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Original research

Taxonomic Identification of Commercial Boswellia spp. Resins by Ambient Ionization Mass Spectrometry

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ABSTRACT

Historically, *Boswellia* resin, commonly known as frankincense, has been used for a variety of applications, including medicinal purposes and in religious ceremonies. These practices continued into the modern era, in which the trade of resin has increased dramatically. The genus *Boswellia* contains many species that are not currently protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), but research has found that some of these populations are predicted to decrease by 50 % in the next two decades due to overexploitation (Bongers et al., 2019). This highlights the need for a method that can identify *Boswellia* resins. In this research, resin from nine different species of *Boswellia* were collected and analyzed in positive ion mode by Direct Analysis in Real Time Time-of-Flight mass spectrometry (DART TOFMS) by dissolving the resin in methanol to obtain a chemotype devoid of background noise. Chemometric analysis of the data indicated that positive ion spectra from the resins separated the species effectively, indicating that DART TOFMS can be used to identify resins in trade.

1. Introduction

Aromatic resins from the family Burseraceae have maintained an important place throughout history and are still used in a variety of applications today, ranging from religious ceremonies, incense, perfumes, foods, aromatherapy, and medicines, to embalming and insecticides ([16,17] pp. 344–374; [12,13]). While the purpose of resin is to act as a defense against environmental threats including insects, parasites, herbivores and drought, [16], the trees that produce this fragrant material hold immense value as a source of income in Arabia, India and countries within the Horn of Africa [11,12] with contemporary trade expanding across range States in central and west Africa. Of the 17 genera within Burseraceae, *Boswellia* and *Commiphora* contain the primary species found in the resin trade [12].

Resin is theorized to have been an important trade item as far back as 3000 BCE [3], and demand has not decreased over time. Today, resin is an important source of revenue for rural communities who harvest the resin during the dry season before it is exported globally as a commercial product ([9,12,4]; Lemenih et al., 2003; [27]). Due to over-exploitation, unsustainable harvesting techniques, clear-cutting for agriculture and also livestock grazing, some resin-producing *Boswellia* populations are forecasted to decrease [12,23,25,5] by as much as 50 % over the next decade [4], which has prompted concern from regulatory agencies such as the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [8].

Previous research on aromatic resins, (also called olibanum, oleogum resin and numerous local names), have shown that the material is composed of essential oils, terpenes, and polysaccharides ([16,2] pp. 363). Despite the popularity of resin in trade and the myriad research publications identifying its chemical makeup, few studies are available that focus on species-level resin identification. In fact, fewer than 25 % of the more than 160 studies examined samples from multiple taxa. Of these, Bekana et al. [2] found that the commonly traded *Boswellia* species, *B. papyrifera*, *B. neglecta*, and *B. rivae*, could be differentiated based on their chemical profiles through gas chromatography-mass spectrometry (GC-MS) [2], while Mathe et al. [18] were able to separate *B. carteri*, *B. sacra*, and *B. frereana* using high-performance liquid chromatography (HP-LC). Both approaches are costly in terms of time

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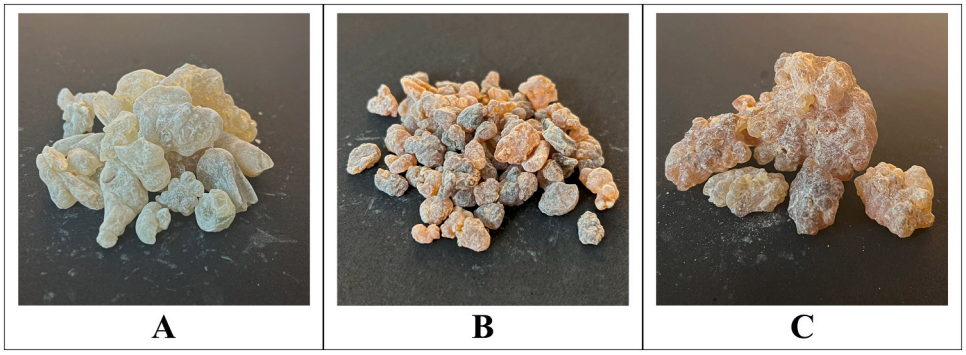


Fig. 1. Example resin samples from *Boswellia sacra* (A), *Boswellia serrata* (B), and *Boswellia papyrifera* (C).

Table 1
Sample information for all *Boswellia* spp. used for identification.

Species	No. of different commercial sources	Declared geographic source	No. of spectra in model	No. of blind test specimens
B. carteri	9	Somalia & Yemen	20	5
B. dalzielii	6	Nigeria	20	5
B. elongata	2	Yemen (Soqotra)	20	5
B. frereana	7	Somalia	21	4
B. neglecta	6	Ethiopia & Somalia	21	4
B. papyrifera	10	Ethiopia & Sudan	20	5
B. rivae	3	Ethiopia	20	5
B. sacra	18	Oman	21	4
B. serrata	6	India	20	5

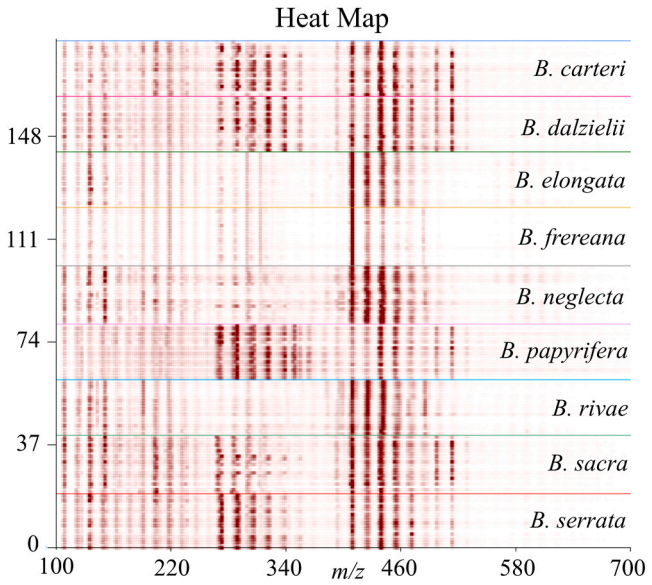


Fig. 2. Heat map of all *Boswellia* species spectra after methanol dilution.

needed to prepare samples and to perform the analysis, leading to the need for a rapid and inexpensive approach. Furthermore, GC-MS is limited to detecting molecules with increased volatility [13] and within a typical range of 30 – 400 *m/z*, though wider scans can go as high as 600 *m/z* if needed [20]. While this may not appear to be a limitation, larger masses (>600 *m/z*) can be found when analyzing plant-based samples (see Figure 6 in [22];) and have been found to be useful for species differentiation (Deklerck et al., 2019). In addition, it is unclear whether claims made about detecting species-level differences through comparing multiple studies are valid due to untested comparability in methodological approaches.

Furthermore, issues of taxonomic complexity, lack of provenance, and some species of *Boswellia* having no information available about the chemical composition of their resins has led to concern over the ability to identify resin samples - especially where no morphological voucher specimens are available to validate species-level identification. For example, the variable *B. sacra* includes populations from Somalia that have been called *B. carteri* – a superfluous and illegitimate name nevertheless used widely in commerce – and despite various claims that these two "species" differ in their chemical composition these studies are currently not backed up by morphological diagnostic characters leading to a lack of clarity as to the provenance of samples labeled as *B. sacra*. A similar situation exists with *B. neglecta* and *B. microphylla* with the latter previously recognized taxonomically within the former. Samples labeled *B. neglecta* may include samples of *B. microphylla* if sub-specific identification was not undertaken at the time of collection or labeling and *B. microphylla* has never been analyzed chemically, meaning any distinguishing characteristics could not be identified. Accurate identification of the provenance of samples and assignment to their correct species – based upon species-level morphology – is critical when global conventions such as CITES work at the species level.

The use of Direct Analysis in Real Time Time-of-Flight Mass Spectrometry (DART TOFMS) for species analysis of commercial timber has been an accepted methodology for over a decade [15,19,6]. During that time, many closely related tree species and their lookalikes have been successfully differentiated using this technology. Benefits of using DART TOFMS are rapid data collection, and relatively cheap analysis, leading to its obvious usage for the identification and separation of *Boswellia* species through resin analysis. Additionally, the addition of the TOF system in conjunction with the DART-MS allows for larger masses (>600 *m/z*) to be analyzed.

In this research, resin from nine different species of *Boswellia* were collected and analyzed by DART TOFMS for species-level identification. This experimental approach consisted of dissolving the resin samples (Fig. 1) in methanol and analyzing the solvent to characterize extracted molecules by ambient ionization mass spectrometry in positive ion mode.

2. Methods

2.1. Sample sources

Boswellia resin samples were collected from commercial sources (Supplemental Table 1). A total of 80 individual plastic bags (Table 1) filled with small pieces of resin were accrued for this research. From each bag, ten pieces were removed and analyzed to ensure that the chemotypes were constant across the samples. Each sample was carefully chosen to ensure there were no impurities such as bark, dirt, or

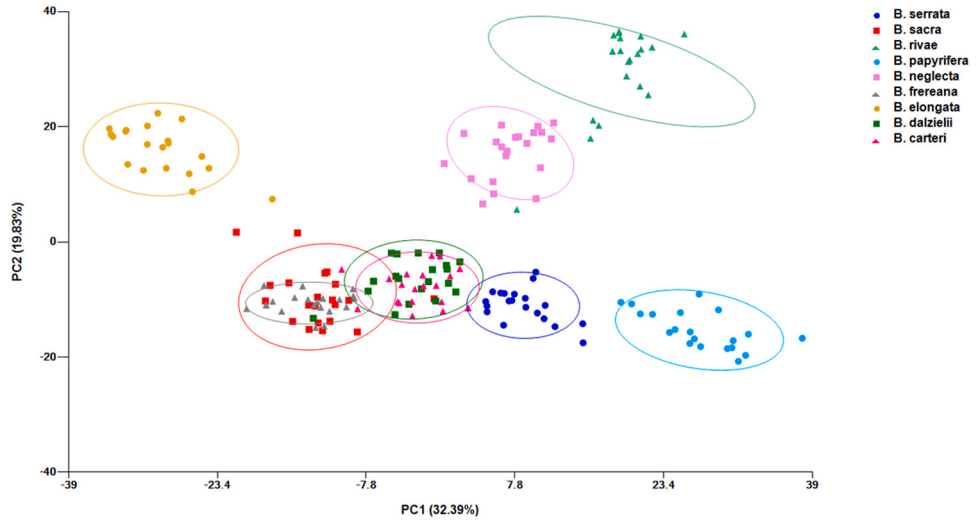


Fig. 3. A PCA model created from the spectra using two principal components. Ovoids with two standard deviations from the center of each group encircle each individual species class.

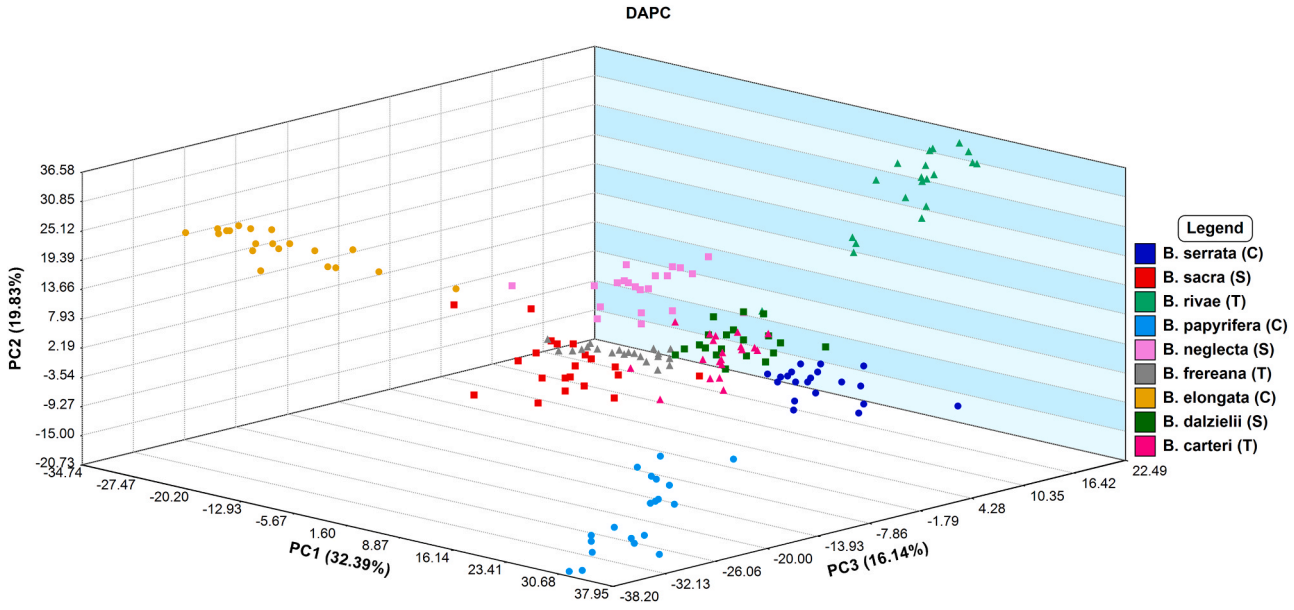


Fig. 4. The DAPC model from mass spectra separated each species by 92.9 % through LOOCV.

insects. Color variation was notable in some sample bags and in these cases, each differently colored piece was analyzed via DART TOFMS to ensure that the color change did not influence the chemotype. In the case of *B. elongata*, only two commercial sources were collected.

A Time-of-Flight mass spectrometer (AccuTOF (JMS-T100LP 4 G), JEOL, USA, INC., Peabody, MA. USA) equipped with a Direct Analysis in Real Time ion source (DART-SVP, IonSense Inc., Saugus, MA. USA) was used to collect mass spectra measurements. The mass spectrometer is stated by the manufacturer to have a resolving power of 10,000 FWHM. All samples were analyzed in positive ion mode from a range of 100 *m/z* to 1000 *m/z*. Orifice 1 was set to a voltage of 30 V, Orifice 2 and the Ring Lens were set to 5 V, and the DART gas heater was set to 350 °C for the helium DART gas.

2.2. Sample preparation for DART TOFMS

Preliminary examination showed that direct surface analysis of the resin samples produced low quality mass spectra. To address for the poor spectra produced by the solid resin samples, we instead dissolved each resin piece in 1.5 mL of methanol and collected mass spectra by dipping an inverted capillary tube in the resulting solution and holding it in the heated helium stream. While collecting the mass spectral data, poly (ethylene glycol) 600 (Sigma-Aldrich, St. Louis, MO, USA) was collected after every fifth sample. The first PEG measurement was used to calibrate the data and the rest were used for drift correction.

All spectra underwent drift correction and calibration before subtracting the background and averaging using msAxel software (version 1.0.5.2, JEOL Ltd.). The spectra were then centroided and exported as text files for analysis via Mass Mountaineer software (massmountaineer.com). A heat map was created using the spectra to examine the chemotypes for each species. The most abundant ions from the spectra were selected using a threshold of 5 % and 5 mmu, and all ions underwent analysis of variance (ANOVA) in order to find the ions that were statistically significant ($p \leq 0.05$) for separating the 9 species of *Boswellia*. This resulted in a total of $n = 1236$ ions that were used for classification modeling. A principal component analysis (PCA) model was constructed

Table 2
Test spectra and their classifications indicated that accuracy of the model was 92.86 %, misclassified samples are indicated through **bold** font.

Test Sample Species	Identifier	Distance	Prob. %	Assigned Species
Boswellia carteri	WD210314–1	26.84342	92.08	*Boswellia carteri*
Boswellia carteri	WD210314–2	20.6431	86.09	*Boswellia carteri*
Boswellia carteri	WD211979–3	18.74534	83.76	*Boswellia carteri*
Boswellia carteri	WD211996–1	33.52779	96.09	*Boswellia carteri*
Boswellia carteri	WD211996–2	32.83296	95.77	*Boswellia carteri*
Boswellia dalzielii	WD210310–4	15.92345	78.68	*Boswellia dalzielii*
Boswellia dalzielii	WD211958–2	20.98327	85.27	*Boswellia dalzielii*
Boswellia dalzielii	WD211958–4	22.53902	86.99	*Boswellia dalzielii*
Boswellia dalzielii	WD211970–1	22.58467	87.03	*Boswellia dalzielii*
Boswellia dalzielii	WD212013–4	42.11049	98.23	*Boswellia dalzielii*
Boswellia elongata	WD212028–12	23.29903	93.87	*Boswellia elongata*
Boswellia elongata	WD212028–3	23.87272	94.31	*Boswellia elongata*
Boswellia elongata	WD212028–4	29.04657	97.29	*Boswellia elongata*
Boswellia elongata	WD212028–6	53.44745	99.98	*Boswellia elongata*
Boswellia elongata	WD212028–7	29.92245	97.63	*Boswellia elongata*
Boswellia frereana	WD211959–3	8.606727	98.82	*Boswellia frereana*
Boswellia frereana	WD211998–2	30.14985	100	*Boswellia frereana*
Boswellia frereana	WD211999–4	14.04739	99.99	*Boswellia frereana*
Boswellia frereana	WD212014–1	11.63421	99.89	*Boswellia frereana*
Boswellia neglecta	WD212000–2	36.98854	85.86	*Boswellia neglecta*
Boswellia neglecta	WD212001–2	33.46362	100	***Boswellia frereana***
Boswellia neglecta	WD212001–4	23.56112	75.31	*Boswellia neglecta*
Boswellia neglecta	WD212015–2	67.05831	97.43	*Boswellia neglecta*
Boswellia papyrifera	WD210315–1	46.75391	94.75	*Boswellia papyrifera*
Boswellia papyrifera	WD211961–2	40.77353	92.13	*Boswellia papyrifera*
Boswellia papyrifera	WD211961–3	15.43744	70.38	*Boswellia papyrifera*
Boswellia papyrifera	WD211964–1	28.45085	83.81	*Boswellia papyrifera*
Boswellia papyrifera	WD211983–2	37.53419	90.35	*Boswellia papyrifera*
Boswellia rivae	WD210313–5	58.8519	99.63	*Boswellia rivae*
Boswellia rivae	WD210313–7	44.3246	97.83	*Boswellia rivae*
Boswellia rivae	WD211972–2	51.70638	99.08	*Boswellia rivae*
Boswellia rivae	WD211972–8	62.22117	99.77	*Boswellia rivae*
Boswellia rivae	WD211984–3	20.99922	83.07	*Boswellia rivae*
Boswellia sacra	WD211985–1	35.29946	93.99	*Boswellia sacra*
Boswellia sacra	WD211988–1	44.1547	97.4	*Boswellia sacra*
Boswellia sacra	WD212005–1	54.71453	99.99	***Boswellia elongata***
Boswellia sacra	WD212008–1	15.07278	78.58	***Boswellia carteri***
Boswellia serrata	WD211974–3	17.53465	83.93	*Boswellia serrata*
Boswellia serrata	WD211992–2	44.81545	99.44	*Boswellia serrata*
Boswellia serrata	WD212010–4	60.92329	99.97	*Boswellia serrata*
Boswellia serrata	WD212011–2	17.64661	84.08	*Boswellia serrata*
Boswellia serrata	WD212025–2	40.72493	98.94	*Boswellia serrata*

with 55 principal components that encompassed 87.2 % of the variance within the data. PCA was used to both reduce the dimensionality of the data and to observe patterns and trends in each species class. The resulting PCA model was used as the foundation for classification of each species class via discriminant analysis of principal components (DAPC).

Table 3
Tentative assignments of selected ions present in all *Boswellia* species.

m/z	Molecular Formula	Tentative Assignment (+H)	As Described By:
457.367	$C_{30}H_{48}O_3$	Boswellic acid	Ur-Rahman et al. [28]; Culioli (2003); Beton (1956)
297.149	$C_{19}H_{20}O_3$	Acerogenin C	Lakshmi Niranjan Reddy et al. [14]
309.149	$C_{20}H_{20}O_3$	Ovalifoliolatin B	Lakshmi Niranjan Reddy et al. [14]
415.394	$C_{29}H_{50}O$	(-)-β-Sitosterol	Ur-Rahman et al. [28]
499.378	$C_{32}H_{50}O_4$	Acetyl-boswellic acid	Singh et al. [24]
513.358	$C_{32}H_{48}O_5$	3α-Acetoxy-11-keto-β-boswellic acid	Corsano & Iavarone [7]
291.264	$C_{20}H_{34}O$	Cembrenol	Pardhy & Bhattacharyya [21]
409.346	$C_{29}H_{44}O$	24-Nor-3,12-ursadien-11-one	Hanus et al. [10]
455.352	$C_{30}H_{46}O_3$	Elemonic acid	Ur-Rahman et al. [28]
471.347	$C_{30}H_{46}O_4$	11-Keto-β-boswellic acid	Ur-Rahman et al. [28]; Corsano & Iavarone [7]
475.374	$C_{30}H_{50}O_4$	Lup-20(29)-ene-3α-acetoxy-24-oic acid	Zhou & Cui [29]

3. Results

3.1. Analysis

Examination of the heat map in Fig. 2 showed the reproducibility of the analysis within each species class. The ubiquitous boswellic acid ($C_{30}H_{48}O_3$ $+H^+$) found in the resins is shown in the heat map at 457.36 *m/z* in accordance with Afendi et al. [1].

Although *B. carteri* and *B. sacra* are considered to be synonymous [26], they trend into separate clusters with slight overlap by 2D PCA (Fig. 3). When DAPC was applied, the accuracy of the model by Leave-One-Out Cross validation (LOOCV) was 92.9 % (Fig. 4).

Test samples not included in the PCA or DAPC models were randomly selected and entered into the DAPC model as unclassified spectra; all spectra were assigned to their correct species class with the exception of a single *B. neglecta* and two *B. sacra* samples. Test statistics, including Euclidean distances from the centroid of each cluster and probability values for each test spectrum are shown in Table 2.

As previously stated, a total of 1236 ions were statistically significant and selected for differentiating between the *Boswellia* spp. resin spectra. A small subset of these ions were searched against the KNApSAcK Family Databases [1] to assess whether the ions were consistent with the masses of compounds previously detected in *Boswellia* species. It should be emphasized that no single ion had the statistical power to separate any single species class. Rather all ions were required for differentiation and identification. Table 3

4. Discussion

Using DART TOFMS, mass spectra from resin samples collected from commercial sources including *Boswellia carteri, B. dalzielii, B. elongata, B. frereana, B. neglecta, B. papyrifera, B. rivae, B. sacra,* and *B. serrata*, were analyzed directly in positive ion mode. Analysis of the mass spectra showed that the specimens collected for this study were in consensus with their commercial classifications and expressed unique chemotypes which were adequately distinct to allow for species identification. Applying PCA followed by DAPC showed that we could classify the *Boswellia* species with a LOOCV accuracy of 92.9 %, and a blind test accuracy of 92.86 %.

The trade in aromatic *Boswellia* resins is unlikely to decrease in the future, and as a result of its popularity, many species are in danger of disappearing from their native habitats. On account of the over-exploitation and rapid habitat loss experienced by certain populations of

Boswellia, international regulatory agencies, such as CITES, have turned their attention to how resin should be regulated in order to ensure its availability for generations to come. Given these facts, it is of the utmost importance that a reliable method for differentiating between species experiencing harvesting pressure and those whose populations are sufficient for continuous international trade to be developed. Our research advances the previous work carried out in the field of chemical analysis for species identification from resins by clearly showing that *Boswellia* species can be separated using multivariate analysis of DART TOF mass spectra.

It should be noted that samples used in this study were from commercial sources and as such have no verifiable species-level information confirmed by botanical vouchers. Despite this, species-level classification was significant and there was clear reproducibility across each species class. Inaccurate identification would invariably lead to misclassification of some samples and potentially a reduction in the resolving power of DART TOFMS if mixed samples were classified together in the test set. In fact, this may have been the case for the misidentified *B. neglecta* and *B. sacra* test samples, as these species do not have overlapping geographic ranges. As a result, a library of vouchered and verified resin samples is required, which we expect will lead to increased resolving power based upon the level of discrimination already observed from commercial samples.

Ethics statement

No animals were harmed in this study and the samples analyzed were purchased through established commercial sources as indicated in Supplemental Table 1: Commercial supplier for each resin species including claimed origin.

CRediT authorship contribution statement

Edgard O Espinoza: Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Conceptualization. **Robert A Running:** Investigation, Formal analysis. **Alan Forrest:** Writing – review & editing, Writing – original draft. **Doina Voin:** Resources. **Pamela J McClure:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Megahn H Pinedo:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Kierra R Cano:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Erin R Price:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis.

Declaration of Competing Interest

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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsiae.2024.100090.

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