

The Dynamic Nature of a Static Headspace Above Frozen Leaf Biomass From a Toxic Brazilian Plant

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Abstract

A static headspace method of sampling coupled to a conventional instrumental analytical probe, gas chromatography mass spectrometry (GCMS), has been applied to examine the volatile fraction above leaf tissue from *Palicourea marcgravii*, a toxic Brazilian plant that causes sudden death in livestock, and two other surrogate species (*Lippia alba* and *Plectranthus* spp.).

The unusual total ion chromatogram obtained for the plant of interest is discussed and explained by considering the “dynamic” nature of the static headspace when intact cellular biomass is present and frozen leaves are analyzed concerning the presence and production of methyl salicylate (MeSA), which is this plants main toxin.

Keywords: analytical chemistry, gas chromatography - mass spectrometry (GC-MS), natural product, toxic Brazilian plant, MeSA toxin

1. Introduction

Several analytical preparatory platforms, such as static headspace (S-HS), simultaneous purge and trap (P&T), and solid-phase micro extraction in the vapor phase (HS-SPME) have been used to isolate and analyze the volatile fraction from different plants species (1, 2, 3, 4, 5). Sampling the headspace is a simple, non-invasive and rapid method (6) that needs minimal sample handling (7) with less degradation of the analytical column and the GC instrument (9). It enables the volatiles to be mainly qualitatively assessed, although the quantification by internal standard technique is still used in many liquid and gas analyses (10). A primary benefit of using headspace sampling is that small amounts of analytes sequestered in a large amount of biomass can be detected without having to homogenize the biomass and extract the volatiles. It can be used on living plant materials, either in the laboratory or in the field and it gives a profile of the compounds in the plant material that are volatile under the conditions of a predefined experiment (8). This qualitative study tests a methodology for the isolation of volatile organic compounds (VOCs) from three different plant species with a notorious odor, including the toxic Brazilian plant *Palicourea marcgravii*. *P. marcgravii* is a member of a group of plants that causes sudden death in livestock. It belongs to the Rubiaceae family which comprises three other species known to be toxic for cattle, i.e., *P. corymbifera*, *P. grandiflora* and *P. juruana* (11, 12). In Brazil, the main cause of death in cattle is by ingestion of poisonous plants. *P. marcgravii* is known as one of the most toxic plants in the Brazilian biomes due to its levels of toxins, their cumulative effect and its palatability (13). Its main toxin, monofluoroacetic acid (MFAA), interferes with cardiac function leading to death through heart failure. The unconventional headspace profile from isolated leaf tissue of *P. marcgravii* is considered in this work in terms of methyl salicylate (MeSA) production in response to the abiotic stress of the excised tissue and a dynamic headspace.

2. Method

2.1 Plant Material

P. marcgravii was harvested from a selected urban region of Brasília (Brasília Botanical Garden), initially on two different occasions. First the plant was cut at ground level and placed in a Mylar bag before being put into a glass container. The Mylar bag provided a relatively contaminant free environment and prevented the excessive loss of moisture. It also minimized the existence of a constant concentration gradient through which endogenous volatiles could be lost. The samples were put in the freezer overnight after which foliage was separated from roots and stems. On the second occasion, approximately 4 leaves of *P. marcgravii* were cut with sterilized scissors, put straight into a 4ml clear glass vial and sealed with a plastic screw cap fitted with Teflon lined, silicone septa.

The field sampling methodology used in this part of the study was tested for reliability by adopting the same regimen for sampling two other plants containing volatiles, as indicated by the localized presence of odors detected by the human olfactory sense. *L. alba* and *Plectranthus* spp. were both harvested around the Embrapa Genetic Resources and Biotechnology property in Brasilia on various occasions. At no point were the surrogate leaf material placed in the freezer. The surrogates were vialled up and the headspace sampled as soon as the prescribed equilibration regimens were completed.

Field samples were collected with attention to the way in which livestock species might forage and remove foliage. Livestock are likely to feed by tearing foliage and stems rather than uprooting plants. Hence, the excising of leaves was preferred to taking whole plants. The samples from *P. marcgravii* were stored frozen to protect the integrity of the biomass and minimize loss of analytes. Lastly, the field samples were sub-sampled for instrumental analysis.

A second round of sampling was conducted approximately fourteen months later to attempt to minimize the impact of seasonal variation. The biomass from this sampling was used to validate the outcomes originally obtained.

2.2 The Static Headspace

The isolation of the volatile compounds from excised tissue was performed using a static headspace procedure. The biomass was not weighed but the number of leaves of similar size was counted. This was a qualitative study and the generation and reproducibility of the headspace above the biomass is dependent on a number of variables, not the least of which is the integrity of the “headspace vial” to hold the headspace statically.

Whole leaves were placed in 4 ml clear glass vials. The vials were capped and allowed to come to ambient temperature on the bench. Prior to injection on to the analytical column of the GCMS, a vial was placed into an elevated isothermal state for a predetermined period.

Integrity of the headspace vial to isolate the headspace from the external environment was evaluated by trapping a headspace and producing a GCMS profile of the two plant surrogate species, *L. alba* and *Plectranthus* spp. Different profiles for each species will indicate that the developed methodology will provide suitable containment for a static headspace.

After suitable equilibration in an elevated isothermal environment, the trapped headspace was sampled using a 10 μ L, glass, Hamilton gas tight syringe fitted with a removable 30mm septum piercing needle. An empty syringe was pushed through the self sealing septum and the plunger extended to full scale draw to sample 10 μ L. The syringe was held *in situ* for 20 seconds to allow equalization of partial pressures and fully fill the syringe. This approach was adopted during the last round of testing with 20 ml glass, Agilent, headspace vials capped with aluminum crimp tops and fitted with Teflon lined septa.

The equilibration temperature of 45 °C was chosen as this intuitively seemed to provide a stable elevated temperature, above ambient, that would contribute to the liberation of low boiling molecules into the headspace such as MeSA, without over heating to the point of thermal degradation of sample components prior to injection.

2.3 Conditions for Sample Component Separation by GCMS

An Agilent 7890A GC was used to separate volatile sample components. A helium carrier gas was delivered at a constant flow rate of 1.0 mL min⁻¹ and a splitless septum purge flow of 3 mL min⁻¹. Chromatographic separations were conducted on a HP-5MS (30 M $\times$ 250 μ m $\times$ 0.25 μ m film thickness) capillary column.

A long, multi-gradient heating schedule was used to separate volatile species and thereby, generate a total ion chromatogram (TIC) profile of the three plants of interest. The heating regimen was 50 °C for 1 minute, then

5 °C/min to 180 °C for 0.1 minutes, then 10 °C/min to 280 °C for 30 minutes. This temperature program had a total run time of 67.1 minutes with an initial detector dead time of 6 minutes.

A second GC was used to validate the separation achieved using the Agilent 7890A. A Shimadzu GC-2010 was equilibrated with a helium carrier stream delivered at a constant flow rate of 1.05 mL min⁻¹ and splitless septum purge of 3 mL min⁻¹. The analytical column was a Phenomenex Zebron, ZB-WAX Plus (30 M × 250 µm × 0.50 µm film thickness) capillary.

A long, single temperature gradient was employed to yield the required resolution of sample components in the headspace above biomass harvested from *P. marcgravii* only. An initial oven temperature of 50 °C was raised to 245 °C at a constant rate of 3 °C/min. The final temperature was held constant for 12 minutes. This regimen required a total runtime of 77 minutes with an initial dead time of 5 minutes.

Plots of both temperature ramps are displayed on Figure 1, together with a plot of the total dead times for each chromatograph.

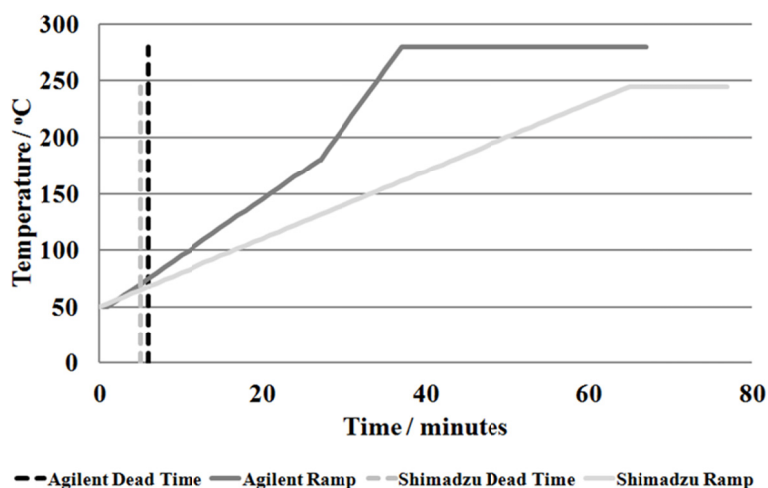


Figure 1. A plot of the temperature ramps and detector dead time used in this study

2.4 Conditions of Ionization and Mass Discrimination

An Agilent 5975C quadrupole mass spectrometer was used for mass discrimination post component separation with the Agilent 7890A. The ionization energy of electrons in the source was 70 eV. And the source and quadrupole temperatures were 230 °C and 150 °C, respectively.

The Shimadzu GC-2010 was coupled to a Shimadzu GCMS-QP2010 quadrupole mass spectrometer. The ionization energy of electrons in the source was 70 eV. The source and quadrupole temperatures were 250 °C and 200 °C, respectively.

2.5 Software Identity and Functionality

Data acquisition, TIC peak picking and integration were accomplished using Agilent ChemStationEnhanced software v.E.02.00.493 to acquire and control output from the Agilent instrumentation. Acquisition and control of the Shimadzu instrumentation was achieved with Shimadzu, GCMS Analysis GCMSsolution V.2.50SU1. OpenChrom Community Edition (Aston) was used to convert native Agilent *.D files and Shimadzu *.qgd files to *.CDF file format. ChemStation and OpenChrom were used to generate flat *.csv files to process and plot TIC data from the Agilent and Shimadzu instruments respectively. Excel in MS Office 2007 was used to plot all TICs.

An arbitrary limit of detection (LOD) of twice the baseline noise for 2 minutes about the retention time of the MeSA peak was applied. A sample of this baseline is shown in black as a trace in Figure 5.

NIST MSSEARCH was used to match spectra against the complete NIST/EPA/NIH Mass Spectral Library (v.NIST08). The search algorithm embedded in MSSEARCH was NIST MSsearch (v.2.0f). Some post processing of data files was also completed using AMDIS v.2.71 to verify retention times.

2.6 Liquid Extraction of *P. marcgravii*

Supplemental to the principal gas phase experiments that are the subject of the work presented, a test for the presence of endogenous MeSA in the leaf tissue post field sampling, was designed. Ten moderately sized leaves from a single *P. marcgravii* were placed into a dry 200 ml Schott bottle. 150 mL of HPLC grade ethyl acetate (Merck) was added and the solvent allowed to leach into the biomass by contact over 48 hours.

After leaching for 48 hours, the ethyl acetate was poured into a 500 ml separating funnel and washed with 80 mL of 35% v/v, 99.9% propan-2-ol (J. T. Baker) in Milli-Q water. This step separated substituted benzenes and organic acids from the chlorophyll bearing ethyl acetate. The ethyl acetate phase was discarded and 35% propan-2-ol phase shaken with an equivalent volume of Supra Solvent n-hexane (Merck).

The non-polar hexane phase was dried over anhydrous $Mg(SO_4)$ as required, and then filtered. The dried hexane filtrate was then added to a 250 mL round bottom flask (RBF) and the flask fitted to the condenser of a Buchi 210 Rotovapour to evaporate the hexane to 1 mL. Chilled water was recirculated through the condenser with an Etica recirculation water bath. Evaporation was completed at 32 °C with the RBF rotating in a Buchi B-149 heating water bath and under reduced pressure generated by a Buchi V-700 vacuum pump.

A 0.5 μ L injection of this concentrate was injected on the analytical column of the Shimadzu GCMS instrument described above.

3. Results

The principal results presented are the TICs from the Shimadzu and Agilent instruments respectively. Figure 2 is a composite plot of the TICs of the three plants under consideration obtained using the 67.1 minutes temperature ramp. MeSA elutes at 14.72 minutes. The plot is detector response (abundance) as a function of time. The TIC for *P. marcgravii* is plotted as empirically obtained.

In Figure 2 it is possible to compare the variation observed in the volatile profiles of the three plant species. The offset observed on the domain of the three chromatograms at 6 minutes is the result of the application of a detector dead time to protect the instrumentation from signal saturation by any early eluting species in the solvent front. The peak exhibiting full scale deflection in the TIC of *P. marcgravii* is MeSA. Although this study is qualitative, the raw signal is disproportionate in comparison to other components in the TIC of *P. marcgravii*, and is unmatched for raw signal by any component in any of the three TICs obtained. It dominates and may even suppress the volatilization of other headspace components by saturating the vapor phase.

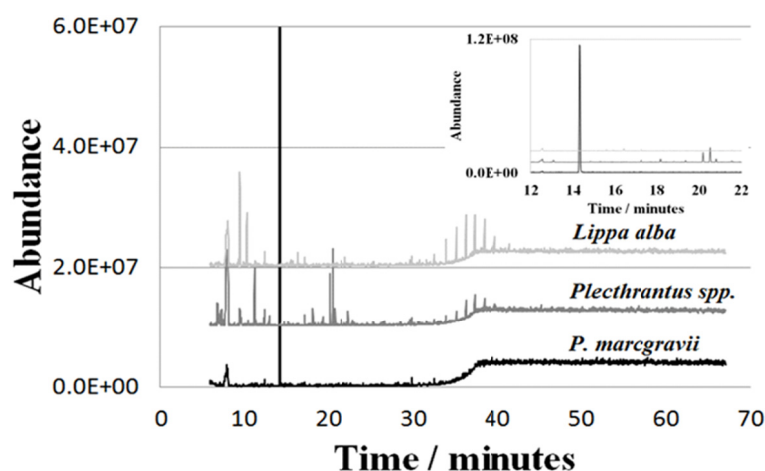


Figure 2. The TIC's of the plant of interest below the two chose surrogate plant species. Inset is the size of MeSA peak relative to other peaks in the headspace of *P. marcgravii* over a time domain of between 12 minutes and 22 minutes. The headspace was sampled from 4 ml HPLC vials. A 67.1 minutes temperature ramp was used to elute all sample components from an Agilent GC then ionized and ions detected with an Agilent quadrupole mass spectrometer

The representative headspace profile of *L. alba* showed the presence of many compounds with an aromatic ring, such as D-Limonene. This compound is known to be in the essential oil of *L. alba* (15). The sample of fresh

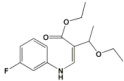
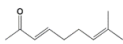
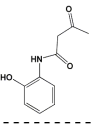
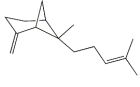
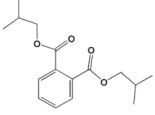
leaves of *Plectranthus* spp. showed the richest volatile profile of the three. The retention times and names of identified components in the three different plant genera are listed in Table 1. Table 2 is a correlation of the retention time with the structure of the molecules identified.

Table 1 lists 17 peaks that were separated with sufficient resolution to provide good spectral purity for library matching against the NIST08 main library. Peaks that were well above the chosen LOD provided good spectral peak abundances. Peaks not fitting the criteria of good resolution and high abundance were considered poor candidates, yielding inaccurate matches and so omitted. Table 2 is dominated by aromatic and heterocyclic structures.

Table 1. Correlation of the retention time with peak identity for the plant of interest and the two pseudo surrogates

I.D.	RT	Compound Name
1	7.02	2-methyl-5-(1-methylethyl)-Bicyclo[3.1.0]hex-2-ene
2	7.46	Camphene
3	9.62	D-Limonene
4	9.63	(S)-1-methyl-4-(1-methylethenyl)-Cyclohexene
5	10.45	1-methyl-4-(1-methylethyl)-1,4-Cyclohexadiene
6	10.93	N-(2-methoxyphenyl)-3-oxo-Butanamide
7	11.37	1,3,3-trimethyl-Bicyclo[2.2.1]heptan-2-one
8	13.09	(1R)-heptan-2-one, 1,7,7-trimethyl-Bicyclo[2.2.1]
9	14.72	MeSA
10	15.20	N-[3-fluorophenyl]-2-Amino-1,1-dicarboxylic acid
11	16.41	(E)-8-methyl-3,7-Nonadien-2-one
12	18.20	3-methyl-6-(1-methylethylidene)-Cyclohexene
12	20.20	[1aR-(1a α ,4a,4a β ,7b α)]-1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl-1H-Cycloprop[e]azulene
14	20.54	Caryophyllene
15	20.82	[1R-(1 α ,5 α ,6 β)]-6-methyl-2-methylene-6-(4-methyl-3-pentenyl)-Bicyclo[3.1.1]heptane
16	22.43	1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-Cyclohexane
17	29.97	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester

Table 2. Relates retention time to structure over the same 17 peaks. The surrogates display a typical multi-component profile

I.D.	RT	<i>P. marcgravii</i>	<i>L. alba</i>	<i>Plecthrantus</i> spp.	I.D.	RT	<i>P. marcgravii</i>	<i>L. alba</i>	<i>Plecthrantus</i> spp.
1	7.02				10	15.20			
2	7.46				11	16.41			
3	9.62				12	18.20			
4	9.63				13	20.20			
5	10.45				14	20.54			
6	10.93				15	20.82			
7	11.37				16	22.43			
8	13.09				17	29.97			
9	14.72								

The representative mass spectrum of MeSA is showed in Figure 3. There, abundance is a function of mass-charge ratio. The spectral data from the peak with a retention time of 14.72 minutes using the Agilent GCMS and 67.1 minutes temperature ramp from the *P. marcgravii* sample, is shown as a positive deflection with the spectral data from the NIST08 Main Library aligned head to tail and oriented in the negative direction. This typical spectrum shows the quality of the match between the sample unknown and the principal library candidate. Good spectral data is acquired both with respect to the ions present and their intensities to yield reliable alignment with the NIST08 libraries.

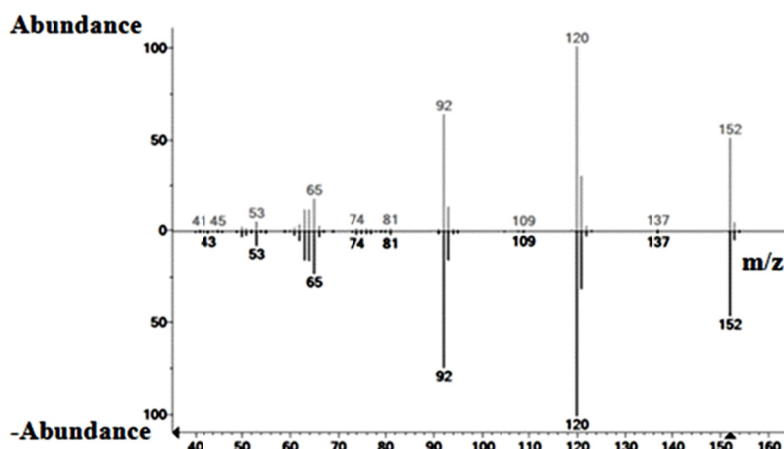


Figure 3. The head-to-tail comparison the unknown (in gray above) and the library (inverted in black below), as modified from the MSsearch program

The difference between the TICs from the Agilent system and that of the Shimadzu system (Figure 4), where MeSA elutes at 34.22 minutes, is the presence of small peaks almost equidistant (homologous peaks) apart. These were aligned with the NIST08 libraries and found to match the spectra of silicone contacting molecules and can be attributed to the conditioning of the new Zebron, ZB-WAX Plus column, especially in the light of the 0.50 μm film thickness.

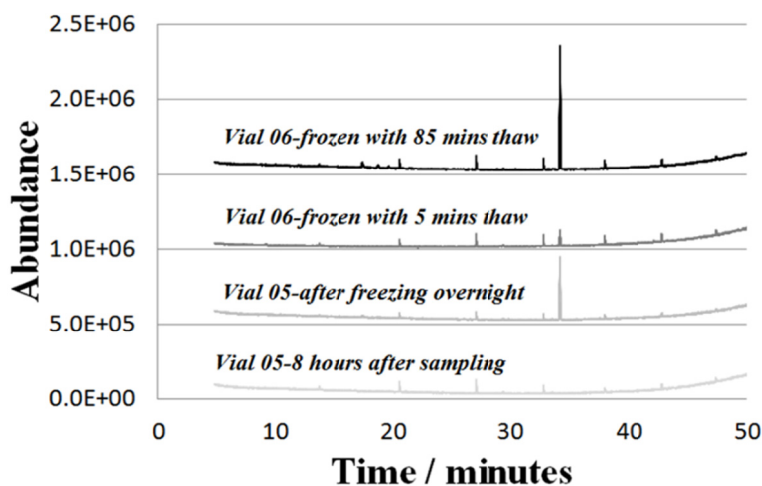


Figure 4. Are typical total ion chromatograms for *P. marcgravii* over various storage scenarios. The predominant feature is the presence of MeSA in all but the “fresh” sample stored at room temperature until injection. All biomass came from of *P. marcgravii* was placed in glass, 20 ml, Agilent headspace vials with self-sealing, septa and aluminum, crimped top caps. All sample components were eluted with a 77 minute temperature ramp from a Shimadzu GC then ionized and ions detected with a Shimadzu quadrupole mass spectrometer

Figure 5 is an overlay of 2 minutes of baseline obtained from the first injection of 10 μl of headspace from Vial 05 (*P. marcgravii*). Upon first injection of the headspace from Vial 05, labeled “Vial05 ambient”, left at room temperature for 8 hours, no discernible MeSA peak is observed (that is < LOD). Freezing Vial 05 overnight at -20°C then thawing and injecting a headspace sample generates a MeSA peak as shown labeled as “Vial 05 after freezing”. In the instance of excising and freezing intact leaf biomass from the plant *P. marcgravii* then thawing directly, yields elevated levels of MeSA in the headspace above the biomass.

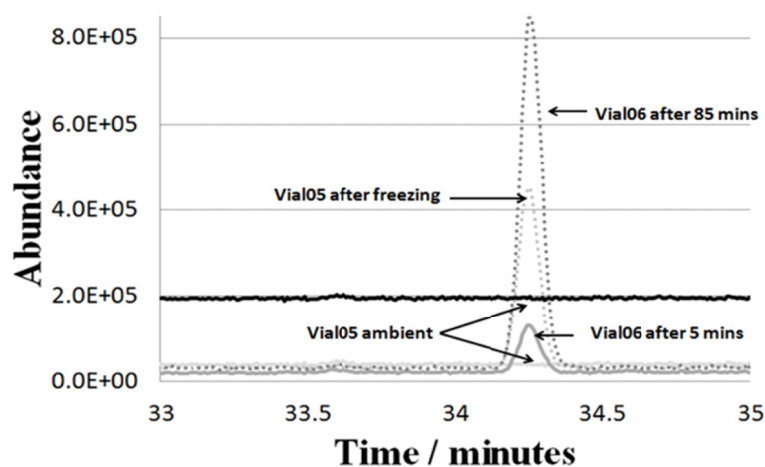


Figure 5. Provides 2 minutes of baseline in the region of the TIC where MeSA elutes from the Shimadzu GCMS instrument. Freezing appears to exacerbate the presence of MeSA in the entrapped static headspace

4. Discussion

It is known that its sodium salt, sodium monofluoroacetate, found in high amounts in *P. marcgravii* leaves is responsible for reducing the production of ATP leading to death (13). MeSA is an indicator of plant stress, not only in the species under consideration but also in many other plants species (14). MeSA is a methyl ester of the plant hormone salicylic acid and it is formed via the shikimic acid pathway (15). The role of plant hormones like salicylic acid (SA), jasmonic acid and ethylene in response to biotic stresses is well documented in the literature (16). Plant response to wounding is reported as an abiotic stress that these phytohormones signal (16) the occurrence of SA and its metabolism to MeSA and, or a glycosylated derivative, methyl salicylate glycosylate (MeSAG) (17).

Figure 1 compares and contrasts the static headspace TICs of leaf biomass from three different plant species. *L. alba* and *Plectranthus* spp., which were chosen to act as sample surrogates for the species of interest. Because sample surrogacy was methodology selective in that both surrogate biomasses were not frozen but used fresh post field sampling, the difference between the TICs is explained. The pseudo-surrogates show typical multi-component headspace profiles. The same cannot be said for the TIC obtained for the plant species of interest. The hypothesis from this observation of the empirical data is simply that the common storage practice of freezing field harvested biomass from some plants has a major impact on the composition of the static headspace in the instance of the species of interest.

To confirm the endogenous presence of MeSA in the field samples a liquid extraction was performed on two biomass samples. One was stored overnight in a glass, screw cap, Schott bottle under refrigeration at 4 °C and a similar amount was stored overnight in a separate, screw cap, Schott bottle in a freezer at -20 °C. 0.5 µl of neat, chlorophyll containing ethyl acetate post leaching and 0.5 µl of analyte bearing hexane were each separately injected on to the Shimadzu GCMS. The TICs and aligned head to tail is showed in the Figure 6. Clearly, the hexane extract contains a peak that elutes at 34.22 minutes, the same time as MeSA with a mass spectrum that aligns well with the library spectrum of MeSA. This implies that MeSA is present in the ethyl acetate in extractable amounts and that the subsequent extractions are effective in separating the MeSA from the chlorophyll and partitioning an amount in the hexane.

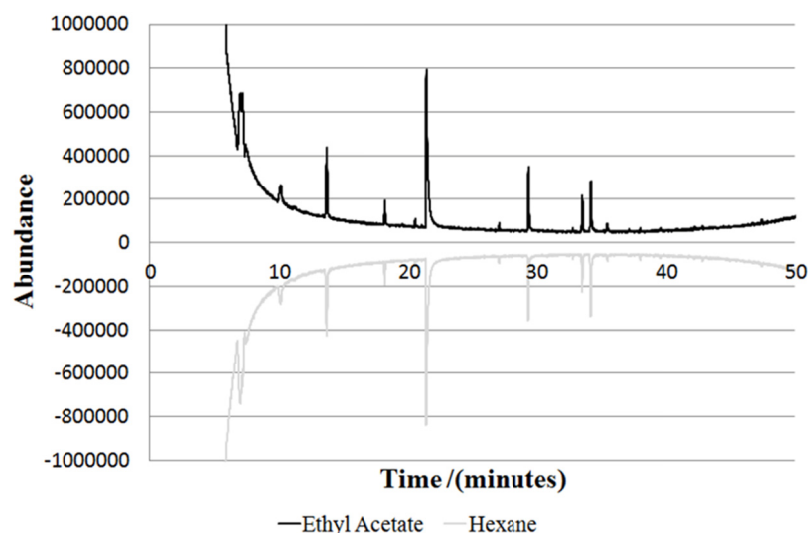


Figure 6. TICs of 0.5 μ l of neat, chlorophyll containing ethyl acetate post leaching and 0.5 μ l of analyte bearing hexane were each separately injected on to the Shimadzu GCMS. The ethyl acetate fraction is the first step of the solvent extraction and the hexane is the last step of the process. The presence of MeSA in the hexane suggests that some fraction of the total amount is extracted

After the hexane extracts of the refrigerated and frozen material are concentrated and injected onto the Shimadzu GCMS column, the frozen extract shows a slightly larger MeSA peak. Although this may be solely the result of the freezing regimen it may also be attributed to inconsistencies in the total volume of solvent removed upon concentration by evaporation under reduced pressure.

Having established that MeSA is present endogenously in the leaf biomass, Figure 2 establishes that the TIC for the plant of interest is real and reproducible and that the TIC can be reproduced on a second analytical instrument with a different column and heating regimen.

Freezing *P. marcgravii* leaf biomass provides a stimulus for the release of MeSA. This may not be the only stimulus but is certainly involved in triggering the release of MeSA into the headspace. The second implication is that the septa are self-sealing and able to hold a static headspace after being pierced by the sampling needle.

If the leaf tissue is allowed to come to equilibrium and left to rest at ambient temperature, after freezing, for a longer period of time then the peak corresponding to MeSA will increase in size (as illustrated by the peak labeled “Vial 06 after 85 mins”). The significance of these results is that freezing may provide the initial stimulus but equilibration time upon thawing may also increase the amount of material detected in the headspace.

Although Miura and Tada (19) suggest the ice forms in “cellular fluids”, it could be argued that this is true of both the intracellular and extracellular fluid and that the rates of this formation would be dependent on the nature and composition of solutes in each. The outcome, as suggested by Miura and Tada (19), is cell death through dehydration. In the case of some cells, cellular disintegration is possible since as the entrained water freezes the growth and expansion of ice crystals could rupture cell membranes and perhaps damage cell walls. This would represent a more catastrophic cell death than desiccation.

Ambient temperature depression increases the endogenous levels of free SA and free glycosyl SA in a range of plant tissues documented by Miura and Tada (19), suggesting the conclusion they draw, “that SA is involved in the regulation of cold responses”.

Although there are numerous opinions and suppositions that could be suggested as extrapolations *in-vivo*, the fundamental aim of this work is to try and adequately explain the TIC provided in Figure 1 for the plant of interest in contrast to the two pseudo-surrogates.

As previously stated, leaf biomass from *L. alba* and *Plectranthus* spp. were used fresh and not frozen. This also explains their typical, multi-component static headspace profiles. The TIC profile from the static headspace of the plant of interest can be explained in terms of the empirical data and literature surveyed.

The dynamic nature of an assumed static headspace-Static headspace samples are often generated with a relatively simple sample matrix. Often these matrices are environmental in origin and typically they are

contaminated soils or liquids. As such, they sit at the bottom of the sealed headspace vial and upon thermal equilibrium with temperatures above ambient selectively release VOCs into the vapor phase above the sample matrix. The headspace is static because the sealed vial prevents the loss of the headspace into the ambient environment. The thermodynamic concentration gradient at the interface between the sample matrix and the headspace effectively prevents exchange of volatile material as vaporization is forcing the movement of material from the sample matrix into the headspace.

Introducing sample biomass with cellular integrity changes the complexity of the sample matrix and although the headspace is static with respect to the ambient environment, the same cannot be said about the interface between the sample matrix and headspace.

On the cellular level, until desiccation causes cellular dysfunction and ultimately death, as long as energy requirements are met, stimuli are applied and the precursor starting materials are available, some biochemical pathways will continue to function and synthesis of some phytochemicals will continue, *de novo*, after material is excised and even post mortem. In the instance of tissue from the plant of interest, *P. marcegravi*, freezing excised leaves inside 4ml HPLC vials or 20ml crimped top headspace vials, provide the abiotic extreme cold stress with wounding, that contributes to a secondary stimulus prompting the generation of the plant hormone SA. The excised tissue is signaling cold stress via mitogen-activated protein kinase (MAPK or MPK) cascade signal transduction pathways. As cold stress proceeds to freezing, SA is metabolized to MeSA or to the glycosyl derivative. In the instance of extreme cold stress, the energy requirement for the two reactions and the deterioration of the cellular state and also the lower molecular weight of the product, suggest the reaction rate for the MeSA pathway will be preferential. Conversion of SA to MeSA is mediated by the enzyme SA methyltransferase. SA-induced protein kinase (SIPK), its Arabidopsis ortholog (AtMPK6), the tobacco wound-induced protein kinase (WIPK) and its Arabidopsis ortholog (AtMPK3) play significant roles in responses to biotic stresses, but more importantly for this work, abiotic stimuli such as wounding and cold (17), among others and reviewed in (18).

As cold stress becomes freezing, the concentration of MeSA will remain constant and possibly be concentrated by the formation of ice crystals on the sub-cellular level. Solid ice at the cellular level will rupture membranes and compromise cellular integrity. Cellular desiccation and nutrient starvation will result in dysfunction and cell death as cold stress becomes complete freezing.

Thawing the frozen biomass will release sub-cellular material into extracellular spaces and ultimately into the headspace around the biomass. Aside from the color of the biomass fading, little visual change is observed between the fresh, frozen and thawed states.

Figure 1 is then the outcome of wounding and extreme cold stress releasing SA, the metabolism of the SA to MeSA in the time frame between the onset of cold stress and freezing; and then release of MeSA that was sequester inside the compromised biomass into the headspace upon thawing.

The results shown as Figure 1 is reproducible independent of the vials used for trapping and sampling the headspace. It is also independent of the instrumentation used for separation and identification of sample components and, somewhat independent of the geospatial distribution of the biomass collected (this is anecdotal based on observed TICs). It is suggested that the release of volatile and semi-volatile molecular components into the headspace make the headspace static with respect to the ambient environment but absolutely dynamic with release of endogenous constituents from the compromised plant tissue.

Usually the approach for preparing leaf biomass is to quench the material with liquid nitrogen and homogenize. These limited results suggest that care needs to be exercised as cold stress can modify the state of the tissue and, therefore the dynamic of the headspace. This study indicates that static headspace sampling may be useful to determine the condition of biomass post storage, for some plant species. More work to establish a method of quantification, further confirm and completely characterize reproducibility in this and other species, and optimize headspace sampling methodology is required.

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Authors contributions

M.sc Mariah Ribeiro Rocha was responsible for the study design, data collection and writing of the manuscript. B.sc Paulo de Moraes Ferreira contributed immensely to the writing as well as revision of the manuscript. M.sc Márcio Wandré Moraes, Ph.D Thales Lima Rocha and Ph.D. Maria Fátima Grossi de Sá were involved in the design of the study and revision of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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