Pyrrolizidine alkaloids (PAs) are small secondary metabolites composed of a 1-hydroxymethylated necine core, which is esterified with a variety of necic acids; they are often obtained as their N-oxides, which may be the result of metabolism or simple air exposure of the corresponding free bases (Hartmann and Witte, 1995). PAs play an important ecological role in mediating plant–herbivore interactions (Hartmann, 1999; Trigo, 2011), as they are highly toxic to mammals (Prakash et al., 1999) and deterrents to generalist insect herbivores (van Dam et al., 1995). Specialist insect herbivores, however, have evolved the ability to detoxify and sequester PAs for their own use (Hartmann, 2009; Trigo, 2011).

**Applications in Plant Sciences**

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**PREMISE OF THE STUDY:** Understanding the phylogenetic distribution of defensive plant secondary metabolites is essential to the macroevolutionary study of chemically mediated plant–animal interactions. The chemical ecology of pyrrolizidine alkaloids (PAs) has been extensively studied in a number of plant–herbivore systems, including Apocynaceae (the milkweed and dogbane family) and Danainae (the milkweed and clearwing butterflies). A systematic survey is necessary to establish a detailed understanding of their occurrence across Apocynaceae. A survey of this species-rich, mainly tropical and subtropical family will rely heavily on small tissue samples removed from herbarium specimens, some of which will be very old and/or preserved with alcohols or mercuric chloride.

**METHODS:** We optimized PA extraction methods from small leaf fragments of recently collected silica-dried leaves of the PA-positive *Echites umbellatus*, varying crushing and extraction time. We then applied our optimized method to leaf fragments from 70–167-year-old herbarium specimens of *E. umbellatus*. To simulate the effect of alcohol treatment on PA detectability in herbarium specimens, we incubated freshly collected leaves of the PA-positive *Parsonsia alboflavescens* in three different alcohols before drying and compared PA recovery to freshly dried controls. PAs were quantified using high-performance liquid chromatography–mass spectrometry analysis. X-ray fluorescence was used to identify mercury-containing specimens.

**RESULTS:** Fifteen seconds of leaf crushing followed by 24 h of extraction were optimal for PA free-base and N-oxide recovery. This method yielded ~50-fold greater PA recovery than prior methods. Half of the herbarium specimens (13 of 23), including the oldest, tested positive for PAs; leaf age did not correlate with success in PA extraction. Treatment of fresh leaves with alcohol before drying did not diminish PA recovery; mercury was observed in both PA-positive and PA-negative specimens.

**CONCLUSIONS:** PAs can be reliably detected in small tissue samples from herbarium specimens up to 167 years old, including specimens that had been treated with alcohol or mercury salts. The variability of PA presence among herbarium specimens of *E. umbellatus* indicates that multiple specimens will need to be tested before a particular species is determined to lack PAs.

**KEYWORDS** Apocynaceae; coevolution; Danainae; HPLC-MS; pyrrolizidine alkaloids; secondary metabolism.
Species of Danainae (milkweed and clearwing butterflies; Nymphalidae) often feed on plants from the Apocynaceae (milkweeds and dogbanes), which are widely hypothesized to be their ancestral larval host plants (Ackery and Vane-Wright, 1984; Wahlberg et al., 2009; Brower et al., 2010). Danainae not only sequester PAs for defense (Orr et al., 1996), but also use them in courtship and mating, as precursors for male mating pheromones (Schneider et al., 1975), and as nuptial gifts (Dussourd et al., 1989). The mode of PA acquisition by most Danainae species is highly unusual. PAs are sequestered via adult pharmacophagy, i.e., feeding to obtain chemicals rather than nutrients, on diverse plant sources (Boppré, 1978, 1984). However, a few Danainae species are documented as obtaining PAs via larval feeding (Trigo and Motta, 1990). These facts led to the proposal of a co-evolutionary hypothesis of “defense de-escalation” for the origin of Danainae PA pharmacophagy (Edgar et al., 1974; Edgar, 1984). The ancestral larval host plants of Danainae are hypothesized to have produced PAs but to have lost them once they were co-opted by Danainae ancestors, causing the Danainae to seek these chemicals elsewhere during the adult life stage. A prediction from the defense de-escalation hypothesis is that PAs were ancestrally present in Apocynaceae but were subsequently repeatedly lost (Livshultz et al., 2018).

Phylogenetic reconstruction of PA evolution in Apocynaceae requires detailed understanding of their presence and absence among the ~4500 extant species. To date, however, only 40 species have been specifically tested for PAs and 15 PA-positive species discovered in seven genera of four tribes (Burzynski et al., 2015; Colegate et al., 2016). Although there is an extensive literature on the secondary chemistry of Apocynaceae (Endress et al., 1990; Agrawal et al., 2012), the distribution of PAs remains poorly known, as evidenced by the recent first report of PAs in Anspheurine (A. DC.) Pichon (Colegate et al., 2016). Apocynaceae are a globally distributed, predominantly tropical and subtropical family; a systematic survey of PA presence and absence across the family will rely heavily on specimens preserved in the world’s herbaria.

Herbarium specimens are an essential source for plant trait data, including secondary metabolites (Phillipson, 1982; Soto-Sobenis et al., 2001; Funk, 2003; Zangerl and Berenbaum, 2005). As a global resource, herbarium specimens must be minimally modified in the sampling process to preserve them for future generations. PAs are highly stable chemicals and have been previously detected in herbarium specimens of Senecio L. (Asteraceae) (Pelser et al., 2005) and Amsinckia Leh. (Boraginaceae) (Colegate et al., 2014). The latter found PAs in specimens up to 100 years old. However, these taxa are temperate, whereas field preservation methods applied to tropical Apocynaceae, such as the Schweinfurth method of alcohol treatment before drying (Forman and Bridson, 1989), may remove some alcohol-soluble PAs. On the other hand, the small volume of alcohol typically used (just enough to saturate the specimens in their newspaper folds [D. J. Middleton, Singapore Botanic Gardens, personal communication]) may extract only a fraction of the PA content of plant tissues.

The preferred method of detection of PAs and their N-oxides is extraction in methanol followed by high-performance liquid chromatography–mass spectrometry (HPLC-MS) (Crews et al., 2010). HPLC-MS couples the separatory capabilities of an HPLC with the mass analysis capabilities of a mass spectrometer, allowing for more efficient and selective detection of PAs (Wuilloud et al., 2004). HPLC requires much less cleanup and derivatization than do other separation methods (Crews et al., 2010). Furthermore, HPLC-MS exploits the ionizable nitrogen in the necine core to allow for resolved detection of any PAs in a sample (These et al., 2013). Because of this structural characteristic, positive electro-spray ionization mass spectrometry is the most common mode used (Avula et al., 2015). The presence of a PA can be corroborated within a mass spectrum by adding the mass of oxygen (16 Da) to the PA free-base mass to give the mass of the N-oxide form; the two analogous chemicals are often found in the same leaf samples (Hartmann and Ober, 2000).

We developed a method to maximize PA yield from small tissue samples and established that samples of this size from herbarium specimens of Apocynaceae can yield detectable PAs. It is not yet known if the amount of time spent in the extraction medium affects PA extraction and if it does, to what extent. Likewise, it is unknown whether or not leaf crushing time has an effect on PA extraction efficacy. We further sought to determine how alcohol treatment and mercuric chloride preservation affect PA yield from herbarium specimens. We hypothesized that a loss of PA content would occur in samples treated with alcohols because of the solubility of PAs in such solvents. Such a finding would be particularly important for future studies of PAs in herbarium specimens from tropical countries. We also suspected that mercuric chloride treatment could lead to reaction with PAs and mask their presence on HPLC-MS.

METHODS

Materials

A monocrotaline standard (Toronto Research Chemicals, Toronto, Ontario, Canada) was used as received. All solvents, including water and methanol, were HPLC-grade. Extractions of dry leaf tissue from Echites umbellatus Jacq. and Parsonsia alboflava vesens (Dennst.) Mabb. (Apocynaceae), both PA-positive species (Burzynski et al., 2015), were made from 4–6 mg for method development (approximately 1 cm²), 22–24 mg for field simulation, and 4–18 mg for herbarium-preserved specimen analysis. All vouchers are listed in Table 1.

Detection of PAs by HPLC-MS

All samples were analyzed by reverse-phase HPLC with an ACE C18 column (3-μm particle, 150 × 4.6 mm; Advanced Chromatography Technologies Ltd., Aberdeen, Scotland). PAs were separated with a binary mobile phase composed of acidified water (0.1% formic acid, v/v; solvent A) and acidified acetonitrile (0.1% formic acid, v/v; solvent B) flowing at 0.5 mL/min. Each chromatographic separation lasted 30 min with the following gradient: 10% B (2-min hold) ramped to a final mobile phase concentration of 100% B for 20 min (5-min hold). MS was performed using an API 2000 triple quadrupole mass spectrometer in positive electrospray ionization mode (Applied Biosystems, Waltham, Massachusetts, USA). The mass analyzer was operated in the single quadrupole mode and scanned over a mass range of 120 to 800 m/z. Data analysis and instrument operations were performed under Analyst 1.6.2 control.

Identification and quantification of PAs

Correlation of retention times to PA structures was accomplished by re-running archived methanol extracts from E. umbellatus analyzed...
by Burzynski et al. (2015), as the LC conditions vary slightly from those previously used; retention times are compiled in Table 2. All samples were weighed before extraction to obtain dry weight. Areas from PAs were obtained from extracted ion chromatograms. Units for area were reported as “area units” (AU). Quantification of PAs from recovered alcohols from the alcohol treatment experiments is AU per milliliter initial volume (AU/mL).

Optimization of PA extraction via crush time and extraction time

The *E. umbellatus* leaf sample was weighed on an analytical balance in a 1.7-mL Eppendorf tube and then crushed in the same tube with a microspatula for 0, 15, 30, 60, or 180 s. Samples were prepared in triplicate. Each specimen was suspended in 750 μL of methanol, vortexed for 5 s, left to rest for time intervals between 1 min and 48 h, and vortexed again for 5 s. The solution was then removed via a 1-mL Norm-Ject disposable syringe (Henke-Sass Wolf, Tuttlingen, Germany) and filtered with a 0.2 μm filter (Nalgene, Rochester, NY, USA).

### Table 1. Voucher specimens of *Echites umbellatus* and *Parsonia alboflavescens* used in this study.

| Species | Catalog no. | Voucher collector/ collector no. | State/province, country | Collection date | PA’s identified (AU/mg) | Mercury analysis (+/-) |
|---------|-------------|---------------------------------|-------------------------|----------------|------------------------|-----------------------|
| *Parsonia alboflavescens* | PH00224583 | Livshultz TL2011-14 | Pingtung, Taiwan | 07/09/2011 | 1 (4.19E+06), 1NO (1.31E+05), 2 (5.24E+06), 3 (1.71E+07), 4 (2.49E+06), 5 (7.98E+06), 6NO (1.48E+05), 6 (1.45E+05), 7NO (5.85E+05), 8 (1.30E+07), 9 (7.93E+06) | + |
| *Echites umbellatus* | PH00358001 | Livshultz s.n. | San Salvador, Bahamas | 12/20/2011 | 4 (1.67E+08), 4NO (1.01E+08), 5 (2.07E+05), 6NO (1.66E+07), 7NO (3.49E+07), 13 (1.68E+07), 14 (2.21E+06) | + |
| *Echites umbellatus* | PH00044929 | Rugel | Florida, USA | 1840–1850 | 4 (2.21E+06), 5 (5.65E+04), 12 (8.35E+04) | – |
| *Parsonia alboflavescens* | PH00044932 | Poiteau | Distrito Nacional, Dominican Republic | 1854 | 1 (8.13E+04), 4 (2.52E+05), 12 (4.02E+04) | + |
| *Echites umbellatus* | PH00044931 | Auber 18 | Cuba | 1860 | 1 (3.30E+04), 4 (1.08E+05), 12 (2.80E+04) | – |
| *Echites umbellatus* | PH00044924 | Wright 1661 | Cuba | 1860 | 4 (4.86E+06), 4NO (4.25E+05), 12 (3.90E+05) | – |
| *Echites umbellatus* | PH00044888 | Curtiss 2266 | Florida, USA | 1875–1887 | – | – |
| *Echites umbellatus* | PH00044933 | Garber | Florida, USA | 8/1877 | – | – |
| *Parsonia alboflavescens* | PH00044930 | Rothrock 375 | North Eleuthera, Bahamas | 11/10/1890 | + |
| *Parsonia alboflavescens* | PH00044926 | Rothrock 375 | North Eleuthera, Bahamas | 11/22/1890 | + |
| *Parsonia alboflavescens* | PH00044885 | Brown 108 | Florida, USA | 6/23/1904 | + |
| *Parsonia alboflavescens* | PH00044886 | Brown 51 | Florida, USA | 6/20/1904 | + |
| *Parsonia alboflavescens* | PH00044918 | Harshberger 61 | Ouest Department, Haiti | 7/4/1901 | 1 (2.16E+05), 4 (3.57E+05), 4NO (4.25E+05), 12 (1.60E+04) | – |
| *Parsonia alboflavescens* | PH00044927 | Brace 4399 | Acklins Island, Bahamas | 12/21/1905–1/6/1906 | 1 (1.02E+06), 4 (1.81E+07), 4NO (2.02E+06), 6 (1.79E+05), 12 (8.03E+05) | + |
| *Parsonia alboflavescens* | PH00044925 | Baker 5331 | La Habana, Cuba | 6/1/1905 | 3 (2.97E+04), 3NO (1.56E+04), 4 (6.06E+05), 4NO (5.33E+04), 12 (2.18E+03) | – |
| *Parsonia alboflavescens* | PH00044920 | Brown 327 | St. James Parish, Jamaica | 3/1–2/1910 | 8 (1.90E+05), 4 (4.66E+02), 4NO (7.94E+06), 11 (2.66E+06), 12 (4.11E+06) | – |
| *Parsonia alboflavescens* | PH00044889 | Small 191 | Florida, USA | 11/1906 | 6 (2.41E+03), 6NO (6.74E+04) | – |
| *Parsonia alboflavescens* | PH00044888 | Small 115 | Florida, USA | 11/1906 | + |
| *Parsonia alboflavescens* | PH00044891 | Harshberger | Florida, USA | 8/18/1911 | – |
| *Parsonia alboflavescens* | PH00044929 | Pennell 11211 | St. James Parish, Jamaica | 10/9/1922 | 1 (1.07E+06), 4 (9.86E+06), 4NO (5.41E+05), 12 (3.23E+05) | + |
| *Parsonia alboflavescens* | PH00044928 | Ruiz 3291 | Cuba | 8/20/1924 | – |
| *Parsonia alboflavescens* | PH00044930 | Pilsby | Cuba | 11/1/1931 | 1 (1.94E+07), 4 (5.36E+07), 4NO (6.92E+06), 12 (3.77E+06) | – |
| *Parsonia alboflavescens* | PH00044881 | Seibert 1281 | Florida, USA | 2/4/1940 | – |
| *Parsonia alboflavescens* | PH00044883 | Dunbar | Florida, USA | 6/26/1946 | 1 (3.77E+06), 4 (6.29E+05), 6 (2.11E+05), 11 (9.33E+04), 12 (4.80E+04) | – |

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a All vouchers are deposited at the Herbarium of the Academy of Natural Sciences (PH), Philadelphia, Pennsylvania, USA.

b Voucher specimen collection dates are given and are presented as month/day/year. For the alcohol experiments, *P. alboflavescens* fresh leaf material from cultivated plants was collected on 25–26 July 2017.

c PAs and their corresponding AU/mg of dry weight (average of the triplicate preparation) from *P. alboflavescens* were taken from the untreated control leaves used in the alcohol treatment simulation, and from *E. umbellatus* (using 15 s crush and 24 h extraction). Tentatively identified PAs and N-oxides are numbered according to Table 2. Area (AU/mg of dry weight) obtained from extracted ion chromatograms.

d Mercury analysis was performed only for the historical *E. umbellatus* specimens.

e Voucher specimen used in methods optimization experiments.

f Voucher specimen used in alcohol treatment experiments.

g Voucher specimen collection dates are given and are presented as month/day/year. For the alcohol experiments, *P. alboflavescens* fresh leaf material from cultivated plants was collected on 25–26 July 2017.

h Mercuru analysis was performed only for the historical *E. umbellatus* specimens.

i Voucher specimen for samples used in alcohol treatment experiments.

j Voucher specimen for samples used in methods optimization experiments.

k Historical *E. umbellatus* specimens.
Germany) attached to a 20-gauge × 1 PrecisionGlide needle (Becton, Dickinson & Company, Franklin Lakes, New Jersey, USA) and transferred to a second 1.7-mL Eppendorf tube, followed by 2 × 250-μL methanol washes. The methanol was evaporated from the crude extracts in vacuo in a DR 120 SpeedVac (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with the drying rate on low. The crude extracts were reconstituted in 250 μL of methanol containing a 1 ppm monocrotaline internal standard and vortexed for 5 s. The reconstituted samples were then transferred to a 0.25-mL plastic autosampler vial with a 1-mL Norm-Ject disposable syringe attached to an Acrodisc CR 13-mm, 0.2-μm PTFE membrane filter (VWR International, Radnor, Pennsylvania, USA). Samples were analyzed via HPLC-MS using the conditions described above.

**Simulation of field preservation with alcohol treatment**

Approximately 2 g of fresh *P. alboflavescens* leaves were torn off the stem, crumpled, and stuffed at the bottom of a 50-mL Falcon tube. All samples were prepared in triplicate with either no treatment, methanol (70% or 100%), ethanol (70% or 100%), or isopropanol (70% or 100%). Four milliliters of alcohol was added to each tube so that the leaves were saturated but not submerged. The leaves were then left to steep in the alcohol, held horizontally on a rotational shaker (60 rpm) at 25°C for 7 days. The alcohol was poured off and the leaves were removed from the Falcon tube and dried in a drying cabinet at 44°C for 24 h. Control samples were dried the day that they were harvested. The recovered alcohol was left to evaporate at room temperature with evaporation completed in vacuo. The crude alcohol extracts were reconstituted in 2 mL of methanol containing a 1 ppm monocrotaline standard. Extraction of PAs in the dried PAs were reconstituted in 250 μL of methanol containing a 1 ppm monocrotaline internal standard and vortexed for 5 s. The crude extracts were reconstituted in 250 μL of methanol containing a 1 ppm monocrotaline internal standard and vortexed for 5 s. The crude extracts were reconstituted in 250 μL of methanol containing a 1 ppm monocrotaline internal standard and vortexed for 5 s. The crude extracts were reconstituted in 250 μL of methanol containing a 1 ppm monocrotaline internal standard and vortexed for 5 s. The crude extracts were reconstituted in 250 μL of methanol containing a 1 ppm monocrotaline internal standard and vortexed for 5 s. The crude extracts were reconstituted in 250 μL of methanol containing a 1 ppm monocrotaline internal standard and vortexed for 5 s.

**Processing of herbarium-preserved *E. umbellatus* leaves**

The extraction of PAs was as described above, employing 15 s of crushing with 24 h of extraction in methanol. The samples were analyzed via HPLC-MS using the conditions described above.

**Determination of mercury species on herbarium-preserved specimens by portable X-ray fluorescence spectrometry**

A handheld Tracer III-SD XRF spectrometer (Bruker Corporation, Billerica, Massachusetts, USA) under reduced pressure was used to determine the presence of mercury on the 23 herbarium specimens. Samples were irradiated with a rhodium target at 40 kV and 13 μA. X-ray fluorescence (XRF) spectra were accumulated for 30 s for solid samples and 60 s for liquid sample extracts. During analysis, the XRF spectrometer was held upright in an acrylic stand and plant samples were placed on a protective Mylar film. Dry leaf samples were placed to cover the XRF beam aperture; for liquid samples, 10 μL of liquid leaf extracts were pipetted on top of the Mylar film. Spectra were collected and interpreted using PXRF1 software (Bruker Corporation). The presence of mercury L emission lines were examined in each specimen at Lα1 = 9.9888, Lβ1 = 11.8226, and Lγ1 = 13.8301.

**RESULTS**

**Crush time and extraction time affect PA and N-oxide recovery**

The results from all methods optimization experiments are presented in Fig. 1. Although considerable variation occurred between samples, likely due to the heterogeneity of the leaf samples, general trends can be inferred from the data shown. First, whereas longer crush times increased the average peak areas of PA free-base and N-oxide recovery up to 24 h (Fig. 1, Appendix S1). For example, this is apparent from the increase of free-base and N-oxide recovery in the uncrushed preparation at 1 min of extraction time (1.81E+06 ± 1.36E+06 AU/mg; 4.25E+05 ± 1.52E+05 AU/mg) compared to the uncrushed preparations at subsequent extraction times, especially at 24 h (1.68E+08 ± 1.84E+07 AU/mg; 8.24E+07 ± 1.12E+06 AU/mg) and 48 h (1.16E+08 ± 1.36E+07 AU/mg; 5.80E+07 ± 1.71E+07 AU/mg). Finally, there was possible decrease in PA free-base and N-oxide yield after 48 h of extraction compared to 24 h (Fig. 1).
Alcohol treatments do not decrease PA and N-oxide recovery

Alcohol treatment (i.e., soaking leaves for a week in alcohol before drying to mimic field preservation conditions) showed no apparent effect on PA free-base extraction with respect to the control (Fig. 2A, Appendix S2). The 70% ethanol ($4.18 \times 10^7 \pm 5.26 \times 10^7$ AU/mL) and 100% ethanol ($4.35 \times 10^7 \pm 2.90 \times 10^7$ AU/mL) exposures seemed to lead to the most PA recovery from leaves compared to the control ($7.66 \times 10^7 \pm 7.84 \times 10^7$ AU/mL).

PA N-oxide recovery in alcohol-pretreated samples (Fig. 2B, Appendix S2) showed an unexpected improvement in samples treated with 100% methanol ($6.03 \times 10^6 \pm 2.05 \times 10^6$ AU/mL), 70% ethanol ($7.36 \times 10^6 \pm 3.47 \times 10^6$ AU/mL), 100% ethanol ($9.71 \times 10^6 \pm 4.09 \times 10^6$ AU/mL), and 70% isopropanol ($6.50 \times 10^5 \pm 2.94 \times 10^5$ AU/mL) with respect to the control ($3.16 \times 10^6 \pm 1.47 \times 10^6$ AU/mL). Much variation can still be seen, but it is not as extreme as the free-base peak areas.

High levels of PAs were found in the leaves even though the recovered soaking alcohols, especially ethanol, showed substantial PA and PA N-oxide extraction from alcohol-treated leaves (70%: $4.01 \times 10^6 \pm 4.60 \times 10^6$ AU/mL; 100%: $7.94 \times 10^5 \pm 2.87 \times 10^5$ AU/mL) (Fig. 2B, Appendix S2).

PAs found sporadically in herbarium-preserved E. umbellatus specimens

Thirteen of the 23 herbarium specimens tested contained PAs (Table 1). PA content did not appear to increase or decrease with time; rather, a sporadic distribution of PAs was apparent among the samples (Table 1). Moreover, specimens showed PA profiles that were distinct from each other in either the variety of PAs identified or the intensity of each metabolite (Table 1). Compared to the recently collected E. umbellatus samples used for method development and the P. alboflavescens leaves used for field preservation studies, fewer PA compounds were identified and N-oxide content was somewhat lower in the herbarium specimens (Table 1).

Mercury species are detectable in E. umbellatus specimens

Mercury species, possibly one of its salts and/or organometallic adducts, were observed in herbarium-preserved specimens, as indicated by XRF spectrometric analysis. There was, however, no strict correlation to samples bearing or lacking PAs, as mercury species were detected in three of 13 PA-positive specimens and six of 10 PA-negative specimens (Table 1).

DISCUSSION

Herbarium specimens are a crucial resource for investigating plant traits (Funk, 2003). Although PAs are well preserved in herbarium specimens (Colegate et al., 2014), destructive sampling must be minimized to conserve these specimens for future researchers; therefore, extractions must be efficient, maximizing PA yield from each fragment of tissue. Burzynski (2014) used a protocol of a 5-min extraction without crushing to extract PAs; this is in contrast to the work of Colegate et al. (2014), who used a copper-coated pellet with shaking to break up plant tissue instead of crushing before extracting in methanol. We found improved yield with both increasing crush times and extraction times (Fig. 1, Appendix S1). However, the marginal improvement from added crush time seemed to diminish as the extraction time lengthened (Fig. 1). Crush time does not appear to be a major determinant of extraction efficiency for the
We found that 15 s of dry leaf crushing followed by 24 h of exposure to methanol were vastly superior to the protocol of Burzynski (2014), with recovery of PA free bases and N-oxides an average of 33× and 69× greater. For both free-base and N-oxide recovery, there was a trend for lower metabolite recovery after 48 h of extraction relative to 24 h (Fig. 1, Appendix S1). The longer crushes (60 and 180 s) may accentuate this trend for lower PA and N-oxide recovery with 48 h of extraction, suggesting degradation of the compounds while steeping in methanol (Fig. 1, Appendix S1). We identified fewer PA and N-oxide species in *P. alboflavescens* and *E. umbellatus* (Table 1) than did Burzynski et al. (2015) from the same source populations. This may be due to the inherent variation in PA profile that can occur between individuals within a species.

Our optimized protocol allowed for the identification of PAs in small samples removed from 70–167-year-old herbarium specimens, with recovery of PAs unlinked to specimen age (Table 1). It is thus anticipated that PA occurrence can be successfully documented based on herbarium specimens; however, fewer PA and N-oxide species were identified in the herbarium specimens than in recently collected samples of *E. umbellatus* (Table 1). Whether this is an artifact of degradation or actual infraspecific variation cannot be determined without comparable sampling of populations between the historical and recent specimen sets.

In our simulation experiments of alcohol field preservation with *P. alboflavescens* leaves, there did not seem to be a negative effect of alcohol on PA recovery (Fig. 2). In fact, there was a trend for greater recovery of the N-oxides after preliminary exposure to (and removal of) alcohols including methanol, ethanol, and isopropanol (Fig. 2). This is not an artifact of the treatment because alcohols are not expected to cause N-oxidation. We interpret this observation as evidence of hardening of the leaf tissues by alcohol exposure, which in turn assists the subsequent crushing/extraction procedure. The leaves that were steeped in alcohol were more brittle and easier to crush, as compared to the control, likely leading to increased PA extraction in the treated samples, despite substantial extraction of PAs by the soaking alcohols (Fig. 2A). Repetition of this experiment with a larger sample size and possibly different PA-producing species is necessary to mitigate variability in the samples and to quantify PA loss due to alcohol preservation.

The historical addition of the toxic preservative mercuric chloride to some of the herbarium specimens was inferred by XRF spectroscopy. Its addition, fortunately, did not preclude the observation of PA species, despite the potential for mercury salts to react with the alkene present in PAs. This suggests either that there is an incomplete or reversible chemical reaction with mercuric chloride, or that this preservative was added in miniscule amounts. Despite our current inability to detect PA-mercury adducts in our mass spectrometric analyses, we will continue to pursue this possibility.

The relative amounts of free-base and N-oxide recovery is also of interest, although we are not able to draw conclusions about their relative mass amounts using mass spectrometry in the absence of authentic samples. It has been reported that plants store more N-oxide than free base, and that N-oxides are less stable than the free-base form and may revert back to the free base, thereby regaining stability (Hartmann and Ober, 2000). We did note that N-oxide content increased more dramatically upon longer extraction times (Fig. 1B); it is not clear why free-base PAs were more readily extractable in short timeframes. Historical samples were observed to display fewer N-oxides than specimens collected in the past decade, both in absolute amounts and in number of different structures (Table 1), but without a time-based decomposition study, it is not clear if this is indicative of chemical degradation.
Confounding our ability to identify all PAs in a sample is the fact that many lycopsamine PAs are structurally similar and often constitutionally isomeric compounds; their identical masses made it impossible at times to identify isomeric PAs using mass spectrometry methods alone. For instance, spiraline and parsonsianidine both have protonated molecular ions at 456.2 m/z, reflecting their shared molecular formula \( \text{C}_{22}\text{H}_{33}\text{NO}_{9} \) (Burzynski et al., 2015). However, without the presence of a strong chromophoric group that would provide additional structural characteristics, or an authentic sample, the identity of the PA remains ambiguous. Burzynski et al. (2015) were able to tentatively identify PAs based on a precursor ion scan followed by a multiple reaction monitoring (MRM) scan, which screened only for compounds that had a 120 m/z necine core moiety common to many PAs. Although this did not offer information that would allow differentiation of isomers, it did confirm the masses of compounds containing the 120 m/z fragment, which at least suggested which compounds were PAs. Fortunately, reanalysis of methanol extracts prepared by Burzynski, as well as the structural analysis therein using MRM scans (Burzynski et al., 2015), allowed determination of retention times of lycopsamine PAs and likely structural assignments in the present study (Table 2). A supporting strategy to corroborate the identities of PAs was to locate their N-oxides (a structural analog with a mass increase of 16 m/z), which consistently elute approximately 0.25–0.5 min after their free-base counterparts (Fig. 3) (Wuilloud et al., 2004).

Inherent biological variation was expected during method development because the samples were of the same species and collected at the same time and place but were not always from the same leaf or individual. This resulted in quite large standard deviations in our small sample sizes (Appendix S1). Infraspecific variation may explain the variable presence of PAs among the historical herbarium specimens, as neither age, mercuric salt treatment (Table 1), nor alcohol treatment (Fig. 2) appear to be factors. The threshold for detection was based on a commercial monocrotaline standard measured in parts per billion (ppb). Because it was not possible to measure a threshold value in area units per milligram of dry weight, this is the best estimate of a minimum threshold value. Thirteen of 23 specimens had PAs above the minimum detection threshold of the 100 ppb monocrotaline commercial standard. Infraspecific variation in PA presence/absence has been previously reported in *Prestonia coalita* (Vell.) Woodson (Burzynski et al., 2015). We cannot, however, exclude the possibility that prolonged storage in a hydrated state before drying resulted in microbial PA degradation, as has been reported for PAs in *Senecio* (Crews et al., 2009; Hough et al., 2010), although none of the specimens looked moldy. We will be following up the hypothesis of infraspecific variation with population studies of PAs in *E. umbellatus* in Florida, where we have both PA-positive and PA-negative specimens (Table 1).

**CONCLUSIONS**

We have adapted and optimized a method (Burzynski, 2014; Colegate et al., 2014) that allowed us to qualify PA presence or absence in small fragments of leaves from 70–167-year-old herbarium specimens. Compared to the previous extraction method of Burzynski et al. (2015), we were able to extract ~33 times more free base and ~69 times more N-oxide by identifying the optimal crush and extraction times. Sample processing was not laborious (15 s crush, 24 h rest) and HPLC-MS analysis was straightforward, allowing for the analysis of many samples in a short time period. We also show that neither alcohol treatment nor mercuric salt treatment of herbarium specimens should impair detection of PAs. This will allow a systematic survey of PA occurrence across the 4500 species of the Apocynaceae based on the specimens preserved in the world’s herbaria, as well as newly collected samples from field and cultivated sources. The variability in PA presence/absence observed among herbarium specimens of *E. umbellatus* cautions us that multiple specimens should be tested before it is concluded that a species is PA-free. In the future, we hope to incorporate precursor ion scan and MRM scan methodology to add structural selectivity to this improved extraction technique (These et al., 2013; Avula, 2015; Burzynski et al., 2015) and ultimately quantify levels of PAs and their N-oxides by exploiting the one piece of molecule they all have in common, the necine core. This will permit a detailed reconstruction of PA evolution in Apocynaceae and a robust test of predictions of the defense de-escalation hypothesis (Edgar, 1984; Livshultz et al., 2018).

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**SUPPORTING INFORMATION**

Additional Supporting Information (Appendices S1 and S2) may be found online in the supporting information tab for this article.

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