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Atmospheric solids analysis probe mass spectrometry for the rapid identification of pollens and semi-quantification of flavonoid fingerprints

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RATIONALE: From allergies to plant reproduction, pollens have important impacts on the health of human and plant populations, yet identification of pollen grains remains difficult and time-consuming. Low-volatility flavonoids generated from pollens cannot be easily characterized and quantified with current analytical techniques.

METHODS: Here we present the novel use of atmospheric solids analysis probe mass spectrometry (ASAP-MS) for the characterization of flavonoids in pollens. Flavonoid patterns were generated for pollens collected from different plant types (trees and bushes) in addition to bee pollens from distinct geographic regions. Standard flavonoids (kaempferol and rhamnazin) and those produced from pollens were compared and assessed with ASAP-MS using low-energy collision MS/MS. Results for a semi-quantitative method for assessing the amount of a flavonoid in pollens are also presented.

RESULTS: Flavonoid patterns for pollen samples were distinct with variability in the number and relative abundance of flavonoids in each sample. Pollens contained 2–5 flavonoids, and all but *Kochia scoparia* contained kaempferol or kaempferol isomers. We establish this method as a reliable and applicable technique for analyzing low-volatility compounds with minimal sample preparation. Standard curves were generated using 0.2–5 µg of kaempferol; from these experiments, it was estimated that there is approximately 2 mg of kaempferol present in 1 g of *P. nigra italica* pollen.

CONCLUSIONS: Pollens can be characterized with a simple flavonoid pattern rather than analyzing the whole product pattern or the products-temperature profiles. ASAP-MS is a rapid analytical technique that can be used to distinguish between plant pollens and between bee pollens originating from different regions. Copyright © 2016 John Wiley & Sons, Ltd.

Recent advancements in mass spectrometry (MS) allow ions to be generated at ambient pressure and consequently mass spectra can be collected on samples with minimal preparation or pre-treatment.^[1–3] Such novel MS instrumentation techniques have enabled analysis of unaltered samples, such as the analysis of chemicals in the solid phase or adsorbed on surfaces of solid materials, pharmaceuticals, synthetic chemicals, hydrocarbons, and *in vivo* biological systems such as cells and tissues.^[4–8] Atmospheric solids analysis probe mass spectrometry (ASAP-MS) is one of these direct ionization techniques.^[9,10] Upon thermal desorption at high temperatures, the vaporized molecules are ionized at ambient pressure before entering the inlet of the mass spectrometer. Although ionization occurs outside the mass analyzer, sensitivity and chemical specificity is not compromised.^[3,9,11] In addition, ASAP provides soft ionization conditions, resulting in little or no ion fragmentation. Elemental compositions have been deduced from the generated molecular ions by high-resolution mass spectrometry with mass accuracies at the 1 ppm level.^[12] 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 suitable for semi- and low-volatility chemical compounds.^[5,6,13,14]

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 ingest pollen as bee pollen and as honey. Additionally, observing 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 would have tremendous benefit for these areas of research.

Yet, because of the complexity and diversity of pollens, identification remains challenging. Optical and electron microscopy can distinguish pollens at a generic level, but analysis is tedious, slow, and requires a high level of expertise.^[15] Pollens contain a wide variety of chemicals, including carbohydrates, organic acids, carotenoids, flavonoids, phospholipids, and proteins. Consequently, identification of pollen via chemical fingerprint has been an area of research interest. Volatile and semi-volatile compounds were found in pyrolysis-gas chromatography/MS (Py-GC/MS) experiments,^[16] while vibrational spectroscopy techniques (IR and Raman) were used to differentiate pollens through the intensity and

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distribution of C–N and C–O single and double bond vibrational modes.^[17,18] 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.^[19,20] Unique molecular variability has also been observed after extensive solvent extraction.^[21–27] 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 using ASAP-MS. Attachment of pollen to the closed end of a glass capillary probe is the only sample preparation required. Upon heating, pollen grains are thermally degraded and the vaporized molecules are ionized before entering the inlet of the mass spectrometer. We report and discuss the observation of unique flavonoid patterns generated from individual windborne pollens (*Ambrosia trifida*, *Populus nigra*, *Artemisia tridentata*, and *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. In addition to generating pollen flavonoid patterns, a semi-quantitative method was used to estimate the amount of the flavonoid kaempferol in *P. nigra italica* pollen.

EXPERIMENTAL

Materials

Pollens (*Populus nigra italica*, *Kochia scoparia*, and *Artemisia tridentata*) were purchased from Sigma Aldrich (St. Louis, MO, USA) and also from Greer Laboratories (Lenoir, NC, USA) (*Populus nigra*) as fine dried powders. The bee pollens were purchased from local grocery stores (Sprouts and Natural Grocers) and originated from bee farms in New Mexico, Colorado, and California. The grain size ranges from a few to 50 micrometers. Mortars and pestles were used to break apart bee pollen pellets before attachment to glass capillaries. Pollens were stored at 4°C until analysis. The flavonoids kaempferol (Fisher Scientific; Pittsburgh, PA, USA) and rhamnazin (Sigma Aldrich) were used as standards.

Instrumentation and sample attachment

ASAP-MS experiments were performed on a Synapt G2-HDMS time-of-flight (TOF) high-resolution mass spectrometer (Waters Corp., Manchester, UK). Mass analysis was performed in positive ionization mode. TOF-MS settings were: source temperature, 120°C, sampling cone, 48.0 V, extraction cone, 4.0 V, corona current, 8 μ A, acquisition mass range, m/z 50–1000, scan time, 0.5 s, and desolvation gas, 1000 L/h. Each mass spectrum presented in the text was averaged from more than 100 scans. The mass accuracy was <5 ppm for flavonoid standards and products. The low-energy fragments had mass uncertainty <50 ppm dependent on their abundances. The m/z windows used for the extracted ion profiles was ± 0.05 u.

In order to establish which compounds were flavonoids, low-energy collision-induced dissociation (CID) was performed for selected mass products and the fragmentation patterns were compared to those of flavonoid standards (kaempferol and rhamnazin). The collision energy was set between 10 and 50 eV, so that the products were fragmented gently to produce high-mass fragments (i.e., less extensively fragmented ions) closer to the products for ease of structural comparison and identification.

Kaempferol and rhamnazin were used as flavonoid standards and their ions at m/z 286.0477 and 330.0740 were applied as lock masses when they were produced from pollen samples. Both flavonoids were dissolved in acetonitrile (Fisher Scientific, HPLC-MS grade) and attached to the ASAP probes by drying ~ 5 μ L solution with concentrations ranging from 1 to 5 mg/mL. Additionally, for the examination of flavonoid productivity in pollen, kaempferol was added directly to pollen before attachment to the probe. The fine pollen powders were attached to the glass capillaries with the help of a thin layer of water. The bee pollens were attached to the probe by physical friction since the grains were not completely dried after being ground. The N_2 gas temperature was increased from 50 to 600°C at 50°C/min or as plotted in the specific figures. The temperature ramping rate was $\sim 5^\circ\text{C/s}$. The actual temperature at the sample probe was increased or decreased rapidly correspondent to the input temperatures.^[28] The experiments were reproduced multiple times (>5) for individual pollen samples. All m/z values were recorded and analyzed with ultimately high accuracy. For simplicity all m/z values throughout this manuscript are reported to four or zero decimals.

RESULTS AND DISCUSSION

Characteristics of flavonoid standards

A variety of flavonoids have been produced from plants, but have not been observed directly in pyrolysis GC/MS studies.^[14,29] Flavonoids are semi-volatile compounds that would be precipitated at the regular source temperatures (below 150°C) before entering the mass detectors. ASAP allows the desorption/ionization of relatively high-mass molecules. These ions will not condense as they are transported to the inlet as gas-phase ions within an electric field. This is advantageous over traditional pyrolysis/GC/MS in which the molecules pass through a long GC column before being ionized and analyzed. The combination of a short diffusion length combined with ambient ionization largely reduces the residence time, preserving the product pattern. To identify the flavonoids produced from the pollen samples, the mass spectra of two flavonoid standards, kaempferol and rhamnazin, were recorded. Flavonoids were subjected to low-energy (<50 eV) CID to generate both high- and low-mass fragments which were used for the structural identification of unknown flavonoids.

Kaempferol ($C_{15}H_{10}O_6$) is a common flavonoid found in plants and plant-derived foods. Flavonoids have been shown to have a wide range of biological and pharmacological activities, including anti-allergic, anti-inflammatory, antioxidant, and anti-cancer properties.^[30–32] Kaempferol has a melting point of 276–278°C and a boiling point close to 600°C. Figure 1(A) shows the mass

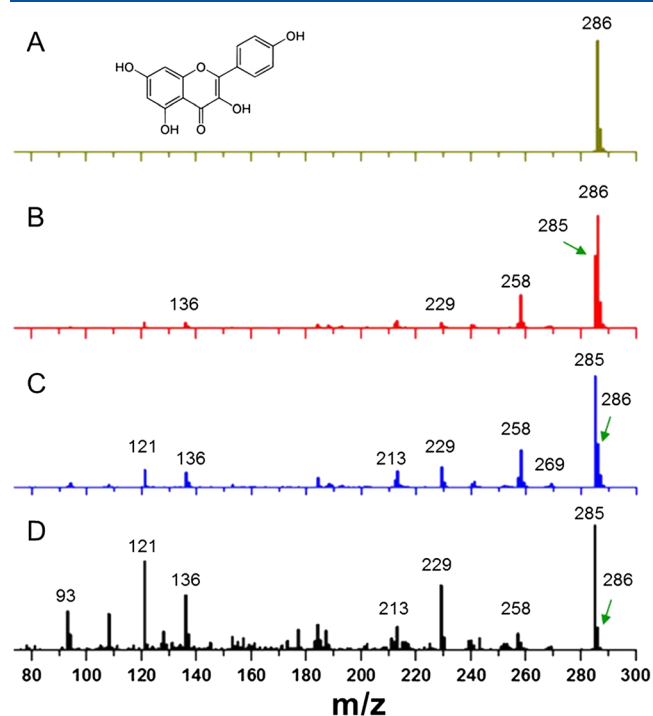


Figure 1. ASAP-MS and low-energy CID spectra of kaempferol heated at 400°C in the absence (A) and presence of increased collision energy: 10 eV (B), 20 eV (C), and 30 eV (D). The chemical structure of kaempferol is included. Y-axis is relative intensity.

spectrum resulting from low-energy fragmentation of kaempferol vaporized at 400°C. Without collision energy applied, the mass spectrum showed a single molecular ion at m/z 286.0477 (See Table 1 for calculated mass and mass uncertainty), confirming that ASAP is a soft ionization technique. When the collision energy was increased, a change of 1 u was observed due to a hydrogen loss. In addition, there was a significant increase in the intensity of mass fragments at m/z 269.0461 ($C_{15}H_9O_5^+$), 258.0582 ($C_{14}H_{10}O_5^+$), 229.0546 ($C_{13}H_9O_4^+$), and 121.0308 ($C_7H_5O_2^+$). Calculated masses and mass uncertainties are presented in Table 1. These ions were observed and discussed by March *et al.*^[33] and Cuyckens *et al.*,^[34] respectively. Figures 1(B) to 1(D) demonstrate the influence of collision energy on both deprotonation and fragmentation processes. Clearly, by increasing the collision energy, the ratio between m/z 285 and m/z 286 increases, illustrating collision energy induced

dehydrogenation of the radical cations (i.e., cleavage of a hydrogen atom from a molecular ion) rather than a thermal process.

Rhamnazin ($C_{17}H_{14}O_7$) is also a natural flavonoid, sharing the same flavonol backbone as kaempferol. Figure 2(A) shows only molecular ions at m/z 330.0740 in the absence of collision energy. Figures 2(B) and 2(C) show the typical fragmentation patterns at m/z 315, 301, 287, 259, 231, and 151. Similarly to kaempferol, the base m/z ratio decreased by 1 u, from m/z 330 to 329, when collision energy was applied.

It is worth noting that the high-mass fragments are representative of flavonoids; for example, the mass fragment at m/z 269 from kaempferol and the fragments at m/z 316, 301, and 287 from rhamnazin are typical of natural flavonoids. In the following discussion, the same mechanism of low collision energy fragmentation mass spectrometry was applied to determine which m/z ratios from different pollen samples are flavonoids.

Two-dimensional temperature-mass spectra profiles of pollens

To illustrate how pollens can be rapidly identified by ASAP-MS, representative pollens (tree pollen [*Populus nigra*] and bee pollen collected from Colorado and named as Colorado bee pollen) were examined. The mass spectra recorded during thermal degradation exhibit a complex composition of compounds at variable abundances and temperature-related production rates. Since ASAP is a soft ionization technique coupled with high mass accuracy, each m/z ratio, with relevant isotopes, represents a specific chemical compound. Below 400°C, the most intense m/z ratios were observed at lower molecular weights ($m/z < 450$). Notable m/z ratios for *P. nigra* pollen (Fig. 3) are present at m/z 95, 129, 147, 161, 177, 194, 208, 254, and 269. These m/z ratios are separated by small mass differences of 14 ($-CH_2$), 15 ($-CH_3$), 17 ($-OH$), and 18 (H_2O). The other main m/z ratios are at m/z 213, 241, 257, 285, 287, 313, 341, 369, and 409. The elemental compositions,

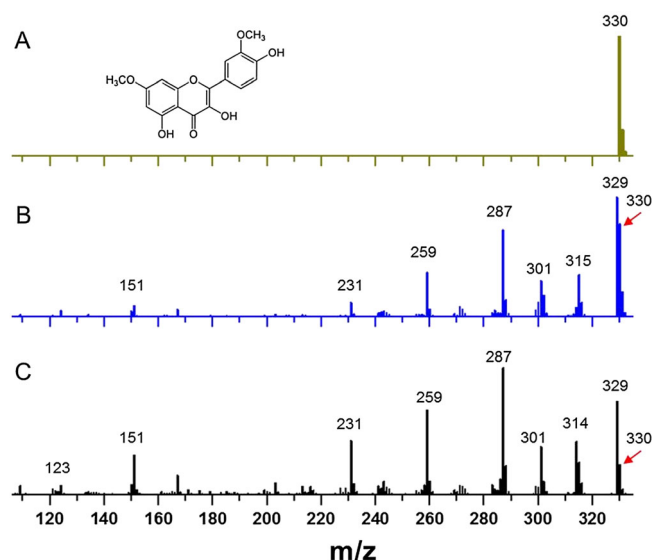


Figure 2. ASAP-MS and low-energy CID spectra of rhamnazin heated at 400°C in the absence (A) and presence of increased collision energy: 10 eV (B) and 30 eV (C). The chemical structure of rhamnazin is included. Y-axis is relative intensity.

Table 1. Observed masses, chemical formulae, calculated masses, and mass uncertainties for selected ions

Observed mass	Formula	Calculated mass	Mass uncertainty (ppm)
121.0308	$C_7H_5O_2^+$	121.0290	14.9
229.0546	$C_{13}H_9O_4^+$	229.0501	19.6
254.0579	$C_{15}H_{10}O_4^+$	254.0579	0.0
258.0582	$C_{14}H_{10}O_5^+$	258.0528	21.0
269.0461	$C_{15}H_9O_5^+$	269.0450	4.1
286.0477	$C_{15}H_{10}O_6^+$	286.0477	0.0
330.0740	$C_{17}H_{14}O_7$	330.0739	0.3

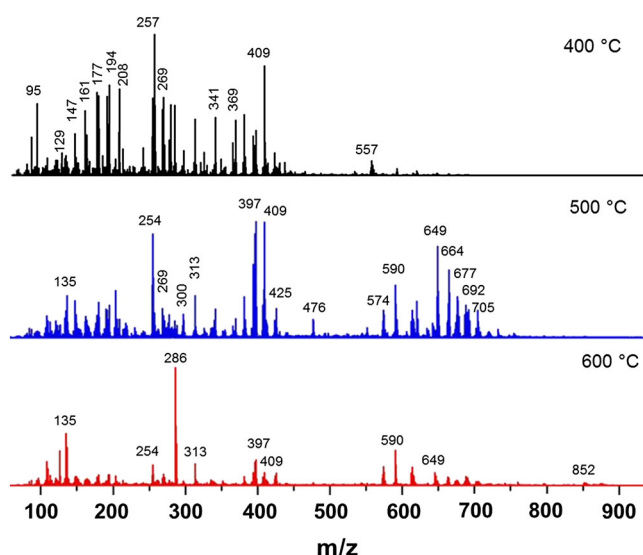


Figure 3. ASAP-MS of *Populus nigra* tree pollens thermally degraded at 400, 500, and 600°C, respectively. Y-axis is relative intensity.

computed from accurate mass measurements, for these compounds indicate that there are more elements present than just C, H, and O. The temperature-dependent mass spectra shown in Figs. 3 and 4 were found to vary with pollen type and species, showing both identical and variable thermal desorption products. Since the spectra were reproducible for individual pollens, these temperature-dependent mass spectra profiles are reliable for rapid identification of pollens.

In the case of Colorado bee pollen (Fig. 4), the intense m/z ratios are present between m/z 109 and 690, with the majority at or below m/z of 409. A few of the m/z ratios are identical to the ions observed from *P. nigra* tree pollens, such as m/z 257 and 409. Some of the product ions are shifted by 2 u,

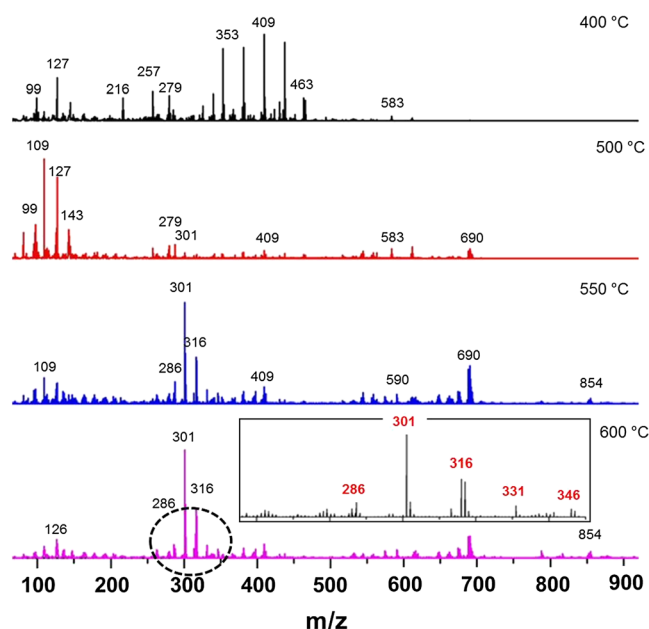


Figure 4. ASAP-MS of Colorado bee pollens thermally degraded at 400, 500, 550, and 600°C, respectively. The expanded spectrum represents the fingerprint of five flavonoids. Y-axis is relative intensity.

presumably due to a double bond ($=2.0157$ u); examples include m/z ratios at m/z 127/129 and 145/147. We did not attempt to positively identify the exact chemical compositions and structures of other m/z ratios, since the flavonoids were the focus of this study.

Note that the m/z ratios at 254 and 269 are not observed for Colorado bee pollen; instead, m/z 301 starts to develop at higher temperatures. When the temperature was increased to 500 and 600°C, some higher molecular weight compounds appeared, first in the m/z range between 500 and 800, then above 800. This is due to the temperature-assisted vaporization and higher temperature thermal degradation. However, at elevated temperatures ($>550^\circ\text{C}$), the m/z 286 became the most intense m/z ratio in the case of *P. nigra* tree pollen, while m/z 301 was the most intense for the Colorado bee pollens.

The expanded spectrum in Fig. 4 shows the five flavonoids identified for Colorado bee pollens at m/z 286, 301, 316, 331, and 346. For *P. nigra* tree pollen, only four flavonoids were observed at m/z 254, 269, 286, and 300. The difference in m/z value and relative intensity indicates that flavonoid patterns are specific and distinct for individual pollens, and, thus, provide a simpler molecular fingerprint for pollen identification than relying on the whole product patterns or the product-temperature profiles. Flavonoids would be difficult to volatilize in most commonly available GC systems due to their high boiling points. Furthermore, our current ASAP-MS instrumentation has also enabled us to fragment individual chemical species simultaneously in a mixture, significantly enhancing the identification of flavonoids based on their unique fragmentation patterns.

The m/z 286 from both *P. nigra* pollen (Fig. 3) and Colorado bee pollen (Fig. 4) shows similar fragmentation patterns to kaempferol that was discussed in Fig. 1. Therefore, both *P. nigra* tree pollens and Colorado bee pollens contain kaempferol but at variable concentrations. Similarly, the m/z 254.0579 is observed from *P. nigra* pollen (Fig. 3) and putatively assigned to chrysin ($\text{C}_{15}\text{H}_{10}\text{O}_4$) with the exact calculated mass of 254.0579. Chrysin has the same flavone backbone structure but two less hydroxyl substituents than kaempferol.

Figure 5 shows interesting mass fragmentation patterns for the four flavonoids produced from Colorado bee pollens. The four flavonoids have some similar fragments in the low m/z range (m/z 95, 121, 147, and 229), indicating that these flavonoids may have similar backbone structures. At higher m/z ratios, the fragment ions at m/z 316, 303, and 286 are observed from the fragmentation of mass product m/z 331, while fragments at m/z 301 and 286 were observed from the fragmentation of mass product m/z 317. Note that these less extensively fragmented ions were also observed as thermal degradation products. These interesting fragmentation patterns illustrate the structural similarity of the observed flavonoids but vary based on the number of hydroxyl ($-\text{OH}$) and methoxy ($-\text{OCH}_3$) functional groups. As discussed before, kaempferol has four hydroxyl groups and rhamnazin has three hydroxyl and two methoxy groups (Figs. 1 and 2). Comparing the spectra shown in Fig. 5 to those of kaempferol and rhamnazin shown in Figs. 1 and 2, the spectra showed similar fragments, indicating that these four flavonoids shown in Fig. 5 share the same backbone structure that kaempferol shares with rhamnazin.

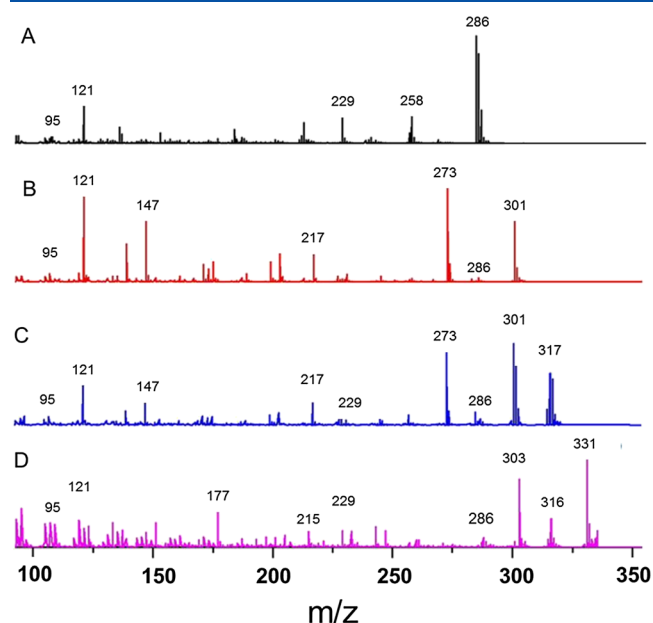


Figure 5. Low-energy CID spectra of four flavonoids produced in thermal degradation of Colorado bee pollens at 550°C: m/z 286 (A), 301 (B), 317 (C), and 331 (D), respectively. Collision energy: 20 eV. Y-axis is relative intensity.

The five flavonoids produced from Colorado bee pollens have m/z 286, 301, 316, 331, and 346 (Fig. 4), all of which vary by 15 or 30 u. One methoxy substitution (mass increases 14 u) accompanied by one hydroxyl group (mass increases 16 u) would result in an overall mass increase of 30 u. Since the replacement of hydroxyl and methoxy functional groups is variable (see the flavonoid structures in Figs. 1 and 2) and can occur at multiple locations, the presence of isomers are possible among these high-mass flavonoid products.^[29] For example, azaleatin, rhamnetin, and isorhamnetin ($C_{16}H_{12}O_7$) are isomers and all have m/z 316. Kaempferol and rhamnazin all have multiple isomers. Isomeric identification would add additional information to pollen fingerprints, and is currently under further investigation.

Flavonoid patterns in other pollens

The typical flavonoid patterns observed for *P. nigra italica*, *Kochia scoparia*, *Artemisia tridentata*, California bee pollens, and New Mexico bee pollens at 550°C are shown in Figs. 6(A)–6(E), respectively. For *P. nigra italica* and *P. nigra* pollens, kaempferol (m/z 286) is the main product. Three other flavonoids are identified at m/z 254, 269, and 300. Pollen from the weed *K. scoparia* has an intense m/z ratio at m/z 316. The other notable flavonoid m/z ratios are present at m/z 271, 302, and 330. Unlike the other pollens, there is no notable m/z ratio at either m/z 286 or 346. Pollen from the weed *Artemisia tridentata*, however, had an intense m/z ratio at m/z 286 with other masses present at m/z 302 and 316. The m/z 302 is assigned to morin or quercetin ($C_{15}H_{10}O_7$), which has one more hydroxyl group (for a total of five hydroxyl groups) than kaempferol. There are not any notable m/z ratios at m/z 254, 271, 330, and 346 for *A. tridentata*.

Among the bee pollens, Colorado bee pollens produce five flavonoids (Fig. 4(D)), while California bee pollens showed notable m/z ratios at m/z 254, 286, 301 and 317 (Fig. 6(D)),

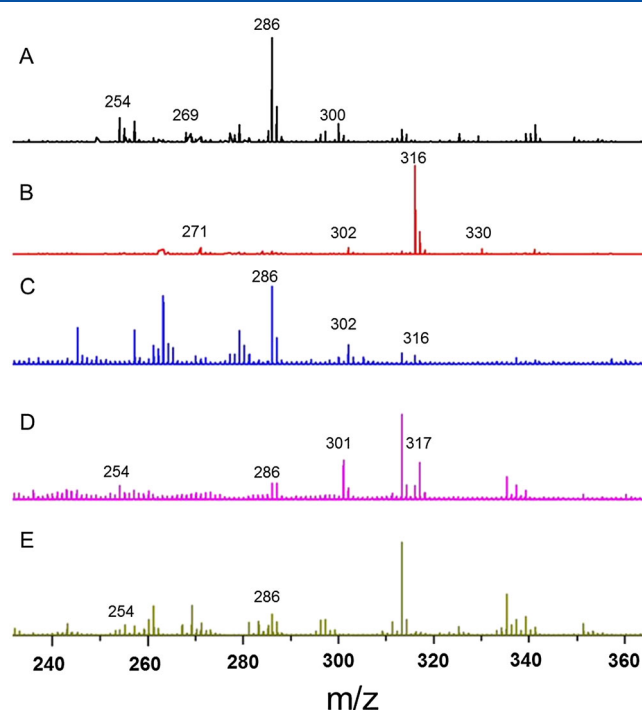


Figure 6. Representative flavonoid mass spectrometric patterns produced at 550°C for *Populus nigra italica* (A), *Kochia scoparia* (B), *Artemisia tridentata* (C), California bee pollens (D), and New Mexico bee pollens (E). Only m/z ratios identified as flavonoids by comparison to kaempferol and rhamnazin standards are labeled. Unlabeled m/z ratios are not flavonoids. Y-axis is relative intensity.

and New Mexico bee pollens only showed very weak m/z ratios at m/z 254 and 286 (Fig. 6(E)). Both California and New Mexico bee pollens show abundant m/z ratios at m/z 313 and 335, which are not assigned to flavonoids. The distribution of flavonoids for all pollens is summarized in Table 2.

Semi-quantification of flavonoid productivity in pollen

ASAP-MS provides a method for the rapid and reliable identification of flavonoid patterns in pollens. Additionally, ASAP-MS can be used to estimate the amount of flavonoids present in a pollen sample without solvent extraction. Figure 7 shows that flavonoids from Colorado bee pollens were produced only at temperatures above 400°C, though some non-flavonoid products (m/z 127 and 409) were produced at lower temperatures. The five flavonoids were produced simultaneously but at a much higher rate as the temperature was increased, while the relative intensity of m/z 301 > 316 > 286 > 331 > 346 was independent of temperature (chromatogram for m/z 346 is not shown in Fig. 7 because low intensity obscured the other curves). Assuming similar ionization and detection efficiency, these production rates and their integrated intensities (i.e. productivity) can be compared to known concentrations of flavonoid standards to estimate relative concentrations. Carrizo *et al.*^[7] also utilized a similar method combined with ASAP-MS to estimate the concentrations of pollutants in complex matrices.

As an example, kaempferol was used as both an external and internal standard, to quantitate the flavonoid productivity from *P. nigra italica* pollen. As an external standard, kaempferol

Table 2. Summary of number and distribution of flavonoids in pollens

Pollen	Number of flavonoids	Highest abundance m/z value or ratio	The pattern of flavonoids (m/z)
<i>Populus nigra</i>	4	286	254, 269, 286, 301
<i>Populus nigra italica</i>	4	286	254, 269, 286, 300
<i>Kochia scoparia</i>	4	316	271, 302, 316, 330
<i>Artemisia tridentata</i>	3	286	286, 302, 316
Colorado bee pollen	5	301	286, 301, 316, 331, 346
California bee pollen	4	317/301	254, 286, 301, 317
New Mexico bee pollen	2	286	254, 286

was evaporated by itself and the ratio between the recorded ions and the total amount of molecules attached to the capillary probe resulted in ion detection efficiency; thus the m/z 286 productivity from pollen samples could be obtained by multiplying the observed total ions by the ion detection efficiency. The ion efficiency, or a calibration curve, was obtained by applying pure kaempferol of mass range from 0.1 to 15 μg , which was not included in Fig. 8. As an internal standard, an equivalent amount of pure kaempferol was spiked into the pollen samples. The increased ions at m/z 286 then could lead to a direct comparison between flavonoids produced from the internal standard and the pollen samples.

Figure 8 shows a typical series of five measurements for kaempferol standard and *P. nigra italica* pollen with five times of sample loading and temperature ramping. In Figs. 8(A) and 8(D) kaempferol was used as an external standard, with 0.4 and 2 μg , respectively, being attached to the probe from the acetonitrile solution. In Figs. 8(B) and 8(E) kaempferol was used as an internal standard by spiking 2 mg pollen with kaempferol at a low dose of 0.4 μg or at a high dose of 2 μg . Figure 8(C) illustrates kaempferol productivity from pollen

alone. All five samples were subjected to the same temperature ramp from 250 to 600°C. As discussed before, four flavonoids (m/z 254, 269, 286, 300) were identified from the thermal desorption of *P. nigra italica* pollen. Figure 8 also shows that flavonoids of m/z 254 and 269 were produced at relatively lower temperatures starting around 250°C than the flavonoids of m/z 286 and 300 at temperatures above 450°C. The productivity of kaempferol is about 10 times greater than that of the other three flavonoids (m/z 254, 268, 300), roughly estimated from their total ion counts.

The ion profiles for kaempferol (Fig. 8A) and pollen with (Fig. 8(B)) or without (Fig. 8(C)) low dose kaempferol illustrate that a much higher temperature is required to produce kaempferol from the pollen samples. The internal kaempferol started to evaporate at ~300°C, while the production of kaempferol from pollens occurred mainly at temperatures above 450°C (Figs. 8(B) and 8(C)). Therefore, Fig. 8(B) shows the separation between the internal kaempferol standard and kaempferol produced from pollen (Fig. 8(B), inset). The ratio of total ions of m/z 286 for 0.4 μg and 2 μg kaempferol was much larger than their mass ratio (i.e., 7.8 vs 5),

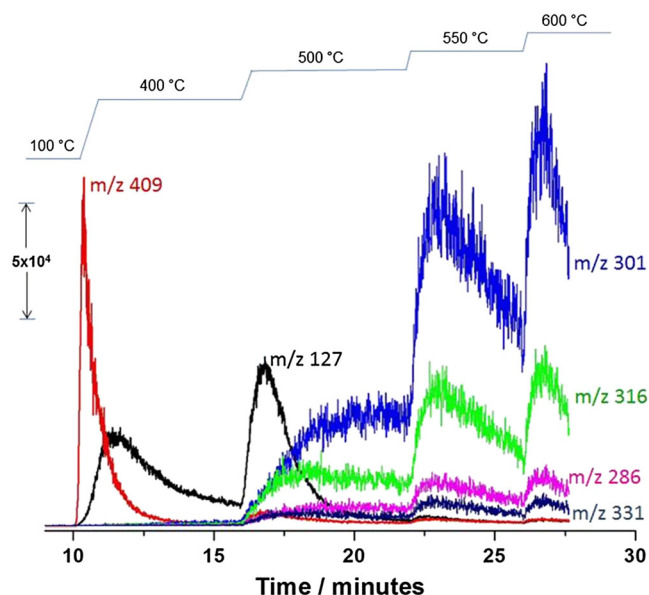


Figure 7. Temperature dependent production rates of four flavonoids (m/z 286, 301, 316, and 331) and non-flavonoid compounds (m/z 127 and 409). The signal of the flavonoids was amplified by 8 times.

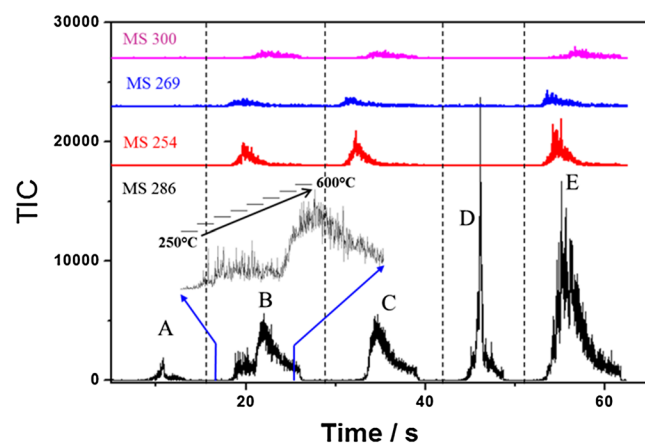


Figure 8. Mass spectrometric analysis of flavonoid production from *Populus nigra italica*, which contains approximately 4 μg kaempferol: (A) 0.4 μg kaempferol standard, (B) 0.4 μg kaempferol standard + 2 mg *Populus nigra italica*, (C) 2 mg *Populus nigra italica*, (D) 2 μg kaempferol standard, and (E) 2 μg kaempferol standard + 2 mg *Populus nigra italica*. The temperature was increased from 250 to 600°C in 50°C increments every 30 s. Inset shows detailed total ion profile for sample B with the temperature ramp.

illustrating nonlinear behavior due to ion suppression that decreases the ion detection efficiency. In the experiments, the background spectra often had ions at m/z 281, 355, and 429 which were comparably intense when less than 1 μg kaempferol was tested. As a result, the flavonoids of lower production from the pollen samples might be more suppressed by other high-yield products.

Using an internal standard with a comparable amount of 2 μg kaempferol spiked into *P. nigra italica* pollen (Fig. 8(E)), the total number of m/z 286 ions was about three times greater than the total ions resulting from 2 μg pure kaempferol (Fig. 8(D)), which indicated that the estimated production yield of kaempferol is $\sim 4 \mu\text{g}$ for a 2 mg pollen sample, or $\sim 2 \text{ mg}$ kaempferol from 1 g *P. nigra italica* tree pollen. The errors from lower doses of external (Fig. 8(A)) and internal (Fig. 8(B)) standards were relatively large in our experiments, possibly because of the ion suppression from background ions.

CONCLUSIONS

We have demonstrated that ASAP-MS is a reliable and applicable tool for rapid generation of flavonoid patterns from pollen samples. With ambient pressure ionization, we were able to identify distinct flavonoid patterns among the complex thermal products resulting from pollens. Pollen samples were analyzed directly, eliminating sample extraction steps required for other MS and chromatography methods such as gas or liquid chromatography MS. By ionizing at ambient pressure and elevated temperature, ASAP-MS is able to capture the full product patterns including both volatile and semi-volatile products. Flavonoids in pollens were identified by low-energy collision-induced dissociation and by comparing their fragmentation patterns to those of flavonol standards. Upon thermal desorption, pollens produce specific and distinct flavonoid patterns that vary in the number and relative intensity of flavonoids, with most samples containing 4 or 5 flavonoids. A method for estimating the amount of a flavonol present in pollen using ASAP-MS was also described.

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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