ARTICLE

Study of annatto from *Bixa orellana* seeds: an application of time-of-flight secondary ion mass spectrometry

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Introduction

As colour is often a key consumer perception for food preference and acceptability, alimentary colourants play a key factor in the food industry. Natural and synthetic colourants have been extensively used to colour food, drugs and cosmetics. Since the first synthetic colourants were introduced into the market at the end of the eighteenth century, a set of prohibition rules have been established by governments worldwide as some of these molecules proved to be harmful to human health. Even so the use of synthetic colourants is still conceived as undesirable and harmful. In contrast, natural colourants, being nature-derived products, are considered by the consumers as healthy and of good quality, so the demand for natural colorants has increased. Hence better understanding of their chemistry and biochemistry is required; in the food industry, the safety of colourants is indeed a very important issue.

Compared with their synthetic counterparts, some natural colourants can be more sensitive to heat, pH or light; i.e. their colouring agent can degrade more easily. The degradation of the colouring agent can be used to tailor some properties of the colourant. However, no matter whether it is of natural or synthetic origin, the degradation of the colourant may generate undesirable products. Consequently, careful analysis of their production and storage is important in order to establish safety specifications.

A trend in the analysis of natural products is the search for new techniques that can be applied to their characterisation without unduly interfering with their structure. During the analyses of natural products, some of the following preventive measures may be essential: reduced time of analysis, elimination of oxygen, protection from light, temperature control and use of high-purity solvents. In this study we applied time-of-flight secondary ion mass spectrometry (ToF-SIMS) in order to study annatto, a natural food colourant. ToF-SIMS, a technique already well developed to analyse organic and inorganic samples, can become a powerful tool for elucidating the chemical and molecular compositions, spatial distribution of elements and degradation characteristics of natural products. ToF-SIMS is a surface analytical technique that uses an ion beam to remove atoms or molecules from the outermost layer of a surface. A short pulse of primary ions strikes the sample surface, and the secondary ions produced during the impact are extracted from the sample surface into a time-of-flight mass spectrometer. These secondary ions are dispersed in time according to their velocities, which are proportional to $(m/z)^{-1/2}$, where m/z is their mass/charge ratio. 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 10,000 atomic mass units (Daltons), at a mass resolution ($m/\Delta m$) of 8000 at mass 29. Pulsed operation of the primary beam allows insulating surfaces to be neutralised between pulses using a low energy electron beam. Among the advantages attributed to the ToF-SIMS, one can cite the possibility to directly analyse the samples, without the need to extract their components; in some cases detection of a concentration lower than 1 ppm can be achieved, the spatial distribution of elements and molecules with 100 nm lateral distribution may be analysed and insulating materials may be analysed. Moreover, measurements are performed under high vacuum avoiding reaction with undesired chemical elements. Large molecular ions and molecular fragmentation patterns (or "fingerprints") are characteristic of the sample. A review of surface analysis with ToF-SIMS can be found in Reference 1.

Among the naturally occurring colourants, an important one is annatto (E160b). It is a carotenoid-based dye, extracted from the surface of the seed of a tropical tree called *Bixa orellana* L. (achiote in Spanish, urucum in Portuguese); named after the 16th century Spanish scientist and explorer of South America, Franscisco de Orellana. This tree is native to the Central and South American rain forest; however, through Spanish influence it became grown in South-East Asia. Annato was classified by the Food and Drug Administration in the USA as a "color additive exempt of certification".[2] Its principal colouring agent is bixin ($C_{25}H_{30}O_4$), a carotenoid having

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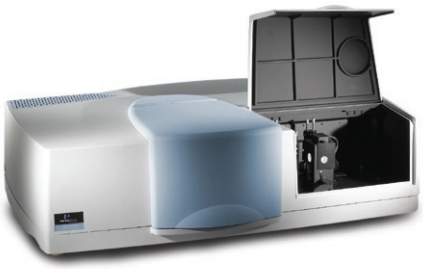

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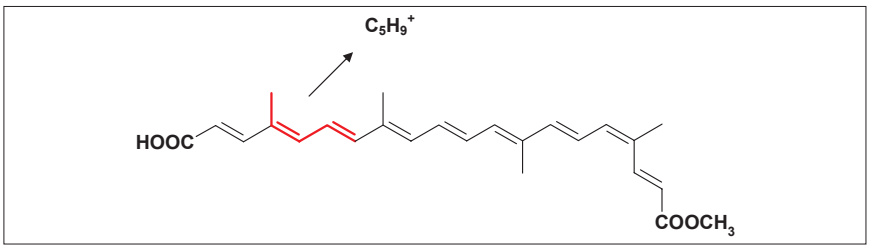

Figure 1. Chemical structure of bixin.

a carboxylic acid and a methyl ester as its end groups (see the chemical structure in Figure 1). The average annual production of annatto seeds is near 10^4 tons, 60% of which comes from Latin America, 27% from Africa and 12% from Asia. Among natural colourants, annatto ranks second in economic importance;[3] it is a substitute of Tartrazine, a synthetic colourant that has been prohibited in several countries.[4]

The use of annatto as a food colourant can be traced back to the ancient Aztecs, whose original chocolate drink is reported to have contained annatto seeds. Given annatto's high fat content and its red colour that could have been associated with blood (which of course had religious connotations in Aztec society) its use is entirely probable. Until the 17th century, the use of annatto in Europe to deepen the colour of chocolate was also common. Nowadays, annatto is primarily used for colouring cheese (50%), other dairy products (butter, margarine, snacks etc.) and body care products. The colouring agent bixin is extracted by heating *in vacuo* a suspension of the seeds in vegetal oil to a maximum temperature of 130°C. At this extraction temperature, bixin undergoes a series of degradation reactions to produce a range of products coloured from pale yellow to orange. The principal thermal degradation product is the yellow coloured $C_{17}O_4H_{20}$ molecule, the formation of which is accompanied by release of *m*-xylene, toluene, toluic acid and toluic acid methyl ester, all undesirable molecules in preparations intended for food use. In considering the use of annatto as a colouring agent, it is necessary to develop suitable methods of analysis to study directly the extracted annatto preparations and relate them to the characteristics of the seeds and the extraction mechanism. In this work we applied ToF-SIMS to determine constituents of *Bixa orellana* seeds, its extract and its degradation products.

Sample preparation

Four sets of samples were analysed. The first one was composed of *Bixa orellana* seeds that were purchased from a local retail outlet in São Carlos (State of São Paulo, Brazil). A second set of samples was composed of the organic extracts obtained by washing seeds of *Bixa orellana* with a (1:3) solution of methanol and dichloromethane at room temperature and which were analysed just after the extraction. To perform the analysis, the extracts were dripped onto a silicon substrate and blown dry under a flow of nitrogen. Finally, to study possible degradation, the previous sample set was divided into two sub-sets: one was analysed after being heated *in vacuo*, *in situ* in the ToF-SIMS spectrometer to different temperatures ranging from room temperature to 150°C; the other set was analysed after exposure in air to ambient light during three months at room temperature. A colour change was observed, which is an indication of degradation.

Experimental

The mass spectra of the samples were recorded on a ToF-SIMS IV instrument from Ion-Tof GmbH, Germany. 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. Typical analysis conditions for this work were 25 keV pulsed Ga^+ beam at a 45° incidence, 2 pA pulsed current rastered over a 130 × 130 µm area. The ion fluence was kept below 3×10^{-12} ions cm^{-2} to ensure static conditions. The background pressure during the experiment was better than 10^{-9} mbar. The mass resolution ($m/\Delta m$) near mass 29 was typically 8000. Electron flood-gun charge compensation was necessary during the measurements recorded directly on the seeds. For thermal degradation study, the samples were heated *in situ* by a regulated resistive heater.

Result and discussion

Annatto seed and extract analysis

In order to determine the spatial localisation of the colourant, ToF-SIMS analyses were performed on sectioned seeds in which the endosperm and the aril were analysed (see the cross-section from the *B. orellana* seed in Figure 2). Organic fragments dominate typical spectra recorded from the endosperm; furthermore, it is important to note the presence of a peak at *m/z* 55.93 (Figure 3), which indicates the presence of Fe inside the seeds. Bixin and its related fragments are found to be present only at the seed's aril. Figure 4 shows the typical positive ToF-SIMS spectrum recorded at the seed aril: this spectrum should be considered as the colourant fingerprint, since no extraction treatment, which could interfere with its structure, was used. The peak at *m/z* 396 is 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 peak at *m/z* 410 is attributed to methyl bixin, a minor carote-

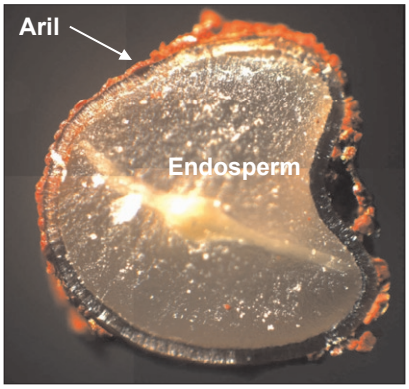

Figure 2. Cross-section from a *B. orellana* seed.

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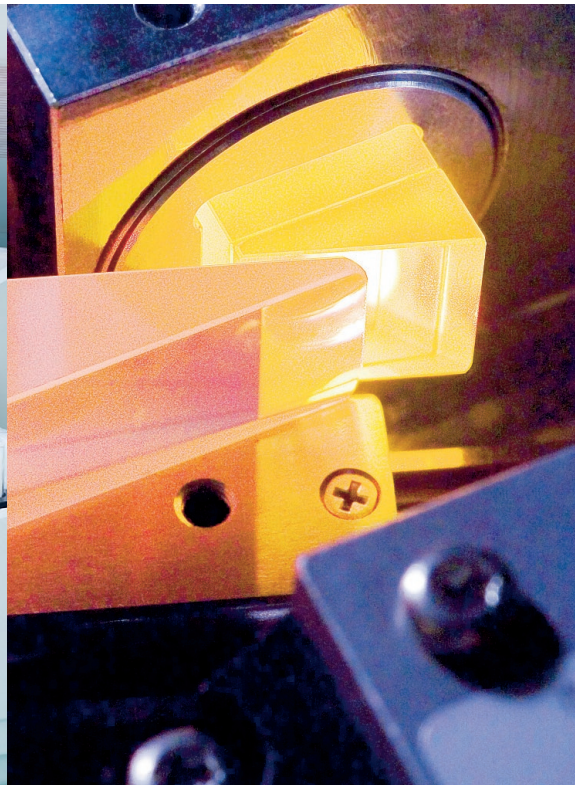

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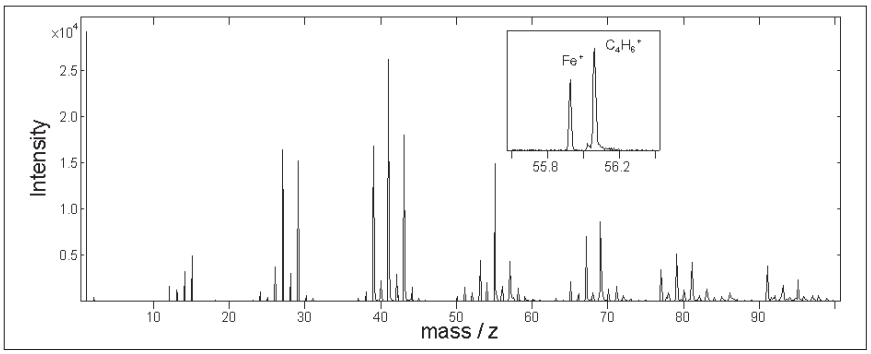

Figure 3. Typical spectrum recorded from the endosperm of the *B. orellana* seed.

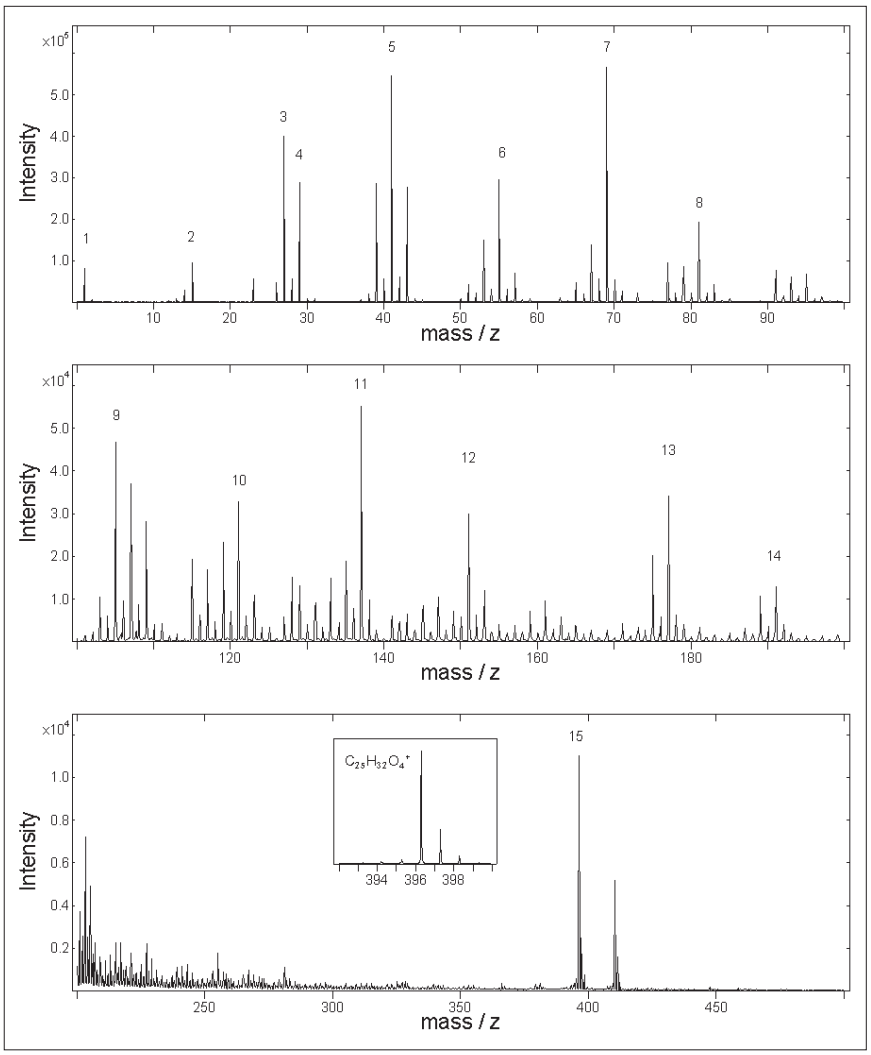

Figure 4. Typical spectrum recorded from the aril of the *B. orellana* seed. Labels (1–15) are described in Table 1.

noid which is known to coexist with bixin in the Annato seeds.

The positive ToF-SIMS spectrum recorded on the extract just after it had been prepared is similar to the one recorded on the aril of the *B. orellana* seed. It is dominated by the bixin molecular peak at *m/z* 396. Moreover, similar characteristic fragments peaks were obtained both from the seed's aril and on the extract. The principal fragments are shown in Table 1 and the possible fragmentation sites in the bixin molecule are shown in Figure 5.

Table 1. Bixin and some of its fragments.

m/z	Fragments	Label
1	H	1
15	CH_3	2
27	C_2H_3	3
29	C_2H_5	4
41	C_3H_5	5
55	C_4H_7	6
69	C_5H_9	7
81	C_6H_9	8
105	C_8H_9	9
121	C_9H_{13}	10
137	$C_8H_9O_2$	11
151	$C_9H_{11}O_2$	12
177	$C_{11}H_{13}O_2$	13
191	$C_{12}H_{15}O_2$	14
396	$C_{25}H_{32}O_4$	15

Thermal and light degradation

In order to quantify the degradation induced by light, the extract was reanalysed after three months of exposure to ambient light. By visually inspecting the appearance of the extract, it was easy to observe that the initially orange pigment had turned to light yellow (Figure 6). The change in the extract colouration is due to the known degradation of the colourant agent.[5] The spectra obtained after light degradation exhibit a severe decrease (by a factor of five) in the intensity of the peak associated to the $[M+2]^+$ molecular ion, indicating degradation of the bixin, along with a two-fold increase of the $C_8H_9^+$ signal, attributed to xylene, an aromatic degradation product not welcome in food!

For the thermal degradation study of bixin, a set of analyses was performed after heating *in situ* the extract to different temperatures. Figure 7 shows the evolution of the normalised intensity to the total spectral intensity of the peaks related to the bixin *m/z* = 396 and the fragments *m/z* = 137 and 177. Three temperature ranges can be distinguished: the first, shows a plateau from 20 to 80°C ; near 80°C, a sharp intensity decrease is observed, accompanied

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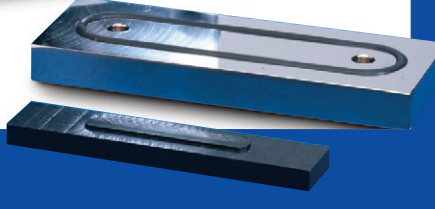

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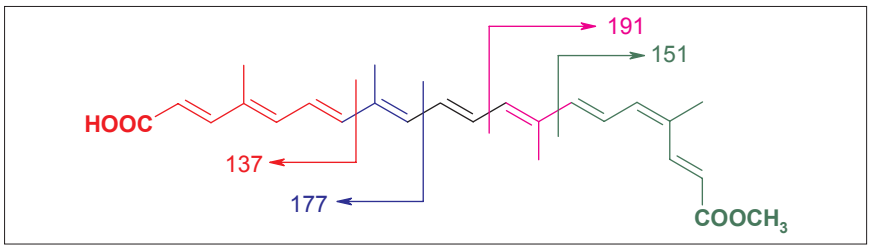

Figure 5. Main degradation sites of *bixin*.

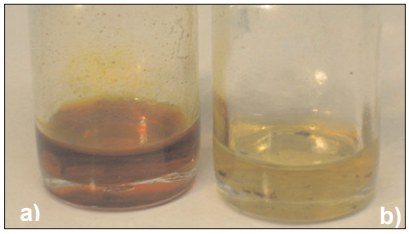

Figure 6. Photograph showing a) fresh annatto extract and b) light degraded annatto extract.

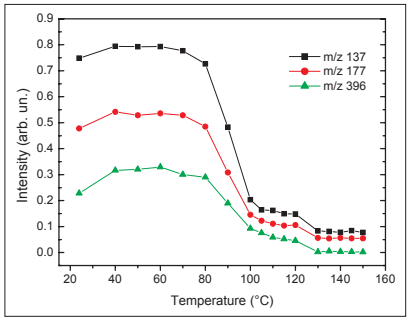

Figure 7. Temperature effect on *bixin* and some high mass fragments.

by another plateau from 100 to 120°C; finally, another sharp intensity drop appears at 120°C, until another plateau is achieved at 130°C. One can deduce from this figure that the colourant agent begins to degrade near 80°C. By visual inspection, it was possible to observe a change in the colourant colour near this temperature. This observation supports the important traditional cooking practice of adding food colourant and some green herbs that need to keep their colour just at the end of the food preparation, in order to avoid their colour degradation, i.e. to grow pale.

Figure 8 shows the evolution of the normalised intensity to the total spectral intensity of the peaks related to some fragments of lower mass for increasing temperatures. One can observe a gradual increase of the intensity in the range 100

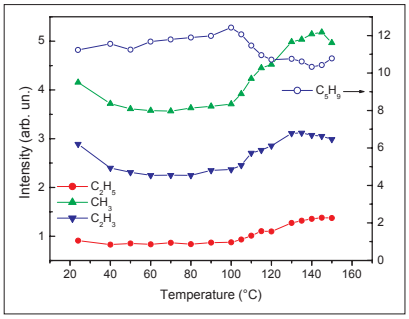

Figure 8. Low mass fragments increase as temperature increases.

to 120°C for CH_3, C_2H_3 and C_2H_5 and 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 the intensities arising from small fragments, but in which the "repetition unit", C_5H_9, remains (Figure 1).

During the heating experiment the formation of aromatic degradation products, as inferred during the degradation by light, is expected. However, these products were not observed during the heating experiment, which is probably due to desorption of volatile compounds caused by heating. It has to be noted that during "real" cooking situations it is reasonably feasible that these volatile compounds will suffer various fates, depending upon the food components, the heating conditions employed and where the degradation occurs. For instance, aromatics formed at the surface of the food are more likely to be volatilised and effectively removed, whereas formation of aromatics within the interior of the food may be trapped.[5] The traditional cooking practice of stirring the food while in the pan may in certain cases helps to avoid the imprisonment of undesired volatile products.

Concluding remarks

The experimental study described in this article shows that ToF-SIMS can be used to analyse natural samples. This technique allows preventive measures, such as protection from light, temperature control, inert atmosphere and no solvent use to be achieved during the analysis. It was shown that the technique allows the direct measurement on the seed; this opens a new possibility to be considered for the analysis of natural samples. By analysing the seed's aril, it was shown that bixin, one of the main colouring constituents of the annatto seed, could be easily detected by ToF-SIMS, without any sample treatment. After prolonged exposure to light, a significant degradation of bixin is observed. It was shown that degradation of the colourant agent occurs by heating near 80°C. The systematic identification of the products of degradation opens a new possibility for ToF-SIMS application. The molecular sensitivity of ToF-SIMS has been shown to allow the straightforward detection of the main constituents present in annatto seeds, with potential applications in food industry. Results also support some traditional cooking practice.

Acknowledgement

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