Rapid Species-level Identification of *Salvias* by Chemometric Processing of Ambient Ionisation Mass Spectrometry-derived Chemical Profiles

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ABSTRACT:

Introduction – The *Salvia* genus contains numerous economically important plants that have horticultural, culinary and nutraceutical uses. They are often similar in appearance, making species determination difficult. Species identification of dried *Salvia* products is also challenging since distinguishing plant morphological features are no longer present.

Objective – The development of a simple high-throughput method of analysis of fresh and dried *Salvia* leaves that would permit rapid species-level identification and detection of diagnostic biomarkers.

Methodology – Plant leaves were analysed in their native form by DART-MS without the need for any sample preparation steps. This furnished chemical fingerprints characteristic of each species. In the same experiment, in-source collision-induced dissociation was used to identify biomarkers. Biomarker presence was also independently confirmed by GC–MS. Chemometric processing of DART-MS profiles was performed by kernel discriminant analysis (KDA) and soft independent modelling of class analogy (SIMCA) to classify the fingerprints according to species.

Results – The approach was successful despite the occurrence of diurnal cycle and plant-age related chemical profile variations within species. In a single rapid experiment, the presence of essential oil biomarkers such as 3-carene, α-pinene, β-pinene, β-thujone, β-caryophyllene, camphor and borneol could be confirmed. The method was applied to rapid identification and differentiation of *Salvia apiana*, *S. dominica*, *S. elegans*, *S. officinalis*, *S. farinacea* and *S. patens*.

Conclusion – Species-level identification of *Salvia* plant material could be accomplished by chemometric processing of DART-HRMS-derived chemical profiles of both fresh and dried *Salvia* material. Copyright © 2016 John Wiley & Sons, Ltd.

▨ Supporting information can be found in the online version of this article.

Keywords: DART-MS; ambient ionisation; sage; *Salvia*; multivariate statistical analysis; triterpenoids; essential oils; SIMCA; KDA

Introduction

The *Salvia* genus, commonly referred to as Sage, is one of the most agriculturally important taxa in the world due to the commercial importance of its plants in the culinary, medicinal, fragrance and horticultural sectors, and to its wide geographic distribution. Among the culinary sages are *Salvia officinalis* L., *Salvia elegans* Vahl (pineapple sage), *S. fruticose* (Greek sage) and *S. melissordora* (grape scented sage). Ornamental sages include *Salvia apiana* Jeps. (white sage), *S. discolor*, *S. greggii*, *Salvia farinacea* Benth., *S. microphylla* and *S. clary* among numerous others. *Salvia* essential oils, particularly those from *S. officinalis* and *S. lavandulaefolia*, have been reported to exhibit memory enhancing effects (Eidi *et al.*, 2006). This has been attributed to the synergistic impact of constituent terpenes and terpenoids such as α-pinene, geraniol, linalool, γ-terpinene, 1,8-cineole and camphor acting on cholinergic receptors (Miroddi *et al.*, 2014). *Salvia* spp. plants have also been reported to exhibit antimicrobial (Jirovetz *et al.*, 2006; Jirovetz *et al.*, 2007; Benkherara *et al.*, 2015), anti-inflammatory (Moretti *et al.*, 1997; Ehrnhöfer-Ressler *et al.*, 2013), anticancer (Russo *et al.*, 2013) and antioxidant activities (Kozics *et al.*, 2013). As it is the essential oils of the plants which are important for many of its culinary and medicinal applications, significant attention has been given to determining the defining features of the essential oil profiles of *Salvia* species plants. In this regard, a number of

trends have been observed. For example, although *Salvias* are often found to contain terpenes and terpene derivatives such as α- and β-pinene, camphene, 1,8-cineole, α- and β-thujone, camphor, borneol, α- and β-caryophyllene and viridiflorol (Oelschlägel *et al.*, 2012), the relative proportions and appearance of these constituents in a particular plant part vary widely depending upon species. Thus, while the leaves of *S. officinalis* have consistently been found to contain the aforementioned terpenes, *S. elegans* leaves have been found to be devoid of β-pinene, 1,8-cineole, α- and β-thujone and camphor (Oelschlägel *et al.*, 2012). It has also been shown that constituent composition can vary as a function of intraspecies genetic variability (Perry *et al.*, 1999), plant part (Perry *et al.*, 1999; Santos-Gomes and Fernandes-Ferreira, 2001), season (Putievsky *et al.*, 1986; Müller-Riebau *et al.*, 1997), flowering versus vegetative stage (Piccaglia *et al.*, 1997) and harvest date (Perry *et al.*, 1999). Despite these observations, it is also clear that

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each *Salvia* spp. should have metabolomic features that define it, regardless of variability in plant age, growth conditions or other parameters.

Since it is the essential oil constituent make-up that defines the commercial importance of the plants, there is considerable interest in the development of methods that can be used to rapidly identify *Salvia* spp. from their small-molecule profiles (Gu *et al.*, 2004; Jiang *et al.* 2014; Luo *et al.*, 2015). Gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been used to identify plants with high concentrations of specific phytochemical constituents (Perry *et al.*, 1999; Jassbi *et al.*, 2012). However, studies have shown that depending upon the method used for their detection, the relative amounts of the compounds observed can also vary widely. For example, in a study of the volatile-component composition of *S. officinalis* essential oils, Baj *et al.* (2013) found that although the same constituents were observed in samples obtained using either the Deryng apparatus or the Clevenger apparatus, the relative amounts of the components were quite different. Additionally, a salient feature of the majority of the methodologies used to determine the small-molecule composition is the inclusion of an extraction step, which is necessitated by the analysis protocol. The most commonly used protocols, which include GC, GC-mass spectrometry (GC–MS), capillary electrophoresis and HPLC, all require that the plant part(s) of interest is/are subjected to extraction in order to convert the sample to a form compatible with the analysis method. The choice of solvent has a significant impact on the profile of compounds observed, as it selects for those metabolites that dissolve in it most readily. Since the utilisation of a common solvent for extraction of a group of *Salvia* species plant parts is likely to select for similar compounds, this step has the potential to yield results that emphasise non-discriminating similarities between species, rather than those that define principal differences (Lesiak *et al.*, 2015). As such, the inclusion of an extraction step can undermine the ability to use the observed chemical fingerprint to distinguish between species, and limits the extent to which extraction-based methods permit species identification and discrimination. Even in those cases where the chromatographic fingerprints of solvent extracts have been used to differentiate species, the time-consuming sample processing steps limit the wide applicability of the methods (Daszykowski *et al.*, 2009).

The *Salvia* genus is comprised of upwards of 900 species, and their categorisation as members of the sage family is based in part on characteristic morphological features of the plant reproductive parts. Specifically, *Salvias* exhibit a distinct staminal structure that includes two stamens with the two thecae on each stamen separated by an elongate connective (Walker *et al.*, 2004). Thus, besides genetic matching, definitive identification of *Salvia* species most often entails examination of the features of mature flowering plants. This approach is time-consuming and impractical, as it necessitates that the plants grow to maturity. Species identification based on DNA profiling requires that the genome for the plant in question be mapped. However, since <1% of *Salvia* spp. genomes have been sequenced, this approach has very limited utility.

We have proposed that chemical profiling could potentially be used for species identification, even in cases where: (1) the species have several biomarkers in common; (2) biomarker identities are not fully known; and/or (3) the small-molecule profile of plants within the same species exhibit seasonal and/or diurnal variations, among other differences. This can be accomplished providing that the small-molecule fingerprint used for the identification spans a broad range of defining compounds whose presence is not pre-selected by solvent choice. Analysis by MS using a Direct Analysis in Real Time (DART®) ion source provides the opportunity to analyse material in its native form in the absence of solvent, and this method has been shown to detect molecules spanning both extremes of the dielectric constant spectrum. It has been demonstrated that the DART high-resolution mass spectrometry (DART-HRMS) derived small-molecule profiles of animals, tree parts and seeds can be used to rapidly identify species (Musah *et al.*, 2015), to the extent that adulteration of plant-based drug products can be revealed by this method (Lesiak *et al.*, 2016).

Given the inherent advantages of being able to accurately and rapidly identify species or determine the defining biomarker profiles of various agriculturally important plants, we sought to determine whether a diversity of representative *Salvia* spp. could be distinguished from one another, despite the fact that they exhibit several compounds in common. We report herein that chemometric processing of rapidly acquired DART-HRMS-derived chemical fingerprints of plant leaves can be used to identify and distinguish between *Salvia* genus plants. The leaves can be analysed in their native form without the use of solvent. The acquired chemical profile information highlights intraspecies similarities and interspecies differences that can be exploited for the purpose of species identification. Within a single experiment, in-source collision-induced dissociation can be used to confirm the identity of important biomarkers, and the relative proportions of these biomolecules can be ascertained. Furthermore, knowledge of biomarker identities is not required for species discrimination. We demonstrate how this approach can be used to distinguish between sages representing culinary (*S. officinalis* and *S. elegans*), medicinal (*S. apiana*) and ornamental (*S. dominica*, *S. farinacea*, *Salvia patens* Cav.) species.

Experimental

Plant materials

Thirty of the analysed *Salvia* plants were purchased from an online vendor (Strictly Medicinal Seeds, formerly Horizon Herbs, Williams, OR): 10 *S. apiana*, 10 *S. dominica* and 10 *S. officinalis* (i.e. garden variety sage). Plants were approximately six months of age. Thirty-six additional *Salvia* plants were purchased from a local vendor (Hoeffner Farms, Montgomery, NY): 10 *S. farinacea*, 12 *S. elegans* (2 of the honey melon variety and 10 of the pineapple variety), 6 *S. patens*, and 8 *S. officinalis* (2 plants each of the garden, purple, tricolour, and berggarten varieties). These plants were between six and eight months of age. All plant material was sampled and analysed between July 2014 and May 2015. Plants were grown in 4″ pots with professional mix soil (ASB Greenworld, New Brunswick, Canada), kept in a glasshouse and watered daily. The average daily temperature of the glasshouse is 70 °F. With the exception of *S. patens*, plants were not flowering at the time of analysis.

Chemical standards

The following chemical standards were obtained from the indicated vendors: carnosic acid, oleanolic acid, ursolic acid, (Cayman Chemical, Ann Arbor, MI); (*Z*)-isoeugenol, borneol, *p*-cymen-8-ol, α-pinene oxide, α-terpinene, and β-caryophyllene (Thermo Fisher Scientific, Rockford, IL); bornyl acetate, (*E*)-β-ocimene, 1,8-cineole, 3-carene, β-thujone, caffeic acid, camphene, camphor, carvacrol, chlorogenic acid, geranial, limonene, myrcene, rosmarinic acid, sabinene hydrate, salvianolic acid A, terpinolenone, α-pinene, β-pinene and γ-terpinene (Krackeler Scientific, Albany, NY). Ethyl acetate was purchased from Pharmco-AAPER (Brookfield, CT). Diethyl ether was purchased from Sigma Aldrich (St Louis, MO).

Leaf trichome imaging

Salvia spp. leaf trichomes were imaged with a Nikon stereozoom SMZ800 microscope. Photographs were taken with a Nikon DS Fi2 microscope camera.

DART-HRMS data collection and processing

Mass spectral data were acquired using a DART-SVP ion source (IonSense, Saugus, MA) attached to a JEOL AccuTOF mass spectrometer (JEOL USA, Peabody, MA) in positive-ion mode. The DART ion source grid voltage was 250 V and the gas heater temperature was set to 350 °C. Mass spectrometer settings were: ring lens voltage, 5 V; orifice 1 voltage, 20 V; orifice 2 voltage, 5 V; and peak voltage, 400 V. Spectra were obtained over the mass range of *m/z* 50–800 at 1 spectrum per second. The helium flow rate for the DART source was 2.0 L/s. The resolving power of the mass spectrometer was 6000 FWHM. Mass calibration was performed using Jeffamine M600 (Huntsman, The Woodlands, TX) or polyethylene glycol (PEG 600).

Salvia spp. were analysed by removing 6 mm diameter circular chads from 10 different leaves within each plant using a metal hole puncher (Staples, Framingham, MA). The chads were held with metal tweezers between the ion source and mass spectrometer inlet. The distance between the chad and the inlet was ~1 cm and all analyses were conducted in positive-ion mode at 350 °C. Dried leaf samples were prepared by removing whole leaves from the plant and placing them inside a 37 °C oven for 10 days, with leaves being turned over once daily. Chads were then removed from dried leaves and held with metal tweezers between the ion source and mass spectrometer inlet. Chemical standards were tested by dipping the closed end of a melting point capillary tube into each sample, and presenting the coated surface to the open air space between the mass spectrometer inlet and ion source.

In-source collision-induced dissociation (CID) was performed on plant material and standards using function switching, in which a pre-defined set of increasing orifice 1 voltages is used to cause increasing degrees of fragmentation of detected molecules, all within a single MS experiment. In these analyses, the orifice 1 voltage was alternated from 20 V, 30 V, 60 V and 90 V. All plant material and chemical standards were analysed by in-source CID.

Data processing including calibration, background subtraction, and peak centroiding were performed using TSSPro3 software (Shrader Analytical Labs, Detroit, MI). Mass spectral analysis, elemental composition, and kernel discriminant analysis (KDA) were conducted using Mass Mountaineer software (Mass-spec-software.com, RBC Software, Portsmouth, NH). Solo (Eigenvector Research, Wenatchee, WA) software was used for multivariate statistical analysis.

Plant material analysis by DART-HRMS

Samples of plant material to be analysed were taken from the leaves of potted plants using a metal paper hole puncher (Staples, Framingham, MA) to generate circular chads that were 6 mm in diameter. For DART-HRMS analysis, each chad was held with stainless steel tweezers between the ion source and the mass spectrometer inlet. The chads were punched from different leaves of the same plant in order to acquire as broad a range of representative spectra as possible. A total of fifty replicates from each species were analysed. In some cases, 10 plants representing a given species were available (i.e. *Salvia apiana, S. dominica* and *S. farinacea*). For these plants, a total of fifty mass spectral replicates were acquired as follows: from each of the 10 plants representing a given species, five leaf chads were taken and each was analysed by DART-HRMS. The resulting five spectra were then averaged into a single spectrum. This approach yielded fifty individual spectra per plant. For *S. patens*, only six plants were available. In this case, a total of fifty mass spectral replicates were acquired as follows: for each of four of the plants, 10 chads were taken and analysed by DART-HRMS. The fifty resulting spectra were then averaged into a single spectrum. For the remaining two plants, five chads were taken from each and the five spectra obtained in each case were averaged into a single spectrum. For some of the other

species, a number of varieties were available (e.g. for *S. elegans*, there were the honey melon and pineapple varieties; for *S. officinalis*, there were the garden, purple, tricolour and berggarten varieties). Five chads from each of the aforementioned plant varieties were analysed, thus providing a dataset that also included the other available parent species (i.e. a total of sixty replicates of *S. elegans* and ninety replicates of *S. officinalis*).

GC–MS experiments were also conducted on plant material as a means of comparison. The analyses were performed with a HP 6890 GC–MS instrument and a Agilent J&W HP-5 ms column. The mass selection detector was operated in electron ionisation mode with ionisation energy of 70 eV and mass range 40–500. The injection temperature was 250 °C and helium flow rate 1 mL/min. Standards were injected with a split ratio of 20:1 and sample volume of 1 μL. Plant extracts were injected splitless and with a volume of 3 μL. The column temperature began at 50 °C and was held for 20 min. It was then gradually increased at 1 °C/min to 100 °C, and then 20 °C/min to 300 °C and held for 5 minutes. The biomarker 3-carene was confirmed using an AccuTOF-GCv 4G GC–MS instrument at a scan range was 40–500. The injector was set to 250 °C and standards were injected with a split ratio of 100:1 and a volume of 1 μL. The column temperature was held at 50 °C for 20 min, and ramped to 100 °C at a rate of 1 °C/min, and then to 300 °C at a rate of 20 °C/min. Finally it was held at 300 °C for 10 min.

The authentic standard samples were prepared for GC–MS analysis in ethyl acetate solutions and examined. *Salvia officinalis* was selected as the representative plant material because it was shown to contain many of the selected biomarkers by DART-HRMS. Plant material extracts were prepared by suspending leaf chads in 1 mL of ethyl acetate followed by sonication for 30 min. The samples were then evaporated to dryness and reconstituted in 500 μL of ethyl acetate for analysis, further concentrated before analysis. Compounds were identified based on GC retention times and matching of mass spectral fragmentation patterns against those in the National Institute of Standards and Technology (NIST) library of authentic standards.

Results and discussion

Each *Salvia* spp. displayed a unique and characteristic mass spectral fingerprint

The essential oils in *Salvia* spp. are manufactured in glandular trichomes that appear on plant aerial parts, most notably the leaves. Thus, chemical profile analysis of the leaves has the potential to provide information that could enable discrimination of one *Salvia* spp. from another. In order to determine whether *Salvia* spp. display unique chemical fingerprints, six species representing a combination of culinary, ornamental and medicinal *Salvias* were analysed. The microscopic leaf trichomes of each of the species are pictured in Fig. 1. *Salvia apiana* is used medicinally and for culinary purposes. *Salvia dominica, S. farinacea* and *S. patens* are ornamental varieties of *Salvia. Salvia elegans* and *S. officinalis* are both used as cooking herbs, with the former having a flavour described as pineapple-like, and the latter being the well-known "garden" sage. For the DART-HRMS analyses, 6 mm diameter-sized samples of fresh material acquired from different sections of the leaves were analysed. Spectra were acquired by holding each chad between the ion source and the mass spectrometer inlet with a pair of stainless steel tweezers. To provide a comprehensive dataset, 5–10 replicates representing different leaves within the same plant were analysed, and a minimum of 10 plants within each distinct species were sampled.

The results of these experiments are shown in Fig. 2 and the corresponding mass spectral data of measured and calculated masses and relative peak abundances are listed in Supporting Information Table S1. Depending upon the species, the spectra displayed between 38 and 164 peaks above a 2% relative abundance threshold,

Figure 1. Micrographs of *Salvia* spp. leaf trichomes observed at 6× magnification.
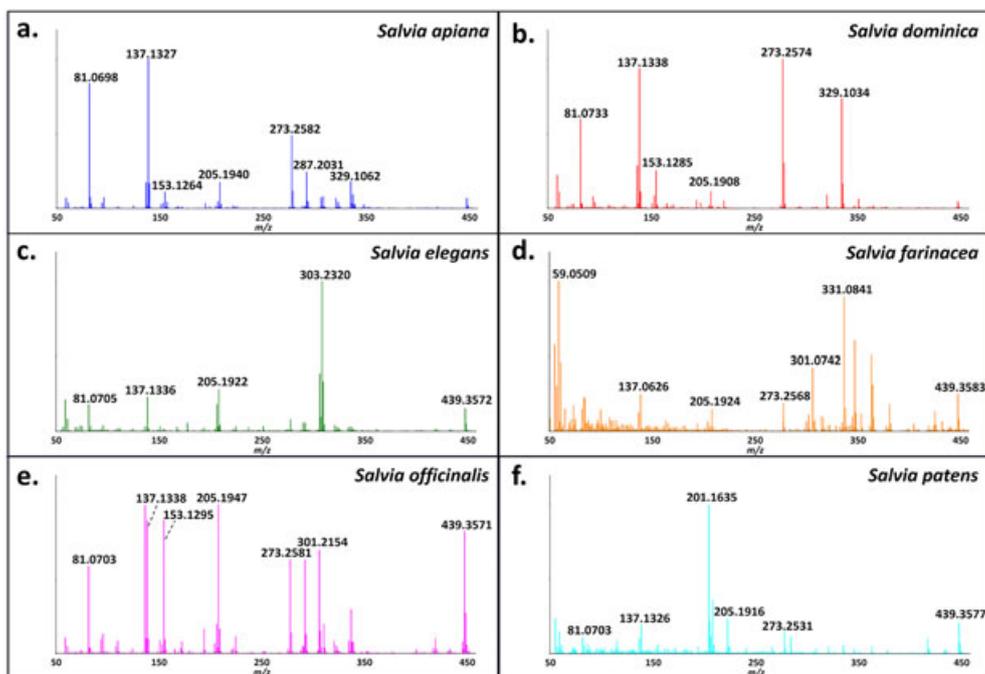

Figure 2. DART-HRMS spectra of the indicated fresh *Salvia* spp. leaf samples analysed in positive-ion mode at 20 V. Panels a, b, c, d and e represent the average of five individual leaf spectra. Panel f represents the average of ten individual leaf spectra. The corresponding mass spectral data can be found in Supporting Information Table S1.

with each peak representing a protonated molecule. *Salvia dominica* [Fig. 2(b)] had the smallest number of peaks and *S. farinacea* [Fig. 2(d)] had the greatest. Visual inspection of the six spectra showed that each species' fingerprint was unique. Nevertheless, some peaks were common to all six species examined. Nominal *m/z* 137, 205 and 439 were observed within all of the species. From the high-resolution data obtained, it was possible to make tentative assignments for several of the observed peaks based on previous reports of compounds detected in *Salvia* species. For example, the peak at *m/z* 137 corresponded to a compound with the formula $[C_{10}H_{16} + H]^+$, which was consistent with the possible presence of 3-carene and/or its isomers α-pinene, β-pinene or α-terpinene. All of these have been isolated from *Salvia* spp. β-Caryophyllene (formula $C_{15}H_{24}$) could be assigned to the peak at *m/z* 205, and *m/z* 439, corresponding to the formula $[C_{30}H_{48}O_3 + H]^+$, was attributed to loss of water from protonated ursolic or oleanolic acid. There were also peaks observed in only a subset of species. For example, nominal *m/z* 153 was detected in *S. apiana*, *S. dominica* and *S. officinalis*, and the exact mass corresponded to molecular formula $[C_{10}H_{16}O + H]^+$. In principle, this formula could represent any of the isomers camphor, citral, α-pinene oxide or β-thujone, all of which have been observed in *Salvia* spp. The peak at *m/z* 155 corresponding to a compound with the formula $[C_{10}H_{18}O + H]^+$ was detected in *S. apiana*, *S. dominica*, *S. farinacea* and *S. patens*, and could be attributed to borneol. In like manner, the presence of *m/z* 169 with formula $[C_{10}H_{16}O_2 + H]^+$, which was detected in *S. apiana*, *S. dominica*,

S. farinacea, *S. officinalis* and *S. patens* could be attributed to campholenic acid. A peak at *m/z* 457 was only detected in *S. officinalis*, and was suspected to represent the protonated parent of ursolic or oleanolic acid. *Salvia farinacea* was one of the more unique species in that it contained a number of distinctive peaks, including those at nominal *m/z* 59, 61, 83 and 331. In addition, it displayed the greatest number of peaks, as well as the largest number of peaks below *m/z* 100.

DART-HRMS-derived chemical fingerprint profiles were minimally affected by diurnal cycles

The small-molecule profile of plants and in particular plant organs is known to be influenced by diurnal cycles (Mirjalili *et al.*, 2006) and the presence or absence of sunlight. To investigate the extent of chemical fingerprint variations as a function of diurnal cycles, a study of the mass spectral signature in the morning and at night was conducted. Fresh plant material was analysed at 9 a.m., and the same plants were also analysed again later the same day at 10 p.m. Chads representing five leaves from different plants belonging to a given species were analysed in the morning by DART-HRMS, and the results were averaged into a single spectrum. A similar analysis was conducted in the evening. The results, presented in head-to-tail plot format, are shown in Fig. 3, and the corresponding mass spectral data are presented in Supporting Information Table S2. Each species is represented by a panel, and
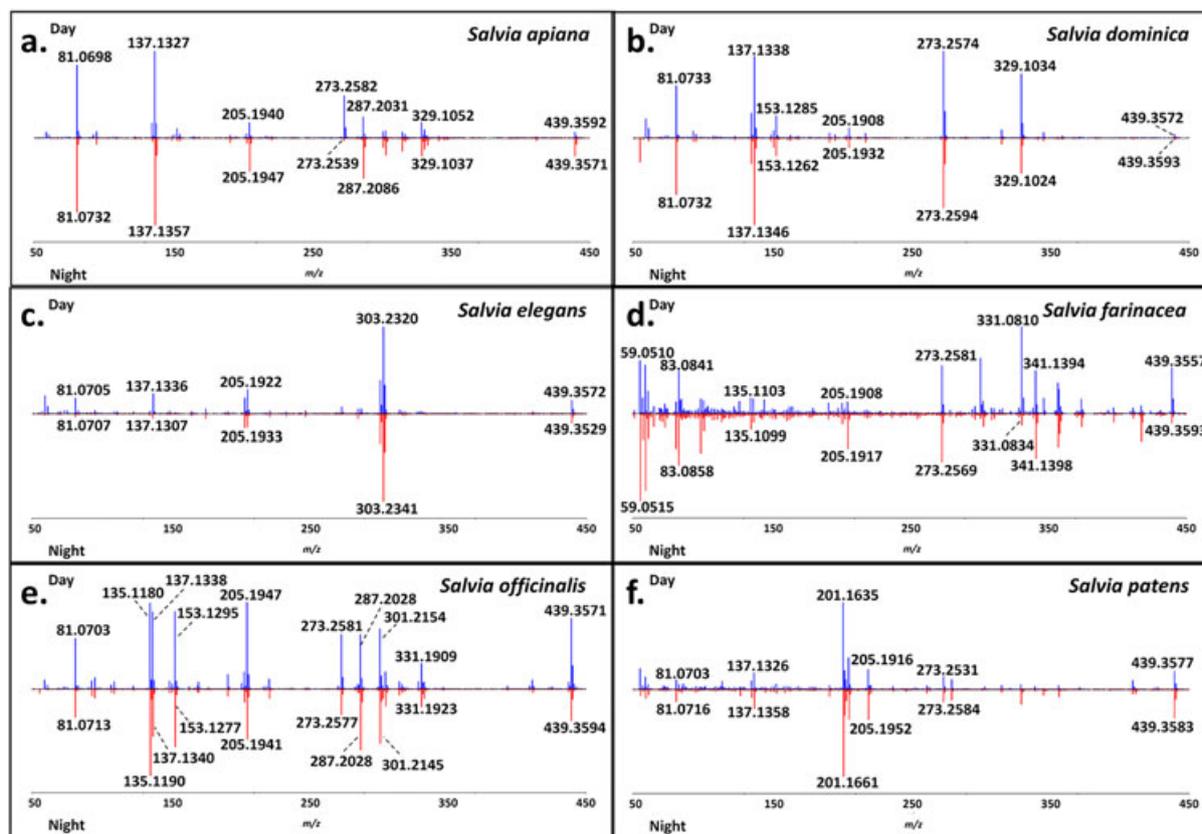

Figure 3. Head-to-tail plots depicting positive-ion mode DART-HRMS spectra of the indicated *Salvia* spp. The top spectrum in each panel was acquired during the day, while the bottom one was measured at night. Each of the spectra in panels a, b, c, d and e represents an average of five individual leaf spectra. The top spectrum of panel f represents an average of ten individual leaf spectra, and the bottom represents an average of five spectra. The corresponding mass spectral data can be found in Supporting Information Table S2.

each panel shows the morning spectrum (top) and the evening spectrum (bottom).

In terms of the *m/z* values of the observed peaks, the overall trend was that the chemical profiles were not greatly affected by day/night cycles. In general, peaks detected in the morning analyses were consistently also seen at night, albeit with occasional variations in intensity. The case of *S. dominica* [Fig. 3(b)] is representative. The major peaks at nominal *m/z* 81, 137, 153, 205, 273 and 329 were observed at similar relative abundances during the day and at night. *Salvia officinalis* [Fig. 3(e)] was similar in that it also exhibited major peaks that were present both during the day and at night (at *m/z* 81, 135, 137, 153, 169, 205, 287, 301 and 331). However, the relative abundancies of *m/z* 153, 273 and 439 were quite different from night to day, being much higher during the day. *Salvia patens* [Fig. 3(f)] behaved similarly, with peaks at *m/z* 81, 137, 201, 205, 273 and 439 appearing both in the morning and evening. Compared to the other species studied, *S. apiana* [Fig. 3(a)], *S. farinacea* [Fig. 3(d)] and *S. elegans* [Fig. 3(c)] displayed some variation in the peak abundances between night and day, although the chemical profiles were very similar. In *S. apiana*, the peak at *m/z* 273 was of 45% higher relative abundance in the morning spectrum than in the evening spectrum, while the peak at *m/z* 153 was 8% more abundant in the morning. The opposite was true for *m/z* 287 (more abundant at night by 22%), and *m/z* 439, which was also more abundant at night (by 14%). For *S. farinacea*, *m/z* 331 was the base peak in the morning spectrum, while the base peak in the evening spectrum was *m/z* 59. *Salvia elegans* showed a similar trend with peaks at *m/z* 81 and 137 being considerably more prominent in the morning (by 15% and 18%, respectively). Nevertheless, regardless of the relative abundances present, the chemical fingerprints representing each species were not affected by time of day.

Age-dependent changes in biomarker profiles vary by species

Plants are known to have life cycles over which the chemical profiles of plant parts can change (Mirjalili *et al.*, 2006). Chemical profiles can also change as a function of the season (Müller-Riebau *et al.*, 1997; Santos-Gomes and Fernandes-Ferreira, 2001). Thus, possible changes in chemical profiles with age were investigated. The spectra presented earlier were for plants that were approximately six months of age when they were analysed in July 2014. The same plants were analysed again the following May when they were about 16 months old, using the same earlier described protocol.

Figure 4 illustrates the impact of plant age on the chemical profiles at the two different time points, and the corresponding spectral data are presented in Supporting Information Table S2. Each panel represents a different species and shows a head-to-tail plot. The top spectrum in each panel shows the results of morning analysis in July 2014, while the bottom spectrum represents morning analysis in May 2015. For *S. apiana* [Fig. 4(a)], *S. dominica* [Fig. 4(b)] and *S. patens* [Fig. 4(f)], the base peaks (nominal *m/z* 137, 273 and 201, respectively) remained the same regardless of plant age. Furthermore, most of the peaks detected at six months of age were also present at 16 months. In contrast, although the *m/z* values observed at the two ages were similar, the base peaks for *S. elegans* [Fig. 4(c)], *S. farinacea* [Fig. 4(d)] and *S. officinalis* [Fig. 4(e)] were different at six months when compared to 16 months. For example, in *S. elegans*, the base peak for the older plant was *m/z* 303, while that for the younger plant was *m/z* 205. In *S. officinalis*, the peak at *m/z* 153 was 11% more abundant in the younger plant. The most

dramatic difference between the chemical profiles of the younger and older plants was observed for *S. farinacea*. There were several peaks observed in the older plants that were absent in the younger plants. These peaks were nominal *m/z* 55, 57, 59, 61, 65, 107, 135, 155, 169, 191, 201 and 331. The relative abundances of masses that were detected in both the older and younger plants were also remarkably different. The peaks at *m/z* 76, 117 (base peak), 287 and 391, which were all above a 12% relative abundance in the July 2014 spectra, were all below a 4% threshold at 16 months. The opposite was true in the case of the peak at *m/z* 273, which was present at 5% relative abundance in the younger plants, but was much more prominent in the spectra of the older plants (55% relative abundance). The peaks at *m/z* 81, 83 and 99 were also observed in much higher abundances in the older plant spectra.

The presence of several tentatively assigned *Salvia* biomarkers was confirmed by in-source collision-induced dissociation and GC–MS

Performing in-source CID allowed us to confirm the presence of several terpenes in the leaves of the selected *Salvia* plants without the need for a chromatographic interface. This approach can be compared to identification of molecules by comparison of electron ionization (EI) fragmentation patterns to those of authentic chemical standards, although with some important differences. In conventional tandem MS, a single precursor ion is sequestered and fragmented by the collision gas, resulting in little ambiguity regarding the origin of the fragment ions. However, when in-source CID is performed by DART-HRMS on a complex plant matrix, all ions become energised and fragment. This can result in the appearance of peaks representing fragments of the same mass that are derived from different precursor molecules. However, this does not prevent identification of individual compounds in the mixture. This is because the fragments of authentic standards are numerous and unique enough that all of them are unlikely to appear in an in-source CID spectrum of a mixture unless the compound is actually present. Thus, by performing in-source CID on a single compound such as an authentic chemical standard, the fragment peaks detected can be used as a diagnostic fingerprint for that specific standard. If the fingerprint is observed in the in-source CID spectrum of plant material acquired under conditions identical to those used to analyse the standard, the presence of that specific compound can be confirmed.

DART-HRMS analyses can be conducted at different orifice 1 voltages, with a low orifice 1 voltage (e.g. 20 V) resulting in the appearance of unfragmented protonated precursor molecules. However, with increasing orifice 1 voltage, varying degrees of in-source CID can occur, with the observed fragmentation pattern serving as a fingerprint for the given analyte. Thus, as previously reported (Lesiak *et al.*, 2015) the presence of specific compounds can be confirmed through comparison of the spectral fragmentation pattern of the analysed sample with that of authentic standards acquired under similar in-source CID conditions. We applied this technique to confirm the identity of tentatively assigned biomarker peaks. First, the optimal orifice 1 voltage (i.e. that which yielded fragment peaks while retaining the protonated parent) was determined. This voltage was found to be 60 V. In order to determine the unique diagnostic fragmentation pattern associated with each of the available authentic biomarker standards, each was analysed by DART-HRMS at an orifice 1 voltage of 60 V. The compounds included 1,8-cineole, 3-carene, α-pinene, β-pinene, α-pinene oxide, α-terpinene, β-caryophyllene, borneol, bornyl
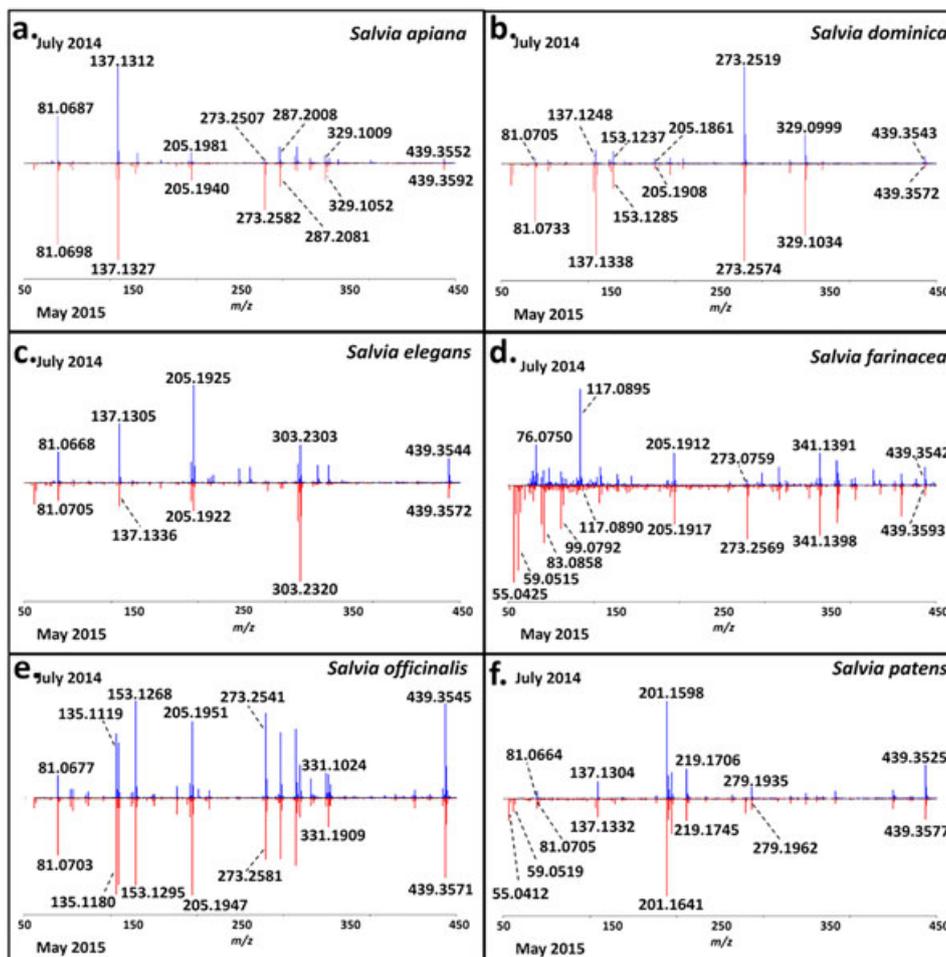
Figure 4. Head-to-tail plots of positive-ion mode DART-HRMS spectra of various *Salvia* spp. at two different growth stages. Within each panel, the spectra on top are of the younger plants analysed in July 2014, and the bottom spectra are of the older plants analysed in May 2015. Each of the spectra of panels a, b, c, d and e represents an average of five individual leaf spectra. Both the top and bottom spectra in panel f represent an average of ten individual leaf spectra. The corresponding mass spectral data can be found in Supporting Information Table S3.

acetate, camphene, camphor, carnosic acid, carvacrol, chlorogenic acid, citral, γ-terpinene, isoeugenol, limonene, myrcene, ocimene, oleanolic acid, *p*-cymen-8-ol, rosmarinic acid, sabinene hydrate, salvianolic acid A, terpinolene, thujone and ursolic acid. The spectra of each of these were then compared to those of each of the *Salvia* spp. also acquired under identical in-source CID conditions. The results, rendered as head-to-tail plots (i.e. top spectrum that of the plant sample, and bottom spectrum that of the authentic

standard), are presented in Fig. 5 and Figs S1–S15 in Supporting Information. The supporting mass spectral data are listed in Table S4.

A representative example of the kind of data obtained from these types of experiments is presented for *S. officinalis* (Fig. 5). Five of the 28 authentic standards analysed under in-source CID conditions were confirmed by this method to be present, including α-pinene oxide, α-terpinene, β-caryophyllene, β-pinene and citral. The presence of β-caryophyllene was confirmed through the
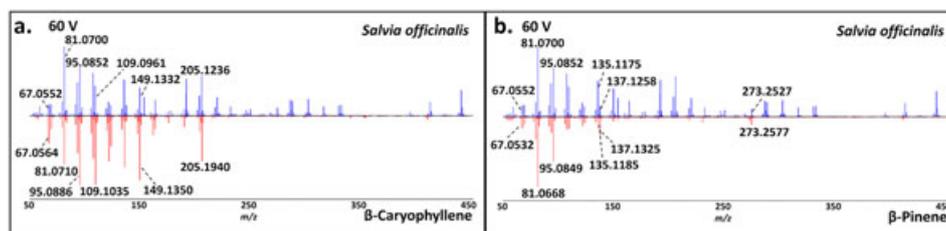

Figure 5. Positive-ion mode spectra of *Salvia officinalis* fresh leaf and various standards measured under in-source CID conditions. Within each panel, the top spectrum is that of the fresh plant material and the bottom is that of the indicated authentic standard at the specified voltage. The presence within the spectrum of the plant, of the peaks observed in the standard, indicates the presence of the specified standard in the plant. The top spectrum in each panel (*S. officinalis* plant material) represents an average of five individual leaf spectra. The bottom spectra (chemical standards) represent an average of three individual analyses. The corresponding mass spectral data can be found in Supporting Information Table S4.

observation in the *S. officinalis* in-source CID spectrum of not only the protonated precursor, but all of the fragment peaks seen in the spectrum of an authentic standard of β-caryophyllene [Fig. 5(a)]. Thus, the fragment peaks observed at *m/z* 67, 81, 93, 95, 107, 109, 121, 135, 149 and 205 for the β-caryophyllene standard were also observed in the spectrum for *S. officinalis*. The presence of β-pinene ($C_{10}H_{16}$; calculated protonated mass of 137.1330) was also confirmed, based on the presence of the diagnostic fragment peaks in the *S. officinalis* spectrum acquired under similar in-source CID conditions [Fig. 5(b)]. By performing similar experiments the results of which are presented in Figs S1–S14, the presence of various biomarkers in the analysed samples was determined to be as follows: α-pinene, β-pinene and β-caryophyllene were detected in *S. apiana*; β-pinene, β-caryophyllene and β-thujone were detected in *S. dominica*; 3-carene and β-caryophyllene were detected in *S. elegans*; β-caryophyllene was detected in *S. farinacea*; β-caryophyllene and β-pinene were detected in *S. officinalis*; and β-caryophyllene was detected in *S. patens*.

In-source CID was also used to rule out some tentative assignments. For example, oleanolic acid and ursolic acid, a pair of constitutional isomers of formula $C_{30}H_{48}O_3$ (calculated mass of 456.3603) have both been reported to be present in *Salvia* spp. (Stesevic *et al.*, 2014). For this reason, it was surmised that the peak at nominal *m/z* 457 (detected only in the spectrum of *S. officinalis*) might have represented the protonated form of either of these compounds. The in-source CID mass spectra of ursolic and oleanolic acids, rendered as a head-to-tail plot in which the spectrum of oleanolic acid appears on top and that of ursolic acid appears on the bottom, is shown in Fig. 6. These experiments were conducted using an orifice 1 voltage of 30 V because at this voltage, fragment ions as well as the protonated parent ion were present. The corresponding mass spectral data are listed in Supporting Information Table S5. As expected, the spectra were similar. Both were characterised by the appearance of the protonated parent (*m/z* 457) and a base peak at *m/z* 439, a result of loss of a hydroxyl from the acid. Other peaks that the compounds had in common included peaks at nominal *m/z* 122, 191, 248 and 411. However, a notable difference between them was the presence of peaks that appeared only in the oleanolic acid spectrum, at *m/z* 58, 372, 454 and 473. Comparison of the in-source CID spectra of the acids to that of *S. officinalis* (Supporting Information Figs S23 and S24) showed that the fragments of neither oleanolic nor ursolic acid appeared in its spectrum. This indicated that the peak at *m/z* 457

in the *S. officinalis* spectrum did not represent either of these acids. Similar comparisons of the in-source CID spectra of the other *Salvia* spp. to those of the acids showed that these acids were also absent in the other *Salvias* analysed in this study (see Figs S15–S26).

The presence of biomarkers was further confirmed by GC–MS analysis of an ethyl acetate extract of *S. officinalis*, which was chosen as a representative species because it exhibited the broadest range of diagnostic terpenes. Confirmations were made through comparisons of GC–MS results (including NIST EI-MS database matching) for the extract to those of authentic standards. The results of these experiments are shown in Supporting Information Figs S27–S31. Observed compounds included α-pinene, β-pinene, β-caryophyllene, thujone and 3-carene, with retention times of 5.59 min, 7.40 min, 13.66, 19.93 min and 65.95 min respectively. We also observed exact masses in the DART-HRMS 20 V spectrum of an ethyl acetate extract of *S. officinalis* that were consistent with the compounds camphor, borneol, and campholenic acid (Fig. 32). The presence of camphor and borneol were also confirmed by matching of their EI mass spectra to those of the corresponding compounds in the NIST library (Figs S34–S35).

Multivariate statistical analysis processing of DART-HRMS-derived mass spectral fingerprints enabled species-level differentiation and species identification

The consistent similarities between spectra representing the same plant species on the one hand, and the contrasts between the spectra of different species on the other hand, implied that the DART-HRMS-derived chemical profiles could be used to identify each species. This hypothesis was explored by subjecting the DART-HRMS data to pattern recognition processing. For this purpose, KDA utilising 14 feature masses representing both compounds common to several *Salvia* spp. and others that were unique to individual species, was performed. These masses are listed in Table 1. The training set was comprised of 380 total spectra representing all six species. The results, which are presented in Fig. 7, showed that five principal components covered 98.37% of the variance, and the leave-one-out cross validation (LOOCV) was 93.59%. As illustrated, the data clustered according to species and all of the clusters were well resolved. Interestingly, in the cases where the mass spectral data from two or more varieties of a particular species were analysed, (i.e. honey melon and pineapple varieties of the *S. elegans* species, and garden, purple, tricolour and berggarten varieties of the *S. officinalis* species), all the varieties representing the particular species clustered tightly together. This finding illustrates the power of this approach in identifying genetic relatedness, particularly in cases where the plants appear very different visually. For example, as illustrated in Fig. 1, the *S. officinalis* garden, purple and tricolour varieties are very different in
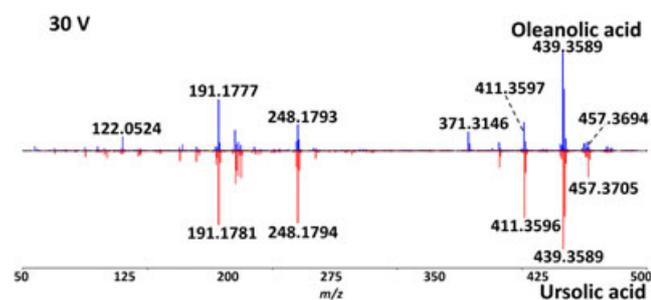

Figure 6. Head-to-tail plot showing a comparison of the positive-ion mode in-source CID DART-HRMS spectra of oleanolic (top spectrum) and ursolic (bottom spectrum) acids measured at 30 V. Although the isomers exhibit similar spectra, there are diagnostic differences in their fragmentation patterns. Both the top and bottom spectra represent an average of three individual analyses of the chemical standard. The corresponding mass spectral data can be found in Supporting Information Table S5.

Table 1. Feature masses used for the kernel discriminant analysis (KDA) plot presented in Figure 7

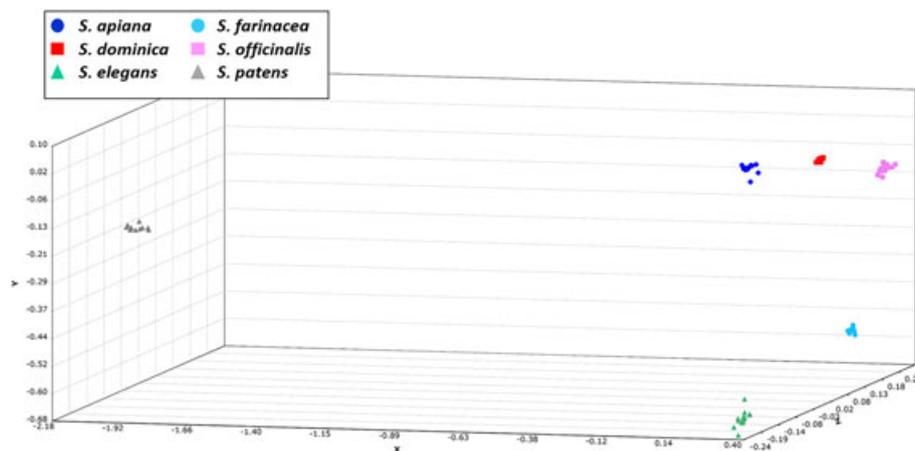|	
81.0698	205.1940
117.0904	273.2582
135.1156	274.2617
137.1327	303.2323
138.1377	329.1052
153.1264	357.1314
201.1620	439.3532

Figure 7. KDA plot created using 14 feature masses from positive-ion mode DART-HRMS analysis of the six indicated *Salvia* spp. The masses used are listed in Table 1. Each of the clusters represents the indicated species and the clusters are well-resolved. Five principal components accounted for 99% of the variance, and the leave-one-out cross validation (LOOCV) was 94%.

appearance, yet their membership within a single species is well-illustrated by the results of the KDA.

The dried leaves of several *Salvia* species are used as spices. These include *S. officinalis* and *S. elegans*. It would therefore be useful to be able to identify dried *Salvia* species products, particularly since diagnostic plant morphological species identifiers are often lost upon drying. Thus, using soft independent modelling of class analogies (SIMCA), we investigated whether the DART-HRMS of dried plant material would be correctly classified by species using the data derived from analysis of the fresh plant material. Dried leaves representing *S. apiana*, *S. dominica*, *S. elegans*, *S. officinalis* and *S. patens* were analysed by DART-HRMS using the parameters described earlier. These were used as "unknowns" in the SIMCA analysis. Since the aforementioned KDA was performed using the data acquired from plant samples collected in the morning, and since we observed that for some species, there were slight fluctuations between detected bio-markers in the morning compared to the evening, we also tested the system using DART-HRMS data from leaf samples collected at night. This group (*S. apiana*, *S. dominica*, *S. elegans*, *S. officinalis* and *S. patens*) comprised the second set of unknowns. Figure 8 shows the results of SIMCA analysis. Each of the colours represents a class, with class membership defined by species (i.e. yellow for *S. patens* based on 50 recorded spectra; pink for *S. officinalis* based on 95 recorded spectra; turquoise for *S. farinacea* based on 50 recorded spectra; blue for *S. elegans* based on 75 recorded spectra, green for *S. dominica* based on 55 recorded spectra; and red for *S. apiana* based on 65 recorded spectra). The results, which showed that 100% of the spectra representing both types of unknowns were correctly classified, suggest that an algorithm can be designed to enable species-level differentiation of sages from DART-HRMS analysis of either fresh or dried leaves.

The importance of real-time determination of the metabolite profiles of agriculturally important plant species in general, and sage species in particular, is exemplified by the sustained appearance of literature reports of analytical methods designed to accomplish this task. The ability to discriminate between *Salvia* spp. is also of forensic relevance because of the increasing use of *S. divinorum*. This plant, which can be difficult to distinguish from other sage species by visualisation alone, contains salvinorin A, a hallucinogenic diagnostic biomarker that is scheduled in several
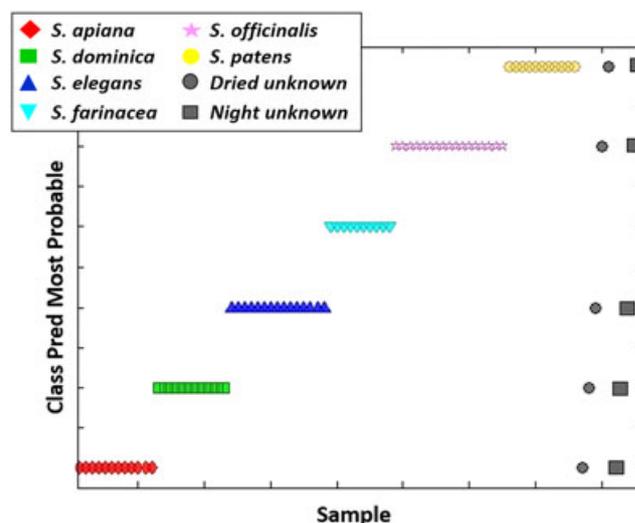

Figure 8. Soft independent modelling of class analogies (SIMCA) plot created using positive-ion mode mass spectra, collected at 20 V. Each of the species is represented by the indicated colours and symbols, and each falls within a distinct class. Unknowns representing the positive-ion mode DART-HRMS 20 V spectra of: (1) dried samples (grey circles); and (2) fresh leaves collected at night (grey squares) were all correctly classified.

countries. Small-molecule fingerprinting protocols extend from the more conventional methods (e.g. GC–MS, HPLC and NMR) (Mirjalili *et al.*, 2006; Oelschlägel *et al.*, 2012) to those that utilise more advanced instrumentation (Gudi *et al.*, 2015). The GC- and HPLC-based methods can be time consuming to perform and require significant methods development. Furthermore, the requisite sample solubilisation step selects the subset of compounds most soluble in the solvent of choice. Indeed, depending upon which subset of secondary metabolites are of interest, different methods may need to be developed for analysis of the same plant-derived substance. Here, we show that despite the small-molecule profile variations that occurred as a function of growth stage and diurnal cycles, and the fact that many compounds were common to all the species analysed, the *Salvias* exhibited consistent interspecies differences and intraspecies similarities. This

finding is consistent with that reported by Sajewicz *et al.* (2011) who observed that the fingerprints acquired by binary HPLC-diode array and HPLC-evaporative light scattering analysis of methanol extracts of six *Salvia* spp. were visually distinguishable. Willard *et al.* (2012) determined that *S. divinorum* could be distinguished from a combination of culinary and ornamental sages by visual inspection of the gas chromatograms of dichloromethane extracts, a result confirmed by various objective statistical analysis treatments. It is because of the intraspecies consistencies and interspecies differences observed among the sages that chemometrics (in the form of KDA and SIMCA in this study) could be applied to DART-HRMS data to rapidly differentiate between several species of *Salvia*. This demonstration of proof of principle implies that a database that can be used to rapidly identify *Salvia* plant material (from DART-HRMS spectra) can be created. Data acquisition is rapid (~3 s) and as such, the large spectral datasets that would be required to increase the robustness and comprehensiveness of such a database can be rapidly acquired. Our approach is further distinguished from others in that: (1) the material can be analysed in its native form, and thus no solvent extraction methods development is required; and (2) since there is no chromatographic interface, several of the pretreatment steps that would normally need to be applied prior to statistical analysis processing of GC or HPLC chromatograms can be circumvented. These include background correction and retention time alignment, among other steps. We observed that the method could be applied to analysis of both fresh and dried plant products, with plant materials representative of all of the analysed plant species being correctly classified. Expansion of the number of plants in the study to include a broader range of *Salvia* spp. representing culinary, ornamental, medicinal and hallucinogenic plants, is the subject of continuing investigations in our laboratory.

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Supporting information

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