Processed stigmas of *Crocus sativus* L. imaged by MALDI-based MS

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The processed, i.e. dried under certain conditions, stigmas of *Crocus sativus* L. are one of the most expensive plant parts used commercially. For the color, aroma and biological activity a very complex mixture of glycolipids termed crocins are responsible. Therefore studying structural composition and distribution in the commercial plant material is of great interest. We showed successfully the application of a MALDI-based mass spectrometric imaging (MSI) approach for stigmas towards different crocin species. MSI opens up the investigation of processed plant materials in various fields allowing studying the processing in detail as well as adulteration attempts (which are quite frequent due to the price of the material). Furthermore, we could demonstrate that a similar number of crocins present in stigmas could be detected by MALDI MSI compared to the classical approach of analyzing the solvent-extract of stigmas by MALDI-MS.

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The introduction of MALDI-TOF-MS for the analysis of non-volatile molecules dates back to the late 1980s [1–3]. Whereas initial work was focused mainly on large proteins, later it was also demonstrated that also “smaller molecules” as peptides [1], oligosaccharides [4] and various classes of lipids as well as glycolipids [5, 6] could be characterized by MALDI-TOF-MS [7]. By the turn of the millennium major improvements of MALDI-TOF-MS instrumentation were realized in terms of, e.g. resolution as well as in applicability of TOF-based MS/MS experiments thus allowing high energy CID in the keV-range for structural elucidation [8–11]. Besides to those instrumental developments during this time period also mass spectrometry imaging (MSI) was adapted to those instruments for the 2-D visualization of analytes as proteins, peptides and lipids present on thin tissue sections after prior MALDI matrix deposition [12, 13]. Whereas a great number of applications was dealing with the analysis of human or animal thin tissue sections within the last few years this technique was increasingly applied to the analysis of secondary metabolites of plant surfaces and sections present in various plant tissues as root, tuber, bulb, stem, leaves, and flower petals. Several reviews on MALDI MSI as well as on other desorption/ionization techniques combined with MSI of different plant tissues (e.g. leaves, petals, pollen, seeds, fruits, fruit skin, root) appeared recently [14–17].

During our efforts to characterize crocetin glycolipids (so-called “crocins”) present in processed, i.e. slowly dried stigmas of the spice/colorant/folk medicine plant saffron from the flowers of *Crocus sativus* L., we initially compared the potential of different desorption/ionization techniques as ESI with either ion trap (IT) or Q (quadrupole)-TOF MS, MALDI with Q-TOF MS as well as vacuum MALDI-TOF-MS [18]. Further investigations included the detailed structural investigation of the aglycon crocetin as well as of selected isomeric glycosylated crocetins [19]. The most abundant crocin glycosides found in the dried stigmas of saffron contain between two and five hexose residues. Structurally identified ester glycosides with a given molecular weight were found to be present with different types of oligosaccharide chain length attached to either one or both of the two attachment sites (the carboxy groups of the crocetin aglycon) as
determined by RP LC in combination with positive ion ESI IT-mass stage MS in our research group [20].

Based on this knowledge of these complex apocarotenoid ester glycosides we got interested in the lateral surface distribution of those crocins across entire processed single stigmas as found in commercial products. Therefore, individual dried stigmas covered with 2,4,6-trihydroxyacetophenone (THAP) as MALDI-MS matrix deposited via airbrush deposition were analyzed by MALDI MSI to obtain lateral distributions of crocins. Furthermore the MALDI mass spectrometric crocin pattern was compared to solvent extracts from processed individual stigmas.

For the MALDI reflectron TOF-MS analysis of an unseparated extract of stigmas a plain methanol solution was prepared. Three stigmas (approx. 1 mg dry weight; dehydration and drying, i.e. processing of stigmas was carried out by storing them at 20°C during the first hours of the process and then at 30–35°C until the moisture reaches the level of 10–12%) were extracted with 1 mL of methanol for 24 h in the darkness. The obtained highly colored solution was filtered through a nylon accrodisc filter (0.45 μm; Whatman-Merck, Darmstadt, Germany) and 1 μL of this solution was mixed with 1 μL of MALDI-MS matrix solution. MALDI reflectron TOF-MS of the nonseparated methanolic extract of three stigmas and MALDI MSI of single stigmas (hand-picked stigmas were dehydrated by maintaining them at 20°C during the first hours of the drying process and then stored at 30–35°C until the moisture level reaches 10–12%) were performed on a AXIMA TOF® tandem TOF instrument (Shimadzu Kratos Analytical, Manchester, UK) using THAP as MALDI-MS matrix (30 mg/mL in methanol doped with NaCl (until saturation)).

MALDI MSI was performed on the aforementioned instrument by mounting a single, ribbon-like stigma onto a plain MALDI-MS target by use of a conductive tape. A methanolic solution of 30 mg THAP in 1 mL solvent saturated with NaCl was deposited onto the fixed stigma using the airbrush technique [21–24]. Subsequently, a MALDI-MS matrix layer could be seen by light microscopy. The area rastered was 2.5 × 2.5 mm by applying 2601 single laser shots with a spacing of 50 μm (laser spot size 45 μm) visualized with BioMAP software version 3.7.5.5 (Novartis, Basel, Switzerland).

The electron microscopic images were obtained on a Quanta 200 SEM instrument (FEI, Hillboro, OR, USA). The resulting micrographs of two different stigmas are shown in Fig. 1A and B exhibiting a relatively flat surface although a cellular structure is clearly visible. A representative height/thickness of an individual stigma, which is exhibiting usually ribbon-like structure was measured to be roughly 80 μm (Fig. 1C). Care has to be taken that the stigma is glued as flat as possible to the metallic MALDI-MS target. After airbrush deposition of THAP for subsequent MS analysis, a significantly changed surface morphology with an islet-like coverage with THAP matrix can be observed (Fig. 1D), which is even more evident at higher magnification factor (× 500) as seen in Fig. 1E. It should be noted that 2, 5-dihydroxy benzoic acid, a quite popular MALDI-MS matrix, was also evaluated as matrix but with no homogeneous surface coverage.

Investigating the terminal end of a stigma covered with the MALDI-MS matrix containing sodium chloride by positive ion MALDI MSI yielded a mass spectrum summed across the whole area (2.5 × 2.5 mm) with a characteristic crocin (and related compounds) pattern (Fig. 2A) where signals are present for the matrix (m/z 359.1, a [2M+Na]+ matrix cluster and fragments thereof) as well as for the saffron-specific analytes the monoterpene glycoside picrocrocin (m/z 353.3, [P1+Na]+, containing one glycosidically bound hexose unit), the secondary metabolite kaempferol (m/z 633.2, [K1+Na]+ containing one hexose and m/z 795.3 [K1+Na]+ containing two hexose units) and various assumed potent neuronal antioxidant species (m/z 675.3 [C2+Na]+, m/z 837.4 [C1+Na]+, m/z 999.4 [C4+Na]+ and m/z 1161.5 [C5+Na]+ containing from two up to five hexose units as determined by LC/ESI-MS). All lower abundant signal indicated by an asterisk (*) indicate additional potassium adducts ions of already described analyte ions spaced by +16 Da from the sodium adduct ions. Without the addition of sodium chloride to the MALDI-MS matrix in some samples the potassiated species were the dominating adduct ions. Based on these data the lateral distribution of the major crocins at m/z 999.4 – mainly bis-gentiobiosyl-crocetin and to a much lower extent gentiotriosyl-glucosyl-crocetin (verified by tandem MS) and m/z 837.4 – gentiotriosyl-glucosyl-crocetin (both, selected m/z range ± 3 Da) of a terminal end of a saffron stigma were determined as shown in Fig. 2B and C. The MALDI-MS based images of these two selected m/z traces (sodiated molecules) show the domination of the bis-gentiobiosyl-crocetin species. Furthermore the islet-like distribution of the MALDI-MS matrix on surface (as seen in Fig. 1D and E) of the ribbon-like stigmas can be observed, too.

In conclusion, the analysis of the methanolic extract of identically processed saffron stigmas was carried out by MALDI reflectron TOF-MS in the positive-ion mode. All molecular ion species of the crocin constituents were predominately detected again as sodiated species as the THAP matrix was doped with sodium chloride in order to suppress again other types of adduct ions like potassiated ones as much as possible, although low abundant [M+K]+ adduct ions were still visible due to the naturally high content of potassium ions in plant tissues (see Fig. 3). The signal-to-noise ratio was of course much better than in the MSI experiments. It should be noted that potassium species have been observed dominating in all MSI analyses directly from stigmas with the non-doped plain MALDI matrix (data not shown). This observation clearly indicates that all analyte adduct ions detected strongly correspond to the ones already described for the MSI experiments.

In conclusion, one can deduce from the MALDI MS images for the dominating crocin species at m/z 837.4 and m/z 999.4 that the selected individual molecular species of crocins are evenly distributed across the stigma surface rather than localized in specific morphological areas. Furthermore, these
Figure 1. EMs of two individual commercial saffron stigmas which were air dried (middle section of the stigma) (A) and (B) as well as a cross-section of a stigma exhibiting the thickness of the target-fixed stigma (C). Image of a stigma after THAP matrix deposition by the airbrush technique at different magnification factors (D, 200 ×) and (E, 500 ×).

Figure 2. Sum of mass spectra of positive ion MALDI MSI of a single terminal stigma area (2.5 × 2.5 mm) (A) and MALDI MSI of the terminal part of the stigma of the trace at m/z 999.4 (sodiated tetrahexosyl-crocetin) (B) and of the trace at m/z 837.2 (sodiated trihexosyl-crocetin) (C). Annotation see text.
MALDI-based MSI “patterns” were first molecular images of processed plant material. This type of approach will allow the investigation of undried stigmas (after hand-collection from the blossom of the plant) as well as during the processing steps (e.g. allowing to determine a potential crocin-based end point determination) collected stigmas. To detect and show the distribution of crocins and their precursors in other parts of the *Crocus* plant might be of commercial interest, too. Furthermore, this approach might be of interest for regulatory authorities in the area of adulteration related to phytomaterials in the field of nutrition and phytopharmaceuticals. The processing of plant materials can be monitored directly regarding the presence/disappearance of compounds of interest. Finally, it is also demonstrated clearly that under well-defined conditions, a similar number of major analytes present in stigma samples can be detected by MALDI reflectron TOF-MS in the solvent extraction mode as well as by MALDI MSI. Different types of MALDI-MS matrix deposits could possibly further improve the quality of mass spectrometric images obtained.

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References


