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Time-of-Flight Secondary Ion Mass Spectrometry and X-ray Photoelectron Spectroscopy Analyses of *Bixa orellana* Seeds

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Three different experiments were performed in order to obtain the major carotenoid composition of the natural colorant annatto (E160b) through ToF-SIMS (time-of-flight secondary ion mass spectrometry) and XPS (X-ray photoelectron spectroscopy) analyses. In the first experiment, *Bixa orellana* seeds aril as well as its interior part were analyzed. The analysis of the seeds aril by ToF-SIMS gives the colorant fingerprint without any sample treatment, showing the presence of bixin and its characteristic fragments. The analysis performed in the interior part of the seeds indicates the presence of Fe. The second set of measurements was conducted on the seeds organic extract right after extraction revealing the same components observed by in situ measurement. A third set of measurements was performed aiming to determine the reason for the organic extract color shift observed after 3 months of exposure to ambient light at room temperature. In this case, it was possible to evidence the degradation of bixin by the loss of xylene molecules through ToF-SIMS and the probable carotenoid oxidation based on the C1s XPS spectrum of the degraded extract.

KEYWORDS: XPS; ToF-SIMS; carotenoid; annatto; bixin; degradation; food colorant

INTRODUCTION

The past few years have seen a growth in worldwide concern for food quality and safety; as a consequence, governments have introduced sets of standards (*1*−*3*). Special attention has been devoted to food colorants; in food industry, the safety of colorants is a very important issue. In one hand, colorants are very important due to the visual impact that food color has on the consumers. On the other hand, some colorants tend to be perceived as undesirable and harmful; some are considered to be responsible for allergenic and intolerance reactions (*2*). Colorants can be natural or synthetic. For the food industry, natural colorants are very important, as nature-derived products are considered by the consumers as healthy and of good quality. Among the known natural colorants, annatto (E160b) appears as an important one for food industry primarily due to its potential use as a substitute of the synthetic colorant Tartrazine that has been prohibited in several countries (*3*). Annato was classified by the Food and Drug Administration in the U.S.A. as a "colour additive exempt of certification" (*4*). This red-orange colorant is extracted from the seeds of *Bixa orellana* L. (Bixaceae), a tropical tree native to the Central and South American rain forest. Its principal coloring agent is bixin ($C_{25}H_{30}O_4$), a carotenoid having a free carboxyl and an esterified carboxyl as the end groups (see the chemical structure in **Figure 1**), present in the seed's aril (see the cross-section from a *B. orellana* seed in **Figure 2**). The hydrolytic removal of the methyl ester group from bixin through saponification produces norbixin ($C_{24}H_{28}O_4$), a water soluble carotenoid also found in annatto preparations. Apart from norbixin, other minor carotenoids have been isolated and identified (*5*−*12*).

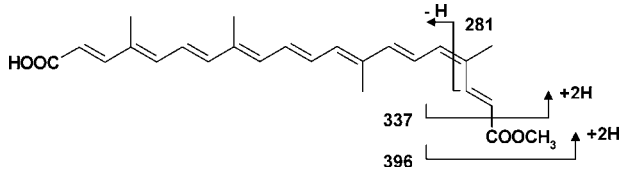

Figure 1. Chemical structure of bixin and main SIMS fragmentation pattern.

In addition to its traditional uses in folk medicine in tropical America, annatto has also been employed worldwide as a color additive for food, drugs, and cosmetics. Although the use of annatto is not restricted at all in the U.S., there are some legislative restrictions of its consumption in Europe. Taking into account toxicological data, an acceptable daily intake (ADI) for annatto as low as 2.5 mg/kg body weight/day (for a preparation containing 2.6% carotenoids expressed as bixin) and as 0.065 mg/kg body weight/day expressed as the pure pigment was established (*5*). However, it is reported that in countries where annatto is used in the preparation of traditional foods, the daily intake can be 150% higher than the ADI (*13*). Notwithstanding the large use of annatto pigment as a food colorant, toxicological

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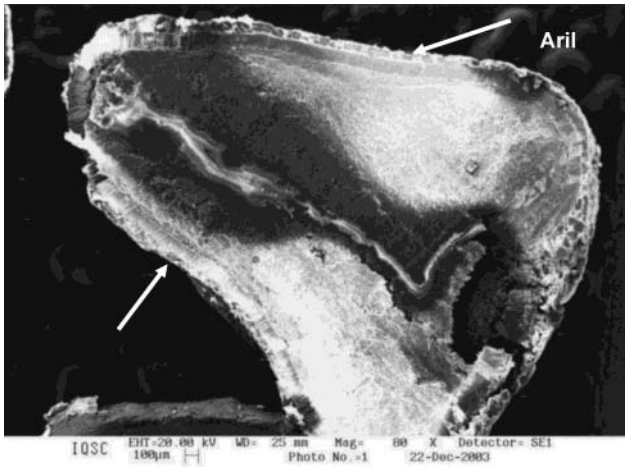

Figure 2. Micrography showing the cross-section from a *B. orellana* seed.

data about annatto pigments are limited and mostly determined in animals using commercial annatto preparations, which are often poorly characterized on a chemical basis (*5*). In considering the use of annatto as a coloring agent, in parallel to the toxicity studies, it is important to determine its constituents in order to validate the toxicity studies, to obtain some information on its nutritional importance, and to establish purity criteria for annatto preparations as some degradation products can release constituents, which raise serious health concerns.

Several techniques, such as UV−visible spectroscopy, proton nuclear resonance (NMR), gas chromatography mass spectrometry, and high-performance liquid chromatography, have been used to study annatto carotenoids (*6*−*19*). In this work, X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (ToF-SIMS) analyses were applied in order to determine the constituents of annatto. The use of these techniques allows the direct measurements on the aril of *B. orellana* seeds and on its organic extract. This opens new possibilities for the analysis of degradation occurring during extraction. By XPS, we inferred the presence of C and O− elements associated to bixin and to other minor carotenoids and N. The ToF-SIMS analyses were used to identify molecules and trace elements in the extract, directly at the aril and at the interior of the *B. orellana* seed.

MATERIALS AND METHODS

Three sets of samples were analyzed. The first one was composed of *B. orellana* seeds that were purchased from a local retail outlet in São Carlos (State of São Paulo, Brazil). Because of its high spatial resolution, ToF-SIMS analyses were performed on the aril as well as in the interior of the seeds in order to determine the spatial localization of the colorant. XPS analyses were performed on the aril only, as it was established by ToF-SIMS that the colorant was localized in this part of the seed. A second set of samples was composed of the organic extracts obtained by washing seeds of *B. orellana* with (1:3) a solution of methanol and chloroform at room temperature and analyzed just after the extraction (*20*). Finally, to detect possible degradation, the previous set was analyzed after it had been exposed to ambient light during 3 months. A color change was observed, which is an indication of degradation (*14*, *15*). To perform the analysis, the extracts were dripped on a silver substrate and blown under dry nitrogen.

The XPS measurements were performed with a system equipped with a hemispherical electron energy analyzer (HP5950A spectrometer). The photon source was a monochromatized Al Kα line ($h\nu = 1486.6$ eV). The resolution of the system (source + analyzer) was 0.7 eV. Charging effects were neutralized using a flood gun operated at 2 eV of kinetic energy. To avoid degradation by heating during the XPS measurements, the samples were cooled to room temperature. The cooling temperature was checked by a thermocouple fixed on the sample holder. A review of surface analysis with XPS can be found in ref *21*. Typical XPS spectra display the electron binding energy on the *x*-axis and the intensity on the *y*-axis. Units of intensity are counts per second, so that the intensity depends on acquisition time and X-ray photon intensity. Because those parameters often change from one spectrum to another, arbitrary units are generally preferred.

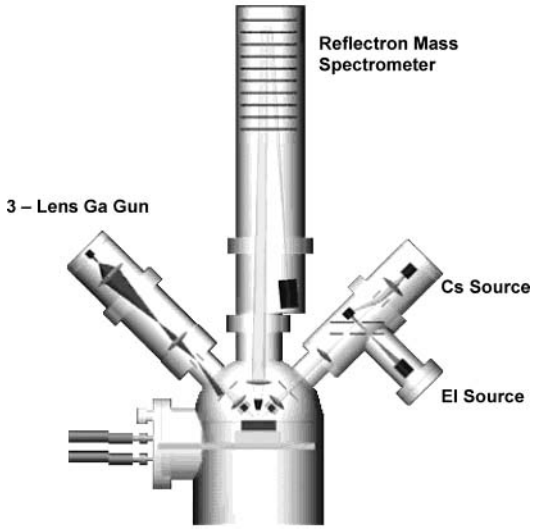

Figure 3. Schematic of the ToF-SIMS instrument.

The ToF-SIMS measurements were performed with a ToF-SIMS IV instrument from ION-TOF. A schematic of the instrument is given in **Figure 3**. The sample was bombarded with a pulsed Gallium ion beam. The secondary ions generated were extracted with a 2 keV voltage and their time-of-flight, from the sample to the detector, was measured in a reflectron mass spectrometer. Other ion sources were also available for depth profiling. A review of surface analysis with ToF-SIMS can be found in ref *22*. Typical analysis conditions for this work were 25 keV pulsed Ga^+ beam at a 45° incidence, 2 pA pulsed current rastered over a 130 μm × 130 μm area. The ion fluence was kept below 3×10^{12} ions/cm^2 to ensure static conditions. The mass resolution ($m/\Delta m$) around mass 30 was typically 8000. Electron floodgun charge compensation was necessary to study the seeds. Typical mass spectra displayed the mass over charge ratio (m/z) on the *x*-axis and the intensity on the *y*-axis. Units of intensity are counts per channel so that the intensity depends on the channel width and analysis beam current. Because those parameters often change from one spectrum to another, arbitrary units are generally preferred.

RESULTS AND DISCUSSION

ToF-SIMS. The analysis of the seeds aril gives the colorant fingerprint in a direct form, i.e., without any sample treatment as extraction is not necessary in this case. **Figure 4** shows the typical ToF-SIMS spectrum recorded at the seed aril, using charge compensation. The peak at m/z 396 can be assigned to the bixin molecular ion plus two hydrogen atoms ($C_{25}H_{32}O_4^+$ or M + 2). The peaks at higher masses (m/z 397 and 398) are (M + 2) molecular ions containing one or two ^{13}C isotopes (the natural abundance of ^{13}C is 1.1%). The presence of characteristic fragments at m/z 337, which is attributed to $C_{23}H_{29}O_2^+$ obtained from the previous molecular ion with loss of a $COOCH_3$ ester group and at m/z 281, a fragment compatible with the $C_{19}H_{21}O_2^+$ ion obtained by the loss of a $C_6O_2H_8$ end group plus one hydrogen atom (see **Figure 1**), confirms the previous assignment. Moreover, a high-intensity peak at m/z 105 compatible with the $C_8H_9^+$ ion indicates the presence of xylene (C_8H_{10}); it is known that the bixin molecule reorganizes by losing a xylene fragment (*8*). The seeds were cut, and their internal parts were analyzed (**Figure 2**). Bixin and its related

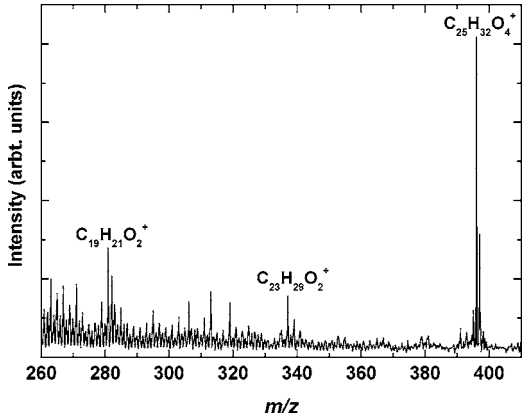

Figure 4. ToF-SIMS spectrum of the *B. orellana* seeds aril, M + 2H region.

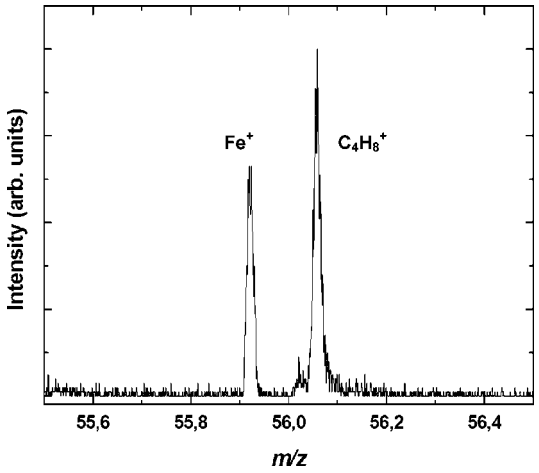

Figure 5. ToF-SIMS spectrum of the *B. orellana* seeds interior, Fe^+ region.

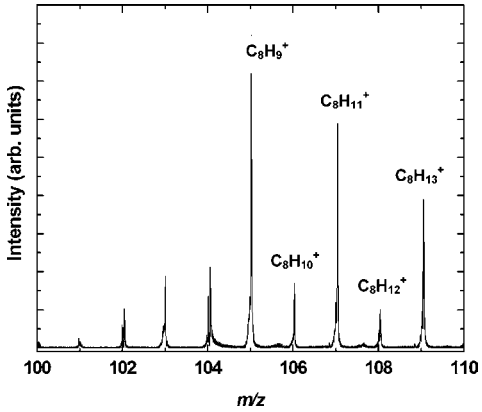

Figure 6. ToF-SIMS spectrum of the annatto extract deposited on Ag, xylene region. The assignment for peaks at mass lower than 105 is not yet clear.

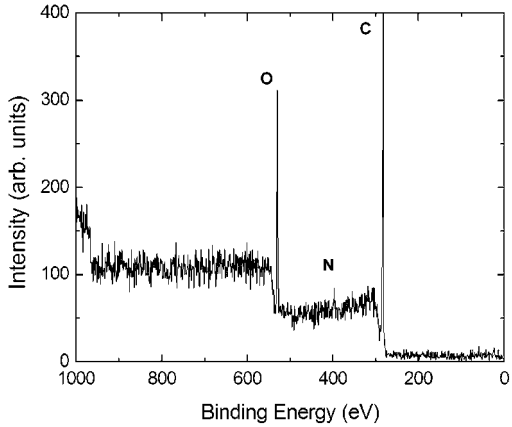

Figure 7. Survey XPS spectrum of the seeds extract.

fragments were not observed in the spectra recorded at the internal part of the seeds. This simply implies that the colorant is only present at the seed's aril, which is red indeed, but not in internal parts of the seed, which are not colored. Typical spectra recorded on this region are dominated by organic fragments. However, it is important to note the presence of a peak at *m*/*z* 55.93 in **Figure 5**, which indicates the presence of Fe inside the seeds. The other peak at *m*/*z* 56.06 is attributed to the fragment $C_4H_8^+$. Because of the technique's high mass resolution, the two ions produce well distinct peaks on the spectrum. The $^{56}Fe^+$ ion does not have any isobaric interference with other elements or fragments, and other isotopes, such as $^{54}Fe^+$, were measured, which makes the assignment unambiguous.

Above *m*/*z* 300, the positive ToF-SIMS spectrum recorded on the extract just after it had been prepared is dominated by the bixin molecular peak at *m*/*z* 396, as it was observed in the spectra recorded on the aril of the seeds. The characteristic fragments obtained on the aril of the seed are all present on the spectrum taken on the extract. The presence of Fe was observed in some of the extract samples even though no traces of Fe were detected on the seed's aril. This observation suggests that during the extraction the solvent was in contact with the inner part of the seed containing Fe. The possibility of having Fe as one of the components of the annatto powder can add important nutritional value to this colorant; this issue has to be explored considering the extraction mechanism of the colorant from the seeds.

The extract was reanalyzed after 3 months of exposure to ambient light. By inspecting the appearance of the extract, it was easy to observe that the initially orange/red pigment turned to light yellow. The change in the extract coloration is due to the known degradation of the pigment (*14*, *15*). The spectra obtained after degradation exhibit a severe decrease (by a factor of 5) in the intensity of the peak associated to the $[M + 2]^+$ molecular ion, indicating a gradual degradation of the bixin, along with a 2-fold increase of the $C_8H_9^+$ signal, attributed to xylene (**Figure 6**). This can be explained by the main degradation pathway of the bixin molecule, which reorganizes into a smaller one by releasing *m*-xylene (*15*). It should be noted that some $C_8H_9^+$ fragments are also probably formed during the analysis as fragmentation products of the bixin molecule, induced by the impinging ions. This explains the already high signal of this ion even on the nondegraded seeds aril. However, the further signal increase on degraded samples is most likely related to the production of *m*-xylene at the surface.

XPS. Figure 7 shows the typical survey XPS spectrum recorded on the annatto samples. It reveals essentially the presence of carbon, oxygen, and nitrogen (*21*). The XPS core level C 1s spectra recorded on the seeds and on the degraded extract are shown in **Figure 8**. The C 1s core level (**Figure 8a**) recorded on the seed can be decomposed into four contributions appearing near 284.8, 286.4, 287.7, and 289.1 eV. The main peak near 284.8 eV was assigned to sp^2-hybridized carbon atoms and to sp^3 carbon atoms bound to hydrogen. Peaks near 286.4, 287.7, and 289.1 eV are typical of carbon atoms bound to oxygen atoms (like in epoxide or enols, aldehyde, and carboxylic acid functions). The C 1s core level XPS spectrum recorded after the degradation by ambient light is shown in **Figure 8b**. It can be seen that after degradation the four components are

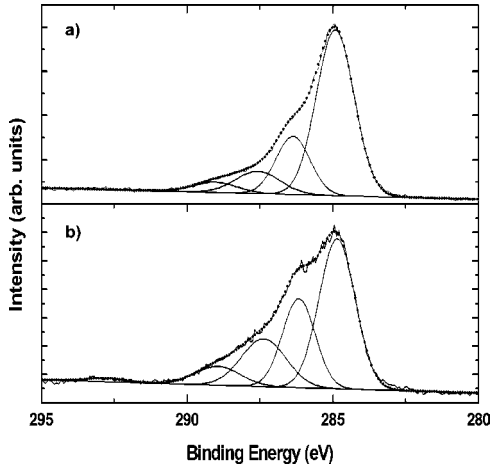

Figure 8. C1s XPS spectrum of (**a**) the aril of the seeds and (**b**) the extract after 3 months exposed to ambient light at room temperature.

still observed but the relative percentage of each contribution has changed. Comparing the relative intensity among the components to the main peak, it can be observed that for the degraded sample the components related to carbon atoms bonded to oxygen atoms show higher relative intensities (0.6, 0.4, and 0.14, respectively, for 284.8, 286.4, and 287.7 eV) than for the case of the data recorded on the seed (0.3, 0.12, 0.06). This indicates that after degradation, a reduction of the sp^2 species with an increase of the carbon atoms present in functions as carboxylic acid, epoxide, enols, and aldehyde is observed. This is an indication of degradation probably due to oxidation of the carotenoid double bonds. It was reported that the color shift observed in cheddar cheese—a dairy product that contains annatto as a colorant—when stored under CO_2 atmosphere and exposed to fluorescent light is due to the oxidation of bixin by free radicals (*21*). On the basis of this assumption, the rise in the peaks of carbon bonded to oxygen atoms can be attributed to oxidation products of the carotenoids present in the annatto extract.

In conclusion, the use of XPS and ToF-SIMS as analysis techniques of natural samples was shown to be important since usual techniques need a set of precautions, such as use of diffuse light, low temperature, inert atmosphere, and solvent free of acids and peroxides, to prevent degradation during the extraction and often demand higher quantities for analysis. It was shown that both techniques allow the direct measurement on the seed; this opens a new possibility considering the analysis of natural samples.

It was shown that bixin, one of the main coloring constituents of the annatto seed, could be easily detected by ToF-SIMS at the seed's aril, without any sample treatment. The spectra obtained on the crude sample were similar to those obtained on a seed extract. After prolonged exposure to light, a significant degradation of the bixin is observed. A systematic identification of the products of degradation is being conducted and should open a new possibility for ToF-SIMS application.

The presence of a peak at mass 56 associated to Fe was observed in the internal part of the seeds but not at the seed's aril, indicating that this element can be found only inside the seed. However, in some extract samples, this element was observed; this is probably due to the presence of cracks in the aril of the seeds used to prepare the extracts. On the basis of this result, depending on the extract mechanism, i.e., if the inner part of the seed is exposed or not during the extraction, the presence of Fe can be tailored. This could add nutritional value to this colorant provided bioavailable species of Fe could be found. We still work in the determination of the Fe species.

The molecular sensitivity of ToF-SIMS has proven to allow the straight detection of the main constituents present in annatto seeds, with potential applications in food industry. Several minor constituents were also detected, but their precise assignment still requires further analysis. The XPS measurements are in accordance with the ToF-SIMS results. After degradation, reductions of the sp^2 species with an increase of the carbon atoms present in functions as carboxylic acid, epoxide, enols, and aldehyde were observed probably due to an oxidation process.

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