (DSM Food Specialties, Menomonee Falls, WI) in 90 mL of sterile DI water. The solution pH was adjusted to 7.4 with 2 N HCl. The stock solution was used directly for bioautography and solid-phase extraction (SPE). To test the effect of pH on the antimicrobial activity of annatto extracts, a series of solutions were prepared from the stock by changing the pH to 9, 8.4, 7, 6, 5, 4, and 3 by adding either 1 N NaOH or 2 N HCl as necessary. Each solution was divided in two: one part was used directly

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10.1021/jf048056q CCC: $30.25
Published on Web 03/11/2005

for the agar diffusion test and the other half was filter-sterilized (ZapCap 0.2 μm pore size membrane) (VWR, Bridgeport NJ). Blank solutions were adjusted for each pH with sterile DI water and either 1 N NaOH or 2 N HCl. All solutions and filtrates were tested for antimicrobial activity by the agar diffusion method (*11*), with *Staphylococcus aureus* (ATCC 25923, American Type Culture Collection, Manassas, VA) as the test organism and antibiotic #1 agar as the growth medium (Difco, Sparks, MD).

Thin-Layer Chromatography. Thin-layer chromatography (TLC) was employed to separate the pigments and other compounds present in annatto. Baker-flex 5 cm × 20 cm, 250 μm analytical layer with IB-2F fluorescent indicator silica gel plates (JT Baker, Phillipsburg NJ) was employed. Samples of 10−15 μL of the stock solution were applied to the Si-gel plates and eluted with a mixture of chloroform/4% acetic acid/acetonitrile/acetone (8:1:0.5:0.5). Spots were visualized with UV light at λ 254 and 365 nm. Most fractions could be visualized without UV light.

Bioautography. This technique has been used for the detection of antimicrobial substances in many biological extracts (*12*). TLC was performed on double-strength commercial water-annatto extracts, by the technique described above. After separation, the retention factor (R_f) of each band was recorded; several plates were kept for reference. Plates were air-dried for 30 min and overlaid with antibiotic #1 agar containing 0.5% calcium carbonate ($CaCO_3$) and 10^6 colony-forming units (cfu) of *S. aureus* (ATCC 25923). Plates were incubated overnight at 35 °C under aerobic conditions and then sprayed with a solution of *p*-iodonitrotetrazolium violet. Areas with bacterial growth appeared violet, while clear zones corresponded to bacterial inhibition. Inhibition zones were compared with the retention factors (R_f) of the related spots on the reference TLC plates. Active fractions were then scraped from the reference plates, dissolved in acetonitrile, and analyzed by liquid chromatography/photodiode array/mass spectrometry (LC/PDA/MS).

Solid-Phase Extraction. Solid-phase extraction (SPE) was used as a cleanup step. A 20 mL volume Supelclean LC-18 cartridge (Supelco, Bellefonte PA) was conditioned with 10 mL of MeOH, followed by 20 mL of 50% MeOH and 20 mL of 5% MeOH solutions. All solutions were acidified with HCl to a final pH of 3.5. The cartridge was loaded with 20 mL of 10% double-strength annatto, washed with 40 mL of 5% methanol, and eluted with a gradient of 5%, 10%, 30%, 50%, 70%, and 90% methanol in acidified DI water (pH 3.5). Each fraction was neutralized to pH 7 and tested for antimicrobial activity with *S. aureus* by agar diffusion. Fractions that presented activity were injected into the LC/PDA/MS. Peaks that matched the retention time of the active compounds found by bioautography were recovered, concentrated by evaporation with nitrogen gas, and retained for NMR analysis.

Liquid Chromatography/Photodiode Array/Mass Spectrometry (LC/PDA/MS). Separation and analysis of annatto pigments were done with a 2690 Alliance separation module high-performance liquid chromatography (HPLC) system equipped with a 996 photodiode array detector (PDA) (Waters Inc., Milford MA) and coupled to a Quattro LC triple-quadrupole mass spectrometer with electrospray interface Z-spray source (Micromass Ltd., Manchester, U.K.). HPLC (20−50 μL injections) was performed on a 250 × 4.6 mm i.d., 5 μm octadecylsilica column (Beckman Instruments Inc., Fullerton, CA) at room temperature with a column flow rate of 1 mL/min. Two methodologies were used for LC separation: the first method consisted of an isocratic elution with 57:43 acetonitrile/acidified water mixture (0.4% glacial acetic acid/5% acetonitrile in DI water), and the second method was run with a gradient of acetonitrile/acidified water. Acetonitrile was increased from 10% to 90% in 30 min, held for 3 min, and then brought back to 10% in 10 min. PDA detection was programmed to scan between 250 and 600 nm. Peak analysis and integration were performed automatically with MassLynx 4.0 (Micromass Ltd., Manchester, U.K.). Mass-spectrometry operation conditions were as follows: source temperature, 140 °C; desolvation temperature, 400 °C; nitrogen nebulization gas, 80 L/h; desolvation gas, 600 L/h; capillary voltage, 3.5 kV in positive mode (ES+) and −2.9 kV in negative mode (ES−); cone voltage 30 kV; start mass 100 and end mass 450 in electrospray positive and negative modes.

Proton Nuclear Magnetic Resonance. ^{1}H NMR spectra were recorded with 1000 acquisitions at a proton spectral width of 3100 Hz and 8K data points with a 300 MHz QE Plus spectrometer (Bruker, Inc., Billerica, MA). All chemical shifts were referenced against the residual proton signal of deuterated methanol.

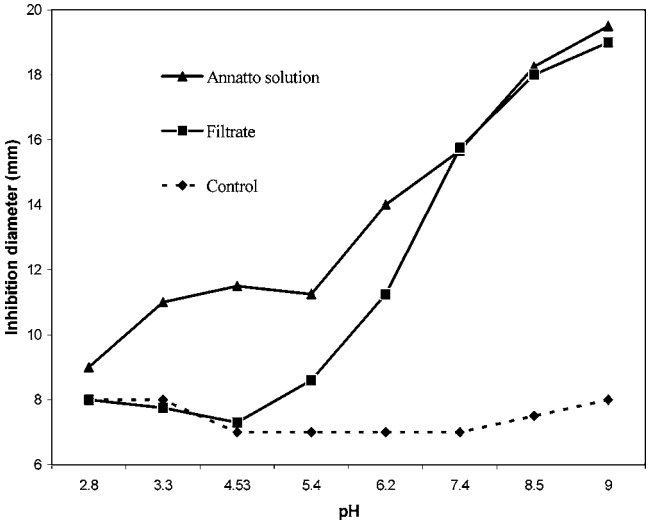

Figure 1. Effect of pH on the antimicrobial activity of commercial water-soluble annatto extracts (SD = 0.88).

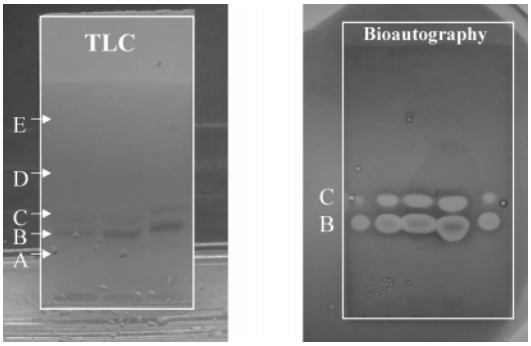

Figure 2. TLC and bioautography of commercial water-soluble annatto extracts.

Spectroscopic Data of Norbixin Isomers: (1) 9′-*cis*-Norbixin (Compound B). UV/Vis λ_{max} 460 and 489 nm; MS ES(−): *m*/*z* 401.5, 380.5, 379.4 (100) [M − H]$^-$, 335.4 [M − CO_2], 291.4 [M − 2CO_2]. MS ES(+): *m*/*z* 382.4, 381.3 (100) [M + H]$^+$, 363.3 [M − H_2O]. ^{1}H NMR (300 MHz, MeOH-d_4): 1.94 (6H, s, -CH_3, H-19 and H-19′), 1.98 (3H, s, -CH_3, H-20′), 2.00 (3H, s, -CH_3, H-20), 5.96 (2H, d, J = 15.5 Hz, H-7 and H-7′), 6.25 (1H, d, H-10′), 6.38 (1H, d, H-10), 6.49 (2H, d, J = 13.6 Hz, H-12 and H-12′), 6.7 (4H, m, H-14, H-14′, H-15, and H-15′), 7.0 (1H, d, J = 13.6 Hz, H-11′), 7.12 (1H, d, J = 15.1 Hz, H-11), 7.8 (1H, d, J = 11.7 Hz, H-8′), 8.54 (1H, s, H-8)

(2) *all-trans*-Norbixin (Compound C). UV/Vis λ_{max} 287, 470, and 494 nm. MS ES(−): *m*/*z* 401.5, 380.5, 379.4 (100) [M − H]$^-$, 335.4 (-CO_2), and 291.4 [M − 2CO_2]. MS ES(+): *m*/*z* 382.3, 381.3 (100) [M + H]$^+$, 380.3, 363.3 [M − H_2O] and 197.0. ^{1}H NMR (300 MHz, MeOH-d_4): 1.94 (6H, s, -CH_3, H-19 and H-19′), 1.98 (6H, s, -CH_3, H-20 and H-20′), 5.96 (2H, d, J = 15.5 Hz, H-7 and H-7′), 6.40 (2H, d, J = 12.1 Hz, H-10 and H-10′), 6.49 (2H, d, J = 15.14 Hz, H-12 and H-12′), 6.7 (4H, m, H-14, H-14′, H-15, and H-15′), 7.12 (2H, d, J = 15.9 Hz, H-11 and H-11′), 8.54 (2H, s, H-8 and H-8′).

RESULTS AND DISCUSSION

In a recent study (*11*), commercial annatto extracts were found to have antimicrobial properties against several Gram-positive bacteria, especially against *Bacillus cereus*, *Clostridium perfringens*, and *S. aureus*. In the present study *S. aureus* was chosen as the test organism, due to its high sensitivity to annatto extracts and its growth properties. *S. aureus* was first used to

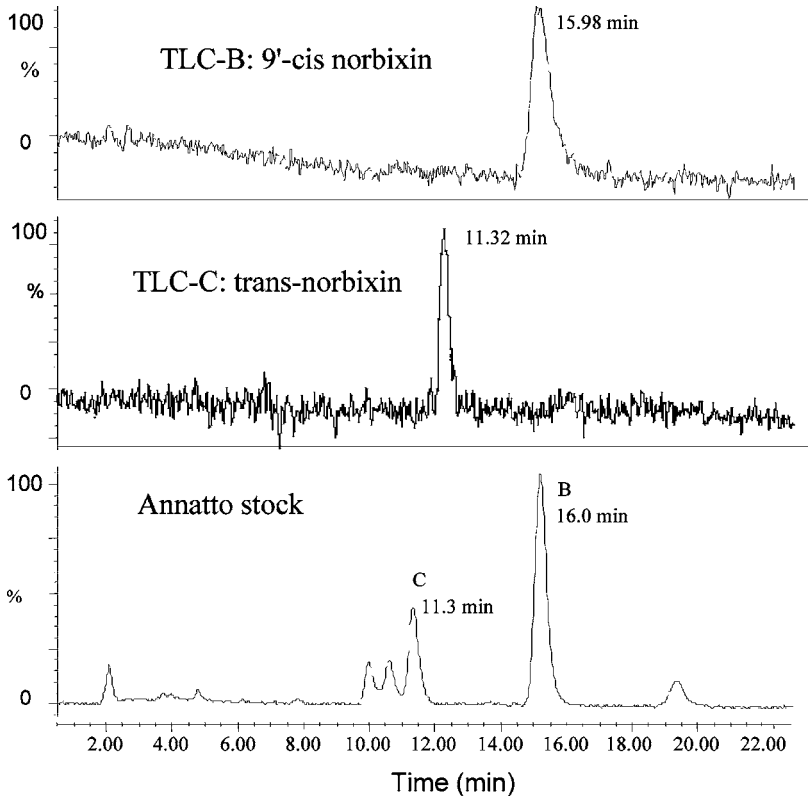

Figure 3. LC/PDA/MS chromatograms of annatto stock solution and TLC fractions B and C under isocratic elution.

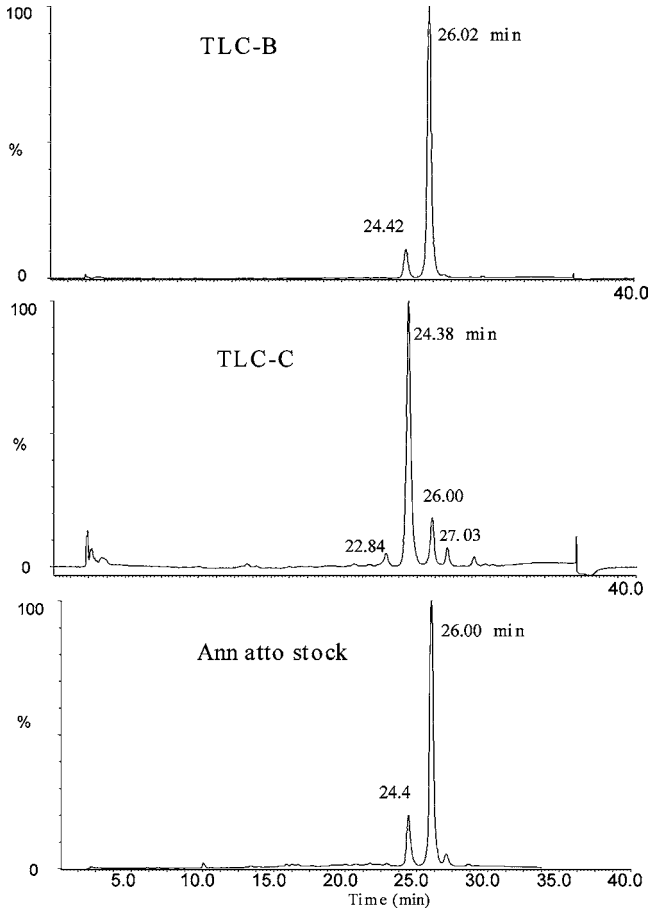

Figure 4. LC/PDA/MS chromatograms of annatto stock and TLC fractions B and C under gradient elution.

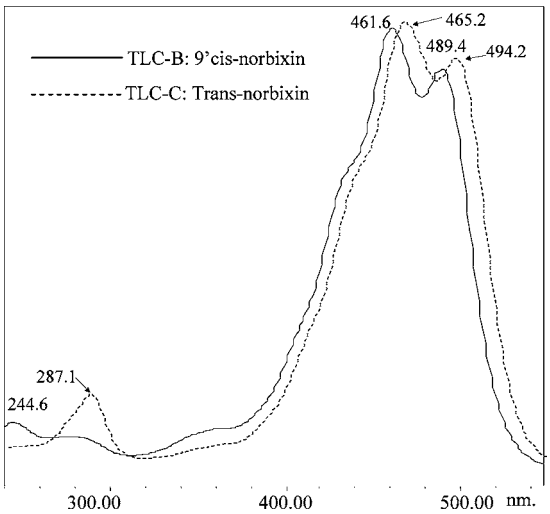

Figure 5. UV/Vis spectra of the major peaks found in fractions B (RT 16.0 min) and C (RT 11.3 min) under isocratic elution.

determine the effect of pH on the antimicrobial activity of annatto extracts. An agar diffusion test was run with a series of annatto solutions whose pH ranged from basic to highly acidic (**Figure 1**). Heat and light can considerably affect the oxidative stability of annatto extracts. When bound to proteins or starch, norbixin is stable to both heat and light, but in aqueous systems, the stability is significantly reduced. Bixin and norbixin suffer degradation at temperatures higher than 100 °C, producing an initial yellow coloration due to the formation of the C_{17} yellow compound among other degradation products and a subsequent loss of color. Light exposure will result in a gradual bleaching of color. In general, annatto pigments are unstable to oxidation, as are all carotenoid compounds, but they are relatively more stable than other carotenoids colorants used in food (*13*).

Commercial water-soluble annatto samples have a pH of 13, which allows the pigment to be in solution; however this is not

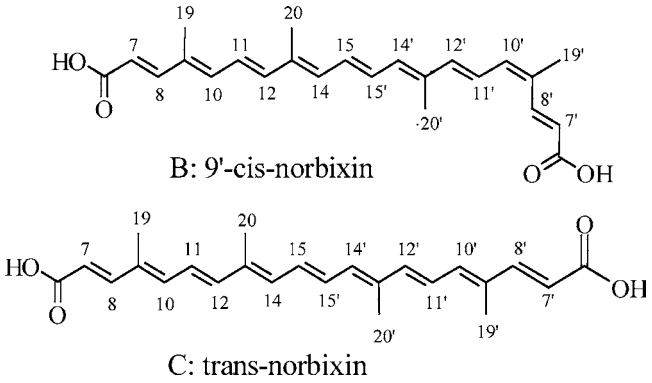

Figure 6. Structures of antimicrobial compounds present in annatto extracts.

usually the final pH of the foods that are colored with annatto. Antimicrobial activity of annatto is dependent on the solution's pH (**Figure 1**). Results showed that annatto has strong antimicrobial activity under alkaline conditions. Below pH 8.5, the pigments start precipitating. At pH 7.4 a strong antimicrobial activity is manifest against *S. aureus* (16 mm diameter of inhibition). Continued lowering of the pH causes a considerable reduction in this activity. At pH 3.3 the diameter of inhibition for the unfiltered sample was 11 mm and there was no detectable inhibition with the filtrate. Due to the extreme pH of some samples, a blank was prepared for each different pH with sterile DI water and tested for inhibition with the same technique. Results obtained for the blanks are included in **Figure 1**.

To determine the partitioning of the active compound when the pH is lowered, different annatto solutions were tested for antimicrobial activity before and after filtration. There is a rapid decrease in the diameter of inhibition with the filtrates at low pH (**Figure 1**). This effect could be due to retention of the active compound on the filter after it comes out of solution at low pH, explaining the low activity obtained with the filtrates. At low pH the pigments aggregate and cannot pass through the 0.2 μm filter; thus from pH 4.5 to 2.8 most of the pigment was retained and the filtrate appeared as a clear solution with no antimicrobial activity. These results confirmed that the active compound(s) reside in the retentate.

Thin-layer chromatography of the stock solution revealed five fractions (A−E) with R_f values of 0.11, 0.24, 0.33, 0.58, and 0.73, respectively (**Figure 2**). Most of the red-orange coloration was found in fractions B and C (R_f 0.24 and 0.33). Bioautography revealed two distinctive areas of bacterial growth inhibition in the TLC plates (**Figure 2**). These areas correspond to fractions B (R_f 0.24) and C (R_f 0.33) in the reference plates. Both B and C fractions were scraped from the TLC plate and analyzed with LC/PDA/MS along with the stock solution and SPE purified fractions.

Two different HPLC methodologies were developed to separate the compounds present in annatto. The isocratic elution method is a modified version of that used by Scotter et al. (*14*) for the analysis of annatto food coloring formulations. This method was used to purify the samples for NMR analysis. A gradient elution method was used in order to improve resolution of peaks that appear in the first 5 min of elution in the isocratic method.

LC/PDA/MS analysis of fraction B by both the isocratic and gradient elution methods revealed one major peak with retention times of 16.0 min (**Figure 3**, isocratic elution) and 26.0 min (**Figure 4**, gradient elution). By comparison of the UV/vis spectra with those reported in the literature (*14*), fraction B presents similar absorption maxima and fine structure as the carotenoid 9′-*cis*-norbixin (**Figures 5** and **6**). Mass spectrometry (MS) showed a major peak at *m*/*z* 379 that corresponds to the quasi-molecular ion $[M - H]^-$ in electrospray negative mode (**Figure 7**). Analyses in electrospray positive mode showed a

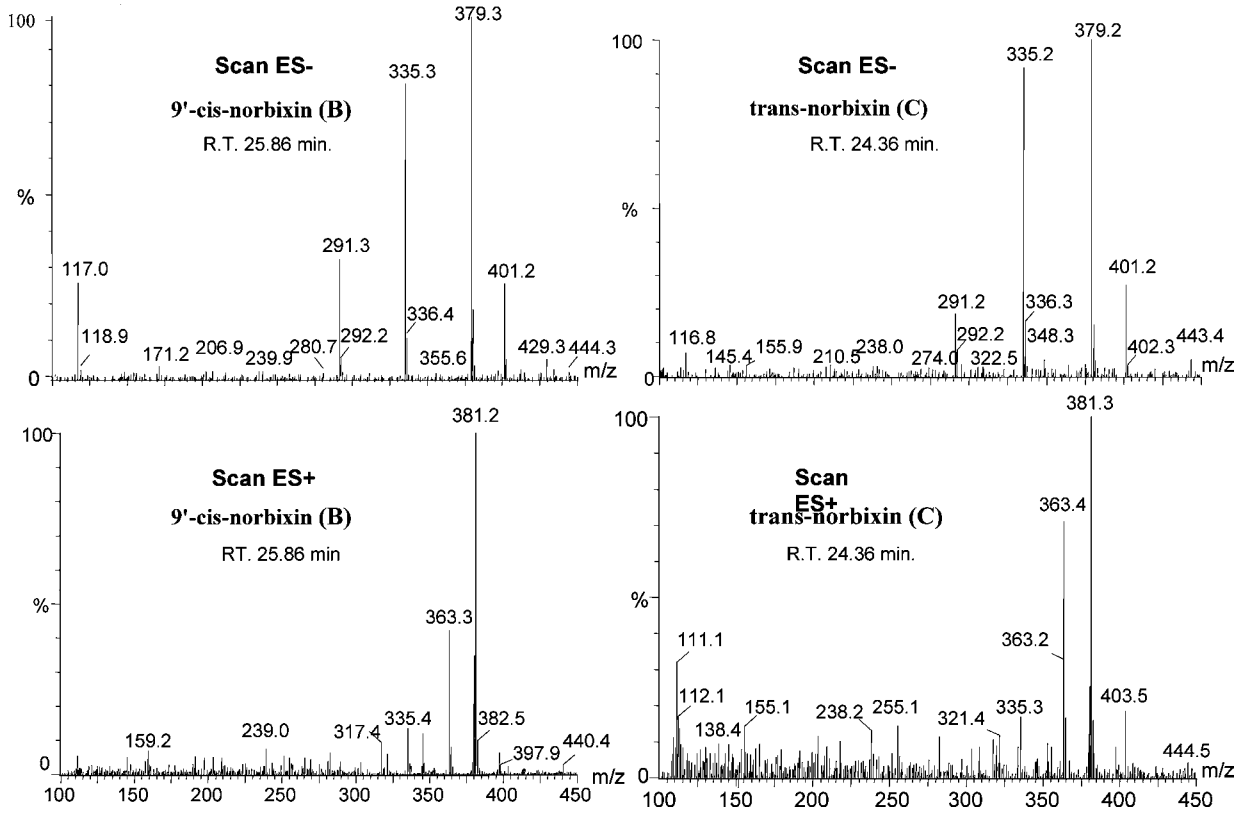

Figure 7. MS spectra of fractions B and C.

major peak at *m*/*z* 381 $[M + H]^+$, both cases indicating the presence of norbixin.

Fraction C revealed a major peak with retention time of 11.3 min (**Figure 3**, isocratic elution), and a mixture of isomers with a major peak at 24.4 min (**Figure 4**, gradient elution). The UV/vis absorption maxima and fine structure were similar to those reported for *all-trans*-bixin (*15*). The peaks that accompany *all-trans*-norbixin in **Figure 4** correspond to di-*cis*-norbixin and 9′-*cis*-norbixin. It is common for carotenoids to undergo spontaneous isomerization to an equilibrium mixture of forms. This can occur at ambient temperatures, is catalyzed by the presence of light or metals, and can explain the different peaks found in **Figure 4**. No major differences were detected on the quasi-molecular ions of fraction C as compared to fraction B when analyzed with MS (**Figure 7**); only small differences can be detected on the residues. The ion *m*/*z* 117 is present in the ES− spectrum of compound 9′-*cis*-norbixin but not in the *all-trans*-norbixin spectrum, in the same way *all-trans*-norbixin shows an ion at *m*/*z* 111.1 in ES+ that is not present in the 9′-*cis*-norbixin spectrum. These differences may be due to different fractionation patterns due to the cis and trans conformational bonds.

To corroborate the results of the UV/vis spectra, proton NMR was performed on fractions B and C. Peak assignments are consistent with previous NMR reports of bixin and its structural analogues (*15*, *16*). NMR spectra confirm the identity of compound B as 9′-*cis*-norbixin and compound C as *all-trans*-norbixin. Structural differences between B and C can be assigned on the basis of the 1.5−2.5 and 6.5−9 ppm regions. Compound B shows two peaks at 1.9 and 2.0 ppm corresponding to the methyl groups C-20′ and C-20, while compound C shows only one peak at 1.9 ppm that integrates for both methyl groups. The differences are due to the symmetry of the molecule: in the case of *all-trans*-norbixin (compound C), the methyl groups are symmetrical and therefore have the same chemical shifts, while the cis bond of 9′-*cis*-norbixin (compound B) provokes a downfield shift of the peak for C-20′. Similar effects can be observed with hydrogens H-8/H-8′ and H-11/H-11′. At 7.11 ppm, *all-trans*-norbixin shows a doublet that integrates for two hydrogens (H-11 and H-11′), while 9′-*cis*-norbixin shows two doublets integrating for one hydrogen each at 6.95 and 7.11 ppm. Finally, 9′-*cis*-norbixin shows a doublet at 7.80 ppm (H-8′) with a coupling constant of 11.7, which reflects the cis bond, and a singlet at 8.5 ppm (H-8), while *all-trans*-norbixin shows both hydrogens as a singlet at 8.5 ppm.

Annatto is known worldwide for its coloring properties, especially in the food industry where it is marketed as a natural food colorant. It is widely used as a condiment in traditional dishes and as a folk remedy, to treat a variety of diseases. Additionally, commercial annatto extracts have antimicrobial properties against important food-borne pathogens such as *B. cereus*, *S. aureus*, and *C. perfringens* (*11*). Our results show 9′-*cis*-norbixin, which constitutes the main component of water-soluble annatto extracts, and *all-trans*-norbixin as the main compounds responsible for the antimicrobial properties found in commercial annatto extracts. It is possible that all norbixin isomers present a certain degree of antimicrobial activity; however, it was not possible to specifically test them during this experiment due to the low concentrations of other norbixin isomers in commercial samples. These findings constitute a novel report of antimicrobial activity from carotenoids. It is known that norbixin binds casein molecules and is thereby stabilized (*17*). Further studies should be performed on the stability of these pigments, the mechanism of microbial inhibition, and the potential inhibitory activity in cheese and other annatto-containing foods.

ACKNOWLEDGMENT

We thank M. Monica Giusti and Dennis C. Westhoff for providing assistance in the laboratory, Walter Schmidt and Ute Klingebiel for conducting the NMR analysis, Cliff Rice and Jorge E. Loyo-Rosales for their assistance with the LC/PDA/MS equipment, and DSM Food Specialties USA Inc. for the contribution of annatto samples.

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Received for review November 19, 2004. Revised manuscript received February 1, 2005. Accepted February 2, 2005. We thank the University of Maryland, Department of Animal and Avian Sciences, the CONACYT/Fulbright Garcia-Robles program for financial support.

JF048056Q