Atmospheric Solids Analysis Probe Mass Spectrometry (ASAP-MS) for the Rapid Identification of Pollens through Flavonoid Fingerprints

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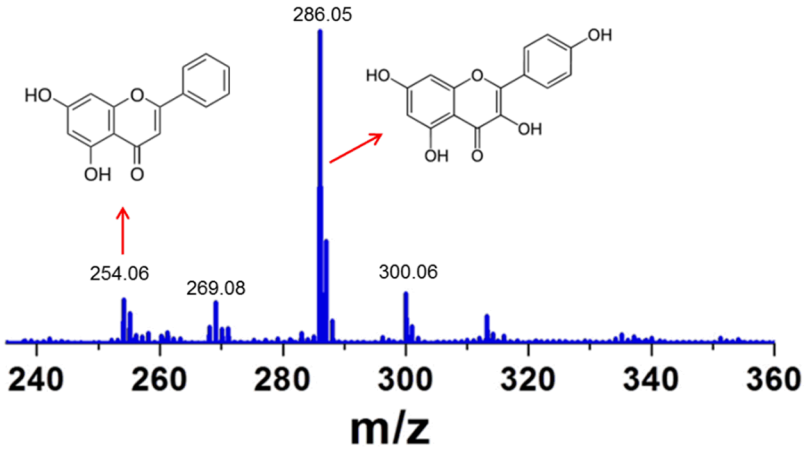

ABSTRACT.

From allergies to reproduction, pollens have important impacts, on the health of human and plant populations, respectively, yet identification of pollen grains remains difficult and time-consuming. Here we demonstrate that the use of atmospheric solids analysis probe mass spectrometry (ASAP-MS) is appropriate for the rapid identification of pollens based on both two-dimensional mass spectra-thermal degradation temperature profiles and flavonoid fingerprints. Flavonoid fingerprints were generated for pollens collected from different plant types (trees and bushes) in addition to bee pollens from distinct geographic regions, and were unique for individual pollen species and also for bee pollens, which represent the flowering plant diversity of micro-regions. Low-energy collision induced fragmentation was applied to identify the flavonoid structures and structural similarity (including isomers). With the development of a

pollen flavonoid database, ASAP-MS offers a rapid identification technique that is able to identify species of plant pollens and regions of origin for bee pollens.

INTRODUCTION

Recent advancements in mass spectrometry (MS) allow ions to be generated at ambient pressure and consequently mass spectra can be collected on ordinary samples, in their native environment, without sample preparation or pre-treatment.[1,2,3] Such novel MS instrumentation techniques have enabled *real-world* sample analysis, such as the analysis of chemicals in the solid phase or adsorbed on surfaces of solid materials, and *in vivo* biological systems such as cells and tissues.[4,5] Atmospheric-solids-analysis-probe mass-spectrometry (ASAP-MS) is one of these direct ionization techniques.[6] Upon thermal desorption at high temperatures, the vaporized molecules are ionized at ambient pressure before entering the time-of-flight mass spectrometer. Since ionization occurs outside the spectrometer, sensitivity and chemical specificity is not compromised. Not only does ASAP-MS simplify and significantly accelerate sample analysis time by eliminating time-consuming sample preparation and solvent-induced extraction problems, such as solubility and reactivity with a solvent, it is also more suitable for semi- and non-volatile chemical compounds.[7,8,9,10]

Pollens are of interest to many scientific disciplines because of their importance in the reproduction of plants, the health of ecological systems, and their impact on human society. Pollen is important not only for plant propagation, but also for consumption by insects, such as bees. Humans also consume pollen in the form of bee pollen and as honey. Additionally, understanding the spread of pollen in local environments and beyond is important for understanding the genetic structure of plant populations. Furthermore, pollens can have deleterious effects on humans because of the allergenic qualities of many pollen species. Identifying pollens can have tremendous benefit for these areas of research.

Yet, because of the complexity and diversity of pollens, identification has been a challenging subject for decades. Optical and electron microscopy can distinguish pollens at a generic level, but analysis is tedious, slow, and requires a high level of expertise.[11] Pollens contain a wide variety of chemicals, including carbohydrates, organic acids, carotenoids, flavonoids, phosphate

lipids, and proteins. Identification of pollen via chemical fingerprint has been an area of research interest for many years. Volatile and semi-volatile compounds were found in pyrolysis-gas chromatography/MS (Py-GC/MS) experiments,[12] while vibrational spectroscopy techniques (IR and Raman) were used to differentiate pollens through the intensity and distribution of C-N and C-O single and double bond vibrational modes.[13,14] Recent high resolution Raman mapping showed patterns of the vibration modes over the surface of pollen grains, which can be used as a fingerprint-like chemical classification of pollens.[15,16] Unique molecular variability or mass spectrometric fingerprints have also been observed after extensive solvent extraction.[17,18,19,20,21,22,23] For example, lipid profiling between phosphatidylserine (PS) and phosphatidylcholine (PC) was reported for different families of pollens after microwave-assisted solvent extraction and formic acid hydrolysis. [24] The disadvantage of these techniques is that sample preparation remains a cumbersome and time-consuming step.

Here we demonstrate the rapid analysis of pollen grains (representing different plant types) using atmospheric solids analysis probe mass spectrometry (ASAP-MS). In our experiments, pollen grains are directly attached to the closed end of a glass capillary probe, eliminating the need for sample preparation. Upon heating, pollen grains are thermally degraded and the vaporized molecules are electrically ionized before entering the mass spectrometer, thus generating two-dimensional mass-temperature profiles. We report and discuss the observation of unique flavonoid fingerprints generated from individual windborne pollens (*Ambrosia trifida*, *Populus nigra*, *Artemisia tridentata*, *Kochia scoparia*) and bee pollens collected from multiple regions in the United States. Bee pollens are collections of the flowering pollens available around a beehive at a given time, and represent the micro-region diversity of plant populations.

EXPERIMENTAL SECTION

Materials

Pollens (*Populus nigra italica*, *Kochia scoparia*, and *Artemisia tridentata*) were purchased from Sigma Aldrich and also from Greer Laboratories in North Carolina (*Populus nigra*). The bee pollens were purchased from local grocery stores (Sprouts and Natural Grocers) and originated from bee farms in New Mexico, Colorado, and California. Mortars and pestles were used to break apart bee pollen pellets before analysis. Pollens were stored at 4°C until analysis. Standard flavonoids of kaempferol (Fisher Scientific) and rhamnazin (Sigma) were used as received.

Instrumentation and Sampling

ASAP-MS experiments were performed on a Waters Corporation (Manchester, UK) Synapt G2-HDMSTM time-of-flight (TOF) high resolution mass spectrometer. Mass analysis was performed in positive ionization mode. TOF-MS settings were: source temperature of 120°C, sampling cone at 48.0 V, extraction cone at 4.0 V, corona current at 8 μA, acquisition mass range of 50-1000 Da, scan time of 0.5 s, and desolvation gas at 1000 L/h. Each mass spectrum presented in the text was averaged from more than 100 scans.

Kaemferol and rhamnazin were dissolved in acetonitrile (Fisher Scientific, HPLC-MS grade). After drying out ~5μl solution from the glass capillary, the sample molecules were evaporated inside the ASAP-MS apparatus by hot N_2 gas. Their MS peak at 286.0477 and 330.0358 was used, respectively, for mass calibration and as internal standards when they were readily generated from pollen degradation. Pollen grains were directly attached to the glass capillary probe by physical forces. The N_2 gas temperature was increased from 50 to 600 °C in 50°C increment per minute or as plotted in the specific figures. The temperature ramping rate is ~ 5°C/s.

RESULTS AND DISCUSSION

The mass spectra recorded during thermal degradation of *Populus nigra* tree pollens and Colorado bee pollens are presented in Figure 1 and 2, respectively. Since ASAP is a soft ionization technique, each mass peak with their relevant isotopes represents a specific chemical compound. In general, Figure 1 and 2 demonstrate a rich composition of compounds at variable concentrations and production rates at different temperatures. At 400°C, the most intense mass peaks are observed at lower molecule weights (m/z <450). Notable peaks are at m/z: 95.02, 129.09, 147.05, 161.06, 177.06, 194.06, 208.08, 254.06, and 269.09, varied by small mass differences of 14 ($-CH_2$), 15 ($-CH_3$), 17 (-OH), and 18 (H_2O). The other main peaks are at m/z: 213.19, 241.22, 257.25, 285.28, 287.28, 313.32, 341.35, 369.38, and 409.41. These mass peaks, however, are due to the compounds that have more elements than just C, H, and O when computing the elemental compositions.

In the case of Colorado bee pollens, the intense mass peaks are at 109.03, 127.05, 143.04, 177.07, 193.05, 257.25, 287.28, 301.04, 341.33, 351.33, and 381.37, 409.41, 437.44, and so on. A few of them are identical to the mass peaks observed from *P. nigra* tree pollens, such as 177.06, 257.25, 381.38, and 409.41, an indication of similarity. Some of them are just shifted by 2 mass units, the difference between a single N and O atom, like 127 versus 129 or 145 versus 147. We did not attempt to positively identify their exactly chemical compositions and structures as it was beyond the scope of this study.

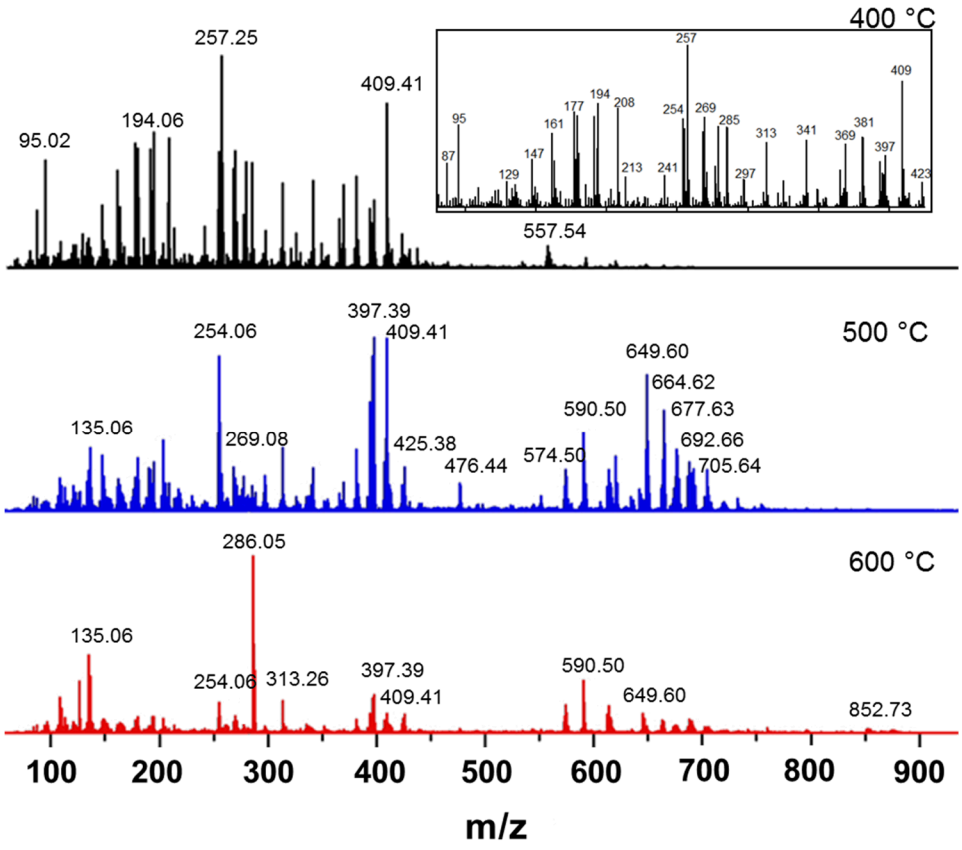

Figure 1. ASAP-MS of *Populus nigra* tree pollens thermally degraded at 400, 500, and 600°C, respectively. The expanded spectrum shows rich chemical products at m/z below 430.

Note that the mass peaks at 254.06 and 269.09 are not the base peaks in Figure 2, instead, a mass peak at 301.04 starts to build up with the increase of the temperature. When the temperature was increased to 500 and 600°C, some higher molecular weight chemicals appeared, first in the mass range between 500 and 800, then above 800. This is mostly due to the temperature assisted

vaporization and deeper thermal degradation. The m/z 286.05 became the most intense one in the case of *P. nigra* tree pollens, while m/z 301.04 was the most intense for the Colorado bee pollens at temperatures above 550°C.

In fact, both m/z 286.05 and m/z 301.04 are observed simultaneously in such two pollen samples. The relative intensities are dramatically different. The m/z 286.06 was identified and assigned as kaempferol ($C_{15}H_{10}O_6$), and m/z 301.04 to kaempferol with the change of a hydroxyl group to methoxy bond, or O-methylated kaempferol ($C_{16}H_{12}O_6$). The identification of these chemicals was based on low-energy collision induced fragmentation techniques, which will be discussed later.

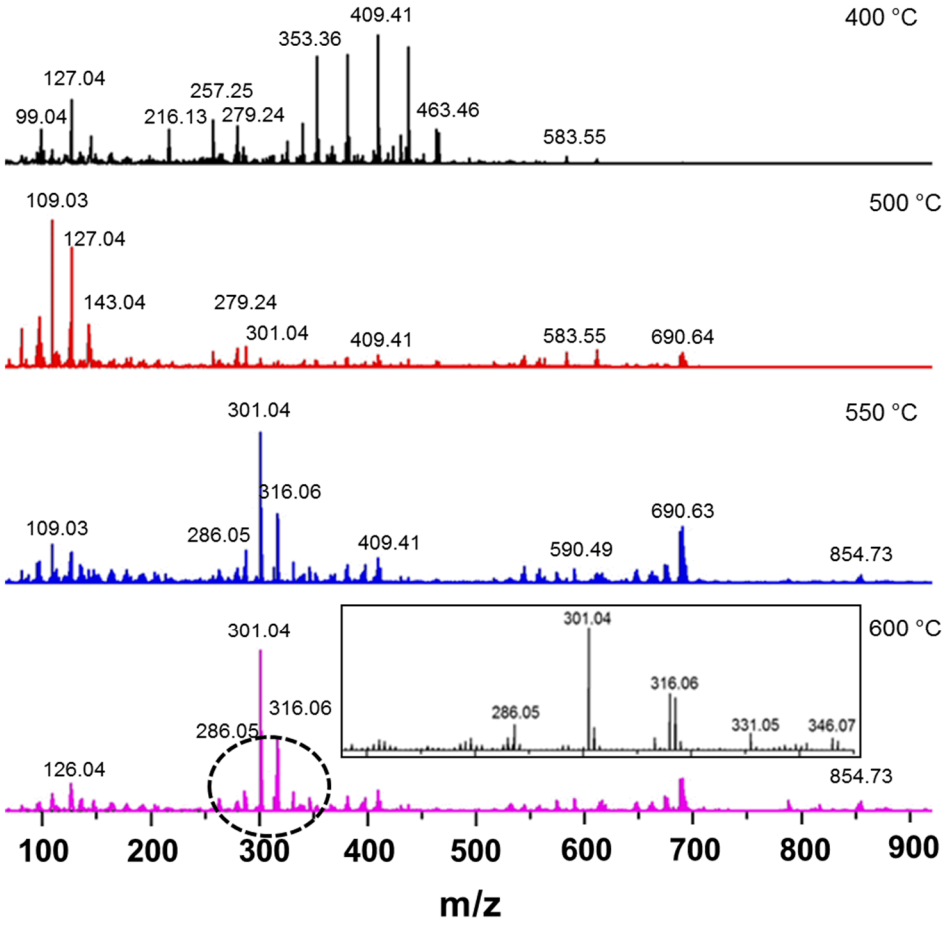

Figure 2. ASAP-MS of Colorado bee pollens thermally degraded at 400, 500, 550, and 600°C, respectively. The expanded spectrum represents the fingerprint of five flavonoids.

The expanded spectrum in Figure 2 represents the five flavonoids identified for Colorado bee pollens and formed as a spectrometric pattern. Five flavonoids were shown as m/z 286.05, 301.04, 316.06, 331.05, and 346.07. Instead, only four flavonoids were observed at m/z 254.06, 269.08, 286.05, and 301.04 in *P. nigra* tree pollens. The difference in mass peak position and relative intensity indicates that the spectrometric pattern of flavonoids is specific for different kind of pollens.

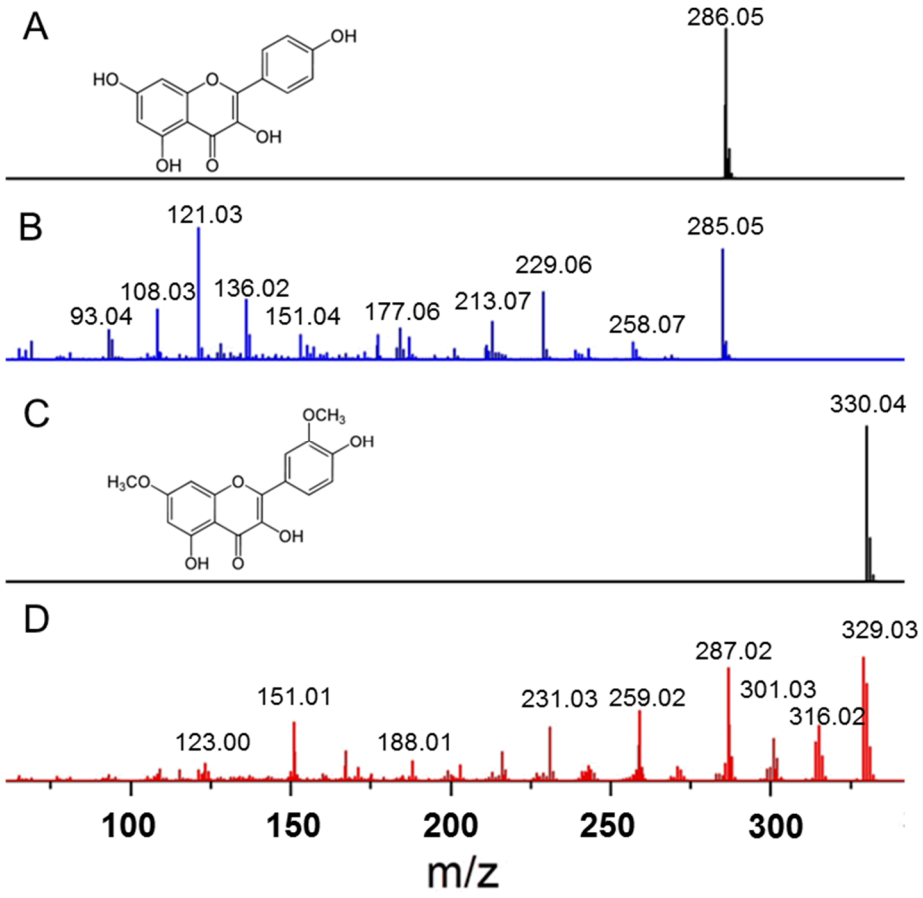

Figure 3. ASAP-MS and low energy collision fragmentation of kaempferol (A, B) and rhamnazin (C, D) heated at 500°C. The chemical structure is included. Collision energy: 20 eV.

A variety of flavonoids have been extracted from plants, but not observed directly in previous py-GC/MS studies. These chemical compounds are volatile only at high temperatures, therefore they would be precipitated before entering the mass detectors at the regularly source temperatures (below 150°C). Kaempferol is a natural flavonoid, found in plants and plant-derived foods. Kaempferol has a melting point of 276-278°C and a boiling point close to 600°C.

Figure 3A shows the low-energy collision mass spectrum of kaempferol vaporized at 500°C. Without collision energy applied, the mass spectrum showed a single molecule radical peak at m/z 286.05, further confirming that ASAP is a soft ionization technique.[25] The parent molecular ion as either the radical cation M^{+*} or the protonated molecule MH^{+} are usually formed without fragmentation or breakdown. With the increase of collision energy, the peak was shifted by one mass unit to m/z 285.05 and with the decreased intensity. The intensity of collision-induced fragments was then increased with the increase of collision energy. The spectrum shown in blue was recorded at the collision energy ~20 eV, which is consistent to that reported in the literature by electrospray ionization (ESI)-MS/MS techniques.[26,27] In reference 26 and 27, March et al. discussed in great detail the fragmentation mechanisms of each fragment ion observed.

Rhamnazin is also a natural flavonoid, sharing the same flavonol backbone as kaempferol. Figure 3C shows only molecular radicals at m/z 330.04 detected in the absence of collision energy. With the applied collision energy, the base peak was shifted to m/z 329.03. At ~ 20 eV, the fragmentation peaks at m/z 316, 301, and 287 are intense. Those peaks are similar to the mass peaks observed for the mixture of flavonoids from thermal degradation of Colorado bee pollens.

The current MS instrumentation has enabled the fragmentation of several chemical species simultaneously in a mixture of flavonoids. The goal of this work was to evaluate the relationship among those chemical compounds without GC or pre-purification processes. On the other hand, the non-volatile flavonoids would be difficult to volatilize in most commonly available GC systems due to high boiling points. Figure 4 shows low-energy collision MS/MS spectrum of individual flavonoids produced from Colorado bee pollens. Interestingly, the four chemicals have very similar fragments at m/z 121, 147, and 229, and low molecular weight peaks [are often included as the fragments of high-molecule weight species] (not sure what you mean here – change for clarity). For example, the fragments at m/z 316.03, 303.06, and 288.03 are observed for m/z=331.05 fragmentation, while the fragments at m/z 301.04 and 285.05 were observed for m/z=317.07. These results illustrate the structural similarity in these four flavonoids. Comparing the spectrum shown in Figure 4A to that of kaempferol shown in Figure 3B, both are almost identical, indicating that these four flavonoids shown in Figure 4 possibly share the same

backbone structure of kaempferol, as it shares with rhamnazin (Figure 3). Four hydroxyl groups in kaempferol are potentially changed to four methoxy bonds to make up m/z from 286 to 301, 316, 331, and even 346.

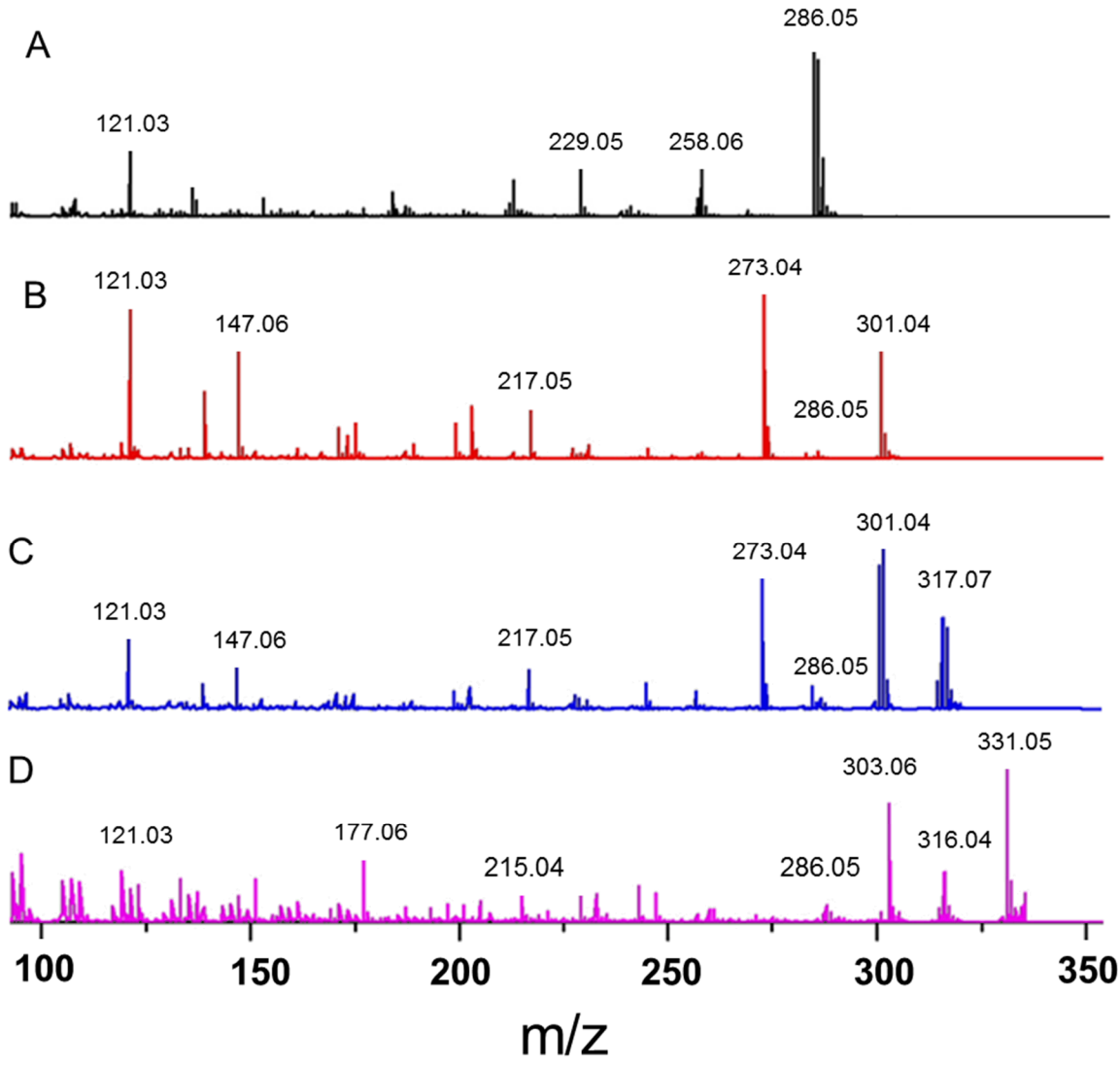

Figure 4. Low energy collision fragmentation mass spectrometry of 4 flavonoids produced in thermal degradation of Colorado bee pollens at 550°C: m/z = 286 (A), 301 (B), 317 (C), and 331 (D), respectively. Collision energy: 20 eV.

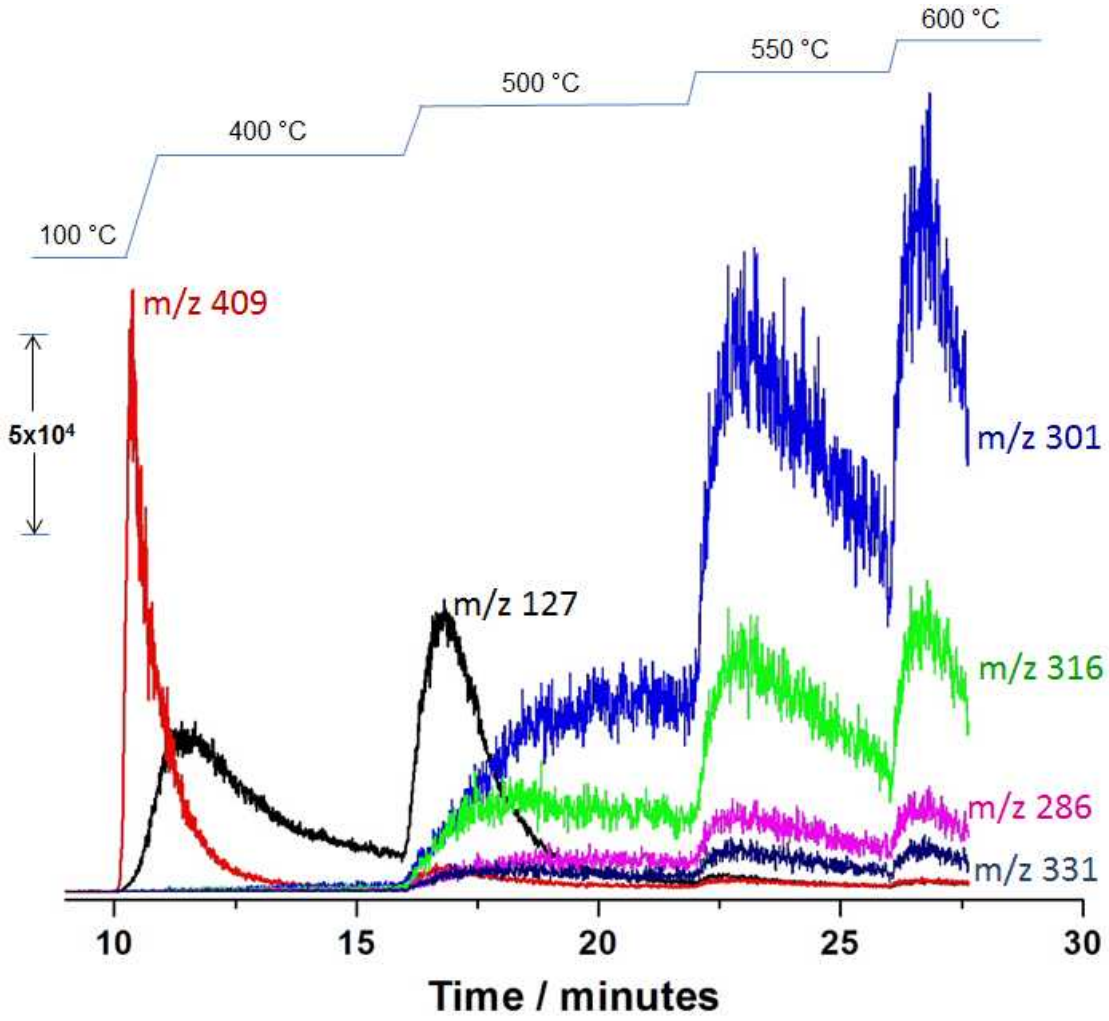

Figure 5. Temperature dependent production rates of the 4 flavonoids (m/z 286, 301, 316, and 331) and nonflavonoid compounds (m/z 127 and 409). The signal of the flavonoids was amplified by 8 times.

The temperature effect on the production rates of flavonoids was compared to the non-flavonoid products (m/z 127.03, and 409.41). Flavonoids were produced only at temperatures above 400°C from Colorado bee pollens. Figure 5 shows that these flavonoids are produced simultaneously and faster at higher temperatures. The relative intensity is kept rather constant, independent on the temperature: m/z=301 > 316 > 286 > 331 > 346. Dependent on the ionization probability of individual flavonoids, the relative intensity may not be directed to the product rates, which will be under further investigation. How these flavonoids are produced and why a constant production ratio is maintained are not yet clear.

Looking back at the spectra of *P. nigra* tree pollens, the most intense mass is at m/z 286.05 with a weak peak at m/z 301. There is not any other peak at m/z= 316, 331, and 346, indicating a rather different flavonoid mixture is generated. The low-energy collision MS/MS spectrum of m/z=286.05 shows very similar fragments as from kaempferol. As mentioned earlier, the mass peaks at m/z 254.06 and 269.09 are significantly intense at temperatures between 400 and 500°C. Those two peaks are assigned to lower molecular weight flavonoids sharing the same backbone structure as chrysin ($C_{15}H_{10}O_4$) and O-methylated chrysin ($C_{16}H_{12}O_4$) based on their similarity in fragmentations.

Figure 6 shows the other typical flavonoid patterns observed for *P. nigra italica* (Figure 6A), *Kochia scoparia* (Figure 6B), *Artemisia tridentate* (Figure 6C), California bee pollens (Figure 6D), and New Mexico bee pollens (Figure 6E) at 550°C. *P. nigra italica* tree pollens are similar to that of *P. nigra*, thus the flavonoid pattern looked alike. Kaempferol at m/z 286.05 is the main product. Three other flavonoids are identified at m/z = 254.06, 269.08, and 300.06. The production ratio also looks similar in both cases (Figure 6A versus Figure 1C).

Weed pollens from *K. scoparia* shows the intense peak at m/z = 316.08 in Figure 6B. The other notable flavonoids are at m/z 254.08, 271.08, 302.06, and 330.09. There is no notable peak at m/z 286 and 346. The fragmentation study shows that m/z 316 is identical to the one observed in Colorado bee pollens, indicating that m/z 316 and 330 may have the same backbone structure of kaempferol.

The weed pollen from *Artemisia tridentata*, however, showed the intense peak at m/z 286.06 with fractions of m/z 302.04 and 316.06. There are not any notable peaks at 254, 271, 330, and 346 in Figure 6C. Flavonoids of m/z 286 and 316 are also correlated to kaempferol. The m/z 302 may be assigned to Morin (MS 302, $C_{15}H_{10}O_7$) or its isomers, which has five hydroxyl groups.

Among three bee pollens from Colorado, California and New Mexico, Colorado bee pollens produce five flavonoids as discussed earlier (Figure 3D), while California bee pollens showed notable peaks at 254, 286, 301 and 317 (Figure 6D). New Mexico bee pollens, however, only

showed very weak peaks at m/z 254 and 286 (Figure 6E), largely different from Colorado and California bee pollens.

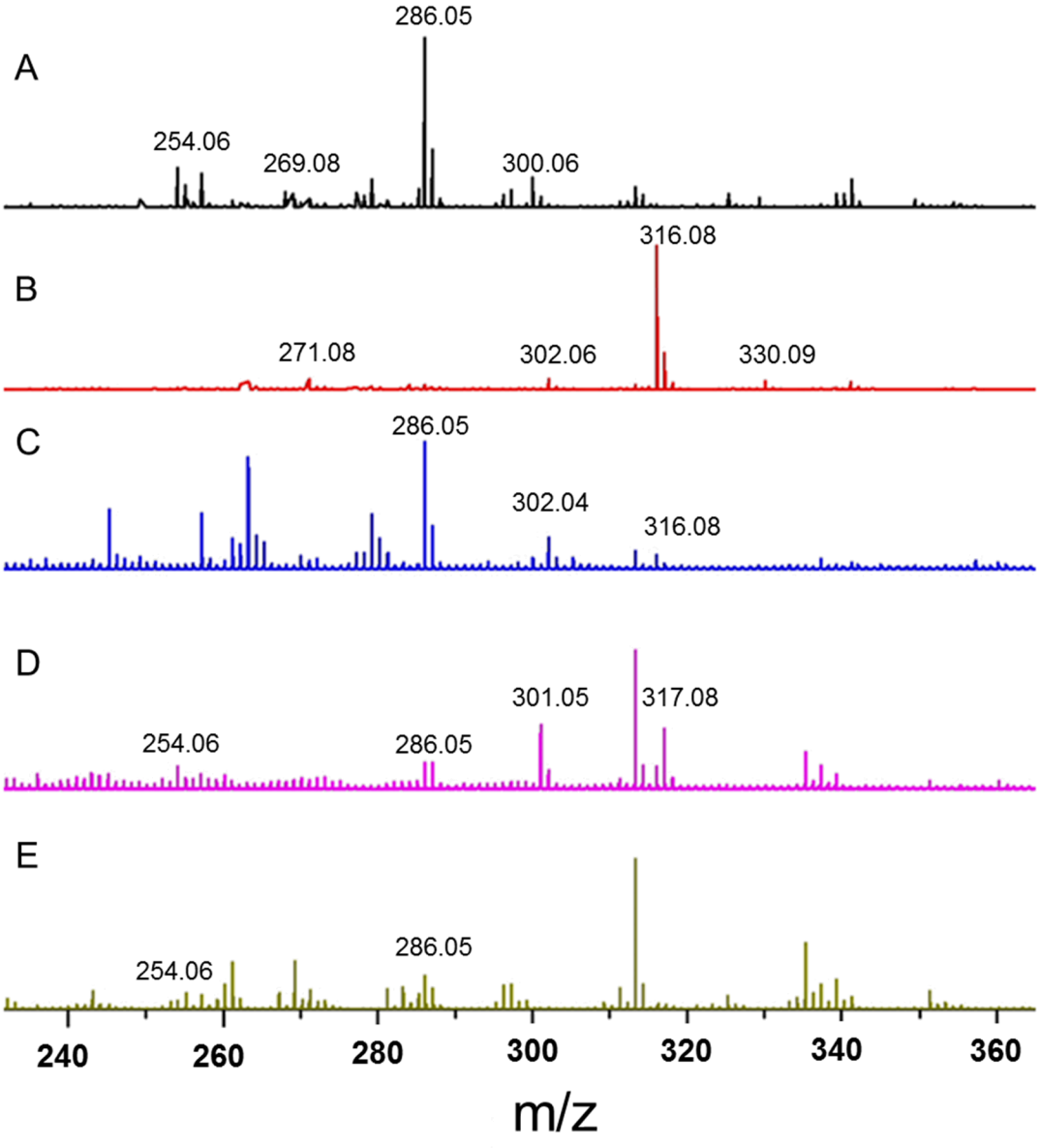

Figure 6. Typical mass spectrometric patterns of flavonoids produced at 550°C for (A) *Populus nigra italic*, *Kochia scoparia* (B), *Artemisia tridentata* (C), California bee pollens (D), and New Mexico bee pollens (E). Mass peaks as identified flavonoids are only designated.

Flavonoids have been shown a wide range of biological and pharmacological activities, including anti-allergic, anti-inflammatory, antioxidant, and anti-cancer.[28,29,30] Many studies suggest that consuming kaempferol may reduce the risk of various cancers and kaempferol is

currently under consideration as a possible cancer treatment. To extend our ASAP-MS application, the production rate and yield of flavonoids was obtained directly. As shown in Figure 7, a calibration experiment was carried out using m/z 286 of kaempferol, which was dissolved in acetone and recrystallized at the glass capillary. The amount of chemicals attached to the capillary was calculated by the concentration and volume. Kaempferol was not observed until the temperature reached 200°C. Increasing the temperature led to higher evaporation rates, especially at the temperature above the melting point. As the temperature was increased to 500°C, which is close to its boiling temperature, the evaporation rate was increased then decreased abruptly. While the evaporation rate was still increased when the temperature was increasing, eventually, all kaempferol molecules were vaporized, led to ion counts dropped to the background level. By integrating the counts, the ratio of the ions detected to the total number of molecules attached to the probe was obtained. Followed the same procedure, the counts of ions were obtained from pollen degradation. We estimated that 200 µg kaempferol could be extracted from 1 g *P. nigra italica* tree pollens. Figure 7 also illustrates the temperature dependent thermal desorption processes.

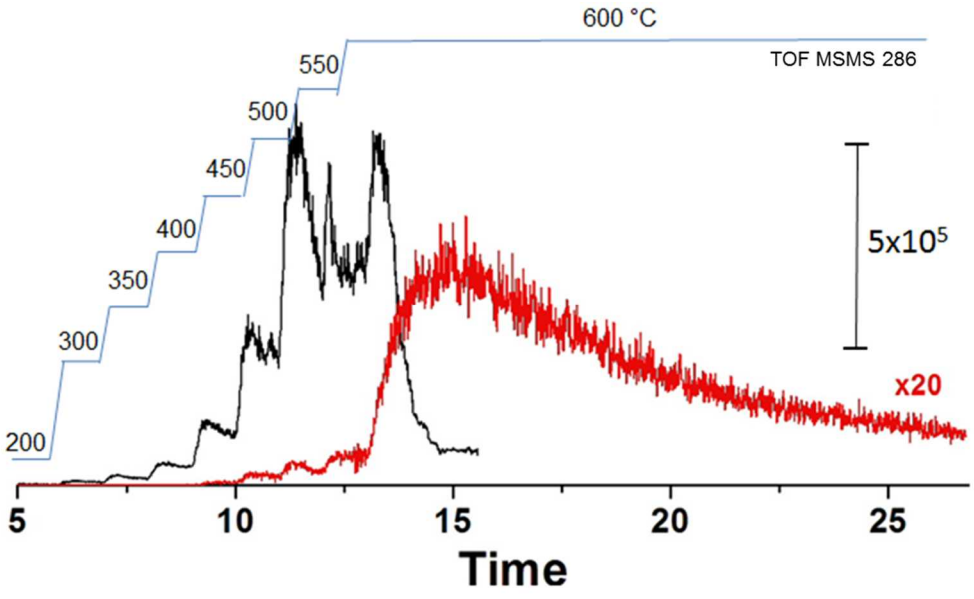

Figure 7. ASAP-MS/MS of m/z 286 for 5µg kaempferol (black) and 1.3 mg *Populus nigra italica* tree pollens (red) in the absence of collision energy. The temperature profile is indicated on the figure.

CONCLUSIONS

In conclusion we have demonstrated that ASAP-MS is a powerful tool for rapid fingerprinting of pollen samples. With ambient pressure ionization, we were able to identify distinct flavonoid signatures for pollens, yet pollen samples were analyzed directly, eliminating sample extraction steps requisite for other MS platforms such as gas or liquid chromatography MS. Upon thermal desorption, pollens produce a wide variety of chemicals, which are temperature dependent and specific to the type of pollens. The mass charge ratio (m/z) region ranging from 250 and 400 is complex due to the production of flavonoids, including their isomers. The masses, along with their relative mass intensities, generate specific fingerprints allowing for identification of pollens. Pollen flavonoids are shown as molecular radicals in our ASAP-MS, confirming soft ionization and ease for molecular interpretation. Low-energy collision induced fragmentation experiments were simultaneously performed for the flavonoid mixtures to identify their structural relationship. Increasing the throughput of pollen identification has implications for many fields including public health, agriculture, and ecology, and will allow for monitoring of changes in pollen densities and assessment of regional changes in plant populations via windborne and bee-borne pollens. We expect that ASAP-MS can also be developed as a simple, direct, and cost-effective technique for obtaining fingerprints for other biological entities such as bacteria and viruses.

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