

ToF-SIMS Characterization of Thermal Modifications of Bixin from *Bixa orellana* Fruit

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Bixa orellana fruit extracts were studied by time-of-flight secondary ion mass spectrometry (ToF-SIMS). The intensity of the peak at m/z 396, assigned to the bixin molecular ion plus two hydrogen atoms ($C_{25}H_{32}O_4^+$), decreased as the extract was heated and nearly disappeared with heating above 150 °C. Simultaneously, the formation of dimers at m/z 790, 804, and 818 was observed. The ToF-SIMS spectrum is characterized by a large amount of peaks generated by the principal ions and their multiple fragmentation patterns. To extract maximum information from the data set, multivariate statistical analysis was applied. Principal component analysis revealed important structural changes of the bixin molecule upon heating at different temperatures. This information can be used by the food industry as by controlling the temperature of the heating process the red/yellow balance of this colorant can be tuned.

KEYWORDS: Bixin; *Bixa orellana*; food colorant; carotenoid; ToF-SIMS; thermal degradation

INTRODUCTION

The prohibition on the use of the tartrazine (E-102) synthetic dye in several countries gave strength to research for possible substitutes. Among the alternatives, annatto (E-160b) appears to be promising. This red-orange colorant, classified by the U.S. Food and Drug Administration as a “color additive exempt of certification”, is extracted from the pericarp of the seeds of *Bixa orellana* L. (Bixaceae), a tropical tree native to the Central and South American rain forest. The major coloring component of annatto is the apo-carotenoid 9'-*cis*-bixin ($C_{25}H_{30}O_4$), a carotenoid having a free carboxyl and an esterified carboxyl as end groups (see the chemical formula in **Figure 1**) (1). The conjugated double-bond system constitutes the light-absorbing chromophore responsible for the red color. Loss or change in its color enables the immediate indication of degradation or structural modification of the molecule. In fact, most carotenoids, not just bixin, are susceptible to isomerization and oxidation due to their highly unsaturated nature. Those reactions can easily occur during the extraction or analysis of bixin. To avoid interference on the structure during the analyses, some preventive measures such as reduced time of analysis, elimination of oxygen, protection from light, temperature control, and use of high-purity solvents are required. On the other hand, the extraction methods of the colorant have to be very well documented to establish safety specification.

An important method applied for the commercial extraction of annatto pigments from the *B. orellana* fruit consists of heating

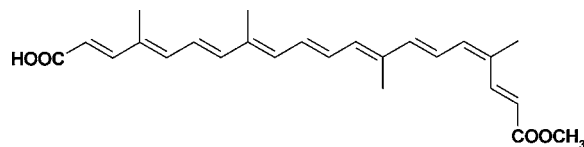


Figure 1. Main coloring component: $C_{25}H_{30}O_4$, mass $M = 394$ Da.

the suspension of seeds in oil to a maximum temperature of 130 °C in vacuo (2, 3). It is important to mention that the temperature appears as one of the main sources of the bixin molecule degradation, that is, molecular structural modification. Near the extraction temperature, bixin undergoes a series of degradation reactions. As mentioned before, the colorant degradation is observable by color changes, which is an important characteristic for the food industry as by controlling the temperature and duration of the heating process the red/yellow balance of this colorant can be tuned (3). The possibility of tailoring the colorant end color opens a vast range of use. Nevertheless, the degradation or structural modification of the bixin is accompanied by the release of *m*-xylene, toluene, toluic acid, and toluic acid methyl ester, all of which are undesirable in preparations intended for food use (4). Therefore, in considering the use of annatto and its related “degraded” products as coloring agents, it is necessary to apply suitable methods of analysis to characterize the annatto preparation and the thermal degradation products of bixin.

Traditionally, carotenoid analysis has relied on gas chromatography and mass spectrometry (MS) (5–15). MS is used to identify volatile and semivolatile organic compounds in complex mixtures. Components of chemical mixtures are separated in the gas chromatograph and identified by their respective masses

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in the mass spectrometer. Organic compounds must be dissolved in volatile and organic solvents for injection into the gas chromatograph.

In the present study, to evaluate the stability of the bixin molecule and the formation of degraded products when heated in vacuo, time-of-flight secondary ion mass spectrometry (ToF-SIMS) was applied. To our knowledge, this is the first time that ToF-SIMS has been used to perform a detailed analysis of the thermal degradation of a carotenoid. ToF-SIMS is a surface analytical technique that uses an ion beam to remove atoms or molecules from the outermost layer of a material surface. A short pulse of primary ions strikes the sample surface, and the secondary ions produced during the impact are extracted into a time-of-flight mass spectrometer and dispersed in time according to their velocities. Discrete packets of ions of differing mass are detected as a function of time at the end of the flight tube. ToF-SIMS is capable of detecting ions over a large mass range, up to 10000 atomic mass. Among the advantages of the ToF-SIMS technique are the possibility to analyze directly the samples, without need of dissolving them in volatile solvents; detection of concentrations as low as 1 ppm; analysis of the spatial distribution of elements and molecules with 100 nm lateral distribution; analysis of insulating materials; and measurements recorded under high vacuum, avoiding reaction with undesired chemical elements. A review of surface analysis with ToF-SIMS can be found in ref 16.

Ion bombardment of the sample leads to molecular fragmentation, generating mass spectra with a large amount of peaks. To extract maximum information from ToF-SIMS spectra, multivariate statistical analyses are very appropriate (17). This statistical tool operates the reduction of the dimensionality of the acquired data. This work describes the application of principal component analysis (PCA) to inspect similarities and trends among ToF-SIMS spectra recorded on bixin at different temperatures in order to reveal changes in structural features aiming to attain information about the degradation path of the bixin molecule.

MATERIALS, EXPERIMENTAL PROCEDURE, AND METHODS

Low-intensity light was used during sample preparation and analysis to avoid sample degradation by light.

Materials. Seeds were removed from *B. orellana* fruits, originating from Sao Carlos (State of Sao Paulo, Brazil), just before the analysis. The bixin extract was obtained by washing the seeds with dichloromethane at room temperature. The extract was not purified; it is therefore likely that it contains in addition to bixin various other extractants such as resinous matter. The extract was then dripped on a silicon substrate and dried under a dry nitrogen flux prior to analysis.

Experimental Procedure. The sample was mounted immediately after preparation in the introduction chamber of the ToF-SIMS equipment, pumped down to 10^{-6} mbar, and then transferred to the analysis chamber, where the background pressure during the experiment was better than 10^{-9} mbar.

The mass spectra of the samples were recorded on a ToF-SIMS IV instrument from ION-TOF GmbH. To perform the measurements, the sample was bombarded with a pulsed gallium ion beam. The generated secondary ions 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. Typical analysis conditions for this work were a 25 keV pulsed Ga^+ beam at a 45° incidence and a 2 pA pulsed current rastered over a $130 \mu\text{m} \times 130 \mu\text{m}$ area. The total ion fluence was kept below 3×10^{-12} ions/cm² to ensure static conditions. The mass resolution ($m/\Delta m$) near mass 29 was typically 8000. For each spectrum, the mass scale was calibrated by using well-identified hydrocarbon fragments, such as CH_3^+ , C_2H_2^+ , and C_3H_4^+ .

For the thermal modification study of the bixin molecule, the samples were heated in situ by a regulated resistive heater attached to the sample holder. The temperature was monitored by a thermocouple attached to the silicon substrate surface. The temperature was increased by steps of 5–10 °C, and the analysis was performed 5 min after the set temperature was reached. The analyses were performed in the range of 23–150 °C on different fresh sample areas each. Acquisition time for each spectrum was 2 min.

Methods. PCA Analysis. PCA is capable of revealing information in a large data set that cannot be accessed by visual examination or univariate analysis; it tries to reduce the dimension of a data set presenting many variables. This is achieved with a mathematical transformation of the original variables generating a new set of variables called principal components (17, 18). Geometrically, new coordinate axes (principal components) are obtained by the rotation of the original axes in a way that the directions of the principal components correspond to the directions of major data spread (variance). The first principal component explains most of the variability present in the data set. Two properties of principal components are that they are orthogonal among them and that each consecutive PC carries less information than the previous one. In this new set of axes, all observations have a new set of coordinates called scores. Depending on the data set a few PCs can already contain a high percentage of information, enabling the analyst to have a good picture of the (dis)similarities among the observations.

Another important feature of PCA regards the loadings, which are defined as the cosine direction between the original variables and the PCs. Loadings are used to determine the contribution of each original variable to the data spread observed in the score graph. High positive or negative loadings for a given variable indicate a high correlation with the PC, whereas loadings close to zero indicate low correlation.

RESULTS AND DISCUSSION

Characterization of the Bixin Extract. Figure 2 shows a typical ToF-SIMS spectrum recorded on the extract just after it had been prepared. It is characterized by a peak at m/z 396 that is assigned to the bixin molecular ion plus two hydrogen atoms ($\text{C}_{25}\text{H}_{32}\text{O}_4^+$ or $M + 2$). In ToF-SIMS, it is a common feature to observe the protonated molecular ion ($M + 1$) as the most intense peak; on the contrary, the ($M + 2$) ion is quite unusual. It is known from organic chemistry that the repetition of conjugated double bonds strongly stabilizes ions and radicals by resonance; this is called the mesomeric effect (19). Therefore, the ($M + 2$) ion is most likely a free radical cation ($M + 2\text{H}$)⁺. It is important to draw attention to the results of bixin analysis obtained by using other mass spectrometries such as electron spray ionization (ESI) and high-resolution matrix-assisted laser desorption ionization (HR-MALDI) (20–24). Due to their different ionization processes those techniques yield results that are not comparable to those of ToF-SIMS. The peaks at higher masses (m/z 397 and 398) are ($M + 2$) molecular ions containing one or two ¹³C isotopes (the natural abundance of ¹³C is 1.1%). The peak at m/z 410 can be assigned to the bixin dimethyl ester ($\text{C}_{26}\text{H}_{32}\text{O}_4$), a minor carotenoid from the seed coat of *B. orellana* fruits (8, 20). Peaks appearing below m/z 396 give information about bixin molecular fragmentation and reorganization. The presence of a characteristic fragment at m/z 337, which is attributed to $\text{C}_{23}\text{H}_{29}\text{O}_2^+$ obtained from the bixin molecular ion with loss of a COOCH_3^- ester group, confirms the previous assignment. This fragment is associated with molecular fragmentation caused by ion impact during the ion emission process; it is not a degradation product from bixin. Other pronounced peaks in the spectra can be associated with the following bixin molecule fragments: m/z 69 to C_5H_9^+ , m/z 137 to $\text{C}_8\text{H}_9\text{O}_2^+$, m/z 151 to $\text{C}_9\text{H}_{11}\text{O}_2^+$, m/z 177 to $\text{C}_{11}\text{H}_{13}\text{O}_{12}^+$, and m/z 191 to $\text{C}_{12}\text{H}_{15}\text{O}_{12}^+$. Moreover, a high-intensity peak at m/z 105 attributed to C_8H_9^+ , compatible with the methyltropylium ion, indicates the presence of xylene (C_8H_{10}); it is known that the

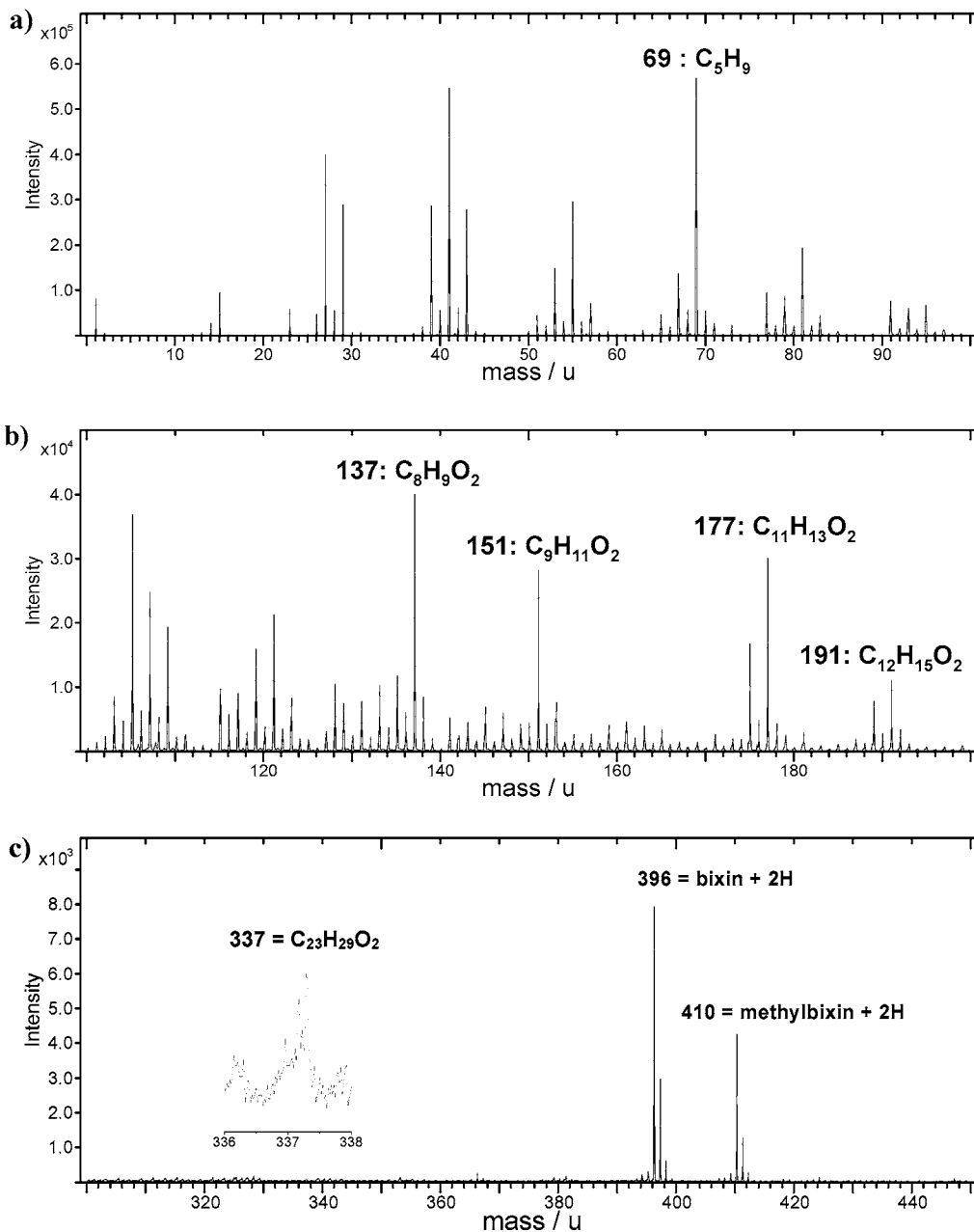


Figure 2. Annatto extract on Si positive ToF-SIMS spectrum: (a) mainly low-mass fragments; (b) mainly related bixin species; (c) bixin and methylbixin.

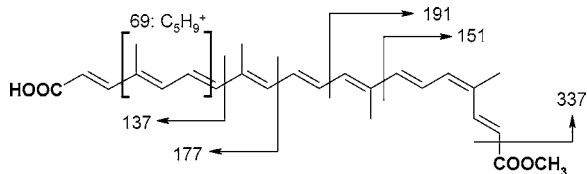


Figure 3. Fragmentation pattern of bixin molecule according to the ToF-SIMS spectrum.

bixin molecule reorganizes by losing a xylene fragment (3). **Figure 3** shows the fragmentation pattern of the bixin molecule based on the positive ToF-SIMS spectrum shown in **Figure 2**. The majority of peaks in the spectra are directly attributed to bixin and its main fragments, produced after ion impact. As the analyzed extract was not purified, peaks associated with contaminants, such as resinous matter or surface airborne contamination, can also be present in the spectra. However their presence does not complicate the analysis owing to the high mass resolution of the instrument. Consequently, the presence

of other extractants is not interfering with the bixin fingerprint spectrum.

Characterization of Temperature Effects on Bixin Extract.

The analysis of the thermal modification of bixin was performed by heating the extract to different temperatures inside the analysis chamber. The background pressure during the analysis at room temperature was 10^{-9} mbar, but a pressure rise was observed when the sample was heated, suggesting that, as expected, volatile species were being formed and released. ToF-SIMS peak intensities were measured at different temperatures and normalized over the total signal to take into account possible beam current fluctuations. The ToF-SIMS spectra recorded as the temperature increased exhibit a severe decrease in the intensity of the peak associated with the $[M + 2]^+$ molecular ion, indicating a gradual thermal degradation of bixin. The evolution of the peak intensities as the temperature increases, for peaks related to bixin m/z 396 and methylbixin m/z 410 and related fragments at m/z 137 and 177, is shown in **Figure 4a**. It can be observed that the evolution of the intensities shows

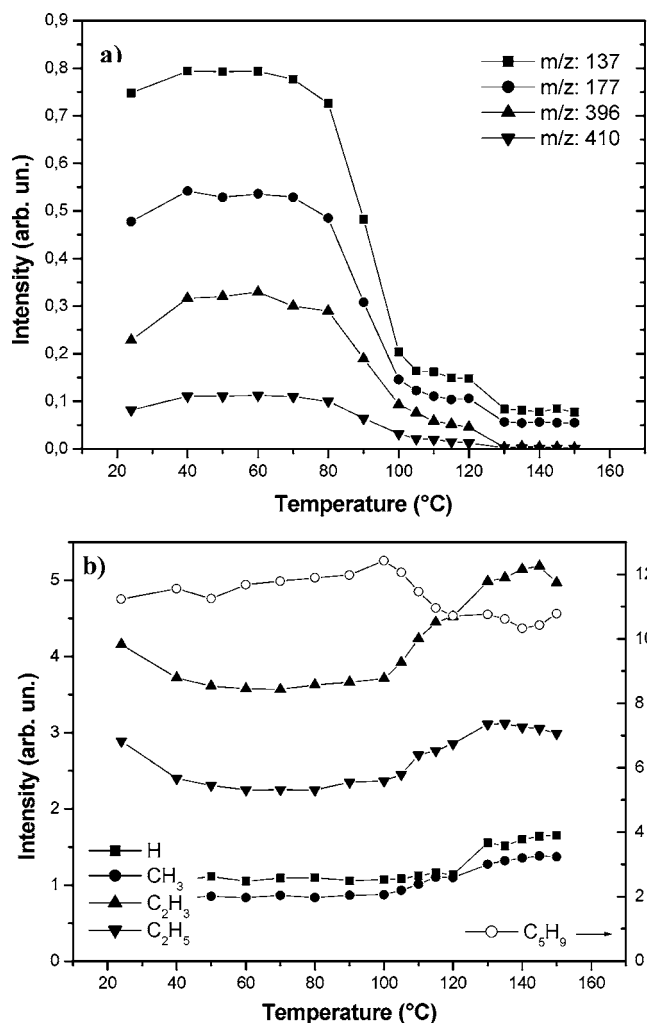


Figure 4. Temperature effect on (a) bixin and related species and (b) low-mass fragments.

three distinct temperature ranges: in the first range, from 20 to 80 °C, no significant variation in the intensity was measured, which indicates that no molecular structural change occurs; in the second range, from 80 to 120 °C, a sharp intensity decrease of the high-mass fragments is first observed, followed by a second plateau from 100 to 120 °C; finally, in the third range, above 120 °C, another sharp intensity drop occurs first, until a last plateau is reached at 130 °C. The drop in the peak intensities suggests changes in the ion molecular structures, such as loss of atoms (degradation) and formation of dimers.

For the lower mass fragments, different trends were found. **Figure 4b** shows the evolution of the peak intensities related to some fragments of lower mass for increasing temperatures. A gradual increase of the intensity in the 100–120 °C range can be observed for CH_3 , C_2H_3 , and C_2H_5 along with a slight decrease for C_5H_9 . This suggests that the thermal degradation of bixin above 100 °C leads to the creation of smaller species, explaining the enhancement of small fragment intensities, but in which the “repetition units”, C_5H_9 , remain (**Figure 3**). During the heating experiment the formation of aromatic degradation products is expected. However, these products were not observed during the heating experiment, which is probably due to the desorption of volatile compounds such as *m*-xylene caused by heating. It was shown in a previous work (24) that the $C_8H_{10}^+$ ion intensity, related to *m*-xylene, increases by a factor of 2 when the extract is exposed to light at room temperature; in that experiment, no heating was done and the volatile species

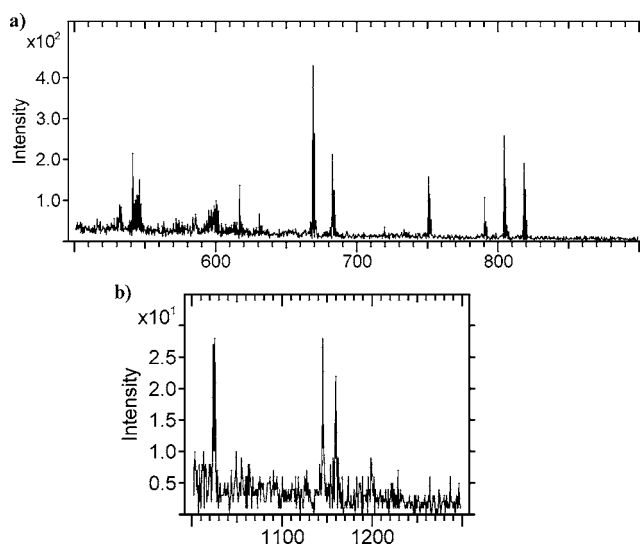


Figure 5. Temperature effects: (a) high-mass species and dimer creation [m/z 668, 682, 790 ($C_{50}H_{62}O_8^+ = 2M + 2H$), 804 ($C_{51}H_{64}O_8^+ = 2M + 2H + CH_2$), 818 ($C_{52}H_{66}O_8^+ = 2M + 2H + 2CH_2$)]; (b) high-mass species.

remained at the surface. There is no evidence on the spectra for the formation of a C17 compound ($C_{17}H_{20}O_4$) at m/z 288 due to thermal degradation, although this has been reported as the major thermal degradation product of bixin solubilized in oil. This is rather surprising but may be understood by the specific heating conditions of our experiment. In our case, a solid film of bixin, deposited on a Si wafer, was heated in vacuo. The thermal degradation mechanism is therefore drastically different from the one for bixin molecules heated in liquid solutions.

Another major effect observed during the heating experiment was the formation of dimers at mass m/z 790 ($C_{50}H_{62}O_8^+ = 2M + 2H$, m/z 804 ($C_{51}H_{64}O_8^+ = 2M + 2H + CH_2$, m/z 818 ($C_{52}H_{66}O_8^+ = 2M + 2H + 2CH_2$, and other high-mass species for which the ion identification is less clear at mass m/z 668, 682, 1023, 1144, and 1158 (**Figure 5**). These species were observed in the temperature range of 90–120 °C, coinciding with the disappearance of the bixin molecule (**Figure 5a**).

PCA. As shown in **Figure 2**, ToF-SIMS spectra contain a huge, but redundant, amount of information. Many peaks are correlated (e.g., m/z 137, 177, 396, and 410), and their evolutions indicate the same trend (see **Figure 4a**). In such a case, PCA is an efficient method to reduce the data without losing much information. PCA applied to this heating experiment allowed us to identify the main structural changes occurring upon heating. The evolution of principal components PC1 and PC2 as a function of temperature is shown in **Figure 6**.

The set of data was produced by selecting all peaks in the spectrum. Because many peaks are highly correlated, 67% of the variance is already described by PC1, meaning that two-thirds of the information contained in each spectrum represents the same trend. In other words, PC1 represents the main thermal structural changes because it explains two-thirds of the data. PC1 remains constant up to 100 °C and then drops sharply (**Figure 6**), which means that the fragmentation associated with PC1 is “activated” at or above 100 °C. The second PC, which still accounts for 10% of the data, also exhibits a strong variation with temperature. It remains fairly constant up to 70 °C; it then drops and reaches a plateau from 100 to 120 °C, and then it rises again (**Figure 6**). The other PCs are not shown, because their variance is low and they exhibit no clear changes with temperature.

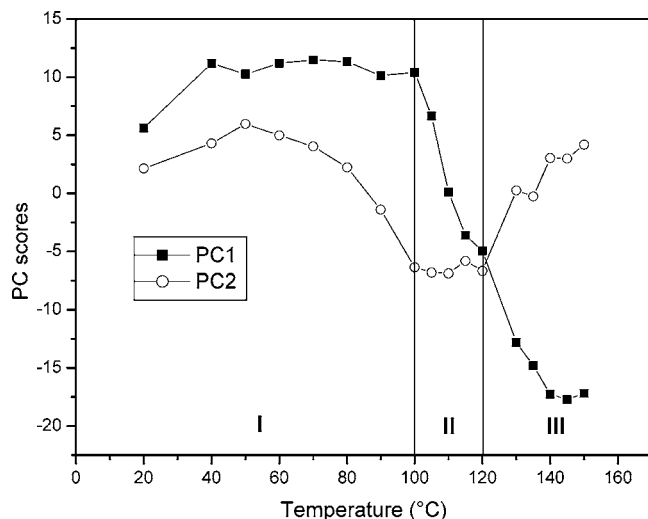


Figure 6. Temperature variation of PC1 and PC2.

PCs are also interpreted by looking at the “loadings”, which are the coefficients multiplying the peak intensities in the linear combination defining each PC. High positive or negative value indicates the peaks that contribute the most to the PC and thus, in this case, to the molecular structural change associated with it. When a PC increases, it means that the ions with high positive loadings are created, whereas the ions (fragments) with high negative loadings are destroyed. Moreover, ions (fragments) with low loadings do not contribute to the PC. The loading plots for PC1 and PC2 are given in Figure 7. The interpretation of these plots is not straightforward, but general trends are identified. For PC1 (Figure 7a), all of the low-mass fragments (below 69

Da) have strong negative loadings, whereas most higher mass fragments, including the molecular ions directly associated with bixin, have strong positive loadings. The sharp drop of PC1 above 100 °C (Figure 6), indicates that the high-mass fragments and the bixin molecule start to disappear, leading to a fragmentation to low-mass ions. This represents the main molecular structural change, triggered at 100 °C. The nature of the mechanism is not fully understood, as the degradation products, which are most likely aromatics, are volatilized at high temperature, but it is clearly related to a thermal fragmentation of the bixin molecule. The PC2 loading graph (Figure 7b) is more complex, but it indicates that the bixin molecular ion has a high positive contribution to PC2, whereas the dimer molecular ion, at m/z 804, has a strong negative contribution to PC2. Therefore, PC2 appears to describe the chemical reactions leading to the formation of dimers and other high-mass molecules. Those reactions are activated above 70 °C, leading to a gradual disappearance of monomers and the creation of dimers, both effects that lower PC2.

A general understanding of the thermal modification induced on the bixin extract emerges from this PCA: the extracts remain thermally stable below 70 °C; above 70 °C, dimerization reactions start and the bixin amount decreases; above 100 °C, the bixin molecules start to degrade, leading to fragmentation; finally, extensive degradation of bixin and its dimers occurs above 120 °C, which is indicated by rapid changes for PC1 and PC2 (Figure 6). Again, the PCA did not suggest the formation of a C17 compound or related fragments from solid bixin; therefore, no evidence is found here for *cis*–*trans* isomerization of bixin, which is known to occur in liquid solutions.

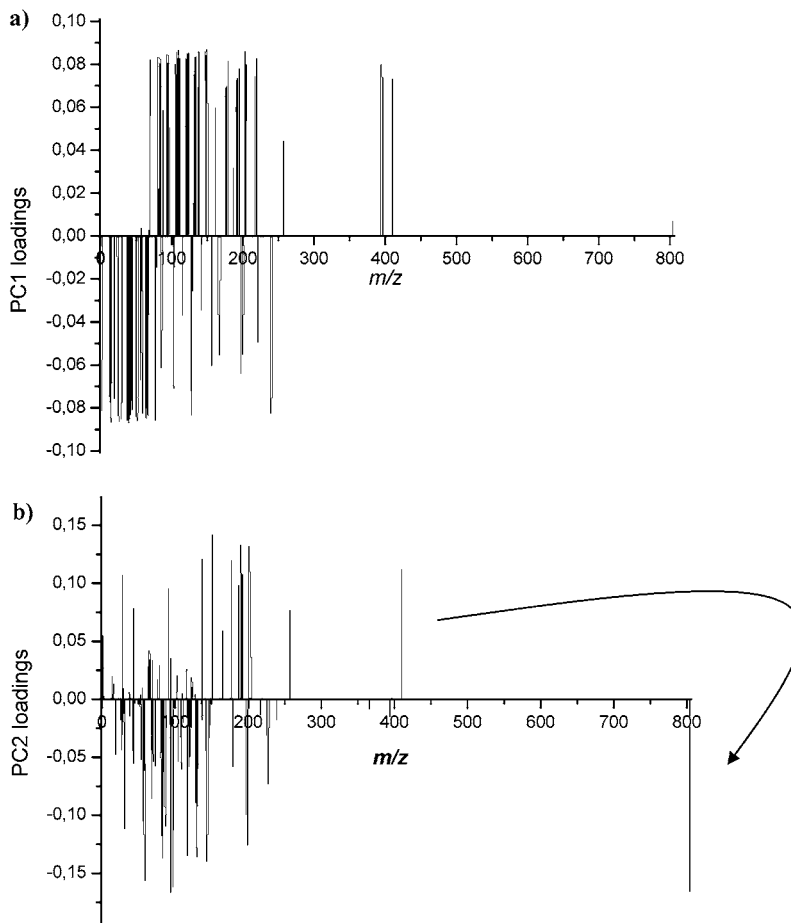


Figure 7. Loading plots of principal components (a) PC1 and (b) PC2.

In conclusion, extracts of *B. orellana* fruits were studied by means of ToF-SIMS associated with PCA. The fast and easy recording of the spectra showed that this technique is powerful for carotenoid analysis. Moreover, PCA proved to be an important tool for the analysis of the ToF-SIMS spectra recorded at different temperatures. This technique allows the use of important preventive measures for carotenoids analyses, such as protection from light, temperature control, and inert atmosphere. By heating the sample in situ, it was possible to follow the changes in the molecular structure of bixin. It was shown that degradation of the colorant agent occurs by heating. Three distinct temperature ranges were found: below 70 °C, the extracts remain thermally stable, that is, no molecular structural change occurs; above 70 °C, dimerization reactions start and the bixin amount decreases; near 100 °C, the bixin molecules start to degrade, leading to fragmentation; finally, extensive degradation of bixin and its dimers occurs above 120 °C. The systematic identification of the products of degradation opens a new possibility for ToF-SIMS application.

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